Journal of Chromatography, 544 (1991) 41–49
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 041

Review

Hydrophilic shielding of hydrophobic, cation- and anion-exchange phases for separation of small analytes: direct injection of biological fluids onto high-performance liquid chromatographic columns

BINYAMIN FEIBUSH* and CARMEN T. SANTASANIA
Supelco, Inc., Supelco Park, Bellefonte, PA 16823 (U.S.A.)

ABSTRACT

Shielded hydrophobic phases (SHPs) have an external hydrophilic network that prevents larger protein molecules from interacting with hydrophobic zones. Smaller analytes are not sterically hindered from interacting with the hydrophobic zones and are retained. This mechanism allows the separation of the proteins from the analytes of interest. SHPs have been shown to be useful for the direct-injection analysis of drugs in biological fluids. In this paper, two kinds of shielded phases are discussed: bonded micellar phases and embedded polymeric phases. For some compounds of interest, the hydrophobic retention and selectivity is insufficient to obtain the desired resolution. To increase selectivity without affecting protein exclusion, shielded phases were prepared with ion-exchange groups added to the hydrophobic zones. Such modified phases with cation-exchange and anion-exchange capabilities were examined for additional selectivities. Usage of these additional selectivities will be demonstrated to achieve better analysis and resolution for basic, acidic, and neutral compounds.

CONTENTS

1. INTRODUCTION

The well established reversed-phase high-performance liquid chromatographic (HPLC) technique resolves many kinds of analytes, but tends to fail when significant

0021-9673/91/$03.50

amounts of proteins are present in the sample. To avoid (almost immediate) clogging of a reversed-phase column due to protein accumulation, techniques were developed to introduce a protein-free sample onto the analytical column. In early studies, the serum proteins were first removed from the aqueous sample by precipitation and centrifugation. The analytes of interest were then extracted into an organic layer and introduced onto the analytical column. Later, a more advanced technique was developed, involving an on-line pre-column separation of the interfering proteins from the analytes of interest. This technique depends on column-switching systems [1].

To overcome the handicap of the previous techniques, new packing materials were designed. These materials retain and resolve the analytes of interest, while eluting the water-soluble proteins in or near the void volume. Excluding the larger proteins from the pores of a packing was the first approach tried. If the retentive interaction could be made to occur only inside the pores, then the excluded proteins would elute unretained, while the included analytes would be retained and eluted later. That such an approach could be successful was indicated in a study in which a polystyrene–divinylbenzene resin was used as the pre-column packing in a column-switching system to analyze the drug methaqualone in serum [2]. Yoshida *et al.* [3] similarly used a C_{18} packing, bonded on a small-pore-size silica support, to adsorb denatured plasma proteins. The plasma proteins were excluded from the small pores and precipitated only on the external surface of the packing. The alkyl ligands, inside the pores, were free and uncontaminated. Using the appropriate amount of plasma proteins, the external surface is saturated with a protein coating. Proteins in a serum-containing drug sample, analyzed on such a packing material, will contact only the previously precipitated proteins and elute unretained. Drugs in such a sample can penetrate into the pores and interact with the C_{18} ligands.

Pinkerton used a similar model in his elegant packing design. Phenylalanine or its dipeptide homologue was bonded to the surface of a small-pore-size (80 Å) silica gel support. This modification was followed by an enzymatic cleavage of the phenylalanine moiety, leaving a digested hydrophilic exterior and a hydrophobic phenylalanine-modified interior [4].

The phases that were developed in our laboratory for the same analytical purpose have bonded ligands that exhibit hydrophilic character toward the larger proteins and hydrophobic character towards the small analytes. We have termed these phases shielded hydrophic phases (SHPs). We discuss in this paper two types of SHPs, described elsewhere [5], and some additional phases in which the active underlayer contains basic or acidic ion-pairing groups for additional interaction with acidic or basic analytes.

2. EXPERIMENTAL

2.1. Chromatographic system

The liquid chromatographic system consisted of a Waters 510 HPLC pump, a Wisp 712 automatic sample injection system, and a 484 tunable absorbance detector. A 720 system controller was used to control the pumps and autosampler. All data were recorded on a 745B data module, at a chart speed of 1.0 cm/min. (All components from Waters, Milford, MA, U.S.A.)

2.2. Columns

Hisep shielded hydrophobic phase columns (5-μm silica packing, 15 cm × 4.6 mm I.D. or 25 cm × 2.1 mm I.D.; Supelco, Bellefonte, PA, U.S.A.) were used in this study. In addition, modified Hisep shielded phases were used. Groups introduced into the phase included CO_2H, SO_3H, $N(CH_3)_2$, $N^+(CH_3)_3$, and $N^+(Bu)_3$ (5-μm silica packing, 15 cm × 4.6 mm I.D. columns). An in-line 0.5-μm frit filter (Supelco) was used to protect the analytical column.

2.3. Mobile phases, reagents, drugs, and test compounds

HPLC-grade acetonitrile, methanol, and ammonium acetate were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Purified water (18 MΩ) from a Milli-Q system (Millipore, Bedford, MA, U.S.A.) was used in the preparation of all mobile phases and samples. Buffers were filtered through 0.45-μm Magna nylon 66 membrane filters (Supelco), and all mobile phases were degassed under vacuum in an ultrasonic bath before use. All pH measurements were made using a Fisher Accumet pH meter (Fisher Scientific, Pittsburgh, PA, U.S.A.) equipped with a standard pH electrode.

Trimethoprim, salicylic acid, propranolol, chloramphenicol and its succinate sodium salt, and benzoic acid were obtained from Sigma (St. Louis, MO, U.S.A.). Stock solutions of each compound were prepared in methanol at 1 mg/ml. These stock solutions were then diluted 1:1 with water. Appropriate dilutions to the desired concentrations were made with water, fetal bovine serum, or human cerebral spinal fluid.

Triple-filtered fetal bovine serum was obtained from Hyclone Labs. (Logan, UT, U.S.A.). Human cerebral spinal fluid (AIDS- and hepatitis-free) was obtained from Immunostics (Ocean, NJ, U.S.A.).

3. RESULTS AND DISCUSSION

It must first be emphasized that SHPs address the same analytical need as do restricted-access phases but consist of a different chemistry. In SHPs, the same phase covers all of the support, while the restricted-access phase has a dual-phase chemistry. The pore size of the support, which is so essential to the restricted phase separation, makes no contribution in the case of the shielded materials. A non-porous support can be used for the shielded phases, as long as the contribution of the surface area to the separation can be neglected.

We previously studied two kinds of silica-supported SHPs, a bonded "micellar layer" and an "embedded network". In the bonded micelle, the bonded hydrophobic alkyl ligands are substituted at the ω-position with a polar hydrophilic group. The stationary phase resembles a low-density micellar layer bonded to the support through the hydrophobic tails. In the embedded-network SHP, a polymeric hydrophilic network embeds hydrophobic moieties.

In our studies, we attempted to mimic the chromatographic behavior of water-soluble proteins on a detergent-modified reversed phase, as shown in Fig. 1A. The use of micellar mobile phases in the HPLC of proteins has been established in a number of studies, including direct plasma and serum injections [6,7]. Under these conditions, using a C_{18} silica column and a surfactant-containing mobile phase, the surfactant

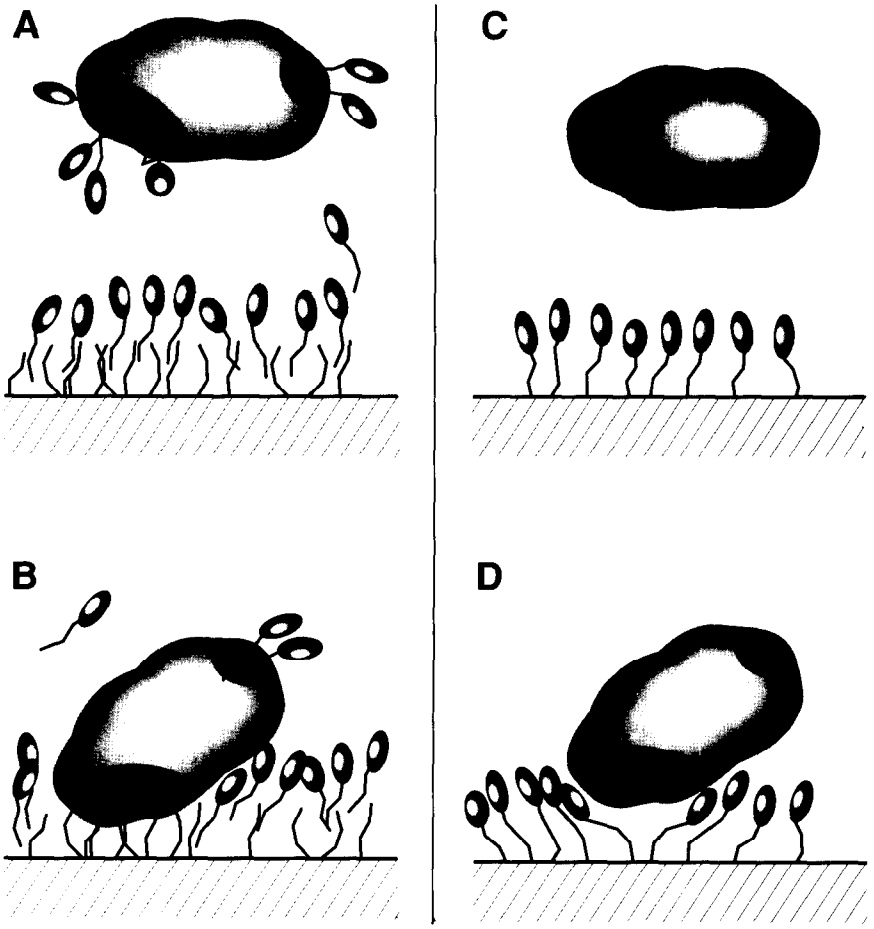

Fig. 1. Schematic representation of an albumin molecule with two hydrophobic patches: (A) as a solute in the presence of a detergent containing mobile phase; (B) adsorbed to a C_{18}-detergent phase; (C) as a solute in a non-detergent mobile phase; (D) adsorbed to a micellar-type bonded phase.

saturates the C_{18} phase to form a layer having a polar hydrophilic external interface.

Under aqueous mobile phase conditions, desorption of many surfactants from a C_{18} phase is very slow, and the detergent layer is maintained long after the additive has been removed from the mobile phase. Many water-soluble proteins elute from such a column in the exclusion volumes when, for example, the non-ionic surfactant Tween®, a fatty acid ester of bispolyethyleneoxide-modified sorbitol, is used [8].

On the other hand, albumins are known to associate with Tween or similar detergents even below the critical micelle concentration (CMC). Interaction occurs through hydrophobic patches located at the surface of these molecules [9]. A schematic illustration of such association is shown in Fig. 1A. A detergent–C_{18} layer can thus be drastically depleted of detergent molecules by injections of large serum samples. In contrast, a bonded detergent-type ligand will sustain its hydrophilic interface character despite continuing serum injections.

Another important difference between an adsorbed and a bonded detergent can be seen in Fig. 1B. Adsorption of a large protein molecule to the stationary phase can displace the detergent from both surfaces, to yield a direct interaction between the alkyl ligands and the hydrophobic patch on the protein. In order to expose the hydrophobic character of a bonded-detergent phase, the protein molecule must be capable of squeezing the hydrophilic moieties out of the way, as shown in Fig. 1D. In contrast, small analytes can undergo mobile–stationary phase distribution without affecting the physical structure of the bonded-detergent stationary phase.

One of the examples of micellar bonded phases described previously [5] is N,N-bis(2′-methoxyethyl)-11-silylundecamide, $\equiv Si(CH_2)_{10}CON(CH_2CH_2OCH_3)_2$. It was bonded to a 100-Å pore-size silica gel to yield a ligand surface density of 3.14 $\mu mol/m^2$. The micellar bonded layer consists of the $-CON(CH_2CH_2OCH_3)_2$ hydrophilic groups in the mobile–stationary phase interface and a hydrophobic decamethylene alkyl chain underlayer.

Direct analyses of drug-spiked serum were performed on a column containing this packing under isocratic mobile phase conditions. Drug retention time varied as the number of injections increased, showing a slow drift to lower values as a result of some protein contamination of the stationary phase. It was obvious from Chang's report [8] that synthesizing a shielded micellar phase with bulkier hydrophilic heads should eliminate the limitation shown in this case. For example, increasing the size of the polar group by increasing the ethylene oxide chains, $CON[(CH_2CH_2O)_mCH_3]_2$, from $m = 1$ to $m \geqslant 2$ could produce an excellent SHP. The reproducibility obtained

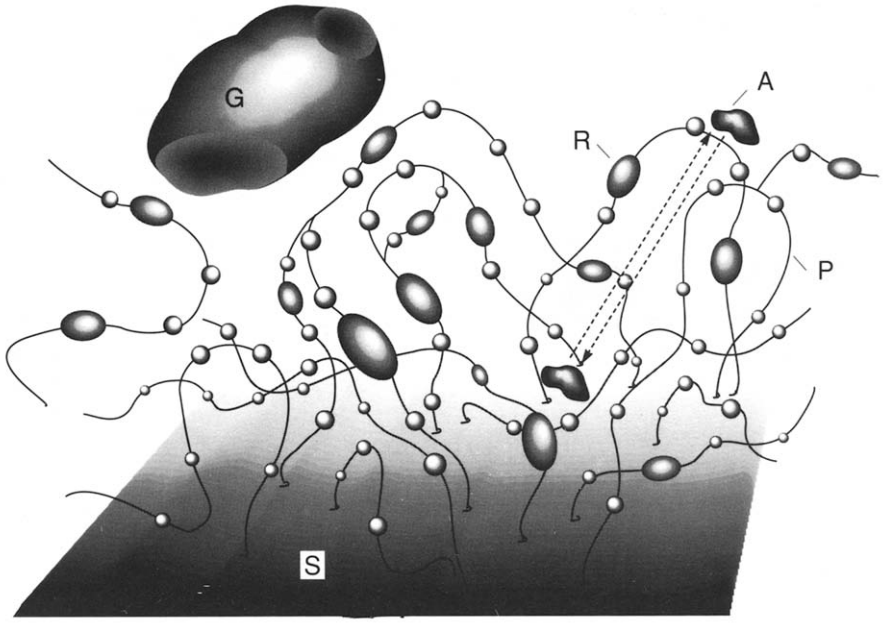

Fig. 2. Schematic representation of an embedded-network shielded hydrophobic phase. A = Small retained analyte; G = unretained protein; P = polyethylene glycol hydrophilic network; R = hydrophobic phenyl groups; S = support matrix.

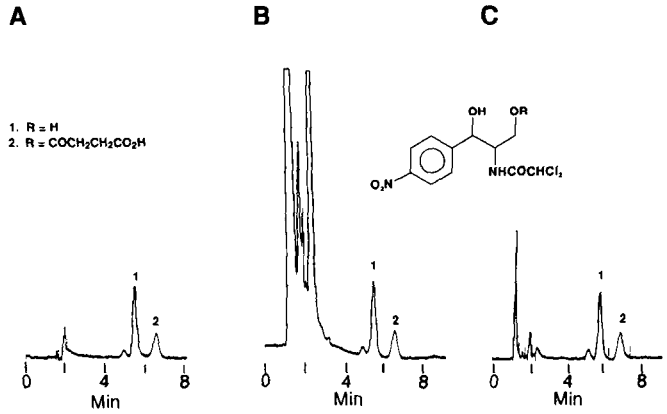

Fig. 3. Separation of 10 μg/ml chloramphenicol (1) and 10 μg/ml of its succinic acid ester derivative (2) on a Hisep 25 cm × 2.1 mm I.D. column. (A) Water solution; (B) serum-spiked sample; (C) cerebral spinal fluid-spiked sample. Mobile phase, 180 m*M* aqueous ammonium acetate–acetonitrile (80:20); flow-rate, 0.3 ml/min; UV detection, 278 nm, 0.008 a.u.f.s.; injection volume, 0.5 μl; temperature, ambient.

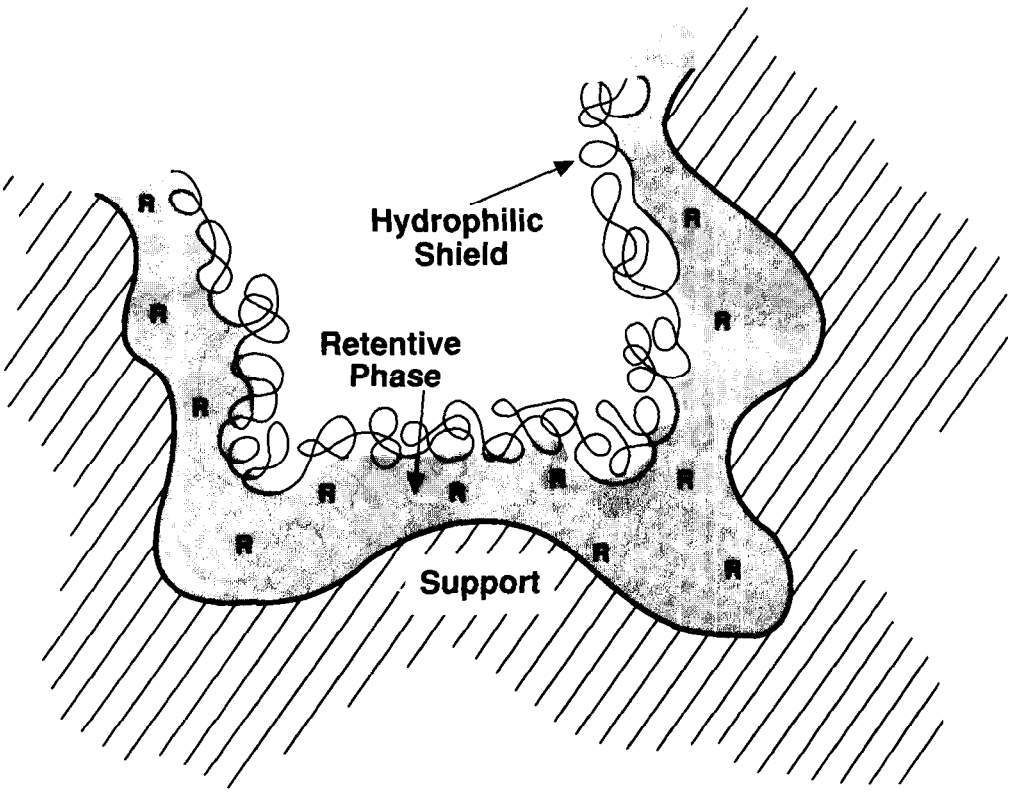

Fig. 4. Schematic representation of shielded hydrophilic phases containing R groups having additional selectivity.

with the other type of SHP, the embedded network, limited the study of micellar phases to the requirements of the patent [5].

Fig. 2 illustrates the concept of an embedded-network SHP, in which a polymeric network is bonded to a support. It can be seen how the hydrophobic moieties (the larger spheres) are entangled in the hydrophilic network. In comparison to a bonded micelle-type phase, the embedded network is much more resistant to the deformation needed to expose hydrophobic protein–stationary phase interactions. Small molecules, on the other hand, can freely interact with the hydrophobic parts of the polymer without the need to affect the shape of the phase. Such a phase will express its hydrophilic character towards the large protein molecules and its hydrophobicity towards small analytes. Supelco's Hisep column is an example of this kind of shielded phase, in which the hydrophilic parts are polyethylene oxide chains and the hydrophobic-enclaved moieties are disubstituted aromatic rings.

The wide application range of the Hisep column for direct serum monitoring has already been discussed in the literature [10–12]. We shall describe here one additional new example, only to illustrate the versatility of this column. Chloramphenicol succinate, a pro-drug, hydrolyses *in vivo* to chloramphenicol. This broad-spectrum antibiotic is frequently used to treat infants and children for ampicillin-resistant *Hemophilus influenza* infections. Therapeutic levels of chloramphenicol in blood are 10–20 μg/ml. Sustained levels above 25 μg/ml have produced anemia, while levels above 50 μg/ml have been associated with cardiovascular collapse [13]. Accurate monitoring is critical because variation in chloramphenicol metabolism may be significant. This drug is also known to cross the blood–brain barrier. We studied the direct analysis of chloramphenicol-spiked serum and cerebral spinal fluid to show that the Hisep column could be used with alternative biological fluids. Fig. 3 shows the analyses of the pro-drug (R = $COCH_2CH_2CO_2H$) and chloramphenicol (R = H) at therapeutic levels, with just 0.5-μl sample injections.

TABLE 1

CAPACITY FACTORS ON THE HISEP SHIELDED PHASE AND RELATED BASIC-MODIFIED PHASES

Chromatographic conditions: mobile phase, 180 m*M* aqueous ammonium acetate (pH 7.0)–acetonitrile (95:5); flow-rate, 2.0 ml/min; injection volume, 10 μl; concentration and detection, chloramphenicol, 10 μg/ml, 278 nm, 0.016 a.u.f.s.; salicylic acid, 25 μg/ml, 280 nm, 0.008 a.u.f.s.; benzoic acid, 10 μg/ml, 254 nm, 0.016 or 0.032 a.u.f.s.; serum, neat, 254 nm, 0.016 a.u.f.s.; temperature, ambient.

Compound[a]	Capacity factor			
	Hisep	R = $N(CH_3)_2$	R = $N^+(CH_3)_3$	R = $N^+(Bu)_3$
Chloramphenicol	2.54	4.04	5.61	5.20
Salicylic acid	2.05	5.20	10.20	23.19
Benzoic acid	1.16	2.19	3.66	4.44
Total serum protein area (in million counts)	11.5	10.8	11.6	12.0

[a] Chloramphenicol, salicylic acid, and benzoic acid values were determined in a serum matrix. The void volume was determined with uracil dissolved in mobile phase.

TABLE 2

CAPACITY FACTORS ON THE HISEP SHIELDED PHASE AND TWO RELATED ACID-MODIFIED PHASES

Chromatographic conditions: mobile phase pH 7.0, 180 m*M* aqueous ammonium acetate (pH 7.0)–acetonitrile (95:5); mobile phase pH 4.0, 90 m*M* aqueous ammonium acetate (pH 4.0)–acetonitrile (95:5); flow-rate, 2.0 ml/min; injection volume, 10 μl; concentration and detection, chloramphenicol, 10 μg/ml, 278 nm, 0.016 a.u.f.s., trimethoprim, 25 μg/ml, 254 nm, 0.016 a.u.f.s.; propranolol, 25 μg/ml, 254 nm, 0.016 a.u.f.s.; serum, neat, 254 nm, 0.016 a.u.f.s.; temperature, ambient.

Compound[a]	Capacity factor					
	Hisep		R = CO_2H		R = SO_3H	
	pH 7.0	pH 4.0	pH 7.0	pH 4.0	pH 7.0	pH 4.0
Chloramphenicol	2.54	2.25	3.79	3.16	4.06	3.53
Trimethoprim	2.56	0.30	4.83	0.25	4.02	2.21
Propranolol	2.94	0.85	8.40	0.76	9.80	6.00
Total serum protein area (in million counts)	11.5	11.1	11.7	12.0	11.4	10.0

[a] Chloramphenicol, trimethoprim, and propranolol values were determined in a serum matrix.

Shielded phases, of course, should not be restricted to shielded hydrophobic phases. Other interacting groups could be added to the stationary phase, as shown in Fig. 4. The shielded R group could be, for example, a basic group, an acidic group, or any other group having additional selectivity. Many hydrophilic analytes contain acidic or basic groups. Having the appropriate ion-pairing shielded R group, the retention time of such analytes could be manipulated for better resolution from the protein front peak and from other analytes.

We synthesized Hisep-type basic phases in which the R groups are $-N(CH_3)_2$, $-N^+(CH_3)_3$, or $-N^+(Bu)_3$, as well as acidic phases in which the group is $-CO_2H$ or $-SO_3H$. Integration values of the frontal peaks are shown in Tables 1 and 2. These values emphasize that all the modified phases elute the total serum protein content in the void volume.

Table 1 shows the capacity factor (k') of two organic acids and a neutral polar reference compound on the Hisep column and on basic-modified columns. The capacity factor of the stronger acid, salicylic acid, increases ten-fold from the Hisep to the $-N^+(Bu)_3$-modified column. The weaker acid, benzoic acid, shows a lesser effect, but even so, with a capacity factor of 4.4 benzoic acid is well resolved from the protein front. Note that the retention time of chloramphenicol, a neutral polar compound, is also affected by the ionic modifications.

Table 2 shows the effect of the acidic modifications on the capacity factors of two organic bases and chloramphenicol. At pH = 7.0, the carboxylic-modified and sulfonic-modified shielded phases show similar k' values for the two bases and the neutral compound. At this pH, both groups are completely ionized and show similar ion-pairing tendency toward the bases. The capacity factor for trimethoprim, the weaker base, increases from 2.56 to 4.83, while the capacity factor for propranolol increases from 2.94 to 9.80. At pH 4.0, only the sulfonic acid is ionized and shows ion-pairing retention of the protonated bases. Chloramphenicol, the neutral com-

pound, is hardly affected by the pH change. The protonated bases elute almost at the void volume for the Hisep column and its carboxylic modification but show appreciable retention on the sulfonic-modified column.

4. CONCLUSION

Introduction of shielded phases to HPLC has added an important analytical tool for the resolution of small analytes in biological media. Direct serum injection analysis, in which the proteins elute in the void volume, while the analytes of interest are retained and separated, is the area in which such columns will make the greatest contribution. Applications in other areas of interest such as food and beverage analyses, fermentation processes, etc. are certain to follow. Addition of ion-pairing groups adds selectivity and retention to the shielded phase to extend the analyses to samples where hydrophobic retention, alone, is inadequate.

REFERENCES

1 W. Roth, K. Beschke, R. Jauch, A. Zimmer and F. W. Koss, *J. Chromatogr.*, 222 (1981) 13.
2 R. A. Hux, H. Y. Mohammed and F. F. Cantwell, *Anal. Chem.*, 54 (1982) 113.
3 H. Yoshida, I. Morita, T. Masujima and H. Imai, *Chem. Pharm. Bull.*, 30 (1982) 3327.
4 H. I. Hagestam and T. C. Pinkerton, *Anal. Chem.*, 57 (1985) 1757.
5 B. Feibush and D. J. Gisch, patent pending
6 F. J. DeLuccia, M. Arunyanart and L. J. Cline Love, *Anal. Chem.*, 57 (1985) 1564.
7 J. G. Dorsey, *Adv. Chromatogr.*, 27 (1987) 167.
8 J. P. Chang, presented at the *8th International Symposium on Column Liquid Chromatography, New York, May 20–25, 1984*, paper 2A-B3.
9 A. Helenius, *Biochim. Biophys. Acta*, 415 (1979) 29.
10 D. J. Gisch, B. T. Hunter and B. Feibush, *J. Chromatogr.*, 433 (1988) 264.
11 C. T. Santasania, *J. Liq. Chromatogr.*, 13 (1990) 2605.
12 S. H. Y. Wong, L. A. Butts and A. C. Larsen, *J. Liq. Chromatogr.*, 11 (1988) 2039.
13 G. Ireland (Editor), *Drug Monitoring Data Pocket Guide*, American Society for Clinical Chemistry, Washington, DC, 1981, p. 52.