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Note

Purification of methylated bile acids recovered from thin-layer chromatograms on silica gel-sintered plates

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Highly purified samples are required for identification by IR spectrophotometry and mass spectrometry; however, it is difficult to purify small amounts of bile acids from biological samples by crystallization. Thin-layer chromatography (TLC) is a convenient technique for separation, but samples recovered from silica gel after TLC seldom provide satisfactory spectra. Extraction from the gel with large volumes of organic solvents often results in contamination with impurities from the solvents and from the gel, and extraction with small volumes of organic solvents may lead to poor recovery of the samples.

Purification of the methyl esters of bile acids by chromatography on organic materials or materials containing organic compounds also leads to poor IR spectra, especially with small samples and a micro-disc technique.

In the present study, purification of the methyl esters of bile acids eluted from silica gel after TLC is carried out on silica gel-sintered plates (SSP)[1]. The method is useful for preparing small amounts (50–100 μg) of samples for IR spectral analysis, gives good recoveries, requires only small volumes of organic solvents and is suitable for repeated use.

MATERIALS AND METHODS

[24-^{14}C]Cholic acid and [24-^{14}C]deoxycholic acid of purity (assessed by TLC and autoradiography) greater than 98%, were obtained from The Radiochemical Centre (Amersham, Great Britain) and International Chemical and Nuclear Corp. (Cleveland, Ohio, U.S.A.), respectively.

Lithocholic and chenodeoxycholic acids were obtained from Weddel Pharmaceuticals (London, Great Britain), and deoxycholic and cholic acids were kindly provided from Professor Haslewood's collection. The methyl esters were prepared with diazomethane.

The purity of the methyl esters was assessed by GLC in a Pye Series 104 dual flame ionization chromatograph with a coiled glass column packed with 3% of poly-

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sulfone on Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.) and by TLC on silica gel G and H (E. Merck, Darmstadt, G.F.R.), with benzene–isopropanol–acetic acid (15:5:1, v/v), modified from Eneroth[2], as developing solvent.

Quantitative assessment was by GLC and by measurement of radioactivity in a liquid scintillation counter (No. 8312; Nuclear Enterprises, Edinburgh, Great Britain).

Aluminium oxide (neutral) was obtained from M. Woelm (Eschwege, G.F.R.) and Sephadex LH-20 from Pharmacia (Uppsala, Sweden).

SSP (re-usable TLC plates) were obtained from Reeve Angel (London, Great Britain). They were soaked in a chromic acid–sulphuric acid bath for 2 h, then the acid was washed out by running water for 30 min. After a final wash with distilled water, the plates were dried in air and then over silica gel.

Solvents for chromatography were redistilled at intervals of 4–6 weeks; methanol and ethanol were redistilled over solid potassium hydroxide.

IR spectroscopy

The material (50–100 μg) in methanol (20 μl) was applied to powdered potassium bromide (15–20 mg) and dried at 40° for 30 min; the mixture was then repowdered and pressed into a disc (diameter, 1.5 mm) in the usual way. Spectra were recorded with a Perkin-Elmer Model 157G spectrophotometer.

Recovery of bile acid methyl esters from silica gels G and H

A 100-μg portion of each bile acid was applied, in as narrow and even a band as possible, about 1.5 cm from one end of the plate (20 × 5 cm; 0.25 mm thick) and the chromatograms were developed with benzene–isopropanol–acetic acid (15:5:1, v/v), dried and allowed to stand in iodine vapour. The defined zones were scraped off, and the esters were eluted from the gel with 4 ml, and then 2 ml of solvent. The mixture from each elution was centrifuged at 1200 *g* for 15 min, and the combined supernatant solutions were evaporated to dryness in a stream of nitrogen.

Purification of bile acid methyl esters on SSP

The SSP (5 × 5 cm, 0.2 mm thick), treated as described above, were eluted with 10 ml of methanol by the descending technique in a tank (25 cm high × 15 cm I.D.). The plate was connected with a glass solvent reservoir (7.0 × 1.5 × 1.5 cm) through filter paper (7.5 × 5 cm, Whatman Grade 113; W. & R. Balston, Maidstone, Great Britain), about 1 cm of one end of which was sandwiched between a piece of glass plate (5 × 1 cm) and sintered silica gel on to the SSP and fixed with a pair of metal clips.

After air-drying of the washed plate, each bile acid ester of those recovered from the silica gel (up to 1.0 mg) in methanol (20–40 μl) was applied, in as narrow and even a band as possible, about 1.5 cm from one end. Another SSP (15 × 5 cm, 0.2 mm thick) was connected layer-to-layer about 1 cm from the end opposite to the loaded end and fixed with a pair of metal clips.

The combined plate was developed with benzene until the solvent front had run about 15 cm. After air-drying, the 5 × 5-cm plate was placed with its loaded end in a glass solvent reservoir (7.0 × 1.5 × 1.5 cm) in the tank; 5.0 ml of ethyl acetate–methanol (100:1, v/v) was pipetted into the solvent reservoir, and the tank was closed.

Chromatography was stopped when the solvent front reached the top of the plate. After air-drying, the plate was eluted in the tank with 4.5 ml of ethyl acetate–methanol (25:1, v/v) by the method described for washing with methanol; for small samples, the plate was so cut as to form a point at the end remote from the start line. The eluate was collected through a Y-shaped funnel, whose outlet was sharpened and so angled that it was able to touch the inside wall of a small test tube (7.5 cm × 8 mm I.D.). Whatman Grade 113 filter paper gave a flow-rate suitable for elution. The resulting eluate was evaporated to dryness in a stream of nitrogen.

RESULTS AND DISCUSSION

Recovery of bile acid methyl esters from silica gels G and H with various solvents

The results are listed in Table I and show that methanol is a suitable solvent for elution, giving constant recovery of the esters from the gel; similar results were found for esters eluted from silica gel H.

TABLE I

RECOVERY OF BILE ACID METHYL ESTERS ELUTED FROM SILICA GEL G WITH VARIOUS SOLVENTS AFTER TLC

Values are the means ± standard errors from three experiments.

Compound	*Recovery (%)*		
	Methanol	*Ethyl acetate*	*Diethyl ether*
Methyl [24-^{14}C]cholate	95.0 ± 2.4	77.5 ± 3.0	14.5 ± 0.3
Methyl [24-^{14}C]deoxycholate	96.0 ± 2.1	87.4 ± 2.6	60.9 ± 2.5
Methyl lithocholate	98.0 ± 4.0	90.4 ± 4.6	100.7 ± 3.5

Recovery of bile acid methyl esters by purification on SSP

From 5–100 μg of each bile acid methyl ester were subjected to the purification procedure described above; the over-all recoveries are shown in Table II.

Purification of bile acid methyl esters recovered from silica gels G and H on SSP

From 50–100 μg of each bile acid methyl ester recovered from silica gels G and H were subjected to purification as described above on SSP.

TABLE II

RECOVERY OF BILE ACID METHYL ESTERS BY PURIFICATION ON SSP

Values are means ± standard errors from three experiments.

Compound	*Amount loaded (μg)*	*Recovery (%)*
Methyl [24-^{14}C]cholate	100	98.0 ± 3.4
	20	98.1 ± 3.2
	5	97.0 ± 2.4
Methyl [24-^{14}C]deoxycholate	100	98.5 ± 2.1
	20	97.5 ± 3.0
	5	97.3 ± 2.3
Methyl lithocholate	100	98.1 ± 2.0
	20	98.0 ± 1.5

The IR spectra of samples so purified are shown in Fig. 1 in comparison with spectra obtained before and after purification of a standard sample on a column of aluminium oxide according to Sandberg *et al*[3]. The method provided good samples for IR spectral analysis. Similar results were obtained with methyl lithocholate, chenodeoxycholate and deoxycholate after recovery from silica gels and purification on SSP.

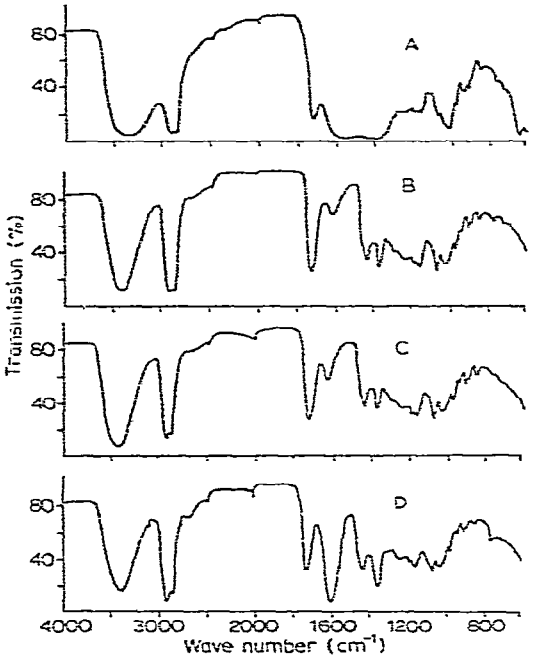

Fig. 1. IR spectra of methyl cholate recovered from silica gel G before and after purification on SSP. (A) Methyl cholate recovered from silica gel G; (B) after purification on SSP; (C) standard methyl cholate; (D) standard methyl cholate after purification on a column of aluminium oxide. Spectra were recorded from a micro-disc made from a mixture of each sample (about 100 μg) with powdered potassium bromide (20 mg).

The bile acid methyl esters recovered from silica gel H showed less absorption in the region 1300–1700 cm^{-1} than did those recovered from silica gel G.

The preliminary development with benzene was carried out to remove compounds less polar than methyl lithocholate, but this process was not usually required.

Purified bile acid methyl esters eluted from a column of aluminium oxide exhibited intense absorption in the region 1530–1670 cm^{-1} and some absorption in the regions 1340–1470 cm^{-1} and 750–780 cm^{-1}. Similarly, a sample eluted from a column of Sephadex LH-20 according to the method of Haslewood and Haslewood[4] exhibited intense absorption in the region 1500–1800 cm^{-1}, as reported, and considerable absorption in the regions 990–1060 cm^{-1} and 910–930 cm^{-1}.

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