Electrophoretic behaviour of pharmacologically active alkylxanthines

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Abstract

The electrophoretic behaviour of ionizable and neutral alkylxanthines commonly used in pharmaceutical preparations was studied. The performance of various separation modes including capillary zone electrophoresis (CZE), cyclodextrin electrokinetic chromatography (CD–EKC), and micellar electrokinetic chromatography (MEKC) with either sodium dodecyl sulphate (SDS) or bile salts as surfactants, was assessed. CZE in an alkaline medium successfully separates ionizable xanthines and dyphylline. The addition of carboxymethyl-β-cyclodextrin to the BGE allows only partial resolution of neutral xanthines. Based on MEKC results, bile salts exhibit more discrimination ability than SDS to separate similar xanthines. The best results are provided by taurodeoxycholic acid, which ensures thorough separation of xanthines to baseline.

Keywords: alkylxanthines, CZE, MEKC, cyclodextrins, bile salts

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1. Introduction

Alkylxanthines constitute a family of compounds widely used in pharmaceutical preparations on account of their bronchodilating and vasodilating properties. The structures of these species arise from replacement of the protons at positions 1,3 and 7 in the parent compound, xanthine, with various substituents. The substitutions yield a series of active principles of similar chemical structure and slightly different, complementary pharmacological activity that has propitiated their joint use in pharmaceuticals. The most common xanthines (caffeine, theophylline) have been successfully separated from pharmaceutical and biological fluids using capillary electrophoresis (CE) [1–4]. In this work, we assessed the ability of the CE technique to resolve other alkylxanthines used in pharmaceuticals, the separation of which has seemingly never to date been addressed.

The purpose was to study the electrophoretic behaviour of a mixture of eight alkylxanthines with pharmaceutical activity in order to find the most suitable working conditions to analyze some pharmaceuticals preparation that contain a mixture of them. To this end, the performance of various separation modes including CZE and MEKC (with dodecyl sulphate and bile salt micelles) was assessed and compared. The influence of other experimental variables such as pH, the nature of the surfactant, the presence of an organic modifier and the use of a charged cyclodextrin was also examined.

2. Materials and methods

2.1. Reagents

Sodium dodecyl sulphate and six xanthines (theophylline, dyphylline, proxyphylline, caffeine, pentoxyphylline and enprophylline) were obtained from Sigma S.A. Theophylline-7-acetic acid, theobromine, bile salts and carboxymethyl-β-cyclodextrin

were purchased from Fluka S.A. A 20 mM solution of disodium tetraborate decahydrate (Merck) dissolved in ultrapure Milli-Q water and adjusted to pH 8.5 unless otherwise noted was used as BGE. The xanthines were directly dissolved at a concentration around 0.5 mM in Milli-Q water.

2.2. Apparatus

Measurements were made on a ^{3D}CE instrument from Hewlett–Packard (Waldbronn, Germany) equipped with a diode array detector. Hydrodynamic injection at the anode, using a pressure of 50 mbar for 5 s, was used in all experiments. Separations were conducted at 25 °C, using a custom-made fused silica capillary (Sugelabor, Spain) of 50 μm ID and 56 cm effective length.

2.3. Procedure

The study was conducted in the above-described buffer, which was supplied with appropriate amounts of the surfactant and cyclodextrin as required. The capillary was conditioned with the BGE for 3 min prior to each injection. Following injection, a voltage of 30 kV was applied (direct polarity) and the electropherogram recorded. Xanthines were detected at 274 nm, where all exhibited maximum absorption. After measurement, the capillary was flushed with 0.1 M NaOH and water for 3 min. Methanol was used as electroosmotic flow (EOF) marker, even though the injection dip recorded at short wavelengths (200 nm) was also useful.

3. Results and discussion

Xanthines can be charged and hence separated by CZE, both in a basic medium (as anions) and in an acid one (as cations). However, the separation of xanthines as cations entails using very low pHs as these compounds are very weak bases and some are unstable[5]. Separating them in a basic medium is much easier as the deprotonation of the nitrogen atoms at positions 1 and 7 (pK_a = 8–10) is favoured by

the delocalization of the negative charge. As a result, xanthines bearing alkyl groups on these nitrogen atoms can not be readily ionized. We studied a mixture of ionizable (1–4) and neutral alkylxanthines (5–8) (Figure 1).

3.1. Capillary zone electrophoresis

The pK_a values for xanthines allow one to use a pH above 8 in order to ensure their ionization. Figure 2 shows changes in migration sequence and analyte resolution in the range of pH 8.5-10. As expected, neutral xanthines (dyphylline excepted) were not resolved, so they appeared alongside the EOF throughout the pH range studied. On the other hand, dyphylline migrated and emerged after the EOF although it possessed no ionizable chemical group. Its charge was generated through complexation with borate ions in the BGE. The reaction of borate ions with vicinal diols (such as dyphylline) in a basic medium is well-known [6,7]. It was found an increase in effective electrophoretic mobility of dyphylline with increase in pH and the BGE concentration that is consistent with this complexation [6] and confirmed by the fact that replacing the tetraborate in the BGE with a phosphate caused the xanthine to migrate with the EOF. On the other hand, those xanthines with a free proton at position 7 (theophylline and enprophylline) or 1 (theobromine) are partially ionized. The migration sequence for these xanthines can be explained on the basis of their degree of deprotonation at different pHs. Thus, the migration sequence at pH 9.3 coincided with that of pK_a, namely: theobromine (pK_a = 10.0) > theophylline (pK_a= 8.8), enprophylline (pK_a = 8.4) > theophylline-7-acetic acid (pK_a ≈ 4.7). Raising the pH increased their migration times through increased deprotonation and selectivity changes. This behaviour was not observed in theophylline-7-acetic acid (3) because the acetic group was fully deprotonated at the working pH, so the electrophoretic mobility of this xanthine remained virtually constant whatever the pH. Best results

were obtained in the range of pH 8.5-9.3 because at higher pHs the overlap between enprofylline (4) and theophylline (1) occurs.

3.2. Cyclodextrin electrokinetic chromatography (CD–EKC)

The inability to separate neutral xanthines led us to test the addition of an anionic cyclodextrin (*viz*. carboxymethyl-β-cyclodextrin,) to the BGE. We conducted tests at pH 8.5 or 9.3 containing the anionic cyclodextrin. At these pHs values, neutral xanthines were in fact included in the cyclodextrin and thus were shifted with respect to the EOF and ionizable xanthines were resolved at longer times with the migration order observed in the CZE experiments. However, the inclusion constants for neutral xanthines were not different enough to allow their separation; this resulted in the obtainment of five partially overlapped peaks (neutral xanthines and theobromine). We increased CM-β-CD concentration to 15 mM in an attempt to improve resolution. The results reflected better separation of neutral xanthines from the EOF but no improved resolution.

3.3. Micellar electrokinetic chromatography (MEKC)

In order to achieve the migration of neutral xanthines, it was studied the electrophoretic behaviour of the analytes in a BGE containing SDS or bile salts, which form micelles of different nature and geometry [8].

3.3.1. MEKC with SDS micelles

We examined the effect of SDS concentrations over the range 10–200 mM in the BGE that was previously found to provide best resolution (20 mM tetraborate, pH 9.3). The variation of the migration time with the SDS concentration (Figure 3) exhibited two different patterns corresponding to neutral and anionic xanthines. The migration times for anionic xanthines varied very little with SDS concentration (they exhibited straight lines of near-zero slope), which suggest a weak interaction of these

analytes with the micelles owing to electric repulsions. The more marked variation of the migration time for theobromine relative to the other ionizable xanthines can be ascribed to its weak ionization and hence to its stronger interaction with the surfactant micelles at pH 9.3.

The migration times for the neutral xanthines increased substantially with increasing SDS concentration. Pentoxyphylline was the xanthine most strongly interacting with SDS. Proxyphylline and caffeine also interacted strongly with the micelles but exhibited constant migration times. The similar polarity of both xanthines is the apparent origin of this behaviour. Again, dyphylline behaved differently from the other neutral xanthines; in fact, it interacted only weakly with SDS as it formed negatively charged complexes with borate ions in the BGE.

A 100 mM SDS concentration was adopted as optimal for further experiments as it provided a wide enough window for elution of the neutral xanthines. However, the conditions used until then (20 mM tetraborate, pH 9.3, 100 mM SDS) resulted in overlap between caffeine and proxyphylline, and between enprophylline and theophylline. In order to resolve these xanthine pairs, we examined the effect of pH on the micelle separation mode over the range 8.5-10. The migration times for the neutral analytes were found not to be affected by the pH (interactions with the micelles were not altered), whereas those for the anionic analytes changed in the same manner as in CZE. The two extreme pHs tested (8.5 and 10) allowed seven of the eight peaks to be resolved (with variable selectivity); those for caffeine and proxyphylline, however, continued to be overlapped. Intermediate pHs resulted in partial or total overlap of the ionizable xanthines.

The addition of methanol to adjust the partition coefficients [9] for caffeine and proxyphylline and improve its resolution was useless because only a partial resolution

at high solvent contents (> 10%) was obtained and the peaks were broader and analysis times longer.

3.3.3. MEKC with bile salt micelles

Bile salts have been used to resolve analytes with aromatic rings or polycyclic structures [8]. Alkylxanthines possess a structure potentially capable of interacting with bile salts. We tested sodium taurodeoxycholate (STDC), sodium taurocholate (STC), sodium deoxycholate (SDC) and sodium cholate (SC) at concentrations exceeding the critical micelle concentration that is in the range 5-15 mM depending on the the bile salt.

In a solution containing 50 mM STDC, the ionizable xanthines migrated in the expected sequence, dictated by their pK_a values, whereas the neutral ones (pentoxyphylline excepted) were partially overlapped (Figure 4). Increasing the STDC concentration increased resolution among the neutral xanthines. With a 125 mM concentration, the eight xanthines were resolved to baseline. The experiments with STC and SC revealed their inability to resolve the neutral xanthines (pentoxyphylline excepted) over the concentration range studied (50–150 mM). The results with SDC were similar to those provided by STDC; however, the resolution between caffeine and proxyphylline was poorer. Raising the SDC concentration to 150 mM failed to improve the situation; it merely altered the migration order between pentoxyphylline and theophylline.

A comparative study of the separation with taurodeoxycholic and deoxycholic acids against their 2-hydroxyl counterparts revealed the absence of the hydroxyl group to result in improved discrimination of caffeine and proxyphylline, and the presence of a taurine residue instead of the carboxyl group to have little effect on the resolution of this analyte pair.

4. Concluding remarks

The electrophoretic behavior of some alkylxanthines have been studied using different operation modes and additives in the BGE. CZE allows to separate charged alkylxanthines and dyphylline that behaves as an anionic substance through complexation with borate ions in the BGE. The neutral xanthines interact with a CM-β-CD, but the interaction is not strong enough to allow its resolution to baseline. In MEKC, bile salts exhibit a higher discrimination ability than SDS to separate similar xanthines. The complete separation of the mixture in a short time is possible with taurodeoxycholic acid. These conclusions have been used to develop a simple and rapid method to analyse a mixture of alkylxanthines in a pharmaceutical preparation [11].

Acknowledgements

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Figure 1

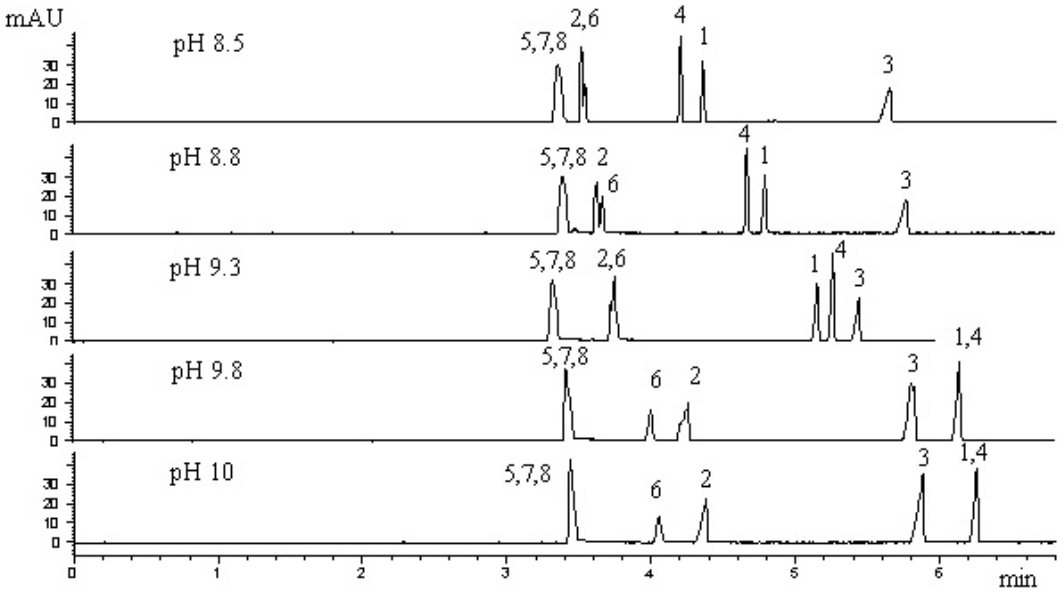

mAU
pH 8.5
5,7,8
2,6
4
1
3
pH 8.8
5,7,8
2
6
4
1
3
pH 9.3
5,7,8
2,6
1
4
3
1,4
pH 9.8
5,7,8
6
2
3
1,4
pH 10
5,7,8
6
2
3
1,4
0
1
2
3
4
5
6
min

Figure 2

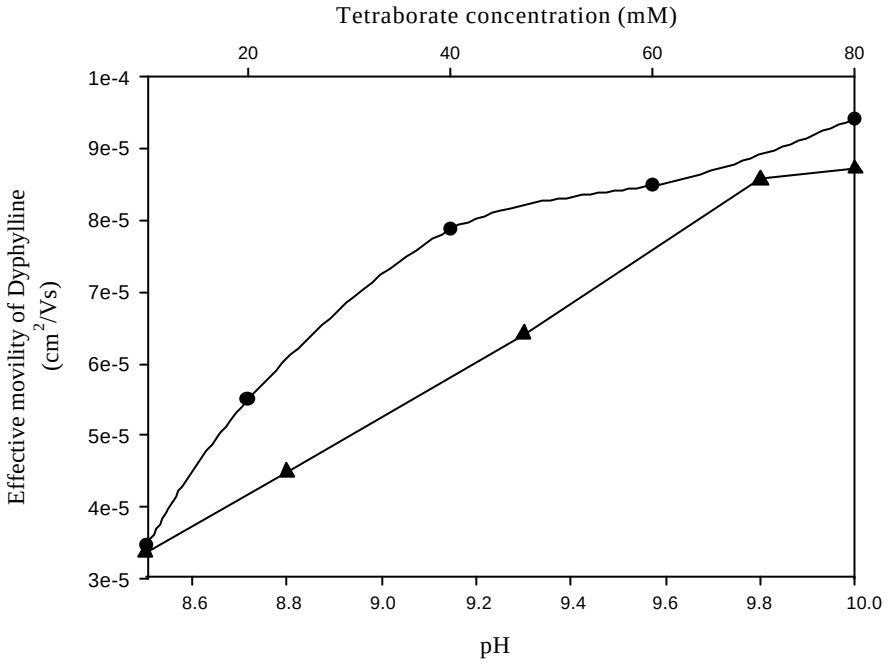

Tetraborate concentration (mM)
20
40
60
80
1e-4
9e-5
8e-5
7e-5
6e-5
5e-5
4e-5
3e-5
Effective movility of Dyphylline
(cm²/Vs)
8.6
8.8
9.0
9.2
9.4
9.6
9.8
10.0
pH

Figure 3

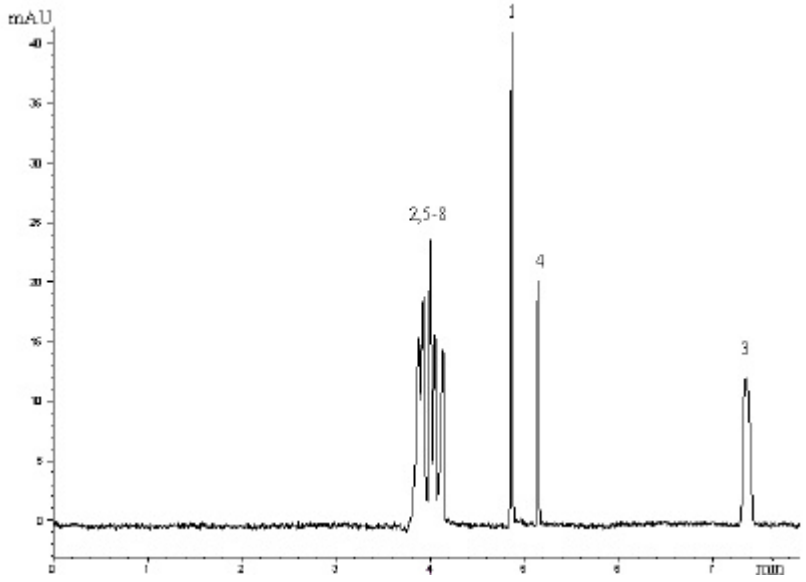
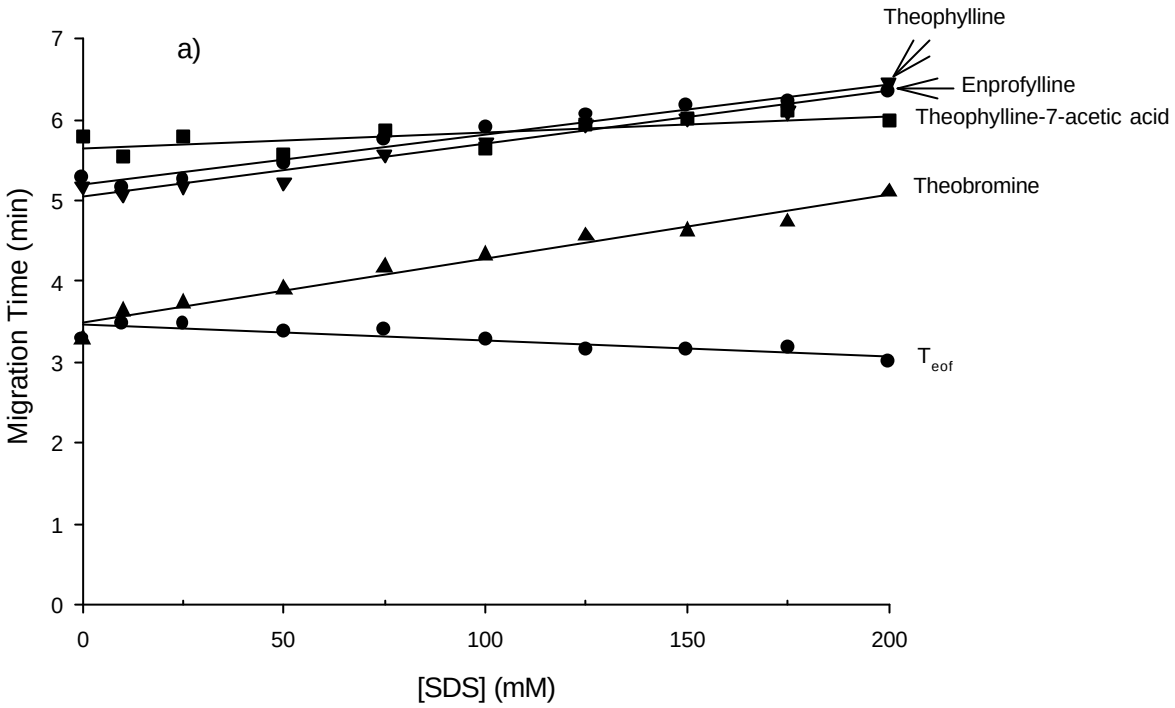


Figure 4


a)
Theophylline
Enprofylline
Theophylline-7-acetic acid
Theobromine
T_{eof}
Migration Time (min)
[SDS] (mM)
0
1
2
3
4
5
6
7
0
50
100
150
200

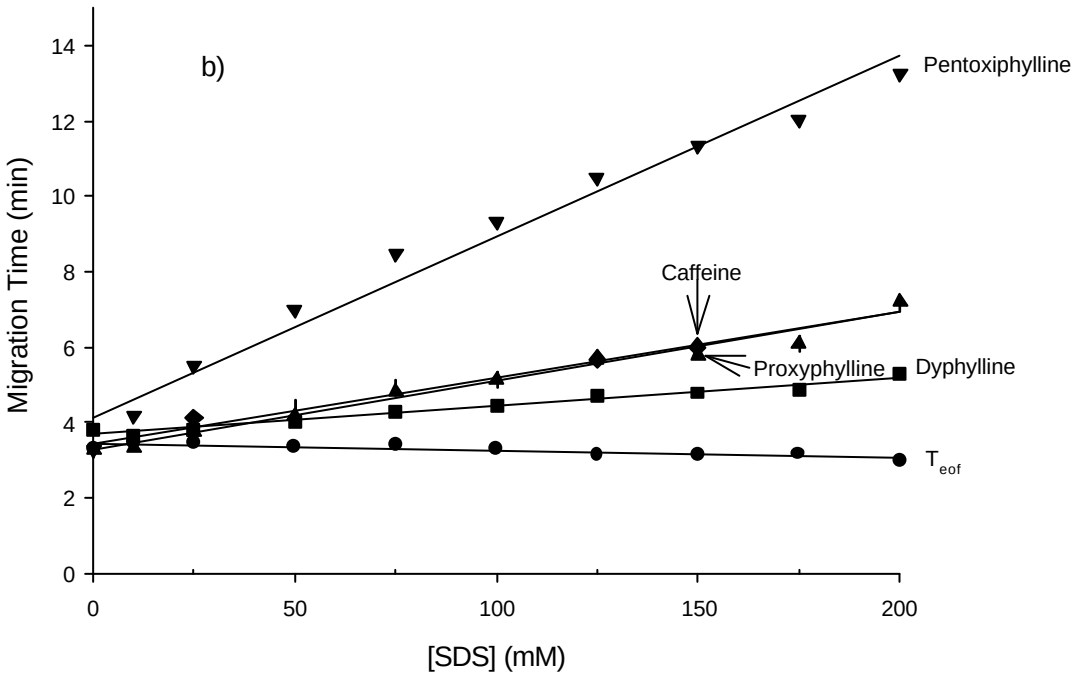

b)
Pentoxiphylline
Caffeine
Proxyphylline
Dyphylline
T_{eof}
Migration Time (min)
[SDS] (mM)
0
2
4
6
8
10
12
14
0
50
100
150
200

Figure 5

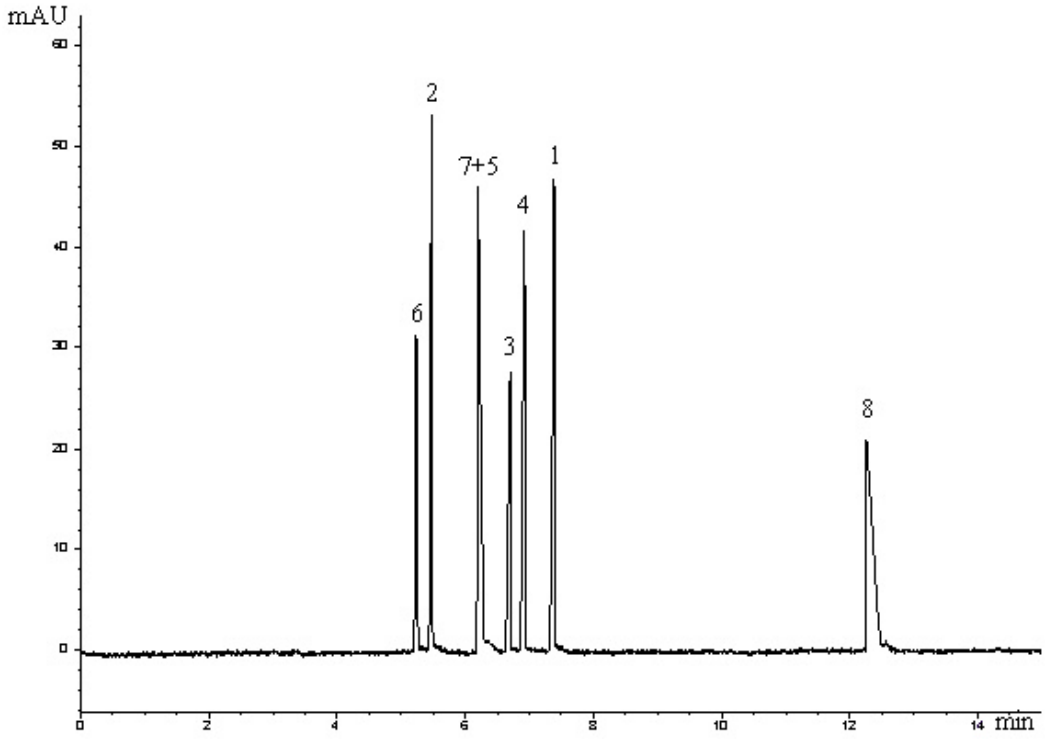


Figure 6

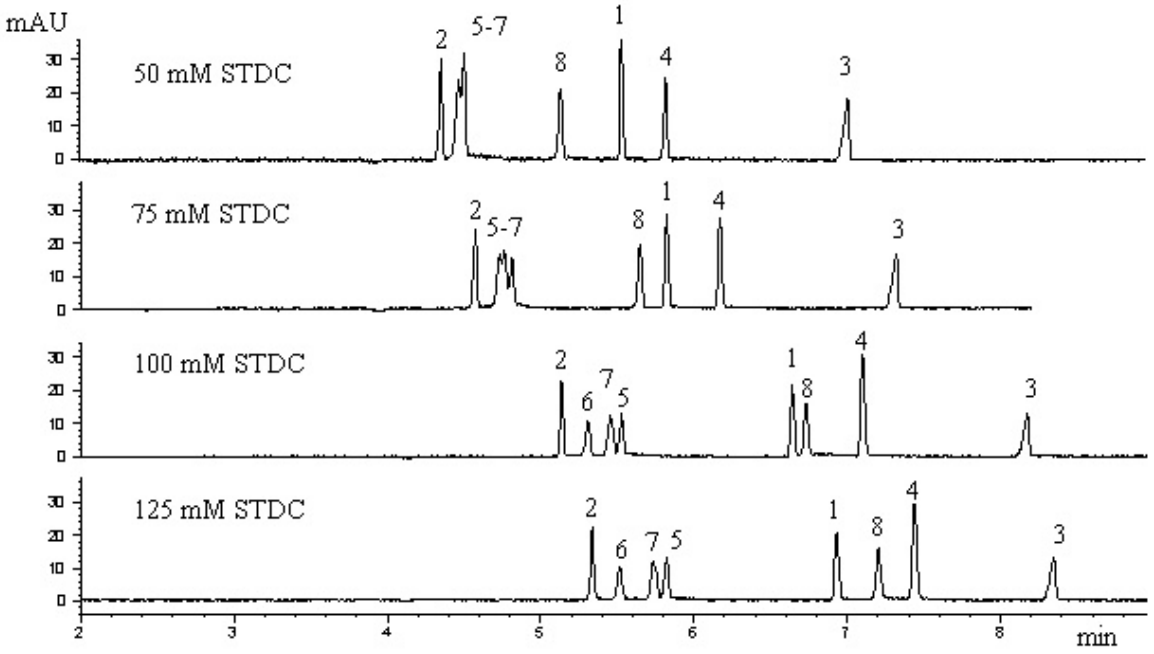
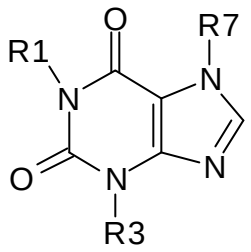

mAU
50 mM STDC
75 mM STDC
100 mM STDC
125 mM STDC
2
5-7
8
1
4
3
6
7
5
min

Table 1



Anionic	R1	R3	R7	pKa
1 Theophylline	-CH3	-CH3	-H	8.8
2 Theobromine	-H	-CH3	-CH3	10.0
3 Theophylline-7-acetic acid	-CH3	-CH3	-CH2COOH	≈ 4.7
4 Enprofylline	-H	-CH2CH2CH3	-H	8.4
Neutral				
5 Caffeine	-CH3	-CH3	-CH3	
6 Diphylline	-CH3	-CH3	-CH2CHOHCH2OH	
7 Proxyphylline	-CH3	-CH3	-CH2CHOHCH3	
8 Pentoxifylline	-$(CH2)_4$COCH3	-CH3	-CH3	

Figure Captions

Figure 1. Variation of the CZE resolution of xanthines with pH using 20 mM sodium tetraborate as BGE. T = 25 °C. V_{app} = 30 kV.

Figure 2. Variation of the effective electrophoretic mobility of dyphylline (absolute value) with the tetraborate concentration in the BGE at pH 8.5 (•, upper scale) and the pH of a BGE containing 20 mM tetraborate (▲, lower scale).

Figure 3. Separation of alkylxanthines by CD–EKC in a BGE of pH 8.5 containing 20 mM tetraborate and 10 mM CM-β-CD. T = 25 °C. V_{app} = 30 kV.

Figure 4. Effect of the SDS concentration on the migration time for the xanthines. (*a*) Ionizable xanthines. (*b*) Neutral xanthines. 20 mM tetraborate BGE of pH 9.3 containing 100 mM SDS. T = 25 °C. V_{app} = 30 kV.

Figure 5. Separation of alkylxanthines by MEKC, using a 20 mM tetraborate BGE of pH 10 containing 100 mM SDS. T = 25 °C. V_{app} = 30 kV.

Figure 6. Effect of the sodium taurodeoxycholate concentration on the separation of xanthines in a BGE consisting of 20 mM tetraborate at pH 8.5. T = 25 °C. V_{app} = 30 kV.

Table 1. Identification numbers and structures of alkyl-xanthines studied