notes on methodology

Extraction of bile acids from rat feces containing cholestyramine

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SUMMARY The fecal extraction procedure described by Evrard and Janssen (1) was inadequate for the complete extraction of conjugated bile acids from feces containing the bile acid sequestrant, cholestyramine. As judged by gas–liquid chromatographic analysis, substitution of 0.5 N HCl in absolute ethanol for glacial acetic acid allowed for complete recovery (98–104%) of three different conjugated bile salts in the presence of the resin.

SUPPLEMENTARY KEY WORDS trifluoroacetates · OV-210 · gas–liquid chromatography

THE METHOD of Evrard and Janssen (1) was used to measure fecal bile acid excretion in several animal species on various dietary regimens, some of which included the bile acid sequestrant, cholestyramine, an insoluble quarternary ammonium anion-exchange resin. However, when known amounts of conjugated bile salts were added to feces of animals fed diets containing cholestyramine, only 35–50% of the bile salts were recovered. Taurine-conjugated bile salts yielded the lowest recovery.

In studies with gallbladder bile, Gordon, Kuksis, and Beveridge (2) noted that acetic acid in ethanol displaced free bile acids but not taurine-conjugated bile acids from Dowex 1 columns. Since Dowex 1 and cholestyramine are quite similar, these observations suggested that the conjugated bile salts, especially taurine conjugates, were not being eluted from the bile acid sequestrant. Therefore, the method of fecal bile acid extraction was examined and an extraction procedure defined that would assure quantitative recovery of all conjugated bile salts.

Procedure. The procedure of Evrard and Janssen (1) for extracting bile acids from feces was modified by substituting 0.5 N HCl in absolute ethanol for the glacial acetic acid. After several trials the following procedure was adopted.

All solvents used were distilled in glass (Burdick & Jackson Laboratories, Muskegon, Mich.). An aliquot of freeze-dried ground feces was accurately weighed (0.25–1 g) into a 16 × 150 mm screw-capped (Teflon-lined) test tube. 1 mg of internal standard, 23-nordeoxycholic acid, and 5 ml of the 0.5 N HCl in absolute ethanol were added to the feces. The tube was capped, agitated for 15 sec, and incubated in a water bath at 37°C for 1 hr with repeated agitation at 15-min intervals. The extract was clarified by centrifugation at 1,000 *g* for 10 min and the liquid was transferred to a 14 × 100 mm screw-capped test tube. 5 ml of the 0.5 N ethanolic HCl was added to the fecal residue and the extraction was repeated. The pooled extract was dried under partial vacuum at 25°C and stored overnight at 4°C. The saponification and subsequent extraction of the bile acids with diethyl ether was completed as described by Evrard and Janssen (1).

The methyl esters of the extracted bile acids were prepared by reacting the bile acids with dry HCl in methanol (5%, w/v) overnight at room temperature. After the HCl and methanol were removed by evaporation with nitrogen, the trifluoroacetates were formed by reacting the bile acid methyl esters with 0.5 ml of trifluoroacetic anhydride (>99% purity, Pierce Chemical Co., Rockford, Ill.) for 30 min at 37°C (3). The excess reagents were removed by evaporation with nitrogen and the trifluoroacetates were resuspended in carbon disulfide.

The gas–liquid chromatographic conditions used were similar to those reported by Kuksis and Gordon (4). However, after some experimentation it was found that superior chromatograms were achieved when a 3% OV-210 phase on 80–100 mesh Supelcoport (Supelco, Inc., Bellefonte, Pa.) was substituted for the QF-1 phase. The gas chromatograph was a Hewlett-Packard (Avondale, Pa.) model 402 equipped with a digital integrator and dual hydrogen flame detectors. The glass columns (1/4 inch O.D., 6 ft U tube) were silanized with freshly prepared 10% dimethylchlorosilane in toluene (v/v). After washing with toluene, the columns were filled with methanol and allowed to stand for 5 min. The columns were rinsed with 75 ml of methanol and dried with nitrogen. The columns were packed by vibration, using approximately 5.5 g of packing, and they were then conditioned for 72 hr at 260°C. After conditioning, the optimal oven temperature was 235°C and the helium flow rate was 60 ml/min.

Results. When 2.5 mg of sodium taurocholate was added to rat feces containing cholestyramine and extracted with glacial acetic acid, according to Evrard and Janssen (1), only 0.8 mg, or 32%, of the added bile salt was recovered. A further single extraction of this fecal residue with 0.5 N HCl in absolute ethanol re-

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TABLE 1 RECOVERY OF TAURINE-CONJUGATED BILE SALTS FROM RAT FECES CONTAINING CHOLESTYRAMINE

Bile Salts	Added	Found*	Average Recovery
	mg	*mg*	%
Taurolithocholate	3.72	3.65	98.6
Taurodeoxycholate	3.77	3.92	104.0
Taurocholate	3.80	3.88	102.0

* Each value is the mean of three separate determinations.

TABLE 2 REPRODUCIBILITY OF THE MEASUREMENT OF FECAL BILE ACIDS EXTRACTED FROM RAT FECES CONTAINING CHOLESTYRAMINE

Bile Acid	Replicates	Mean ± SD
		mg/g of feces
Lithocholic	9	6.24 ± 0.48
Deoxycholic	9	11.20 ± 0.76
Cholic	5	2.38 ± 0.32

moved 1.25 mg, or 73%, of the remaining bile salt. This indicated that the glacial acetic acid did not completely displace the conjugated bile salts from the resin.

Data showing the extraction and recovery of conjugated bile salts with 0.5 N HCl in absolute ethanol followed by saponification and gas–liquid chromatographic analysis as described above are presented in Table 1. When aqueous solutions (1 mg/ml) of sodium taurolithocholate, taurodeoxycholate, and taurocholate were added to rat feces containing cholestyramine, recoveries ranged from 99 to 104%.

The reproducibility of this extraction technique was also studied. The data obtained from replicate analyses performed on a homogeneous pool of finely ground rat feces are tabulated in Table 2. They demonstrate good reproducibility, the average coefficient of variation being less than 10%.

The above data demonstrate that the solvent used for extraction of conjugated bile acids from feces in the presence of the bile acid sequestrant, cholestyramine, must displace the bound bile acids in addition to providing solvation. The 0.5 N HCl in absolute ethanol appears to fulfill these criteria by providing the necessary anions (Cl^-) to exchange with the bile acids on the resin and a solvent for the conjugated bile acids.

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