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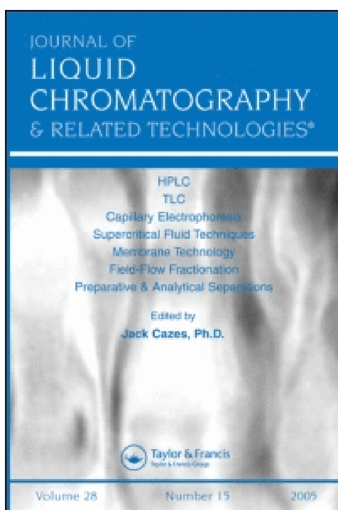

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Ghoos, Yvo , Rutgeerts, Paul and Vantrappen, Gaston(1982) 'The use of SEP-PAK™ C_{18} Cartridges in the Preparation of Bile Acid Methyl Ester Acetates', Journal of Liquid Chromatography & Related Technologies, 5: 1, 175 — 186

To link to this Article: DOI: 10.1080/01483918208068829

URL: http://dx.doi.org/10.1080/01483918208068829

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JOURNAL OF LIQUID CHROMATOGRAPHY, 5(1), 175-186 (1982)

THE USE OF SEP-PAK™ C_{18} CARTRIDGES IN THE PREPARATION OF BILE ACID METHYL ESTER ACETATES

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ABSTRACT

A method is described for the rapid and quantitative extraction of bile acid derivates by Sep-Pak™ C_{18} cartridge. The method is used for the preparation of bile acid methyl ester acetates. The method was validated by determining the efficiency and the recovery of radiolabelled taurine-conjugated and free bile acids and of bile acid containing biological samples, by thin-layer chromatography with zonal scanning after each step and by internal standardization withing the gas-chromatographic analysis. The recovery of bile acids after hydrolysis amounted to 93.7% ± 2.7%, 97.7% ± 3.6% and 100% ± 2.3% for gallbladder bile, serum bile and mixtures of pure bile acids resp. The recovery of cholic acid methyl ester acetate and chenodeoxycholic acid methyl ester acetate after the entire procedure, including hydrolysis, Sep-Pak™-extraction, methylation, acetylation and again Sep-Pak™ - extraction, amounted to 85.6% ± 4.6%, 88.4% ± 5.3% and 89.3% ± 3.5% for gallbladder bile acids, serum bile acids and bile acid mixtures resp. It is concluded that Sep-Pak™ can efficiently be used in the preparation of bile acid methyl ester acetates, thereby avoiding time-consuming and inconsistent extractions.

INTRODUCTION

Since the introduction of reversed phase chromatography, XAD-2 columns have been used by several authors for extraction of bile acids from biological fluids (1,2). This extraction is

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time-consuming, because the resin needs prepurification and large volumes of solvents. The quantitative isolation of hydrophobic or semi-hydrophobic compounds from biological media has become simpler and easier since a non-selective octadecyl-silyl microparticulate phase (Sep-Pak™ C_{18}) has become available. The Sep-Pak™ C_{18} cartridge has been used for the extraction of free and conjugated steroids from urine (3), and for the extraction and concentration of bile acids from biological fluids (4). The Sep-Pak™ C_{18} cartridge resulted in a very good recovery of bile acids in analytical procedures i.e. in the preparation of bile acid methyl ester trimethylsilyl ethers (5) for gas-liquid chromatography. We studied the usefulness of the Sep-Pak™ C_{18} cartridge in the preparation of the bile acid methyl ester acetate. This bile acid derivative is the chemical form of choice for mass spectroscopy (6) because it it stable, has a low molecular weight and a low isotopic complexicity. The Sep-Pak™ C_{18} cartridge seemed to be the appropriate resin to overcome the long-lasting and error-inducing liquid-extractions, needed after the hydrolysis and the acetylation steps. Moreover, we studied the possibility to hydrolyse directly the bile acids in the biological sample without prior separation.

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MATERIALS AND METHODS

The Resin

The Sep-Pak™ C_{18} cartridge (Waters Associates, Milford,MA) is a small cylindrical column (1cm x 1cm), containing an octadecylsilane bounded phase packing, retained between filters. The Sep-Pak™ C_{18} cartridges were primed with 5 ml of methanol, followed by 5 ml of water. Since the same cartridge was used for multiple extractions (i.e. after hydrolysis, and after acetylation) of the same sample, the cartridge was reconditionned between the extractions in the same manner as described above.

The flow was always maintained at 2 ml/min. with the aid of a Harvard pump,model 954 (Millis, Mass., USA).

Thin-layer Chromatography Systems (TLC)

Tauro- and glyco- conjugates were separated from free bile acids in the solvent system : propionic acid - isoamyl-acetate - propanol - water (15/20/10/5) (i.e. solvent A) (7). Bile acid methyl ester acetates were run in the solvent system : heptane - ether (60/40) (i.e. solvent B) (8). The TLC-support in all cases was Kieselgel G-plates (Merck, Darmstadt, W. Germany).

Enzymes and enzymatic Procedures

Bile acids were assayed quantitatively by the enzyme 3α -hydroxysteroid dehydrogenase (E.C. 1.1.1.51), type STDHMP, (Worthington, Freehold, N.J., U.S.A.) following a slight modification of the method of Koss et al. (9). Bile acids were deconjugated enzymatically by the enzyme cholylglycine hydrolase (E.C. 3.4.22.8.), lyophilized powder (35-50 Units/mg solid) (Sigma, St. Louis, Miss.). To hydrolyse the bile acids 0.1 ml of an enzyme solution (14 mg enzyme in 1 ml 0.2 M acetate buffer pH 5.6) was used.

Gas-liquid Chromatography

Gas-liquid chromatography was carried out in a gas-chromatograph Packard-Becker, model 433 (Delft, The Netherlands). The apparatus is equipped with a flame ionization detector and the separation is achieved on a Gas-Chrom Q 100-120 mesh column, activated by 3% OV-17.23-Nordeoxycholic acid was used as internal standard (10).

Bile acids and organic Solvents

The following bile acids were purchased from Steraloids (Wilton, N.H.) : 5β -cholanic acid- 3α , 7α , 12α -triol

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N-(2-sulfoethyl)-amide(=TC), 5β -cholanic acid - 3α , 7α -diol N-(2-sulfoethyl)-amide(=TCDC), 5β -cholanic acid - 3α -ol N-(2-sulfoethyl)-amide(=TLC), 5β -cholanic acid - 3α ,7α , 12α triol N- (carboxymethyl)-amide (=GC), 5β -cholanic acid - 3α , 7α -diol N- (carboxymethyl)-amide (=GCDC), 5β -cholanic acid - 3α , 12α -diol N- (carboxymethyl)-amide (=GDC), 5β - cholanic acid - 3α -ol N- (carboxymethyl) amide (=GLC) and 23- nor - 5β -cholanic acid - 3α , 12α -diol (=nor-DC). All compounds were controlled for purity on TLC in the solvent A.

The organic solvents, analytical grade, were UCB-products (Brussels, Belgium), and were used without further purification.

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Radiolabeled Bile Acids

Tauro-(carboxyl)-^{14}C cholic acid and (11,12(n)-^{3}H) cheno-deoxycholic acid were purchased from the Radiochemical Centre (Amersham, Eng.) . The products were controlled for chemical identity by thin-layer chromatography on Kieselgel-G plates (Merck, Darmstadt, W. Germany) in the solvent system A and for radiochemical purity by zonal scanning (Berthold, model LB 2723, W. Germany). The compounds were found to be pure for more than 99%. Radioactivity was counted in a liquid scintillation counter (Packard, model 2450 Pale Alto, Ill.). ^{3}H- and ^{14}C-activities were separated by oxidyzing the samples prior to counting (Packard, oxidizer, model 306).

Biological Samples

Bile samples were obtained by duodenal intubation and stimulation of gallbladder contraction by intravenous injection of Cerulein (Montedison) 0.0064 μg/kg body weight in 10 normals.

Procedure

Preparation of the Samples

40 mMolar, 20 mMolar and 10 mMolar solutions of all previously mentionned conjugated bile acids were prepared. To 1 ml solution 18.5 kBq (=0.5 µCi) tauro-(carboxyl-^{14}C)-cholic acid and 18.5 kBq (=0.5 µCi) ^{3}H-chenodeoxycholic acid were added. 200 µl of each solution was taken to prepare the methyl ester acetate of the bile acid. To 2 ml serum or 200 µl duodenal aspirate the same amount of radioactive labeled bile acid was added.

Multistep Procedure in the Preparation of Bile Acid Methyl Ester Acetates

The basic concepts for hydrolysis of bile acids were introduced by Nair et al. (11) and further developped by Ross et al. (12). We used the acetate buffer medium as proposed by van Berge Henegouwen et al. (13) and we adapted the enzyme concentration and the incubation time. The serum samples were diluted with 5 ml 0.2 M acetate buffer (pH 5.6), and the pH was adjusted to 5.6 with 0.02 N HCl. The 200 µl duodenal samples were also diluted with 5 ml 0.2 M acetate buffer (pH 5.6), and EDTA and β -mercaptoethanol were added (20 µmoles of each) to activate enzymatic hydrolysis.
The solution was made up with 0.1 ml of the cholylglycine hydrolase solution, and incubated overnight at 37°C in a shaking bath, and brought at pH 3-4 with 0.2 N HCl, and the bile acid solutions were passed over a Sep-Pak™ C_{18} cartridge for concentrating the free bile acids on the resin. The free bile acids were then extracted from the cartridge with 10 ml methanol, dried under nitrogen stream, and redissolved in 1 ml of methanol. To check the efficiency of the procedure an aliquot of this methanolic solution was taken for counting the radioactivity. Another aliquot was run on TLC in the solvent A and scanned for

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radioactivity. For the methylation 0.5 ml of the methanol solution was dried, the bile acids were methylated with etherea diazomethane. The dried bile acid methyl esters were acetylated with 1 ml of a solution composed of 5 ml of acetic acid, 5 ml of acetic anhydride and 0.05 ml of perchloric acid (10). After 15' at 25°C in a shaking bath, 10 ml of 20% NaCl was added and the pH was brought to 3-4. The solution was passed through the cartridge for the second time and the bile acid methyl ester acetates were regained by a 5 ml methanol wash. This solution was dried, and the bile acid esters were dissolved in 1 ml of acetone. An aliquot was taken for counting radioactivity, another part was screened by TLC in the solvent B and by subsequent zonal scanning, 2 µl was injected for gaschromatographic analysis.

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RESULTS

Recovery Studies

The results on the efficiency of the hydrolysis and the recovery of the bile acids, as controlled by thin-layer chromatography, radioactivity counting and enzymatic assay, are summarized in table 1. The free bile acids were extracted with the cartridge from different media i.e. bile acid mixtures, serum and gallbladder bile. After hydrolysis and extraction no conjugated bile acids could be detected on thin-layer chromatography.

Table 2 shows the recovery of the bile acid methyl ester acetates after extraction by Sep-Pak™ C_{18}, as controlled by thin-layer chromatography (for chemical purity) and counting of the radioactivity present on ^{14}C-cholic and ^{3}H-chenodeoxycholic methyl ester acetates. The results are given as cumulative values (i.e. the losses induced by the previous steps are included) and are expressed in mean ± s.d.

TABLE 1

Hydrolysis and Recovery of Bile Acids : mean ± s.d.

Control	Bile acid mixture (n=32)	Serum (n=10)	Gallbladder bile (n=10)
Radioactivity counting	100 ± 2.3	97.7 ± 3.6	93.7 ± 2.7
3α -hydroxysteroid dehydrogenase	97.6 ± 1.8	96.8 ± 4.8	93.6 ± 3.1

TABLE 2

Recovery of Bile Acid Methyl Ester Acetates: mean ± s.d.

Control	Bile acid mixture (n=20)	Serum (n=10)	Gallbladder bile (n=10)
Radioactivity counting	89.3 ± 3.5	88.4 ± 5.3	85.6 ± 4.6

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The recoveries of ^{14}C-cholic acid methyl ester acetate and of ^{3}H-chenodeoxycholic acid methyl ester acetate were equal. Zonal scanning revealed that less than 1% radioactivity was present as unidentified compounds. Eluants, other than the methanol fraction (i.e. ethanol, acetone) accounted for less than 1% also. The Sep-Pak^{TM} resin itself was not controlled for radioactivity.

Gas-chromatographical Analysis

Figure 1 shows a typical gas-chromatogram. The main bile acids are well separated from the internal standard, 23-nor-deoxycholic acid.

Reproducibility Study

Three gallbladder bile samples (200 µl each) not included in the results mentionned above, were done in triplicate.

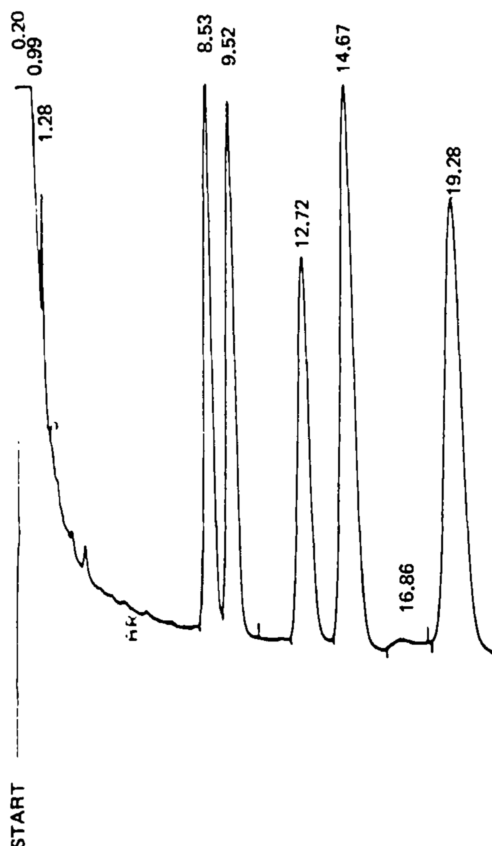

FIGURE 1. Gas chromatograph of bile acid methyl ester acetates on Gas-Chrom Q (100-200 mesh) column, 3% OV-17. From top to the bottom can be seen :lithocholic acid (8.53 min), 23-nor-deoxycholic acid (9.52 min), deoxycholic acid (12.72 min), chenodeoxycholic acid (14.67 min) and cholic acid (19.28 min).

The coefficient of variation was not greater than 3%, and the mean recovery of the bile acids as methyl ester acetates was 86.0 $\pm$ 2.9. The original concentrations of the bile samples, as determined by the 3α -hydroxysteroid dehydrogenase method, was 10.1, 13.7 and 20.1 mmol respectively.

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DISCUSSIONS

Gas-chromatographic analysis of individual bile acids is a valuable tool for the study of malabsorption syndromes and liver diseases. Several bile acid derivatives have been used in these studies, such as trifluoroacetates (13), methylestertrimethylsilyl ether (2), heptafluoroburyrates (14) and permethylated derivatives (15). The bile acid methyl ester acetates, are known to be very stable and hence most suitable to be used as reference compounds and for reproducibility studies and mass-spectrometric analysis. The preparation procedure however is cumbersone and the reproducibility of the results is delicate because at least two extractions are needed.

The present study indicates that the extractions needed in the conventional method for the synthesis of bile acid methyl ester acetates can be replaced by the use of Sep-Pak™ C_{18} cartridges. The extraction rate of the bile acids proved to be excellent. Thin-layer chromatography showed complete hydrolysis : Sep-Pak™ recovery rate on the basis of radiolabelled taurocholic acid is 100%. The enzymatic method gives a slightly lower yield. This is not surprising because the enzymatic method measures all bile acids including free bile acid which are not so reactive with the enzyme as conjugated bile acids.

The enzyme, cholylglycine hydrolase, was used in a very low concentration because it is so expensive that a long (overnight) incubation was preferred over a higher enzyme concentration. The results obtained with the gallbladder bile samples (200 µl) indicate that the selected conditions are sufficient for routine analysis of biological samples. After the extraction of the free bile acids the Sep-Pak™ C_{18} cartridges can again be used for the extraction of the bile acid methyl ester acetates. The very high coincidence of

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extraction yield between standard bile acid solution, serum samples and gallbladder aspirates proves that the method is very reproducible and reliable; although about 10% of the compound is lost during the methylation-acetylation procedure. Unidentified side-products and the eluants only account for about 2%. This means that 8% of the radioactivity, derived from either ^{14}C-cholic acid or ^{3}H-chenodeoxycholic acid, could not be demonstrated. We assume that this amount sticks to the resin, although further extraction with methanol does not reveal any activity. Soot formation in the oxidizer inhibited complete exploration of the resin.

The parallelism between ^{14}C-cholic acid and ^{3}H-chenodeoxycholic acid indicates that the extraction method we used does not introduce any enrichment for cholic acid or chenodeoxycholic acid. The gas-liquid chromatography of the bile acid methyl ester acetates versus nor-deoxycholic acid methyl ester acetate as internal standard shows that this parallelisme can be extended to all bile acid studied.

We conclude that the Sep-Pak^{TM} C_{18} cartridge is very useful in the preparation of bile acid methyl ester acetates : the procedure is clean, does not require large amounts of organic solvents,is reproducible and reliable. The method can be standardized, and yields bile acid compounds that are suitable for storage or further spectroscopic analysis.

ACKNOWLEDGEMENTS

This research was supported by grants from the FWGO, Brussels, Belgium. Mrs. S. Rutten, Miss. L. Swinnen and Mr. L. Verhaegen provided excellent technical assistance.
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