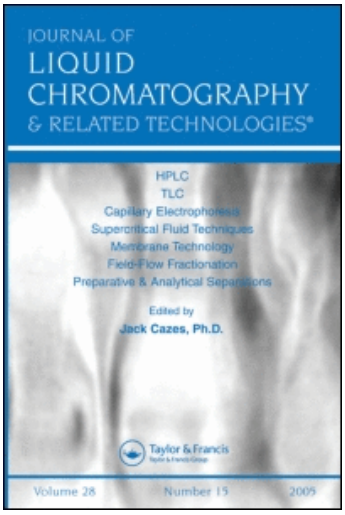

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Ion-Paired Liquid Chromatographic Determination of Phenylglyoxal Bis(Guanylhydrazone) in Serum and Urine

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Original Article

ION-PAIRED LIQUID CHROMATOGRAPHIC DETERMINATION OF PHENYLGLYOXAL BIS(GUANYLHYDRAZONE) IN SERUM AND URINE

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ABSTRACT

Phenylglyoxal bis(guanylhydrazone) (PGBG),shows a strong inhibitory activity of the same order and pattern as Methylglyoxal bis (guanylhydrazone) (MGBG), against Diamine oxidase from the small intestine of the rats and against S-adenosylmethionine decarboxylase (one of the key enzymes of polyamine biosinthesis) from *Eimeria stiedai* cysts, and from partially purified rabbit liver enzyme. The therapeutic interest of polyamine biosynthesis inhibitors in tumoral, infectious and parasitic processes and the lesser toxicity of PGBG than of MGBG favour the use of this compound as an alternative treatment for this kind of disease.

For this study a paired-ion HPLC method was developed to measure concentrations of PGBG in serum and urine samples. The method involved ultrafiltration of diluted serum and urine,through a 30,000-Dalton molecular weight cut-off filter.

Recoveries for PGBG in serum and urine were 97-99%. Ultrafiltered samples were then injected into a C_{18} column and eluted isocratically with a mobile phase consisting of a mixture of phosphate buffer 0.05 M, pH = 3.5, methanol (83:17) and 1.5 mM

sodium butane sulfonate. The ultraviolet absorbance of the column effluent was monitored at 280 nm. The lower limit of detection for PGBG was 1.0 μg/ml in urine and 0.1 μg/ml in serum.

INTRODUCTION

Earlier reports exist of the role of phenylated analogs of Methylglyoxal bis (guanylhydrazone) (MGBG), as therapeutic agents against such proliferative processes as parasitic protozoa infections (e.g. rabbit coccidiosis and *Leishmania infantum* promastigote cultures) (1) (2).

The IUPAC name of Phenylglyoxal bis(guanylhydrazone) (PGBG) is 1,1'-[(phenylethanediylidene)dinitrilo] diguanidine dihydrochloride (Figure 1). PGBG has shown a strong inhibitory activity,of the same order and pattern as MGBG, against diamine oxidase from the small intestine of the rat (3) and against S-adenosylmethionine decarboxylase (one of the key enzymes of polyamine biosynthesis) from *Eimeria stiedai* cysts (4) and from partially purified rabbit liver enzyme (5).

The therapeutic interest of polyamine biosynthesis inhibitors in tumoral (6), infectious (7) and parasitic processes and the lesser toxicity of PGBG than MGBG (1), favour the use of this compound as an alternative treatment for diseases of this kind.

The purpose of the present study is to determine quantitatively the influence of pH, organic solvent quantity and the concentration and nature of sodium alkyl sulfonate in the mobile phase (8) on the retention of PGBG by the stationary phase,with a view to having sufficient data available to establish analytical HPLC methods for the said compound in serum and urine.

MATERIAL AND METHODS

Reagents and Materials

Standard PGBG was synthetized in our Department according to Balochi's method (9). Its purity and chemical stucture were tested by UV,IR and H-RMN.

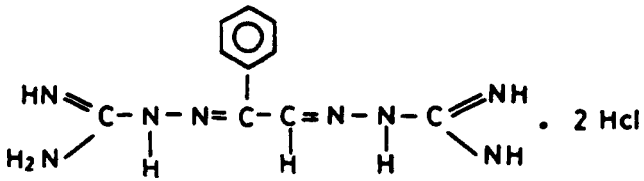

Figure 1. Structure of phenylglyoxal bis(guanylhydrazone) (PGBG)

All the water used was purified by the Milli-Q, Water Purification System. (Millipore Corporation Bedford, MA, USA). The sodium salts of butane, pentane, hexane and heptane sulfonic acids were purchased from Fluka Chemie (Buchs, Switzerland). Methanol of HPLC grade was supplied by Merck (Darmstardt, RFA). All other chemicals were reagent grade.

The microseparation system with a molecular weight cut-off of 30,000 Daltons (Ultrafree-MC,UFC3 TTK 00) was purchased from Millipore Corporation.

Instruments

Chromatography was performed on a component system consisting of a Konik KNK-500-A pump (Konik Instruments, Barcelona, Spain). A Rheodyne Model 7210, 20 μl loop injector and a Waters Model 441 absorbance detector operating at 280 nm. Peak areas were measured with a Waters 745-B (Waters Assoc. Molford, Mass. USA). The column used was C_{18} Spherisorb ODS-2 (25 x 0.4 cm, 10 μparticle size) purchased from Teknokroma.Coop. (Barcelona, Spain).

Chromatographic Procedure

The different mobile phases used were prepared by using phospate buffer 0.05 M, and varying the pH, methanol percentage and sodium alkyl sulfonate quantity and nature for each case (10). The mobile phase used for final analysis consisted of a mixture of phospate buffer 0.05 M pH = 3,5 methanol (83:17) and 1.5 mM sodium

butane sulfonate, with the flow-rate set at 1.0 ml/min. Injection volumes of 20 μl were used for all HPLC analyses. All chromatographic operations were carried out under ambient conditions.

Preparation of Standard Solutions

Stock solutions of PGBG for assay in serum and urine were prepared by dissolving the compound in 2N hydrochloric acid to obtain a solution of 500 μg/ml, which solution was diluted with water to obtain solutions in the concentration range 0.01-300 μg/ml.

Preparation of Serum and Urine Samples

Standard calibration solutions were prepared by adding 0.2 ml of aqueous PGBG stock solution, and 0.2 ml of a mixture (11) (12) of ethanol, acetonitrile, water (40:10:50) to 0.2 ml of serum or urine to obtain solutions in the concentration range 0.01-100 μg/ml. These samples were "Vortexed" for 20 seconds, and were added to the Ultrafree-MC, ultrafiltration system with a molecular weight cut-off filter of 30,000 Daltons, and centrifuged for 15 min. at 3000 g, the clear filtrate being used directly for chromatographic analysis.

RESULTS AND DISCUSION

Influence of Mobile Phase pH

This was studied by using mobile phases comprising 0.05 M phosphate buffer of different pH = 2.5 ; 3.5 ; 5.0 ; 6.0 ; 7.0 and 8.0, methanol (85:15) and 2.5 mM sodium alkyl sulfonate (butane,pentane or hexane). The representation of k' values contrasted with pH is shown in Figure 2. It will be noticed that k' values reach a minimum at pH ≈ 3.5 for sodium butane sulfonate and pH ≈4.5 for sodium pentane and hexane sulfonates, after which the k' values increase markedly in all cases as the pH increases.

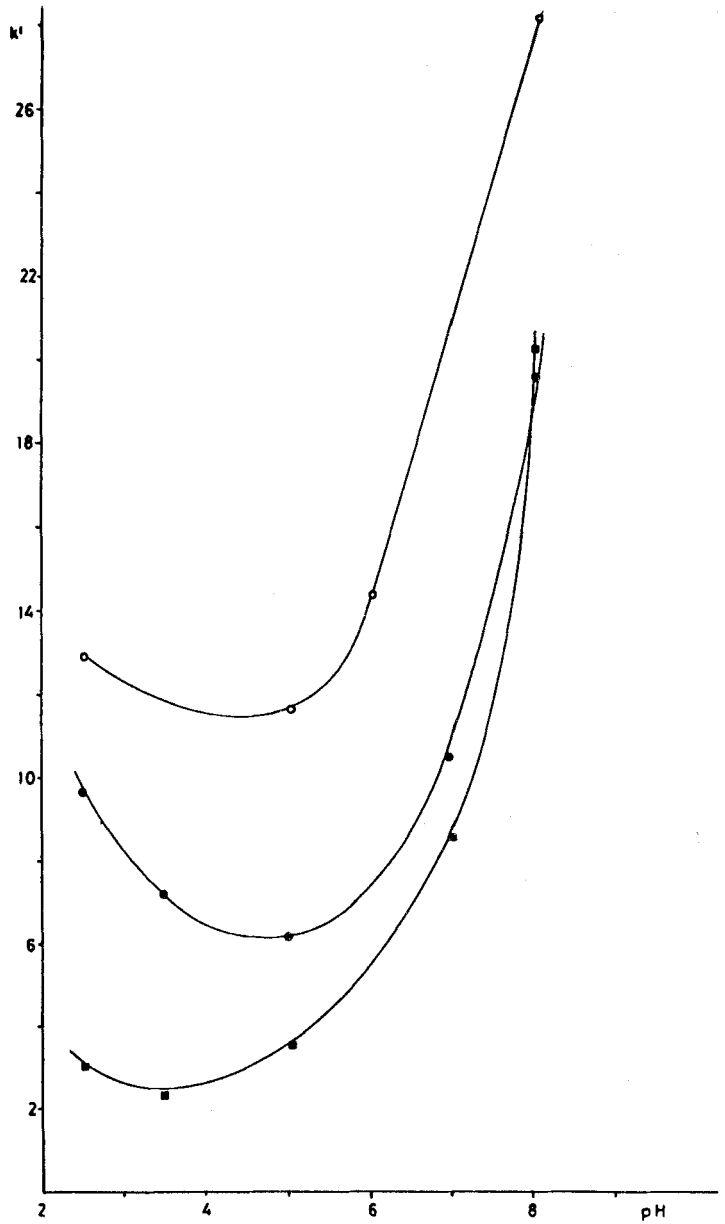

Figure 2. Effect of variation of the capacity factor of (PGBG) in mobile phase containing 0.05 M phospate buffer with different pH = 2.5; 3.5 ; 5.0 ; 6.0 ;7.0 and 8.0 , methanol (85:15) and 2.5 mM sodium alkyl sulfonates. (■) Butane; (●) Pentane ; (O) Hexane.

Influence of Methanol Contents

Mobile phases comprised 0.05 M phosphate buffer pH=5.0, methanol in proportions of 10 ; 20 ; 30 ; 40 ; 50 and 60 % , and 2.5 mM of sodium alkyl sulfonate (butane,pentane,hexane or heptane). The representation of k' values contrasted with the percentage of methanol for (C_4 - C_8) sodium alkyl sulfonates is given in Figure 3. A noticeably sharp increase occurs in k' values in all cases when methanol decreases to below 30 % . From the shape of these curves regions can be selected where great incremental changes in methanol concentration have great effects on the capacity factor, and therefore on chromatographic separations.

Influence of Sodium Alkyl Sulfonate Concentration

This was determined by working with mobile phases constituted by 0.05 M phosphate buffer pH=3.5 , methanol (85:15) and sodium alkyl sulfonate (butane, pentane or hexane) in concentrations of : 0.0 ; 1.0 ; 2.0, 2.5 ; 3.0 ;4.0 and 5.0 mM. Figure 4, gives the values of the capacity factor obtained by varying these concentrations , it being observed that the presence of these salts remarkably increases the value of the capacity factor,as was to be expected, since chromatographic interactions of this kind in the presence of sodium alkyl sulfonates proceed from an ion-pair formation mechanism. In the case of sodium hexane sulfonate the interaction with the apolar stationary phase is much greater than in the other two alkyl sulfonates studied, owing to the hydrophobicity caused by the greater length of its side chain.

In the absence of sodium alkyl sulfonate,it is observed that PGBG is retained to a much lesser extent than when mobile phases containing sodium alkyl sulfonate are used, which is interesting,since alkyl sulfonates can be used to alter the retention times of PGBG to suit our needs in chromatographic determinations in serum and urine.

Chromatographic Separation

The results obtained from the chromatographic study allow us to select the best chromatographic conditions for the determination by HPLC of PGBG in serum and

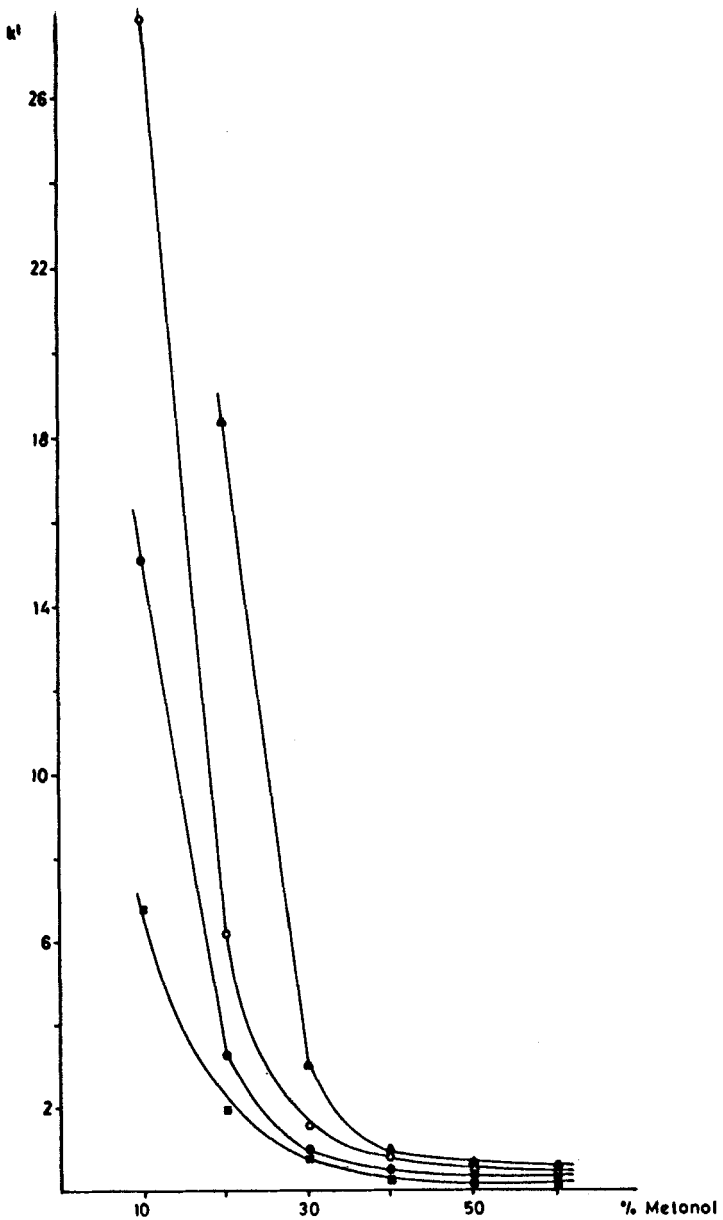

Figure 3. Effect of variation of the percentage of methanol content in the mobile phase on the capacity factor of PGBG. Mobile phase containing 0.05 M phosphate buffer pH = 5.0, methanol in proportions : 10 ; 20 ; 30 ; 40 ; 50; and 60 % and 2.5 mM of sodium alkyl sulfonate. (■) Butane; (●) Pentane ; (○) Hexane; (▲) Heptane.

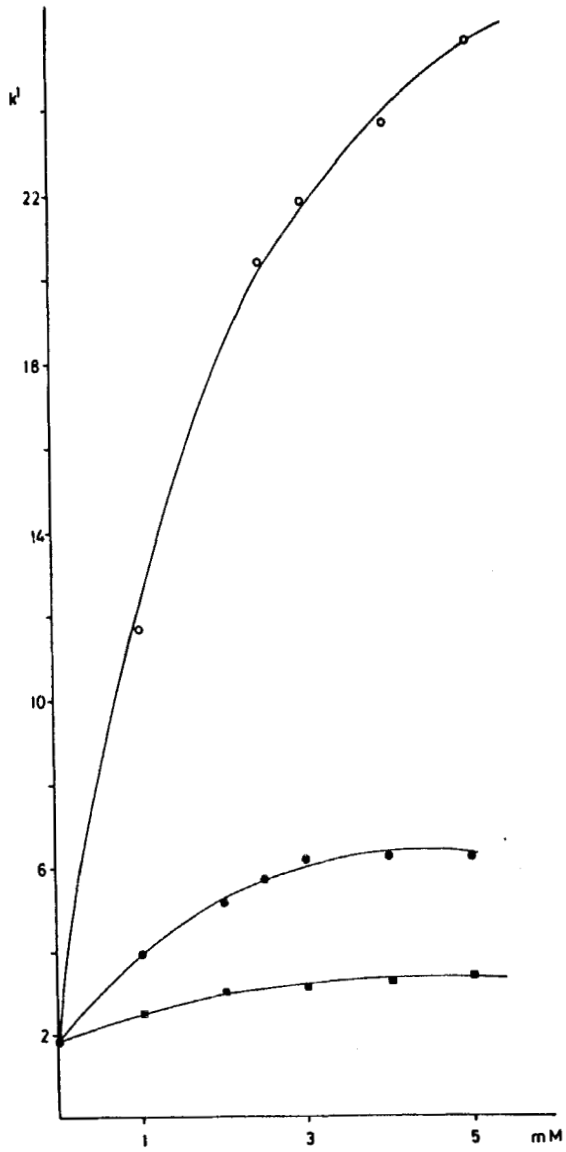

Figure 4. Effect of sodium alkyl sulfonate concentration in mobile phases on the capacity factor of PGBG.Mobile phase containing 0.05 M phosphate buffer pH = 3.5, methanol (85:15) concentration of sodium alkyl sulfonate : 0.0 ; 1.0 ; 2.0 ; 2.5 ; 3.0 ; 4.0 and 5.0 mM. (■) Butane ; (●) Pentane ; (○) Hexane.

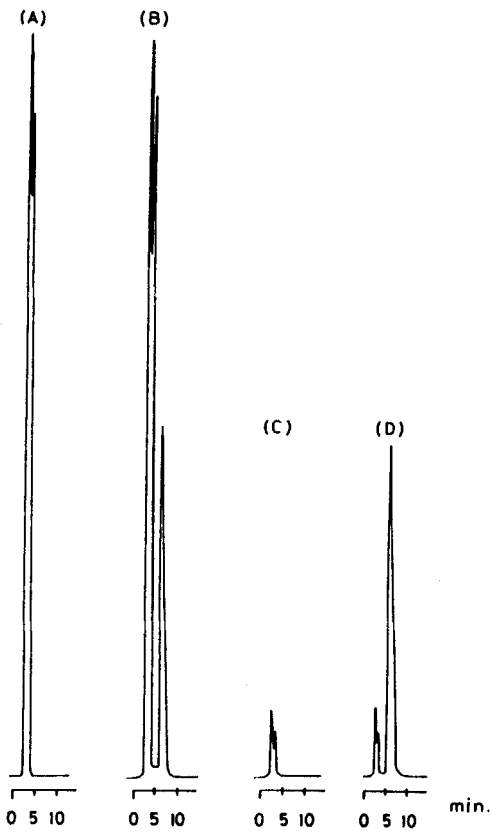

Figure 5. Chromatograms: (A) Blank urine. (B) Urine containing PGBG. (C) Blank serum. (D) Serum containing PGBG. Separation carried out on octadecylsilane bonded-phase column. Mobile phase containing 0.05 M phosphate buffer pH = 3.5, methanol (83:17) and 1.5 mM sodium butane sulfonate. Flow-Rate:1.0 ml/min.

urine. We have established that the most suitable mobile phase is that formed by mixing 0.05 M phosphate buffer pH=3.5 , methanol (83:17) and 1.5 mM sodium butane sulfonate. The chromatograms obtained for serum and urine samples are shown in Figure 5.

Extraction of PGBG

The extraction of PGBG from serum and urine samples was carried out using Ultrafree-MC ultrafiltration units with low-binding polysulphone 30,000-Dalton membranes, a solution made up of ethanol,acetonotrile, water (40:10:50) being used to avoid the binding of PGBG to serum and urine proteins (11). Analysis by HPLC of the filtrate after application of the serum or urine samples to the Ultrafree-MC, units revealed no binding of PGBG to proteins and that nothing was retained in the ultrafiltration membrane.

Sensitivity, Recovery and Linearity.

The limits of detection for PGBG (determined by a signal-to-noise ratio greater than 3) were 1.0 μg/ml in urine and 0.1 μg/ml in serum, as determined by direct analysis of PGBG-supplemented urine and serum samples . (n=5, standard deviation: serum ±3.0 ; urine ±5.0 %).

Detector responses of serum and urine samples spiked with PGBG were compared with detector responses of directly injected aqueous solutions that had identical concentrations of the compound in question. Recovery monitored in serum and urine ranged from to 97 to 99 % with a coefficient of variance never exceeding ±3 %.

Linearity was checked measuring 10 different concentrations in the range 0-100 μg/ml for samples of PGBG in water, serum and urine. The resulting lines of representations of peak areas of PGBG contrasted with concentrations of PGBG is represented by the equations : $y = 0.45 + 1.6\,x$; coefficient correlation 0.998 for water ; $y = 0.77 + 1.02\,x$; CC = 0.999 . for serum and $y = 0.65 + 1.34\,x$; CC = 0.997 for urine samples.

CONCLUSION

An isocratic, ion-paired HPLC method was establised for monitoring phenylglyoxal bis(guanylhydrazone) (PGBG) in serum and urine in both therapeutic and pharmacokinetic studies. The drug was retained as an ion- pair, with sodium butane sulfonate and determined by UV absorbance detection at 280 nm. The method involves

a simple ultrafiltration sample, Ultrafree-MC, system with a molecular weight cut-off of 30,000 Daltons.

The proposed method is practical,quick and accurate and requires small sample volumes to determine PGBG in serum and urine.

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