Title: Application of micellar electrokinetic chromatography to the quality control of a pharmaceutical preparation containing three bronchodilators

Authors: Marcelo Blanco and Ismael Valverde

Affiliations: Departamento de Química, Unidad de Química Analítica, Universidad Autónoma de Barcelona, E-08193 Bellaterra, Barcelona, Spain.

Running Title: Development and validation of a new MEKC method

Keywords: MEKC, method validation, bronchodilators, alkylxanthines

Abstract

Theophylline(1,3-dimethylxanthine), dyphylline [7-(2,3-dihydroxypropyl)theophylline] and proxyphylline [7-(β-hydroxypropyl)theophylline] are three bronchodilators administered jointly in a single pharmaceutical preparation used against asthma. A micellar electrokinetic chromatography (MEKC) method for their resolution using a background electrolyte consisting of 20 mM tetraborate at pH 8.5 and 100 mM sodium dodecyl sulphate is proposed. The method was used to determine the three active principles in a pharmaceutical preparation. The small amount of sample required and the expeditiousness of the procedure allow content uniformity to be determined in individual tablets. The values of the validation parameters for the method (*viz.* selectivity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness) are reported. A complete factor design ($2^3 \times 2$) including pH, the surfactant concentration and the ionic strength of the background electrolyte as factors was used to estimate robustness. Based on the results, the method is robust enough for quantitation purposes.

1. Introduction

The use of capillary electrophoresis (CE) for the determination of drugs is rapidly gaining acceptance in the pharmaceutical industry. Capillary electrophoresis is an interesting alternative to classical chromatographic techniques, which it usually surpasses in expeditiousness, flexibility and resolving power. The CE technique has so far been successfully used in the analysis of enantiomeric purity in active principles [1], the determination of drug-related impurities [2], and the qualitative and quantitative separation of mixture components [3,4].

CE separations are usually performed in the capillary zone electrophoresis (CZE) mode, which is the most simple and flexible. In this mode, charged analytes can be separated by virtue of their differential rate of migration. Resolving neutral analytes entails using and additional separation process such as partitioning with a micellar or stationary phase. This has given rise to two separation modes in between capillary electrophoresis and liquid chromatography, namely: micellar electrokinetic chromatography (MEKC) and capillary electrochromatography (CEC).

The separation of active principles with highly similar chemical structures and also similar therapeutic effects is a frequent analytical need, even if the substances are not administered jointly [5,6]. However, the presence in the same pharmaceutical of several active principles from the same family makes their determination difficult owing to their structural similarity. Theophylline (1,3-dimethylxanthine), dyphylline [7-(2,3-dihydroxypropyl)theophylline] and proxyphylline [7-(β-hydroxypropyl)theophylline] are three bronchodilators of the alkylxanthine family that act by relaxing the muscles that govern the airways, so they are used in combination against asthma [7].

There is only one reference to the determination of the three active principles at once, *viz*. their quantitation in serum using HPLC [8]. Theophylline and dyphylline have been determined jointly by HPLC [9], and theophylline individually by CE [10]. A range of other xanthines were previously resolved by MEKC[11]. An UV–Vis spectrophotometric method for determining theophylline in the presence of proxyphylline and dyphylline has also been reported [12].

To date, no CE method for determining the three above-mentioned xanthines jointly appears to have been developed. This paper reports on the development and validation of a CE method for determining theophylline, dyphylline and proxyphylline in the same pharmaceutical preparation. Because xanthines with an hydrogen at position 1 or 7 (*e.g*. theophylline) are ionizable, and those with a methyl group at such positions (*e.g*. dyphylline and proxyphylline) are neutral [13], MEKC appeared to be the most suitable choice for their joint determination.

A method validation study was conducted in order to ascertain whether the proposed method was fit for its purpose [14]. This was a required preliminary step with a view to its use in routine analyses. Standard issuing organizations such as IUPAC [15], AOAC [16] and EURACHEM [17] have released guidelines for method validation. In this work, we used the analytical method validation guidelines issued by ICH in 1996 [18] that recommends that validation be based on method accuracy, precision, selectivity, limit of detection, limit of quantification, linearity and robustness.

2. Material and methods

2.1. Reagents

Theophylline, dyphylline, proxyphylline and sodium dodecyl sulphate were purchased from Sigma. The background electrolyte (BGE) was a 20 mM solution of disodium tetraborate decahydrate (Merck) and 100 mM sodium dodecyl sulphate adjusted to pH 8.5 with 0.1 M NaOH.

2.2. Apparatus

Measurements were made on a ^{3D}CE HPCE 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 fused silica capillary (Sugelabor, Spain) of 50 μm ID and 56 cm length. pH measurements were made with a Crison micropH 2001 pHmeter (Alella, Spain).

2.3. Samples

Ten tablets were ground and mixed. 0.05 g of the resulting powder was weighed and dissolved in 70 ml of water. The suspension was magnetically stirred for 5 min, sonicated for 15 min, passed through a 0.22 μm membrane filter and made up to 100 ml with Milli-Q water. Prior to analysing the pharmaceutical preparation (Novofilin® Retard, from Laboratorios Ferrer), ten tablets were weighed in order to calculate the average tablet mass.

2.4. Procedure

Before each injection, the capillary was conditioned with the BGE for 3 min. After injection, a voltage of 30 kV was applied (direct polarity) and the corresponding electropherogram recorded. The xanthines were detected at 274 nm, where all exhibited an absorption maximum. Finally, the capillary was flushed with 0.1 M NaOH and water for 3 min each. Methanol was used as EOF

marker, even though the injection dip recorded at short wavelengths (200 nm) was also useful for this purpose.

2.5 Software

Modde 5.0 (Umetrics, Umeå, Sweden) was used for experimental design

3. Results and discussion

Preliminary tests were carried out by using a BGE consisting of 20 mM tetraborate at pH 9.3. Proxyphylline gave a distorted peak close to the EOF, whereas theophylline and dyphylline migrated as anions and yielded well-resolved peaks. Based on these results, theophylline is deprotonated and dyphylline, a neutral xanthine, migrates between the other two, which suggests the presence of negative charge in its structure. Such negative charge can be ascribed to the formation of negatively charged complexes with borate ions [19,20]. Although the three analytes were successfully separated by CZE, the micellar mode was used in order to allow proxyphylline to migrate from the EOF and increase the selectivity of the separation as a result.

3.1. Optimization of the experimental conditions

The influence of the addition of SDS concentrations over the range 10–200 mM to the BGE (20 mM tetraborate, pH 9.3) was studied. The mobility of the EOF remained virtually constant, whereas the migration times for theophylline and dyphylline increased slightly with increase in surfactant concentration. On the other hand, proxyphylline, interacted strongly with the micelles, so its migration time increased markedly with increasing SDS concentration. This differential behaviour in three structurally related bronchodilators can be ascribed to the presence of negative charge in both theophylline and dyphylline, which reduces their interaction with micelles through electric repulsion. An SDS concentration

of 100 mM was chosen for subsequent experiments as it ensured acceptable resolution of the three analytes (which migrated in the sequence dyphylline > proxyphylline > theophylline).

The effect of the BGE pH was studied over the range 8.5–10. Only the migration time for theophylline was found to increase with increasing pH. The best results were obtained in a BGE consisting of 20 mM tetraborate at pH 8.5 containing 100 mM SDS; under these conditions, the migration sequence was dyphylline > theophylline > proxyphylline (see Fig. 3) and the three species were accurately resolved.

3.2. Application of the analytical method

The structural similarity of the three xanthines reflects in their UV–Vis spectrum: all three exhibit an absorption maximum at the same wavelength (274 nm). Under the previously established optimum conditions, calibration curves were run at such a wavelength by plotting areas (corrected for migration times) against the concentrations of the corresponding standards. Method development was done by using artificial mixtures containing the three analytes at concentrations over the same range (10^{-4}–10^{-3} M) as the mole content of the three xanthines in the pharmaceutical was identical. The values of the calibration parameters are given in Table 1. Aliquots of two ground samples from different batches were analysed and the results found to be consistent, both mutually and with the composition stated on the pharmaceutical label (see Table 2).

The small amount of sample required and the short analysis time allowed content uniformity to be assessed in individual tablets. To this end, 10 tablets from the same batch were analysed on an individual basis. Based on the results, the content of each analyte in each tablet differed by less than ± 5% from the average content

in the batch; the batch contents in the three principles can thus be deemed uniform as pharmacopoeias typically accept variations of up to 10% [21].

3.3. Validation of the proposed method

Any analytical method should be validated in order to obtain objective evidence for the quality of its results and that these can be used to solve the analytical problem from which the method originated (*i.e.* fitness for purpose).

3.3.1. Selectivity

The selectivity of chromatographic procedures should be demonstrated with representative chromatograms. In the proposed MEKC method, the electropherogram (Fig. 2) reveals complete resolution of the three bronchodilators with no interference from other compounds present in the pharmaceutical preparation. The three analytes were identified by comparison of their migration times and UV–Vis spectra with those for pure standards. In order to assess the repeatability of the selectivity thus measured, a solution of the pharmaceutical preparation was injected nine times and the repeatability of the migration times and resolution established (see Table 3). The results reflected very good repeatability of resolution and the migration times, which allows the accurate identification of peaks.

3.3.2. Precision

Precision in corrected areas was established in terms of repeatability (within-day precision) and intermediate precision (between-day precision). The repeatability study was split into injection repeatability and method repeatability. The former was assessed by consecutively injecting the same pharmaceutical aliquot nine times. On the other hand, method repeatability was assessed by injecting nine

identical aliquots of the pharmaceutical in duplicate. Finally, intermediate precision was calculated by injecting three aliquots of the pharmaceutical in duplicate on three consecutive days. These precision parameters are expressed as the relative standard deviations (% RSD) of the corrected areas for the three analytes in Table 3. Based on the injection and method repeatability obtained, reproducibility in the CE injection [22] was the principal source of imprecision in analyses performed on the same day.

3.3.3. Linearity

Solutions for each of the six points in the 0.1–1 mM range of the calibration curve were prepared in duplicate. The ANOVA [23] of the curve revealed that the lack of fit was always statistically smaller than the pure error, which confirmed that a straight line was a good model for the three analytes. In addition, the lack of significance of the intercept revealed the absence of systematic calibration errors.

3.3.4. Limits of detection and quantitation

Limit of detection (LOD) and quantitation (LOQ) were defined as the analyte concentration giving a signal exceeding that of the blank by 3 times or 10 times respectively its standard deviation. The signal and standard deviation of the blank were calculated as follows: time intervals on the baseline of the same width as the analyte peak and close to it in the electropherograms were integrated and their mean corrected area (blank signal) and standard deviation calculated. The values thus obtained are given in Table 3.

3.3.5. Accuracy

One of the ICH proposals for checking the accuracy of a new method involves spiking a sample with known amounts of analyte and calculating the recovery. To this end, 6 aliquots containing *ca*. 0.05 g of the pharmaceutical were supplied with accurately known amounts of theophylline, proxyphylline and dyphylline in the region of 30% of the amount of analyte presumably contained in the aliquot. A difference test (*viz*. the paired *t*-test) was used to check that mean recovery was not statistically different from 100% and hence the absence of systematic errors in the quantitations. The difference test provided *t* values smaller than their tabulated counterparts for the three analytes; this, together with the recoveries obtained, confirms the accuracy of the proposed method (see Table 3).

3.3.6. Robustness

The robustness of a method is identified through the effect of small changes in the experimental conditions on the quality of its results. We studied the effect on corrected areas (responses) of small variations in the pH and ionic strength of the background electrolyte, and of the surfactant concentration (variables). We also examined other responses such as the resolution of the dyphylline–theophylline (R1) and theophylline–proxyphylline (R2) pairs. Figure 3 summarizes the experimental conditions, the domain tested and the experimental design used (complete experimental design $2^3 \times 2$). The advantadges of this design and the relationship beetween response and variables has been described somewhere in the literature[24]. The interaction between the three variables was considered in the initial analyses; however, it was scarcely significant and chemically unlikely. Consequently, subsequent analyses only included the original variables and their second-order interactions. Based on the results, none of the variables or their interactions compared with the confidence interval are significant in relation to

the corrected areas for the three analytes, which reveals the robustness of the proposed method for quantitative analysis. By way of example, Fig. 4 shows the results for dyphylline. The bars represent the variable regression coefficient, the confidence interval being expressed by error bars over the coefficient.

The study of the variation of resolution between analytes confirms the results examined in the method development section. As can be seen from Fig. 5, the buffer pH had a significant, positive effect on R1 and a negative effect on R2. This was a result of increasing pH values causing theophylline to migrate at longer times without altering those for dyphylline and proxyphylline. On the other hand, the positive effect of the variable SDS on R2 shows that a decreased SDS concentration reduced the migration time for proxyphylline and hence resolution between theophylline and proxyphylline.

4. Concluding remarks

The proposed MEKC method is suitable for the rapid, reliable quality control of commercially available pharmaceutical formulations containing theophylline, dyphylline and proxyphylline. In addition, the minimal amount of sample required and short analysis times required allow content uniformity to be confirmed in individual tablets. The method was validated in terms of selectivity, accuracy, precision, linearity and robustness. The robustness study revealed it to be robust enough for quantitative purposes within the experimental domain tested. In summary, the proposed method is an effective alternative to existing (basically HPLC) methods for the determination of the three bronchodilators.

5. Acknowledgements

The authors are grateful to Spain's DGICyT (Project BQU2000-0234) for funding this research.

6. References

[1] Blanco, M.,Coello, J., Iturriaga, H., Maspoch, S., Pérez-Maseda, C. *J. Chromatogr. A.* 1998,*799*, 301-307.

[2] Altria, K.D. *J. Chromatogr.*, 1996, *735*, 43-56.

[3] Donato, M.G., Beyens, W., Van den Bosche, W., Sandra, P. J.Pharm.Biomed. Anal. 1993, *11*,197-201.

[4] Li,Yong-M., Vanderghinste, D., Pecanac, D., Van Schepdael, A., Roets, E., Hoogmartens, J. *Electrophoresis*. 1998, *19*, 2890-2894.

[5] Fillet, M.,Bechet, I., Piette, V., Crommen, J. *Electrophoresis*.1999, *20*, 1907-1915.

[6] Hirai,T., Matsumoto,S., Kishi, I. J.Chromatogr.B. 1997, *692*, 375-388.

[7] www.people.virginia.edu/~smb4v/tutorials/asthma/bdilate.html

[8] Valia, K. H., Hartman, C. A., Kucharczyk, N., Sofia, R. D. *J. Chromatogr*. 1980, *221*, 170-175.

[9] Ou, C.N., Frawley, V.L. *Clin. Chem*. 1982., *28*, 2157-2160.

[10] Haque, A., Xu, X.H, Stewart, J.T. J.Pharm. Biomed. Anal. 1999, *21*, 1063-1067.

[11] Korman, M., Vindevogel,J., Sandra, P. *Electrophoresis*. 1994, *15*, 1304-1309.

[12] Martin Saez,R., Llobat Estelles, M., San Martín Ciges, M.D., Mauri Acejo, A.R. *Anal. Lett.* 1993, *26* (4), 641-655.

[13] Joule, J.A., Mills, K., Smith, G.F. *Purines: reactions and synthesis In: Heterocyclic Chemistry*. Chapman & Hall, London 1995, 3rd Edition, 409-433.

[14] Mark Green, J. *Anal. Chem.* 1996, *68*,305A-309A.

[15] IUPAC recommendations. *Pure & Appl.Chem*. 1995, *67*(4), 1699.

[16] IUPAC,ISO & AOAC Int. *Pure & Appl.Chem*. 1995, *67*(4), 649.

[17] The Fitness for Purpose of Analytical Methods-a laboratory guide to method validation and related topics. www.vtt.fi/ket/eurachem/publications.htm, 1998

[18] Guidance for the industry.Q2B Validation of Analytycal Procedures: Methodology.ICH, 1996.

[19] Hoffstetter-Kuhn, S., Paulus, A., Gassmann E., Widmer M.H. *Anal. Chem*. 1991, *63*, 1541-1547.

[20] Soga, T. Hewlett Packard Application Note. Capillary zone electrophoresis of carbohydrates by borate complexation utilizing EOF reversal. 1995.

[21] British Pharmacopoeia. Vol. II, 1993, 753-755.

[22]Heiger, D.N. High Performcance Capillary Electrophoresis-An introduction. Hewlett-Packard Company, 1992,2nd Edition,77-84.

[23] Deming, S.N., Morgan, S.L. Experimental design: a chemometric approach. Elsevier, Amsterdam 1987, 135-150.

[24] Goupy, J.L. Methods for experimental Design. Elsevier, Amsterdam 1993, 49-63.

Figure captions

Figure 1. Chemical structures of the bronchodilators studied.

Figure 2. Electropherogram for the pharmaceutical preparation Novofilin® Retard in 20 mM tetraborate containing 100 mM SDS, pH 8.5, 30 kV. The concentration of analytes is approximately 0.5 mM.

Figure 3. Variables and levels as executed in the ($2^3 \times 2$) robustness test. SDS, sodium dodecyl sulphate concentration. Bor, tetraborate concentration.

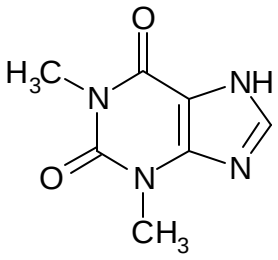
Figure 4. Regression coefficient plot for the corrected area of dyphylline (CAD) and the resolution of the dyphylline–theophylline (R1) and theophylline–proxyphylline (R2) pairs. Bor, tetraborate concentration. SDS, sodium dodecyl sulphate concentration.

Table 1. Figures of merit of the calibration curves used for quantitation. (*a*) Expressed as corrected area *versus* concentration over the range 0.1–1 mM. (*b*) Confidence interval as calculated from the standard deviation of the intercept and slope, respectively. (*c*) Standard error of residuals.

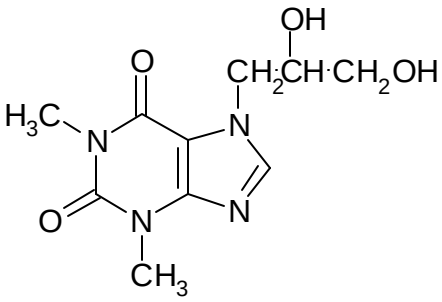
Table 2. Analysis of the pharmaceutical preparation Novofilin® Retard. Data are the averages for aliquots injected in duplicate and confidence intervals are given as standard deviations.

Table 3. Figures of merit of some validation parameters. Precision parameters are given as relative standard deviations (% RSD).

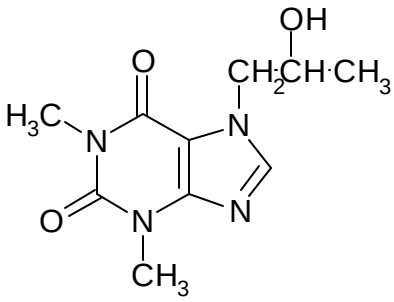
Figure 1



Theophylline



Dyphylline



Proxyphylline

Figure 2

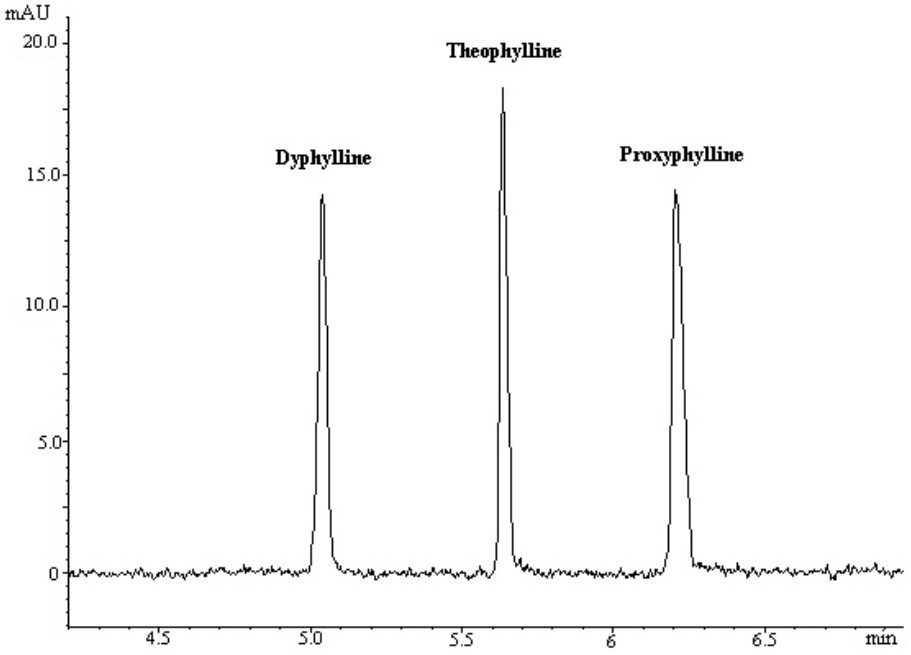

mAU
20.0
15.0
10.0
5.0
0
Dyphylline
Theophylline
Proxyphylline
4.5
5.0
5.5
6
6.5
min

Figure 3

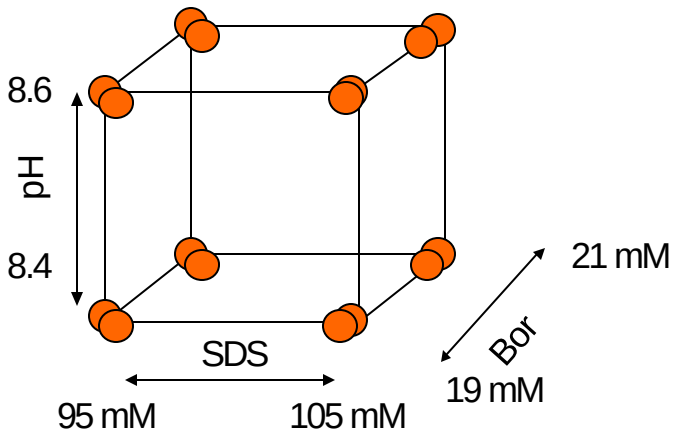
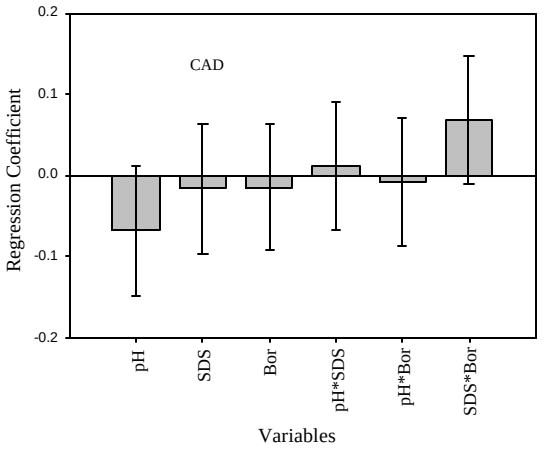
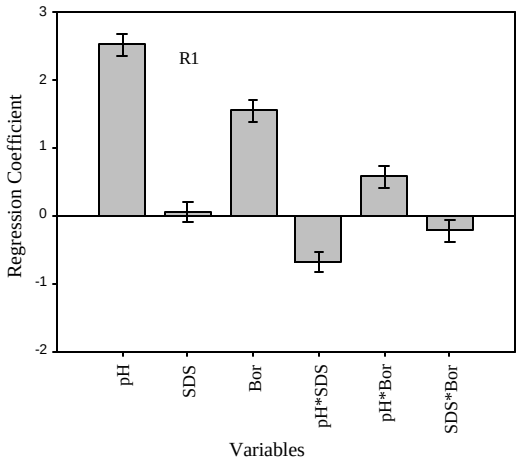


Figure 4


CAD
Regression Coefficient
0.2
0.1
0.0
-0.1
-0.2
pH
SDS
Bor
pH*SDS
pH*Bor
SDS*Bor
Variables


R1
Regression Coefficient
3
2
1
0
-1
-2
pH
SDS
Bor
pH*SDS
pH*Bor
SDS*Bor
Variables

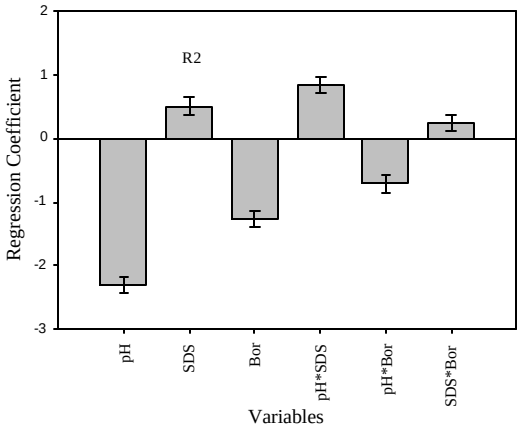

R2
Regression Coefficient
2
1
0
-1
-2
-3
pH
SDS
Bor
pH*SDS
pH*Bor
SDS*Bor
Variables

Table 1

Curve [a)]	Intercept [b)]	Slope [b)]	Correlation Coefficient	$S_{y/x}$ [c)]
Dyphylline	0.166 ± 0.281	14033.527 ± 456	0.9998	0.134
Theophylline	0.107 ± 0.351	17304.849 ± 588	0.9999	0.142
Proxyphylline	0.177 ± 0.224	14493.005 ± 372	0.9997	0.115

Table 2

Batch	Theophylline[a)] found (mg/g)	Dyphylline[b)] found (mg/g)	Proxyphylline[b)] found (mg/g)
L-1	74.7±0.1	113.1±0.2	112.8±0.8
N-1	72.2±1.3	115.2±0.6	113.8±0.6

a) Nominal value 75mg/g

b) Nominal value 112.5 mg/g

Table 3

	Dyphylline	Theophylline	Proxyphylline
Average Migration Times (min)	4.95	5.57	6.12
Repeatability	0.8	0.9	0.6
Intermediate Precision	1.8	1.9	2.2
Average Corrected Area	6.44	6.69	6.54
Injection Repeatability	1.9	1.9	2.0
Method Repeatability	1.2	2.2	1.6
Intermediate Precision	2.5	4.0	3.0
Average Resolution		11.29	9.81
Repeatability		6.0	2.5
Intermediate Precision		7.1	3.3
Average Recovery (%)	103	103	105
LOD (mM)	0.013	0.010	0.011
LOQ (mM)	0.023	0.018	0.021