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Short communication

Development of a Micellar electrokinetic capillary chromatography method for the determination of three drugs employed in the erectile dysfunction therapy

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Abstract

A Micellar electrokinetic capillary chromatography method is proposed for the determination of sildenafil, vardenafil and tadalafil, which are employed in oral therapy for erectile dysfunction. Optimum conditions for the separation were investigated. A background electrolyte solution consisting of 10 mM phosphate buffer adjusted to pH 12.0, sodium dodecyl sulfate (SDS) 25 mM, hydrodynamic injection, and 25 kV as separation voltage were used. Relative standard deviations (R.S.D.s) were 1.0, 1.0, 0.4% and 2.9, 2.9, 1.9% for migration time and corrected peak area (CPA) (n = 9) for sildenafil, vardenafil and tadalafil, respectively. Detection limits obtained for the three drugs ranged from 0.19 to 0.61 mg L^{-1}. A linear concentration range between 1 and 20 mg L^{-1} was obtained. A ruggedness test of this method was checked using the fractional factorial model of Plackett–Burman, in which the influence of six factors at three different levels was tested on different electrophoretic results: resolution and corrected peak area. The statistical evaluation of the electrophoretic results was achieved by Youden and Steiner method. The described method is rapid, sensitive and rugged and it was tested in the pharmaceutical formulations analysis obtaining recoveries between 98 and 107% respect to the nominal content

Keywords: Micellar electrokinetic capillary chromatography; Electrophoresis; Sildenafil; Vardenafil; Tadalafil; Erectile dysfunction; Ruggedness; Pharmaceutical formulations

1. Introduction

Since the early 1980s, research on the mechanisms of penile erection has done much to clarify erectile physiology and pathophysiology [1]. More recent studies have identified the importance of neurochemical mediators in erection. These include the nitric oxide–cyclic guanosine monophosphate (cGMP) cell-signaling system, a complex molecular pathway that mediates smooth muscle relaxation in the corpus cavernosum. Phosphodiesterase 5 (PDE5) inactives cGMP, which terminates nitric oxide–cGMP-mediated smooth muscle relaxation. Inhibition of PDE5 is expected to enhance penile erection by preventing cGMP degradation. Development of pharmacologic agents with this effect has closely paralleled the emerging science.

The prototype of this new therapeutic class of PDE5 inhibitors is sildenafil, which was approved for treatment of erectile dysfunction in 1998. Vardenafil and tadalafil have been the second and the third oral product approved for the Food and Drug Administration for the treatment of erectile dysfunction in men (impotence).

The bioavailability, maximum plasma concentration (C_{max}), the time (T_{max}) required for attaining C_{max}, and time ($t_{1/2}$) required for elimination of one half of the inhibitor from plasma are all important factors [2]. Sildenafil, vardenafil and tadalafil have broadly similar C_{max} and T_{max}, but the $t_{1/2}$ of tadalafil is considerably longer than other two

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PDE5 inhibitors. The extended $t_{1/2}$ of Tadalafil could provide a longer therapeutic effect, which may be preferred for spontaneous sexual activity but could expose the patient to greater risk of side effects.

No previous report has presented the separation of vardenafil and tadalafil. For the determination of sildenafil there are around 40 previous publications. The widely used technique is liquid chromatography [3–7], but techniques such as electroanalytical methods [8,9], gas chromatography [10,11] and capillary electrophoresis have also been reported [12–15]. In the case of capillary electrophoresis, there are two reports using CZE [13,15] and two others [12,14] describing MEKC.

Li et al. employ 60 mM phosphate buffer pH 5.0 as running buffer, voltage of 30 kV and the method is applied to pharmaceutical preparations, while Qin et al. use an ionic liquid which is covalently bonded onto the silica capillary surface and determination by MS, where the running buffer contain 10 mM acetic acid adjusted to pH 4.5 with 1 M ammonia and sildenafil with its metabolite are separated within 14 min. Berzas et al. [12,14] propose two method for determining sildenafil and its metabolite by MEKC. One method uses 10 mM phosphate buffer pH 12.3/30 mM sodium dodecyl sulfate (SDS); and in the other MEKC is used with sample stacking and polarity switching for the determination in human serum in the concentration range of clinical interest and the analysis takes about 6 min.

In this work, our objective is to propose a new method providing simplicity, rapidity and ruggedness for the routine pharmaceutical control of these three drugs used in erectile dysfunction therapy.

Up to now, we have found no report for analytical determination of vardenafil and tadalafil. It is the first report for the simultaneous determination of sildenafil, vardenafil and tadalafil by capillary electrophoresis.

2. Experimental

2.1. Chemicals

Milli-Q water was used throughout this study.

Sildenafil, vardenafil and tadalafil were obtained from Pfizer, Bayer and Lilly pharmaceutical companies, respectively.

The stock standard solution of 100 mg L^{-1} sildenafil and vardenafil were prepared in water, whereas tadalafil (100 mg L^{-1}) was prepared in methanol. Working standard solutions (10–20 mg L^{-1}) were prepared daily by diluting of the stock standard solutions with purified water.

Dilute Na_2HPO_4 solutions (0.5 M) were prepared in deionised water and the pH was adjusted to 12 using NaOH (1.0 M).

2.2. Instrumentation

A Beckman P/ACE System MDQ (Fullerton, CA, USA) equipped with a diode-array detector was used. Beckman capillary electrophoresis software controlled the system. Separation was carried out on a 60 cm long (10 cm to the detector, short way) × 75 μm i.d. fused-silica capillary housed in a cartridge with a detector window 100 μm × 800 μm.

2.3. Stability of solutions

The stability of the stock standard solutions were followed spectrophotometrically by preparing every day in water three different standard solutions of 10 mg L^{-1} for each component. In all cases, the obtained UV–vis absorption spectra remained unaffected at least 3 weeks latter to its preparation.

For the evaluation of the stability of working standard solutions (10 mg L^{-1}), the UV–vis absorption spectra were recorded every 15 min during a period of 3 h. In all cases, the absorption spectra remained unaffected.

2.4. Operating conditions

The wavelength selected for the electropherograms was 222 nm. The capillary was conditioned prior to its first use by rinsing with 0.5 M NaOH for 20 min, water for 10 min and finally phosphate buffer (pH 12, 10 mM) with 25 mM of SDS for 10 min. At the start of each sequence, the capillary was washed with NaOH 0.1 M during 2 min and with the separation electrolyte during 4 min.

Electrophoretic separation was performed using a 10 mM phosphate buffer, pH 12 with 25 mM of SDS. For sample loading, an hydrodynamic injection time of 3 s (0.5 psi) was applied at the detector end of the capillary. Separation was performed by applying a voltage of −25 kV. Under the selected conditions, the current was around 50 μA. Duplicate injections of the solutions were performed and average-corrected peak areas (CPA) (area/migration time) were used for quantitative analysis. The data generated from the first two injections of a sequence were not used on account of the necessary equilibration system. The rinse step was carried out using vials different from the separation vials in order to keep the level of buffer constant during the separations. The separation vial set was changed after every six runs.

2.5. Preparation of the samples

Two tablets of the pharmaceutical preparation were weighed and grounded in a mortar. A single tablet was pulverized and dissolved with approximately 90 mL of methanol. This solution was sonicated for 10 min and centrifuged during 10 min. The supernatant solution was transferred to 100 mL volumetric flask, and adjusted to the mark with the same solvent.

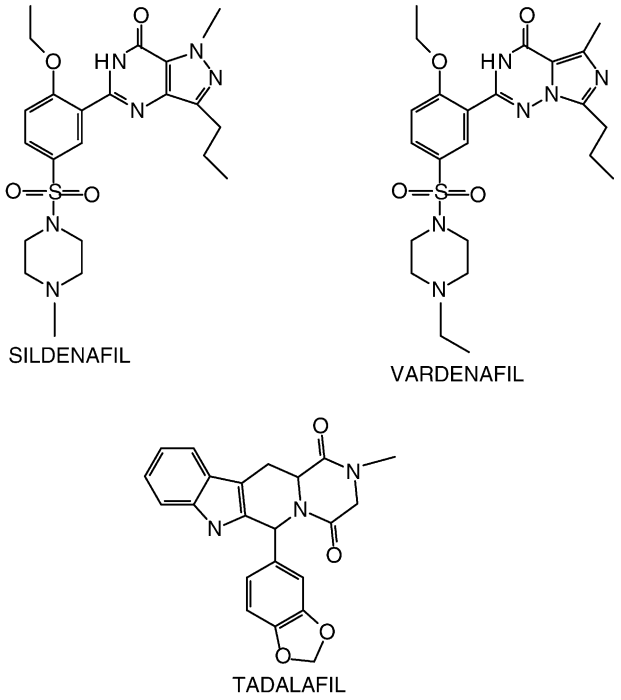

Fig. 1. Structures of sildenafil, vardenafil and tadalafil.

3. Results and discussion

3.1. Optimisation of the MEKC method

3.1.1. Preliminary experiments

The pH of the running electrolyte has a significant impact on the ionization of the acidic silanols of the capillary wall and on the electrophoretic mobilities of the compounds studied. Based on the structure of the analytes (Fig. 1), either basic or acidic buffers can be used to promote their ionization. The migration behaviour of the target analytes was first examined with CZE for pH buffer ranging from 2.2 to 13.0. In very acidic media, sildenafil and vardenafil were found to migrate before the electroosmotic flow. On the other hand, in very basic media sildenafil and vardenafil migrated after the electroosmotic flow. In contrast, tadalafil remained neutral under all pH conditions examined and migrated with the EOF. Then, MEKC was investigated in order to the simultaneous determination of the three analytes.

3.1.2. Influence of nature of surfactants and pH of background electrolyte

Some experiments were carried out using different surfactants such as sodium dodecyl sulfate, cholic acid and deoxicholic acid. In all cases, the concentrations of the surfactant were varied between 10 and 40 mM, for pH varying from 6.5 to 12.0.

In the case of cholic acid no satisfactory separation was observed for the conditions examined. By using among of all surfactant buffers examined, SDS gave the best separation with narrow and well-resolved peaks for all three analytes.

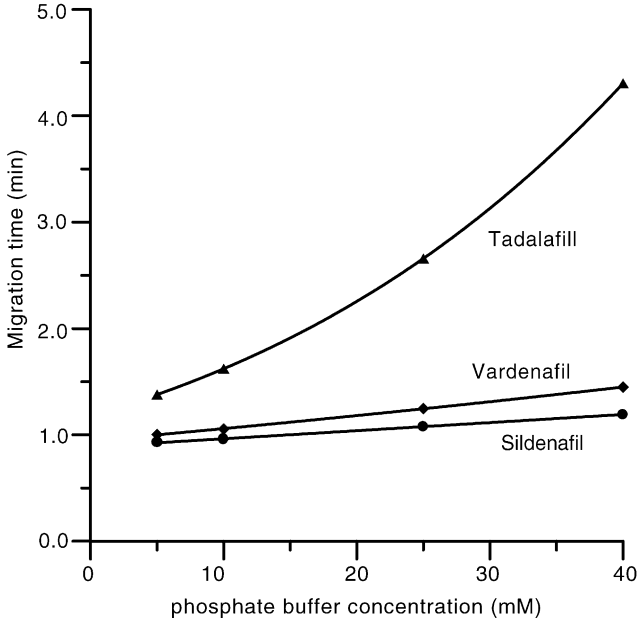

Fig. 2. Influence of phosphate buffer concentration.

Then, the optimal separation conditions were SDS at a concentration of 25 mM and pH 12 by using phosphate buffer solution.

3.1.3. Influence of phosphate buffer concentration

The ionic strength of the buffer has significant effects on solute mobility and separation efficiency [16]. The effect of the buffer concentration on the migration time and peak dispersion for the compounds examined was evaluated for concentrations 5–40 mM. As expected, the migration times of the three analytes and the separation current increase with the buffer concentration (Fig. 2). A buffer concentration of 10 mM was selected to maintain good peak shape, low current and the better resolution between all peaks.

3.1.4. Optimisation of injection time

In order to decrease the detection limits, the injection time was varied between 0.6 and 7 s at 0.5 psi (1 psi = 6894.76 Pa). It was found that up to 3 s the peak area of all compounds increased, but for injection times higher the electrophoretic peaks became not so narrows with the correspondent loss of resolution. For this reason, 3 s of injection time was chosen as optimal value.

3.1.5. Effect of the voltage applied

The effect of the voltage applied from 5 to 30 kV was investigated. When the voltage increases, the migration time of all the drugs decreases together with the corresponding peak resolution. A voltage of 25 kV yielded the best compromise in terms of run time, separation current and resolution and was used for all experiments stages of the development method. The total separation time takes less than 2 min.

3.1.6. Selected conditions

From these studies, the following electrophoretic conditions selected were:

- electrolyte: 10 mM phosphate buffer pH 12.0 with 25 mM of SDS;
- voltage: 25 kV, current: −50 μA;
- injection: hydrodynamic, 3 s (0.5 psi);
- detection wavelength: 222 nm.

3.2. Validation of the electrophoretic procedure

3.2.1. Precision

The precision of this method for the analysis of the three drugs is expressed in terms of relative standard deviation (R.S.D.). Nine samples of analytes prepared at a concentration of 8 mg L^{-1} each were prepared and injected sequentially under the conditions described in Section 2. The precision of migration times and corrected peak areas (ratio between peak area and migration time) was 1.0, 1.0, 0.41% and 2.9, 2.9 and 1.9% for sildenafil, vardenafil and tadalafil, respectively.

These performances suggest that the method is suitable for quantitative determination of the drugs.

3.2.2. Linear concentration range

The detector response, for all the drugs studied, in presence of the excipients of their pharmaceutical formulations, was determined for concentrations ranging from 1 to 20 mg L^{-1} for all the compounds examined (Fig. 3). The linear concentration range was determined by repeated injections of eight different concentrations of each drug. The results are given in terms of corrected peak areas. The linear regression equations obtained using the least-square method and coefficients of determinations are presented in Table 1.

3.2.3. Specificity

Specificity can also be determined by measurement of peak homogeneity. Because of the different techniques available in a DAD are not equally effective for the detection of possible impurities or interferences in an electrophoretic peak, the use of several techniques is recommended [17].

In this work, peak purity was assessed for both the working standard and samples of sildenafil, vardenafil and tadalafil. The techniques used to validate the peak purity of the drugs present in each pharmaceutical formulation were [18]:

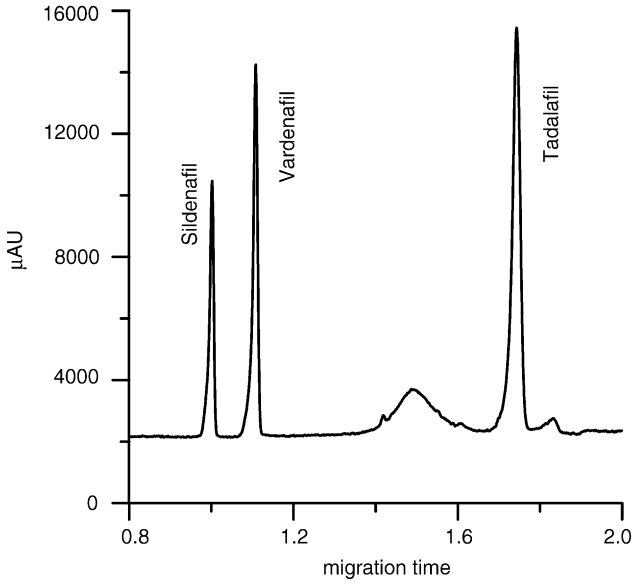

Fig. 3. Sample of 18 mg L^{-1} of sildenafil, vardenafil and tadalafil in presence of the excipients included in their pharmaceutical formulations. Operating conditions: 10 mM of phosphate buffer pH 12 with 25 mM SDS, 25 kV of voltage, 3 s injection time (0.5 psi).

- Normalization and comparison of spectra from different peak sections was done by using a typical overlay of the spectra captured at the apex, upslope and downslope.
- Absorbance at two wavelengths.

Both techniques showed that the purity of the peaks corresponding to the compounds studied present a high level of purity. Therefore, no interferences by matrix effect were observed.

3.2.4. Limit of detection (LOD) and limit of quantification (LOQ)

Limits of detection (LOD) and quantification (LOQ) were estimated in the usual way. LOD was obtained as the concentration of drug resulting in a peak of height three times the baseline noise level; LOQ was calculated as 10 times the baseline noise level. LOD and LOQ for each compound are shown in Table 1.

3.2.5. Ruggedness

The purpose of a ruggedness test is to identify possible sources of error when changes occur in the specified method

Table 1
LODs and LOQs for the three studied drugs

	Equation[a]	Coefficient of determination (r^2)	LOD (mg L^{-1})	LOQ (mg L^{-1})
Sildenafil	$y = (114.9 \pm 53.9) + (356.6 \pm 4.7)\,x$	0.998	0.19	0.63
Vardenafil	$y = (21.1 \pm 89.6) + (524.6 \pm 7.4)\,x$	0.998	0.32	1.08
Tadalafil	$y = (-6.14 \pm 152.6) + (599.2 \pm 12.9)\,x$	0.994	0.61	2.00

[a] Concentration (x = mg L^{-1}) vs. corrected peak area (y). $y = (a \pm \mathrm{Sa}) + (b \pm \mathrm{Sb})\,x$, a: intercept, Sa: standard deviation of intercept, b: slope, Sb: standard deviation of slope. Linearity (n = 8).

Table 2
Variables selected as factors and chosen values as levels

Factors	Minimum values (−1)	Optima values (0)	Maximum values (+1)
(A) pH	11.5	12	12.5
(B) Concentration of phosphate buffer (mM)	8	10	12
(C) SDS concentration (mM)	20	25	30
(D) Injection time (s)	2	3	4
(E) Voltage (kV)	22	25	28
(F) Wavelength (nm)	220	222	224

conditions [19]. Fractional factorial designs developed by Plackett and Burman [20] were used, based on balanced incomplete blocks. Main effects and standard errors were calculated according to procedures described by Youden and Steiner [21].

The choice of variables and the levels at which to test them is very important if the ruggedness test is to be of value. Variables must be those which are likely to be significant in practice and the levels must reflect the variations which are usually observed. The variables selected and the levels are shown in Table 2.

The three values used for each variable are shown in parentheses, where level (−1) is the minimum value studied, level (0) is the optimal value for the method, and level (+1) is the maximum value tested in this experiment.

The mean value of each variable is the average difference between observation made at the extreme levels and those made at the optimal level. Mean effects and standard errors (DA, DB, DC, ...) were calculated using the procedures described by Youden and Steiner.

The ruggedness was determined, in our case, from triplicate injections of a solution of 10 mg L^{-1} of sildenafil, vardenafil and tadalafil in presence of excipients. Results of the effect of each factor levels over resolution and corrected peak areas are shown in Table 3. As an example, Fig. 4

Table 3
Deviations for each result obtained using Youdner and Steiner's statistical method

	Corrected peak area			Resolution	
	Sildenafil	Vardenafil	Tadalafil	Sildenafil–Vardenafil	Vardenafil–Tadalafil
$DA_{(+1)}$	−864	−1271	−835	0.15	0.21
$DB_{(+1)}$	433	623	123	0.009	−0.51
$DC_{(+1)}$	119	74	−391	−0.41	−0.98
$DD_{(+1)}$	1323	−2682	−2339	0.37	1.50
$DE_{(+1)}$	149	308	512	0.11	0.91
$DF_{(+1)}$	370	468	27	−0.28	−0.94
$DA_{(-1)}$	270	240	6.5	0.22	3.2
$DB_{(-1)}$	68	6.8	−7.6	−0.076	0.054
$DC_{(-1)}$	−90	−115	−2.4	0.073	−0.67
$DD_{(-1)}$	−1284	1886	1491	−0.096	0.96
$DE_{(-1)}$	−11	−91	507	−0.20	−1.06
$DF_{(-1)}$	−878	−1229	−1080	0.19	−0.033
$\sqrt{2} \times S$	2162	3127	2614	0.61	3.5

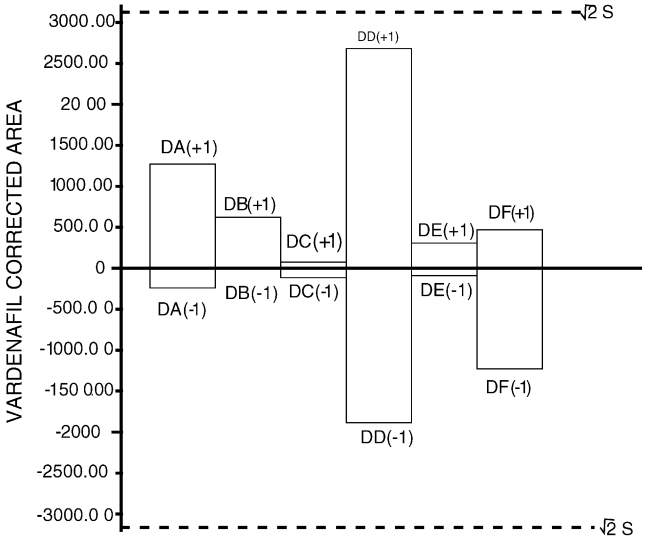

Fig. 4. Level variation effects (−1, 0, 1) on the selected six operating factor on vardenafil corrected area.

shows graphically these effects over vardenafil corrected area.

Taking into account the deviations shown in Table 3 for the different checked results when the selected operating factors were tested upon the experimental design of Placket–Burman and the statistical treatment of Youden–Steiner, this analytical method for measuring the three drugs, has proved to be rugged to all the variations tested in this work. The validity of the Plackett–Burman design is confirmed for the purpose of ruggedness testing.

The main interaction effects are produced by injection time (DD) over the corrected peak areas measures; and by the pH of buffer electrolyte (DA) over resolution. But the ruggedness obtained in all cases, allows to use this method by different laboratories, analysts or instrument without any appreciable error (because in all cases $\sqrt{2} \times S >$ | deviation |).

3.3. Applications

To demonstrate the usefulness of the developed electrophoretic procedure, the method was applied to the determination of the analytes in pharmaceutical preparations, which each contain one drug with other components (excipients). In addition to the active ingredient, each tablet contains microcrystalline cellulose, crosporidone, colloidal silicon dioxide, magnesium stearate, hydropromellose, polyethylene glycol, titanium dioxide, yellow ferric oxide and red ferric oxide as excipients. Preparation of the sample is described in Section 2. The pharmaceutical preparations (in tablets) are: Viagra 25 mg (which contain sildenafil), Levitra 10 mg (which contain vardenafil) and Cialis 20 mg (which contain tadalafil). Determination of the content of each drug in the pharmaceutical formulations was performed in triplicate by external standardization using a typical sequence of calibration solutions and sample solutions (calibration 1, sample 1, calibra-

Table 4
Results from analysis of pharmaceutical formulations

Commercial product (mg)	Active principle	Recovery (%) ± R.S.D. (%)
Viagra (25)	Sildenafil	107 ± 4
Levitra (10)	Vardenafil	99 ± 2
Cialis (20)	Tadalafil	98 ± 2

tion 2, sample 2, calibration 3, sample 3). The corrected peak area for the calibration was used to calculate the response factor and the concentration of the analytes in the sample were obtained using the mean response factor of each drug. The results obtained are given in Table 4, with recoveries range from 98 to 107% of the values declared by manufacturers.

4. Conclusions

The MEKC method described for the simultaneous determination of sildenafil, vardenafil and tadalafil is very simple and rapid (less than 2 min).

The experimental obtained results with respect to linear concentration range, specificity, sensitivity, precision and ruggedness for the simultaneous determination of the three drugs in presence of a big variety of excipients show that the proposed method can be used for routine quality control of commercial formulations as an alternative method to HPLC (it is more rapid, flexible and economical).

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References

[1] R.C. Rosen, J.B. Kostis, Am. J. Cardiol. 92 (Suppl.) (2003) 9M.
[2] J.D. Corbin, S.H. Francis, J. Androl. 24 (6) (2003) S38.
[3] M.C. Tseng, J.H. Lin, Yaowu. Shipin. Fenxi. 10 (2) (2002) 112.
[4] M.T. Sheu, A.B. Wu, G.C. Yeh, A. Hsia, H.O. Ho, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 791 (1/2) (2003) 255.
[5] J. Kim, H. Ji, S.I. Kim, H.W. Lee, S.S. Lee, D.S. Kim, M. Yoo, W.B. Kim, H.S. Lee, J. Pharm. Biomed. Anal. 32 (2) (2003) 317.
[6] A. Tarcqui, B. Ludes, J. Anal. Toxicol. 27 (2) (2003) 88.
[7] H.Y. Aboul-Enein, M.M. Hefnawy, J. Liq. Chromatogr. Relat. Technol. 26 (17) (2003) 2897.
[8] J.J. Berzas, J. Rodriguez, G. Castaneda, M.J. Villasenor, Anal. Chim. Acta 417 (2) (2000) 143.
[9] S.A. Ozkan, B. Uslu, P. Zuman, Anal. Chim. Acta 501 (2) (2004) 227.
[10] J.J. Berzas-Nevado, M.J. Villasenor-Llerena, A.M. Contento-Salcedo, J. Rodriguez-Flores, J. Sep. Sci. 25 (12) (2002) 767.
[11] A. Pelander, I. Ojanpera, J. Sistonen, I. Rasanen, E. Vuori, J. Anal. Toxicol. 27 (4) (2003) 226.
[12] J.J. Berzas-Nevado, J. Rodriguez-Flores, G. Castaneda-Penalvo, N. Rodriguez-Farinas, Electrophoresis 22 (10) (2001) 2004.
[13] R.K. Li, T. Bo, H.W. Liu, K.A. Li, Sepu 20 (4) (2002) 335.
[14] J.J. Berzas-Nevado, J. Rodriguez-Flores, G. Castaneda-Penalvo, N.R. Rodriguez-Farinas, J. Chromatogr. A 953 (1/2) (2002) 279.
[15] W.D. Qin, S.F.Y. Li, Electrophoresis 23 (24) (2002) 4110.
[16] S.F.X. Li, Capillary Electrophoresis Principles, Practice and Applications, Journal of Chromatography Library, vol. 52, Elsevier, Amsterdam, 1992.
[17] H. Farbe, A. LeBris, M.D. Blanchin, J. Chromatogr. A 697 (1995) 81.
[18] L. Huber, Applications of Diode-Array Experience, Hewlett-Packard Publication No. 12, 1989, p. 5953.
[19] M. Mulholland, J. Waterhouse, J. Chromatogr. 395 (1987) 593.
[20] R.L. Plackett, J.P. Burman, Biometrika 33 (1946) 305.
[21] W.J. Youden, E.H. Steiner, The Association of Official Analytical Chemistry, Washington, DC, 1975, pp. 33–41.