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Quantification of ephedrines in urine by column-switching high-performance liquid chromatography

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Abstract

A method for the quantification of five congener ephedrines in urine samples without sample preparation was developed. The analytes were trapped on a C_{18} precolumn and separated on a C_{18} BDS analytical column. Baseline separation was achieved for all analytes. The method meets the requirements of the International Olympic Committee (IOC) medical commission regarding cut-off limits for positive doping cases with ephedrines. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Ephedrines

1. Introduction

Ephedrines are classified as prohibited substances according to the International Olympic Committee (IOC) list of prohibited classes of substances due to their stimulating potency to the central nervous system. As they are contained in many pharmacological preparations commonly used for influenza, asthma, colds, etc. the IOC has set threshold levels for each of these substances above which a doping sample is considered positive.

The relevance of this class of substances for doping purposes is best shown by the latest IOC statistics of positive cases of 1999. A total of 375 out of 532 (70%) positive cases for stimulating agents were caused by ephedrines, with pseudoephedrine being the most frequently misused stimulant. A reliable and cost-effective quantification method for these compounds is therefore of the utmost interest to doping control laboratories.

Usually ephedrines are determined by gas chromatography and nitrogen or mass selective detection [1–3]. Derivatisation as silyl- or fluoracetylderivatives after extractive clean-up leads to excellent separation and reliable quantification results. High-performance liquid chromatography (HPLC) with appropriate sample clean-up to remove disturbing matrix compounds is a viable alternative [4,5].

Anyway, sample clean-up is a time-consuming step and the steadily increasing number of samples calls for as much automatisation as possible. Column switching techniques offer a high potential in this respect, reducing the need for personal resources and simultaneously increase the reliability of analysis [6,7].

This paper presents a column-switching HPLC

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method for the quantification of all five ephedrines contained in the IOC list of prohibited substances. The analytes are extracted on a precolumn and subsequently back-flushed to the analytical column. Besides aliquoting and the addition of internal standards, no further sample preparation is necessary.

2. Experimental

2.1. Materials and equipment

Cathine (norpseudoephedrine) and ephedrine were kindly provided by Knoll AG (Ludwigshafen, Germany), methylephedrine by Klinge Pharma (Munich, Germany) and pseudoephedrine by Glaxo Welcome (Greenford, UK). Norephedrine (phenylpropanolamine) and bamethan were purchased from Sigma (St. Louis, MO, USA) and etilefrine from Boehringer Ingelheim (Vienna, Austria). All those reference substances were certified and had more than 99% purity. Acetonitrile and methanol (Scharlau, Barcelona, Spain) and sulphuric acid (Merck, Darmstadt, Germany) were of HPLC-grade. Purified water was obtained by a Milli-Q reagent-grade water system (Millipore, Bedford, MA, USA).

2.2. Column-switching procedure

The HPLC system consisted of a Model AS3000 autosampler (AS), a Model P4000 quaternary pump (P1), a Model UV6000L diode array detector (DAD; all components from Thermo Quest, Vienna, Austria) and a six-port valve (V; VICI AG, Schenkon, Switzerland). The system was controlled by a ChromQuest data system (Thermo Quest, Vienna, Austria). Pump 2 (P2) was a Model 112 solvent delivery module (Beckman, San Ramon, CA, USA). Fig. 1 shows the back flush arrangement.

The precolumn (PC) was filled with Hypersil ODS C_{18} adsorbent (3-μm particle size, 20×3 mm) and the analytical column (AC) with Hypersil BDS C_{18} adsorbent (3-μm particle size, 150×3 mm). Both columns were prepared in the laboratory.

2.2.1. Sample loading mode

Water (solvent 1) was delivered by P2 at 1 ml/min for sample loading. AC was flushed by P1 at 1 ml/min with 0.1% of sulphuric acid containing acetonitrile (3%) (solvent 2). After injection of a 5-μl sample volume, the PC was washed for 5 min and the matrix was directed into the waste (W).

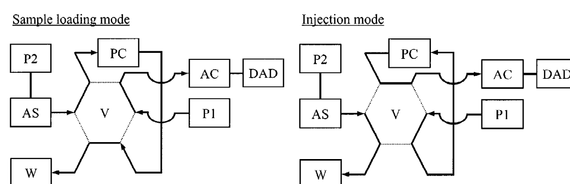

Fig. 1. Back flush arrangement. Abbreviations are given in the text.

2.2.2. Injection mode

By switching the valve (V), the components retained on C1 were back flushed by P1 delivering solvent 2 for successive isocratic separation on AC. The next run was prepared by switching back the valve after 20 min with a re-equilibration period of 5 min.

The diode array detector was set to 205 and 214 nm with additional scan from 195 to 280 nm at a 1-Hz scanning rate. The AC was maintained at 35°C.

2.3. Sample preparation

About 1 ml of the urine sample was placed in an autosampler vial and spiked with 10 μl of a methanolic solution of the internal standards, etilefrine (IE) and bamethan (IB) (1 mg/ml each), to give a total concentration of 10 μg/ml urine. For calibration purposes, urine was spiked with ephedrines at five concentration levels as shown in Table 1 (all concentrations are given in μg/ml). The lower working range limit (LWR) corresponds to about 0.25 times

Table 1
Quantification information

Compound	IOC cut-off (μg/ml)	LWR (μg/ml)	UWR (μg/ml)
Norephedrine (NE)	25	6.18	123.5
Cathine (CA)	5	1.17	23.4
Ephedrine (EP)	10	2.64	52.8
Pseudoephedrine (PE)	25	6.23	124.5
Methylephedrine (ME)	10	2.48	49.7

LWR=Lower working range limit, UWP=upper working range limit.

the IOC cut-off limit, the upper working range limit (UWR) to approximately five times the IOC limit.

Etilefrine and bamethan were not used for quantitative purposes but as retention time markers only to indicate the retention window of all five determined ephedrines. As the volume taken for analysis was determined by the autosampler's injection syringe setting, no manual aliquoting of the urine sample is required.

3. Results and discussion

3.1. General considerations

To guarantee optimal reproducibility of the retention times, the HPLC columns were thermostated at 35°C. The injection volume of 5 μl of urine yielded sufficient peak areas in the target concentration range. No carry over from previous injections was visible even at the upper limit of the working range.

Taking into consideration the potential pH range of real urine samples, the recovery of all target analytes and the internal standards was checked at pH 5, 6, 7, 8 and 9. For this experiment, phosphate buffer solutions were spiked at about the IOC cut-off level. Fig. 2 represents the averages of three consecutive determinations.

No significant pH dependence of the absolute areas was recorded, only a small decrease in the absolute area of the signal for etilefrine was noticeable.

3.2. Selection of precolumns and analytical columns

Two different stationary phase materials, octadecylsilica (ODS) and base deactivated octadecylsilica (C_{18} BDS) were tested as well for analyte extraction on the precolumn as for separation on the analytical column. A particle size of 3 μm was chosen in any instance.

3.2.1. Precolumn

Both materials showed quantitative recoveries of the target analytes. The BDS stationary phase material exhibited higher background signals due to less specificity for ephedrines than the ODS precolumn chosen for further method optimisation.

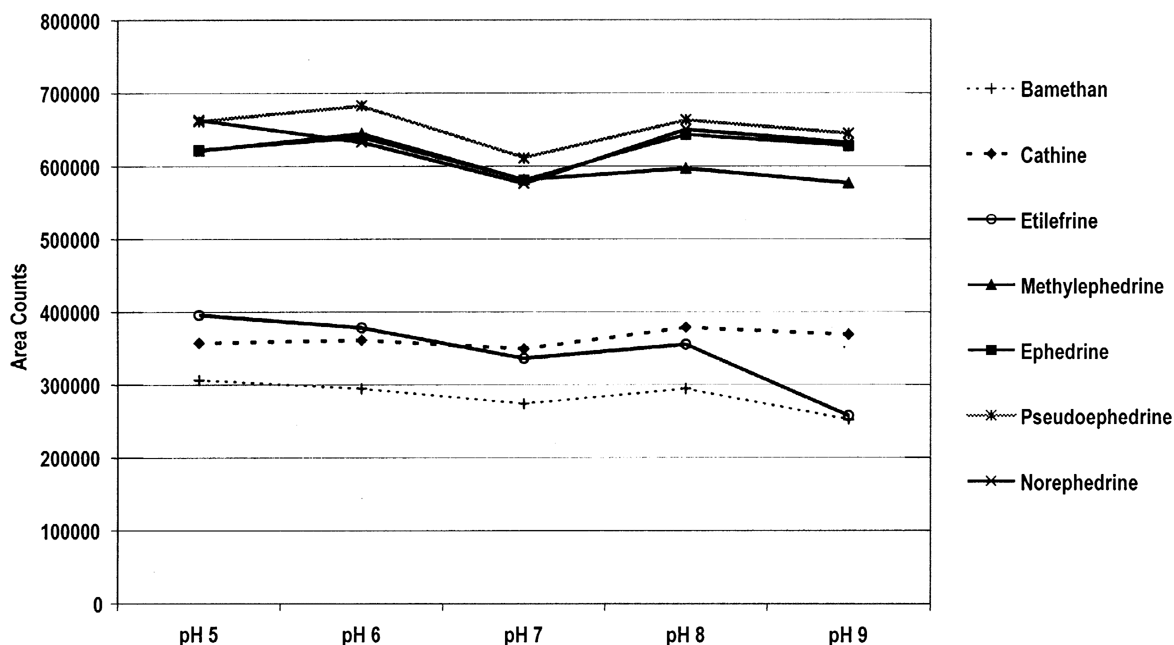

Fig. 2. Dependence of the areas of the target analytes as function of pH.

3.2.2. Analytical column

BDS showed better separation due to less peak tailing, especially in the case of ephedrine and pseudoephedrine. Fig. 3 shows a chromatogram of the five target ephedrines and both internal standards with their retention times as indicated. The concentrations given in the figure reflect the IOC cut-limits for positive cases. All substances are baseline separated, exhibiting symmetrical peaks. As already mentioned, etilefrine and bamethan are used for the determination of the retention time window and for calculation of the relative retention times and not for quantification purposes.

3.3. Composition of solvents

To study possible interferences by matrix compounds, about 40 urine samples were analysed by the described method as matrix blanks. Some of these exhibited a rather high background, mainly in the region of norephedrine and cathine (as can be seen in the example shown in Fig. 4). The most abundant signals are labelled with M1–M4.

A straightforward possibility to separate these matrix compounds from the target analytes consists in varying the acetonitrile concentration in the eluent mixture between 2 and 4%, which changes the retention behaviour of the matrix compound compared to the ephedrines: With 2% acetonitrile in 0.1% sulphuric acid, the matrix peaks M1–M3 move behind cathine, resulting in rather undisturbed signal in the norephedrine/cathine region. With 4% acetonitrile, some of these peaks are shifted to just in front of norephedrine.

M4 reacts in the opposite way: with 2% acetonitrile this peak approaches methylephedrine, whereas 4% acetonitrile in 0.1% sulphuric acid moves it even behind bamethan (see Figs. 5 and 6).

3.4. Figures of merit

To describe quantitatively the performance of the entire procedure, certain analytical quality criteria

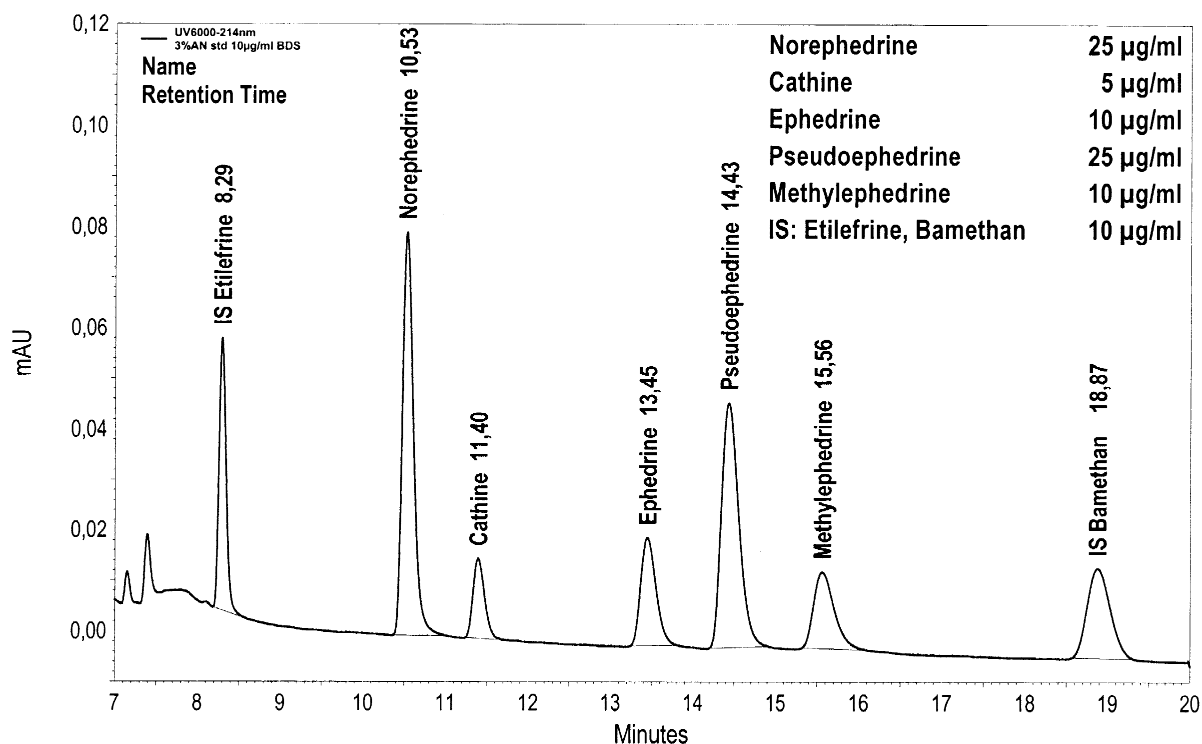

Fig. 3. HPLC chromatogram of a water sample spiked with ephedrines. Analytical conditions are given in Section 2.

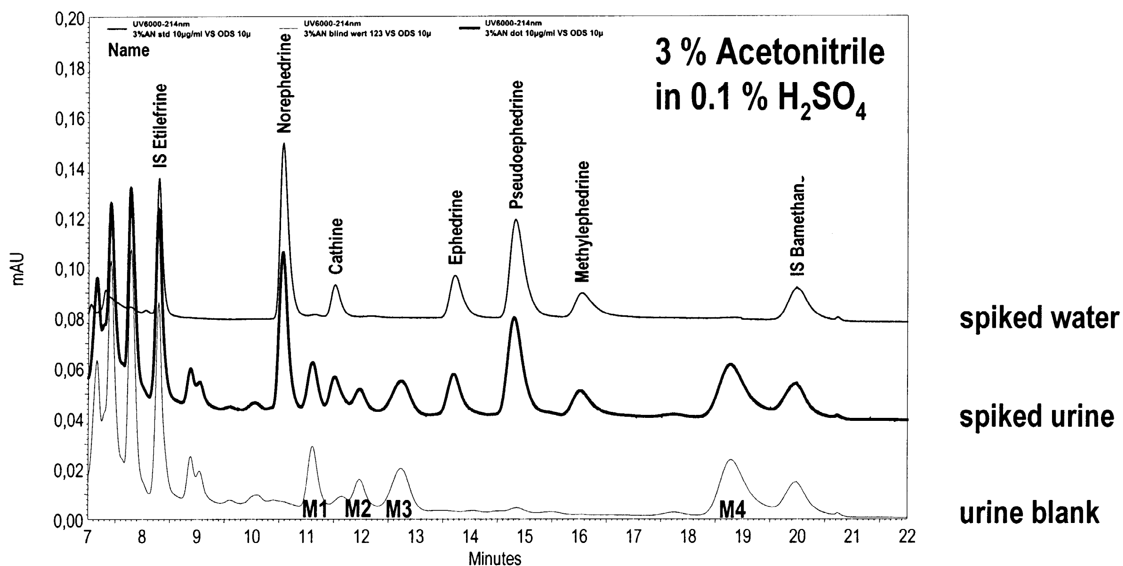

Fig. 4. Matrix background of urine: comparison of blank urine, spiked urine and spiked water samples. M1–M4: Matrix compounds; eluent: 3% acetonitrile in 0.1% sulphuric acid. For detailed analytical conditions see Section 2.

were determined [8]. In Table 2, the limit of quantification, correlation coefficient, relative standard deviation and the recovery are given. The recoveries (column *R* in Table 2) were determined by relating the results obtained from the spiked urine to spiked water samples.

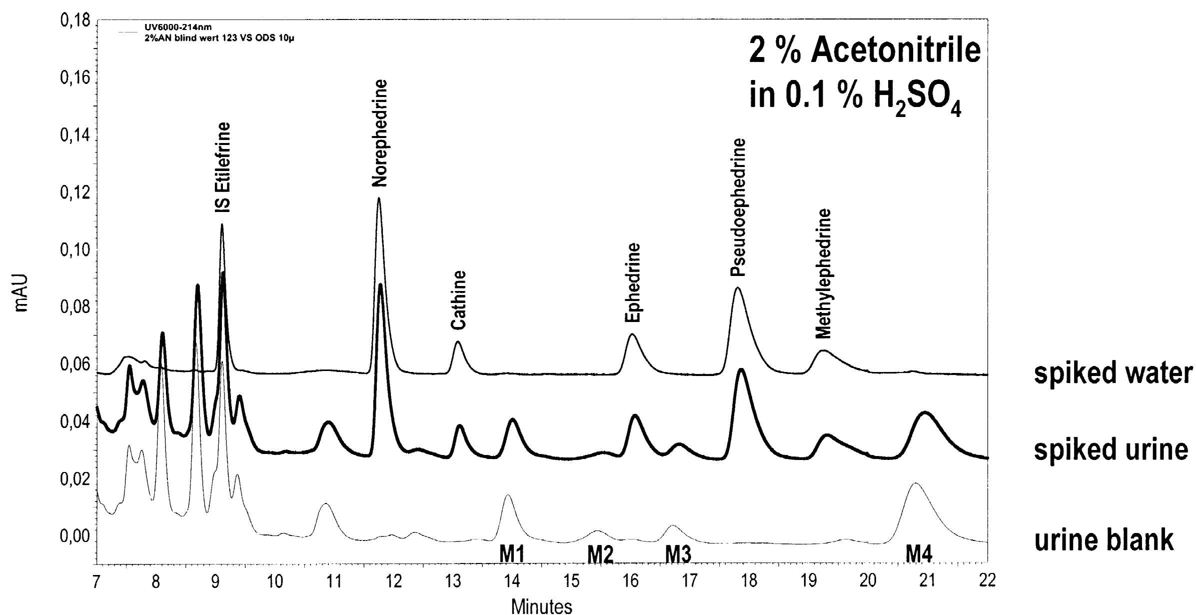

Fig. 5. Matrix background of urine: comparison of blank urine, spiked urine and spiked water samples. M1–M4: Matrix compounds; eluent: 2% acetonitrile in 0.1% sulphuric acid. For detailed analytical conditions see Section 2.

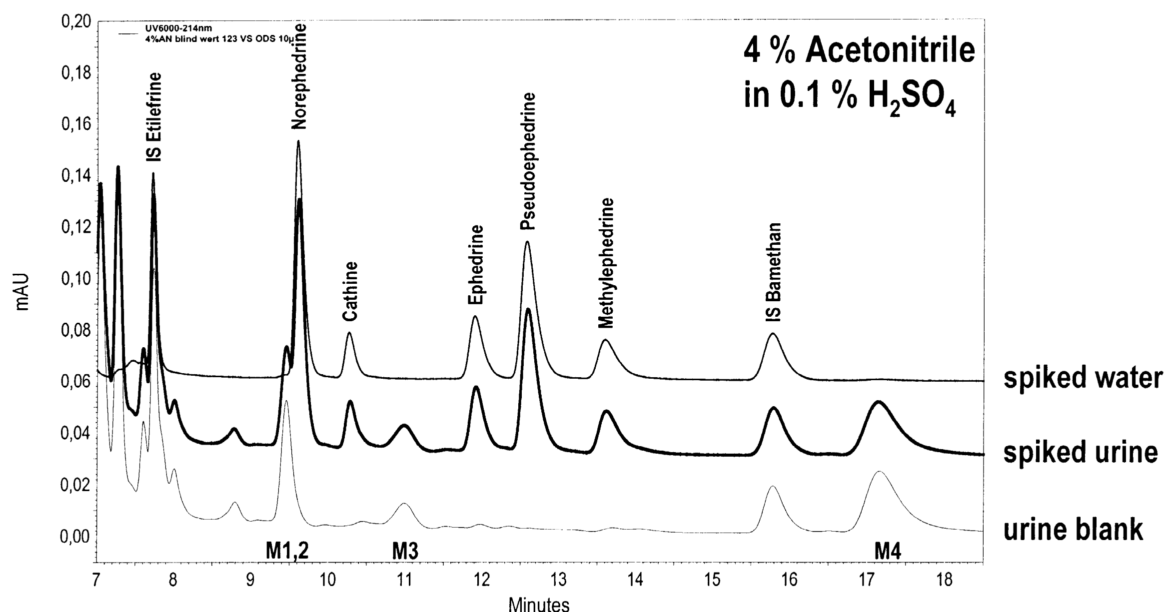

Fig. 6. Matrix background of urine: comparison of blank urine, spiked urine and spiked water samples. M1–M4: Matrix compounds; eluent: 4% acetonitrile in 0.1% sulphuric acid. For detailed analytical conditions see Section 2.

Taking into account the working range as outlined in Table 1, all limits of quantification are well beyond the actual IOC cut-off limits, with standard deviations lower than 6% for all target compounds. As evidenced by the correlation coefficients, linearity is very good. Likewise, nearly quantitative recovery compared to spiked water samples is achieved.

In a laboratory comparison test, the pseudoephedrine concentration obtained by our method to amounted to 99% of the mean value of all participating laboratories, being well within the relative standard deviation of ±7% [9]. These results may be considered as a further proof for the accuracy and precision of the method.

3.5. Relative retention behaviour of selected drugs

To investigate potential interference, some drugs with similar chemical or analytical properties were analysed with the presented method. Table 3 shows a list of the examined compounds. For all substances eluting in the monitored time window, their retention times, absolute and relative to ephedrine, are given.

4. Conclusion

By the presented method, all five ephedrines appearing in the IOC list of prohibited substances

Table 2
Figures of merit

Compound	IOC cut-off (μg/ml)	LOQ (μg/ml)	SDP (%)	CC	*R* (%)
Norephedrine (NE)	25	18.0	5.3	0.9987	97
Cathine (CA)	5	2.3	2.9	0.9998	95
Ephedrine (EP)	10	5.5	3.8	0.9993	98
Pseudoephedrine (PE)	25	13.2	5.2	0.9990	97
Methylephedrine (ME)	10	6.2	3.9	0.9990	101

LOQ=Limit of quantification, SDP=standard deviation of the entire procedure, CC=correlation coefficient, *R*=recovery.

Table 3
Retention times and relative retention times of selected drugs to ephedrine

	RT	RRT
Acebutolol	–	–
Alprenolol	–	–
Amphetamine	14.94	1.26
Atenolol	11.71	0.98
Betaxolol	–	–
Bisoprolol	–	–
Bunolol	–	–
Caffeine	22.6	1.90
Dimetamphetamine	–	–
Ephedrine	11.89	1.00
Ethylephedrine	–	–
Labetalol	–	–
Mepindolol	18.64	1.57
Methamphetamine	18.5	1.56
Metoprolol	–	–
Morphine	8.05	0.68
Nadolol	–	–
Octapamine	–	–
Oxprenolol	–	–
Penbutolol	–	–
Phentermine	–	–
Pindolol	–	–
Propranolol	–	–
Salbutamol	10.45	0.88
Sotalol	11.02	0.93
Synephrine	–	–
Terbutaline	10.01	0.84
Timolol	–	–
Tyramine	–	–

can be quantified. The limits of quantification are well below the cut-off limits decreed by the IOC medical commission. The relative standard deviations of the entire method are lower than 6% and the recoveries better than 95%. The method proved to be reliable, accurate and precise. Interfering matrix components can be disposed of by changing the solvent composition. Further potential applications of the method were anticipated by investigating the relative retention behaviour of related pharmacological compounds.

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