Reprint from

10th Cologne Workshop On Dope Analysis 7th to 12th June 1992 - Proceedings -

M. Donike
H. Geyer
A. Gotzmann
U. Mareck-Engelke
S. Rauth
(Editors)

Sport und Buch Strauß, Köln, 1993

C. IMAZ, D. CARRERAS, R. NAVAJAS, C. RODRIGUEZ, A.F. RODRIGUEZ, J. MAYNAR, R. CORTES:

Determination of Ephedrines in Urine by HPLC

In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.) 10th Cologne Workshop On Dope Analysis 7th to 12th June 1992. Sport und Buch Strauß, Köln, (1993) 269-280

C. Imaz, D. Carreras, R. Navajas, C. Rodriguez, A.F. Rodriguez, J. Maynar and R. Cortes

Determination of Ephedrines in Urine by HPLC

Laboratorio de Control del Dopaje, Madrid, Spain

Abstract

An improved high-performance liquid chromatographic method with ultraviolet detection, for the simultaneous quantification of ephedrines (norephedrine, norpseudoephedrine, ephedrine, pseudoephedrine, methylephedrine and ethylephedrine) in urine is described. The six substances were seperated on reserved phase column with phosphate buffer-TEA (pH 5.5) as mobile phase. The linearity and reproducibility was very satisfactory for levels usually found in urine (1-30 mg/l).

Introduction

Ephedrine (EPH)-pseudoephedrine (PEPH), and norephedrine (NEPH) norpseudoephedrine (NPEP) are pairs of diasteroisomerous (1) compounds which are included in the dopping list of pharmacological forbidden substances indicated by the Medical Commission of IOC (2). As they are widly available in asthma, ophtalmic, cold and allergy products (3) and as they are found in more than 100 pharmaceutical formulations, the mentioned Commission has setup concentrations above which they are considered positive. For this reason both, the determination of such pairs of diastereoisomers as well as their quantification are necessary.

The most often used technique for such determination and quantification is by gas chromatography coupled to mass spectrometry (4). In this case a previous selective derivatization (5) is needed (O-TMS,N-TFA), but not always the reproducibility obtained is enough because more than one derivate for the same compound is achieved. In this paper attempts have been made to develope a simple, rapid, selective and accurate HPLC method, where a previous derivatization process isn`t necessary. It has been determinated the two pairs of diastereoisomers and also methylephedrine (MEPH) and ethyelphedrine (ETEP) are included (Fig.1).

Studies with positive urines proceeding from athletes have been carried out and very good results have been obtained.

Experimental

Reagents

NEPH, NPEP, EPH, PEPH, MEPH, ETEP were obtained from Sigma (St. Louis, Mo, USA). Diethylether was purchased from Carlo Erba (Milan, Italy). Phenylpropylamine (IS), KH_2PO_4, H_3PO_4 and triethylamine (TEA) were from E. Merck (Darmstadt, F.R.G.). Water was double-destilled, deionized and purified by Milli-Q system (Millipore, Milford, MA, USA). All other regents and solvents were analytical-reagent grade.

Standard solutions and clibration standards

Stock solutions of EPH, PEPH, NEPH, NPEP, MEPH, ETEP and IS were prepared in mobile phase described below at a concentration of 1000 mg/l. These stock solutions were then diluted further to yield appropriate working solutions for the preparation of the calibration standards. The solutions were sealed and refrigerated at 4°C until use.

Preparation of mobile phase

The pH 5.5 buffer solution was adjusted by the appropriate addition of 200 mM phosphoric acid - 150 mM TEA to 200 mM potassium dihydrogen phosphate - 150 mM TEA. Before analysis, this mobile phase was filtered through 0.22 μm filter and pumped through the column for 30 min. At the end of each chromatographic session, the column was washed for 15 min. with deionized water, and then with methanol.

Analytical procedure

To 5 ml of urine in a 15 ml glass tube were added 25 μl of IS (1000 mg/l), 100 μl of 10 M NaOH and 2 ml of diethylether, then the urine was saturated with 3 g of Sodium sulphate and shook for 20 min. Tubes were centrifuged at 1200 g for 5 min and the organic layer was taken and evaporated to dryness. The extract was dissolved in 100 μl of the mobile phase and 20 μl of the solution was injected into the liquid chromatograph.

Chromatographic conditions

The chromatographic system consisted of a Hewlett-Packard (Palo Alto, CA, USA) HP 1090 series, a liquid chromatograph equipped with an autosampler/autoinjector and an HP1040A diode-array UV detector. Chromatography was performed at 40° C on a (Hewlet-Packard) reversed-phase analytical column (Lichrospher 60 RP select B, 5 μm, 125 mm X 4 mm I.D.). The mobile phase was 200 mM phosphate buffer (pH = 5.5) with

150 mM triethylamine at a flow-rate of 1.3 ml/min. The injection volume was 20 μl and the column effluent was monitored at 215 nm (4 nm band width), where the ephedrines exhibit a maximum absorption. For data evaluation a HP 79994A Chemstation was used, which consisted of an HP 900 Series 300 computer, a 10 Mbytes Winchester disc drive and a Thinkjet printer.

Results

Representative chromatograms for urine analyses are shown in Fig.2. The peaks of interest were well seperated from potential interferences. Preliminary experiments led us to the conclusion that the seperation depends significantly on the column used. The best results were obtained when a Select B column was used.

The composition of the mobile phase was selected in such a way that all the ephedrines were resolved in the shortest analysis time possible. As it is shown in Fig. 1, ephedrines have hydroxilated carbon at position B. For this reason they can interact with the free silanol groups. Using TEA in the mobile phase those interactions decreased because such silanol groups are masked (4). As long as the concentration of TEA of the mobile phase is increased, the K' values is decreased (Fig.3).

The pH of the mobile phase also influences on the determination of the six ephedrines (Fig.4). The K'values increased with increasing pH of the mobile phase, but a better resolution was achieved, above all with the pair of compounds pseudoephedrine and methylephedrine where the resolution was total at pH 55.5 (Rs=1.25).

Precision and accuracy were carried out from spiked urine samples at concentration of 2, 8, 24 mg/l. The samples were extracted and subjected to HPLC. Each concentration was calculated on bases of the peak-height ratios against the IS. The results are listed in Table I. The linearity was evaluated over the range of concentrations from 1 to 30 mg/l using duplicate spike samples at levels of 1, 5, 10, 15 and 30 mg/l. The calibration curves obtained and the equations of the mean plots are shown in Fig. 5 and Table II, respectively.

The limit of detection was defined as the lowest concentration of each ephedrine resulting in a single-to-noise ratio of three. For NEPH, NPEP and EPH the limit was 0.2 mg/l and for PEPH, MEPH and ETEP their limit was 0.5 mg/l.

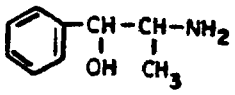
Other pharmacological substances were examined in order to establish possible interferences. Substances tested are shown in Table III. None of these interfered the ephedrines determination.

Conclusion

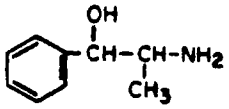
In conclusion, the elution and separation of ephedrins were clearly affected by the column used and the concentration of TEA as well as pH of the mobile phase. As it was not necessary the use of a modifier, endogenous compounds were eluted in very long times resulting very clear chromatograms where interferences of such endogenous compounds weren't observed. This method has been used in urines proceeding from athletes, obtaining very satisfactory results.

References

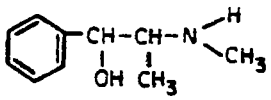
1. I.W. Wainer and A.L. Marcotte, in I.W. Wainer and D.E. Drayer (Editors) Drug Stereochemistry, Analytical Methods and Pharmacology, Marcel Dekker, New York, 1988, pp 31-41
2. The Pharmaceutical Society of Great Britain, Martindale. The Extra Pharmacopeia 28th edition, The Pharmaceutical Press, London, 1982.
3. List of Doping Classes and Methods, International Olympic Committe (1991).
4. M. Donike, 9th Workshop in Dope Analyses, Köln, March 17-22, 1991.
5. M. Donike and J. Derenbach, Z. ANAL. CHEM., 279 (1976).
6. Z. Varga - Puchony an Gy. Vigh, J. Chromatogram. 257 (1983) 380.



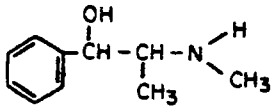
Norephedrine



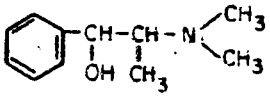
Norpseudoephedrine



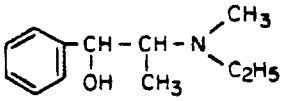
Ephedrine



Pseudoephedrine



N-Methylephedrine



Ethylephedrine

Fig. 1

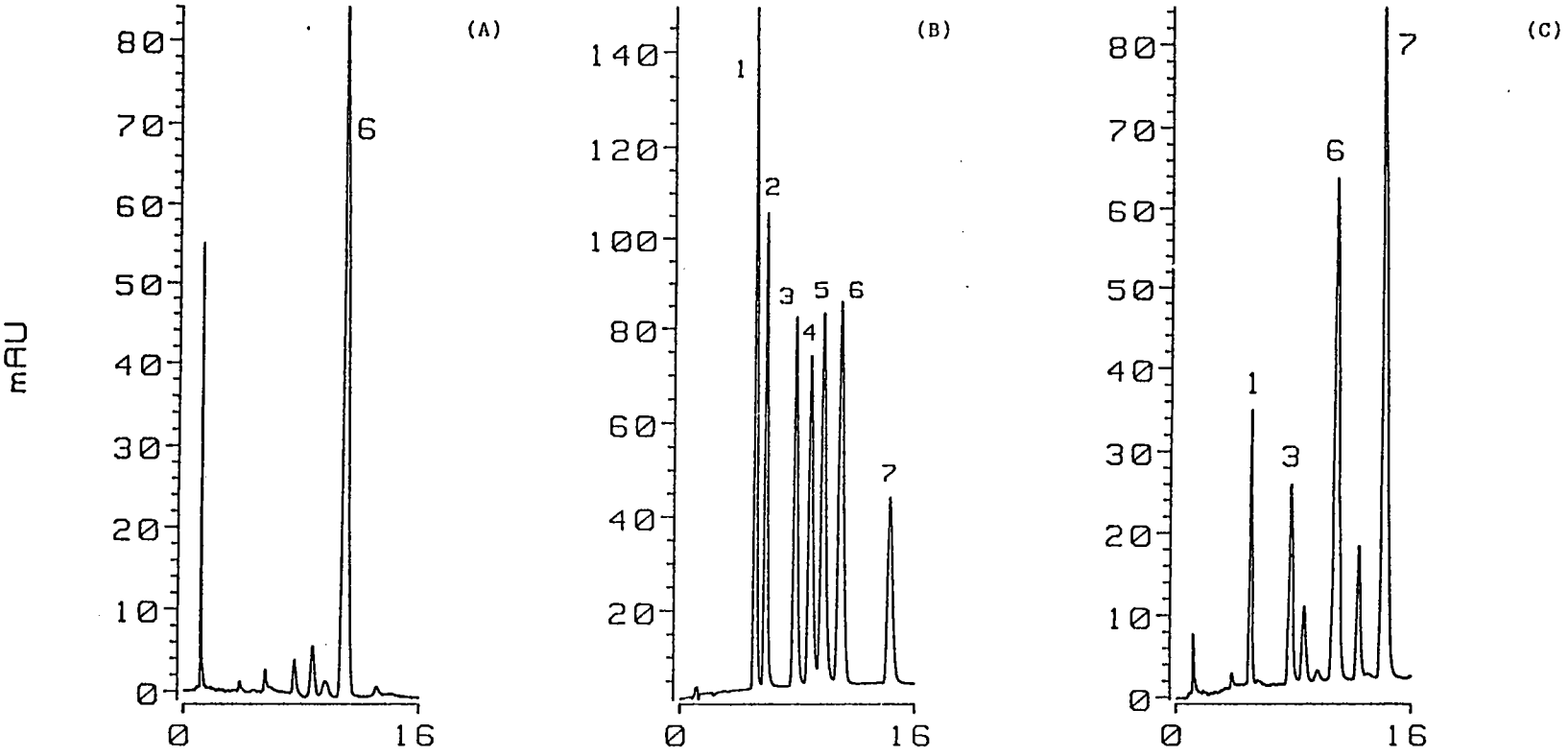


Fig. 2. Chromatograms obtained after analysis of (A) blank urine to which internal standard (peak 6) was added, (B) urine standard spiked with NEPH (peak 1), NPEP (peak 2), EPH (peak 3), PEPH (peak 4), MEPH (peak 5), ETEP (peak 7) and IS (peak 6), and (C) urine sample from an athlete who took ETEP.

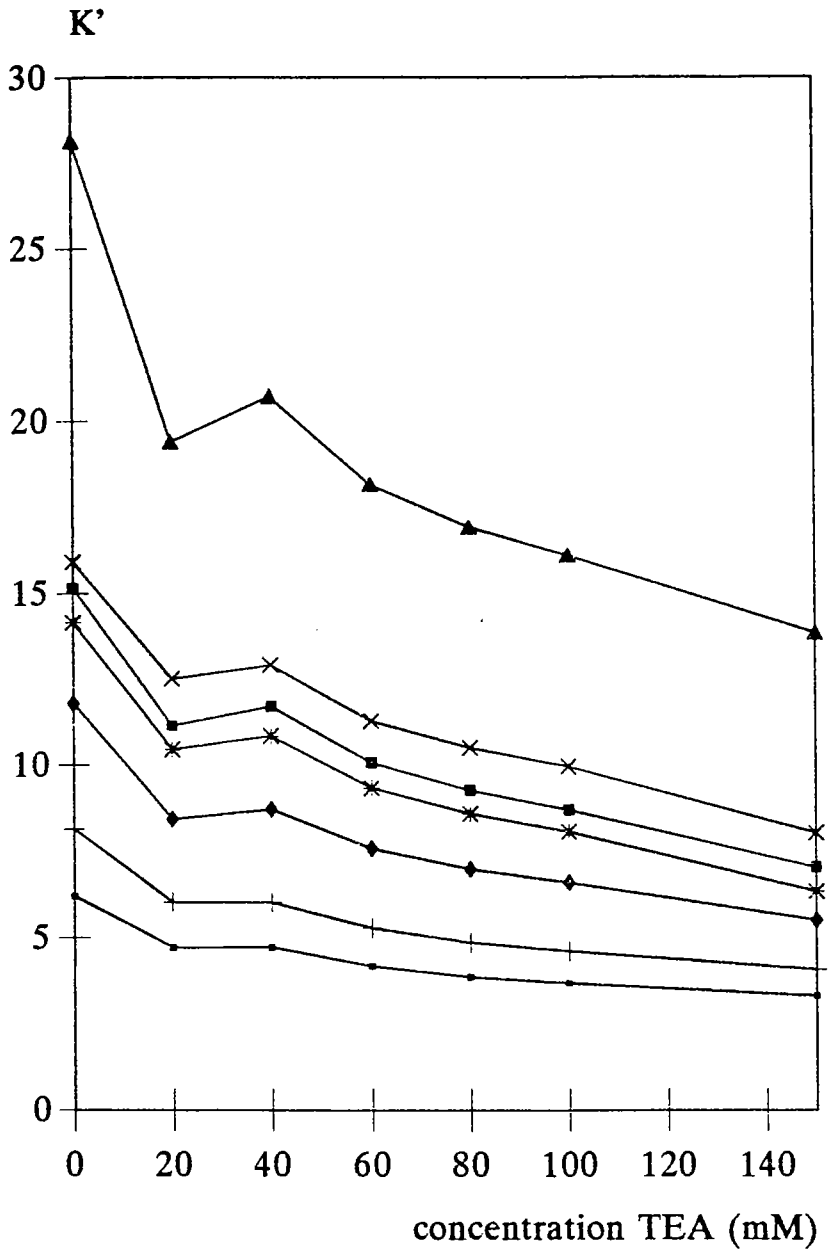


Fig. 3. Effect of the concentration of TEA in the column on K' values of ephedrines. Mobile phase, 200 mM phosphate (pH 4); column, lichrospher 60 RP Select B, 5 um (125 mm x 4 mm I.D.)

NEPH NPEPH PEPH MEPH IS EPH ETEPH

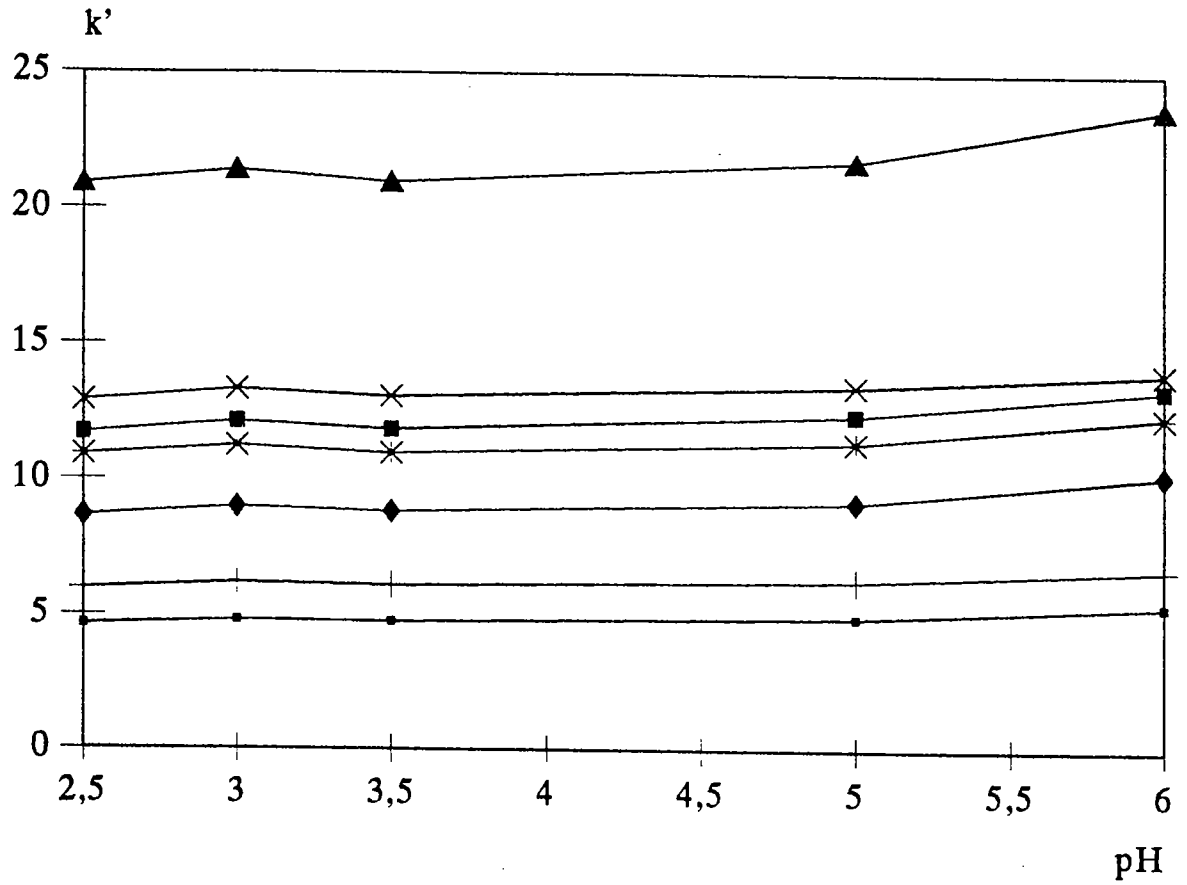


Fig. 4. Effect of pH on K' values. Concentration of TEA of the mobile phase, 150 mM; other conditions and compounds as specified in Fig. 3.

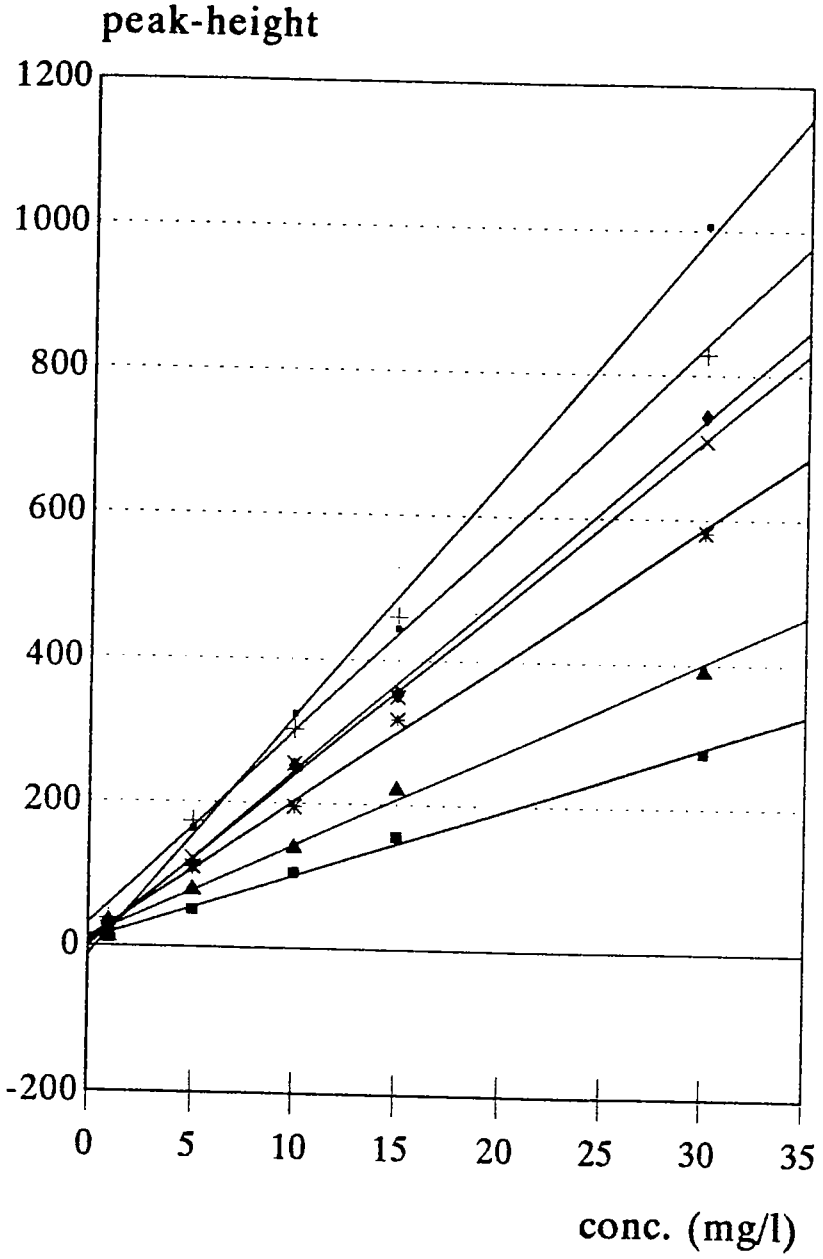


Fig. 5. Linear calibration curves for the ephedrines specified in

ANALYTICAL ACCURACY AND REPRODUCIBILITY OF THE ANALYSIS OF EPHEDRINES IN HUMAN URINE

Concentration Added (µg/ml)	NEPH		NPEP		EPH		PEPH		MEPH		ETEP	
	Mean ± S.D. (µg/ml)	C.V. (%)	Mean ± S.D. (µg/ml)	C.V. (%)	Mean ± S.D (µg/ml)	C.V (%)	Mean ± S.D. (µg/ml)	C.V. (%)	Mean ± S.D. (µg/ml)	C.V. (%)	Mean ± S.D. (µg/ml)	C.V. (%)
2	1.98±0.07	3.5	2.05±0.03	1.3	1.96±0.09	2.2	1.92±0.02	1.0	1.97±0.05	2.69	1.87±0.12	3.1
8	8.04±0.05	5.3	8.22±0.06	0.7	8.27±0.04	0.4	8.07±0.06	0.7	7.95±0.04	0.56	7.23±0.21	4.4
24	25.93±0.26	0.9	24.34±0.28	1.1	26.00±1.17	4.5	25.59±0.23	0.9	22.47±0.28	1.26	25.14±1.49	6.4

Table I.

EQUATIONS OF LINEAR CALIBRATION CURVES

NEPH	y=33.38x-13.63	rr=0.9978
NPEP	y=27.02x+30.64	rr=0.9983
EPH	y=24.58x-0.92	rr=0.9991
PEPH	y=19.27x+10.82	rr=0.9987
MEPH	y=9.12x+8.78	rr=0.9975
IS	y=23.55x+3.66	rr=0.9993
ETEP	y=12.93x+13.08	rr=0.9971

Tabla II.

DRUGS TESTED FOR POSSIBLE INTERFERENCE

HEPTAMINOL
AMPHETAMINE
METHYLAMPHETAMINE
FENCAMFAMINE
DIMETHYLAMPHETAMINE
NICOTINE
PHENDIMETRAZINE
METHOXAMINE
LEPTAZOL
NIKETAMINE
PETIDINE
CAFFEINE
LIDOCAINE
PROCAINE
CODEINE
STRYCHNINE
ETAMIVAN
PHENMETRAZINE
METHYLPHENIDATE
PROLINTANE
PIPRADOL
AMFEPRAMONE
CHLORPHENTERMINE
COCAINE

Table III.