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Determination by capillary zone electrophoresis of berenil, phenamidine, diampron and dibromopropamidine in serum and urine

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Abstract

A quick, simple and reliable analysis method has been developed in order to determine berenil, phenamidine, diampron and dibromopropamidine by capillary zone electrophoresis in samples of serum and urine. In order to define the operation parameters in CZE, we have carried out a study on how the apparent electrophoretic mobility (μ_{app}) varies when pH, buffer concentration, voltage and temperature are modified. Ohm's law plot has been studied, too. With the data obtained from this study we have determined the optimum work conditions, which are: citrate buffer 25 m*M*, pH=3.70, 14 kV, 30°C, wavelength of the UV detector: 200 nm, capillary tube: 570 mm×75 μm. Under these conditions, all the products appear in times between: 7.6 min phenamidine and 8.8 min dibromopropamidine, limits of detection being: berenil: 0.50, phenamidine: 0.25, diampron: 0.40 and dibromopropamidine: 0.80 $\mu g\,ml^{-1}$. We have carried out a recovery study with three kinds of extraction cartridges: Sep-pak C-18 plus, Sep-pak C-8 plus and Oasis HBL for each one of the products in blood and urine.

Keywords: Berenil; Phenamidine; Diampron; Dibromopropamidine

1. Introduction

The chemical–therapeutic activity of the group of compounds generically called aromatic diamidines is very broad; these molecules are active against *Trypanosoma rhodesiense,* and *Trypanosoma congolense*; they are also effective against infections by *Babesia canis* in dogs or *Lehismania donovani* in hamsters. These results in animals are the experimental background for the therapeutic use of diamidines in the treatment of lehismaniasis and trypanosomiasis in humans. Aromatic diamidines interact in the metabolism of polyamines; the complex regulation of the level of polyamines and their transport is evidenced by the administration of α-difluoromethylornithine, an inhibitor of ornithine decarboxylase (ODC), the first and key enzyme in the synthesis of polyamines of the biosynthesis of polyamines [3,4] provoking a decrease in the intracellular levels of putrescine and spermidine, simultaneously increasing very markedly the activity

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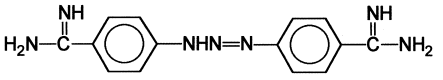

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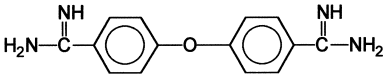

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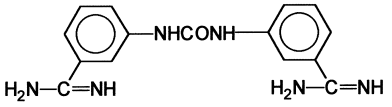
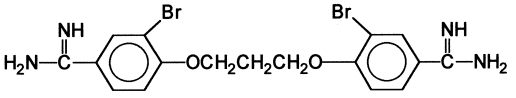


DIAMPRON



DIBROMOPROPAMIDINE

Fig. 1. Structure of diamidines: (1) Berenil (diminacene aceturate): 4,4′(1triazene1,3diyl)bis (benzenecarboximidamide) *N*-acetylglycine; (2) Phenamidine: 4,4′-oxybisbenzenecarboximidamide; (3) Diampron (amicarbalide): 3,3′-(carbonyldiimino)bisbenzenecarboximidamide; (4) Dibromopropamidine: 4,4′[1,3 propanediylbis(oxy)]bis (3-bromo benzene carboximidamide).

of the transporter [5]. Aromatic diamidines inhibit *S*-adenosyl-L-methionine decarboxylase (SAMDC) and can, therefore, slow down or prevent the growth of tumours [1,2], even ODC can act as an oncogene since its overexpression confers a phenotype of transformed tumour cell.

With this viewpoint, different structural analogues of aromatic diamidines have been synthesized in the last twenty years, like berenil, phenamidine, diampron, dibromopropamidine, etc. with a view to obtain medicines increasingly effective against tumours, with high stability, low toxicity and easy to administrate.

The aim of this work is to obtain an analysis method by CZE of a series of aromatic diamidines which are (Fig. 1):

berenil:

4,4′-(1-triazene-1,3-diyl)bis(benzenecarboximidamide) *N*-acetylglycine

phenamidine:

4,4′-oxybisbenzenecarboximidamide

diampron: (amicarbalide)

3,3′-(carbonyldiimino)bisbenzenecarboximidamide

dibromopropamidine:

4,4′-[1,3-propanediylbis(oxy)]bis(3-bromobenzenecarboximidamide).

In order to design a reliable, effective analysis method by CZE with high resolution and low limits of detection for these products, it is necessary to study how the apparent electrophoretic mobility (μ_{app}) of these products is affected by several parameters which directly influence in the electrophoretic process. The parameters that have been studied are: Variation of electrolyte pH, variation of electrolyte concentration, Ohm's law plot, voltage variation and temperature variation. With the data obtained from this study, we suggest a set of analytical conditions which allow a good separation and quantification of these products in serum and urine of patients treated with these products. In order to obtain a good analysis method, having the most adequate conditions of CZE is as important as making a good and, if possible, simple preparation of the sample. Therefore, we have included in this paper data about different methods tried for treating the samples of serum and urine before their analysis by CZE.

We have also experimented in vivo with phenamidine in rats in order to confirm that the method we suggest is perfectly valid for in vivo pharmacological studies of these kind of products.

2. Experimental

2.1. Equipment

For all experiments a P/ACE System 2000 HPCE instrument (Beckman, Palo Alto, CA, USA) was used. An untreated fused-silica capillary tube (Beckman Instruments), 570 mm×75 μm ID, effective length to detector 500 mm, enclosed within a temperature controlled, liquid-filled cartridge, was employed for separation. The wavelength of the UV

detector was set at 200 nm. System Gold Nouveau software (Beckman Instruments) was used to control the equipment, the data acquisition and the analysis of the results.

For solid-phase extraction a Manifold (Waters, Milford, MA, USA) was used and a SC110A Speed Vac plus concentrator (Savant Instruments, Farmingdale, NY, USA) for solvents evaporation.

2.2. Reagents and materials

The water was purified with a Milli Q II water purification system purchased from Millipore (Bedford, MA, USA). Berenil were obtained from Sigma-Aldrich. All other reagents of electrophoresis reagent grade and pentobarbital were obtained from Sigma-Aldrich Química, (Madrid, Spain). Phenamidine, diampron, and dibromopropamidine were generous gifts from Rhône Poulenc Rorer (Dagenham, UK). Sep-pak Cartidge C-18 Plus, Sep-Pak Cartridge C-8 Plus, Oasis HLB (Waters).

2.3. Preparation of standard solutions

The standard solutions were prepared by dissolving the necessary amount of berenil, phenamidine, diampron and dibromopropamidine in water. All the standard solutions were stored away from light at 4°C.

2.4. Preparation of serum and urine samples

Serum was prepared from human blood by centrifugation, different amounts of each product studied here were dissolved in the serum in order to obtain the desired concentrations.

Each one of the products was dissolved in human urine in the amounts necessary to obtain the desired concentrations.

We have studied three systems of extraction in the solid-phase as preparation methods for samples of serum and urine previous to the analysis by CZE. The procedures used for extraction cartridges Sep-Pak C-18 Plus and Sep-Pak C-8 Plus are: activation first with 8 ml of methanol, then with 10 ml of water, load with 1 ml of serum or urine marked with the product, elution of unwanted components with 1 ml of water and elution of the components of interest with 1 ml of methanol which will be evaporated by Speed-Vac and then reconstituted with 1 ml of a dilution 1:10 of the work buffer to be injected in CZE.

Cartridges Oasis HLB: The conditioning of the cartridge is carried out by passing through it 1 ml of methanol and then 1 ml of water. It is then loaded with 1 ml of urine or serum and washed with 1 ml of methanol–water 5%, elution of the product with 1 ml of methanol which will be evaporated in Speed-Vac and reconstituted in the same way as in the previous case before initiating the analysis by CZE. In this case, it is necessary to add 10 μl of phosphoric acid per ml to the serum samples, before initiating the process of extraction in order to prevent the loss of the product because of its union to proteins

2.5. Electrophoretic procedures

In order to achieve a good repeatability, it was necessary that the process be always carried out in identical conditions of the capillary. To achieve this, it was necessary to have an unchanging work routine, which includes a cycle of washing and regeneration of the previously used capillary in every electrophoretic process. The steps of the process we have used are:

1. Regeneration of the capillary with NaOH 0.1 *M* (2 min).
2. Filling the capillary with work buffer (2 min).
3. Introduction of the sample into the capillary by pressure at 0.5 p.s.i. for 5 s.
4. Introduction of the buffer into the capillary by pressure at 0.5 p.s.i. for 1 s.
5. Introduction of the capillary extremes into two vials with new buffer for each process to avoid ‘Buffer Depletion’ [6,7] and initiate the electrophoretic process.

When the electrophoretic process is over the capillary is washed with water for 2 min.

2.6. In vivo study of phenamidine

Adult male Wistar rats, purchased from IFFA CREDO (Barcelona, Spain) were used as experimen-

tal subjects. They were housed, three per cage, in a temperature-controlled room (21–23°C), with a 40–60% humidity atmosphere and a 12–12 h light–dark circle. Rats were acclimatised to these conditions for a minimum of 1 week before the study, being fed with a standard rodent diet (Panlab, Barcelona, Spain) and water ad libitum.

Animals were anesthesized with an intraperitoneal injection of a solution of pentobarbital in distilled water (600 mg/100 ml), being administered 1 ml of the solution per each 100 g of body weight. Then, the animal was kept warm at a site by a light focus. When the degree of the anesthesia is the adequate, the rat is placed in a surgical board in supine position, hold by an adhesive tape through the extremities. Jugular vein and carotida artheria were cannulated.

3. Results and discussion

In this paper we have studied the variation of several CZE operating parameters and their influence on apparent electrophoretic mobility (μ_{app}) of the products studied.

The time required for a solute to migrate to the point of detection is called the migration time (t) and is given by the quotient of migration distance and velocity. The migration time and other experimental parameters can be used to calculate the μ_{app} using [8]:

$$\mu_{app} = L_t \cdot L_d / t \cdot V (cm^2\ V^{-1} \cdot s^{-1})$$

L_t = Total capillary length; L_d = length capillary to the detector; t = migration time; V = applied voltage.

3.1. Influence of pH electrolyte

The influence of electrolyte pH on the electrophoretic process is of capital importance, since when the electrolyte pH varies, so does the EOF and the state of ionization of the different analytes contained in the sample and their effective charge change too, therefore modifying their apparent mobility (μ_{app}), the solubility of the analyte in the electrolyte and all the parameters affecting the efficiency and selectivity of the separation, like: number of theoretical plates, peak height and area of every peak [9].

In this paper we have studied how μ_{app} varies as electrolyte pH changes. In Fig. 2 we can observe that there is a small decrease of μ_{app} as pH passes from 3.5 to 4.0 in all the products studied here, and, from pH 4.0 onwards, there is an important increase of $\mu_{app.}$ Taking into account that all these products behave very much in the same way and that we are interested in low levels of μ_{app} we have selected pH = 3.70 as the most adequate buffer pH.

3.2. Influence of electrolyte concentration

In most cases, low buffer concentrations (10–50 mM) are utilised, but with buffer concentrations of 100 mM or more, an important amount of heat is generated, even with low voltages, due to Joule heat [10]. If the equipment in which CZE is carried out doesn't have the capability to eliminate this heat, it brings about several undesired effects like variation of viscosity and electrolyte pH with the subsequent variation of EOF and the resulting loss of resolution of the analysis method.

When electrolyte concentration is higher, its regulation capacity also grows bigger and the interaction

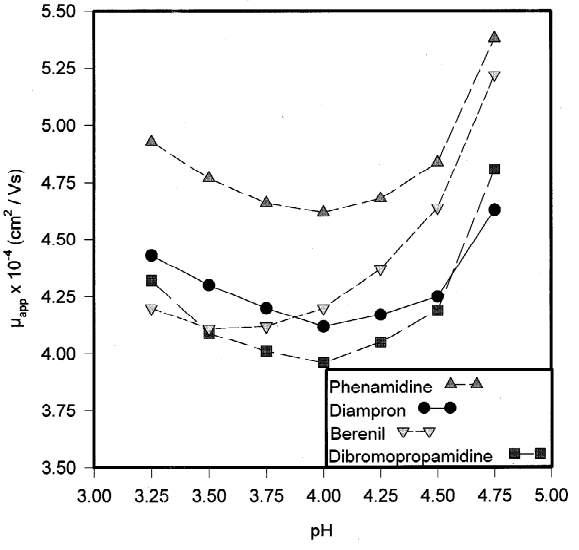


Fig. 2. Influence of the electrolyte pH in μ_{app}. CZE conditions: citrate buffer 25 mM, V = 14 kV, T = 30°C, pH: 3.25, 3.50, 3.75, 4.0, 4.25, 4.50, 4.75.

between the analyte and the capillary walls decreases [11]. At higher electrolyte concentration, there will be bigger regulation capacity, less interaction between the analyte and the capillary walls, and lower EOF due to an increase in the thickness of the double ionic layer. All of these could lead to a rise in the analysis times and therefore to a fall in $\mu_{app.}$ When electrolyte concentration gets higher, so does resolution and the interaction between analytes fall, both aspects leading to a better analysis method.

In order to accurately know the effects brought about by variation of electrolyte concentration on the compounds being studied, we have investigated the variation of μ_{app} in each one of them when electrolyte concentration changes. This study has been carried out using the following electrolyte concentrations: 10, 20, 30, 40, 50 m*M*; Voltage: 14 kV and keeping the temperature of the capillary constant at 30°C.

The data thus obtained are shown in Fig. 3, in which we can observe that the behaviour of the four products is very similar. In all four cases, the apparent electrophoretic mobility (μ_{app}) decreases significantly as electrolyte concentration gets higher; resulting in an increase of the analysis time and a significant improvement of resolution.

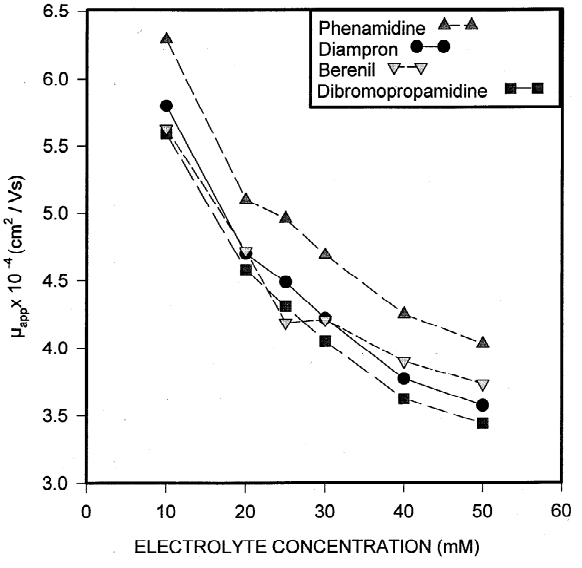


Fig. 3. Variation in μ_{app} with variation in electrolyte concentration. CZE conditions: citrate buffer pH=3.70, V=14 kV, T=30°C, electrolyte concentration: 10, 20, 30, 40, 50 m*M*.

3.3. Ohm's law plot

As we have said in the previous paragraph, it is advisable to work with a high electrolyte concentration since it improves resolution, but a high electrolyte concentration leads to a low resistance and, according to Ohm's law, to a high intensity which in turn leads to an important generation of heat called Joule heat [12,13] which can rise the temperature of the capillary. Thus, the temperature of the electrolyte inside is not homogeneous but the temperature in the capillary centre is higher than the temperature near the capillary wall. The radial temperature profile brings in, of course, homogeneities of the physico–chemical parameters such as mobility, pH, density, viscosity, etc. Modern equipment for Capillary Electrophoresis (CE) has very efficient systems of thermostats in the capillary that make it possible to eliminate the heat, provided that we work within the adequate limits of voltage and electrolyte concentration.

To determine the maximum buffer concentration and the maximum voltage that can be applied in the analytical method by CE, Nelson et al. [14] suggested Ohm's law plot that makes it easy to determine the maximum buffer concentration and maximum voltage that can be applied by plotting the intensity values obtained at different voltages for different electrolytes or, as in this paper, for different concentrations of the same electrolyte. The range of concentrations that in this chart yields a straight line as a result, indicates that capillary temperature is correct, that is to say, that the Joule heat is being effectively dissipated by the system. The voltage at which the linearity is lost, indicates that the capacity of the equipment's thermostat has been overrun and that, therefore, the conditions are no longer adequate to work. We recommend that the power associated to the buffer [15] should be less than 1 to get good conditions of separation, and never bigger than 5 $W\,m^{-1}$.

In this work we have studied how intensity varies with voltage changes at: 8, 11, 14, 17, 20, 23, and 26 kV. We have worked with citrate buffer 25 m*M*; pH=3.7; T=30 °C. In Fig. 4 Ohm's law plot is shown in these circumstances observing that this electrolyte keeps linearity up to 23 kV and, from this voltage onward, it deviates from linearity. Therefore,

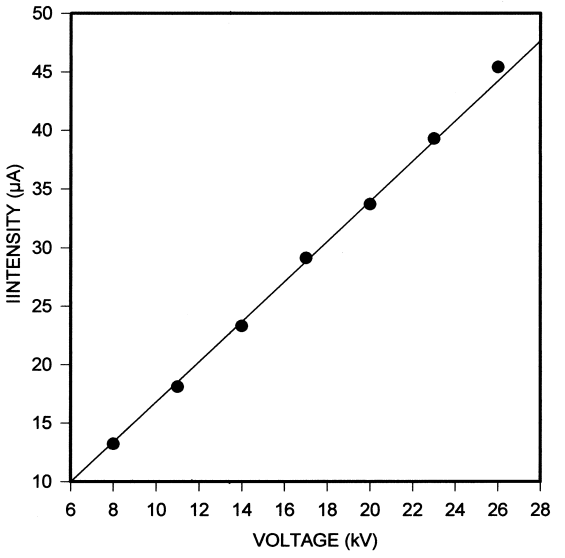


Fig. 4. Ohm's law plot. CZE conditions: citrate buffer 25 m*M*, pH = 3.70, T = 30°C, applied voltage: 8, 11, 14, 17, 20, 23, 26 kV.

it is not adequate to work with a voltage higher than 23 kV.

Applying the equation that allows us to obtain the power associated to the electrolyte in the conditions described above and with a voltage of 14 kV, the results are:

$$\text{Watts m}^{-1} = (\text{Voltage})\,(\text{Amperage})/\text{Column length (m)} \cdot 1000$$

$$\text{Watts m}^{-1} = 14\ \text{kV} \cdot 23\ \mu\text{A}/0.57 \cdot 1000$$

$$= 0.56\ \text{W m}^{-1}$$

The result is less than 1, so the condition we commented above is fulfilled and, in principle, it would be acceptable to work at 14 kV, further studies notwithstanding.

3.4. Influence of applied voltage

The effects of the increase of voltage on the CZE process are multiple; in the first place the electrosmotic flow rises, migration times fall and, as a consequence, μ_{app} rises. Since migration times are smaller, the peaks are thinner and the resolution and efficiency are higher [16].

Rising the voltage also has disadvantages, since working with a high voltage produces a lot of Joule heat that, if not dissipated efficiently, can negatively affect the accomplishment of a good method in CZE.

In order to determine the best voltage for the study of these products by CZE, we have studied how μ_{app} is affected by voltage variation (Fig. 5). We have worked with buffer citrate 25 m*M*; pH = 3.70 and applied voltages of: 8, 11, 14, 17, 20, 23 and 26 kV; observing no important changes in μ_{app} values until 14 kV and onwards, we can see a progressive increase when voltage rise; reaching very high values at 26 kV, specially in phenamidine which already had higher values than the rest of products at all voltages.

With the data obtained from this study and Ohm's law plot and considering that we are interested in short analysis times and that high voltages are less convenient, we have chosen 14 kV as the most adequate voltage.

3.5. Influence of temperature

The temperature at which CZE is carried out has to be carefully selected since it is one of the most influential parameters in the process of CZE and a

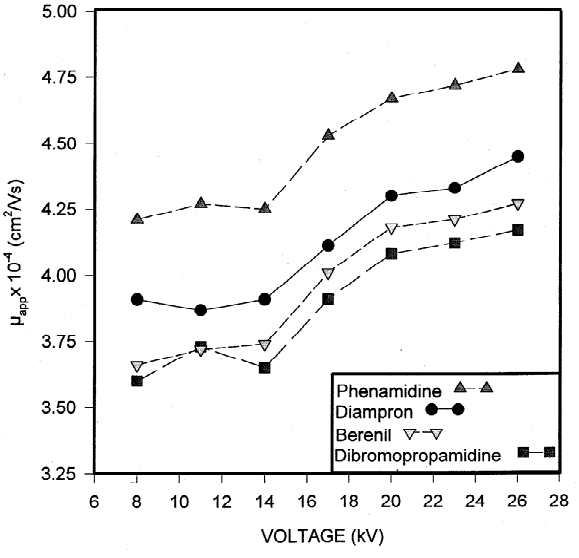


Fig. 5. Variation of μ_{app} with variation of voltage. CZE conditions: citrate buffer 25 m*M*, pH = 3.70, T = 30°C, applied voltage: 8, 11, 14, 17, 20, 23, and 26 kV.

good method of analysis can only be obtained working at an adequate temperature [17].

CE equipment has very advanced thermostatic systems which allow us to work with no problems in a range between room temperature and 50–60°C. Working at a high temperature (50°C) reduces the migration times of analytes and allows for a slightly bigger sample to be loaded into the capillary, since the viscosity of the sample and that of the buffer are smaller. However, working at high temperatures can have negative effects like affecting the stability of the sample or of the analytes or limiting the voltage we can use since, at high temperatures, the Joule heat produced by high voltages will be difficult to evacuate and we could have to face the problems that have been commented in the section about Ohm's law plot.

In order to obtain data to choose the most convenient temperature, we have represented (Fig. 6) the μ_{app} values obtained working with citrate buffer 25 m*M*; pH = 3.70, voltage: 14 kV, at different temperatures: 25, 28, 31, 34, 37, 40°C. As we expected, when temperatures raised, migration times fell and, conversely, μ_{app} values raised in all the products.

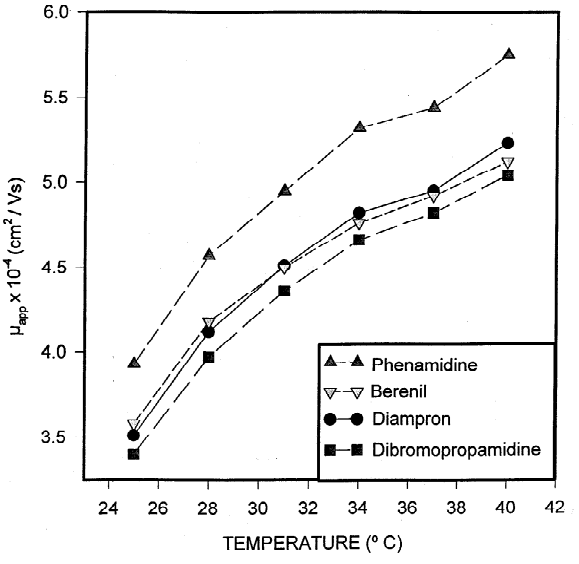


Fig. 6. Variation of μ_{app} with variation of temperature. CZE conditions: citrate buffer 25 m*M*, pH = 3.70, 14 kV, Temperature: 28, 31, 34, 37, 40°C.

Since we are interested in low μ_{app} values, we consider that the most adequate work temperature is 30°C.

3.6. Optimal Conditions for CZE

From the data obtained from the previous studies, we suggest that the most adequate conditions for the study of this products in serum and urine are: electrolyte citrate 25 m*M*; pH = 3.70, 14 kV, *T* = 30°C. The electrical intensity generated under this conditions is 23.8 μA.

Under this conditions, we have obtained the electropherogram shown in Fig. 7 from a watery sample containing 7.5 $\mu g\,ml^{-1}$ of each one of the products being studied; we have observed a good separation between them in a period between 7.6 and 8.8 min, which is intentionally long so that the analytes are not interfered with by the components of the serum and urine matrixes in which they were dissolved in real situation.

3.7. Precision

In order to determine the values of standard deviation we have analysed, under the analysis conditions we propose, six replicates of each one of the products dissolved in water in a concentration of 4 and 7.5 $\mu g\,ml^{-1}$, obtaining the results shown in Table 1.

3.8. Linearity

For the establishment of linearity, at least five concentrations level (1, 2.5, 5, 7.5 and 10 $\mu g\,ml^{-1}$) have been used for each one of the products dissolved into water.

Data are to be analysed by linear regression, obtaining the equations and regression coefficients corresponding to each product, which are shown in Table 2.

3.9. Preparation of samples

We have studied three extraction systems in the solid-phase in order to prepare the samples of serum and urine for the analysis by CZE.

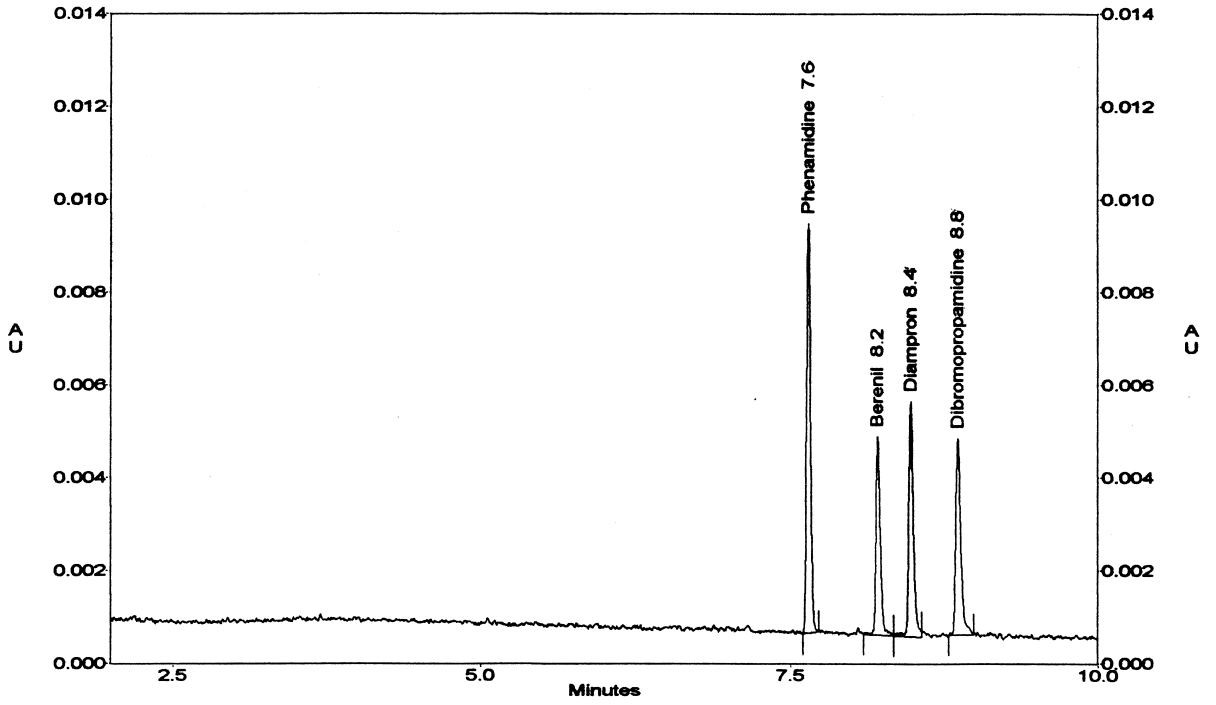


Fig. 7. Electropherogram from a watery sample containing 7.5 μg ml^{-1} of berenil, phenamidine, diampron, dibromopropamidine in optimal conditions for CZE: citrate buffer 25 m*M*, pH = 3.70, 14 kV, 30°C.

Table 1
Precision assay

	Level (μg ml^{-1})	Calculated average	SD	% C.V.
Berenil	4	3.85	0.09	2.40
	7.5	7.7	0.15	2.00
Phenamidine	4	3.9	0.15	4.00
	7.5	7.65	0.20	2.60
Diampron	4	4.05	0.38	4.10
	7.5	7.55	0.34	4.40
Dibromopropamidine	4	3.9	0.09	2.45
	7.5	7.7	0.39	5.10

Table 2
Equations for the straight line relationships and the correlation coefficients

	Equation	Correlation coefficient
Berenil	$y = 40.28x + 0.44$	0.997
Phenamidine	$y = 22.25x + 0.27$	0.999
Diampron	$y = 33.22x + 0.31$	0.998
Dibromopropamidine	$y = 34.17x + 1.02$	0.997

We have used samples of serum and urine supplemented with the products in concentrations of 5 and 10 μg ml^{-1}. We carried out 6 complete processes of extraction and analysis with every sample and the data of recovery we obtained are shown in Table 3.

3.10. Determination of analytes in serum and urine

Once the analysis method has been tested with all the products by means of patterns dissolved in water; we made a separate study for each one of them in serum and urine. We added by means of a watery dissolution the amount necessary to obtain a concentration of 5 μg ml^{-1}. Then, we have treated the samples using Oasis HLB cartridges for urine and Sep-pak C-18 for serum, in the manner described, and we have carried out the analysis by CZE obtaining the electropherograms shown in Fig. 8.

Table 3
Recovery test of solid-phase extraction systems

Concentration ($\mu g\ ml^{-1}$)		Sep-pak C_{18} plus				Sep-pak C_8 plus				Oasis HLB			
		Serum		Urine		Serum		Urine		Serum		Urine	
		5	10	5	10	5	10	5	10	5	10	5	10
Berenil	% Rec.	–	–	98.7	58.2	–	–	83.1	75.0	53.8	17.3	96.2	59.3
	% C.V.	–	–	3.2	5.2	–	–	3.1	2.9	4.0	5.3	1.8	1.9
Phenamidine	% Rec.	95.6	83.2	78.7	59.4	72.6	57.1	95.3	83.9	26.3	11.4	102.0	95.8
	% C.V.	2.6	2.3	1.9	2.4	5.8	6.2	2.4	2.4	7.6	8.5	1.4	1.9
Diampron	% Rec.	97.4	74.4	–	–	76.5	59.8	78.3	76.2	69.3	56.8	103.0	89.5
	% C.V.	1.6	2.3	–	–	4.6	4.8	3.0	3.7	3.6	3.9	1.9	2.1
Dibromopropamidine	% Rec.	95.9	75.7	–	–	83.3	70.8	48.6	45.6	103.0	105.0	83.4	74.2
	% C.V.	2.2	2.9	–	–	3.2	3.9	5.1	4.3	1.5	1.7	3.1	3.4

3.11. Limit of detection

Considering as limit of detection the concentration at which the analyte peak is three times the noise, the values obtained in this work utilising watery solutions, serum and urine are the following: berenil 0.50 $\mu g\ ml^{-1}$, phenamidine 0.25 $\mu g\ ml^{-1}$, diampron 0.40 $\mu g\ ml^{-1}$ and dibromopropamidine 0.80 $\mu g\ ml^{-1}$.

4. Application of the method to experimental samples

An animal experiment was designed to test the new method and provide useful information on peak serum concentration and the rate of drug elimination from serum. Four rats (between 550 and 710 g of weight) were chosen as the experimental animal so

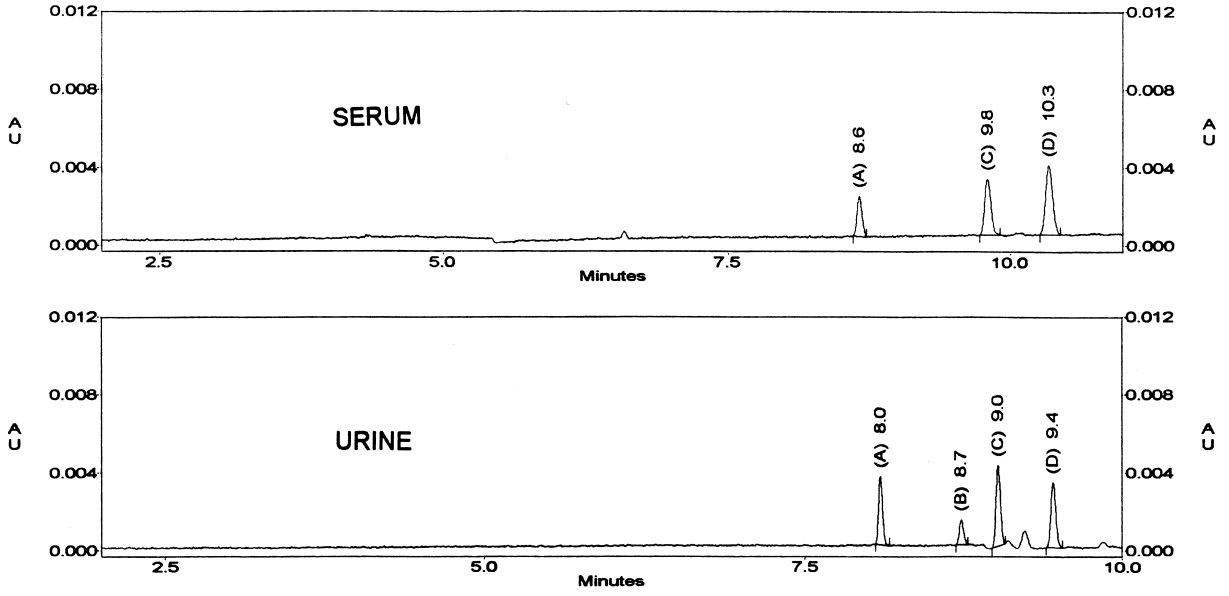


Fig. 8. Electropherograms of serum and urine. (A) Phenamidine, (B) Berenil, (C) Diampron, (D) Dibromopropamidine.

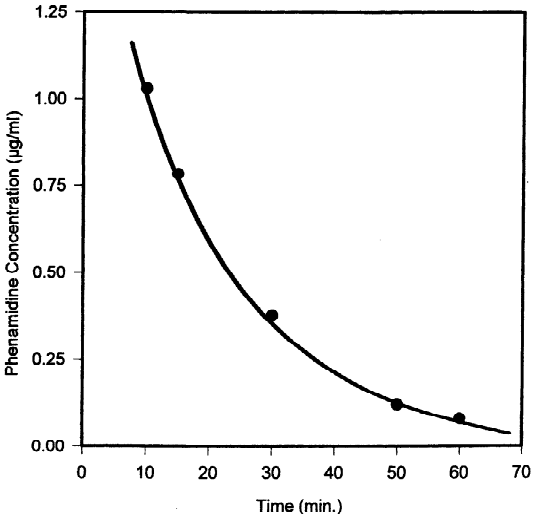


Fig. 9. Plot of serum concentration versus time after administration of phenamidine.

that sufficient serum would be available to run triplicate analyses for each data point.

A 4 mg kg^{-1} of phenamidine were administered intravenously in the jugular vein and blood samples were collected from carotida artheria at different times (10, 15, 30, 50 and 60 min) and treated using Sep-pak C-18 plus cartridges and analysed. Plot of serum concentration versus time after administration of phenamidine is shown in Fig. 9.

5. Conclusions

We have studied the influence of several factors which are most important in the electrophoretic process by CZE: pH of electrolyte, buffer concentration, Ohm's law plot, voltage applied and temperature of the capillary, on the apparent electrophoretic mobility of several aromatic diamidines with therapeutic interest: berenil, phenamidine, diampron and dibromopropamidine. The data obtained with this study allow us to gain a deep insight of the behaviour of these products in CZE. This understanding makes it possible to easily design analysis methods for all four products in serum and urine, in this case, or in different biological matrixes that could be interesting in other cases.

We suggest an analysis method for each product in serum and urine. The analytical conditions which this study shows as most adequate are: citrate buffer 25 m*M*; pH = 3.70, 14 kV, $T = 30°C$, the wavelength of the UV detector: 200 nm, capillary tube 570 mm × 75 μm. Under these conditions all products appear after between 7.6 and 8.8 min, thus avoiding interference with the components of the serum and urine matrix.

From the study carried out for the preparation of the sample, it follows that Oasis HLB is a quick and suitable method, with good recovery results, for the preparation of urine samples and Sep-pak C-18 plus for serum samples except for Berenil that it is necessary used Oasis HLB and with a poor recovery.

We consider this analysis method to be quite reliable, offering low limits of detection, and requiring a simple preparation of the sample. We believe that this method is an interesting alternative for the analysis by HPLC formerly suggested for these products in serum and urine.

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