ELSEVIER

Journal of Chromatography B, 758 (2001) 311–314

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

Determination of ephedrines in urine by gas chromatography–mass spectrometry

Maria-Helen E. Spyridaki[a], Christina J. Tsitsimpikou[a], Panayotis A. Siskos[b], Costas G. Georgakopoulos[a,*]

[a]*Doping Control Laboratory of Athens, Olympic Athletic Center of Athens 'Spiros Louis', Kifissias 37, 151 23 Maroussi, Athens, Greece*

[b]*Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Panepistimiopolis, 157 71, Zographou, Athens, Greece*

Received 16 January 2001; received in revised form 14 March 2001; accepted 15 March 2001

Abstract

A selective gas–liquid chromatographic method with mass spectrometry (GC–MS) for the simultaneous confirmation and quantification of ephedrine, pseudo-ephedrine, nor-ephedrine, nor-pseudoephedrine, which are pairs of diastereoisomeric sympathomimetic amines, and methyl-ephedrine was developed for doping control analysis in urine samples. *O*-Trimethylsilylated and *N*-mono-trifluoroacetylated derivatives of ephedrines — one derivative was formed for each ephedrine — were prepared and analyzed by GC–MS, after alkaline extraction of urine and evaporation of the organic phase, using d_3-ephedrine as internal standard. Calibration curves, with r^2>0.98, ranged from 3.0 to 50 μg/ml depending on the analyte. Validation data (specificity, % RSD, accuracy, and recovery) are also presented. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, GC; Ephedrines

1. Introduction

Ephedrine, pseudo-ephedrine, nor-ephedrine, nor-pseudoephedrine are pairs of diastereoisomeric sympathomimetic amines [1] known to have central nervous system stimulating properties [2] and are therefore included — along with methyl-ephedrine — in the doping list of pharmacological forbidden substances indicated by the Medical Commission of the International Olympic Committee (IOC) [3]. From an analytical perspective, in doping control analysis, the use of chromatography coupled with mass spectrometry for the final confirmation of suspected compounds is mandatory [3,4]. The problem with gas chromatography–mass spectrometry (GC–MS) in the determination of ephedrines is that the reproducibility obtained is not always sufficient, because more than one derivative may be obtained for the same compound [5,6].

In this work, an accurate, selective, rapid GC–MS method with derivatization (*O*-trimethylsilylated and *N*-mono-trifluoro-acetylated derivatives) was developed for the confirmation and quantification of ephedrine, nor-ephedrine, pseudo-ephedrine, nor-pseudo-ephedrine and methyl-ephedrine in urine and

*Corresponding author. Fax: +3-1-6868-549.
E-mail address: oaka@compulink.gr (C.G. Georgakopoulos).

0378-4347/01/$ – see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(01)00174-8

was applied to doping control analysis. It is important to emphasize that only one derivative was formed for each ephedrine and the analysis of the samples was carried out by using the same instrumentation as for screening analysis. This work was occurred in the framework of the preparation of the doping control laboratory of Athens for the Olympic Games 2004.

2. Experimental

2.1. Chemicals and reagents

Ephedrine–HCl (purity 99%), 1*S*,2*S*-(+)-pseudoephedrine–HCl (purity 98%), 1*S*,2*R*-(+)-norephedrine–HCl (purity 99%), *S*,*S*-norpseudoephedrine–HCl (purity 98%), 1*S*,2*R*-(+)-*N*-methylephedrine (purity 99%) and hepta-fluorobutyric anhydride (HFBA) were obtained from Sigma–Aldrich (Athens, Greece). 1*S*,2*R*(+)-d_3-Ephedrine–HCl in methanol (purity 99%) was obtained from Promochem (Welwyn Garden City, UK). Potassium hydroxide, diethylether, anhydrous sodium sulphate were of analytical reagent grade and provided from commercial sources. Chlortrimethylsilane and dichlordimethylsilane were purchased from Merck (Darmstadt, Germany). *N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), trimethyl-silyl-imidazole (TSIM), *N*-methyl-bis-trifluoroacetamide (MBTFA), trifluoroacetic anhydride (TFAA) were purchased from Macherey–Nagel (Postfach, Düren). *N*-*tert*.-Butyl-dimethyl-silyl-*N*-methyl-trifluoro-acetamide (MTBSTFA) and *N*,*O*-bis(trimethylsilyl)-trifluoro-acetamide (BSTFA) were obtained from Fluka (Athens, Greece) and Serva Feinbiochemica (Heidelberg), respectively.

2.2. Apparatus

A Hewlett-Packard 6890 gas chromatograph coupled with a 5973 quadrupole mass spectrometric detector (MSD) with a crosslinked 5% diphenyl–95% dimethylsiloxane capillary column (12.5 m×0.2 mm I.D.; 0.33-μm film thickness, HP Ultra 2) was used. Helium was used as carrier gas at flow 1.0 ml/min. Injection port and transfer zone temperatures were maintained at 250°C. A 1-μl sample volume was injected in the split mode (30:1). Split inlet liner with cup (Agilent Technologies, Hellamco, Athens, Greece, part no. 18740-80190) was used. Initial oven temperature was set at 80°C for 2 min, then ramped at 10°C/min to 150°C and at 10°C/min to 310°C and held for 1 min. Time of sample analysis 13.2 min. MSD was run in a scan mode (mass range 70–400) with electron impact ionization (70 eV).

2.3. Analytical procedure

Standard aqueous solutions of ephedrine, norephedrine, pseudoephedrine, norpseudoephedrine and methylephedrine were prepared, sealed and refrigerated at 4°C until used.

Blank urine spiked with ephedrines or urine obtained from positive doping case were extracted as follows: to 1.0 ml of urine was added 10 μl d_3-ephedrine–HCl 1000 μg/ml (internal standard) and 0.250 ml potassium hydroxide 5.0 *M*. The mixture was extracted twice with 5.0 ml diethyl ether, in the presence of 1.5 g anhydrous sodium sulphate, by vortexing for 1 min. After centrifuging at 1900 *g* for 10 min, the organic phase was gently evaporated to dryness under stream of nitrogen. Samples were kept in a desiccator for 30 min. A 75-μl volume of MSTFA:TSIM (100:10) was added to the dried residue, vortex-mixed and kept at 80°C for 15 min to obtain the trimethylsilyl derivatives of hydroxyl group. After cooling at room temperature, 30 μl MBTFA were added and the mixture was vortexed and incubated for 15 min at 80°C to obtain trifluoroacetamide (TFA) derivatives of primary and secondary amines. One μl of the mixture was subjected to GC–MS analysis.

3. Results and discussion

3.1. Optimisation of the analytical procedure

The solvent of choice for the extraction of ephedrines is diethylether due to its low boiling point (34.4°C) and its analytical properties for this purpose. Recovery studies for the extraction procedure

proved that a double extraction with 5.0 ml of diethylether was needed.

Evaporation step is very crucial for ephedrines due to their high volatility [6]. Triplicate experiments were carried out in order to examine three ways of evaporation: (a) evaporation under gentle stream of nitrogen at room temperature, (b) evaporation by using rotavapor, (c) over night evaporation at room temperature. Furthermore, in order to prevent loss of ephedrines, the presence of 20 μl MBTFA [4] or two to three drops of chlortrimethylsilane or dichlordimethylsilane was also examined, but no improvement has been shown. The loss of ephedrines was less in the case of evaporation under gentle stream of nitrogen at room temperature.

The classical derivatization reaction of ephedrines is with MSTFA/MBTFA [7]. However, with the above procedure, nor-ephedrine and nor-pseudoephedrine form two derivatives, *N*-trifluoroacetyl-*O*-trimethylsilyl- and *N*-trifluoroacetyl-bis-*N*,*O*-trimethylsilyl derivatives, which is not desirable [6,8].

Many derivatization reagents were examined, like HFBA [9], MSTFA, MTBSTFA, BSTFA. In all the above cases, either derivatives of nor-ephedrine and nor-pseudo-ephedrine were not separated, either two derivatives were formed. In order to prevent hydrogen atom on amino group from receiving a trimethylsilyl (TMS) group, TSIM was added to the derivatization mixture. Several ratios MSTFA:TSIM were tested in different temperatures and heating-time periods. The best results were obtained by using MSTFA:TSIM (100:10), heating at 80°C for 15 min and then addition of MBTFA, heating at 80°C for 15 min (Fig. 1).

3.2. Detection criteria

3.2.1. Chromatography

Ephedrines derivatives from spiked with ephedrines blank urine samples, which were analysed according to the procedure described in Section 2.3, were detected at retention times relative to d_3-ephedrine of 0.833 for methyl-ephedrine-*O*-TMS, 0.893 for nor-pseudo-ephedrine-*N*-TFA-*O*-TMS, 0.902 for nor-ephedrine-*N*-TFA-*O*-TMS, 0.999 for ephedrine-*N*-TFA-*O*-TMS and 1.02 for pseudo-ephedrine *N*-TFA-*O*-TMS.

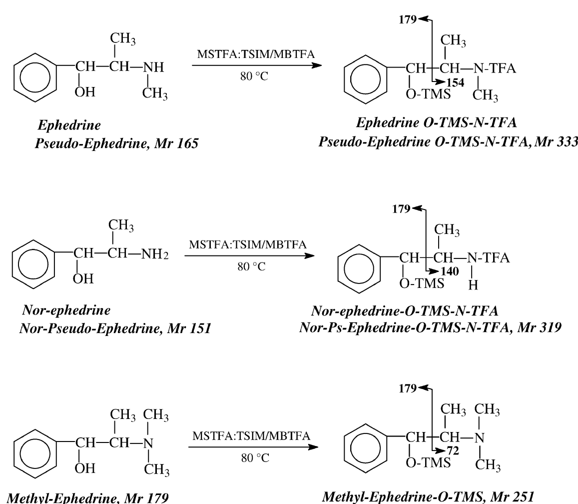

Fig. 1. Structures of ephedrines, ephedrines *O*-trimethylsilylated and *N*-mono-trifluoro-acetylated derivatives and fragmentation pattern of structural diagnostic ions *m*/*z*.

3.2.2. Mass spectrometry, full scan mode (low resolution)

The following ions are of structural diagnostic importance: *m*/*z* 179, 154, 227, 318 for ephedrine-*N*-TFA-*O*-TMS and pseudo-ephedrine-*N*-TFA-*O*-TMS, *m*/*z* 179, 163, 304 for nor-ephedrine-*N*-TFA-*O*-TMS and nor-pseudo-ephedrine-*N*-TFA-*O*-TMS, *m*/*z* 72, 163, 236 for methyl-ephedrine-*O*-TMS. Ephedrine and pseudo-ephedrine have the same mass spectrum, as it is expected, and the same exists for the pair nor-ephedrine/nor-pseudo-ephedrine.

3.3. Validation of the method

For the validation of the method for the determination of the mixture of ephedrines, data of calibration curves, specificity, precision and accuracy of the method were calculated [10]. Analysis of blank urine samples ($n=10$) and spiked blank urine samples with ephedrines proved that there is not interference from urine matrix in the determination of ephedrines. Five calibration curves ($n=5$ standards×2×2 injections), were established in the range 5.0–30 μg/ml for ephedrine and methyl-ephedrine, 10–50 μg/ml for pseudo-ephedrine and nor-ephedrine and 3.0–20 μg/ml for nor-pseudo-ephedrine, showing to be linear with $r^2>0.99$ for all ephedrines. Quantification was achieved using peak

area ratios of the ephedrines to internal standard. For the quantification of pseudo-ephedrine, nor-ephedrine, nor-pseudo-ephedrine the most abundant ion *m/z* 179 was used, whereas for ephedrine and d_3-ephedrine ions *m/z* 227 and 230 were used, respectively. The signal-to-noise ratio of the above ions was greater than 10:1.

Precision (% RSD) within day ranged from 2.0 to 7.0% for all ephedrines at a concentration of 10 μg/ml ($n=10$). Precision (% RSD) between days ranged 1.6–2.9% for ephedrine, 2.1–3.8% for pseudo-ephedrine, 3.3–9.4% for nor-ephedrine, 4.0–14% for nor-pseudo-ephedrine and 2.9–7.7% for methyl-ephedrine, calculated from all standards of the five calibration curves.

The accuracy of the method was calculated taking into consideration the mean analytical error of the calibration curves for all ephedrines. Mean analytical errors were found to be 2.0, 3.0, 5.6, 6.1 and 3.9% for ephedrine, pseudo-ephedrine, nor-ephedrine, nor-pseudo-ephedrine and methyl-ephedrine, respectively.

In order to calculate analytical recovery, relative areas obtained from fortified urine samples with ephedrines and analyzed according to procedure Section 2.3, were compared to relative areas obtained from ephedrine standard solutions, in which only evaporation and derivatization were carried out. Analytical recovery ranged from 86 to 119%.

3.4. Application of the method

In this paper, a validated, simple GC–MS method is presented for the confirmation and quantification of ephedrines in urine samples subjected to doping control analysis. Results can be obtained within 24 h. Positive urine samples containing ephedrines, which were obtained from competitors in sporting events, were analyzed by this method and very good results were obtained in comparison to quality control samples (accuracy, % RSD<10%), which were included in the analysis. Since the concentrations of these ephedrines are usually higher than 5.0 μg/ml, the products were easy to detect. Several positive urine samples were analysed and the method has been shown no interference from endogenous compounds in urine or from a variety of drugs with similar structure.

Acknowledgements

M-E. S. is grateful to the Greek State Scholarships Foundation for financial support.

References

[1] L. Lasagna, in: Phenylpropanolamine. A Review, Wiley, New York, 1988.
[2] D. Palm, in: P. Bellotti, G. Benzi, A. Ljungqvist (Eds.), Official Proceedings of the International Athletic Foundation World Symposium on Doping in Sport, Florence, 1987, p. 111.
[3] International Olympic Committee Medical Code and Explanatory Document, International Olympic Committee, Lausanne, 1997.
[4] P. Hemmersbach, R. de la Torre, J. Chromatogr. B 687 (1996) 221.
[5] Imaz, D. Carreras, R. Navajas, C. Rodriguez, A.F. Rodriguez, J. Maynar, R. Cortes, J. Chromatogr. 631 (1993) 201.
[6] P.J. van der Merwe, S.E. Hendrikz, J. Chromatogr. B 663 (1995) 160.
[7] J.M. Halket, Derivatives for gas chromatography–mass spectrometry, in: K. Blau, J.M. Halket (Eds.), Handbook of Derivatives for Chromatography, Wiley, New York, 1993.
[8] M. Donike, J. Chromatogr. 103 (1975) 91.
[9] E. Brendel, I. Meineke, E.-M. Henne, M. Zschunke, C. De Mey, J. Chromatogr. 426 (1988) 406.
[10] J.K. Taylor, in: Quality Assurance of Chemical Measurements, CRC Press, Boca Raton, FL, USA, 1987.