NOTE

HIGH YIELD SULFONATION OF [^{14}COOH]-LITHOLCHOLIC AND [^{14}COOH]-TAUROLITHOCHOLIC ACIDS

SUMMARY

Sulfate esters of [^{14}COOH]-lithocholic and taurolithocholic acids have been prepared using a sulfur trioxide·pyridine complex as the sulfonating reagent. The utilization of liquid-liquid extraction or reversed-phase silica gel and Amberlite XAD-2 chromatographic techniques gave pure sulfate esters which did not contain inorganic salt impurities. These methods offer a useful one-step approach to the synthesis of sulfate esters at the milligram level using commercially-available labeled precursors.

Key Words: Bile acid sulfates, SO_3·pyridine, Lithocholate, Taurolithocholate

INTRODUCTION

Bile acid sulfate esters of lithocholic acid are found in the bile of both humans and animals and are believed to be detoxified metabolites which are formed to facilitate excretion (1). Sulfolithocholic acid can also become conjugated with glycine or taurine with continued enterohepatic cycling (2).

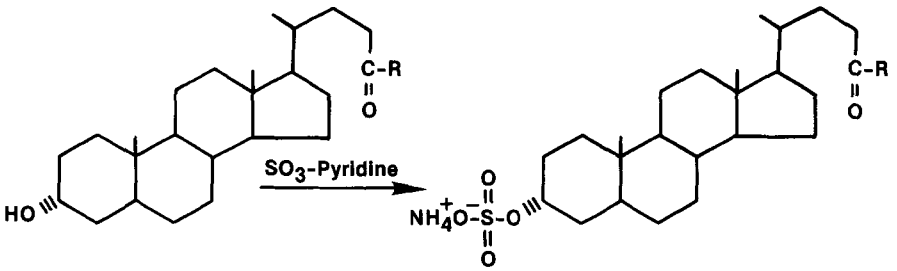
The first observation of the existence of bile acid sulfates in human bile was reported by Palmer (3), and details of their metabolites produced in the gut require elucidation. Labeled lithocholic acid sulfates prepared by methods described in this report have been used in metabolism studies with human intestinal microflora (4) in order to identify possible new mutagenic/carcinogenic products. The parent compound, lithocholic acid, enhances the bacterial mutagenicity of certain known chemical carcinogens including 2-aminoanthracene (5) and benzo(a)pyrene (6). In addition, this major fecal bile acid enhances tumorigenesis in the liver (7) and colon (8) and has been implicated as a contributing factor to human colon carcinogenesis (9).

0362-4803/79/0316-0507$01.00

Received June 28, 1978
Revised September 3, 1978

For metabolic studies we prepared [^{14}COOH]-labeled substrates using [24-^{14}C]-lithocholic acid as the starting material (Figure 1) since these radioactive

Figure 1



R = OH, Lithocholic acid

R = $\overset{-+}{O}NH_4$, sulfolithocholate, diammonium salt

R = NH-CH_2-CH_2-$\overset{-+}{SO_3}$Na, Taurolithocholate

R = NH-CH_2-CH_2-$\overset{-+}{SO_3}NH_4$, sulfotaurolithocholate, diammonium salt

sulfate esters are not available commercially.

The synthesis of sulfate esters of monohydroxy bile acids such as lithocholic acid has been described by Tserng and Klein (10) using a number of alternative procedures including a one-pot preparation of sulfolithocholate from lithocholic acid as well as a two-step synthesis of sulfotaurolithocholate. Our work was in progress when this report (10) was published, and our methods were designed to use commercially-available labeled and unlabeled reagents with a minimum of chemical steps under mild reaction conditions. In addition, we prepared the diammonium salts of our products in order to compare the properties of our labeled derivatives with those already published by Palmer (11) as a further check on the suitability of our methods. This report describes an improved rapid synthesis and clean-up protocol for these bile salts and offers a practical approach to preparing these labeled derivatives at the milligram level.

MATERIALS AND METHODS

Chemicals

Unlabeled lithocholic acid was obtained from Caribbean Research Laboratories (Mayaguez, Puerto Rico) and was purified by Sephadex LH-20 column (column volume = 250 ml) chromatography using isooctane:chloroform:methanol (2:1:1) as the eluting solvent (12). [^{14}COOH]-lithocholic acid (sp. act. = 59 mCi/mmole) was purchased from Amersham-Searle (Arlington Hts., IL.) and was 98% pure by thin-layer chromatographic analysis using system A (Table 1). All R_f values for each system studied are summarized in Table I.

Table I. R_f values of lithocholic acid and its sulfate derivatives on ITLC-SG and ITLC-SA sheets.

	SG				SA		
Compound	A	B	C	D	B	D	E
lithocholic acid	0.31	0.77	0.94	0.55	0.20	0.98	0.53
sulfolithocholic acid	0	0.36	0.44	0.03	0.04	0.79	0.11
taurolithocholic acid	0	0.87	0.05	0.43	0.42	0.71	0.53
sulfotaurolithocholate	0	0.52	0	0	0.11	0.57	0.10

The following solvent systems were used:

A = isooctane:diisopropyl ether:acetic acid (75:30:0.3)

B = isooctane:isopropanol:ammonium hydroxide (20.80:0.5)

C = chloroform:diisopropyl ether:isopropanol:acetic acid (50:30:15:1)

D (13) = chloroform:methanol:acetic acid:water (65:24:15:9)

E = isooctane:isopropanol:ammonium hydroxide (5:80:5)

F = isooctane:isopropanol:ammonium hydroxide (40:20:0.1)

Taurolithocholic acid sodium salt (Calbiochem, San Diego, CA.) and [^{14}COOH]-taurolithocholate (California Bionuclear Corp.; 50 μCi, 4.2 mCi/mmole) required further purification as described below. The sulfur trioxide•pyridine complex obtained from Upjohn Co. (Kalamazoo, MI.) was used as the sulfonating reagent and pyridine (Fisher Chemical Co., Pittsburgh, PA.) was dried and stored over potassium

hydroxide pellets (Fisher Scientific, Fairlawn, NJ.). Other solvents were purchased from Fisher or Burdick-Jackson (Muskegon, MI.).

Amberlite XAD-2 resin (Mallinckrodt Chemical Co., St. Louis, MO.) was purified by extraction with methanol overnight using a standard soxhlet apparatus. Reverse-phase silica gel used to purify the taurolithocholates consisted of a C_{18} phase-bonded Hi-Flosil (80/100) as supplied by the manufacturer (Applied Science Laboratories; State College, PA.).

The liquid scintillation cocktail used for radiological assays was Amersham-Searle's PCS, and liquid scintillation counting was performed on a Nuclear-Chicago Isocap 300.

Thin-layer Chromatography

All analyses were performed on ITLC-SG or ITLC-SA sheets (Gelman Instrument Co., Ann Arbor, MI.) and are summarized in Table 1. Sulfolithocholic and lithocholic acids were separated on ITLC-SG sheets developed in systems A, B and C.

Sulfotaurolithocholate and taurolithocholate were analyzed on ITLC-SA sheets using systems D and E. Assay of taurolithocholate for non-polar impurities was performed on ITLC-SG sheets developed in system F.

All solvent mixtures are expressed as volume/volume proportions. Spots were visualized by charring the glass fiber coated sheets after spraying with a solution of ethanol:sulfuric acid:water (2:2:1). Absolute R_f values did change slightly as a result of batch variations in the Gelman sheets, but the quality of the separations between the respective metabolites remained the same.

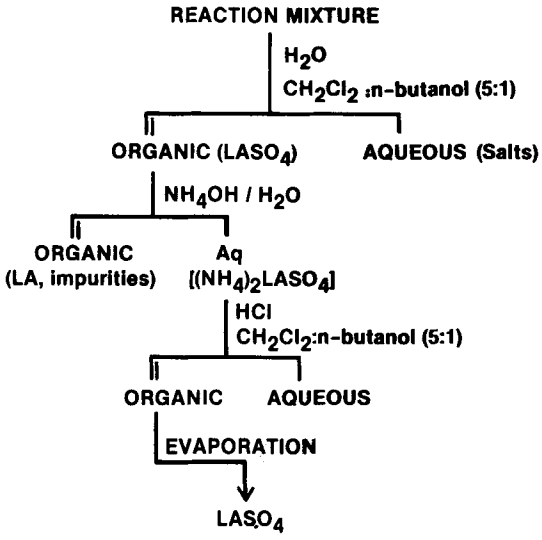
[^{14}COOH]-Sulfolithocholic Acid

The pyridine·SO_3 complex (128 mg, 0.8 mmole) was stirred with 3 ml of pyridine in an ice bath for 10 minutes. Radiolabeled lithocholic acid (0.3 mg, 50 μCi) diluted with 51 mg of purified unlabeled bile acid was dissolved in 2 ml of pyridine (55^{o}) and added dropwise to the cold solution of reagent. The reaction was continued at 25^{o} for 20 minutes followed by an additional 2.5 hours at 60^{o}C. All procedures were performed under yellow fluorescent lights. After removal of

the pyridine with nitrogen, the solid residue was dried overnight at 40°C under vacuum.

Purification of the product from inorganic salts and traces of unreacted lithocholic acid required a liquid-liquid extraction procedure outlined in Figure 2. The reaction mixture was dissolved in 10 ml of methylene chloride: n-butanol (5:1) and washed twice with 2 ml of distilled water to remove inorganic salts. This organic layer was then washed with 3 ml of water and 1 ml of concentrated ammonium hydroxide to extract out the sulfolithocholic acid. The aqueous layer was again gently extracted with 10 ml of organic solvent to remove non-polar impurities (such as traces of unreacted lithocholic acid) and was

Figure 2



acidified with concentrated hydrochloric acid. This acid solution was quickly extracted twice with 10 ml aliquots of methylene chloride:butanol (5:1). Both organic phases were evaporated to dryness by first adding 0.5 ml of concentrated ammonium hydroxide to each solution and then removing the solvent by rotary evaporation (11). The solid residue was dried at 40°C overnight in a vacuum dessicator.

Crystallization was performed by dissolving the residue in 1 ml of hot absolute ethanol containing 5 drops of ammonium hydroxide and chilling the solution at -5°C overnight. The mother liquor was removed carefully, and the crystals were washed with 1 ml of cold acetone. After drying the crystals at 40°C under vacuum, the yield of product was 60 mg (90%) and had the following properties: m.p. = 183-185° [lit. 181° - (11)]; ir (KBr) = 3250 (broad), 1550, 1225, 1055, 970 cm^{-1} which agrees with the literature (11); radiochemical purity of 98% in solvent systems A and C; and specific activity = 0.30 mCi/mmole (0.38 mCi/mmole calculated).

Purification of Commercial Taurolithocholate

Assay of the commercial labeled taurolithocholate by thin-layer chromatography in system F revealed about 30% impurities. To purify this sample, approximately 6 mg of [^{14}COOH]-taurolithocholate (33 μCi) was dissolved in 0.5 ml of methanol: water (2:1) and placed on a column (0.5 X 12.5 cm) containing 3.5 g reverse-phase silica gel. Fractions of 1 ml were collected and assayed for activity by ITLC-SG using system F, and 5 mg of product (33 μCi, 98% pure) was obtained. A 9 gram reverse-phase column was used to purify 60 mg of unlabeled taurolithocholate, and 44 mg of pure product was recovered by this procedure.

[^{14}COOH]-Sulfotaurolithocholic Acid

Purified [^{14}COOH]-taurolithocholate (5 mg; 33 μCi) was diluted with unlabeled taurolithocholate (44 mg) to give a final specific activity of 0.34 mCi/mmole. The combined taurolithocholate was dissolved in 2 ml of warm pyridine (55°C) and added dropwise to a solution of 90 mg of sulfur trioxide·pyridine complex in 3 ml of cold (0°C) pyridine. All procedures were conducted under yellow fluorescent lights. After continuous mixing at 25°C for 15 minutes, the reaction mixture was heated at 60° for 60 minutes. An additional 60 mg of sulfur trioxide·pyridine was added, and the reaction was again maintained at 60°C for two hours. The solvent was removed at 50°C under nitrogen, and the residue was dried at 40°C overnight in a vacuum dessicator.

The reaction mixture was then dissolved in 3 ml of 10% ammonium hydroxide and added to a column (0.75 X 40 cm) containing 50 g of purified (see Methods) Amberlite XAD-2 resin. Sequential elutions with water (140 ml) and methanol (140 ml) gave inorganic salts and sulfotaurolithocholate, respectively. The yield of the product as determined by radioactivity was 74%, and ITLC-SA analyses using system E indicated that the sulfotaurolithocholate was 91% pure.

Recrystallization of the product from methanol or ethanol (2 ml) containing a few drops of ammonium hydroxide and 4 ml of acetone gave crystals within 15-30 minutes at -5°C. After removal of the mother liquor, the crystals (20 mg) were washed with 1 ml of cold acetone and the supernatant removed. A second batch of crystals (10 mg) was obtained from recrystallization of the mother liquor, and the isolated products had specific activities of 0.36 (first crystals) and 0.34 mCi/mmole, respectively (0.34 mCi/mmole expected). ITLC-SA assay of both crystalline products using solvent systems D and E revealed a purity of 97-98% and the melting points of the products were 190-191° [lit. = 189-190° (11)].

ACKNOWLEDGMENTS

This research was sponsored by the National Cancer Institute under Contract No. NO1-CO-75380 with Litton Bionetics, Inc.

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Morris I. Kelsey and Jorge E. Molina
Chemical Carcinogenesis Program, Frederick Cancer Research Center,
Frederick, Maryland 21701