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Determination of pentamidine in serum and urine by micellar electrokinetic chromatography

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Abstract

A number of parameters influencing the electrokinetic processing of pentamidine by micellar electrokinetic chromatography (MEKC) were studied in order to develop an analytical method for this compound. The parameters considered were: pH, ionic strength, and SDS concentration of electrolyte, temperature and working voltage. On the basis of the results obtained, the best analytical conditions for the detection of pentamidine in serum and urine by MEKC were determined. Analysis by MEKC permitted determination of the drug in 10 min. Good linearity, reproducibility and accuracy were obtained in the range 0–30 μg/ml for both samples, with a correlation coefficient $r \geq 0.9998$ and a recovery of 87–92% in serum and 90–108.9% in urine. We examined the metabolism of pentamidine using rat liver homogenates in order to exclude any possible interference of metabolites in the analysis of pentamidine.

Keywords: Pentamidine

1. Introduction

The metabolism of the polyamines in lower eukaryotes proceeds via a tightly regulated route of biosynthesis and is involved in proliferative processes. It has thus been widely used for therapeutic purposes in infectious parasitic diseases [1]. The enzymes involved in the biosynthesis of polyamines may be inhibited by a large number of chemicals: some specifically synthesized for this purpose, and others having chemical structures which resemble the polyamines [2]. The aromatic diamidines belong to this second group. These drugs were designed as antiparasitic compounds, and are used successfully against infections caused by some species of *Trypanosoma*, *Leishmania* and *Babesia* geni, either in human or veterinary medicine [3]. Pentamidine is a micromolar inhibitor of *S*-adenosyl-L-methionine decarboxylase (SAMDC), the key enzyme of spermidine biosynthesis, and of diamine oxidase (DAO), the first enzyme of terminal polyamine oxidation in mammalian and in lower eukaryotes [4,5]. The aromatic diamidines have recently been shown to be non-competitive inhibitors of the take-up of putrescine in parasitic protozoans [6].

Several HPLC methods have been developed to determine pentamidine in serum and urine [7–13], and in *Leishmania infantum* promastigotes [14]. MEKC, which was introduced by Terabe and his co-workers in 1984 [15] for the analysis of electrically neutral substances, is nowadays used to enhance the selectivity of separation of both neutral and ionic

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solutes [16]. The technique is based on the addition of a surfactant in sufficient quantity to form micelles. Separation is based on the different distribution coefficients of the injected solutes between the micelle and the buffer. MEKC has some advantages over HPLC. These are: lower operating costs, because of the low price of the capillary and solvents used, and very high plate numbers, coupled with a requirement for only a small amount of sample and the fact that in some case pretreatment of the sample is minimal [17].

The intention of the present work was to study how variation of the different parameters (pH, ionic strength and SDS concentration of electrolyte, and voltage and temperature used in the experiments) influence the capacity factor (k') of pentamidine using MEKC and, further, to use these data as a starting point to develop a specific MEKC method for the determination of pentamidine in serum and urine. We believe that this is of interest, as it would provide us with a new analytic method for determining pentamidine. This would, furthermore, be complementary to HPLC, as MEKC is a highly efficient technique with very different selectivity from that of HPLC.

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 I.D.; 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 214 nm. System Gold software (Beckman Instruments, Fullerton, CA, USA) was used to control the equipment, the data acquisition and the analysis of the results.

2.2. Reagents and materials

SDS pentamidine isethionate salt and corn oil were obtained from Sigma (St. Louis, MO, USA). The water was purified with a Milli Q II water purification system purchased from Millipore (Bedford, MA, USA). All other reagents and solvents of analytic reagent grade were obtained from Merck (Darmstadt, Germany). The ultrafiltration systems, with a 30 000 relative molecular mass cut-off (Ultrafree-MC UFC3 LTK 00 and UFC3 TTK 00) were purchased from Millipore. Aroclor 1254 was purchased from IFA-CREDO (Madrid, Spain). Albendazole and its metabolites (sulphoxide and sulphone) were supplied by Smith-Kleene Animal Health Products (Sucy en Brie, France). NADP and D-glucose-6P were obtained from Boehringer Mannheim (Mannheim, Germany).

2.3. Preparation of standard solutions

The standard solutions were prepared by dissolving the necessary amount of pentamidine isethionate in water. All the standard solutions were stored away from light at 4°C.

2.4. Preparation of samples

2.4.1. Serum

Serum was prepared from human blood by centrifugation. Several amounts of pentamidine isethionate were dissolved in the serum in order to obtain different concentrations. A volume of 138 μl of each dissolution was treated with 12 μl of 0.5 *M* H_3PO_4, vortexed and filtrated using the Ultrafree-MC ultrafiltration system of low protein binding regenerated cellulose with a molecular mass cut-off filter of 30 000 by means of centrifugation at 5000 *g* for 10 min. The filtrate was directly used for analysis.

2.4.2. Urine

Several amounts of pentamidine isethionate were dissolved in human urine to provide the desired concentrations. A volume of 120 μl of each disolution was filtrated using Ultrafree-MC ultrafiltration system of polysulphone with a molecular mass cut-off filter of 30 000 by means of centrifugation at 5000 *g* for 5 min. The filtrate was discarded and the filter then treated in a sonication bath with 60 μl of 0.01 *M* H_3PO_4 and again centrifuged. The filter was treated in the same way with 60 μl of 0.02 *M* H_3PO_4, and the two 60-μl fractions mixed and used for analysis.

2.5. In vitro study of pentamidine metabolism

The supernatant of the post-mitochondrial liver fraction from two male healthy Sprague–Dawley rats (weighing approximately 200 g), induced with Aroclor 1254 (a polychlorinated biphenyl, PCB, mixture), was used to provide in vitro metabolism of the pentamidine. The animals were housed in a controlled environment with a 12 h light–12 h dark cycle and with access to food and water ad libitum. Aroclor 1254 was diluted in corn oil to a concentration of 200 mg/ml and a single i.p. injection of 500 mg/kg was administered to each rat five days before the homogenate preparation date. Food was removed 24 h before sacrificing the animals. The rats were killed by cervical dislocation and immediately exsanguinated. The livers were removed, freed of extrahepatic tissue, washed with an ice-cold saline solution and blotted free of excess moisture. All subsequent operations were performed at 4°C. Samples of liver (7 g) corresponding to aliquots from the pooled liver samples were prepared by homogenization with a Teflon pestle with 21 ml of ice-cold 0.15 *M* ClK in 5 m*M* Na–K phosphate buffer (pH 7.4), in a glass potter homogenizer. The homogenates were centrifuged for 20 min at 9000 *g* in a refrigerated centrifuge. The supernatant fraction was carefully transferred and was used as the enzyme source. Protein concentration was determined by the Coomasie blue method [24].

2.6. Pentamidine incubation

The reaction mixture contained, in a final volume of 4 ml, 0.5 ml of liver homogenate supernatant (7.5 mg of protein), 0.5 ml of cofactor solution (5 m*M* NADP, 7 m*M* glucose-6-phosphate), and 2 ml of 0.133 *M* Na–K phosphate buffer (pH 7.4) containing 25 m*M* KCl and 8 m*M* $MgCl_2$, was preincubated for 10 min at 37°C in a shaking water bath. The metabolism experiments were conducted at 37°C in a shaking water bath (89 U/min). One millilitre of pentamidine stock solution (260 μg/ml) was then added to initiate the reaction under an atmosphere of 95% O_2–5% CO_2. Aliquots (200 μl) of the incubation mixture were taken at timed intervals (0, 10, 20, 30, 45, 60 and 90 min). The reactions were terminated by adding 50 μl of CH_3OH, and centrifuged for 15 min at 5000 *g* in a microfuge. The supernatants were inmediately analysed using MECK. Control experiments to measure the nonenzymatic degradation were done simultaneously using enzyme solution pre-boiled at 100°C for 10 min. To confirm the enzymatic activity of the liver homogenates, the metabolism of albendazole to albendazole-SO and albendazole-SO_2, was measured in simultaneous experiments.

3. Results and discussion

It is generally accepted [16] that hydrophobic interactions are the main force leading to the formation of micelles of a surfactant in an aqueous medium. The structure of the micelle is also affected by factors such as pH, ionic strength, concentration of the surfactant oin the electrolyte, temperature of work and others. The most important factor in the formation of the micelle is the concentration of the surfactant. At low concentrations and with a temperature above the critical value (Kraft point), the surfactant remains dispersed in the aqueous medium, while as the concentration goes above a given minimum value the molecules aggregate to form micellar structures. The average number of molecules per micelle is known as the aggregation number. The most common micelles are those composed of between 40 and 140 molecules [16]. The minimum concentration of surfactant needed for the formation of micelles is called the critical micelle concentration (CMC). At 25°C and under a pressure of one atmosphere, CMC is usually less than 20 m*M* [16]. Both the aggregation number and the CMC value depend on a range of physiochemical parameters which includes the addition of organic solvents, ionic strength, pH, temperature and others. Separation in MEKC is due to the different distribution coefficients of the injected solutes between the micellar pseudophase and the solvent, and the difference in mobility between these two phases. Thus, selectivity may be adjusted by altering the type and composition of the surfactant and varying the other parameters previously listed (see Section 2). It is commonly accepted that in MEKC, by analogy with classic HPLC, the concepts of retention time (in MEKC, migration time) and capacity factor are

suited to the quantitative description of the separation process [15,18]. t_0 (min) is the migration time of a solute with no interaction with the micelles (electroendosmotic flow marker). Methanol was used to measure t_0, in view of its availability and because its distribution coefficient in the SDS micelles is almost negligible. Furthermore, it can be detected by UV absorption, thanks to a change in refractive index as the methanol peak passes through the detection zone. The measurement of t_{mc} (migration time of the micelle) is analogous, and was carried out by measuring the retention time of a neutral, fully solubilised substance, Sudan III, migrating at the speed of the micelles. In MEKC the capacity factor (k') is defined for a given solute as the ratio of the total moles of solute in the micelles (n_{mc}) to those in the aqueous phase (n_{aq})

$$k' = \frac{n_{mc}}{n_{aq}}$$

The migration time (t_R) and the capacity factor of the solute are then expressed [18];

$$t_R = \frac{1 + k'}{1 + (t_o/t_{mc})k'} t_o \qquad k' = \frac{t_R - t_o}{t_o(1 - t_R/t_{mc})}$$

t_R being the solute migration time in min, and t_{mc} the retention time of a neutral, fully solubilized substance migrating at the speed of the micelles. As t_{mc} tends to infinity (the micellar phase becomes stationary) the expression of k' reduces to the equivalent equation for conventional chromatography.

$$k' = \frac{t_R - t_o}{t_o}$$

Many parameters may be varied in MEKC to achieve the desired efficiency, selectivity or resolution. We studied how these variations affected k' for pentamidine.

3.1. Influence of pH

One parameter having considerable influence in MEKC is the pH of the electrolyte. If it is wrongly set, then the possibility of a good separation is drastically reduced. It is often the case that the separation of analogous compounds is based on small differences in their pK_a and, therefore, on small variations in the pH of the electrolyte. In general it is worthwhile undertaking a study of the behaviour of the substance under analysis both in high- and low-pH zones.

The behaviour of pentamidine in MEKC was studied at 214 nm and 30°C using a 10 m*M* phosphate–50 m*M* borate buffer with various pH values: 7.0, 7.5, 8.0, 8.5 and 9.0. The SDS concentration was 50 m*M* and the separation voltage 15 kV. Variations in the capacity factor are shown in Fig. 1. In this range, the pentamidine peak showed good symmetry. The lowest k'-value appears at a pH close to 8.0. At pH higher than this value, a rapid increase in the current is observed, probably due to the increase in the sodium ion concentration of the NaOH used to adjust the pH.

3.2. Influence of ionic strength

Study of the variation of ionic strength of the electrolyte is important because it influences the efficiency, sensitivity and resolution of an MEKC method. Since an increase in ionic strength produces higher intensities and higher Joule heating, effective temperature control becomes essential. The variation of k' of pentamidine with ionic strength (Fig. 2) was studied at 214 nm and with a voltage of 15 kV, in a

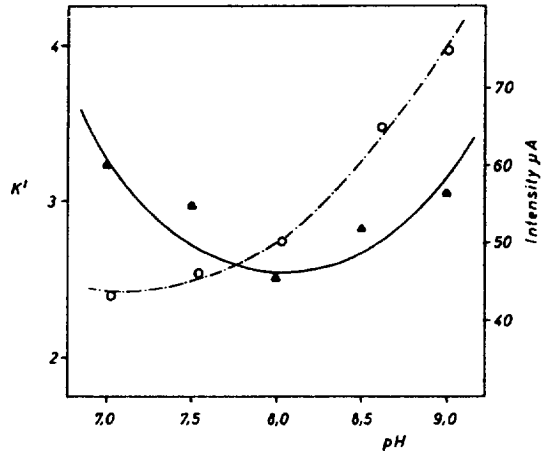

Fig. 1. Variation in the capacity factor of pentamidine (▲) and in intensity (○) with pH. Separation was carried out with 15 kV at 30°C in a 10 m*M* phosphate–50 m*M* borate buffer. SDS concentration is 50 m*M*.

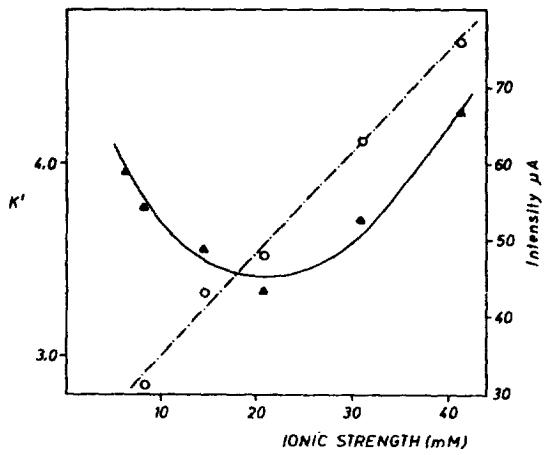

Fig. 2. Variation in the capacity factor of pentamidine (▲) and in current (○) with variation in ionic strength. Separation was carried out with 15 kV at 30°C in a phosphate–borate buffer at pH 8.0 and with an SDS concentration of 50 m*M*.

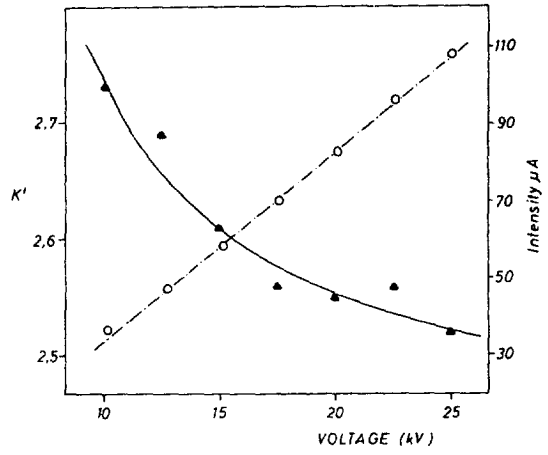

Fig. 3. Variation in the capacity factor of pentamidine (▲) and in intensity (○) with voltage. Separation was carried out at 30°C in a 10 m*M* phosphate–50 m*M* borate buffer at pH 8. SDS concentration 50 m*M*.

phosphate–borate buffer at pH 8.0 and with an SDS concentration of 50 m*M*. The molarities of both buffers were proportionately increased to obtain ionic strengths between 6 and 40 m*M*. It can be seen that k' descends until the ionic strength value is 20 m*M*, as a result of increased mobility of the substance caused by a decrease in the Z potential of the wall of the capillary. From 20 m*M* onwards, mobility decreases (k' increases), due to a drop in buffer viscosity caused by greater heat [19].

3.3. Influence of voltage

Increased voltage brings about a greatly increased efficiency and resolution, together with shorter analysis times, but more heat is produced. When Joule heat cannot be removed effectively, the number of theoretical plates and the resolution decrease. Conditions for maximum resolution are thus obtained when current and Joule heating are not limiting factors. Monitoring of the current is, therefore, extremely useful in determining voltage conditions. Proper voltage selection can be achieved using Ohm's law, maximum efficiency being attained at the point where Ohm's law just deviates from linearity [20]. Fig. 3 shows the effects of voltage on the k' for pentamidine and Ohm's law plot obtained from the intensities registered. From these results we may conclude that there is no deviation from linearity in the range 10–25 kV, which allows the use of 25 kV for separation, provided that the heat generated in these working conditions does not give rise to other problems in separation.

3.4. Influence of SDS concentration

The most important parameter with respect to the formation of micelles in the electrolyte is the concentration of surfactant. At low concentrations and at temperatures above the critical micelle temperature (Kraft point), the surfactant is dispersed in the aqueous medium at a molecular level. As the surfactant concentration goes above a given minimum value, the molecules associate to form micellar assemblies. The concentration of surfactant at the beginning of micelle aggregation is termed the critical micelle concentration (CMC). At 25°C and a pressure of one atmosphere, CMC is typically less than 20 m*M*, the effects of the concentration of SDS on the k' of pentamidine (Fig. 4) was studied at 214 nm and 15 kV in a 10 m*M* phosphate–50 m*M* borate buffer pH 8 at 30°C; k' increases linearly with the increase in SDS concentration, indicating that there is greater interaction with the micelles. A noteworthy

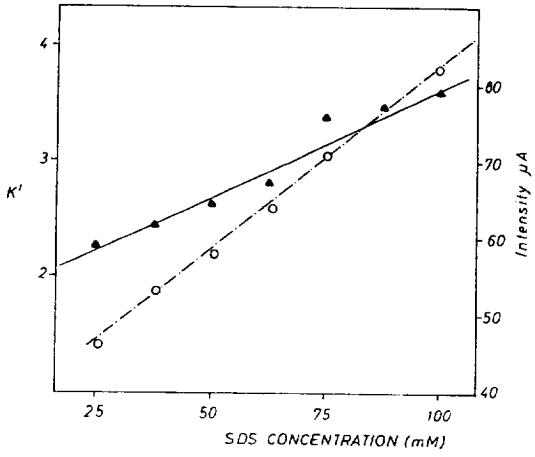

Fig. 4. Variation in the capacity factor of pentamidine (▲) and in intensity (○) with SDS concentration. Separation was carried out at 15 kV and 30°C in a 10 m*M* phosphate–50 m*M* borate buffer at pH 8.

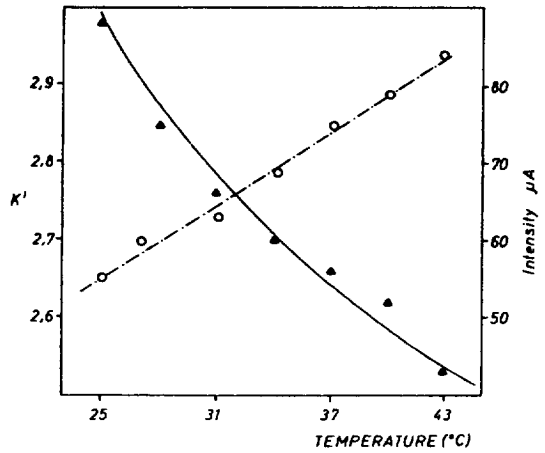

Fig. 5. Variation in the capacity factor of pentamidine (▲) and in intensity (○) with temperature. Separation was carried out at 15 kV. in a 10 m*M* phosphate–50 m*M* borate buffer at pH 8. SDS concentration 50 m*M*.

rise in current was observed with the increase of SDS concentration.

3.5. Influence of temperature

The importance of temperature control has been described by Nelson [21]. Good temperature control is vital for better reproducibility, as temperature changes may affect a number of physical parameters which can alter the migration velocities of substances under analysis through changes in their electrophoretic mobility, the electroendosmotic flow-rate, or both. Buffer pH, dielectric constant, viscosity, adsorption to capillary walls of the substance being analysed, dissociation of ionizable groups in the substance under analysis, and the configuration of biomolecules also depend upon temperature [19].

The variations with temperature in k' for pentamidine are shown in Fig. 5. The temperature was increased stepwise from 25 to 45°C. Pentamidine migrates faster as the temperature rises, and so k' drops. Moreover, it can be seen that current increases linearly as the temperature rises, while viscosity decreases, also linearly, for the temperature range studied; since there is a direct link between the viscosity and migration times of pentamidine, this major decrease is justified.

3.6. Separation procedure

The data obtained from previous work have allowed us to get close to the optimal conditions for analysis. In order to analyse real samples of serum and urine, it proved necessary to make some adjustments in the characteristics of the buffer used in the study: the 10 m*M* phosphate–50 m*M* borate buffer was replaced with a 100 m*M* borate buffer; the optimal pH was 8.35 and the optimal SDS concentration was 50 m*M*; the detection wavelength was set at 214 nm and the voltage at 25 kV. The separations were carried out at 30°C. Before each separation, the capillary was cleaned with 0.1 *M* NaOH for 1 min, followed by a rinse lasting 2 min with the buffer used for analysis. After the rinse cycle, the capillary was filled with the separation buffer. Samples were introduced into the capillary under pressure for 5 s. The electropherograms obtained for serum and urine samples are shown in Fig. 6.

3.7. Selectivity, recovery and linearity

The BET detection limits for pentamidine (established as a signal-to-noise ratio of 3) were 0.3 μg/ml in both serum and urine, as indicated by direct analysis of pentamidine-supplemented urine and

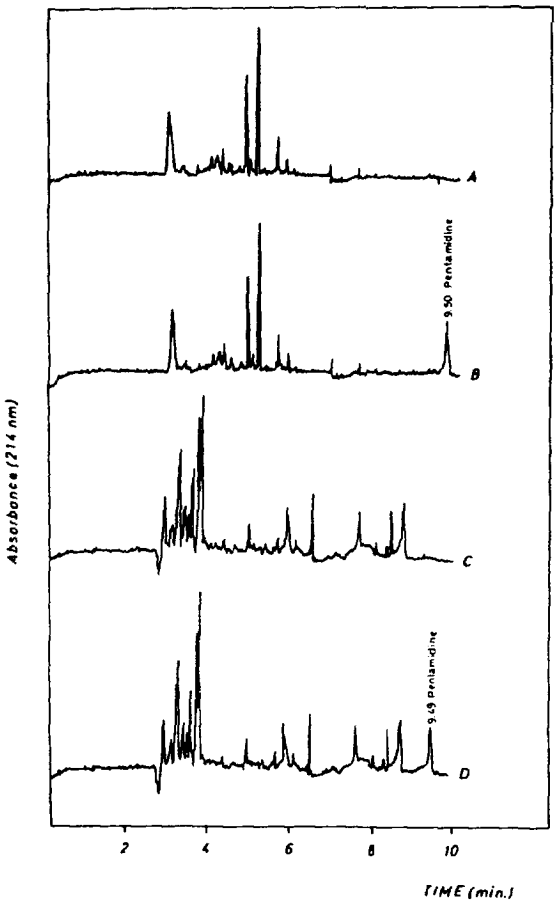

Fig. 6. Electropherograms: (A) blank urine; (B) urine containing pentamidine; (C) blank serum; (D) serum containing 1 μg/ml of pentamidine. Separation carried out at 25 kV a 30°C in a 100 m*M* borate buffer at pH 8.35. SDS concentration, 50 m*M*. Detection wavelength, 214 nm.

serum samples ($n=5$; standard deviation: serum, ±5%; urine, ±3%). Detector responses of the serum and urine samples spiked with pentamidine and treated as previously described were compared with detector responses for directly injected aqueous solutions having identical concentrations of the substance in question. Recovery monitored in serum ranged between 87% and 92%, and in urine between 90% and 108.9%, with a coefficient of variation never exceeding ±3% (Table 1). Linearity was checked by measuring five different concentrations in the range 0 to 30 μg/ml for samples of pentamidine in water, serum and urine. The measurements

Table 1
Recovery test

Pentamidine added (μg ml^{-1})	*n*	Recovery (%)	
		Serum	Urine
1	5	91.1	90.2
5	5	91.7	91.4
10	5	87.4	108.9
20	5	92.0	94.4
30	5	88.3	92.2

of the amounts of pentamidine were determined using the peak areas, and the coefficient of correlation obtained was ≥0.9998.

3.8. Possible interference by metabolites in the analytic method

A bibliographic search concerning the existence of pentamidine metabolites that might interfere with the analytic method proposed revealed that most authors consulted found no pentamidine metabolites in serum or urine in vitro tests [23–35]. Nevertheless, in vitro tests were performed to check whether in our case any interference occurred. In the results obtained, not only under the same conditions as in the method proposed but also with considerably altered electrophoretic conditions (lower voltages, longer capillaries) so as to improve separation conditions for this technique, no indication was found of the peak relating to pentamidine being interfered by any of its metabolites.

4. Conclusion

We have studied the behaviour of pentamidine in relation to the most important variables having an effect on its separation by MEKC, using for this purpose the variation in the capacity factor (k'). On the basis of the data obtained we have optimized the analytic conditions so as to be able to detect as little as 0.3 μg/ml of pentamidine in serum and urine in less than 10 min. MEKC offers an alternative to HPLC in determining pentamidine in serum and urine samples, and has the advantage of being a highly efficient technique, but with very different selectivity. Its main drawback, to which attention has

already been paid by a number of authors (e.g. [22]), is that, for some substances, it does not give a low detection limit using UV detection.

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