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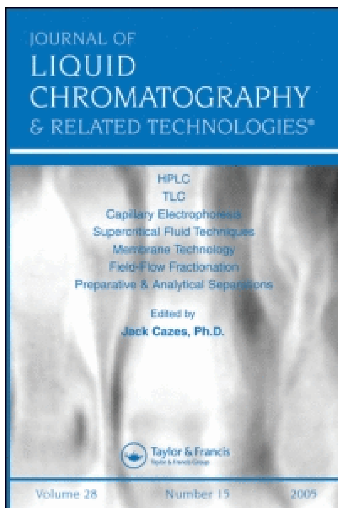

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The Use of Internal Surface Reversed-Phase Packing for the Solid Phase Extraction of Drugs from Serum

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Original Article

THE USE OF INTERNAL SURFACE REVERSED-PHASE PACKING FOR THE SOLID PHASE EXTRACTION OF DRUGS FROM SERUM

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The utilization of Internal Surface Reversed Phase (ISRP) packing as a solid phase extraction (SPE) matrix was investigated. Evaluation of the relative retention of nineteen medicinal agents on ISRP material was monitored using HPLC. The effects of altering pH and/or buffer concentration on retention were further studied using verapamil, phenelzine and tamoxifen as model compounds. Spiked serum samples containing the model compounds plus amitriptyline were also subjected to ISRP-SPE and HPLC. Verapamil, phenelzine, and tamoxifen were all strongly retained on ISPR as pH increased. The buffer concentration of the sample was not as critical on retention of these compounds as pH. Verapamil was quantitatively recovered from spiked serum samples (103 ± 8.5%, *n* = 6), with a limit of detection of 10 ng/mL using fluorescent detection (λex = 280 nm, λem = 310 nm). Amitriptyline recovery was also quantitative (99.0± 5.3%, n = 6), and its limit of detection was 10 ng/mL employing short wavelength UV detection (λ = 214 nm). The ISRP packing as an SPE matrix failed to adequately disrupt the binding of either phenelzine or tamoxifen from serum proteins; therefore, recoveries of these compounds were poor (<50%). A comparison of ISRP to conventional C18 and C2 SPE columns indicated that the ISRP packing eliminated interferences as well or better than the conventional columns.

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INTRODUCTION

In 1985, Hagestam and Pinkerton [1] introduced an Internal Surface Reverse Phase (ISRP) column (also known as the Pinkerton column), which allowed the direct injection of plasma or serum samples for HPLC analysis. The column consisted of modified 5 µm silica particles whose internal pore surfaces possess bound, slightly hydrophobic tripeptides (Gly-Phe-Phe), but whose external surfaces are hydrophilic diol-glycine moieties. Small analytes may enter the pores and be preferentially retained; whereas, the larger serum proteins are excluded from the pores and simply elute at the solvent front. To date, the Pinkerton columns have been utilized in the analysis of a wide variety of drugs in plasma and serum samples [2-6]. The retention properties of ISRP-HPLC columns and the effects of altering chromatographic conditions have been well characterized [7]. The use of ISRP columns involves certain restrictions with respect to mobile phase pH and organic modifier [8]. The manufacturer recommends that organic solvent content not exceed 20%, which in some cases may result in rather long retention times. In order to shorten retention times and increase detectability via sample concentration, column-switching techniques have been employed [1, 6, 9].

In this study, the use of ISRP packing material as a pre-column solid phase extraction (SPE) matrix is investigated. Serum samples containing an analyte are diluted with water or buffer, passed slowly through a small column containing ISRP material, washed to remove

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interferences, and eluted with a small volume of eluent prior to injection into an HPLC or GC.

The results of an initial screening of the retention of 19 pharmaceuticals on the ISRP-SPE column are reported. From these compounds, verapamil, phenelzine, and tamoxifen were chosen as model compounds to further determine the effects of pH and buffer concentration on retention on ISRP-SPE columns. In addition, spiked serum samples containing verapamil, amitriptyline, phenelzine and tamoxifen were subjected to ISRP solid phase extraction followed by HPLC analysis. The recoveries of these analytes were calculated, and the efficiency of the ISRP sample cleanup was compared to that obtained with conventional C18 and C2 solid phase extraction columns.

EXPERIMENTAL

Reagents and Chemicals

Acetonitrile (HPLC grade), monobasic sodium phosphate, phosphoric acid, and sodium hydroxide were obtained from the J.T. Baker Chemical Company (Phillipsburg, NJ). Phenelzine sulfate, verapamil hydrochloride, d-amphetamine sulfate, imipramine hydrochloride, ibuprofen, pargyline, tranylcypromine, and 17 α-methyltestosterone were obtained from Sigma Chemical Company (St. Louis, MO), Procainamide hydrochloride and tamoxifen from Aldrich Chemical Company (Milwaukee, WI), Amitriptyline hydrochloride and probenecid from Merck, Sharpe, and Dohme (Rahway, NJ), Prochlorperazine dimaleate and cimetidine from Smith Kline and French Labs (Philadelphia, PA), Nifedipine from Pfizer, Inc. (New York,

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NY), Propanolol hydrochloride from Ayerst Laboratories (New York, NY), Diltiazem hydrochloride from Marion Merrell Dow, Inc. (Kansas City, MO), Desipramine hydrochloride from Geigy Pharmaceuticals (Ardsley, NY),and Aspirin was synthesized in-house.

Empty 1.5 mL solid phase extraction reservoirs (Cat. No. 1213-1007) and packed solid phase extraction columns (C18, Cat. No. 1210-2001 and C2, Cat.No. 1210-2003, 100 mg/1.0 cc) were obtained from Analytichem International (Harbor City, CA). Mobile phases were filtered through 0.45 µm nylon filters obtained from Micron Separations, Inc. (Westboro, MA), Drug free human serum was obtained from Biological Specialties Corporation (Lansdale, PA), HPLC grade water was obtained using a Continental Water System (Atlanta, GA). The internal surface reversed phase material (40 µm particle size, 100 A pore size) was a gift from Regis Chemical Company (Morton Grove, IL).

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Apparatus

The HPLC system consisted of a Beckman Model 110B pump (Fullerton, CA) and a Rheodyne Model 7125 injector (Cotati, CA) equipped with a 100 µL sample loop. In some applications, a 20 µL loop was employed. Detection was accomplished using either a Varian Model 2550 variable wavelength UV detector (Sunnyvale, CA), a Bioanalytical Systems Model LC-4B amperometric detector (West Lafayette, IN), or a Hitachi Model F1000 fluorescence spectrophotometer (Tokyo, JAPAN). Data collection was performed using a Spectra-Physics Model 4290 integrator (San Jose, CA).

A Brownlee (Santa Clara, CA) Spheri-5 Phenyl cartridge column (5 µm, 100 mm X 4.6 mm i.d.) equipped with a Brownlee RP-18 Newguard cartridge was employed for all analytical separations except tamoxifen where a Brownlee cyano cartridge (5 µm, 100 mm X 4.6 mm i.d.) was employed. An Upchurch Scientific (Cat. No. A-314) in-line filter was placed between the injector and the HPLC column. A Fiatron (Oconomowoc, WI) TA-50 temperature controller was used to maintain the HPLC column temperature at 30°C.

An Analytichem International Vac-Elut apparatus (Cat. No. 1223-4001) was employed during the sample cleanup steps. Amicon (Danvers, MA) MPS-1 micropartition units and a Sorvall (Wilmington, DE) Model T6000B centrifuge were employed for the ultrafiltration of serum samples.

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Preparation of ISRP - Solid Phase Extraction Columns

Solid phase extraction columns containing ISRP packing were prepared as follows: after removing the upper or inlet frit from an empty solid phase extraction reservoir, a weighed amount of the ISRP packing (either 100 or 200 mg) was transferred to the reservoir. Portions weighing 100 mg were used for the initial retention screening of drugs, while 200 mg portions were used for the quantitative procedures. The material was wet with one column volume of acetonitrile and then the reservoir was submerged into a glass test tube containing acetonitrile. The column was sonicated for 10 seconds to help rid the packing of trapped air. The packing material was stirred using a small piece of metal tubing, and then the column was again sonicated for

10 seconds. The upper frit was then pushed slowly down towards the packing, taking care not to trap additional air between the frit and the packing.

Prior to the addition of a drug sample, the ISRP-solid phase extraction column was conditioned by sequentially passing 5 mL each of acetonitrile, water, and phosphate buffer through the packing. During the conditioning and the sample extraction procedures, care was taken to keep the packing wet.

Mobile Phases

The following mobile phases were prepared for use in this study: Mobile Phase A - 50:50 acetonitrile - 0.05M NaH_2PO_4, pH 2.5; Mobile Phase B - 40:60 acetonitrile - 0.05; NaH_2PO_4, pH 2.5; Mobile Phase C - 20:80 acetonitrile - 0.05M NaH_2PO_4 containing 0.02 M heptane sulfonic acid sodium salt, pH 2.5; Mobile Phase D - 15:85 acetonitrile - 0.05M NaH_2PO_4 containing 0.02M heptanesulfonic acid sodium salt, pH 2.5; Mobile Phase E - 20:80 acetonitrile - 0.05M NaH_2PO_4, pH 2.5; and Mobile Phase F - 10:90 acetonitrile - 0.05M NaH_2PO_4, pH 2.5. The pH of the NaH_2PO_4 solution was adjusted to 2.5 by the addition of concentrated phosphoric acid. Acetonitrile was added to the phosphate solution and the resulting solvent mix filtered and degassed by sonication prior to use.

Screening of Compounds for Retention on ISRP Material

Samples of each of the nineteen drugs were prepared by transferring a weighed aliquot of 10 mg to a 100 mL volumetric flask, and, if sufficiently water soluble,

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diluting to volume with HPLC grade water. Some of the more lipophilic compounds required dissolution in approximately 25 to 50 mL of acetonitrile prior to dilution with water. For the acidic drugs, the initial dilution used 0.1 M NaH_2PO_4, pH 7.4 rather than water. Then two successive 1:10 dilutions of each drug solution (the first with water, the second with 0.1 M NaH_2PO_4, pH 7.4) yielded a final drug concentration of 1 µg/mL.

A 1.0 mL aliquot of each drug solution was pipetted onto a conditioned ISRP column using a volumetric pipet. The sample was passed slowly through the column (1 mL/min) using positive pressure applied on the column by a 6 mL plastic syringe fitted with an adapter (Analytichem International, Cat. No. 636001). The sample fraction was collected in a 2 mL volumetric tube and diluted to volume with 0.1 M NaH_2PO_4, pH 7.4. The column was then washed with 2 mL of the phosphate buffer which was also collected in a 2 mL volumetric tube. All sample and wash fractions were chromatographed on a phenyl HPLC column. Mobile phases were chosen such that analyte retention times were no more than 10 minutes (Table 1). Peak responses were compared to those obtained by injecting unextracted drug solutions. The percentage of each drug retained on the ISRP material was determined by quantitating the amount found in each eluted sample and wash fraction and subtracting the amounts lost in those fractions from the original amount of the drug applied to the ISRP column. Quantitation was based on peak height.

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TABLE 1

HPLC Parameters for Selected Pharmaceuticals[a]

Compound	UV nm	Mobile Phase[b]	Flow Rate mL/min	Retention Time, min
Verapamil Hydrochloride	232	A	1.0	4.8
Imipramine Hydrochloride	214	A	1.0	5.2
Amitriptyline Hydrochloride	214	A	1.0	5.8
Desipramine Hydrochloride	214	A	1.0	4.0
Tamoxifen	207	A	2.0	7.2
Prochlorperazine Dimaleate	254	A	1.5	3.3
Propranolol Hydrochloride	254	A	1.0	2.6
Nifedipine	254	A	1.0	3.0
Diltiazem Hydrochloride	254	A	1.0	3.6
Amphetamine Sulfate	210	E	1.0	2.8
Phenelzine Sulfate	209	F	1.0	3.3
Procainamide Hydrochloride	209	E	1.0	2.5
Tranylcypromine	209	F	1.0	4.1
Pargyline	209	F	1.0	4.8
Ibuprofen	209	A	1.0	3.2
Aspirin	209	F	2.0	4.0
Probenecid	242	A	1.0	2.7
Cimetidine	209	F	1.0	3.9
17 α Methyl-testosterone	240	A	1.0	3.2

[a] A Brownlee 10 cm phenyl column coupled to an RP-18 guard cartridge was employed for all chromatograms.
[b] Mobile phase compositions are described in the Experimental section.

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Determination of pH Effects and Buffer Concentration

To test for pH effects, aqueous 0.1 M sodium phosphate buffers at pH 4, 5, 6, 7, and 8 were prepared. The pH was adjusted with either concentrated phosphoric acid or 5 N sodium hydroxide. To test the effects of varying the salt concentration of the sample solutions, 0.01, 0.05, 0.1 and 0.2 M solutions of pH 7 phosphate buffer were also prepared. The pH of each solution was adjusted to 7 using 5 N sodium hydroxide.

Samples of verapamil, phenelzine, and tamoxifen were prepared in each of the phosphate buffers such that the final concentration was 1.0 µg/mL. These samples also served as standards for quantitating the amounts of each drug in the sample and wash fractions collected. A 1.0 mL aliquot of each drug sample was applied to a conditioned ISRP-SPE column. The sample fraction was passed slowly through the column using positive pressure, collected in a 2 mL volumetric tube, and diluted to volume with the buffer solution used for the sample preparation. The column was then washed with 2 mL of the buffer, and the wash was collected in a separate 2 mL volumetric tube. Each fraction was injected into the HPLC and the amount of drug in each fraction was determined.

ISRP Extraction of Human Serum containing Verapamil, Amitriptyline, Phenelzine, or Tamoxifen

1. Verapamil

Verapamil base solutions in water containing 0.1, 0.2, 0.5, 1, 2, and 5 µg/mL. were prepared using verapamil

hydrochlonde. Then, solutions representing 10-500 ng/mL of verapamil base were prepared by transferring 100 μL aliquots of each solution to disposable test tubes containing 1 mL of serum. Each tube was vortexed, and 1 mL of 0.2 M NaH_2PO_4, pH 8 was added. Using a disposable pipet, each sample was then applied to an ISRP - solid phase extraction column (200 mg) which was mounted in a Vac-Elut apparatus and which had been previously conditioned with 5 mL each of acetonitrile, water, and 0.2 M NaH_2PO_4, pH 8. The sample was passed slowly through the column using a weak vacuum (2-3 mm Hg). The test tube was rinsed with 2 mL of the phosphate buffer which was then added to the solid phase column and passed through. The column was then removed from the Vac-Elut apparatus and, employing positive pressure from a syringe, the analyte was eluted into a volumetric tube with 1 mL of mobile phase A. A 20 μL aliquot was injected into the HPLC. Detection was accomplished using fluorescence detection with λex = 280 nm and λem = 310 nm.

A serum sample was obtained from a male volunteer who had taken a daily oral dose of 480 mg of sustained-release verapamil for one year. A 1 mL portion of the serum was subjected to the ISRP solid phase extraction procedure and analyzed by HPLC.

A 100 ng/mL spiked sample of verapamil in serum was also subjected to solid phase extraction on a C2 SPE column as a comparison to the ISRP procedure. The SPE method developed by Claxton and Tackett for verapamil in serum was followed [10] and HPLC analysis of the sample was performed.

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2. Amitriptyline

Aqueous solutions (1-10 µg/mL) of amitriptyline base were prepared from amitriptyline hydrochloride. In disposable test tubes, 1 mL aliquots of human serum were each spiked with 100 µL of the diluted amitriptyline solutions followed by 1 mL of 0.2 M NaH_2PO_4, pH 8. Each serum sample was loaded onto a conditioned ISRP solid phase extraction column (200 mg) fitted to a Vac-Elut apparatus using a disposable pasteur pipet. The sample was pulled slowly through the SPE column with a weak vacuum (2-3 mm Hg). The test tube was rinsed with 2 mL of the phosphate buffer which was then used to wash the solid phase extraction column and passed through. The analyte was then eluted from the SPE column into 1 mL volumetric tubes with 1 mL of Mobile Phase B. A 20 µL aliquot was injected into the HPLC. UV detection at 214 nm was utilized.

3. Phenelzine

A phenelzine base stock solution (10 µg/mL) in water was prepared from phenelzine sulfate. Spiked serum samples (1 µg/mL) were prepared by transferring 1 mL of human serum to a disposable test tube and adding 100 µL of the 10 µg/mL phenelzine solution. Each test tube was vortexed and a 1 mL aliquot of 0.1 M NaH_2PO_4, pH 8 was added.

A solid phase extraction column containing 200 mg of ISRP packing was connected to a Vac-Elut apparatus and conditioned with 5 mL each of acetonitrile, water, and 0.1 M phosphate buffer, pH 8. The sample was loaded onto the column using a disposable pasteur pipet, and then pulled

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slowly through the column using a weak vacuum (2-3 mm Hg). Two milliliters of the pH 8 buffer were pipetted into the test tube to rinse any remaining phenelzine from the walls, and were then used to wash the ISRP solid phase column and passed through. The drug was then eluted from the column with 10 mL of 0.05 M NaH_2PO_4, pH 2.5, and collected in a 10 mL volumetric flask. The flask was shaken and a 20 µL aliquot was injected into the HPLC. Mobile phase C was used for the assay along with amperometric detection (applied voltage = + 1300 mV vs Ag/AgCl, detector output + 10 mV, range = 10 nA).

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To compare the ISRP-SPE procedure to a conventional solid phase extraction column, a C18 (100 mg) solid phase column was employed to analyze serum spiked with phenelzine. The conditioning and extraction steps were identical to those used with the ISRP column except that the eluting solvent consisted of 20:80 acetonitrile - 0.05 M NaH_2PO_4, pH 2.5.

Ultrafiltration of spiked serum was used to determine the extent of protein binding of phenelzine. A volume of protein free serum was obtained by placing serum in Amicon ultrafiltration units and centrifuging at 3000 rpm for 15 min. A 100 µL aliquot of the 10 µg/mL phenelzine solution was transferred to a 1 mL volumetric tube and diluted to volume with the protein free serum (Standard Solution). An 800 µL sample of spiked serum containing 1 µg/mL phenelzine base was then transferred to an Amicon unit and centrifuged at 3000 rpm for 15 min. Twenty microliter aliquots of the

filtrate and the standard solution were injected into the HPLC. Mobile phase D and amperometric detection as described above were used for the assay.

4. Tamoxifen

A tamoxifen stock solution (100 µg/mL) in 50:50 acetonitrile/water was prepared. A 1:10 dilution in water gave a final drug concentration of 10 µg/mL. Spiked serum samples containing 1 µg of tamoxifen were prepared by transferring 1 mL of serum to a disposable test tube and adding 100 µL of the 10 µg/mL tamoxifen solution. A 1 mL aliquot of 0.2 M NaH_2PO_4, pH 8 was used to dilute the serum sample prior to extraction. The samples were pipetted onto solid phase extraction columns containing 200 mg of ISRP packing and which had been previously connected to a Vac-Elut apparatus and conditioned with 5 mL each of acetonitrile, water, and pH 8 phosphate buffer. The sample was pulled slowly through the SPE column with a weak vacuum (2-3 mm Hg). Each column was washed with 2 mL of the pH 8 buffer, and the analyte was eluted with 2 mL of mobile phase A into a 2 mL volumetric tube. A cyano HPLC column and UV detection at 207 nm were used.

To determine the serum protein binding of tamoxifen, a 1 mL sample of serum containing 1 µg of tamoxifen was diluted with 1 mL of pH 8 phosphate buffer and passed slowly through a conditioned ISRP-SPE column. The sample fraction was collected in a centrifuge tube, and a 2 mL aliquot of acetonitrile was added. The tube was vortexed for 5 sec and centrifuged for 10 min. The centrifugate was decanted, filtered, and injected into the HPLC.

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TABLE 2

Retention of Selected Drugs on ISRP-SPE at pH 7.4

Compound	MW[a]	log P[b]	% lost in sample fraction	% lost in wash fraction	% retained on column[c]
Verapamil Hydrochloride	454.59	7.5	0.21	2.66	97.3
Imipramine Hydrochloride	280.40	5.5	---[d]	8.9	91.1
Amitriptyline Hydrochloride	277.39	7.0	---	---	100.0
Desipramine Hydrochloride	266.37	5.0	---	24.0	76.0
Tamoxifen	371.53	8.0	---	---	100.0
Prochlorperazine Dimaleate	373.94	5.5	---	---	100.0
Propranolol Hydrochloride	259.34	7.0	---	36.8	63.2
Nifedipine	346.34	3.8	---	33.5	66.5
Diltiazem Hydrochloride	414.52	3.8	---	21.7	78.3
Amphetamine Sulfate	135.20	2.5	70.9	32.5	---
Phenelzine Sulfate	136.19	3.0	60.0	---	40.0
Procainamide Hydrochloride	253.33	2.5	24.6	71.6	6.8
Tranylcypromine	133.19	2.5	46.5	53.2	0.3
Pargyline	159.22	3.5	---	74.6	25.4
Ibuprofen	206.27	4.3	25.6	64.6	9.8
Aspirin	180.15	1.1	60.5	17.6	21.9
Probenecid	285.36	1.4	26.2	78.4	---

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TABLE 2 (continued)

Compound	MW[a]	log P[b]	% lost in sample fraction	% lost in wash fraction	% retained on column[c]
Cimetidine	252.34	-1.0	41.6	52.6	5.8
17α Methyl-testosterone	302.44	8.0	---	67.0	33.0

[a] molecular weight of unionized drug
[b] log P estimates based on π values [15]
[c] % retained = 100% - (% lost in sample fraction + % lost in wash fraction).
[d] No detectable signal

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RESULTS AND DISCUSSION

The goal of this study was to evaluate the use of Internal Surface Reversed Phase (ISRP) packing material as a solid phase extraction matrix. Nineteen drugs with differing chemical structures were screened for retention on the ISRP material to provide insight to the size and chemical nature of compounds best suited for this new mode of sample cleanup. Extractions of four selected drugs from spiked serum were also performed to illustrate the efficiency of the cleanup and accuracy of the resulting assays.

In our initial studies, it was decided to keep the extraction conditions constant so that differences in retention could be attributed to characteristics of the drugs themselves. The nineteen drugs used for the retention screening, their molecular weights, estimated log P values, and percent retention on the ISRP column are shown in Table 2. Basic drugs were found to be the best retained. Both the sample matrix solution and wash solutions were buffered to pH 7.4 for this application. This pH appeared to favor

retention of the basic compounds since the drug would be positively charged while the carboxyl groups on the ISRP surface would have a negative charge. Acidic compounds were not as well retained at pH 7.4 since they would be negatively charged and, therefore, electronically repulsed by the ionized carboxyl groups. Surprisingly, methyltestosterone, a highly lipophilic and neutral compound, was not well retained on the ISRP material at pH 7.4. When the pH of a methyltestosterone sample was adjusted to 2.5, the retention of the steroid on the ISRP material improved to 75%. A low pH was also favorable for acidic drugs. However, low pH can be a problem with serum samples due to protein precipitation.

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From the 19 drugs initially studied, verapamil, phenelzine, and tamoxifen were chosen as model compounds to further determine the effects of pH and buffer concentration on retention by ISRP solid phase extraction columns. Table 3 shows the percent retention of these drugs on ISRP material as a function of the pH of the sample and wash solutions. Verapamil and tamoxifen have reported pka values of 8.6 [11] and 8.85 [12], respectively. Phenelzine was found to have a pka of 5.2 by potentiometric titration in this laboratory. All three drugs were better retained as the pH of the sample and wash solutions increased. The stationary phase of the ISRP packing terminates in a carboxylic acid, which will become ionized as the pH becomes more basic. The tertiary amine groups of verapamil and tamoxifen are protonated below pH 8; therefore, in addition to Van der Waals attractions, there will be ion-exchange

TABLE 3

Retention of Verapamil, Phenelzine, and Tamoxifen on ISRP Solid Phase Extraction Columns as a Function of Sample pH

Sample Solution pH[a]	% Retained[b,c]		
	Verapamil	Phenelzine	Tamoxifen
4	40.0	5.2	88.3
5	70.6	36.2	84.6
6	90.6	79.1	92.3
7	93.9	99.1	98.1
8	92.3	100.0	95.4

[a]All buffers contained 0.1 M NaH_2PO_4.

[b]% Retained = 100% - (Amounts lost in sample and wash fractions).

[c]Based on peak height data.

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interactions between these molecules and the stationary phase. Even though phenelzine (with its lower pka value) becomes unionized as pH increases, it was still observed to be better retained at a pH of 7 or higher. Its retention on ISRP, therefore, must not be dependent upon ion-exchange interactions.

Table 4 shows the retention of phenelzine, verapamil and tamoxifen on ISRP material as a function of the buffer concentration of the sample and wash solutions. At pH 7 all three drugs are well retained at the four buffer concentrations tested. Phenelzine is essentially unionized at pH 7 and is not subject to changing buffer concentrations. Verapamil and tamoxifen, however, are ionized and would be expected to be responsive to changes in ionic strength. Nakagawa and coworkers [7] observed that

TABLE 4

Retention of Verapamil, Phenelzine, and Tamoxifen on ISRP Solid Phase Extraction Columns as a Function of Buffer Concentration

Buffer Concentration[a]	% Retained[b,c]		
	Verapamil	Phenelzine	Tamoxifen
0.01 M	92.5	98.9	93.5
0.05 M	95.2	97.4	90.9
0.10 M	93.9	99.1	98.1
0.20 M	90.0	100.0	91.2

[a] All buffer solutions contained NaH_2PO_4, pH 7.

[b] % Retained = 100% - (Amounts lost in sample and wash fraction).

[c] Based on peak height data.

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the retention time of phenethylamine on an ISRP- HPLC column decreased as the ionic strength of the mobile phase increased. The larger molecular size and lipophilicity of both verapamil and tamoxifen apparently diminish the effects of increasing the buffer concentration on the retention of these compounds.

Using the ISRP-SPE procedure, spiked serum samples containing verapamil, amitriptyline, phenelzine, and/or tamoxifen were assayed to illustrate the applicability of ISRP sample clean-up prior to HPLC determination. The recovery of verapamil from spiked serum samples was quantitative (101.2 $\pm$ 7.3% based on peak area and 103 $\pm$ 8.5% based on peak height, n = 6). A standard curve of verapamil in spiked serum was linear over the range of 10-500 ng/mL (r^2 = 0.9996 based on peak area, r^2 = 0.9997 based on peak height, n = 6). The limit of detection (S/N = 2) was 10

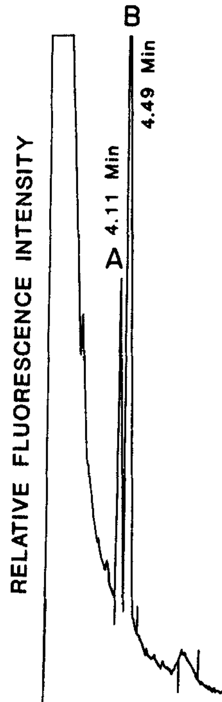

Fig. 1. HPLC chromatogram of actual human serum sample containing verapamil following ISRP solid phase extraction. Volunteer received 240 mg of verapamil HCl as sustained release tablets twice daily. Column: 10 cm Brownlee Phenyl (5 µm) coupled with RP-18 guard cartridge. Mobile phase A: 50:50 acetonitrile - 0.05M sodium dihydrogen phosphate, pH 2.5. Flow rate: 1.0 mL/min. Sample size: 20 µL. Detection: fluorescence, $\lambda ex = 280$ nm, $\lambda em = 310$ nm. Key: (A) norverapamil, (B) verapamil.

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ng/mL using this method. Verapamil was observed to elute at 4.4 minutes, free of any interferences from serum proteins.

A serum sample collected from a male volunteer who was a chronic user of verapamil was also subjected to the ISRP solid phase extraction procedure and chromatographed. The

resulting chromatogram (Figure 1) shows verapamil (4.5 min) to be well resolved from norverapamil (4.1 min), its primary metabolite. The concentration of verapamil in the serum sample was calculated to be 285.6 ng/mL based on peak area and 283.6 ng/mL based on peak height.

It appears that the ISRP material packed in the SPE column sufficiently disrupts drug binding from serum proteins to allow excellent recovery of verapamil, which is reported to be 90% bound to serum proteins. It was found that 200 mg of ISRP material was necessary for a successful recovery. When 100 mg of the ISRP packing was employed, only a 70 to 80% recovery of the drug was obtained.

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Amitriptyline is extensively protein bound (96%), and the ISRP solid phase procedure also gave excellent recoveries of the drug from serum (94.0 $\pm$ 5.4% based on peak area and 99.0 $\pm$ 5.3% based on peak height, n = 6). A standard curve of amitriptyline in spiked serum was linear over the 100 to 1000 ng/mL range (r^2 = 0.9989 based on peak area and r^2 = 0.9974 based on peak height, n = 6). The limit of detection (S/N = 2) was 10 ng/mL. Amitriptyline was observed to elute at 4.6 min (Fig. 2).

Samples of human serum spiked with 1 µg/mL phenelzine were subjected to ISRP solid phase extraction, and phenelzine recoveries were found to be only 30-40%. A serum sample containing 1 µg/mL of phenelzine was further subjected to ultrafiltration. It was found that phenelzine was 90-94% protein bound in serum. The ISRP material apparently does not disrupt the protein binding of

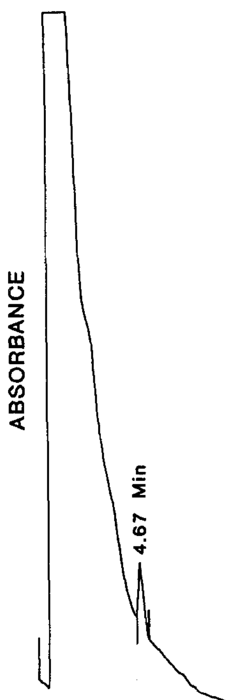

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Fig. 2. HPLC chromatogram of amitriptyline (200 ng/mL) following ISRP solid phase extraction from human serum. Column: 10 cm Brownlee Phenyl (5 μm) coupled with RP-18 guard cartridge. Mobile phase B: 40:60 acetonitrile - 0.05 M sodium dihydrogen phosphate, pH 2.5. Flow rate: 1.5 mL/min. Sample size: 20 μL. Detection: UV at 214 nm.

phenelzine enough to allow its retention on the solid phase matrix.

In addition to low recoveries, phenelzine presented another problem when subjected to ISRP solid phase extraction. When buffered samples of phenelzine are chromatographed on 5 μm ISRP-HPLC columns, the drug

is observed to elute very quickly using a mobile phase of aqueous 0.05 M NaH_2PO_4, pH 2.5. When phenelzine was applied to our 200 mg ISRP-SPE solid phase column, it failed to elute in a reasonably small volume of the same phosphate buffer. Typically, a 10 mL aliquot of the eluting solvent was required to remove all the phenelzine from the ISRP packing.

ISRP-SPE of spiked serum containing tamoxifen yielded less than 10% recoveries of drug tamoxifen. However, tamoxifen was extracted from water on the ISRP packing with approximately 98% recovery. Obviously, the strong serum protein binding of drug is not disrupted by the ISRP matrix, and thus, the tamoxifen is not retained on the solid phase extraction matrix. A serum sample containing 1 µg of tamoxifen was passed through a conditioned ISRP solid phase extraction column and collected in a centrifuge tube. The proteins were precipitated using acetonitrile and the mixture was centrifuged. Analysis of the centrifugate showed that over 80% of the tamoxifen had passed through the ISRP material unretained.

Figure 3 and 4 show comparisons of HPLC chromatograms obtained using ISRP versus conventional SPE methods for serum sample clean-up of verapamil and phenelzine, respectively. Using fluorescent detection, the chromatograms in Fig. 3 for verapamil with ISRP and C2 SPE columns are comparable. Using electrochemical detection, ISRP solid phase extraction produced a cleaner chromatogram for phenelzine than did C18 SPE extraction (Fig. 4).

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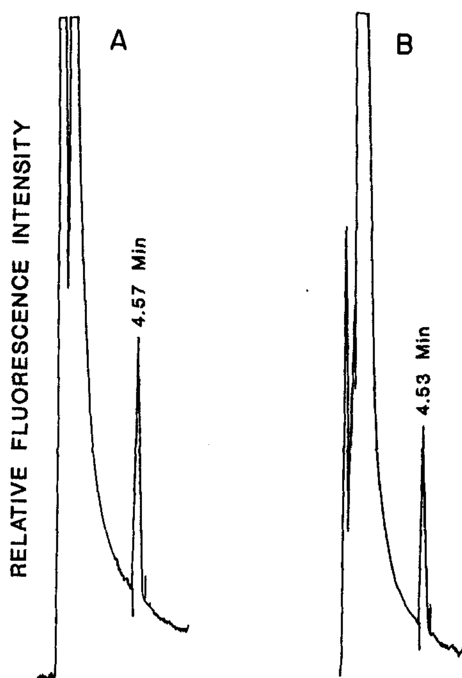

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Fig. 3. HPLC chromatograms showing comparison of solid phase extraction of verapamil from human serum. Column: 10 cm Brownlee Phenyl (5 μm) coupled with RP-18 guard cartridge. Mobile phase A: 50:50 acetonitrile - 0.05 M sodium dihydrogen phosphate, pH 2.5. Flow rate: 1.0 mL/min. Sample size: 20 μL. Detection: fluorescence, λex = 280 nm, λem 310 nm. Key: (A) ISRP; (B) C2 column.

The usefulness of ISRP material as a solid phase extraction matrix for an analyte depends upon: (1), the protein binding of a drug must be adequately disrupted to enable the drug to interact with the ISRP surface; (2), a drug must be retained by the ISRP material long enough to allow serum proteins to be washed from the column; and (3),

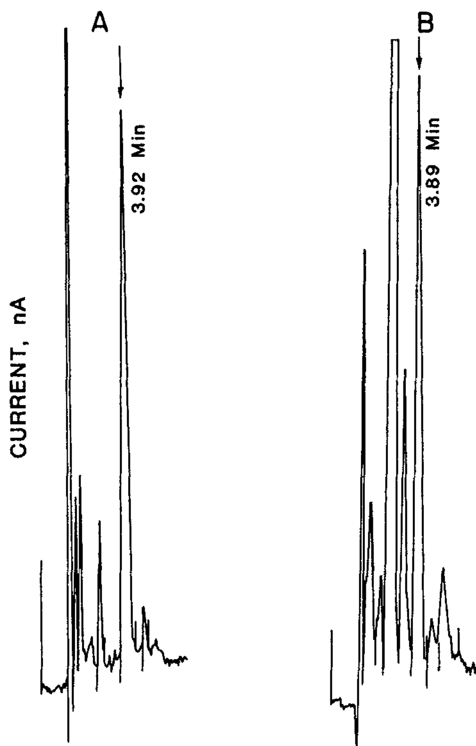

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Fig. 4. HPLC chromatograms showing comparison of solid phase extraction of phenelzine from human serum. Column: 10 cm Brownlee Phenyl (5 μm) coupled with RP-18 guard cartridge. Mobile phase C: 20:80 acetonitrile - 0.05 M NaH_2PO_4, 0.02 M HSA, pH 2.5. Flow rate: 1.0 mL/min. Sample size: 20 μL, Detection: Amperometric, +1300 mV vs Ag/AgCl. Key: (A) ISRP, (B) C18 column.

a drug must be eluted in a relatively small volume of eluent to ensure adequate sensitivity.

Several factors determine whether these three requirements can be met. To obtain a quantitative recovery of highly protein bound drugs when ISRP-HPLC columns are

used, the ionic strength of the mobile phase must be 0.1 M or greater and there should be at least 25% organic solvent present in the mobile phase [8]. Because of the short length of the ISRP-SPE column and its moderate hydrophobicity, some analytes will not be sufficiently retained in the presence of any organic modifier. Using 3% v/v acetonitrile in our phenelzine or tamoxifen serum samples did not appreciably increase their recoveries.

Shibukawa and coworkers [13] found that the injection volume of serum samples greatly influenced the release of bound drug when ISRP-HPLC was employed. When small aliquots (<100 μL) were injected, the drug was released from the proteins and eluted separately as a single peak. When injection volumes of 100 μL or greater were used, the drug eluted as two peaks. The earlier eluting peak represented strongly bound drug molecules which were not quickly released after injection, and the later eluting peak represented free drug and drug molecules which had been quickly released. Solid phase extraction using ISRP material involves a very large sample volume (1-2 mL) being passed through 100-200 mg of packing material. It may be necessary to either greatly reduce the serum sample size or to increase the amount of ISRP material used in the column. When analyzing spiked serum containing verapamil, the latter approach was successful. When 100 mg of ISRP packing was used, recovery was 70-80%; however, when 200 mg of packing were used, a quantitative recovery of verapamil was obtained.

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Pore size and particle size of the ISRP packing are also important. The 40 µm ISRP material used in this study had a nominal pore diameter of 100 A compared to the 54 A median pore diameter of the 5 µm ISRP material used in HPLC columns. The larger pore diameter can result in a greater amount of cleavage of the stationary phase phenylalanine moieties near the openings of the pore. This will present less opportunity for the preferential retention of drug molecules. In addition, there is a greater chance of serum proteins coming into contact with the GLY-PHE-PHE internal surface and blocking pore entrances.

If a drug is to be successfully separated from serum proteins using ISRP material in a solid phase extraction mode, it is imperative that the drug have sufficient opportunity to interact with the hydrophobic phase within the pores. The size of the silica particles used in the production of the ISRP packing will influence the interactions between drug molecules and the GLY-PHE-PHE stationary phase. Many of the factors which adversely affect chromatographic separations may be minimized by simply reducing particle size. In addition, the interstitial volume between the particles is on the order of the same size as the particles themselves [14]. The larger the interstitial volume, the farther a drug molecule must diffuse in order to enter a pore. With ISRP packings, therefore, smaller particles will result in smaller interstitial volumes and greater interaction between unbound drug and internal surface hydrophobic phase. As more and more drug molecules enter pores and are retained, the

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equilibrium between bound and free drug is altered in such a way as to drive the release of more drug molecules. The fact that tamoxifen is quantitatively recovered from serum using a 1 cm guard cartridge of 5 µm ISRP material in an on-line HPLC system, but is carried bound through a solid phase column of 40 µm ISRP packing serves as an example of the importance of particle size.

In conclusion, solid phase extraction using 40 µm ISRP packing has been shown to be useful as a clean-up procedure in the assay of certain basic drugs in serum samples. Varying degrees of success were achieved in extracting the four model drugs from spiked serum samples. In addition, the effects of pH and buffer concentration on the retention of basic compounds have been illustrated. Solid phase extraction columns containing a smaller particle size of ISRP material may prove even more useful as a sample clean-up procedure for highly protein bound drugs in serum.

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