ELSEVIER

Journal of Chromatography B, 760 (2001) 255–261

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Simultaneous quantitation of ephedrines in urine by gas chromatography–nitrogen–phosphorus detection for doping control purposes

P. Van Eenoo, F.T. Delbeke*, K. Roels, P. De Backer

Ghent University, Doping Control Unit, Department of Pharmacology, Pharmacy and Toxicology, Salisburylaan 133, B-9820 Merelbeke, Belgium

Received 13 February 2001; received in revised form 29 May 2001; accepted 29 May 2001

Abstract

A gas chromatographic method for the simultaneous quantitation of ephedrine, pseudoephedrine, norephedrine (phenylpropanolamine), norpseudoephedrine (cathine) and methylephedrine in urine is described. The method consists of a liquid–liquid extraction with *tert*.-butyl methyl ether at pH 14. The extracts are analysed on a GC system equipped with an Rtx-5 Amine column and a nitrogen–phosphorus detector. Method validation shows excellent separation, linearity, specificity, accuracy, precision, intra-laboratory repeatability and reproducibility, making the method especially suitable for quantitation of ephedrines in urine samples for doping control purposes. A statistical analysis on the abuse of the different ephedrines in urine from athletes controlled in the Flemish doping control laboratory during the period 1993–2000 is included.

Keywords: Doping control; Ephedrines

1. Introduction

Ephedrine and the related compounds norephedrine, pseudoephedrine, norpseudoephedrine and methylephedrine (Fig. 1) are sympathomimetic amines with central nervous system stimulating properties [1,2]. These substances are ingredients of many medicines commonly used in the treatment of flu, rhinusitis, colds and allergy [3]. They are also ingredients of several dietary supplements and are found in a diverse range of sports nutritional supplements. In many cases manufacturers of these products only indirectly refer to the presence of ephedrines by mentioning the botanical source (Ma Huang, Ephedra, etc.).

The International Olympic Committee [4], the Flemish government [5] and most international sports federations have put the ephedrines on their list of prohibited doping substances and have adopted urinary threshold concentrations, above which an athlete is regarded as positive. Nowadays, these thresholds are 5 μg/ml for norpseudoephedrine, 10 μg/ml for ephedrine and methylephedrine and 25 μg/ml for norephedrine and pseudoephedrine [4,5]. Therefore, a fast, simple and reliable method

*Corresponding author. Tel.: +32-9-2647-347; fax: +32-9-2647-497.

E-mail address: frans.delbeke@rug.ac.be (F.T. Delbeke).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00275-4

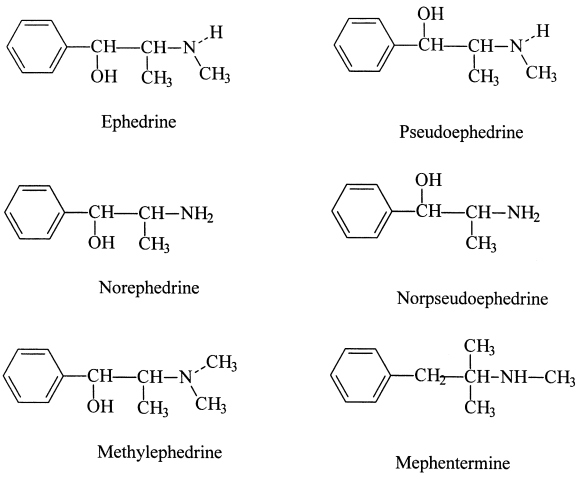

Fig. 1. Chemical structures of ephedrines and mephentermine (internal standard).

for the simultaneous quantitation of these substances is needed.

Various methods for the simultaneous quantitation of ephedrines in body fluids and pharmaceuticals using high-performance liquid chromatography have been described [1–3,6–10]. Gas chromatography (GC) methods for the analysis of ephedrines have also been described [1,11–14]. However, these methods require a derivatisation step to allow for separation of the diastereoisomeric substances.

In this study, a simple, rapid, selective and accurate GC–nitrogen–phosphorus detection (NPD) method for the quantitation of ephedrines without prior derivatisation is described.

2. Experimental

2.1. Chemicals

KOH, NaCl and anhydrous Na_2SO_4 were purchased from Merck (Darmstadt, Germany), *tert*.-butyl methyl ether was obtained from Fluka (Buchs, Switzerland), methanol was from Panreac (Barcelona, Spain). All chemicals were analytical grade.

2.2. Standards

Ephedrine·HCl and methylephedrine were from Fluka. Norephedrine·HCl was from Sigma (St. Louis, MO, USA), pseudoephedrine·HCl was from Aldrich (Steinheim, Germany), norpseudoephedrine·HCl and mephentermine sulfate (internal standard, I.S.) were a generous gift from Merck.

Stock solutions of all compounds were made by dissolving the substances in methanol and were stored at −20°C.

2.3. Calibration graphs and quality control samples

Calibration curves for the different ephedrines were generated by spiking blank urine samples with methanolic standard solutions. The final concentrations for each compound at each calibration point (three replicates/point) are given in Table 1.

Quality control samples were made by spiking blank urine samples at the threshold concentrations. All samples were analysed according to the described procedure.

2.4. Extraction

To 0.5 ml of urine were added 2.0 ml of bidistilled water, 2 g NaCl, 50 μl of the I.S. solution (mephentermine, 50 μg/ml) and 250 μl KOH (5 *M*). The mixture was extracted with 1.0 ml *tert*.-butyl methyl ether by rolling during 20 min on a CAT RM5 (M. Zipperer, Staufen, Germany). After centrifugation at 1200 *g* for 5 min, the organic layer was transferred into a new tube and dried over anhydrous Na_2SO_4.

2.5. Gas chromatographic conditions

GC analysis was performed on a HP 6890 GC system (Hewlett-Packard, Waldbronn, Germany) equipped with a nitrogen-selective detector. An Rtx-5 Amine (Restek, Bellefonte, PA, USA) crosslinked

Table 1
Calibration curve levels

	Level (μg/ml)				
	1	2	3	4	5
Norpseudoephedrine	2	4	6	8	10
Ephedrine	4	8	12	16	20
Methylephedrine	4	8	12	16	20
Pseudoephedrine	10	20	30	40	50
Norephedrine	10	20	30	40	50

5% diphenyl–95% dimethylpolysiloxane column (15 m×0.25 mm I.D., 1.0 μm film thickness) was used at a column head pressure of 24 p.s.i. (1 p.s.i.=6894.76 Pa).

Helium was used as carrier gas. A 2-μl volume of the organic layer was injected (split 2:1), the injector temperature was 280°C and the detector temperature was maintained at 250°C. The GC oven temperature program was as follows: 100°C – 0.5°C/min→105°C – 2°C/min→118°C – 50°C/min→280°C (2 min).

3. Results and discussion

3.1. Chromatography

Retention times (t_R) and relative retention times (RRTs) of the different ephedrines are shown in Table 2. A representative chromatogram of a quality control sample is given in Fig. 2 indicating that all substances are sufficiently resolved.

Chromatographic separation was checked by analysing quality control samples spiked with each ephedrine separately and with a combination of all ephedrines. The mean and standard deviation of the quantitative results obtained with both types of quality control urine samples were statistically compared (*t*-test, $\alpha=0.05$). The results revealed no differences, indicating that the method allowed for adequate separation.

3.2. Quantitation

Calibration curves were generated by plotting the peak area ratios of the analytes and the internal standards versus the concentration. Linear regression using an unweighted least-squares fit showed good linearity for each substance (Table 3). Using the described method, approximately 20% of the positive samples need to be diluted to allow for quantitation.

Table 2
Retention time (t_R) and relative retention time (RRT) for different ephedrines

	t_R (min)	RRT
Mephentermine (I.S.)	8.522	1.000
Norpseudoephedrine	12.078	1.417
Norephedrine	12.248	1.437
Ephedrine	14.837	1.741
Pseudoephedrine	15.086	1.770
Methylephedrine	17.228	2.022

3.3. Accuracy, precision, repeatability and reproducibility

Accuracy and precision of the method was established at the threshold levels, as required by the ISO guidelines, and at the lowest level of the calibration curve. Precision was assessed as both repeatability (within-day) and reproducibility (between-day and sample preparation by different analysts). The results are summarised in Table 4.

Acceptable tolerances (%) for reproducibility and repeatability can be calculated from $RSD_{max}=2^{(1-0.5\log C)}$. The maximum allowed tolerance for reproducibility and repeatability is RSD_{max} and 2/3 RSD_{max}, respectively [15]. As shown in Table 4, the reproducibility and repeatability of the method, expressed in terms of RSD, are within these limits.

The between-day accuracy (Table 4), expressed as mean error, is within the acceptable deviation of 15% [16].

These results demonstrate that this method is suitable for quantitation of ephedrines with satisfactory accuracy and precision.

3.4. Selectivity and specificity

The selectivity and specificity of the method was tested by analysing 20 different urine samples with and without internal standard. No matrix interferences are detected at the retention times of the different ephedrines or the internal standard. Analysis of urine samples spiked with structurally related compounds (final concentration: 50 μg/ml) also demonstrate the selectivity and specificity (Table 5). The difference in RRT between methoxyphenamine and pseudoephedrine is 3.2%, well beyond the criteria from the International Olympic Committee (IOC), allowing for a maximum of 1% deviation for positive identification [17].

Validation of this method, using liquid–liquid extraction, is excellent. Moreover, the method is economically and ecologically competitive or even

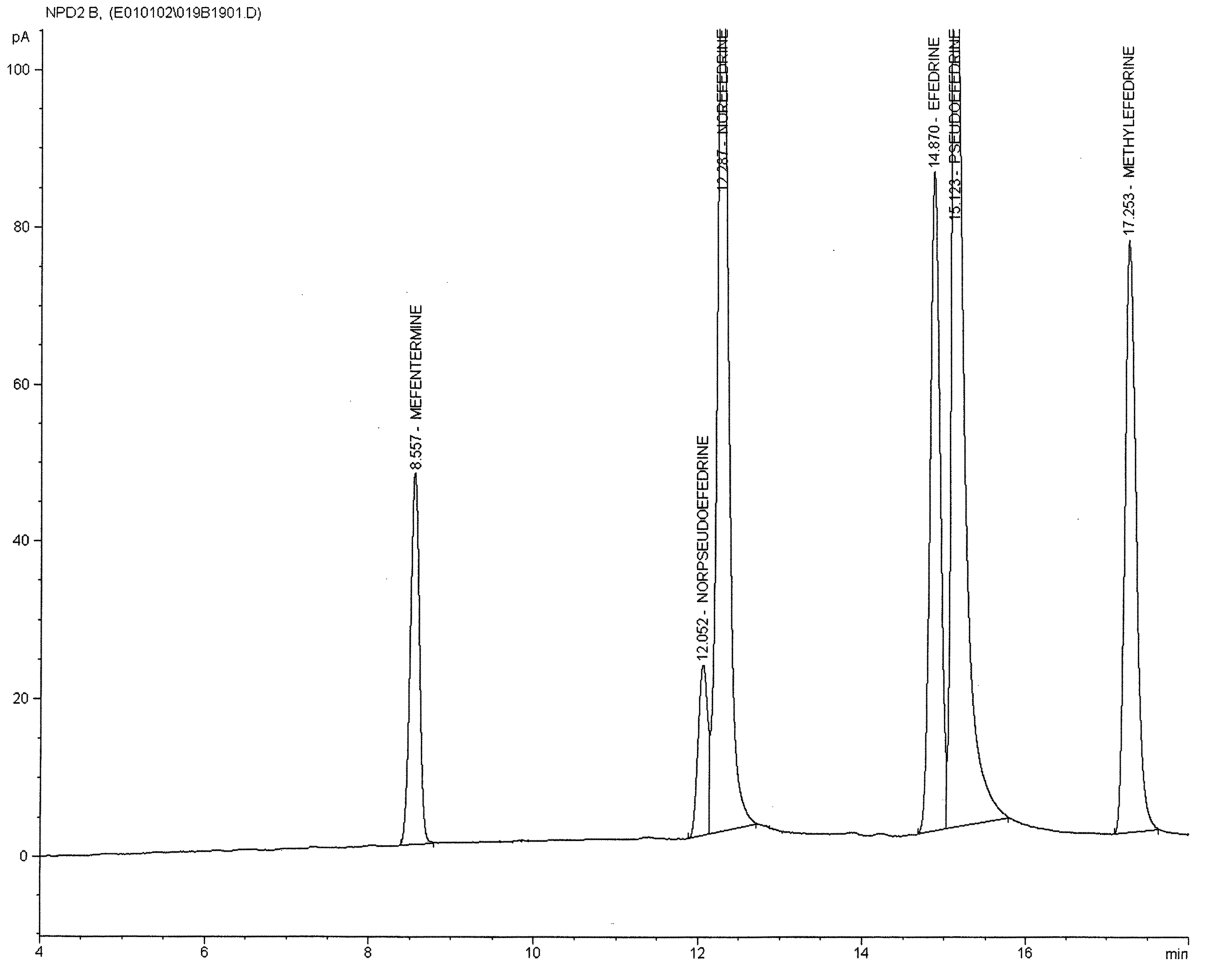

Fig. 2. Gas chromatogram of a quality control sample.

better than methods using solid-phase extraction, because only 1 ml of solvent is used and no solid-phase extraction column needs to be discarded. Furthermore the use of liquid–liquid extraction improves the robustness of the method as compared to non-automated solid-phase extraction.

Table 3
Equations and correlation coefficients of the calibration curves

	Working range (μg/ml)	Equation	Correlation coefficients (r^2)
Norpseudoephedrine	2–10	$y=0.130x-0.137$	0.996
Ephedrine	4–20	$y=0.215x-0.159$	0.998
Methylephedrine	4–20	$y=0.218x-0.148$	0.999
Pseudoephedrine	10–50	$y=0.177x-0.726$	0.998
Norephedrine	10–50	$y=0.146x-0.389$	0.996

Table 4
Accuracy (between-day), repeatability, reproducibility and tolerance limits of the method at the threshold level and the lowest level of the calibration curve

	Concentration (μg/ml)	Accuracy (%)	Repeatability (%, $n \geq 6$)	Reproducibility (%, $n \geq 12$)	RSD_{max} (%)	2/3 RSD_{max} (%)
Norpseudoephedrine	2	8.5	2.1	7.6	14.4	9.6
	5	5.9	1.9	8.0	12.6	8.4
Ephedrine	4	−5.8	6.0	8.2	13.1	8.7
	10	2.0	3.5	2.5	11.3	7.5
Methylephedrine	4	−2.8	5.0	6.4	13.1	8.7
	10	4.4	2.1	2.0	11.3	7.5
Pseudoephedrine	10	−1.7	4.1	5.2	11.3	7.5
	25	−2.3	2.0	4.6	9.9	6.6
Norephedrine	10	1.2	3.1	6.8	11.3	7.5
	25	8.4	3.4	6.9	9.9	6.6

3.5. Statistical analysis of samples collected for doping analysis

It was previously reported that the misuse of ephedrines in sports had a high incidence in Flanders in several sports [18,19].

During the period 1993–2000, 14 995 urine samples, routinely collected for doping control purposes, were analysed in our laboratory. Ephedrines were detected in 275 samples (1.8%). Prior to 2000 threshold levels were not applied for these substances in the Flemish Doping Decree, contrary to the rules of the IOC and international federations [5].

Table 5
Retention time (t_R) and relative retention time (RRT) of structurally related compounds

Substance	t_R (min)	RRT
Amphetamine	4.266	0.498
Phentermine	5.166	0.603
Methylamphetamine	5.781	0.675
Mefenorex	7.304	0.856
Ethylamphetamine	7.656	0.893
Fenfluramine	7.878	0.919
Dimethylamphetamine	8.200	0.957
Chlorphentermine	8.569	0.619
Methoxyphenamine	15.667	1.828

Nevertheless quantitation was done with the described method for all samples in which ephedrines were detected.

In 60% of the cases (165 samples) the concentration of one or more substance from the ephedrine group exceeded the threshold level of the IOC. In 2000, the Medical Commission of the IOC increased the threshold concentrations for ephedrine and methylephedrine from 5 to 10 μg/ml and the threshold concentrations for norephedrine and pseudoephedrine from 10 to 25 μg/ml. Using these new threshold levels 113 samples (41%) could still be regarded as positive. As shown in Fig. 3, the highest incidence for exceeding the threshold levels was found for ephedrine, followed by norephedrine and pseudoephedrine.

The incidence of ephedrines in urine samples from athletes in Flanders during 1993–2000 in major sports (number of samples tested >200) are shown in Table 6. Most positive cases (%) were detected in body building, followed by powerlifting. A previous report on the abuse of doping agents in body builders in Flanders [19] already indicated the widespread use of ephedrines in body building, often in combination with anabolic steroids. These findings are further supported by reports in newsgroups on the internet indicating that ephedrines, often in combination with caffeine and salicylic acid, are commonly used

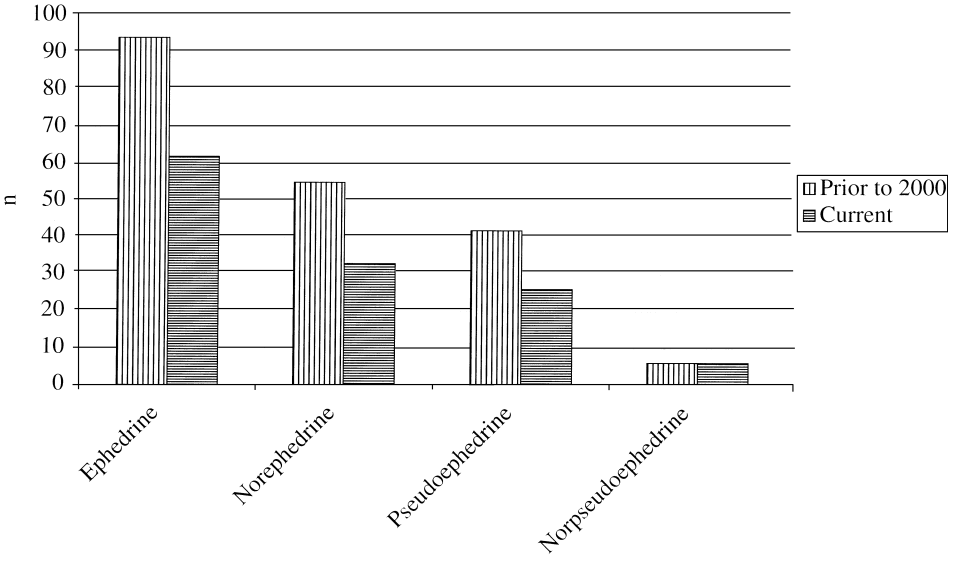

Fig. 3. Number of samples above the threshold levels for ephedrines collected during the period 1993–2000 for doping control purposes in Flanders according to substance and according to threshold level (current and threshold at time of analysis). Total number of samples analysed: 14 995.

among body builders. Stimulants are frequently abused in cycling [18]. However the data presented here, suggest that misuse of ephedrines in cycling is only slightly above the mean percentage of positives in the tested population. Contrary to volleyball, where the incidence of ephedrines misuse is low, the misuse in basketball and handball are rather substantial.

Table 6
Incidence of ephedrines in samples collected for doping analysis in Flanders (1993–2000) according to sport

Sport	Samples tested	Samples positive	% Positives
Volleyball	874	2	0.23
Judo	287	1	0.35
Tennis	280	1	0.36
Gymnastics	232	1	0.43
Athletics	1196	9	0.75
Swimming	411	4	0.97
Indoor soccer	267	3	1.12
Soccer	1870	30	1.60
Table tennis	290	5	1.72
Cycling	5951	111	1.87
Basketball	686	17	2.48
Handball	328	9	2.74
Powerlifting	286	13	4.55
Body building	533	35	6.57

4. Conclusion

Using the described gas chromatographic method, ephedrines can be quantitated in urine for doping control purposes in a fast, precise and accurate way without the need for derivatisation. The selectivity of the method was satisfactorily tested by analysing blank urine samples, spiked with several structurally related substances. The incidence of ephedrines in athletes controlled in Flanders is high and approximately half of the samples containing ephedrines exceed the doping threshold level.

Acknowledgements

The technical assistance of Mrs. G. Demey was greatly appreciated. Part of this study was financed by the Flemish Ministry of Health.

References

[1] P.J. van der Merwe, S.E. Hendrikz, J. Chromatogr. B 663 (1995) 160.
[2] W. König, K. Ernst, J. Chromatogr. 280 (1983) 135.
[3] J. Gal, J. Chromatogr. 307 (1984) 220.
[4] List of Prohibited Classes of Substances and Prohibited Methods, International Olympic Committee, Lausanne, 2001.
[5] Decree of the Flemish Executive C-2000/36128 of 31 October 2000 (B.S. 25.11.2000).
[6] I.W. Wainer, T. Doyle, F. Fry, Z. Hamidzadeh, J. Chromatogr. 355 (1986) 149.
[7] P.J. van der Merwe, L.W. Brown, S.E. Hendrikz, J. Chromatogr. B 661 (1994) 357.
[8] C. Imaz, R. Navajas, D. Carreras, C. Rodríguez, A.F. Rodríguez, J. Chromatogr. A 870 (2000) 23.
[9] S. Barkan, J.D. Weber, E. Smith, J. Chromatogr. 219 (1988) 81.
[10] C. Imaz, D. Carreras, R. Navajas, C. Rodríguez, A.F. Rodríguez, J. Maynar, C. Cortes, J. Chromatogr. 631 (1993) 201.
[11] K. Kruse, W. Francke, W. König, J. Chromatogr. 170 (1979) 423.
[12] R.T. Coutts, R. Dawe, G.R. Jones, S.-F. Liu, K.K. Midha, J. Chromatogr. 190 (1980) 51.
[13] F.T. Delbeke, M. Debackere, Biopharm. Drug Dispos. 12 (1991) 37.
[14] R. Kazlauskas, A. Lisi, G. Trout, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Proceedings of the Manfred Donike Workshop, Cologne, March 1998, Recent Advances in Doping Analysis, Vol. 6, 1998, p. 431.
[15] W. Verwaal, M. van Bavel, A. Boot, J. Bravenboer, F. de Goey, C. Maas, A. van der Putten, De Ware(n)-Chemicus 26 (1996) 17.
[16] F. Bressolle, M. Bromet-Petit, M. Audran, J. Chromatogr. B 686 (1996) 3.
[17] Meeting of Working groups of Heads of Laboratory, Lausanne, Recommendations For Reporting Prohibited Substances And/or Metabolites, International Olympic Committee, 21–23 March 1998.
[18] F.T. Delbeke, N. Desmet, M. Debackere, Int. J. Sports Med. 16 (1995) 66.
[19] F.T. Delbeke, Int. J. Sports Med. 17 (1996) 434.