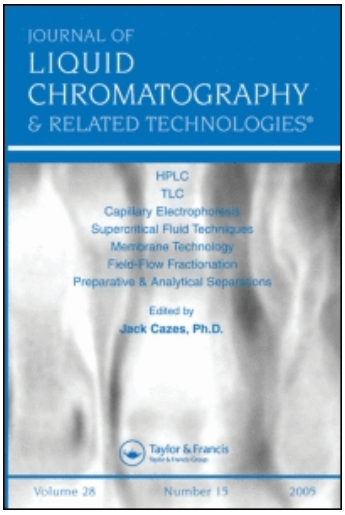

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation of Small Molecules in the Presence of Proteins Using Conducting Polymer Stationary Phases

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To cite this Article Ge, H. , Gilmore, K. , Ashraf, A. , Too, C. O. and Wallace, G. G.(1993) 'Separation of Small Molecules in the Presence of Proteins Using Conducting Polymer Stationary Phases', Journal of Liquid Chromatography & Related Technologies, 16: 1, 95 — 108
To link to this Article: DOI: 10.1080/10826079308020899
URL: http://dx.doi.org/10.1080/10826079308020899

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(1), 95-108 (1993)

SEPARATION OF SMALL MOLECULES IN THE PRESENCE OF PROTEINS USING CONDUCTING POLYMER STATIONARY PHASES

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ABSTRACT

Polypyrrole has been found to be selective for basic organic compounds. In this work, separation of basic drugs in the presence of proteins was investigated. The results indicated that under reversed phase conditions proteins had no retention on polypyrrole while basic drugs did. Proteins eluted at dead time while high retention was observed for caffeine and theophylline. Therefore the presence of proteins did not affect the separation of basic drugs.

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INTRODUCTION

In order to determine small molecular species in biological samples it is often necessary to remove proteins present in the sample [1]. This is even more important for reversed phase HPLC analysis of such samples because the existing proteins may be adsorbed on the column permanently causing deterioration of the column performance [2,3]. Two approaches have been taken in an attempt to overcome such problems. The first involves sample pretreatment to remove proteins before HPLC, while the second uses some new stationary phases to allow direct injection of protein-containing sample on to the columns since the proteins elute unretained. Among these stationary phases, internal surface reversed phases (ISRP) have been most widely used [4-8]. ISRP packing materials have been prepared by cleavage of the hydrophobic stationary phases on the external surface of the particles by various methods [9-10]. Small pore silica particles of typically 80Å pore size have been used as supports. Small molecules diffuse into the pores and interact with the hydrophobic surface, while large protein molecules elute in the void volume due to the fact that they are not attracted to hydrophilic surfaces. Other stationary phases including a shielded hydrophobic phase (SHP) [11] and a hydrophilic polymeric phase for high-performance frontal analysis (HPFA) [13] have been used to enable direct injection of protein containing samples.

Separation of caffeine and theophylline from protein-containing samples has been studied previously [13-15]. Following a sample clean-up procedure, direct injection analysis of caffeine and theophylline in serum was possible using a mixed function silica material [16] or ISRP [17].

It has been shown previously that polypyrrole modified silica has selectivity for basic compounds such as caffeine and theophylline [18]. The stationary phase also shows a degree of hydrophilicity which makes it possible to use this stationary phase for a mixed-functional separation of caffeine and theophylline from proteins.

In this work a polypyrrole based stationary phase has been considered for the separation of small molecules in prctein containing samples.

EXPERIMENTAL

Materials & Reagents

Pyrrole (Sigma) was distilled before use. Silica (Ultrasphere, 10 μm, 80Å pore size, 220 m^2/g specific surface area) was supplied by Beckman Instruments USA. Ferric chloride (GPR, BDH) was used without pre-treatment. Human serum albumin (HSA), bovine serum albumin (BSA), and ovalbumin were

obtained from Sigma. 0.5% (W/V) protein solutions were prepared using de-ionized water. Eluent consisted of 35% water and 65% methanol (HPLC) with 0.1% trifluoroacetic acid. NH_4Ac and acetate buffer solutions were used as eluents for protein retention tests. All other reagents were of analytical grade.

Preparation of Stationary Phases

Polypyrrole coated silica (PP/Si) was prepared using the following procedure:

1. 10 g silica was placed in a sample vial and dried at 100 °C over night, cooled, and after cooling, mixed with 20 g distilled pyrrole.

2. The mixture was treated in an ultrasonic bath for 5 minutes and then in vacuum for 20 minutes. This procedure was repeated three times.

3. The residual liquid pyrrole was removed and the remaining mixture was transferred to a 2 L beaker containing a stirred 1.5 L of 0.5 mol dm^{-3} $FeCl_3$ solution.

4. The polymerisation was allowed to continue for 30 minutes and the products were thrice washed each with water and then with acetone.

5. The products were dried at 100 °C for 30 minutes and gently abraded using a mortar and pestle.

6. The pure polymer particles and polymer coated silica particles were separated by flotation.

   The coated particles were packed into a cartridge column (5 mm ID x 100 mm) and a stainless steel column (4.6 mm ID x 100 mm).

Instrumentation

The cartridge column was placed in a RCM 8 X 10 cartridge holder (Waters). The chromatographic system included a Dionex Series 4000i gradient pump and injector, an ICI SD 2100 UV-VIS variable wavelength detector and a Kipp and Zonen BD41 chart recorder. The pH of buffers was measured using an ORION model SA 520 pH meter. Retention times were recorded using a stopwatch.

RESULTS AND DISCUSSION

An electron micrograph of the stationary phase is depicted in Figure 1. A brush type of polymer was observed on the silica surface. Elemental analysis showed that C, H, N and Cl comprised

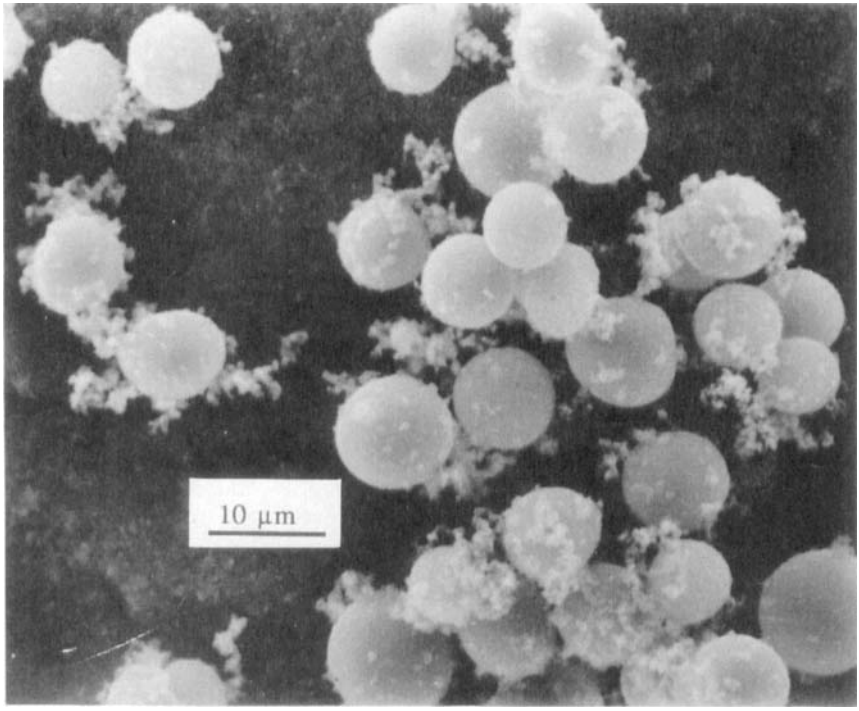

Figure 1 Scanning electron micrograph of polypyrrole chloride modified silica

21.8, 1.88, 6.27 and 3.72% respectively such that 33.67% of total weight consisting of polymer coating. This indicated that a thick coating was obtained. The surface area analysis confirmed that the polymer coated silica had a low specific surface area of 59 m^2/g compared to the uncoated silica, (220 m^2/g) since the polymer coating reduced the pore size.

The retention of small molecules on the PP/Si stationary phase was determined using the test compounds previously

TABLE 1 Capacity Factors for Small Molecules

Molecules	65% MeOH +35%H_2O	65% MeOH + 35% H_2O with 0.1% TFA
Benzene	1.657	0.9238
Toluene	1.841	1.848
Dimethylphthalate	1.893	1.502
Diethylphthalate	1.318	1.077
Phenol	0.6542	0.6162
Aniline	0.3963	-
N, N-Dimethylaniline	1.365	-
Caffeine	> 20	7.470
Theophylline	> 20	4.798
Benzoic Acid	> 20	3.18

Column: 4.6 mm ID x 100 mm

employed [18,19] (Table 1). The effect of adding trifluoroacetic acid (TFA) to the eluent was investigated because TFA is a reagent normally used to dissolve proteins in methanol solution. It was found that retention times of all compounds especially caffeine, theophylline and benzoic acid decreased with the

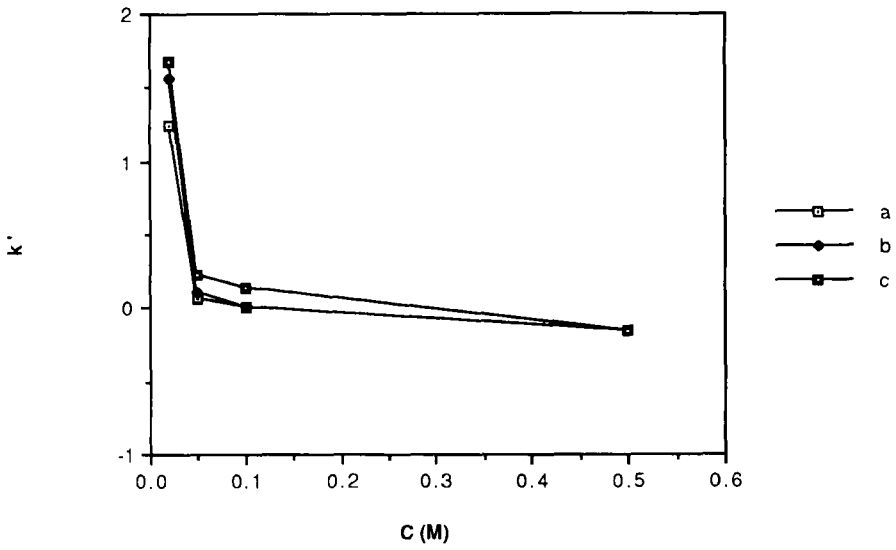

Figure 2 Effect of salt concentration on protein retention

Column: 4mm ID x 100 mm, PP/Si, 10 μm;
Eluent: NH_4Ac (pH = 6.0 adjusted with HAc) in H_2O;
Sample: 0.05 mol dm^{-3} protein solutions, 50 μl;
Detector: UV, 280 nm.
a. BSA; b. HSA; c. Ovalbumin.

addition of TFA. Such salt effects have been observed previously [18,19]. Aniline and N,N-dimethyl aniline had no UV responses after addition of TFA, due to protonation. All proteins had no retention in the presence of 0.1% TFA. This indicated that the stationary phase is hydrophilic enough to exclude proteins.

Various conditions were investigated in order to retain proteins on the column. It was found that the retention of

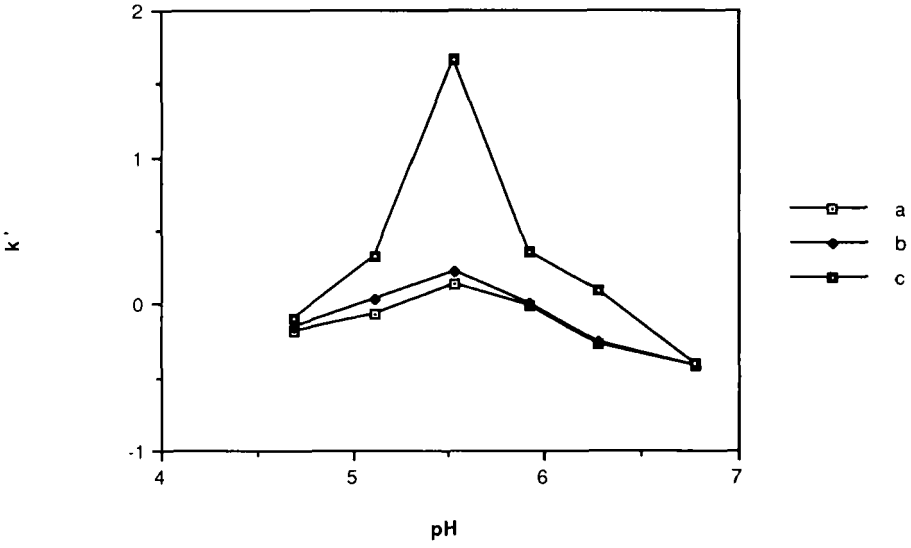

Figure 3 Effect of pH on protein retention

Column: As in Figure 2
Eluent: 0.05 mol dm^{-3}, NaAc - HAc buffer;
Sample: 0.05% protein solutions, 50 µl;
Detector: UV, 280 nm.
a. BSA; b. HSA; c. Ovalbumin.

proteins was dependent on the salt concentration of the eluent (Figure 2). This behaviour was observed previously on this phase using anion exchange test compounds [18,19]. However, in the case of proteins, the overall interaction was weak since retention could only be obtained at very low salt concentration. The effect of pH on the retention of proteins is shown in Figure 3. Retention was again low. Maximum retention was at pH approximately 5 (near the isoelectric points for these proteins).

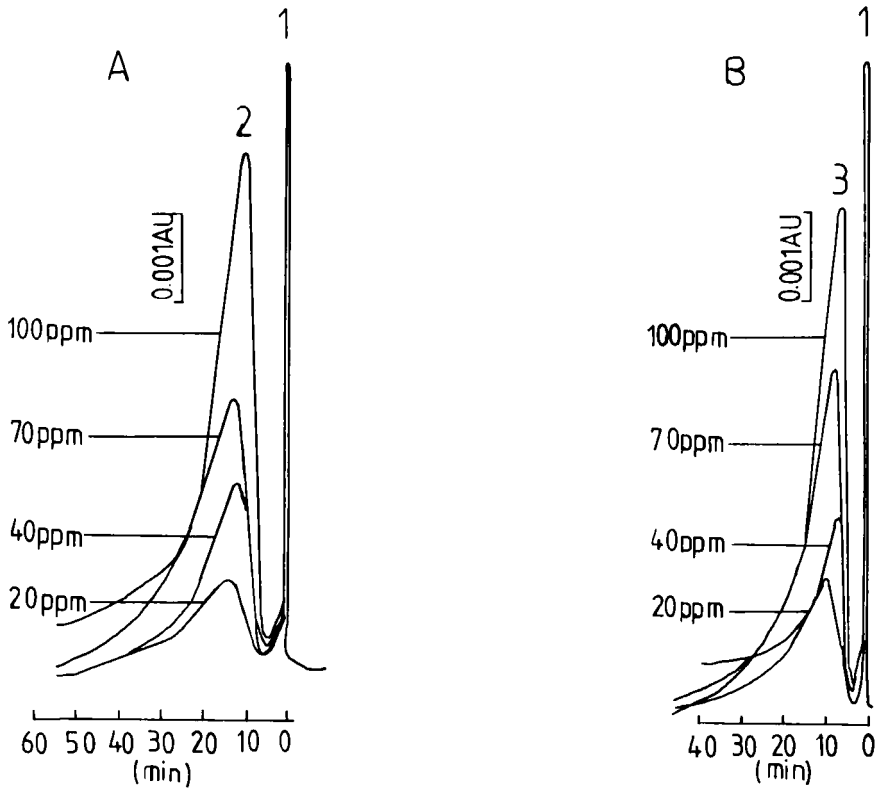

Figure 4 Effect of drug concentration on responses

Column: 4.6mm IDx100mm, PP/Si, 10μm
Eluent: 65% MeOH and 35% H_2O with 0.1% TFA at 1.0 ml/min.
Detector: UV, 280 nm
1. 250 μg HSA or BSA
2. caffeine
3. theophylline

TABLE 2 Effect of Amount of Protein on Drug Responses

HSA(μg)	70 ppm Caffeine response (mm)	70ppm Theophylline response (mm)
50	64	94
250	61	80
500	68	94

Eluent: 65% MeOH and 35% H_2O with 0.1% TFA at 1.0 ml/min.
Column: 4.6 mm ID x 100 mm

It was noted that k' was lower than 0, under certain conditions, ie retention of the proteins was less than the solvent front. This is probably due to size exclusion effects. Hydrophobic interaction chromatography was also investigated using 2.0 mol dm^{-3} $(NH_4)_2SO_4$ as eluent. Again no retention was observed. The above results indicated that chromatographic separations similar to that obtained using ISRP or mixed functional stationary phases could be achieved using this column. The stationary phase may be used to separate proteins from small molecules and hence may be employed in either purification of proteins or analysis of small molecules in biosamples.

The separation of small molecules from proteins was tested. Examples of chromatograms are shown in Figure 4. The broad peaks obtained were due to the low column efficiency

which is caused by the high polymer loading and brush type deposition as shown in Figure 1. This should be improved in future work.

The chromatographic peak responses were found to increase with increased drug concentration (Figure 4). This is further evidence that no interactions between drugs and proteins occurred [12]. The recoveries of HSA (250 μg) and BSA (250 μg) under the present conditions were 73% and 100% respectively. This data was obtained by comparing protein peak areas with and without the column.

Changes in the amount of protein injected over the range 50 μg to 500 μg were found to have no effect on the drug responses (Table 2).

CONCLUSION

Polypyrrole can be used as a stationary phase for separation of basic drugs such as caffeine and theophylline in the presence of proteins. Proteins do not interact strongly with the stationary phase whereas basic drugs are retained. No drug-protein interaction was observed under the chromatographic conditions employed. High protein recoveries were obtained. These results hold promise for the analyses of small molecular species in the presence of protein found in biological samples.

ACKNOWLEDGEMENTS

This research work was funded by a GIRD grant from the Department of Industry, Technology and Commerce, Australia

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Received: March 25, 1992
Accepted: April 8, 1992