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Short communication

## Simultaneous quantification of ephedrines in urine by high-performance liquid chromatography

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### Abstract

A high-performance liquid chromatographic method for the simultaneous determination of ephedrine, pseudoephedrine, phenylpropanolamine, norpseudoephedrine and methylephedrine in urine is described. Samples were extracted at basic pH into diethyl ether and then back extracted into a small volume of acetic acid. These extracts were chromatographed on a Phase Sep Spherisorb ODS 1 column with tetraethylammoniumphosphate–methanol mobile phase and the analytes were detected by UV absorbance at 214nm. Calibration curves were linear over the range 0.5–100  $\mu\text{g/ml}$  for each analyte. The selectivity of the method was demonstrated for several drugs found with the ephedrines in pharmaceutical formulations. This method has an excellent accuracy, precision and recovery for all the ephedrine at the cut-off concentrations as set by the IOC for a positive doping case and can thus be used to quantify these ephedrines when present in the urine obtained from sportspersons.

### 1. Introduction

Ephedrine and its congeners pseudoephedrine, norephedrine (phenylpropanolamine), norpseudoephedrine and methylephedrine are all potential central nervous system stimulants and are therefore forbidden by the International Olympic Committee (IOC) for sports participation. These ephedrines are ingredients of many medicines commonly used for flu, hay fever, colds, rhinitis etc., and the IOC has set permitted levels below which the presence of these ephedrines in urine will not be regarded as a positive doping case. These limits are 5  $\mu\text{g/ml}$  for ephedrine, norpseudoephedrine and methyle-

phedrine and 10  $\mu\text{g/ml}$  for norephedrine and pseudoephedrine.

It is therefore necessary to have a fast, reliable and selective method available for the simultaneous quantitation of the mentioned ephedrines. Various methods have been published for the quantitation of one or two of these ephedrines in urine at a time. These methods include gas chromatography (GC) with electron-capture detection [1–4], gas chromatography with nitrogen-specific detection [5], all after derivatisation prior to GC analysis, and high-performance liquid chromatography [6–8]. Sagara et al. [9] described a method for the simultaneous detection of norephedrine, pseudoephedrine, ephedrine and methylephedrine but the method is only suitable for pharmaceutical preparations and not

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for urine. Imaz et al. [10] quantified the ephedrine simultaneously by high-performance liquid chromatography but they evaporate the samples to dryness after extraction which may cause some problems.

The purpose of this study was to develop a fully validated method for the simultaneous determination of all the ephedrine in urine without evaporation or derivatisation.

## 2. Experimental

### 2.1. Materials

Ephedrine-HCl, pseudoephedrine-HCl, norephedrine-HCl, norpseudoephedrine-HCl, methylephedrine and ethylephedrine were all from our departmental reference substance collection. Tetraethylammoniumhydroxide (TEAH, 20% in water), acetic acid, phosphoric acid and methanol were all reagent grade (Merck, Darmstadt, Germany). Water was purified and deionized using a Milli-Q reagent-grade water system (Millipore, Bedford, MA, USA).

### 2.2. Apparatus

A modular high-performance liquid chromatographic system consisting of a Series 1050 Hewlett-Packard pump and auto-injector (Hewlett-Packard, Avondale, PA, USA) was used. A Phase Sep Spherisorb ODS 1 column, 5  $\mu\text{m}$  particle size, 150  $\times$  4.6 mm I.D., was coupled to the system. The column effluent was monitored with an ultraviolet absorbance detector (Waters Assoc., Milford, MA, USA) operated at 214 nm. The results were processed using a Hewlett-Packard Model HP3396A integrator.

### 2.3. Chromatography

The mobile phase consisted of tetraethylammoniumphosphate (TEAP)-methanol (98:2, v/v). The buffer was prepared by adding 15.54 g TEAH and 2.8 g phosphoric acid (89%) and made up to a final volume of 500 ml with water. The pH of the buffer was 2.8. A constant flow-

rate of 1.0 ml/min was maintained through the HPLC column at ambient temperature.

### 2.4. Standards

The internal standard solution contained 1 mg of ethylephedrine in 1 ml of methanol. A stock solution A containing ephedrine, pseudoephedrine, norephedrine, norpseudoephedrine and methylephedrine was prepared at a concentration of 2 mg/ml methanol of each substance. Stock solution A was diluted to give stock solution B with a concentration of 0.1 mg/ml methanol. These stock solutions were spiked to blank urine to produce 11 calibration standards covering the range 0.5–100  $\mu\text{g/ml}$ .

### 2.5. Extraction procedure

To 1 ml of urine was added 40  $\mu\text{l}$  of internal standard solution and 100  $\mu\text{l}$  of 20% NaOH solution in a 5-ml ampoule. The mixture was extracted with 4 ml of distilled diethyl ether by vortex-mixing for 30 s. After centrifugation at 1285 g for 5 min the aqueous phase was frozen and the ether layer decanted to a second ampoule containing 100  $\mu\text{l}$  of 1% acetic acid. The mixture was vortex-mixed again for 30 s, centrifuged and the aqueous layer was frozen. The ether layer was discarded and the remaining ether evaporated under a stream of high purity nitrogen. The acetic acid layer was diluted with 100  $\mu\text{l}$  of mobile phase and a 5- $\mu\text{l}$  aliquot of this mixture was injected onto the HPLC column.

## 3. Results and discussion

### 3.1. Chromatography

Representative chromatograms are shown in Fig. 1 which demonstrate the lack of interfering compounds. All the peaks are symmetrical and well resolved with the following retention times: norephedrine 3.05 min, norpseudoephedrine 3.55 min, ephedrine 4.43 min, pseudoephedrine 5.21 min, methylephedrine 6.10 min and ethylephedrine (internal standard) 9.35 min,

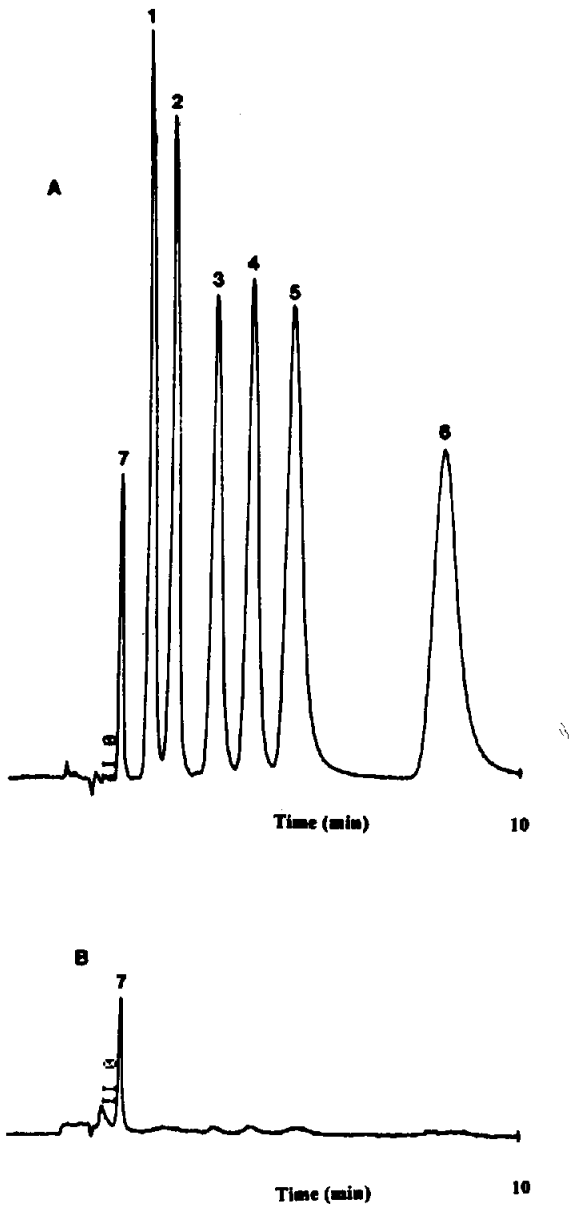


Fig. 1. Chromatograms of urine standard of 20  $\mu\text{g}/\text{ml}$  of each analyte (A) and blank urine (B). Peaks: 1 = norephedrine, 2 = norpseudoephedrine, 3 = ephedrine, 4 = pseudoephedrine, 5 = methylephedrine, 6 = internal standard, 7 = endogenous compound.

respectively. Fig. 2 shows chromatograms of urine extracts obtained from a person who took Colcaps (containing phenylpropanolamine-HCl, phenylephrine-HCl, chlorpheniramine maleate,

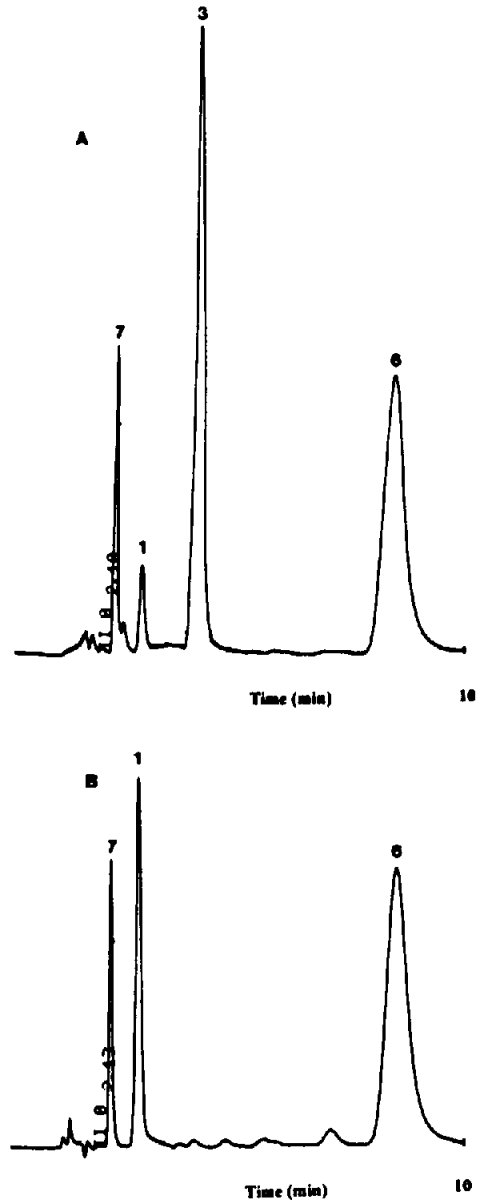


Fig. 2. Chromatograms of urine from a person who took Bronchilate Linctus(A) and a person who took Colcaps (B). Peaks: 1 = norephedrine, 6 = internal standard, 3 = ephedrine, 7 = endogenous compound.

mepyramine maleate, salicylamide and caffeine) and from a person who took Bronchilate Linctus (containing pholcodine, ephedrine-HCl, cetrimide, mepyramine maleate and menthol). The chromatograms demonstrate the absence of any

interferences from these compounds. Positive urine samples obtained from competitors in sporting events were analysed and very good results were obtained.

### 3.2. Quantitation

Peak-heights of the analytes and internal standard were calculated by the integrator. The calibration curves of analyte/internal standard peak-height ratio versus concentration were linear over the range 0.5–100  $\mu\text{g/ml}$  for each analyte, using an unweighted linear least-squares fit. Table 1 shows the slopes, intercepts and regression coefficients of the calculated calibration curves for each analyte (11 standards).

### 3.3. Recovery

The recoveries for all five the ephedrines, obtained with spiked urine using the complete extraction procedure using ethylephedrine as an external standard and external calibration, are given in Table 2. The recovery of internal standard at 40  $\mu\text{g/ml}$  using ephedrine as external standard is also given in Table 2. In all cases  $n = 3$ .

### 3.4. Accuracy and precision

The accuracy and precision of the assay procedure was monitored by preparing quality control samples containing known concentrations of each analyte. The quality control samples were prepared in five-fold in the same fashion as the

Table 1  
Statistical evaluation of the analytical parameters for the calibration curves

Compound	Slope	Intercept	Regression coefficients ( $r^2$ )
Ephedrine	18.34	0.158	0.9995
Pseudoephedrine	21.04	0.363	0.9993
Norephedrine	16.75	0.786	0.9980
Norpseudoephedrine	17.26	0.609	0.9983
Methylephedrine	25.17	0.073	0.9997

Table 2  
Recovery of analytes from urine ( $n = 3$ )

Compound	Added ( $\mu\text{g/ml}$ )	Absolute recovery (%)	C.V. (%)
Ephedrine	40	100	3.1
	20	95	2.2
	2	97	11.7
Pseudoephedrine	40	95	12.3
	20	95	1.5
	2	96	11.5
Norephedrine	40	92	2.6
	20	88	2.8
	2	95	2.4
Norpseudoephedrine	40	81	11.7
	20	90	3.2
	2	88	11.4
Methylephedrine	40	81	11.7
	20	88	1.1
	2	87	11.2
Internal standard	40	93	4.8

standards used for the calibration curves and over the same concentration range.

The quality control specimens and the standards were prepared by two different persons, each using his own stock solutions. The standards and quality control samples were all assayed on the same day by the same analyst. Table 3 shows the within-day validation for the accuracy and precision of the urine quality control samples. The cut-off concentrations as set by the IOC for a positive doping case have a coefficient of variation of 1.0% at 5.2  $\mu\text{g/ml}$  for ephedrine, 8.5% at 8.9  $\mu\text{g/ml}$  for pseudoephedrine, 3.7% at 9.5  $\mu\text{g/ml}$  for norephedrine, 1.4% at 5.2  $\mu\text{g/ml}$  for norpseudoephedrine and 0.6% at 4.5  $\mu\text{g/ml}$  for methylephedrine. From this data it is clear that, by using the present method, all the ephedrines can be quantitated with satisfactory accuracy and precision in doping cases. Therefore the actual limits of quantification were not determined. However from Table 3 it can be deduced that all the ephedrines can easily be quantified down to at least 2.5  $\mu\text{g/ml}$ .

Table 3

Within-day precision and accuracy of the assay for the quality control samples ( $n = 5$ )

Compound	Normal urine concentration ( $\mu\text{g/ml}$ )	Nominal/found (mean) (%)	C.V. (%)
Ephedrine	48.2	98.2	2.0
	9.5	92.0	1.5
	2.7	99.0	0.2
Pseudoephedrine	44.0	96.6	3.5
	8.9	92.2	8.5
	2.5	99.2	1.7
Norephedrine	47.0	99.4	5.3
	9.5	88.4	3.7
	2.7	84.2	1.1
Norpseudoephedrine	47.0	98.0	4.3
	9.5	88.4	2.9
	2.7	87.6	1.8
Methylephedrine	44.8	95.1	1.0
	9.1	92.8	0.8
	2.3	99.5	0.2

### 3.5. Selectivity

The selectivity was tested by injection of several structurally related compounds as well as of common drugs found together with the ephedrine in various formulations, and evaluation of their retention times. The drugs examined for interferences with the assay were: amphetamine, paracetamol, pheniramine, brompheniramine, triprolidine, phenyltoloxamine, codeine, chlorpheniramine, phenylephrine, mepyramine, diphenhydramine, caffeine, dextromethorphan, salicylamide and promethazine. The retention times of all these compounds were either longer than 10 min or no peaks were detected.

### 4. Conclusions

With the method presented here all tested ephedrine can be separated when injected simultaneously. It has the advantage of fast and repeatable extraction without the use of evaporation or derivatisation. The accuracy and precision are satisfactory which makes the method suitable for quantitation. The selectivity is satis-

factory with no interfering endogenous compounds or interferences from other substances occurring with the ephedrine in medicines.

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