ELSEVIER

Journal of Chromatography A, 778 (1997) 355–361

JOURNAL OF CHROMATOGRAPHY A

Direct determination of prolintane and its metabolite oxoprolintane in human urine by capillary zone electrophoresis and β-cyclodextrin-modified micellar electrokinetic chromatography

A.G. Espartero[b], J.A. Pérez[a], A. Zapardiel[a], E. Bermejo[a], L. Hernández[a,*]

[a]*Departamento de Química Analítica y Análisis Instrumental, Universidad Autónoma de Madrid, 28049 Madrid, Spain*

[b]*CIEMAT-ITN, Avda Complutense 22, 28040 Madrid, Spain*

Abstract

This paper describes a fast and simple method for the direct determination of the stimulant prolintane and its principal urinary metabolite, oxoprolintane, in human urine by capillary zone electrophoresis and β-cyclodextrin-modified micellar electrokinetic chromatography. The determination was performed in phosphate buffer, pH 8.5, with UV detection at 211 nm. The effect of the ionic strength ratio between sample and running electrolyte, pH, sodium dodecyl sulphate and β-cyclodextrin concentrations, and other factors, on the electrophoretic signals of these drugs was examined. This method can be applied for doping control. The determination limits are 1.0 μg ml^{-1} for prolintane and 0.7 μg ml^{-1} for oxoprolintane. © 1997 Published by Elsevier Science B.V.

Keywords: Prolintane; Oxoprolintane

1. Introduction

Prolintane (Fig. 1) is a stimulant of the central nervous system and an antidepressant, whose stimulating action and molecular structure are similar to amphetamine. Like other stimulants, prolintane is a tertiary amine whose substituted pyrrolidine ring is essential for its stimulant action.

This stimulant is used pharmacologically in the treatment of cardiac circulatory disorders, low arterial pressure and generally in alterations of the cardiac function. Against narcotic drugs, prolintane shows a labelled analeptic action, increasing the effects produced by adrenaline and noradrenaline [1,2].

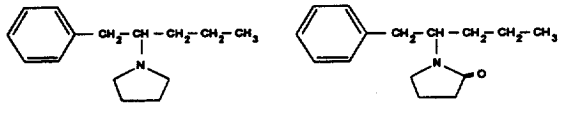

Fig. 1. Chemical structures of prolintane (P) and its metabolite oxoprolintane (O).

Therefore, prolintane is considered a doping substance by the International Olympic Committee owing to its stimulant activity and it is forbidden in sports [3].

In human urine, 24 h after oral administration of prolintane, different metabolites and unchanged prolintane have been identified [4]. The major metabolic oxidation occurs at the α-carbon of the pyrrolidine ring, forming the lactam oxoprolintane (Fig. 1).

Different methods have been published on prolintane determination, such as TLC [5–11], GLC [5,7–9,12–16], HPLC [5–9,11,17–20], GC–MS [4,21,22], high-voltage paper electrophoresis [5] and adsorptive

*Corresponding author.

PII S0021-9673(97)00478-0

stripping voltammetry [23]. Oxoprolintane has been determined together with prolintane, in human urine [4] and in biological fluids of animals by different chromatographic methods [7–9].

Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC), recently developed analytical methods, have demonstrated to be attractive tools for the analysis of charged and uncharged substances of biological interest, because of their high separation efficiency, easy operation, small injection volumes, high resolution, low consumption of polluting solvents, and rapid and inexpensive analysis. Recent reviews of Lloyd [24], and Nishi and Terabe [25], summarise the development of new methods of drug analysis in body fluids by CE and MEKC, respectively. To our knowledge, studies of prolintane and oxoprolintane by CZE and MEKC have not been published.

The aim of this work was to study the electrophoretic behaviour of prolintane and its metabolite, oxoprolintane, in order to develop a direct determination method in human urine by CZE and β-cyclodextrin-modified MEKC.

2. Experimental

2.1. Apparatus

Electrophoretic measurements were done using a Europhor (Toulouse, France) equipped with a manual injector (Prime Vision I), high voltage source (Prime Vision V) and UV-Vis detector (Prime Vision IV). Supelco (Bellefonte, PA, USA) untreated silica [Cat. No. 77500 (64 cm to detector) 1 m×75 μm I.D.×363 μm O.D.] was used as capillary. Electropherograms were registered with a Varian 4290 integrator (Palo Alto, CA, USA).

2.2. Chemicals and solutions

Prolintane hydrochloride was obtained from Boehringer Ingelheim (Barcelona, Spain). Oxoprolintane was synthesised by the authors following the procedure described by Rücker et al. [26]. Stock standard solutions were prepared in water for prolintane, and in methanol for oxoprolintane, and stored in the dark under refrigeration to avoid possible decomposition. More dilute solutions were prepared daily from the stock solutions.

Sodium dodecyl sulphate (SDS; Sigma, St. Louis, MO, USA), β-cyclodextrin (β-CD; Fluka, Buchs, Switzerland), and Sudan III (Aldrich, Milwaukee, WI, USA), of different concentrations, were used.

Buffer solutions were prepared by dissolving the corresponding salts in water.

All the chemicals used were of analytical reagent grade and were used without further purification. Water was obtained from Milli-Q/Milli-RO system (Millipore, Bedford, MA, USA).

Samples of human urine from volunteers were collected for a period of 24 h.

Samples and electrolytes were filtered through a 0.45-μm filter before use and degassed by ultrasonic bath.

2.3. Procedures

In order to establish the analytical conditions, aqueous solutions of $1.0 \cdot 10^{-4}$ *M* prolintane and oxoprolintane in 40 m*M* borate buffer, pH 8.5, were used. After 2 s of hydrodynamic injection in vacuum (0.27 bar), detection was performed at $\lambda = 211$ nm, using 50 m*M* borate buffer, pH 8.5, as running electrolyte, applying a constant voltage of 15 kV (2 μA current intensity) during the run.

For the direct determination, urine samples containing prolintane and oxoprolintane were diluted with phosphate buffer, pH 8.5, in a ratio 3:10 (v/v). These solutions (10 m*M* phosphate buffer) were hydrodynamically injected in vacuum (0.27 bar) over a 2-s period and detected at 211 nm, using 50 m*M* phosphate buffer, pH 8.5 (56 μA), for prolintane and 50 m*M* phosphate buffer, pH 8.5, modified with 4 m*M* SDS, 2 m*M* β-CD (49 μA) for oxoprolintane, as running electrolytes, and 15 kV as running voltage.

The capillary column was cleaned by successive flushing under pressure during 2 min with 0.1 *M* sodium hydroxide, 2 min with purified water, and 4 min with the buffer used in each analysis.

3. Results and discussion

3.1. Analytical conditions

Different 50-m*M* buffers, pH 8.5, such as phos-

phate, borate, ammoniacal and carbonate were tested, dissolving the drugs in the corresponding buffer. Comparing the number of theoretical plates, migration times, heights and areas of the electrophoretic peaks obtained with each buffer, the conclusion was that borate and phosphate gave the best results, although borate showed the shortest elution times.

When the borate concentration was increased, from 10 to 100 m*M*, the peak widths and the migration times increased slightly, and the resolution remained practically constant. A concentration of 50 m*M* borate buffer was chosen in order to avoid possible Joule's heating effects.

When the drugs were dissolved in water, an electrostacking effect [27] in the prolintane peak was observed, whereas the oxoprolintane peak showed, under these conditions, an adverse effect of relatively long sample width. For this reason, the influence in the concentrating capabilities of the relative differences between the ionic strength of the running electrolyte and the sample was studied (Fig. 2), obtaining as a compromise a ratio of buffer concentrations of 40 m*M* for the drug sample and 50 m*M* for the running buffer.

The study of the pH influence, between 3.0 and 10.0, with phosphate buffer, using benzyl alcohol as electroosmotic flow marker, showed that prolintane was positively charged, and that oxoprolintane remained as a neutral substance in this pH range.

Resolution improved with increasing voltage from 5 to 25 kV, selecting a running voltage of 15 kV (2 μA) for the subsequent studies, in order to avoid the possibility of Joule's heating and allowing that the time of analysis be sufficiently short (12 min). The value of the resolution obtained under these conditions was 5.1. The representative electrophoretic parameters of these drugs were calculated following the expressions deduced by Jorgenson and Lukacs [28]. The number of theoretical plates calculated for 15 kV was $2.2 \cdot 10^4$ for prolintane and $1.3 \cdot 10^4$ for oxoprolintane. The apparent mobilities and the diffusion coefficients were estimated: $9.7 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1} and $4.6 \cdot 10^{-4}$ cm^2 s^{-1} for prolintane, and $7.6 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1} and $6.8 \cdot 10^{-4}$ cm^2 s^{-1} for oxoprolintane, respectively. Taking into account that oxoprolintane is a neutral substance and, because of this, that it moves with the velocity of the electroosmotic flow, the electrophoretic mobility of prolintane can be calculated: $2.1 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}.

The reproducibility calculated with electrokinetic (3.5% for heights and 7.3% for areas) and hydrodynamic injection (1.2% for heights and 3.7% for areas), indicated that the latter method is more precise and this corresponds to the literature [29].

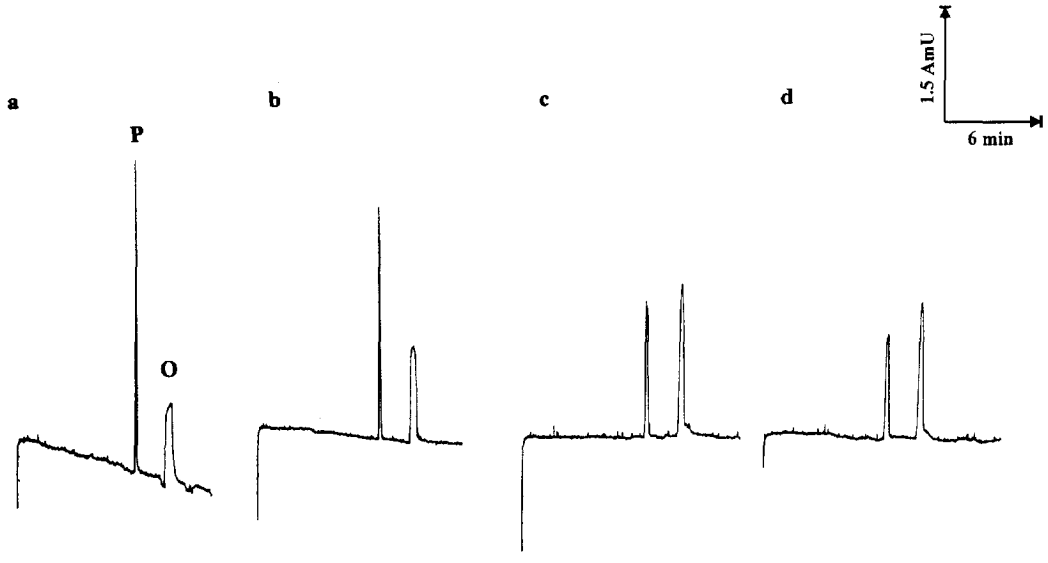

Fig. 2. CZE electropherograms obtained at 211 nm of a solution $1.0 \cdot 10^{-4}$ *M* prolintane and oxoprolintane dissolved in water (a), and in 20 (b), 40 (c) and 50 m*M* (d) borate buffer, pH 8.5. Sampling time, 2 s hydrodynamic. Separation voltage, 15 kV; running buffer, 50 m*M* borate, pH 8.5.

The hydrodynamic method was chosen as the injection mode, and the highest signal and the best precision were obtained with a 2-s period.

The influence of modifiers [acetonitrile, methanol, 2-propanol and tetrahydrofuran (THF)], up to 5% (v/v), was also studied. The migration times remained practically constant with the addition of these alcohols and THF, whereas with acetonitrile they were reduced slightly. Because these organic modifiers, in the concentrations indicated, did not affect the electrophoretic signal noticeably, and drugs were demonstrated to be well separated without any addition of organic modifiers, we decided not to use them in the subsequent studies.

The heights and the areas of the electrophoretic peaks increased when the concentration of prolintane and oxoprolintane was increased. The response was linear in the concentration range from $2.0 \cdot 10^{-6}$ to $1.0 \cdot 10^{-4}$ *M*; being the calibration plots:

Prolintane: height (AmU; absorbance milli-units)$=0.003+1.74 \cdot 10^{4}$ *M*, $r=0.9992$ ($n=10$)
area (μV s)$=-200+1.59 \cdot 10^{8}$ *M*, $r=0.998$ ($n=10$)

Oxoprolintane: height (AmU)$=0.017+1.92 \cdot 10^{4}$ *M*, $r=0.9998$ ($n=10$)
area (μV s)$=-390+2.96 \cdot 10^{8}$ *M*, $r=0.995$ ($n=10$)

In the range of concentrations studied, the relative errors and the relative standard deviations were lower than 2.4 and 3.2%, respectively.

3.2. Direct determination in human urine

The previous tests carried out comparing a blank sample of diluted urine with a similar sample containing prolintane and oxoprolintane (Fig. 3), showed that the prolintane peak is not affected by the components of the urine, whereas oxoprolintane migrates at the same time as the neutral components of this fluid. The use of borate buffer in urine samples did not give good results, because several of the urine components were adsorbed onto the walls of the silica column [30], and this adsorption was prevented by using phosphate buffer. Due to the high concentration of salts in the urine, it was necessary to reduce the buffer concentration in the sample solution in order to improve the prolintane peak.

In order to achieve the separation of oxoprolintane from the urine components, the mobility of this drug was studied adding different amounts of SDS to the running buffer. The migration time of oxoprolintane increased from 2 m*M* SDS (Fig. 4A) with high capacity factors, calculated using Sudan III and methanol to determine micellar and electroosmotic flow migration times, respectively [31]. Since cyclodextrins form selective inclusion complexes with lipophilic compounds [32,33], different amounts of β-CD were added to the running buffer containing 4m*M* SDS, in order to avoid large analysis times and coincidences with the electrophoretic peaks from the numerous urine components which absorb at 211 nm. When the β-CD concentration was 3 m*M*, oxoprolintane was totally included into the β-CD cavity and it migrated with the electroosmotic flow (Fig. 4B). A spiked urine sample was run with 50 m*M* phosphate buffer modified with 4 m*M* SDS and 2 m*M* β-CD. Under these conditions, oxoprolintane was completely separated from the urine components, with a short analysis time.

When SDS and β-CD were added to the electrolyte, the migration time of oxoprolintane was not affected with pH between 7.0 and 10.0.

In urine from different donors, the influence of the differences in ionic strength between the running electrolyte and the sample was studied. The best results were obtained with a phosphate concentration ratio of 10 m*M* for the sample solution and 50 m*M* for the running electrolyte. Urine samples had to be diluted to carry out the direct analysis due to the salt content (peak broadening) and the numerous interferences; a dilution of 3:10 (v/v) was selected.

Different modifiers (methanol, 2-propanol, acetonitrile and THF) up to 5% (v/v), did not improve the electrophoretic peaks of the drugs and intensified those of urine, increasing the interferences; therefore modifiers were not added to the running buffer.

From the results obtained at different running voltages, the apparent mobilities of prolintane ($5.2 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}) and oxoprolintane ($3.2 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}), and the diffusion coefficients ($3.9 \cdot 10^{-4}$ and $3.6 \cdot 10^{-5}$ cm^2 s^{-1}, respectively), were deduced. Prolintane peak height was not affected with the

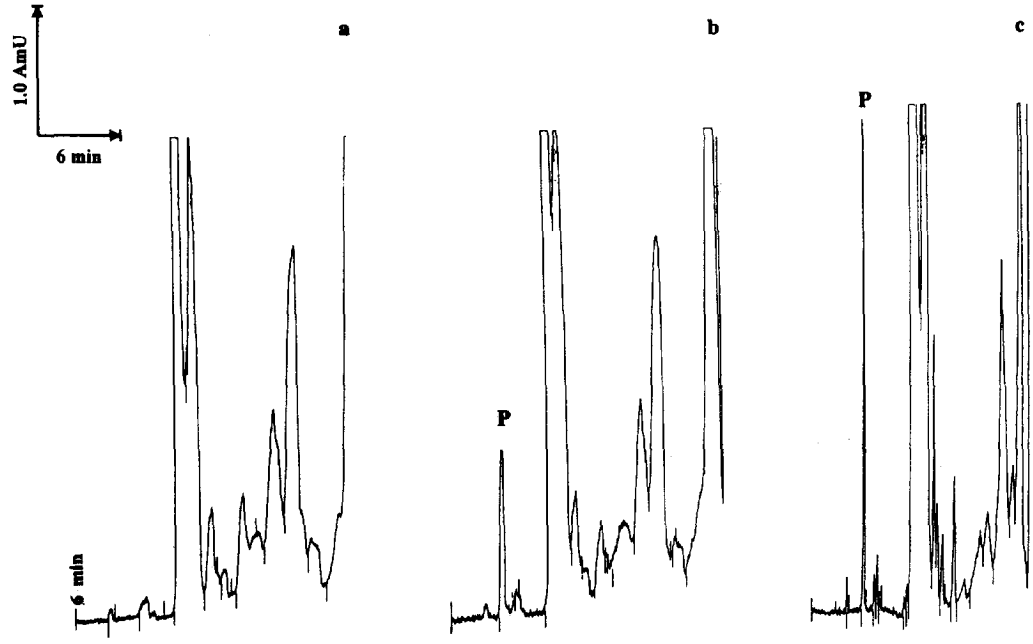

Fig. 3. CZE electropherograms obtained from an urine sample 3:10 diluted with 40 m*M* phosphate buffer, pH 8.5 (a,b) and with water (c). Without drugs (a) and containing $1.0 \cdot 10^{-4}$ *M* prolintane and oxoprolintane (b,c). Running buffer, 50 m*M* phosphate, pH 8.5. Other conditions as in Fig. 2.

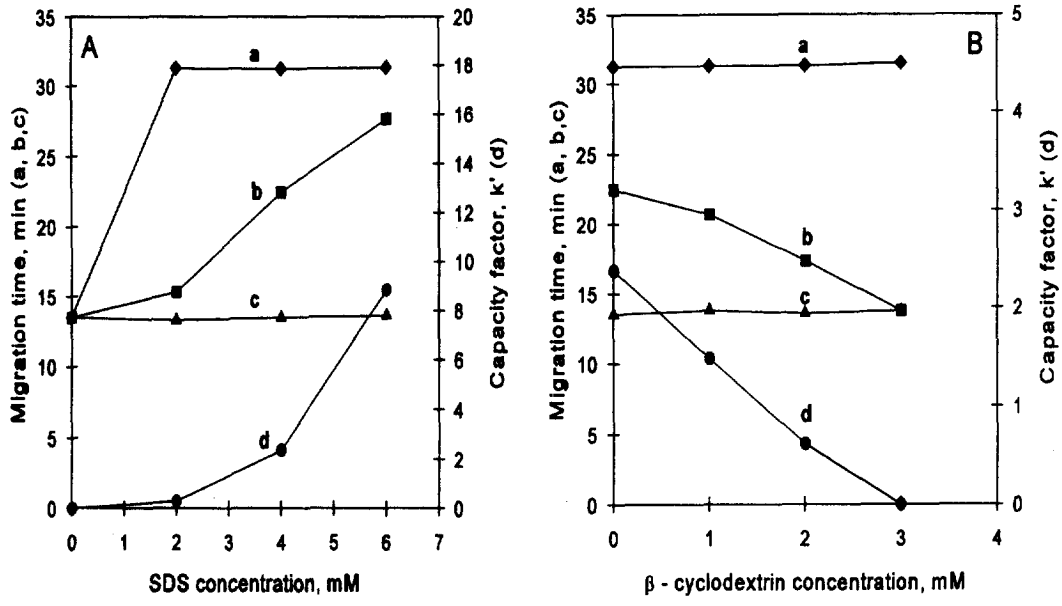

Fig. 4. Influence of SDS (A) and β-CD (B) concentrations on the migration time of Sudan III (a), oxoprolintane (b) and methanol (c) and on the capacity factor of oxoprolintane (d). Drug concentration $4.0 \cdot 10^{-5}$ *M* in 40 m*M* phosphate buffer, pH 8.5. Running buffer, 50 m*M* phosphate, pH 8.5, modified with SDS and β-CD. Other conditions as in Fig. 2.

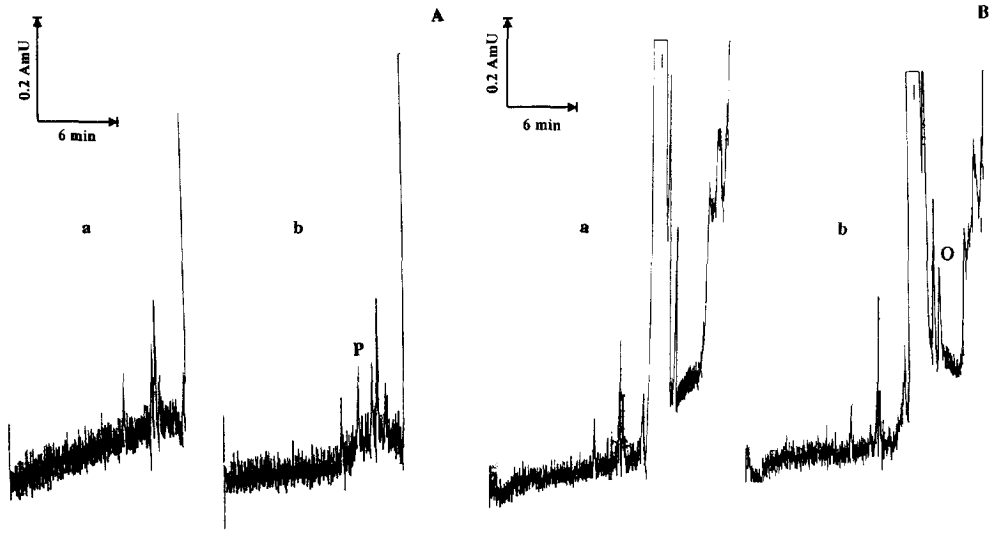

Fig. 5. Electropherograms obtained from urine samples 3:10 diluted with 10 m*M* phosphate buffer, pH 8.5, without drugs (a) and containing 2.5 μg ml^{-1} prolintane and oxoprolintane (b). Running buffer, pH 8.5, (A) 50 m*M* phosphate, (B) 50 m*M* phosphate modified with 4 m*M* SDS and 2 m*M* β-CD. Other conditions as in Fig. 2.

running voltage, whereas the oxoprolintane height was increased slightly.

Although the efficiency improved with the voltage applied, the best resolution in relation to the endogenous urine peaks was achieved at 15 kV. The numbers of theoretical plates at this voltage were $1.2 \cdot 10^4$ for prolintane and $1.0 \cdot 10^5$ for oxoprolintane.

To study the influence of prolintane and oxoprolintane concentrations in human urine on the electrophoretic peaks, spiked urine samples with different amounts of this drug and its metabolite (lower than 30 μg ml^{-1}) were analysed. The samples were diluted in a 3:10 ratio in 10 m*M* phosphate buffer, pH 8.5.

Fig. 5 shows the electropherograms obtained by CZE and MEKC from samples containing prolintane and oxoprolintane. Table 1 summarises the results obtained with the calibration plots. Repetition of analysis of three spiked urine samples with different concentrations of prolintane (1.2, 3.7, 6.2, 12.4 μg ml^{-1}) and oxoprolintane (0.8, 3.0, 6.0, 14.9 μg ml^{-1}), allowed an estimation of the relative errors

Table 1
Results obtained from the direct determination in urine (conditions as in Fig. 5)

Compound	Peak parameter	Sensibility[a] (mean±S.D.)	Correlation coefficient	Determination limit[b] (μg ml^{-1}) (mean±S.D.)	Detection limit[c] (μg ml^{-1}) (mean±S.D.)
Prolintane	Area (μV s)	450±10	0.998	1.0±0.1	0.3±0.1
	Height (AmU)	0.030±0.001	0.9995	1.0±0.1	0.3±0.1
Oxoprolintane	Area (μV s)	870±15	0.9990	0.7±0.1	0.2±0.1
	Height (AmU)	0.076±0.001	0.9993	0.7±0.1	0.2±0.1

[a] The adjustment was performed with eight different concentrations, each one repeated five times. Sensibility is the slope of the calibration curve.
[b] Signal-to-noise ratio, 10:1.
[c] Signal-to-noise ratio, 3:1.

and the relative standard deviations, which did not exceed 4.2 and 3.6%, respectively.

In order to minimise the effect of the sample matrix on the electrophoretic response, a standard additions method was used. The electrophoretic peaks were measured and plotted for standard additions of 1.0 μg ml^{-1} of stimulants to urine samples containing 1.2 and 2.5 μg ml^{-1} of each drug.

Repeating the standard additions method three times with drug concentrations of 1.2 and 2.5 μg ml^{-1} of urine ($n=5$), we obtained relative errors of less than 4.8% and relative standard deviations of less than 3.9%.

Comparing the CE method proposed in this paper, with the GC–MS method [4] in spiked urine samples with different amounts of prolintane and oxoprolintane, differences in results of less than 7.8% were obtained.

4. Conclusions

The results obtained in this study establish that the CE method proposed for the direct determination of prolintane and its metabolite, oxoprolintane, in human urine is easy to carry out, reliable and accurate for drug concentrations between 1.0 and 30 μg ml^{-1} of urine. The method is also quite fast and a complete analysis can be performed in 40–45 min.

This method can be used for doping control since there is a positive concordance between the results obtained by electrophoresis and GC–MS.

Acknowledgments

The authors thank the CICYT (Spain) for financial support (Project DEP 91-103) and Boehringer Ingelheim (Spain) for the kind supply of prolintane.

References

[1] R. Kadatz, E. Pötzsch, Arzneim.-Forsch. 7 (1957) 344.
[2] K. Takagi, H. Saito, Y. Higuchi, A. Yamaguchi, Oyo Yakuri 5 (1971) 5.
[3] C. Rodríguez Bueno, Dopaje, McGraw-Hill Interamericana, Madrid, 1992.
[4] G. Rücker, M. Neugebauer, D. Zhong, Xenobiotica 22 (1992) 143.
[5] T. Daldrup, F. Susanto, P. Michalke, Fresenius Z. Anal. Chem. 308 (1981) 413.
[6] G.P. Cartoni, M. Lederer, F. Polider, Chromatography 71 (1972) 370.
[7] S. Yoshihara, H. Yoshimura, Chem. Pharm. Bull. 20 (1972) 1906.
[8] S. Yoshihara, H. Yoshimura, Chem. Pharm. Bull. 22 (1974) 714.
[9] S. Yoshihara, H. Yoshimura, Xenobiotica 4 (1974) 529.
[10] H. Vapaatalo, S. Karkkainen, K.E.O. Senius, Int. J. Clin. Pharmacol. Res. 4 (1984) 5.
[11] G. Assumarra, G. Scarlata, G. Cirma, G. Asmano, S. Palazzo, S. Clementi, L. Giulietti, J. Chromatogr. 350 (1985) 151.
[12] B. Caddy, F. Fish, D. Scott, Chromatographia 6 (1973) 251.
[13] E. Marozzi, V. Gambaro, F. Lodi, Il Farmaco 32 (1977) 330.
[14] M. Auer, G.L. Dadisch, B. Kolb, G. Machata, P. Pospisil, Angew. Chromatogr. 30 (1977) 12.
[15] R. Dugal, R. Masse, G. Sanchez, M.J. Bertrand, J. Anal. Toxicol. 4 (1980) 1.
[16] J. Schmid, J. Chromatogr. 222 (1981) 129.
[17] Y. Jane, A. Mckinnon, R.J. Flanagan, J. Chromatogr. 323 (1985) 191.
[18] T. Dapdrup, P. Michaike, W. Boehme, Chromatogr. Newsl. 10 (1982) 1.
[19] D.H. Catlin, R.C. Kammerer, C.K. Hatton, M.H. Sekera, J.L. Merdink, Clin. Chem. 33 (1987) 319.
[20] C.M.C. Whittlesea, S.P. Lam, J.W. Gorrod, Prog. Pharmacol. Clin. Pharmacol. 8 (1991) 49.
[21] D.S. Lho, H.S. Shin, B.K. Kang, J. Park, J. Anal. Toxicol. 14 (1990) 73.
[22] P. Lillsunde, T. Korte, J. Anal. Toxicol. 15 (1991) 71.
[23] A.G. Espartero, J.A. Pérez, A. Zapardiel, E. Bermejo, L. Hernández, Electroanalysis 9 (1997) 416.
[24] D.K. Lloyd, J. Chromatogr. A 735 (1996) 29.
[25] H. Nishi, S. Terabe, J. Chromatogr. A 735 (1996) 3.
[26] G. Rücker, M. Neugebauer, D. Zhong, Arch. Pharm. 325 (1992) 47.
[27] F.E.P. Mikkers, F.M. Everaerts, Th.P.E.M. Verheggen, J. Chromatogr. 169 (1979) 11.
[28] J.W. Jorgenson, K.D. Lukacs, Anal. Chem. 53 (1981) 1298.
[29] D.J. Rose, J.W. Jorgensen, Anal. Chem. 60 (1988) 642.
[30] D. Perret, G. Ross, Trends Anal. Chem. 11 (1992) 156.
[31] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem. 56 (1984) 111.
[32] H. Nishi, M. Matsuo, J. Liq. Chromatogr. 14 (1991) 973.
[33] C.L. Copper, M.J. Sepaniak, Anal. Chem. 66 (1994) 147.