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Evaluation of restricted access media for high-performance liquid chromatographic analysis of sulfonamide antibiotic residues in bovine serum

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ABSTRACT

Three commercially-available high-performance liquid chromatographic columns packed with restricted access media were evaluated for suitability in multi-residue direct injection analysis at the ng/ml level. The internal surface reversed-phase and shielded hydrophobic phase columns were not sufficiently retentive to separate all analytes from the tail of the matrix peak. Coelution of some of the analytes was also observed with these columns. The semi-permeable surface column was significantly more retentive and selective, providing good separation of analyte and matrix peaks. With this column, an analytical protocol requiring no organic solvents was developed for the assay of six sulfonamides at a detection limit of 25 ng/ml.

INTRODUCTION

The injection of biological fluids into conventional high-performance liquid chromatographic (HPLC) systems results in rapid degradation of performance, typically evidenced by increased backpressure and reduced efficiency. These effects are usually attributed to precipitation and irreversible or slowly-reversible adsorption of proteins on the stationary phase. Sample clean-up procedures which remove protein prior to injection are therefore needed for conventional HPLC analysis of small molecules in biological samples. These clean-up steps are generally slow, difficult to automate, and employ large quantities of toxic organic solvents. As a result, much of the time and cost of current methods for drug residue analysis are associated with sample preparation, and the full capabilities of modern, automated HPLC systems are seldom realized.

One approach to removing the sample preparation bottleneck is to automate the clean-up operation [1], preferably utilizing methods which reduce [2] or eliminate [3,4] organic solvent consumption. Another approach is the use of stationary phases which permit the direct injection of proteinaceous samples. Several dozen phases have been developed specifically for HPLC of protein-containing samples [5,6]. These phases are generally referred to as restricted access media (RAM), and a number of such columns are now commercially available. Direct injection HPLC on RAM columns has been applied to the analysis of a large number of pharmaceuticals [5] at therapeutic levels (10–100 μg/ml) in human body fluids, but to our knowledge it has not been used for quantitative determinations at concentrations (1–100 ng/ml) relevant to residue analysis (a detection limit of 5 ng/ml has been claimed in one report [7], but quantitative results were demonstrated only at μg/ml levels). The ability to determine traces of drug and pesticide residues in foods without sample preparation would greatly enhance the speed and cost-effectiveness of food safety monitoring programs. Elimination of sample treatment steps would also reduce the time required for development and optimization of new analytical methods. We therefore initiated a study of several commercially available columns in order to assess

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the use of RAM for detection of ng/ml levels of drug residues, using the sulfonamide antibiotics in bovine serum as a test system. We briefly discuss here the columns used in this study, and refer the reader to recent review articles [5,6] for detailed information on history, nomenclature, synthesis, mode of operation, and applications of restricted access media. Three different types or classes of packings were studied: the internal surface reversed-phase (ISRP) type [8], the shielded hydrophobic phase (SHP) type [9], and the semi-permeable surface (SPS) type [10]. ISRP packings consist of small-pore silica particles having a bonded phase with hydrophilic properties at the outer surface and hydrophobic properties within the pores. The packings are generated in three steps: bonding of a hydrophilic group to the silica; capping the hydrophilic group with a hydrophobic species (polypeptide in the original work); and enzymatic cleavage of the hydrophobic end group at the particle surface to expose the hydrophilic sub-phase. The commercial ISRP column used here (GFF-II) employs a glycidoxypropyl hydrophilic sub-phase and a glycine–phenyalanine–phenylalanine hydrophobic cap. The SHP uses hydrophobic moieties (disubstituted aromatic rings) embedded by a proprietary process in a hydrophilic polyethylene oxide polymer network bonded to a silica support. The SPS phase utilizes hydrophilic polyoxyethylene "tails" bound to alkylated silica particles. The column used in this work had a C_{18} alkyl group, but packings with other hydrophobic moieties are available. Size exclusion plays the dominant role in restricting retention of protein in the ISRP, while chemical effects (*e.g.* hydrophobic interactions) are claimed to predominate with the SHP and SPS phases. However, it has been pointed out that size exclusion may also be the dominant mechanism for limiting protein retention in the SPS and SHP packings due to the small pore size of the silica used for these phases [6]. Despite differences in structure and chemistry, all three columns exhibit similar behavior: large molecules (proteins) are prevented from interacting with the stationary phase and are eluted in or near the void volume, while low-molecular-weight analytes are retained and separated by mechanisms similar to those operating in conventional reversed-phase HPLC. Comparison of the columns was facilitated by the fact that all had identical dimensions (150 × 4.6 mm) and support particle size (5 μm), and exhibited similar efficiency for test compounds (plate count > 50 000 m^{-1}).

EXPERIMENTAL

Reagents and solutions

Sulfamethazine (SMZ), sulfamerazine (SM), sulfapyridine (Sp) and sulfathiazole (STZ) were obtained from Sigma (St. Louis, MO, USA) and sulfanilamide (SA) was from J. T. Baker (Phillipsburg, NJ, USA). N^4-acetylsulfamethazine (N4-ASMZ) was prepared from sulfamethazine and acetic anhydride (gift of Dr. Owen W. Parks, USDA, Philadelphia, PA, USA). Reagent grade dibasic potassium phosphate, phosphoric acid, sodium acetate, ammonium acetate and acetic acid were obtained from Thomas Scientific (Swedesboro, NJ, USA). Dimethylsulfoxide and Tween 40 (Aldrich, Milwaukee, WI, USA) were used as received. Spectrapor dialysis tubing, 10 000 molecular weight cutoff (Arthur H. Thomas Co., Philadelphia, PA, USA) was rinsed in distilled water before use. House-purified HPLC grade water was used in all mobile phases and solutions. Mobile phases were filtered through 0.45-μm membranes and degassed with helium before use. Bovine serum albumin (Sigma) was received in freeze-dried form and reconstituted as needed using HPLC-grade water. Stock solutions of the sulfonamide drugs were prepared in methanol and added to serum to generate the spiked serum samples. Serum samples were stored at 4°C and filtered through 0.45-μm syringe-top filters prior to injection. Beef and pork samples were prepared by blending 2.5 g muscle tissue with 15 ml water for 1 min in a Polytron mixer (Brinkmann Instr., Westbury, NY, USA), centrifuging the homogenate 5 min at 3000 *g*, and adding stock solutions of the sulfonamide drugs to the supernatant.

Apparatus

The HPLC system consisted of an HP 1050 quaternary pump (Hewlett-Packard, Avondale, PA, USA), a Model 7125 loop injector (Rheodyne, Cotati, CA, USA), a Spectroflow 773 variable-wavelength detector (Kratos Analytical, Ramsey, NJ, USA), and an HP 3396A integrator (Hewlett-Packard). A Fiatron CH-30 column heater (Alltech, State College, PA, USA) was used for some analy-

ses. The shielded hydrophobic phase (SHP) column used was the Hisep (Supelco, Bellefonte, PA, USA), the internal surface reversed-phase (ISRP) column was the GFF-II (Regis, Morton Grove, IL, USA), and the semi-permeable surface (SPS) column was the SPS-C18 (Regis). All columns were packed with 5-μm particles and had dimensions of 150 × 4.6 mm I.D.

Procedures

Each analytical column was protected by a commercially-packed guard column containing the same packing and by a 2-μm in-line filter. Unless otherwise noted, mobile phase flow-rate was 1.0 ml/min, temperature was ambient (20–30°C), injection size was 20 μl, and detection was performed at 265 nm with a 1-s detector risetime. Phosphate buffers were used in the pH range 5.7–7.5, and sodium acetate buffers were used for lower pH. For analyses at levels below 100 ng/ml, the column was temperature-controlled at 30°C and a detector rise time of 5 s was used. For studies of injection size response, the injection valve was fitted with a 100-μl loop which was partially filled with a syringe to obtain the desired injection volume. Chromatographic performance was evaluated periodically using benzene and toluene as analytes with a 70% methanol mobile phase. Guard columns were backflushed or replaced when elevated backpressure or reduced efficiency were observed.

RESULTS AND DISCUSSION

The nominal goal of this work was development of an HPLC method for direct injection analysis of a mixture of sulfonamides in bovine serum with a detection limit below 100 ng/ml. The success of RAM technology in determinations of individual drugs at therapeutic levels (*ca.* 10 μg/ml) suggested that this goal could be readily met. However, initial experiments showed that simple extrapolation from results obtained at therapeutic levels was not possible. A typical chromatogram of phenobarbital in serum at the 10 μg/ml level (Fig. 1a) showed an isolated analyte peak on a flat baseline. At the 100 ng/ml level (Fig. 1b), the long tail of the matrix peak became apparent, and the steeply sloping background under the analyte peak (SMZ) made detection and quantitation very difficult. The need for

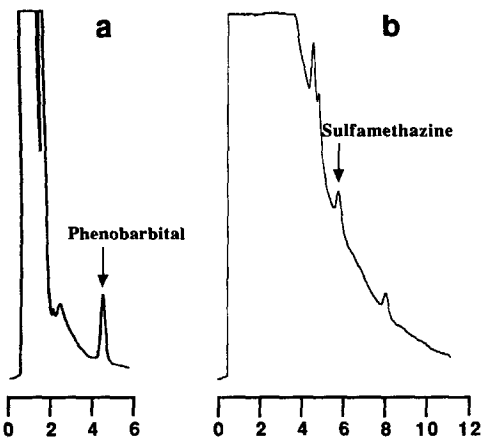

Fig. 1. RAM analyses at therapeutic and trace levels. (a) Phenobarbital (12.5 μg/ml) in human serum, 625 ng injected. SHP column, 150 × 4.6 mm I.D.; mobile phase, 180 m*M* ammonium acetate–acetonitrile (95:5); flow-rate 2.0 ml/min; wavelength, 240 nm; injection volume, 50 μl. (Reproduced with permission from Supelco Chromatography Products Catalog 28, p. 178). (b) Sulfamethazine (100 ng/ml) in bovine serum, 2 ng injected. SHP column, 150 mm × 4.6 mm I.D.; mobile phase, 100 m*M* pH 7 phosphate–acetonitrile (90:10); flow-rate, 1.0 ml/min; wavelength, 265 nm; injection volume, 20 μl. Time in min.

significant reduction in the tailing of the matrix peak and/or much greater retention of the analyte was clear.

At the time this work was initiated, two types of RAM phases, the ISRP and the SHP, were commercially available. The performance of these columns was similar, and we discuss them together below. Several months later, a series of SPS columns having C_8, C_{18}, CN, and phenyl functionality was introduced. We chose to evaluate the C_{18} column from this series as it was expected to provide the greatest degree of analyte retention and similarity to the conventional C_{18} columns used for sulfonamide analysis [11,12].

The sulfonamide antibiotics were chosen as model compounds for several reasons: they are widely used in animal husbandry and of considerable regulatory interest: they are often administered in mixtures of several sulfonamide types; they exhibit a fairly wide range of capacity factors in reversed-phase HPLC; and their chromatographic behavior on reversed-phase columns is well known. The principal metabolite of SMZ, N4-ASMZ, was included as a representative metabolite which may be used as a marker SMZ exposure.

ISRP and SHP columns

In order to identify optimum separation conditions, the chromatographic behavior of the matrix and analytes was studied as a function of pH, organic modifier concentration, and organic modifier type. In an effort to reduce tailing of the matrix peaks a number of mobile phase additives were also examined. Dialysis of the sample to remove lower-molecular-weight matrix components suspected of causing tailing was also investigated.

Organic modifier type. A number of organic modifiers, including methanol, acetonitrile, isopropanol, THF, and dioxane were evaluated. In the narrow range of modifier concentrations (0–10%) which gave acceptable capacity factor (k') values, none of the modifiers offered any significant improvement in selectivity or efficiency over acetonitrile. Little or no effect on elution or tailing of the matrix peaks was observed with variation in modifier type.

Organic modifier concentration and pH. The retention times of the sulfonamides on the SPS column as a function of pH and acetonitrile concentration are shown in Fig. 2. Retention times varied little with pH, even at pH values above the pK_a of the analytes. Capacity values for the analytes were much lower than those observed with conventional C_{18} phases under similar conditions, and coelution of analytes prevented full resolution of the mixture. The retention time of the matrix peaks was also a slowly varying function of mobile phase composition, although the presence of several large matrix peaks presented problems with analyte inteference.

Retention times of the sulfonamides on the ISRP column as a function of pH and acetonitrile concentration are shown in Fig. 3. In constrasts to the SPS phase, analyte retention behavior varied considerably with changes in pH and modifier concentration. However, the matrix peaks also exhibited complex changes in retention time and peak height when mobile phase composition was varied, making selection of conditions which minimized matrix interference difficult. Similar complex behavior has been observed in separations of peptides on ISRP columns [13]. The pH dependence of analyte retention was qualitatively similar to that observed with conventional C_{18} phases and reflected the ionization of the drugs at high pH. Elution order was similar to that observed with C_{18} phases, but k' values were much smaller. The effect of low pH was studied for the ISRP column with 0.1% trifluoroacetic acid (pH 2) as the mobile phase. Under these conditions, the free aromatic amine of the antibiotics was protonated, and all the analytes except N4-ASMZ eluted within 2 to 3 min. Even without organic modifier, the early-eluting compounds could not be resolved from the major matrix peak, and the steeply sloping tail of the matrix peak prevented reliable detection and integration of the analytes at low concentration. No conditions were found which gave complete resolution of all six analytes.

Mobile phase additives. The addition of protein

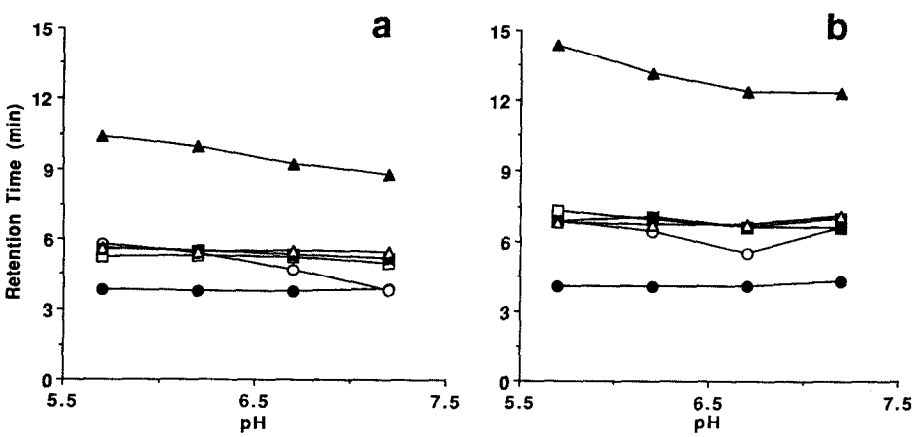

Fig. 2. Retention of sulfonamide antibiotics on SHP column as a function of mobile phase pH. Buffer, 100 mM phosphate; flow-rate, 1.5 ml/min; wavelength, 265 nm. (a) mobile phase, buffer–acetonitrile (90:10); (b) mobile phase, buffer–acetonitrile (95:5). ● = SA, ○ = SM, ■ = SMZ, □ = N4-ASMZ, ▲ = STZ, △ = SP.

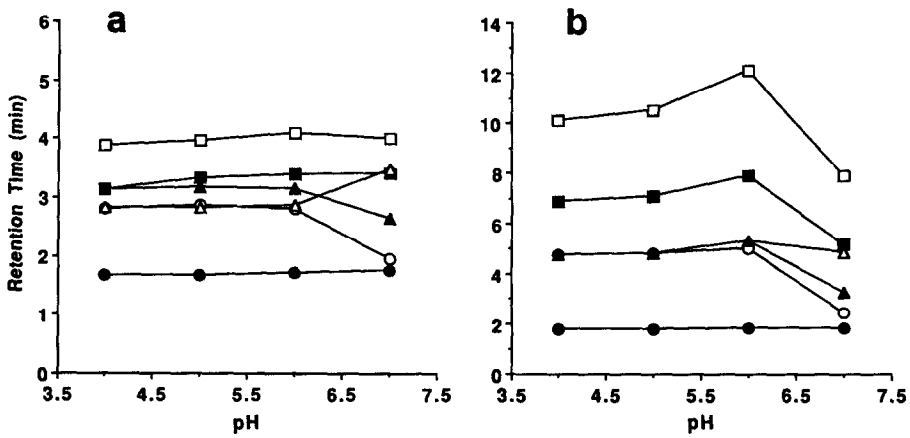

Fig. 3. Retention of sulfonamide antibiotics on ISRP column as a function of mobile phase pH. Buffers, 100 m*M* phosphate (pH 6, 7), 100 m*M* sodium acetate (pH 4, 5); flow-rate, 1.5 ml/min; wavelength, 265 nm. (a) mobile phase, buffer–acetonitrile (95:5), (b) mobile phase, buffer. ● = SA, ○ = SM, ■ = SMZ, □ = N4-ASMZ, ▲ = STZ, △ = SP.

solubilizing agents such as dimethylsulfoxide (2%) and Tween (0.1%) to the mobile phase as a means of reducing the tailing of the matrix peaks was explored without success. Addition of 50 ng/ml of serum to the mobile phase did result in noticeable suppression of matrix peak tailing, but was not sufficient to permit reliable detection/quantitation of the early eluting components. Suppression of matrix peak tailing diminished slowly over several hundred column volumes after switching to mobile phase without added serum.

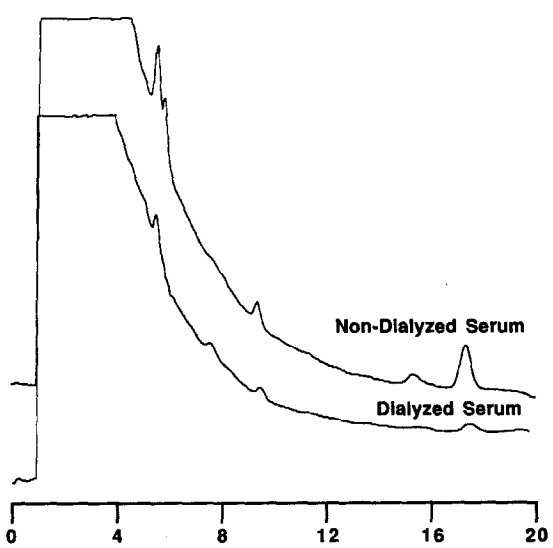

Fig. 4. Effect of dialysis on serum. Column, SHP, 150 × 4.6 mm I.D.; mobile phase, 100 m*M* pH 7 phosphate–acetonitrile (90:10); flow-rate, 1.0 ml/min; wavelength, 265 nm; injection volume, 20 μl. Time in min.

Dialysis. It was hypothesized that the tailing of the matrix peak was due to slow elution of small proteins and peptides which had penetrated into the pores or hydrophobic region of the stationary phase. To test this theory, a sample of serum was dialysed overnight against pH 6.0 buffer with a 10 000 molecular weight cutoff cellulose membrane to remove low- and intermediate-molecular-weight components. Chromatograms of dialysed and undialysed serum on the SHP column are shown in Fig. 4. Two sharp peaks at 2.1 and 2.5 min (off scale at this attenuation) were eliminated by dialysis, but no significant reduction in matrix peak tailing was apparent.

Buffer concentration. Buffer concentration was varied from 10–500 m*M* at pH 6 with minimal effect on either the serum peak shape or analyte retention times for buffer concentration greater than 50 m*M*. Small shifts in retention time observed at lower buffer concentrations could be counteracted by slightly acidifying the serum before injection. The retention time changes were attributed to lack of buffer capacity resulting in transient alteration of mobile phase pH by the sample.

Optimum results. Based on the retention behavior of the analytes and matrix constituents, optimum

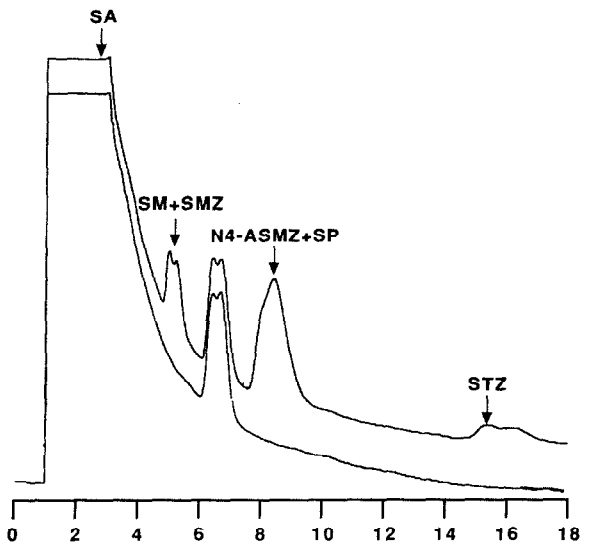

Fig. 5. Optimum separation of sulfonamides on SHP column. Sample, sulfonamide mixture (300 ng/ml) in serum; column, SHP, 150 × 4.6 mm I.D.; mobile phase, 100 m*M* pH 6 phosphate–acetonitrile (95:5); flow-rate, 1.0 ml/min; wavelength, 265 nm; injection volume, 20 μl; temperature 30°C. Upper chromatogram, sample; lower chromatogram, serum blank. Time in min.

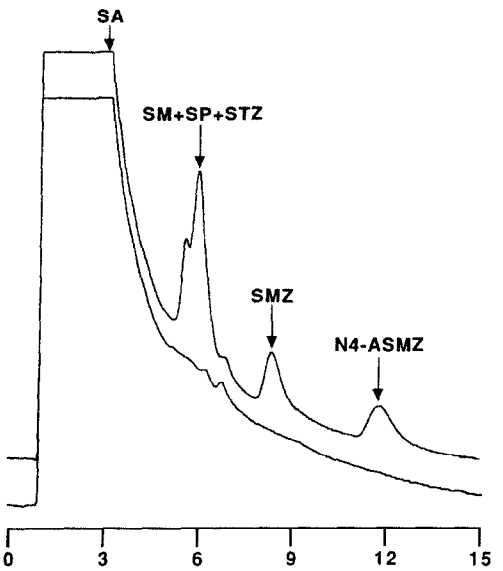

Fig. 6. Optimum separation of sulfonamides on ISRP column. Sample, sulfonamides (300 ng/ml) in serum; column, ISRP, 150 × 4.6 mm I.D.; mobile phase, 100 m*M* pH 6 phosphate–acetonitrile (99:1); flow-rate, 1.0 ml/min; wavelength, 265 nm; injection volume, 20 μl; temperature, 30°C. Upper chromatogram, sample; lower chromatogram, serum blank. Time in min.

conditions for analyte detection were determined. Choice of optimum conditions required some compromise between maximum resolution of analytes from each other, and minimum interference from matrix peaks. A chromatogram illustrating the optimum separation obtained with the SHP column is shown in Fig. 5. The large matrix peaks at 6.5 min were not identified. Only three analytes could be resolved, and the SA peak was not separated from the matrix. However, five of the analytes could be detected individually or in appropriate mixtures, without matrix interference.

A chromatogram of the sulfa mixture on the ISRP column at pH 6 is shown in Fig. 6. SA coeluted with the matrix peaks on the ISRP column, and three of the analytes (SM, SP and STZ) coeluted. While other pH values afforded better resolution of the analytes from each other, severe matrix interferences at those pH values prevented detection of several analytes. The conditions shown permitted five of the analytes the be detected (though not simultaneously) without matrix interference. Both columns were exposed to over 100 injections of serum (20 μl) without appreciable degradation of performance.

SPS-C18 column

The chromatographic behavior of the matrix and analytes was also studied as a function of pH, organic modifier concentration and organic modifier type for the SPS column. Because the analytes were adequately separated from the matrix peaks with this column, the use of mobile phase additives was not explored. Several other matrices were examined with promising results.

Organic modifier type. Methanol, acetonitrile, propanol, tetrahydrofuran and dioxane were evaluated as organic modifiers. Over the limited range of modifier concentrations (0–10%) which gave acceptable k' values, none of the modifiers offered any significant improvement in selectivity or efficiency over acetonitrile. Little or no effect on elution or tailing of the matrix peaks was observed.

Organic modifier concentration and pH. The retention times of the analytes as a function of pH and acetonitrile concentration are shown in Fig. 7. Retention of the analytes on the SPS column was significantly greater than with the ISRP and SHS columns, though still considerably less than observed with conventional C_{18} phases. The pH depend-

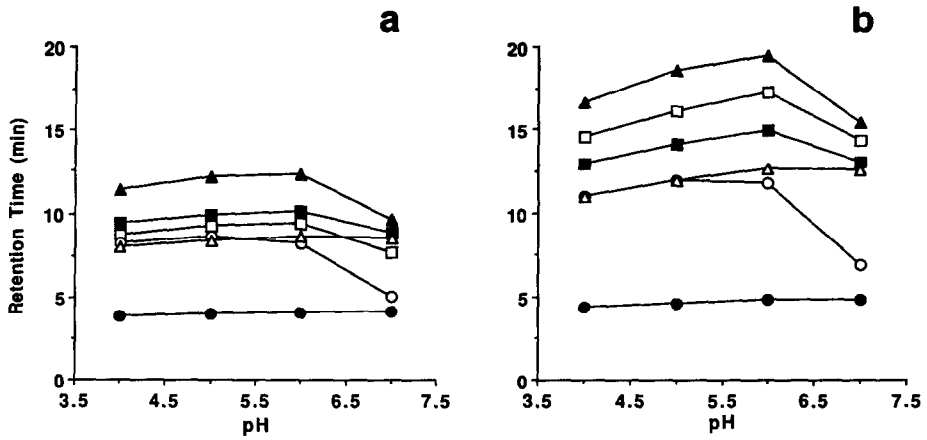

Fig. 7. Retention of sulfonamide antibiotics on SPS column as a function of mobile phase pH. Buffers, 100 m*M* phosphate (pH 6.7), 100 m*M* sodium acetate (pH 4.5); flow-rate, 1.0 ml/min; wavelength, 265 nm. (a) mobile phase, buffer–acetonitrile (95:5); (b) mobile phase, buffer. ● = SA, ○ = SM, ■ = SMZ, □ = N4-ASMZ, ▲ = STZ, △ = SP.

ence was qualitatively similar to that observed with C_{18} phases and indicated decreased retention due to ionization of the drugs at high pH. Over the first several weeks of column use, retention times for the sulfonamides decreased approximately 30%, and then reached a steady value which remained unchanged for months. Virtually no change in retention time for benzene and toluene was observed over this time frame, however. SMZ and STZ, which initially coeluted, were well resolved following this "conditioning" period. We are working with the column manufacturer to determine protocols for conditioning columns in order to obtain stable retention rapidly, and results of these studies will be reported elsewhere. Resolution of all six sulfonamides could be achieved at pH 6 with both 0.5% acetonitrile and unmodified mobile phases. The separation is relatively robust, and pH and modifier concentration may be altered to avoid interferences without compromising resolution of the analytes. A chromatogram of the test mixture using unmodified buffer as the mobile phase is shown in Fig. 8.

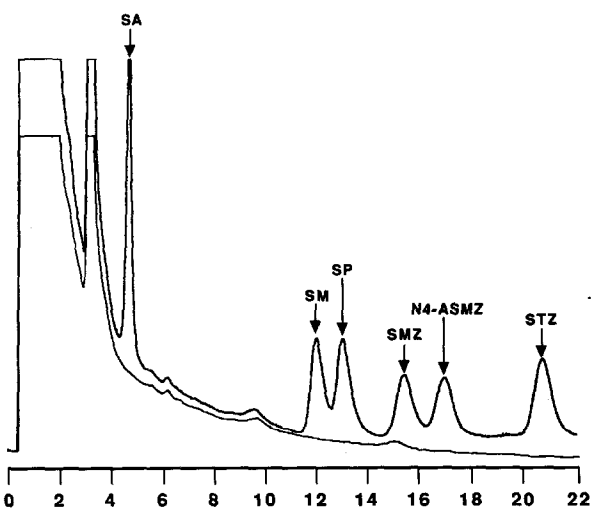

Fig. 8. Sulfonamides on SPS column. Sample, sulfonamides (300 ng/ml) in serum; column, SPS, 150 × 4.6 mm I.D.; mobile phase, 100 m*M* pH 6 phosphate; flow-rate, 1.0 ml/min; wavelength, 265 nm; injection volume, 20 μl; temperature, 30°C. Upper chromatogram, sample; lower chromatogram, serum blank. Time in min.

Temperature and ionic strength. Temperature and ionic strength variations were studied for the SPS-C18 column and found to provide minimal improvement in the separation. Temperature changes in the range 25–45°C resulted in predictable reduction of k' with increasing temperature, but did not improve resolution or peak shape significantly. Ionic strength was studied by holding buffer concentration at 50 m*M*, pH at 6.0, and acetonitrile concentration at 2%, and varying KCl concentration from 0 to 1 *M*. Retention time increases ranging from 0.2 min for sulfanilamide to 2.7 min for sulfathiazole

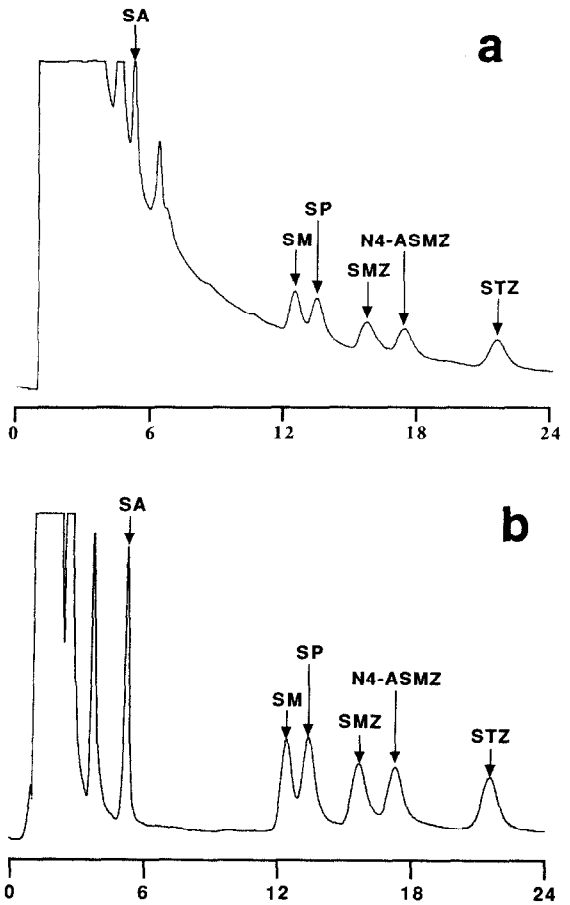

Fig. 9. Analysis of milk and beef homogenate on SPS column. (a) 300 ng/ml sulfonamide mixture in beef tissue homogenate; (b) 300 ng/ml sulfonamide mixture in milk. Mobile phase, 100 m*M* pH 6 phosphate; flow-rate, 1.0 ml/min; wavelength, 265 nm; injection volume, 20 μl; temperature, 30°C. Time in min.

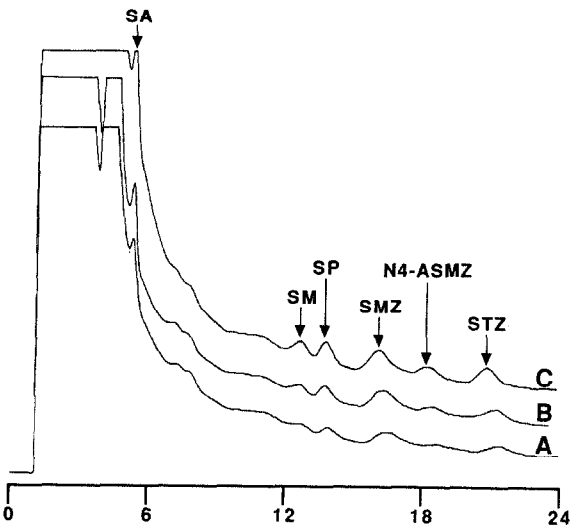

Fig. 10. Limit of detection for sulfonamides on SPS column. Bovine serum spiked with sulfonamides at concentrations of (A) 25 ng/ml, (B) 50 ng/ml, (C) 75 ng/ml. Mobile phase, 100 m*M* pH 6 phosphate; flow-rate, 1.0 ml/min; wavelength, 265 nm; injection volume, 50 μl; temperature, 30°C. Time in min.

were observed for all components as KCl concentration was varied from 0 to 1 *M*. While retention time could be manipulated by adjusting ionic strength, little gain in selectivity between analytes and virtually no effect on matrix tailing was observed.

Injection size. The effect of sample size on peak area was investigated using 300 ng/ml sulfamethazine in serum. Peak area increased linearly with injection volume over the range tested (10–100 μl). Injection volumes greater than 50 μl resulted in an initial 200–400 p.s.i. pressure increase which decayed over several minutes. Additional peaks also appeared at unpredictable times in the chromatograms, apparently due to late eluting species from earlier injections. With 100-μl injections, the precolumn became partially plugged and separation efficiency was noticeably reduced within 3–5 injections. This behavior was in contrast to that of ISRP columns, which are known to accomodate injection sizes up to 660 μl [14]. Injection size was subsequently limited to 50 μl to maximize column performance and lifetime.

Other matrices. Beef tissue homogenate, pork tissue homogenate and milk were spiked with the sulfonamide mixture and chromatographed under the same conditions used for bovine serum. Results for the beef and milk samples are shown in Fig. 9. Chromatograms of pork homogenate were very similar to those of beef. It should be noted that the beef homogenate contained only 2.5 g tissue per 15 ml, and that the sulfonamides were added at a level of 300 ng/ml to the homogenate. The matrix peaks were therefore much smaller relative to the analyte peaks than in the case of the serum or milk samples. Nonetheless, direct injection analysis of milk and homogenized tissue using RAM columns appears to be very promising as a method for residue analysis in these matrices.

Limit of detection. A series of serum samples spiked with 10–100 ng/ml of the sulfonamides was analysed to estimate the limit of detection and limit of quantitation for 50-μl injections. Chromatograms of spiked serum at 25, 50, and 75 ng/ml levels are shown in Fig. 10. From this data, a limit of detection of 25 ng/ml and a limit of quantitation of 50 ng/ml was conservatively estimated for all of the components. Other techniques such as electrochemical detection might be used to achieve lower detection limits.

CONCLUSIONS

The utility of RAM for analysis of multiple analytes in biological matrices at ng/ml levels has been demonstrated for sulfonamide antibiotics in serum. At the sensitivity required to detect these low concentrations, a steeply sloping background due to tailing of the serum matrix was observed which prevented reliable detection of analytes with $k' < 5$ at low (50 ng/ml) concentrations. Due to limited retention and selectivity, the ISRP and SHP columns were unsuitable for analysis of all the sulfonamides, but could be used to determine some individual drugs or mixtures with detection limits below 100 ng/ml. The SPS column exhibited the greatest analyte retention and selectivity of the columns tested, and could be used for direct injection, multi-residue analysis of all six sulfonamides in serum with an estimated detection limit of 25 ng/ml, using minimal sample preparation and no organic solvents.

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