Journal of Chromatography, 631 (1993) 201–205
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2650

Determination of ephedrines in urine by high-performance liquid chromatography

C. Imaz, D. Carreras, R. Navajas, C. Rodriguez, A. F. Rodriguez, J. Maynar and R. Cortes

Laboratorio de Control del Dopaje, ICEF y D, Consejo Superior de Deportes, Greco s/n, 28040 Madrid (Spain)

ABSTRACT

An improved high-performance liquid chromatographic method with ultraviolet detection for the simultaneous determination of norephedrine, norpseudoephedrine, ephedrine, pseudoephedrine, methylephedrine and ethylephedrine in urine is described. The six substances were separated on a reversed-phase column with phosphate buffer–triethylamine (pH 5.5) as the mobile phase. The linearity and reproducibility were satisfactory for the levels usually found in urine (1–30 μg/ml).

INTRODUCTION

Ephedrine (EPH)–pseudoephedrine (PEPH) and norephedrine (NEPH)–norpseudoephedrine (NPEPH) are pairs of diastereoisomeric compounds [1] which are included in the doping list of pharmacological forbidden substances indicated by the Medical Commission of the International Olympics Committee (IOC) [2]. As they are widely available in asthma, ophthalmic, cold and allergy products [3] and as they are found in more than 100 pharmaceutical formulations, the Commission has defined concentrations above which they are considered positive. For this reason the determination of such pairs of diastereoisomeric compounds is necessary.

The most commonly used technique for such determinations is gas chromatography coupled with mass spectrometry [4]. In this event a previous selective derivatization [5] is needed (O-trimethylsilyl, N-trifluoroacetic acid), but the reproducibility obtained is not always sufficient because more than one derivative may be obtained for the same compound. Several HPLC methods have been reported for the determination of pseudoephedrine [6,7] and also for the pair pseudoephedrine–norpseudoephedrine [8] in pharmaceutical preparations and biological fluids. In this work, attempts were made to develop a simple, rapid, selective and accurate HPLC method without any previous derivatization process for the determination of the two pairs of diastereoisomers and also methylephedrine (MEPH) and ethylephedrine (ETEPH) (Fig. 1).

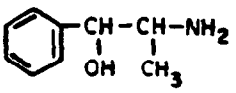
Positive urine samples taken from athletes were also analysed and very good results were obtained.

Correspondence to: C. Imaz, Laboratorio de Control del Dopaje ICEF y D, Consejo Superior de Deportes, Greco s/n, 28040 Madrid, Spain.

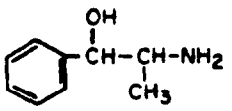
EXPERIMENTAL

Reagents

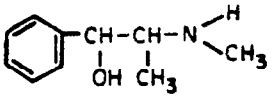
NEPH, NPEPH, EPH, PEPH, MEPH, ETEPH were obtained from Sigma (St. Louis, MO, USA). Diethyl ether was purchased from Carlo Erba (Milan, Italy) and phenylpropylamine [internal standard (I.S.)], KH_2PO_4, H_3PO_4 and triethylamine (TEA) from Merck (Darmstadt, Germany). Water was doubly distilled, deionized and purified with a Milli-Q system (Millipore, Milford, MA, USA). All other reagents and solvents were of analytical-reagent grade.



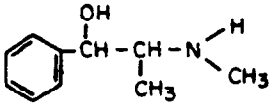
Norephedrine



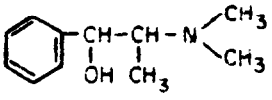
Norpseudoephedrine



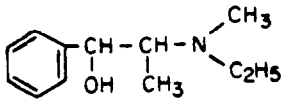
Ephedrine



Pseudoephedrine



N-Methylephedrine



Ethylephedrine

Fig. 1. Structures of ephedrines.

Standard solutions and calibration standards

Stock solutions of EPH, PEPH, NEPH, NPEPH, MEPH, ETEPH and the I.S. were prepared in the mobile phase described below at a concentration of 1000 μg/ml. These stock solutions were then diluted further to yield appropriate working solutions for the preparation of the calibration standards. The solutions were sealed and refrigerated at 4°C until used.

Preparation of mobile phase

The mobile phase was 200 m*M* phosphate buffer with TEA added to a final concentration of 150 m*M*. The pH was adjusted to 5.5. Before analysis, this mobile phase was filtered through a 0.22-μm filter and pumped through the column for 30 min. At the end of each chromatographic session, the column was washed for 15 min with deionized water and then with methanol.

Analytical procedure

To 5 ml of urine in a 15-ml glass tube were added 25 μl of I.S. solution (1000 μg/ml), 100 μl of 10 *M* NaOH and 2 ml of diethyl ether, then the urine was saturated with 3 g of sodium sulphate and shaken for 20 min. The tubes were centrifuged at 1200 *g* for 5 min and the organic layer was removed and evaporated to dryness. The residue was dissolved in 100 μl of the mobile phase and 20 μl of the solution was injected into the liquid chromatograph.

Chromatographic conditions

The chromatographic system consisted of a Hewlett-Packard (Palo Alto, CA, USA) HP 1090 Series A liquid chromatograph equipped with an autosampler/autoinjector and an HP 1040 A diode-array UV detector. Chromatography was performed at 40°C on a Hewlett-Packard reversed-phase analytical column (LiChrospher 60 RP Select B, 5 μm) (125 mm × 4 mm I.D.). The mobile phase was 200 m*M* phosphate buffer–150 m*M* TEA (pH 5.5) at a flow-rate of 1.3 ml/min. The injection volume was 20 μl and the column effluent was monitored at 215 nm (band width 4 nm), where the ephedrine exhibit

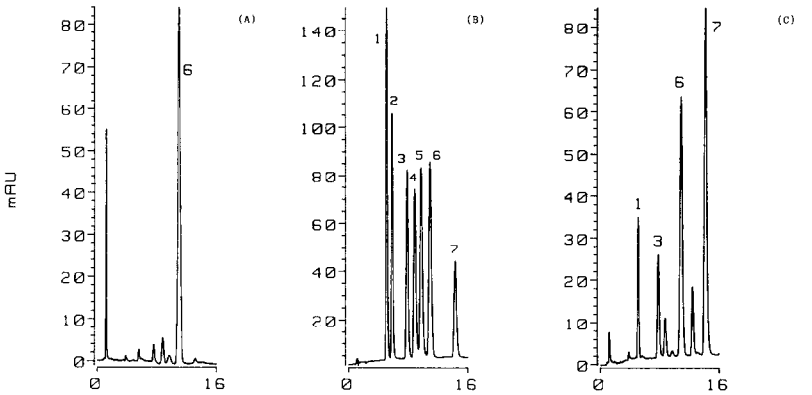


Fig. 2. Chromatograms obtained after analysis of (A) blank urine to which internal standard at a concentration of 5 μg/ml (peak 6) was added; (B) urine standard spiked with (1) NEPH, (2) NPEPH, (3) EPH, (4) PEPH, (5) MEPH, (7) ETEPH and (6) I.S., each at a concentration of 5 μg/ml; (C) urine sample from an athlete who had taken ETEPH, where levels close to 10 μg/ml for ETEPH and 1 μg/ml for its metabolites (NEPH and EPH) were found.

maximum absorption. For data evaluation and HP 79994 A Chemstation was used, which consisted of an HP 900 Series 300 computer, a 10 Mbyte Winchester disk drive and a Thinkjet printer.

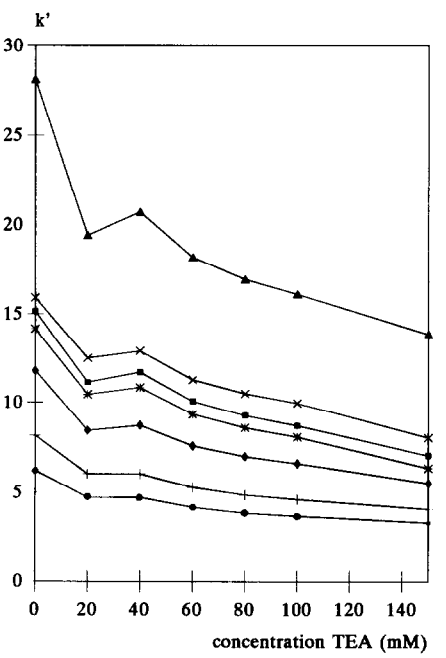


Fig. 3. Effect of the concentration of TEA in the column on k' values of ephedrines. Mobile phase, 200 mM phosphate (pH 4); column, LiChrospher 60 RP Select B, 5 μm (125 mm × 4 mm I.D.). ● = NEPH; + = NPEPH; ✳ = PEPH; ■ = MEPH; × = I.S.; ◆ = EPH; ▲ = ETEPH.

RESULTS

Representative chromatograms for urine analyses are shown in Fig. 2. The peaks of interest were well separated from potential interferences. Preliminary experiments indicated that the separation depends significantly on the column used. The best results were obtained when a Select B column was used.

The composition of the mobile phase was selected in such a way that all the ephedrines were resolved in the shortest analysis time possible. As it is

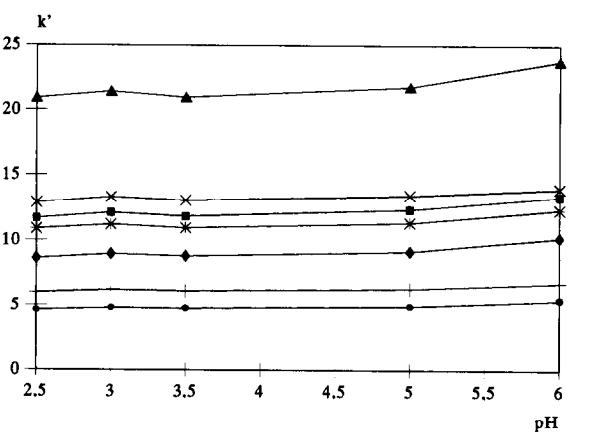


Fig. 4. Effect of pH on k' values. Concentration of TEA in the mobile phase, 150 mM; other conditions and compounds as in Fig. 3.

TABLE I

BETWEEN-DAY ACCURACY AND REPRODUCIBILITY OF THE DETERMINATION OF EPHEDRINES IN HUMAN URINE OVER A PERIOD OF 2 WEEKS (n = 6)

Concentration added (μg/ml)	NEPH		NPEPH	
	Mean ± S.D. (μg/ml)	R.S.D. (%)	Mean ± S.D. (μg/ml)	R.S.D. (%)
2	1.98 ± 0.07	3.5	2.05 ± 0.03	1.3
8	8.04 ± 0.05	5.3	8.22 ± 0.06	0.7
24	25.93 ± 0.26	0.9	24.34 ± 0.28	1.1
	EPH		PEPH	
	Mean ± S.D. (μg/ml)	R.S.D. (%)	Mean ± S.D. (μg/ml)	R.S.D. (%)
2	1.96 ± 0.09	2.2	1.92 ± 0.02	1.0
8	8.27 ± 0.04	0.4	8.07 ± 0.06	0.7
24	26.00 ± 1.17	4.5	25.59 ± 0.23	0.9
	MEPH		ETEPH	
	Mean ± S.D. (μg/ml)	R.S.D. (%)	Mean ± S.D. (μg/ml)	R.S.D. (%)
2	1.97 ± 0.05	2.69	1.87 ± 0.12	3.1
8	7.95 ± 0.04	0.56	7.23 ± 0.21	4.4
24	22.47 ± 0.28	1.26	25.14 ± 1.49	6.4

shown in Fig. 1, ephedrines have a hydroxyl group on the β-carbon and hence they can interact with free silanol groups. With TEA in the mobile phase these interactions decrease because such silanol groups are masked [9]. When the concentration of TEA in the mobile phase is increased, the k' values decreased (Fig. 3).

The pH of the mobile phase also influences the determination of the six ephedrines (Fig. 4). The k' values increased with increase in the pH of the mobile phase, but a better resolution was achieved, especially for the pseudoephedrine–methylephedrine pair, where resolution was complete at pH 5.5 (R_s = 1.25).

The precision and accuracy were measured using urine samples spiked at concentrations of 2, 8 and 24 μg/ml. The samples were extracted and subjected to HPLC. Each concentration was calculated on the basis of the peak-height ratio with respect to the I.S. The results are given in Table I. The linearity was

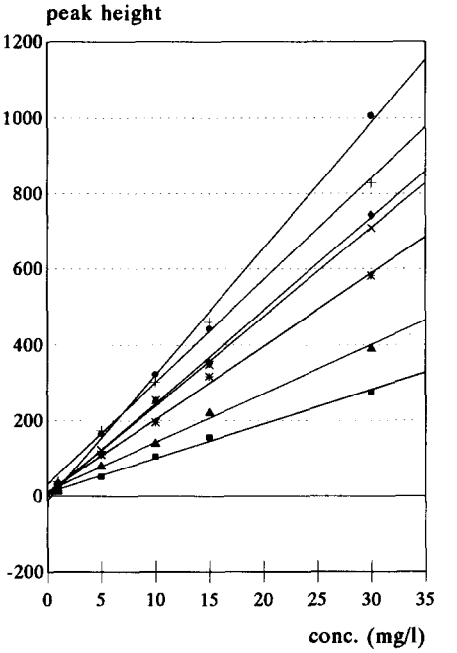


Fig. 5. Linear calibration graphs for the ephedrines specified in Fig. 3.

evaluated over the range of concentrations 1–30 μg/ml using duplicate samples spiked at levels of 1, 5, 10, 15 and 30 μg/ml. The calibration graphs obtained and the equations of the mean plots are shown in Fig. 5 and Table II, respectively.

The limit of detection was defined as the lowest concentration of each ephedrine resulting in a signal-to-noise ratio of 3. For NEPH, NPEPH and EPH the limit of detection was 0.2 μg/ml and for

TABLE II

EQUATIONS OF LINEAR CALIBRATION GRAPHS

Compound	Equation[a]	r^2
NEPH	$y = 33.38x - 13.63$	0.9978
NPEPH	$y = 27.02x + 30.64$	0.9983
EPH	$y = 24.58x - 0.92$	0.9991
PEPH	$y = 19.27x + 10.82$	0.9987
MEPH	$y = 9.12x + 8.78$	0.9975
I.S.	$y = 23.55x + 3.66$	0.9993
ETEPH	$y = 12.93x + 13.08$	0.9971

[a] y = Peak height (arbitrary units); x = concentration (mg/l).

PEPH, MEPH and ETEPH it was 0.5 μg/ml. The limit of detection in urine is dependent on the amount of interferences present, but in all instances it was less than the stated limits.

Other pharmacological substances examined in order to establish possible intereferences were amfepramone, amphetamine, caffeine, chlorphentermine, cocaine, codeine, cropropamide, crotethamide, dimethylamphetamine, etamivan, fencamfamine, heptaminol, leptazol, lidocaine, methoxamine, methylamphetamine, methylphenidate, nicotine, niketamine, pethidine, phendimetrazine, phenmetrazine, pipradol, procaine, prolintane and strychnine. None of these interfered with the determination of the ephedrines.

CONCLUSION

The elution and separation of ephedrines were clearly affected by the column used, the concentration of TEA and the pH of the mobile phase. As the use of a modifier was not necessary, endogenous compounds were eluted at very long times, resulting in very clear chromatograms where no interferences from such endogenous compounds were observed. This method has been applied since January 1992 to urine samples taken from athletes. Over this period, we have analysed more than 20 physiological samples with different levels of ephedrines.

REFERENCES

1 I. W. Wainer and A. L. Marcotte, in I. W. Wainer and D. E. Drayer (Editors), *Drug Stereochemistry, Analytical Methods and Pharmacology*, Marcel Dekker, New York, 1988, pp. 31–41.
2 *List of Doping Classes and Methods*, International Olympic Committee, Lausanne, 1991.
3 J. E. F. Reynolds (Editor), *Martindale. The Extra Pharmacopoeia*, Pharmaceutical Press, London, 28th ed., 1982.
4 M. Donike, presented at the *9th Workshop on Dope Analysis, Cologne, March 17–22, 1991.*
5 M. Donike and J. Derenbach, *Fresenius' Z. Anal. Chem.*, 279 (1976) 128.
6 I. L. Honigberg, J. T. Stewart, and A. P. Smith, *J. Pharm. Sci.*, 63 (1974) 766.
7 A. Yacobi, Z. M. Look and C. M. Lai, *J. Pharm. Sci.*, 67 (1978) 1668.
8 C. M. Lai, R. G. Stoll, Z. M. Look and A. Yacobi, *J. Pharm. Sci.*, 68 (1979) 1243.
9 Z. Varga-Puchony and Gy. Vigh, *J. Chromatogr.*, 257 (1983) 380.