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The synthesis of taurine–conjugated bile acids and bile acid sulfates labeled with ^{14}C or ^{3}H in the taurine moiety

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SUMMARY

Studies of bile acid transport systems require radio–labeled taurine–conjugated bile acids with high specific activity. An established synthetic procedure was optimized to provide mild, fast, and effective conjugation of radio–labeled taurine with different types of bile acids, including those with labile 7α–hydroxy–3–oxo–Δ^4 or 3β,7α–dihydroxy–Δ^5 structures. Taurine labeled with ^{14}C or ^{3}H was reacted with excess bile acid anhydride formed from the tributylamine salt and ethylchloroformate (2/1 M/M) in aqueous dioxane for 15 min at room temperature. The yields were higher than 95% and less than 2% side products were formed. Bile acid sulfates were conjugated with ^{14}C– or ^{3}H– labeled taurine by using N–ethoxycarbonyl–2–ethoxy–1,2–dihydroquinoline as the coupling reagent. The products were effectively purified by chromatography of the sodium salts on Sephadex LH–20. The yields of taurine–conjugated bile acid sulfates were 65–70%.

Key words: [1,2–^{14}C]taurine, [2–^{3}H]taurine, taurine–conjugated bile acids, taurine–conjugated bile acid sulfates, synthesis.

INTRODUCTION

Patients with a genetic lack of the 3β–hydroxy steroid dehydrogenase/isomerase (1) or 5β–reductase (2), enzymes required for normal bile acid biosynthesis, develop liver disease and cholestasis. This has been suggested to depend on the toxicity of the bile acids with 3β–hydroxy–Δ^5 or 3–oxo–Δ^4 structures produced in these patients (1,2). In order to study the effects of such bile acids on hepatic transport systems, a method was needed to prepare labeled versions of their water–soluble taurine conjugates.

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Several methods have been used to synthesize taurine-conjugated bile acids (3-6). However, no method is able to couple trace amounts of taurine in high yields with bile acids. Kramer and Kurz (7) used a modification of Norman's method (4) to prepare radio- labeled taurine-conjugated bile acids, but a side reaction was a limiting factor. Furthermore, the time course of the reaction and the ability to conjugate chemically labile bile acids were not studied. The present paper describes a quantitative and qualitative study of this method, resulting in a mild and rapid procedure with little or no formation of side products. Taurine-conjugated bile acid sulfates were synthesized using a modified procedure based on the method of Tserng et al (6).

MATERIALS AND METHODS

Materials

All solvents were redistilled before use. Dimethylformamide (DMF) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) were from Sigma (St. Louis, MD). [1,2-^{14}C]Taurine (3.4 GBq/mmol) and [2-^{3}H(N)]taurine (810.3 GBq/mmol) were from New England Nuclear (Boston, MA). [24-^{14}C]cholic acid (1.99 GBq/mmol) was from Amersham International Plc. (Amersham, UK). Lithocholic acid 3-sulfate was from Calbiochem (San Diego, USA). 3β,7α-dihydroxy-5-cholenoic and 7α-hydroxy-3-oxo-4-cholenoic acids were synthesized from methyl 3β-acetoxy-5-cholenoic acid (Steraloids, Wilton, NH) (8). Chenodeoxycholic acid 3-sulfate was prepared from chenodeoxycholic acid using the sulfation procedure described by Mumma et al (9). A limiting amount of sulfuric acid was used to give the monosulfate as the main product. This was extracted on octadecylsilane (ODS)-silica and unreacted and disulfated chenodeoxycholic acids were removed by chromatography on Lipidex-DEAP (10). Other bile acids were from previous studies in this laboratory.

Columns of ODS-bonded silica (Preparative C_{18}, Waters Associates, Milford, MA) and Lipidex-DEAP were prepared as described previously (3). Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was used as described by Almé et al. (10).

Chromatographic and Instrumental Methods

Thin-layer chromatography (TLC) was carried out using plates precoated with silica gel 60 and the solvent system was butanol-acetic acid-water 10:1:1 (by vol). Radioactivity on TLC plates was determined by a Berthold Dünnschichtscanner II connected to a Hewlett-Packard Model 3396A integrator.

High-performance liquid chromatography (HPLC) was performed with an LKB 2150 pump (Pharmacia-LKB, Bromma, Sweden), a model 201 fraction collector (Gilson, Villies Le Bel,

France), a μ-Bondapak C18 steel column (300 x 3.9 mm, particle size 10 μM, Waters). The mobile phase was methanol-acetic acid-water 70:1:30 (by vol). Radioactivity in HPLC fractions was determined by liquid scintillation counting (1211 Minibeta, Wallac, Sollentuna, Sweden) using Optiphase "HiSafe" II (Wallac) as scintillation liquid.

Fast-atom bombardment mass spectrometry (FAB MS) was carried out as described previously (3,11,12). Gas chromatography-mass spectrometry (GC/MS) was performed as described (13).

Synthesis, Extraction, and Purification

Taurine-conjugated bile acids Free bile acids (1 μmole) were dissolved in 50 μl of dioxane containing 1 μmole tri-n-butylamine, to which 50 μl of dioxane containing 0.5 μmol ethylchloroformate was added. To the resultant solution, 1-100 μl of [1,2-^{14}C]- or [2-^{3}H(N)]taurine (0.05-100 nmol) in 0.01 M hydrochloric acid, as supplied by the manufacturer, was added. The mixture was left for 15 min at room temperature. It was then diluted with 1.0 ml of rwater and passed through a column of ODS-silica (1.5 x 0.8 cm), which was washed with 5 ml of water. The bile acids were eluted with 8 ml of methanol.

The eluate from the ODS-silica was taken to dryness in vacuum. The residue was dissolved in 1 ml of 72 % aqueous ethanol, and applied to a column (25 x 0.4 cm) of Lipidex-DEAP in acetate form in 72% aqueous ethanol. After washing with 15 ml of 72% aqueous ethanol, unconjugated bile acids were eluted with 7.4 ml of 0.1 M acetic acid in 72% aqueous ethanol, and taurine-conjugated bile acids with 11 ml of 0.15 M acetic acid/ammonium hydroxide in 72% aqueous ethanol (pH 6.6). Ethanol was removed under vacuum, and the remaining solution passed through an ODS-silica column. After washing with of 5 ml water, taurine-conjugated bile acid were eluted with 5 ml of methanol. The product was further purified by HPLC.

When optimal conditions had been extablished, syntheses with unlabeled taurine were performed and the purified taurine-conjugated bile acid was analyzed by FAB MS (11) and by GC/MS (13) after an enzymatic hydrolysis (14)

Taurine-conjugated bile acid sulfates To chenodeoxyocholic acid 3-sulfate or lithocholic acid 3-sulfate (5 μmol), 100 μl of DMF containing 7 μmol EEDQ, 0.9 μl triethylamine and 1-20 μl of [1,2-^{14}C]- or [2-^{3}H(N)]-taurine (0.05-20 nmol) in 0.01M HCl solution was added. The mixture was left for 0.5 h at 90°C. The reaction was stopped by the addition of 1 ml water, and bile acids were extracted on an ODS-silica column as above. The extracted bile acids were dissolved in 1 ml chloroform/methanol, 3:2 (v/v), saturated with sodium chloride, and this solution was then applied to a column of Sephadex LH-20. Unreacted bile acid sulfate was eluted with 60 ml of chloroform/

methanol 3:2 (v/v) saturated with sodium chloride and taurine-conjugated bile acid sulfate was eluted with 40 ml of chloroform/methanol 2:3 (v/v) containing 0.02 M sodium chloride.

The fractions containing taurine-conjugated bile acid sulfate were combined and taken to dryness. The residue was dissolved in 2 ml water and sodium chloride was removed by an ODS-bonded silica extraction. The products of syntheses with unlabeled taurine were analyzed by FAB MS (11).

RESULTS AND DISCUSSION

The aim of the present study was to find a mild reaction that could be used to conjugate radio-labeled taurine in high yields with different types of bile acids. Different methods (3-6) were tested, and the best results were obtained with the method of Norman (4) after suitable modifications. The reaction was performed by mixing taurine with bile acid anhydride formed by reaction of ethylchloroformate with the tributylamine salt of the bile acid. The side-product, N-ethoxycarbonyl-2-aminoethanesulfonic acid (7), was easily formed when the reaction was performed on a small scale. However, we found that this loss of labeled taurine was avoided by using an excess of bile acid anhydride with tributylamine and ethylchloroformate in a molar ratio of 2 to 1.

The experimental conditions were optimized by varying the concentrations of bile acid, tributylamine and ethylchlorofomate, the amount of taurine, reaction time, and types of bile acid. The results were evaluated by monitoring the radioactivity on TLC before extraction and by HPLC after extraction with ODS-silica and purification on Lipidex-DEAP. The reaction conditions finally adopted gave the highest yield of taurine conjugated with the bile acid and a minimum amount of sideproducts.

In previous studies (4,7), the amount of ethylchloroformate used was the same or higher than that of the bile acid tributylamine salt. Our experiments showed that these conditions gave about 50% of side products in the small scale (tracer) reactions. When 1.2 μmol of ethylchloroformate was mixed with 1, 1.2, 2 and 3 μmol of tributylamine cholate, 11%, 46%, 94%, and 96% of 0.1 μCi [1,2-^{14}C]-taurine (1.1 nmol) were converted to cholyltaurine in 15 min, and the yields of the side product were 87%, 52%, 5%, and 1%, respectively. The time course studies showed that the reaction was completed in 15 min.

Tributylamine played a important role in the reaction. Free tributylamine increased the formation of the side product, and the bile acid salt should be prepared with an equimolar amount of tributylamine. We also noticed that the yields were decreased using triethylamine instead of tributylamine.

It was not necessary to prepare the sodium salt of taurine by addition of sodium hydroxide solution as done in previous methods (4,7). This is a practical advantage since the [1,2-^{14}C]- or [2-^{3}H(N)]taurine are supplied in 0.01M hydrochloric acid. A suitable volume of the commercial solution could be directly added to the reaction mixture. When 1 to 100 μl (0.1-100 μCi) were used, higher than 95% yields were obtained. However, we found that the addition of taurine as the sodium salt gave similar results.

The common bile acids, cholic, chenodeoxycholic, lithocholic acids, as well as the chemically labile structures, i.e. 3β,7α-dihydroxy-5-cholenoic acid and 7α-hydroxy-3-oxo-4-cholenoic acid were subjected to the reaction and in all cases, the yields were higher than 95%.

In order to detect structural changes or presence of side reactions other than that mentioned above, the reaction products were analyzed by FAB MS and after enzymatic hydrolysis by GC/MS. By comparing the retention indices and spectra with those of standard bile acids and calculating the recoveries of bile acids, the results confirmed the expected products, quantitatively and qualitatively.

Taurine-conjugated bile acid sulfates are another important group of bile acid conjugates. The method described above was not successful for conjugation of bile acid sulfates with taurine due to the poor solubility of bile acid sulfates in dioxane. In 1977, Tserng et al (6) described a method for the synthesis of glycine and taurine conjugates of bile acids using DMF as solvent and EEDQ as the reagent to form a mixed anhydride. We tested the possibility to use this method to conjugate bile acid sulfates on the nmol scale. By varying the concentration of bile acid sulfates, triethylamine, EEDQ and taurine, optimum condition could be established. The yields were 65-70% when lithocholic acid sulfate and chenodeoxycholic acid 3-sulfate were substrates. Higher yields of taurine-conjugated bile acids without the sulfate group (80-85%) were obtained using the same conditions. It is possible that the charged sulfate group limits the displacement of the mixed anhydride by taurine.

The taurine-conjugated bile acid sulfate was readily separated from unreacted bile acid sulfate by chromatography of their sodium salts on Sephadex LH-20 in chloroform/methanol containing an excess of sodium chloride (9). This separation was not achieved by anion exchange chromatography on Lipidex-DEAP.

In summary, we have described two simple procedures for the synthesis and purification of radio-labeled taurine-conjugated bile acids and bile acid sulfates with good yields. Both methods can be used with varying amounts of radio-labeled taurine and on different types of bile acid.

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