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SIMULTANEOUS DETERMINATION OF EPHEDRINES IN URINE BY RP-HPLC

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INTRODUCTION

Ephedrine (EPH), pseudoephedrine (PEPH), norephedrine (NEPH), norpseudoephedrine (NPPH), methylephedrine (MEPH) and ethylephedrine (ETEPH) are all sympathomimetic amines known to have central nervous system stimulating properties and are therefore included in the list of doping substances by the Medical Commission of the International Olympic Committee (MC-IOC).

The MC-IOC has defined concentrations in urine above which these ephedrines (except ETEPH) are considered as a positive doping case. Several chromatographic methods have been reported for the separation and determination of these compounds in urine. The GC methods are time consuming since they require derivatisation prior to analysis (1-5) for obtaining either acceptable resolution between all ephedrines or improved sensitivity. HPLC separations on the other hand, have been performed by using reversed phase packing with mobile phases containing less than 5% organic modifier to achieve sufficient retention and organic amines as masking agent to reduce peak tailing (6-8). However, operating under such "high aqueous" mobile phase conditions can lead to poor chromatographic reproducibility since the alkyl bonded phases of C18 columns undergo "phase collapse" or "matting". The purpose of this study was to develop a fully validated HPLC method for the simultaneous determination of all the ephedrines using a simple mobile phase with a good reproducibility of the retention times. Several parameters affecting separation and elution were developed.

EXPERIMENTAL

1.Apparatus and chromatographic conditions

A High –Performance Liquid Chromatographic system consisting of a LC 10 ATVP SHIMADZU pump with a manual injector (20 µl loop) was used. A phase separation PRISM RP (KEYSTONE SCIENTIFIC) column, 5 µm particle size, 250 x 4.6 mm i.d. and a guard column (5 µm, 10 x 4.6 mm) are coupled to the system. The column effluent was monitored with a UV-VIS detector SPD-10A VP SHIMADZU operating at 215 nm. The results were processed using a SHIMADZU CR6A integrator. The mobile phase consisting to a mixture of phosphate buffer / methanol (95/5, v/v). The buffer was prepared by dissolving adequate amount of KH2PO4 in distilled water. To determine the effect of the buffer pH on the separation of the ephedrines five different eluents were prepared at pHs 2.5, 3.5, 4.5, 5.5, and 6.5. In addition, the influence of phosphate buffer level was studied at a concentrations of 50 mM, 100 mM and 150 mM .The flow rate of the mobile phase was 1.5 ml/min.

2.Preparation of drugs solution and urine calibration standard

Internal standard (IS) solution contained phenypropylamine at 1 mg/ml in methanol. Stock solution of ephedrines were prepared at a concentration of 1 mg/ml in methanol. These Stock solutions were spiked to blank urine to produce calibration standards in the range of 1 – 60 µg/ml.

3.Extraction procedure

Procedure A: to 1 ml of urine, was added 20 µl of IS and 100 µl of 5N NaOH in 10 ml glass tube. The mixture was extracted with 4 ml of diethyl ether by vortex-mixing for 30 s. After centrifugation at 1500 g for 5 min, the ether layer was transferred to a second tube containing 100 µl of 1% acetic acid. The mixture was vortex-mixing again for 30 s and centrifuged. The ether layer was discarded and the acidic layer was diluted with 100µl of the mobile phase. 20 µl aliquot of this mixture was injected onto the HPLC column.

Procedure B:To 1 ml urine, was added 20 µl of IS, 100 µl of 10N NaOH and 4 ml of

diethyl ether, then the urine was saturated with 1 g of sodium sulphate and shaken for 20 min. The tubes were centrifuged at 1500 g for 5 min. The organic layer was transferred to a second tube and evaporated to dryness at room temperature. The residue was dissolved in 200 µl of the mobile phase and 20 µl aliquot of this mixture was injected onto the HPLC column.

RESULTS AND DISCUSSION

1.Extraction procedures comparison

Significantly difference was obtained between the two procedures in terms of both recovery and relative standard deviation (Table 1). Best result were obtained with procedure A. Thus, This extraction method was used for further continuation of the study.

Table 1: Recovery of extractions (n=5).

	Procedure A		Procedure B	
	Recovery (%)	RSD%	Recovery (%)	RSD%
EPH	95.3	3.1	61.4	8.4
NEPH	87.9	2.3	39.6	14.4
PEPH	85.9	5.2	63.4	11.6
NPEPH	97.7	1.6	52.1	11.1
IS	91.1	0.8	52.8	23.1

2.Optimization of the Chromatographic conditions and method validation

The ephedrines were well resolved at a pH higher than 3. However, the increase of both pH and the concentration of the phosphate buffer led to an increase of the retention time of the different ephedrines. On the other hand, a poor reproducibility of the elution time was observed when methanol was used on the mobile phase at a proportion of 3% indicating

thus, that the column underwent a phase collapse while a good result was obtained with a methanol proportion of 5%.

Taking into account the different parameters, the optimum conditions for the separation of ephedrines in the shortest analysis time were potassium phosphate monobasic (50 mM, pH 3.5) – Methanol (95:5 v/v). The complete separation under the selected conditions is depicted in Fig.1.

Three 5 point calibration curves for each substance performed on three different days, were plotted as the peak area ratio versus concentration. Each concentration was injected in diplucate. The linear regression results (Table 2) showed a good linear relationship over a concentration range of 1 – 60 µg/ml for the different components.

Table 2: Linearity parameters.

	Range	Slope	Intercept	R
EPH		0.034	0.017	0.9995
NEPH		0.019	0.029	0.9986
PEPH	1-60 µg/ml	0.032	0.024	0.9982
NPEPH		0.022	0.021	0.9986
MEPH		0.037	0.037	0.9989

Table 3 lists the results of the intra-day and the inter-day variability of the method from samples containing 2, 20 and 60 µg/ml for the different substances. The obtained RSD values indicate a satisfactory results. The recovery of each ephedrine at three concentrations is presented in table 4. The obtained RSD values indicate a satisfactory results.

Table 3: Precision of the method. Variation was estimated by RSD%.

	Repeatability (1day, n=5)			Reproducibility (3 days, n= 15)		
Concentration (µg/ml)	2	20	60	2	20	60
EPH	2.8	0.9	0.6	5.7	2.9	2.9
NEPH	1.3	2.6	1.7	5.6	2.9	2.9
PEPH	2.8	2.5	1.7	5.7	2.4	2.8
NPEPH	3.5	3.6	3.1	4.9	2.8	2.8
MEPH	4.2	3.2	3.2	6.6	1.7	2.6

Table 4 : Accuracy/Recovery (%) of ephedrines quantitation (mean of 3 determinations).

	2 µg/ml	20 µg/ml	60 µg/ml
EPH	104	98.7	96
NEPH	106	104.5	97.4
PEPH	120	105.7	100.1
NPEPH	110	103	100.5
MEPH	109.5	98.8	98.8

The limit of detection at a signal to noise ratio of 3 (S/N = 3) were 1 µg/ml and 0.3 µg/ml for ethylephedrine and all the remaining substances respectively. The corresponding limit of quantitation at a signal to noise ratio of 10 was 1 µg/ml.

CONCLUSION

A simple and accurate HPLC method with good precision has been developed for simultaneous determination of ephedrines. The selectivity is satisfactory with no interfering endogenous compounds or interferences from others substances. The method was applied successfully in doping analysis.

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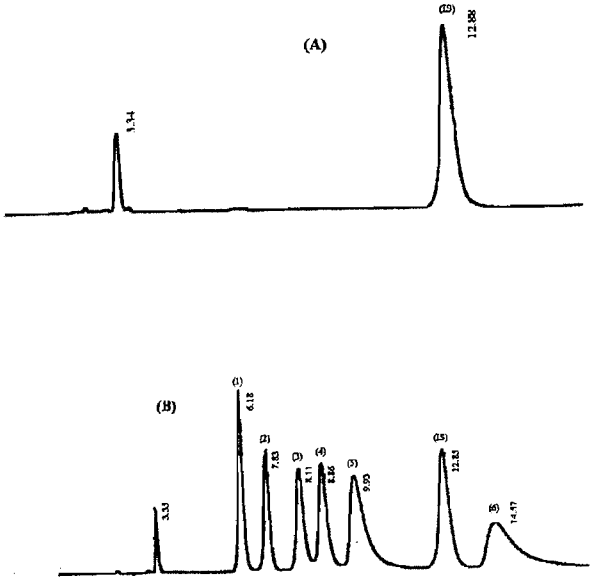

Fig.1 : Chromatograms of (A) an extracted blank urine and (B) a spiked urine with ephedrines 1= NEPH, 2= NPEH, 3= EPH, 4= PEHP, 5= MEPH, 6= ETEPH and IS. The concentrations in the sample were 5 µg/ml and 8 µg/ml for of all the ephedrines and the internal standard (IS) respectively.