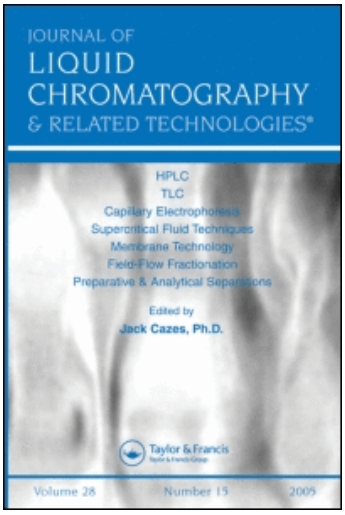

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Liquid Chromatographic Studies of Silica-Immobilized Bovine Serum Albumin Under Normal-Phase Conditions: Separation of Phenolic Solutes Using Ternary Mixtures of Hydrogen Chloride-Diethyl Ether-Hexane as Eluents

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LIQUID CHROMATOGRAPHIC STUDIES OF SILICA-IMMOBILIZED BOVINE SERUM ALBUMIN UNDER NORMAL-PHASE CONDITIONS: SEPARATION OF PHENOLIC SOLUTES USING TERNARY MIXTURES OF HYDROGEN CHLORIDE-DIETHYL ETHER-HEXANE AS ELUENTS

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ABSTRACT

The capacity factors, k', for multifunctional solutes with hydroxyl and carboxyl groups are measured on silica-immobilized bovine serum albumin (BSA) using various compositions of hydrogen chloride saturated diethyl ether, hexane, and neat diethyl ether. The values of k' are smaller at higher concentrations of free ether and larger as more of the ether is complexed with hydrogen chloride. Additionally, solute retention increases with increasing hydroxyl and carboxyl substituents in the molecule. The data indicate that solute retention is consistent with normal-phase behavior, which, to our knowledge, makes this work the first successful normal-phase application of silica-immobilized BSA to be reported.

INTRODUCTION

Due to their importance, there exist an extensive literature on the adsorption and chromatographic properties of proteins (e.g., 1-9). Although

chromatography has been employed primarily to purify and separate this class of compounds (e.g., 5-7), it also has played a significant role in studying their structure and interactions at various liquid/solid interfaces (e.g., 4, 8, 9). In each of these cases the protein serves as the solute molecule. Alternately, proteins may be immobilized to a suitable surface and either used as a separation medium or their physicochemical properties characterized by measuring the interaction of selected test solutes with the biomolecules.

Bovine serum albumin (BSA) is one of the proteins which has been employed most often as a stationary phase in liquid chromatography. In its native state the protein can bind a variety of compounds and in many cases has the ability to selectively recognize chirality (10). Allenmark and co-workers (11-15) were the first to use the BSA immobilized to silica as a high performance liquid chromatographic packing for separating N-aroyl D,L-amino acids and related enantiomers (15-22). However, because temperature, pH, and other solution equilibria can dramatically effect the size, shape, and conformation of bovine serum albumin (23,24) and hence change its binding properties, chromatographic reproducibility may be difficult (22).

Recently, various physicochemical aspects of enantiomeric recognition of silica-immobilized BSA have been studied by Guiochon et al. (25-27) and by Gilpin et al. (22,28). Adsorption heats and chromatographic band profiles have been measured for N-benzoyl-alanine (25,26) and leucine (27) enantiomers on BSA columns using phosphate buffers which contained small amounts of 1-propanol as the mobile phase. In addition, the effect of temperature on chiral recognition of tryptophan enantiomers has been examined under totally aqueous mobile phase conditions as a function of pH (22) as well as when small amounts of methanol were added to the mobile phase (28).

Over the last decade there has been an increasing number of studies that have been concerned with the behavior of proteins in nonaqueous solvents. Potential advantages may be gained by removing a protein from its native aqueous environment (29-31). Under nonaqueous conditions the new solvent can: 1) induce conformational rigidity into the protein which may either enhance or create new interactions; 2) facilitate the dissolution of compounds that are insoluble under aqueous conditions; and 3) increase the

thermal stability of the biomolecule. In addition, recently reported ligand memory effects (29,30) open up the possibility of initially exposing and/or binding a target molecule to a protein in an aqueous media which alters the biomolecule's structure and then to 'freeze' into place the newly induced conformation via use of a nonaqueous solvent after the water is removed (31). This process has been used by Braco et al. to carry out 'ligand-imprinting' and binding experiments on BSA and lysozyme (31). Although there are a number of questions and concerns which arise concerning the extent of and the mechanism involved in 'ligand-imprinting', the use of protein modifiers in combination with nonaqueous conditions does seem to be an interesting and novel approach to develop new chromatographic adsorbents.

In 1953 Carr (32) treated BSA with hydrogen chloride in order to decrease the activity of the amide groups. In a similar fashion Ehtesham and Gilpin (33) have demonstrated that the strong interactions of aminopropyl bonded phases may be attenuated significantly via converting the basic chains to their corresponding hydrochloride salt. Further they have shown that the bonded phase could be cycled reproducibly between the free base and HCl salt forms. Similarly, in the current study this approach has been applied to silica-immobilized BSA and the resulting HCl altered protein phase used to separate a series of simple mono, di and trifunctional solutes. The chromatographic properties of the HCl-modified BSA phase have been studied using various combinations of hydrogen chloride-diethyl ether-hexane as eluents. As an initial approach, since the retention data are consistent with normal-phase behavior, they have been discussed in terms of a simple displacement model. To our knowledge the work presented here represents the first successful application of silica-immobilized BSA as a normal-phase packing.

EXPERIMENTAL

Materials

The bovine serum albumin (fatty acid free), sodium phosphate (reagent grade) and sodium cyanoborohydride were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). The HPLC grade hexane and 2-propanol as well

as the reagent grade anhydrous diethyl ether were from Fisher Scientific (Pittsburgh, PA, USA), the LiChrospher Si-300 silica from EM Science (Gibbstown, NJ, USA), and the 3-aminopropyltriethoxysilane from Huls America (Piscataway, NJ, USA). The remaining chemicals were purchased from the Aldrich Chemical Co. (Milwaukee, WI, USA). These included the hydrogen chloride (220 g lecture cylinder), the glutaric dialdehyde (25 Wt.% solution in water) and the various organic compounds used as test solutes.

The deionized water was purified in-house using a Millipore (El Paso, TX, USA) MilliQ reagent water system. The HCl-saturated diethyl ether was prepared by slowly bubbling the gas through the solution overnight at ambient temperature.

Instrumentation

The liquid chromatograph consisted of a Spectra-Physics (San Jose, CA, USA) SP8810 precision isocratic pump, a SpectroMonitor III variable wavelength UV detector, and a DataJet integrator. Other system hardware included a Rheodyne (Cotati, CA, USA) injector with a 20 μl loop, a Fisher Scientific 730 isotemp immersion circulator set at 25 °C, a Neslab Instruments (Portsmouth, NH, USA) EN-350 cryocooler and a Phase Separation (Queensberry, Clwyd, UK) FLOSOA1 flowmeter.

The 2.1 mm i.d. x 150 mm columns were packed using a Haskel (Burbank, CA, USA) DST-52 air driven fluid pump and a custom built dynamic slurry reservoir. The packing procedure was similar to that reported previously (22,28), except the slurry and delivery solvent was anhydrous ethanol. The solution pH was measured with an Orion Research 701A meter and freeze drying was carried out with a Labconco (Kansas City, Missouri) 8 dryer.

Column Preparation

Lichrospher Si-300 silica (2.5 g) was rinsed with deionized water (25 ml), the solution centrifuged, and the supernatant decanted off. The resulting silica was transferred to a special reaction flask and 10 ml of an aqueous

solution of 3-aminopropyltriethoxysilane (10 % v/v silane in deionized water) were added. The pH of this mixture was adjusted to 3.4 via addition of phosphoric acid and then the reaction was allowed to proceed for 3 hr at 75°C while stirring the contents of the vessel with a stream of bubbling nitrogen. The resulting aminopropyl-derivatized silica was washed six times using 25 ml portions of deionized water. The product was dried at 100°C overnight. A small portion of this material was removed for elemental analysis which was carried out by Huffman Laboratories (Golden, CO, USA).

The aminopropyl-silica (2.4 g) was reacted for 3 hr at room temperature with 100 ml of a 2.5% aqueous solution of glutaric dialdehyde (10 ml of 25% glutaric dialdehyde in 90 ml of 0.05 M pH 7 phosphate buffer) and 0.126 g of sodium cyanoborohydride. The resulting aldehyde-activated silica was washed with five 30 ml portions of deionized water and then freeze dried. Again a small sample of this material was removed for elemental analysis.

The final synthetic step involved the coupling of the bovine serum albumin to the aldehyde-activated silica. This was done by combining 2.0 g of it with 84 ml of 0.05 M pH 7 phosphate buffer followed by the dropwise addition of 30 ml of a 1% buffered solution of bovine serum albumin (pH 7) and 53 ml of phosphate buffer (pH 7). Two portions of 0.1 g sodium cyanoborohydride were used as a reducing reagent during the reaction which took 12 hr. The final silica-immobilized BSA was washed thoroughly with deionized water and freeze dried. Again elemental analysis was carried out on a portion of the material. Based on carbon, nitrogen and sulfur analysis a coverage of 67 mg of BSA/g of silica was obtained.

RESULTS AND DISCUSSION

The immobilized BSA support was prepared by initially derivatizing silica with aminopropyl groups followed by protein attachment using glutaric dialdehyde. The resulting surface was then treated with hydrogen chloride dissolved in diethyl ether under completely anhydrous conditions. The rational in doing this is similar to that discussed previously for aminopropyl

bonded phases (33). The strong interactions which arise from the amide groups in the protein are attenuated by converting them to their corresponding HCl form and phenolic and aromatic acids can be eluted from the chromatographic column in a reasonable time. A series of retention measurements were made using various combination of HCl-saturated diethyl ether, hexane, and neat diethyl ether as the eluents. In all cases at least three replicate determinations were made.

In treating the various equilibria involved in the current study, the role of HCl in the mobile phase must be considered. The solubility of HCl in diethyl ether varies from a molar ratio of approximately 1 at 0 °C to 0.7 at 25°C (34), the temperature of the current study. Thus, at lower temperatures the system is mainly bimolecular complexes composed of one HCl molecule and one ether molecule. However, at higher temperatures, since the molar ratio of HCl to ether is less than unity, the composition of the solvent is more complex. The remaining ether molecules are either unassociated and/or form other complexes. In discussing the various trends observed in the current study the former case has been assumed to be the dominate species and the system has been treated as a three component systems comprised of simple HCl-ether complexes, hexane, and free diethyl ether molecules.

Based the above simplified ternary model for the eluent, the relative eluting power of the HCl-ether complex was evaluated. Although it is well established that diethyl ether is a more polar solvent than hexane, the eluting strength of the HCl-ether complex has not been determined previously. In order to address this question, solute retention was measured as a function of increasing amounts of the complex. This was done in two ways, by using mobile phases: 1) that were prepared from hexane and HCl-saturated diethyl ether, and 2) that were prepared from neat diethyl ether, HCl-saturated diethyl ether and a fixed volume of hexane.

Shown in Figure 1 are the experimentally observed relationships between the capacity factors for three selected solutes and the volume fraction of HCl-saturated diethyl ether in the mobile phase. Although the ordinate of this plot has not been corrected in terms of the level of complex present (i.e., a molar ratio of 0.7), it should be proportional to the HCl-diethyl ether. The capacity factors decreased rapidly at low levels of HCl-saturated ether and

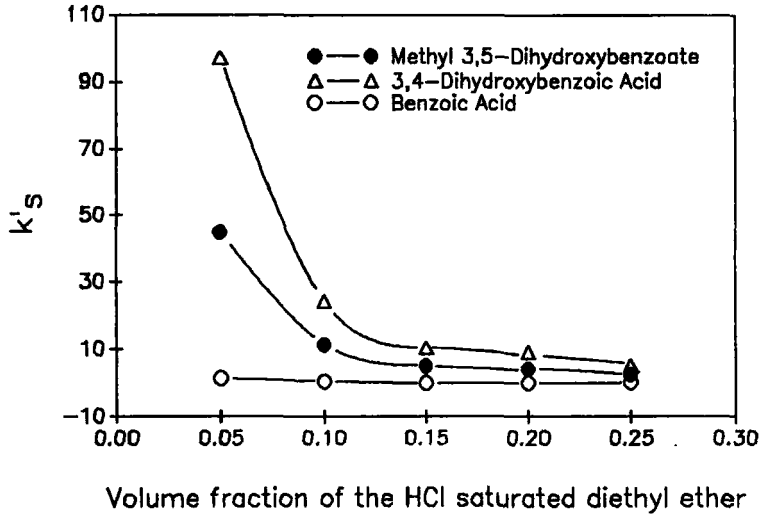

FIGURE 1. Experimental dependence of the capacity ratio on the volume fraction of the HCl saturated ether in n-hexane for three selected solutes at 25°C.

more gradually at concentrations higher than 10%. Although the general shapes of the curves shown in Figure 1 appear to be fitted by more than one relationship, the reasons for which are unknown at the present and warrant further study, the overall decreases in the capacity factors indicate that hexane is less polar than the HCl-ether complex. Further, the small slopes of the curves at higher concentrations of HCl-saturated ether suggests that values of k'_s in the pure HCl-complex are higher than those in neat diethyl ether. This idea is supported further by the data shown in Figure 2, which were measured at a fixed volume (80%) of hexane and varying amounts of neat and HCl-saturated diethyl ether. Increases in the level of free ether results in decreasing solute retention which is consistent with the idea that ether is the more polar component and influences elution to greatest extent.

In an effort to simplify the physical interpretation of the complex equilibria involved in the system studied, another set of measurements were made using a fixed small volume (i.e., 5%) of the HCl-saturated ether. In

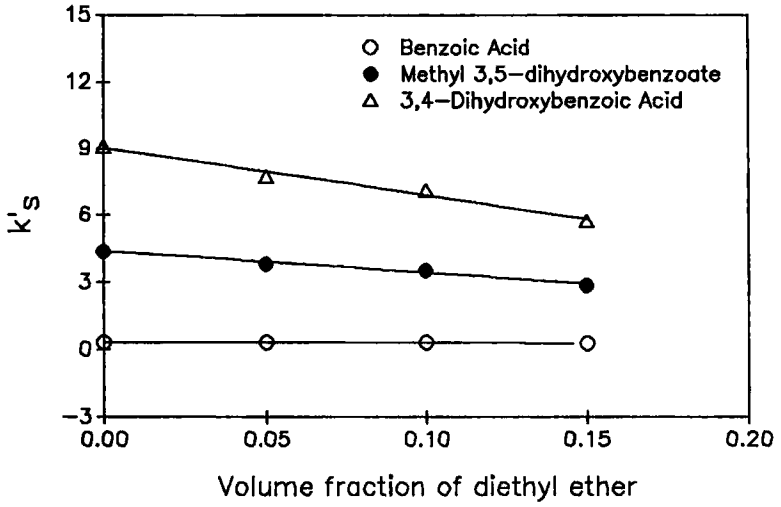

FIGURE 2. Experimental dependence of the capacity ratio on the volume fraction of ether for three selected solutes. The total volume fraction of ether and the HCl saturated ether was equal 0.2 and kept constant during measurements.

doing this it was assumed that under these conditions the concentration of the (1:1) HCl-ether complex would remain constant as the amount of free ether was varied in the mobile phase. Based on this initial assumption, the capacity factors were analyzed as a function of the volume fraction of free ether, which as demonstrated above, is the most polar component in the mobile phase.

The capacity ratios, k'_s, were measured for a total of ten simple aromatic solutes. Summarized in Table 1 are the values of k'_s for toluene, benzoic acid, four monohydroxyl and four dihydroxyl compounds chromatographed as a function of increasing amounts of diethyl ether and a fixed amount of HCl-saturated ether. A brief inspection of Table 1 shows that: 1) the nonpolar solute, toluene, is nearly unretained under any of the conditions studied, 2) increasing functionality in a solute results in increased retention, and 3) increasing amounts of the polar component, diethyl ether, in the mobile phase results in decreased retention. These general trends,

TABLE 1
Capacity Factors for Simple Aromatic Solutes Containing Hydroxyl and Carboxyl Subsitutents Chromatographed under Normal-Phase Conditions on a Silica-Immobilized BSA Column Treated with Hydrogen Chloride.

Solute	Capacity Factors [volume fraction of neat diethyl ether in the mobile phase]*				
	0.15	0.20	0.25	0.30	0.35
Toluene	0.01	0.01	0.01	0.01	0.01
Benzoic acid	0.47	0.35	0.28	0.26	0.23
Phenol	0.48	0.26	0.25	0.20	0.19
o-Hydroxybenzoic acid	1.63	1.11	1.00	0.66	0.59
m-Hydroxybenzoic acid	1.66	1.09	1.02	0.65	0.63
p-Hydroxybenzoic acid	1.71	1.10	0.93	0.66	0.66
Resorcinol	1.85	1.31	1.09	0.75	0.75
Methyl 3,5-dihydroxybenzonate	4.02	1.19	1.07	0.64	0.64
3,4-Dihydroxybenzoic acid	8.84	6.25	3.69	3.24	2.39
3,5-Dihydroxybenzoic acid	11.93	8.01	3.98	3.49	2.48

*All mobile phases contain 5 v/v% HCl-saturated diethyl ether and varying amounts of diethyl ether and hexane as given; values obtained at 25°C, and 1 ml/min.

which are illustrated for three of the test solutes in Figure 3, are consistent with those expected under normal-phase conditions.

As noted above, the magnitude of the capacity factor was dependent on functional structure of the solute. At a given mobile phase composition, the k'_s increased with increasing numbers of polar groups. This behavior has been examined further by calculating the capacity factors ($k'_{s(h)}$-values) for a mobile phase which contained no added free ether. Graphically this was done by extrapolating the experimental dependencies illustrated in Figure 3 to the

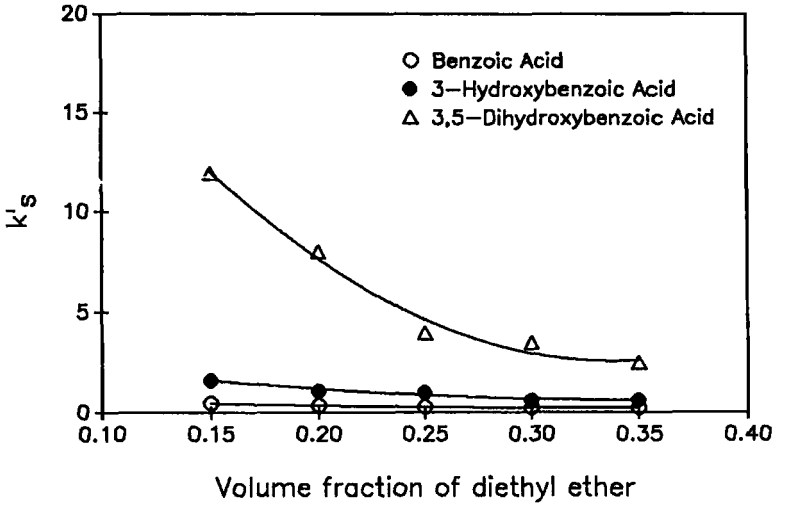

FIGURE 3. Experimental dependences of the capacity ratio on the volume fraction of ether in the mobile phase for selected solutes chromatographed on the BSA column at 25°C.

mobile phase containing only HCl-saturated ether. Again it should be noted that this approach assumes that the amount of the ether complex remains constant, which in the strictest sense, is probably incorrect. A second limitation of these values results from the fact that the experimental range lies relatively far from the extrapolation point and hence there is a statistically large error associated with these values irrespectively of any physical and chemical influences. Consequently the $k'_{s(h)}$-values, which appear in the first column of Table 2, should be treated only as very crude approximations of the values of k'_s in a mobile phase devoid of free ether. With this in mind, two trends were apparent from the data in Table 2: 1) The elution order increased with increasing functionality as has already been noted, and 2) the hydroxyl functional group contributed more to retention than the carboxyl group. This latter trend can be seen by comparing the elution behavior of each group of solutes. For the monofunctional solutes, benzoic acid and phenol, the k'_s-values were respectively 0.98 and 1.22. Similarly, the $k'_{s\,(h)}$-values for solutes which contained two functional groups were between 3.36

TABLE 2

Calculated Values of ($k'_{s(h)}$), ($k'_{s(p)}$), and r with a Constant 5 v/v% HCl-Saturated Diethyl Ether in the Mobile Phase.

Solute	$k'_{s(h)}$	$k'_{s(p)}$	r
Benzoic acid	0.98	0.09	0.83
Phenol	1.22	0.06	1.04
o-Hydroxybenzoic acid	3.36	0.17	1.21
m-Hydroxbenzoic acid	3.57	0.18	1.17
p-Hydroxybenzoic acid	4.08	0.18	1.17
Resorcinol	4.10	0.21	1.25
Methyl 3,5-dihydroxybenzonate	14.0	0.20	1.90
3,4-Dihydroxybenzoic acid	22.0	0.47	1.59
3,5-Dihydroxybenzoic acid	33.0	0.34	1.89

and 4.10 with resorcinol, which was the only solute to contain two hydroxyl groups, eluting last. The highest values of $k'_{s(h)}$ were obtained for solutes with three-functional groups. Each of these contained two hydroxyl groups. In the case of methyl 3,5-dihydroxybenzonate, conversion of the acid group to its corresponding ester resulted in decreased retention which indicates that each of the three polar subsituents contributes to retention.

Since free ether was the most polar component of the mobile phase and the HCl-saturated ether was held at a constant low level, the k'-values have been analyzed as a function of the volume fraction of diethyl ether assuming a simple displacement model (35-40). Accordingly, the active sites in the stationary phase (i.e., sites where the solutes interact in the immobilized BSA) are occupied mainly by ether molecules which are displaced when a strongly polar solute interacts. This process can be described by the following equilibrium constant K (37):

$$K = (\phi_s^s/\phi_s^m)\ (\phi_p^m/\phi_p^s)^r \quad (1)$$

where ϕ_i^ρ denotes the volume fraction of the i-th component in the ρ-th phase, r is the statistical number of the functional groups in a solute molecule that interact with the active sites in the stationary phase. The superscripts s and m refer respectively to the stationary and mobile phases, and the subscripts s and p refer respectively to the solute and polar solvent. Equation (1) is the mathematical description of a simple displacement mechanism which neglects non-ideality of both phases. In applying this model to the current study it is recognized that at best the derived quantities represent only very qualitative descriptors of an extremely complex set of equilibria and thus serve only as a guide to understanding the mechanisms involved.

By assuming that the solute-solvent competition for the active sites in the BSA phase can be represented by equation (1) and the solute phase ratio, ϕ_s^s/ϕ_s^m, is proportional to k_s' (35), the following expression can be obtained (37,38):

$$\log k_s' = \log k_{s(p)}' + r \log (\phi_p^s/\phi_p^m) \quad (2)$$

When the solute is infinitely dilute and the concentration of the polar component of the solvent is sufficiently high that it occupies all active sites, $\phi_p^s = 1$, equation (2) reduces to the Snyder-Soczewinski relationship (40):

$$\log k_s' = \log k_{s(p)}' - r \log \phi_p^m \quad (3)$$

This expression has been used frequently to describe normal-phase retention behavior (38).

Shown in Figure 4 are log-log plots of the curves which appear in Figure 3. Similar plots were constructed for the remaining data in Table 1. In all cases, equation (3) was found to provide a good linear fit to the retention data measured for the hydroxylated solutes on the silica-immobilized BSA surface. Summarized in Table 2 are the calculated values of the capacity factor, $k_{s(p)}'$, and r for a mobile of 95% diethyl ether and 5% HCl-saturated diethyl ether. There appear to be two generalization which can be made concerning the calculated values of r in Table 2: 1) The solutes that contain

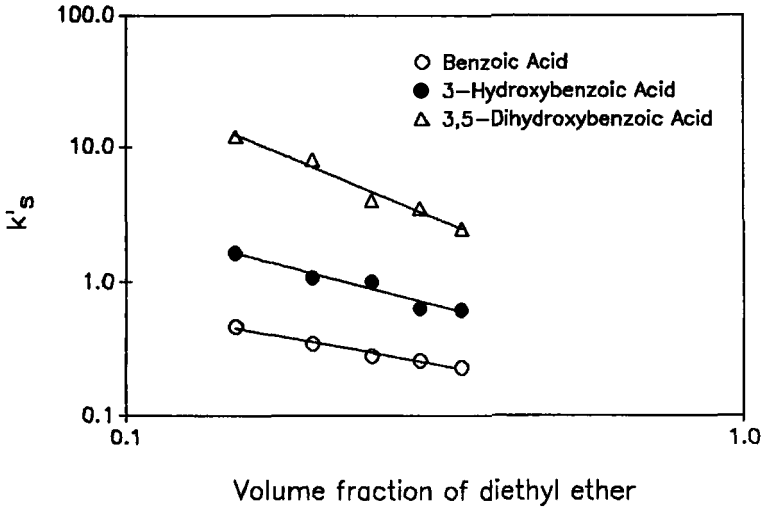

FIGURE 4. Experimental dependences shown in Fig. 3 plotted in the log-log coordinates.

a single functional group have r values close to the theoretically expected value of one, and 2) the r values for the multifunctional solutes have r values considerably smaller than the number of functional groups present. In a very general sense, these results are typical of systems where many of the sites of interactions are isolated compared to the size of the solute molecule and/or a good steric fit between the multifunctional groups in the solute and the active sites exists only in a few cases. Each of these seem reasonable based on the complex structure of BSA which is a "cigar-shaped" protein makeup of 582 amino acid residues with varying α-substituents and folded into three domains which are held together by 17 disulfide bridges (23). In a stricter sense, as noted above, the simple displacement model used to calculate the r-values likely neglects many important aspects and thus a more rigorous evaluation of the results in Table 2 is unwarranted at the present time. For example, it is unclear why both 3,4-dihydroxybenzoic acid and methyl 3,4-dihydroxybenzoate on a relative basis have higher values of r compared to resorcinol if the meta arrangement of the OH groups is important.

Nevertheless, the relative elution order and values of r indicate that interactions of solutes with the immobilized BSA phase are controlled mainly by the polarity of the functional groups (specific interactions) and by their position in the solute (steric effect).

CONCLUSIONS

The current study to our knowledge represents the first successful attempt to use an immobilized protein as a normal-phase separation medium. In order to accomplish this, the amide groups which act as very strong adsorption sites in the protein and hence make elution mode experiments difficult were converted to their corresponding hydrochloride salts. Under these conditions aromatic solutes which contain multifunctional groups can be eluted within a reasonable time using combinations of diethyl ether, hexane, and HCl-saturated diethyl ether as eluents. Further it has been shown that under such conditions the bound protein system behaves like a normal-phase system and that a relatively simple displacement model can be used to describe the elution behavior. A number of questions have arisen during the current work, as well as, treatment of the work, especially in terms of a more exact description of the mobile phase equilibria and in terms of stereochemistry of the solutes and surface. Many of these questions are now being examined chromatographically, as well as, by other techniques. The use of protein modifier may be a useful means of forming a variety of novel chromatographic surfaces.

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REFERENCES

1. W. Norde, Advan. Colloid and Interface Sci., 25: 267-340 (1986)
2. S.S. Saavedra, W.M. Reichert, Langmuir, 7: 995-999 (1991)

3. R. Blanco, A. Arai, N. Grinberg, D.M. Yarmush, B.L. Karger, J. Chromatogr., 482: 1-12 (1989)

4. J. Stahlberg, B. Jonsson, Cs. Horvath, Anal. Chem., 63: 1867-1874 (1991)

5. K.G. Briefs, M.R. Kula, Chem. Eng. Sci., 47: 141-149 (1992)

6. S. Yamamoto, M. Nomura, Y. Sano, Chem. Eng. Sci., 47: 185-188 (1992)

7. P.M. Boyer, Hsu, J.T., Chem. Eng. Sci., 47: 241-251 (1992)

8. N.K. Boardman, S.M. Partridge, J. Biochem., 59: 543 (1955)

9. W.R. Melander, Z.J. El Rassi, Cs. Horvath, J. Chromatogr., 469: 3-27 (1989)

10. R.H. McMenamy, in Albumin Structure, Function and Uses, V.M. Rosenoer, M. Oratz, M.A. Rothschid, eds., Pergamon, Oxford, 1977, pp. 27-51.

11. S. Allenmark, B. Bomgren, J. Chromatogr., 237: 473-477 (1982)

12. S. Allenmark, B. Bomgren, H. Boren, J. Chromatogr., 316: 617-624 (1984)

13. S. Allenmark, S. Anderson, J. Chromatogr., 351: 231-238 (1986)

14. S. Allenmark, S. Anderson, J. Bojarski, J. Chromatogr., 436: 479-483 (1988)

15. S. Allenmark, B. Bomgren, H. Boren, J. Chromatogr., 264: 63-68 (1983)

16. M.T. Aubel, L.B. Rogers, J. Chromatogr., 392: 415-420 (1987)

17. M.T. Aubel, L.B. Rogers, J. Chromatogr., 408: 99-113 (1987)

18. G. Gubitz, Chromatographia, 30: 555-563 (1990)

19. L.W. Wainer, Trends Anal. Chem., 6: 125 (1987)

20. P. Erlandsson, S. Nilsson, J. Chromatogr., 482: 35-51 (1989)

21. Z. Simek, R. Vespalec, J. High Resolution Chromatogr., 12: 61-62 (1989).

22. R.K. Gilpin, S.E. Ehtesham, R.B. Gregory, Anal. Chem., 63: 2825-2828 (1991)

23. T. Peters, Jr., Advan. in Protein Chem., 37: 161-245 (1985)

24. C. Tanford, S.A. Swanson, W.S. Shore, J. Amer. Chem. Soc., 77: 6414-6421 (1955)

25. S. Jacobson, S. Golshan-Shirazi, G. Guiochon, J. Chromatogr., 522: 23-36 (1990)

26. S. Jacobson, S. Golshan-Shirazi, G. Guiochon, J. Amer. Chem. Soc., 112: 6492-6498 (1990)

27. S. Jacobson, S. Golshan-Shirazi, G. Guiochon, Chromatographia 31: 323-328 (1991)

28. R.K. Gilpin, S.B. Ehtesham, S.T. Liao, Chromatographia, in press.

29. A. Zaks, A.M. Klibanov, Science, 224: 1249-1251 (1984)

30. A.M. Klibanov, Trends Biochem., 14: 141-144 (1989)

31. L. Braco, K. Dabulis, A.M. Klibanov, Proc. Natl. Acad. Sci. USA 87: 274-277 (1990)

32. C.W. Carr, Arch. Biochem. Biophys., 46: 417-81 (1953)

33. S.B. Ehtesham, R.K. Gilpin, Chromatographia, 32: 79-81 (1991)

34. International Critical Tables of Numerical Data; Physics, Chemistry and Technology, E.W. Washburn, C.J. West, N.E. Dorsey, F.R. Bichowsky, A. Klemenc, eds., McGraw-Hill, New York, 1928, p. 264.

35. Snyder, L.R., Principles of Adsorption Chromatography, Marcel Dekker, New York, 1968.

36. L.R. Snyder, H. Poppe, J. Chromatogr., 184: 363-413 (1980)

37. M. Jaroniec, J.K. Rozylo, B. Oscik-Mendyk, J. Chromatogr., 179: 237-245 (1979)

38. M. Jaroniec, D.E. Martine, M. Borowko, Adv. Colloid Interface Sci., 22: 177-227 (1985)

39. M. Jaroniec, D.E. Martire, J. Chromatogr., 351: 1-16 (1986)

40. E. Soczewinski, J. Chromatogr., 130: 23-28 (1977)