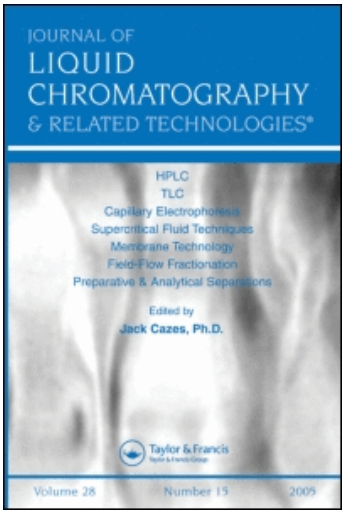

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Study of Nine Aromatic Diamidines Designed to Optimize Their Analysis by HPLC

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ABSTRACT

The aromatic diamidines are a series of chemicals with high basicity. Among other properties they have, is that of intervening in the metabolism and transport of polyamines, since they inhibit *s*-adenosyl-*L*-methionine decarboxylase (SAMDC) and diamine oxidase (DAO). They are of importance in pharmacology. Examples of this are: that pentamidine is used, for instance, in treating pneumonia in patients affected by the human immunodeficiency virus (HIV), that propamidine is utilized, among other things, to treat cornea infections, and that berenil is employed in veterinary medicine, though not in humans. Owing to their considerable interest, further chemicals belonging to

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this family are constantly being synthesized, with the aim of ensuring greater pharmacological capacities, better stability, and fewer secondary effects. It is essential to have available analytical methods for detecting these drugs in a range of media. In this study, attention was paid to nine aromatic diamidines that are heads of series: pentamidine, stilbamidine, DAPI, propamidine, hydroxystilbamidine, phenamidine, diampron, berenil, and dibromopropamidine, with the aim of proposing analytical methods employing HPLC. Use was made of ion-pairing for each of them, and for others of similar structure, in aqueous solutions and in biological media, such as serum and urine. To achieve the purpose of the study, the effects on the capacity factor k' of varying the chief chromatographic parameters were tracked. These parameters were the influences exercised by: concentration and chain length of the ion-pair-forming agent, pH in the mobile phase, methanol percentage, buffer concentration, and temperature. The data obtained from this work suggest general conditions for the analysis of each of these substances in aqueous solution would be a mobile phase consisting of 25.0 mM citrate buffer, with pH = 3.25, methanol 45%, ultrasphere ODS column (5 μm particle size,15 cm × 4.6 mm I.D.), temperature 30°C, flow 1.00 mL/min, and specific conditions for individual chemicals as laid out in Table 1. Under these conditions, detection limits ranging from 45.0 ng/mL for berenil to 5.00 ng/mL for DAPI can be attained.

Key Words: Aromatic diamidines; HPLC.

INTRODUCTION

The chemical and 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 *Leishmania dovani* in hamsters. These results in animals are the experimental background for the therapeutic use of diamidines in the treatment of leishmanianiasis and trypanosomiasis in humans. Aromatic diamidines interact with the metabolism of polyamines, whose levels and transport are subject to complex regulation. This aspect is evidenced by the administration of α-difluoromethylornithine, an inhibitor of ornithine decarboxylase (ODC), the first, and key enzyme in the biosynthesis of polyamines.[1,2] This triggers a decrease in the intracellular levels of putrescine and spermidine, simultaneously, increasing transporter activity very markedly.[3] Aromatic diamidines inhibit *s*-adenosyl-*L*-methionine decarboxylase (SAMDC) and diamine oxidase (DAO), enzymes whose activity increases in the presence of

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Table 1. Specific analytic conditions for each substance, showing linearity and detection limits.

	Ion-pair reagent	Concentration (mM)	Correlation coefficient (R^2)	Detection limit (ng/mL)
Pentamidine	hexane sulphonate	4.00	0.9995	20
Stilbamidine	octane sulphonate	4.00	0.9991	10
DAPI	heptane sulphonate	8.00	0.9988	5
Propamidine	heptane sulphonate	6.00	0.9992	30
Hydroxystilbamidine	octane sulphonate	4.00	0.9988	15
Phenamidine	octane sulphonate	4.00	0.9995	20
Diampron	octane sulphonate	4.00	0.9997	10
Berenil	octane sulphonate	4.00	0.9994	45
Dibromopropamidine	hexane sulphonate	3.00	0.9997	35

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growth-affecting enzymes, found in higher amounts in tumour cells. They can, therefore, slow down or prevent the growth of tumours.[4,5] Even (ODC) can act as an oncogene since its over-expression confers a phenotype of transformed tumour cell.

In view of this, various structural analogues of aromatic diamidines have been synthesized in recent years, such as: Pentamidine: 4,4′-[1,5-pentanediyl-bis(oxy)]bis-benzenecarboximidamide; Stilbamidine: 4,4′-(1,2-ethenediyl)bis-benzenecarboximidamide; DAPI: 4′,6-diamidino-2-(4-amidino-phenyl)indole dilactate; Propamidine: 4,4′-[1,3-propanediylbis(oxy)]bis-benzene carboximidamide; Hydroxystilbamidine: 4-[2-[4-(aminoiminomethyl)phenyl] ethenyl]-3-hydroxy-benzenecarboximidamide; Phenamidine: 4,4′-diamidinodiphenylether Diampron: 3,3′-diamidinocarbanilide; Berenil 4,4′-diamidinodiazoamino benzene; and Dibromopropamidine: 2′,2″-dibromo-4′,4″-diamidino-1,3-diphenoxypropane.

The aim has been to produce medicines that are increasingly effective against tumours, with high stability, low toxicity, and easy administration.

The objective of the present study, is to achieve a method of analysis, using HPLC, of the aromatic diamidines[6,7] listed in Fig. 1.

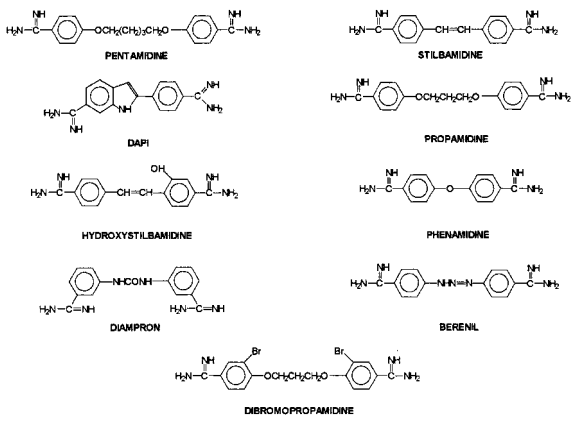

Figure 1. Structure of diamidines, Pentamidine: 4,4′-[1,5-pentanediylbis(oxy)]bis-benzenecarboximidamide. Stilbamidine: 4,4′-(1,2-ethenediyl)bis-benzenecarboximidamide. DAPI: 4′,6-diamidino-2-(4-amidinophenyl)indole dilactate; Propamidine: 4,4′-[1,3-propanediylbis(oxy)]bis-benzenecarboximidamide. Hydroxystilbamidine: 4-[2-[4-(aminoiminomethyl)phenyl]ethenyl]-3-hydroxybenzenecarboximidamide. Phenamidine: 4,4′-diamidinodiphenylether. Diampron: 3,3′-diamidinocarbanilide. Berenil: 4,4′-diamidinodiazoamino benzene. Dibromopropamidine: 2′,2″-dibromo-4′,4″-diamidino-1,3-diphenoxypropane.

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EXPERIMENTAL

Chemicals and Reagents

Pentamidine isethionate salt, berenil diaceturate salt, and DAPI dihydrochloride salt were obtained from Sigma-Aldrich Química S.A. (Madrid, Spain). Diampron isethionate salt, hydroxystilbamidine isethionate salt, propamidine isethionate salt, dibromopropamidine isethionate salt, phenamidine isethionate salt, and stilbamidine isethionate salt, were generously donated by Rhône Poulenc Rorer (Dagenham, UK). The ion-pairing agents, pentane, hexane, heptane, octane, and decane sulphonate sodium salts were supplied by Sigma-Aldrich Química S.A. HPLC-grade methanol and other chemicals of analytical grade were supplied by Merck (Darmstadt, Germany). The water was purified with a Milli-Q element purchased from Millipore (Bedford, Massachussets).

Chromatograph System

The HPLC system incorporated a Beckman 116 programmable solvent pump and Beckman 168 photodiode detector. These were checked and data were processed with the Gold Nouveau software system (Beckman Coulter, Palo Alto, California). It also had a Reodyne 7125 automatic injector with a 100 μL loop, and a column furnace. Analyses were carried out in an ultrasphere ODS column (5 μm particle size,15 cm × 4.6 mm I.D.) purchased from Beckman. A guard column (2 cm × 2 mm I.D.) packed with Sperisorb RP-18 (30–40 μm) was supplied by Upchurch Scientific (Oak Harbor, Washington).

The HPLC–MS Integrity system consists of a low dispersion gradient HPLC pump, an automatic injector module, a column bypass module, a 996 photodiode array detector, and a ThermaBeam mass detector. The Millennium software used to control the different modules for data acquisition and interpretation was purchased from Waters Corporation.

Standard Solutions

The standard solutions for each chemical were prepared by dissolving, in pure water, the requisite quantity of the diamidine under study. These standard solutions were kept refrigerated in total darkness at 4°C.

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RESULTS AND DISCUSSION

In order to design a reliable, effective analysis method using HPLC and ion-pair-forming agents, with high resolution and low detection limits for these substances, it was necessary to study the effect on capacity factor (k') of varying certain chromatographic parameters. These were: concentration and chain length of the ion-pair-forming agent, pH of the mobile phase, percentage of methanol, buffer concentration, and temperature.

Influence of the Concentration and Chain Length of the Ion-Pair-Forming Agent

The characteristics of aromatic diamidines make it possible to introduce long-chain ionic alkyl sulphonates into the mobile phase in order to form ion pairs.[8,9] Such a formation of ion pairs renders it easy to alter the capacity factor k', while also making it adaptable for use with various biological systems. The effect of sulphonate salt concentration and chain length on k' was studied by measuring the capacity factor using berenil as a reference chemical, with a mobile phase consisting of 25 mM citrate buffer, pH = 3.25, methanol 45%, containing pentane, hexane, heptane, octane, and decane sulphonate sodium salts at concentrations of: 0.00; 1.00; 2.00; 3.00; 4.00; 5.00; 6.00; 9.00, and 12.0 mM. The data obtained are shown in Fig. 2. On the basis of these data, and taking into account that it is desirable to have values for k' in the range 6–10 so as to be able to analyze these substances in blood and urine, it can be deduced that use of pentane sulphonate would require very high concentrations, use of decane sulphonate would need excessively low concentrations, but any of the other salts included in the study would be suitable. This is because the differences in k' from one to another are slight. Careful attention was paid to optimizing the concentrations and the ion-pairing agent most suited for each chemical, yielding the results indicated in Table 1.

Influence of pH in the Mobile Phase

The pH value is one of the parameters with the greatest impact on the separation of ionizable molecules. To keep aromatic diamidines ionised, it is necessary to work at very low pH levels, since the diamidine groups that are twice present in each molecule are strongly basic, $pK_a = 13{,}86$ (10), owing to the fact that the cation formed in an acid medium is given great stability by resonance and the symmetry of the molecular structure. The effect of pH on k' was studied using a mobile phase consisting of 25 mM citrate buffer, methanol

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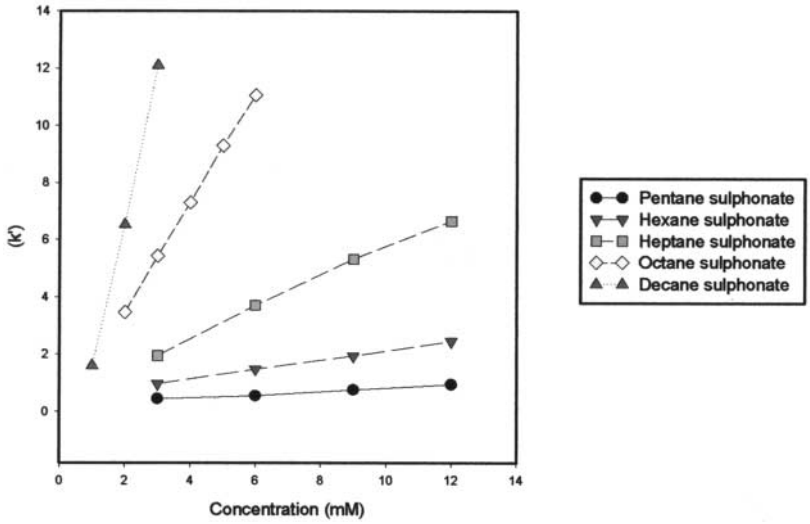

Figure 2. Influence of length and concentration of the ion-pair-forming agent on k'. This effect was studied using berenil in a mobile phase consisting of 25 mM citrate buffer with pH = 3.25, methanol 45% containing pentane, hexane, heptane, octane, and decane sulphonate sodium salts at concentrations of: 0.00; 1.00; 2.00; 3.00; 4.00; 5.00; 6.00; 9.00, and 12.0 mM, temperature 30°C, flow 1.00 mL/min.

45%, 4.00 mM octane sulphonate, at pH values ranging between 3.00 and 4.50. Figure 3 shows a decrease in k' when pH is varied over this range, the result of the larger number of diamidine groups ionized at pH = 3.00 forming more links with the sulphonate chains and. hence, yielding higher figures for k'. From pH = 4.00 upwards, diamidines are not strongly retained. This is not efficient for analysis of these substances in serum and urine, as they produce an initial front of considerable size in HPLC. In the light of these circumstances and the data obtained, the value selected as most appropriate was pH = 3.25.

Influence of Methanol Content

The effect of the percentage of methanol in the mobile phase on k' is shown in Fig. 4. This was studied using a mobile phase consisting of 25 mM citrate buffer, pH = 3.25, 4.00 mM octane sulphonate, and a percentage of methanol varying over the range 42% to 57%. The results obtained showed that over the interval from 42% to 54% there was a noteworthy decrease

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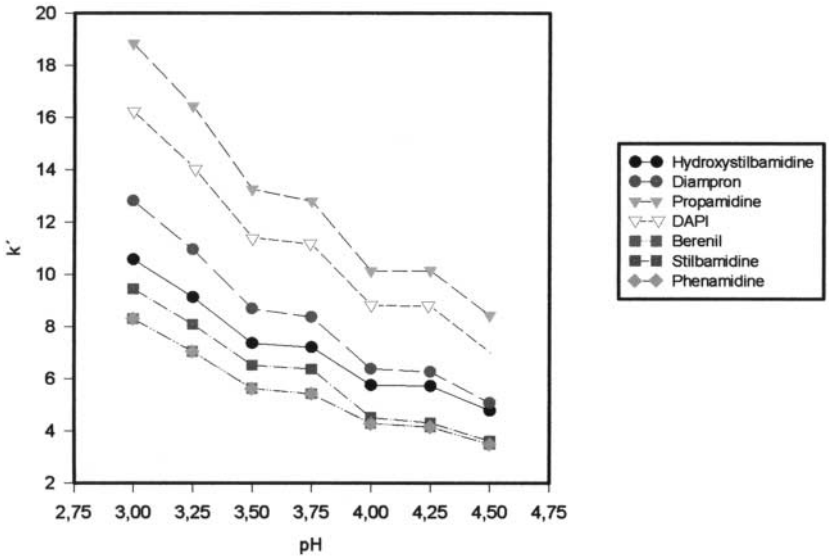

Figure 3. Influence of pH on k'. This effect was studied using a mobile phase consisting of 25 mM citrate buffer, methanol 45%, 4.00 mM sodium octane sulphonate, and pH values of: 3.00; 3.25; 3.50; 3.75; 4.00; 4.25; and 4.50, temperature 30°C, flow 1.00 mL/min.

in k' when the percentage of methanol dropped. In contrast, k' remained practically constant when the quantity of methanol increased above 54%. On the basis of these data, the most suitable level of methanol was considered to be 45%.

Influence of Buffer Concentration

To check on the influence of buffer concentration on k' a mobile phase was used that consisted of 45% methanol, 4.00 mM octane sulphonate, and citrate buffer with a concentration varying between 5.00 and 100.0 mM, with pH remaining at 3.25 in all cases. The results obtained are shown in Fig. 5, in which it may be observed, that increasing the concentration over the range up to 60.0 mM brings about a large decrease in k', but also that there is a considerable improvement in the symmetry of the peaks, these values remaining constant from 60.0 to 100.0 mM, the latter being the highest value used in the study. From these data it was concluded that the most appropriate figure for use would be 25.00 mM.

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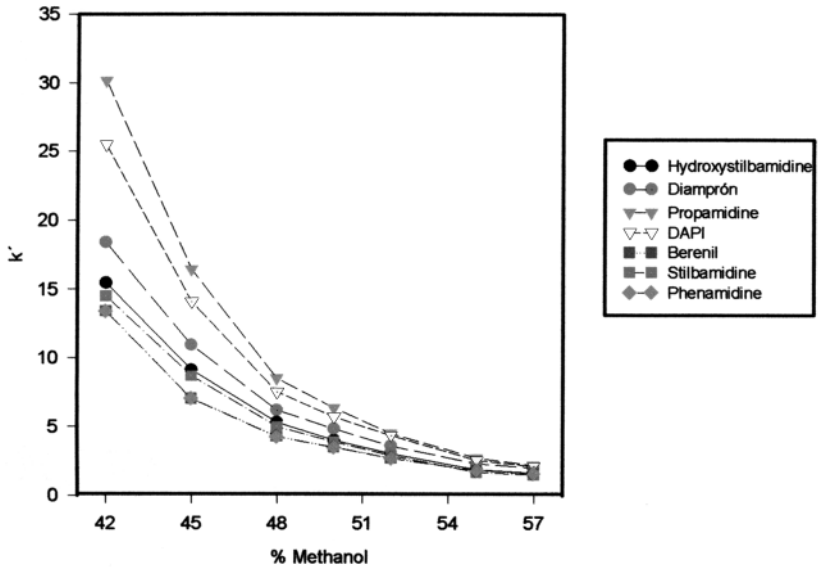

Figure 4. Influence of the percentage of methanol on k'. This effect was studied using a mobile phase consisting of 25 mM citrate buffer, pH = 3.25, 4.00 mM sodium octane sulphonate, and methanol percentages of: 42.0; 45.0; 48.0; 50.0; 53.0; 55.0; and 57.0. Temperature 30°C, flow 1.00 mL/min.

Influence of Temperature

Chromatographic separations in which ion pairs are utilized may be exothermal or endothermal processes, with noteworthy changes in entalpy occurring as the absolute temperature varies.[11,12] This is linked to a major drop in values for k' as temperature rises, as indicated by the equation:

$$\ln k' = \frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{T} + \ln \phi \text{[13]}$$

where ΔH° and ΔS° are the variations in these figures during the process of solute-column retention and ϕ is the phase ratio, when $\ln k'$ is shown against $1000/T\,\mathrm{K}^{-1}$. In Figure 6 it is possible to observe that straight lines are yielded for all the diamidines, and from these it is feasible to determine the values for ΔH° and ΔS° corresponding to given chromatographic processes.[14] In the light of these data, and in view of the values for k' that are of interest in

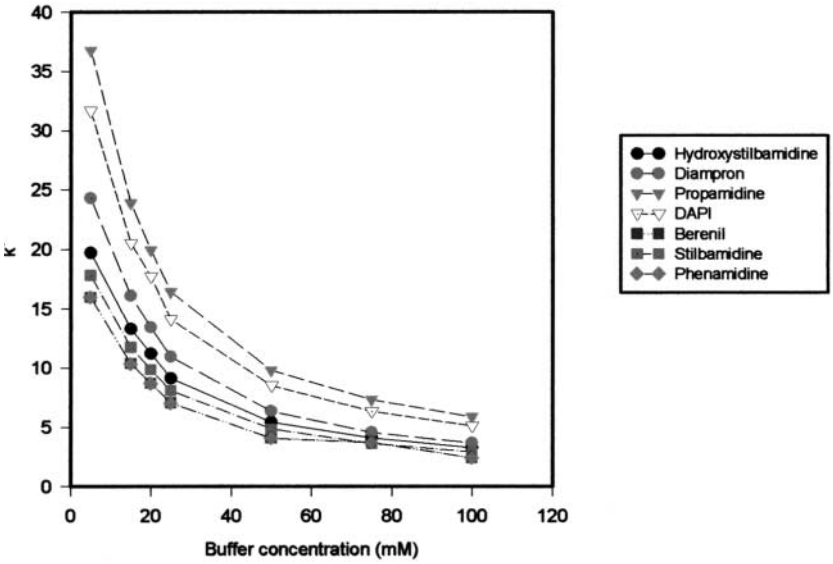

Figure 5. Influence of buffer concentration on k'. This effect was studied using a mobile phase consisting of 45% methanol, 4.00 mM sodium octane sulphonate, citrate buffer pH = 3.25 in all cases, and concentrations of: 0.00; 5.00;15.0; 20.0; 25.0; 50.0; 75.0; and 100.0 mM, temperature 30°C, flow 1.00 mL/min.

this type of analysis, it was deduced that the best working temperature would be 30°C.

Optimal Conditions for Analyses: Linearity and Detection Limit

From the data relating to all the chromatographic parameters obtained from the study recounted above, it is possible to establish the most appropriate conditions for analyzing the nine diamidines concerned in aqueous solutions. These conditions are not exactly the same for all nine substances, owing to their structural differences. The analytical conditions common to all are the following: mobile phase consisting of 25.0 mM citrate buffer, pH = 3.25, methanol 45%, column ultrasphere ODS (5 μm particle size,15 cm × 4.6 mm I.D.), flow 1.00 mL/min, temperature 30°C. In Table 1, the specific conditions for each individual chemical are displayed. Figure 7 gives chromatograms for the various chemicals under the conditions established and in an aqueous solution with concentration 2.00 μg/mL.

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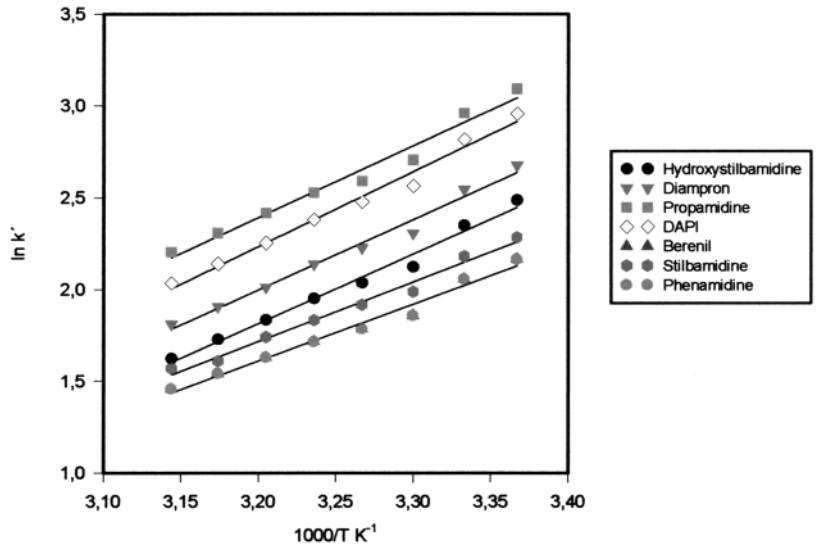

Figure 6. Influence of temperature on k'. This effect was studied using a mobile phase consisting of 25 mM citrate buffer, pH = 3.25, 45% methanol, 4.00 mM sodium octane sulphonate. Flow 1.00 mL/min, and temperatures ranged between 3.00 and 3.40 $(1000/T)$ (K^{-1}).

From the data arising from this study, it is possible to work out the conditions for analyzing each of these substances in other media such as serum, urine, and so forth. This requires the use of k' values that are generally higher, and can be achieved by varying one or more of the parameters studied, and then using the data from the relevant section to determine the figure for k' needed in each case. Further, Table 1 sets out the linearity and detection limits for each of the substances. In calculating linearity, analyses were performed on six aqueous solutions with concentrations lying between the detection limit and thirty times this figure for each of the chemicals. The detection limit in an aqueous solution, was taken here as being the concentration of the given substance that yielded a peak three times bigger than the background noise level of the base line.

HPLC–MS

To round out the chromatographic study of this type of chemical, mass spectrometry was undertaken for each substance, utilizing aqueous

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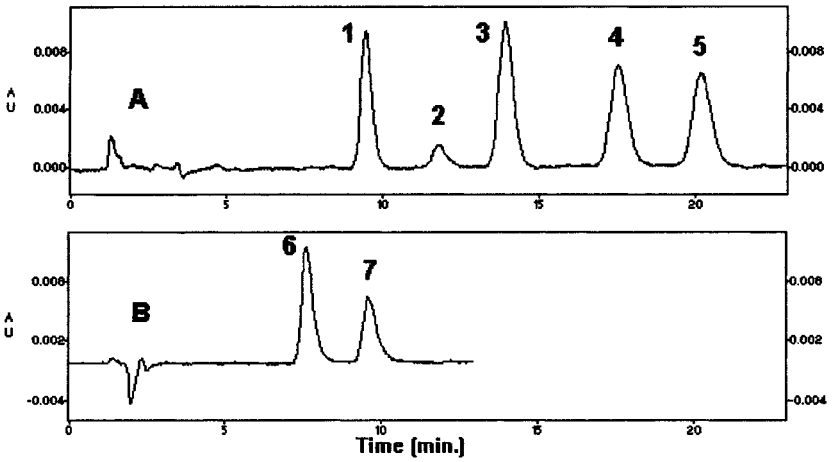

Figure 7. Chromatograms of seven diamidines, using a mobile phase consisting of 25 mM citrate buffer, pH = 3.25, 45% methanol, 4.00 mM sodium octane sulphonate in A, and 3.00 mM hexane sulphonate in B, flow 1.00 mL/min, temperature 30°C, concentration 2.00 μg/mL. (1) Phenamidine. (2) Hydroxystilbamidine. (3) Diampron. (4) DAPI. (5) Propamidine. (6) Pentamidine. (7) Dibromopropamidine.

concentrations of 200 ppm. In view of the low sensitivity of this equipment, samples were introduced directly into the mass detector, without use of a chromatographic column. Mass spectra were obtained for all of them that were similar to that shown in Fig. 8, which relates to pentamidine and DAPI. Working conditions were: 50% methanol–water mobile phase, flow rate of 0.25 mL/min, capillary diameter 51 μm, temperatures: nebulizer 80°C, expansion region 80°C, and ion source 200°C. MS detector settings, scan from 40 to 500 AMU, 1 scan per second. Volume of sample injected is 10.00 μL.

CONCLUSION

The behavior of nine aromatic diamidines in relation to the most important variables having an effect on their separation by HPLC was studied; for this purpose, use was made of variations in the capacity factor k'. On the basis of the data obtained, optimization of analytical conditions was undertaken; with these detection limits, over the range from 45.0 ng/mL (for berenil) to 5.00 ng/mL (for DAPI), an aqueous solution was achieved.

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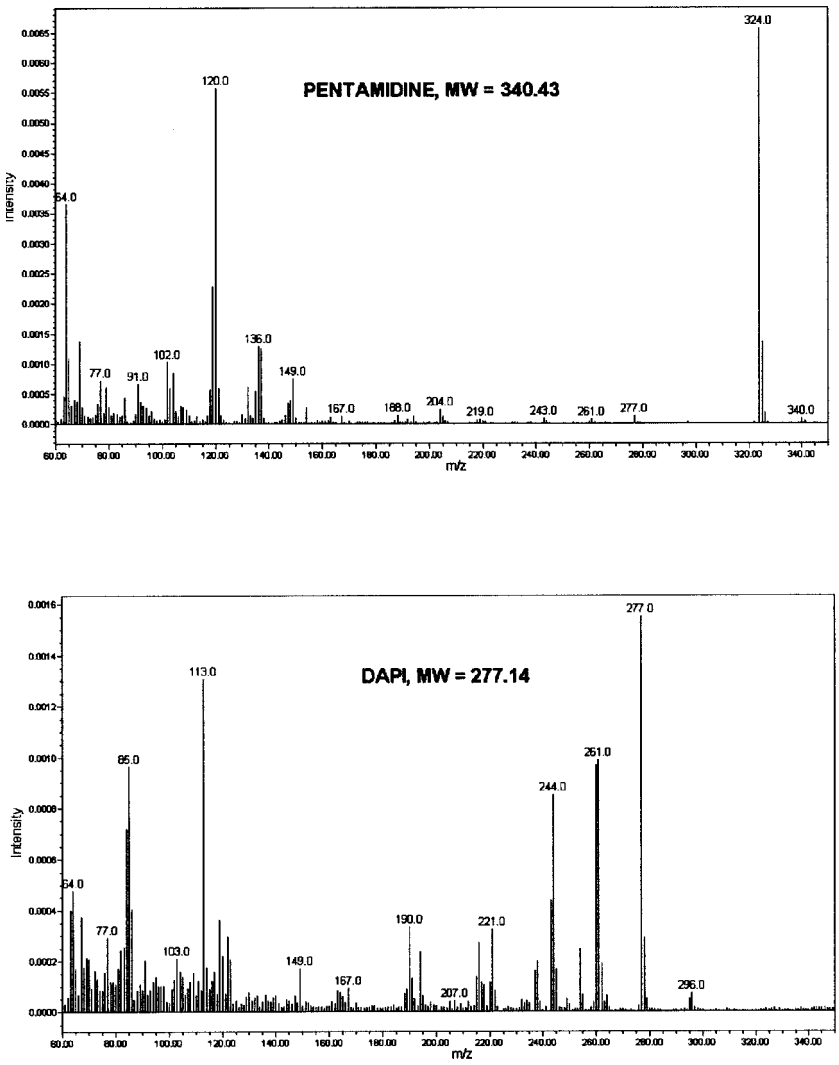

Figure 8. HPLC–MS of pentamidine and DAPI. The working conditions for obtaining these spectra were: methanol–water 50% mobile phase, flow 0.25 mL/min, capillary 51 μm. The temperatures used were: nebulizer 80°C, expansion region 80°C, and ion source 200°C. MS detector settings, scan from 40 to 500 AMU, 1 scan per second. Volume of sample injected was 10.00 μL.

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The data recorded make it easy to establish the most appropriate analytical conditions for determining each of these substances in other biological media, such as serum, urine, and the like. Figure 8 give the HPLC–MS data for pentamidine and DAPI.

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