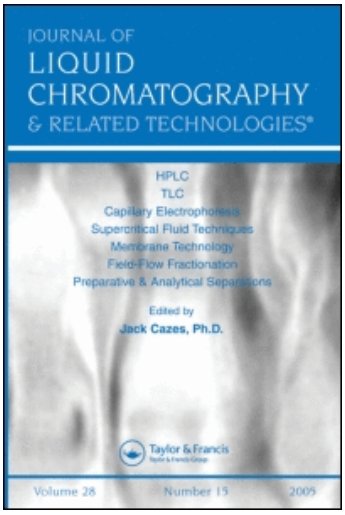

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A Simple Liquid Chromatographic Method for Quantitative Extraction of Hydrophobic Compounds from Aqueous Solutions

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A SIMPLE LIQUID CHROMATOGRAPHIC METHOD FOR QUANTITATIVE EXTRACTION OF HYDROPHOBIC COMPOUNDS FROM AQUEOUS SOLUTIONS

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ABSTRACT

We are reporting a rapid, high-capacity liquid chromatographic method for quantitative extraction and concentration of hydrophobic compounds from biological fluids and aqueous solutions. Samples are injected into commerically-available cartridges (Sep-Pak C^{18R}) containing a microparticulate, reversed phase packing which retains hydrophobic compounds. Inorganic salts and organic hydrophilic contaminants are removed with a water wash. Hydrophobic compounds are eluted quantitatively with minimal volumes (~5 ml) of organic solvents. As demonstrated with radiolabeled taurocholate, thin-layer chromatography, enzymatic fluorimetry and capillary gas chromatography, complete recovery of bile salts from large volumes of urine, serum, amniotic fluid and hydrolysis reaction mixtures was achieved at flow rates up to 20 ml/min. A single cartridge concentrated approximately 50 mg of either taurocholate or the more polar bile salt, taurolithocholate sulfate. The technique is simple and applicable to the isolation of a wide range of hydrophobic compounds from aqueous solutions.

INTRODUCTION

Quantitative isolation of hydrophobic compounds from biological fluids and other aqueous solutions is

technically difficult, time-consuming and often expensive. Liquid extraction requires large volumes of potentially toxic organic solvents and may introduce significant contamination (1). Solid extraction on Amberlite XAD-2 columns (2) necessitates elaborate prepurification of the resin (3,4), copious solvents and careful control of both flow rate and quantity of sample (5). We have developed a method using commercially available cartridges packed with a non-selective octadecylsilyl microparticulate phase (Sep-Pak C^{18R}), for the quantitative solid extraction of bile salts from urine, serum, amniotic fluid and hydrolysis reaction mixtures. The technique is rapid, requires minimal equipment, uses limited volumes of organic solvent and should find wide application in the isolation of hydrophobic compounds from aqueous solutions.

MATERIALS AND METHODS

Sep-Pak C^{18R} bonded cartridges (approximately 1 cm long x 1 cm I.D., with a void volume of $\sim$ 0.44 ml), were purchased from Waters Associates, Inc. (Milford, MA.). Normal urine, urine from patients with liver disease, normal adult serum, and pooled amniotic fluid collected for diagnostic purposes, were used with permission of the Human Experimentation Committee at U.C.S.F.

Recovery was monitored by addition of approximately 4.0×10^5 dpm of (^{3}H) taurocholate to the sample

before extraction except in one experiment where urine (50 ml) was labeled with 3.6×10^4 dpm of (^{35}S) taurolithocholate-3-sulfate. Urine and amniotic fluid were adjusted to pH 4.0 with 4N HCl, and filtered through 0.45 μ millipore filters. Serum was diluted with an equal amount of water. Standard solutions containing 5.0 mg of taurocholate were hydrolyzed by refluxing for 10 hrs at 120° in 6 ml of 15% (w/v) NaOH in 50% aqueous ethanol (5). Extracted amniotic fluid was hydrolyzed as above and solvolyzed for 16 hrs in ethyl acetate which had been equilibrated with 2 N aqueous sulfuric acid (5). After hydrolysis, the alcohol concentration of the hydrolysis mixture was adjusted to 10% by addition of 30 ml water, and the mixture was brought to pH 4.0 with 4N HCl. Previously extracted urine (20 ml) was dried under vacuum, solubilized in 0.5 M Na acetate buffer (pH 5.4) and treated with 50 mg of sulfatase (Sigma Co., St. Louis, MO.) at 37° for 72 hrs (6). The reaction mixture was clarified by the addition of 15 ml of water at the end of incubation.

The total capacity of the extraction system was assessed with 10 ml samples of 1.25 to 25.0 mM taurocholate added to normal urine and with 20 ml samples of unlabeled 12.5 mM taurolithocholate sulfate. The total 3α-hydroxysteroid content before and after extraction of serum standards containing a known amount of bile salt (Seronorm lipid - Accurate Chemical and Scientific

Co., Hicksville, N.Y.), normal adult serum, normal and cholestatic urine, and amniotic fluid, was determined at different dilutions by enzymatic fluorimetry using Sterognost reagents (7). Glass capillary gas chromatography was performed on 25 meter wall-coated open tubular glass capillaries (0.25 mm I.D.) coated with Carbowax 20M. 22-bisnor-5-cholenoic acid (10 µg) was added as internal standard before derivatization. Peaks were identified by relative retention time, and quantitation was by peak height relative to height of the internal standard. All results are reported as mean±standard deviation (S.D.).

Procedure

A Sep-Pak C^{18R} cartridge was connected to a glass syringe and washed sequentially with 2.0 ml of methanol and 5.0 ml of water. The sample was placed in the syringe and injected through the cartridge. When volume exceeded 100 ml, the sample was drawn through the cartridge by suction. Flow rates up to 20 ml/min were used. After injection of the sample, the cartridge was washed with 10-20 ml of water. Bile salts were eluted by pumping 5.0 ml of methanol through the cartridge. Following elution of bile salts, the cartridges were washed with additional methanol (5-10 ml) and regenerated with water for further use. In

experiments in which unlabeled taurolithocholate sulfate was passed through the cartridge, the sample effluent and the water wash were acidified and extracted with ethyl acetate (1). Aliquots of the ethyl acetate extracts and of the methanol effluent were analyzed by thin layer chromatography on nanograde silica gel G using a $CHCl_3$:MeOH:HAc:H_2O (65:24:15:9) solvent system (8). 10% phosphomolybdic acid in ethanol was used for visualization. Aliquots of methanol extracts of biological fluids were dried under nitrogen and dissolved in 0.15 M NaCl for enzymatic fluorimetry. After hydrolysis/solvolysis, an aliquot of amniotic fluid extract was methylated with diazomethane and trimethylsilylated with pyridine-hexamethyldisilazane-trimethylchlorosilane (5) for capillary gas chromatography.

RESULTS AND DISCUSSION

Table 1 demonstrates that taurocholate was isolated quantitatively from urine, serum, amniotic fluid and bile salt hydrolysates by the Sep-Pak C^{18R} procedure. Generally, less than 5% of radiolabeled taurocholate was lost in the sample effluent. Losses in the water wash never exceeded 1%. Elution of over 90% of the radioactivity added to the sample was achieved with 2.0 ml of methanol while 5.0 ml gave complete recovery.

TABLE 1
Recovery of Taurocholate from Biological Solutions and Analytical Reaction Mixtures

SAMPLE	VOLUME (ml)	RECOVERY (percent±SD)
Urine	20 (4)	99±4
	50 (6)	96±4
	500 (1)	95
	750 (1)	92
Serum	5 (6)	99±3
Amniotic Fluid	30 (5)	94±2
Hydrolysis Reaction[a]	80 (5)	94±5
Sulfatase Reaction[b]	25 (2)	94±3

Numbers in parentheses represent individual determinations.
[a]Hydrolsis of 5.0 mg taurocholate.
[b]Sulfatase treatment of 20 ml of extracted urine as described in text.

Even large volumes of urine (over 750 ml) could be extracted without significant loss. Both unaltered taurocholate and released cholic acid were recovered from the hydrolysates.

The more polar bile salt, taurolithocholate sulfate, was also quantitatively extracted from urine by the Sep-Pak procedure. When 6 50 ml urine samples containing labeled taurolithocholate sulfate were extracted, the average recovery was 95%±5%.

As shown in Table 2, flow rates up to 20 ml/min could be applied to the cartridge without significant loss of bile salt.

These results clearly show that Sep-Pak C^{18R} cartridges can be used for rapid, quantitative extrac-

TABLE 2
Effect of Flow Rate through Sep-Pak C^{18R} Cartridges on Recovery of (^{3}H) Taurocholate from 20 ml of Urine

FLOW RATE (ml/min)	RECOVERY (percent±SD)
2.0 (3)	100±2
10.0 (6)	95±2
20.0 (6)	94±1
50.0 (3)	86±4

Numbers in parentheses represent individual determinations.

tion and concentration of even relatively polar and acidic hydrophobic compunds such as bile salts from aqueous solutions. Considerable purification is accomplished in the washing step which removes organic hydrophilic impurities and inorganic salts. The procedure is simple and inexpensive since Sep-Pak cartridges can be reused, limited volumes of organic solvent are required and no elaborate equipment is necessary.

The excellent recovery in the hydrolysis and sulfatase experiments indicates that Sep-Pak C^{18R} cartridges are suitable for extraction of hydrophobic compounds from aqueous reaction mixtures used in analytical procedures. Such extractions are usually performed by solvent partitioning methods requiring large quantities of solvent, and are frequently complicated by formation of intractable emulsions. Liquid chromatography on Sep-Pak C^{18R} cartridges eliminates these disadvantages.

TABLE 3
Recovery of Taurocholate at Various Concentrations in Normal Urine

TAUROCHOLATE SOLUTION (μM)	RECOVERY[a] (percent)
1.25	97.0
2.5	97.5
5.0	95.5
7.5	98.5
10.0	96.5
12.5	97.0
25.0	52.0

[a]Means of duplicate determinations.

Ten ml of a 12.5 mM solution of taurocholate in normal urine could be passed through a single cartridge without significant loss (Table 3). Since only 52% of a 25.0 mM solution was retained on the cartridge, the total capacity of a single cartridge for bile salts in urine must be in the range of 50 mg.

By comparing the densities of spots, with known standards, after thin layer chromatography of the sample effluent, water wash and methanol extract, it was estimated that over 95% of a 50 mg sample of taurolithocholate sulfate was extracted whereas less than 2% was lost in the procedure.

The high capacity of a single C^{18} Sep-Pak^{R} cartridge easily covers the range for complete extraction of hydrophobic substances in standard collections of biological fluids, such as 24 hr urine collections. Sufficient amounts of materials can be rapidly prepared

TABLE 4
3α-Hydroxysteroid Content of Biological Fluids before and after Extraction on Sep-Pak C^{18R} Cartridges

SAMPLE	BEFORE EXTRACTION (μM±SD)	AFTER EXTRACTION (μM±SD)
Seronorm Lipid (8)	26.8±1.6	25.0±1.8
Adult Serum (4)	4.5±0.2	4.4±0.2
Adult Urine (6)	5.1±1.2	5.0±0.5
Cholestatic Urine (2)	36.0	37.0
Amniotic Fluid (2)	5.0	5.0

Numbers in parentheses represent individual determinations.

by this procedure for structural analyses of unidentified compounds by techniques which require substantial quantities of material (e.g., nuclear magnetic resonance, infra-red and mass spectrometry).

The 3α-hydroxy steroid content of Seronorm lipid, normal serum, normal and cholestatic urine, and amniotic fluid, determined by enzymatic fluorimetry before and after Sep-Pak C^{18R} liquid chromatographic extraction is shown in Table 4. In all cases, the calculated molarities were comparable to those of the original samples, indicating that this isolation technique can be used for routine concentration of samples which might otherwise be too dilute for measurement.

As shown in Figure 1, the bile acids, cholic, deoxycholic, chenodeoxycholic, lithocholic and 3β-hydroxy-5-cholenoic acid from extracted amniotic fluid were well-separated by capillary gas chromatography.

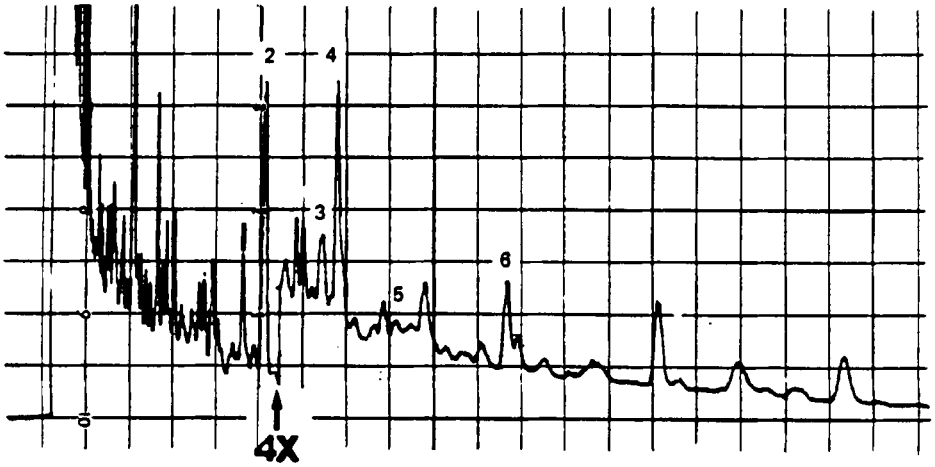

FIGURE 1. Capillary gas chromatograph of bile acids (as methyl/ester trimethylsilyl ethers) from amniotic fluid. Column temperature 230°. The arrow indicates a 4-fold increase in sensitivity. The bile acid peaks are numbered as follows: cholic acid, 1; 22 bisnor-3β-hydroxy-5-cholenoic acid (internal standard), 2; deoxycholic acid, 3; chenodeoxycholic acid, 4; lithocholic acid, 5; 3β-hydroxy-5-cholenoic acid, 6.

Several as yet unidentified compounds in amniotic fluid which may be physiologically important are presently being analyzed by mass spectrometry. The molar concentration of bile acid present in the pooled amniotic fluid samples based on the gas chromatographic data was 2.5 μM/l. The percent molar concentration contributed by cholic acid was 47%, deoxycholic 9.7%, chenodeoxycholic 27%, lithocholic acid 3% and 3β-hydroxy-5-cholenoic acid 21.7%. These values are consistent with those reported by Deleze et al. obtained with conventional extraction procedures (9).

Extraction and purification from aqueous solution can be performed by liquid chromatography on Sep-Pak C^{18R} cartridges for any compound which is sufficiently hydrophobic to be retained on the packing material. For example, we are currently evaluating the extraction of urinary steroids and steroid conjugates. The simplicity, rapidity, high capacity and broad range of Sep-Pak C^{18R} extraction combined with relatively low expenditures for cartridges and organic solvents suggest that the procedure described in this paper should be broadly applicable for separation of hydrophobic compounds from aqueous solutions of biological, environmental, or analytical origins.

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