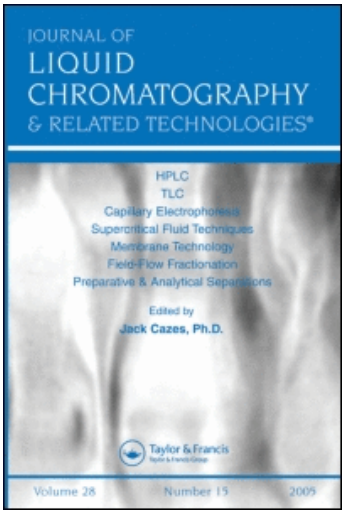


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# Determination of Pentamidine in *Leishmania* Infantum Promastigotes by Ion-Paired Liquid Chromatography

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# DETERMINATION OF PENTAMIDINE IN *LEISHMANIA* INFANTUM PROMASTIGOTES BY ION-PAIRED LIQUID CHROMATOGRAPHY

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## ABSTRACT

Pentamidine is an aromatic diamidine, which have recently been shown to be non-competitive inhibitors of putrescine uptake in parasitic protozoa. In order to understand the mechanism involved in Pentamidine uptake by parasitic protozoa cells, a HPLC analytical method for determination of pentamidine in *Leishmania* infantum promastigote cultures was developed.

The influence on the capacity factor of various characteristic parameters in the mobile phase such as pH, percentage of methanol and temperature of column is studied, taking into account that pentamidine can form ion-pairs with sulphonic compounds, the influence of the length of the lateral chain and concentration of various compounds of this type are also studied.

The results obtained in this study allow us to know the best chomatographic conditions for the determination of pentamidine in *Leishmania* infantum promastigotes and they can also be used for the optimization of analytical methods in order to determine pentamidine in other biological media or other complex mixtures.

The chromatographic procedure uses a reversed-phase column Nucleosil $C_{18}$ 5$\mu$m, the column effluent was monitored by ultraviolet-visible spectrophotometry at 261 nm. The procedure involves a simple method of the cleaning-up *Leishmania* promastigote extract samples cultured with pentamidine

by ultrafiltration through a polysulfone membrane with 100,000 relative molecular mass cut-off.The method shows good recovery, precision and accuracy. The limit of detection for pentamidine is 5.70 ng/ml in *Leishmania* cells culture.

## INTRODUCTION

Polyamine (putrescine, spermidine and spermine) metabolism in lower eukaryotes is a highly regulated biosynthetic pathway involved in proliferative proccesses which have been widely used for therapeutic purposes in parasitic infection diseases (1). The enzymes involved in polyamine biosynthesis have been targeted with a large number of compounds either specifically synthesized to inhibit these enzymes or those whose chemical structure resembles higher polyamines (2). Aromatic diamidines belong to this last kind of compounds. These drugs were designed as antiparasitic compounds, being successfully used against infections produced by species of *Trypanosoma, Leishmania* and *Babesia* genus both in human and veterinary medicine (3). Pentamidine is one of the most important drugs of this family, which is micromollar inhibitors of S-adenosyl-L-methionine decarboxylase (SAMDC), the enzyme involved in spermidine biosynthesis, and Diamine Oxidase, the key enzyme of polyamine terminal catabolism in mammals and lower eukaryotic cultures (4,5). Recently, aromatic diamidines have been shown to be non-competitive inhibitors of putrescine uptake in parasitic protozoa (6).

Several chromatographic methods have been developed in order to determine pentamidine in serum and urine (7,8,9). The aim of this work was to develop a HPLC analytical method specific with regard to retention times and sample preparation for the determination of pentamidine in *Leishmania* infantum promastigotes cultures so as to study the mechanisms implied in pentamidine accumulation by parasitic protozoa cells. In order to determine the optimal chromatographic conditions we carried out a wide study on the influence which it has on the capacity factor of various parameters related to the mobile phase such as, pH variation, methanol variation percentage, temperature of column and the influence of the chain length and concentration of different sulphonate salts which form ion-pairs with the pentamidine guanidinio groups.

## MATERIALS AND METHODS.

### Chemicals and materials

Pentamidine Isethionate salt is (1,5-bis [p-amidinophenoxy] pentane bis [2-hydroxyethanesulfonate salt] was supplied by Sigma Chemical Co. (St. Louis. USA). HPLC-grade methanol was supplied by Farmitalia Carlo Erba (Milan. Italy). The ion-pairing agents pentane, hexane, heptane, octane, and decane sulphonates sodium salts were supplied by Sigma Chemical Co. Medium 199 and gentamicin sulphate (150 $\mu$g/ml) were purchased from Sigma Chemical Co. (St Louis. USA). The foetal calf serum came from Boehringer Manhein. The water was purified using a Milli-Q II water purification system purchased from Millipore (Bedford MA. USA). All other chemicals were of analytical grade. The ultrafiltration system with a 100 000 relative molecular mass cut-off polysulfone membrane, (Ultrafree MC UFC3THK 25), was purchased from Millipore.

### Chromatographic System and Conditions

The HPLC system consisted of a Beckman 116 programable solvent pump. Beckman 116 absorbance detector with "System Gold" chromatography software and a Reodyne 7125 20 $\mu$l loop injector. Analyses were carried out on a reversed-phase Nucleosil $C_{18}$ column ( 5 $\mu$m particle size, 20 cm x 0,2 cm I.D) purchased from Teknokroma (Barcelona. Spain). A guard column ( 2 cm x 2 mm I.D ) packed with Spherisorb RP-18 (30-40 $\mu$m pellicular) was supplied by Upchurch Scientific (Oak Harbor. WA. USA). The most adequate mobile phase and the one which was used in the analysis of real samples of Leishmania cultures is made up of 5.0 mM of citric acid-methanol (50 : 50 v/v) and pentane sulphonate sodium salt 5.0 mM adjusted to pH = 4.0 with NaOH. The flow-rate was 0.5 ml/min. The detection wavelength was 261 nm All injection volumes for HPLC analysis were 40 $\mu$l (a 20 $\mu$l loop being used). The whole system was kept at room temperature (22° ± 3° C)

### Standard Solutions

Standard solutions of pentamidine were prepared by dissolving pentamidine isethionate salt in Milli Q II purified water in order to obtain

solutions of the concentrations in pentamidine (172.0 ; 17.20 ; 8.60 ; 4.30 ; 1.72 and 0.43 $\mu$g/ml ). All the standard solutions were stored at 4.0° C.

Solutions for the calibration curve

Solutions for the calibration curve were pentamidine-free *Leishmania* extracts mixed with the appropiate quantity of an aqueous 17.2 $\mu$g/ml standard solution of pentamidine and with 10 $\mu$l of ortophosphoric acid - water 10 % (p/p) to obtain 400 $\mu$l of final mixture of acidulated extracts with pentamidine concentrations of 8.60 ; 4.30 ; 1.72 ; 0.43 and 0.0129 $\mu$g/ml to be obtained. The resultant mixture was vortex-mixed and transfered to an Ultrafree-MC ultrafiltration system with a 100,000 relative molecular mass cut-off polysulphone membrane and centrifuged for 5 min. at 5 000 g. The clear filtrate was used directly for chromatographic analysis.

Samples of Leishmania treated with pentamidine

Extracts of *Leishmania* infantum promastigotes cultured with pentamidine in concentrations 0.03 ; 0.06 ; 0.14 ; 0.29 and 0.57 mM were treated using the same procedure as the solutions for the calibration curve and were without the addition of pentamidine.

Cell culture

*Leishmania* infantum promastigotes obtained by thermic transformation of amastigotes (PEP1G11 line) from lymph nodes of a naturally infected dog, were kindly supplied by Dr. J.M. Alunda and L.Carrera , Dpto. Patología Animal I , Universidad Complutense de Madrid. Cells were routinally grown at 26.5 °C in medium 199 supplemented with 10% heat inactivated foetal calf serum and gentamicin sulphate (150 $\mu$g/ml). Promastigotes were subcultured once a week and used with a low number of subpassages. Cell growth was estimated by counting in improved Neubauer chambers.Pentamidine isethionate was dissolved in water purified by Milli Q II and sterilised prior to its addition to the cultures. In order to determine the inhibitory effect of pentamidine, this compound was added at several concentrations to the cultures, and after four days subpassage, cell viability was estimated. In order to evaluate the pentamidine internalized by

cells, late lag phase promastigotes (3rd day post inoculation) were pulsed with 0.03 ; 0.06 ; 0.14 ; 0.29 and 0.59 mM pentamidine. Twenty four hours afterwards, cells were harvested, washed in phosphate saline-glucose (99:1 w/v) buffer, and resuspended in the same buffer up to a soluble protein concentration of 1.00 mg/ml.

## RESULTS AND DISCUSION

Pentamidine (1,5-bis [p-amidinophenoxy] pentane) (Fig.1) is an aromatic diamidine, making it possible to introduce long-chain ionic alkyl sulphonates into the mobile phase in order to form ion- pairs (10) (11). That formation of ion-pairs allows the capacity factor (k') to be easily altered while making it adaptable for use with different biological systems (12) (13) (14). In order to obtain an efficient method for the determination of pentamidine in *Leishmania,* we studied the effect on the capacity factor (k') by varying some chromatographic parameters: concentration and chain length of the ion-pairing forming agent, pH, percentage of methanol in the mobile phase and temperature of column. The influence of the length and concentration of the side chain of the ion-pair forming agent is shown in Fig. 2. The effect of sulphonates salt concentrations on (k') was studied by measuring capacity factor using mobile phase consisting of 5.0 mM citric acid - methanol (50:50 v/v) containing pentane, hexane, heptane and octane sulphonate sodium salts at concentrations of 0.0 ; 5.0 ; 10.0 ; 20.0 and 30.0 mM respectively and adjusting to pH = 4.0 with NaOH. A lineal increase in the capacity factor on adding different sulphonic salts formed from ion-pairs studied is observed. This increase is pronounced as hydrocarbonated chain length increases, which is to be expected.

The effect of pH on k' is shown in Fig.3 and was studied using a mobile phase consisting of 5.0 mM citric acid - methanol (50:50 v/v) and 1.0 mM hexane sulphonate sodium salt., adjusted with NaOH to pH = (3.5 ; 4.5 ; 5.5 and 6.5. A decrease in k' when pH varied from 3.5 to 4.8 is observed; from this value an increase in k' is produced.

The effect of methanol percentage in the mobile phase is shown in Fig.4 and was studied using mobile phase consisting of 5.0 mM citric acid methanol 40 ; 50 ; 60 and 70 %, 1.0 mM hexane sulphonate sodiun salt, adjusted with NaOH to pH = 3.5 . The results obtained show a notable decrease of k'

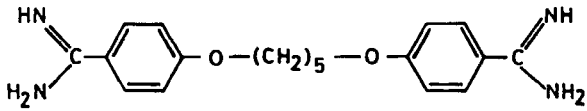


Fig. 1. Structure of pentamidine.

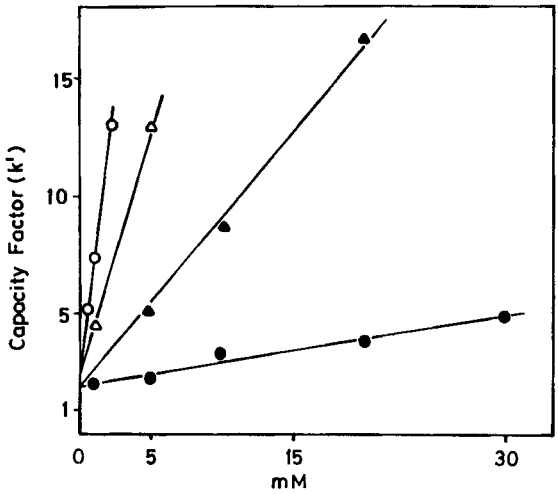


Fig. 2. Influence of length and concentration of the side chain in the capacity factor (k'). This effect was studied using a mobile phase consisting of 5.0 mM citric acid - methanol (50:50 v/v) containing 0.0 ; 5.0 ; 10.0 ; 20.0 and 30.0 mM of pentane (●), hexane (▲), heptane (△) and octane (○) sulphonate sodium salts and adjusting to pH = 4.0 with NaOH.

when the percentage of methanol in the mobile phase drops from 30 % to 50%, preactically maintaining a constant k' value when the quantity of methanol increases from 50 % to 70 %.

Figure 5 shows the variation of k' when the temperature of the column varies between 25 and 65° C. The mobile phase used to carry out this test was made up of 5.0 mM citric acid - methanol (50 :50 v/v) and 5.0 mM pentane sulphonate sodiun salt, adjusting to pH = 4.0 with NaOH. The results obtained show that the increase in temperature from 25 to 65°C makes the values of k'

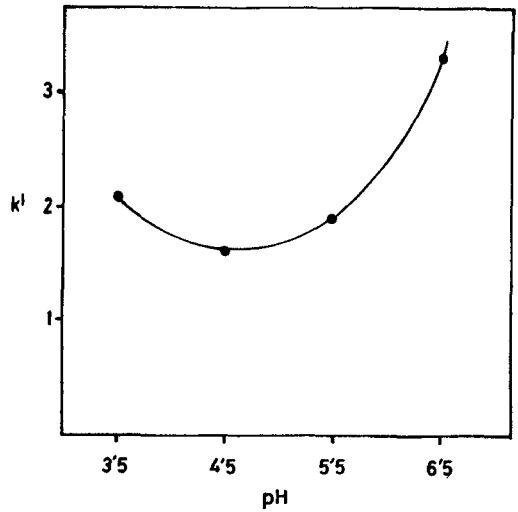


Fig. 3. Effect of variation of pH in mobile phase in the capacity factor of pentamidine. This effect was studied using a mobile phase consisting of 5.0 mM citric acid - methanol (50 : 50 v/v) and ajusting with NaOH to pH = ( 3.5 ; 4.5 ; 5.5 and 6.5 ) and 1.0 mM hexane sulphonate sodiun salt.

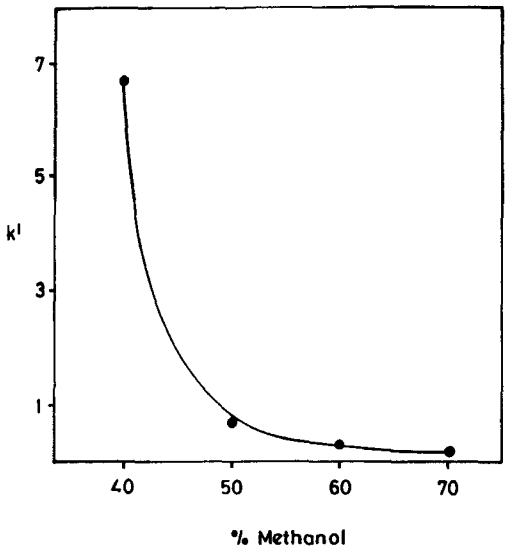


Fig.4 Effect of variation of percentage of methanol content in the mobile phase on the capacity factor of pentamidine. Mobile phase consisting of 5.0 citric acid - methanol 40 ; 50 ; 60 and 70 % and 1.0 mM hexane sulphonate sodium salt adjusting with NaOH to pH = 4.0

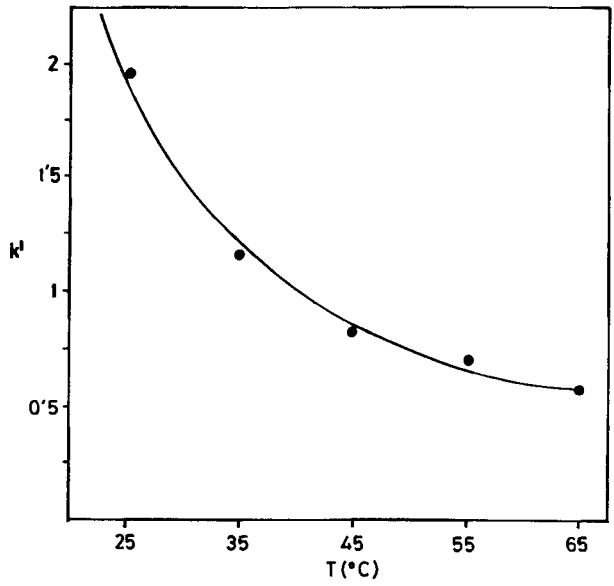


Fig. 5 Effect of column temperature on the capacity factor. Mobile phase consisting in 5.0 mM citric acid - methanol (50 :50 v/v) and 5.0 mM pentane sulphonate sodium salt, adjusting with NaOH to pH = 4.0.



decrease in only 1.5 units. The use of this parameter does not turn out to be of interest in this case as greather values of k' can be obtained on varying other chromatographic factors which are easier to modify. However, the data may be of interest in other cases.

The results of the study allows us to select the optimum conditions for the HPLC determination of pentamidine in *Leishmania* extracts, a mobile phase consisting of 5.0 mM of citric acid - methanol (50:50 v/v) containing 5.0 mM of pentane sulfonate sodium salt, adjusting to pH = 4.0 with NaOH and temperature of column from (22±3° C)

The chromatograms obtained for a blank extract of *Leishmania* and *for* a extract of *Leishmania* cultured with pentamidine are shown in Fig.6.

Linearity, Precision and Detection Limit

Linearity, accuracy and precision were determined with blank extracts of *Leishmania* treated, as previously described, with known amounts of

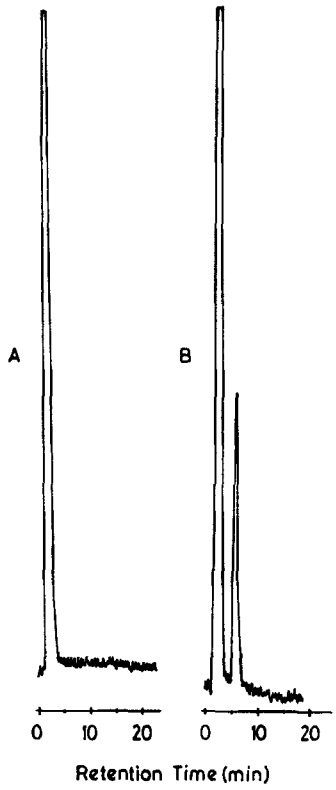


**Fig. 6. Chromatograms. (A) Extract of *Leishmania* culture. (B) Extract of *Leishmania* culture with 6.02 ng/ml of pentamidine. The mobile phase used for analysis is 5.0 mM of citric acid - methanol (50:50 v/v) and pentane sulphonate sodium salt 5.0 mM final pH = 4.0. Flow-rate 0.5 ml/min. Column Nucleosil $C_{18}$ (5 $\mu$m particle size, 20 cm x 0.2 cm I.D)**

**pentamidine in order to obtain concentrations of pentamidine in the range 0.0 - 4.30 $\mu$g/ml. A linear relationship was observed between the pentamidine peak areas and the concentrations of pentamidine in the extracts. The linear relationship can be expressed by the equation Y = 170419 X - 8798 where Y and X are, respectively, the peak area and the amount of pentamidine in $\mu$g/ml. The correlation coefficient was 0.9998.**

The accuracy and precision (C.V) of the method (Table I) were determined in extracts with pentamidine concentrations at 8.60 ; 4.30 ; 1.72 and

TABLE I

**Accuracy and precission of determination of pentamidine in *Leishmania* culture samples.**

| Spiked concentration ($\mu$g/ml) | n | Observed concentration ($\mu$g/ml) | CV (%) | Accuracy (%) |
|---|---|---|---|---|
| 8.60 | 6 | 8.94±0.51 | 4.5 | 104.0 |
| 4.30 | 6 | 4.64±0.02 | 0.7 | 108.0 |
| 1.72 | 6 | 1.71±0.20 | 3.8 | 99.4 |
| 0.43 | 6 | 0.37±0.02 | 7.7 | 86.7 |



0.43 $\mu$g/ml. The coefficient of variation (C.V) was determined from the equation C.V = ( Stardard deviation / Mean value) x 100 and the accuracy of the assay was estimated using the following equation:

Accuracy (%) = ( Observed concentration/Spiked concentration) x 100

Accuracy ranged from 87 % to 108 % with a coefficient of variation never exceeding ± 7.7 The detection limit of analytical method in *Leishmania* samples ( protein concentration of 1.00 mg/ml ) is 5.70 ng/ml with a signal to noise ratio 5:1.

With the aim of proving the interest and validity of the analytical method in real samples, a study of the inhibition of pentamidine in *Leishmania* growth was carried out and the results are shown in Fig. 7. The quantity of pentamidine internalized by *Leishmania infantum* promastigotes grow cultured with pentamidine concentrations 0.03 ; 0.06 ; 0.14 ; 0.29 and 0.57 mM was determined by this analitical method. The results are shown in Table II.

As a system of sample preparation ultrafiltration was used. The advantages of ultrafiltration are that no compounds that might interfere with the HPLC analysis are added to the sample, proteins and macromolecules are removed and the method is quick and efficient. In order to facilitate the passing of all the pentamidine contained in the *Leishmania* sample through the filter and obtain

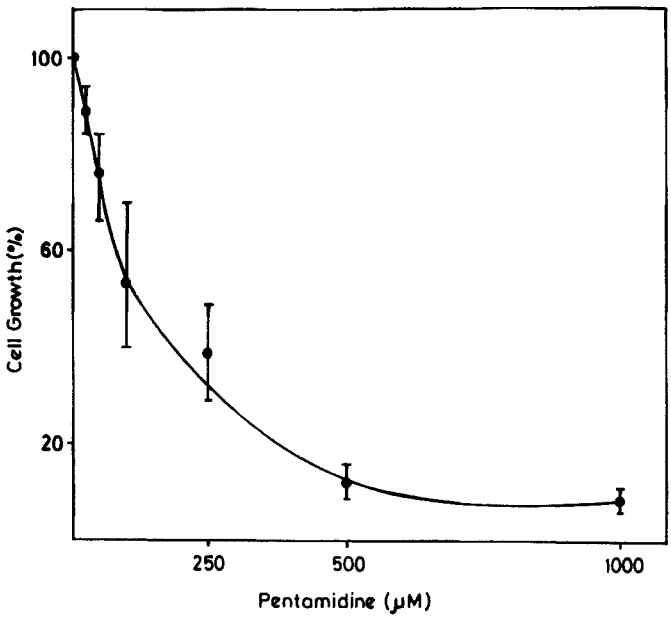


Fig.7. Inhibitory effect of pentamidine in *Leishmania* growth.



TABLE II

**Analysis of Pentamidine internalized by cells of *Leishmania*.**

| Sample | Pentamidine added to the culture (mM) | Protein concentration of the extract (mg/ml) | Pentamidine observed concentration (μg/ml) | Pentamidine internalized* |
|---|---|---|---|---|
| 1 | 0.03 | 1.34 | 0.12 | 0.09 |
| 2 | 0.06 | 1.39 | 0.15 | 0.11 |
| 3 | 0.14 | 1.36 | 0.17 | 0.12 |
| 4 | 0.29 | 1.19 | 0.16 | 0.14 |
| 5 | 0.57 | 0.89 | 0.66 | 0.74 |

*μg/mg Protein.

good recoveries, we have proved that it is necessary to acidify the medium. In order to do so we added small amounts of phosphoric acid-water as has been previously described.

To sum up, we used a wide study of various chromatographic parameters which affect pentamidine analysis using ion-pairs. the data obtained allow us predict which are the most adequate chromatographic conditions to carry out quantitative analysis of pentamidine in *Leishmania* infantum promastigotes. These data can also be used to create an analitical method which would allow us to analyze pentamidine in any other biological matrix or complex mixture in which it is found.

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