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Note

Shielded hydrophobic phase: a new concept for direct injection analysis of biological fluids by high-performance liquid chromatography

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Unless special precautions are taken, injection of untreated biological samples onto a reversed-phase high-performance liquid chromatography (HPLC) column causes column clogging. This results in increasing pressure drop, peak broadening, variation of retention times, etc. To avoid clogging and rapid deterioration of the analytical column, a sample preparation step is needed to remove the proteins (e.g., precipitation followed by extraction of the compound(s) of interest into a protein-free sample). Although such multi-step preparation techniques are time-consuming, they still attract much of the practical attention of analysts.

To shorten and simplify sample handling, considerable effort has been dedicated to the development of direct sample injection HPLC analyses for drugs, drug metabolites, etc. in biological matrices. Methods for direct injection have been summarized in a recent review [1]. In the earliest applications, the matrix of interest was simply injected and the column was thoroughly washed after every few injections to remove precipitated proteins [2,3]. More recently, micellar mobile phases that solubilize the proteins have been used with direct injection analysis on standard columns [4]. Precolumn or two-column techniques have been applied, using a variety of column switching methods that allow only the drug(s) to elute onto the analytical column [5–9]. In addition to reversed-phase packings (e.g., C_8 or C_{18} bonded silica supports), special column packings, such as a polymer of styrene–divinylbenzene [10] or butyl-modified methacrylate [11], have been used in the precolumn.

A completely different approach was undertaken by two other groups. These investigators designed packings to elute proteins in the excluded volume, while

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small analytes were retained and separated. Yoshida et al. [12] modified a reversed-phase packing by precipitating denatured plasma proteins on a C_{18} phase bonded to a silica support with a small pore diameter. Hagestam and Pinkerton [13] prepared a so-called internal surface reversed-phase (ISRP) packing by using a hydrophobic oligopeptide to chemically modify a hydrophilic diol phase bonded on a ≥ 80 Å pore silica gel support. An enzymatic cleavage with a protease is carried out, producing a packing with hydrophilic ligands on the external surface (exposed to the protease) and internal pores having the uncleaved hydrophobic oligopeptide.

In this paper we will describe a novel type of phase which also excludes proteins, while interacting with small molecules. We have termed the new material a shielded hydrophobic phase (SHP). Several examples will illustrate the application of SHP-type phases for direct injection HPLC analysis of drugs in biological fluids.

EXPERIMENTAL

Materials

A 150 mm × 4.6 mm Hisep™ SHP column (Supelco, Bellefonte, PA, U.S.A.) was used for the direct injection chromatography of serum and plasma samples spiked with several drugs. Reagents, standards and other chemicals were obtained from the following sources: ammonium acetate, carbamazepine and trimethoprim from Sigma (St. Louis, MO, U.S.A.), phenobarbital from Supelco, triply filtered fetal bovine serum from HyClone Labs. (Logan, UT, U.S.A.) and HPLC-grade acetonitrile from Anachemia (Champlain, NY, U.S.A.). All other solvents were of HPLC grade. Human serum and plasma were obtained fresh in-house.

Chromatography

The analyses were performed on a Spectra Physics SP-8800 liquid chromatograph equipped with an SP-8780 autosampler, an SP-4290 integrator interfaced with LABNET/RS-232 (Spectra Physics, San Jose, CA, U.S.A.) and a Kratos 757 Spectraflow variable-wavelength UV detector (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.).

RESULTS AND DISCUSSION

Chromatographic separations on an SHP represent a new design for direct injection analysis. The SHP material consists of a polymeric bonded phase containing hydrophobic regions (R) enclaved by a hydrophilic network (P), as shown in Fig. 1. The hydrophilic network forms a water-solvated interface through which small analytes, such as drugs (A), penetrate and interact with the hydrophobic groups, while larger water-solvated molecules, such as proteins (G), are prevented from such interactions by hydrophilic shielding. Under appropriate chromatographic conditions, the bulk of the protein matrix will elute as an unretained — or nearly unretained — band without affecting the retention of the smaller

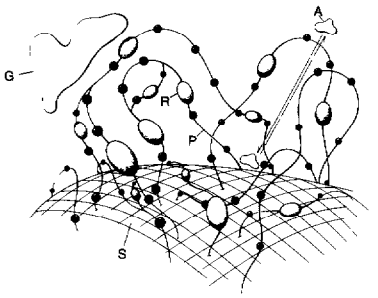

Fig. 1. Schematic representation of a shielded hydrophobic phase. S = silica gel matrix; R = hydrophobic pocket; P = hydrophilic network; G = large unretained protein; A = small retained analyte.

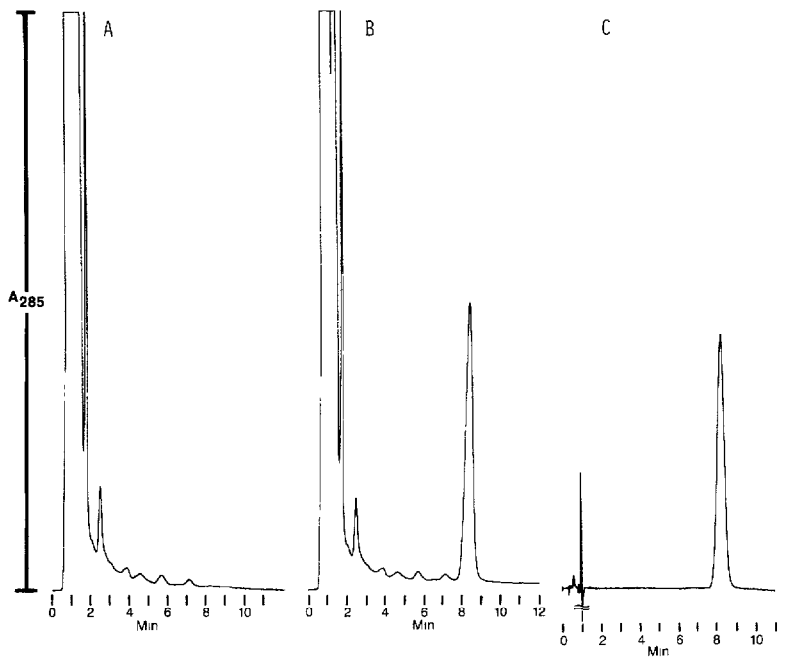

Fig. 2. Chromatograms of blank human serum (A), human serum spiked with 5 μg/ml carbamazepine (B) and a 5 μg/ml carbamazepine standard (C). Chromatographic conditions: column: Hisep (150 mm × 4.6 mm); mobile phase: acetonitrile–180 m*M* ammonium acetate (12:88); flow-rate: 2.0 ml/min; temperature: ambient; detection: UV at 285 nm, 0.032 a.u.f.s.; injection volume: 50 μl.

analytes. In the Hisep packing, the hydrophilic network consists of bonded polyethylene oxide with embedded hydrophobic phenyl groups. Further chemical details of the SHP surface will be published elsewhere [14].

Hisep column usefulness for analyses of drugs in human serum and plasma was demonstrated with samples spiked with therapeutic levels of carbamazepine and phenobarbital [15]. Fig 2 shows chromatograms of blank serum, carbamazepine-spiked serum (5 μg/ml) and a carbamazepine standard (5 μg/ml). Fig. 3 shows chromatograms of phenobarbital-spiked plasma (12.5 μg/ml) and a phenobar-

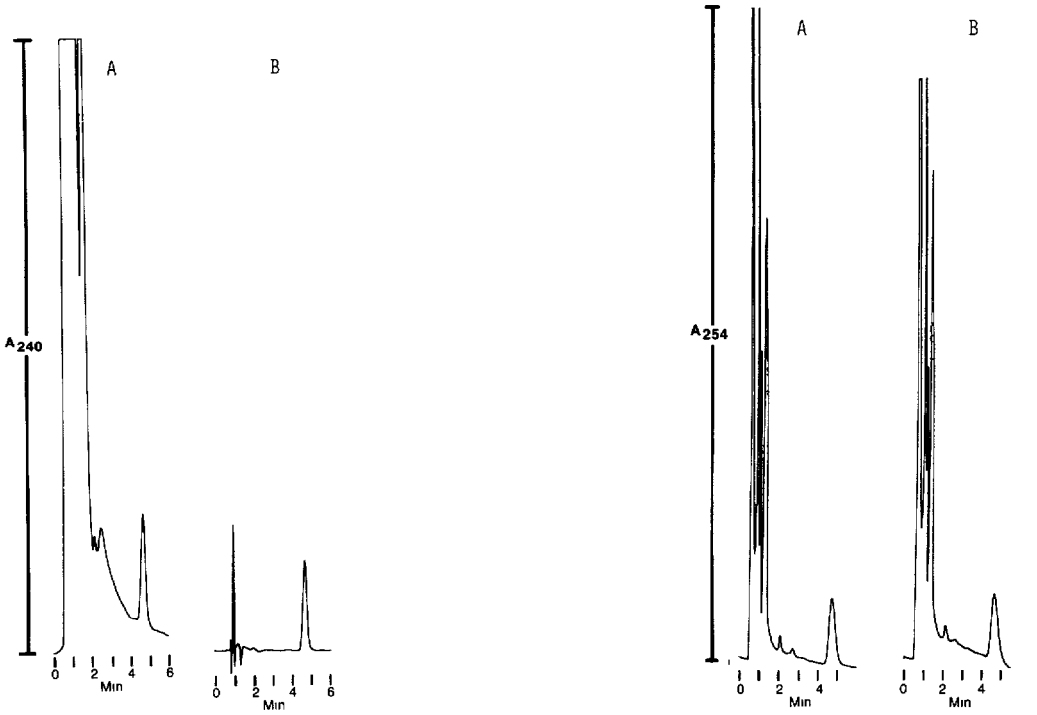

Fig. 3. Chromatograms of human plasma spiked with 12.5 μg/ml phenobarbital (A) and a 12.5 μg/ml phenobarbital standard (B). Chromatographic conditions: column: Hisep (150 mm×4.6 mm); mobile phase: acetonitrile–180 m*M* ammonium acetate (5:95); flow-rate: 2.0 ml/min; temperature: ambient; detection: UV at 240 nm, 0.064 a.u.f.s.; injection volume: 50 μl.

Fig. 4. Chromatograms of trimethoprim-spiked bovine serum (25 μg/ml) after 3 injections (A) and after 1008 injections (B). Chromatographic conditions as for Fig. 3, except: detection: UV at 254 nm, 0.008 a.u.f.s.; injection volume: 10 μl.

bital standard (12.5 μg/ml). The bulk of the proteins was eluted unretained from the serum and plasma matrices. Retention of the low-molecular-mass components of such matrices can be adjusted by modifying mobile phase conditions. A wide range of mobile phase changes can be made, as long as protein precipitation is avoided. Typically, the mobile phase should contain no more than 15–20% organics and 0.5 *M* buffered solutions. Because the SHP is based on a silica support, the pH range of the mobile phase is limited to approximately 2.5–7.

Chromatographic stability and reproducibility of the SHP, after more than 1000 injections of trimethoprim-spiked bovine serum, is illustrated by Fig. 4. Retention of the drug did not change significantly, nor was there any significant change in column back-pressure. This high degree of physical stability and chromatographic reproducibility, when used with large numbers of serum injections, shows that the Hisep column will be useful for routine or experimental work involving separating proteins from smaller analytes.

By embedding hydrophobic regions in a polymeric hydrophilic network, a shielded hydrophobic support has been synthesized. This material retains drugs

while excluding the proteinaceous matrix of serum and plasma samples, making it most useful for direct injection analysis of complex biological samples.

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