SYNTHESIS AND CHARACTERISTICS OF THE SPECIFIC MONOSULFATES OF CHENODEOXYCHOLATE, DEOXYCHOLATE AND THEIR TAURINE OR GLYCINE CONJUGATES

G. Parmentier and H. Eyssen

The Rega Institute, University of Leuven,
10 Minderbroedersstraat, B-3000 Leuven, Belgium.

Rec'd. 5-30-77.

ABSTRACT

The isomeric monosulfates of chenodeoxycholate, deoxycholate, and their taurine or glycine conjugates, were synthesized and characterized. Reaction with chlorosulfonic acid in pyridine for 2 minutes mainly afforded the 3-monosulfates. To prepare the 7- or the 12-monosulfates, the 3-hydroxyl group was protected by carbethoxylation prior to sulfation of the 7- or 12-hydroxyl group for 24 h to 5 days; after sulfation, the protecting 3-carbethoxy function was removed by mild alkaline hydrolysis. The crude bile salt monosulfates were purified by chromatography on silica gel and on Sephadex LH-20 and were crystallized from methanol-ethanol-ethyl acetate. The results of elemental analysis demonstrated that the compounds were disodium dihydroxy bile salt monosulfates. Thin layer chromatography of the sulfates, and gas-liquid chromatography after oxidation and solvolysis, showed that the substances were pure and that the sulfate group was at the expected position.

INTRODUCTION

Bile salt sulfates are produced in a variety of hepato-biliary diseases in man (1-5). Sulfation of bile salts also occurs in rats (6,7) and in mice (8,9). However, in spite of the increased interest in this aspect of bile salt metabolism, adequate reference substances are not yet available and the TLC-characteristics of bile salt sulfates are largely undefined. The sulfates of lithocholic acid and of its glycine or taurine conjugates were first prepared by Palmer and Bolt (10). In a previous paper we reported on the synthesis of the isomeric monosulfates of cholic acid (11). Haslewood and Haslewood prepared the 3-monosulfates of cholic acid, chenodeoxycholic acid and deoxycholic acid (12). Chenodeoxycholate 7-sulfate was synthesized by Summerfield *et al.* (13). In the present paper we describe the synthesis and characteristics of the

specific monosulfates of chenodeoxycholic and deoxycholic acid and of their taurine and glycine conjugates.

MATERIALS AND METHODS

Materials. Solvents and reagents were as described previously (11). Cholylglycine hydrolase was obtained from Schwarz-Mann, Orangeburg, N.Y., USA. Reference 3α-hydroxy-7-oxo-5β-cholanoic acid, methyl 7α-hydroxy-3-oxo-5β-cholanoate and 3α-hydroxy-12-oxo-5β-cholanoic acid were obtained from Steraloids, Pawling, N.Y., USA. All other bile acids were supplied by Maybridge, Trevilett, Tintagel, U.K.

Thin-layer chromatography (TLC). Unsulfated conjugated bile salts, and unconjugated or glycine conjugated bile salt sulfates were chromatographed in solvent 1 : ethyl acetate-butan(1)-ol-acetic acid-water (40:30:15:15, by vol.) (14). Taurine conjugated bile salt sulfates were better resolved with solvent 2 : butan(1)-ol-acetic acid-water (10:5:1, by vol.). Solvent 3 : isooctane-ethyl acetate-acetic acid (5:5:1, by vol.) (15), was used for chromatography of unconjugated bile acids, their methyl esters and the 3-carbethoxy derivatives.

Solvolysis. Sulfated bile salts were solvolyzed for 18 h at 37°C in 10 ml methanol-acetone (1:9, v/v) plus 0.1 ml of 6N HCl.

Gas-liquid chromatography (GLC). Bile acids were chromatographed as the methyl esters and methyl ester acetates on 5 ft columns with 3 % OV-1, 3 % OV-17 or 1 % QF-1 as stationary phase (9).

EXPERIMENTAL PROCEDURE

1. Preparation of bile salt 3-sulfates

A pyridine-SO_3 complex was prepared by adding dropwise 0.6 ml of chlorosulfonic acid to 10 ml of dry pyridine cooled in ice; 1 g of bile salt in 20 ml of dry pyridine was added to this complex. After 2 min, the reaction was terminated by adding 20 ml water and 40 ml of 2N NaOH. The bile salt 3-sulfates were contaminated with minor amounts of starting product, of 7- or 12-sulfate, and of disulfate. Therefore, after desalting on a column of 40 g Amberlite XAD-2 (11), the bile salts were taken up in 20 ml chloroform-methanol (1:1, v/v), containing 0.01 mole NaCl per liter, and were chromatographed on a column of 70 g Sephadex LH-20 in the same solvent system (16). Fractions containing pure bile salt 3-monosulfate were desalted and the product was crystallized from methanol-absolute ethanol-ethyl acetate.

Conjugated bile salt 3-sulfates contained small amounts of unconjugated 3-sulfates which could be eliminated by chromatography on silica gel. Therefore, prior to chromatography on Sephadex LH-20, the desalted preparation was dissolved in 5 ml of chloroform-methanol-acetic acid-water (65:25:2:4, by vol.) and applied to a column of 120 g silica gel 60 (0.063-0.2 mm, Merck). With the same solvent, the unconjugated bile salt monosulfate moved in front of the conjugated form.

2. Preparation of bile salt 7- or 12-sulfates

Carbethoxylation of the 3-hydroxyl group. The methyl esters (1 g) of deoxycholic or chenodeoxycholic acid were dissolved in 5 ml of dioxane containing 0.8 ml of dry pyridine. The 3-hydroxyl group was carbethoxylated with 1 ml ethyl chlorocarbonate, as described by Fieser et al. (17). The reaction product was extracted with diethyl ether and TLC with solvent 3 showed a main spot with Rf 0.73 for both 3-carbethoxy derivatives. To prepare the 3-carbethoxy derivatives of the glycine- or the taurine-conjugates, 1 g of bile salt was suspended in 6 ml pyridine plus 36 ml dioxane; 7.5 ml of ethyl chlorocarbonate were added under cooling in ice and the mixture was kept for 2.5 h at room temperature. TLC with solvent 1 showed a main spot with Rf 0.90 and Rf 0.63 for the glycine- and the taurine-conjugated 3-carbethoxy derivatives, respectively.

Sulfation and purification. The crude 3-carbethoxy derivative was dissolved in 20 ml pyridine and was sulfated for at least 24 h with 1 ml of chlorosulfonic acid in 10 ml of dry pyridine. After adding 60 ml of 2N NaOH and evaporation to dryness, the residue was taken up in 200 ml water and desalted. The 3-carbethoxy group was removed by hydrolysis in 80 ml methanol and 20 ml of 50 % aq. NaOH for 4 h at 65°C; the crude bile salts were desalted on Amberlite XAD-2. Bile salt 7- or 12-monosulfates were contaminated with minor amounts of starting product, the 3-sulfate and the disulfate, which could be removed by chromatography on Sephadex LH-20. Conjugated bile salt monosulfates also contained small amounts of unconjugated monosulfates and required an additional purification by chromatography on silica gel, as described for the 3-sulfates. The products were crystallized from methanol-absolute ethanol-ethyl acetate.

3. Characterization of the bile salt sulfates

All preparations were characterized by their mobility on TLC, their melting point, elemental analysis and infrared spectrometry. Bile salt sulfates were very soluble in methanol and water, sparingly soluble in ethanol, and insoluble in ethyl acetate and ether. The infrared spectra of all preparations showed the broad absorption band at 1200-1270 cm^{-1} and the sharp band at 1060 cm^{-1}, which are characteristic for the sulfate ester group. No striking individual differences were seen.

The position of the sulfate group was determined by oxidation of the free hydroxyl group followed by solvolysis of the resulting sulfoxy-oxo-5β-cholanoate (11). The hydroxy-oxo-5β-cholanoic acid so obtained was identified by GLC. Conjugated bile salt sulfates were deconjugated with cholylglycine hydrolase (18) for 15 h at 37°C prior to determination of the position of the sulfate group.

As reference 12α-hydroxy-3-oxo-5β-cholanoic acid was not available, the location of the sulfate group in sulfate esters of deoxycholic acid was confirmed by another reaction sequence. Approximately 2 mg of the product was acetylated with 1 ml of pyridine-acetic anhydride (1:1, v/v) for 16 h at room temperature. After solvolysis, the resulting hydroxy-acetoxy-cholanoic acid was extracted and methylated. Oxidation in acetone with Jones reagent (19) yielded a methyl acetoxy-oxo-5β-cholanoate, which was identified by GLC.

Disodium chenodeoxycholate 3-sulfate

Sulfation of 1.01 g chenodeoxycholic acid for 2 min yielded 730 mg of pure disodium chenodeoxycholate 3-sulfate (Rf 0.68 in solvent 1) after chromatography on Sephadex LH-20 and desalting. On crystallization from methanol-absolute ethanol-ethyl acetate (1:5:10, by vol.) 690 mg of a white, crystalline compound was obtained which shrank and cleared at 197-198°C.

Anal. Calcd. for $C_{24}H_{38}O_7SNa_2$: 55.79 % C, 7.41 % H, 6.22 % S. Found : 55.65 % C, 7.54 % H, 6.03 % S.

The bile acid obtained after oxidation and solvolysis was identified by GLC as 3α-hydroxy-7-oxo-5β-cholanoic acid and no 7α-hydroxy-3-oxo-5β-cholanoic acid was detected.

Disodium chenodeoxycholate 7-sulfate

The methyl ester from 1.02 g chenodeoxycholic acid was carbethoxylated at C-3 prior to sulfation for 24 h. After removal of the carbethoxy group and chromatography on Sephadex LH-20, 940 mg of pure disodium chenodeoxycholate 7-sulfate (Rf 0.63 in solvent 1) was obtained. Crystallization from methanol-absolute ethanol-ethyl acetate (1:2:5, by vol.) afforded 710 mg of a white crystalline compound; m.p. 190-194°C (decomp.).

Anal. Calcd. for $C_{24}H_{38}O_7SNa_2$: 55.79 % C, 7.41 % H, 6.22 % S. Found : 55.59 % C, 7.35 % H, 6.11 % S.

The bile acid obtained after oxidation and solvolysis was identified by GLC as 7α-hydroxy-3-oxo-5β-cholanoic acid; no 3α-hydroxy-7-oxo-5β-cholanoic acid was detected.

Disodium deoxycholate 3-sulfate

Deoxycholic acid (598 mg) was sulfated for 2 min, yielding 448 mg of disodium deoxycholate 3-sulfate (Rf 0.71 in solvent 1) after purification on Sephadex LH-20. Crystallization from methanol-absolute ethanol-ethyl acetate (1:2:20, by vol.) afforded 359 mg of white crystals; m.p. 204-205°C (decomp.).

Anal. Calcd. for $C_{24}H_{38}O_7SNa_2$: 55.79 % C, 7.41 % H, 6.22 % S. Found : 53.93 % C, 7.30 % H, 6.33 % S.

After oxidation and solvolysis, only 3α-hydroxy-12-oxo-5β-cholanoic acid was detected by GLC. Acetylation followed by solvolysis and oxidation yielded no 3α-acetoxy-12-oxo-5β-cholanoic acid.

Disodium deoxycholate 12-sulfate

Treatment of 1.025 g deoxycholic acid as described for chenodeoxycholate 7-sulfate yielded 1.06 g of pure disodium deoxycholate 12-sulfate (Rf 0.66 in solvent 1). Crystallization from methanol-absolute ethanol-ethyl acetate (1:5:10, by vol.) yielded 898 mg of a white crystalline product; m.p. 205-206°C (decomp.).

Anal. Calcd. for $C_{24}H_{38}O_7SNa_2$: 55.79 % C, 7.41 % H, 6.22 % S. Found : 54.16 % C, 7.47 % H, 5.77 % S.

Acetylation and solvolysis followed by oxidation yielded 3α-acetoxy-12-oxo-5β-cholanoic acid. Oxidation followed by solvolysis yielded no 3α-hydroxy-12-oxo-5β-cholanoic acid.

Disodium taurochenodeoxycholate 3-sulfate

Sulfation of 1.01 g sodium taurochenodeoxycholate for 2 min yielded 818 mg of disodium taurochenodeoxycholate 3-sulfate (Rf 0.47 in solvent 2) after purification on Sephadex LH-20. Crystallization from methanol-absolute ethanol-ethyl acetate (2:2:15, by vol.) yielded a white crystalline product (720 mg) that decomposed at 178°C without melting.

Anal. Calcd. for $C_{26}H_{43}O_9NS_2Na_2$: 50.06 % C, 6.95 % H, 2.24 % N, 10.28 % S. Found : 50.24 % C, 6.94 % H, 2.32 % N, 10.10 % S.

After enzymic deconjugation, TLC in solvent 1 showed a main spot of chenodeoxycholate 3-sulfate (Rf 0.68); chenodeoxycholate 7-sulfate (Rf 0.64) was not detected. Oxidation and solvolysis of the deconjugated product yielded only 3α-hydroxy-7-oxo-5β-cholanoic acid.

Disodium taurochenodeoxycholate 7-sulfate

The 3-carbethoxy derivative of 1.04 g sodium taurochenodeoxycholate was sulfated for 5 days. After removal of the carbethoxy group and chromatography on silica gel and on Sephadex LH-20, 707 mg of pure disodium taurochenodeoxycholate 7-sulfate (Rf 0.39 in solvent 2) was obtained. Crystallization from methanol-absolute ethanol-ethyl acetate (1:1:10, by vol.) yielded 588 mg of a white crystalline product, which decomposed at 174°C without melting.

Anal. Calcd. for $C_{26}H_{43}O_9NS_2Na_2$: 50.06 % C, 6.95 % H, 2.24 % N, 10.28 % S. Found : 48.20 % C, 6.94 % H, 2.17 % N, 9.03 % S.

After enzymic deconjugation, only chenodeoxycholate 7-sulfate was detected on TLC (Rf 0.64 in solvent 1). Oxidation of the deconjugated product followed by solvolysis yielded 7α-hydroxy-3-oxo-5β-cholanoic acid; no 3α-hydroxy-7-oxo-5β-cholanoic acid was detected by GLC.

Disodium taurodeoxycholate 3-sulfate

Sulfation of sodium taurodeoxycholate (1.11 g) for 2 min yielded 778 mg of disodium taurodeoxycholate 3-sulfate (Rf 0.47 in solvent 2) after purification on Sephadex LH-20. After crystallization from methanol-absolute ethanol-ethyl acetate (1:1:10, by vol.), 638 mg of a white crystalline product was obtained which decomposed at 186°C without melting.

Anal. Calcd. for $C_{26}H_{43}O_9NS_2Na_2$: 50.06 % C, 6.95 % H, 2.24 % N, 10.28 % S. Found : 48.22 % C, 6.88 % H, 2.14 % N, 9.12 % S.

Enzymic deconjugation yielded deoxycholate 3-sulfate (Rf 0.71 in solvent 1); no deoxycholate 12-sulfate (Rf 0.66) was detected on TLC. After oxidation and solvolysis of the deconjugated product, GLC showed only 3α-hydroxy-12-oxo-5β-cholanoic acid.

Disodium taurodeoxycholate 12-sulfate

Sodium taurodeoxycholate (1.08 g) was treated as described for taurochenodeoxycholate 7-sulfate and yielded 978 mg of disodium taurodeoxycholate 12-sulfate (Rf 0.39 in solvent 2). Crystallization from methanol-absolute ethanol-ethyl acetate (1:1:5, by vol.), yielded 722 mg of white crystals which decomposed at 185°C without melting.

Anal. Calcd. for $C_{26}H_{43}O_9NS_2Na_2$: 50.06 % C, 6.95 % H, 2.24 % N, 10.28 % S. Found : 48.25 % C, 6.90 % H, 2.15 % N, 9.22 % S.

Enzymic deconjugation yielded deoxycholate 12-sulfate (Rf 0.66 in solvent 1); deoxycholate 3-sulfate (Rf 0.71) was not detected. Acetylation of the deconjugated bile salt followed by solvolysis and oxidation yielded 3α-acetoxy-12-oxo-5β-cholanoic acid.

Disodium glycochenodeoxycholate 3-sulfate

Sulfation of sodium glycochenodeoxycholate (1.00 g) for 2 min yielded 1.22 g of pure disodium glycochenodeoxycholate 3-sulfate (Rf 0.42 in solvent 1) after chromatography on silica gel and on Sephadex LH-20. Crystallization from methanol-absolute ethanol-ethyl acetate (1:2:10, by vol.) afforded 774 mg of a white crystalline product; m.p. 196°C (decomp.).

Anal. Calcd. for $C_{26}H_{41}O_8NSNa_2$: 54.43 % C, 7.20 % H, 2.44 % N, 5.59 % S. Found : 54.33 % C, 7.32 % H, 2.32 % N, 5.40 % S.

Enzymic deconjugation yielded chenodeoxycholate 3-sulfate (Rf 0.68 in solvent 1); no chenodeoxycholate 7-sulfate (Rf 0.63) was seen. Oxidation of the deconjugated product followed by solvolysis yielded 3α-hydroxy-7-oxo-5β-cholanoic acid and no 7α-hydroxy-3-oxo-5β-cholanoic acid was detected by GLC.

Disodium glycochenodeoxycholate 7-sulfate

Treatment of 1.045 g of sodium glycochenodeoxycholate as described for taurochenodeoxycholate 7-sulfate yielded 356 mg of disodium glycochenodeoxycholate 7-sulfate (Rf 0.38 in solvent 1). Crystallization from methanol-absolute ethanol-ethyl acetate (1:1:20, by vol.) yielded 325 mg of white crystals; m.p. 179-180°C.

Anal. Calcd. for $C_{26}H_{41}O_8NSNa_2$: 54.43 % C, 7.20 % H, 2.44 % N, 5.59 % S. Found : 54.24 % C, 7.18 % H, 2.41 % N, 5.45 % S.

After enzymic deconjugation, TLC in solvent 1 showed a single spot of chenodeoxycholate 7-sulfate (Rf 0.63). Oxidation of this product followed by solvolysis yielded 7α-hydroxy-3-oxo-5β-cholanoic acid and no 3α-hydroxy-7-oxo-5β-cholanoic acid was detected by GLC.

Disodium glycodeoxycholate 3-sulfate

Sodium glycodeoxycholate (1.54 g) was sulfated for 2 min and yielded 1.14 g of disodium glycodeoxycholate 3-sulfate (Rf 0.42 in solvent 1) after purification. Crystallization from methanol-absolute ethanol-ethyl acetate (1:2:20, by vol.) yielded 748 mg of white crystals which decomposed at 188°C without melting.

Anal. Calcd. for $C_{26}H_{41}O_8NSNa_2$: 54.43 % C, 7.20 % H, 2.44 % N, 5.59 % S. Found : 54.18 % C, 7.09 % H, 2.39 % N, 5.36 % S.

Enzymic deconjugation yielded deoxycholate 3-sulfate (Rf 0.71 in solvent 1); no deoxycholate 12-sulfate (Rf 0.66) was seen. After oxidation of the deconjugated product followed by solvolysis, GLC only showed 3α-hydroxy-12-oxo-5β-cholanoic acid.

Disodium glycodeoxycholate 12-sulfate

Treatment of sodium glycodeoxycholate (1.01 g) as described for taurochenodeoxycholate 7-sulfate yielded 328 mg of disodium glycodeoxycholate 12-sulfate (Rf 0.38 in solvent 1) after purification. Crystal-

lization from methanol-absolute ethanol-ethyl acetate (1:1:10, by vol.) provided 272 mg of a crystalline product which decomposed at 210°C without melting.

Anal. Calcd. for $C_{26}H_{41}O_8NSNa_2$: 54.43 % C, 7.20 % H, 2.44 % N, 5.59 % S. Found : 54.30 % C, 7.13 % H, 2.49 % N, 5.39 % S.

Enzymic deconjugation yielded only deoxycholate 12-sulfate (Rf 0.66 on TLC in solvent 1). The deconjugated bile salt sulfate was acetylated and the compound obtained after solvolysis and oxidation was identified by GLC as 3α-acetoxy-12-oxo-5β-cholanoic acid; oxidation prior to solvolysis yielded no 3α-hydroxy-12-oxo-5β-cholanoic acid.

DISCUSSION

The equatorial 3-hydroxyl group of deoxycholic and chenodeoxycholic acid was more reactive toward sulfation than the axial 7- or 12-hydroxyl groups and a short reaction for 2 min yielded mainly the 3-sulfates. Small amounts of other isomers and of unreacted starting product were efficiently removed by chromatography on Sephadex LH-20. Unconjugated bile salt monosulfates, which contaminated the preparations of conjugated monosulfates, could be removed by chromatography on silica gel prior to the purification on Sephadex LH-20 and crystallization.

In order to prepare the 7- or the 12-monosulfates, the 3-hydroxyl group was protected by reaction with ethyl chlorocarbonate. Fieser demonstrated that, in cholic acid, this reaction resulted in selective carbethoxylation of the equatorial 3α-hydroxyl group (17); similar results were obtained in the present studies on chenodeoxycholic and deoxycholic acid. After sulfation of the free hydroxyl at C-7 or C-12, the protecting 3-carbethoxy group could be removed by alkaline hydrolysis in 10 % NaOH in 80 % ethanol; under these conditions the sulfate ester function was not hydrolyzed, and deconjugation of the taurine or glycine conjugates did not occur.

The results of the elemental analysis were consistent with disodium salts of monosulfated dihydroxy bile acids. The position of the sulfate group was determined by a method involving oxidation of the unsubstituted hydroxyl group followed by solvolysis of the sulfate ester, or alternatively, by acetylation and solvolysis prior to oxidation. The hydroxy-oxo-5β-cholanoic acid so obtained was identified by GLC. The results confirmed that the sulfate group was at the expected position.

The purity of the crystalline products was further demonstrated by TLC. The isomeric chenodeoxycholate monosulfates were separated from each other and from the disulfate on TLC in solvent 1. This system also separated the isomeric deoxycholate monosulfates. It could also be used to separate the isomeric monosulfates of glycochenodeoxycholate and of glycodeoxycholate, respectively. The sulfates of taurine conjugated bile salts migrated very slowly in solvent 1, and separation between the isomers was inadequate. In solvent 2, however, taurochenodeoxycholate 3-sulfate was well separated from the 7-sulfate; likewise, taurodeoxycholate 3-sulfate and the 12-sulfate were completely resolved.

REFERENCES

(1) Palmer, R.H., Proc. Nat. Acad. Sci. USA, 58, 1047 (1967).
(2) Stiehl, A., Digestion, 11, 406 (1974).
(3) Back, P., Sjövall, J. and Sjövall, K., Med. Biol., 52, 31 (1974).
(4) Makino, I., Hashimoto, H., Shinozaki, K., Yoshino, K. and Nakagawa, S., Gastroenterology, 68, 545 (1975).
(5) Van Berge Henegouwen, G.P., Brandt, K-H., Eyssen, H. and Parmentier, G., Gut, 17, 861 (1976).
(6) Palmer, R.H., J. Lipid Res., 12, 680 (1971).
(7) Fisher, M.M., Price, V.M., Magnusson, R.J. and Yousef, I.M., Lipids, 9, 786 (1974).
(8) Parmentier, G., Mertens, J. and Eyssen, H., In : Advances in Bile Acid Research. S. Matern, J. Hackenschmidt, P. Back & W. Gerok (eds.), pp. 139-143. F.K. Schattauer Verlag, Stuttgart - New York (1975).
(9) Eyssen, H.J., Parmentier, G.G. and Mertens, J.A., Eur. J. Biochem., 66, 507 (1976).
(10) Palmer, R.H. and Bolt, M.G., J. Lipid Res., 12, 671 (1971).
(11) Parmentier, G. and Eyssen, H., Steroids, 26, 721 (1975).
(12) Haslewood, E.S. and Haslewood, G.A, Biochem. J., 155, 401 (1976).
(13) Summerfield, J.A., Gollan, J.L. and Billing, B.H., Biochem. J., 156, 339 (1976).
(14) Briggs, T. and Bussjaeger, C., Comp. Biochem. Physiol., 42B, 493 (1972).
(15) Eneroth, P., J. Lipid Res., 4, 11 (1963).
(16) Sjövall, J. and Vihko, R., Acta Chem. Scand., 20, 1419 (1966).
(17) Fieser, L.F., Herz, J.E., Klohs, M.W., Romero, M.A. and Utne, T., J. Amer. Chem. Soc., 74, 3309 (1952).
(18) Nair, P.P. and Garcia, C., Anal. Biochem., 29, 164 (1969).
(19) Eneroth, P., Gordon, B., Ryhage, R. and Sjövall, J., J. Lipid Res., 7, 511 (1966).