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Comparison of various reversed-phase columns for the simultaneous determination of ephedrines in urine by high-performance liquid chromatography

C. Imaz*, R. Navajas, D. Carreras, C. Rodríguez, A.F. Rodríguez

Laboratorio de Control de Dopaje, Consejo Superior de Deportes, C/Greco s/n, 28040 Madrid, Spain

Abstract

Different reversed-phase columns for basic analytes were compared for the simultaneous determination of ephedrines in urine, such as LiChrospher 60 RP-Select B, LiChrospher 100 RP18, Hypersil BDS-C18, Inertsil ODS-2, Spherisorb ODS-B and Symmetry Shield RP8. Symmetry Shield was the only column which did not require the use of high concentrations of buffer and triethylamine. With this column, a good separation of the six ephedrines and the internal standard was achieved using 50 m*M* phosphate buffer–25 m*M* triethylamine as a mobile phase. Linearity, precision and accuracy were satisfactory for the levels usually found in urine. Due to these all parameters the developed analytical method was found to be suitable for the application in the doping field.

Keywords: Ephedrines

1. Introduction

The Medical Commission of the International Olympic Committee included the quantification of ephedrines in the list of forbidden substances in 1990. Nowadays, this Commission has established the following limits of concentration in urine above which they are considered positive: for phenylpropanolamine (NEPH) and pseudoephedrine (PEPH) 10 μg/ml, for cathine (NPEPH), ephedrine (EPH) and methylephedrine (MEPH) 5 μg/ml, and as long as the sum of all those ephedrines in the sample is above 10 μg/ml [1].

The separation of ephedrines in urine by high-performance liquid chromatography (HPLC) is especially difficult because they have similar chemical structures and are basic compounds with polar functional groups (Fig. 1). Typically, such compounds exhibit severe band tailing, broad bands and low plate numbers. For this reason, the determination of ephedrines requires reversed-phase packings based on high purity silicas with a low level of silanol activity (endcapped bonded phases), and mobile phases with high ionic strength and amine modifiers [2,3].

Several HPLC methods have been reported for the determination of ephedrines in pharmaceutical preparations and in biological fluids [4–6].

In 1993, we performed a method for the simultaneous determination of ephedrines in urine by HPLC, using a LiChrospher 60 RP-Select B column and 200 m*M* phosphate buffer–150 m*M* triethylamine (TEA) (pH 5.2) as a mobile phase [7].

The aim of this work was the comparison of different reversed-phase columns and the optimi-

*Corresponding author.

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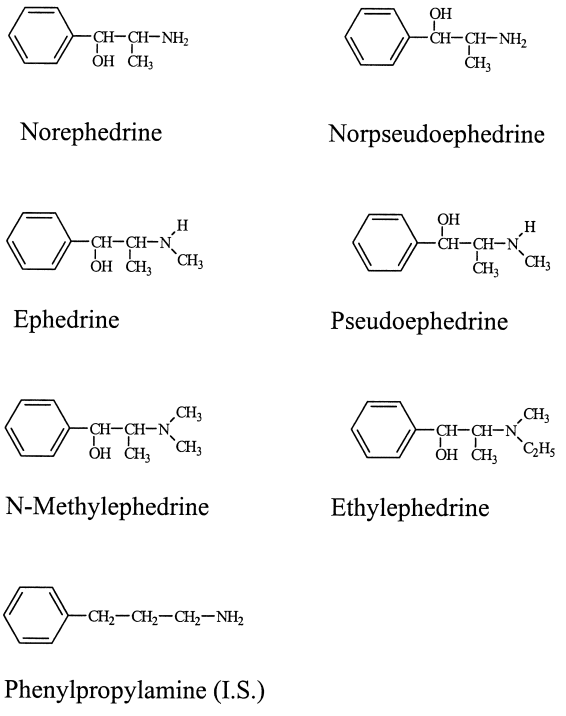

Fig. 1. Structures of ephedrines and phenylpropylamine (IS).

sation of the best chromatographic conditions, for the determination of ephedrines.

2. Experimental

2.1. Reagents and chemicals

NEPH, NPEPH, EPH, PEPH, MEPH and ethylephedrine (ETEPH) were obtained from Sigma (St. Louis, MO, USA). Diethyl ether was purchased from Carlo Erba (Milan, Italy) and phenylpropylamine (internal standard), KH_2PO_4 and TEA from Merck (Darmstadt, Germany). Water was doubly distilled, deionized and purified with a Milli-Q system (Millipore, Milford, MA, USA). All other reagents and solvents were of analytical-reagent grade.

2.2. Stock solutions and internal standard solution

Stock solutions were prepared in bidistilled water at a concentration of 1000 μg/ml. These solutions were sealed and refrigerated at 4°C until use.

Phenylpropylamine was used as an internal standard (I.S.). It was also dissolved in bidistilled water at a concentration of 1000 μg/ml.

2.3. Calibration standards

Calibration graphs were obtained by adding known amounts of ephedrines from 1 to 60 μg/ml in blank urines. Quantification was based on peak-area ratios of compound-to-I.S. versus concentration of compound spiked.

2.4. Equipment

The chromatographic system consisted of a Hewlett-Packard (Palo Alto, CA, USA) HP 1090 Series, a liquid chromatograph equipped with an autosampler/autoinjector and a HP 1040 A diode-array UV detector. For data evaluation an HP Vectra XM serie 4 Chemstation was used.

2.5. Sample preparation

To 2 ml of urine in a 15-ml glass tube were added 20 μl of I.S. solution (1000 μg/ml), 100 μl of 10 *M* NaOH and 2 ml of diethyl ether, then the urine was saturated with 1 g of sodium sulfate and shaken for 20 min. The tubes were centrifuged at 1200 *g* for 5 min and the organic layer was removed and evaporated to dryness. Because of the volatility of ephedrines, it was necessary to control the temperature of the evaporation process. Therefore, a vacuum evaporator at a temperature up to 18°C was used. The residue was dissolved in 100 μl of the mobile phase and 20 μl of the solution was injected into the liquid chromatograph.

2.6. Chromatographic conditions

Chromatography was performed at 40°C on a reversed-phase column. The columns used were as follows: LiChrospher 60 RP-Select B, 5 μm (250×4 mm I.D.), Hewlett-Packard; LiChrospher 100 RP18,

5 μm (250×4 mm I.D.), Hewlett-Packard; Hypersil BDS-C18, 5 μm (250×4 mm I.D.), Hewlett-Packard; Inertsil ODS-2, 5 μm (250×4.6 mm I.D.), Chrompack (The Netherlands); Spherisorb ODS-B, 5 μm (250×4.6 mm I.D.), Sugelabor (Spain) and Symmetry Shield RP8, 5 μm (250×4.6 mm I.D.), Waters (USA). The mobile phase was 50 m*M* phosphate buffer with TEA added to a final concentration of 25 m*M*. This phosphate buffer solution was prepared by adding certain volume of 50 m*M* phosphoric acid–25 m*M* TEA solution to 50 m*M* potassium dihydrogen phosphate–25 m*M* TEA solution and adjusting the pH to 6.5, which was measured simultaneously with a pH meter. Before analysis, this mobile phase was filtered through a 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. The initial flow-rate was 1.5 ml/min and then was increased to 2.0 ml/min in 10 min. The injection volume was 20 μl and the column effluent was monitored at 215 nm (band width 4 nm). The dead time (t_0) of each column was determined by injection of potassium nitrate. Capacity factors (k) were calculated as $k=(t_R-t_0)/t_0$.

3. Results and discussion

3.1. Optimisation of chromatographic conditions

The following reversed-phase columns for basic analytes were tested: LiChrospher 60 RP-Select B, LiChrospher 100 RP18, Hypersil BDS-C18, Inertsil ODS-2, Spherisorb ODS-B and Symmetry Shield RP8. All the stationary phases (except for Symmetry Shield RP8) showed the same behaviour. They required the use of high concentrations of buffer (high ionic strength) and TEA to improve the peak shape, although a good separation was not always achieved. Fig. 2 shows the k values of the ephedrines for the different stationary phases, using mobile phase without TEA. Symmetry Shield was the only column that could be used without TEA, in which the analysis time for the separation of the six ephedrines and the internal standard took less than 20 min. Therefore, this column was used for further experiments.

The composition of the mobile phase was selected in such a way that all the ephedrines were resolved in the shortest analysis time possible. Fig. 3 shows the effect of TEA concentration on the k values of

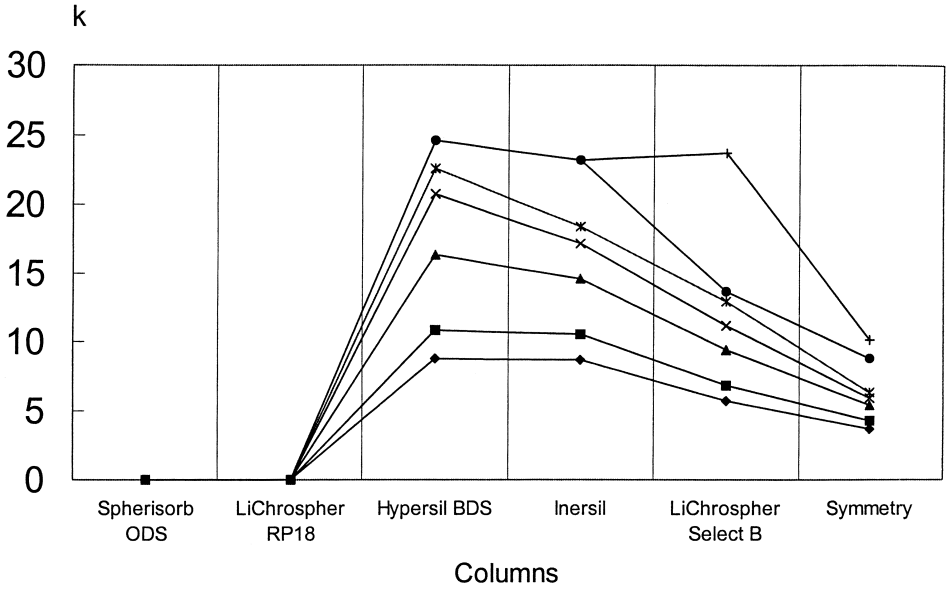

Fig. 2. Comparison of the different reversed-phase columns for the determination of six ephedrines and the I.S. using 200 m*M* phosphate buffer with no TEA (pH 5.2) as a mobile phase. ♦=NEPH; ■=NPEPH; ▲=EPH; ×=PEPH; ★=MEPH; ●=IS; +=ETEPH.

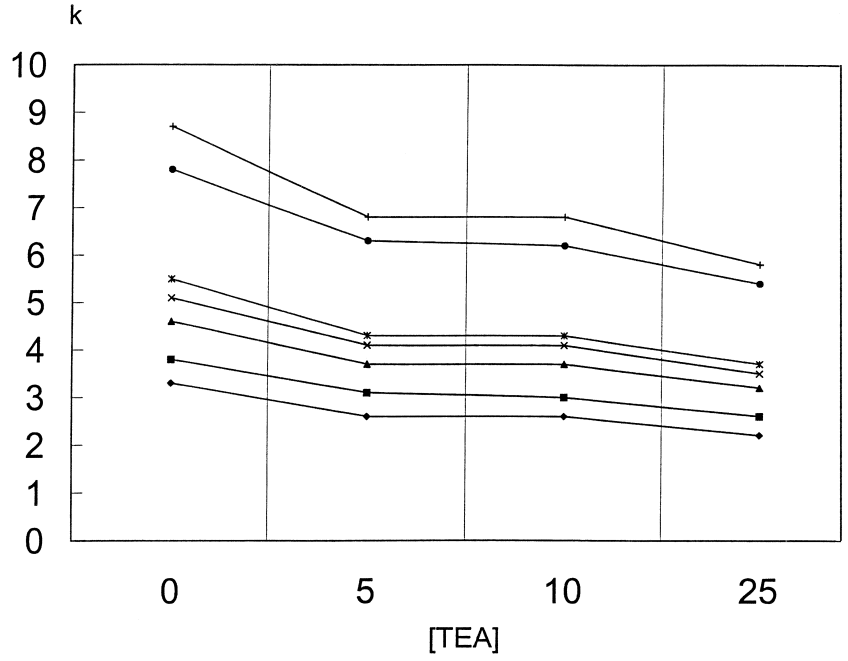

Fig. 3. Effect of TEA concentration on *k* values of ephedrines, using Symmetry Shield RP8 column and 50 m*M* phosphate buffer (pH 5.2). ♦=NEPH; ■=NPEPH; ▲=EPH; ×=PEPH; ★=MEPH; ●=IS; +=ETEPH.

the ephedrines. There was a slight effect of TEA concentration on the separation, because of the high purity silica which was also well endcapped. Therefore use of high concentrations of amine modifier was not necessary. The pH of the mobile phase also influenced the determination of the ephedrines, as shown in Fig. 4.

Having considered the different parameters, the optimum conditions for the separation of the ephedrines using the Symmetry Shield RP8 column, were 50 m*M* phosphate buffer–25 m*M* TEA (pH 6.5) as a mobile phase. Representative chromatograms for urine analyses are shown in Fig. 5.

3.2. Precision and accuracy

The precision and accuracy were estimated using five different replicate samples containing 3, 20 and 50 μg/ml, respectively. Each concentration was calculated on the basis of the peak-area ratio with respect to the I.S. Statistical results are given in Table 1.

3.3. Linearity

The linearity was evaluated over the range of concentrations 1–60 μg/ml using duplicate samples spiked at levels of 1, 5, 10, 20, 40 and 60 μg/ml. The linear regression equations are shown in Table 2.

3.4. Limits of detection

The limit of detection was defined as an analyte signal-to-background noise (S/N) ratio of 3. The limits of detection were 0.2 μg/ml for NEPH, 0.3 μg/ml for NPEPH and EPH, 0.5 μg/ml for PEPH and MEPH, and 1 μg/ml for ETEPH.

4. Conclusion

The reversed-phase separation of basic analytes such as ephedrines often results in broad, tailing bands, which are caused by acidic sites present in the column packing. The use of less acidic columns, as

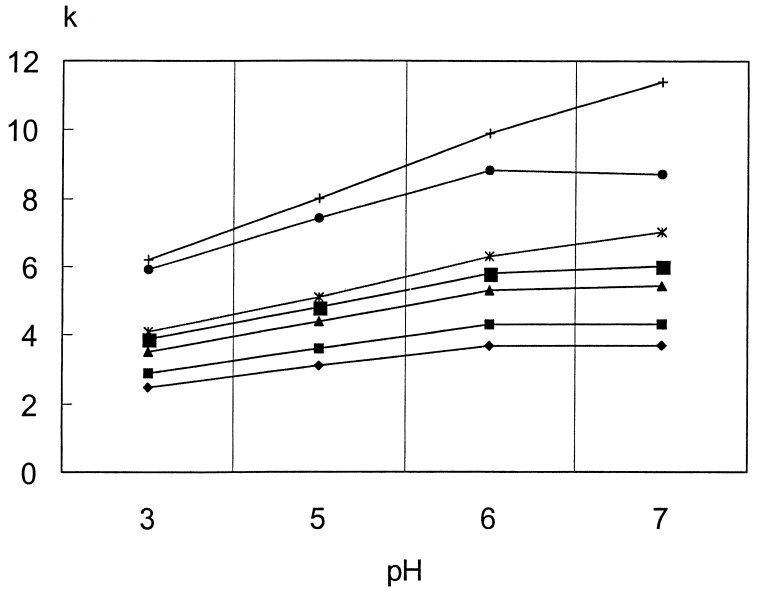

Fig. 4. Effect of pH on *k* values of ephedrines using mobile phase with no TEA; other conditions and symbols as in Fig. 3.

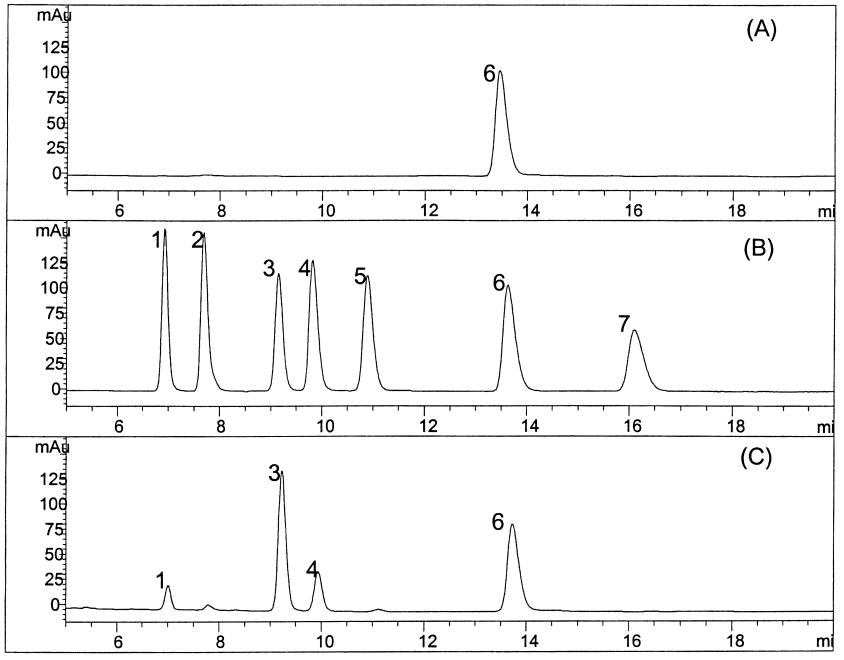

Fig. 5. Chromatograms obtained after analysis of (A) blank urine to which I.S. at a concentration of 10 μg/ml (peak 6) was added; (B) urine standard spiked with (1) NEPH, (2) NPEPH, (3) EPH, (4) PEPH, (5) MEPH, (7) ETEPH and (6) I.S., each at a concentration of 10 μg/ml; (C) urine sample from an athlete where NEPH, EPH and PEPH were found, each at a concentration of 2.3, 18.1 and 3.9 μg/ml, respectively; the concentration of the I.S. was 10 μg/ml.

Table 1
Analytical accuracy and reproducibility for ephedrines in spiked urine ($n=5$)

	Concentration (μg/ml)	Estimated concentration (mean±SD)	Precision (RSD, %)	Accuracy (relative error, %)
NEPH	3	3.1±0.3	8.5	3.3
	20	21.2±0.5	2.4	6.0
	50	51.1±1.2	2.4	2.2
NPEPH	3	2.8±0.1	3.6	6.6
	20	20.7±0.5	2.3	3.5
	50	51.8±0.6	1.1	3.6
EPH	3	2.6±0.1	2.2	13.3
	20	20.7±1.2	5.9	3.5
	50	51.3±1.3	2.5	2.6
PEPH	3	2.6±0.2	6.5	13.3
	20	21.5±0.6	2.7	7.5
	50	53.6±1.3	2.5	7.2
MEPH	3	2.6±0.2	8.4	13.3
	20	19.7±0.2	0.8	1.5
	50	49.7±4.0	8.0	0.6
ETEPH	3	2.9±0.3	8.6	3.3
	20	19.8±1.2	6.0	1.0
	50	51.8±1.6	3.1	3.6

well as mobile phases with high ionic strength and amine modifiers are necessary in order to minimise this problem.

Best results are obtained using Symmetry Shield RP8 column with 50 m*M* phosphate buffer–25 m*M* TEA (pH=6.5) as a mobile phase.

The proposed method is sensitive, reproducible and accurate enough for the determination of ephedrines in the doping control field. We have tested this method by analysing over 50 physiological samples with different levels of ephedrines, and in all cases good results have been obtained.

Table 2
Equations of linear calibration graphs

Compound	Equation[a]	r^2
NEPH	$y=64.7x-13.1$	0.9980
NPEPH	$y=78.1x-64.4$	0.9990
EPH	$y=62.1x-34.4$	0.9992
PEPH	$y=70.4x-12.3$	0.9998
MEPH	$y=57.6x-66.9$	0.9996
I.S.	$y=57.6x-35.1$	0.9975
ETEPH	$y=61.2x-72.8$	0.9990

[a] y=Peak height (arbitrary units); x=concentration (μg/ml).

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