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Quantification of ephedrines in urine without sample preparation

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Abstract

An HPLC method for the quantification of five congener ephedrines in urine samples without sample preparation is presented. The analytes are trapped on a C18 precolumn and separated on a C18 BDS analytical column. Baseline separation is achieved for all analytes. The method meets the requirements of the IOC medical commission regarding cut-off limits for positive doping cases with ephedrines. The presented method can easily be modified for the quantification of caffeine and salbutamol.

1 INTRODUCTION

Ephedrines are classified as prohibited substances according to the IOC - list due to their stimulating potency on the central nervous system. As many pharmaceutical preparations commonly used for influenza, asthma, colds etc. contain ephedrines the IOC has set threshold levels for 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. Indeed 375 out of 532 (70%) positive cases for stimulats 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 utmost interest to doping control laboratories.

Usually ephedrines are determined by gas chromatography and nitrogen or mass selective detection[1,2,3]. Derivatisation as silyl- or fluoracetyl derivatives 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 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. The scope of this method is shown by its extension to the analysis of salbutamol, a ß2-agonist with stimulating and anabolic effects, and of caffeine. Both analytes can be quantified without changing the system.

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, United Kingdom). Norephedrine (phenylpropanolamine), caffeine, bamethan was purchased from Sigma (St. Louis, MO) 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 Thermo Quest, Vienna, Austria) and a 6-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).

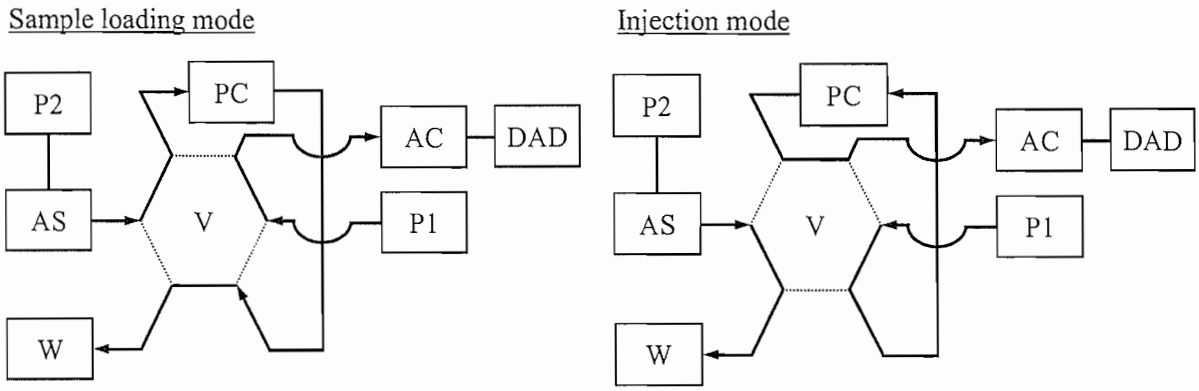

Fig. 1: Back flush arrangement; abbreviations are described in the text.

The precolumn (PC) was filled with Hypersil ODS C18 adsorbent (3 µm particle size, 20 mm x 3 mm) and the analytical column (AC) with Hypersil BDS C18 adsorbent (3 µm particle size, 150 mm x 3 mm). Both columns were prepared in the laboratory.

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 3 % of acetonitrile (solvent 2). After injection of 5 µl sample volume, PC was washed for 5 min, the matrix was directed into the waste (W).

Injection mode: By switching the valve (V), the components retained on PC 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 at 205 nm and 214 nm with additional scan from 195 - 280 nm at 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 internal standards etilefrine (IE) and bamethan (IB) (1 mg/ml each) to give a total concentration of 10 µg/ml urine. For calibration purposes water was spiked with ephedrine at five concentration levels. The lower working range limit (LWR) corresponds to about 0.25 times the IOC cut-off limit, the upper working range limit (UWR) to approximately 5 times the IOC limit.

For method validation the same concentration levels were prepared in urine. Etilefrine and bamethan are 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 is

determined by the autosampler's injection syringe setting, no manual aliquoting of the urine sample is required.

3 RESULTS AND DISCUSSION

Fig. 2 shows a chromatogram of the five target ephedrines and both external standards with their retention times as indicated. The concentrations reflect the IOC cut-off limits for positive cases. All substances are baseline separated exhibiting symmetrical peaks. As already mentioned, etilefrine and bamethan are used for determination of the retention time window and for calculation of the relative retention times and not for quantification purposes.

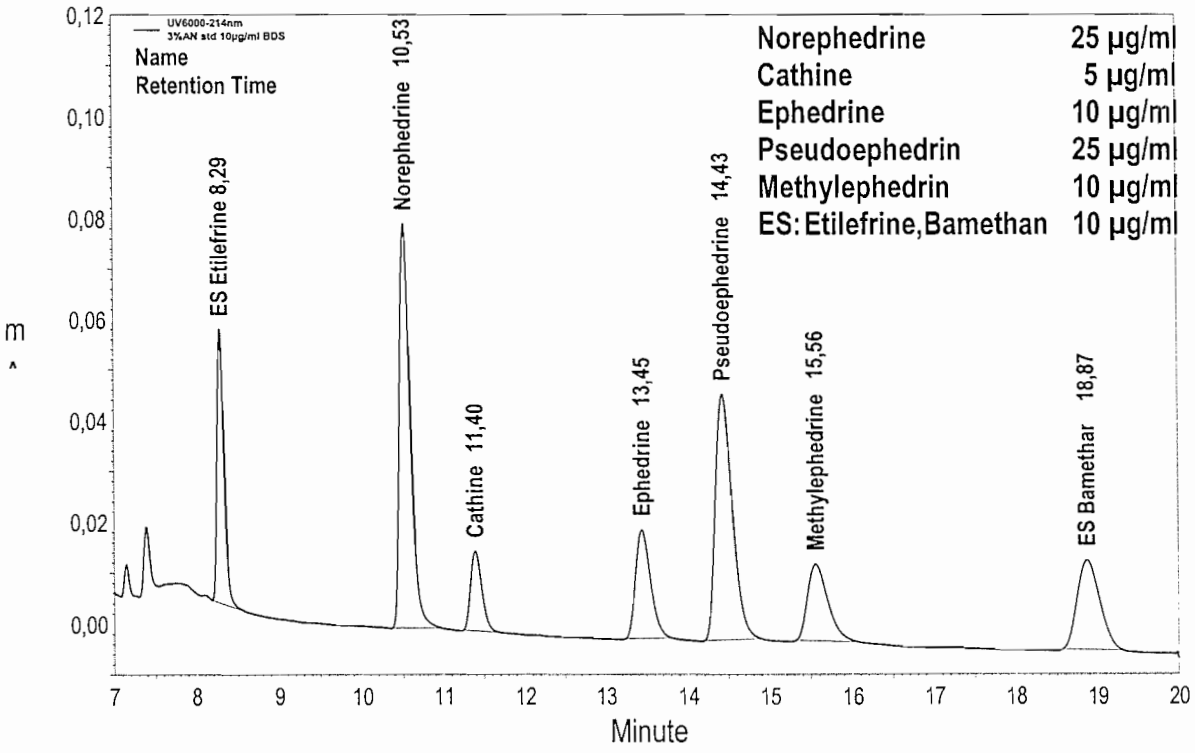

Fig. 2: HPLC - chromatogram of a water sample spiked with ephedrines. Analytical conditions are given in section 2.2.

To study possible interferences by matrix compounds, about 40 urine samples from an occupational monitoring program were analysed by the described method as matrix blanks. Some of these exhibit a rather high background, mainly in the region of norephedrine and cathine (as can be seen in the example shown in Fig. 3). The most abundant signals are labelled with M1 - M4.

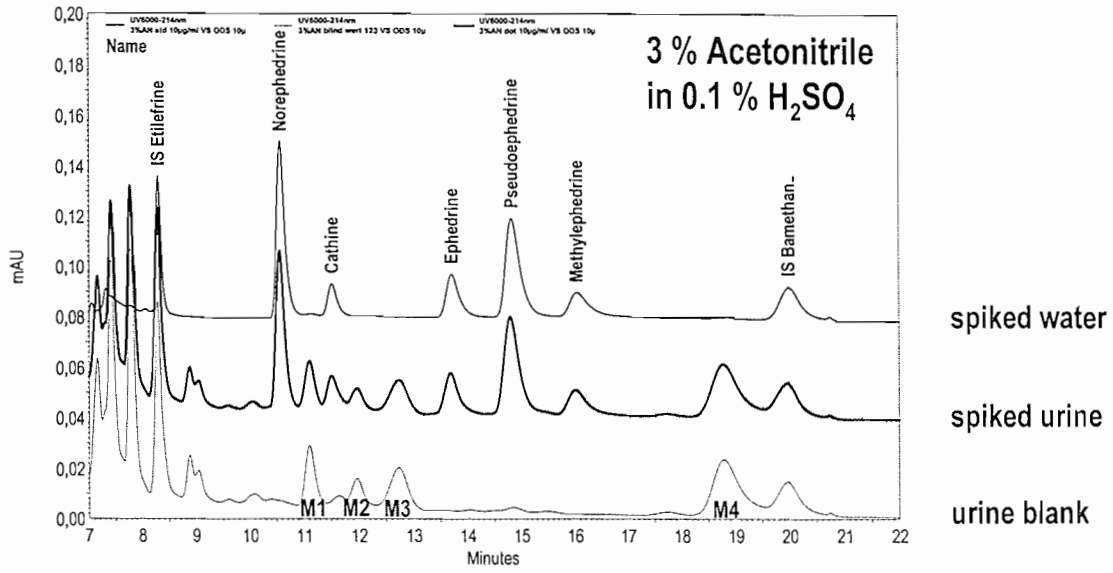

Fig. 3: Matrix background of urine: comparison of blank urine, spiked urine and spiked water; M1-4: matrix compounds; eluent: 3 % acetonitrile in 0.1 % sulphuric acid; for detailed analytical conditions see section 2.2.

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 - 3 elute behind cathine, resulting in a rather undisturbed signal in the norephedrine / cathine - region while with 4 % of acetonitrile some of these peaks move 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 4 and 5).

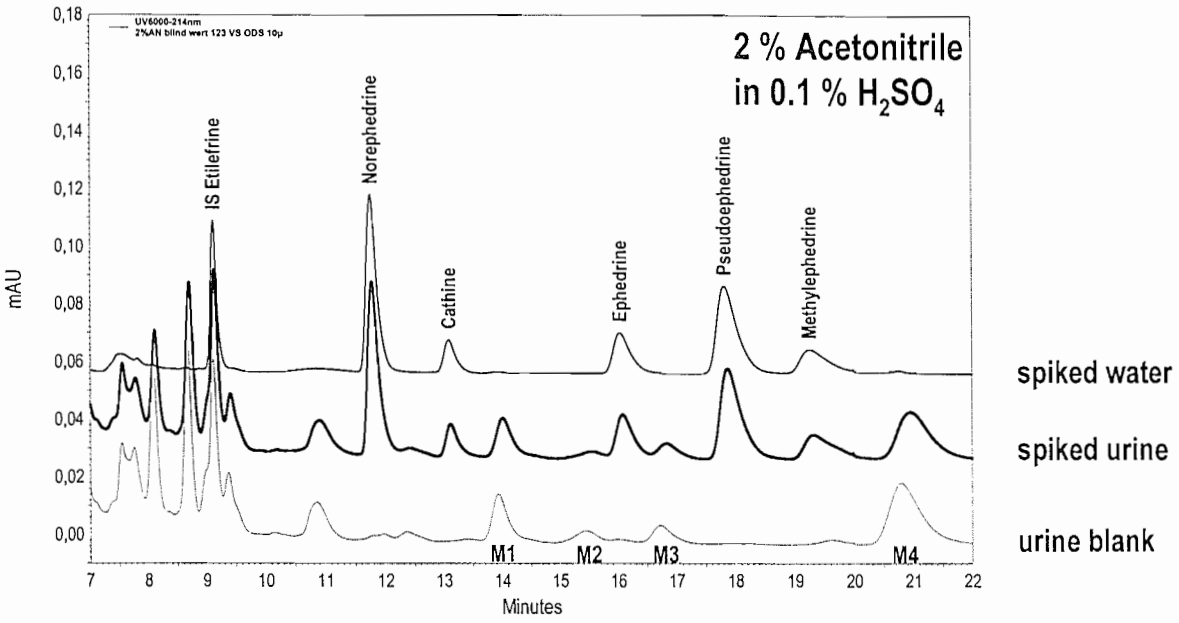

Fig. 4: Matrix background of urine: comparison of blank urine, spiked urine and spiked water; M1-4: matrix compounds; eluent: 2 % acetonitrile in 0.1 % sulphuric acid; for detailed analytical conditions see section 2.2.

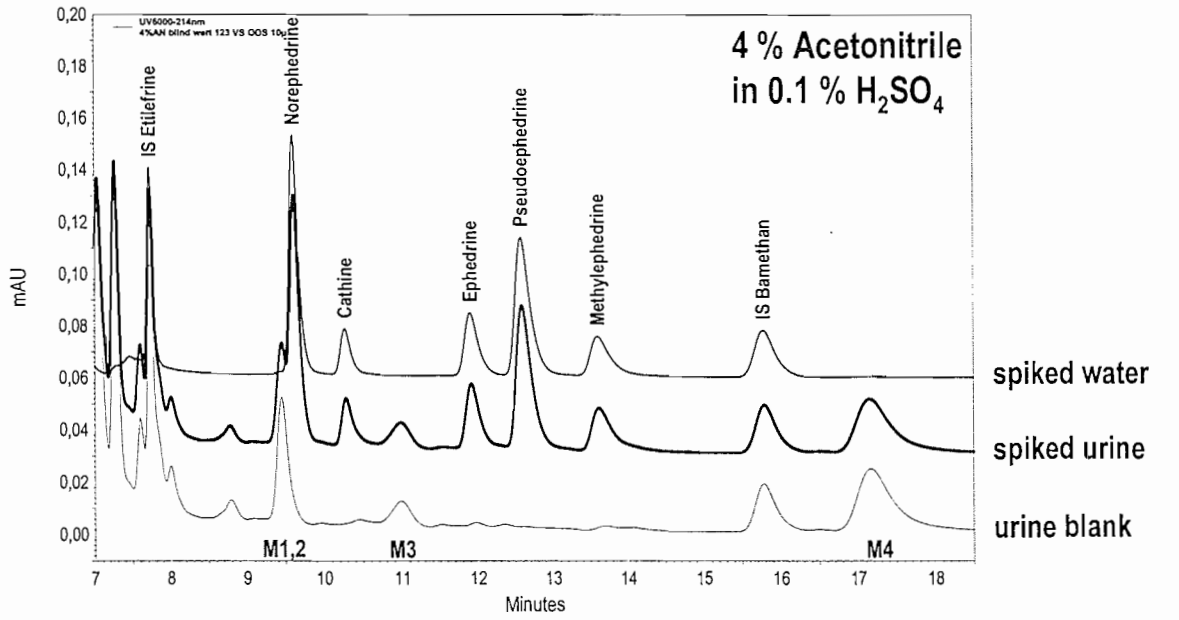

Fig. 5: Matrix background of urine: comparison of blank urine, spiked urine and spiked water; M1-4: matrix compounds; eluent: 4 % acetonitrile in 0.1 % sulphuric acid; for detailed analytical conditions see section 2.2.

In order to valueta the method, certain analytical quality criteria were determined[8]: In Table 1, the limit of quantification, the correlation coefficient, the relative standard deviation, and the recovery are given. The recoveries (column R in Table 1) were determined by relating the results obtained from the spiked urine to spiked water samples.

Table 1: Validation

Compound	IOC cut-off µg/ml	LOQ µg/ml	RSD (%)	CC r	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
Caffeine	12	2.3	3.2	0.9990	101

LOQ: limit of quantification; RSD: Relative standard deviation; CC: correlation coefficient; R: recovery

For ephedrines, all limits of quantification are well beyond the IOC cut-off limits, with relative 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.

According to Gotzmann et al.[9] the column switching arrangement was used for the quantification of caffeine, too. In this case, the mobile phase was changed to a gradient consisting of water and acetonitrile, as mentioned in the literature. Fig. 6 shows the analytical quality of the method by comparing spiked water, spiked urine and a blank urine sample. Figures of merit are presented in Table 1.

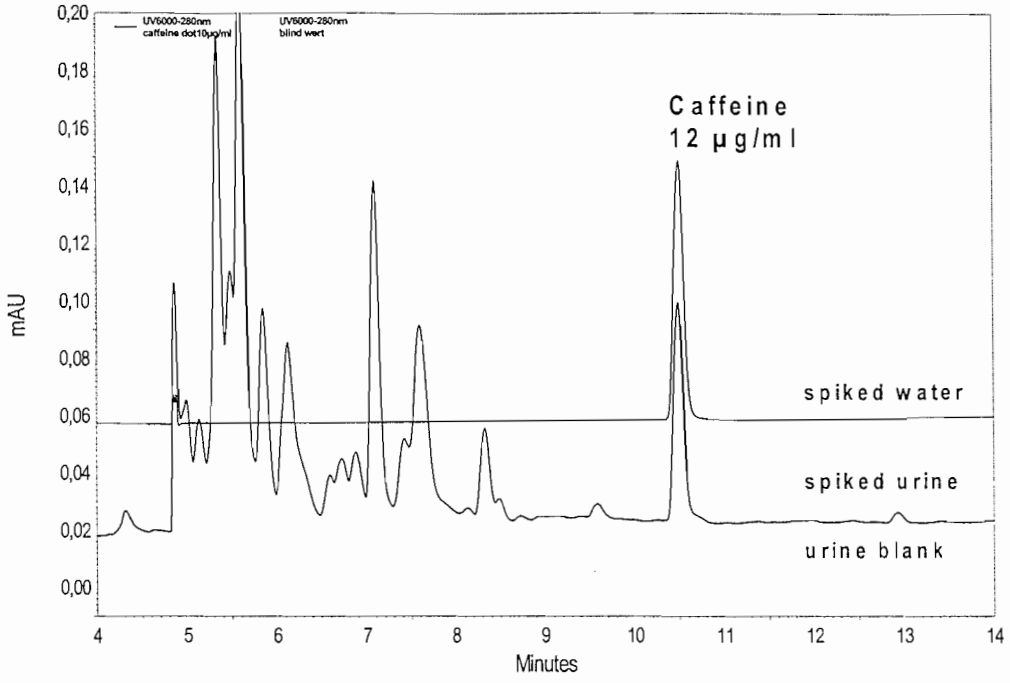

Fig. 6: Quantification of caffeine: comparison of spiked water, spiked urine and blank urine.

4 CONCLUSION

By the presented method, all five ephedrines appearing in the IOC list of prohibited substances can be quantified. The limits of quantification are well below the IOC cut-off limits. The relative standard deviations are lower than 6 % and the recoveries better than 95%. The method proved to be reliable. 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 %[10]. These results may be considered as a further proof for the accuracy and precision of the method.

5 REFERENCES

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