SOLVOLYSIS OF CHENODEOXYCHOLIC ACID SULFATES

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ABSTRACT

Chemical solvolysis of chenodeoxycholic acid sulfates was studied using 4 published methods. Quantitative recovery of chenodeoxycholic acid from the 3-sulfate was obtained with each method. However, only 2 methods yielded chenodeoxycholic acid after solvolysis of the 7-sulfate. In each instance a compound resembling lithocholic acid by GLC but identifiable as a derivative of chenodeoxycholic acid by mass spectrometry was obtained and represents a product formed during solvolysis. Failure to obtain adequate solvolysis of chenodeoxycholic acid 7-sulfate can lead to false identification of monohydroxy bile acids and apparent absence of the 7-sulfate and disulfate esters.

INTRODUCTION

The occurrence of the 3- and 7-sulfate and the 3, 7-disulfate of chenodeoxycholic acid in biological fluids has not been determined fully. Thus, in some studies the presence of the 3, 7-disulfate has been reported (1) and in others, neither the 3, 7-disulfate nor the 7-sulfate have been found (2). In an attempt to resolve these problems in our laboratory, we were surprised to find that the 7-sulfate, compared to the 3-sulfate, is resistant to chemical solvolysis using the method previously reported by us (3) but could be improved by extending the length of the reaction time. Further, we have found that other solvolysis methods (2, 5) have unsatisfactory recoveries of chenodeoxycholic acid from chenode-

oxycholic acid 7-sulfate. Comparison of the various methods of solvolysis is the subject of this report.

METHODS AND MATERIALS

Chenodoxycholic acid (Canada Packers, Toronto, Canada) methyl ester 3, 7-diacetate was crystallized from methanol and then hydrolyzed in methanol KOH, and again crystallized from methanol/water as the free acid.

The specific 3- and 7-sulfates of chenodeoxycholic acid were prepared using chlorosulfonic acid as described by Parmentier and Eyssen (6) and Haslewood and Haslewood (7). Each of the synthetic sulfates was chromatographically homogeneous.

Chenodeoxycholic acid 3-sulfate or chenodeoxycholic acid 7-sulfate (25μg) were solvolyzed using the following published conditions: Method 1 (3) Reagents: 25μg bile acid sulfate, 2, 2-dimethoxypropane 4 ml, methanol 3 ml, ethyl acetate 3 ml, conc. HCl 0.1 ml. Conditions: 25° for 4 hours or 24 hours. Method 2 (5) Reagents: 25μg bile acid sulfate, ethanol 1 ml/acetone 9 ml, 2N HCl 4 drops. Conditions: 25° for 2 days. Method 3 (4) Reagents: 25μg of bile acid sulfate, acetone 1 ml/methanol 9 ml, 6N HCl 3 drops. Conditions: 37° for 18 hour. Method 4 (2) Reagents: absolute ethanol 3 ml and ethyl acetate 27 ml equilibrated with 10 ml of 2M aqueous H_2SO_4. Conditions: 39° for 16 hr.

To each sample the internal standard 3α, 7α, -dihydroxy-12-oxo-5β-cholan-24-oic acid (Steraloids) equimolarly equivalent to the bile acid sulfate was added to correct losses during the procedure and for quantitative GLC analysis.

After the solvolysis reactions were terminated, the volumes were reduced to 0.5 ml and 2 ml of 1.25N NaOH was added to each tube. The samples were heated at 100° for 1 hr. They were subsequently cooled in ice and acidified to pH 2-3 with 12N HCl. Each sample was extracted 3X with 3 ml ethyl acetate. The ethyl acetate was washed with H_2O and evaporated to dryness under nitrogen. Methyl esters were prepared with diazomethane (Diazald-Aldrich). The solvents were evaporated and the acetate derivatives of the methyl esters were prepared by the method of Roovers et al (8).

GAS LIQUID CHROMATOGRAPHY

Samples were analyzed as the bile acid methyl ester acetates on 6 ft. glass column packed with 3% SP2250 on 80/100 Gas Chrom Q. All analyses were seen at 275° using flame ionization detector and electronic integration of the peak areas.

GAS LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY

Samples were analyzed on a Hewlett Packard 5992 B Mass Spectrometer. The following conditions were employed. Source pressure, 1×10^{-6}, electron impact voltage 70 E.V.; oven temp. 260, injector temp., 265; flow rate 20 ml/min. Carrier gas, helium; column, glass packed with 2% SP 2250 on 80/100 Gas Chrom Q. Spectral data were acquired on a Hewlett Packard 9825A computer calibrated against perfluorotributylamine (PFTBA) using the standard software provided by the manufacturer.

RESULTS

Solvolysis of chenodeoxycholic acid 3-sulfate was obtained using all 4 published methods (Table I). Recovery was greater than 90% except for method 4. In addition to quantitation by GLC analysis, each sample was analyzed by mass spectrometry. In each instance chenodeoxycholic acid methyl ester diacetate was identified by the characteristic fragments at m/z 430 (M - 60), 370 (M - 2X60), 355 (M - 2X60+15), 315 (M - 60+ ring cleavage), 255 (M - 2X60+ side chain), 228 (M - A+B ring cleavage), 213 (M - 2X60+ side chain + CO ring cleavage) and 201 (M - 2X60 + side chain + A ring).

TABLE I
Recovery of chenodeoxycholic acid from chenodeoxycholic acid 3-sulfate

Method #	Reaction Time HR	Chenodeoxycholic acid (RRT=0.578) AUC+	Internal Standard (RRT=1.0) AUC+	% Recovery Cheno
1 (3)	4	21.0	22.7	93
	24	20.4	23.0	89
2 (5)	48	21.7	23.4	93
3 (4)	18	21.5	21.5	100
4 (2)	16	15.0	18.0	83

( ) Reference Numbers
+ AUC = Area under the peak determined by electronic integration
RRT = relative retention time

Solvolysis of chenodeoxycholic acid 7-sulfate gave results quite different from the 3-sulfate. Only method 1 extended to 24 hours or method 3 yielded recoveries of 90% or more. It was noted that in all procedures a new peak appeared (Compound I) with a relative retention time (RRT) much less than chenodeoxycholic acid. Under the GLC conditions used Compound I has an RRT identical to a standard of lithocholic acid methyl ester acetate. However, mass spectral analysis yielded major fragments at m/z 255 and m/z 370 (Figure 1) indicating an unsaturated monohydroxy bile acid rather than lithocholic acid. It is of interest that the amount of Compound I appears to be inversely proportional to the yield of chenodeoxycholic acid.

TABLE II
Recovery of chenodeoxycholic acid from chenodeoxycholic acid 7-sulfate

Method #	Reaction Time HR	Compound I (RRT=0.360) AUC+	Chenodeoxycholic acid (RRT=0.578) AUC	Internal Standard (RRT=1.0) AUC	% Recovery Cheno
1 (3)	4	7.9	1.0	7.9	13
	24	2.1	19.5	20.0	98
2 (5)	24	11.4	5.3	17.7	30
3 (4)	18	1.3	4.3	4.8	90
4 (2)	16	22.9	8.1	47.6	17

( ) Reference Numbers

\+ AUC = Area under the peak determined by electronic integration

RRT = relative retention time

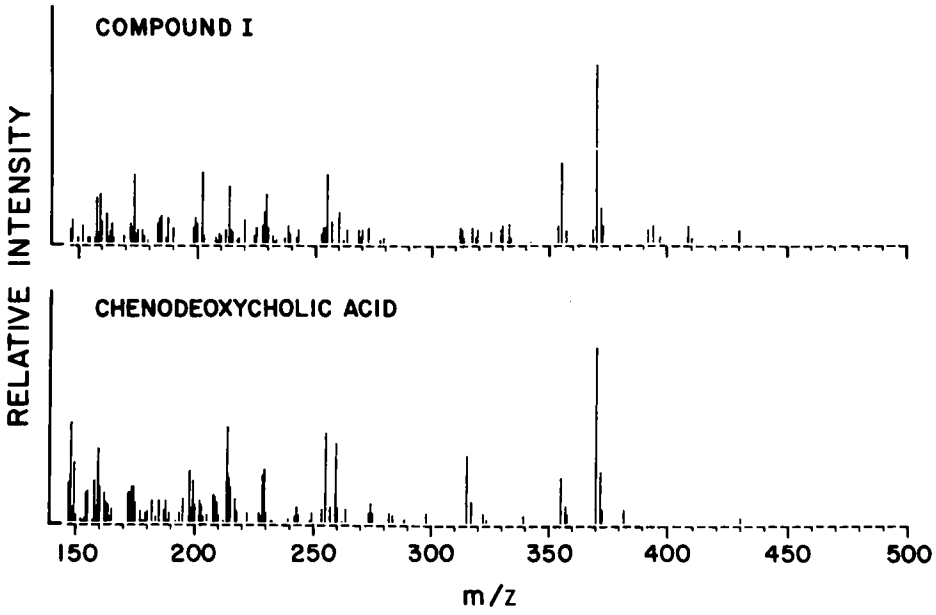

FIGURE 1. COMPARISON OF MASS SPECTRUM OF COMPOUND I TO CHENODEOXYCHOLIC ACID. The presence of major peaks at m/z 370 and m/z 255 indicates an unsaturated monohydroxy bile acid derived from chenodeoxycholic acid.

DISCUSSION

It is apparent from the findings that some of the methods currently in use do not provide satisfactory yields of chenodeoxycholic acid from the 7-sulfate and accounts, at least in part, for the differences in the amounts reported in urine (1, 2). Extending the reaction time of method 1 to 24 hours (3) provides a technique for obtaining satisfactory recovery. The formation of relatively large amounts of Compound I during solvolysis procedures that yield only small amounts of chenodeoxycholic acid from the 7-sulfate can give

spurious results in regard to the bile acid composition of biological fluids. Although mass spectral analysis indicates that Compound I is derived from chenodeoxycholic acid, its exact structure is unknown and the mechanism of its formation has not been studied.

Principles of chemical solvolysis were introduced by Burstein and Lieberman (9), who emphasized the need for establishing reaction conditions for each sulfate. The findings in these studies reaffirm this principle and delineate methods for the quantitation of the 7-sulfate of chenodeoxycholic acid.

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