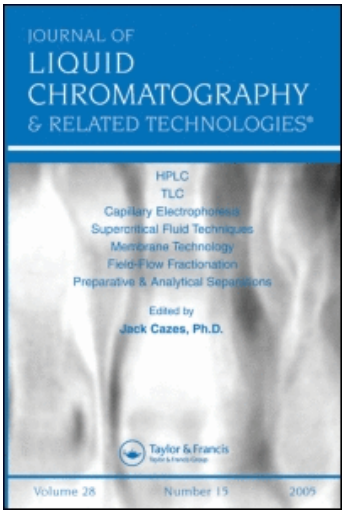

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DIRECT DETERMINATION OF EPHEDRINE ALKALOIDS AND EPINEPHRINE IN HUMAN URINE BY CAPILLARY ZONE ELECTROPHORESIS

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ABSTRACT

The complete separation of a mixture of six phenylamines and epinephrine in human urine was achieved by Capillary Zone Electrophoresis (CZE) in 15 min. For the CZE separation of the compounds, electrophoretic media with phosphate-borate and phosphate-acetonitrile buffer at different pH were used. A buffer solution that contained 40 mM phosphoric acid and 10 mM boric acid adjusted to pH 9.7 with NaOH 1 N, was found to be the most suitable electrolyte for epinephrine separation. The results successfully demonstrated the use of CZE with UV detection for screening and quantification of phenylamines and epinephrine in human urine without previous treatment, in concentration lower than 35.0 µg/ml, and quantification limit of 2.0±0.1 µg per millilitre of urine.

INTRODUCTION

Sympathomimetic or adrenergic drugs play important roles as neurotransmitter in the central and peripheral autonomic nervous systems and as hormones exerting endocrine and exocrine effects. It is not

surprising, therefore, that the lack of sufficient amounts of some of these drugs in the body can have a severe impact on both the quality and duration of life. These drugs have a stimulating effect on several systems of the body, mainly the central nervous, respiratory and the vasomotor systems. Consequently, the phenylamines are considered to be doping substances by the International Olympic Committee, and their maximum amount allowed per millilitre of urine is 5 μg/ml (for ephedrine).

These stimulants have been widely studied through different analytical techniques, and several methods have been reported for the determination of some of these compounds: for ephedrine, gas chromatography *(1-3)*, thin-layer chromatography *(4)*, spectrophotometry *(5)*, radioinmunoassays *(6)*, electrochemical methods with solid electrodes *(7,8)* and with selective liquid membrane electrodes *(9)*; for ephedrine and pseudoephedrine, ^{13}C-NMR-spectrometry *(10)*, and for different ephedrine alkaloids high performance liquid chromatography HPLC *(11-14)*, isotachophoresis *(15)* and the use of joint techniques such as HPLC-capillary electrophoresis *(16)*.

Capillary zone electrophoresis is a recently developed separation technique based on the differences in the mobility exhibited by different molecules in an electric field. It has many attractive features, including being a simple, fast and highly efficient technique applicable to a wide variety of analytes (for recent reviews, see refs. *(17,18)*). The development of effective methods of drugs separation and determination are important in pharmaceutical analysis as well as for the screening and quantification of drugs in biological fluids.

Several studies have shown that the application of CZE in real samples creates a great number of difficulties basically due to changes in the behaviour of substances *(19)*. They can be summarized as follows: (a) the

effect on the matrix on the charge of the analytes when performing the injection by electromigration, (b) the high concentration of salts presents in biological samples, (c) charges in the capillary walls, (d) adsorption of undesirable analytes on the capillary walls, and (e) overcharge in the capillary, caused by the total amount of components in the sample.

The analysis of several sympathomimetic drugs, such as the catecholamines, has been studied by CZE using electrochemical *(20)*, and spectrophotometric detection *(21-23)*. On the other hand, the simultaneous separation and quantification of this six phenylamines and epinephrine in human urine by CZE have not been reported. Only a recent method has been developed for the determination of ephedrine and pseudoephedrine in *Ephedra-Erba (24)*.

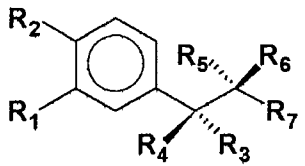
Following our previous paper *(25,26)*, we wish to report here a fast, simple and accurate method for the separation and direct determination of a mixture of six phenylamines and epinephrine in human urine by CZE. This method offers many advantages over some other methods described in literature, including that the urine samples can be directly injected without previous separation, so the procedure of analysis was greatly simplified.

EXPERIMENTAL SECTION

Chemicals

All chemicals were of analytical-reagent or research grade, Merck. The compounds investigated were obtained from Aldrich and Sigma. The formulae and abbreviations used in this paper are given in Table I. Stock

TABLE 1



ABBREVIATION	R_1	R_2	R_3	R_4	R_5	R_6	R_7	NAME	REFERENCE
(+)-E	H	H	OH	H	H	CH_3	$NH(CH_3)$	(+)-(1S,2R)-Ephedrine	Aldrich- 85-735-5
(+)-PE	H	H	OH	H	CH_3	H	$NH(CH_3)$	(+)-(1S,2S)-Pseudoephedrine	Aldrich- 29-461-6
(+)-NE	H	H	OH	H	H	CH_3	NH_2	(+)-(1S,2R)-Norephedrine	Aldrich- 19-362-3
(-)-NPE	H	H	H	OH	H	CH_3	NH_2	(-)-(1R,2R)-Norpseudoephedrine	Sigma- N-2758
(-)-ME	H	H	H	OH	CH_3	H	$N(CH_3)_2$	(-)-(1R,2S)-Methylephedrine	Aldrich- 25-521-0
(-)-MPE	H	H	H	OH	H	CH_3	$N(CH_3)_2$	(-)-(1R,2R)-Methylpseudoephedrine	Aldrich- 29-003-3
(-)-EPI	OH	OH	H	OH	H	H	$NH(CH_3)$	(-)-1R-Epinephrine	Aldrich- 21-930-4

solutions (0.01 M) of compounds were prepared in purified water (Milli-Q/Milli-RO, Millipore) and kept in the dark and under refrigeration.

Purified water was used to prepare all aqueous solutions, which were filtered through 0.45-μm filter Dynagard (Laguna Hills, CA, USA) before use. Samples of urine were similarly filtered before CZE analysis.

Electrophoretic Instrumentation and Running Conditions

All experiments have been performed in an EUROPHOR (Toulouse, France), equipped with a manual injector (Prime Vision I), high voltage source (Prime Vision V) and UV-VIS detector (Prime Vision II) adjusted to 210 nm. Supelco (Bellefonte, PA, USA; Cat. No. 77500) untreated silica (1 m x 75 μm I.D.; 363 μm O.D.) was used as capillary. The effective separation distance was 65 cm. Electropherograms were monitored with a Varian 4290 integrator. A constant voltage of 20 kV was applied. The cathode was on the detector side. Sample application occurred by hydrodynamic injection for a specified period of time (typically, 2 s).

Conditioning between runs was achieved by rinsing the capillary with 0.1 N NaOH for 2 min, purified water for 2 min and with buffer used in the analysis for another 2 min.

Procedure

Our own urine was employed as blank matrices. For the analysis of human urine, 2 ml of blank sample or one spiked with the seven drugs (see Table 1) was diluted to 10 ml with purified water.

The CZE data in this paper were obtained from three consecutive runs, and the run-to-run relative standard deviation (RSD) of the migration times was less than 2.3%.

RESULTS AND DISCUSSION

Optimum conditions for the separation were obtained by examining the effects of buffer pH, ionic strength and applied voltage. Of these, increasing the ionic strength of the buffer did not affect the migration time, but it did give a constant increment in the width of the peaks. Our studies showed that an ionic strength of 50 mM, produce the best separation of the phenylamines peaks. On the other hand, the buffer pH was found to be the most critical factor affecting the resolution.

The study of pH, was performed in urine samples spiked with the six phenylamines (E, PE, NE, NPE, ME, MPE) and epinephrine (EPI) using a buffer solution containing 50 mM phosphoric acid and adjusted the different pH with NaOH 1N.

Figure 1, shows the influence of pH on the migration times of 6 phenylamines and EPI. The electrophoretic peak of EPI was not visible for pH higher than 7.0 because this appears simultaneously on an endogenous peak of the urine.

It was found that at pH higher than 7.0 the separation between phenylamines was good. At pH lower than 7.0, only one electrophoretic peak was visible and the analysis time was increased two or three times. This is due to the fact that the amino groups of the stimulants are charged at this pH, making their separation impossible under these conditions. As the pH of the electrophoretic media increases, protonation of these amino groups would be inhibited, at a pH closer to the pK_a of the compounds the drugs were separated.

A pH of 9.7 was selected for the process because it produces the best resolution of compounds.

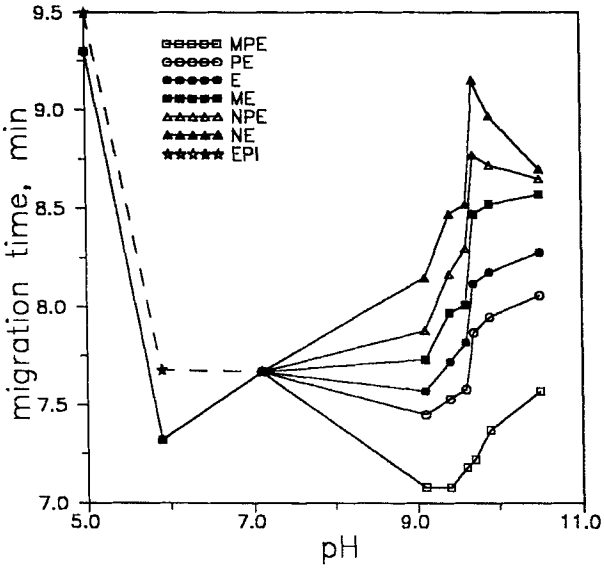


Figure 1.- EFFECT OF pH ON THE MIGRATION TIME OF PHENYLAMINES AND EPINEPHRINE IN HUMAN URINE.
Buffer: 50 mM phosphoric acid, UV detection: 210 nm, Hydrodynamic injection: 2 s., Running voltage: 20 kV, Urine sample five-fold diluted. Drug concentration $1.0x10^{-4}$ M of each one.

The effect of applied voltage on separation and resolution was investigated for the six phenylamines in phosphate buffer 50 mM at pH 9.7. By all accounts, experimentally determined resolution improves with increasing voltage up to a certain point (20 kV) beyond which the voltage is so high as to contribute to zone broadening by Joule´s heating effect *(27)*. A voltage of 20 kV (current: 0.074 mA) was chosen, because it produces the shortest analysis time together with best efficiency (No. theoretical plates: $2.5x10^5$).

In a series of experiments the influence of organic solvent in the carrier electrolyte was investigated. Figure 2, shows the influence of acetonitrile on

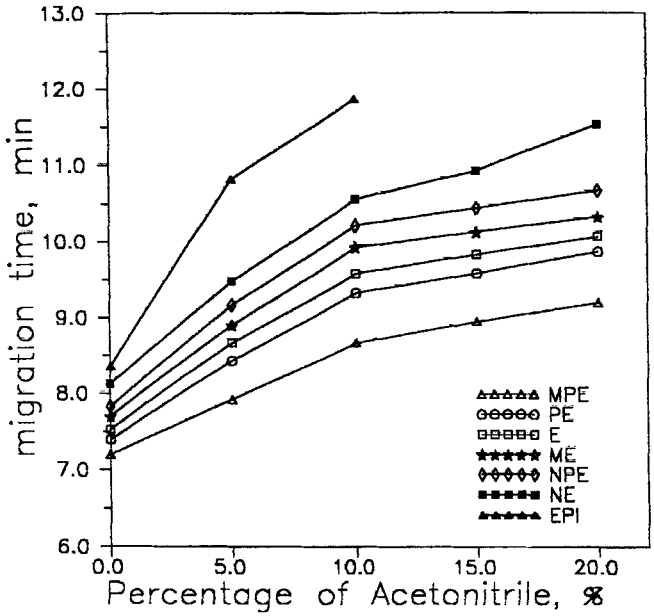


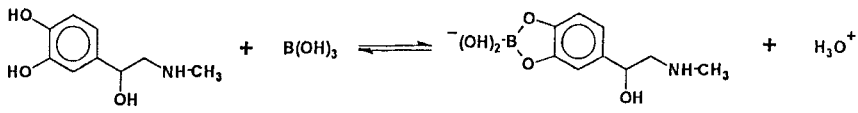
Figure 2.- **EFFECT OF ACETONITRILE ON THE MIGRATION TIME OF PHENYLAMINES AND EPINEPHRINE IN HUMAN URINE.**
Buffer: 50 mM phosphoric acid adjusted to pH 9.7. Other conditions as in figure 1.

the migrationtimes of phenylamines and EPI. Up to a 20% acetonitrile was used, from the results obtained, migration order of compounds in urine are not influenced by the content of organic solvent in the buffer. Furthermore, organic solvent caused a general increase in the migration times of all compounds, which can be attributed to two factors: on the first, the acetonitrile decreases the electrical conductivity, thereby decreasing the current (for a running voltage of 20 kV; buffer without acetonitrile, current: 0.074 mA; buffer with 20% acetonitrile, current: 0.047 mA); On the second, the acetonitrile possibly decreases the amount of electroosmotic flow modifier adsorbed onto the inner wall of the fused-silica capillary. Increasing amounts of acetonitrile produced a slight increase on the migration time of the compounds, improving the electrophoretic separations.

Thus, 10% of acetonitrile has been selected as modifier. Figure 3, shows typical electropherograms of the six phenylamines and epinephrine in human urine diluted 2:10 with purified water, using a 50 mM phosphate buffer at pH 9.7 with 10% acetonitrile. Under these conditions the electrophoretic peak of epinephrine was only visible for concentration higher than 10.0 µg/ml of this compound.

On the other hand, a matrix effect that slightly reduces the migration times of the all drugs and endogenous components of urine was observed when the samples were analyzed in this buffer. This effect disappeared with careful rinsing of the capillary and change of buffer vial in anode, with this the migration times returned to normal values in subsequent runs with the standard drug mixture.

In this paper, we have altered the migration behaviour of epinephrine through complexation with boric acid *(28,29)*. The complexation of boric acid with ortho-dihydroxy compounds such as catechols and certain carbohydrate has been studied extensively *(30,31)*. The complex forms via a reversible reaction with strongly pH dependent equilibrium indicated by the reaction scheme:



Of importance in this reaction is the negative charge on the boron atom which, when complexed with catechols, transforms cationic species into zwitterions and non ionic species into anions.

By adding boric acid to the running buffers, the retention time of a some solute is increased as they react with boric acid to form borate complex. To

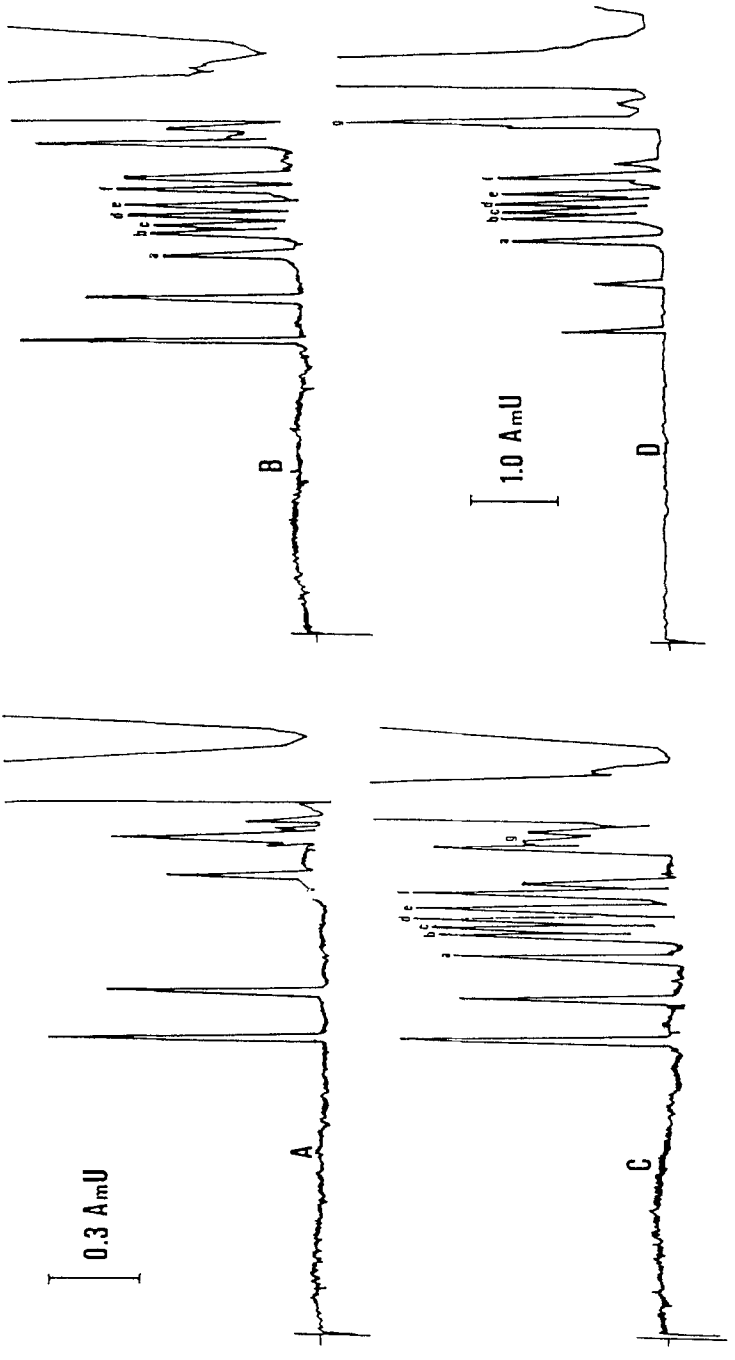


Figure 3.- **ELECTROPHEROGRAMS OF (A) FIVE-FOLD DILUTED BLANK URINE, URINE SPIKED WITH (B) 1.2×10^{-6} M, (C) 2.0×10^{-6} M, (D) 4.0×10^{-6} M OF EACH DRUGS.** Electrophoretic conditions: buffer, 50 mM phosphoric acid adjusted to pH 9.7 with 10% acetonitrile. Peak identification: a: MPE, b: PE, c: E, d: ME, e: NPE, f: NE, g: EPI. Other conditions as in figure 1.

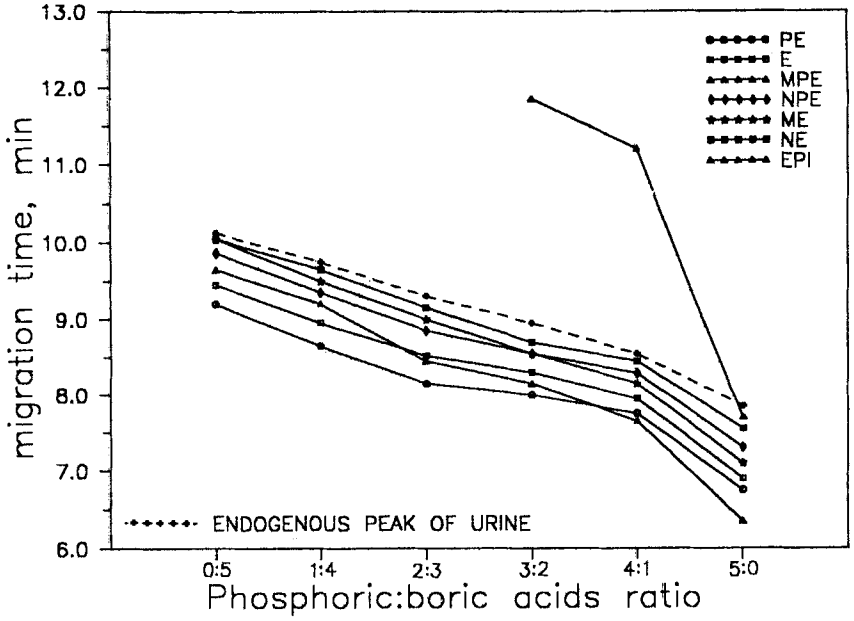


Figure 4.- EFFECT OF DIFFERENT 50 mM PHOSPHORIC-BORIC ACIDS RATIOS ADJUSTED TO pH 9.7 ON THE MIGRATION TIME OF PHENYLAMINES, EPINEPHRINE AND ONE ENDOGENOUS PEAK OF URINE SAMPLE.
Drugs concentration: 1.0×10^{-5} M of each one. Other conditions as in figure 1.

control the best separation of EPI from the endogenous peak of human urine, different buffer solutions of concentration 50 mM with different ratios phosphoric acid-boric acid adjusted to pH 9.7 with NaOH 1 N were prepared.

Figure 4, shows the effect of different ratios phosphoric-boric acids (0:5; 1:4; 2:3; 3:2; 4:1; 5:0) on the migration times of seven drugs under study (phenylamines and EPI) and the endogenous component of urine. In all cases, increasing the concentration of phosphoric acid in the total composition of buffer, gave a reduction of the migration time of drugs. This effect is more pronounced for EPI, due to the complexation of boric acid

with ortho-dihydroxy groups is highly favorable. On the other hand, a large variation of migration times was observed for MPE and ME. It is very important to note that in a buffer 50 mM boric acid adjusted to pH 9.7, the effective mobilities of ME and NE are too close for them to be separated. Therefore, the resolution of this drugs can be improved by simply adding a little amount of phosphoric acid to the total buffer concentration.

When the buffer containing ratios of phosphoric-boric acids 3:2, 4:1 and 5:0, adjusted at pH 9.7 with NaOH 1 N, the electrophoretic peak of EPI was visible and separated from endogenous peak on the urine. When the buffer ratios containing more amounts of boric acid, ratios phosphoric-boric acids such as 2:3, 1:4 or 0:5, the electrophoretic peak of EPI was not observed, because the migration time increases due to the reaction with boric acid, (see figure 4).

Figure 5 shows the electropherograms obtained with different ratios of phosphoric-boric acids adjusted at pH 9.7 with NaOH 1 N.

In all the cases studied in 50 mM phosphoric acid with 10% acetonitrile adjusted to pH 9.7, electroosmotic mobility was 7.8×10^{-8} $m^2V^{-1}s^{-1}$, the resolution and efficiency of six phenylamines in human urine samples were very good, (No. theoretical plates: MPE, 3.4×10^5; PE, 2.1×10^5; E, 1.9×10^5; ME, 3.1×10^5; NPE, 2.6×10^5; NE, 2.0×10^5) but the resolution between EPI and endogenous peak of urine was poor, (No. theoretical plates: EPI, 1.0×10^4). However in 4:1 phosphoric-boric acids relation adjusted to pH 9.7, electroosmotic mobility was 6.9×10^{-8} $m^2V^{-1}s^{-1}$, resolution and efficiency between six phenylamines were slightly poor comparing with the previous buffer, but the resolution of EPI peak was good (No. theoretical plates: EPI, 1.6×10^5).

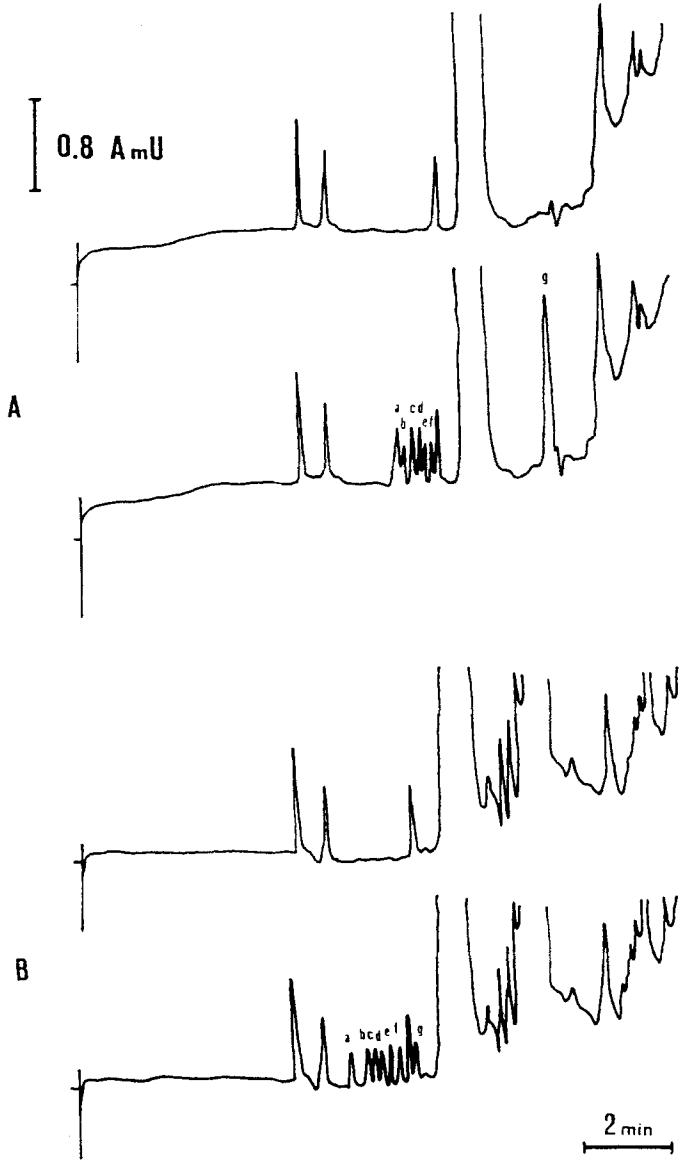


Figure 5.- **ELECTROPHEROGRAMS OF BLANK URINE FIVE-FOLD DILUTED, AND URINE SPIKED WITH 1.0×10^{-6} M OF PHENYLAMINES AND EPINEPHRINE AT TWO DIFFERENT RUNNING BUFFERS ADJUSTED TO pH 9.7.**
(A) 40 mM phosphoric acid + 10 mM boric acid, (B) 50 mM phosphoric acid. Other conditions as in figure 1.

Direct injection of urine provides complex electropherograms within the first half of the elution ranges, making unambiguous identification of zones and complete separation difficult *(32)*. However the last years different advantageous aspects of capillary electrophoresis for the determination of drugs in body fluids have been reported *(33)*.

Our studies have shown that the urine sample does not require any pretreatment, except dilution, before CZE process. For this reason, two millilitres of urine samples were diluted to ten millilitres with purified water, so the linearity was assessed in the concentration range 2.0 to 35.0 µg/ml, under next conditions: running voltage 20 kV, hydrodynamic injection 2 s, detection at 210 nm, buffers at pH 9.7 containing a) 40 mM phosphoric acid + 10 mM boric acid; b) 50 mM phosphoric acid + 10% acetonitrile. In these conditions, the migration order of drugs was: MPE, PE, E, ME, NPE, NE and EPI, and the analysis can be completed within 15 min in both of them.

The studies of influence of concentration were performed in the two previous buffers, with ten different concentrations, and each one of then was repeated three times. Quality parameters of proposed methods are shown in table II and III.

Determination of EPI in 50 mM phosphate buffer (pH 9.7, 10% acetonitrile) was only possible for concentrations between 13.0 to 40.0 µg/ml, for this conditions, the heigh of peak vs. concentration is linear with a sensitivity of 0.141±0.004 AmU.ml.μg^{-1}, r=0.998, with relative standard deviation lower than 8.9%, and a relative error no higher than 7.2%.

The determination of EPI in the electrophoretic media with a relation phosphoric-boric acid (4:1) adjusted to pH 9.7, was possible. The EPI peak increases linearly with the EPI concentration up to 10 µg/ml, with a sensitivity of 0.115±0.005 AmU.ml.μg^{-1}, r=0.992, with a relative standard

TABLE 2
Analytical Characteristics Obtained for the Determination of MPE, PE, E, ME, NPE, NE and EPI in Human Urine by Direct Injection. 50 mM phospate buffer (ph 9.7, 10% Acetonitrile)

COMPOUND	LINEAR RESPONSE (µg/mL)	SENSITIVITY[a] (Mean±RSD) ($AmU.ml.\mu g^{-1}$)	CORRELATION COEFFICIENT	DETECTION LIMIT[b] (Mean±RSD) (µg/ml)	MEAN RELATIVE ERROR %	RSD %
MPE	2.5 - 40.0	0.059±0.001	0.9993	0.8±0.1	4.2	3.8
PE	2.6 - 40.0	0.067±0.001	0.9996	0.8±0.1	4.3	3.5
E	2.3 - 40.0	0.065±0.001	0.9990	0.7±0.1	3.9	3.1
ME	2.3 - 40.0	0.058±0.001	0.9994	0.7±0.1	4.1	4.2
NPE	1.9 - 40.0	0.078±0.001	0.9995	0.6±0.1	4.5	4.2
NE	2.0 - 40.0	0.076±0.001	0.9990	0.6±0.1	4.6	4.2
EPI	13.0 - 40.0	0.141±0.004	0.998	3.9±0.2	7.2	8.9

[a] SLOPE OF THE CALIBRATION PLOT.
[b] SIGNAL-TO-NOISE RATIO= 3:1

TABLE 3
Analytical Characteristics Obtained for the Determination of MPE, PE, E, ME, NPE, NE and EPI in Human Urine by Direct Injection. 50 mM phosphoric-boric acids (4:1) adjusted to ph 9.7

COMPOUND	LINEAR RESPONSE (µg/mL)	SENSITIVITY[a] (Mean±RSD) ($AmU.ml.\mu g^{-1}$)	CORRELATION COEFFICIENT	DETECTION LIMIT[b] (Mean±RSD) (µg/ml)	MEAN RELATIVE ERROR %	RSD %
MPE	1.8 - 40.0	0.065±0.001	0.9993	0.6±0.1	1.9	2.1
PE	1.7 - 35.0	0.069±0.001	0.9992	0.5±0.1	2.3	2.4
E	1.4 - 35.0	0.088±0.001	0.998	0.4±0.1	2.4	2.4
ME	1.7 - 40.0	0.071±0.001	0.9991	0.5±0.1	2.3	2.6
NPE	1.4 - 35.0	0.085±0.001	0.9990	0.4±0.1	2.0	2.1
NE	1.3 - 35.0	0.092±0.001	0.9991	0.4±0.1	2.4	2.8
EPI	1.0 - 10.0	0.115±0.005	0.992	0.3±0.2	6.9	7.9

[a] SLOPE OF THE CALIBRATION PLOT.

[b] SIGNAL-TO-NOISE RATIO= 3:1

deviation no higher than 7.9% and a relative error lower than 6.9%. Comparison of the urine blank and urine spiked only with EPI reveals the presence of this drug in human urine.

The standard addition method used for urine samples spiked with drugs, gave similar results as the method using the calibration curves.

CONCLUSIONS

Because of its specific advantages, including automation, small sample size, easiness buffer change, direct injection of urine samples, when compared with chromatographic approaches, capillary zone electrophoresis appears to be very attractive for the monitoring of several drugs.

The separation of six phenylamines and epinephrine has been achieved using capillary zone electrophoresis. The addition of boric acid to the buffer system, containing phosphoric acid, has overcome the difficulty associated with the epinephrine peak in urine samples.

It can be concluded that the method developed is fast, simple and reliable, allowing simultaneous and direct determination of six phenylamines and epinephrine in human urine samples for concentration between 2.0 to 35.0 µg per millilitre of urine.

ACKNOWLEDGEMENT

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