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Development and validation of a capillary zone electrophoresis method for the determination of ephedrine and related compounds in urine without extraction

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Abstract

A capillary zone electrophoresis (CZE) method, with UV detection and in the presence of dimethyl-β-CD, was optimized by means of an experimental design for the separation and the simultaneous quantitation of ephedrine, pseudoephedrine, norephedrine (phenylpropanolamine) and norpseudoephedrine (cathine) in urine without any extraction. In this application, the optimization of the analytical conditions with an experimental design was preferred to a univariate study. Therefore, a central composite design was used and the following factors were investigated and varied simultaneously: buffer concentration, buffer pH and dimethyl-β-CD concentration. In order to evaluate the influence of each experimental parameter on the analytical separation, the resolutions between the four compounds, as well as the separation time and generated current were observed and established as responses of the experimental design. A model was obtained for each response by linear multiple regression of a second-degree mathematical expression. After acceptance of the mathematical models, the most favorable conditions were determined by maximizing the resolutions between the four compounds and by setting the other responses at threshold values. Successful results were obtained with a 260 m*M* Tris–phosphate buffer at pH 3.5 in the presence of 13.3 m*M* dimethyl-β-CD at 25 °C and with an applied voltage of 30 kV. Under these optimized conditions, a baseline separation of the four compounds was achieved in less than 6 min. The method was validated in terms of precision, linearity, accuracy and successfully applied for the determination of these compounds in urine samples without any extraction as well as in nutritional supplements.

Keywords: Validation; Ephedrine; Pseudoephedrine; Norephedrine; Norpseudoephedrine

1. Introduction

Ephedrine, pseudoephedrine, norephedrine and norpseudoephedrine are pairs of diastereoisomeric sympathomimetic amines [1] known to have central nervous system stimulating properties [2,3]. These substances are ingredients of many pharmaceuticals commonly used in the treatment of flu, rhinusitis, colds and allergy [4]. They are also found in various dietary and nutritional supplements. In almost all of these cases, the manufacturers refer to the presence of ephedrines by mentioning only the botanical source (*Ephedra*, *Ma-Huang*, etc . . .) [5–7].

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Olympic Committee included the ephedrines in the list of forbidden substances in 1990 [8]. Nowadays, the commission has adopted the following limits of concentration in urine above which an athlete is considered as ''positive'': for pseudoephedrine and norephedrine 25 μg/ml, for ephedrine 10 μg/ml and for norpseudoephedrine 5 μg/ml. Antidoping controls are routinely carried out on the urines and thus a fast, simple and reliable analytical method is needed for identifying the abuse of such compounds by athletes.

The chromatographic separation of ephedrines is especially difficult because of their similarity in chemical structure. High-performance liquid chromatography (HPLC) methods have been reported for the determination of ephedrines in pharmaceutical preparations and in biological fluids [9,10]. Gas chromatography (GC) methods for the analysis of ephedrines have also been described [11,12]. However, these methods require a derivatisation step or exotic mobile phases to allow for separation of the diastereoisomeric substances.

Capillary electrophoresis (CE) has become an interesting alternative to classical chromatographic techniques, particularly for the separation of diastereoisomers. Indeed, CE provides several advantages: short analysis time, high resolution power and low operational cost. Enantioseparations are generally performed by adding a chiral selector to the running buffer. Various additives acting as chiral selectors have been reported in the literature, such as cyclodextrins (CDs), crown ethers, proteins, antibiotics, bile salts and chiral micelles [13–15]. Nevertheless, CDs are the most widely used selectors in chiral CE. Neutral CDs and derivatives presenting various functional groups have been developed to induce different stereoselective interactions and thus, to enhance selectivity.

CE has been successfully applied to the analysis of pharmaceutical products, but the first reviews on the application of CE to the analysis of plant secondary metabolites have only been published recently [16,17]. Surprisingly, only few reports concerning the analysis of ephedrines are found in the literature [18–23].

Screening designs are mainly used to estimate the effects of factors in experimental studies on a statistical basis [24–28]. Two main designs can be selected according to the number of factors. As long as this number is small, full factorial designs can be used, but in the case of multiple factors, Plackett and Burman designs should be preferred [29]. Whereas the latter allow only to estimate main effects, full factorial designs enable to determine the most relevant factors and their influence range as well as the factor's interactions. However, both present a major limitation as they are first-degree factorial designs which implies that the estimated variables are linear over the whole range of experimentation. Second-degree designs such as central composite designs [24] and Doehlert designs [30] allow to model non-linear variables and determine optimal conditions [31]. The prediction quality of the model is verified by the good agreement between the experimental and predicted responses. Furthermore, the central composite design offers the possibility to evaluate the method robustness by drawing response surfaces [32].

In this paper, a central composite design was carried out in order to find the optimal electrophoretic conditions for the simultaneous analysis of ephedrine, pseudoephedrine, norephedrine and norpseudoephedrine using dimethyl-β-CD. The method was validated and successfully applied to the determination of these compounds in urine without any extraction and/or pretreatment of the sample. The method was also applied to the qualitative analysis of nutritional supplements.

2. Experimental

2.1. Chemicals and samples

(−)-Ephedrine hydrochloride was purchased from Siegfried (Zofingen, Switzerland). (−)-Norpseudoephedrine hydrochloride, (+)-phenylpropanolamine hydrochloride and diphenylamine were obtained from Sigma (St. Louis, MO, USA). (+)-Pseudoephedrine hydrochloride was obtained from Aldrich (Milwaukee, WI, USA). Scopolamine hydrobromide, used as internal standard, was provided by Acros (Geel, Belgium). α-CD, β-CD, γ-CD, hydroxypropyl-β-CD and dimethyl-β-CD were purchased from Acros, whereas, methyl-β-CD was obtained from Aldrich (Steinheim, Germany). All chemicals were of analytical grade: tris(hydroxymethyl)-aminomethane (Tris) was obtained from

Aldrich. Phosphoric acid was provided by Merck (Darmstadt, Germany). Methanol (MeOH) was purchased from Biosolve (Valkenswaard, Netherlands). Chloroform ($CHCl_3$), *tert*.-butyl methyl ether (TBME), potassium hydroxide (KOH) and sodium chloride (NaCl) were obtained from Acros, whereas dichloromethane (CH_2Cl_2) and sodium sulfate (Na_2SO_4) were provided by BDH (Poole, UK). Concentrated ammonia (NH_4OH) was purchased from Merck. Ephedra 850 dietary supplement capsules containing *Ephedra* extract were manufactured by NVE Pharmaceuticals (Newton, NJ, USA) and Kwik Energy dietary supplement tablets containing *Ma-Huang* extract were manufactured by Universal Nutrition (New Brunswick, NJ, USA). Ultrapure water, provided by a Milli-Q RG unit from Millipore (Bedford, MA, USA), was used for standard and sample preparation. Electrolyte solutions were filtered through a 0.20 μm microfilter (Supelco, Bellefonte, PA, USA) before use.

2.2. Gas chromatography–nitrogen-phosphor detection

GC–NPD analyses were carried out using a Hewlett-Packard 5890 Series II gas chromatograph (HP Analytical Division, Waldbronn, Germany), equipped with a HP 7673 autosampler and a nitrogen-phosphor detector. Instrument control and data processing were performed with a HP Vectra VL Computer and ChemStation software. GC separation was achieved on a DB-XLB J&W Scientific (BGB Analytik, Anwil, Switzerland) fused-silica column (25 m×0.2 mm I.D., 0.33 μm film thickness) operated with a helium inlet pressure of 175 kPa and temperature programming: 60 °C for 0.5 min, ramped at 20 °C/min to 150 °C, ramped at 30 °C/min to 320 °C and held for 1 min. Injections of 1-μl samples were made at 250 °C in the splitless mode (0.8 min) into a split-splitless injection port with an inner quartz liner and the nitrogen-phosphor detector was heated at 300 °C.

2.3. Capillary electrophoresis instrumentation and electrophoretic procedure

Electrophoresis was carried out on a Hewlett-Packard capillary electrophoresis system equipped with an on-column diode-array detector (DAD). The capillary (BGB Analytik) was 48.5 cm long (40 cm effective length) with a 75 μm internal diameter (I.D.). An alignment interface containing an optical slit matched to the internal diameter of the capillary was used. Detection at 40 cm from the point of sample introduction was set at 195 nm with a bandwidth of 10 nm. A CE Chemstation (Hewlett-Packard) allowed instrument control, data acquisition and data handling.

All experiments were carried out in cationic mode (anode at the inlet and cathode at the outlet). The capillary was thermostated at 25 °C and a constant voltage of 30 kV, with an initial ramp of 1 min, was applied during analysis. Sample injections (ca. 16 nl injection volume) were achieved with pressure mode for 20 s at 25 mbar.

The carrier buffer was obtained by dissolving a suitable amount of dimethyl β-cyclodextrin in a solution prepared by mixing tris(hydroxymethyl)-aminomethane (Tris) and phosphoric acid solutions in an appropriate ratio to give a suitable pH value between 2 and 4. Each day, the capillary was rinsed with 0.1 *M* sodium hydroxide for 10 min followed by water for 5 min. Before each run, the capillary was equilibrated with the running buffer for 3.5 min. Before first use, the capillary was flushed with 0.1 *M* sodium hydroxide for 30 min followed by water for 15 min.

As electrolysis can alter the running buffer and subsequently change the electroosmotic flow (EOF), a replenishment system was also applied to maintain reproducibility. Prior to each sequence, two ''blank'' injections were performed to stabilize the capillary wall surface and allow the buffer and sample solutions to reach a constant temperature on the autosampler tray.

2.4. Standard and sample solutions

Stock standard solutions of ephedrine, norpseudoephedrine, phenylpropanolamine, pseudoephedrine and scopolamine were prepared by dissolving each compound in methanol in order to give a concentration of 1.0 mg/ml. Working standard solutions were prepared by diluting the stock standard solution with water. Water was used as a diluent and allowed sample stacking which was effective in enhancing sensitivity by on-column preconcentration of the sample within the capillary.

Calibration curves for ephedrine, norpseudoephedrine, phenylpropanolamine and pseudoephedrine were established over the sample concentration range of 5–45 μg/ml and in the presence of 10 μg/ml scopolamine, used as internal standard.

2.5. Extraction and isolation of ephedrines from dietary supplements

For Kwik Energy dietary supplement, three tablets were finely powdered and the equivalent of one tablet was accurately weighed for extraction. For Ephedra 850 dietary supplement, the content of one capsule was directly extracted. Both dietary supplements were extracted with 8 ml $CHCl_3$–MeOH-concentrated NH_4OH (15:5:1 v/v/v), with sonication for 15 min and vortex mixing at 5-min intervals to avoid aggregation of the powdered sample. After centrifugation (2500 *g* for 5 min), supernatants were collected and evaporated under nitrogen to dryness. The residue was basified with 3 ml distilled water-concentrated NH_4OH (50:50 v/v) and applied on an Extrelut 3 column (Merck). Ephedrines were eluted with 3×8 ml CH_2Cl_2.

In all cases, the solvent was evaporated to dryness. The residue was suitably diluted with water in order to obtain ephedrine at a final concentration between 5 and 45 μg/ml. Each sample was filtered before injection.

2.6. Extraction and isolation of ephedrines from urine for GC–NPD analysis

Five ml of urine were basified with 0.5 ml 5 *M* KOH and ~3 g of NaCl were added. After addition of 25 μl of a solution of diphenylamine (1 mg/ml), used as internal standard, the urine was extracted with 2 ml TBME by shaking during 10 min. After centrifugation (2500 *g* for 5 min), the supernatant was collected, dried with Na_2SO_4 and 1 μl was injected.

2.7. Computation

Coefficients for the regression models and optimized conditions were calculated with the NEMROD software package (LPRAI, Marseille, France). Three-dimensional response surfaces were drawn with Microsoft Excel (version 7.0).

3. Results and discussion

The separation of the four ephedrines was achieved through complexation with cyclodextrins. After optimization with an experimental design, the analytical method was then validated. The chemical structure of the investigated compounds is given in Fig. 1.

3.1. Choice of the chiral selector

Despite the large number of applications concerning the use of CDs and their derivatives, there is no general rule for linking the stereoselectivity of these selectors to their chemical structure. Thus, for CE separation of diastereoisomeric drugs, the choice of a suitable chiral selector remains of crucial importance. Therefore, various CDs and derivatives, namely α-CD, β-CD, γ-CD, methyl-β-CD, dimethyl-β-CD and hydroxypropyl-β-CD were investigated at a concentration of 15 m*M* using a 200 m*M* Tris–phosphate buffer at pH 2.5, with an applied voltage of 30 kV and a temperature of 25 °C. Under these conditions, the best separation of the four compounds was obtained with dimethyl-β-CD as shown

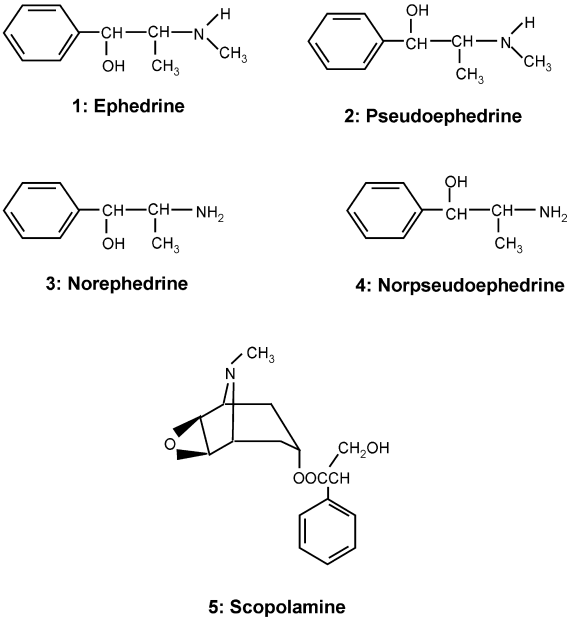

Fig. 1. Structure of investigated ephedrines.

in Fig. 2. It can be noted that the migration times and the migration orders depend on the cyclodextrin used in relation to the degree of interactivity between each ephedrine and the specific cyclodextrin. Thus, dimethyl-β-CD was selected for subsequent investigations.

3.2. Method optimization

Traditionally, analytical methods are optimized by a univariate study. This classical approach involves the systematic alteration of a single variable whilst maintaining the others constant, with no straightforward prediction of the separation conditions. It ensues that a univariate optimization generates a large number of experiments whereas experimental designs allow a high quality in optimization processes with a smaller number of experiments [24–28].

3.2.1. Design of experiments

The method was optimized using a central composite design, as already described [33,34]. Three relevant factors were simultaneously investigated: buffer concentration (X_1), buffer pH (X_2) and dimethyl-β-CD concentration (X_3). Indeed, preliminary investigations show that the separation between positional isomers can only be achieved in the presence of cyclodextrin. Levels of the three experimental factors are listed in Table 1.

The experimental conditions for the 20 experiments are listed in Table 2. The experiment at the center was carried out six times in order to obtain an estimation of the experimental error. The 20 experiments were performed randomly.

The effect of each factor was examined by means of five responses: the resolution between norpseudoephedrine and norephedrine (Rs_1), between norephedrine and ephedrine (Rs_2) and between ephedrine and pseudoephedrine (Rs_3). The resolution is calculated with the following equation:

$$Rs = 2[(t_2 - t_1)/(W_{b(1)} + W_{b(2)})]$$

where t_1 and t_2 are the migration times of the two investigated compounds, W_1 and W_2 are the peak widths measured at the baseline. In order to represent the quality of the separation, the analysis time (Time), measured by the migration time of the last compound (pseudoephedrine in this case), as well as the current (Current) generated during electrophoresis were also examined.

3.2.2. Regression modeling

Multiple regression enables the mathematical relationship between the responses and the independent variables. A central composite design provides sufficient data for fitting a second-degree expression to each response, such as given below:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3$$

where Y represents the experimental response, X_i the independently evaluated factors (in coded variables), b_0 the intercept and b_{ij} the parametric coefficients of the model obtained by multiple regression. Table 3 shows the regression parameters calculated for each response.

The coefficients of determination (R^2), the adjusted coefficients of determination (R_a^2), the residual standard deviation (SD_{res}) were satisfactory, as shown in Table 4, indicating the good predictability of the models.

Moreover, the residual error values were contained within the range of ± 2 SD_{exp}, where SD_{exp} is the experimental standard deviation obtained through the experiments at the center ($n = 6$), as illustrated in Fig. 3. Thus, the responses were sufficiently explained by the regression models allowing to establish response surfaces and to predict any response within the experimental domain.

3.2.3. Determination of the optimal conditions

The mathematical model allowed to determine optimal conditions by maximizing the resolutions and minimizing the migration time and current to avoid excessive Joule effect. As a result, optimal conditions were reached with a 260 m*M* Tris–phosphate buffer at pH 3.5 and 13.3 m*M* dimethyl-β-CD. All experiments were performed at 25 °C and 30 kV.

The good predictability of the model was experimentally verified by the good agreement between the experimental and predicted responses under the optimized conditions (Table 5). The residual error value was contained within a range of ± 2 SD_{exp} for

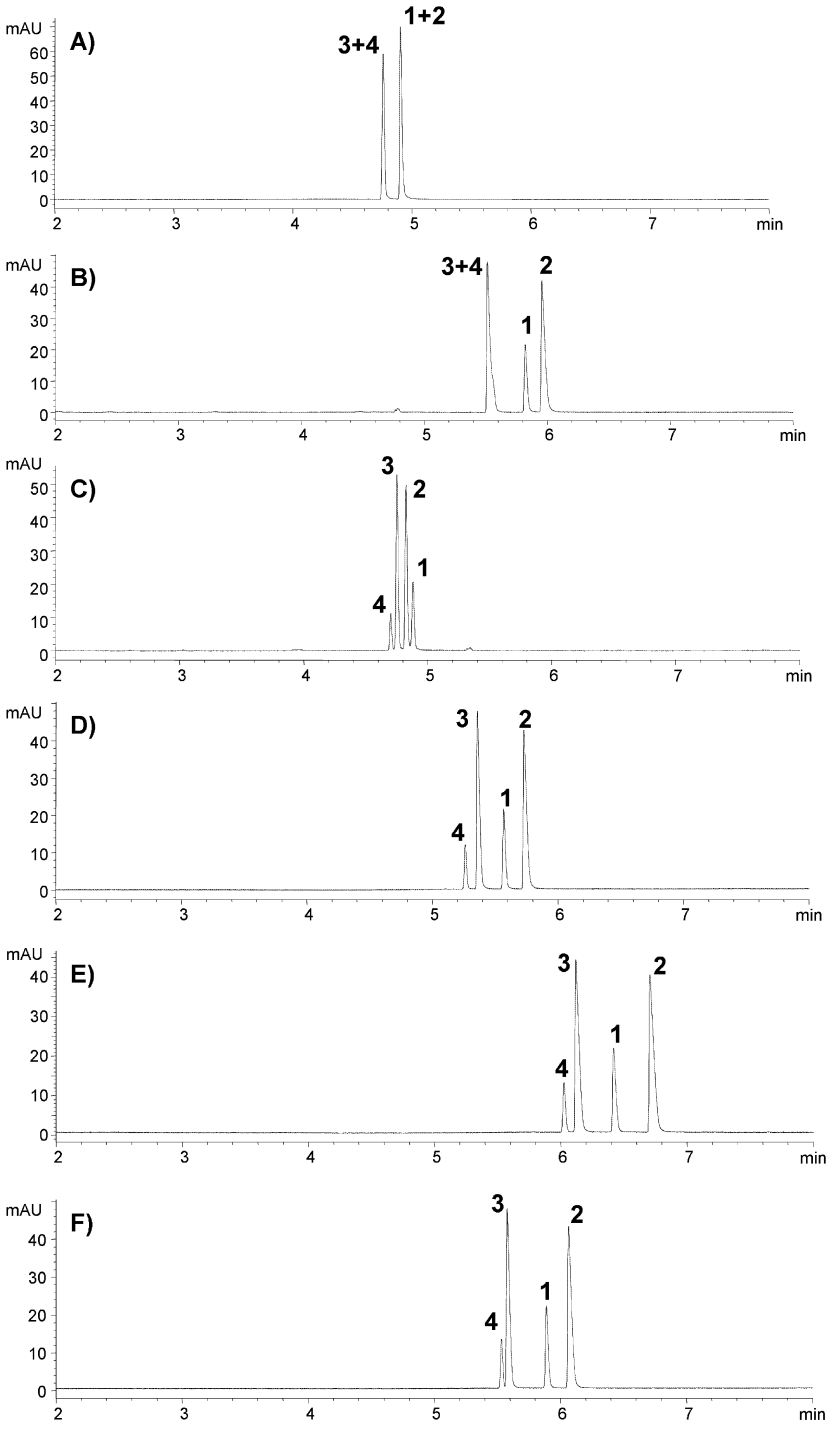

Fig. 2. Effect of cyclodextrin type on separation of ephedrine, pseudoephedrine, norephedrine and norpseudoephedrine (standard solution with the substances at the CIO limits). Buffer: 200 m*M* Tris–phosphate, pH 2.5 and 15 m*M* of (A) α-CD, (B) β-CD, (C) γ-CD, (D) methyl-β-CD, (E) dimethyl-β-CD, (F) HP-β-CD. Electrophoretic conditions: applied voltage 30 kV, temperature 25 °C. Uncoated fused-silica capillary: $L = 48.5$ cm, $l = 40$ cm, I.D. = 75 μm. Peak numbering is the same as in Fig. 1.

Table 1
Coded values of experimental factors

Level	X_1 Buffer (mM)	X_2 pH	X_3 Dimethyl-β-CD (mM)
−1	100	2	5
0	200	3	15
+1	300	4	25

each response. Under these optimal conditions, baseline separation of the four compounds was achieved in less than 6 min, as shown in Fig. 4.

Moreover, it is possible to draw surface responses (Rs_1, Rs_2 and Rs_3) as a three-dimensional plot of two relevant factors, while keeping the third and less influencing factor constant at its optimal value (Fig. 5).

Table 2
Three-factor central composite design with the corresponding responses

Trial	Run order	Experimental factors			Measured responses				
		X_1	X_2	X_3	Rs_1	Rs_2	Rs_3	Time (min)	Current (μA)
1	11	−1	−1	−1	0.94	1.79	1.10	4.62	78
2	12	1	−1	−1	0.94	2.43	1.42	4.54	143
3	13	−1	1	−1	0.95	1.71	0.95	4.37	36
4	4	1	1	−1	1.32	2.17	0.92	4.26	113
5	16	−1	−1	1	0.89	1.95	2.16	6.60	72
6	17	1	−1	1	0.93	2.9	2.74	6.68	130
7	19	−1	1	1	0.88	1.80	2.10	6.11	34
8	20	1	1	1	1.31	2.64	2.84	6.31	102
9	5	−1	0	0	1.00	2.11	2.28	6.35	37
10	18	1	0	0	1.35	2.68	2.59	6.53	103
11	6	0	−1	0	1.13	2.5	2.44	5.86	102
12	3	0	1	0	1.15	2.35	2.39	5.49	72
13	7	0	0	−1	1.26	2.24	1.33	5.33	71
14	9	0	0	1	1.28	2.70	3.00	7.89	66
15	15	0	0	0	1.26	2.59	2.67	6.85	69
16	8	0	0	0	1.33	2.70	2.73	6.86	69
17	2	0	0	0	1.27	2.60	2.68	6.66	72
18	14	0	0	0	1.28	2.62	2.72	6.85	69
19	10	0	0	0	1.27	2.62	2.67	6.85	69
20	1	0	0	0	1.17	2.35	2.41	6.30	72

Table 3
Regression coefficients estimated for each response

Coefficient	Rs_1	Rs_2	Rs_3	Time	Power
b_0	1.2689	2.5736	2.6384	6.7037	69.51
b_1	0.1190	0.3460	0.1920	0.0264	33.45
b_2	0.0780	−0.0900	−0.0660	−0.1757	−16.75
b_3	−0.0120	0.1650	0.7120	1.0470	−3.75
b_{11}	−0.1023	−0.1691	−0.1909	−0.2285	1.09
b_{22}	−0.1373	−0.1391	−0.2109	−0.9940	18.09
b_{33}	−0.0073	−0.0941	−0.4609	−0.0555	−0.41
b_{12}	0.0950	−0.0636	−0.0238	0.0115	2.69
b_{13}	0.0125	0.0863	0.1288	0.0585	−1.94
b_{23}	−0.0025	−0.0088	0.0862	−0.0425	0.81

Table 4
Statistical data

	Rs_1	Rs_2	Rs_3	Time	Current
SD_{exp} [a]	0.0520	0.1192	0.1188	0.2236	1.5492
SD_{res} [b]	0.0290	0.0519	0.0510	0.0951	0.7446
R^2 [c]	0.9371	0.9598	0.9827	0.9783	0.9982
R^2_{adj} [d]	0.8805	0.9237	0.9671	0.9588	0.9965

[a] Experimental standard deviation in % ($n=6$).
[b] Residual standard deviation in %.
[c] Coefficient of determination.
[d] Adjusted coefficient of determination.

Table 5
Comparison of predicted and measured results ($n=3$) under optimal conditions

	Rs_1	Rs_2	Rs_3	Time (min)	Current (μA)
$2\times SD$ [a]	0.104	0.238	0.238	0.447	3.098
Predicted	1.23	2.46	2.79	6.95	58
Measured	1.32	2.68	2.92	6.51	61

[a] SD represents the standard deviation obtained by performing the central point in replicate ($n=6$).

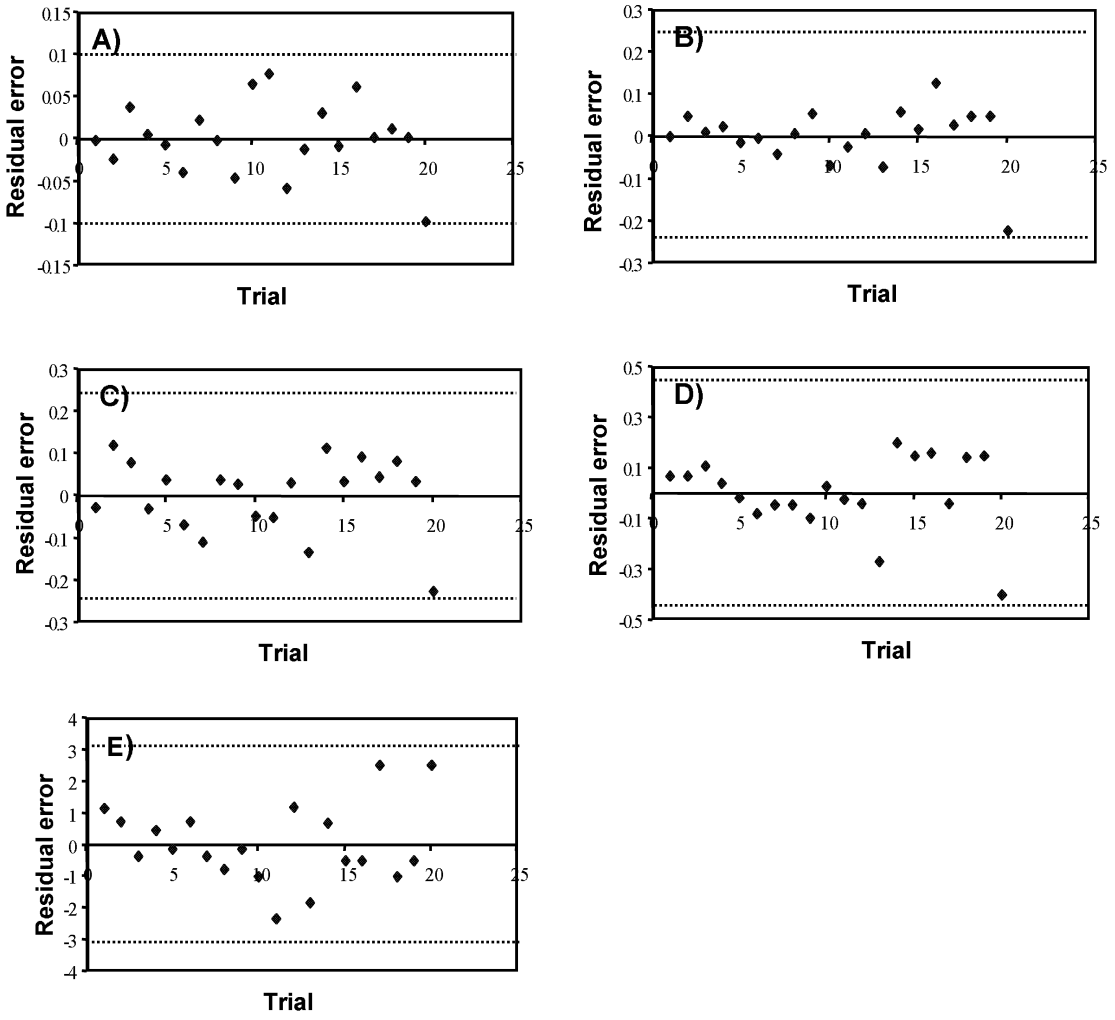

Fig. 3. Residual error for (A) Rs_1, (B) Rs_2, (C) Rs_3, (D) time and (E) current.

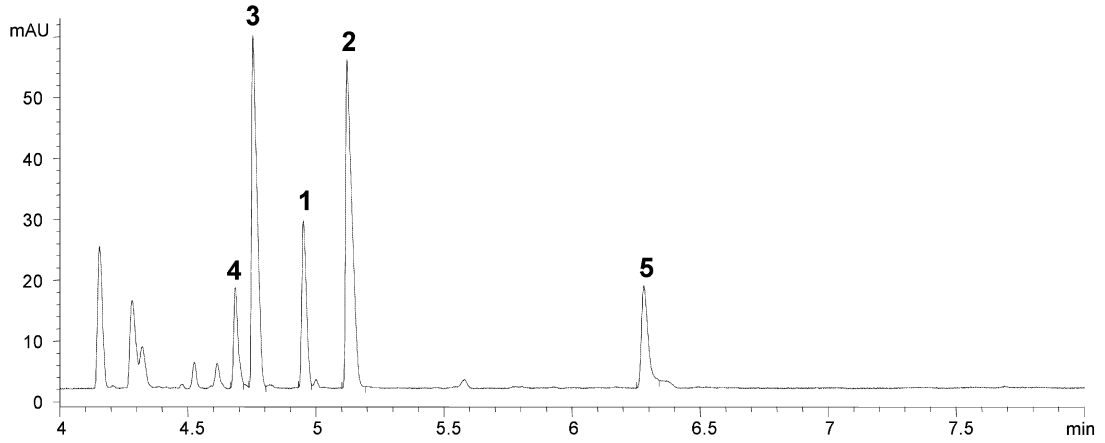

Fig. 4. Typical electropherogram of ephedrine, pseudoephedrine, norephedrine and norpseudoephedrine (spiked in urine at 10 μg/ml, 25 μg/ml, 25 μg/ml and 5 μg/ml, respectively) obtained by CZE using the optimized conditions: 260 m*M* Tris–phosphate at pH 3.5 and 13.3 m*M* dimethyl-β-CD. Applied voltage 30 kV (I = 61 μA), temperature 25 °C. Uncoated fused-silica capillary: L = 48.5 cm, l = 40 cm, I.D. = 75 μm. Peak numbering is the same as in Fig. 1.

For the sake of simplicity, other surface responses are not reported. The three response surfaces are almost similar and show an identical mechanism of interaction of dimethyl-β-CD with each ephedrine.

These response surfaces allow not only the determination of an optimal zone, where a good quality of the separation can be established for further validation, but also prove the robustness of the method in the tested domain.

It can be noted that the resolution between norpseudoephedrine and norephedrine (Rs_1) is the most difficult to achieve, whereas the values obtained for Rs_2 and Rs_3 are superior to 1.9 and 1.5, respectively, over all the tested experimental domain. Consequently, Rs_1 is the most important response surface to be taken into account and hopefully the surface reaches a plateau where the optimal zone can be perfectly defined and where a good robustness of the separation is assured. Moreover, in this case, it was determined that the use of more than 13.3 m*M* dimethyl-β-CD is useless and does not improve the resolution between the two compounds.

3.3. Method validation

Once the optimized conditions were selected, the method was validated for the determination of ephedrine, norpseudoephedrine, phenylpropanolamine and pseudoephedrine directly in urine without any extraction or sample pretreatment, using scopolamine as internal standard. The validation was carried out in a similar way to that generally adopted for HPLC and now employed to validate CE methods [35]. The procedure requires the assessment of migration time and peak area precision, detector response linearity with sample concentration and accuracy. It has to be noted that the sensitivity of the method was not evaluated because the considered substances are authorized by the IOC until 5 μg/ml, 10 μg/ml or 25 μg/ml as already stated in the Introduction, concentrations well detected with the diode-array detector.

3.3.1. Precision

Method precision was determined by measuring repeatability and intermediate precision (between-day precision) of relative migration times and normalized peak areas for each compound.

In order to determine the repeatability of the method, replicate injections (n = 6), of a urinary sample spiked with the four ephedrines at the concentration authorized by the IOC and scopolamine used as internal standard at 10 μg/ml, were carried out. In Table 6, relative standard deviations (RSD) are given for migration time and peak area ratio. In all cases, repeatability was better

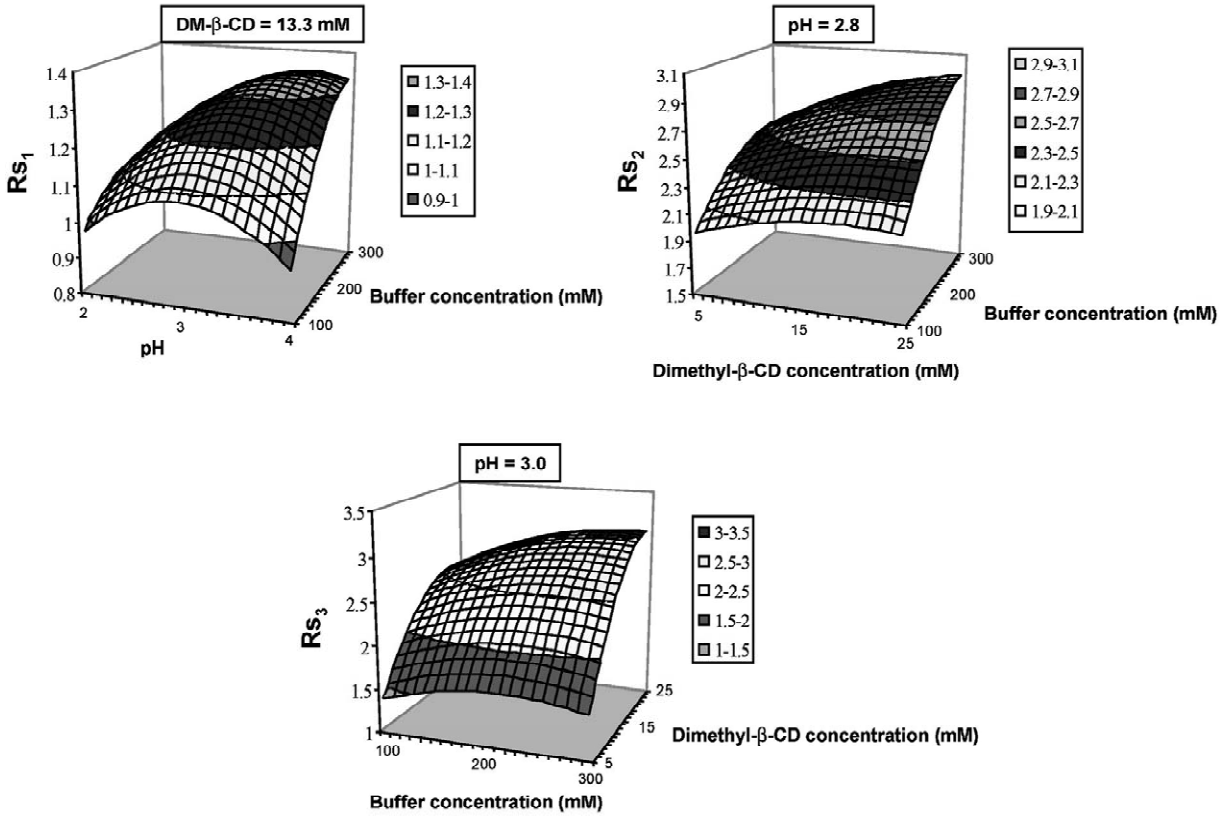

Fig. 5. Surface response plots for Rs_1, Rs_2 and Rs_3 as a function of two factors. The third and less influent factor is set at its optimal level.

than 1% for the migration time and 5% for the peak area ratio.

The intermediate precision was evaluated over 3 days by performing six successive injections daily. Results (Table 6) show that RSD values are in this case slightly superior than those obtained for repeatability. The use of an internal standard is necessary in order to compensate the variability of the

Table 6
Precision of CE expressed as RSD values for relative migration time and peak area ratio

	Norpseudoephedrine	Norephedrine	Ephedrine	Pseudoephedrine
Repeatability				
Relative migration time[a]	0.45%	0.42%	0.33%	0.27%
Peak area ratio[b]	5.28%	3.13%	4.09%	2.70%
Intermediate precision				
Relative migration time[a]	2.74%	2.61%	2.28%	1.97%
Peak area ratio[b]	8.21%	5.68%	6.03%	5.04%

[a] The relative migration time is defined as the analyte migration time divided by the internal standard (scopolamine) migration time.
[b] The peak area ratio is defined as the analyte peak area divided by the internal standard (scopolamine) peak area.

migration times and the poor precision observed with the hydrodynamic injection, and hence to achieve a good method precision [26].

3.3.2. Linearity

Detector response linearities were assessed by preparing five spiked urines covering the concentration range 5–45 μg/ml. Each sample was injected in duplicate. Regression curves were obtained by plotting peak area ratios (analyte peak area divided by internal standard area) versus concentration, using the least square method. In all cases, the correlation coefficient (Table 7) is superior to 0.999 and was improved by using peak area ratios. For all the substances, the intercept is not significantly different from zero (Student's *t*-test). Then, from these results, it can be said that the response of the detector is directly proportional to the amount of the corresponding substance in urine and consequently there is no influence of the urinary matrix on the detected signal.

3.3.3. Accuracy

As the validation is directly performed in urine, the method accuracy was evaluated by performing the dosage of each ephedrine in the spiked urines (5–45 μg/ml), previously prepared for the assessment of linearities, by GC–NPD after extraction (actual reference method used at the laboratory for the quantification of various compounds and ephedrines in particular). Each sample was also injected in duplicate. Regression curves were obtained by plotting peak area ratios (analyte peak area divided by internal standard area) versus concentration, using the least square method. The correlation coefficients were also superior to 0.999. Results generated by the developed CZE method were then compared with those obtained by GC–NPD: all results were in the same order of magnitude (slopes of the regression curves and target values for each spiked urine).

These findings suggest that the urinary matrix not pretreated does not influence significantly the quantification of the investigated compounds and therefore the developed method is accurate.

3.4. Applications

3.4.1. Analysis of a positive urinary sample

Firstly, the optimized method was applied to the analysis of a ''positive'' urine sample received at the laboratory and proved to contain ephedrine and pseudoephedrine, after the screening analysis at the laboratory. Both compounds were quantified by GC–NPD after extraction of the urine sample and identified by GC–MS after derivatization (methods currently used at the laboratory). Afterwards, the sample was analyzed by capillary electrophoresis with the method previously optimized. The urine was directly injected, after filtering through a 0.2 μm filter and without any extraction and pretreatment. The two compounds were clearly separated and quantified without interference from the urinary matrix (Fig. 6A). The concentrations of the two compounds are reported in Table 8 and results were similar to those found by GC–NPD. Then, in accordance with the IOC rules, this sample has been declared ''positive'' to the sport federation for the presence of ephedrine at a concentration superior to the authorized limit.

3.4.2. Analysis of two dietary supplements

Secondly, the optimized method was applied to the analysis of two dietary supplements (Figs. 6B

Table 7
Regression data

Regression parameters		Compound			
		Norpseudoephedrine	Norephedrine	Ephedrine	Pseudoephedrine
Regression equation					
($y = ax + b$)	a	0.083	0.086	0.089	0.096
	b	0.022	0.025	0.038	−0.006
r^2		0.9992	0.9996	0.9994	0.9993

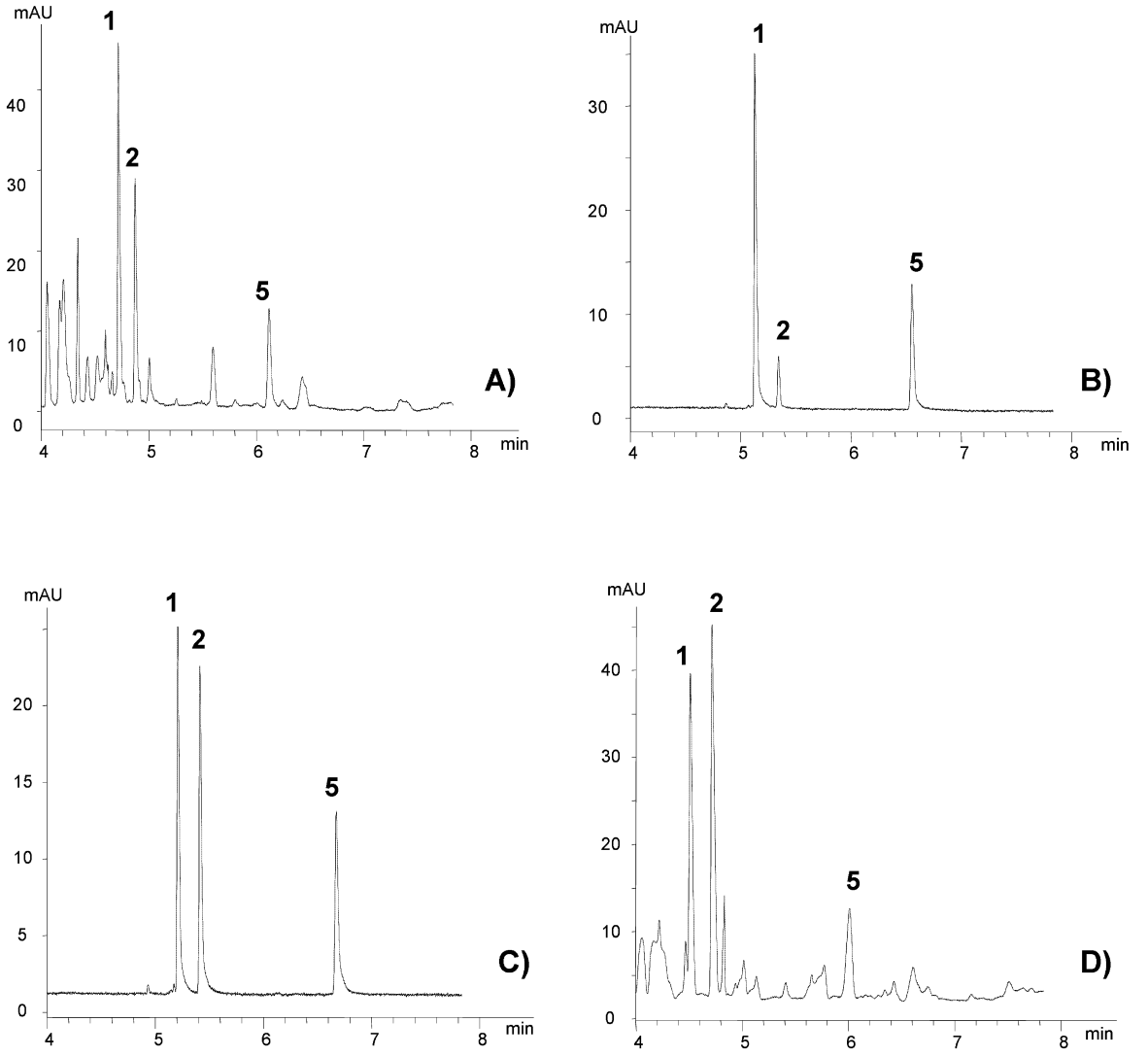

Fig. 6. Typical electropherograms of (A) positive urine, (B) and (C) dietary supplement extracts (Ephedra 850 and Kwik Energy, respectively), (D) excretion urine from Kwik Energy, analyzed under optimized conditions: 260 m*M* Tris–phosphate at pH 3.5 and 13.3 m*M* dimethyl-β-CD. Applied voltage 30 kV ($I = 61$ μA), temperature 25 °C. Uncoated fused-silica capillary: $L = 48.5$ cm, $l = 40$ cm, I.D. = 75 μm. Peak numbering is the same as in Fig. 1.

and C) bought via the Internet: Ephedra 850 and Kwik Energy. The first one is labeled as containing *Ephedra* extract whereas the second one contains, among other components, *Ma-Huang* and Guarana extracts, Royal jelly, oriental Ginseng root, bee pollen, Goldenseal root, Cola Nitida, Spirulina, Cayenne fruit and citrus bioflavonoids.

In this case, the matrices were extracted and it can be noted that ephedrine and pseudoephedrine are present in both dietary supplements as already described [5–7] and then the compounds could be easily determined and no disturbance was observed due to the complexity of the products contained (Table 8).

3.4.3. Analysis of urine after taking Kwik Energy

Two tablets of Kwik Energy dietary supplement were administered to a healthy volunteer. Urines

Table 8
Results of CZE assay of ephedrine and pseudoephedrine in various samples

Sample	Weight per unit	Component[a]	Amount found
Positive urine	–	Ephedrine	25.4 μg/ml
		Pseudoephedrine	16.0 μg/ml
Ephedra 850	664.8 mg/capsule	Ephedrine	24.8 mg/capsule
		Pseudoephedrine	2.9 mg/capsule
Kwik Energy	1.546 g/tablet	Ephedrine	7.5 mg/tablet
		Pseudoephedrine	6.9 mg/tablet
Urine from Kwik Energy[a]	–	Ephedrine	21.9 μg/ml
		Pseudoephedrine	26.6 μg/ml

[a] Urine obtained 11 h after oral administration of two tablets of the dietary supplement.

were collected during 48 h and analyzed in the same way as in Section 3.4.1. For the sake of simplicity, only the urinary sample obtained 11 h after the administration of the supplement is shown in Fig. 6D. At this time, which corresponds approximately to the peak of the excretion study, the concentrations found in the urinary sample were 21.9 μg/ml and 26.6 μg/ml of ephedrine and pseudoephedrine, respectively (Table 8). Therefore, in the case of an unexpected control, this person could have been declared as positive for these two substances.

Then, through this application, it can be said that this method is appropriate for the direct analysis of ephedrines in urinary samples without any pretreatment.

4. Conclusion

A capillary zone electrophoresis method has been developed for the simultaneous determination of ephedrine, norephedrine, pseudoephedrine and norpseudoephedrine. In particular, the electrophoretic behavior of the four ephedrines was critically affected by the nature and concentration of the chiral selector. A central composite design was preferred to a univariate study and was applied for the optimization of the experimental parameters. Under the optimal conditions, the separation of the four tested ephedrines was performed in less than 6 min. The method was then validated showing satisfactory validation data for linearity, precision and accuracy.

Traditionally, in the doping control laboratories, ephedrines are quantified by HPLC or GC–NPD after extraction and the pairs of stereoisomers are chromatographically separated and each compound identified by GC–MS after derivatization. These two steps are necessary and time-consuming for the confirmation of a positive sample.

Presently, the CZE method is suitable for the determination of the investigated ephedrines in urinary samples without any extraction or pretreatment of the sample and if necessary in dietary supplements. This can be very interesting for a doping control laboratory where a lot of urinary samples have to be investigated in a short time, but also to avoid any contamination or alteration of the urinary sample to be analyzed, for confirmation of the positive result, that can happen during the extraction steps. This last aspect could lead to the analytical result being called into question.

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