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Semipermeable-surface reversed-phase media for high-performance liquid chromatography

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ABSTRACT

Polyoxyethylene was both adsorbed hydrophobically (through the use of non-ionic surfactants) and covalently bonded to reversed-phase high-performance liquid chromatographic packings, thereby establishing a semipermeable hydrophilic layer over the alkylsilane surface. This layer restricts proteins from adsorbing to the alkylsilane phase while permitting penetration and chromatographic separation of small molecules. Biological fluids containing low-molecular-weight analytes may be injected directly, without sample pretreatment or the use of micellar eluents. In the case of adsorbed coatings, surfactant loading was determined primarily by the surface area (over the reversed phase) occupied by the polyoxyethylene head group. Semipermeability of the hydrophilic layer was demonstrated by observing changes in retention of both small molecules and proteins with increasing eluent ionic strength. Coated column stability was evaluated with regard to cumulative eluent volume and repeated serum injections.

INTRODUCTION

The past decade has witnessed a proliferation of methods and media for direct injection of biological fluids onto high-performance liquid chromatographic (HPLC) systems. (Recent reviews of direct injection have been published by Shihabi [1] and Westerlund [2]). The primary impetus for this development has been the rising need for simple, fast, and automated analyses of drugs in biological fluids. Conventional reversed-phase chromatographic media are not designed to tolerate direct injection of small analytes in protein-containing matrices. Upon contact with the hydrophobic surface of the packing under typical reversed-phase elution conditions, most proteins are denatured and irreversibly adsorb, resulting in drastic deterioration of chromatographic efficiency and rapid plugging of the column. In addition to the use of precolumn switching systems [3–5], methods have been developed in which either the proteins or the packing materials themselves are modified to prevent the deleterious effects of protein adsorption. Efforts have included the use of micellar concentrations of surfactants as eluent modifiers and the creation of specialized chromatographic packings. Such "restricted access"sorbents include protein-coated reversed-phase

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media [6–8], Internal Surface Reversed-Phase columns [9] (ISRP, a trademark of Regis Chemical Company, Morton Grove, IL, U.S.A.) and Shielded hydrophobic phase packings [10] (SHP, a trademark of Supelco, Bellefonte, PA, U.S.A.).

Even though the work described in this paper did not require the use of surfactants in the chromatographic mobile phase, consideration of the mechanisms involved in micellar chromatography directed much of this research. Most published work on the subject deals with the use of anionic surfactants, usually sodium dodecyl sulfate, in eluents to preemptively denature and solubilize proteins as samples are injected. Most of the attention has been focused on chromatographic effects of the concentration of micelles in the eluent. Several reviews examine this and related topics [11,12]. Although non-ionic surfactants have been used as micellar eluent modifiers for direct injection in the same manner as anionic detergents, there are two significant differences. First, because they lack charged head groups, non-ionic surfactants form micelles at much lower concentrations than do anionic detergents. Makino *et al.* [13] have shown that because they cannot achieve sufficient monomer concentrations, non-ionic surfactants do not bind cooperatively to proteins and, therefore, do not cause denaturation. More importantly, as demonstrated by Chang [14], these surfactants substantially and almost irreversibly coat reversed-phase surfaces, creating a protective hydrophilic layer which does not retain proteins. Several researchers [15,16] have noted decreased retention and changes in selectivity for test analytes on reversed-phase columns during micellar liquid chromatography performed with non-ionic surfactant in the eluent. Again, attention was focused on the effect of micelle concentration in the mobile phase. Borgerding *et al.* [17], however, have recently examined the adsorption of two polyoxyethylene (POE) non-ionic surfactants (Brij-22 and -35) and one anionic surfactant (sodium dodecyl sulfate) on a C_{18} column packing material. Surface area and pore volume measurements indicated that the surfactants adsorbed as a film on the surface of a reversed-phase column.

Restricted access column packings also address the problems inherent to direct chromatographic analysis of biological fluids for small molecules. Japanese workers [6–8] found that small-pore, reversed-phase columns repeatedly exposed to dilute solutions of bovine serum albumin (BSA) and other proteins eventually exhibited good retention and recovery of small solutes. Plasma proteins were excluded from the pores due to irreversibly-adsorbed BSA on external surfaces of the packing material. This approach, however, has not been widely used.

Hagestam and Pinkerton [9] developed a novel restricted-access packing by attaching a hydrophobic tripeptide to glycerylpropyl-derivatized, small pore silica. An enzyme was then used to cleave the tripeptide from the external surface, leaving this surface hydrophilic and non-adsorptive to proteins. Plasma proteins are further prevented from entering the pore matrix by a size exclusion mechanism. Small analytes, which can access the internal pore surface, are adsorbed and thereby separated from one another. This material has come to be known as "Internal-Surface Reversed-Phase" (ISRP). Another type of restricted-access media has recently been reported [10]. Termed a "Shielded Hydrophobic Phase" (SHP), this silica-packing contains a polymeric hydrophilic network of bonded polyethylene oxide with embedded hydrophobic (phenyl) groups [10].

The work described in this paper was undertaken to determine whether a layer of hydrophilic polymer, *i.e.* polyoxyethylene, over the surface of a conventional C_8 or

TABLE I

SURFACTANTS EMPLOYED IN THIS STUDY

Commercial name	Code[a]	*m*	*n*	HLB[b]
G7606J	P-10-SM-12	10	12	14.9
Tween-20	P-20-SM-12	20	12	16.7
G4280	P-80-SM-12	80	12	19.1
Tween-40	P-20-SM-16	20	16	15.6
Tween-60	P-20-SM-18	20	18	14.9 (stearate)
Brij-35	P-23-AE-12	23	12	16.9
Brij-700	P-100-AE-18	100	18	18.8 (stearate)

[a] Surfactants are coded based on their chemical structures (see Fig. 1): Polyoxyethylene (*m*) Sorbitan Monoalkylates (*n*) or Polyoxyethylene (*m*) Alkyl Ethers (*n*). Letters "*m*" and "*n*" denote number of oxyethylene units per head group and number of carbon atoms per hydrocarbon tail, respectively.

[b] HLB = Hydrophilic/lipophilic balance [(weight % OE)/5].

C_{18} reversed-phase can restrict access of proteins to the underlying hydrophobic stationary phase.

EXPERIMENTAL

Materials

Monomeric reversed-phase packings used for hydrophobic adsorption were purchased in bulk by special arrangement with Supelco. These materials were end-capped octyldimethyl (Supelcosil LC-308; designated "C_8") and octadecyldimethyl (Supelcosil LC-318; "C_{18}") phases on 5-μm silica particles with 300 Å pores. The non-ionic surfactants used in this study are listed in Table I. These water-soluble members of the Tween and Brij series (see Fig. 1 for structures) were used because they can be reproducibly adsorbed onto reversed-phase packings from aqueous solution. All surfactant samples were kindly provided by ICI Specialty Chemicals Division, ICI Americas (Wilmington, DE, U.S.A.) and were used as received.

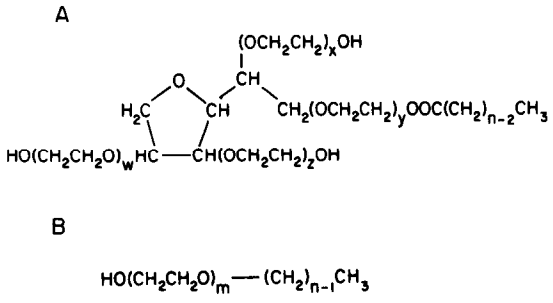

Fig. 1. (A) Chemical structure of Tween series of polyoxyethylene sorbitan monoalkylates. $m = w + x + y + z$ = number of oxyethylene units ($O-CH_2-CH_2$). (B) Chemical structure of Brij series of polyoxyethylene alkyl ethers.

The column of covalently-bonded semipermeable-surface reversed-phase media used to examine retention of selected drugs was graciously supplied by Regis (Morton Grove, IL, U.S.A.), as were most of the drugs used.

Model analytes and drugs, inorganic salts and solvents were all of analytical reagent grade or higher quality. Methanolic stock solutions of analytes were diluted to desired concentrations using either filtered eluent or serum prior to injection. Hen egg-white lysozyme and human serum albumin (Pentex brand) were obtained from Sigma (St. Louis, MO, U.S.A.) and Miles Scientific (Naperville, IL, U.S.A.), respectively. Pooled human serum was purchased from Pel-Freez Biologicals (Brown Deer, WI, U.S.A.) and single-donor serum was provided by the laboratory of Dr. P. Low (Chemistry Department, Purdue University, W. Lafayette, IN, U.S.A.). Bradford reagent for protein assays was purchased from either Bio-Rad (Richmond, CA, U.S.A.) or Pierce (Rockford, IL, U.S.A.).

The mobile phase used for chromatographic evaluation of all adsorbed surfactant columns consisted of 0.05 *M* potassium dihydrogen phosphate (in double-deionized water; pH adjusted to 6.5 or 6.8 with 3 *M* sodium hydroxide) containing 3% (v/v) 1-propanol. Buffer was filtered through a 0.2-μm nylon 6,6 membrane and degassed thoroughly prior to addition of 1-propanol; the final mixture was degassed only briefly.

Apparatus

Isocratic chromatography was performed using a single-piston minipump (Model 396, LDC-Milton Roy, Riviera Beach, FL, U.S.A.) at 1.0 ml/min. One of the two systems used contained a tenport manual injection valve with 25- and 100-μl sample loops (Model C10-U, Valco Instruments, Houston, TX, U.S.A.). The other contained a six-port manual injection valve with a 20-μl sample loop (Rheodyne, Cotati, CA, U.S.A.). As needed, either system was connected to a differential refractometer (Model R-401, Waters Assoc.), Milford, MA, U.S.A.) or to a fixed-wavelength (254 nm) UV detector (Model 153, Beckman, Berkeley, CA, U.S.A.). The repetitive serum injection study utilized a programmed HPLC pump and automatic injection valve system (Model 590 programmable solvent delivery module, Waters Automatic Valve Station, Waters Assoc., fitted with a 20-μl sample loop. The loop was filled by a small laboratory pump (Model RH-SY, Fluid Metering, Oyster Bay, NY, U.S.A.) controlled by the programmed main pump. No guard or precolumns were used.

Gradient chromatography (drug retention study) was performed using an LDC Constametric I and IIIG pumping system with a Gradient Master controller. A Varian (Walnut Creek, CA, U.S.A.) 634 Series UV–VIS spectrophotometer was used to quantitate serum protein recovery.

Procedures

Surfactant coating and quantitation. Micellar solutions of the surfactants described in Table I were made by dissolving 0.5% (w/w), accounting for any water content, of the desired surfactant in filtered (0.2 μm), double-deionized water. Coating of the reversed-phase (C_8 or C_{18}) packing materials was carried out by pumping surfactant solution through either small cartridges or chromatographic columns (described below). In order to quantitate adsorbed surfactant, media (0.1 g) were slurry-

packed from methanol, by vacuum, into empty plastic cartridges from a Waters Guard-Pak precolumn module. The filled cartridge was washed with methanol, placed in the holder and attached to a chromatographic pump. Following a wash with (15 ml) double-deionized water, surfactant solution was pumped through the cartridge at 1.0 ml/min for 30 min. (Initially, this process was monitored using a differential refractometer to insure complete coating.) The coated cartridge was then washed with water (1.0 ml/min) for 60 min to remove excess surfactant. Surfactant-coated packing material was subsequently removed from the cartridge, dried overnight at 83°C, under vacuum, and subjected to elemental analysis (Purdue University, Chemistry Department). The increase in percent carbon and decrease in percent silicon, relative to uncoated controls was used to calculate the amount of surfactant adsorbed. The coating procedure described above was carried out at least twice for each surfactant on both C_8 and C_{18} materials, except that Brij surfactants were not tested on C_{18} packing.

Chromatographic columns. Quantities of the same reversed-phase materials were also slurry-packed into 5 or 15 cm (× 0.46 cm I.D.) stainless-steel columns using a Shandon (Sewickley, PA, U.S.A.) HPLC packing pump at 7500 p.s.i. (Columns were initially packed using methanol; however, improved bed stability was achieved by pumping isopropanol for 200 ml, followed by methanol–water, 50:50 for 400–500 ml.) Columns were then washed with water and coated at 1.0 ml/min using the desired 0.5% surfactant solution. Preliminary experiments indicated that pumping times of 60 and 90 min were more than sufficient to coat 5- and 15-cm columns, respectively. After use, the surfactant-coated columns were stripped of surfactant by washing with pure methanol at 1.0 ml/min for at least 4 h.

Serum protein recovery. A 5-cm column of reversed-phase packing coated with each surfactant was evaluated for serum protein recovery. Undiluted, filtered (0.45 μm), pooled human serum was injected (20 or 25 μl) onto the column, then eluted at 1.0 ml/min using the mobile-phase described previously. Triplicate samples and controls (injector connected directly to detector) were collected in 10-ml volumetric flasks and protein content determined using the Bradford [18] assay. Recovery of individual, pure proteins was not investigated.

Evaluation of the semipermeable hydrophilic surface. Retention of small solutes (benzyl alcohol and phenol) and proteins (hen egg-white lysozyme and human serum albumin) on surfactant-coated columns was measured as a function of eluent ionic strength. A 5-cm column of C_8 reversed-phase packing coated with P-20-SM-12 (and, later, with P-100-AE-18) was evaluated using duplicate 20 μl injections of each of the four samples. Eluents of increasing ionic strength were made by dilution from a stock solution containing 0.6 moles of KH_2PO_4 and K_2HPO_4 per liter of water (pH values were near neutral). Dilutions, each containing 3% 1-propanol, were made to total ionic strengths of 2.4, 1.8, 1.2, 0.6, 0.3 and 0.15, according to eqn. 1:

$$([K^+] + H_2PO_4^-])/2 + (2[K^+] + [HPO_4^{2-}]^2)/2 = 2.4 \quad (1)$$

The experiment was also conducted using 2 *M* $(NH_4)_2SO_4$.

Chromatographic retention and efficiency. A variety of model analytes were used to measure chromatographic retention and efficiency on uncoated and surfactant-

coated columns. Efficiency, expressed as N/m, plates per meter, was calculated using the equation:

$$N = 5.54(t_r/w_{1/2})^2 \quad (2)$$

where t_r is the retention time from injection and $w_{1/2}$ is peak width measured at half-height.

Coated-column lifetime studies. Experiments were conducted: (1) to examine the stability of the surfactant coating and serum protein recovery after long periods of continuous pumping without surfactant in the eluent; and (2) to observe coated-column efficiency and backpressure with repetitive serum injection.

The first experiment was carried out using, in tandem, a precolumn module and a 15-cm analytical column. Both contained C_8 media, packed and coated with P-20-SM-12 surfactant as previously described. This precolumn cartridge and column were washed for 22 h with mobile phase (0.05 *M* phosphate buffer, pH 6.5, containing 3% 1-propanol) at 1.0 ml/min. At that time, both cartridge and column were washed with water to remove eluent. Packing material in the cartridge was removed, dried overnight and submitted for elemental analysis. The analytical column was evaluated for serum protein recovery, then stripped free of all surfactant (using methanol). This column was subsequently recoated, a new cartridge placed in-line (same type of packing and surfactant as used initially) and the above procedure repeated, washing for 40 h this time.

The serum injection study was carried out using a (5 × 0.46 cm I.D.) column packed with C_8 reversed-phase material and coated with P-100-AE-18. Phenol (*ca.* 100 μg/ml) was added to single-donor serum (centrifuged and filtered, 0.45 μm) in order to monitor column efficiency for small molecules. A programmed pump and automatic valve system (see *Apparatus*) was used to inject 500 20-μl loads, at 10-min intervals, of phenol-enriched serum onto the surfactant-coated column. Eluent was pumped at 1.0 ml/min. To prevent excessive leaching of surfactant from the column during the serum injection study, it was recoated with surfactant solution after each liter of mobile phase had been used. It should be emphasized, however, that no surfactant was included in the eluent.

RESULTS AND DISCUSSION

Two approaches were taken to achieve reversed-phase media with a hydrophilic surface layer. Initially, polyoxyethylene-containing non-ionic surfactant were hydrophobically adsorbed to selected C_8 and C_{18} HPLC packings as described in *Experimental.* The resultant "semipermeable-surface" reversed-phase sorbents were then evaluated for properties necessary to the chromatographic separation of small molecules in the presence of proteins.

Factors governing surfactant coating

Surface area per surfactant molecule. Elemental analyses revealed that the parameter having the greatest influence on surfactant density was the area over the reversed-phase surface occupied by the surfactant head group. Fig. 2 shows an inverse linear relationship between the amount of surfactant coated and the square root

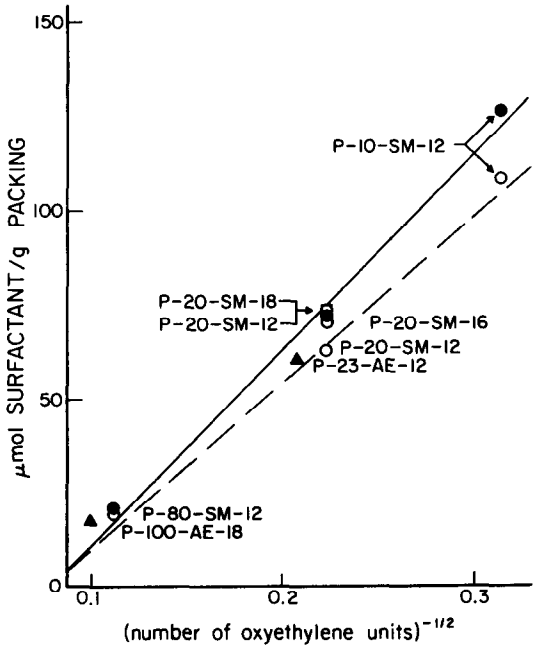

Fig. 2. Surfactant coverage on reversed-phase silica as a function of the inverse square root of the number of oxyethylene units per surfactant head group. Surfactants represented by solid symbols and the solid line were on C_8 silica. Those indicated by open symbols and a dashed line were on C_{18} silica. Tween and Brij surfactants are represented by circles and triangles, respectively.

of the number of oxyethylene units per molecule for all surfactants studied. That the area occupied by the hydrated POE chains predominates in determining saturation adsorption at liquid–solid, liquid–liquid, and liquid–air interfaces is a concept supported by Van Voorst Vader [19]. (Saturation adsorption on non-polar adsorbents is equivalent to complete monolayer coverage [20].) He states that "...because of hydration, at saturation adsorption, the ether groups can only occur in specific maximal density, the surface area per non-ionic molecule must increase proportionally to $n/n^{1/2} = n^{1/2}$, as found experimentally" and concludes that the shape and size of the hydrophobic group have relatively minor influence on surfactant saturation adsorption [19]. Fig. 3 provides evidence that the area occupied by surfactant head groups on reversed-phase surfaces is proportional to the square root of the number of oxyethylene units per molecule, and determines the maximum amount of surfactant adsorbed. Data for the adsorption from water of P-10-SM-12, P-20-SM-12, P-80-SM-12, P-23-AE-12, and P-100-AE-18 onto C_8 silica are compared to data for saturation adsorption of POE nonylphenols from water onto carbon [21] and at an air–water interface [22]. The relationship between surface area and square root of the number of oxyethylene units per molecule is linear in each case. Differences in slope may indicate a different mode of packing of surfactant molecules. On hydrophobic surfaces, polyethylene glycol is adsorbed in random coils, even without an attached hydrophobic moiety [20]. At the air–water interface, POE chains do not spread out,

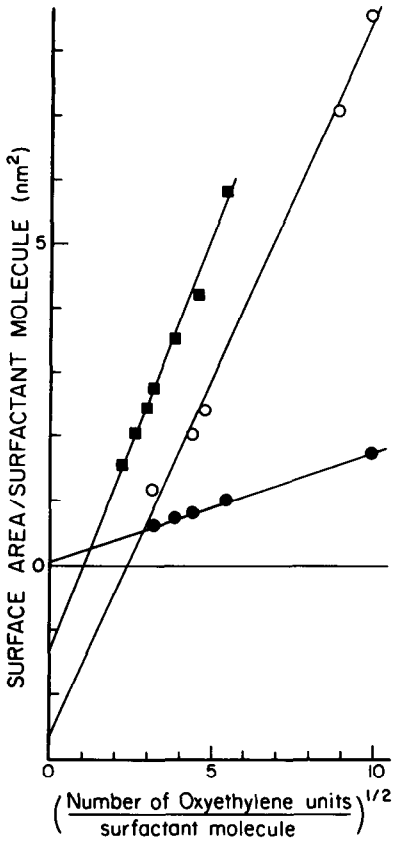

Fig. 3. Plot of surface area occupied per surfactant molecule *vs.* the square root of the number of oxyethylene units per head group. Open circles represent adsorption from water of non-ionic surfactants (P-10-SM-12, P-20-SM-12, P-80-SM-12, P-23-AE-12 and P-100-AE-18) onto C_8 reversed-phase silica. Comparative data are for the saturation adsorption of POE nonylphenols from water onto carbon (■) and at an air–water interface (●) (replotted from refs. 21 and 22, respectively).

but coil more vertically into the water [20]. Based on the slopes, it would appear that the mode of packing on C_8 reversed-phase silica is similar to that on the solid, flat carbon surface. Saturation adsorption at the interface between water and a non-polar solid is believed to occur as a planar monolayer in which the hydrocarbon chains of the surfactant approach a vertical orientation [20]. (Negative y-intercepts of the lines for surfactant adsorption on solids may indicate overlapping of the extended, coiled POE chains).

Surfactant head-group structure. Data in Fig. 2 also permit comparison of coating with branched (Tween) *vs.* linear (Brij) surfactants (on C_8 silica). Points for the two selected Brij molecules (P-23-AE-12 and P-100-AE-18) fall close to the lines in Figs. 2 and 3, indicating that the structure of the head group —whether branched or linear— has little effect on the amount of surfactant coated. Branched and linear POE chains are flexible in aqueous solution and can rearrange to fill equal areas over the reversed-phase thereby attaining specific maximal densities of oxyethylene units.

Hydrocarbon surface area of reversed phase. Fig. 2 also shows a slight difference in the amounts of surfactants coated on C_8 *vs.* C_{18} materials. This difference varies from almost none for surfactants having the largest head groups to 15% for the smallest species. An explanation of this phenomenon is complicated by the fact that the base silicas used for the two reversed-phase materials have different surface areas, as reported by the manufacturer. The lot of silica used for the C_8 material is 88.4 m^2/g and the C_{18}, 95.0 m^2/g. However, this relatively small difference is offset somewhat by the lengths of the two reversed-phase ligands. Where surfactant adsorption is concerned, the important surface area is not that of the bare silica, but hydrocarbon surface area available after silanization. The longer C_{18} ligands will take up more space, decreasing the amount of available pore volume and surface area inside the pores. Also, C_{18} ligands can block access to more small pores at the low end of the pore-size distribution. These factors combined contribute to decreased surfactant

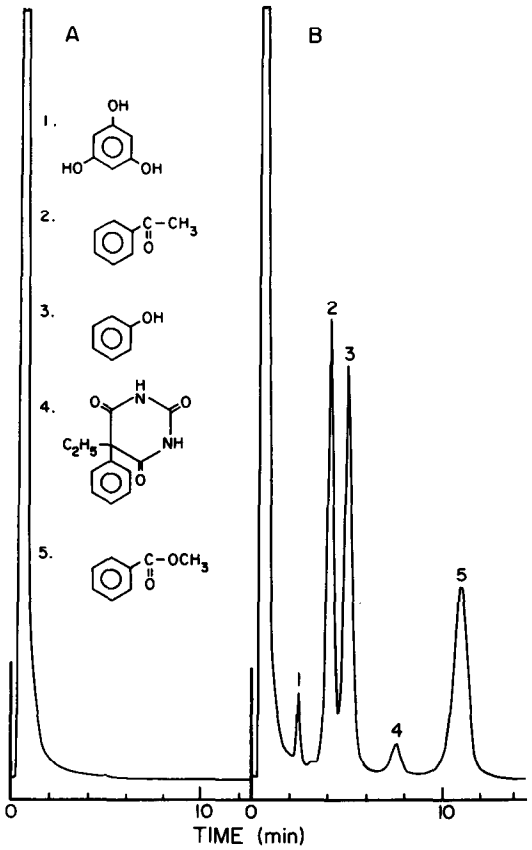

Fig. 4. Separation of model analytes from human serum on a C_8 reversed-phase column coated with P-100-AE-18 surfactant. Human serum (A) was spiked with five model analytes (B) at concentrations ranging from 20–200 μg/ml. Chromatographic conditions: 25 μl injection volume; mobile phase: 0.05 *M* phosphate buffer (pH 6.5) containing 3% 1-propanol; elution from column (5 × 0.46 cm I.D.) at 1.0 ml/min; detection at 254 nm, 0.04 a.u.f.s.

coverage on the C_{18} packing. The smaller surfactants are more sensitive to this effect since they have greater access to available surface area.

Ratio of reversed-phase ligands to surfactant molecules. Coverage data used to construct Figs. 2 and 3 was also combined with reversed-phase ligand density values for the C_8 and C_{18} materials to determine the ratios of reversed-phase ligands to surfactant molecules. Reversed-phase ligand density values reported in Table II were calculated using elemental analysis data and manufacturer's surface area measurements. (The amount of endcapping silane on these monomeric phases was assumed to be negligible.) For the Tween series with 12-carbon "tails", both C_8 and C_{18} packings showed a five-to-six-fold increase in the number of alkylsilane residues per surfactant as head group size increased from 10 to 80 oxyethylene units. This supports the hypothesis that it is the amount of surface area covered by the head group that determines surfactant density, and not the degree of hydrophobicity (length of the hydrocarbon chain) of the surface or the surfactant.

Chromatographic performance of surfactant-coated reversed-phase columns

Figs. 4 and 5 show that surfactant-coated reversed-phase packings, while nonretentive to proteins (as in serum), are still capable of separating small molecules. Applications similar to those achieved on ISRP columns are anticipated. Recoveries of whole serum from columns coated with all of the surfactants listed in Table I were 97 ± 3% or greater.

Semipermeability and hydrophilicity of surfactant layer.

Fig. 6 illustrates the effect of increasing eluent ionic strength on retention (expressed as k') of two small solutes and two proteins on a Tween-coated C_8 column. The dashed line (data points for lysozyme and human serum albumin are superimposed) indicates that, as ionic strength increases, neither protein is retained. Increasing ionic strength, however drives the small solutes to stronger hydrophobic interaction with the column. Similar results were obtained on a C_8 column coated with P-100-AE-18 (data not shown). These results are interpreted to mean that proteins do not have any significant degree of contact with the alkylsilane phase. Small

TABLE II

RATIOS OF REVERSED-PHASE LIGANDS TO SURFACTANT MOLECULES ON COATED REVERSED-PHASE PACKINGS

Ligand density based on elemental analysis: C_8, 3.26 μmol/m^2; C_{18}, 2.32 μmol/m^2. Surface area specified by manufacturer: C_8, 88.4 m^2/g; C_{18}, 95.0 m^2/g.

Surfactant	C_8	C_{18}
P-10-SM-12	2.28	2.04
P-20-SM-12	3.98	3.54
P-80-SM-12	13.88	11.41
P-23-AE-12	4.76	–
P-100-AE-18	16.79	–
P-20-SM-16	–	3.12
P-20-SM-18	–	3.03

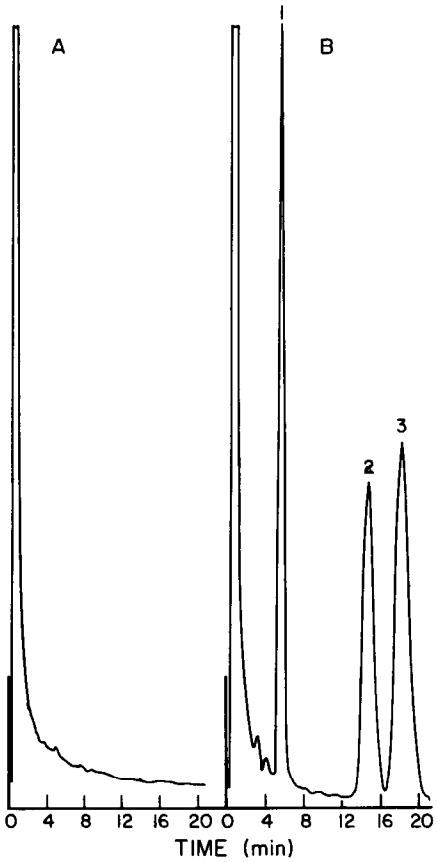

Fig. 5. Separation of diuretics from human serum on a C_8 reversed-phase column coated with P-100-AE-18 surfactant. Human serum (A) was spiked with (1) hydrochlorothiazide (50 μg/ml), (2) chlorthalidone (100 μg/ml) and (3) furosemide (100 μg/ml) (B). Chromatographic conditions same as in Fig. 4.

molecules, in contrast, appear to have good access to the underlying hydrophobic stationary phase. Because the hydrophilic polymer in this case is POE, non-retention of proteins does not continue to extreme ionic strengths. Both lysozyme and human serum albumin are retained in 2 *M* $(NH_4)_2SO_4$. This is not surprising, since several POE-derivatized phases alone have been used for hydrophobic interaction chromatography of proteins [23–25].

Chromatographic efficiency. Newly-packed, uncoated columns exhibited efficiencies of 25 000–35 000 plates/m for polar solutes (*e.g.* phenol, phloroglucinol, aniline) and 35 000–45 000 plates/m for non-polar analytes (*e.g.* benzene, toluene, ethyl benzene). Although chromatographic efficiency decreased upon coating, efficiencies for the surfactant-coated columns were approximately equal to those achieved on ISRP columns [26]. The effects of surfactant head-group size and structure on chromatographic efficiency were assessed by comparing plates/m before and after coating (data not shown). The degree to which efficiency decreased depended on both surfac-

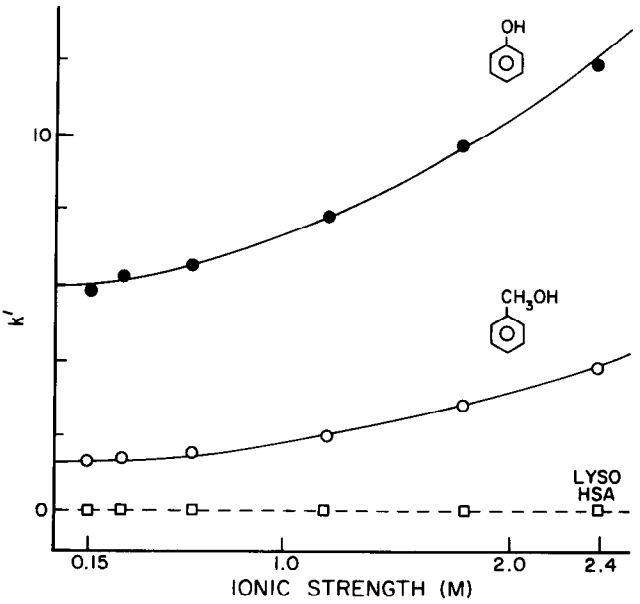

Fig. 6. Capacity factor (k') of selected small solutes and proteins on a surfactant-coated column as a function of eluent ionic strength. A C_8 column (5 × 0.46 cm I.D.), coated with P-20-SM-12, was eluted with *ca.* pH 7 phosphate buffer (containing 3% 1-propanol) of increasing ionic strength at a flow-rate of 1.0 ml/min. Small molecules chromatographed were phenol (●) and benzyl alcohol (○). Data for the two proteins, lysozyme (LYSO) and human serum albumin (HSA), are superimposed (□).

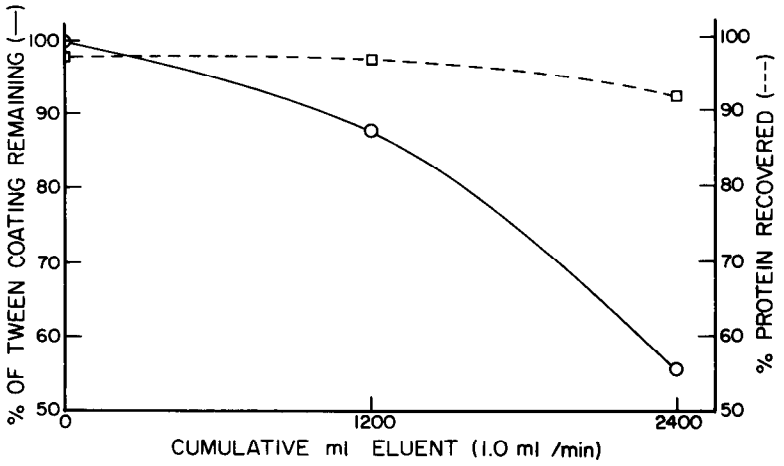

Fig. 7. Effect of cumulative eluent volume on an adsorbed non-ionic surfactant coating. The stability of a surfactant (P-20-SM-12)-coated reversed-phase (C_8) packing to continuous use without surfactant in the mobile phase was evaluated as described in *Experimental*. The amount of coating which remained and serum protein recovery were measured after exposure to a total of 1200 or 2400 ml of mobile phase.

tant and solute; losses were smallest with surfactants having the largest head groups and efficiency decreased less for non-polar than polar analytes. For example, coating C_8 media with P-20-SM-12 resulted in a 15–30% decrease in efficiency as measured with polar analytes, whereas a P-80-SM-12 coating decreased efficiency by 10% or less in the case of non-polar solutes.

Stability of surfactant coating. Coating stability in the presence of the surfactant-free eluent was evaluated as described in *Experimental.* Almost half of the surfactant load can be lost before protein recovery is reduced (Fig. 7). However, in order to maintain reproducible retention of small molecules, columns were normally recoated after each liter of eluent was used.

Effects of repetitive serum injection. A P-100-AE-18-coated C_8 column was subjected to 500 20-μl injections of serum. Periodic increases in backpressure were entirely due to plugging of the stainless steel frits, and not to the coated packing material. Occasional losses in efficiency (*ca.* every 100 injections) were accompanied by the buildup of a very thin grey-brown film at the column head. Removal of the film and replacement of the packing with fresh coated material completely restored efficiency. (This indicates the benefits of a guard column.) After experiencing a total of 10 ml of serum, this coated column retained its original backpressure and efficiency.

Covalently-bonded semipermeable-surface packing

Based on the data obtained from surfactant-coated reversed-phase packings, methods were developed to synthesize alkylsilane-derivatized silica with covalently-

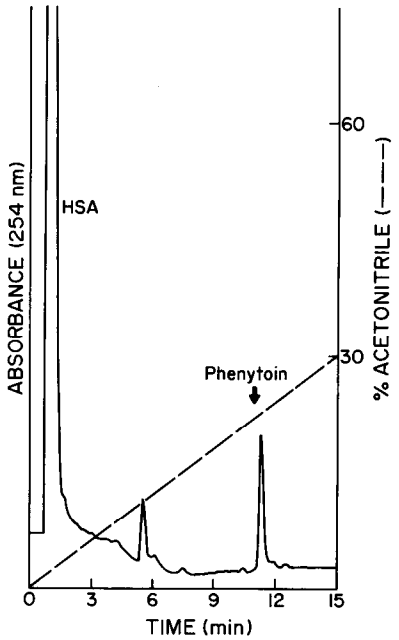

Fig. 8. Gradient elution of the anticonvulsant, phenytoin, from a covalently bonded semipermeable-surface reversed-phase column (15 × 0.46 cm I.D.). A sample (20 μl) of human serum albumin (HSA; 12 mg/ml) and phenytoin (200 μg/ml) was chromatographed using a 30-min linear gradient from 0 to 60% acetonitrile in 0.1 *M* KH_2PO_4 buffer (final pH = 6.8) at a flow-rate of 1 ml/min.

bonded polyoxyethylene; details of the coupling chemistry used are described elsewhere [27,28]. A durable semipermeable-surface sorbent with chromatographic retention and protein recovery analogous to that of surfactant-coated C_8 columns was achieved. Unfortunately, the rather broad particle-size distribution (revealed by microscopic examination) of silica used in our synthetic work precluded good chromatographic efficiencies. However, a commerical column based on the semi-permeable surface concept provides *ca.* 50 000–60 000 plates/m, depending on the solute analyzed [29].

Since the hydrophilic layer is covalently attached, it is stable to organic solvents. Fig. 8 illustrates the separation of phenytoin, a strongly-retained drug, from human serum albumin via a gradient of increasing acetonitrile concentration. Gradient chromatography circumvents the need (as in isocratic elution) to limit mobile phase organic content in order to prevent protein denaturation and precipitation within the column or system [30,26].

A preliminary study using several "weakly-retained" drugs [26,30], *e.g.* acetaminophen, indicated that a covalantly bonded semipermeable-surface reversed-phase media can be 2–3 times more retentive than ISRP or SHP columns. This is significant with regard to hydrophilic species which have been difficult to resolve from serum proteins on other restricted access sorbents. Details of this work and other studies will be the subject of future publications.

CONCLUSIONS

Restricted access media that perform the same function as internal surface reversed-phase columns were prepared by either adsorbing or chemically bonding polyoxyethylene moieties to the surface of alkylsilane-based reversed-phase packings. Direct injection of biological samples is possible because proteins are prevented from contacting the hydrophobic stationary phase by a layer of hydrophilic polymer. Repetitive injection of 20-μl serum samples did not cause plugging of either the surfactant-coated or covalently bonded polyoxyethylene packing material. (Original column efficiency and backpressure were maintained after injection of a total of 10 ml of serum in the case of the adsorbed surfactant coating.)

Water-soluble non-ionic surfactants were adsorbed from aqueous solution onto reversed-phase surfaces in amounts determined primarily by the number of oxyethylene units in their head groups, and surface area over the reversed phase which they occupy. Head-group structure (branched *vs.* linear) was not a factor in adsorption. The hydrocarbon tail functions as an anchor, ensuring that a maximum number of surfactant molecules are arranged on the packing surface. The data suggests that surfactant packing density is sterically regulated by the size of the head group. Surfactant adsorption sometimes resulted in decreased chromatographic efficiency. This effect was variable, depending on the combination of solute and surfactant involved. In general, a longer, linear polyoxyethylene appeared to reduce efficiency less than a shorter, branched one.

When surfactants were hydrophobically adsorbed, use of eluent organic modifiers was restricted. Instead, changes in retention and selectivity were achieved by changing the size of the surfactant head group. The surfactant leaching problem was overcome by covalently bonding polyoxyethylene to the reversed-phase. This permits

the use of organic solvent, including gradient elution, in the case of strongly retained analytes.

Preliminary studies indicated that the covalently-bonded semipermeable-surface reversed-phase packing material was analogous in chromatographic retention and protein recovery to surfactant-coated C_8 columns. Furthermore, a commercially-manufactured column of covalently-bonded media was reasonably retentive to several hydrophilic drugs which have been difficult to resolve from serum proteins on other restricted access columns. Thus, it is concluded that the semipermeable surface concept can be utilized for separating low-molecular-weight analytes from proteins in the direct injection of biological fluids.

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