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Direct serum injection in ion chromatography on packing materials with a semi-permeable surface

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ABSTRACT

Reversed-phase packing materials with restricted access of proteins to the hydrophobic sites were tested for their applicability in the ion-interaction chromatography of anions. Especially C_8-modified silica, which had been coated with a hydrophilic polymer acting as a semi-permeable barrier, could be used successfully for the separation of several anions in a proteinaceous matrix without removing the proteins prior to injection. In combination with UV or conductivity detection, this technique allows the determination of some physiologically important anions in serum samples.

INTRODUCTION

The determination of anions or cations in biological fluids by ion chromatography (IC) generally requires pretreatment procedures to remove proteinaceous sample components. Direct injection of biological samples on to conventional packing materials may cause denaturation and accumulation of proteins, resulting in severe degradation of the system performance. Various sample preparation steps are possible to remove sample proteins prior to IC, such as protein precipitation or ultrafiltration. In many instances, these sample pretreatment procedures are disadvantageous because of low recoveries, difficulties with reproducibility or consumption of time.

More recently, direct injection of serum samples on to specially prepared columns has been described as an alternative to sample pretreatment. This approach has gained some popularity for the detemination of drugs in biological fluids by reversed-phase high-performance liquid chromatography (HPLC). Typical packings designed for this purpose include internal surface reversed-phase materials [1–3], semi-permeable surface materials [4], dual-zone materials [5], shielded hydrophobic phase materials [6,7] and mixed functional phase materials [8–10]. Common to all these packing materials is the restricted access of proteins to the hydrophobic sites of the particle. Ideally, proteins will elute in the void volume. Although these techniques have successfully proved their advantages for typical reversed-phase HPLC separations, no applications have been reported in IC.

This paper reports the determination of anions in proteinaceous samples using ion-interaction chromatography with semi-permeable surface (SPS) packings. C_8-modified silica was used, which had been coated with a hydrophilic polymer. This hydrophilic layer acts as a semi-permeable barrier which prevents proteins from adsorbing irreversibly on the packing. A commercially available SPS phase and laboratory-made phases coated with polyethylene glycol were tested and compared with respect to the separation efficiency for anions in serum samples. Further, an internal surface reversed-phase (ISRP) material was investigated for its applicability in ion-interaction chromatography.

EXPERIMENTAL

Instrumentation

The chromatographic instrumentation consisted of a Perkin-Elmer (Norwalk, CT, USA) Series 3B

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HPLC pump, a Rheodyne (Cotati, CA, USA) Model 7125 injection valve with a 10- or 20-μl loop, a Perkin-Elmer LC 75 UV detector and a Waters (Milford, MA, USA) M430 conductivity detector. The following separation columns were used: a 5-μm SPS RP-8 column (250 × 4.6 I.D.), obtained from Regis (Morton Grove, IL, USA); a column (250 × 4 mm I.D.) packed with C_8-modified silica coated with polyethylene glycol according to the procedure given below; and a Pinkerton GFF-S5-80 ISRP column (100 × 4.6 mm I.D.), obtained from Regis.

The mobile phase was prepared by adjusting an aqueous 5, 10 or 15 m*M* octylamine solution to pH 6.5 with phosphoric acid.

Serum samples were passed through a Schleicher & Schuell (Keene, NH, USA) 0.45-μm Spartan-3 filter prior to injection.

The characterization of coated particles by infrared spectrometry was carried out with a Bio-Rad Labs. (Cambridge, MA, USA) FTS-45 Fourier transform infrared spectrometer.

Coating of C_8-modified silica with polyethylene glycol

A 10-g amount of polyethylene glycol (PEG) with a molecular weight of 1000 (Carbowax 1000) obtained from Supelco (Bellefonte, PA, USA), was heated to about 100°C and mixed with 3 g of LiChrospher RP-8 silica, 5-μm particle size, 100-Å pore diameter (obtained from Merck, Darmstadt, Germany). The temperature was raised to 280°C and the suspension stirred for 3 h. The excess of PEG was dissolved by addition of 20 ml of methylene chloride and the particles were separated by centrifugation. This step was repeated three times. Finally, the particles were washed with isopropanol.

RESULTS AND DISCUSSION

The first investigations dealt with the behaviour of a commercially available SPS C_8 column in ion-interaction chromatography. The analysis of proteinaceous samples requires the use of a mobile phase with little or no organic modifier and a pH around neutral to avoid protein precipitation. Skelly [11] suggested a mobile phase containing an octylammonium salt for use in ion-interaction chromatography. In our experiments, a 5 m*M* octylamine solution adjusted to pH 6.5 with phosphoric acid was found to give satisfactory separations of inorganic anions on LiChrospher C_8 silica. Therefore, this mobile phase was chosen for separations on the SPS column. The separation pattern of chloride, bromide, nitrite, nitrate, iodide and thiocyanate on the SPS column was similar to that on the uncoated C_8 column and the capacity factors were 1.8, 2.5, 2.8, 3.6, 7.1 and 19.5, respectively. This result confirmed that the semi-permeable surface does not interfere with the separation mechanism of ion-interaction chromatography.

In the next step, the separation of an anion standard in a 5% albumin matrix was investigated. Fig. 1 shows a typical chromatogram with UV detection at 200 nm. The peak eluting in the void volume corresponds to albumin. Fractions of the eluate were collected and the protein content was determined by the method of Hartree [12]. These experiments confirmed that the injected protein is eluted quantitatively and no protein is adsorbed irreversibly on the stationary phase. A graph of peak area *versus* concentration showed linearity of the reponse of nitrite, nitrate, bromide, iodide and thiocyanate anions in the albumin matrix in the range 1–100 ppm.

Finally, the direct injection of human serum samples was investigated. The broad peak of the serum proteins and also peaks of serum components eluting near the void volume cause problems for the separation of early-eluting anions such as nitrite, bromide and nitrate if UV detection is used. The separation can be improved to some extent if the concentration of octylamine in the mobile phase is increased to 10 m*M*, but then the retention of anions such as thiocyanate becomes unacceptably long.

Considering that the concentrations of many anions in serum are in the low ppm or ppb range, UV detection obviously is not the optimum detection mode. Nevertheless, one should remember that the aim of this work was the investigation of the general behaviour of proteinaceous samples on SPS C_8 silica under ion-interaction chromatographic conditions and not the optimization of sensitivity. Selective and sensitive detection modes are known for several anions of physiological or medical importance, such as postcolumn reaction detection or

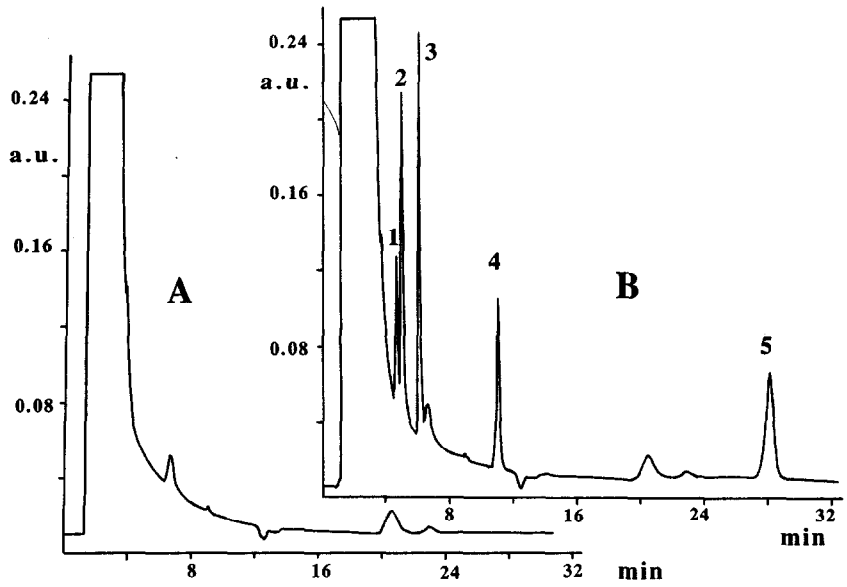

Fig. 1. Chromatogram of a standard mixture of anions (10 ppm each) in a proteinaceous matrix using a Regis semi-permeable surface C_8 packing. (A) 5% albumin solution; (B) anion standard in a 5% albumin matrix. Peaks: 1 = bromide; 2 = nitrite; 3 = nitrate; 4 = iodide; 5 = thiocyanate. Injection volume, 20 μl. UV detection at 200 nm. Mobile phase, 5 m*M* octylamine adjusted to pH 6.5 with phosphoric acid. Flow-rate, 1.0 ml/min.

electrochemical detection, which can easily be combined with direct injection of proteinaceous samples on to SPS phases. In some instances, however, direct UV detection at 210 nm can be used successfully for certain anions, as can be seen from Fig. 2, which shows the determination of iodide and thio-

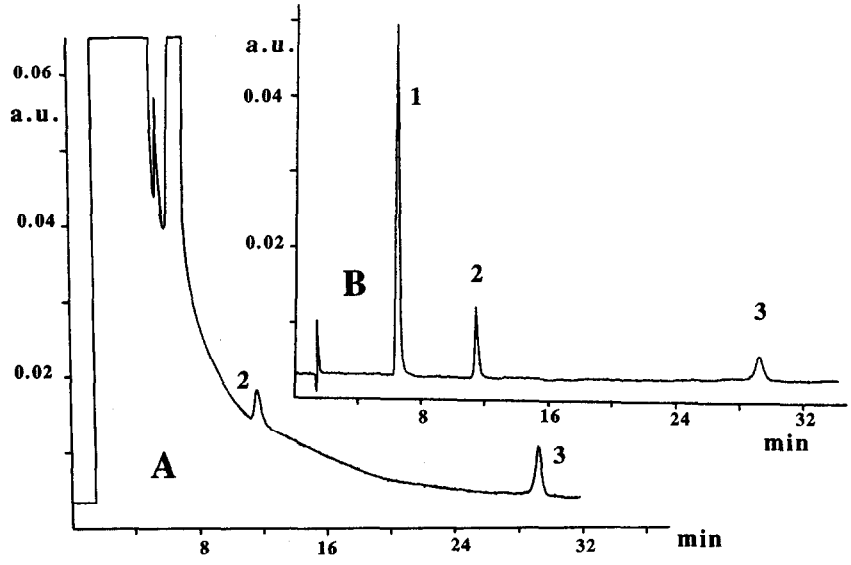

Fig. 2. Determination of anions in human serum. (A) Serum sample; (B) anion standard mixture (5 ppm each). Peaks: (1) nitrate; (2) iodide; (3) thiocyanate. UV detection at 210 nm. Other conditions as in Fig. 1.

cyanate in a serum sample from a subject after treatment with iodide-containing drugs; the thiocyanate concentration is also relatively high, indicating that the subject was a heavy smoker; the peak at the retention time of nitrate is caused by interfering serum components.

In a series of experiments, the coating of RP-8 particles with PEG was investigated in order to produce stationary phases with properties similar to those of commercially available SPS phases. The idea was to bind PEG via its alcohol group to free silanol groups of the silica particle (after partial removal of C_8 groups already bound to the silica). The mechanism of the coating procedure described under Experimental is not fully clear. Nevertheless, after extensive washing of the coated phase with different organic solvents there were still PEG groups present at the surface of the particle. This could be verified by diffuse reflectance IR spectrometry. The spectrum obtained after subtraction of a spectrum of RP-8 silica from a spectrum of PEG-coated RP-8 silica showed characteristic bands at wavenumbers of 1457, 1349, 1326, 1299 and 951 cm^{-1}, which were identical with the bands of a PEG standard. Therefore, it is unlikely that the polymer is adsorbed only physically. Further information on the extent and nature of PEG binding could not be deduced from the IR measurements carried out so far.

The separation properties of the PEG-coated RP-8 silica were similar to those of the commercially available SPS column, the only difference being the order of elution of sulphate and iodide. Sulphate eluted before iodide on the SPS column but after iodide on the PEG-coated column. During the first injections of proteinaceous samples on to a new PEG-coated column, the elution of the protein was incomplete. Only after the fifth injection was the injected protein eluted quantitatively. Obviously, some active sites for irreversible protein adsorption are still present. Nevertheless, the amount of protein adsorbed seems to be small enough to avoid column deterioration.

Physiological amounts of sulphate can be detected in serum by using conductivity detection. Such measurements are essential for investigations of factors controlling the rate of sulphoconjugation in the organism. Fig. 3 shows a typical chromatogram of a human serum sample containing *ca.* 30 ppm of sulphate. This concentration was found after calibration with aqueous sulphate standards. Comparisons with other established procedures are still to be done in order to assess the accuracy of the method. The main advantage of this HPLC procedure is that no sample pretreatment is necessary except filtration through a 0.45-μm filter. Therefore, the method is simpler than other HPLC procedures reported recently [13].

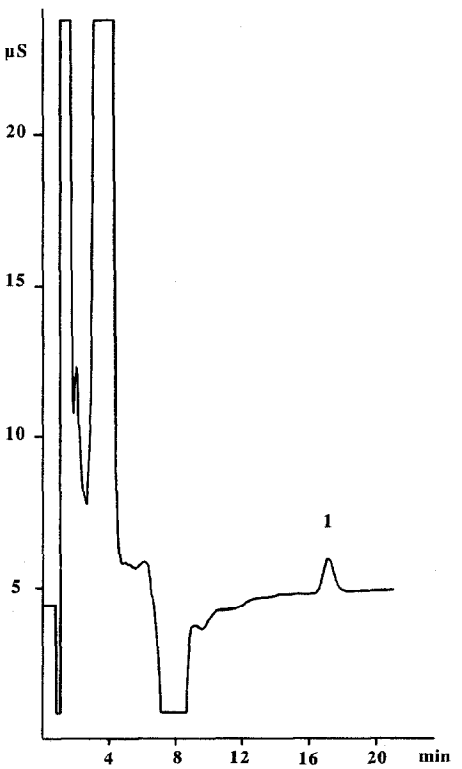

Fig. 3. Determination of sulphate in human serum using a C_8 packing coated with PEG and conductivity detection. Peak: (1) sulphate, *ca.* 30 ppm. Flow-rate, 1.5 ml/min. Other conditions as in Fig. 1.

The long-term behaviour of PEG-coated C_8 silica was also investigated and compared with an uncoated C_8 silica. Seventy replicate 10-μl injections of a serum sample were carried out. The efficiency of the coated material (number of theoretical plates) remained roughly the same whereas the efficiency of the uncoated material changed in an unpredictable way. The performance tended to decrease dramatically after a few injections, but could

be restored to some extent by extensive washing of the column with water. The uncoated material cannot be recommended for routine analytical work.

Preliminary investigations were carried out on coating a silica-based anion-exchange material with PEG. A 40-μm Bondesil SAX material (obtained from Analytichem International, Harbor City, CA, USA) was treated in the same way as described above for RP-8 material. The separation of anions was possible using sodium sulphate as eluent, but owing to the particle size it was not possible to fill a high-performance column. Further experiments will be done with a material of smaller particle size and lower capacity, as the capacity of the SAX material is too high to use eluents with low concentration, which are compatible with conductivity detection.

Some investigations were carried out with a commercially available ISRP column. This packing material has a hydrophilic diol phase on the external surface of the silica particle and a hydrophobic phase (glycerylpropylglycyl-L-phenylalanyl-L-phenylalanine) on the internal surface (pores) [1,2]. Serum proteins are excluded from the small pores, whereas small molecules penetrate and may be retained at the hydrophobic sites. Unfortunately, the use of the mobile phase described above containing 5 m*M* octylammonium phosphate as the ion-interaction reagent resulted in almost no retention of anions such as chloride, nitrate, iodide and thiocyanate. A threefold increase in the concentration of the ion-interaction reagent did not improve the separation. Obviously, the hydrophobicity of the internal polypeptide phase is considerably lower than that of typical reversed-phase materials such as C_8- or C_{18}-modified silica. Therefore, its applicability in ion-interaction chromatography seems to be limited. Nevertheless, the concept of ISRP packings is worth developing further in order to obtain materials with new internal phases of higher hydrophobicity.

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