SENSITIVITY ENHANCEMENT STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF DRUGS OF ABUSE AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

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Igor Botello González

DOCTORAL THESIS

Supervised by

Dr. Marta Calull and Dr. Carme Aguilar

Departament de Química Analítica i Química Orgànica

UNIVERSITAT ROVIRA I VIRGILI

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2012

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La Dra. MARTA CALULL BLANCH, Professora Titular del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili, i

La Dra. CARME AGUILAR ANGUERA, Professora Agregada del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili,

CERTIFIQUEM:
Que la present Tesi Doctoral, que porta per títol: "SENSITIVITY ENHANCEMENT STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF DRUGS OF ABUSE AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS", presentada per IGOR BOTELLO GONZÁLEZ per optar al grau de Doctor per la Universitat Rovira i Virgili, ha estat realitzada sota la nostra direcció, a l'Àrea de Química Analítica del Departament de Química Analítica i Química Orgànica d'aquesta universitat, i que tots els resultats presentats són fruit d'experiències realitzades per l'esmentat doctorand.

I, per a que consti, expedim aquest certificat a Tarragona, 4 de juliol de 2012.

Dra. Marta Calull Blanch Dra. Carme Aguilar Anguera

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A mis padres

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Hoy que puedo ver mi sueño de ser doctor en química hecho realidad, miro hacia atrás en el tiempo y me doy cuenta de que sólo no lo hubiese logrado. Quisiera entonces que estas líneas sirvan para expresar mi más profundo agradecimiento a todos los que directa o indirectamente han contribuido a que llegase a la meta.

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El soporte emocional ha sido también responsabilidad de mi familia y amigos, que aún en la distancia me han apoyado siempre. Nunca han faltado las palabras de ánimo y su ayuda incondicional. A mis primos Luisa y Enrique, a Mayra, Camelia, Julito, Betty, Jesús, Mauren, Yoan, Magaly Guerra, Yuliat, Rosa, Merci, Ibis, Jordito y muchos otros que no menciono pues se haría interminable la lista. A mis vecinos de Masó, en particular a Vivian. Dedicada también a mi abuela y a mi tía Zenaida, que aunque ya no están sé que estarían muy felices por mí. En especial, agradezco a mis padres por estar siempre cerca, aun estando tan lejos y a mi hermanito Iván por ser amigo y consejero. A todos, muchas gracias.

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1. INTRODUCTION

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Emerging organic contaminants (EOC) are defined as pollutants that were previously unknown or unrecognized as being of concern, because they have barely been investigated due to the absence of environmental data and the lack of appropriate analytical methods. These kinds of compounds include a diverse range of groups such as pharmaceuticals, drugs of abuse, personal care products, steroids and hormones, surfactants, flame retardants, and industrial additives and agents, among others [1].

Among the different EOCs, pharmaceuticals and drugs of abuse are the most widely studied compounds at present. These compounds are excreted unchanged or as metabolites and eventually reach wastewater treatment plants (WWTPs), where a fraction of these compounds can be removed, but some residues may be released into the aquatic environment, ending up in surface water and even in drinking water [1,2]. The continuous environmental input of such compounds may lead to relatively long-term concentration and thereby promote continuous but unnoticed adverse effects on aquatic and terrestrial organisms. As a consequence, the development of suitable analytical methodologies in order to determine their presence is an essential preliminary step to measure their concentration levels in different ecosystems [2,3].

In addition, pharmaceuticals and drugs of abuse have become increasingly relevant in fields like doping control and forensic analysis. A common task in forensic laboratories involves the determination of these kinds of compounds and their metabolites in biological samples for investigation of intoxication or to determine the causes and circumstances of death [4-7].

The main problem in the analysis of environmental and biological samples lies in the fact that, in these kinds of complex matrices, interfering endogenous compounds are usually present at a higher concentration in comparison to the target analytes and so the concentration sensitivity is an important factor to be taken into account with respect to the analytical methods to be developed for its application in environmental, forensic, toxicological and doping fields [2,7-9].

Liquid chromatography (LC) and gas chromatography (GC) continue to be the predominant techniques for the determination of organic pollutants, their metabolites and transformation products in different matrices [2,10]. However, recent advances in capillary electrophoresis (CE) have made this technique a more promising alternative for the analysis of environmental and biological samples [6-8,11,12]. The growing interest in CE as an analytical technique is certainly due to its high efficiency, high resolution power, low reagent consumption, automation and low cost compared with chromatographic techniques.

In order to achieve the separation of a wide variety of compounds, different modes of capillary electrophoresis can be used, such as capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MEKC), microemulsion electrokinetic chromatography (MEEKC), capillary isotachophoresis (ITP), capillary electrochromatography (CEC) and capillary isoelectric focusing (CIEF) [4,8,13-16]. Despite the advantages of using these modes for separation purposes, CE has less-than-desirable sensitivity based on concentration, especially when compared to chromatographic techniques. In the majority of practical applications, this is far more important, particularly for the analysis of compounds present at low concentrations. The concentration sensitivity problem is mainly due to two sources, namely the low sample injection volume (three orders of magnitude smaller than the typical injection volume for conventional LC) and the short optical path-length for on-capillary detection determined by the capillary inner diameter (25-100 μm) [9,17].

Applied researchers have become increasingly interested in the development of several approaches to overcome sensitivity issues in CE over the last few decades [9,17]. From among the many different attempts, two main approaches have emerged for increasing the sensitivity of CE. The first consists of the use of more sensitive detection systems, such as laser-induced fluorescence (LIF) or mass spectrometry (MS) [18-20]. However, these detection systems are relatively expensive to purchase and maintain, which is why absorbance detection is still the most widely used detection system in CE [21,22]. The second approach, an area of major interest in CE over the past two decades, involves the improvement of the low concentration sensitivity associated with this technique through the development of various high sensitivity preconcentration strategies [17,23-25]. The importance of the development of these kinds of methodologies based on strategies that allow a considerable increase in the sensitivity of CE lies in the possibility of applying them in various fields, such as biological and environmental analysis [17,24].

In light of the above facts, in the first part of the introduction of this Doctoral Thesis, a detailed description is presented of the strategies developed to increase the sensitivity of CE for the preconcentration of the studied compounds. The second part of this section is related to pharmaceuticals and drugs of abuse, describing in detail the groups of compounds that have been studied, which include non-steroidal anti-inflammatory drugs (NSAIDs), barbiturate drugs and opioids, among others. The section includes an outline of their main characteristics, some examples of the results of research regarding their presence in various types of environmental and biological samples and an overview of the use of CE for their determination. In the case of drugs of abuse, the related subsection is presented in review article format. After the introduction, the main objectives of this Doctoral Thesis are set out. The third chapter presents the results and discussion of the studies derived from the experimental

research, which are included in paper format. Finally, the main conclusions that can be drawn from these studies are presented.

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1.1. Strategies to increase sensitivity in capillary electrophoresis

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As mentioned above, the limited concentration sensitivity is one of the most important issues when CE is applied to the determination of analytes at low concentration levels. With this in mind, a number of strategies can be used to improve sensitivity and these can be performed either through preconcentration strategies or through improved detection hardware [9,11,12,17,26]. There are promising indications for the use of these preconcentration techniques, which allow signal-to-noise ratios comparable to those in LC to be obtained. Some simple preconcentration strategies are capable of providing sensitivity improvements of 10- to 100-fold, while more sophisticated techniques that involve multiple mechanisms can improve the sensitivity by over 100,000-fold [17,27]. These techniques can be subdivided into two groups. The first group, known as stacking preconcentration techniques (or electrophoretic preconcentration techniques), involves methods based on electrophoretic phenomena [9,24,28]. The second group, known as extraction preconcentration techniques, includes all those techniques which involve partitioning onto or into a distinct phase to concentrate the analytes prior to CE separation [17,25,29].

The term 'stacking' has widely been used to group together approaches for preconcentration that generate changes in electrophoretic velocity. In this kind of preconcentration technique, the key requirement is that there is an electrophoretic component in the preconcentration mechanism and that the analytes concentrate on a boundary through a change in velocity [17,24].

In some types of stacking methodologies, a long plug of a low-conductivity sample is injected into the capillary. When the voltage is switched on across the capillary, sample ions migrate rapidly as a result of higher electric field strength in the injection zone. Subsequently, the sample ions pass through the boundary between the sample zone and the BGE and enter the BGE region. Here, the sample ions are slowed down, due to the lower electric field strength and, consequently, they are concentrated into a narrow zone. They then migrate in the BGE and are separated and detected [9,24]. There are different strategies that have been reported in the literature that work using this principle, such as field-amplified sample stacking (FASS), field-amplified sample injection (FASI) and large volume sample stacking (LVSS) [30,31].

Other stacking strategies are based on differences in the pH value, depending on the capillary zone. Some examples that focus on this principle are pH-mediated stacking and dynamic pH junction techniques [9,24]. In pH-mediated stacking, the sample is injected into the capillary and when a plug of a strong acid or base is electrokinetically introduced into the capillary, the counter ions of the BGE migrate into the sample zone. As a consequence, titration of the BGE counter ions occurs in the sample zone and then a decrease in the conductivity is experienced with an

increase in the mobility of the analytes. In contrast, dynamic pH junction is used to focus weak electrolytes selectively, as their effective charges and hence their effective mobilities are pH dependent. The BGE has a pH value that differs significantly from that of the sample which is injected hydrodynamically into the capillary. Therefore, when the analytes enter the BGE by electromigration across the pH junction, their effective mobilities change suddenly and they focus into a short zone [9,17,32].

Sweeping is a preconcentration strategy which is usually performed in MEKC and it is based on the interaction between the pseudostationary micellar phase and the compounds [28,33]. The basic condition for sweeping is that the BGE contains micelles of a surfactant, with a concentration above its critical micelle value, while the sample solution is free of micelles. The electric voltage brings about a situation in which sample analytes and micelles meet and migrate across each other. The micelles effectively extract analytes from the aqueous phase into their cavities. In other words, the micellar phase efficiently 'sweeps' the injected analytes. Since the concentration of micelles is high, the capacity of this phase is large and the micelles may move through a large injected volume of the sample and sweep the analytes distributed in the injected volume into a narrow concentrated zone.

Another interesting stacking approach is known as transient isotachophoretic (t-ITP) stacking [12,34,35]. This strategy is useful for the analysis of complex samples because its effectiveness is not dependent on the sample conductivity. In this technique, the sample zone is stacked between the leading (ions with a higher mobility than the analytes) and terminating (ions with a lower mobility than the analytes) zones that are created in the capillary. The leading and terminating electrolytes can be formed by macrocomponents from the sample, by the BGE co-ions or they can also be injected separately before and after the sample injection. After the t-ITP step is finished, destacking proceeds and the individual destacked zones migrate and separate in the CZE mode.

Solid-phase extraction (SPE) and liquid-phase extraction (LPE) are the most widely used extraction preconcentration strategies in CE. These preconcentration techniques have a wide range of applicability and enable high preconcentration factors to be achieved [17,29,36]. Of all the techniques that may be included in this group, it should be highlighted that SPE is the predominant extraction-based preconcentration technique. There are four main modes of combining SPE and CE: off-line, at-line, on-line or in-line. Off-line SPE-CE has widely been used and, in this case, the two steps (preconcentration and separation) are performed separately and the transfer of analytes to the CE system is carried out manually. If a robotic or a similar system is used to transfer the samples from SPE to the CE system, an at-line combination is achieved. In both off-line and at-line systems, there is no direct stream of liquid between the two parts. Despite the great number of applications involving both

combinations, over the last few years, there has been increasing interest in the development of preconcentration strategies that integrate SPE extraction procedures with capillary electrophoresis [17,25,29]. The growing awareness of these forms of on-line and in-line sample preconcentration is likely to be due to the difficulty faced with almost all stacking methods with respect to dealing with complex sample matrices and the fact that many of these methods still require exhaustive off-line treatment for complex samples.

The on-line coupling SPE-CE comprises physical connection and contact between the capillary and the stream flowing from the SPE column. This coupling is performed via an interface that automatically transfers the analytes from the SPE column to the separation capillary [9,25]. In in-line SPE–CE systems, the preconcentration column is an integral part of the CE system. Both integrated approaches are more effective, due to the possibility of performing the complete analysis with a minimum amount of manual sample handling [17].

In the following subsections, we will focus on the techniques employed in the present Doctoral Thesis in order to increase the sensitivity of CE. The first subsection involves an overview of the theory and some relevant applications of different preconcentration techniques based on t-ITP, while the second subsection, is focused on the use of an extraction-based preconcentration strategy, in particular in the in-line coupling between SPE and CE.

1.1.1. Stacking techniques based on transient isotachophoresis

The principal condition for intrinsic stacking, namely the low conductivity of the sample matrix, is not valid for most real samples of biological, environmental or industrial origin. These kinds of samples contain a huge amount of other chargeable components, whereas the analytes of interest are present at very low concentration levels. Thus, the ionic strength (and conductivity of the sample) is usually higher than the ionic strength of the BGE, so in order to decrease the sample conductivity below the value of conductivity of the BGE, a large dilution of the sample is necessary. Another option would be to increase the BGE concentration. However, under these conditions, a substantial improvement of the stacking cannot be expected, as the negative effects of Joule heating would result in enhanced dispersion, bubble formation and, eventually, the collapse of the analysis. To solve this problem, either extraction of the analytes from the matrix or desalting of the sample can be performed. Another approach that is currently popular for the analysis of this kind of complex samples is the use of an ITP methodology as, in this case, the conductivity of the sample does not affect the preconcentration and separation process [22].

The name ITP is derived from three Greek terms, *iso* meaning equal, *tachos* meaning speed and *phoresis* meaning migration. In this technique, a multi-analyte sample is introduced between an electrolyte with a higher mobility than the analytes (leading electrolyte: LE) and an electrolyte with a lower mobility than the analytes (terminating electrolyte: TE). As a requirement for the proper functioning of this strategy, the leading ion, the terminating ion and the sample components must have the same charge polarity [12,17,34]. When an electric field is set up and a current passes through the system, initially a uniform electric field will be formed across the sample and the individual ions will separate from one another according to their different migration rates (based on their differing mobilities). This means that the faster moving ions will be slowed down by the weaker field around them and the slower moving ions will be accelerated by the stronger field around them. Subsequently, following a brief transient period in which the discrete solute zones are formed, this ITP 'stack' achieves a fixed concentration profile with a constant velocity moving in the direction of the leading ion [12,35].

As mentioned above, in ITP, the leading ion of the LE must present an electrophoretic mobility higher than that of the analytes to be preconcentrated. This leading ion can be present in the sample, added to the sample or injected hydrodynamically into the capillary prior to the sample. Inorganic ions, such as Li^+, Na^+, Cl^- or NH_4^+, are typically used as leading ions [37]. In contrast, the terminating ions of the TE must have lower electrophoretic mobility than the analytes. The choice of the TE is generally more difficult than the selection of the LE because, depending on the analytes' mobility, it is sometimes hard to find a compound with the appropriate mobility. The most commonly used TEs are generally organic compounds such as HEPES, CHES and MES, among others [38,39].

ITP can mainly be performed in two different modes [38,40]. The first consists of two coupled capillaries, one of which is used for stacking by ITP and the other for separation by CE. This mode is known as column-coupling ITP-CE and it enables sample volumes exceeding the total volume of the CE capillary to be stacked into nanolitre volumes for separation. The preconcentration efficiency of this mode easily leads to 10,000-fold sensitivity enhancements factors (SEFs). The second mode consists of the use of a single capillary for both stacking by ITP and CE separation. This last mode, known as transient-ITP, is the most frequently reported in the literature and, in the present Doctoral Thesis, stacking methodologies based on its principles have been developed. Figure 1 shows a schematic diagram of the different steps involved when this strategy is used for the preconcentration of cationic analytes. In Figure 1A, the LE, the sample solution containing the analytes (S_1, S_2, S_3) and the TE are injected in turn after filling the capillary with BGE. Figure 1B represents the first stage in the ITP performance, in which the analytes are stacked

between the LE and TE when a high positive voltage is applied. Finally, the concentrated analyte zones are separated by CZE (Figure 1C).

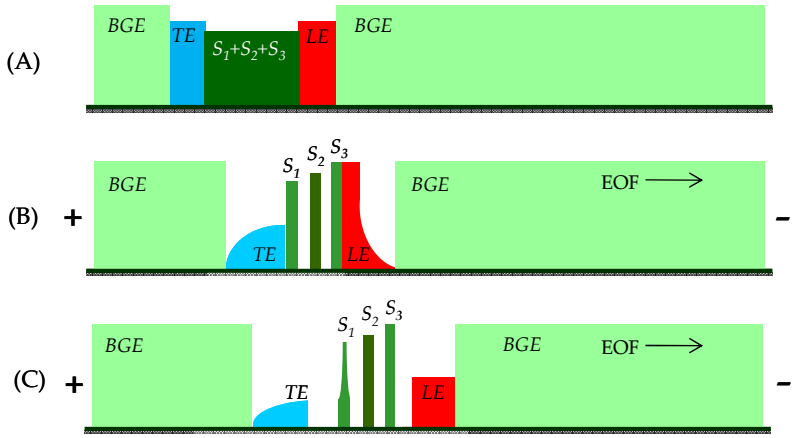

Figure 1. Schematic diagram of a transient isotachophoresis process for cations. (**A**) The leading electrolyte, the sample solution and the terminating electrolyte are injected in turn. (**B**) A high positive voltage is applied and the preconcentration of the analytes begins between the leading and terminating ions during t-ITP migration. (**C**) The concentrated analytes zones are separated by CZE. EOF refers to the electroosmotic flow.

There are other important approaches based on t-ITP principles, such as the so-called transient pseudo-ITP and electrokinetic supercharging (EKS). The main differences between these techniques lie in the fact that, for transient pseudo-ITP, an organic solvent acts as the TE, while for EKS performance, the strategy is similar to t-ITP but with electrokinetic injection of the sample. Table 1 shows a summary of a number of relevant applications reported in the literature, using these different t-ITP methodologies.

Table 1. Overview of methodologies based on t-ITP

Analytes	CE conditions	D [a]	LOD	Sample	Sample treatment	Ref.
		Transient-ITP				
Glutathione (reduced form) Glutathione (oxidized form)	**CZE** Capillary: 50.2 cm x 50 µm; 25 kV, 25 ºC BGE: 50 mM NH_4Ac (pH 7.5) LE: 50 mM NH_4Ac (pH 7.5) TE: 10 mM HEPES (pH 7.5)	UV	18.0 – 23.4 µg/L [b]	Tomato and human serum	Filtration and dilution	[38]

[a] Detector
[b] Standard samples
[c] Real samples

Table 1. Overview of methodologies based on t-ITP (Cont.)

Analytes	CE conditions	D [a]	LOD	Sample	Sample treatment	Ref.
Phosphate	**CZE** Capillary: 97 cm x 75 μm; 30 kV, 25 ºC BGE: 5 mM PDC + 0.01 % HPMC (pH 3.5) TE: 500 mM MES (pH 4.0)	UV	16 μg/L [b]	Seawater	Filtration	[39]
Melamine Aniline 2,4-diaminotoluene 4,4´-diaminophenylmethane	**CZE** Capillary: 60 cm x 75 μm; 20 kV, 25 ºC BGE (TE): 60 cm H_3PO_4 + Tris (pH 2.65) LE: 80 mM H_3PO_4-KH_2PO_4 (pH 2.85)	UV	0.02 – 0.05 μM [b]	Milk	Solvent extraction	[41]
Nitrite Nitrate	**CZE** Capillary: 97 cm x 75 μm; -11 kV, 30 ºC BGE: 3 mM CTAC (pH 7.9) TE: 150 mM sulphate + 10 mM bromate	UV	2.2 and 1.0 μg/L [b]	Seawater	Filtration	[42]
Alkyl methylphosphonic acids	**CZE** Capillary: 90 cm x 50 μm; 20 kV, 22 ºC BGE (LE): 30 mM or 50 mM ammonium acetate (pH 8.8) in (35:65 v/v) MeOH/H_2O TE: 200 mM glycine (pH 10.0) in (35:65 v/v) MeOH/H_2O	MS	4 – 75 μg/L [c]	Soil and rat urine	Pressure-assisted solvent extraction (soil) and filtration (urine)	[43]
Fe^{2+} Ni^{2+} Zn^{2+}	**CZE** Capillary: 50 cm x 50 μm; 20 kV, 20 ºC BGE : 150 mM *N*-tris(hydroxymethyl) methyl-3-aminopropanesulfonic acid + 127 mM triethylamine + 0.1 mM 4-(2-pyridylazo) resorcinol (pH 9.0) LE: 30 mM or 50 mM sodium chloride TE: 200 mM glycine (pH 10.0)	UV	0.16 – 0.8 μg/L [b]	Urine	Dilution and filtration	[44]
Transient pseudo-ITP						
Mycophenolic acid Mycophenolic acid glucoronide	**CZE** Capillary: 35 cm x 50 μm; 10 kV, 25 ºC BGE: 75 mM phosphate (pH 7.5)	UV	-	Human serum	Precipitation	[45]

[a] Detector
[b] Standard samples
[c] Real samples

Table 1. Overview of methodologies based on t-ITP (Cont.)

Analytes	CE conditions	D [a]	LOD	Sample	Sample treatment	Ref.
Cysteamine	**CZE** Capillary: 64.5 cm x 50 μm; 30 kV, 25 ºC BGE: 0.2 mM Tris/HCl (pH 2.1)	UV	0.8 μM [b]	Human plasma	Precipitation	[46]
Glutathione (reduced form) Glutathione (oxidized form)	**CZE** Capillary: 31 cm x 75 μm; 5 kV, 25 ºC BGE: 300 mM sodium tetraborate (pH 8.0)	UV	0.3 -0.5 μM [b]	Human plasma	Precipitation	[47]
Myosmine Anabasine	**CZE** Capillary: 48 cm x 50 μm; 10 kV, 25 ºC BGE: 380 mM tartaric acid (pH 3.7)	UV	0.1 -0.3 ng/mL [b]	Cigarette	Ultrasonic extraction	[48]
Mycophenolic acid	**MEKC** Capillary: 50 cm x 75 μm; 30 kV, 25 ºC BGE: 2 % boric acid + 10 % methanol + 0.1 % SDS	UV	95 ng/mL [c]	Serum	Precipitation	[49]
±1,1-binaphthyl diyl hydrogen phosphate ± 1,1'-bi-2-naphthol Dansyl-DL-tryptophan	**MEKC** Capillary: 50 cm x 50 μm; 25 kV, 25 ºC BGE: 60 mM sodium cholate + 20 mM CAPS (pH 9.0) + 20 % acetonitrile	UV	-	-	-	[50]
	EKS					
Diclofenac Diflunisal Fenoprofen Ibuprofen Indomethacin Ketoprofen Naproxen	**CZE** Capillary: 85 cm x 50 μm; -28 kV, 25 ºC BGE: 15 mM disodium tetraborate + 10 % methanol + 0.1 % HDMB LE: 100 mM sodium chloride TE: 100 mM CHES	UV	50 -180 ng/L [b]	Wastewater	Filtration	[14]
β-Lactoglobulin peptides	**CZE** Capillary: 60 cm x 50 μm; 30 kV, 25 ºC BGE (TE): Acetic acid 10% (v/v) LE: 935 mM NH_4Ac (pH 9.3)	UV	5.4 – 10 nM [b]	-	-	[37]

[a] Detector
[b] Standard samples
[c] Real samples

Table 1. Overview of methodologies based on t-ITP (Cont.)

Analytes	CE conditions	D [a]	LOD	Sample	Sample treatment	Ref.
K^+, Na^+, Li^+, La^{3+} Ce^{3+}, Pr^{3+}, Nd^{3+} Sm^{3+}, Eu^{3+},Gd^{3+} Tb^{3+}, Dy^{3+},Ho^{3+} Er^{3+}, Tm^{3+}, Yb^{3+} Lu^{3+},Y^{3+}	**CZE** Capillary: 100 cm x 75 μm; 20 kV, 25 ºC BGE: 30 mM creatinine, 4 mM 2-hydroxyisobutyric acid + 0.4mM malonic acid (pH 4.8) LE: 100 mM aqueous NH_3 + 7.5 mM 2-hydroxyisobutyric acid + 2.0 mM malonic acid + 2-ethyl-*n*-butyric acid (pH 4.8) TE: 1 M HCl	UV	-	-	-	[51]
Diclofenac Diflunisal Fenoprofen Ibuprofen Indomethacin Ketoprofen Naproxen	**CZE** Capillary: 85 cm x 50 μm; -28 kV, 25 ºC BGE (LE): 15 mM disodium tetraborate + 10% methanol + 0.1% HDMB TE: 100 mM CHES	UV	10.7 – 47.0 ng/L [b]	Wastewater	Filtration	[52]
K^+, Na^+, Li^+, La^{3+} Ce^{3+}, Pr^{3+}, Nd^{3+} Sm^{3+}, Eu^{3+},Gd^{3+} Tb^{3+}, Dy^{3+},Ho^{3+} Er^{3+}, Tm^{3+}, Yb^{3+} $Lu^{3+}Y^{3+}$	**CZE** Capillary: 100 cm x 75 μm; 20 kV, 25 ºC BGE: 30 mM creatinine, 4 mM 2-hydroxyisobutyric acid + 0.4mM malonic acid (pH 4.8) LE: 100 mM aqueous NH_3 + 7.5 mM 2-hydroxyisobutyric acid + 2.0 mM malonic acid + 2-ethyl-*n*-butyric acid (pH 4.8) TE: 1 M HCl	UV	-	Monazite ore	-	[53]
La^{3+} Nd^{3+} Sm^{3+} Gd^{3+} Y^{3+} Er^{3+} Yb^{3+}	**CZE** Capillary: 50 cm x 75 μm; -28 kV, 25 ºC BGE (LE): 10 mM 4-methylbenzylamine + 4 mM 2-hydroxyisobutyric acid + 0.4 mM malonic acid + 0.1 % hydroxypropyl cellulose (pH 4.8) TE: 2-ethylbutyric acid	UV	0.4 -1.3 ng/L [b]	-	-	[54]
Pyromelitic acid Trimellitic acid Phthalic acid Naphthalenedisulfonic acid	**CZE** Capillary: 55.5 cm x 25 μm; 20 kV, 25 ºC BGE: triethanolamine (pH 7.8) LE: 100 mM sodium nitrate TE: 100 mM camphor sulfonic acid	UV	8.73 -12.9 ng/mL [b]	Urine	Filtration	[55]

[a] Detector
[b] Standard samples
[c] Real samples

Table 1. Overview of methodologies based on t-ITP (Cont.)

Analytes	CE conditions	D [a]	LOD	Sample	Sample treatment	Ref.
NO_2^- NO_3^- SCN^- CrO_4^{2-} MoO_4^{2-} Naphthalenedisulfonate	**CZE** Capillary: 55.5 cm x 25 μm; -28 kV, 25 ºC BGE (TE): 100 mM CHES (pH 8.05) LE: 100 mM HCl + 200 mM Tris (pH 8.05)	UV	6.3 -24 ng/L [b]	-	-	[56]
NO_2^- NO_3^- SCN^-	**CZE** Capillary: 34.5 cm x 25 μm; -20 kV, 25 ºC BGE (LE): 100 mM NaCl + 200 mM Tris (pH 8.05) TE: 100 mM MES	UV	0.05 -0.66 ng/mL [b]	-	-	[57]
Diclofenac Diflunisal Fenoprofen Ibuprofen Indomethacin Ketoprofen Naproxen	**CZE** Capillary: 80 cm x 50 μm; 28 kV, 25 ºC BGE (LE): 50 mM ammonium hydrogencarbonate (pH 9.2) + 10% methanol TE: 8 mM CAPS	UV	6.7 -18.7 ng/L [b]	-	-	[58]
Fe^{2+} Co^{2+} Ni^{2+}	**CZE** Capillary: 68.5 cm x 75 μm; 30 kV, 25 ºC BGE: 30 mM creatinine + 18 mM lactic acid (pH 4.9) LE: 200 mM ammonia + 215 mM lactic acid (pH 4.9) TE: 1 M HCl	UV	30 ng/L [b]	Heat exchanger fluids	-	[59]

[a] Detector
[b] Standard samples
[c] Real samples

Transient-ITP

Transient-ITP (t-ITP) can be considered the most commonly used stacking technique based on ITP principles over the last ten years [12,35]. Figure 2 shows the diagrams of different possibilities of discontinuous electrolyte systems in which conditions for t-ITP stacking can be obtained. In the different configurations, the LE, TE or both are injected separately before and after the sample, respectively. In Figure 2A, the diagram shows the configuration in which the capillary is filled with a BGE which also has an LE character. After the sample introduction, the injection of a plug of TE is performed. Both ends of the capillary are then inserted into vials with BGE that serves as LE (BGE + LE). Another possible configuration is obtained when the capillary is filled with a solution which has a TE character and then a plug of a LE is introduced into the capillary to create the potential jump necessary for the ITP migration mode (Figure 2B), prior to the sample injection. In Figure 2C, the diagram

of the arrangement is shown, with the sample sandwiched between LE and TE, both being different from the BGE used for further CE analysis.

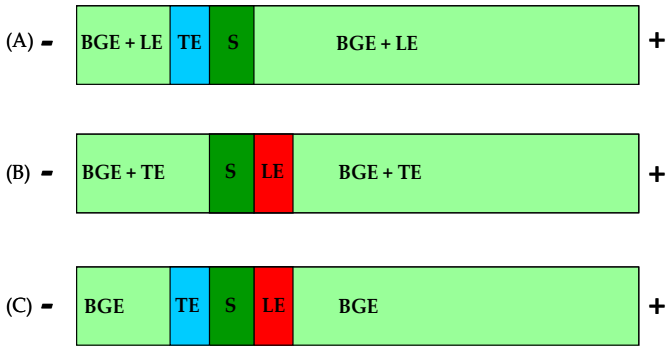

Figure 2. Schematic diagrams of discontinuous electrolyte systems that can be used to create a t-ITP state. (**A**) The BGE co-ion acts as the leading ion and, after its introduction, the sample is injected followed by a plug of the TE. (**B**) The BGE co-ion acts as the terminating ion and, prior to the sample injection, a plug of LE must be introduced into the capillary. (**C**) LE and TE, different from BGE, are injected before and after the sample plug, respectively.

This stacking methodology has been applied to the analysis of different kinds of analytes in complex matrices, such as food [38,41], seawater samples [39,42] and soil [43]. For example, Wang *et al.* [41] developed a strategy based on t-ITP to assay aromatic amines in milk samples. Using K^+ as the LE and Tris as the TE, a plug of 10 cm of sample solution was introduced into a 60 cm capillary, giving LODs as low as 0.02 μM. The method allowed about 200-fold improvement in sensitivity for the primary aromatic amines studied. Okamoto *et al.* [39] also used t-ITP as a preconcentration procedure with indirect UV detection for the determination of phosphate in seawater. In the method, no LE was hydrodynamically injected prior to the sample because the chloride ions in the sample matrix acted as the leading ions for t-ITP. The TE solution consisted of 500 mM MES (pH 4.0). The LOD for phosphate in artificial seawater samples was 16 μg/L. The method was applied to the determination of phosphate in a seawater certified reference material for nutrients and the results were very similar to certified values. An interesting approach was proposed by Fukushi *et al.* [42], in which a combination of different terminating ions was evaluated for the determination of nitrite and nitrate in seawater. In addition to 150 mM sulphate as the principal TE, 10 mM bromate was added to the sample solution as the additional TE. As the result of this strategy, the authors concluded that the LODs diminish with respect to the use of a unique TE. This combination was effective for the preconcentration and determination of nitrite and nitrate, with LODs of 2.2 and 1.0 μg/L (as with nitrogen), respectively, for standard samples.

Generally, t-ITP stacking is performed in a suppressed electroosmotic flow (EOF) to minimize zone-broadening, due to an EOF mismatch occurring from variations in the conductivities of sample zones. However, some authors have reported the application of t-ITP under strong-EOF conditions (sEOF-t-ITP) [38,44]. For example, Ríaz and Chung [44] reported the use of this strategy for the preconcentration of anionic complexes of various heavy metals. To be specific, they compared different conditions and developed a method using a bare capillary and a high pH BGE in order to have a strong EOF, and another method for which a coated capillary under negligible EOF was employed. When they used the bare capillary, the strong EOF pulled the analytes against their mobilities towards the outlet side of the capillary, allowing separation in the normal polarity mode. Stacking efficiency, reproducibility, analysis time and sample loading capacity in the case of coated and bare capillaries were compared. The authors concluded that even though the stacking efficiency and reproducibility were higher and the analysis time was shorter in the case of the coated capillary, when the bare capillary was used, a larger sample volume could be introduced into the capillary and the LODs obtained were comparable to those for the coated capillary without compromising the resolution between the analyte peaks. Yan *et al.* [38] also applied t-ITP under strong counter-EOF for the preconcentration of reduced glutathione (GSH) and oxidized glutathione (GSSG). The sensitivity enhancement factors were 320-fold for GSH and 280-fold for GSSG, obtaining LODs of 23.4 and 18.0 μg/L for GSH and GSSG, respectively.

In some cases, the TE has to be injected prior to the sample, if the process occurs under counter-EOF conditions. Lagarrigue *et al.* [43] used counter-EOF t-ITP to enhance the sensitivity of a method based on CE-ESI-MS for the determination of nerve agent degradation products (alkyl methylphosphonic acids) contained in high-conductivity matrices. In this case, the preconcentration by t-ITP was induced by the BGE (ammonium acetate) acting as the LE, while the TE (glycine) was loaded before the sample. Using this method, an enhancement in detection sensitivity of 40-fold was achieved and the method was successfully applied to soil extracts. The best results for the analysis of soil extracts fortified with the analytes were obtained with an LE composed of 30 mM CH_3COONH_4, adjusted to pH 8.8 with ammonium hydroxide in (35:65 v/v) MeOH/H_2O mixture. The TE consisted of 200 mM glycine adjusted to pH 10.0 with ammonium hydroxide in the same solvent mixture. The LODs obtained in the soil extract were between 4 and 70 μg/L. This t-ITP-CZE-MS method was further adapted for the analysis of rat urine samples fortified with the analytes and, for example, in this case, they required the use of a more concentrated LE (50 mM), due to the strong diffusion of the analytes observed when a concentration of 30 mM was used. The LODs for rat urine ranged between 5 and 75 ng/mL.

Transient pseudo-ITP

In 2002, Shihabi [60] proposed a methodology known as transient pseudo-ITP, which is especially suitable for samples containing high levels of salts and proteins. In some papers, this technique is also referred to as acetonitrile stacking, because acetonitrile is the most commonly used organic solvent for this strategy. In this case, the stacking of the analytes can be achieved if the sample has a high concentration of chloride (to function as a leading ion) and about 60 to 70% acetonitrile in the sample (which can function as a terminating ion). These requirements make the technique suitable for the analysis of biological fluids because acetonitrile, added 2:1 to the sample (making 66% acetonitrile), is used for protein precipitation and it is therefore possible to inject the supernatant directly and achieve a 2- to 3-fold improvement in sensitivity [45-49]. The low conductivity of the organic solvent provides the high field strength necessary for band sharpening, similar to that provided by the terminating ion. In contrast, when salts are present in the sample, they act as leading ions, migrating rapidly in the organic solvent until they are slowed down at the interface of the separation buffer. When the organic solvents are added to the sample, both the migrations and the stacking of the analytes are affected by the concentration of salts (leading ions) in the sample, similar to that observed in ITP.

In practice, transient pseudo-ITP stacking is much easier to perform compared to typical t-ITP, because, in some applications with the latter approach, the choice of terminating ions is limited by the electrophoretic mobility of the target analytes to be studied [61]. Figure 3 shows a diagram of the different steps involved in this preconcentration strategy using cations as model compounds. In Figure 3A, the capillary is filled with a BGE. Subsequently, the sample, containing the analytes (S_1, S_2, S_3), the LE and the organic solvent (Sl), which acts as TE, are introduced. Both ends of the capillary are then inserted into vials with BGE and, after applying an appropriate voltage, the ITP stage is generated and the stacking of the analytes is performed (Figure 3B). Finally, in Figure 3C, the analytes are separated through the capillary by CZE.

For a better understanding of the principles involved in this preconcentration strategy, some authors have reported different theoretical studies about the transient pseudo-ITP mechanism [61-64]. For example, Osbourn *et al.* [62] considered that the mechanism in transient pseudo-ITP was similar to a LVSS stacking procedure due to the addition of the solvent, which reduces the conductivity of the sample plug with respect to the BGE. However, the effect of salts was not mentioned in that work. Subsequently, Shihabi [61,63] investigated the early steps of stacking without the influence of the separation step (the separation buffer). In those studies, the author demonstrated the importance of the concentration of salts in the sample.

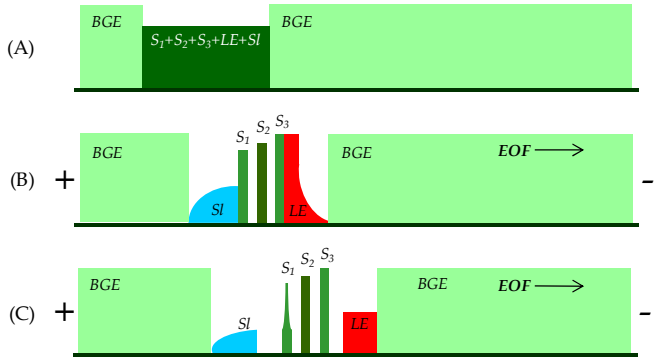

Figure 3. Schematic diagram of a transient pseudo-ITP process for the preconcentration of cationic analytes. (**A**) The sample solution containing the LE and the organic solvent (Sl) is injected. (**B**) A high positive voltage is applied and the preconcentration of the analytes begins between the leading ions and the organic solvent acting as a terminating agent during t-ITP migration. (**C**) The concentrated analyte zones are separated by CZE.

As the concentration of the leading ions (salts) was increased, higher stacking efficiency was obtained. In the same way, in the absence of salts or acetonitrile, the stacking was greatly reduced. Another interesting study was reported by Yu [64], who evaluated the influence of different salts ($NaNO_2$, CH_3COONa, Na_2SO_4, Na_2CO_3, $CaCl_2$, NH_4Cl, and KCl) on stacking in the analysis of anions by transient pseudo-ITP. For salts which had different cations (but the same anions), the values for peak efficiency were similar. This indicated that the difference of the cations seemed to have no relation to the stacking effects. In the opposite case, for the salts containing different anions, it was found that the stacking effects increased as the mobilities increased. In addition, the authors also suggested that the stacking effect is larger when the concentration of the leader ion is increased. With respect to the organic solvent added to the sample, even though acetonitrile is the most commonly used organic solvent for the development of transient pseudo-ITP, Shihabi [60] showed that many other water miscible organic solvents, such as acetone and small alcohols, can successfully act as terminating ions.

Apart from these theoretical studies, there are a number of interesting reports in which transient pseudo-ITP has been applied as a preconcentration approach for different compounds in biological samples. For example, Kubalczyk *et al.* [46] used transient pseudo-ITP for the determination of total cysteamine in human plasma samples. A large volume of sample was injected in order to achieve analyte preconcentration directly on the capillary. The procedure was applied to the analysis of plasma samples of healthy volunteers spiked with known amounts of cysteamine standard solution. Through the use of the stacking methodology, the authors achieved sensitivity comparable to that typical of LC, with LODs of 0.8 μM. Kong *et al.* [47] also analysed human plasma using this methodology for the determination of

low concentrations of both reduced form glutathione and oxidized form glutathione. The plasma samples were deproteinized with acetonitrile and centrifuged. A total of 25% of the capillary length was filled with sample and the sensitivities of both target analytes increased between 15- and 20-fold.

A novel strategy in the use of transient pseudo-ITP and its application for other complex matrices has been proposed by Zhang *et al.* [48]. The approach consisted of a combination of field-amplified sample injection (FASI) induced by acetonitrile and transient pseudo-ITP for the preconcentration of alkaloid cations in a high-salt sample matrix. In this case, the acetonitrile added to the sample induces field-amplified sample stacking by decreasing conductivity and, at the same time, acts as a terminating electrolyte for the transient pseudo-ITP process. A plug of H^+ was injected electrokinetically after sample injection and a neutralization reaction between H^+ and tartrate from the buffer solution produced a low conductivity zone, in which the injected cations were further concentrated. With this sequential preconcentration method, 1,400-fold concentration was obtained compared with the conventional electrokinetic injection method. The LODs for myosmine and anabasine were 0.1 and 0.3 ng/mL, respectively. The method was used to determine trace alkaloids in cigarette samples.

In the literature, there are also some examples of transient pseudo-ITP in combination with MEKC as a separation mode [49,50,65]. For example, Choy *et al*. [50] employed this strategy for the preconcentration and separation of enantiomers. The authors demonstrated the effectiveness of the addition of acetonitrile and NaCl to the sample matrix in order to induce narrowing of the analyte bands in the presence of sodium cholate as the chiral surfactant micelle in the separation buffer. Significant improvement of the peak height and resolution for the MEKC separation of enantiomers was achieved when both acetonitrile and 1% NaCl (2:1 v/v) were added to the sample matrix. Shihabi [49] applied this technique for the analysis of mycophenolic acid in serum. In that paper, acetonitrile was used to remove serum proteins and supernatant was injected directly by filling 5 to 21% of the capillary volume. The use of this strategy yielded sensitivity comparable to or better than that of LC, with good separation and a better theoretical plate number.

Electrokinetic supercharging

The main limitation when using electrokinetic injection (EKI) in CE is that it is strongly dependent on the sample conductivity. In contrast, t-ITP is not affected by this property of the sample. Moreover, in intrinsic EKI, the injected analyte amount is limited to a certain extent, but the t-ITP process allows the introduction of much larger sample amounts. Therefore, combining EKI and t-ITP, the latter can remedy the shortcomings of the former, and higher increases in sensitivity can also be

expected than in EKI or t-ITP, when applied separately. This combination, known as electrokinetic supercharging (EKS), is one of the recent preconcentration strategies for CE based on ITP and it shows great promise for the analysis of complex samples for the determination of compounds at low concentration levels [12].

EKS has been used over the last few years as a powerful tool for the preconcentration and separation of different types of analytes and was first reported by Hirokawa *et al.* [51] in 2003. EKS is generally performed by hydrodynamic injection of a LE, followed by the electrokinetic injection of the analytes and, finally, the hydrodynamic injection of a TE. The application of a separation voltage causes the diffused band of analytes introduced during electrokinetic injection to stack between the LE and TE by t-ITP. Subsequently, the ITP stage destacks and the analytes are allowed to separate by conventional CE. Figure 4 shows a diagram of this preconcentration strategy using cationic analytes as model compounds. Firstly (Figure 4A), the capillary is filled with a BGE and the LE is injected hydrodynamically. The sample is then injected electrokinetically (Figure 4B). In Figures 4C and 4D, the TE is injected and then, after applying a high positive voltage, the preconcentration of the analytes begins between the LE and TE during t-ITP migration. After the stacking process, the analytes are separated by CZE (Figure 4E).

Hirokawa *et al.* [51] described the principle of EKS, based on computer simulation. The simulation of the electrokinetic injection showed that EKS was effective for the preconcentration of analytes with wide mobility ranges. In that case, EOF was suppressed to increase the amount of analytes that could be injected under constant voltage mode. A test mixture of rare-earth chlorides was used to demonstrate the use of EKS with high preconcentration factors for this kind of compounds. Furthermore, as a complementary theoretical contribution to the EKS field, Xu *et al*. [66] studied the effect of the change of the distance between the electrode and the capillary end in the quantitative repeatability of EKS-CZE. The authors found that this distance might seriously affect repeatability. By using a Teflon spacer to fix the distance between the electrode and the capillary end to 1.1 mm, the RSD of peak area (n=5) was decreased from 20 to 3.4% for several metal cations. Thus, the authors applied the methodology to the detection of ions from atmospheric electrolytes in high-purity water exposed to ambient air for 2 hours. Anions (chloride, sulphate, nitrate, formate, acetate and lactate) and cations (ammonium, calcium, sodium and magnesium) were detected at microgram per litre levels using a conventional UV detector.

After its introduction, EKS has been applied to the analysis of peptides [37], inorganic compounds [51,53,54,56,57,59] and organic compounds [14,52,55,58].

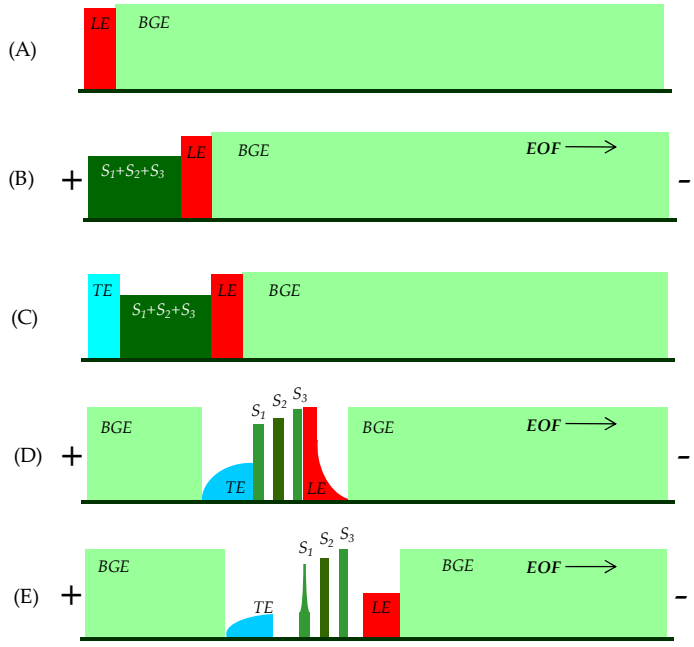

Figure 4. Schematic diagram of an EKS procedure for cations. (**A**) The leading electrolyte is injected hydrodynamically. Afterwards (**B**), the analytes are injected electrokinetically and then (**C**) the terminating electrolyte is injected in turn. (**D**) A high positive voltage is applied and the concentration of the analytes begins between the leading and terminating ions during t-ITP migration. (**E**) The concentrated analytes zones are separated by CZE.

Furthermore, the applicability of this technique has been demonstrated in complex samples of environmental, biological and industrial-type origin. For example, Okamoto *et al.* [53] applied EKS for the determination of rare-earth in model samples of rare-earth ores (xenotime and monazite) and a real sample of monazite ore. When EKS-CZE was applied, it was possible to determine the minor components (Er, Tm and Yb) with contents less than 0.025% (rare-earth/total rare-earth). The authors reported that the analytical sensitivity of EKS-CZE was comparable with that of ICP-AES. Urbanek *et al.* [59] determined Fe^{+2}, Co^{+2}, and Ni^{+2} at ppb levels using EKS in CZE. In addition, the authors described an in-capillary derivatization with ortho-phenanthroline for direct UV detection. An interesting approach in that paper involved the preconcentration of the derivatization reagent by inducing a second t-ITP, before labelling analytes that were already separated in CZE mode. This strategy was applied in order to sharpen the analyte zones during the in-capillary derivatization step for improving sensitivity and resolution further. The LODs obtained were as low as 30 ng/L in standard samples.

Dawod *et al.* [14] applied EKS for the analysis of NSAIDs in water samples. The best results were obtained by the hydrodynamic injection of LE (100 mM NaCl), followed

by the electrokinetic injection of the sample (200 s, - 10 kV) and, finally, the injection of the TE (100 mM CHES). With this method, the sensitivity was improved by 2,400-fold, giving detection limits of 50 to 180 ng/L in standard samples. The developed method was applied to the analysis of wastewater samples from a local sewage treatment plant without the use of an off-line sample clean-up. In later work [52], the same authors developed a modification for EKS system, namely counter-flow EKS (CF-EKS), and applied it for the on-line preconcentration and separation of seven NSAIDs in water samples. The EKS modification proposed by the authors consisted of applying a hydrodynamic counter-flow during the electrokinetic injection of the analytes. This counter-flow enabled the injection of a high amount of analytes but, at the same time, the sample matrix introduction is minimized. In that work, the authors stated the possibility that the BGE (sodium tetraborate) also acted as the LE and, therefore, no plug of NaCl was used prior to the introduction of the sample. The sensitivity was enhanced up to 11,800-fold and the LODs obtained ranged between 10.7 and 47.0 ng/L for the selected NSAIDs in standard samples. The developed method was applied for the determination of the studied NSAIDs in drinking water and wastewater samples.

Recently, Breadmore [55] reported another interesting approach as a variation of the original EKS performance. In particular, the author developed an EOF counter-balanced ITP boundary method to stack anions from high conductivity samples during continuous electrokinetic injection of the sample. The method consisted of hydrodynamically filling the whole capillary with sample containing a suitable leading ion with a terminating ion placed in the outlet vial. Then, upon application of a voltage, an ITP boundary is generated and moves back towards the inlet of the capillary. Ions with mobility between the leading and terminating zones get stacked on the boundary. For this, a polystyrene sulphonate/poly(diallyldimethylammonium chloride) polyelectrolyte-coated capillary was prepared in order to give a low and pH-independent EOF. The entire capillary was filled with sample and the sample vial was in the inlet position during ITP stacking allowing an additional electrokinetic injection of the sample. The sensitivity of a mixture of organic acids prepared in 100 mM sodium chloride (LE) was improved by 700- to 800-fold, using bis-tris-propane as the BGE with a whole-capillary hydrodynamic injection of the sample and further electrokinetic injection (5 min at 28 kV). SEFs ranging between 1,100- and 1,300-fold were obtained using triethanolamine as the BGE under the same conditions but applying 60 min of electrokinetic injection. The prospective of the method was demonstrated by filling the whole capillary with urine spiked with naphthalenedisulphonic acid, with limits of detection 450 times lower than those reachable with normal hydrodynamic injection.

The same research group reported an electroosmotic flow (EOF) controlled counter-flow isotachophoretic stacking boundary (cf-ITPSB) system under field-amplified

conditions as a preconcentration technique of anions in CE [56]. This method consists of the balance of the electrophoretic movement of an ITPSB with the EOF in order to allow larger sample injections. The cf-ITPSB remained inside the capillary at a position defined by the velocities of leading anion and EOF, and the boundary reached a stationary point within the capillary. The system consisted of a high concentration of a high-mobility leading ion (100 mM chloride) and a low concentration of low-mobility terminating ion (1-3 mM MES or CHES), added to the sample in an unmodified fused-silica capillary. The authors demonstrated the potential of this approach for sensitivity enhancement for the co-EOF determination of a mixture of six inorganic and small organic ions, with LODs in the ng/L level, which are 100,000 times better than those achieved by normal hydrodynamic injection for standard samples. In another interesting approach, the same group also presented an on-line technique for stacking based on the generation of a stationary isotachophoretic (sITP) boundary [57]. By balancing the anodic migration of an ITP boundary with a cathodic EOF, a stationary boundary is formed and can be used to concentrate analytes indefinitely according to ITP principles during electrokinetic injection. The ITP boundary was created by using an electrolyte containing a leading ion (chloride) and a suitable terminating ion added to the sample (MES). This provided LODs for NO_2^-, NO_3^- and SCN^- of between 0.05 and 0.66 ng/mL, which are 10,000 times lower than in the case of conventional hydrodynamic injection. The main limitation of this methodology is that hyphenation with a MS detector is not possible. In order to overcome this issue, the same research group, Meighan *et al.* [58], used a positive pressure in EKS (pressure-assisted EKS, or PA-EKS) during injection. Moreover, by using this method an improvement in the stacking of NSAIDs can be also achieved. After the optimization of several parameters related to the EKS, an enhancement in sensitivity of almost 50,000-fold was obtained in the LODs for seven NSAIDs. Therefore, the PA-EKS method developed has the potential to be a MS-compatible preconcentration method for improving the sensitivity of CE.

1.1.2. Extraction techniques based on in-line SPE-CE

As mentioned in a previous section, SPE and LPE are the most commonly used extraction preconcentration techniques in CE. Using them, in particular SPE, a broad range of applicability and high concentration factors can be achieved [17,29,36,67]. Even though some stacking techniques allow larger amounts of sample to be loaded into the CE capillary, it is generally not possible to inject a volume greater than the entire capillary volume. In contrast, it is possible to load multiple capillary volumes into a CE capillary when a SPE sorbent is used in combination with CE and this allows the analytes to be concentrated several-fold with minimal adverse effect on the CE separation process. Several strategies have been developed for the coupling between SPE and CE, and the on-line and in-line modes are preferred, as they can easily be automated, require less sample handling and have a short analysis time. In

on-line SPE-CE, a preconcentration column is usually connected to a CE system via a valve or tee joint interface that automatically transfers the analytes from the SPE column to the separation capillary. However, in these systems, band broadenings are sometimes inevitable because the connection of the SPE column and the CE capillary generally produces dead volume at the interface of the valve or tee joint used. In addition, the desorption solvent required is usually in the order of microlitres, much greater than the acceptable injection volume in CE. Therefore, only a small part of the eluted sample could be injected into the separation capillary after the SPE sample preconcentration, resulting in a drop in preconcentration efficiency.

In-line SPE-CE is the alternative which presents the highest degree of integration and, since the separation voltage is applied across the SPE material, no further additional equipment is needed [7,25,29]. The main advantages of in-line SPE-CE are that it can be easily automated with fewer sample-handling steps, and, unlike at-line and on-line systems, the complete desorbed volume from the SPE column is analysed by CE. Moreover, since SPE and CE are carried out in a single capillary, dead volume can be completely eliminated and there is no sample loss during transfer between sample pretreatment and the analysis. However, there are also some drawbacks related to this coupling, such as the limitation in the choice of the separation electrolytes because some analytes could be eluted in the preconditioning step if the BGE contains, for example, organic solvents. Moreover, the peak efficiency depends on the desorption volume and on the composition of the solvent used for desorption of the analytes from the SPE device. In addition, difficulty in analysing complex samples can be observed, as problems can occur with clogging, reversed EOF or irreproducible results caused by adsorption of sample matrix components to the capillary wall, which result in poor separations.

In the literature, there are different possible designs for performing this in-line SPE-CE coupling [29,67]. Four main setups have been reported as shown in Figure 5. In the first setup (Figure 5A), the SPE column is an open tubular (OT) capillary that is coated with the SPE sorbent. The preparation of OT wall-coated SPE columns inside fused-silica capillaries involves the modification of the capillary wall with several reagents followed by coating the capillary wall with a SPE material. The second setup consists of a packed bed material placed in a capillary (Figure 5B). In this setup, the packing material can be kept in place by retaining frits near the inlet side of the CE capillary, if the sorbent particles diameter is small, but in the case of a higher diameter, a frit-free device can also be constructed. In general, these packed bed columns have higher retention capacity than OT columns due to the larger amount of SPE material present. In the third setup, the principles are the same as for packed beds but in this case, a monolithic material (also known as continuous beds) is employed (Figure 5C). In this in-line mode, the preconcentration bed does not contain individual stationary-phase particles or require any retaining end frits to

operate. Finally, in the last setup (Figure 5D), a thin impregnated membrane (disk) of SPE material is positioned in the middle of a Teflon sleeve between two fused-silica capillaries.

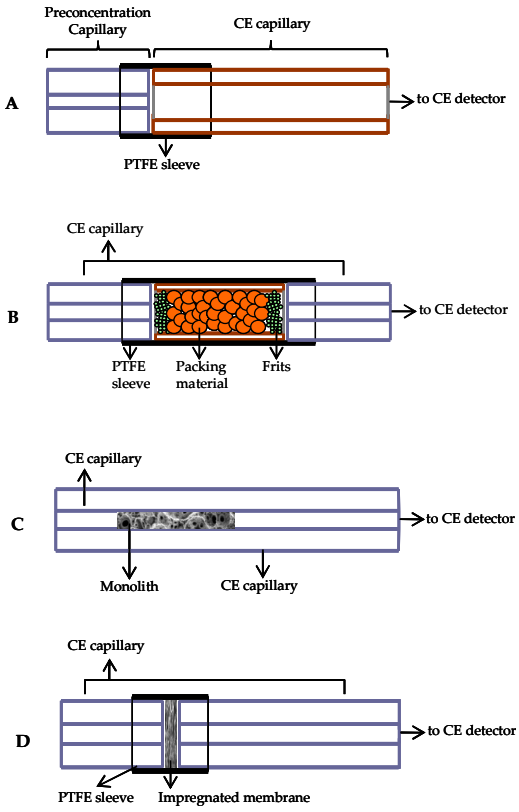

Figure 5. Schematic representation of different in-line SPE-CE designs: A) Open tubular B) Packed bed C) Monolith and D) Impregnated membranes (disks).

As this is an area of considerable interest in CE research, a number of papers reporting in-line SPE–CE methods have been reviewed in recent years [25,29,36,67]. Table 2 shows an overview of some selected applications of in-line SPE-CE methodologies published in recent years. As can be observed from the table, different sorbent materials have been used for the construction of the in-line concentrator device. In addition, it should be highlighted that the packed bed setup is the most commonly used design. Regarding the applicability of the developed methodologies based on in-line SPE-CE, this has been widely demonstrated in the analysis of samples of biological and environmental origin. Moreover, this methodology has been useful for the determination of different kind of compounds such as pharmaceuticals, peptides, weak bases and herbicides, among others.

Table 2. Overview of in-line SPE-CE applications

Analytes	Sample	SPE sorbent	Detection	LODs	Ref.
Open Tubular					
Amino acids	Standards	Sol-gel C18-TMS	UV	24.5 – 60.7 nM[a]	[68]
Myoglobin, aspargine	Standards	Sol-gel C18-TEOS	UV	-	[69]
Proteins	Horse skeletal muscle	Glycidyl-methacrylate	UV	-	[70]
Packed bed					
Naproxen	Tap water	C18	UV	10 ng/L[b]	[71]
Ceftiofur	River water	C18	UV	10 pg/mL[b]	[72]
Impurities	Synthetic peptides	C18	UV	1-5 ng/mL[a]	[73]
Neuropeptides	Human plasma	C18	ESI-MS	0.1 -10 ng/mL[b]	[74]
Opioids peptides	Human plasma	C18	ESI-MS	0.1 – 10 ng/mL[a]	[75]
Oxprenolol	Urine	C18	UV	250 ng/L[a]	[76]
Peptides	Standards	C18	UV	100 pM[a]	[77]
Opioids peptides	Human plasma	C18	ESI-MS	1 – 10 ng/mL[b]	[78]
Tryptic peptides	Biological fluids	C18	ESI-MS	-	[79]

[a] Standard samples
[b] Real samples

Table 2. Overview of in-line SPE-CE applications (Cont.)

Analytes	Sample	SPE sorbent	Detection	LODs	Ref.
Opioid peptides	Human plasma	C18	MS	0.01-0.1 ng/mL[a]	[80]
Rare earth	Standards	C18-derivatized	UV	20 – 60 pg/L[a]	[81]
Sulfonamides	Tap, bottle and river water	Oasis HLB	UV	0.3 – 0.6 ng/mL[b]	[82]
Pharmaceuticals	River water	Oasis HLB	UV	0.19 – 1 ng/mL[b]	[83]
3-nitrotyrosine	Urine	Oasis MCX	UV	4.4 μM	[84]
Quinolones	Meat	Oasis MCX	MS/MS	40 – 140 ng/kg[b]	[85]
Chlorophenols	River water	Abselut NEXUS	UV	0.07 - 0.10 ng/mL[a]	[86]
Chlorophenols	River water	Abselut NEXUS	UV	17 - 25 μg/L[a]	[87]
Triazine herbicides	Human urine	Molecularly imprinted polymer	UV	0.2 – 0.6 ng/mL[a]	[88]
Aromatic amine	Urine	Blue chitin	MS/MS	0.065 nM[a]	[89]
Peptides	Biological fluids	Inmuno-affinity sorbent	ESI-MS	-	[90]
Fluorescein isothiocyanate (FITC)-labelled testosterona	Standards	Chelating Sepharose modified	LIF	-	[91]

[a] Standard samples
[b] Real samples

Table 2. Overview of in-line SPE-CE applications (Cont.)

Analytes	Sample	SPE sorbent	Detection	LODs	Ref.
Parabens NSAIDs	Aqueous sample	Funtionalized magnetic silica-coated iron oxide particles	UV	-	[92]
Opioid peptides	Biological fluids	Inmuno-affinity sorbent	MS	100 ng/mL[b]	[93]
Monoliths					
DNA fragments	Escherichia coli crude lysate	Amino silica monolith	LIF	65 – 123 ng/L[a]	[94]
Pirimicarb Carbendazim	Drinking and river water	Polymeric monoliths	UV	0.01 μg/L[a]	[95]
Neutral and ionized model compounds	Standards	Acrylate-based monolith	UV	-	[96]
Weak bases	Standards	Poly(MA-co-EGDMA) monolith	UV	8.0 – 30 ng/mL[a]	[97]
Carbamate pesticides	Standards	Divinylbenzene-based monolithic polymer	UV	1 – 16 ng/mL[a]	[98]
Organic ions	Standards	Latex AS18	UV	1.5 – 12 nM[a]	[99]
Disks					
Peptides Proteins	Bovine serum	C18-impregnated extraction disk	MS/MS	500 amol[a]	[100]
Peptides	Bovine serum	PS-DVB copolymer Filterdisk	MS/MS	100 amol/μL[a]	[101]

[a] Standard samples
[b] Real samples

Open tubular columns

OT columns are considered a simple way of performing an in-line preconcentration procedure in CE. In this approach, generally a small section of the capillary is coated with a stationary phase and positioned at the injection end of the separation capillary (Figure 5A). This capillary section works as an SPE column integrated into the CE system in such a way that, when the sample is loaded by flushing, the analytes are retained [25]. After the elution solvent plug is injected, a voltage is then applied and the analytes are simultaneously eluted and separated. Between two analyses, the technique requires the stabilization of the OT capillary column and a time-consuming flushing procedure, which can take up to 10–20 min. Even so, the OT capillary is easily contaminated by (non-soluble) sample components. Another disadvantage is that the capacity of the open tubular cartridge is limited due to the low surface area and this is associated with a restricted gain in sensitivity. Despite these disadvantages, this kind of in-line setup has been used repeatedly and the good performance of the system has been demonstrated through its application in the analysis of several analytes in different kind of matrices [68-70]. For example, Li *et al*. [68] reported a method for the first time for the extraction and preconcentration of amino acids using a sol-gel-coated column coupled to a UV detector. Capillaries were coated with the use of N-octadecyldimethyl (3-(trimethoxysilyl)propyl) ammonium chloride in the sol-gel coating solutions. For extraction, after ensuring a net negative charge of the amino acids by adjusting the pH, a long plug of sample was then passed through the sol-gel-coated capillary to facilitate extraction via electrostatic interaction between the positively charged sol-gel coating and the negatively charged amino acid molecules. Desorption and preconcentration of the extracted amino acids was carried out by local pH change. The presented methodology provided SEFs of 150,000-fold for alanine. The same authors also used a negatively charged sol-gel coating for the in-line preconcentration of zwitterionic biomolecules in CE [69]. In that paper, the resulting surface-bonded sol-gel coating was able to carry a negative charge over a wide range of pH values due to the presence of deprotonated sulphonic acid groups. The focusing of desorbed analytes was achieved by taking advantage of the dynamic pH junction between the sample solution and the background electrolyte zone. This in-line preconcentration by the combination of the sulphonated sol-gel columns and dynamic pH-junction gave SEFs around 3,000-fold for myoglobin, and 7,000-fold for asparagine.

Packed beds

The most common design for in-line SPE-CE procedures involves the use of small packed beds. This kind of setup has been used extensively since the 1990s. Figure 5B shows a schematic representation of this setup. As in OT systems, the capillary that contains the SPE sorbent can be the same as that used for the CE separation or it may

also be a different one which is coupled in-line with the separation capillary. In the first approach using this setup, frits were used in order to retain the sorbent particles in a section of the capillary. However, the use of these frits can lead to increases in backpressure, long analysis times and irreproducible EOFs [25,67,84]. When the size of the particles of the sorbent are greater than the separation capillary diameter, the use of frits can be avoided and this strategy can overcome the problems associated with the presence of frits. This frit-free configuration consists of coupling the concentrator device between two capillaries. In this case, the preconcentrator device is constructed by using a small piece of capillary, which generally has a length between 2 mm and 1 cm, and internal diameters (ID) (150 – 250 μm) higher than the ID of the separation capillary (50 – 75 μm) [83,85].

In recent years, the number of papers using the design based on packed beds has increased in the literature, demonstrating its great potential for a wide range of applications [71-93]. The most commonly used sorbents are reverse-phase sorbents such as Oasis HLB [82,83] and C18 [71-80]. Oasis MCX as a mixed-mode cation exchange sorbent [84,85], MIPs [88] and also inmuno-affinity stationary phases have been reported [90,93]. For example, Hernández *et al.* [74] employed microcartridges containing C18 as the SPE sorbent, in this case using frits, for the study of the suitability of an in-line-SPE-CE methodology coupled to MS in the determination of neuropeptides in human plasma. Sample pretreatment based on precipitation with ACN was used for the clean-up of the plasma samples and the LODs were noticeably improved compared with CE-MS, ranging between 0.1 and 10 ng/mL. Maijó *et al*. [83] used Oasis HLB as a sorbent for a frit-free in-line SPE-CE system in order to preconcentrate and separate various pharmaceutical compounds. The LODs reached for standard samples were in the range of 0.06 to 0.5 ng/mL, providing SEFs around 5,900-fold compared with normal CZE. The developed in-line SPE-CE-UV method was applied to fortified river water samples, obtaining LODs in the range of 0.19 to 1 ng/mL. Lara *et al.* [85] used sorbent particles of Oasis MCX to construct a frit-free concentrator device for an in-line SPE-CE-MS/MS methodology in the determination of quinolones in meat. Optimization of a pressurized liquid extraction (PLE) method was used to extract the antibiotics from the chicken muscle samples. By the application of the entire method, the LODs obtained ranged from 40 to 140 ng/kg.

An interesting approach based on the use of the packed bed design was reported by Medina-Casanellas *et al.* [80], in which they combined two preconcentration methodologies in CE. A method based on t-ITP with in-line SPE-CE coupled to time-of-flight mass spectrometry was developed to improve the sensitivity of three opioid peptide analyses. Using the combination of both electrophoretic and chromatographic preconcentration techniques, LODs were decreased 10-fold in relation to SPE-CE-TOF-MS for two of the studied peptides, while no improvements were obtained for the third. Giménez *et al.* [90] also employed a combination of in-

line SPE-CE-MS with further focusing by dynamic pH junction to detect peptide markers from recombinant human erythropoietin. In that paper, an inmuno-affinity stationary phase was prepared from a custom-made polyclonal anti-EPO (81-95) antibody immobilized onto a solid support of CNBr-Sepharose 4B. This highly selective sorbent was packed in the microcartridge and used as the preconcentrator device. Elution with a buffer at a low pH value allowed the focusing by the dynamic pH junction mechanism. Although the limits of detection for the peptide marker were similar to those obtained with CE-MS (about 25 mg/L), the authors stated that this novel approach had the potential to detect rhEPO and its analogues selectively and unambiguously at the levels expected in biological fluids.

Recently, magnetic particles have also been reported as a novel SPE material for CE applications by Tennico *et al.* [92], who used functionalized magnetic beads for in-line sample extraction in CE. To achieve this, silica-coated iron oxide particles were used as a solid support and further functionalized with octadecylsilane. Magnets were used to immobilize the magnetic SPE particles inside the separation capillary. The authors assess that this methodology allows the injection of the sample without any previous pretreatment and it eliminates the need of using frits to immobilize the sorbent.

Monoliths

Over the last few years, the potential of monoliths in in-line SPE preconcentration in capillary electrophoresis has also been demonstrated by several authors. These materials are considered one of the most promising materials for in-line SPE–CE, since they are highly permeable and have high surface areas [94-99]. Moreover, these monolithic materials allow high flow rates, so sample loading time can be minimized. The monoliths are attractive as preconcentration supports because they consist of a continuous piece of highly porous microstructure prepared by in situ polymerization within the capillary. Since monolithic columns allow always frit-free operation, they are less susceptible to bubble-formation problems during high-voltage operations. Two types of monoliths have been introduced to date, namely silica-based (inorganic) monoliths and polymer-based (organic) monoliths. Usually, silica monoliths are prepared using sol–gel technology, whereas polymer-based monoliths are typically prepared by in situ polymerization of monomers and cross-linkers. This latter type includes acrylamide-, styrene-, and acrylate or methacrylate-based polymers.

Despite the advantages of monoliths as preconcentration sorbents, their use as SPE sorbents in the in-line SPE coupling is still not widespread. Up to now, there are only a few papers in the literature which show their applicability. The first application regarding the use of a monolithic methacrylate polymer as a preconcentrator was

demonstrated by Baryla and Toltl [102], who prepared a methacrylate monolith at the inlet end of a capillary by photoinitiated polymerization. This system demonstrated the in-line sample enrichment of standard propranolol. Recently, in a study reported by Feng *et al*. [94], for the first time, an amino silica monolithic column was prepared in situ inside the separation capillary for the development of an in-line SPE-CE method for the sensitive analysis of DNA fragments. Using this method, preconcentration factors of over 100-fold were obtained for all DNA fragments compared to conventional CE. LODs from 65 to 123 ng/L were achieved for six DNA fragments when this methodology was applied. The applicability of this in-line SPE-CE method was demonstrated in the analysis of *E. coli crude lysate* samples. A similar methodology was proposed by Rodríguez-Gonzalo *et al.* [95], in which a monolithic polymer formed in situ within the capillary as a medium for analyte retention was used to preconcentrate carbamate pesticides. The synthesized monolithic bed exhibited high porosity and allowed samples to be loaded at flow rates of about 65 μL/min by applying a pressure of 12 bar. Using this methodology, the determination of these pesticides in drinking water at a concentration level of 0.01 μg/L was achieved.

The application of monolithic materials has also been successfully implemented in microchip devices. For example, Proczek *et al.* [96] developed an integrated microdevice to couple on-chip SPE to separation by channel electrochromatography. An acrylate-based monolith was used for both preconcentration and separation of the analytes. The composition of the polymerization mixture was chosen to achieve a monolithic material containing both hydrophobic and charged moieties to ensure an electroosmotic flow for separation. Neutral, ionizable and charged compounds were successfully preconcentrated and separated within the microdevice through electrochromatographic mechanisms. As a result, the preconcentration of a mixture of PAHs led to SEFs of 270-fold.

Disks

Disks or impregnated membranes loaded with a solid sorbent are another format for in-line SPE (Figure 5D). By using this format, higher flow rates and smaller elution volumes can be used and this is an advantage over the packed bed setup. The use of disks offers improved performance due to the use of smaller beds and higher flow rates, even at the relatively low pressures applied. In addition, it requires significantly smaller elution volumes and frits are not needed to hold the sorbent material in position. Analytes can therefore be eluted more efficiently during the desorption step and with less organic solvent. However, disk devices have a lower capacity than sorbents disposed in packed beds, which can concentrate a higher amount of analytes. This disadvantage is especially important for analytes with a relatively low retention in the SPE sorbent [25,67].

Only a few papers using this in-line SPE format have been reported in the literature. For example, Janini *et al.* [100] developed a method to preconcentrate a sample directly within the CE capillary followed by its electrophoretic separation and detection using a true zero dead-volume sheathless CE-MS interface. For the construction of the preconcentrator device, multiple pieces of C18-impregnated extraction disks were inserted into the fused-silica capillary sleeve up to a length of around 3 mm. Using this method, the mass limit of detection obtained was 500 amol for tandem MS analysis of a standard peptide. Pelzing *et al.* [101] also studied the potential of in-line SPE–CE in a disk device coupled to MS. To this end, the authors proposed the use of an in-line microfilter, such as an SPE cartridge filled with a plug of polystyrene divinylbenzene (PS-DVB) copolymer disk, for the analysis of a tryptic digest of bovine serum albumin as a reference standard. This SPE-CZE-MS/MS setup was compared directly to nanoLC/nanoESl, using the same sample of the tryptic digest. The sensitivity for nanoLC-MS was about five times higher than for CZE-MS. However, the CZE-MS technique showed reduced loss of peptides, especially for larger peptides (missed cleavages) and was about four times faster than the nanoLC-MS approach.

1.2. Determination of drugs by capillary electrophoresis

UNIVERSITAT ROVIRA I VIRGILI
SENSITIVITY ENHANCEMENT STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF DRUGS OF ABUSE AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS
Igor Botello González
Dipòsit Legal: T. 1299-2012

The group known as pharmaceutically active compounds (PhACs) includes medical prescription pharmaceuticals, recreational drugs of abuse and hormone-based compounds. Due to the potential toxicity of these PhACs, the study of these compounds and their presence in the environment and biological specimens is needed. In general, pharmaceuticals are substances used in the diagnosis, treatment, alteration or prevention of abnormal health conditions in the body, and there are a great number of compounds included in this group. Of all of them, analgesics (e.g. acetylsalicylic acid) are the most commonly used agents for relieving pain. Of the group of analgesics, NSAIDs (e.g. naproxen, ketoprofen, ibuprofen, diclofenac, piroxicam, indomethacine and phenazone) are widely used nowadays [10,103]. Other analgesics, such as morphine, methadone and codeine, are included in the group known as drugs of abuse, because of the frequency of their illicit use.

Over recent years, society's awareness of the issues of environmental impact and health safety has increased. Therefore, the reliable control of residues of these kinds of compounds in different matrices must be established. One of the most important conditions of efficiency for this control is the application of accurate, precise and fast methods of analysis.

The following sections present the recent studies based on the use of CE in the literature for the determination of the groups of compounds that have been studied in the present Doctoral Thesis, which include non-steroidal anti-inflammatory drugs (NSAIDs), barbiturate drugs and opioids, among others.

1.2.1. Determination of non-steroidal anti-inflammatory drugs by capillary electrophoresis

NSAIDs are a group of analgesic, antipyretic and anti-inflammatory drugs which have been extensively used in human and farm animals for several decades in order to treat the inflammatory process and any related clinical effects. Although they provide some benefits for health and well-being, these compounds need to be used carefully and in the proper dosage because of their adverse effects on the digestive system. In terms of their chemical structure, these pharmaceuticals are weak acidic compounds, polar, and highly soluble in aqueous media. Due to their high polarity and poor degradability in water, NSAIDs are not removed well during wastewater treatment [10,104]. Similarly to other polar organic contaminants, NSAIDs are regarded as a group of emerging chemical contaminants due to their high environmental distribution and potential ecotoxicological effects. In addition, the determination of NSAIDs in biological fluids is also important because it provides useful information for assessing their safety, therapeutic effects and mechanism of action [105,106].

Efficient methods are necessary for the determination of the occurrence and fate of NSAIDs in environmental and biological matrices. To this end, the use of GC

[107,108] or LC [109-112] is widely reported. CE has also been extensively used for that purpose and, with this in mind, in 2007, Macià *et al.* [103] reviewed the use of this technique for the determination of NSAIDs. Since then, a number of papers have been published based on the use of CE. Table 3 shows an overview of some of these recent applications.

Table 3. Overview of the determination of NSAIDs by CE

Analytes	CE conditions	Detection	LODs	Sample	Sample pretreatment	Ref.
Oxytocin Norfloxacin Diclofenac	**CZE** Capillary: 50 cm x 75 μm; 30 kV, 25 ºC BGE: 50 mM sodium tetraborate (pH 10)	UV	0.05 – 1 μg/mL[a]	Milk	Protein precipitation	[113]
Ibuprofen Naproxen Ketoprofen Diclofenac Ketorolac Aceclofenac Salicylic acid	**CZE** Capillary: 50 cm x 75 μm; 25 kV, 20 ºC BGE: 30 mM sodium acetate (pH 4) + 25 % acetonitrile	UV	0.25 - 0.86 ng/mL[a]	Wastewater	HF-LPME	[114]
Ibuprofen Ketoprofen Flurbiprofen Fenbufen	**MEKC** Capillary: 40 cm x 75 μm; 10 kV, 20 ºC BGE: 50 mM boric acid + 50 mM SDS + 1.25 mg/L C_{60} (pH 8.5)	UV	0.34 – 0.90 mg/L[a]	Urine and tablets	SPE	[105]
Indomethacin	**MEKC** Capillary: 31.2 cm x 75 μm; 10 kV, 25 ºC BGE: 30 mM Tris (pH 8.0) + 100 mM octanesulfonate	UV	0.1 μg/mL[b]	Plasma	Protein precipitation	[106]
Ketoprofen Metylparaben Propylparaben	**MEKC** Capillary: 60 cm x 75 μm; 30 kV, 25 ºC BGE: 50 mM tricine buffer + 30 mM SDS + 15 % (v/v) of methanol (pH 8.3)	UV	0.38 – 0.82 μg/mL[a]	Phamaceutical preparations	Filtration and dilution	[115]
Fenoprofen Flurbiprofen Indoprofen Ketoprofen Naproxen Suprofen	**MEKC** Capillary: 70 cm x 75 μm; -15 kV, 30 ºC BGE: 75 mM sodium tetraborate (pH 10) + MeOH (5%, v/v) + 0.02 g/L f-MWCNTs	UV	-	Urine	-	[116]
Ibuprofen Tetrazepam	**MEKC** Capillary: 60 cm x 75 μm; 30 kV, 25 ºC BGE: 15 mM sodium tetraborate (pH 10.2) + 40 mM SDS	UV	0.2 and 0.3 μg/mL[b]	Urine	SPE	[117]

[a] standard samples
[b] real samples

Table 3. Overview of the determination of NSAIDs by CE (Cont.)

Analytes	CE conditions	Detection	LODs	Sample	Sample pretreatment	Ref.
(S/R)-flurbiprofen	**NACE** Capillary: 50 cm x 75 μm; 30 kV, 25 ºC BGE: 40 mM ammonium acetate in metanol + 10 mM PA-β -CD	UV	0.06 μg/mL[b]	Plasma	Protein precipitation and SPE	[118]
Sulindac Indoprofen Ketoprofen Naproxen Fenoprofen Flurbiprofen Ibuprofen Indomethacin Diclofenac	**CEC** Capillary: 30 cm x 75 μm; 20 kV, 25 ºC Mobile phase: 50 % of 5 mM sodium dihydrogen phosphate (pH 3.0) + 50 % ACN Column: poly(SMA–DVB) monolithic material	MS	0.01–0.19 μg/L[a]	River water	SPE	[119]
Naproxen	**In-line SPE-CZE** Capillary: 36 cm x 75 μm; -20 kV, 20 ºC BGE: 20 mM of ammonium acetate in methanol/water, 70:30 v/v)	UV	10 ng/L[b]	Tap water	SPE	[71]
Benzafibrate Piroxicam Diclofenac Naproxen Clofibric acid	**In-line SPE-CZE** Capillary: 112.5 cm x 50 μm; 28 kV, 25 ºC BGE: 50 mM ammonium hydrogencarbonate (pH 9.2) + 10 % methanol	UV	0.19 – 1 ng/mL[b]	River water	Filtration	[83]
Ketoprofen Tolmetin Indomethacin	**On-line SPE-CZE** Capillary: 60 cm x 50 μm; 20 kV, 25 ºC BGE: 25 mM ammonium acetate (pH 6.9)	MS	1.6 – 2.6 ng/mL[a]	Urine	Filtration	[120]
Diclofenac Diflunisal Fenoprofen Ibuprofen Indomethacin Ketoprofen Naproxen	**PA-EKS** Capillary: 80 cm x 50 μm; 28 kV, 25 ºC BGE: 50 mM ammonium hydrogencarbonate, pH 9.2 + 10 % MeOH	UV	6.7 – 18.7 ng/L[a]	-	-	[58]
Diclofenac Diflunisal Fenoprofen Ibuprofen Indomethacin Ketoprofen Naproxen	**EKS** Capillary: 85 cm x 50 μm; -28 kV, 25 ºC BGE: 15 mM sodium tetraborate + 10 % methanol and 0.1 % HDMB LE: 100 mM sodium chloride TE: 100 mM CHES	UV	50 -180 ng/L[a]	Wastewater	Filtration	[14]

[a] standard samples
[b] real samples

Table 3. Overview of the determination of NSAIDs by CE (Cont.)

Analytes	CE conditions	Detection	LODs	Sample	Sample pretreatment	Ref.
Diclofenac Diflunisal Fenoprofen Ibuprofen Indomethacin Ketoprofen Naproxen	**CF-EKS** Capillary: 85 cm x 50 μm; -28 kV, 25 ºC BGE (LE): 15 mM sodium tetraborate + 10 % methanol and 0.1 % HDMB TE: 100 mM CHES	UV	17.1 – 47.0 ng/L [a]	Wastewater	Filtration	[52]
Gemfibrozil Fluvastatin Atorvastatin Diflunisal Naproxen Ketoprofen Indoprofen Indomethacin Fenoprop Mecoprop	**Sweeping-MSS-CZE** Capillary: 50 cm x 50 μm; 18 kV, 20 ºC BGE: 10 mM CTAB and 20 mM NH_4HCO_3 (pH 9.5)	UV	1.23 – 11.72 μg/mL[a]	Wastewater	LLE	[121]
Diclofenac Ibuprofen Fenoprofen Naproxen Ketoprofen	**ASEI-sweeping-MEKC** Capillary: 60 cm x 50 μm; -27 kV, 25 ºC BGE: 50 mM SDS in 20 mM sodium dihydrogenphosphate (pH 2.5) + 30 % acetonitrile	UV	29 – 58 ng/mL[b]	River water	Filtration	[13]
Ketoprofen Fenbufen Indomethacin	**LVSS-MEKC** Capillary: 60.2 cm x 75 μm; 20 kV, 25 ºC BGE: 20 mM disodium hydrogen phosphate + 50 mM SDS (pH 9)	UV	0.1 ng/mL[a]	Saliva	SPE	[122]
Ibuprofen Diclofenac Flurbiprofen Indomethacin Fenoprofen Sulindac Naproxen Ketoprofen Idoprofen Piroxicam	**FASI-MEEKC** Capillary: 48.5 cm x 75 μm; -15 kV, 30 ºC BGE: 3.31 % SDS + 1.01 % PGDA + 8.61 % 2-propanol + 15 % acetonitrile + and 72.1 % of 50 mM sodium dihydrogen phosphate (pH 2)	UV	0.03 – 0.3 ng/mL[a]	River, tap and ground water	SPE	[123]

[a] standard samples
[b] real samples

As can be observed in the table, NSAIDs have been analysed by different CE modes, such as CZE [14,52,58,71,83,113,114,120,121], MEKC [13,105,106,115-117,122], MEEKC [123], NACE [118] and CEC [119]. In almost all of these reported publications, the use of organic solvents added to the BGE is a common strategy in all capillary electrophoresis separation modes in order to improve resolution. This requirement was reported by Macià *et al.* [103], who pointed out that a separation system with higher selectivity is needed in certain cases for the resolution, due to structural similarities of a group of compounds.

Generally, for those strategies reported in the bibliography which focus on the separation of NSAIDs, the LODs obtained are at mg/L levels [105,106,113,115-118], which are not low enough to be able to determine these compounds at the usual desirable concentration levels at which NSAIDs are normally present in biological and environmental samples. Due to this fact, it is necessary to include a preconcentration technique, mainly when UV detection is used, with the ability to obtain high enrichment factors. On this point, it is worth mentioning several preconcentration strategies based on t-ITP principles for the determination of NSAIDs in water samples, in particular EKS [14,52,58]. Even though these papers have been detailed in Section 1.1, it should be pointed out that this kind of preconcentration technique has become a very promising tool for the determination of NSAIDs at low concentration levels, due to the low LODs that can be achieved in the low ng/Ml levels. For example, the EKS methodology presented by Dawod *et al.* [14] was applied to the analysis of wastewater after a simple filtration step in order to eliminate the particulate matter. The LODs achieved with EKS for real samples were approximately 10 times higher than when compared to those obtained using standards. The authors stated that this was due to the effect of the salt content in wastewater samples on the electrokinetic injection step. In order to overcome this issue, in a more recent paper [52], the same authors applied a LLE step prior to the analysis of wastewater samples, using a similar methodology. Despite this, the authors also pointed out that, in the case of samples of low conductivity, direct injection is possible without the need of a clean-up step.

Another trend when using CZE is its in-line combination with SPE [71,83]. For example, Maijó *et al.* [83] applied an in-line SPE-CE methodology for the preconcentration and separation of diluted solutions of pharmaceutical compounds (bezafibrate, piroxicam, diclofenac sodium, naproxen and clofibric acid). The authors used a packed bed design in which the frit-free analyte concentrator was packed with Oasis HLB sorbent. The method provided SEFs around 5,900-fold in peak height compared with the normal hydrodynamic injection. LODs ranging between 0.19 and 1 ng/mL were obtained when the method was finally applied to river water samples. In that work, only a simple filtration was necessary as sample pretreatment prior the analysis of the river waters.

In recent years, there has been a trend of using environmentally friendly sample preparation techniques in order to avoid the use of toxic organic solvents. For example, Villar-Navarro *et al.* [114] describe a CE determination for several NSAIDs in environmental waters using hollow fibre membrane liquid-phase microextraction (HF-LPME). Compared with LLE and SPE, HF-LPME gives satisfactory sensitivity and similar enrichment of analytes. The consumption of solvent is significantly reduced, up to several hundred or several thousand times. The extraction of the studied compounds was carried out using a polypropylene membrane supporting

dihexyl ether and the LODs obtained for standard samples using the whole methodology ranged between 0.25 and 0.86 ng/mL. The method was then applied to the direct determination of the seven NSAIDs in wastewaters and five of them were quantified in different urban wastewaters.

Recently, the effectiveness of the use of carbon nanotubes (CNTs) as sorbent material has been demonstrated for a wide variety of compounds. The use of this kind of material for the extraction of NSAIDS was reported by Suárez *et al.* [120]. The authors utilized carboxylated single-walled carbon nanotubes (c-SWNTs) as on-line SPE sorbent coupled to CE in order to show the potential of this new sorbent for the preconcentration of NSAIDs from urine samples. The system consists of a peristaltic pump, an injection valve to introduce the eluent in the system, a minicolumn containing the immobilized c-SWNTs, and two selection valves. The first valve is for selecting sample or cleaning solution and the other is used to select waste or to direct the purified sample to the CE-MS equipment. Using this methodology, LODs ranging from 1.6 to 2.6 ng/mL were obtained. The reliability of the proposed method was evaluated by applying it to a set of spiked real urine samples with recoveries values between 98.6 and 102.2%.

Over the last few years, preconcentration methodologies based on MEKC in CE have also been reported for the determination of NSAIDs. For example, Maijó *et al.* [13] developed an ASEI-sweeping-MEKC method for the determination of NSAIDs in river water samples. After univariate optimization of different parameters affecting the preconcentration factors, the method was validated for the river water samples obtaining LODs in the range of 29 to 58 ng/mL. In this case, only a filtration step was necessary before the sample injection. However, the resulting separation remained poor and the sweeping effect was almost negligible, since the sample matrix composition has an important influence on this process. For that reason, a 10-fold dilution was necessary for the effective performance of the method in the analysis of river waters. In another study, Almeda *et al.* [122] used a LVSS methodology in MEKC for determining traces of NSAIDs in saliva samples. Prior to the sample analysis, two off-line preconcentration steps were applied. Firstly, a commercial centrifugal filter device was used in order to reduce the viscosity of saliva and exclude the substances with the highest molecular mass. This initial pretreatment was then followed by off-line SPE to isolate and concentrate the analytes. The authors stated that the whole methodology provides an effective method for the routine determination of ketoprofen, fenbufen and indomethacin at levels above 0.1 ng/mL in saliva samples.

With respect to the use of preconcentration strategies in MEEKC, Kuo *et al.* [123] determined ten NSAIDs in river, tap and ground water samples. In order to increase the detection sensitivity factors for the analytes, FASI was used in conjunction with

MEEKC. Applying this preconcentration technique, the LODs were reduced to the range of 0.03 to 0.3 ng/mL, providing SEFs of around 1,400- to 6,100-fold. The methodology was applied to the determination of the studied NSAID residues in water samples after a SPE pretreatment procedure.

To the best of our knowledge, in recent years, only one application based on the use of CEC for the determination of NSAIDs has been reported. In that work by Hsu *et al.* [119] , the determination of nine NSAIDs in river water was reported using CEC-MS and, even though the presented strategy did not include a preconcentration strategy, the overall approach results in a useful method for the determination of NSAIDs at low concentration levels achieving LODs in the range of 0.01 to 0.19 μg/L in standards. To this end, a series of poly(stearyl methacrylate-divinylbenzene) (poly(SMA-DVB)) monolithic columns were prepared in situ by a single step polymerization of divinylbenzene (DVB), stearyl methacrylate (SMA) and vinylbenzenesulphonic acid (VBSA, charged monomer) and were applied, for the first time, as separation columns. The best results were obtained by the use of monolithic columns prepared with a short reaction time (3 h) and low SMA:DVB ratio (40/60 ratio of SMA:DVB).

1.2.2. Determination of drugs of abuse by capillary electrophoresis

Over the last few decades, the use of drugs of abuse has been a growing problem and is often accompanied by a devastating social impact upon community life. The abuse of these kinds of substances involves significant costs to communities, including violent and property crimes, prison expenses, court and criminal costs, emergency room visits, child abuse and neglect, lost child support, reduced productivity, unemployment, and victimization. In consequence, it is important to achieve the determination of these drugs and their main metabolites in biological matrices, seized illegal preparations and also in environmental samples. The increasing use of CE, due to its unique separation mechanism, speed, efficiency and versatility, makes this technique an important analytical tool in the determination of these drugs in different matrices. Moreover, recent advances in the coupling of preconcentration strategies to CE has developed into a significant field of research for the determination of compounds at low concentration levels, mainly when environmental samples are analysed.

A review related to the determination of these drugs by capillary electrophoresis has been completed and submitted to '*Trends in Analytical Chemistry*' as a revision paper. Therefore, in order to avoid repetition of information, we attach a copy of the manuscript that has been accepted in this journal for its publication. In this paper, the most relevant results of this Doctoral Thesis are also included because this revision was carried out after the experimental part had been conducted.

UNIVERSITAT ROVIRA I VIRGILI
SENSITIVITY ENHANCEMENT STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF DRUGS OF ABUSE AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS
Igor Botello González
Dipòsit Legal: T. 1299-2012

THE CURRENT ROLE OF CAPILLARY ELECTROPHORESIS IN THE DETERMINATION OF DRUGS OF ABUSE AND THEIR METABOLITES

Igor Botello, Francesc Borrull, Marta Calull, Carme Aguilar
Departament de Química Analítica i Química Orgànica. Facultat de Química.
Universitat Rovira i Virgili. Marcel·lí Domingo s/n, 43007 Tarragona, Spain

Abstract

Nowadays, so-called drugs of abuse are widely used and pose an undeniable problem at a social level. These compounds have incalculable consequences for the society, such as the cost of medical treatment, higher incidence of criminality and economic repercussions. As a result, it is important to be able to determine these drugs and their main metabolites in biological matrices, in seized illegal preparations and in environmental samples. The present review outlines the recent advances in the use of capillary electrophoresis (CE) in the determination of drugs of abuse, in particular amphetamines, benzodiazepines, barbiturates, cannabinoids, heroin, methadone, cocaine and their metabolites in different samples. The review is based on the papers published during the period from 2007 to March 2012. These kinds of drugs have been selected for study due to their relevance and widespread use.

Keywords: *Biological samples, capillary electrophoresis, drugs of abuse, environmental samples, forensic analysis, preconcentration*

1. Introduction

Drug of abuse is a term given to drugs that are taken for non-medicinal reasons, usually in order to exploit their mind-altering effects. The abuse of a drug, in general, can lead to physical and mental damage and, in some cases, dependence and addiction can develop. The determination of these drugs has important clinical and forensic applications, from diagnosis of acute/lethal intoxication to identification of drugs affecting performance at work (workplace drug testing), while driving (driving under the influence of drugs) and in sports (doping control).

These drugs are excreted unchanged or as metabolites and eventually reach wastewater plants (WWTPs). These plants can remove a fraction of these compounds but some residues can be released into the aquatic environment, ending up in surface water and even in drinking water [1]. Due to this fact, the determination of their presence is an essential preliminary step towards the measurement of concentration levels in different ecosystems [1,2]. These drugs have been found in different surface water samples at ng/L levels. For example, cocaine has been found in concentrations ranging from 6 to 678 ng/L in different European river waters [2-5] and codeine has also been detected in river water at concentration levels around 150 ng/L [5].

In the analysis of drugs and pharmaceuticals in a clinical or forensic context, the most common analysed matrices are blood and urine. In addition, other biological specimens such as hair, oral fluid, meconium and tissue have also been analysed. Blood is the most versatile specimen for drug analysis, not only in clinical chemistry, but also in forensic science [6,7]. Urine is the most commonly analysed matrix, as it provides direct evidence for the short-term use of illicit drugs. However, the target analytes will remain in urine no longer than 10 days after use [8]. Like urine, hair belongs to the group of non-invasive samples and is considered another biological specimen suitable for drug testing. Hair samples present many advantages, such as ease of preservation and a longer retrospective period (months to years).

Different analytical methods, mainly based on liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectrometry (MS), have been developed for the determination of drugs of abuse and their metabolites [2,3,9,10]. Most of these methods are applied to the analysis of biological and environmental samples. Capillary electrophoresis (CE) is another alternative due to its wide range of applications [7,11-15]. The increasing interest in CE as an analytical technique is certainly based on its high efficiency, high resolution power, low reagent consumption, automation and the fact that it is a low-cost alternative compared to other chromatographic techniques, such as LC.

The aim of this review is to discuss the recent advances in capillary electro-

phoresis for the determination of drugs of abuse and their metabolites, focusing on papers published in the period from 2007 to March 2012. To this end, we have selected the group of drugs considered to be of the greatest significance nowadays from both analytical and society perspectives, as well as because these groups involve the most frequently used illicit drugs (amphetamines, benzodiazepines, barbiturates, cannabinoids, opiates and cocaine). In the following subsections, the most relevant papers regarding the determination of different groups of drugs of abuse are highlighted and the information is also given in table format. Please note that, since some of the published papers focus on the determination of several drugs belonging to different groups, the corresponding information is reported in different tables.

2. Amphetamines

Generally speaking, the term amphetamine refers to a whole family of synthetic drugs with stimulant effects, which are related to the stimulation of the central nervous system and they have a notorious reputation in the illicit drug market [16]. Owing to their psychoactive properties and euphoric effects, they are often abused as recreational drugs, especially popular among young people [17]. Amphetamines increase self-confidence and alertness and improve physical performance. An overdose of amphetamines can cause serious seizures, coma or even death. Abuse of amphetamines is relatively common, particularly of amphetamine itself, which is a controlled drug. However, there are other amphetamine-like drugs of abuse of which significant use is widespread, such as 3,4-methylene dioxy amphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') and 3,4-methylene dioxyethylamphetamine (MDEA). Analytical procedures of high efficiency need to be developed for the determination of these drugs in order to fight the increasing trend in the abuse of amphetamines around the world [18,19].

Table 1 shows an overview of the most relevant papers regarding the determination of amphetamines by CE in different kinds of samples during the period covered by this review. As shown in the table, amphetamines have been widely studied by CE and, as can be observed, most of the published papers used CZE [16,17,20-28] as the separation mode, even though MEEKC [29], CEC [30,31] and MEKC [8,32,33] have also been reported as strategies to separate these compounds. The most extensi-vely used detector is UV but there are also some alternative detectors, such as the use of electrochemical [17], conductivity [16] and MS detection [21,26,27,31]. With respect to the samples analysed, the existing literature is mainly focused on biological samples, in particular in urine [8,17,20-23,25-28,30,31,33] and also in hair samples [8,21]. However, there are other types of sample, such

Table 1. Overview list of CE methodologies for amphetamines determination

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
2-(4-methoxyphenyl) ethylamine 2-bromo-N-methylbenzylamine 2-methoxy-N-methylaniline 2-Phenethylamine 5-aminomethyl-7-chloro-1,3-benzodioxole 2-methoxyphenethylamine 3-methyl-N-methylbenzylamine Amphetamine Dextroamphetamine Methamphetamine 3,4-methylene dioxymethamphetamine	**CZE** Capillary: 90 cm x 50 μm; 30 kV, 25 ºC BGE: 30 mM hydroxypropyl-β-cyclodextrin + 75 mM acetic acid + 25 mM sodium acetate (pH 4.55)	C^4D - UV	1.3 μg/mL [a]	Street-grade tablet samples	Dilution	[15]
Methamphetamine 3,4-methylene dioxyamphetamine 3,4-methylene dioxymethamphetamine	**CZE** Capillary: 50 cm x 25 μm; 20 kV, 25 ºC BGE: 0.05 M PBS Detection buffer: 0.1 M PBS (pH 9.0) + 5.0 10^{-3} M Ru $(bpy)_3^{2+}$	EC/ECL	1.6 10^{-7} - 3.3 10^{-8} mol/L [a]	Urine	LLE	[16]
Methamphetamine Amphetamine Dimethylamphetamine β-hydroxymethamphetamine Ephedrine Norephedrine Methylephedrine 3,4-methylene dioxymethamphetamine 3,4-methylene dioxyamphetamine 3,4-methylene dioxy-N-ethyl-amphetamine	**CZE** Capillary: 64.5 cm x 50 μm; 30 kV, 25 ºC Capillary coated- diol and capillary coated-PVA BGE: 125 mM Tris (sodium dihydrogen phosphate (pH 6.15)) + 6 mM DM-β-CD + 12 mM β-CD	UV	-	Urine	LLE	[19]

Table 1. Overview list of CE methodologies for amphetamines determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Amphetamine Methamphetamine 3,4-methylenedioxyamphethamine 3,4-methylenedioxymethamphethamine Ephedrine Cocaine Morphine Codeine 6-monoacetylmorphine Benzoylecgonine	**CZE** Capillary: 100 cm x 75 μm; 15 kV, 20 ºC BGE: 10 mM sodium dihydrogen phosphate (pH 4.5)	MS	0.006 - 0.100 ng/mg [b]	Hair	Digestion and LLE	[20]
Amphetamine Ketamine Methamphetamine 3,4-methylenedioxyamphethamine 3,4-methylenedioxymethamphethamine	**CZE** Capillary:60 cm x 75 μm; 20 kV, 25 ºC BGE: 30 mM PBS (pH 2.0) + 15 % v/v ACN	UV	4.0 - 6.0 ng/mL [b]	Urine	MSPE	[21]
Amphetamine DL-p-chloroamphetamine, 2-amino-1,2-diphenylethanol	**CZE** Capillary: 60 cm x 50 μm; 25 kV, 25 ºC BGE: 50 mM Bis(2-hydroxyethyl) iminotris(hydroxymethyl) methane + 0.8 mM (1)-(18-crown-6)-tetracarboxylic acid (pH 4)	UV	0.4 – 2.0 ng/mL [b]	Urine	SDME	[22]
Heroin DL-methamphetamine DL-3, 4-methylenedioxymethamphetamine DL-ketamine	**CZE** Capillary: 37 cm x 50 μm; 20 kV, 25 ºC BGE: 0.1 M potassium dihydrogenphosphate and 0.1M phosphoric acid (pH 3.23) + 20 mM β-CD	UV	0.05 - 0.20 ng/mL [a]	Bankanotes, kraft paper, plastic bag and silver paper	DLLME	[23]

Table 1. Overview list of CE methodologies for amphetamines determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Amphetamine Methamphetamine Ephedrine Psilocin Cocaine Cocaethylene Methadone PCP Pheniramine Diphenhydramine Oxycodone Thebaine Fentanyl Codeine Morphine 6-monoacetylmorphine Heroin Noscapine Papaverine	**CZE** Capillary: 67 cm x 50 μm; 25 kV, 25 ºC BGE: 100 mM sodium dihydrogen phosphate (pH 6.0) + 20 mM β -CD + 5 % acetonitrile + 20 % isopropanol	UV	5 - 30 ng/mL [b]	Urine	SI-SPE [c]	[24]
2,5-Dimethoxy-4-methylamphetamine 2,5-Dimethoxy-4-ethylamphetamine 2,5-Dimethoxy-4-propylamphetamine	**CZE** Capillary: 120 cm x 50 μm; 10 kV, 25 ºC BGE: 10 mM sodium dihydrogen phosphate (pH 4.5)	MS	3.9 - 4.6 ng/mL [b]	Urine	SPE	[25]

Table 1. Overview list of CE methodologies for amphetamines determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Morphine Codeine 6-monoacetylmorphine Ethylmorphine Fentanyl Pethidine Buprenorphine Nalbuphine Dextromethorphan Methadone 2-ethylidene-1,5-dimethyl-3,3 diphenylpyrrolidine D-Propoxyphene Anphetamine Methamphetamine 3,4-methylenedioxyamphethamine 3,4-methylenedioxymethamphethamine 3,4-methylenedioxyethylamphethamine N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine Ephedrine Pseudoephedrine Norephedrine Methylphenidate Cocaine Cocaethylene Anhydroecgonine methyl ester Metoprolol Procaine Ketamine Trimipramine	**CZE** Capillary: 80 cm x 50 µm; 30 kV, 25 ºC BGE: 20 mM ammonium formate (pH 2.5)	MS	0.25 - 10 ng/mL [b]	Urine	DLLME	[26]

Table 1. Overview list of CE methodologies for amphetamines determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Caffeine Theophylline Barbital Phenobarbital Morphine O^6-monoacetylmorphine O^3- monoacetylmorphine Codeine Ephedrine Heroin Amphetamine Acetylcodeine Methamphetamine Thebaine Papaverine Narcotine	**MEEKC** Capillary: 40 cm x 75 µm; 20 kV, 25 ºC BGE: 89.8 % 5 mM sodium tetraborate (pH 9.5) + 3.3 % SDS + 6.0 % 1-butanol / 0.9 % octane	UV	1 - 1.5 µg/mL [a]	-	-	[28]
Amphetamine Methamphetamine 3,4-methylenedioxyamphetamine 3,4-methylenedioxymethamphetamine 3,4-methylenedioxyethylamphetamine Cocaine Codeine Heroin Morphine 6-monoacethylmorphine	**CEC** Capillary: 33 cm x 75 µm, packed 3 µm Pinnacle II Cyano (cyano silica stationary phase; 10 kV, 20 ºC Mobile phase: 20 mM sodium phosphate (pH 2.5) + acetonitrile (80/20, v/v)	UV	5 - 12 ng/mL [a]	Urine	SPE	[29]
Amphetamine Methamphetamine 3,4-methylene dioxyamphetamine 3,4-methylene dioxymethamphetamine 3,4-methylene dioxyethylamphetamine Cocaine Morphine Heroin Codeine	**CEC** Capillary: 30 cm x 100 µm, packed 3 µm cyano derivatized silica stationary phase; 12 kV, 20 ºC Mobile phase: 25 mM ammonium formate pH 3 + 30 % ACN	MS	0.78 - 3.12 ng/mL [a]	Urine	SPE	[30]

Table 1. Overview list of CE methodologies for amphetamines determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Amphetamine Ketamine Methamphetamine 3,4-methylenedioxyamphethamine 3,4-methylenedioxymethamphethamine Acetylcodeine Codeine Heroin	**FASS-CZE** Capillary:60 cm x 75 μm; 20 kV, 25 ºC BGE: 30 mM PBS (pH 2.0) + 15% v/v ACN	UV	0.015 - 0.105 μg/mL [b]	Urine	MSPE	[27]
Amphetamine Methamphetamine 3,4-methylenedioxymethamphetamine	**CSEI-Sweeping-MEKC** Capillary: 60 cm x 75 μm; 18 kV, 25 ºC BGE: 100 mM sodium dihydrogen phosphate (pH 3) + 20 % methanol + 20 mM SDS	UV	6 - 8 pg/mL [a]	-	-	[31]
Methamphetamine Ketamine Morphine Codeine	**CSEI-sweeping-MEKC** Capillary: 40 cm x 50 μm; -20 kV, 25 ºC BGE: 25 mM sodium dihydrogen phosphate (pH 2.5) + 20 % methanol and 100 mM SDS	UV	50 - 200 pg/mg [b]	Hair	Acid extraction with US	[8]
Alprazolam 7-aminoclonazepam 7-aminoflunitrazepam Clonazepam Diazepam Flunitrazepam N-demethylflunitrazepam Nordiazepam α-hydroxyalprazolam Oxazepam Codeine Morphine Methamphetamine Ketamine	**SRMM-sweeping-MEKC** Capillary: 50.4 cm x 50 μm; -15 kV, 25 ºC BGE: 75 mM phosphate buffer (pH 2.5) + MeOH (70:30 v/v) sweeping buffer: 75 mM phosphate buffer (pH 2.5) + MeOH (90:10 v/v) + 65 mM SDS	UV	20 - 50 ng/mL [b]	Urine	LLE	[32]

[a] standard samples
[b] real samples
[c] sequential injection-solid phase extraction

as street-grade tablets, banknotes, kraft paper, plastic bags and silver paper, have also been studied [16,24]. In this table as well as in the rest of the tables presented in this paper, unless otherwise indicated, the term urine refers to human urine. For the analysis of biological samples, different sample pretreatment procedures have been reported such as LLE [17,20,21], SPE [25,26,30,31], SDME [23], acidic digestion [8,21], MSPE [22,28] and DLLME [24,27].

With respect to the use of CZE, one interesting approach is the use of two detection systems because confirmatory analysis can be performed using a single instrumentation. In one such study, Epple *et al*. [16] determined some amphetamine-type substances with the use of capacitively coupled contactless conductivity detection (C^4D) and UV as a simultaneous detection system. As an example, The method was applied to the analysis of real illicit samples of ecstasy and dexamphetamine and the average LOD in standard samples was 1.3 μg/mL. The method was shown to be suitable for the analysis of street-grade ecstasy and dexamphetamine tablets. Sun *et al*. [17] also demonstrated that multiple detection design can supply complementary information and enlarge the range of analytes when compared to a single detection mode. In this case, the authors reported the determination of amphetamine drugs in urine by CE using simultaneous electrochemical and electrochemiluminescence detection (CE-EC/ECL). LODs for standards of $3.3\ 10^{-8}$ mol/L, $1.6\ 10^{-7}$ mol/L and $3.3\ 10^{-8}$ mol/L were obtained for the three compounds studied. When urine samples were analysed, a prior pretreatment based on LLE using ethyl acetate was used with extraction efficiencies of over 90%.

Another interesting approach using CZE was reported by Iwamuro *et al*. [20], with the aim of increasing the reproducibility in terms of migration times, which is an important advantage, since the lack of reproducibility usually occurs when very complex samples, such as urine, are analysed. In this case, the authors tested two chemically modified capillaries, one with diol groups and the other with poly(vinylalcohol) (PVA) for the chiral separation of amphetamines and related compounds. In both cases, a low pH Tris buffer containing heptakis (2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD) and β-cyclodextrin (β-CD) was used. The results obtained show that the diol-coated capillary gave very reproducible migration times compared to those obtained for the uncoated and poly(vinyl alcohol)-coated capillaries.

With regard to the use of CEC, an interesting approach was proposed by Aturki *et al*. [31], who described a method for the separation and determination of nine drugs of abuse in human urine. Among these drugs, five amphetamine-type compounds were included. The method was based on a previous sample pretreatment by SPE on a strong cation exchange cartridge and further CEC-MS performance. Hyphenation of CEC system

with MS was achieved using a liquid junction interface. LODs were in the range of 0.78 to 3.12 ng/mL for all compounds in standard samples. The method was successfully applied to spiked urine samples and recovery values were between 80 and 95%.

Another promising approach reported by different authors is the use of different preconcentration techniques, such as field-amplified sample stacking (FASS) [28] or sweeping [8,32,33], in order to increase concentration sensitivity. For example, Lin *et al*. [8] used a combination of two preconcentration strategies for the determination of drugs of abuse. In this case, cation-selective exhaustive injection (CSEI) and sweeping were combined in MEKC for the determination of methamphetamine and other drugs of abuse in hair samples. Under the optimal conditions, the LODs were in the range of 50 to 200 pg/mg in hair for the drugs studied. The same strategy was used by Fang *et al.* [32] for determining a group of amphetamine drugs. The authors demonstrated that by controlling the sample conductivity it was possible to achieve highly reproducible results, while still maintaining the sensitivity of field-amplified sample injection in the determination of amphetamine drugs. This procedure provides sensitivity improvement of several thousand-fold and the LODs obtained were from 6 to 8 ng/L in standard samples.

3. Benzodiazepines

Benzodiazepines are used as hypnotics, tranquilizers, anticonvulsants or muscle relaxants. Nowadays, with the frequent occurrence of anxiety and depression, the rate of benzodiazepine consumption is increasing. These compounds are also used in the treatment of sleep disorders, status epilepsy, insomnia, early morning awakenings or other convulsive disorders. Overdose of these drugs can cause acute symptoms and it is therefore important to monitor their concentration in biological specimens [34]. The physico-chemical nature of benzodiazepines may, however, give rise to some problems if using capillary electrophoresis as an analytical technique, as these compounds are difficult to ionize due to their fairly low pKa values, which makes MEKC the preferred CE separation mode [33-38], as shown in Table 2. However, some authors have used CZE [39,40] or CEC [41]. As can be observed in Table 2, UV has been the most commonly used detection system [33-40], although the use of MS has also been reported [41]. With respect to application to real samples, most of the papers report the determination of benzodiazepines in urine samples and, therefore, a pretreatment has been included which generally involves the use of SPE [33,41] or LLE [34,38] procedures.

With regard to the use of CZE for the determination of benzodiazepine drugs, Webb et al. [39] reported a rapid CZE method for the

Table 2. Overview list of CE methodologies for benzodiazepines determination

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Nitrazepam Oxaxepam Alprazolam Flunitrazepam Temazepam Diazepam 7-aminoflunitrazepam 7-aminonitrazepam 7-aminoclonazepam	**CZE** Capillary: 69 cm x 50 μm; 28 kV, 25 ºC BGE: 100 mM ammonium phosphate (pH 2.5) Capillary double coated with poly(diallyldimethylammonium chloride and dextran sulfate)	UV	2.7 - 41.5 μg/mL [a]	Beverages	-	[38]
Bromazepam Diazepam Chlordiazepoxide Flunitrazepam	**CZE** Capillary: 50 cm x 50 μm; 15 kV, 25 ºC BGE: 6 mM sodium dihydrogen phosphate and 33 mM orthophosphoric acid (pH 2.0)	UV	1.57 - 4.27 μg/mL [a]	Powdered tabled samples	Dissolution and Filtration	[39]
Clordiazepoxide Clorazepate Nitrazepam Diazepam Alprazolam Flunitrazepam Bromazepam	**MEKC** Capillary: 50 cm x 50 μm; 15 kV, 25 ºC BGE: 20 mM sodium dihydrogen phosphate (pH 7.0) containing 400 mM ($LiNTf_2$)	UV	-	-	-	[34]
Lorazepam Bromazepam Clorazepate Nitrazepam Diazepam Alprazolam Flunitrazepam	**MEKC** Capillary: 50 cm x 50 μm; 20 kV, 25 ºC BGE: 20 mM disodium hydrogen phosphate (pH 7.0) (55 % methanol) + 170 mM ($BMIM)(NTf_2$) + 10 mM SDS	UV	1.0- 4.42 μg/mL [a]	Urine	SPE	[33]

Table 2. Overview list of CE methodologies for benzodiazepines determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Alprazolam Bromazepam Medazepam Nitrazepam Chlorazepate Chlordiazepoxide Diazepam Oxazepam	**MEKC** Capillary: 50 cm x 50 µm; 25 kV, 25 ºC BGE: 25 mM sodium tetrabotate + 50 mM SDS + 15 mM β-CD + 2 M urea (pH 9.3)	UV	0.58 - 1.42 µg/mL [a]	-	-	[35]
Chlordiazepoxide Diazepam Oxazepam Medazepam Nitrazepam Flunitrazepam Bromazepam Alprazolam	**MEKC** Capillary: 64.5 cm x 50 µm; 25 kV, 20 ºC BGE: 25 mM sodium tetraborate + 50 mM SDS (12 % methanol) (pH 9.5)	UV	-	-	-	[36]
Alprazolam Triazolam Chlordiazepoxide Lorazepam Nitrazepam Clonazepam Flunitrazepam Clorazepate Diazepam Prazepam	**CEC** Capillary: 50 cm x 100 µm; 30 cm packed with hexyl acrylate-based porous monolith 20 kV, 25 ºC Mobile phase: 5 mM ammonium acetate (pH 7.0) + ACN (30:70 v/v)	MS (TOF)	0.6 - 1.8 ng/mL [a]	Urine	LLE	[40]
Chlordiazepoxide Clorazepate Nitrazepam Diazepam Alprazolam Flunitrazepam	**Sweeping-MEKC** Capillary: 50 cm x 50 µm; -25 kV, 25 ºC BGE: 15 mM sodium tetraborate + 20 mM C_{16}MPYB (30 % methanol) (pH 9)	UV	4.7 - 9.8 ng/mL [a]	Urine	SPE	[37]

Table 2. Overview list of CE methodologies for benzodiazepines determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Alprazolam 7-aminoclonazepam 7-aminoflunitrazepam Clonazepam Diazepam Flunitrazepam N-demethylflunitrazepam Nordiazepam α-hydroxyalprazolam Oxazepam Codeine Morphine Methamphetamine Ketamine	**Sweeping-MEKC** Capillary: 50.4 cm x 50 µm; -15 kV, 25 ºC BGE: 75 mM phosphate buffer (pH 2.5) + MeOH (70:30 v/v) sweeping buffer: 75 mM phosphate buffer (pH 2.5) + MeOH (90:10 v/v) + 65 mM SDS	UV	20 - 50 ng/mL [b]	Urine	LLE	[32]

[a] standard samples
[b] real samples

simultaneous determination of nine benzodiazepines in beverages. In this case, a double-coated capillary coated with poly(diallyldimethylammonium chloride) and then dextran sulphate was used. The validated method was applied to beverages spiked with benzodiazepines. This capillary coating enabled the stabilization of the EOF and eliminated associated peak tailing and peak area variability by reducing the adsorption of buffer or sample components into the capillary surface.

However, due the abovementioned characteristics of these compounds, the use of CZE has barely been reported. In addition, different authors have proposed other methodologies mainly based on MEKC and CEC. For example, an interesting approach for improving the resolution in the simultaneous determination of several benzodiazepines by MEKC was proposed by Su *et al*. [35]. The authors used chaotropic salts to modify the BGE in the separation of seven benzodiazepines. Three chaotropic salts (lithium trifluoro-methanesulphonate (LiOTf), lithium hexafluorophosphate ($LiPf_6$) and lithium bis(trifluoromethanesulphonyl)imide ($LiNTf_2$) were compared in terms of separation efficiencies. The best results were achieved when $LiNTf_2$ was used as the BGE modifier. In a separate work [34], the same authors also reported an improvement in the separation of benzodiazepines using 1-butyl-3-methylimidazolium-based ionic liquids (ILs) and sodium dodecyl sulphate (SDS) as modifiers of the BGE. In both works, a separation buffer consisting of 20 mM phosphate (pH 7.0) with the appropriate amount of modifiers was used. With regard to the use of CEC, Blas *et al*. [41] described a method for the determination of benzodiazepines in urine by CEC-MS (TOF) using a hexyl acrylate-based porous monolith. LLE using ethyl acetate was performed in order to extract and concentrate the analytes prior to the analysis. Using the monolithic stationary phase enabled an improvement in sensitivity of 75- to 140-fold with the injection of the sample diluted in low conductivity buffer. The proposed method made the quantification of these drugs possible at low ng/mL levels in urine samples.

In order to increase the sensitivity in CE for the determination of benzodiazepines, some authors have also developed methodologies in which different preconcentration strategies based on sweeping have been used [33,38]. For example, Chiang *et al*. [33] reported a method based on sweeping-MEKC for the determination of different kinds of drugs of abuse in urine samples. Using SDS to sweep and stack, the analytes were determined with LODs in the range of 20 to 50 ng/mL. In addition, Su *et al.* [38] reported a method based on the same preconcentration mechanism for the determination of benzodiazepines. In this paper, ionic liquid-type cationic surfactants were used as sweeping carriers in order to test different separation efficiencies in

comparison with the commonly employed cationic surfactant cetyltrimethylammonium bromide (CTAB). Adding these ionic liquid surfactants to the background electrolyte enhanced the separation efficiency and detection sensitivity during the sweeping-MEKC separation of benzodiazepines. The experimental results showed that the cationic surfactant *N*-cetyl *N*-methylpyrrolidinium bromide exhibited superior sweeping power relative to those of 1-cetyl-3-methylimidazolium bromide and CTAB. The LODs achieved were in the order of 4.7 to 9.8 ng/mL for standard samples.

4. Barbiturates

Barbiturates are substituted pyrimidine derivatives from barbituric acid (2,4,6-trioxypyrimidine) and this results from the condensation of malonic acid and urea. Barbiturates are mainly used as hypnotics in the short-term treatment of insomnia and, preoperatively, to relieve anxiety and provide sedation. Barbital (Veronal) was the first physiologically active drug introduced in 1903. Their medical application has declined since widespread street abuse has aroused public concern [42]. The barbiturates exhibit a wide variety of responses in the body, depending mostly on the substituting groups. Barbiturates are classified into four categories, which are based on the time required for them to take effect and the duration of their activity: long-acting, intermediate-acting, short-acting and ultra short-acting. Compounds with phenyl or ethyl groups seem to have the longest duration. In large doses, barbiturates act as anaesthetics and, at high levels, can cause death [43]. Complications arise from the fact that abused barbiturates often occur as complex mixtures and other drugs and/or excipients are also present. This necessitates the continued development of methods for their efficient separation and precise identification [44].

As shown in Table 3, separation of barbiturate drugs by CE has been performed by CZE [42,45] and by MEEKC [29,46], using UV as the detection system. In most of these papers, the developed methods have been applied to the analysis of biological samples. For example, Jiang *et al*. [42] used a capillary dynamically coated with a polycationic polymer for the direct determination of nine barbiturates in urine samples. The use of the coated capillary allowed the determination of these compounds in this complex matrix at therapeutic levels without any sample pretreatment. The procedure proved to be simple and the authors reported LODs for nine barbiturates in the range of 0.87 to 3.5 μg/mL in urine samples.

Some authors have reported different strategies in order to increase the concentration sensitivity of barbiturates. For instance, various preconcentration methodologies such as LVSS in CZE [45] and sample stacking induced by reverse migrating pseudostationary phase (SRMP) in MEEKC [46] have been developed. For example,

Table 3. Overview list of CE methodologies for barbiturates determination

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Barbital acid Barbital Phenobarbital Pentobarbital Amobarbital Thiobarbituric acid Butobarbital N-methyl-5-phenyl-ethyl barbital acid 5-cyclohexenyl-5-ethyl barbital acid	**CZE** Capillary: 50 cm x 75 μm; -25 kV, 25 ºC BGE: 20 mM sodium tetraborate (pH 10) + 0.04 % HDB + 2.06 mM α-CD	UV	0.87 - 3.5 μg/mL [b]	Urine	Filtration	[41]
Caffeine Theophylline Barbital Phenobarbital Morphine O^6-monoacetylmorphine O^3- monoacetylmorphine Codeine Ephedrine Heroin Amphetamine Acetylcodeine Methamphetamine Thebaine Papaverine Narcotine	**MEEKC** Capillary: 40 cm x 75 μm; 20 kV, 25 ºC BGE: 89.8 % 5 mM sodium tetraborate (pH 9.5) + 3.3 % SDS + 6.0 % 1-butanol + 0.9 % octane	UV	1 - 1.5 μg/mL [a]	-	-	[28]
Barbital Amobarbital Phenobarbital Secobarbital	**LVSS-CZE** Capillary: 60.5 cm x 75 μm; 20 kV, 25 ºC BGE: 40 mM sodium tetraborate (pH 8) (containing 20 % methanol, v/v)	UV	0.015-0.057 μg/mL [a]	Plasma	LLE	[44]
Phenobarbital p-hydroxyphenobarbital	**SRMP-MEEKC** Capillary: 50 cm x 75 μm; 25 kV, 25 ºC BGE: 0.8 % (v/v) ethyl acetate + 6.6 % (v/v) butan-2-ol + 1.0 % (v/v) ACN + 2.0 % (w/v) SDS + 89.6 % (v/v) of 7.5 mM ammonium formate (pH 8)	UV	16.8 ng/mL [b]	Rat urine	SPE	[45]

[a] standard samples
[b] real samples

Kadi *et al*. [46] reported a methodology based on MEEKC with SRMP for the determination of barbiturates in rat urine samples. Before the electrophoretic separation performance, a pretreatment for the purification and preconcentration of the samples was carried out by off-line SPE using C18 as a sorbent. The developed method achieved LODs of 16.8 ng/mL in the urine samples.

5. Cannabinoids

The term cannabis is mainly used to refer to preparations of the cannabis plant intended for use as either a psychoactive drug or a medicine. The primary psychoactive ingredient of cannabis is Δ^9-tetrahydrocannabinol (THC), which accumulates in the body fat because of its high lipid solubility. The amount stored in body fat is a function of the quantity, frequency and potency of cannabis used. Cannabis sativa L. is classified as a drug (e.g. marijuana) due to the psychoactive effect of some of its varieties. THC is the major psychoactive component of marijuana [47]. It is present in various parts of the plant, principally in the dried flowers and trichome of the female. When the plant is administrated orally or by smoking, THC can cause euphoria, hallucination, difficulties in concentration and impairment of memory. THC is metabolized to 11-hydroxy-Δ^9-tetrahydrocannabinol (THC-OH), which is further oxidized to 11-nor-9-carboxy-Δ^9-tetra hydro cannabinol (THC-COOH) by cytochrome 450 isoenzymes in the liver. In its unmodified state, THC is barely detectable in urine, while THC-OH accounts for 2% of the total dose amount. The most abundant urinary metabolite is THC-COOH, which is present in urine as its glucuronide (THC-COOH-glucuronide) [48]. Due to the therapeutic value of Cannabis sativa L. and the increasingly widespread abuse of marijuana, there is a need for rapid, sensitive and accurate analytical methods for the analysis of these compounds [47].

To the best of our knowledge, in the period covered by this review, only two papers have been reported applying CE for the determination of cannabinoids or related compounds (Table 4) [48,49]. In one of these papers, Iwamaru *et al*. [49] reported a CZE method for the direct analysis of major metabolites of THC, THC-COOH and its glucuronide in urine by CZE-MS. Using this combination, due the high selectivity of the MS detector, the only pretreatment needed was dilution with methanol. In another study, Su *et al*. [48] applied sweeping-MEKC for the simultaneous determination of THC and its major metabolites, 11-hydroxy-Δ^9-tetrahydrocannabinol and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol. Bond Elut Certify column-type cartridges for SPE were used for clean-up and preconcentration of the urine samples. The LODs for these samples were in the range of 17.2 to 23.3 ng/mL with sensitivity enhancement factors in the range of 77- to 200-fold.

Table 4. Overview list of CE methodologies for cannabinoids determination

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid glucuronide	**CZE** Capillary: 85 cm x 50 µm; 30 kV, 25 ºC BGE: 40 mM ammonium formate (pH 6.4)	MS	50 ng/mL [b]	Urine	Dilution	[48]
Δ^9-tetrahydrocannabinol 11-hydroxy-Δ^9-tetrahydrocannabinol 11-nor-9-carboxy- Δ^9-tetrahydrocannabinol	**Sweeping-MEKC** Capillary: 60 cm x 50 µm; -20 kV, 25 ºC BGE: 25 mM citric acid/disodium hydrogen phosphate (pH 2.6) + 75 mM SDS (40 % methanol)	UV	17.2 - 23.3 ng/mL [b]	Urine	SPE	[47]

[a] standard samples
[b] real samples

6. Opiates

Opium gum, as it is known, is partly dried latex obtained from the unripe capsules of Papaver somniferum L. It is cultivated mainly in the Indian subcontinent, Turkey, China, Australia, Japan and also in some parts of Europe. The major alkaloids of opium, such as morphine, codeine, thebaine, papaverine and narcotine have a wide range of physiological activities on analgesia, sedation, respiratory depression and cough depression [50-52]. Due to its narcotic nature, illicit trafficking of opium is a global problem. As such, it is important to develop methodologies for the analysis of opium based on the determination of the principal alkaloids present, as listed above [6,53]. In addition, the determination of the synthetic opiates heroin and methadone is also important due to their widespread abuse nowadays. Due to this particularity of both heroin and methadone and their significance as illicit drugs of the opiate type drug family, they will be discussed below in two different subsections (6.1 and 6.2, respectively).

As shown in Table 5, separation of opiates has been reported by using different CE modes, such as CZE [21,25,27,28,54-57], MEKC [8,33,58-60], MEEKC [29] and CEC [30,50,61]. With respect to the detection system, UV detection has been the preferred choice [8,28-30,33,50,55,57-61], although MS [21,27,54,56] has also been applied. For the extraction of the target compounds from the diverse studied matrices, such as opium gum, hair and urine samples, different extraction procedures have been used, such as ultrasound (US) extraction [8,50], DLLME [27], LLE [21,33,54,60], SDME [61] and SPE [28,30,55,59].

With regard to separation, a remarkable method was developed by Lin *et al*. [50] for the determination of five major opium alkaloids by the use of pressurized capillary electrochromatography (pCEC). The application of pressure via a micro-HPLC pump allowed a higher resolution combined with a considerable reduction in analysis time. Moreover, the LODs achieved by the use of this method in *Pericarpium papaveris* samples were in the range of 1.5 to 6.0 μg/mL.

In order to increase the sensitivity in CE for the determination of opiates, some authors have developed methodologies by the use of preconcentration techniques, such as sweeping [8,33,59,60], FASS [28] and in-line SPE [56,57], or by the use of sensitive detection systems as MS [27,54]. For example, Lin *et al*. [59] proposed a combination of two stacking methodologies based on CSEI and sweeping-MEKC for the determination of morphine and its four metabolites in human urine. Optimization of the procedure allowed an increase in sensitivity of around 2,500-fold, compared with normal injection by CZE. After SPE using Oasis HLB as a sorbent and after US extraction, the urine samples were analysed and LODs in the range of 10 to 35 ng/mL were achieved.

Table 5. Overview list of CE methodologies for opium related compounds determination

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
3,4-methylene-dioxyamphetamine 4-methylenedioxy-methamphetamine Methadone Cocaine Morphine Codeine 6-monoacetylmorphine	**CZE** Capillary: 100 cm x 75 μm; -15 kV, 25 ºC BGE: 50 mM ammonium phosphate (pH 6.5)	TOF-MS	-	Hair	Digestion and LLE	[53]
Lidocaine Bupivacaine Noscapine Papaverine	**CZE** Capillary: 48.5 cm x 75 μm; 25 kV, 25 ºC BGE: 150 mM sodium citrate (pH 2.5)	UV	300 ng/mL [b]	Urine	SPE	[54]
Morphine Codeine 6-monoacetylmorphine Ethylmorphine Fentanyl Pethidine Buprenorphine Nalbuphine Dextromethorphan Methadone 2-ethylidene-1,5-dimethyl-3,3 diphenylpyrrolidine D-Propoxyphene Anphetamine Methamphetamine 3,4-methylenedioxyamphethamine 3,4-methylenedioxymethamphethamine 3,4-methylenedioxyethylamphethamine N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine Ephedrine Pseudoephedrine Norephedrine Methylphenidate Cocaine Cocaethylene Anhydroecgonine methyl ester Metoprolol Procaine Ketamine Trimipramine	**CZE** Capillary: 80 cm x 50 μm; 30 kV, 25 ºC BGE: 20 mM ammonium formate (pH 2.5)	MS	0.25 - 10 ng/mL [b]	Urine	DLLME	[26]

Table 5. Overview list of CE methodologies for opium related compounds determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Amphetamine Methamphetamine Ephedrine Psilocin Cocaine Cocaethylene Methadone PCP Pheniramine Diphenhydramine Oxycodone Thebaine Fentanyl Codeine Morphine 6-monoacetylmorphine Heroin Noscapine Papaverine	**CZE** Capillary: 67 cm x 50 μm; 25 kV, 25 ºC BGE: 100 mM sodium dihydrogen phosphate (pH 6.0) + 20 mM β-CD + 5 % acetonitrile + 20 % isopropanol	UV	5 - 30 ng/mL [b]	Urine	SI-SPE [c]	[24]
Amphetamine Methamphetamine 3,4-methylenedioxyamphethamine 3,4-methylenedioxymethamphethamine Ephedrine Cocaine Morphine Codeine 6-monoacetylmorphine Benzoylecgonine	**CZE** Capillary: 100 cm x 75 μm; 15 kV, 20 ºC BGE: 10 mM sodium dihydrogen phosphate (pH 4.5)	MS	0.006 - 0.100 ng/mg [b]	Hair	Digestion and LLE	[20]
Paclitaxel Morphine Codeine	**MEKC** Capillary: 57 cm x 75 μm; -25 kV, 20 ºC BGE: 20 mM sodium tetraborate (pH 9.2) + 60 mM SDS + 5 % methanol	UV	0.03 - 0.09 μg/mL [a]	Urine	Centrifugation and filtration	[57]

Table 5. Overview list of CE methodologies for opium related compounds determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Caffeine Theophylline Barbital Phenobarbital Morphine *O*^{6}-monoacetylmorphine *O*^{3}- monoacetylmorphine Codeine Ephedrine Heroin Amphetamine Acetylcodeine Methamphetamine Thebaine Papaverine Narcotine	**MEEKC** Capillary: 40 cm x 75 µm; 20 kV, 25 ºC BGE: 89.8 % 5 mM sodium tetraborate (pH 9.5) + 3.3 % SDS + 6.0 % 1-butanol + 0.9 % octane	UV	1 - 1.5 µg/mL [a]	-	-	[28]
Amphetamine Methamphetamine 3,4-methylenedioxyamphetamine 3,4-methylenedioxymethamphetamine 3,4-methylenedioxyethylamphetamine Cocaine Codeine Heroin Morphine 6-monoacethylmorphine	**CEC** Capillary: 33 cm x 75 µm, packed 3 µm Pinnacle II Cyano (cyano silica stationary phase; 10 kV, 20 ºC Mobile phase: sodium dihydrogen phosphate (pH 2.5) / ACN (80/20, v/v)	UV	5 - 12 ng/mL [a]	Urine	SPE	[29]
Narcotine Papaverine Thebaine Codeine Morphine	**pCEC** Capillary: 55 cm x 100 µm; 30 cm packed with polymeric monolith -13 kV, 25 ºC Mobile phase: 5 mM sodium dihydrogen phosphate (pH 4.0) in 90 % v/v ACN	UV	1.5 - 6.0 µg/mL [b]	pericarpium papaveris	US extraction	[49]

Table 5. Overview list of CE methodologies for opium related compounds determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Ephedrine Cocaine Strychnine Morphine Caffeine Theophylline Piroxicam	**OT-CEC** Capillary: 57 cm x 50 µm; 15 kV, 25 ºC BGE: 20 mM sodium tetraborate	UV	0.94 - 17.64 ng/mL [a]	Horse urine	SDME	[60]
Amphetamine Ketamine Methamphetamine 3,4-methylenedioxyamphethamine 3,4-methylenedioxymethamphethamine Acetylcodeine Codeine Heroin	**FASS-CZE** Capillary:60 cm x 75 µm; 20 kV, 25 ºC BGE: 30 mM PBS (pH 2.0) + 15% v/v ACN	UV	0.015 - 0.105 µg/mL [b]	Urine	MSPE	[27]
2-ethylidene-1,5-dimethyl-3,3 diphenylpyrrolidine 6-monoacetylmorphine Codeine Hydrocodeine	**In-line SPE-CZE** Capillary: 100 cm x 50 µm; 30 kV, 25 ºC BGE: 60 mM ammonium acetate (pH 3.8)	MS	13 - 210 ng/L [b]	Urine	Dilution	[55]
Cocaine 2-ethylidene-1,5-dimethyl-3,3 diphenylpyrrolidine Codeine 6-monoacetylmorphine	**In-line SPE-CZE** Capillary: 65.5 cm x 50 µm; 30 kV, 25 ºC BGE: 80 mM disodium phosphate anhydrous + 6 mM HCl (pH 3)	UV	70 - 270 ng/L [b]	Tap and river water	-	[56]
Morphine Codeine Normorphine Morphine-3-glucuronide Morphine-6-glucuronide	**CSEI-Sweeping-MEKC** Capillary: 40 cm x 50 µm; -20 kV, 25 ºC BGE: 25 mM sodium dihydrogen phosphate (pH 2.5) + 22 % methanol + 100 mM SDS	UV	10 - 35 ng/mL [a]	Urine	SPE	[58]

Table 5. Overview list of CE methodologies for opium related compounds determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Heroin Morphine Codeine 6-monoacetylmorphine	**CSEI–sweeping-MEKC** Capillary: 38 cm x 50 μm; 20 kV, 25 ºC BGE: 20 mM sodium dihydrogenphosphate (pH 2.5) + 80 mM SDS	UV	10 ng/mL [b]	Urine	LLE	[59]
Methamphetamine Ketamine Morphine Codeine	**CSEI-Sweeping-MEKC** Capillary: 40 cm x 50 μm; -20 kV, 25 ºC BGE: 25 mM sodium dihydrogen phosphate (pH 2.5) + 20 % methanol and 100 mM SDS	UV	50 - 200 pg/mg [b]	Hair	Acid extraction with US	[8]
Alprazolam 7-aminoclonazepam 7-aminoflunitrazepam Clonazepam Diazepam Flunitrazepam N-demethylflunitrazepam Nordiazepam α-hydroxyalprazolam Oxazepam Codeine Morphine Methamphetamine Ketamine	**SRMM-sweeping-MEKC** Capillary: 50.4 cm x 50 μm; -15 kV, 25 ºC BGE: 75 mM phosphate buffer (pH 2.5) + MeOH (70:30 v/v) sweeping buffer: 75 mM phosphate buffer (pH 2.5) + MeOH (90:10 v/v) + 65 mM SDS	UV	20 - 50 ng/mL [b]	Urine	LLE	[32]

[a] standard samples
[b] real samples
[c] sequential injection-solid phase extraction

6.1 Heroin

Heroin (diacetylmorphine) is an illegal and highly addictive drug [62]. However, it is still the most commonly sold opiate on illicit markets. This is because the substance its more lipophilic than morphine due to the two additional acetyl groups present in its structure, which enables it to cross the blood-brain barrier more easily [63]. This drug rapidly metabolizes to 6-monoacetylmorphine by enzymatic hydrolysis and its plasma half-life has been estimated at about 2 to 8 minutes. Morphine results from the further hydrolysis of 6-monoacetylmorphine which has a plasma half-life of 10 to 40 minutes. Morphine may be further metabolized to codeine in the liver and intestine [60,64].

Abuse of heroin is still widespread, although abuse of other opiates such as codeine and, more recently, morphine is also common. Following heroin usage, only morphine is commonly detected in urine, although 6-monoacetylmorphine and codeine may also be detected following relatively recent heroin usage. Following codeine usage, a small amount of morphine as a minor metabolite is commonly observed. Therefore, it may be difficult to distinguish between the abuse of heroin-plus-codeine and the abuse of codeine alone, when 1 or 2 days have elapsed after the time of the ingestion.

In the last years, different analytical methods in CE have been developed for the determination of heroin, its metabolites and basic impurities, mainly in preparations and biological samples. As can be observed from Table 6, determination of heroin has been reported mainly using CZE [24,25,28,63,65], MEEKC [29], CEC [30] and MEKC [60]. UV detection has been most commonly used as the detection system, although MS [63,65] has also been reported. For the extraction of heroin and its metabolites from biological matrices, there are different sample pretreatments, such as LLE [60], DLLME [24], SPE [25,30], MSPE [28] and protein precipitation [65].

As mentioned above, a promising way to obtain faster and more precise migration times and higher plate counts is based on the use of coated capillaries. Recently, Zhang *et al*. [63] reported an interesting approach for the determination of heroin and seven basic impurities (papaverine, narcotine, 6-monoacetylmorphine, acetylcodeine, morphine, thebaine and codeine). For the first time, the authors used charged polymer-protected gold nanoparticle-coated capillaries with mass spectrometry detection. With this methodology, a highly reproducible EOF and a capillary surface with more favourable kinetics can be obtained. Under the optimal conditions, a stable and repeatable capillary coating generating reverse EOF was obtained using a single rising step. Figure 2 shows a typical electropherogram obtained by the developed methodology for the determination of heroin and its impurities.

Table 6. Overview list of CE methodologies for heroin determination

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Papaverine Narcotine Heroin 6-monoacetylmorphine Acetylcodeine Morphine Thebaine Codeine	**CZE** Capillary: 68 cm x 50 μm coated with charged polymer-protected gold nanoparticles; -20 kV, 20 ºC BGE: 120 mM ammonium acetate (pH 5.2) (13 % methanol)	MS	-	-	-	[62]
Amphetamine Ephedrine Methadone Pethidine Tetracaine Codeine Heroin	**CZE** Capillary: 70 cm x 50 μm; 22 kV, 20 ºC BGE: 20 mM ammonium acetate (pH 9.0)	MS	0.40 - 1.0 ng/mL [a]	Urine	Protein precipitation	[64]
Heroin DL-methamphetamine DL-3, 4-methylenedioxymethamphetamine DL-ketamine	**CZE** Capillary: 37 cm x 50 μm; 20 kV, 25 ºC BGE: 0.1 M potassium dihydrogenphosphate and 0.1M phosphoric acid (pH 3.23) + 20 mM β-CD	UV	0.05 - 0.20 ng/mL [a]	Bankanotes, kraft paper, plastic bag and silver paper	DLLME	[23]
Amphetamine Methamphetamine Ephedrine Psilocin Cocaine Cocaethylene Methadone PCP Pheniramine Diphenhydramine Oxycodone Thebaine Fentanyl Codeine Morphine 6-monoacetylmorphine Heroin Noscapine Papaverine	**CZE** Capillary: 67 cm x 50 μm; 25 kV, 25 ºC BGE: 100 mM sodium dihydrogen phosphate (pH 6.0) + 20 mM β -CD + 5 % acetonitrile + 20 % isopropanol	UV	5 - 30 ng/mL [b]	Urine	SI-SPE [c]	[24]

Table 6. Overview list of CE methodologies for heroin determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Caffeine Theophylline Barbital Phenobarbital Morphine O^6-monoacetylmorphine O^3- monoacetylmorphine Codeine Ephedrine Heroin Amphetamine Acetylcodeine Methamphetamine Thebaine Papaverine Narcotine	**MEEKC** Capillary: 40 cm x 75 μm; 20 kV, 25 ºC BGE: 89.8 % 5 mM sodium tetraborate (pH 9.5) + 3.3 % SDS + 6.0 % 1-butanol + 0.9 % octane	UV	1 - 1.5 μg/mL [a]	-	-	[28]
Amphetamine Methamphetamine 3,4-methylenedioxyamphetamine 3,4-methylenedioxymethamphetamine 3,4-methylenedioxyethylamphetamine Cocaine Codeine Heroin Morphine 6-monoacethylmorphine	**CEC** Capillary: 33 cm x 75 μm, packed 3 μm Pinnacle II Cyano (cyano silica stationary phase; 10 kV, 20 ºC Mobile phase: 20 mM sodium phosphate (pH 2.5) + acetonitrile (80/20, v/v)	UV	5 - 12 ng/mL [a]	Urine	SPE	[29]
Amphetamine Ketamine Methamphetamine 3,4-methylenedioxyamphethamine 3,4-methylenedioxymethamphethamine Acetylcodeine Codeine Heroin	**FASS-CZE** Capillary:60 cm x 75 μm; 20 kV, 25 ºC BGE: 30 mM PBS (pH 2.0) + 15 % v/v ACN	UV	0.015 - 0.105 μg/mL [b]	Urine	MSPE	[27]

Table 6. Overview list of CE methodologies for heroin determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Heroin Morphine Codeine 6-acetylmorphine	**CSEI-sweeping-MEKC** Capillary: 38 cm x 50 µm; 20 kV, 25 ºC BGE: 20 mM sodium dihydrogenphosphate (pH 2.5) + 80 mM SDS	UV	10 ng/mL [b]	Urine	LLE	[59]

[a] standard samples
[b] real samples

In order to increase the sensitivity, on-line preconcentration techniques have also been employed [60,65]. For example, Jong *et al*. [60] developed a method for the simultaneous determination of heroin and its metabolites in urine samples. To achieve an enhancement in the sensitivity of the analysis, the authors used CSEI-sweeping-MEKC. In that work, urine samples of addicts were analysed after a LLE pretreatment and the LODs achieved were of 10 ng/mL. Lower LODs were achieved by Lu *et al.* [65] in standard samples by the use of MS as the detection system in a method in which several drugs of abuse including heroin were determined without a preconcentration strategy. Even though the validation of the method was carried out for standard samples, the applicability of the method was demonstrated in urine samples.

6.2 Methadone

Methadone (MET) is a synthetic opiate agonist that was first synthesized in Germany in 1937 and is used in drug addict maintenance programs and the management of severe pain. Side effects of methadone are similar to those caused by other opiates but there may be a greater impact on the cardiovascular system. When compared with morphine, methadone causes a greater degree of cardiovascular depression, including greater and more prolonged reductions in heart rate and cardiac index, a greater increase in systemic vascular resistance, and a greater decrease in oxygen partial pressure.

As can be observed in Table 7, separation of methadone and metabolites has mainly been reported by CZE [12,13,25,27,54,56,66,67]. However, a paper using a methodology based on MEKC has also been reported [68]. In all of these studies, UV detection has been the preferred choice [13,25,67,68], although MS [12,54,56] and ECL [66] have also been employed. With respect to the analysis of real samples, a few papers have been reported for the determination of this drug or metabolites in serum [68], urine [25,27,56], hair [54], saliva [13] and plasma [12] after a DLLME, LLE, SPE procedure or simple dilution of the sample.

An interesting approach for a fast separation of MET and its two major metabolites, 2-ethylidene-1,5-dimethyl 3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP), was reported by Kelly *et al*. [67], who used dynamic doubly coated capillaries. These capillaries, which result in pH-independent EOF, allowed the possibility of very fast cationic separations. Therefore, even under low pH conditions, the provision of a considerable EOF enhances the apparent mobility of charged basic drugs, reducing the analysis time to around 3.6 minutes.

In order to increase the sensitivity in CE for the determination of methadone and metabolites, some authors have developed methodologies by using preconcentration techniques, such as in-line SPE-CE [56]

Table 7. Overview list of CE methodologies for methadone determination

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Pethidine Methadone	**CZE** Capillary: 67.5 cm x 25 µm; 14 kV, 25 ºC BGE: 30 mM sodium dihydrogen phosphate (pH 6.0)	ECL	0.5 µM [a]	-	-	[65]
(R,S)-Methadone (R,S)-2-ethylidene-1,5-dimethyl-3,3 diphenylpyrrolidine (R,S)-2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline	**CZE** Capillary: 65 cm x 50 µm; 30 kV, 25 ºC BGE: 100 mM sodium dihydrogen phosphate (pH 2.6)	UV	-	-	-	[66]
(R,S)-Methadone (R,S)-2-ethylidene-1,5-dimethyl-3,3 diphenylpyrrolidine	**CZE** Capillary: 40.2 cm x 50 µm; 20 kV, 20 ºC BGE: 50 mM sodium dihydrogen phosphate (pH 4.5) + 0.2 % HS-γ-CD	UV	2.3 - 2.4 ng/mL [b]	Saliva	LLE	[13]
(R,S)-3,4-methylene-dioxymethamphetamine (R,S)-Methadona	**CZE** Capillary: 40.2 cm x 50 µm; 20 kV, 20 ºC BGE: 15 mM ammonium formate (pH 2.5) + 0.08 % HS-γ-CD	MS	<0.25 µg/mL [a]	Plasma	LLE	[12]
Amphetamine Methamphetamine Ephedrine Psilocin Cocaine Cocaethylene Methadone PCP Pheniramine Diphenhydramine Oxycodone Thebaine Fentanyl Codeine Morphine 6-monoacetylmorphine Heroin Noscapine Papaverine	**CZE** Capillary: 67 cm x 50 µm; 25 kV, 25 ºC BGE: 100 mM sodium dihydrogen phosphate (pH 6.0) + 20 mM β-CD + 5 % acetonitrile + 20 % isopropanol	UV	5 - 30 ng/mL [b]	Urine	[c] SI-SPE	[24]

Table 7. Overview list of CE methodologies for methadone determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Morphine Codeine 6-monoacetylmorphine Ethylmorphine Fentanyl Pethidine Buprenorphine Nalbuphine Dextromethorphan Methadone 2-ethylidene-1,5-dimethyl-3,3 diphenylpyrrolidine D-Propoxyphene Anphetamine Methamphetamine 3,4-methylenedioxyamphethamine 3,4-methylenedioxymethamphethamine 3,4-methylenedioxyethylamphethamine N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine Ephedrine Pseudoephedrine Norephedrine Methylphenidate Cocaine Cocaethylene Anhydroecgonine methyl ester Metoprolol Procaine Ketamine Trimipramine	**CZE** Capillary: 80 cm x 50 μm; 30 kV, 25 ºC BGE: 20 mM ammonium formate (pH 2.5)	MS	0.25 - 10 ng/mL [b]	Urine	DLLME	[26]
3,4-methylene-dioxyamphetamine 4-methylenedioxy-methamphetamine Methadone Cocaine Morphine Codeine 6-monoacetylmorphine	**CZE** Capillary: 100 cm x 75 μm; -15 kV, 25 ºC BGE: 50 mM ammonium phosphate (pH 6.5)	TOF-MS	-	Hair	Digestion and LLE	[53]

Table 7. Overview list of CE methodologies for methadone determination (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
2-ethylidene-1,5-dimethyl-3,3 diphenylpyrrolidine 6-acetylmorphine Codeine Hydrocodeine	**In-line SPE-CZE** Capillary: 100 cm x 50 µm; 30 kV, 25 ºC BGE: 60 mM ammonium acetate (pH 3.8)	MS	13 - 210 ng/L [b]	Urine	Dilution	[55]
Methadone 2-ethylidene-1,5-dimethyl-3,3 diphenylpyrrolidine 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline	**CSEI-Sweeping-MEKC** Capillary: 60 cm x 50 µm; -15 kV, 25 ºC BGE: 100 mM phosphoric acid (pH 4) + 100 mM SDS + 20 % tetrahydrofuran	UV	200 - 400 ng/L [a]	Serum	LLE	[67]

[a] standard samples
[b] real samples
[c] sequential injection-solid phase extraction

and CSEI-sweeping [68], or by the use of a sensitive detection system, such as MS [12,56]. For example, Wang *et al*. [68] reported a method based on CSEI and sweeping-MEKC for the determination of methadone and its two metabolites in human serum samples. The LODs achieved were between 200 and 400 ng/L in standard samples. Botello *et al*. also [56] reported an interesting approach for the determination of EDDP and other drugs of abuse in urine samples. In this case, in order to increase the detection sensitivity of conventional CE-UV, an in-line SPE method was applied and also hyphenation with MS was performed. To achieve this, the SPE extractor device was filled with Oasis HLB sorbent and inserted into the inlet section of the capillary. Due to the use of a highly selective detector such as MS, it was possible to apply the developed method to 10-fold diluted urine samples. The LODs achieved were between 13 and 210 ng/L.

7. Cocaine

Cocaine is an alkaloid of the Erythroxylum coca plant. It is a stimulant of the central nervous system, an appetite suppressant and a topical anaesthetic. Cocaine and its metabolites, in their unchanged form, are excreted in the urine. This drug has two metabolic pathways: hydrolysis (non-enzymatic or enzymatic with esterases) and oxidation with oxidases. The former case occurs in blood and tissues and the metabolic products are ecgonine methyl ester and benzoylecgonine. In the latter metabolic pathway, the main product is norcocaine. The use of cocaine leads to increasing levels of alertness, temperature and euphoria, rapid breathing and rapid pulse rate. Long term effects involve cardiovascular complications, loss of appetite, restlessness, insomnia, increasing the spread of human immunodeficiency virus (HIV) infection and drug-resistant tuberculosis, among others [69,70].

As shown in Table 8, separation of cocaine and metabolites has been reported by the use of different CE modes, such as CZE [21,25,27,54,57,71], CEC [30,61] and MEKC [72]. In all of these studies, UV detection has been the preferred choice [57,61,72], although MS [21,27,54,71] has also been employed. The analysis of real samples has been reported in human urine [27,71,72], horse urine [61], hair [21,54] and surface water samples [57] after an appropriate pretreatment procedure. For the extraction of heroin and metabolites from the studied matrices, LLE [21,54,71], SPE [72], DLLME [27] and SDME [61] have been used.

Da Costa *et al*. [71] described a method for the simultaneous determination of cocaine and five metabolites in human urine using CE-MS via electrospray ionization. The LODs obtained were between 100 and 250 ng/mL. The method was applied to urine samples and, in the case of positive samples, the presence of cocaine and its metabolites was

Table 8. Overview list of CE methodologies for cocaine

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Cocaine Benzoylecgonine Cocaethylene Anhydroecgonine Anhydroecgonine methyl ester Ecgonine methyl ester	**CZE** Capillary: 80 cm x 50 µm; 30 kV, 25 ºC BGE: 1 M formic acid (pH 1.9)	MS	100 - 250 ng/mL [a]	Urine	LLE	[70]
Morphine Codeine 6-monoacetylmorphine Ethylmorphine Fentanyl Pethidine Buprenorphine Nalbuphine Dextromethorphan Methadone 2-ethylidene-1,5-dimethyl-3,3 diphenylpyrrolidine D-Propoxyphene Anphetamine Methamphetamine 3,4-methylenedioxyamphethamine 3,4-methylenedioxymethamphethamine 3,4-methylenedioxyethylamphethamine N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine Ephedrine Pseudoephedrine Norephedrine Methylphenidate Cocaine Cocaethylene Anhydroecgonine methyl ester Metoprolol Procaine Ketamine Trimipramine	**CZE** Capillary: 80 cm x 50 µm; 30 kV, 25 ºC BGE: 20 mM ammonium formate (pH 2.5)	MS	0.25 - 10 ng/mL [b]	Urine	DLLME	[26]

Table 8. Overview list of CE methodologies for cocaine (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Amphetamine Methamphetamine 3,4-methylenedioxyamphethamine 3,4-methylenedioxymethamphethamine Ephedrine Cocaine Morphine Codeine 6-monoacetylmorphine Benzoylecgonine	**CZE** Capillary: 100 cm x 75 μm; 15 kV, 20 ºC BGE: 10 mM sodium dihydrogen phosphate (pH 4.5)	MS	0.006 - 0.100 ng/mg [b]	Hair	Digestion and LLE	[20]
Amphetamine Methamphetamine Ephedrine Psilocin Cocaine Cocaethylene Methadone PCP Pheniramine Diphenhydramine Oxycodone Thebaine Fentanyl Codeine Morphine 6-monoacetylmorphine Heroin Noscapine Papaverine	**CZE** Capillary: 67 cm x 50 μm; 25 kV, 25 ºC BGE: 100 mM sodium dihydrogen phosphate (pH 6.0) + 20 mM β -CD + 5 % acetonitrile + 20 % isopropanol	UV	5 - 30 ng/mL [b]	Urine	SI-SPE [c]	[24]
3,4-methylene-dioxyamphetamine 4-methylenedioxy-methamphetamine Methadone Cocaine Morphine Codeine 6-monoacetylmorphine	**CZE** Capillary: 100 cm x 75 μm; -15 kV, 25 ºC BGE: 50 mM ammonium phosphate (pH 6.5)	TOF-MS	-	Hair	Digestion and LLE	[53]

Table 8. Overview list of CE methodologies for cocaine (Cont.)

Substance	CE conditions	Detection	LOD	Sample	Sample pretreatment	Ref.
Amphetamine Methamphetamine 3,4-methylenedioxyamphetamine 3,4-methylenedioxymethamphetamine 3,4-methylenedioxyethylamphetamine Cocaine Codeine Heroin Morphine 6-monoacethylmorphine	**CEC** Capillary: 33 cm x 75 μm, packed 3 μm Pinnacle II Cyano (cyano silica stationary phase; 10 kV, 20 ºC Mobile phase: 20 mM sodium phosphate (pH 2.5) + acetonitrile (80/20, v/v)	UV	5 - 12 ng/mL [a]	Urine	SPE	[29]
Ephedrine Cocaine Strychnine Morphine Caffeine Theophylline Piroxicam	**OT-CEC** Capillary: 57 cm x 50 μm; 15 kV, 25 ºC BGE: 20 mM sodium tetraborate	UV	0.94 - 17.64 ng/mL [a]	Horse urine	SDME	[60]
Cocaine 2-ethylidene-1,5-dimethyl-3,3 diphenylpyrrolidine Codeine 6-acetyl morphine	**In-line SPE-CZE** Capillary: 65.5 cm x 50 μm; 30 kV, 25 ºC BGE: 80 mM disodium phosphate anhydrous + 6 mM HCl (pH 3)	UV	70 - 270 ng/L [b]	Tap and river water	Filtration	[56]
Cocaine Benzoylecgonine Norcocaine Cocaethylene	**CSEI-Sweeping-MEKC** Capillary: 60 cm x 50 μm; -20 kV, 25 ºC BGE: 100 mM phosphoric acid (pH 1.8) + 75mM SDS + 10 % 2-propanol + 10 % tetrahydrofuran	UV	29.7 - 236 ng/L [a]	Urine	SPE	[71]

[a] standard samples
[b] real samples

further confirmed by MS/MS experiments. However, Kolher *et al*. [27] obtained lower LODs in urine samples by the combination of dispersive liquid-liquid microextraction (DLLME) with CE and a MS-TOF for the toxicological screening of drugs of abuse. The performance of the entire procedure was evaluated using CE-ESI-TOF-MS with a preconcentration factor of more than 130. The highly sensitive DLLME-CE-ESI-TOF-MS method allowed the detection of 30 toxicological compounds in urine with LODs in the sub-ng/mL level.

Different methodologies for increasing sensitivity in CE have also been employed [57,72]. Su *et al*. [72] used CSEI-sweeping-MEKC for the determination of cocaine and its metabolites in urine samples. The sensitivity enhancements for cocaine, norcocaine, and cocaethylene ranged from 20,600- to 39,600-fold while for benzoylecgonine, the SEF was 1,750-fold. The LODs obtained by CSEI-sweeping-MEKC were in the range of 29.7 to 236 ng/L. After an optimization process, cocaine in a human urine sample was analysed. To achieve this, an off-line SPE was performed in order to minimize the influence of the matrix. Recently, the use of a chromatographic preconcentration technique has been reported by Botello *et al*. [57] for the determination of cocaine and other drugs of abuse in water samples. In order to increase the detection sensitivity of conventional CE-UV, an in-line SPE-CE method was applied. In this case, the SPE extractor device was filled with Oasis HLB sorbent and inserted into the inlet section of the capillary. Using this method, the SEFs achieved were around 2,300- to 5,300-fold compared to normal CE. The applicability of the developed method was demonstrated in tap and river water samples which were directly analysed without any off-line pretreatment and the LODs were between 70 and 270 ng/L.

8. Concluding remarks

The potential of CE for the determination of drugs of abuse in different matrices has been shown. As can be observed, CZE is still the most commonly used CE separation mode. However, for some kinds of compounds, MEKC and CEC have been also employed. Due to the lack of sensitivity of CE when a UV detector is used, some papers have reported the use of preconcentration techniques. Several detection systems have been also coupled to CE as an alternative to UV detection. For instance, the use of MS, LIF, C^4D, EC, FL, ED and ECL have been employed for the determination of drugs of abuse with good results. The amount of applications to the analysis of drugs of abuse confirms that CE is now a widely accepted alternative for the analysis of this kind of compound in different matrices, mainly biological samples. Since the determination of this group of compounds is also important in environmental samples, we believe that future trends in CE applications should address this issue.

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1.3. References

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SENSITIVITY ENHANCEMENT STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF DRUGS OF ABUSE AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS
Igor Botello González
Dipòsit Legal: T. 1299-2012

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2. OBJECTIVE

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The objective of this Doctoral Thesis is the development of analytical methods to increase sensitivity in capillary electrophoresis for the determination of pharmaceuticals and drugs of abuse in environmental and biological samples. To do that, several preconcentration techniques either stacking (based on isotachophoresis principles) or chromatographic (based on in-line SPE) have been studied. The compounds selected for the different studies in this Doctoral Thesis belong to the pharmaceutical compound group (in particularly in non-steroidal anti-inflammatory drugs (NSAIDs)) and to the drugs of abuse group.

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3. EXPERIMENTAL, RESULTS AND DICUSSION

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As mentioned in the Introduction section, EOCs are pollutants that include a diverse range of compounds such as pharmaceuticals, drugs of abuse, personal care products, steroids and hormones, surfactants and flame retardants, among others. The constant emission of these compounds into the environment leads to continuous but often overlooked adverse effects on aquatic and terrestrial organisms. Therefore, determining their presence is an essential preliminary step in order to measure their concentration levels in different ecosystems. The determination of these compounds is also very important in biological samples. The abuse of drugs, for example, can lead to physical and mental damage and in some cases, dependence and addiction may develop. Thus, the determination of these drugs is of great importance in fields such as clinical, forensic and doping control.

CE is a highly promising analytical separation technique with a wide range of applications in biological and environmental fields. However, CE suffers from a lack of sensitivity when compared with chromatographic techniques because, in general, it is not possible to inject high sample volumes and also due to the short optical path-length for on-capillary detection. In order to overcome this issue, in recent decades, a number of strategies for improving sensitivity in CE have been reported in the literature. The most commonly used approaches involve the development of different preconcentration strategies and the use of more sensitive detection systems, such as MS.

The research included in this Thesis was carried out with the aim of developing preconcentration methodologies in CE in order to improve LODs for the determination of pharmaceuticals and drug of abuse in different samples. These more sensitive analytical methods in CE can provide adequate methodologies when these kinds of compounds are present in biological and environmental samples at low concentration levels.

The present chapter includes the experimental part, results and discussion from the different studies that have been carried out through the course of the Doctoral Thesis period. These studies have been classified into two sections and, for each one, a brief introduction is included to establish the context of the research. Moreover, the most remarkable results are also discussed at the end of each section. The results are presented in article format and they have already been published or are in process of being published in several international scientific journals. In addition, the list of all the articles derived from this Doctoral Thesis research project is included in Appendix III.

The first section includes three studies related to different preconcentration techniques based on t-ITP for the determination of NSAIDs and various types of drugs of abuse in environmental waters and biological samples. In the case of

NSAIDs, two different preconcentration strategies were used: transient pseudo-ITP and EKS. The methods developed were validated with the analysis of urine and plasma samples in the case of the first strategy and with the analysis of river water and plasma samples in the case of the second strategy. EKS was also studied for the determination of barbiturate drugs in urine samples and, in this study, the influence of different parameters affecting the EKS performance were evaluated using both univariated and multivariated optimization processes.

The second section involves the development of different methods based on the in-line coupling between SPE and CE for the determination of various types of drugs of abuse in environmental and biological samples. In particular, three different studies are presented in this section and in all cases, Oasis HLB was the sorbent used to construct the analyte concentrator devices. In two of these studies, UV was employed as the detection system and, in the third case, detection sensitivity and selectivity was improved by coupling the in-line SPE-CE system to a MS detector. In addition, for these studies, the developed methodologies were applied to the analysis of environmental water (tap and river waters) and biological samples (urine).

All of the studies reported in this Doctoral Thesis were financially supported by the General Research Directorate of the Spanish Ministry of Science and Technology, projects CTM2008-06847-C02-01/TECNO, CTM2008-0825, CTQ2011-24179, and by *Generalitat de Catalunya, Departament d'Innovació, Universitats i Empreses*, project 2009 SGR 223.

3.1. Sensitivity improvement in capillary electrophoresis by transient isotachophoresis based techniques for the determination of drugs

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The determination of pharmaceuticals and drugs of abuse and their metabolites in environmental waters and in biological fluids is important in fields such as doping control and forensic analysis [1,2]. Although LC and GC are the most commonly used techniques for the determination of these compounds and their metabolites in environmental and biological samples, CE offers an excellent and promising alternative [1-3]. Since the major limitation in CE is related to the lack of sensitivity, over recent decades, efforts have focused on the development of numerous approaches to overcome this issue [4-6].

Among the different strategies for increasing sensitivity in CE, an interesting approach is based on t-ITP stacking techniques. As mentioned in previous sections, in this kind of strategy, the sample zone is stacked between the LE and TE zones that are created in the capillary [6,7]. Two important approaches based on t-ITP principles are worth highlighting. The first is known as transient pseudo-ITP and the second approach is EKS. Even though both strategies are based on the same principles, in the case of EKS, electrokinetic injection of the sample is performed. Based on the encouraging results with respect to the increase of sensitivity reported in the literature when using both approaches, they were selected in this Doctoral Thesis in order to evaluate their potential in the preconcentration of pharmaceuticals and drugs of abuse by CE in biological and environmental analysis.

In this section, the results of three studies which focused on the determination of pharmaceuticals and drugs of abuse in both environmental waters and biological samples are presented. The selection of the target compounds for the different studies included in this section was based on their occurrence and their environmental and health impact. In the first and second studies, a group of NSAIDs (diclofenac, fenoprofen, naproxen, ketoprofen and piroxicam) were selected while in the third study, three barbiturate drugs (barbital, phenobarbital and secobarbital) were chosen. The molecular structures of the different compounds are presented in Appendix II.

The first study focused on the enhancement of sensitivity by using transient pseudo-ITP as the preconcentration strategy. This technique overcomes one of the problems encountered in the case of most of the preconcentration techniques that exist, namely the negative effect produced by the high ionic strength of some samples, such as biological samples. With this in mind, several authors [8,9] have added an organic solvent to the sample to decrease its conductivity and overcome the limitation associated with the presence of a high salt content. Hence, the focus of this study was the development of a sensitive method for determining a group of NSAIDs in samples with a different complexity. In this stacking technique, many factors related to the sample and the BGE affect the solubility, ionization and migration velocities of the different compounds during transient pseudo-ITP preconcentration and the

subsequent separation. As a result, the main parameters affecting the preconcentration performance were studied, such as BGE composition, sample injection, sample matrix composition and pH. To the best of our knowledge, this study represents the first time that transient pseudo-ITP has been used as a preconcentration strategy for this purpose.

In the second study, the performance of a methodology based on EKS was evaluated for the preconcentration and separation of the abovementioned NSAIDs. EKS is based on the combination of EKI and t-ITP, and it is important to note that while EKI is strongly dependent on the sample conductivity, t-ITP acts regardless of this property of the sample. In addition, in intrinsic EKI, the injected amount of analytes is limited to a certain extent, but the t-ITP process allows the introduction of much larger sample amounts. Therefore, it can be concluded that, when combining EKI and t-ITP, the latter remedies the shortcomings of the former and also allows higher increases in sensitivity compared with the sole application of EKI or t-ITP [6]. Thus, EKS as a preconcentration technique makes the analysis of different kinds of matrices possible, such as biological samples with high conductivity, a factor which negatively affects many other preconcentration techniques. Three different possible setups of the EKS technique were assayed. These setups were selected because of the differences between them with respect to the design of the discontinuous electrolyte system. After selecting the setup that provides the best performance, the evaluation of the different parameters affecting the separation and preconcentration efficiency was performed in order to get the maximum preconcentration factors. The optimized variables were the sample pH, the concentration of the leading stacker, BGE composition, the electrokinetic injection time of the sample, the composition and hydrodynamic injection of the solvent plug and of the terminating stacker. In this study, a validation for standards as well as for river water and human plasma samples was performed demonstrating the applicability and reliability of the proposed methodology.

The last study consisted of the development of an EKS-based method for the determination of barbital, phenobarbital and secobarbital in urine samples. In order to determine the optimal performance conditions, the influence of different parameters affecting the preconcentration were evaluated, using both univariated and multivariated optimization processes. The parameters studied were sample pH, concentration and length of the leading and terminating electrolytes, electrokinetic injection of the sample and composition and hydrodynamic injection of the solvent plug. To the best of our knowledge, this preconcentration technique has not been previously reported in the literature for the determination of barbiturates.

The results of these three studies have been published or submitted for publication in different analytical journals: *Electrophoresis 2010, 31, 2964-2973, Anal. Bioanal. Chem. 2011, 400, 527-534,* and *J. Sep. Sci., 2012* (submitted).

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SENSITIVITY ENHANCEMENT STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF DRUGS OF ABUSE AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS
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Dipòsit Legal: T. 1299-2012

3.1.1 Simultaneous determination of weakly ionizable analytes in urine and plasma samples by transient pseudo-isotachophoresis in capillary zone electrophoresis.

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SIMULTANEOUS DETERMINATION OF WEAKLY IONIZABLE ANALYTES IN URINE AND PLASMA SAMPLES BY TRANSIENT PSEUDO-ISOTACHOPHORESIS IN CAPILLARY ZONE ELECTROPHORESIS

Igor Botello, Francesc Borrull, Marta Calull, Carme Aguilar
Departament de Química Analítica i Química Orgànica. Facultat de Química. Universitat Rovira i Virgili. Marcel·lí Domingo s/n, 43007 Tarragona, Spain

Abstract

A rapid method for the simultaneous determination of several non-steroidal anti-inflammatory drugs (NSAIDs) in human plasma and urine has been developed using transient pseudo-isotachophoresis in capillary zone electrophoresis (CZE). The influence of different parameters on resolution and preconcentration efficiency such as BGE composition, sample injection, sample matrix composition and pH were studied in order to optimize transient pseudo-ITP performance. Optimized conditions were reached with a background electrolyte (BGE) consisting of 100 mM $Na_2B_4O_7$ in 10% of MeOH aqueous solution and with the hydrodynamic injection of the sample at 50 mbar for 90 seconds. The sample was prepared in a solution mixture of 1% NaCl/ethanol (30/70 v/v) at pH = 10. Our results show that this simple strategy offers improved sensitivity compared to conventional CZE analysis, reaching a 45-fold preconcentration factor. The detection limits (LODs) were as low as 0.07 mg/L for standard samples with good repeatability (values of relative standard deviation, %RSD < 11%). The method was applied to the analysis of NSAIDs in biological samples. Validation for human plasma and urine samples demonstrated good linearity, low detection limits and satisfactory repeatability values.

Keywords: *Capillary electrophoresis, non-steroidal anti-inflammatory drugs, plasma samples, transient pseudo-isotachophoresis, urine samples*

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are pharmaceuticals with antirheumatic, analgesic, anti-pyretic and anti-inflammatory (in higher doses) effects. These compounds are weak acids with pK_a values ranging between 3 and 7 [1].

The use of NSAIDs in human and veterinary medicine dates to the latter half of the 19th century [1]. The determination of these compounds in biological fluids yields useful information for assessing their safety, therapeutic effect and mechanism of action. The expected levels of these drugs in serum samples are close to 1 mg/L [2, 3]. For example, Hundal *et al*. [4] report a concentration range of piroxicam in human plasma after oral administration of between 0.5 and 8.3 mg/L.

NSAIDs have been analyzed by means of various different capillary electrophoresis (CE) modes including CZE, MEKC, MEEKC, CEC and ITP [5-10]. It is commonly recognized that the main drawback of CE is its poor sensitivity, particularly when compared to chromatographic techniques. However, in recent years, with the development of several on-line preconcentration techniques, CE has become a useful and powerful separation technique [7-15]. For example, Dawod *et al*. [8] used counter-flow electrokinetic supercharging (EKS) to determine non-steroidal anti-inflammatory drugs in water samples, yielding detection limits in the ng/L range. More recently, Kuo *et al*. [9] used microemulsion electrokinetic chromatography (MEEKC) coupled with field-amplified sample injection to determine NSAIDs, also in water samples. The method provided preconcentration factors between 1400 and 6100-fold, reaching LODs in the range of 0.03–0.3 μg/L.

However, the efficacy of most of preconcentration techniques can be affected by the characteristics of the sample being analyzed, especially in the case of sample matrices with high ionic strengths, such as many environmental and biological samples. A high concentration of salts or proteins greatly influences the performance, resolution and quantification of the CE analysis. These sample components can cause band-broadening and lack of sensitivity in the separation process – effects that become more pronounced as the size of the sample in the capillary volume increases [16, 17].

Several of the authors included in the bibliography added an organic solvent to the sample to decrease its conductivity and thereby solve the limitation associated with the presence of a high salt content [18-21]. For example, Shihabi [16] demonstrated the applicability of this method in the determination of weakly cationic compounds such as tyramine, dopamine, and metanephrine in serum samples. This strategy has been called transient pseudo-isotachophoresis (transient pseudo-ITP) and it makes use of a sample matrix consisting of a mixture of salt and an organic solvent, mainly aceto-

nitrile (ACN). A large volume of sample is injected into the capillary, which has previously been filled with the background electrolyte (BGE). After the sample has been injected, the sample vial is replaced with the BGE vial and separation voltage is applied. The salt coion (e.g. chloride for anionic separation) present in the sample zone acts as the leading ion, and the organic solvent works as a terminating electrolyte. The presence of an organic solvent in the sample generates low conductivity zones (high field strength) which increase analyte velocities to achieve stacking. Kong [22] recently proposed the possible mechanism of transient pseudo-ITP. The author stated that after applying the voltage, the ions migrate faster in the sample zone than in the BGE zone. When these ions move through the sample zone and reach the BGE, they do so at a reduced migration velocity and they stack.

Transient pseudo-ITP has been used to determine different compounds. For example, Shihabi [19] studied the mechanism of this method using tyrosine and theophylline as model compounds. In that work, 20% of the capillary volume was filled with the sample, which contained Cl^- as the leading ion and acetonitrile (ACN) as the pseudo-terminating ion. The optimum concentration of ACN was found in the range of 50–70%, while for the leading ion, good separations were observed when chloride was present in the sample in the range of 0.2–1%. Shihabi [18] also applied a similar method using a longer capillary length for the determination of mycophenolic acid in serum samples. The author compared the results for standard samples using capillaries of different lengths. With a capillary length of 50 cm, 9.7% of the capillary volume was filled with sample without detriment to the separation efficiency. On the other hand, when a longer capillary (80 cm) was used, injections of up to 21% of the capillary volume were possible, increasing the sensitivity of the method and reaching 70-fold preconcentration factors. Kubalczyk *et al*. [23] used transient pseudo-ITP for the determination of homocysteine in plasma samples. After reduction and derivatization, the plasma samples were mixed with ACN 1:2 (v/v) and injected into the capillary as a long plug. In that work, the LOD was set at the level of 1 μmol /L. Friedberg *et al*. [24] demonstrated the application of transient pseudo-ITP in the determination of ketoprofen in serum samples. These samples were mixed with ACN and centrifuged for 30 s at 14 000 g. The supernatant was then removed and injected into the capillary, filling 10% of the capillary volume. The LOD for ketoprofen using this approach was 1 mg/L.

Many factors related to the sample and the BGE – such as ionic strength, pH and the type of salts added – affect the solubility, ionization and migration velocities of the different compounds during transient pseudo-ITP preconcentration and the subsequent separation. Consequently, stacking can be different for each compound

even when the same conditions are applied [17, 18].

The main objective of our paper is to propose a fast and simple capillary electrophoresis method based in transient pseudo-ITP-CZE for the simultaneous determination of five NSAIDs in human plasma and urine samples. To the best of our knowledge, this method has not been applied previously for this purpose.

2. Experimental

2.1 Standards and reagents

All reagents were of analytical reagent grade. Ultrapure reagent water purified by a MilliQ gradient system (Millipore, Bedford, MA, USA) was used throughout. Naproxen, fenoprofen, diclofenac, ketoprofen, piroxicam, sodium chloride (99.999%), hexadimethrine bromide (HDMB) and disodium tetraborate anhydrous were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethanol, 2-propanol, acetonitrile and methanol were purchased from SDS (Peypin, France).

Standard stock solutions of 1000 mg/L were prepared in methanol for each NSAID and stored at 4 ºC. Standard solutions of the mixture of all the compounds were diluted in ultrapure water at a concentration of 100 mg/L.

The separation buffer (BGE), consisted of 100 mM disodium tetraborate anhydrous and 10% (v/v) MeOH (adjusted to pH 9.4 with 0.1 M NaOH).

2.2 Instrumentation

For electrophoretic separation, we used an Agilent 3D CE (Agilent Technologies, Waldbroon, Germany) equipped with UV diode-array detection (DAD). The detection of the NSAIDs was performed at 214 nm.

A fused silica capillary (Agilent Technologies, Waldbroon, Germany), 88.5 cm in length (80 cm effective length) with an internal diameter (ID) of 50 μm was used. The capillary chamber was set at 25 ºC in all experiments.

2.3 Transient pseudo-ITP

New capillaries were conditioned with 1M NaOH for 90 min prior to use and daily with 1M NaOH for 10 min and H_2O for 10 min. Between separations the capillary was conditioned with 1M NaOH for 4 min, H_2O for 4 min, HDMB 0.01% in water for 5 min and BGE for 4 min. Injections were performed by placing the sample in the inlet vial and applying 50 mbar for 90 seconds. Reversed polarity of -30 kV was used for the separation of NSAIDs with UV detection. Fresh sample and BGE solutions were used for each experiment.

The transient pseudo-ITP procedure consisted of preparing the working standard samples in a solution mixture of 1% NaCl/ethanol (30/70 v/v) adjusted to pH 10 with 0.1 M NaOH. After hydrodynamic injection of the sample at 50 mbar for 90 seconds, a separation voltage of -30 kV was applied.

2.4 Plasma and urine samples

All plasma and urine samples were obtained from a healthy volunteer in our laboratory. The samples were stored at -18 ºC and then, before the extraction step, the samples were acidified with HCl 1M until pH 2. Sample pretreatment was carried out by adding 1 mL of ethyl acetate/*n*-hexane (20/80 v/v) to 200 μL of spiked sample [25]. After vortex mixing, the sample was centrifuged for 5 min at 9000 rpm. The supernatant solution was collected and evaporated until dry under a gentle stream of dry nitrogen (UHP grade). The residue was reconstituted with a mixture of water/ethanol (30/70 v/v) (200 μL) and adjusted to pH 10, and then transferred to a micro vial for transient-pseudo ITP analysis.

3. Results and discussion

3.1 Separation of NSAIDs by CZE

The separation of the five NSAIDs (naproxen, fenoprofen, diclofenac, ketoprofen and piroxicam) was optimized in a previous work [11]. Briefly, the method consists of a previous preconditioning step by flushing the capillary for 5 min with HDMB 0.01% in MilliQ water for reversing EOF. Optimum conditions for CZE co-electrosmotic separation of the five NSAIDs studied were obtained using 60 mM $Na_2B_4O_7$ (pH = 9.4) with 10% of MeOH at -30 kV of separation voltage. Under these conditions run time was around 10 minutes.

3.2 Transient pseudo-ITP performance

When small volumes of the sample prepared in MilliQ water at pH 6.15 were injected (less than 1% of the capillary) under non-stacking conditions using 60 mM $Na_2B_4O_7$ as BGE and -30 kV of separation voltage, all five compounds obtained good resolution. However, sensitivity was quite poor (Figure 1A). Under these conditions, if injection time is increased to over 10% of the capillary, the overloading of the analytes in the capillary column causes broadened peaks and a loss of resolution (data not shown).

The aim of this work was the simultaneous determination of five NSAIDs at low concentration levels in biological samples, which have a high saline content. In order to decrease the detection limits for these types of complex samples, we selected transient pseudo-ITP as the on-line preconcentration technique because it allows a large volume of the sample to be injected without detriment to the resolution. In the presence of salts in the samples, acetonitrile as well as other water-soluble alcohols can act as "pseudo" terminating ions due to their low conductivity value. This is related to a high field strength that can speed up the migration velocity of the analytes in this region where the terminating ion is present. In our initial experiments, ACN was selected as the "pseudo" terminating ion. NaCl was selected as the leading ion because it is the salt usually present in

biological samples such as plasma and urine [16].

The standard sample was prepared in a manner similar to that reported previously by other authors included in the bibliography, in a mixture of 1% NaCl/ACN (30/70 v/v) in order to induce the transient pseudo-ITP mode [19, 24]. Under these conditions, it is possible to increase the sample volume injected without a loss in resolution. Also, an increase in the detector signal was observed when the injection time of the sample was 50 seconds (Figure 1B) compared to the typical value for injection time in CZE analysis (10 seconds) (Figure 1A).

There are many factors that can affect the transient pseudo-ITP process including BGE composition, sample pH, ionic strength and the type of organic solvent in the sample. Since these factors greatly influence efficiency, resolution and migration, these variables must be studied in order to introduce a high sample volume and thus achieve higher preconcentration factors.

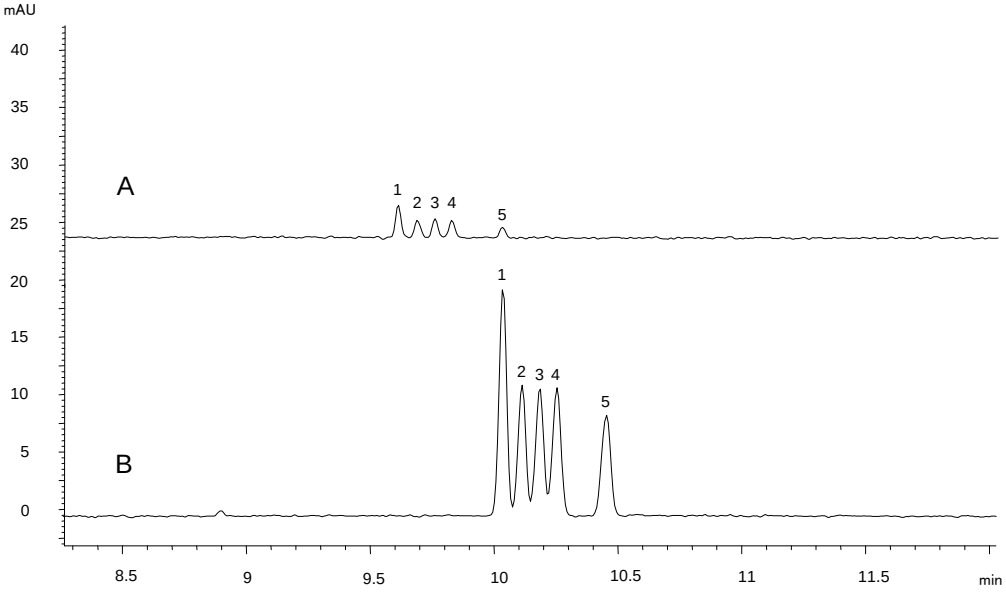

Figure 1. Effect of transient pseudo-ITP on stacking. Standard solution of naproxen (1), fenoprofen (2), diclofenac (3), ketoprofen (4) and piroxicam (5) (5 mg/L) at pH = 6.15. BGE: 60 mM $Na_2B_4O_7$ +10% MeOH (pH = 9.4). Separation voltage of -30 kV. Hydrodynamic injection of: **A)** 50 mbar for 5 seconds (sample prepared in MilliQ water) **B)** 50 mbar for 50 seconds (sample prepared in a mixture of 1% NaCl/ACN (30/70 v/v)).

3.3 Study of the BGE composition

There are many weakly acidic compounds which stack well in ACN in the presence of salts in the sample; however, they require separation buffers with high ionic strengths [16]. As the stacking phenomena in transient pseudo-ITP seems to be related to the difference in analyte velocity between the sample zone and the BGE, stacking efficiency is affected

by BGE composition [22]. In order to verify the influence of this on the transient pseudo-ITP process, different borate concentrations were tested ranging between 60 and 100 mM.
In these experiments, samples were prepared in a mixture of 1% NaCl/ACN (30/70 v/v) at pH = 6.15 and they were injected for 50 seconds at 50 mbar.
Long separation times were obtained through increasing the concentration of the running buffer, but a slight increase in sensitivity enhancement factors (SEFs) was also observed up to a value of 80 mM (data not shown). However, higher concentrations did not increase SEF values. SEF values were calculated according to the equation (1):

$$SEF_{height} = \frac{h_{prec}}{h_{HD}} f \qquad (1)$$

where h_{prec} is the peak height of the preconcentrated analyte, h_{HD} the peak height of the non-preconcentrated analyte detected after a conventional hydrodynamic injection of 10 seconds at 50 mbar, and f is the dilution factor.
As a compromise between resolution and sensitivity we selected 100 mM of $Na_2B_4O_7$ as the optimum BGE concentration. With over 100 mM $Na_2B_4O_7$, current problems occurred, probably due to the increase in the Joule effect. Therefore, 100 mM $Na_2B_4O_7$ + 10% MeOH as a running buffer was selected for further studies.

3.4 Study of sample volume

One of the main advantages of transient pseudo-ITP is that allows a high sample volume to be introduced into the capillary without a loss in resolution or efficiency [16, 18].
In order to examine the effect of injection time on transient *pseudo*-ITP stacking, a range of 50–130 seconds was studied, applying an injection pressure of 50 mbar. After sample injection, a negative voltage of -30 kV was applied for both sample stacking and the subsequent separation. The peak area increased as the injection time increased, as expected (Figure 2). However, injection times exceeding 90 seconds (7.5% of the capillary volume) resulted in poorer resolution and overloading, leading us to establish a 90-second injection time for our study.

3.5 Study of pH and the composition of the sample

According to the Kohlrausch regulating function, the concentration of the stacked sample is proportional to the concentration of the leading ion [26]. When voltage is applied, the anions present in the sample in high concentrations and with high mobilities move ahead of the analytes

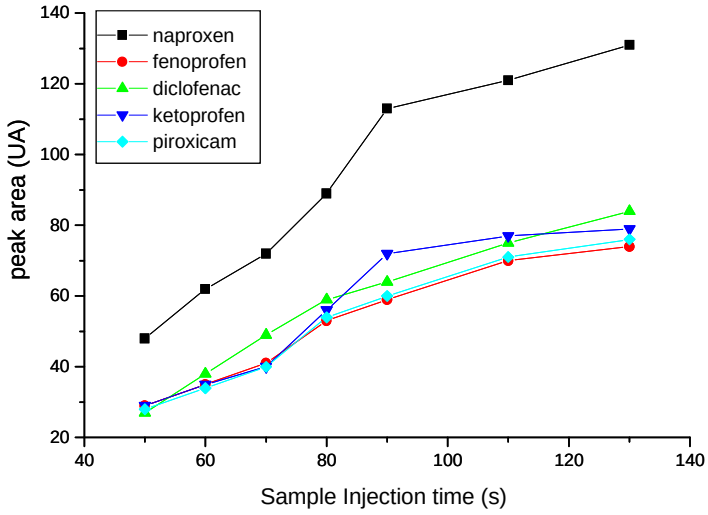

Figure 2. Effect of sample injection time on the sensitivity for the five NSAIDs in transient pseudo-ITP. Standard solution of 3 mg/L prepared in a mixture of 1% NaCl/ACN (30/70 v/v). Other conditions are the same as in Figure 1.

as a wide band acting as a leading electrolyte [22]. The Cl^- ion was selected as the leading ion in this work because its mobility (7.9 10^{-4} cm^2/Vs) is higher than those of the studied NSAIDs (1.52-1.99 10^{-4} cm^2/Vs). In order to study the effect of the salt concentration in the sample on the transient pseudo-ITP process, different solutions of NaCl were prepared at concentrations ranging from 0.2 to 2.5 % (m/v) in MilliQ water. Standard samples were prepared in a mixture of 30% NaCl (for each NaCl solution used in the experiment) and 70% ACN (v/v). In all the experiments the pH was adjusted to 6.5 with 0.1 M NaOH. We found that the peak area values remained constant when the concentration of salt in the sample was increased in the studied concentration range (data not shown). However, in absence of NaCl peaks broadening and lack in resolution showed the important effect of the leading ion in the migration of the analytes. It was not possible to test higher concentrations of NaCl (over 2.5%) due to the formation of two immiscible phases when the standard sample solutions were prepared. Even when increased NaCl concentration in the sample did not significantly affect the transient pseudo-ITP process, we used 1% NaCl/ACN (30/70, v/v) solution in the standard sample preparation for the validation of the method (for standard samples).

As well as ACN, many other water miscible solvents can function as "pseudo" terminating ions in transient pseudo-ITP. Using organic solvents such as ACN and small alcohols in the preparation of the sample

can speed up the velocity of the analytes in this zone due to their low conductivity. The presence of these organic solvents would lead to a marked difference in analyte mobility in the sample zone and in the BGE with the subsequent improvement in the stacking process. Shihabi [19] tested a variety of alcohols and water miscible organic solvents including methanol, ethanol, propanol, isopropyl alcohol and acetone for the determination of tyrosine and theophylline in standard samples and found that all of them produced stacking similar to that of acetonitrile for the studied compounds.

In our work, we tested a few different hydrogen bondable organic solvents (methanol, ethanol and 2-propanol) to study the effect of stacking when different organic solvents are used in sample preparation. In all these experiments, standard samples were prepared in a solution of 1% NaCl/organic solvent 30/70 v/v adjusted to pH 6.15. With methanol the resolution and sensitivity were notably deteriorated. When ethanol and 2-propanol were used, no differences in the stacking effect were observed. However, although the use of ACN tends to be preferred when analyzing biological samples due to its double function as a deproteinization solvent and a "pseudo" terminating ion, in this study we found ethanol to be the optimal organic solvent (data not shown) because it provided higher resolution than ACN. However, it is important to point out that similar stacking was found for all the tested solvents, as reported previously by Shihabi [19].

The studied NSAIDs are weak organic acids with pK_a values between 4 and 6. Therefore, the stacking process is strongly dependent on the acidity of the sample solution. It has been reported that the mobility of weak acids is dependent on the degree of dissociation. Friedberg *et al*. [24] as well as Kong *et al*. [21] stated the importance of sample pH adjustment in order to improve preconcentration enhancement. Due to the large sample volume, pH values have a great influence on ionization and overall separation, including migration and stacking. In our work, the effect of sample pH on transient pseudo-ITP performance was also studied. In the pH range studied, from 6 to 11, we found that the preconcentration was affected by the pH of the medium. At lower pH values (below 6), current losses occurred, possibly due to the mismatch between EOF magnitude in the run buffer and in the sample zone. Higher pH values improved the sensitivity due to the increase in the peak area. However, no significant differences were observed in stacking when pH was above 10 (data not shown). Thus, a pH of 10 was chosen as the optimum pH sample value for further experiments. In these conditions sensitivity enhancement factors were in the range between 40 and 46 fold.

3.6 Validation

Standard solutions for naproxen, feno-

profen, diclofenac, ketoprofen and piroxicam were prepared by diluting appropriate quantities of concentrated stock solutions in 1% NaCl/ethanol (30/70 v/v) solution. The regression equations, the values of repeatability, as well as the RSD, SEFs and LODs for the five NSAIDs are summarized in table 1. Calibration graphs for the studied NSAIDs were linear in the tested range (0.1–20 mg/L). RSD values for repeatability of the peak areas did not exceed 11% when the concentration of the compounds was of 1 mg/L. The accuracy was presented as the ratio of the determined and nominal values of concentration of the studied compounds and then multiplied by 100. For all the compounds the accuracy was between the 97 -100%. The LODs were in the range between 0.07–0.15 mg/L. Figure 3 shows the electropherogram obtained from a standard sample at 5 mg/L following the optimized method.

Table 1. Regression equations, repeatability (% RSD), SEFs and LODs for the five NSAIDs obtained for standard samples, human plasma and urine samples.

	Naproxen	Fenoprofen	Diclofenac	Ketoprofen	Piroxicam
Standard samples					
Linearity (mg/L)	0.1 – 20	0.2 – 20	0.2 – 20	0.2 – 20	0.2 – 20
Calibration curve	y=0.44 + 20.1x	y=0.60 + 10.8x	y=0.97 + 11.8x	y=1.29 + 10.1x	y=0.92 + 7.9x
r^2	0.999	0.999	0.999	0.995	0.998
Repeatability (%RSD)*	6	8	5	10	11
SEF	46	41	46	40	44
LOD (mg/L)	0.07	0.09	0.10	0.11	0.15
Plasma samples					
Linearity (mg/L)	0.5-20	0.5-20	1-20	0.5-20	1-20
Calibration curve	y=0.04 + 3.27x	y=-0.16 + 1.97x	y=-0.39 + 2.01x	y=-0.11 + 2.05x	y=0.08 + 1.56x
r^2	0.992	0.998	0.990	0.994	0.985
Repeatability (%RSD)*	10	11	9	15	14
LOD (mg/L)	0.16	0.20	0.25	0.20	0.26
Urine samples					
Linearity (mg/L)	1-20	1.5-20	1.5-20	1.5-20	1.5-20
Calibration curve	y=1.66 + 12.5x	y=-0.19 + 7.94x	y=-0.25 + 8.35x	y=-0.54 + 7.47x	y=0.57 + 5.44x
r^2	0.998	0.998	0.997	0.994	0.995
Repeatability (%RSD)*	12	10	8	13	13
LOD (mg/L)	0.32	0.56	0.53	0.60	0.75

* interday analysis (n = 3)

3.7 Analysis of real samples

In order to explore the usefulness of transient pseudo-ITP in the analysis of NSAIDs in biological samples we validated the method with human and urine plasma samples.

3.7.1 Human plasma

Deproteinization was first performed with ACN, a procedure that is well documented in the literature [18], and blanks with interfering endogenous

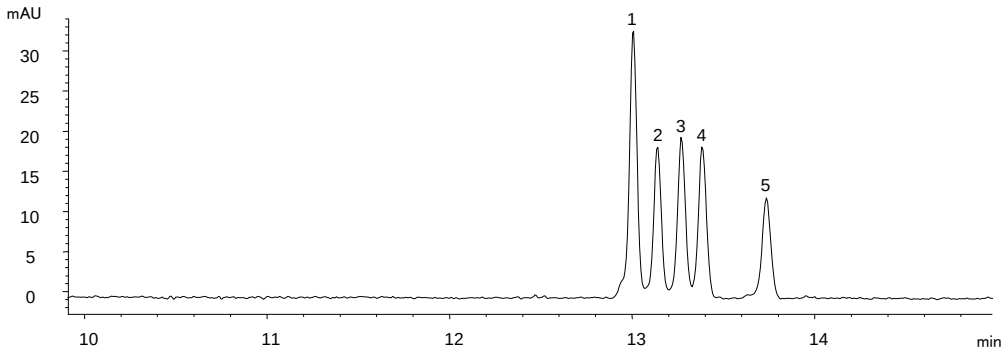

Figure 3. Electropherogram of a standard sample analyzed by transient pseudo- ITP at a concentration of 5 mg/L for the five NSAIDs prepared in 1% NaCl/EtOH (30/70) and injected 90 seconds at 50 mbar. BGE: 100 mM $Na_2B_4O_7$ +10% MeOH at pH = 9.4. Separation voltage of -30 kV. Peak identification as in Figure 1.

compounds were obtained. Next we followed the procedure described in section 2.4, which consists of a simple liquid-liquid extraction using ethyl acetate/*n*-hexane (20/80 v/v). Figure 4A shows the electrophe-rogram obtained from the blank of a human plasma sample following the optimized transient pseudo-ITP method. Figure 4B shows the electropherogram obtained when 200 μL of plasma sample was spiked at a concentration of 2 mg/L for the five NSAIDs after the extraction step. Linearity was determined through triplicate injections of the samples in the range of 0.5 and 20 mg/L. LODs ranged between 0.16 and 0.30 mg/L

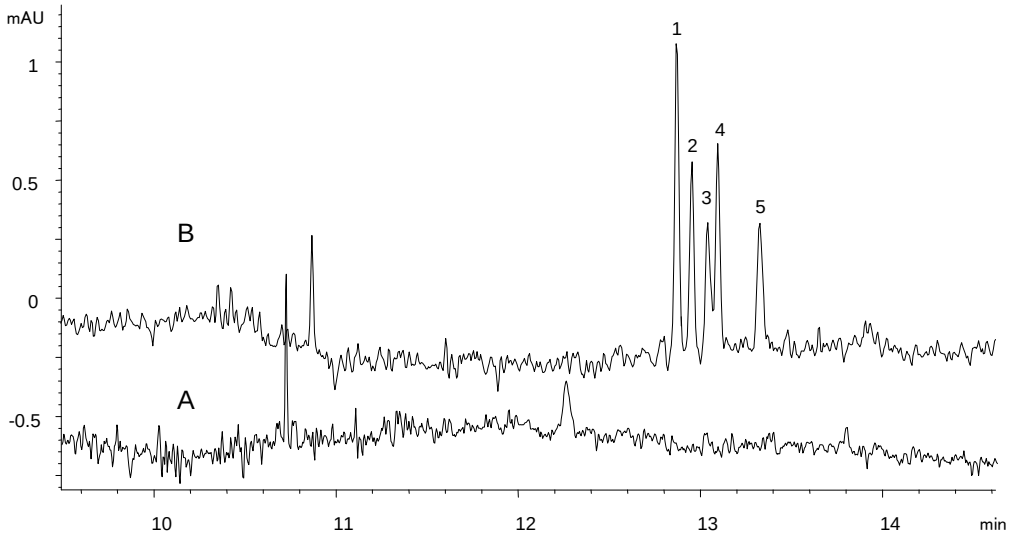

Figure 4. Electropherogram obtained when human plasma sample was analyzed by transient pseudo-ITP. **A**: blank human plasma sample. **B**: human plasma sample spiked at a concentration of 2 mg/L of NSAIDs. Other conditions are the same as in Figure 3.

calculated based on a signal-to-noise ratio of 3:1.As shown in table 1, for intraday (n=3), RSD values were below 15% and good correlation coefficients were obtained. This stacking method has been applied previously for the determination of different compounds in plasma samples. The main advantages of the application of this technique for plasma samples are related to the simplicity of sample preparation as well as the short analysis time for these types of compounds.

Kubalczyk *et al*. [23] recently validated a method for the determination of total cysteamine in human plasma, for which a LODs of 0.8 μM (around 62 ppb) were obtained. Furthermore, Shihabi [18] demonstrated the applicability of this kind of technique in the analysis of serum samples for drug monitoring. A LOD of 95 μg/L for mycophenolic acid in the serum samples was reached by direct injection, filling 21% of capillary volume.

In our work, the LODs obtained using this preconcentration technique, transient pseudo-ITP, were similar to or lower than those reported in the literature when similar methods were used to analyze NSAIDs. Friedberg *et al*. [24] reported the determination of ketoprofen in human serum reaching LODs of 0.6 mg/L. In this approach, 200 μL of ACN were added to 100 μL of serum, the supernatant resulting from a centrifugation step was then added into the capillary as a long plug. However, it is important to note that the simultaneous determination of a group of these types of pharmaceutical compounds has not been previously reported.

3.7.2 Urine

Figure 5A shows the electropherogram obtained from a blank of human urine sample following the optimized transient pseudo-ITP method. Figure 5B shows the electropherogram obtained when 200 μL of urine sample was spiked at a concentration of 2 mg/L for the five NSAIDs after the deproteinization and extraction step described in section 2.4.1. Linearity was determined through the triplicate injection of samples ranging between 1 and 20 mg/L. LODs ranged between 0.32 and 0.75 mg/L calculated based on a signal-to-noise ratio of 3:1. As shown in table 1, for intraday (n=3), RSD values were less than 13%. To the best of our knowledge, the transient pseudo-ITP validation for the simultaneous determination of NSAIDs in human urine samples has not been reported before in the literature. However, more complex methods have been used. For example, Veraart *et al*. [27] analyzed ibuprofen, naproxen, fenoprofen and ketoprofen in urine using an on-line dialysis-SPE-CE method with UV detection. Although those authors reached lower detection limits (0.05–0.1 mg/L), in our work the LODs were low enough to analyze these drugs in urine using a simple and fast method without an off-line preconcentration step, such as solid phase extraction.

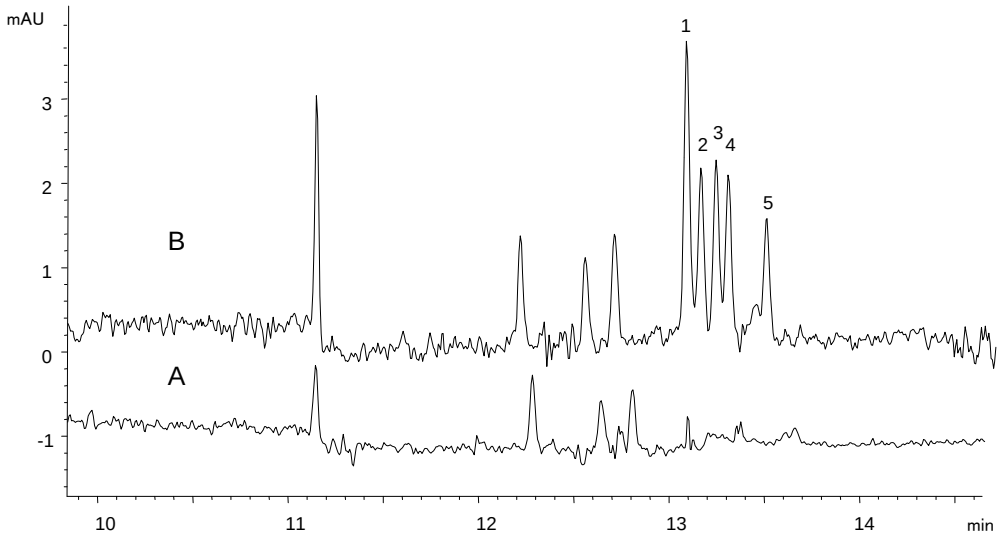

Figure 5. Electropherogram obtained when human urine sample was analyzed by transient pseudo-ITP. **A**: blank human urine sample. **B**: human urine sample spiked at a concentration of 2 mg/L of NSAIDs. Other conditions are the same as in Figure 4.

Also, Pai *et al*. [20] determined indoprofen, ketoprofen, naproxen, ibuprofen, fenoprofen, flurbiprofen and suprofen through coupling electrochromatography (CEC) with field-amplified sample stacking (FASS) in urine samples. In that work, although the LODs for standard samples were lower than in our case, data values for real samples were not reported.

4. Conclusions

The advantages of using transient pseudo-ITP as a stacking preconcentration technique lie in its simplicity, short analysis time, and applicability to a high number of compounds. Although a clinical prescription for more than one NSAID is unusual, a simultaneous method for their determination could be useful for routine clinical analysis. This type of stacking produces low enough LODs for the determination of these kinds of drugs in biological samples because their therapeutic levels in biological samples are typically over 1 mg/L. The method developed in this paper was validated in real urine and plasma samples.

Acknowledgment

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The authors have declared no conflict of interest

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3.1.2 Electrokinetic supercharging focusing in capillary zone electrophoresis of weakly ionizable analytes in environmental and biological samples.

UNIVERSITAT ROVIRA I VIRGILI
SENSITIVITY ENHANCEMENT STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF DRUGS OF ABUSE AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS
Igor Botello González
Dipòsit Legal: T. 1299-2012

ELECTROKINETIC SUPERCHARGING FOCUSING IN CAPILLARY ZONE ELECTROPHORESIS OF WEAKLY IONIZABLE ANALYTES IN ENVIRONMENTAL AND BIOLOGICAL SAMPLES

Igor Botello, Francesc Borrull, Carme Aguilar, Marta Calull
Departament de Química Analítica i Química Orgànica. Faculty of Chemistry. Universitat Rovira i Virgili. Marcel·lí Domingo s/n, 43007 Tarragona, Spain

Abstract

Five non-steroidal anti-inflammatory drugs (NSAIDs), naproxen, fenoprofen, ketoprofen, diclofenac and piroxicam, were separated and analyzed by electrokinetic supercharging (EKS) in capillary zone electrophoresis (CZE). Three different setups of the isotachophoresis (ITP) technique were assayed for the separation and preconcentration of these five NSAIDs. For the setup which gave the best results, we evaluated the influence of different parameters on separation and preconcentration efficiency such as sample pH, concentration of the leading stacker, BGE composition, electrokinetic injection time of the sample, composition and hydrodynamic injection of the solvent plug and of the terminating stacker. In the selected setup the background electrolyte (BGE) (10 mM $Na_2B_4O_7$ + 50 mM NaCl in 10% of MeOH aqueous solution) contained the leading electrolyte while the terminating electrolyte, hydrodinamically injected after the sample (50 mbar x 12 s), was 50 mM of 2-(cyclohexylamino) ethanesulphonic acid (CHES). Prior to sample injection at (700 s at -2 kV) a short plug of MeOH (50 mbar x 3 s) was hydrodinamically injected. The results show that this strategy enhanced detection sensitivity 2000-fold compared with normal hydrodynamic injection, providing detection limits of 0.08 μg/L for standard samples with good repeatability (values of relative standard deviation, %RSD < 1.03%). Method validation with river water samples and human plasma demonstrated good linearity, with detection limits of 0.9 μg/L and 2 μg/L for river water samples and human plasma samples, respectively (as well as satisfactory precision in terms of repeatability and reproducibility).

Keywords: *electrokinetic supercharging, isotachophoresis, non-steroidal anti-inflammatory drugs, plasma samples, water samples*

1. Introduction

In recent years, applied researchers have become increasingly interested in the development of several on-line preconcentration techniques based on stacking in capillary electrophoresis (CE). The main aim in this field is to improve the low concentration sensitivity of CE analysis due to the limited light-path length of the detector and the small injected sample volume [1, 2]. In all these on-line preconcentration techniques, a significant step which characterizes the stacking process is the sample injection mode. There are two methods for introducing the sample into the capillary: hydrodynamic injection (HD) and electrokinetic injection (EKI) [3]. The latter method provides some advantages of sample stacking over similar approaches which use hydrodynamic injection [4]. In EKI the introduction of the analytes into the capillary occurs through electroosmosis and also by their migration velocities in the electric field. This latter particularity offers the advantage of increasing the amount of injected analytes, avoiding the application of large sample volumes which usually affect the separation [3]. However, EKI is very dependent on sample conductivity, and EOF might have negative effects on the EKI process.

Field amplified stacking procedures based on EKI are only possible when the conductivity in the sample solution is lower than in the run buffer [5]. Among the great variety of on-line preconcentration techniques, transient isotachophoresis (t-ITP) is a stacking procedure that may increase the concentration of analyte regardless of the sample conductivity. This technique can be applied when the samples contain highly mobile ions such as biological materials and also some samples of environmental and industrial origin [1].

t-ITP requires the use of a discontinuous buffer system, and is applicable to a wide range of low and high molecular weight ionic compounds [6-9]. In the most common set-up for t-ITP the sample is introduced between two buffers, one with a higher (leading electrolyte, LE) and another with a lower (terminating electrolyte, TE) mobility than the analytes [1]. The t-ITP stacking process occurs when, after the separation voltage is applied, analytes with mobilities between those of LE ion and TE ion undergo temporary focusing until the separation mode changes from ITP to CZE.

It is important to note that while EKI is strongly related to the sample conductivity, t-ITP acts regardless of this sample property. Also in intrinsic EKI, the injected analyte amount is limited to a certain extent, but the t-ITP process allows the introduction of much larger sample amounts. Therefore, combining EKI and t-ITP, one may conclude that t-ITP remedies the shortcomings of EKI, and also that higher increases in sensitivity can be expected than in EKI or t-ITP when applied alone. This combination was first introduced by Hirokawa *et al.*

[10] and was named Electrokinetic Supercharging (EKS). In that work a mixture of 18 inorganic cations was analyzed with detection limits of 0.3 μg/L when the sample was injected by EKI at 20 kV for 150 seconds. Following the successful introduction of this new preconcentration strategy, EKS has been applied for the analysis of other kinds of compounds in different matrices [11-14]. For example, Busnel *et al.* [11, 12] used this preconcentration technique to analyze peptides in biological samples. The authors stated the importance of the selection of a proper LE, since the type and concentration can affect the response. In these approaches, ammonium acetate and lithium chloride were tested as leading electrolyte resulting in an increase in the sensitivity enhancement factors (SEFs) with the increment of the concen-tration of the leading ion. The SEFs were in the range between 1000 and 10,000 and the authors pointed out that the magnitude of SEF depends on the electrophoretic mobility of the analytes.

Non-steroidal anti-inflammatory drugs (NSAIDs) have been used widely as pain relievers and, due to their antipyretic effect, for the treatment of different diseases. The continuous environmental input of such drugs may lead to a relatively long term concentration and thereby promote continuous but unnoticed adverse effects on aquatic and terrestrial organisms. Elimination of acidic pharmaceutical in sewage treatment plants was found to be rather low and consequently sewage effluents are one of the main sources of these compounds and their metabolites [13, 15-17] .

NSAIDs have been analyzed by using different CE modes such as CZE, microemulsion electrokinetic capillary chromatography (MEEKC), micellar electrokinetic chromatography (MECK), capillary electrochromatography (CEC), isotachophoresis (ITP) and trasient-isotachophoresis (t-ITP) [17-26]. In some of these works, different stacking strategies were applied in order to improve the sensitivity, reaching LOD values as low as 0.1 μg/L in standards with an enhancement in sensitivity of 1800-fold.

The possibility of electrokinetically injecting a larger amount of sample is one of the main advantages of EKS as a preconcentration technique. Some authors have applied EKS for the analysis of NSAIDs in water samples [13, 14]. For example, Dawod *et al.* [13] analyzed seven NSAIDs by EKS using NaCl as leading electrolyte and CHES as terminating electrolyte. Sensitivity was improved 2400-fold with an EKI injection of 200 seconds at -10 kV. LODs were as low as 0.05 μg/L in standards. However, the authors stated that they obtained instability current problems and band broadening related to an increase of the time or the voltage in the electrokinetic injection step. In order to allow the use of higher voltages for longer injection times without current instabilities and band broadening due to the injection of large volumes of the

low-conductivity sample by the EOF, they applied a modification of EKS using a counter-flow in the injection step [14]. The LOD values achieved were 5 times higher with respect to the previous work. In this second approach, the authors pointed out that the use of the leading electrolyte (NaCl) was not necessary in the ITP process.

The aim of this work was to explore the applicability of the EKS-CZE preconcentration technique to determine NSAIDs in different sample matrices, in particular environmental and biological samples. In this paper we examine three different setups of t-ITP applied to EKS-CZE. For the setup which produces better responses, different parameters such as injection time, sample pH, low conductivity plug prior to the injection step and BGE composition, which affect the preconcentration factors and the separation, were optimized. The proposed method was validated and applied to the analysis of the NSAIDs in river water samples and human plasma.

2. Materials and methods

2.1 Reagents and standards

All reagents were of analytical-reagent grade. Ultrapure reagent water purified by a MilliQ gradient system (Millipore, Bedford, MA, USA) was used throughout. Naproxen, fenoprofen, diclofenac, ketoprofen, piroxicam, sodium chloride (99.999%), hexadimethrine bromide (HDMB), sodium hydroxide (NaOH), methanol (MeOH), disodium tetraborate anhydrous and 2-(cyclohexylamino) ethanesulphonic acid (CHES) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Standard stock solutions for each NSAID of 1000 ng/L were prepared in methanol and stored at 4 ºC. Working standard solutions of the mixture of all the compounds at a concentration of 100 ng/L were prepared weekly by diluting the standard solutions in ultrapure water. The solutions with a lower concentration were prepared daily by diluting appropriate volumes of the working stock solution in MilliQ water and then adjusted to pH 8.5 with 0.1 M NaOH.

The separation buffer (BGE), which consisted of 10 mM sodium tetraborate anhydrous and 10% (v/v) MeOH (adjusted to pH 8 with 0.1 M NaOH), was prepared by dissolving the appropiate amount of disodium tetraborate anhydrous in MilliQ water and mixing with MeOH.

2.2 Instrumentation

The instrumentation used for electrophoretic separation was an Agilent 3D CE (Agilent Technologies, Waldbroon, Germany) equipped with UV diode-array detection (DAD). The detection of the NSAIDs was performed at 214 nm.

A fused silica capillary with 88.5 cm length (80 cm effective length) and 50 μm of internal diameter (ID) was used. The capillary chamber was heated at 25 ºC in all experiments. The applied

voltage was -2 kV and -30 kV for electrokinetic injection and separation respectively.

2.3 Capillary electrophoresis

2.3.1 CZE

New capillaries were conditioned with 1M NaOH for 90 min prior to use and daily with 1M NaOH for 10 min and water for 10 min. Between separations the capillary was conditioned with 1M NaOH for 4 min, water for 4 min, HDMB 0.01% in water for 5 min and BGE for 4 min. Reversed polarity of -30 kV was used for the separation of NSAIDs with UV detection. Fresh sample and BGE solutions were used for each experiment.

2.3.2 EKS

As t-ITP technique can be performed in different ways [27], three different setups were examined in the present work. The first setup assayed was designed according to the classical ITP procedure where small volumes of leading electrolyte (50 mM NaCl) and terminating electrolyte (50 mM CHES) are introduced hydrodinamically (HD) into the capillary before and after the electrokinetic injection (EKI) of the sample. In the second setup the leading electrolyte was added to the BGE and in this way the t-ITP state is created only by the HD injection of a suitable TE zone after EKI of the sample. The last setup tested was performed adding the terminating (CHES) to the BGE only in the cathodic vial so the leading ion was HD injected prior to the EKI of sample. The anionic vial and capillary contained only BGE.

2.4 Sample pretreatment

2.4.1 River water samples

River water samples were collected and filtered through a 0.2 μm nylon membrane filter to eliminate particulate matter. One milliliter of water sample spiked with an NSAID mix standard solution was diluted to a final volume of 10 mL in MilliQ water and then 10 mL of dichloromethane (DCM) were added. The mixture was vortexed and centrifuged at 3000 rpm for 3 min. Organic phase was collected and evaporated to dryness under gentle stream of dry nitrogen (UHP grade). The residue was reconstituted with 1 mL of MilliQ water adjusted to pH of 8.5 with 0.1 M NaOH and transferred to a glass vial for EKS-CZE analysis.

2.4.2 Plasma samples

Plasma samples were obtained from a healthy volunteer in our laboratory. All samples were stored at -18 ºC until their analysis. Plasma samples were acidified with 25 μL of HCl 1M for neutralizing analytes.
Deproteinization and extraction was then carried out by adding 1 mL of ethyl acetate/n-hexane (20:80 v/v) to 200 μL of spiked plasma [28]. After vortex mixing, the sample was

centrifuged for 10 min at 9000 rpm. Supernatant solution was collected and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted with adjusted pH 8.5 MilliQ water (1mL) and transferred to a glass vial for EKS-CZE analysis.

3. Results and discussion

3.1 Coelectrosmotic separation of NSAIDs

The first stage of this work consisted in the optimization of the separation conditions of the five NSAIDs by CZE. Since the NSAIDs studied are negatively charged, when negative voltage is applied, reverse EOF is necessary to guarantee the analytes' migration towards the detection window when the velocity of the EOF is higher than the velocities of the compounds.

In capillary electrophoresis, it is generally possible to obtain more efficient separations when the electroosmotic flow has the same direction as the migration of the target analytes. Co-EOF analysis makes it possible to reduce the analysis run time and an improvement of resolution can be achieved due to a reduction of the baseline instability in comparison with counterelectrosmotic mode [29].

To reverse EOF to achieve fast separations for ionic analytes, some authors added the polycationic surfactant HDMB into the BGE solution [13, 18, 30]. HDMB has been effective in reversing EOF at low concentrations, providing a net positive charge to the wall capillary, which suggests a change in both the magnitude and sign of the zeta potential. Also Macià *et al.* [18] used a methanol solution of HDMB (0.001%) as the preconditioning step for capillary coating in order to reverse EOF.

In this work, the use of HDMB in the capillary preconditioning step was studied in order to reverse the zeta potential sign on the silica capillary wall. To evaluate its effect, a flow of 0.01% HDMB in water was flushed through the capillary during different periods of time from 3 to 30 minutes. The results showed that in all cases a reversed EOF is reached. However, conditioning times under 4 minutes affected the reproducibility with regard to the analysis of migration times (results not shown). For further studies, 5 min of preconditioning step with HDMB 0.01% in MilliQ water for reversing EOF was used. It is important to note that the effect of the HDMB as modifier of the capillary wall disappears after the post conditioning step with 1 M NaOH and conditioning with HDMB solution is necessary between runs.

As BGE, we used borate, based on previous studies in the bibliography for NSAID analysis [31-34]. We selected a low concentration of $Na_2B_4O_7$ in order to allow further addition of salts (EKS setups) without an excessive increase in the Joule effect. Thus, optimum conditions for CZE coelectrosmotic separation of the five NSAIDs studied were obtained using 10 mM $Na_2B_4O_7$ (pH = 9.4) at -30 kV of separation voltage. Under these

conditions run time was around 8 minutes.

3.2 Different setups of EKS for NSAIDs

The main advantage of the isotachophoretic preconcentration techniques is that they may increase the concentration of an analyte regardless of the conductivity of the sample [5]. Isotachophoretic sample stacking can be achieved in different ways and the proper selection of the discontinuous electrolytic design is an important issue if better results are to be obtainned, not only in preconcentration factors but also in the separation. In theory, in order to generate an ITP state is necessary the presence of a leading and a terminating electrolytes in the discontinuous electrolyte system. In t-ITP, after the preconcentration process a transition to CZE mode occurs when the leading electrolyte (due to its high mobility) spread into de background electrolyte. Even that the electrophoretic mobility of borate ($3.62\ 10^{-4}$ cm^2/Vs) ion is higher than in the case of the NSAIDs ($1.52 – 1.99\ 10^{-4}$ cm^2/Vs), we believe that the difference is not great enough due to the proximity of the values. Thus, we selected the Cl^- ($10.69\ 10^{-4}$ cm^2/Vs) as typical leading electrolyte in order to guarantee the generation of the ITP state.

In this report three different setups (described in the experimental section part) are evaluated. These setups were selected since the differences between them are in the design of the electrolyte system without any changes in sample composition. In the three configurations tested, ion chloride was used as leading ion because it has a higher mobility than those of analytes and CHES was selected as terminating ion based on previous studies due to its lower electrophoretic mobility respect to the analytes of concern [13, 35].

In the first setup, a plug of 50 mM NaCl was hydrodinamically injected at 50 mbar for 15 seconds. Following, electrokinetic injection of the NSAIDs' standard mixture at -12 kV for 15 seconds was carried out. Finally, hydrodynamic injection of a solution of 50 mM CHES at 50 mbar for 12 seconds was performed. In the second setup tested, the leading ion was in the BGE at 50 mM of concentration. Thus, after the electrokinetic injection of the standard mixture at -12 kV for 15 seconds, a hydrodinamic plug of 50 mM CHES at 50 mbar for 12 seconds was injected. Finally, the third setup consisted in the hydrodinamic injection of a plug of 50 mM NaCl at 50 mbar for 15 seconds and, after the electrokinetic injection of the standard mixture at -12 kV for 15 seconds, the BGE containing 50 mM CHES was used in the outlet vial in the separation step.

Figure 1 shows the electropherograms obtained by the analysis of a standard sample containing the NSAIDs at concentration of 1 mg/L for the three tested setups. As can be seen, the design of the configuration of the EKS procedure affects the separation and the sensitivity of the analysis. The best

results regarding resolution were obtained in the setup where the BGE contains the leading ion (Fig. 1B). We also determined the sensitivity enhancement factors (SEFs), which were calculated according to equation (1):

$$SEF_{height} = \frac{h_{prec}}{h_{HD}} f \qquad (1)$$

where h_{prec} is the peak of the preconcentrated analyte, h_{HD} the peak of the non-preconcentrated analyte, detected after a conventional hydrodynamic injection of 10 seconds at 50 mbar, and f the dilution factor.

In this second setup, the estimated SEF for the five analytes were also higher (data not shown). Since the greatest suitability of the EKS method for the selected NSAIDs was reached in this setup, this second configuration was selected for further studies.

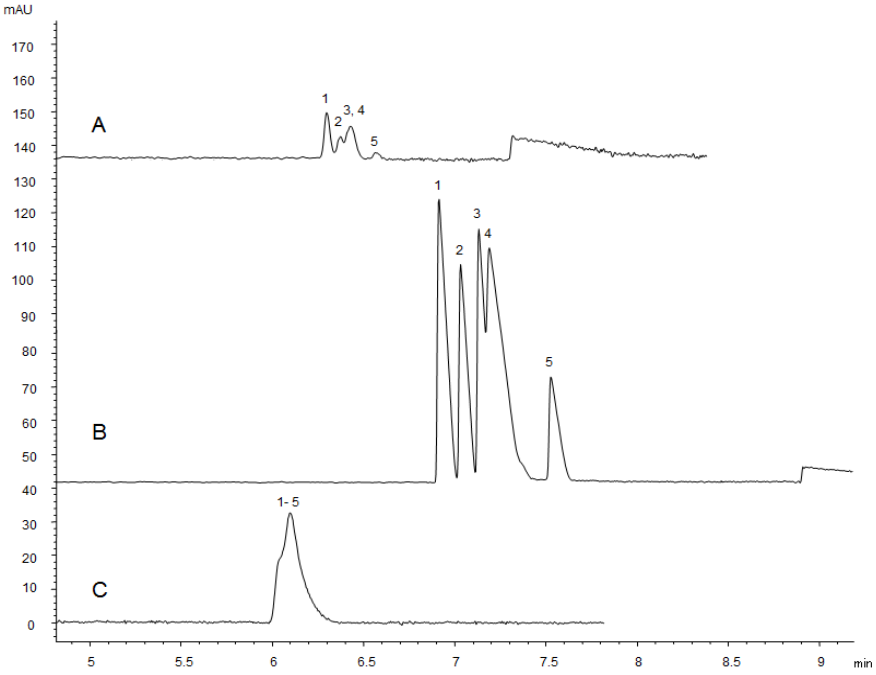

Figure 1. Electropherograms of five NSAIDs at a concentration of 1 mg/L obtained for the three different setups. The sample injection voltage was of -12 kV for 15 s. Separation voltage of -30 kV. (**A**) Conventional setup (T=terminating electrolyte; S=sample; L=leading electrolyte), BGE: 10 mM $Na_2B_4O_7$; (**B**) EKS with the BGE acting as leading electrolyte, BGE: 10 mM $Na_2B_4O_7$ + 50 mM NaCl; (**C**) EKS with BGE (inlet vial only) acting as terminating electrolyte, BGE: 10 mM $Na_2B_4O_7$ + 50 mM CHES. Peak identification: 1, naproxen; 2, fenoprofen; 3, diclofenac; 4, ketoprofen; 5, piroxicam.

3.3 Optimization of the conditions in the separation and preconcentration by EKS

Under the electrophoretic conditions of the selected setup, the NSAIDs studied were not completely resolved (Figure 1B). An optimization of some parameters affecting the separation and preconcentration in EKS was thus necessary. First we focused on the study of the CZE separation in the new run buffer system (10 mM $Na_2B_4O_7$ + 50 mM NaCl) prior to the study of the EKS conditions.

3.3.1 Influence of the NaCl concentration on the separation

The first parameter studied was the concentration of NaCl in the BGE. The leading ion concentration has to be high enough (at least 50 times higher than the analytes concentration) to attain an effective stacking process when the EKS technique is applied [5]. The high concentration of NaCl does not allow an additional increase in the concentration of $Na_2B_4O_7$. The presence of a high salt concentration in the BGE may excessively increase the current during the analysis and losses of current would occur due to the increase of the Joule effect.

The study of the influence of NaCl concentration as leading electrolyte in the BGE was carried out by varying the concentration of NaCl between 10 and 110 mM in the buffer solution. This concentration range of NaCl was selected in order to guarantee that the concentration of LE was higher than the concentration of the analytes in the sample (more than 50 times higher). These experiments were conducted under hydrodynamic injecttion of the sample (50 mbar for 10 seconds) using a standard NSAIDs mixture of 10 mg/L.

The results showed that when the NaCl concentration was increased, an increase in the resolution was obtainned for the five compounds up to the value of 50 mM of NaCl. However, for higher concentrations a decrease in resolution for diclofenac and ketoprofen was observed. Thus, optimum conditions were fixed at 50 mM NaCl in the separation electrolyte.

3.3.2 Influence of the addition of MeOH on the separation

The addition of MeOH on the BGE was studied in order to achieve a baseline separation between peaks, particularly for diclofenac and ketoprofen with the lower resolution value. Effective mobility of the analytes depends on the strength of the buffer electrolyte. Adding a water soluble solvent to the BGE affects not only the ionic strength but also the dielectric constant. Consequently, varying the percentage of this solvent in the buffer is a tool that can be exploited to alter the relative mobility of ions and improve the selectivity [36, 37].

To investigate the effect of the organic modifier on the NSAIDs separation, the fraction of MeOH in the BGE was increased from 0 to 25 % v/v, since this solvent has been widely used to

improve BGE selectivity in the analysis of NSAIDs [31, 33]. Therefore, with the addition of MeOH, a decrease of the absolute mobility is verified for the five compounds and consequently the analysis time increased. With respect to the resolution, an increase was observed up to the addition of 10% MeOH v/v. When the percentage of this organic solvent exceeded 10% v/v, the resolution for ketoprofen and diclofenac decreased. Hence, 10% v/v MeOH was chosen for further work.

3.3.3 Solvent plug of low conductivity

When the sample is electrokinetically injected into the capillary, usually a short plug of water is injected previously in order to enhance the sensitivity of the analysis. Generally, this low conductivity plug provides a higher electrical field at the beginning of the capillary and ensures the fast migration of the analytes across this plug. In this way, it is possible to improve the sample stacking efficiency between this zone and the BGE zone [12, 38, 39]. Additionally, if there are salts present in the matrix, this plug helps to maintain reproducibility [40].

Four different plugs of a low conductivity solution were tested in order to ascertain whether there was an increase in stacking. The plugs of water, acetonitrile: water (1:1 v/v) and MeOH were injected hydrodynamically at 50 mbar for 2, 3 and 4 seconds each one. Under the same experimental conditions the influence of a plug of 10 mM $Na_2B_4O_7$ was also studied because at this concentration the conductivity is lower than in the BGE due to the presence of 50 mM NaCl.

After the low conductivity plug was injected, the inlet end of the column was switched to the reservoir containing the five NSAIDs' standard mixture of 100 μg/L. After sample injection (-2 kV x 80 s), a hydrodynamic injection (50 mbar x 12 s) of 50 mM CHES was performed. Finally, the capillary was switched back to the BGE vial and a negative separation voltage (-30 kV) was applied.

Whereas in other stacking techniques there is a greater effect on preconcentration factors with the use of a low conductivity solvent plug [26], this seems not to be so significant when EKS-CZE is applied. Dawod *et al.* [14] observed the same enhancement in sensitivity with and without using a short water plug (10 mm). However, in our case, even though the results obtained indicated that there was not a significant improvement in the SEF values for the analytes using a low conductivity plug prior to the sample injection, when we applied a short solvent plug of MeOH for 3 seconds, the stacking improved slightly. Thus, these conditions were used for further experiments.

3.3.4 Sample pH

The pH value of the sample regulates the ionization of the compounds and

consequently their absolute mobility [30]. The values of pK_a of the NSAIDs studied are all between 4 and 6. Thus, when the pH is above 6 all the NSAIDs studied are negatively charged. We examined the influence of sample pH in the range from 5.9 to 10.5 (adjusted with 0.1 M NaOH), while maintaining constant the composition of the BGE and the proportion of organic modifier (10% MeOH). Increasing the pH to 8.6 enhanced the intensities of the analyte peaks due to the increase in the degree of ionization of the acidic compounds. On increasing the degree of analyte deprotonation, a larger number of sample ions are injected electrokinetically into the EKS-CZE system. However, a similar tendency was not observed for sample pH over 8.6. This behavior can be explained by the presence of increasing amounts of OH^- in the sample solution when pH is adjusted to the desired value. Therefore, at values of pH greater than 8.6, the NSAIDs were introduced into the capillary in lower amounts because OH^- ions, with a greater mobility, reach the capillary first and consequently the amount of analyte injected decreases.

3.3.5 Study of sample electrokinetic injection time

To improve sensitivity we studied the effect of increasing the volume of sample injected into the capillary in the range between 300 - 800 seconds. It has been demonstrated that stacking efficiency is proportional to the injection time of the sample and to the voltage applied due to the increase in the amount of analyte injected. However, if the injection time is too long the peaks' width may broaden and split [39] and for this reason this parameter needs to be optimized. With respect to the injection voltage, first experiments were done at -12kV, but longer injection times and improvements in the current stabilities were reached when low voltage values (-2kV) were applied in the electrokinetic injection step.
With an increment of the injection time, a higher sensitivity for the five compounds is observed, as expected. Over 700 seconds, problems of instability of the current occur due to the conductivity mismatch between the long plug of the sample injected and the BGE. Therefore, 700 s of injection time (at -2 kV) was selected as the optimum for further experiments.

3.3.6 Influence of terminating electrolyte plug length

The effect of the plug length of CHES as the terminating electrolyte was also examined. Thus, the injection time of 50 mM CHES at 50 mbar was increased over the range 5 - 37 seconds. The peak height for the NSAIDs slightly increased with the injection time of CHES. However, also the run time analysis was larger when a greater plug length was used. The optimum injection time of the terminating electrolyte adopted for the subsequent experiment was

therefore 12 seconds. It is important to point out that, as the LE is included in the separation buffer, changes in its concentration can simultaneously affect the CZE separation step. For this reason, the influence of the leading ion on the stacking was not studied.

3.4 Validation

Figure 2 shows the electropherogram corresponding to the analysis of the NSAIDs at a concentration of 25 μg/L

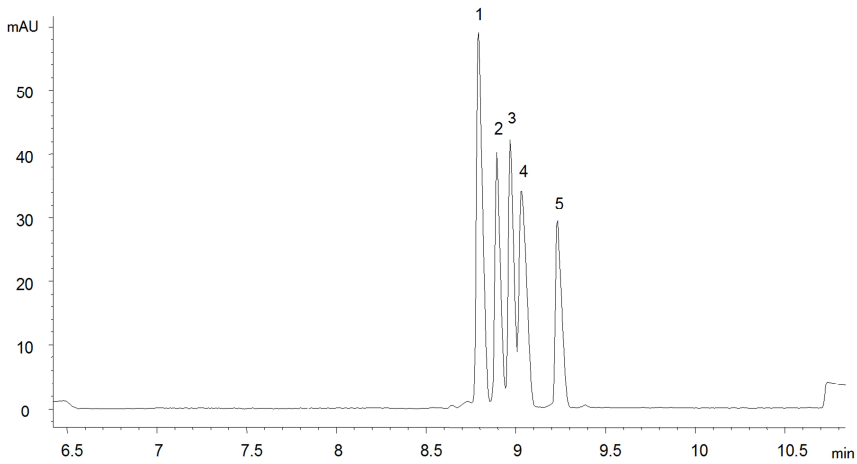

Figure 2. Electropherogram of the optimized EKS-CZE method. In all experiments standard concentrations of 25 μg/L at pH = 8.6 were used. BGE: 10 mM $Na_2B_4O_7$ + 50 mM NaCl + 10% MeOH. Solvent plug of MeOH (50 mbar x 3 s). Sample injection voltage was of -2 kV for 700 s. Terminating electrolyte 50 mM CHES (50 mbar x 12 s). Separation voltage was of -30 kV.

responses were good in the concentration range of 0.3 to 50 μg/L. The correlation coefficients were greater than 0.998 for all the compounds. The RSD in terms of reproducibility were below 2% and the detection limits calculated using a signal-to-noise criterion of 3 were in the 0.08 to 0.1 μg/L range. Applying this configuration of EKS, in which the BGE contains the LE, it is possible to inject up to 700 seconds of the sample at low values of voltage without instability of the current, as was previously reported [14]. The LODs obtained by this method are similar to those obtained by Dawod *et al.* [13] being slightly lower for diclofenac. How-

of the NSAIDs with this sample preconcentration technique under optimum conditions. The sensitivity was enhanced by 1615 to 2167-fold in comparison with normal hydrodynamic injection.

The suitability of the method for quantitative analysis was studied by testing linearity, LODs, LOQs, repeatability and reproducibility for standard samples (Table 1). The calibration curves were obtained by plotting the ratio of peak areas of each analyte versus concentration. Linear

Table 1. Sensitivity enhancement for EKS-CZE in standard samples and linearity, intra- and interday reproducibilities (RSD) of peak area and LODs for standard samples, river water samples and human plasma samples.

	Standard samples					River water samples				Human plasma samples			
Analyte	**Linear range tested µg/L**	**%RSD [a] (n=5)**	**%RSD [b] (n=5)**	**LOD µg/L**	**SEF**	**Linear range tested µg/L**	**%RSD [a] (n=5)**	**%RSD [b] (n=5)**	**LOD µg/L**	**Linear range tested µg/L**	**%RSD [a] (n=5)**	**%RSD [b] (n=5)**	**LOD µg/L**
Naproxen	0.3 - 50	0.94	1.19	0.08	1800	1.5 - 50	3.5	4.9	0.9	3.5 - 50	5.2	8.6	2
Fenoprofen	0.5 - 50	0.97	1.29	0.10	2102	1.5 - 50	4.9	5.2	1.0	3.5 - 50	8.3	14.9	3
Diclofenac	0.5 - 50	0.98	1.51	0.10	2167	1.5 - 50	5.8	7.8	1.0	3.5 - 50	9.1	11.1	3
Ketoprofen	0.5 - 50	0.99	1.10	0.10	1615	1.5 - 50	7.4	7.3	1.0	3.5 - 50	14.9	19.4	3
Piroxicam	0.5 - 50	1.03	1.36	0.10	1815	2 - 50	4.6	5.3	1.5	10 - 50	14.4	18.8	8

[a] Intraday analysis
[b] Interday analysis

ever, our results (LODs) were lower than those obtained by other authors who also analyzed NSAIDs in standard samples by using different stacking techniques [26, 31].

3.5 Analysis of real samples

Once our EKS-CZE method was validated for standards, we tested the applicability of the method in river water samples as well as in human plasma samples.

3.5.1 River water

The matrix composition of the river sample affected the stacking process and it was not possible to introduce the sample directly into the CE system. Thus, an extraction step prior to injection is necessary to obtain clean sample matrices. Among the sample preparation techniques, liquid-liquid extraction (LLE) is an efficient clean-up procedure.

Since dichloromethane (DCM) has been used satisfactorily as solvent in liquid-liquid extraction of NSAIDs from aqueous matrices, we followed a similar extraction procedure to that reported in the literature [14]. Recoveries were determined by comparing the peak area from drug-free river water spiked with standard mixture of 25 μg/L NSAIDs versus the peak area of the sample concentration prepared in MilliQ water injected directly into the capillary. To examine the effect of the extraction solvent and sample volumes, experiments involving two different volumes of DCM and sample were conducted with the same LLE procedure. 10 mL and 1 mL of spiked sample were extracted with 10 mL and 1 mL of DCM, respectively, and the results showed that using 10 mL of DCM for the extraction of 10 mL of sample the recoveries improved. Thus, these volumes were selected as an optimum volume of extraction solvent. Subsequently, also in order to improve the recoveries, the sample was diluted 1:10 prior to the LLE procedure. Applying this step, the matrix effect was reduced and better recoveries (53 – 90 %) were obtained for the five compounds.

Figure 3A shows the electropherogram obtained from a blank of diluted 1:10 river water sample after LLE procedure. Figure 3B shows the EKS-CZE electropherogram obtained when 1 mL of river water sample spiked at a concentration of 2 μg/L for the five NSAIDs and then diluted 1:10 in MilliQ water was extracted following the liquid - liquid extraction step described in section 2.4.1. Table 1 shows the linearity, reproducibilities (RSD) intra- and interday of the peak area and LODs for EKS-CZE of river samples analysis. The correlation coefficients were greater than 0.998 for all compounds. The RSDs of the peak areas in terms of reproducibility intra- and interday were below 7.4% and 7.8% respectively. LODs were lower than 1,5 μg/L calculated based on signal-to-noise 3:1.

LODs obtained by EKS-CZE in the present work proved similar to those obtained by Dawod *et al*. [13]. These

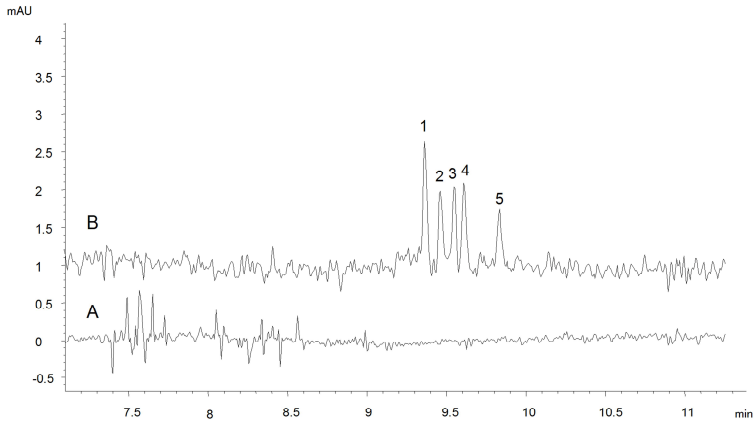

Figure 3. Electropherogram obtained when river water was analyzed by EKS-CZE (Conditions as Figure 2). **A**: blank river water sample. **B**: river water sample spiked at a concentration of 2 μg/L for the five NSAIDs. For peak assignment see Figure 1.

authors applied a second approach in which they introduced a counter flow. However, even though their LODs for standards were lower than in our case, data values for real samples were not reported [14]. In addition, our LODs were also 100-fold higher than those obtained recently by Pedrouzo *et al.* using SPE - HPLC (ESI) - MS in river water samples [41]. We also find that lower LODs were obtained applying CE-MS in river water samples previously preconcentrated by SPE [42]. However, we would like to emphasize that in the mentioned works a preconcentration step based on the use of off-line SPE implied a higher preconcentration of the sample. In our case, even though we only include a simple LLE step, our LODs are comparatively lower than the ones obtained in those works that use a more exhaustive pretreatment of the sample.

3.5.2 Human plasma

To explore the usefulness of the EKS-CZE in the analysis of NSAIDs in biological samples we applied the validated method to human plasma samples.

To the best of our knowledge, the potential of the EKS-CZE in the analysis of NSAIDs in biological samples has not been previously exploited. Figure 4A shows the electropherogram obtained from a blank of human plasma sample. Figure 4B shows the EKS-CZE electropherogram obtained when 200 μL of plasma sample was spiked at a concentration of 10 μg/L for the five NSAIDs after the deproteinization and extraction step described in section 2.4.2.

The linearity was determined from triplicate injection of standards in the range of 1 and 50 μg/L. LODs were

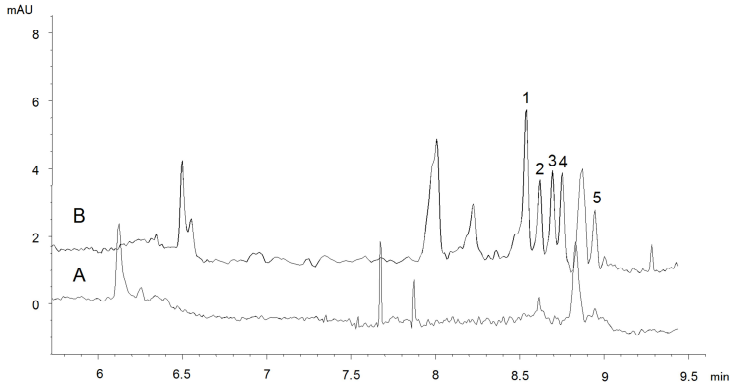

Figure 4. Electropherogram obtained when human plasma sample was analyzed by EKS-CZE (Conditions as Figure 2). **A**: blank human plasma sample. **B**: human plasma sample spiked at a concentration of 10 μg/L for the five NSAIDs. For peak assignment see Figure 1.

lower than 8 μg/L calculated based on a signal-to-noise 3:1 (Table 1). Recoveries were determined by comparing the peak area from drug free plasma spiked with standard mixture of 10 μg/L NSAIDs versus the peak area of the sample concentration prepared in MilliQ water injected directly into the capillary. Recoveries values for the five NSAIDs were between 56% and 73%. As seen in Table 1, for intra- and interday (n=3), tha values of RSD were below 19.4%.

The LODs obtained with this preconcentration technique (EKS-CZE) were lower than reported in literature using different methodologies for the analysis of NSAIDs in human plasma samples by CE [34, 43, 44]. In addition, these results are also lower than those obtained in recent reports when these drugs were analyzed in spiked serum by reversed-phase LC-UV [45] and in spiked plasma directly analyzed under different dilution factors by LC coupled with chemiluminescence detection [46].

4. Concluding remarks

EKS-CZE as a preconcentration technique has been shown to be effective for the analysis of NSAIDs in aqueous media as well as in more complex matrices such as plasma. This simple and fast method allows the loading of a much larger amount of sample without affecting the resolution. This technique also makes possible the analysis of different kinds of matrices such as biological samples with high conductivity, a factor which negatively affects many other preconcentration techniques.

The methodology proposed provided around 2000-fold sensitivity enhancement comparing with the results obtained by conventional CZE without any off-line preconcentration procedure for standard samples. For the

analysis of real samples, a simple LLE step was necessary in order to clean up the sample. The LODs obtained from the five studied NSAIDs were between 0.9 and 1.5 μg/L in river water extract and between 2 and 8 μg/L in plasma samples extract.

Acknowledgment

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The authors have declared no conflict of interest.

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3.1.3 Electrokinetic supercharging in capillary electrophoresis for the separation and preconcentration of barbiturate drugs in urine samples.

UNIVERSITAT ROVIRA I VIRGILI
SENSITIVITY ENHANCEMENT STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF DRUGS OF ABUSE AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS
Igor Botello González
Dipòsit Legal: T. 1299-2012

ELECTROKINETIC SUPERCHARGING IN CAPILLARY ELECTROPHORESIS FOR THE SEPARATION AND PRECONCENTRATION OF BARBITURATE DRUGS IN URINE SAMPLES

Igor Botello, Francesc Borrull, Marta Calull, Carme Aguilar
Departament de Química Analítica i Química Orgànica, Faculty of Chemistry, Universitat Rovira i Virgili, Marcel·lí Domingo s/n, 43007 Tarragona, Spain

Abstract

Three barbiturate drugs, barbital, phenobarbital and secobarbital were separated and analysed by electrokinetic supercharging (EKS) in capillary zone electrophoresis (CZE). In order to determine the optimal conditions of the method, the influence of different parameters on EKS performance was evaluated using both univariated and multivariated optimization processes. The parameters studied were sample pH, concentration and length of the leading and terminating electrolytes, electrokinetic injection of the sample and composition and hydrodynamic injection of the solvent plug. The capillaries were coated with HDMB and the background electrolyte used (BGE) was 20 mM $Na_2B_4O_7$ at a pH value of 9.15. The leading electrolyte (50 mM NaCl) was hydrodynamically injected (50 mbar x 120 s) prior to the sample which was adjusted to pH 9.6 and electrokinetically injected at -8.5 kV for 300 seconds. The terminating electrolyte (100 mM of 2-(cyclohexylamino) ethanesulphonic acid (CHES)) was then hydrodynamically injected (50 mbar x 140 s). The results obtained show that this strategy enhanced detection sensitivity around 1050-fold compared with normal hydrodynamic injection, providing detection limits ranging between 1.5 and 2.1 ng/mL for standard samples with good repeatability in terms of peak area (values of relative standard deviation, %RSD < 3). The applicability of the optimized method was demonstrated by the analysis of human urine samples spiked with the studied compounds at different concentration levels. The estimated detection limits obtained in the urine samples extract ranged between 8 and 15 ng/mL.

Keywords: *Electrokinetic supercharging, isotachophoresis, drugs of abuse, urine samples.*

1. Introduction

The derivatives of barbituric acid (2,4,6-trioxypyrimidine), commonly known as barbiturates, are the most widely used sedative-hypnotic drugs. These compounds, depending mostly on the substituting groups, exhibit a wide variety of responses in the body. Barbiturates act by depressing the activity of the central nervous system and, in high doses, also depress the respiratory system, which accounts for their toxicity [1]. The abuse of barbiturates is widespread nowadays. Complications arise from the fact that abused barbiturates often occur as complex mixtures and other drugs and/or excipients are also present. This requires the continuous development of methods for their efficient separation and precise identification and quantification [2]. The presence of this kind of drugs in urine samples is variable with respect to time after administration. In the analysis of urine samples in clinical cases of patients, the concentration of these drugs has been reported ranging between 0.2 and 21 μg/mL [3]. However, these values depend on the individual's metabolism.

Barbiturates have mainly been determined in biological fluids by liquid chromatography (LC) and gas chromatography (GC) [4-6]. For example, Ye *et al*. [7] developed a LC-APCI-MS method for the determination of phenobarbital, barbital, amobarbital and secobarbital in human plasma. The lowest limits of detection (LODs) achieved in that work ranged between 4 and 5 ng/mL for standard samples. Real samples were also analysed and the range of concentration found was between 451.2 ng/mL and 631.7 ng/mL. Martín-Biosca *et al*. [8] determined six barbiturates in urine using micellar liquid chromatography performing a direct sample injection. The LODs obtained in that case ranged between 130 and 2700 ng/mL. Capella-Peiró *et al*. [9] also used micellar liquid chromatography with direct injection to determine amobarbital, barbital, secobarbital and hexobarbital in serum samples. The method obtained LODs for standards ranging between 30 and 70 ng/mL. The authors also showed the applicability of the method by the analysis of spiked serum samples.

The potential of CE for the determination of barbiturates in biological samples has also been demonstrated by some authors [10-16]. For example, Jiang *et al*. [13] reported the determination of barbiturates in urine by CE using a dynamically coated capillary with a polycationic polymer. In that case, hexadimethrine bromide (HDMB) was employed as an EOF modifier in order to reverse the EOF and reduce the analysis time. The LODs for standard samples ranged between 870 and 3500 ng/mL. Wang *et al*. [12] used a moving reaction boundary (MRB)-induced stacking procedure in combination with CZE for the determination of barbital and phenobarbital in urine samples. In that case, LODs were 270 ng/mL for barbital and 260 ng/mL for phenobarbital. Ferslew *et al*. [15] used

micellar electrokinetic capillary chromatography (MEKC) for the determination of barbiturate drugs in various biological fluids. Applying the method to forensic cases of butalbital intoxication detected concentrations ranging from 1.5 to 7.6 μg/mL in urine. Ohyama *et al*. [11] applied capillary electrochromatography for the separation of barbiturates. However, in that case, no validation or application of the method for standard or real samples was reported.

The determination of drugs and other exogenous compounds in body fluids at low concentration levels is essential in modern forensic toxicology and assessment of drug abuse. Even with the promising results obtained by CE, the LODs achieved when using this technique in comparison with LC and GC are generally higher. This problem is due to the limited light-path length of the UV detector and the small injected sample volume into the capillary [17,18]. In recent years, several on-line preconcentration techniques based on stacking in CE have been developed to overcome sensitivity issues in CE. Among those techniques, those which involve electrokinetic injection (EKI) of the analytes offer the advantage of increasing the amount of injected analytes, avoiding the application of large sample volumes which usually affects the separation [19]. However, EKI is highly dependent on sample conductivity, and EOF might have negative effects on the EKI process. Transient isotachophoresis (t-ITP) may be the only stacking procedure that can increase the concentration of analyte regardless of the sample conductivity. This technique can be applied when the samples contain highly mobile ions such as biological materials and also some samples of environmental and industrial origin [17]. The t-ITP procedure requires the use of a discontinuous buffer system, and is applicable to a wide range of low and high molecular weight ionic compounds [20-23]. In t-ITP, the sample is introduced between two electrolytes, one with a higher (leading electrolyte, LE) mobility and the other with a lower (terminating electrolyte, TE) mobility than the analytes [17].

The combination of EKI and t-ITP allows the disadvantages of EKI to be overcome and also higher increases in sensitivity can be expected than in either EKI or t-ITP when applied alone. This combination was first introduced by Hirokawa *et al.* [24] and it was named Electrokinetic Supercharging (EKS). Following the successful introduction of this new preconcentration strategy, EKS has been applied for the analysis of other kinds of compounds in different matrices [20,25-34]. For example, in previous work by our group, this preconcentration technique was used to determine non-steroidal anti-inflammatory drugs (NSAIDs) in biological and environmental samples achieving sensitivity enhancement factors (SEFs) of around 2000-fold, with LODs as low as 0.9 and 2 ng/mL for river water and human plasma,

respectively [29]. Busnel *et al.* [25] also used this preconcentration technique in the determination of peptides in biological samples. In that case, the SEFs ranged between 1000 and 10,000 and the authors demonstrated that the magnitude of SEFs depends on the electrophoretic mobility of the analytes. Dawod *et al.* [27] analysed seven NSAIDs by EKS with a 2,400 fold improvement in sensitivity with an EKI injection of 200 seconds at -10 kV. LODs were as low as 0.05 ng/mL for standards. In further work by the same authors, the use of counter-flow pressure [28] and pressure assisted injection [35] was applied in order to allow the use of higher voltages for longer injection times. This modifycation of the EKS performance reduced problems of current instabilities due to the injection of large volumes of the low conductivity sample. The LOD values achieved were lower in comparison to the previous work and an enhancement in sensitivity of almost 50,000-fold was obtained [35]. Xu et al. [31] also demonstrated the versatility of an EKS-CE approach for sample concentration for a mixture of seven rare-earth metal ions obtaining LODs at or below 1.3 ng/L. In this case the LODs were over 100000-folds better compared with normal hydrodynamic injection.

The aim of this work is to explore the applicability of the EKS-CZE preconcentration technique to determine barbiturates in urine samples. To the best of our knowledge, this preconcentration technique has not been reported for the determination of barbiturates. In this paper, different parameters which affect the EKS performance are examined using both univariated and multivariated optimization. The variables studied were sample injection time and voltage, BGE composition, sample pH, low conductivity plug prior to the sample injection step and concentration and length of both LE and TE. The proposed method was applied to the analysis of barbiturates in human urine samples.

2. Experimental

2.1 Standards and reagents

All reagents used were of analytical-reagent grade. Ultrapure reagent water purified by a MilliQ gradient system (Millipore, Bedford, MA, USA) was used throughout. Barbital, phenobarbital and secobarbital, hexadimethrine bromide (HDMB), sodium hydroxide (NaOH), disodium tetraborate anhydrous and 2-(cyclohexylamino) ethanesulphonic acid (CHES) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Standard stock solutions for each barbiturate at a concentration of 1000 mg/L were prepared in methanol and stored at 4 ºC. Working standard solutions of the mixture of all the compounds at a concentration of 100 µg/L were prepared weekly by diluting the standard solutions in ultrapure water. The separation buffer (BGE), which consisted of 20 mM sodium tetraborate anhydrous (pH

9.15), was prepared by dissolving the appropriate amount of disodium tetraborate anhydrous in MilliQ water.

2.2 Instrumentation

The instrumentation used for electrophoretic separation was an Agilent 3D CE (Agilent Technologies, Waldbronn, Germany) equipped with UV diode-array detection (DAD). The detection of the barbiturates was performed at 214 nm.
A fused silica capillary of 100 cm in length (91.5 cm effective length) and an internal diameter (ID) of 50 μm was used.

2.3 Capillary Electrophoresis

2.3.1 CZE

New capillaries were conditioned by flushing at 930 mbar with 1M NaOH for 40 min prior to use and daily with 1M NaOH for 10 min and water for 10 min. Between separations, the capillary was conditioned with 1M NaOH for 3 min, water for 4 min, HDMB 0.01% in water for 5 min and BGE for 4 min at 930 mbar. Injections were performed by placing the sample (prepared in MilliQ water) in the inlet vial and applying 50 mbar for 10 seconds. Reversed polarity of -30 kV was used for the separation of the drugs with UV detection.

2.3.2 EKS

The applied EKS technique consisted of the hydrodynamic (HD) introduction of 50 mM NaCl as the leading electrolyte (50 mbar for 120 sec) and 100 mM CHES as the terminating electrolyte (50 mbar for 140 sec) before and after the electrokinetic injection (EKI) of the sample (adjusted to pH 9.6 with 0.5 M NaOH), respectively. The sample injection voltage was -8.5 kV for 300 s and the separation voltage was -30 kV. Fresh sample and BGE solutions (20 mM $Na_2B_4O_7$ at pH 9.15) were used for each experiment.

2.4 Experimental design and analysis of results

For chemometric analysis optimization, the set-up of the statistical analysis of the response variables was supported by the statistical graphics software system STATGRAPHICS plus 5.1 (STSC, Rockville, MD, USA). In all the studies, a three-level full-factorial design was applied. These designs required nine runs. Individual runs of the designs were carried out in a randomized sequence, guaranteeing that the uncontrolled variations would not influence the estimation.

2.5 Human urine samples pretreatment

Urine samples were obtained from a healthy volunteer. The extraction procedure used was based on literature [12] with modifications. Before the extraction of the drugs from the

samples, the pH value was adjusted to 4.0 with 1M HCl. Spiked urine samples were prepared by adding a standard solution containing the analytes at different concentrations into 500 μL blank urine in one Eppendorf tube. Sample pretreatment was carried out by adding 500 μL of ethyl acetate/*n*-hexane (40/60 v/v) to the spiked urine samples for the extraction of the barbiturates. After vortex mixing, the sample was centrifuged for 10 min at 9000 rpm (Hettich Zentrifugen, Germany). The organic phase containing the barbiturates was then transferred to another Eppendorf tube and a second extraction of the residual was performed by 500 μL of ethyl acetate/*n*-hexane (40/60 v/v) and repeating the procedure previously described. Finally, the two organic phases were combined and then evaporated to dryness under a gentle stream of dry nitrogen (UHP grade). The final residue was then dissolved with 100 μL of MeOH and diluted to 500 μL with 0.1 M NaOH (final pH of 9.6). This solution was transferred to a micro vial for EKS-CZE analysis.

3. Results and discussion

3.1 Coelectroosmotic separation of barbiturates

The values of pK_a of the studied barbiturates are all between 7 and 8. As the barbiturates are negatively charged at basic pH values, the electrokinetic injection of the analytes is not possible at positive voltages due to the counter electroosmotic flow. Several pieces of work have demonstrated the effectiveness of HDMB for reversing EOF at low concentrations, providing a net positive charge to the wall capillary, which suggests a change in both the magnitude and sign of the zeta potential [13,27,28,36]. Based on previous work [29], 5 min of preconditioning step with HDMB 0.01% in MilliQ water for reversing EOF was used. A negative voltage was then applied for the separation of the compounds being studied. The migration of the analytes in the same direction as EOF (Co-EOF) allowed the analysis run time to be reduced and also an improvement of resolution was achieved due to a reduction of the baseline instability in comparison with counterelectroosmotic mode.

Based on literature, two different BGE were tested in order to select the most appropriate separation buffer [13,37]. Sodium tetraborate anhydrous was tested at different concentration values ranging from10 to 50 mM and adjusted in all cases to pH 9.7 with NH_3. In the other hand, Tris was also tested at different concentration values ranging from 100 to 200 mM adjusted in all cases to pH 7.8 with concentrated HNO_3. Also, for the two BGE, a study of the separation voltage was done in the range between 20 and 30 kV. Optimum conditions for CZE coelectroosmotic separation of the three barbiturates, in terms of analysis time, resolution and peak shapes, were obtained using 20 mM $Na_2B_4O_7$

(pH 9.15) at -30 kV of separation voltage. Under these conditions, run time was around 8 minutes.

3.2 EKS performance for barbiturates

Prior to the study of the EKS performance of barbiturates in standard samples, EKI of the sample was tested by analysing a standard sample containing the analytes at a concentration of 0.1 μg/mL. An important consideration when performing EKS is the degree to which the analytes are ionized because this technique involves the electrokinetic injection of the sample. The pH value of the sample regulates the ionization of the compounds and consequently their absolute mobility. In our case, at values of pH greater than 9, the total ionization of the barbiturates for the electrokinetic injection study was guaranteed. In all experiments, the standard sample solution was adjusted to a pH value of 9.6 with 0.5 M NaOH. Figure 1A shows an electropherogram using electrokinetic injection of the sample at -5 kV for 80 seconds. The peaks in the electropherogram resulted split, poor in efficiency and sensitivity.

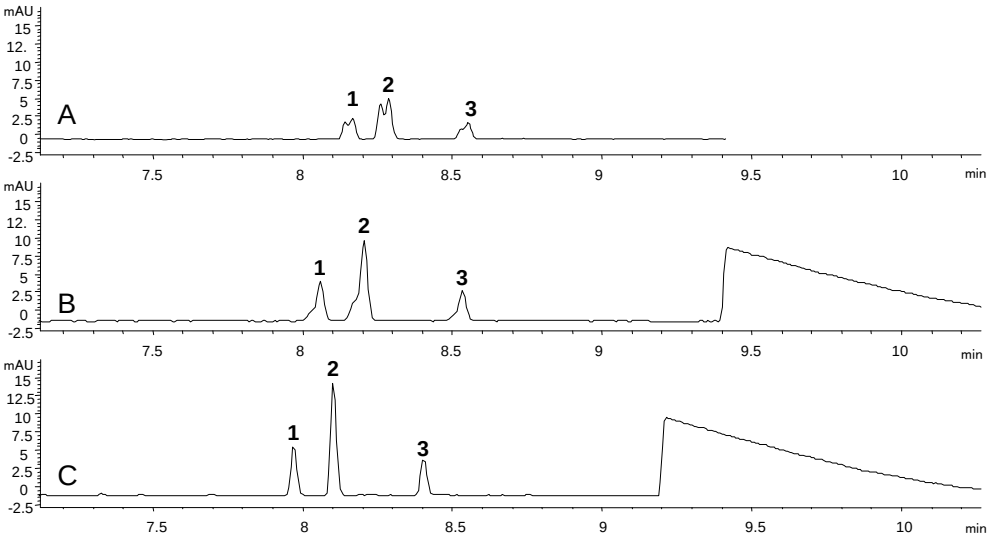

Figure 1. Electropherograms of three barbiturates at a concentration of 0.1 μg/mL (pH 9.6). BGE: 20 mM $Na_2B_4O_7$ (pH 9.15). The sample injection voltage was of -5 kV for 80 s. Separation voltage of -30 kV. (**A**) Normal electrokinetic injection (-5 kV for 80 s). (**B**) sample injection and 50 mM CHES as terminating electrolyte (50 mbar for 50 s) (**C**) EKS, 50 mM NaCl as leading electrolyte (50 mbar for 50 s), sample injection and 50 mM CHES as terminating electrolyte (50 mbar for 50 s). Peak indentification: barbital (1), phenobarbital (2) and secobarbital (3).

In order to generate an ITP state, the presence of LE and TE in the discontinuous electrolyte system is necessary. In t-ITP, after the preconcentration process, a transition to CZE mode occurs when the LE (due to its high mobility) spreads into the background electrolyte (BGE). The LE has to be faster in mobility than the target analytes and the co-ion of the BGE. At the same time, the terminating electrolyte has to have lower mobility than the analytes and the co-ion of the BGE.

An optimization of some parameters affecting the separation and preconcentration in EKS was then performed in order to improve the sensitivity of the method. The optimization process was carried out using a sequential univariated method for the choice of the more suitable LE, the study of the concentration of LE and TE, and the use of a low conductivity plug prior to the sample injection step. Multivariated methodology was then used to optimize parameters such as sample injection time, injection voltage and plug length of LE and TE.

3.2.1 Study of the effect of different leading electrolytes

The appropriate selection of the LE and TE is essential to guarantee the optimum performance of the EKS because the discontinuous electrolyte system governs the range of ions that can be stacked and also the extent of the stacking. In some cases, if the mobility of the co-ion in the BGE is higher than the mobility of the analytes, this co-ion can act as leading electrolyte [28]. Based on our experience in EKS methodology and on the literature [27,29] CHES was selected as TE and introduced after sample injection at 50 mbar for 50 seconds. As borate (electrophoretic mobility -36.2 10^{-5} cm^2/Vs) was used in the BGE, it was tested as LE, since it has higher electrophoretic mobility than the barbiturates (-20.4 10^{-5}, -19.11 10^{-5} and -16.5 10^{-5} cm^2/Vs for barbital, phenobarbital and secobarbital, respectively). Under these conditions and using CHES as TE (50 mbar x 50 s), some problems with peak shape were observed (Figure 1B), similar to those observed when simple electrokinetic injection was performed. Thus, other different leading electrolytes (NaCl, $NaNO_3$, $NaSO_4$ and NaAc) were evaluated. Their selection was based on their co-ion mobility, as this was also higher than the mobility of the BGE and also than the mobility of the barbiturates under study. The effective mobilities values were obtained by the software PeakMaster 5.3 [38]. Therefore, different sodium salts were prepared at a concentration of 50 mM. In all experiments, hydrodynamic injection of the LE was performed at 50 mbar for 50 seconds before sample injection. For this study, the electrokinetic injection of the sample which contains the barbiturates at a concentration of 0.1 μg/mL was performed at -5 kV for 80 seconds. Moreover, TE, 50 mM CHES was introduced after sample injection at 50 mbar for 50 seconds. For this

study, the SEFs were calculated according to equation (1):

$$SEF_{height} = \frac{h_{prec}}{h_{HD}} f \quad (1)$$

where h_{prec} is the peak height of the preconcentrated analyte, h_{HD} is the peak height of the non-preconcentrated analyte, detected after a conventional hydrodynamic injection of 10 seconds at 50 mbar, and f is the dilution factor.

The SEFs obtained when $NaNO_3$ and NaCl were used as leading electrolytes were similar due to the similar values of their effective mobilities (-74.1 10^{-5} cm^2/Vs and -79.1 10^{-5} cm^2/Vs respectively). However, when higher mobility ions such as SO_4^{2-} (-82.9 10^{-5} cm^2/Vs) were used, a decrease in the stacking effectiveness occurred. This fact can be probably attributed to the ion diffusion through the BGE before the efficient stack of the analytes and also due to a fast transition between ITP state and CZE. When the value of the effective mobility of the LE was close to the mobility of the co-ion of the BGE, as in the case of NaAc (-42.4 10^{-5} cm^2/Vs), the stacking effect seemed to be lower. We suggest that this happens because if the diffusion of the leading electrolyte is too low, the analytes can unstack before the CZE transition occurs. However, when chloride is used as LE, an improvement in peak shape and sensitivity was observed (Figure 1C). Therefore, as the greatest suitability of the EKS method for the selected barbiturates was reached when chloride and CHES were both used as LE and TE respectively, this configuration was selected for further studies.

3.2.2 Study of the effect of different concentrations of NaCl and CHES as leading and terminating electrolytes

In this study, different concentrations of the leading (NaCl) and the terminating (CHES) electrolytes were evaluated. In all the experiments, electrokinetic injection of the sample was performed at -5 kV for 100 seconds and the plugs of the LE and TE were injected at 50 mbar for 50 seconds.

Firstly, the concentration of the leading electrolyte was tested between 10 and 200 mM. For this study, the concentration of CHES was kept constant at 50 mM. When the LE (NaCl) is not used or a plug of low concentration is introduced before the sample injection, no or low stacking effect is observed. However, for a higher concentration of the LE an increase in the sensitivity was obtained, being highest for a concentration of NaCl in the range 50-65 mM. As a result, 50 mM NaCl was selected for further experiments. For higher LE concentrations (above the optimum), the sensitivity decreased in terms of peak height in all the experiments, even when no significant changes in the efficiency were observed.

The concentration of CHES as terminating electrolyte was also evaluated with concentrations ranging between 10 and 200 mM using 50 mM

NaCl as leading electrolyte. In this case, the behaviour was similar to that previously observed for the leading electrolyte concentration study. When the TE was not used, no stacking effect was observed. However, higher concentrations of CHES also resulted in an increase in sensitivity. The maximum sensitivity was obtained when the concentration of CHES was 100 mM. This was therefore chosen as the concentration for further experiments. Above the optimum value, the behaviour of the analytes was the same as in the LE study.

3.2.3 Study of the sample injection in EKS

In the optimization of the sample injection step, the main goal was to define the best experimental conditions that allow sufficient efficiency of the relevant peaks and that provide, at the same time, the highest sensitivity. At this stage of the method optimization, a response surface design was applied. The response surface required at least three levels for each factor to enable modelling curvature in the response. The response surface design results were analysed by building a polynomial model describing the relation between the responses and the factors under consideration. The evaluation of the fit of the calculated model was then made by a residual analysis. Graphically, the response surface can be visualized by drawing 2D contour plots or 3D response surface plots. A 2D response shows the isoresponse lines as a function of the level of two variables, while a 3D response surface represents the response, on a third dimension, as a function of the level of two variables.

In the three-level full-factorial design applied, the variables evaluated were sample injection voltage and injection time. The output responses analysed were the peak height and the efficiency for each compound. The range of values of the injection voltage was fixed between -12 kV and -5kV. For the injection time the range of the domain was between 30 and 300 seconds. In all the experiments, a standard sample containing the analytes at a concentration of 0.4 μg/mL (adjusted at pH 9.6) was analysed and hydrodynamic injection of the leading and terminating electrolyte was 50 seconds at 50 mbar in both cases.

After obtaining the model that predicts peak height and peak efficiency as function of the injection voltage and injection time, in the following step, the fit of the calculated model to the experimental data was evaluated by a residual analysis. Here, the experimental response and the response predicted by the model were compared for each experimental design point. Small residuals and no tendencies in the residuals were observed for the three analytes. The model was therefore considered to be adequate.

The analysis of the model (data not shown) showed clearly that the sensitivity (in terms of peak height) increased proportionally when the sample injection time increased as

well as the injection voltage. These increases in sensitivity were expected due to the higher amount of analyte injected when these both variables are increased. However, the peak efficiency decreased with the increment of the two studied factors for the three compounds. By making a compromise between high sensitivity and high peak efficiency, 300 s of injection time and -8.5 kV for injection voltage were selected as the optimum values for further experiments.

3.2.4 Study of the plug length of the leading and terminating electrolytes.

A multivariate optimization (three-level full-factorial experimental design) was also applied in order to obtain the optimal values for the LE and TE plug lengths. The output responses analysed were the peak height and the efficiency for each compound. The range of values for each factor was fixed between 30 seconds (injection plug at 50 mbar) and 150 seconds. Values over 150 seconds of both plugs were not considered since under these conditions, 15% of the capillary volume would be filled by these electrolytes and then not enough capillary length would be available for the separation of the analytes. In all experiments, standard samples containing the analytes at a concentration of 0.4 μg/mL (pH 9.6) were injected at -8.5 kV for 300 seconds.

In this study, the optimal value for the two parameters studied was selected based on a compromise between maximum sensitivity (in terms of peak height) and the best efficiency of the peaks. In the analysis of the results, the highest efficiency zone was close to the optimal zone of peak height. It was observed that, whereas the peak height increased with the increment of the two studied factors in the entire design domain, the peak efficiency had a maximum zone predicted by the model ranging between 80 and 130 seconds of hydrodynamic injection at 50 mbar of the LE and between 90 and 150 seconds for the TE. Figure 2 shows the graphical overlapped representtation of peak height and efficiency as 2D contour plots for each compound. The responses are presented as a function of LE plug length and TE plug length. The results show that both parameters had an influence on the responses. Hence, a simultaneous increase in the length of the LE and TE plug led to an increase in the peak height and efficiency, as can be observed from Figure 2. However, for the efficiency values, an optimum zone was observed. Overlapping the entire surface it was possible to find a compromise in optimal working area (represented by the striped area in Figure 2) between high sensitivity and high peak efficiency. The selected conditions were obtained using a hydrodynamic injection at 50 mbar for 120 seconds in the case of LE and 140 seconds for the TE.

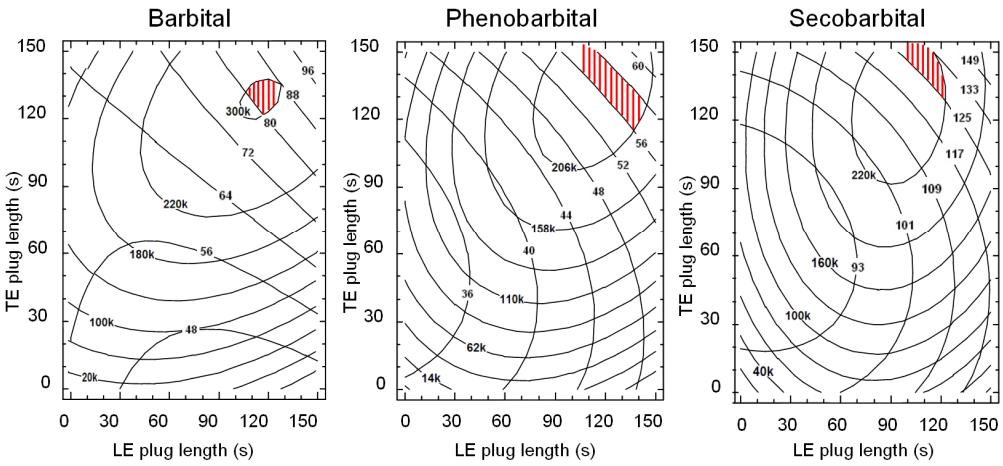

Figure 2. Graphical overlapped representation of the two responses (peak height and efficiency) as 2D contour plot for barbital, phenobarbital and secobarbital. Labeled lines correspond to the fitted model values for peak height and efficiency values (k = 1000). The responses are presented as a function of factors 1 (leading plug length (s)) and 2 (terminating plug length (s)). In all experiments introduction of the LE and TE plugs were done at 50 mbar.

3.2.5 Solvent plug of low conductivity

In some works, it has been reported that a short plug of a low conductivity solution, injected previously to the sample, enhanced the sensitivity of the analysis. Generally, this low conductivity plug provides a higher electrical field at the beginning of the capillary and ensures the fast migration of the analytes across this plug. In this way, it is possible to improve the sample stacking efficiency between this zone and the BGE zone [25,39,40].

In this work, two different plugs of a low conductivity solution were tested in order to verify whether there was an increase in stacking. The plugs of water and MeOH were injected hydrodynamically at 50 mbar for 20 seconds each. After the low conductivity plug was injected, the inlet end of the column was switched to the reservoir containing three barbiturates in a standard mixture of 0.4 μg/mL and the electrokinetic injection was performed at -8.5 kV for 300 seconds. The use of a low conductivity water plug did not seem to be significant when EKS-CZE was applied. Dawod *et al.* [28] observed the same enhancement in sensitivity with and without the use of a short water plug (10 mm). However, when the MeOH plug was used, the stacking improved but with a consequent irreproducibility in the injection current. Taking into account these considerations, no solvent plug was used for validation and application of the method.

3.3 Validation

The proposed EKS-CZE method was evaluated in terms of linearity, repeatability, reproducibility and limit of detection and quantification. Six standard solutions containing the studied barbiturates in a concentration range of 3 to 400 ng/mL were prepared and injected by triplicate. Calibration curves were constructed by plotting peak areas as a function of the concentration. The limit of detection (LOD) was considered as the minimum analyte concentration yielding an S/N ratio equal to three. The repeatability (intraday) was assessed as the relative standard deviation (RSD) of the peak area of replicate experiments (n = 5) at three different concentrations: 10, 100 and 300 ng/mL. The reproducibility (interday) was evaluated by using the results of the repeatability study over five consecutive days with the five replicates of each day. The recovery values for the developed EKS-CZE methodology for standard samples at different concentration levels were calculated. The corresponding values are given in Table 1.

Table 1. Calibration curves, values for reproducibility, repeatability, percent of recovery, SEFs and LODs for the studied drugs obtained for standard samples by EKS-CZE.

	Barbital	**Phenobarbital**	**Secobarbital**
Linearity (ng/mL)[a]	3 -400	3 -400	3 -400
Calibration curve	y=-1.3 + 3.4E2x	y=-4.5 + 5.3E2x	y=-11.5 + 10.3E2x
r^2	0.9998	0.9998	0.9989
Reproducibility (%RSD)[b]			
10 ng/mL	4.3 (102)[d]	3.2 (100)[d]	3.4 (97)[d]
100 ng/mL	5.1 (98)[d]	3.9 (99)[d]	3.1 (101)[d]
300 ng/mL	4.7 (101)[d]	3.8 (103)[d]	3.8 (100)[d]
Repeteability (%RSD)[c]			
10 ng/mL	2.7	1.5	2.8
100 ng/mL	2.5	1.5	2.7
300 ng/mL	2.9	1.9	2.9
LOD (ng/mL)	1.9	1.5	2.1
SEF	1115	1144	875

[a] y: peak area value (mAU x seconds); x: concentration (μg/mL).
[b] peak area (Interday analysis, n = 5)
[c] peak area (Intraday analysis, n = 5)
[d] recovery expressed as percent of theory

3.4 Application of the EKS-CZE method to urine samples

Once the EKS-CZE method was validated for standards, we tested the applicability of the method for human urine samples in order to demonstrate the potential of this method for the analysis of barbiturates in biological samples. The sample pretreatment required is detailed in section 2.5 of the experimental part. Figure 3A shows the electropherogram obtained from the injection of the extract corresponding to a blank urine sample while Figure 3B shows the electropherogram of the extract of urine spiked with 40 ng/mL of each barbiturate.

The estimated LODs of the three barbiturates in urine sample ranged between 8 and 15 ng/mL. The study of recoveries were carried out by comparing the peak area from drug free urine spiked with standard mixture of 100 ng/mL versus the peak area of the standard sample prepared in MilliQ water injected directly into the capillary. Recoveries in urine samples were calculated and the values were of 90%, 94% and 98% for barbital, phenobarbital and secobarbital respectively. Differences in the migration times of the analytes in standards and real samples were observed. This shift in the migration times suggests that the results depend on the nature of the sample matrix due the presence of some endogenous compounds that could have a small influence on the behaviour of the tested analytes.

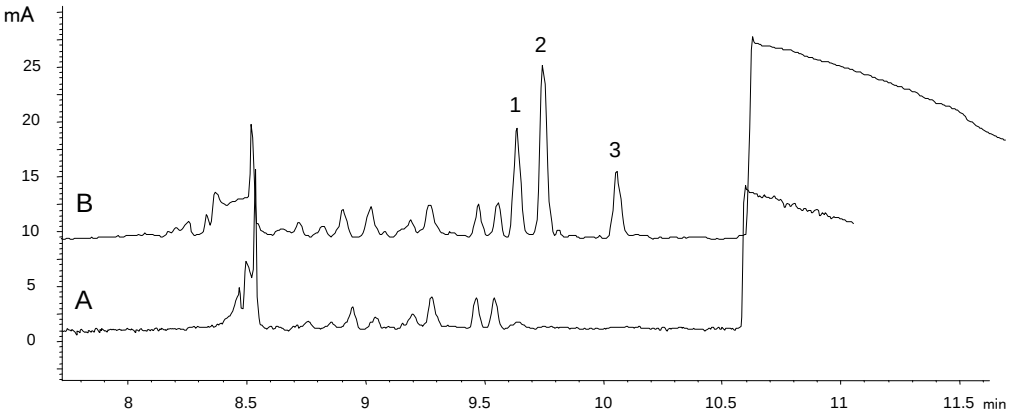

Figure 3. Electropherogram obtained from EKS of (A) blank urine sample and (B) urine sample spiked with 40 ng/mL of the studied barbiturates (pH 9.6). BGE: 20 mM $Na_2B_4O_7$ (pH 9.15). The sample injection voltage was of -8.5 kV for 300 s. Separation voltage of -30 kV. EKS, 50 mM NaCl as leading electrolyte (50 mbar for 120 s), sample injection and 100 mM CHES as terminating electrolyte (50 mbar for 140 s). Peak indentification: barbital (1), phenobarbital (2) and secobarbital (3).

4. Concluding remarks

EKS-CZE as a preconcentration technique makes the analysis of different kinds of matrices possible, such as biological samples with high conductivity, a factor which negatively affects many other preconcentration techniques. This simple and fast method allows the loading of a much larger amount of sample without affecting the resolution and has been shown to be effective for the analysis of barbiturates in urine. The methodology proposed provided around 1050-fold sensitivity enhancement compared with the results obtained by conventional CZE with hydrodynamic injection. For the analysis of real samples, a simple liquid-liquid extraction step was necessary in order to clean up the urine sample. The estimated LODs obtained from the three barbiturates were between 8 and 15 ng/mL in the urine samples extracted.

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The authors have declared no conflict of interest.

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Dipòsit Legal: T. 1299-2012

3.1.4. Discussion of results

UNIVERSITAT ROVIRA I VIRGILI
SENSITIVITY ENHANCEMENT STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF DRUGS OF ABUSE AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS
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Although the experimental results included in this section have been discussed individually in the corresponding papers, this section presents the most important aspects of them. The results obtained in the three studies presented have demonstrated the great potential of t-ITP-based preconcentration techniques in CE for the analysis of complex samples.

In the three studies included in this section, transient pseudo-ITP and EKS preconcentration techniques were investigated for preconcentrating pharmaceuticals and drugs of abuse. In the first study, a group of NSAIDs were analysed by transient pseudo-ITP. The method was optimized and applied to the analysis of biological samples, in particular, urine and plasma samples. The optimized parameters which affect the methodology performance were the composition of the BGE, sample volume and the composition and pH of the sample. Since stacking efficiency is affected by the composition of the BGE, because it is related to the difference in the analyte velocity between the sample zone and the BGE, optimization of this parameter was accomplished [1]. The most suitable BGE composition for the preconcentration and separation of the NSAIDs consisted of 100 mM anhydrous disodium tetraborate and 10% (v/v) of methanol (adjusted to pH 9.4). Separation was carried out in reversed EOF conditions in order to perform a co-EOF separation of the studied compounds.

Optimization of the sample composition was also important in order to generate an appropriate ITP state. To this end, the effect of the concentration of salt (NaCl) was studied, as well as the content of acetonitrile. The initial composition of the sample was then fixed at 1% NaCl/acetonitrile (30:70, v/v) because NaCl is the most abundant salt present in biological samples and 70% acetonitrile is frequently employed for the deproteinization of biological samples. In such a sample composition, the chloride ion acts as leading ion while the organic solvent acts as a terminating ion. Even though acetonitrile is the most commonly used organic solvent for transient pseudo-ITP, other water-miscible solvents can also act as pseudo-terminating electrolytes [2]. Therefore, different organic solvents were tested and with the use of ethanol, an improvement in resolution was observed, although stacking efficiency was similar to the case when acetonitrile was used. As a result, the optimal sample composition was 1% NaCl/ethanol (30:70, v/v).

One of the main advantages of transient pseudo-ITP is that it allows the introduction of a large volume of sample into the capillary without a loss in resolution and efficiency. In our case, the maximum volume of the sample injection was obtained at 50 mbar for 90 seconds, which correspond to 7.5% of the capillary volume.

Using this methodology, enhancement in sensitivity ranging between 40- and 46-fold was obtained and LODs for standard samples were in the range of 70 to 150 ng/mL.

Even though these LODs were not significantly low, they were suitable for the determination of these compounds in urine and plasma, as their occurrence in these matrices is usually higher. In this sense, the determination of NSAIDs in both urine and plasma samples, after deproteinization and extraction of the analytes, was also evaluated. For plasma samples, the LODs obtained ranged between 160 and 260 ng/mL and were similar to those reported in the literature using a similar methodology for ketoprofen [3]. In the case of the analysis of urine samples, LODs ranging between 320 and 750 ng/mL were achieved. However, to the best of our knowledge, the transient pseudo-ITP validation for simultaneous determination of NSAIDs in human urine samples has not previously been reported in the literature.

In the case of the last two studies, a considerable increase in the sensitivity was achieved using EKS in comparison with the transient pseudo-ITP technique. In these studies, the potential of the application of EKS in the analysis of complex matrices was clearly demonstrated, despite the use of an electrokinetic injection of the sample. Table 3.1 shows the main conditions for the performance of each preconcentration strategy, as well as the SEFs in terms of peak height and the LODs obtained by the developed transient pseudo-ITP and EKS methodologies.

Table 3.1 Experimental conditions and results for the t-ITP-based preconcentration strategies.

		Transient pseudo-ITP (NSAIDs)	**EKS (NSAIDs)**	**EKS (Barbiturates)**
Capillary		85.5 cm (50 μm ID)	88.5 cm (50 μm ID)	100 cm (50 μm ID)
Analysis time		14 min	10 min	10 min
Sample injection		50 mbar (90 s)	-2 kV (700 s)	-5 kV (300 s)
SEF $_{\text{peak height}}$		40 - 46	1,615 – 2,167	875 – 1,144
LOD (ng/mL)	Standards	70 - 150	0.08 - 0.10	1.5 – 2.1
	Plasma	160 - 260	2 - 8	-
	Urine	320 - 750	-	8 -15
	River water	-	0.9 - 1.5	-

As can be observed in the table, the preconcentration factors obtained by the use of EKS are approximately 45-fold higher than those obtained with transient pseudo-ITP and this can be explained by the electrokinetic injection of the sample.

In the second study, different possible configurations for the EKS performance were tested and the most suitable in terms of the SEFs achieved was the setup in which the leading ion was in the BGE while the TE was injected hydrodynamically after the injection of the sample. The selected setup was then optimized, taking into account the main variables affecting the preconcentration and separation processes. The optimized parameters were the concentration of NaCl (which was the selected LE), the influence of the presence of methanol in the BGE, the volume of a low conductivity solvent plug, the sample pH and the sample injection time, as well as the influence of the volume of the terminating electrolyte. Regarding the leading electrolyte, the best results were reached by the addition of 50 mM of NaCl to the BGE (10 mM $Na_2B_4O_7$ + 50 mM NaCl in 10% of MeOH aqueous solution) while 50 mM of CHES, as the terminating electrolyte, was injected hydrodynamically (50 mbar by 12 seconds) after the electrokinetic injection of the sample. With respect to the introduction of a low conductivity solvent plug, whereas in other stacking techniques there is a greater effect on preconcentration factors with the use of this solvent plug, it was found that this factor does not seem to be as significant in this case when EKS is applied.

As EKS can be considered as a combination of EKI and t-ITP, the role of the electrokinetic injection of the sample is important in order to reach considerably high preconcentration factors [4]. In comparison with the transient pseudo-ITP, the limitation in the injection time using EKS lies in the instability problems in the current for long injection times. However, in this case, at low voltages such as -2 kV, injection times up to 700 seconds could be achieved. Using this methodology, the sensitivity was enhanced by 1,615- to 2,167-fold and the LODs obtained in standard samples ranged between 0.08 and 0.10 ng/mL.

The applicability of the method was then tested for river water samples, as well as in human plasma samples. For river water samples, since the matrix composition affected the stacking process, a 10-fold dilution and further LLE step was necessary to obtain fully cleaned up sample matrices. The recoveries obtained ranged between 53 and 90%, while the LODs were as low as 0.9 ng/mL. These LODs obtained by EKS were similar to those obtained by Dawod *et al.* [5] using the same preconcentration strategy but with a different configuration of the discontinuous electrolyte system. The same authors presented a second approach in which they introduced a counter flow in order to increase the sample injection time. However, even though their LODs for standard samples were lower than in our case, the authors did not report data values for real samples [6].

To the best of our knowledge, the potential of EKS in the analysis of plasma samples has not previously been explored. In this case, integration of deproteinization and extraction into one single step, based on the use of LLE, provided extracts that were ready for analysis and the LODs obtained were in the range of 2 to 8 ng/mL, which were lower than those reported in literature using different methodologies for the analysis of NSAIDs in human plasma samples by CE [7,8].

In the third study, the potential of EKS in the analysis of urine samples for the determination of barbiturate drugs was investigated. The conventional configuration of EKS performance was selected, in which both leading and terminating electrolyte were hydrodynamically injected before and after the sample injection. In this work, optimization of the different parameters affecting separation and preconcentration in EKS was also performed in order to improve the sensitivity of the method. This optimization process was carried out using a sequential univariated method for the choice of the most suitable LE, the study of the concentration of the LE and TE, and the use of a low conductivity solvent plug prior to the sample injection step. Multivariated methodology was then used to optimize parameters such as sample injection time, injection voltage and plug length of the LE and TE. Different leading electrolytes were tested. However, NaCl was chosen as the best leading electrolyte due to the high sensitivity observed when it was used. With respect to the terminating electrolyte, 50 mM of CHES was injected hydrodynamically (50 mbar by 50 seconds).

Under the optimal conditions, the developed method provided SEFs of around 1,045 fold compared with conventional hydrodynamic injection. Validation of the methodology was achieved for standard samples and the LODs obtained ranged between 1.5 and 2.1 ng/mL. Even though validation of real samples was not performed, the applicability of the optimized methodology was demonstrated in urine samples. In this case, prior to the analysis, a simple LLE step was necessary in order to clean up the urine sample. The estimated LODs for the real samples ranged between 8 and 15 ng/mL with recoveries higher than 90%. Differences in the migration time of the compounds in standard and urine samples were observed and this behaviour suggests that the sample matrix composition plays an important role in the analysis of complex samples, such as urine.

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SENSITIVITY ENHANCEMENT STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF DRUGS OF ABUSE AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS
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3.2. Sensitivity improvement in capillary electrophoresis by in-line SPE-CE for the determination of drugs of abuse

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As mentioned in early sections, the abuse of illicit drugs is widespread nowadays and, consequently, these compounds are excreted in unchanged molecular form or as metabolites. The problems related to the consumption of these illegal substances make its determination necessary in medical, doping control and forensic analysis. In addition, as a consequence of their use, these compounds can reach wastewater treatment plants and can eventually be released into the environmental waters. As a result, these drugs have been found in different surface waters at ng/L concentration levels [1]. Different analytical methods have been reported for the determination of drugs of abuse in different sample matrices. Among them, the use of CE is highly promising as a separation technique and there is growing interest in its applicability in the analysis of biological samples [2,3]. However, to the best of our knowledge, the use of CE for the determination of these drugs in environmental samples has not previously been reported. One of the major limitations of CE relates to its lack of sensitivity. Therefore, the development of strategies which increase this sensitivity has become an important issue for those applications in which the compounds are present at low concentration levels.

Among the different strategies for increasing sensitivity in CE, extraction preconcentration techniques using SPE have proven to be excellent approaches, particularly those involving in-line integration with CE [4-7]. The main advantages of this technique lie in the possibility of higher sample injection volumes, the wide range of applicability and the high preconcentration factors that can be obtained [6,7]. Due to these benefits, in recent years, a number of papers using this approach have been reported in the literature, obtaining high preconcentration factors [8,9]. The most common setup for the in-line SPE-CE device involves the use of small packed beds with Oasis HLB, Oasis MCX and C18 [4,9,10] being the most commonly used sorbents in this approach. In this setup, the packing material can be retained in place by frits in order to prevent small-diameter particles from passing through the separation capillary. However, some authors have reported problems caused by the use of these frits, including irreproducibility with EOF and, in some cases, loss of current or band broadening due to bubble formation [11]. In response to this, a strategy has been reported in the literature in which the particle size of the sorbent is a key parameter, as the use of frits can be avoided if the particles are large enough [9]. This setup was selected for the development of the methods by in-line SPE-CE presented in this Doctoral Thesis.

In this section, the results of three studies are presented, which focus on the determination of drugs of abuse in both environmental waters and biological samples. With regard to the selected compounds, in the first study, cocaine, codeine, EDDP and 6-acetylmorphine were selected as the target compounds and they were determined in tap and river water samples. In the second study, four drugs of abuse (EDDP, codeine, hydrocodeine and 6-acetylmorphine) were also preconcentrated

and separated from urine samples. In the third study, a group of barbiturate drugs (barbital, phenobarbital and secobarbital) were also determined in urine samples. To the best of our knowledge, this is the first time that in-line SPE-CE methodology has been applied to the determination of these drugs of abuse in environmental and biological samples. The importance of these compounds has already been mentioned in the Introduction section and their molecular structures are presented in Appendix II.

In the three studies, different methodologies based on in-line SPE-CE were used, using OASIS HLB as the sorbent for the construction of the concentrator device in order to preconcentrate and separate the abovementioned drugs of abuse. This kind of polymeric solid-phase extraction material, particularly hydrophilic-lipophilic balanced (HLB), tends to be the preferred choice for extracting these compounds [12-15]. Optimization of the in-line SPE-CE performance involves the evaluation of different parameters which influence the preconcentration factors, as well as the separation of the target compounds. The most important parameters optimized in all the studies were sample pH, elution plug volume and sample loading time. Moreover, validation in standards and in real samples was performed to demonstrate the applicability and reliability of the proposed methodologies.

In the first study, the potential of this preconcentration methodology was investigated using UV as the detection system for the determination of a group of drugs of abuse. As tap and river water were analysed, only a filtration step was performed prior to the sample analysis. The second study focused on enhancing sensitivity by both the in-line SPE-CE device and the use of a MS as a powerful detection system for the determination of a similar group of compounds. In this case, the applicability of the method was evaluated for urine samples. The high selectivity obtained by the use of MS detector allowed the analysis of urine without any previous off-line pretreatment. As in the first study, the main parameters affecting the preconcentration were optimized in order to achieve the best performance of the in-line SPE-CE-MS system. The third study consisted of the application of this preconcentration strategy for the determination of barbiturates in urine samples with UV detection. In this case, due to the complexity of the matrix analysed using UV detection, a previous LLE procedure was necessary in order to clean up the sample matrix.

The results of these three studies have been published or submitted for publication in different analytical journals: *Electrophoresis 2012, 33, 528-535*, *Anal. Bioanal. Chem. 2012, 403, 777-784* and *Anal. Bioanal. Chem., 2012 (*submitted). The second study was carried out in collaboration with the research group of Professor Gerhardus J. de Jong of the Department of Pharmaceutical Sciences during a research stay at the University of Utrecht, The Netherlands.

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3.2.1 Investigation of in-line solid phase extraction capillary electrophoresis for the analysis of drugs of abuse and their metabolites in water samples.

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INVESTIGATION OF IN-LINE SOLID PHASE EXTRACTION CAPILLARY ELECTROPHORESIS FOR THE ANALYSIS OF DRUGS OF ABUSE AND THEIR METABOLITES IN WATER SAMPLES

Igor Botello, Francesc Borrull, Carme Aguilar, Marta Calull
Departament de Química Analítica i Química Orgànica, Faculty of Chemistry, Universitat Rovira i Virgili, Marcel·lí Domingo s/n, 43007 Tarragona, Spain

Abstract

In this study, in-line solid phase extraction (SPE) was used as an enrichment technique in combination with capillary electrophoresis (CE) for the preconcentration and separation of 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), cocaine (COC), codeine (COD) and 6-acetylmorphine (6AM). The separation buffer (BGE) used was 80 mM disodium phosphate anhydrous and 6 mM of HCl (final BGE pH of 3). The SPE extractor consists of a small segment of capillary filled with Oasis HLB sorbent and inserted into the inlet section of the electrophoretic capillary. Different parameters affecting preconcentration were evaluated, such as sample pH, the volume of the elution plug and sample injection time. The detection limits (LODs) reached for standard samples by in-line SPE-CE-UV ranged between 50 and 200 ng/L, with sensitivity enhancement factors ranging from 2300 to 5300. Reproducibility values (expressed in terms of relative standard deviation) were below 7.6% for standard samples. This is a simple and effective method for the determination of the studied drugs of abuse and their metabolites. The applicability of the developed method was demonstrated in tap and river water samples which were directly analyzed without any off-line pretreatment. Analytical parameters were evaluated and LODs were between 70 and 270 ng/L with relative recoveries between 85 and 97%.

Keywords: *Drugs of abuse, capillary electrophoresis, in-line solid phase extraction, preconcentration, water samples*

1. Introduction

The use of illicit drugs is widespread in today's society and the abuse of these compounds constitutes an irrefutable social problem with serious consequences such as higher health-care costs, increased crime rates and greater economic hardship [1]. Pharmaceutical and illicit drugs are excreted unchanged or as metabolites and eventually reach wastewater plants (WWTPs). These plants can remove a fraction of these compounds, but some residues can be released into the aquatic environment, ending up in surface water and even in drinking water [2]. Moreover, these drugs and their main metabolites have been classified as a group of emerging contaminants so determining their presence is an essential preliminary step to measure their concentration levels in different ecosystems [2, 3]. These drugs have been found in different surface water samples at ng/L levels. Cocaine has been reported in the concentration range between 6 and 678 ng/L in different European river waters [4-7]. Codeine has also been detected in river water at concentration levels around 150 ng/L [6].

Different analytical methods, such as those based on liquid chromatography or gas chromatography coupled to mass spectrometry, have been developed for the determination of drugs of abuse and their metabolites in environmental samples [1, 2, 4, 8]. Capillary electrophoresis (CE) is an attractive alternative due to its extreme efficiency, short analysis time and wide scope of application [9-12]. However, the application of CE for drugs of abuse have been mainly focoused to the optimization of the separation step [9, 10, 13] and to the analysis of biological samples. As an example, Gottardo et al. [11] described a new method for the determination of illicit drugs by capillary electrophoresis – electrospray ionization-time of flight- mass spectrometry in blood samples reaching limits of quantification ranging from 10 to 30 ng/mL. Also, da Costa et al. [14] and Alnajjar et al. [15] developed methodologies for the determination of illicit drugs and its metabolites in urine samples. The LODs achieved by da Costa et al. were in the range between 100 and 250 ng/mL. Lin et al. [16] established a method for the analysis of codeine in human hair by CSEF – sweep – MEKC using phosphate buffer as BGE. A stereoselective determination of methadone, EDDP and EMDP using cyclodextrine derivatives as BGE additives was developed by Kelly et al. [17].

Poor concentration sensitivity constitutes a major drawback to CE as these compounds are normally present in the environment at low concentration levels. Several strategies for improving this limited sensitivity have been proposed in the literature [18-23]. Among them, chromatographic preconcentration methods, including solid-phase extraction (SPE), allow high sample volumes to be injected because the analytes are absorbed onto a stationary phase. Moreover,

this phase allows an effective clean up of the sample matrix [18, 19, 24]. Although SPE performed off-line is very easy to implement, the increasing trend towards fully automated analytical methods has supported the development of on-line, at-line and in-line methods in SPE coupled to CE [18, 19, 21, 24, 25]. When in-line SPE-CE is used, the preconcentration column is an integrated part of the CE system. The main advantages of this coupling are that it is easily automated, requires low volume of organic solvent consumption throughout the process, needs relatively little sorbent material for the construction of the SPE device, and is capable of analyzing the complete eluate from SPE by CE [18, 19, 24].

There are several designs reported in the literature for the construction of the in-line SPE concentrator, such as open-tubular columns [26], small packed beds [27, 28], disks [29] and monolithic materials [30]. The most common approach for in-line SPE-CE designs is the use of small packed beds. In this setup, the packing material is usually retained by frits near the inlet end of the capillary in order to prevent small-diameter particles from passing through the separation capillary. The most commonly used sorbents in this approach are Oasis HLB, Oasis MCX and C18 [27, 28, 31, 32]. For example, Benavente *et al*. [32] described the development of an in-line SPE-CE method for the analysis of peptides in diluted standard solutions using mass spectrometry detection. In the construction of the analyte concentrator the authors used polyethylene frits (0.1 cm) in order to retain the irregularly shaped C_{18} end-capped silica material. Zhang *et al*. [33] also reported a similar method for the determination of chlorophenols in water. In that study, the frits were made using a sol-gel method before packing a SPE sorbent (Abselut Sorbent, Varian, USA) in the inlet end of the capillary. Although the first studies working with the packed bed design used frits, some authors have reported problems when these are used, including irreproducibility with EOF and, in some cases, loss of current or band broadening due to bubble formation. To solve these issues, some authors have adopted a strategy in which the particle size of the sorbent is a key parameter. If the particle size of the material is large enough, the use of frits can be avoided [27]. For example, Saavedra *et al*. [34] used Oasis MCX in the construction of a frit-free concentrator device for the determination of 3-nitro-L-tyrosine in biological samples by capillary electrophoresis.

The principal aim of this work is the evaluation of different parameters affecting preconcentration of EDDP, COC, COD and 6AM such as sample pH, the volume of the elution plug and sample injection time using a frit-free in-line SPE-CE-UV procedure. We selected the SPE sorbent based on previously published studies in which Oasis HLB and Oasis MCX were used for off-line SPE in the determination of drugs of abuse in surface water by

LC-MS with good results [2, 4, 5]. To the best of our knowledge, the application of a similar preconcentration system for drugs of abuse (in-line SPE-CE-UV) has not yet been reported in the literature. Different parameters affecting preconcentration were evaluated to achieve optimum performance in the determination of the four studied compounds. Finally, to show the applicability, the method was evaluated in the analysis of tap and river water samples without any offline pretreatment.

2. Materials and methods

2.1 Standards and reagents

All reagents were of analytical-reagent grade. Ultrapure reagent water purified by a MilliQ gradient system from Millipore (Bedford, MA, USA) was used throughout. 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP), cocaine (COC), codeine (COD) and 6-acetyl-morphine (6AM) were purchased from Cerilliant (Round Rock, TX, USA). Methanol was purchased from SDS (Peypin, France). Oasis HLB solid phase extraction sorbent with an average particle size of 60 μm was used for the construction of the SPE device and was supplied by Waters (Milford, MA, USA).

Stock standard solutions of 100 mg/L for each compound were prepared in methanol and stored at -18 ºC. Working standard solutions of the mixture of all the compounds at a concentration of 100 μg/L were prepared weekly by diluting the standard solutions in ultrapure water. The solutions with a lower concentration were prepared daily by diluting appropriate volumes of the working standard stock solution in MilliQ water.

2.2 Instrumentation

The instrument used for the electrophoretic separations was an Agilent 3D CE from Agilent Technologies (Waldbroon, Germany) equipped with an UV diode-array detector (DAD). The compounds studied were detected at 200 nm. The capillary chamber was set at 25 ºC for all the experiments.

2.3 CZE separation

We used a fused silica capillary supplied by Agilent Technologies (Waldbroon, Germany) measuring 65.5 cm in length (53 cm effective length) with an internal diameter (ID) of 50 μm and an external diameter (OD) of 360 μm. Separation was performed using an applied voltage of 30 kV. The separation buffer (BGE) consisted of 80 mM disodium phosphate anhydrous and 6 mM of HCl (final BGE pH of 3). Prior to the first use, capillaries were conditioned with 1M NaOH for 40 min and daily with 1M NaOH for 10 min and H_2O for 10 min. Between separations the capillary was conditioned with 1M NaOH for 4 min, H_2O for 4 min, and BGE for 4 min. Injections were performed by placing the sample in

the inlet vial and applying 50 mbar for 5 seconds.

2.4 Construction of the SPE-CE concentrator device

The first step in the construction of the SPE-CE device consisted on cutting 2 mm of bare fused-silica capillary of 150 μm I.D. and 360 μm O.D. A proper cut on both sides of the capillary is essential to obtain an optimum performance of the concentrator. This analyte concentrator (AC) was introduced 1 mm into a 0.5 cm piece of PTFE tubing (Grupo Taper S.A., Madrid, Spain) with an I.D. of 0.250 mm. PTFE material can expand to fit the outer diameter of the bare fused-silica capillary. Then, a piece of 7.5 cm of the separation capillary was introduced at the other end of the PTFE tubing until connect with the AC (inlet), and the free end of this capillary of 7.5 cm was connected to a vacuum pump using a syringe. Afterwards, the AC was introduced into the vial that contained the Oasis HLB sorbent, and this was loaded into the AC. In order to guarantee the homogeneity of the particle size, the sorbent had previously been sieved through a 50 μm steel sieve (the fraction with a particle size greater than 50 μm was then used). Then, the capillary of 7.5 cm and the AC were moved until the preconcentrator was placed in the half-way of the PTFE tubing. Finally, the CE separation capillary of 57 cm was introduced into the other part of the PTFE tubing until to join the other side of the AC (outlet). The entire process of fabricating the concentrator was monitored under a microscope. Finally, the assembly was installed in a CE cartridge and it was checked for abnormal flow by filling the whole capillary with water and applying a pressure of 930 mbar to a vial containing MeOH. The time needed for MeOH to reach the detector was measured by monitoring the signal at 200 nm. This time was about 43 s in a properly constructed capillary (in-line SPE-CE), which is the same that in a capillary without an in-line SPE.

2.5 In-line SPE-CE-UV procedure

New capillaries were conditioned with 1M NaOH for 40 min and H_2O for 10 min before coupling to the SPE-CE extractor device. Daily, and between separations, the capillary with the SPE extractor device was conditioned at 930 mbar with MeOH for 3 min and BGE for 4 min. Figure 1 shows a diagram of the in-line SPE-CE-UV procedure. The conditioning step was performed by rinsing and equilibrating the capillary containing the SPE sorbent with methanol for 3 min and then with BGE for 4 min. Then the water sample (adjusted to pH 7.2 with a solution of 1M of HCl) was loaded at 930 mbar for 30 min (A). The next step was a clean-up with the BGE solution at 930 mbar for 2 minutes to remove the unretained molecules and to equilibrate the capillary prior to elution and separation (B). Then, for the elution step, a plug of MeOH was injected at

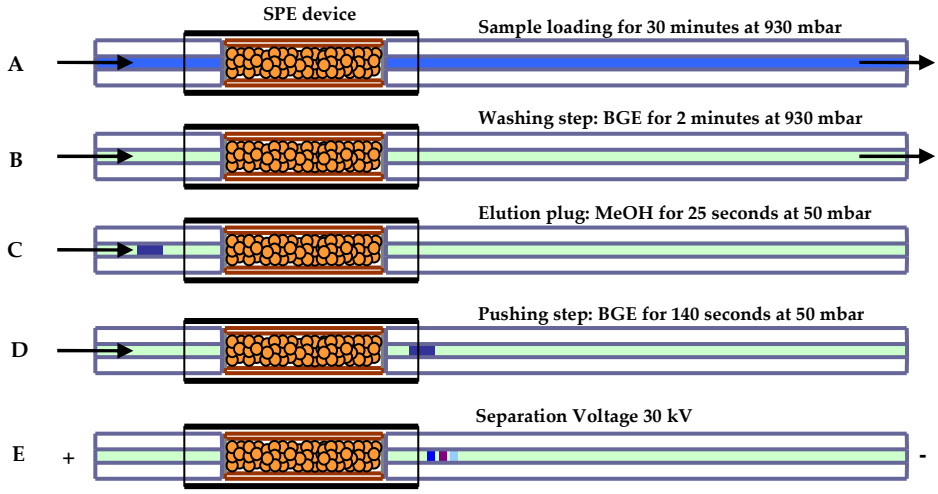

Figure. 1. Diagram of the in-line SPE-CE-UV procedure developed for the EDDP, COC, COD and 6AM preconcentration. From (A) to (E) the different steps followed to preconcentrate and separate the analytes are shown: (A) sample loading; (B) clean-up step; (C) introduction of the elution plug; (D) elution of the analytes from the SPE and (E) separation process. For a detailed description of the method, please see section 2.4.

50 mbar for 25 seconds (C) and this was subsequently pushed by the BGE solution at 50 mbar for 140 seconds (D). Normal polarity of 30 kV was used for the separation of the four analytes (E).

2.6 Tap and river water samples

Tap water samples were collected from the laboratory water supply (Tarragona city). Surface water samples were collected from the Ebro River. Samples were filtered through 0.22 μm nylon membranes (Supelco, Bellefonte, PA, USA) and stored in dark bottles for a maximum of three days at -18 Cº. Prior to the analysis, the samples were adjusted to pH 7.2 with a solution of 1 M of HCl.

3. Results and discussion

3.1 Separation of drugs of abuse and metabolites by CZE

To optimize the separation of the studied drugs by CZE, the selection of the background electrolyte was based on previously published studies in which sodium phosphate buffers were used to analyze different drugs [9, 10, 13]. In our work, we used disodium phosphate and tested concentrations ranging from 50 to 100 mM. As the studied compounds are weak bases, the pH of the BGE was adjusted to 3 in order to guarantee the positive ionization of the drugs. A Na_2HPO_4 concentration of 80 mM and 6 mM of HCl (final BGE pH of 3) was selected

because better resolution was obtained without an excessive increase in the Joule effect for the separation of EDDP, COC, COD and 6AM at 30 kV of separation voltage. Under these conditions run time was around 6.5 minutes.

3.2 Drug analysis by in-line SPE-CE-UV

In this work Oasis HLB was selected as the SPE sorbent to enrich EDDP, COC, COD and 6AM due the good recoveries previously reported in the literature for the analysis of these drugs [2, 4-6]. Oasis HLB is sold in different particle size ranges, something which has to be taken into account for the construction of the concentrator device when no frits are used, as in our case. The main consideration is that the size of the particles must be large enough in relation to the ID of the capillary to prevent the sorbent particles from escaping the system. If the particles are too small the use of frits is usually recommended. In our case, as the separation capillary had a inner diameter of 50 μm, a sorbent Oasis HLB with an average particle size of 60 μm was chosen.

Different parameters affecting the in-line SPE-CE-UV procedure were studied in order to obtain the optimum response in terms of sensitivity and resolution for the selected drugs.

3.2.1 Effect of sample pH

As mentioned earlier, the selected drugs are weak bases (pK_a values between 8 and 10) so sample pH can greatly affect their retention in the selected sorbent. Oasis HLB is a divinylbenzene/N-vinylpyrrolidone copolymer with hydrophilic and lipophilic properties. In the literature, different sample pH values have been reported when Oasis HLB is used to determine these drugs. For example, good retention of these compounds in this polymeric material has been reported at acidic and neutral pH values [1, 4] as well as at basic pH values [6] for the off-line preconcentration of water samples prior to liquid or gas chromatographic analysis. Based on this, and in order to study the effect of pH value on the retention of the analytes in our dynamic conditions using the in-line SPE device, different pH values ranging from 2 to 9 were tested. The study was performed under the following conditions: a standard mixture containing the analytes at a concentration of 100 μg/L (prepared by an appropriate dilution in MilliQ water) was loaded at 930 mbar for 5 minutes; then, a washing step with the BGE solution was carried out at 930 mbar for 2 minutes; and finally, for the elution of the compounds from the SPE sorbent, a plug of methanol was introduced into the capillary at 50 mbar for 10 seconds and this plug was then pushed through the SPE sorbent by introducing the BGE solution at 50 mbar for 200 seconds. This pushing step is necessary to displace the plug of elution solvent through the SPE device in order to elute the analytes

from the sorbent. To do this, the existing volume from the capillary inlet end to the SPE device must be taken into account. When using BGE for 200 s at 50 mbar as a pushing step, the volume of the capillary filled is approximately 0.235 µL, which corresponds to 11.9 cm of the capillary length (calculated through the Poiseuille's equation). This is enough to move the elution solvent out of the SPE sorbent.

The signal response in terms of peak area for all the studied analytes was higher when the pH value of the sample was 7.2 (see Figure 2), so this was the value chosen for further studies. This was in agreement with the results reported by González-Mariño *et al.* [6], who observed, after conducting an off-line SPE pretreatment with Oasis HLB, that the recoveries for compounds such as COC and COD were lower when the sample pH was below 6 than for higher pH values. The authors explained that those results were due to the high positive ionization of the compounds at lower pH values and, consequently, to their high polarity, which hinders their retention in this sorbent.

The optimization of the pushing step is important because the minimum volume that allows good resolution between peaks should be used but enough to assure that the elution plug is displaced out of the sorbent. We first performed different experiments consisting of introducing this pushing plug at different times between 100 and 200 seconds. We based our experiments on the theoretical calculation of the volume of the plug injected at 50 mbar (calculated through the Poiseuille's equation). The best conditions were obtained with 140 seconds at 50 mbar, which corresponds to a capillary volume of 0.165 µL (8.4 cm capillary length, which is enough to ensure that the methanol is displaced out of the SPE device). Problems with current losses occurred at lower injection times for the pushing plug, and this may be due to the fact that a part of the organic solvent was still inside the SPE device when the separation voltage was applied.

3.2.2 Study of the elution step

Pure methanol was selected as the elution solvent. This choice was based on previously published studies which demonstrated the potential of this organic solvent as a means of eluting analytes retained in Oasis HLB sorbent [2, 4, 35]. For example, Boleda *et al*. [2] used off-line SPE with Oasis HLB for the preconcentration of COD, 6AM, EDDP and other depressor drugs prior to UPLC-MS/MS analysis, and MeOH was used as the eluent. The authors reported recoveries for the different compounds in the range of 69 – 95%. In our case, different plugs of methanol were introduced into the capillary at 50 mbar for different periods of time from 5 to 30 seconds, and then the organic solvent was pushed through the SPE sorbent by the BGE solution at 50 mbar for 200 seconds.

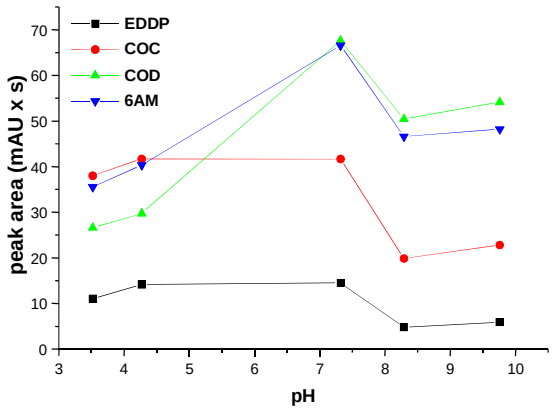

Figure. 2. Effect of sample pH on the peak area of the analytes by using in-line SPE-CE-UV. The concentration of the analytes in standard samples was 100 μg/L. Sample loading time was 5 minutes. Other experimental conditions are reported in the text.

The best results in terms of peak areas were obtained with a MeOH plug of 25 seconds at 50 mbar. When larger plugs of elution solvent were used, we did not see any improvement in the response and in fact a decrease in resolution was observed, probably due to the instability in the current profile caused by the presence of this plug of very low conductivity inside the separation capillary.

3.2.3 Effect of sample loading time

Increasing the injection time is the simplest way to improve sensitivity. However, it is important to ensure that the breakthrough volume of the SPE sorbent is not exceeded [28]. In order to examine the breakthrough volume, the sample loading time at 930 mbar was tested using a 0.5 μg/L mixture solution of the four compounds. In the range of loading times tested (from 20 to 60 minutes), the peak area increased in keeping with the loading time up to 30 minutes. For higher injection times, the responses obtained (peak areas) remained constant for the four compounds. If under these conditions we increase the volume of methanol to elute the analytes, we achieve a higher volume of sample that can be introduced into the system. However, we would like to remark that at higher elution plug volumes current disruption can occur in the system. In the end, taking into account all these considerations, we selected a sample loading time of 30 minutes as an optimum value because this time period provided maximum sensitivity in a reasonable analysis time.

3.3 Validation

We prepared standard solutions for EDDP, COC, COD and 6AM at different concentrations in MilliQ water at a pH of 7.2. The regression equations (which represent the peak

area versus concentration values), the values for reproducibility, repeatability, SEFs, LODs and absolute recoveries for the studied drugs are summarized in Table 1. The calibretion graphs generated were linear in the tested range. SEF values were calculated according to the equation (1):

$$SEF_{height} = \frac{h_{prec}}{h_{HD}} f \quad (1)$$

where h_{prec} is the peak height of the preconcentrated analyte, h_{HD} the peak height of the non-preconcentrated analyte detected after a conventional hydrodynamic injection of 10 seconds at 50 mbar and f is the dilution factor.

The repeatability of the calibration curves was determined by injecting five replicates on the same day (n=5) and reproducibility was evaluated on different days (n=5). The relative standard deviation (RSD) values for reproducibility of the peaks area did not exceed 7.6% when the concentration of the compounds was 1 μg/L. The SEFs (relating to the peak height) reached were between 2282 and 5337. Absolute recoveries for the analytes ranged between 72% and 96%.The study of the absolute recoveries was performed by comparing the peak area obtained for the eluted analytes from the SPE device with the response of the same moles of analytes injected in a capillary without the concentrator. The LODs ranged between 50 and 200 ng/L. Figure 3 shows the electropherogram obtained from a standard sample containing the analytes at 5 μg/L following the optimized in-line SPE-CE-UV method.

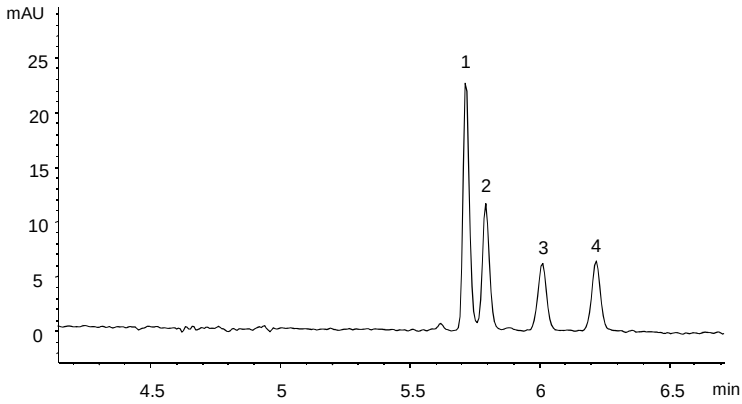

Figure. 3. Electropherogram of a standard sample containing the analytes at a concentration of 5000 ng/L at pH = 7.2 analyzed by in-line SPE-CE-UV in the optimized conditions. Peak assignments: 1, EDDP; 2, COC; 3, COD; 4, 6AM. Other experimental conditions are reported in the text.

3.4 Application

In order to explore the usefulness of the in-line SPE-CE analytical method developed in this study, it was used to analyze tap water and Ebro River water samples from Catalonia (Spain). Figure 4A shows the electropherogram obtained from a blank of tap water sample following the optimized

in-line SPE-CE method with the four drugs. The results indicate that no residues of the studied compounds were found in the samples. Figure 4B shows the electropherogram obtained for a tap water sample spiked at a concentration of 1 μg/L.

Table 1. Regression equations, reproducibility and repeatability values, SEFs, LODs and absolute recoveries for the four drugs in standard samples.

	EDDP	COC	COD	6AM
Linearity (ng/L)	100 - 5000	200 -5000	300 - 5000	300 - 5000
Calibration curve [c]	y=1.91 + 22.20x	y=1.12 + 11.22x	y=0.07 + 6.13x	y=0.82 + 9.04x
r^2	0.9998	0.9986	0.9998	0.9999
Reproducibility (%RSD, n = 3)[a]	4.7	6.3	7.6	7.1
Repeteability (%RSD, n = 3)[b]	1.6	2.4	3.2	4.8
SEF	5337	4352	2282	2341
LOD (ng/L)	50	140	200	200
Absolute recoveries (%, n = 3)	90.2	79.6	72.0	96.4

[a] interday analysis (n = 5) at 1 μg/L
[b] intraday analysis (n = 5) at 1 μg/L
[c] y: peak area value (mAU x s); x: concentration (ng/L)

Table 2 shows the validation parameters obtained for tap water. Linearity was determined from triplicate injections of the samples spiked between 0.1 and 5 μg/L. Relative recoveries were calculated by comparing the areas obtained for the standard mixtures (with AC) with the areas resulting from the analysis for tap and river water samples (with AC) containing the tested analytes at the same concentrations. These relative recoveries were higher at lower concentrations, ranging from 102 to 110%. The relative standard deviations (RSD) in terms of repeata-bility and reproducibility were below 4.3% and 9.1%, respectively. The LODs reached were between 70 and 256 ng/L calculated based on a 3:1 signal-to-noise ratio.

Figure 5A shows the electropherogram obtained from a blank river water sample following the optimized SPE coupled in-line in CE method. The electropherogram obtained for the river water sample spiked with the four drugs at a concentration of 1 μg/L is shown in Figure 5B. Linearity was determined from triplicate injections of the samples ranging between 0.15 and 5 μg/L. The relative recoveries for river water samples were calculated with the same procedure used for tap water. As in the tap water samples, the recovery values were higher at low concentrations, ranging from 96.5 to 105.8%. The relative standard deviations(RSD) in terms of repeatability and reproducibility were below 5.3% and 10.2%, respectively. The LODs rea-

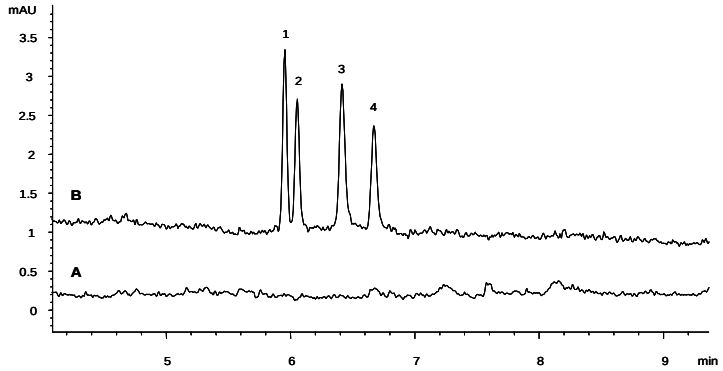

Figure. 4. A) Electropherogram of a blank of tap water sample. B) Electropherogram of a tap water sample containing the analytes at a concentration of 1000 ng/L at pH = 7.2 analyzed by in-line SPE-CE-UV. B) Spiked tap water sample. Other experimental conditions are reported in the text.

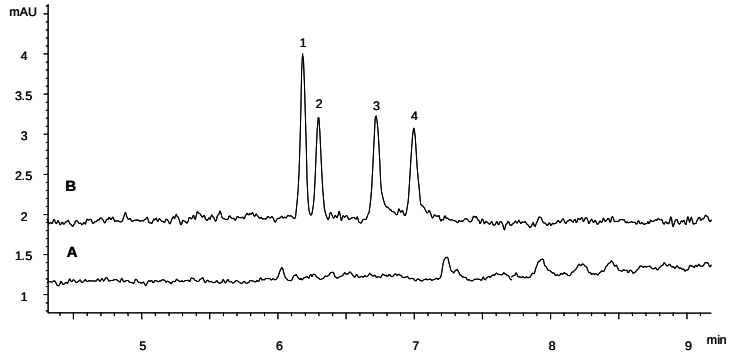

Figure. 5. A) Electropherogram of a blank of river water sample. B) Electropherogram of a river water sample containing the analytes at a concentration of 1000 ng/L at pH = 7.2 analyzed by in-line SPE-CE-UV. Other experimental conditions are reported in the text.

ched were between 100 and 270 ng/L, calculated based on a 3:1 signal-to-noise ratio. Even that the LODs obtained for river water were in general higher than the concentration found in some European rivers for the same compounds [4-7], we would like to emphasize that in our case, the LODs, as the best of our knowledge, are the lowest obtained by capillary electrophoresis for these drugs. In addition, it would be possible to decrease our LODs values applying an additional off-line SPE step prior the methodology proposed or by the combination of electrophoretic pre-concentration techniques with the in-line SPE-CE method.

4. Concluding remarks

A method has been developed for the preconcentration and separation of drugs of abuse using the in-line coupling of SPE to CE. The main advantages of the entire methodology are the complete automation of the process, low cost and the high SEFs

Table 2. Regression equations, reproducibility and repeatability values, LODs and the relative recoveries for the four drugs in real water samples.

	EDDP	COC	COD	6AM
TAP WATER				
Linearity (ng/L)	100 - 5000	250 - 5000	300 - 5000	300 - 5000
Calibration equation curve	y = -0.04 +4.75x	y = -0.002 + 3.63x	y = 0.22 + 4.64x	y = -0.07 + 3.86x
r^2	0.9987	0.9996	0.9998	0.9988
Reproducibility (%RSD)[a]	8.8	6.5	8.6	9.1
Repeatibility (%RSD)[b]	3.1	2.3	4.1	4.3
LOD (ng/L)	70	200	250	260
Relative recoveries (%, n = 3)				
1000 ng/L	105.7	110.1	108	102
5000 ng/L	89.7	97.2	89.6	104.2
RIVER WATER				
Linearity (ng/L)	150 - 5000	300 - 5000	300 - 5000	300 - 5000
Calibration equation curve	y = 0.45 + 5.01x	y = -0.07 + 3.81x	y = 0.13 + 4.53x	y = -0.06 + 4.10x
r^2	0.9995	0.9975	0.9988	0.9988
Reproducibility (%RSD)[a]	7.9	9.5	8.5	10.2
Repeatibility (%RSD)[b]	4.5	3.1	4.9	5.3
LOD (ng/L)	100	270	250	260
Relative recoveries (%, n = 3)				
1000 ng/L	103.8	97.8	96.5	105.8
5000 ng/L	95.4	97.2	85.5	95.5

[a] interday analysis (n = 5) at 1 μg/L
[b] intraday analysis (n = 5) at 1 μg/L
[c] y: peak area value (mAU x s); x: concentration (ng/L)

obtained due to the great volume of sample that can be injected. The procedure can also be considered highly environmentally friendly due to the low quantity of organic solvent consumed for each analysis. High recoveries and good values of RSDs for intra and interday analysis were obtained when tap water and river water samples were analyzed under the optimized conditions without any previous pretreatment. Further works will be focused applying off-line SPE steps methodology and combining electrophoretic preconcentration techniques with the in-line SPE-CE method in order to decrease the LODs for the studied drugs of abuse.

Acknowledgments

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The authors have declared no conflict of interest.

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3.2.2 In-line solid-phase extraction-capillary electrophoresis coupled with mass spectrometry for the determination of drugs of abuse in human urine.

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IN-LINE SOLID PHASE EXTRACTION-CAPILLARY ELECTROPHORESIS COUPLED WITH MASS SPECTROMETRY FOR THE DETERMINATION OF DRUGS OF ABUSE IN HUMAN URINE

Igor Botello[a], Francesc Borrull[a], Marta Calull[a], Carme Aguilar[a],
Govert W. Somsen[b], Gerhardus J. de Jong[b]
[a]Departament de Química Analítica i Química Orgànica, Faculty of Chemistry, Universitat Rovira i Virgili, Marcel·lí Domingo s/n, 43007 Tarragona, Spain
[b]Division of Biomedical Analysis, Department of Pharmaceutical Sciences, Utrecht University, 3584 TC Utrecht, The Netherlands

Abstract

An in-line solid phase extraction -capillary electrophoresis coupled with mass spectrometry detection (SPE-CE-MS) method has been developed for the determination of 2-ethylidene-1,5-dimethyl-3,3-diphenylpirrolidine (EDDP), codeine (COD), hydrocodeine (HCOD) and 6-acetylmorphine (6AM) in urine. The developed preconcentration system consists of a small capillary filled with Oasis HLB sorbent and inserted into the inlet section of the electrophoresis capillary. The SPE-CE-MS experimental conditions were optimized as follows: the sample (adjusted at pH 6.0) was loaded at 930 mbar for 60 minutes, elution was performed with methanol at 50 mbar for 35 seconds, 60 mM ammonium acetate with pH 3.8 was used as running buffer, the separation voltage was 30 kV and the sheath liquid at a flowrate of 5.0 μL/min was constituted by isopropanol/water (50:50 v/v) containing 0.5% of acetic acid. The analysis of 1:1 v/v diluted urine samples spiked with the four drugs was studied in the linearity range of 0.08 – 10 ng/mL. Detection limits (LODs) (S/N = 3) were between 0.013 – 0.210 ng/mL. Repeatability values (expressed in terms of relative standard deviation) were below 7.2 %. The methodology developed is simple and effective for the determination of the studied drugs of abuse in urine samples at levels required in toxicological and doping fields.

Keywords: *Drugs of abuse, capillary electrophoresis, in-line solid phase extraction, preconcentration, urine, mass spectrometry*

1. Introduction

Nowadays, consumption of drugs of abuse is a serious problem around the world and still represents an analytical challenge for doping control and forensic analysis. A common task in forensic laboratories involves the determination of drugs of abuse and their metabolites in biological fluids for investigation of intoxication or to determine the causes and circumstances of death. The main problem in the analysis of biological samples lies in the fact that in these kinds of complex matrices, interfering endogenous compounds are present in a higher concentration than the target analytes. Different analytical methods based on liquid chromatography or gas chromatography coupled to mass spectrometry as identification tool have been developed for the determination of drugs of abuse in environmental and biological samples [1-4]. Capillary electrophoresis (CE) has been proven to have a great potential in the determination of drugs of abuse due to its excellent separation efficiency, reduced sample and solvent volumes and high versatility in terms of separation modes [5-7]. These characteristics of CE make it suitable for the needs of forensic science laboratories [6, 8, 9].

Usually, when biological samples are analyzed, ultrafiltration, protein precipitation, liquid-liquid extraction and solid-phase extraction are commonly required for sample cleanup and preconcentration of several compounds prior to CE analysis. Nevertheless, sample preparation steps are normally performed off-line and manipulation could be a problem when low sample volumes and low analytes concentration are analyzed [10]. Moreover, the lack of sensitivity in CE is an important issue regarding to the application in forensic, toxicological and doping fields [11]. To reach low limits of detection in such complex matrices is possible by the combination of an effective preconcentration technique with a high sensitive detection system [12]. Therefore, it is necessary to develop sensitive and selective methods for extraction, preconcentration, separation and determination of these drugs in human body fluids when these compounds are present at low concentrations [8].

In CE, there are two main groups of methodologies which can be used in order to preconcentrate the target analytes in the sample: those in which the preconcentration is based on electrophoretic mechanisms and those based on chromatrographic methods. The main electrophoretic preconcentration mechanisms found in the literature are sample stacking, field-amplified sample injection and isotachophoresis [13]. However, one of the main drawbacks of using these techniques for complex matrices (as some biological or environmental samples) implies that a great amount of endogenous compounds are preconcentrated simultaneously with the analytes of interest and consequently the separation is affected. So, an exhaustive clean up of the samples is recommended to solve this problem

[13, 14].

In this regard, chromatographic preconcentration methods in CE, in particular in-line and on-line SPE, are an effective alternative to overcome the lack of sensitivity when complex matrices are analyzed. With the use of these approaches is possible to avoid losses of analytes, reduce the cleanup time and the risk of sample contamination [15].

In in-line SPE-CE, a hand-made microcartridge is inserted near to the inlet end of the separation capillary [16, 17]. This concentrator device contains a solid-phase extraction sorbent which retains the analytes. After the conditioning step, a large volume of the sample can be introduced into the capillary. The retained analytes are eluted displacing a small plug of the elution solvent through the SPE device before the application of an appropriate voltage for the separation step. The method then results in an enhancement on the sensitivity with minimum sample handling [12]. This methodology has been successfully used in the analysis of biological samples for different groups of compounds and the most commonly used sorbents in this approach are Oasis HLB, Oasis MCX and C18 [12, 16, 18-23].

Capillary electrophoresis coupled to mass spectrometry (CE-MS) has been increasingly used as this hyphenation combines the high efficiency of CE with the universality, selectivity and sensitivity of the MS [7, 9, 24]. Since the development of electrospray ionization (ESI) in which ionized molecules can be efficiently transferred from a liquid to a gas phase, MS has become a valuable tool as detection system for CE, in particular for doping control and forensic screening [7, 11]. However, when MS is used as detection system some variables have to be taken into account in order to guarantee the good performance of the hyphenated system. In this sense, the method is constricted to the use of some kinds of BGE solutions with different concentration [25]. Nevertheless these limitations, some authors have applied successfully CE-MS for biological samples [7-9]. For example, da Costa et al. [7] proposed a method for the determination of cocaine and its principal metabolites in urine by CE coupled to MS *via* electrospray ionization. Gottardo et al. [9] applied capillary zone electrophoresis–electrospray ionization–time-of-flight mass spectrometry for the determination of amphetamines in blood. Hernández et al. [10] developed a SPE-CE-ESI-MS methodology in order to explore the preconcentration and separation of dilute solutions of six opioid peptides in plasma samples.

In this study, a SPE microcartridge containing Oasis HLB as extraction sorbent was used for the in-line preconcentration and analysis of EDDP, COD, HCOD and 6AM by SPE-CE-MS. Several operational parameters affecting SPE-CE performance were studied to get the optimum conditions in the determination of the four studied compounds. Finally, the method was validated for the analysis of

urine samples with MS detection after an optimization of different parameters which affect the MS response. The main goal of this work was to establish a method based on in-line SPE CE-MS for the simultaneous determination of these drugs in a complex matrix such as urine.

2. Experimental

2.1 Standards and reagents

All reagents were of analytical reagent grade. Ultrapure reagent water purified by a MilliQ gradient system (Millipore, Bedford, MA, USA) was used throughout. 2-ethylidene-1,5-dimethyl-3,3- diphenylpirrolidine (EDDP), codeine (COD), hydrocodeine (HCOD) and 6-acetylmorphine (6AM) were purchased from Cerilliant (Round Rock, TX, USA). Ammonium acetate was pur-chased by Sigma Aldrich (St. Louis, MO, USA). Methanol and isopropanol were purchased from SDS (Peypin) France. Oasis HLB solid phase extraction sorbent with an average particle size of 60 μm was used for the construction of the SPE device and was supplied by Waters (Milford, MA, USA).
For the ESI-MS infusion experiments, 1 ng/mL solution of test compounds was prepared in methanol. The composition of the sheath liquid was isopropanol-water (1:1 v/v) containing 0.5% of acetic acid (Sigma Aldrich (St. Louis, MO, USA)). Stock standard solutions of 100 mg/L for each compound were prepared in methanol and stored at -18 ºC. Working standard solutions of the mixture of all the compounds at a concentration of 100 ng/mL were prepared weekly by diluting the standard solutions in ultrapure water. The solutions with a lower concentration were prepared daily by diluting appropriate volumes of the working standard stock solution in MilliQ water.

2.2 Instrumentation

The used instrumentation for electrophoretic separation was an Agilent 3D CE (Agilent Technologies, Waldbroon, Germany) equiped with UV diode-array detection (DAD). A fused silica capillary (Agilent Techno-logies, Waldbroon, Germany) of 100 cm length (91.5 cm effective length for UV experiments) with an inner dia-meter (ID) of 50 μm and an outer diameter (OD) of 360 μm was used. The capillary chamber was set at 25 ºC for all the experiments. SPE-CE-MS experiments were performed using an Agilent Technologies 1100 Series LC/MSD SL ion-trap mass spectrometer (Waldbronn, Germany) equipped with an Agilent Technologies ESI. The coupling of ESI-MS with CE was achieved through a coaxial sheath-flow interface (CE–MS sprayer of Agilent Technologies). The CE system was positioned in the way that both-capillary inlet and the tip of the sprayer needle were at the same height. The MS instrument was operated in positive ion mode and the scan range was 250 – 350 m/z.

2.3 CE-ESI-MS

Separation was performed at 30 kV with both UV and MS detection. The separation buffer (BGE) consisted of 60 mM ammonium acetate adjusted at pH 3.8 with concentrated acetic acid. Prior to the first use, capillaries were conditioned with 1M NaOH for 40 min and daily with MeOH for 10 min and H_2O for 10 min. Between separations the capillary was conditioned with MeOH for 4 min, H_2O for 4 min, and BGE for 6 min. Injections were performed by placing the sample in the inlet vial and applying 50 mbar for 5 seconds. For SPE-CE-MS a sheath liquid of isopropanol/water (50:50 v/v) containing 0.5% of acetic acid was used. MS detection was performed in the ESI positive ionization mode with electrospray voltage of 3500 V. MS was operated in SIM mode and nitrogen was used as nebulizer gas. All the experimental conditions for MS are summarized in Table 1.

Table 1. Optimized MS conditions.

Parameters	ESI
Spray potential	3500 V
Capillary exit potential	162.5V
Skimmer potential	39.1V
Sheath liquid composition	isopropanol/water (50:50 v/v) + HAc (0.5 % v/v)
Sheath liquid flow rate	5 μl/min
Dry gas flow rate	5 l/min
Nebulizer gas pressure	5 psi

2.4 Construction of the SPE-CE concentrator device

The first step in the construction of the SPE-CE device consisted on cutting 2 mm of bare fused-silica capillary of 150 μm I.D. and 360 μm O.D. This capillary was the analyte concentrator (AC). A proper cut on both sides of the capillary is essential to obtain an optimum performance of the concentrator. The AC was introduced 1 mm into a 0.5 cm piece of PTFE tubing (Grupo Taper S.A., Madrid, Spain) with an I.D. of 0.250 mm. PTFE material can expand to fit the outer diameter of the bare fused-silica capillary. Then, a piece of 7.5 cm of the separation capillary was introduced at the other end of the PTFE tubing until connection with the AC (inlet), and the free end of this capillary of 7.5 cm was connected to a vacuum pump using a syringe. Afterwards, the AC was introduced into the vial that contained the Oasis HLB sorbent, and this was loaded into the AC. In order to guarantee the homogeneity of the particle size, the sorbent had previously been sieved through a 50 μm steel sieve (the fraction with a particle size greater than 50 μm was then used). Then, the capillary of 7.5 cm and the AC were

moved until the preconcentrator was placed in the half-way of the PTFE tubing. Finally, the CE separation capillary of 91.5 cm was introduced into the other part of the PTFE tubing until to join the other side of the AC (outlet). The entire process of fabricating the concentrator was monitored under a microscope. Finally, the assembly was installed in a CE cartridge and it was checked for abnormal flow by filling the whole capillary with water and applying a pressure of 930 mbar to a vial containing MeOH. The time needed for MeOH to reach the detector was measured by monitoring the signal at 200 nm. This time was about 43 s in a properly constructed capillary (in-line SPE-CE), which is the same as that for a capillary without an in-line SPE.

2.5 In-line SPE-CE procedure

New capillaries were conditioned with 1M NaOH for 40 min and H_2O for 10 min before the coupling with the SPE-CE extractor device. Daily, and between separations, the capillary with the SPE extractor device was conditioned at 930 mbar with MeOH for 3 min and BGE for 4 min. Then, the sample (adjusted to pH 6.0 with a solution of 1M of NaOH) was loaded at 930 mbar for 60 min. The next step was a clean-up with BGE solution at 930 mbar for 2 minutes to remove the unretained molecules and to equilibrate the capillary prior to elution and separation. Then, for the elution step, a plug of MeOH at 50 mbar for 35 seconds was injected and this was subsequently pushed by the BGE solution at 50 mbar for 220 seconds. Normal polarity of 30 kV was used for the separation of the four analytes.

2.6 Urine samples

Human urine were collected from a healthy volunteer, filtered through 0.22 μm nylon membranes (Supelco, Bellefonte, PA, USA) and stored at -18 ºC. Before analysis samples were diluted 1:1 in MilliQ water and then adjusted to pH 6 with 1 M NaOH.

3. Results and discussion

3.1 CE conditions

To optimize the separation of the studied drugs by CE, a volatile background electrolyte should be selected for the coupling with the MS. Some papers report the use of this type of volatile electrolyte in CE separation of drugs of abuse when hyphenation with MS detection is used. For example, Aturki et al. [24] used ammonium formate buffer at pH 3 containing ACN as mobile phase for CEC-ESI ion trap MS for determination of drugs of abuse in human urine samples. Da Costa et al. [7] employed formic acid (1 M) for the separation of cocaine and main metabolites in urine by CE coupled to MS. In our work, ammonium acetate was selected as BGE and the pH was adjusted to 3.8 in order to guarantee the positive ionization and good separation of the analytes. To optimize the separation of the compo-

unds, different BGE concentrations in the range from 20 to 100 mM were tested. Increasing the BGE concentration an improvement of resolution and peak shape was observed, however, the analysis time also increased, and for that we tested also the separation voltage from 20 to 30 kV. Finally, an ammonium acetate concentration of 60 mM adjusted at pH 3.8 with concentrated acetic acid was selected because good resolution was obtained in a reasonable analysis time for the separation of EDDP, COD, HCOD and 6AM at 30 kV of separation voltage.

3.2 SPE-CE parameters

Oasis HLB was selected as the SPE sorbent to enrich EDDP, COD, HCOD and 6AM due the good results obtained for this kind of drugs in our previous work focused on the analysis of environmental water samples (Botello et al. [23]). Different parameters affecting the in-line SPE-CE performance (using UV detection) were studied in order to obtain the optimum sensitivity and resolution for the four drugs in study. Although the optimization of those parameters was already performed in the previous paper [23], we evaluated again the performance of in-line SPE-CE since in the present study the compounds are not all the same and this can mean a different behaviour in the extraction process. Moreover, the dimensions of the capillary were not the same as in our previous work and this can also affect the obtained results.

The sample pH can greatly affect the retention of the drugs in the Oasis HLB sorbent since the studied drugs are weak bases (pK_a values between 8 and 10). In the literature, different sample pH values have been reported when Oasis HLB is used to determine these drugs. For example, good retention of these compounds in this polymeric material have been reported at acidic and neutral pH values [1, 26] as well as at basic pH values [4] for the off-line preconcentration of water samples followed by to liquid or gas chromatographic analysis. In order to study the effect of pH value on the retention of the analytes different pH values ranging from 3.5 to 10.5 were tested. For this study, standard mixtures of 10 ng/mL were injected at 930 mbar for 10 minutes. As is shown in Figure 1, the signal response for the studied analytes was higher when the pH value of the sample was in the pH range between 5.5 and 7, and pH 6 was chosen for further studies.

Pure methanol was selected as the elution solvent based on a previous study which demonstrated the potential of this organic solvent to elute the drugs of abuse retained in Oasis HLB sorbent [23]. In this work, different plugs of methanol were introduced into the capillary at 50 mbar for different periods of time from 20 to 40 seconds, and then the organic solvent was pushed through the SPE sorbent by the BGE solution at 50 mbar for 220 seconds. For this study, standard mixtures of 10 ng/mL at pH value of 6 were injected at 930 mbar for 10 minutes. For 40 seconds of elution

plug, some instability in the current profile occurred, probably by the presence of this large plug of very low conductivity inside the separation capillary. For that reason, the methanol plug was reduced to 35 seconds at 50 mbar and, in this case, the reproducibility was improved and also the peak area values were higher than for lower methanol plug. Coupling in-line SPE to CE allows the injection of a high sample volume.

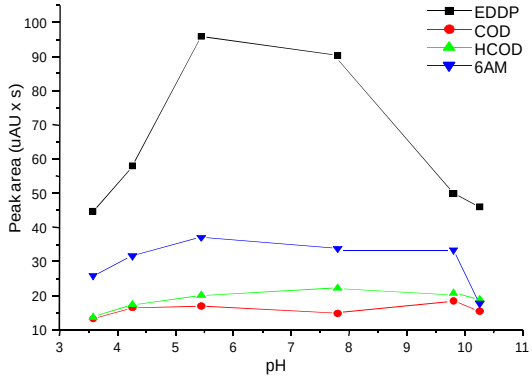

Figure 1. Effect of sample pH on the peak area of the analytes by using in-line SPE-CE-UV. The concentration of the analytes in standard samples was 10 ng/mL. Sample loading time was 10 minutes. Other experimental conditions are reported in the text.

However, it is important to ensure that the breakthrough volume of the SPE sorbent is not exceeded. To examine the breakthrough volume, the sample loading time at 930 mbar using a 10 ng/mL mixture solution of the four compounds, was evaluated in the range between 10 to 60 minutes. The peak area increased with the loading time up to 60 minutes. However, as a compromise between this parameter and the analysis time, 60 minutes of loading time were selected as the optimum value because it provided a high sensitivity in a reasonable analysis time. Figure 2 shows the typical electropherogram of a standard mixture of the studied drugs under optimum conditions. Once the principal parameters which affect the in-line SPE performance were optimized, initial experiments for the analysis of human urine were performed. For that, the urine samples were diluted 1:1 v/v with purified water, spiked with 10 ng/mL of the four drugs and adjusted to pH 6.0 with a solution of 1M of NaOH. Following, the sample was loaded on the SPE sorbent at 930 mbar for 60 minutes keeping constant the rest of the optimized parameters. Results demonstrated that some interfering endogenous compounds were also preconcentrated with the analytes. The signals of these endogenous compounds overlapped with the signals obtained from the analytes, and the resolution was not enough to detect and quantify the drugs in the urine sample when UV detection was used.

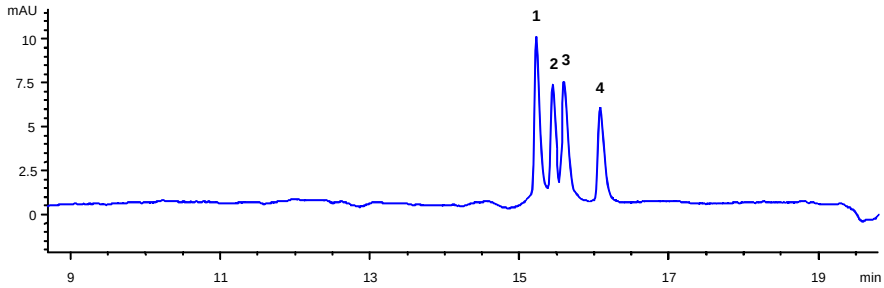

Figure 2. Electropherogram of standard mixture containing 1-EDDP (1.5 ng/mL), 2- COD (7 ng/mL), 3-HCOD (7 ng/mL) and 4-6AM (3 ng/mL) obtained by in-line SPE-CE-UV. Experimental conditions are detailed in section 2.

3.3 Drug analysis by in-line SPE-CE-MS

3.3.1 Optimization of in-line SPE-CE-MS

The optimized methodology using UV detection was transferred to MS detection. Firstly, we optimized different MS parameters in order to obtain the best responses for the four analytes by the infusion of each compound separately at 1 µg/mL in ammonium acetate using the tuning software of the MS instrument. The sheath liquid can affect the analyte transferring from the liquid phase into the gas phase, thus it has significant effect on the MS signal response. Normally, the sheath liquid is made up of organic solvents and water in various ratios and an additive providing conductivity and the adequate pH for ionization. In our case we compared formic acid and acetic acid as additives for the sheath liquid using isopropanol-water (50:50 v/v) as solvent mixture. When isopropanol-water (50:50 v/v) containing 0.5% of formic acid as a sheath liquid was used the signals were not stable. This may be due to the mismatch between the sheath liquid composition and the BGE. Thus, a sheath liquid composition of isopropanol-water (50:50 v/v) containing 0.5% of acetic acid delivered at a flow rate of 5 µl/min was selected in a combination with a nebulizer gas pressure of 5 psi because an stable electrospray was yielded under these MS conditions. Regarding to the sheath liquid flow rate, the optimum value should minimize the dilution effect of the analyte in order to obtain good sensitivity while maintaining a stable spray. Also, capillary position and nebulizing gas pressure were carefully adjusted. In order achieve a stable spray is necessary to remove the polyimide coating from the outlet edge of the capillary and the capillary tip should have a straight cut. Table 1 shows the MS optimized parameters settings selected based on a compromise between all the highest sensitivity values for individual compounds. Using the separation BGE, the drugs

in study were detected as protonated molecules ($M+H^+$) for all the compounds (EDDP: 278 m/z, COD: 300 m/z, HCOD: 302 m/z and 6AM: 328 m/z). Figure 3 shows the extracted ion electropherograms obtained from a standard sample containing the EDDP, COD, HCOD and 6AM at 0.83, 1.7, 1.7 and 1.25 ng/mL respectively following the optimized in-line SPE-CE-MS methodology.

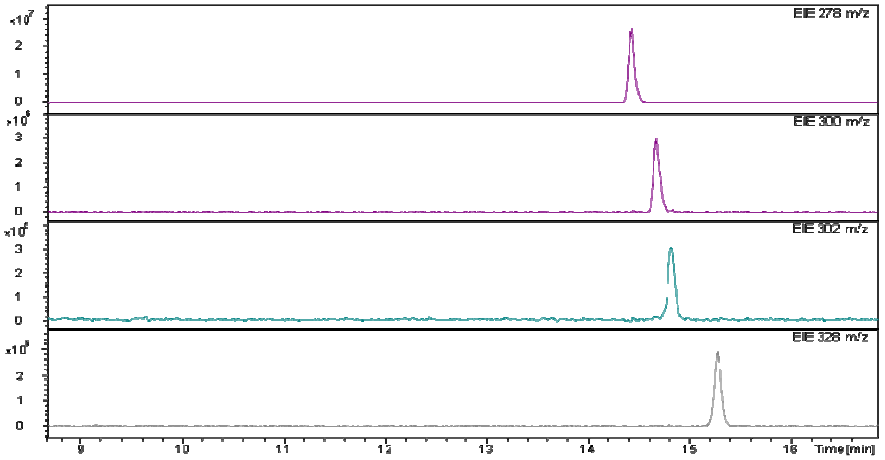

Figure 3. Extracted ion electropherograms obtained from a standard sample containing EDDP, COD, HCOD and 6AM at 0.83, 1.7, 1.7 and 1.25 ng/mL respectively. Other experimental conditions are detailed in the text (section 2).

Previously to the analysis of the urine samples by SPE-CE-MS we validated the method for standards using mass spectrometry. Different concentrations of standard solutions for the four were drugs in MilliQ water at a pH of 6 prepared. The regression equations, the values for reproducibility, repeatability and LODs for the studied drugs are summarized in Table 2.

Table 2. Regression equations, values for reproducibility, repeatability and LODs obtained for standard samples by SPE-CE-MS.

	EDDP	**COD**	**HCOD**	**6AM**
Linearity (ng/mL)	0.05 -10	0.20 – 10	0.20 – 10	0.20 - 10
Calibration curve [c]	y=2.2E7 + 1.2E8x	y=-2.1E6 + 5.8E6x	y=-1.2E6 + 6.2E6x	y=-1.8E6 + 8.9E6x
r^2	0.9998	0.9997	0.9988	0.9996
Reproducibility (%RSD) [a]	6.3	5.2	6.4	7.9
Repeatability (%RSD) [b]	2.7	3.5	4.1	5.1
LOD (ng/L)	8	115	115	78

[a] interday analysis (n = 5)
[b] intraday analysis (n = 5)
[c] y: peak area value (mAU x s); x: concentration (ng/mL)

The calibration graphs were linear in the tested range.The repeatability of the calibration curves was determined by injecting five replicates on the same day (n=5) and reproducibility was evaluated on different days (n=5). The relative standard deviation (RSD) values for reproducibility of the peaks area did not exceed 7.9% when the concen-tration of the compounds was 1 ng/mL. The LODs ranged between 0.008 and 0.115 ng/mL. The increase in sensitivity using MS detection was around 25 fold compared with SPE-CE-UV for standard samples.

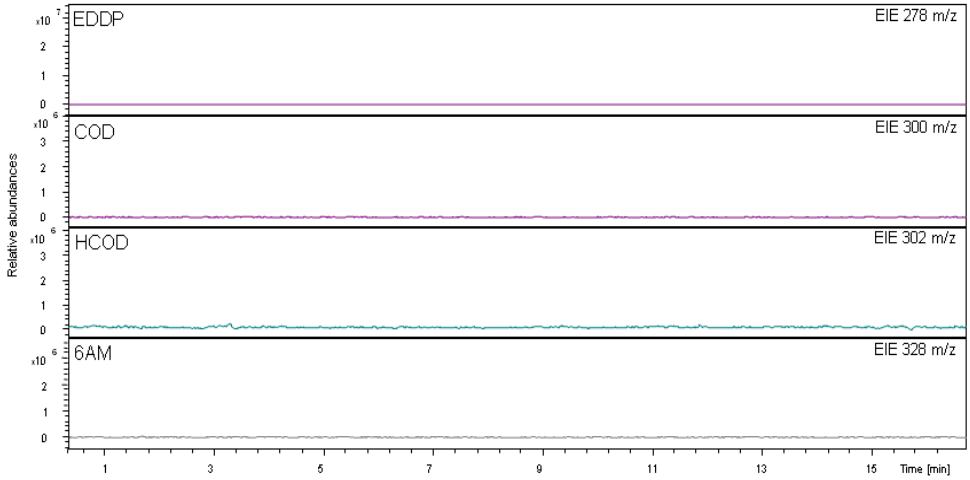

Figure 4. Extracted ion electropherograms obtained from urine diluted 1:1 (range of mass 200 – 400 m/z). Other experimental conditions are detailed in the text (section 2).

3.3.2 Analysis of urine by in-line SPE-CE-MS

As we mentioned in the experimental section, urine samples were diluted 1:1 with MilliQ water, spiked with appropriate amounts of the four drugs and adjusted to pH 6 before analysis. As it has been pointed out before, the analysis of urine by CE-UV was not possible due to the presence of endo-genous compounds in the matrix. However, using MS coupled to the SPE-CE system it is possible to determine the four compounds in urine despite the complexity of the sample matrix. As can be seen in Figure 4, no interferences were obser-ved in the range of ions selected. Figure 5 shows the extracted ion electropherograms obtained from a urine sample containing EDDP, COD, HCOD and 6AM at concentration of 3.3, 6.7, 6.7 and 5 ng/mL, respectively, following the optimized in-line SPE-CE-MS methodology. Recoveries rela-ted to the differences between the areas obtained by SPE-CE-MS for standard samples and those obtained for urine samples were in the range between 40 and 80%. The differences between the behavior of standards and urine samples with respect to the responses and the migration times

could be attributed to the sample matrix. In the case of urine, the matrix can have an important influence on the SPE process and can also influence the MS signal by the suppression of the ionization. Validation data for urine samples are summarized in Table 3. Linearity was determined from five injections of the drug-free urine samples were spiked with standard solution of the compounds in the range between 0.08 and 10

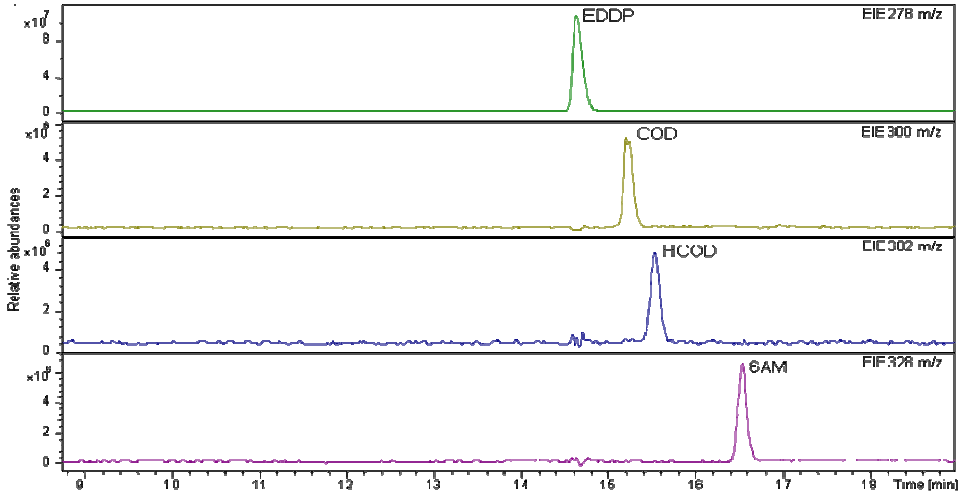

Figure 5. Extracted ion electropherograms obtained from urine diluted 1:1 containing the EDDP, COD, HCOD and 6AM at 3.3, 6.7, 6.7 and 5 ng/mL respectively. Other experimental are detailed in the text (section 2).

ng/mL. Relative standard deviation (RSD) in terms of repeatability was slightly higher than the values obtained for standards under the same conditions. However, the repeatability study showed good results since all the values were below 7.2%. The LODs reached were between 0.013 and 0.210 ng/mL calculated based on a 3:1 signal-to-noise ratio.

Table 3. Regression equations, repeatability and LODs obtained for spiked urine samples by SPE-CE-MS.

	EDDP	COD	HCOD	6AM
Linearity (ng/mL)	0.08 -10	0.40 – 10	0.40 – 10	0.30 - 10
Calibration curve [b]	y=2.5E7 + 9.4E7x	y=9.9E5 + 2.3E6x	y=3.0E5 + 2.1E6x	y=7.2E5 + 4.0E6x
r^2	0.9996	0.9971	0.9988	0.9996
Repeatability (%RSD) [a]	5.1	4.9	5.7	7.2
LOD (ng/L)	13	196	210	161

[a] intraday analysis (n = 5)
[b] y: peak area value (mAU x s); x: concentration (ng/mL)

4. Concluding remarks

In this work a method has been developed for the determination of drugs of abuse in urine samples using the in-line coupling of SPE to CE with MS detection. This coupling yielded excellent results for the determination of EDDP, COD, HCOD and 6AM in urine. This study presents an interesting approach in which a substantial decrease of the LODs, by means of the in-line coupling of SPE and CE, and very good selectivity due to the coupling with MS detection in the analysis of urine samples has been obtained without any previous pretreatment. The main advantages of the presented method are the complete automation of the process and high power of preconcentration due to the large volume of sample that can be injected. The hyphenation with MS also makes possible the accurate identification of the target compounds in this kind of complex samples. To the best of our knowledge, applying in-line SPE-CE-MS we have obtained the lowest detection limits for these drugs in urine samples [2, 6, 8]. These LODs are very useful for rapid screening of illegal drugs in doping control or forensic analysis. Generally the SPE-CE-MS system is very powerful and offers a high potential for trace-level analysis of biological samples. In the near future special attention will be paid to the influence of matrix effects.

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The authors have declared no conflict of interest.

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3.2.3 In-line solid-phase extraction capillary zone electrophoresis for the determination of barbiturate drugs in human urine.

UNIVERSITAT ROVIRA I VIRGILI
SENSITIVITY ENHANCEMENT STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF DRUGS OF ABUSE AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS
Igor Botello González
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IN-LINE SOLID-PHASE EXTRACTION CAPILLARY ZONE ELECTROPHORESIS FOR THE DETERMINATION OF BARBITURATE DRUGS IN HUMAN URINE

Igor Botello, Francesc Borrull, Carme Aguilar, Marta Calull
Departament de Química Analítica i Química Orgànica, Faculty of Chemistry, Universitat Rovira i Virgili, Marcel·lí Domingo s/n, 43007 Tarragona, Spain

Abstract

Three barbiturate drugs, secobarbital, phenobarbital and barbital were preconcentrated and determined by in-line solid phase extraction (SPE) capillary electrophoresis (CE) in urine samples. The separation buffer (BGE) used was 20 mM sodium tetraborate anhydrous (pH 9.2). The analyte concentrator (AC) consists of a small segment of capillary filled with Oasis HLB sorbent and inserted into the inlet section of the electrophoretic capillary. Different parameters affecting preconcentration were evaluated, such as sample pH, the volume of the elution plug and sample injection time. The obtained results show that this strategy enhanced detection sensitivity in the range between 170 and 1840-folds compared with normal hydrodynamic injection. The developed method provides detection limits (LODs) for standard samples in the range between 0.5 and 5 ng/mL with good repeatability (values of relative standard deviation, %RSD < 7). Reproducibility values (expressed in terms of relative standard deviation) were below 9% for standard samples. The applicability of the optimized method was demonstrated by the validation with human urine samples spiked with the studied compounds. Repeatability and reproducibility values were under 8.6 and 10.4 respectively. The LODs obtained for urine samples were in the range between 5 and 60 ng/mL.

Keywords: *Barbiturates, drugs of abuse, capillary electrophoresis, in-line solid phase extraction, urine samples.*

1. Introduction

The increasing interest in CE as analytical technique is certainly based on its high efficiency, high resolution power, low reagent consumption, automation and in that it is a low-cost alternative compared with other chromatographic techniques. However, CE suffers from inherent low concentration sensitivity and the LODs achieved when using this technique in comparison with liquid chromatography (LC) and gas chromatography (GC) are generally higher. This problem is due to the limited light-path length of the UV detector and the small injected sample volume into the capillary [1,2]. In order to overcome that issue, it has been an increasingly interest in the development of several on-line preconcentration techniques in CE [1,3-7]. Among them, solid phase extraction (SPE) coupled to CE is a very attractive technique because low LODs can be achieved due to the high sample volume that can be injected [4-9]. SPE can be performed mainly in four different setups, off-line, on-line, at-line and in-line. The last three setups have been particularly interesting due to the increasing trend towards fully automated analytical methods [4-7,10]. Moreover, the main used setup is when the preconcentration column is an integrated part of the CE system (in-line SPE-CE). This coupling has several advantages such as it requires low volume of organic solvent, is easily automated, needs little amount of sorbent material for the construction of the SPE device, and is capable of analyzing the complete eluate from SPE by CE [4-9].

The most common setup as in-line SPE-CE design is the use of a small packed bed in which the sorbent is placed near of the inlet tip of the capillary. The mainly reported sorbents for this approach are Oasis HLB, Oasis MCX and C18 [8-15]. Some studies in this field reported the use of frits in the packed bed design [11,12,14,15]. These frits are normally used in order to prevent small-diameter particles of the sorbent passing through the separation capillary. However, the use of these frits could generate irreproducibility with EOF and loss of current due to bubble formation. To solve these problems, some authors developed a free-frit in-line SPE in which as the particle size of the sorbent is large enough compared to the inner diameter of the separation capillary, the use of frits is not necessary [8,9,11,16]. For example, Saavedra *et al*. [16] used Oasis MCX in the construction of a frit-free concentrator device for the determination of 3-nitro-L-tyrosine in biological samples by capillary electrophoresis. Experimental SEFs of 100-folds were obtained and the LOD for 3-nitrotyrosine was 4.4 μM in spiked rat urine. Lara et al. [11] also developed an in-line SPE-CE method for the monitoring of residues of sulfonamides in different water samples. The proposed method, using Oasis HLB as SPE sorbent, allowed the direct injection of the water samples without any off-line pretreatment and the achieved

LODs were between 0.3 and 0.6 μg/L. More recently, Maijó et al. [9] evaluated the use of an in-line SPE-CE device for the preconcentration and separation of a group of pharmaceutical compounds (benzafibrate, piroxicam, diclofenac sodium, naproxen and clofibric acid) also using Oasis HLB. The developed strategy provided LODs for standard samples in the range between 0.06 and 0.5 ng/mL and SEFs were around 5900-folds.

Barbiturate drugs are typical sedative-hypnotic drugs and, depending on the substituting groups, exhibit a wide variety of responses in the body. These kind of drugs, in high doses, depress the respiratory system, which accounts for their toxicity [17]. Barbiturates are abused for recreational purposes and, in relatively low doses, they cause relaxation and sleepiness. Moreover, barbiturates may result in drug tolerance and dependence, and they may show additive effects with other central nervous system depressants [17]. The abuse of barbiturates is now widespread and the development of methods for their efficient separation and precise identification and quantification is needed [18]. Barbiturate drugs as other drugs of abuse are mainly excreted in the urine in its original form, so this sample is very suitable for the determination of these drugs in medical and forensic science [19].

The occurrence of these drugs in urine samples is variable in time after administration. In the analysis of this biological sample in clinical cases of patients the concentration of these drugs has been reported in the range between 0.2 and 21 μg/mL [20]. However, these values depend on the individual metabolism and could be lower in combination with alcohol and/or amphetamines. These compounds have been determined in biological fluids by LC and GC [21-25]. For example, Martín-Biosca et al. [25] used micellar liquid chromatography for the determination of barbiturates in urine with a direct injection of the sample with LODs ranging between 130 and 270 ng/mL. Ye et al. [24] developed a LC-APCI-MS method for the determination of barbital, amobarbital and secobarbital in human plasma. The LODs obtained were in the range between 4-5 ng/mL for standard samples. Plasma samples were also analyzed and in this case, the range of concentration found was between 451.2 ng/mL and 631.7 ng/mL. The potential of CE for the determination of barbiturates in biological samples has also been demonstrated by some authors [19,26-32]. For example, Jiang et al. [28] reported the determination of barbiturates in urine by CE using a dynamically coated capillary with polycationic polymers. The LODs for standard samples were in the range between 870 and 3500 ng/mL. Wang et al. [27] used moving reaction boundary (MRB)-induced stacking procedure in combination with CZE for the determination of barbital and phenobarbital in urine samples. In that case, LODs were 270 ng/mL for barbital and 260 ng/mL for phenobarbital. Ferslew et al. [29] used micellar electrokinetic capillary chromatogra-

phy (MEKC) for the determination of barbiturate drugs in various biological fluids. Applying the method to forensic cases of butalbital intoxication detected concentrations ranging from 1.5 to 7.6 μg/mL in urine.

The principal aim of this work is the determination of three barbiturate compounds in urine samples by in-line SPE-CE. The optimization of the in-line SPE-CE system in the determination of the studied barbiturates is performed by the evaluation of different parameters affecting preconcentration factors. Thus, the study of sample pH, the volume of the elution plug and the sample loading time has been carried out. Urine samples from a healthy volunteer have been analysed with the proposed method. To the best of our knowledge, this study represents the first time that a method based on in-line SPE-CE for the determination of barbiturates has been reported.

2. Experimental

2.1 Standards and reagents

All reagents used were of analytical-reagent grade. Secobarbital, phenobarbital and barbital, sodium hydroxide (NaOH) and disodium tetraborate anhydrous were purchased from Sigma Aldrich (St. Louis, MO, USA). Ultrapure reagent water purified by a MilliQ gradient system (Millipore, Bedford, MA, USA) was used throughout. Methanol was purchased from SDS (Peypin, France). Oasis HLB solid phase extraction sorbent with an average particle size of 60 μm was used for the construction of the SPE device and was supplied by Waters (Milford, MA, USA).

Standard stock solutions for each barbiturate were prepared by dissolving each compound in MeOH at a concentration of 1000 mg/L and storing at 4 ºC. Working standard solutions of the mixture of all the compounds at a concentration of 10 μg/mL were prepared weekly by diluting the standard solutions in ultrapure water. Working solutions were prepared daily by diluting these solutions with an appropriate volume of MilliQ water. The separation buffer (BGE), which consisted on 20 mM sodium tetraborate anhydrous (pH 9.2), was prepared by dissolving the appropiate amount of disodium tetraborate anhydrous in MilliQ water.

2.2 Instrumentation

The instrumentation used for electrophoretic separation was an Agilent 3D CE (Agilent Technologies, Waldbroon, Germany) equipped with UV diode-array detection (DAD). The detection of the barbiturates was performed at 214 nm.

A fused silica capillary of 100 cm length (91.5 cm effective length) and 50 μm of internal diameter (ID) was used.

2.3 CZE separation

New capillaries were conditioned with 1M NaOH for 40 min prior to use

and daily with 1M NaOH for 10 min and water for 10 min. Between separations the capillary was conditioned with MeOH for 4 min, water for 4 min, and BGE for 4 min. Injections were performed by placing the sample in the inlet vial and applying 50 mbar for 10 seconds. Normal polarity of 30 kV was used for the separation of the drugs with UV detection.

2.4 Construction of the SPE-CE concentrator device

The construction of the SPE device was performed in a similar way to the described in a previous paper of our group [8]. For this, 2 mm of bare fused-silica capillary of 150 μm ID (the AC device) was properly cutted. Then it was introduced 1 mm into a 0.5 cm piece of PTFE tubing (Grupo Taper S.A., Madrid, Spain) with an ID of 0.250 mm. Then, a piece of 7.5 cm of the separation capillary was introduced at the other end of the PTFE tubing until connect with the AC (inlet), and the free end of this capillary of 7.5 cm was connected to a vacuum pump using a syringe. Following, the AC was introduced into a vial that contained the Oasis HLB sorbent (particle size higher than 50 μm), and this was loaded into the AC device. Then, the capillary of 7.5 cm and the AC were moved until the preconcentrator was placed in the half-way of the PTFE tubing. Finally, the CE separation capillary of 92.5 cm was introduced into the other part of the PTFE tubing until to join the other side of the AC (outlet). The entire process of fabricating the concentrator was monitored under a microscope. Finally, the assembly was installed in a CE cartridge and it was checked for abnormal flow by filling the whole capillary with water and applying a pressure of 930 mbar to a vial containing MeOH. For the capillary dimensions chosen, the time needed for MeOH to reach the detector was measured by monitoring the signal at 200 nm. This time was about 70 seconds in a properly constructed capillary (in-line SPE-CE), which is the same that in a capillary without an in-line SPE device.

2.5 In-line SPE-CE-UV procedure

Capillaries with the AC were, daily and between separations, conditioned at 930 mbar with MeOH for 3 min and BGE for 4 min. Figure 1 shows the schematic diagram of the in-line SPE-CE-UV procedure. First, the sample (adjusted to pH 6 with a solution of 1M of HCl) was loaded at 930 mbar for 60 min (A). The next step was a clean-up with the BGE solution at 930 mbar for 3 minutes to remove the unretained molecules and to equilibrate the capillary prior to elution and separation (B). Then, for the elution step, a plug of MeOH was injected at 50 mbar for 40 seconds (C) and this was subsequently pushed by the BGE solution at 50 mbar for 220 seconds (D). Normal polarity of 30 kV was used for the separation of the three analytes (E).
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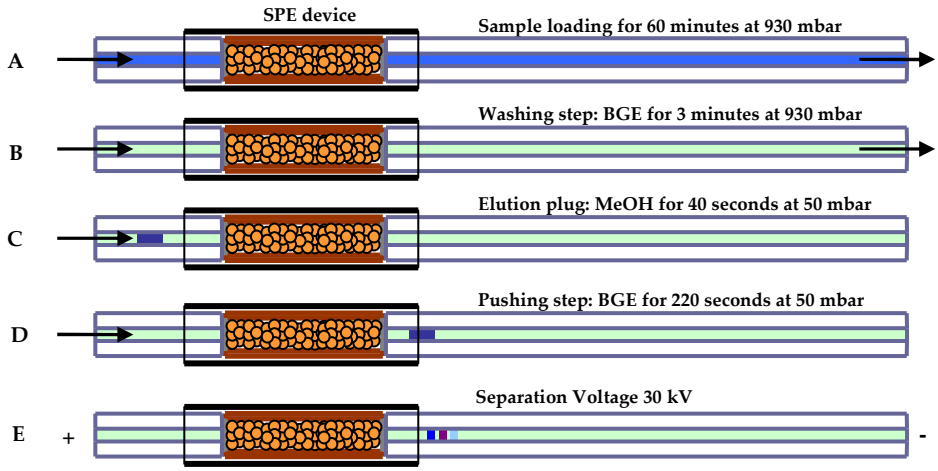

Figure 1. Diagram of the in-line SPE-CE-UV procedure developed for the secobarbital, phenobarbital and barbital preconcentration. From (A) to (E) the different steps followed to preconcentrate and separate the analytes are shown: (A) sample loading; (B) washing step; (C) introduction of the elution plug; (D) elution of the analytes from the SPE sorbent by means of the pushing step, and (E) separation process. For a detailed description of the method, please see section 2.5.

2.5 Human urine samples pretreatment

Urine samples were obtained from a healthy volunteer. The used extraction procedure was based in literature [27] with modifications. Before the extraction of the drugs from the samples, the pH value was adjusted to 4.0 with HCl 1M. Spiked urine samples were prepared by adding a standard solution containing the analytes at different concentrations into 500 µL blank urine in one eppendorf tube. Sample pretreatment was carried out by adding 500 µL of ethyl acetate/*n*-hexane (40/60 v/v) to the spiked urine samples for the extraction of the barbiturates. After vortex mixing, the sample was centrifuged for 10 min at 9000 rpm. The organic phase containing the barbiturates was then transferred to another eppendorf tube and a second extraction of the residual was performed by 500 µL of ethyl acetate/*n*-hexane (40/60 v/v) and repeating the procedure previously described. Finally, the two organic phases were combined and then evaporated to dryness under a gentle stream of dry nitrogen (UHP grade). The final residue was then dissolved with 500 µL with 10^{-6} M HCl (final pH of 6). This solution was transferred to a micro vial for in-line SPE-CE analysis.

3. Results and discussion

3.1 Separation of barbiturates

The pK_a values of the studied barbiturates are between 7 and 8. Since the barbiturates are negatively charged at basic pH values, separation of these compounds at positive

voltages was performed under counter EOF conditions. Different BGEs have been reported in literature for the separation of barbiturate drugs in CE being the most used borate and Tris based buffers [27-33]. Hence, in order to optimize the separation, three different BGEs were tested. The electrolytes studied were: 20 mM $Na_2B_4O_7$ (pH 9.2), 150 mM Tris buffer (adjusted at pH 7.8 with 5M HNO_3) and 50 mM ammonium acetate (adjustted at pH 9.7 with NH_4OH 32%). The last electrolyte was considered taking into account the possibility of coupling CE with MS detection since in this case the use of volatile buffer is highly recommendded. Even when baseline separations were obtained with all the studied BGEs, the best results in terms of sensitivity (peak height) and short analysis time were obtained by using 20 mM $Na_2B_4O_7$ (pH 9.2) as BGE. Under these conditions run time was around 13 minutes.

3.2 Drug analysis by in-line SPE-CE-UV

Different papers in which barbiturates have been extracted from biological samples by off-line SPE [31,34] and our previous knowledge led us to choose a polymeric sorbent as Oasis HLB for the SPE procedure. As it was mentioned in the introduction section, when no frits are used, the main consideration for the construction of the AC device lies on the fact that the size of the particles of the sorbent have to be large enough in relation to the ID of the separation capillary to prevent the sorbent particles escaping out of the system. In our case, as the separation capillary had an ID of 50 μm, Oasis HLB with an average particle size of 60 μm was chosen. Different parameters affecting the in-line SPE-CE-UV procedure were studied in order to obtain the optimum response in terms of sensitivity for the selected drugs.

3.2.1 Effect of sample pH

One of the main factors that influence the extraction efficiency of analytes is the sample pH, which plays a critical role in the SPE procedure, because its value determines which the state of the analytes is. Depending on sample pH, the analytes can be in a charged or in a neutral form and this determines its retention in the SPE sorbent. Based on this, different pH values in the range between 2 and 10 were tested. The study was performed using a standard mixture containing the analytes at a concentration of 0.2 mg/L for secobarbital, 0.1 mg/L for phenobarbital and 0.5 mg/L for barbital; (prepared by an appropriate dilution of the working standards in MilliQ water and adjusted to the desired pH). This mixture was then loaded into the capillary at 930 mbar for 5 minutes; then, a washing step with the BGE solution was carried out at 930 mbar for 5 minutes; and finally, for the elution of the compounds from the SPE sorbent, a plug of methanol was introduced into the capillary at 50 mbar for 30 seconds and this plug was

then pushed through the SPE sorbent by introducing the BGE solution at 50 mbar for 220 seconds. Based in previous works of our group [8], for the capillary dimensions used, we found that the selected pushing conditions with BGE were enough to move the elution solvent out of the SPE sorbent.

Figure 2 shows the diagram of the variation in the peak area of the three barbiturates loaded at different pH values in the SPE device. As can be seen from the figure, the signal response in terms of peak area was higher when the pH value of the sample was below 7. At higher pH values, lower retention of the compounds was observed and this can be explained by the negative charge of the barbiturates at these pH values. For secobarbital and barbital, the differences in the retention behaviour were not significant for pH values under 7 but in the case of phenobarbital, higher retention can be observed at pH 6, so this was the value chosen for further studies.

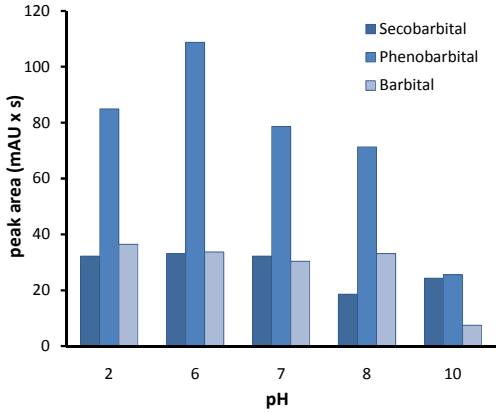

Figure 2. Effect of sample pH on the peak area of the barbiturates by using in-line SPE-CE-UV. The concentration of the analytes in the standard samples was 0.2 mg/L for secobarbital, 0.1 mg/L for phenobarbital and 0.5 mg/L for barbital. Sample loading time was 5 minutes at 930 mbar. Other experimental conditions are reported in the text.

3.2.2 Study of the elution step

Different organic solvents were tested for the elution of the three barbiturates from the Oasis HLB sorbent. For this study, MeOH, basic MeOH (pH 9), MeOH/Isopropanol (80:20, v/v), MeOH/Isopropanol (50:50, v/v) and MeOH/ACN (80:20, v/v) were evaluated. This selection was based on the existing literature regarding the off-line SPE process involving barbiturates and Oasis HLB [34-36]. For example, Hori et al. [34] reported the use of MeOH as elution solvent in SPE for the determination of some barbiturate drugs in serum by LC-ESI-MS with good recoveries values. In the other hand, in the application manual of Waters Corporation, basic MeOH is

the recommended solvent for the elution of barbiturates in urine by LC-UV [36].

The elution plug for the different mixtures tested was introduced into the capillary at 50 mbar for 30 seconds and then it was pushed through the SPE sorbent by introducing the BGE solution at 50 mbar for 220 seconds. Results demonstrated that the differences in the organic solvent and mixtures tested, in terms of peak area, were not significant. So, MeOH was chosen as elution solvent for further experiments.

Following, a study of the MeOH plug time was performed in order to obtain higher sensitivity in terms of peak area. For this, different plugs of methanol were introduced into the capillary at 50 mbar for different periods of time ranging from 20 to 60 seconds, and then the organic solvent was pushed through the SPE sorbent by the BGE solution (at 50 mbar for 220 seconds). The results showed that the peak areas for the studied compounds increased with the increment of the MeOH plug time. However, over 40 seconds, there was an increase of the time required to reach the current stabilization probably caused by the presence of this plug of very low conductivity inside the separation capillary. So, 40 seconds was the selected elution time for following studies.

3.2.3 Study of sample loading time

In order to improve sensitivity and consequently decrease LODs, the simplest way consists on the increase of the injection time. In this sense, the sample loading time at 930 mbar was tested using a standard mixture containing the analytes at a concentration of 0.2 mg/L for secobarbital; 0.1 mg/L for phenobarbital and 0.5 mg/L for barbital (pH 6). In the range of loading times tested (from 10 to 60 minutes), the peak area increased with the loading time. Figure 3 shows the signal response in terms of peak area for the barbiturates *versus* the sample loading time. From the figure is observed that for secobarbital and phenobarbital, peak area increased with the sample loading time, and this indicated that breakthrough volume of the SPE sorbent was not exceeded. However, for barbital, over 40 minutes of sample loading, the responses obtained (peak areas) remained constant. Taking into account all these considerations, we selected a sample loading time of 60 minutes as an optimum value because this time period provided maximum sensitivity (in terms of peak area) in a reasonable analysis time.

4. Validation

The proposed in-line SPE-CE method was evaluated in terms of linearity, repeatability, reproducibility and LODs (calculated using S/N>3). For this, we prepared standard solutions for the three barbiturates at different concentrations in MilliQ water at pH 6. The obtained values are summarized in Table 1. SEF values were

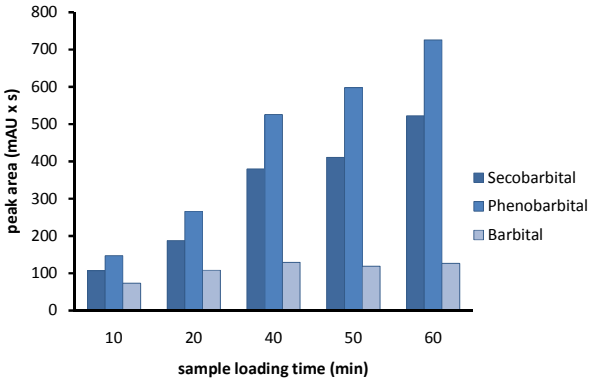

Figure 3. Effect of the sample injection time on the peak area of the eluted analytes by using the optimum conditions for in-line SPE-CE-UV. The concentration of the barbiturates in the standard sample was 0.2 mg/L for secobarbital, 0.1 mg/L for phenobarbital and 0.5 mg/L for barbital. Other experimental conditions are reported in the text.

Table 1. Regression equations, values for reproducibility, repeatability, SEFs and LODs for the studied drugs obtained for standard samples by in-line SPE-CE.

	Secobarbital	**Phenobarbital**	**Barbital**
Linearity (ng/mL)	2 - 500	2 - 500	10 - 500
Calibration curve	y=26.3 + 3621x	y=6.9 + 6809x	y=23.3 + 438x
r^2	0.9999	0.9998	0.9989
Reproducibility (%RSD)[b]			
100 ng/mL	5.8	7.1	7.5
300 ng/mL	7.9	8.7	8.9
Repeteability (%RSD)[c]			
10 ng/mL	3.7	2.4	-
100 ng/mL	4.1	3.9	4.4
LOD (ng/mL)	1	0.5	5
SEF	1410	1840	173

[a] y: peak area value (mAU x s); x: concentration (μg/mL)
[b] interday analysis (n = 5)
[c] intraday analysis (n = 5)

calculated according to the equation (1):

$$SEF_{height} = \frac{h_{prec}}{h_{HD}} f \quad (1)$$

where h_{prec} is the peak height of the preconcentrated analyte, h_{HD} the peak height of the non-preconcentrated analyte detected after a conventional hydrodynamic injection of 10 seconds at 50 mbar, and f is the dilution factor. The calibration graphs generated were linear in the tested range with correlation factors greater than 0.9989.

The repeatability and reproducibility between days of the method, expressed as relative standard deviation (%RSD) of five analysis of standard samples containing the analytes at a two concentration levels (100 ng/mL and 300 ng/mL) were lower than 4.4% and 8.9% respectively for all the compounds. The SEFs (relating to the peak height) reached were of 1410, 1840 and 173 for secobarbital, phenobarbital and barbital respectively. Reached LODs in standard samples were in the range between 0.5 and 5 ng/mL.

Figure 4 shows the electropherogram obtained from a standard sample containing the analytes at 20 ng/mL for secobarbital, 5 ng/mL for phenobarbital and 50 ng/mL for barbital; and following the optimized in-line SPE-CE-UV method.

5. Urine samples

Once the performance of the developed method based on in-line SPE-CE for preconcentration and separation of a group of barbiturates has been demonstrated, we tested with urine samples. The motivation for selecting such kind of matrix arose because urine analysis constitutes a good way of monitoring the intake of drugs since this biological sample is one of the main routes of excretion in the body.

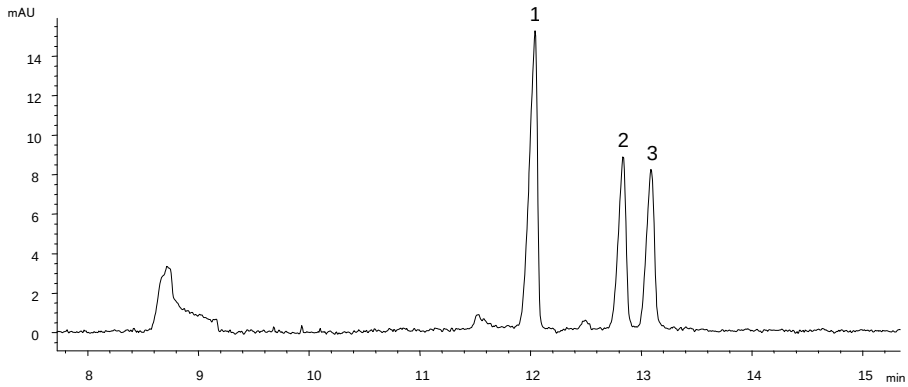

Figure 4. Electropherogram of a standard sample containing the analytes at a concentration of 20 ng/mL for secobarbital, 5 ng/mL for phenobarbital and 50 ng/mL for barbital at pH 6 analyzed by in-line SPE-CE-UV in the optimized conditions. Peak assignments: 1, secobarbital; 2, phenobarbital; 3, barbital. Other experimental conditions are reported in the text.

As the complexity of this kind of matrix could affect the performance of the SPE device, a simple LLE pretreatment was carried out prior to the analysis in order to clean up the samples. The LLE procedure was based in a method already reported in the literature [27]. Figure 5A shows the electropherogram obtained from an extract of a blank urine sample following the optimized in-line SPE-CE method. In the other hand, Figure 5B shows the electropherogram obtained for an extract of urine

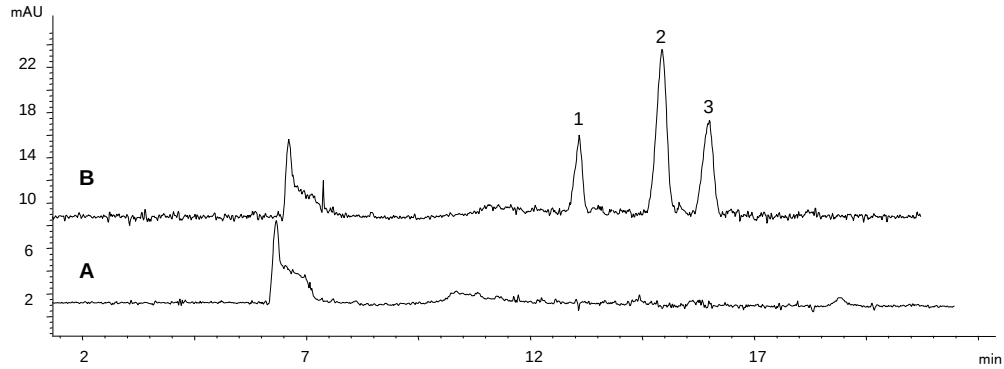

Figure 5. A) Electropherogram of a blank of an of urine sample after LLE. B) Electropherogram of a an extract of urine sample previously spiked with the analytes at a concentration of 50 ng/mL for secobarbital, 50 ng/mL for phenobarbital and 400 ng/mL for barbital at pH 6 analyzed by in-line SPE-CE-UV. Other experimental conditions are reported in the text.

sample spiked at a concentration of 50 ng/mL for secobarbital, 50 ng/mL for phenobarbital and 400 ng/mL for barbital. Differences in the migration times of the analytes in standard samples and urine samples can be observed comparing Figures 4 and 5B. This suggests that some endogenous compounds also retained in the concentrator device and further eluted could have that influence in the behavior of the tested analytes.

Table 2 shows the validation parameters obtained for urine samples.

Table 2. Regression equations, values for reproducibility, repeatability and LODs obtained for urine samples by in-line SPE-CE.

	Secobarbital	**Phenobarbital**	**Barbital**
Linearity (ng/mL)	20 - 500	10 -500	100 - 500
Calibration curve [a]	y=-62.1 + 2973x	y=10.2 + 4595x	y=40.6 + 265x
r^2	0.9975	0.9978	0.9967
Reproducibility (%RSD) [b]			
100 ng/mL	9.1	8.5	-
300 ng/mL	8.9	9.8	10.4
Repeatability (%RSD) [c]			
100 ng/mL	7.2	5.2	-
300 ng/mL	8.0	6.9	8.6
LOD (ng/mL)	10	5	60

[a] y: peak area value (mAU x s); x: concentration (μg/mL)
[b] interday analysis (n = 5)
[c] intraday analysis (n = 5)

The LODs reached were between 5 and 60 ng/mL calculated based on a 3:1 signal-to-noise ratio.

In the literature, different techniques like LC, GC and CE have been used for determination of barbiturate drugs

Table 3. Comparison of methods for the determination of barbiturates in biological samples.

Substance	Technique	LOD	Sample studied	Sample pretreatment	Ref.
Barbital acid Barbital Phenobarbital Pentobarbital Amobarbital Thiobarbituric acid Butobarbital N-methyl-5-phenyl-ethyl barbital acid 5-cyclohexenyl-5-ethyl barbital acid	CE-UV	0.87 – 3.50 mg/L [a]	Urine	Filtration	[28]
Pentobarbital Mephobarbital Phenobarbital Secobarbital Amobarbital Hexobarbital	CE-UV	5 μg/g [a]	Meconium	SPE	[31]
Barbital Phenobarbital Secobarbital	MRB-CE-UV	0.26 and 0.27 μg/mL [a]	Urine	LLE	[27]
Barbital Amobarbital Phenobarbital Secobarbital	LVSS-CE-UV	0.015-0.057 μg/mL [a*]	Plasma	LLE	[19]
Barbital Phenobarbital Secobarbita	CEC-UV	0.83-1.39 μg/mL [a]	Serum	LLE	[26]
Metharbital Primidone Phenobarbital Mephobarbital Pentobarbital	HPLC-UV	0.1-0.2 μg/mL [a]	Urine	10-fold dilution	[23]

[a] real samples
[*] quantification Limits

Table 3. Comparison of methods for the determination of barbiturates in biological samples. (Cont.)

Barbital Amobarbital Phenobarbital Secobarbital	HPLC-MS	4-5 ng/mL[a]	Plasma	LLE	[24]
Barbitone Allobarbitone Phenobarbitone Cyclobarbitone Hexobarbitone Pentobarbitone Secobarbitone Methohexitone	HPLC-UV	-	-	-	[18]
Barbital Diallyl barbituric acid Phenobarbital Butabarbital Amobarbital Pentobarbital	HPLC-UV	0.13-2.7 μg/mL [a]	Urine	Filtration	[25]
Barbital Amobarbital Phenobarbital	GC-MS	0.5 μg/Kg [a]	Pork	ASE and SPE	[21]
Barbital Amobarbital Phenobarbital	GC/MS/MS	0.1-0.2 μg/Kg [a]	Pork	Ultrasonic and SPE	[22]

[a] real samples

[*] quantification Limits

in biological samples [17-19,21-23,25-28,31] as summarized in Table 3. In these papers samples such as urine [23,25,27,28], plasma [19,24], serum [26], meat [21,22] or meconium [31] are analized. When CE or CEC have been used, UV has been selected as detection system. For techniques such as LC and GC the use of MS as detector has also been reported [21,22,24]. As can be observed from that table, the LODs obtained in our study are in general lower than the reported in other papers focused in the determination of barbiturate drugs in biological samples by CE. For instance, Wang et al. [27] using a stacking procedure for the determination of barbiturates in urine samples resulted in 20.5-fold and 22.6-

fold of improvement in concentration sensitivity and the obtained LODs of 0.27 μg/mL for barbital and 0.26 μg/mL for phenobarbital for urine samples. On the other hand, when a preconcentration strategy as LVSS has been combined with CE for the determination of barbiturates in urine samples [19], the reported limits are similar than the obtained for us. When other chromatographic techniques such as LC and GC have been used, the LODs in general are higher than in the present paper. However, the use of MS coupled to LC allowed lower LODs for barbital, amobarbital, phenobarbital and secobarbital in the analysis of human plasma samples [24].

6. Concluding remarks

An in-line SPE-CE method has been developed for the preconcentration and separation of barbiturate drugs in urine samples. This simple method allows the loading of a much larger amount of sample than conventional CE analysis without affecting the resolution and has been shown to be effective for the analysis of barbiturates in urine. Other advantages of this methodology are the complete automation of the process, low cost and the high SEFs obtained. The procedure can also be considered highly environmentally friendly due to the low quantity of organic solvent consumed for each analysis. For the analysis of real samples, a LLE step was necessary in order to clean up the urine samples. The LODs obtained from the three barbiturates were between 5 and 60 ng/mL in urine samples extract.

Acknowledgments

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3.2.4. Discussion of results

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In this section, three methodologies based on the use of in-line SPE-CE have been presented for increasing sensitivity in the determination of drugs of abuse in environmental and biological samples. Even though the experimental results included in this section have been discussed individually in the previously presented papers, this section presents the most important aspects of the results.

The main goal of an in-line SPE-CE method is to optimize the extraction process by maximizing the recovery of the target analytes under conditions which provide efficient and reproducible CE separations. A variety of materials are used as sorbents in SPE for the extraction of compounds of interest. The choice of sorbent is critical in SPE because it has a great influence on selectivity, as well as on capacity. A generic SPE-CE procedure consists of the following basic steps: conditioning, equilibration, sample loading, clean-up and conditioning, elution, pushing and electrophoretic separation.

Some of these steps are only involved in SPE extraction, such as the conditioning of the SPE and the sample loading. However, the other steps are involved in both the SPE extraction and the electrophoretic separation. After the sample loading, a clean-up step with BGE is necessary, in order to remove unretained molecules, as well as to condition the CE system in order to apply the separation voltage. The volume of the elution solvent is a key parameter for in-line SPE, as generally this volume is higher than the conventional hydrodynamic volumes introduced in CE. This can lead to lower peak efficiency and resolution, as well as problems with the stability of the current. Finally, the pushing step is necessary in order to push the elution solvent through the SPE before CE separation.

In the three papers presented, the in-line SPE-CE methodologies were developed using the same concentrator device arrangement. To be specific, a 2 mm length piece of capillary of 150 μm packed with 60 μm particle size Oasis HLB sorbent was used in all cases. Oasis HLB sorbent (a copolymer of vinylpyrrolidone and divinylbenzene) was selected because it is reported in the literature that it provides good recoveries for these kinds of compounds in off-line pretreatment procedures [1-5].

The first study focused on the development of an in-line SPE-CE strategy for enhancing sensitivity for the determination of drugs of abuse (EDDP, cocaine, codeine and 6-acetylmorphine) in tap and river water samples. Optimal conditions for the electrophoretic separation were 80 mM of Na_2HPO_4 and 6 mM of HCl at a final pH value of 3 in order to guarantee the positive ionization of the drugs.

In the study of different parameters affecting the in-line SPE-CE-UV procedure in order to obtain the optimal response in terms of sensitivity and resolution, the

sample pH was an important parameter to be studied in order to achieve better retention of the compounds in the polymeric material. Based on the good results reported in the literature of these compounds at different pH values [4-6], a study of the effect of the pH in our dynamic conditions was performed in the range of pH 2 to 9. The optimal conditions were achieved at pH 7.2. With respect to the optimization of the pushing step, the best conditions were obtained with 140 seconds at 50 mbar, which corresponds to a capillary volume of 0.165 µL (8.4 cm capillary length, which is enough to ensure that the methanol is displaced out of the SPE device). In the study of the elution step, optimal results in terms of peak areas were obtained with a MeOH plug of 25 seconds at 50 mbar. Larger plugs of elution solvent led to a decrease in resolution, which is probably due to the instability in the current profile caused by the presence of this very low conductivity plug inside the separation capillary. For the study of the sample loading time, it was found that over 30 minutes, the increase in sensitivity was not significant. The maximum loading time achievable, in this case, was limited by the volume of the selected elution plug. This fact can be justified because, even though higher volumes of sample could be introduced into the system if there were an increase in the volume of the elution plug, at higher elution plug volumes, current disruption occurred in the analysis.

The determination of cocaine, EDDP, codeine and 6-acetylmorphine was validated in standard samples with good values of repeatability and reproducibility. The SEFs reached (relating to the peak height) were between 2,282 and 5,337. The LODs obtained ranged between 0.05 and 0.2 ng/mL. Absolute recoveries, calculated by comparison of the peak area obtained for the eluted analytes from the SPE device with the response of the same moles of analytes injected into a capillary without the concentrator, were in the range of 72 to 96%.

In order to explore the potential of the in-line SPE-CE analytical method developed in this study, it was used to analyse tap water and river water samples from the Ebro River. Relative recoveries obtained for tap water samples, ranging from 102 to 110%, were calculated by comparing the areas obtained for the standard mixtures (with AC) with the areas resulting from the analysis for tap water samples (with AC) containing the tested analytes at the same concentrations. These relative recoveries were higher at lower concentrations.

For river water analysis, the relative recoveries were calculated with the same procedure used for tap water and ranged between 96.5 and 105.8%. Even though the LODs obtained for river water were generally higher than the concentration of the same compounds in other European rivers [4,5,7,8], it should be highlighted that in our case, to the best of our knowledge, the LODs are the lowest obtained by capillary electrophoresis for these drugs.

In the second study, the same in-line SPE-CE coupling was investigated but MS detection was used in order to determine EDDP, codeine, hydrocodeine and 6-acetylmorphine in urine samples. Different conditions affecting the in-line SPE-CE performance (first using UV detection) were studied in order to obtain the optimal sensitivity and resolution for the four drugs. Although the optimization of these compounds in a similar system had already been performed in the first study of this section, these parameters were evaluated again because, in this study, the selected group of compounds was slightly different from the previous study and this may result in different behaviour in the extraction process. Moreover, the length of the capillary was longer due to the coupling with a MS detector and this also may affect the results.

The LODs in standard samples ranged between 0.008 and 0.0115 ng/mL and the increase in sensitivity due to the use of the MS detector was around 25-fold compared with the use of UV. The analysis of urine samples diluted 1:1 with MilliQ water spiked with appropriate amounts of the four drugs and adjusted to pH 6 was not possible by in-line SPE-CE-UV because of the presence of endogenous compounds in the sample matrix. However, through the use of MS coupled to the SPE-CE system, it was possible to determine the studied drugs despite the complexity of the sample matrix. Recovery based on the differences between the areas obtained by SPE-CE-MS for standard samples and those obtained for urine samples were in the range of 40 to 80%. Differences were observed between the behaviour of standard and urine samples with regard to responses and migration times, which could be attributed to the sample matrix.

This study presents an interesting approach in which a substantial decrease of the LODs (0.013 – 0.210 ng/mL) and very good selectivity were obtained. Due to the coupling with MS detection, the analysis of urine samples without any previous pretreatment could be achieved. To the best of our knowledge, by applying in-line SPE-CE-MS, the lowest detection limits for these drugs in urine samples have been achieved. These LODs are very useful for rapid screening of illicit drugs in doping control or forensic analysis.

In the third study, three barbiturate drugs (secobarbital, phenobarbital and barbital) were preconcentrated and determined by in-line SPE-CE-UV in urine samples. Different parameters affecting preconcentration were evaluated, such as sample pH, the volume of the elution plug and sample injection time. As well as in the previous works, in this case, depending on sample pH, the analytes can either be in their charged or neutral form and this determines its retention in the SPE sorbent. In this case, the signal response in terms of peak area was higher when the pH value of the sample was below 7. At higher pH values, lower retention of the compounds was observed and this can be explained by the negative charge of the barbiturates at

these pH values. For secobarbital and barbital, the differences in the retention behaviour were not significant for pH values under 7 but, in the case of phenobarbital, higher retention was observed at pH 6, so this was the value chosen.
Regarding the study of the elution step, different organic solvents were tested for the elution of the three barbiturates from the Oasis HLB sorbent. For this study, MeOH, basic MeOH (pH 9), MeOH/Isopropanol (80:20, v/v), MeOH/Isopropanol (50:50, v/v) and MeOH/ACN (80:20, v/v) were evaluated. Since the results demonstrated that the differences in the organic solvent and mixtures tested in terms of peak area were not significant, MeOH was chosen as elution solvent for further experiments.

The study of sample loading time was carried out by evaluating the response in terms of peak area for different loading times (from 10 to 60 minutes). For secobarbital and phenobarbital, the peak area increased with the sample loading time, indicating that the breakthrough volume of the SPE sorbent was not exceeded. In the case of barbital, after 40 minutes, the responses obtained remained constant. Taking all of these considerations into account, a sample loading time of 60 minutes was selected as the optimal value because this time period provided high sensitivity in a reasonable analysis time.

The LODs for standard samples ranged between 0.5 and 5 ng/mL, with SEFs ranging from 174- to 4840-fold. Since UV detection was used and due to the complexity of the sample matrix when urine samples were analysed, a previous LLE was necessary as a clean-up step. The LODs obtained ranged between 5 and 60 ng/mL, which were the lowest obtained by CE in this kind of matrix for the barbiturate drugs studied [9-11].

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4. CONCLUSIONS

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The principal conclusions from the studies presented in this Doctoral Thesis can be summarized as follows:

1. The proposed methodologies developed in this Doctoral Thesis for the determination of pharmaceuticals and drugs of abuse were suitable and effective for overcoming the main drawback of CE, namely its lack of sensitivity.

2. The application of preconcentration techniques based on ITP, and also on in-line SPE, allowed LODs for the studied analytes at ng/mL level.

3. Among the techniques based on t-ITP, EKS provides the highest preconcentration factors and, as a consequence, the lowest LODs, as this technique combines an electrokinetic injection of the sample with stacking by t-ITP.

4. The sensitivity enhancement factors obtained for the determination of NSAIDs when using transient pseudo-ITP were relatively low in comparison with those obtained by EKS. This fact can be attributed to the different sample injection mode used (hydrodynamic for the former strategy and electrokinetic for the latter). Despite this difference in sensitivity, transient pseudo-ITP can be considered a good option as a preconcentration strategy when pharmaceuticals have to be determined in biological samples.

5. Although EKS allows low LODs to be obtained when environmental waters are analysed, a previous preconcentration step would be needed in order to determine NSAIDs at the levels in which these compounds are present in these kinds of samples. In contrast, in the case of biological samples, the LODs reached for NSAIDs and barbiturates were low enough for the determination of these compounds in this kind of sample, even though a pretreatment was also usually necessary to clean up the sample matrix.

6. The use of in-line coupling between SPE and CE enables the injection of higher volumes compared with electrophoretic preconcentration techniques and, in general, higher preconcentration factors can also be obtained. Moreover, for this technique, the matrix effect is lower due to the partial clean-up of the sample that takes place in the preconcentration device. In this Doctoral Thesis, a commercially available polymeric sorbent, Oasis HLB, was successfully used to extract and preconcentrate drugs of abuse from water and biological samples.

7. The applicability of the in-line SPE-CE has been demonstrated to be effective for the determination of drugs of abuse in tap water, river water and urine samples.

8. CE hyphenation with MS is a promising coupling, which allows good selectivity of drugs of abuse in complex matrices without the need of laborious sample pretreatments, and the increase in sensitivity, compared with the use of UV as detection system, was over 25-fold. Moreover, the coupling of this hyphenated system with an extraction technique for preconcentration in CE, such as in-line SPE, enabled LODs in the range of low ng/L for urine samples. Therefore, the overall system is very powerful for the determination of illegal drugs in doping and forensic analysis.

9. Even though the LODs obtained for drugs of abuse in river water samples by in-line SPE-CE were generally higher than the concentration levels at which these compounds are usually present in this kind of sample, they are the lowest obtained by CE for these drugs compounds.

10. Although in the case of using in-line SPE-CE, a sample pretreatment was not needed when environmental waters were analysed, for the biological samples, LLE was necessary when UV was used as the detector. In contrast, when MS was the detection system used, a simple dilution of the samples was required.

11. All of the results obtained during this Doctoral Thesis encourage us to continue studying the preconcentration techniques applied in CE in order to decrease the detection limits. Future research into SPE coupled in-line with CE will focus on using more selective sorbents, such as MIPs, and the combination between different electrophoretic and extraction preconcentration techniques, as these factors have considerable potential for improving the sensitivity of electrophoretic analysis.

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APPENDIX

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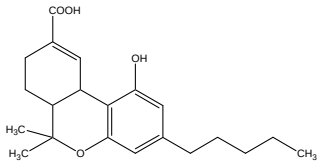
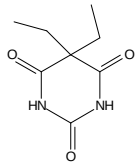
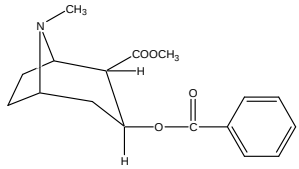
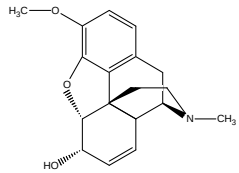
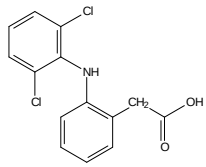
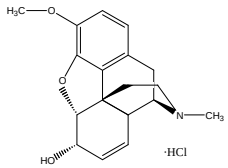
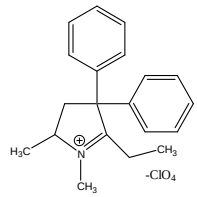
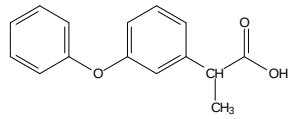
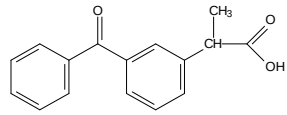
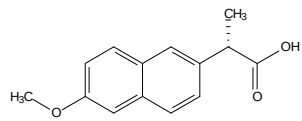
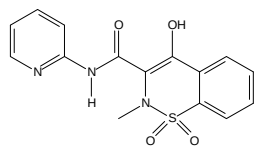
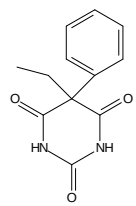
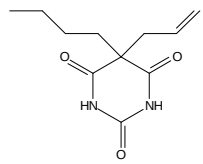
Appendix I. Abbreviations used in this Doctoral Thesis.

6AM	6-acetylmorphine
AC	Analyte concentrator
ACN	Acetonitrile
AES	Atomic emission spectrometry
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
BGE	Background electrolyte
CAPS	3-cyclohexyl-amino-1-propanesulfonic acid
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CF-EKS	Counter-flow electrokinetic supercharging
CGE	Capillary gel electrophoresis
CHES	2-(cyclohexylamino) ethanesulphonic acid
CIEF	Capillary isoelectric focusing
COC	Cocaine
COD	Codeine
CTAC	Cetylmethylammonium
CZE	Capillary zone electrophoresis
GC	Gas chromatography
DAD	Diode array detection
DCM	Dichloromethane
EDDP	1,5-dimethyl-3,3-diphenylpyrrolidine
EGDMA	Ethylene glycol dimethacrylate
EKI	Electrokinetic injection
EKS	Electrokinetic supercharging
EOF	Electroosmotic flow
ESI	Electrospray ionization
FAB	Fast atom bombardment
FASI	Field-amplified sample injection
FASS	Field-amplified sample stacking
FT-ICR	Fourier transform ion cyclotron resonance
HEPES	4-(2-hydroxyethyl)-1-perazineethane sulphonic acid
HD	Hydrodynamic injection
HDMB	Hexadimethrine bromide
HF-LPME	Hollow fiber liquid phase microextraction
HPMC	Hydroxypropylmethylcellulose
ICP	Inductively coupled plasma
ID	Internal diameter
IT	Ion trap
ITP	Isotacophoresis

LE	Leading electrolyte
LC	Liquid chromatography
LIF	Laser-induced fluorescence
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LPE	Liquid phase extraction
LVSS	Large volume sample stacking
MALDI	Matrix assisted laser desorption ionization
MEEKC	Microemulsion electrokinetic capillary chromatography
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
MES	2-morpholinoethanesulphonic acid
MRB	Moving reaction boundary
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NSAIDs	Non-steroidal anti-inflammatory drugs
OT	Open tubular
PDC	2,6-pyridinedicarboxylic acid
PI	Isoelectric point
PTFE	Politetrafluorethylene
Q	Quadrupole
RSD	Relative standard deviation
SEF	Sensitivity enhancement factor
SPE	Solid-phase extraction
tITP	Transient isotachophoresis
TE	Terminating Electrolyte
TEOS	Triethoxysilane
TMS	Trimethoxysilane
TOF	Time of flight
TQ	Triple-quadrupole
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
WWTPs	Wastewater treatment plants

Appendix II. Names and structures of the compounds determined in the Doctoral Thesis.

Name	Structure
6-acetylmorphine (metabolite of morphine)	
Barbital (drug of abuse)	
Cocaine (drug of abuse)	
Codeine (drug of abuse)	
Diclofenac (NSAID)	
Dihydrocodeine (metabolite of codeine)	

Name	Structure
EDDP (drug of abuse)	
Fenoprofen (NSAID)	
Ketoprofen (NSAID)	
Naproxen (NSAID)	
Piroxicam (NSAID)	
Phenobarbital (drug of abuse)	
Secobarbital (drug of abuse)	

Appendix III. List of publications obtained in this Doctoral Thesis

I. Botello, F. Borrull, C. Aguilar, M. Calull, *"The Current role of capillary electrophoresis in the determination of drugs of abuse and their metabolites."* Trends in Anal. Chem., (2012) (accepted) (section 1.2.2).

I. Botello, F. Borrull, M. Calull, C. Aguilar, *"Simultaneous determination of weakly ionisable analytes in urine and plasma samples by transient pseudo-isotachophoresis in capillary zone electrophoresis."* Anal. Bioanal. Chem., 400 (2011) 527-534 (section 3.1.1).

I. Botello, F. Borrull, C. Aguilar, M. Calull, *"Electrokinetic supercharging focusing in capillary zone electrophoresis of weakly ionisable analytes in environmental and biological samples.*" Electrophoresis, 31 (2010) 2964-2973 (section 3.1.2).

I. Botello, F. Borrull, C. Aguilar, M. Calull, *"Electrokinetic supercharging in capillary electrophoresis for the separation and preconcentration of barbiturate drugs in urine samples."* J. Sep. Sci., (2012) (submitted) (section 3.1.3).

I. Botello, F. Borrull, C. Aguilar, M. Calull, *"Investigation of in-line solid phase extraction capillary electrophoresis for the analysis of drugs of abuse and their metabolites in water samples."* Electrophoresis, 33 (2012) 528-535 (section 3.2.1).

I. Botello, F. Borrull, M. Calull, C. Aguilar, G. W. Somsen, G. J. de Jong, *"In-line solid phase extraction-capillary electrophoresis coupled with mass spectrometry for the determination of drugs of abuse in human urine."* Anal. Bioanal. Chem., 403 (2012) 777-784 (section 3.2.2).

I. Botello, F. Borrull, C. Aguilar, M. Calull, *"In-line solid phase extraction capillary electrophoresis for the determination of barbiturate drugs in human urine."*Anal. Bioanal. Chem., (2012) (submitted) (section 3.2.3).

UNIVERSITAT ROVIRA I VIRGILI
SENSITIVITY ENHANCEMENT STRATEGIES IN CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF DRUGS OF ABUSE AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS
Igor Botello González
Dipòsit Legal: T. 1299-2012