Derivatization of bile acids with taurine for analysis by fast atom bombardment mass spectrometry with collision-induced fragmentation[1]

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Abstract When analyzed by fast atom bombardment mass spectrometry, taurine-conjugated bile acids give intense $[M-H]^-$ pseudomolecular ions that can be subjected to collision-induced fragmentation to give structural information. A method has been developed that permits rapid coupling of taurine to unconjugated, glycine-conjugated, sulfated, and glucuronidated bile acids. The reaction is performed for 2 h at room temperature in aqueous pyridine hydrochloride buffer, with or without dioxane, using 0.1 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as the coupling agent and 0.2 M taurine. The yields are higher than 95%. In contrast to published coupling reactions, the method permits conjugation of bile acids with the labile 7α-hydroxy-3-oxo-4-ene structure.—**Zhang, J., W. J. Griffiths, T. Bergman, and J. Sjövall.** Derivatization of bile acids with taurine for analysis by fast atom bombardment mass spectrometry with collision-induced fragmentation. *J. Lipid Res.* 1993. **34:** 1895–1900.

Supplementary key words conjugation • taurine-conjugated bile acids

Fast atom bombardment (FAB) (1, 2) is one of the favored ionization methods for the mass spectrometric analysis of involatile and polar molecules (3). Both negative and positive ion FAB mass spectra show strong pseudomolecular ions that permit facile determination of molecular weights of compounds within a biological mixture. Structural information can be obtained by collision-induced dissociation (CID) of the pseudomolecular ions. FAB mass spectrometry (FABMS) is an important method for rapid screening of profiles of bile acids and their conjugates in biological extracts (4, 5). Because of the low abundance of adduct ions, spectra of negative ions are simpler than those of positive ions and are preferred in these applications. The most intense negative-ion spectra are obtained with bile acids containing a sulfonic acid or sulfate ester group (4, 5). Thus, taurine-conjugated bile acids give strong $[M-H]^-$ ion currents in FABMS. When subjected to CID at high collision energy, these ions undergo charge-remote fragmentation (CRF) which provides important structural information (6). For example, FAB/CID spectra from $[M-H]^-$ ions permit differentiation of positional isomers of taurine-conjugated bile acids (7). Similar information can be obtained from positive-ion B/E linked scans without use of collision gas, although at much lower sensitivity (8). Daughter ion spectra produced by CID of $[M-H]^-$ ions at low energy have also been shown to be structurally informative and useful in diagnostic applications (9). Our studies have shown that taurine-conjugated bile acids give more intense and informative spectra than unconjugated, glycine-conjugated, or glucuronidated bile acids (W. J. Griffiths, J. Zhang, and J. Sjövall, unpublished results), and are the conjugates of choice for FAB/CID studies. A convenient method for conversion of different types of bile acids into taurine conjugates would therefore be of value for structure determination by FAB/CID mass spectrometry.

Several methods have been described for the synthesis of conjugated bile acids (10–12). The more recent methods are not sufficiently mild to permit conjugation of labile acids, e.g., those containing a 7α-hydroxy-3-oxo-4-ene structure, which occur in plasma and urine of patients with liver disease (13, 14) and as intermediates of bile acid biosynthesis (15). The solubilities of taurine and polar bile acid derivatives in the organic solvents used are also limiting factors. Furthermore, these methods were developed for synthesis on a larger scale (1–5 mmol) than that required for analytical purposes, and their ability to conjugate sulfated and glucuronidated bile acids has not been studied.

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is a water-soluble coupling agent that promotes the reaction between free amino and carboxyl groups to form

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Abbreviations: FAB, fast atom bombardment; CID, collision-induced dissociation; MS, mass spectrometry; CRF, charge-remote fragmentation; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; TLC, thin-layer chromatography; ODS, octadecyl silane.

[1]This paper is dedicated to Professor Gustav Paumgartner in honor of his 60th birthday.

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a peptide linkage (16). The present paper describes the use of this reagent in a new procedure for the synthesis of taurine-conjugated bile acids. The method is simple, mild, and rapid, and the reaction can be performed on a small scale and on different types of bile acids.

MATERIALS AND METHODS

Chemicals

All solvents were redistilled before use. Dioxane was refluxed for 2 h over lithium aluminum hydride before distillation. Lipidex-DEAP was from Packard Instruments Co. (Downers Grove, IL). Precoated thin-layer plates (Silica gel 60) and molybdatophosphoric acid were from Merck (Darmstadt, Germany). Octadecyl silane-bonded (ODS) silica (Preparative C18) was from Waters-Millipore (Milford, MA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and taurine were from Sigma (St. Louis, MO). [24-^{14}C]Cholic acid (1.99 GBq/mmol) and [1-^{14}C]glyco-labeled glycocholic acid (0.65 GBq/mmol) were from Amersham International Plc (Amersham, UK). The 3-, 7-, and 12-sulfates of cholic acid and the glucuronidated bile acids were kind gifts from Professor H. Eyssen, Louvain, Belgium and Dr. Peter Back, Freiburg, Germany, respectively. 3β,7α-Dihydroxy-5-cholenoic and 7α-hydroxy-3-oxo-4-cholenoic acids were synthesized from methyl 3β-acetoxy-5-cholenoate (Steraloids, Wilton, NH) (17). Other bile acids were from previous studies in this laboratory.

Synthesis and extraction

Free bile acid (5 μmol or less) was dissolved in 0.2 ml of dioxane, to which 0.2 ml of buffer (0.1 M pyridine hydrochloride in water, pH 5.0) was added. To the resultant solution, 50 μmol EDC and 100 μmol taurine were added in 0.1 ml water. The mixture was left for 2 h at room temperature and was then diluted with 4.5 ml of water and passed through a column of ODS-silica (1.5 × 0.8 cm). After a wash with 10 ml of water to remove excess EDC and taurine, the bile acids were eluted with 10 ml methanol. The same procedure was used for the derivatization of glycine-conjugated, sulfated, and glucuronidated bile acids with taurine, except that water was used as solvent instead of aqueous dioxane. The products were analyzed by thin-layer or ion-exchange chromatography and by mass spectrometry.

Thin-layer chromatography (TLC)

TLC was carried out using plates precoated with silica gel 60 and the solvent system butanol–acetic acid–water 10:1:1 (by vol). The zones were visualized by spraying with 10% molybdatophosphoric acid in ethanol and heating for 15 min at 130°C.

Ion-exchange chromatography

Lipidex-DEAP in acetate form was used to separate taurine-conjugated from unreacted bile acids to determine the yields of conjugated bile acid (18). The eluate from the ODS-silica was taken to dryness in vacuo and the residue was dissolved in 1 ml 72% aqueous ethanol and applied to a column, 30 × 0.4 cm, of the acetate form of Lipidex-DEAP in 72% aqueous ethanol. After washing with 15 ml 72% aqueous ethanol, unconjugated bile acids were eluted with 7.4 ml 0.1 M acetic acid in 72% aqueous ethanol, glycine-conjugated bile acids with 16.5 ml 0.3 M ammonium acetate, pH 5.0, in 72% ethanol, and taurine-conjugated bile acids with 11 ml 0.15 M ammonium acetate, pH 6.6, in 72% ethanol.

Radioactivity

Radioactivity was determined by liquid scintillation counting (1211 Minibeta, LKB Wallac, Sweden) using OptiPhase "HiSafe" II (LKB Wallac) as scintillation liquid.

Mass spectrometry

Negative ion FAB spectra were obtained on a VG 70-250 mass spectrometer (VG Analytical, Manchester, U.K.) fitted with a VG FAB source and an Ion Tech atom gun (Teddington, U.K.). Xenon atoms were used to bombard the sample, the ion gun condition typically being 8 kV accelerating potential and 1–2 mA discharge current. The source accelerating potential was 6 kV. Conjugated bile acids were dissolved in 70% methanol (10 ng–1 μg/μl) and 1–5 μl of solution was placed on the probe tip which was previously coated with glycerol.

CID spectra were generated using helium as the collision gas in the first field-free region gas cell at a pressure that gave a reading of 2 × 10^{-6} torr on the nearby analyzer ion gauge (this pressure of collision gas was sufficient to cause a 50% reduction in parent ion beam intensity). Daughter-ion (B/E is a constant) linked scans were recorded of the $[M-H]^-$ pseudomolecular ions. Approximately 20 scans of 20 s each, were recorded in the multichannel-analyzer mode.

RESULTS AND DISCUSSION

The mechanism of the coupling reaction can be thought of in two steps. First, EDC reacts with the carboxylic acid group and activates it. Second, EDC is released as a water-soluble isourea derivative after displacement by the taurine. One of the advantages of the method is that the purification procedure is simple and fast. The reaction mixture is extracted on a column of ODS-silica, and the isourea, excess of EDC, and taurine are removed by washing with water. The methanol eluate

can then be directly used for analysis by FABMS. Experimental conditions were optimized by varying the concentrations of EDC and taurine, solvents, pH, reaction time, and the amount and type of bile acid. The results were evaluated by TLC and FABMS. These analyses showed that for all bile acids studied, the conditions finally adopted resulted in almost complete disappearance of unreacted bile acid. In all cases, with the exception of the glucuronidated bile acids, only one reaction product appeared giving the pseudomolecular ion expected for the taurine-conjugated bile acid. The glucuronidated bile acids gave a mixture of two isomeric mono- and the di-taurine conjugated bile acid.

A more detailed study of yields was performed using [24-^{14}C]cholic acid. The unreacted and taurine-conjugated bile acids were separated by ion-exchange chromatography. The conversion of 5 μmol cholic acid into cholyltaurine was 78%, 96%, and 99% after 0.1, 1, and 2 h, respectively, at room temperature in 0.5 ml 40% aqueous dioxane containing 50 μmol EDC and 100 μmol taurine. The yield at 1 h was less than 50% with only 25 μmol EDC and 75% with 50 μmol taurine. A 3-fold lower concentration of taurine could be used when added as the sodium salt instead of the acid. Thus, the yield of taurocholate was 99% after 2 h using 50 μmol EDC and 30 μmol sodium taurate. The pH of the reaction mixture was important and had to be kept between 5.0 and 6.0. The presence of 2-propanol, ethanol, or methanol instead of dioxane decreased the yields in this order by 20–80%. However, the reaction was quantitative in the absence of organic solvent as tested with ^{14}C-labeled cholylglycine which yielded 99% of cholylglycyltaurine after 1 h of reaction. The yields of taurine conjugates from small amounts of ^{14}C-labeled cholic acid (64 ng) and cholylglycine (1.3 μg) were also quantitative using the conditions above.

The derivatization with taurine was studied with unconjugated, glycine-conjugated, sulfated, and glucuronidated bile acids. The negative ion FAB mass spectra were recorded for all products and the major ions formed are listed in **Table 1.** No peak corresponding to the pseudomolecular ion of unreacted starting material was observed in any of the spectra. After derivatization with taurine, glycine-conjugated and glucuronidated bile acids gave similar yields of pseudomolecular anions as taurine-conjugated or sulfated bile acids.

The mass spectra of the first eight taurine-conjugated bile acids listed in Table 1 are dominated by $[M-H]^-$ pseudomolecular ions. Other major ions correspond to loss of 16 Da and 18 Da. Shown in **Fig. 1** is the negative ion FAB spectrum of 7α-hydroxy-3-oxo-4-cholenoyl-taurine. The preparation of this compound illustrates the mild condition of the coupling reaction. When published methods were tried (11, 12), the hydroxyl group was eliminated, resulting in formation of 3-oxo-4,6-choladienoyltaurine as the only conjugated bile acid.

In agreement with previous results (7), positional bile acid isomers can be differentiated from each other by the CID spectra of the $[M-H]^-$ pseudomolecular ions of their taurine conjugates. This is shown in **Fig. 2** for the three dihydroxy bile acids carrying hydroxyl groups in different rings. The CID spectrum of hyodeoxycholyltaurine was also slightly but significantly different from that of

TABLE 1. The major peaks in the negative ion FAB mass spectra of taurine-conjugated bile acids synthesized by the present method

Compound	$[M-H]^-$ *m/z* (RA)[a]	Other Ions *m/z* (RA)[a]
Cholyltaurine	514 (100)	498 (8)[b]
Chenodeoxycholyltaurine	498 (100)	482 (8)[b]
Deoxycholyltaurine	498 (100)	482 (6)[b]
7α,12α-Dihydroxycholanoyltaurine	498 (100)	482 (6)[b]
Hyodeoxycholyltaurine	498 (100)	482 (6)[b]
3β,7α-Dihydroxy-5-cholenoyltaurine	496 (100)	480 (10)[b]
7α-Hydroxy-3-oxo-4-cholenoyltaurine	494 (100)	478 (10)[b]
Lithocholyltaurine	482 (100)	466 (8)[b]
Cholylglycyltaurine	571 (100)	555 (5)[b]
Chenodeoxycholylglycyltaurine	555 (100)	539 (5)[b]
Deoxycholylglycyltaurine	555 (100)	539 (5)[b]
Lithocholylglycyltaurine	539 (100)	523 (5)[b]
3-Sulfocholyltaurine	594 (40)	514 (100)
7-Sulfocholyltaurine	594 (50)	514 (100)
12-Sulfocholyltaurine	594 (80)	514 (100)
3-(Glucuronidyltaurine)-chenodeoxycholyltaurine	781 (100)	674 (10)[c]
3-(Glucuronidyltaurine)-deoxycholyltaurine	781 (100)	674 (20)[c]

[a] *m/z*, mass/charge ratio; RA, relative abundance, %.
[b] Ions due to loss of 16 and 18 Da had similar intensities in all cases.
[c] Mono-taurine derivative.

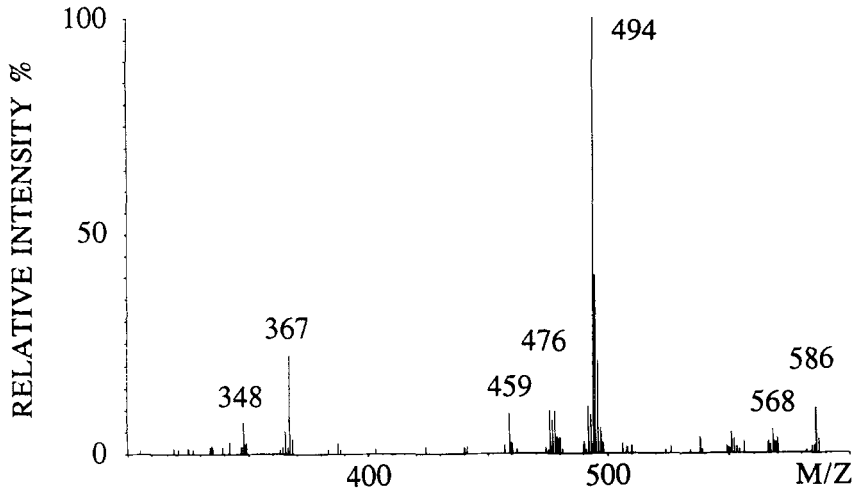

Fig. 1. High mass region of the negative ion FAB mass spectrum of 7α-hydroxy-3-oxo-4-cholenoyltaurine. The pseudomolecular ion at *m/z* 494 is accompanied by its glycerol adduct at *m/z* 586. Peaks due to loss of 16 and 18 Da are seen (cf. Table 1). The peaks at *m/z* 367 and 459 are from the glycerol matrix.

chenodeoxycholyltaurine, showing that 6α- and 7α-hydroxylated bile acids can be differentiated from each other. The spectra of chenodeoxycholyltaurine and ursodeoxycholyltaurine were not significantly different. Thus, determination of the stereochemistry requires analysis by gas chromatography and mass spectrometry of suitable derivatives. As the chromatographic behavior is usually more sensitive to stereochemical differences than the mass spectra, the combination of high-performance liquid chromatography with FAB/CID mass spectrometry may be an alternative. Ongoing studies have shown that FAB/CID spectra that show clear CRF patterns can be obtained with 5 ng taurine-conjugated bile acid using the VG 70-250 instrument. More recent equipment can be expected to give higher sensitivity.

Glycine-conjugated bile acids give less intense $[M-H]^-$ (4), and less informative and intense CRF patterns than taurine-conjugated bile acids (W. J. Griffiths, J. Zhang, and J. Sjövall, unpublished results). When conjugated with taurine, however, the resultant glycyltaurine conjugates show intense $[M-H]^-$ ions and informative CRF patterns.

The three sulfated bile acid isomers (Table 1) were quantitatively converted into taurine conjugates. In agreement with previous studies of sulfated taurine conjugates, the FABMS spectra showed strong $[M-H-80]^-$ ions (4). These correspond to loss of SO_3 in the ionization process. It was possible to differentiate the three isomers from the FAB/CID spectra of the $[M-H]^-$ pseudomolecular ions.

The mass spectra of the derivatized glucuronidated bile acids show intense $[M-H]^-$ ions of mono- and di-taurine conjugates. It is probably possible to achieve complete derivatization with longer reaction times. Taurine can react with the carboxylic acid group of the bile acid or glucuronic acid moieties or with both. CID of the $[M-H]^-$ ions reveals that both mono-taurine conjugates are formed as a mixture. The FAB/CID spectra of the di-taurine conjugates show that ionization can occur at either of the sulfonic acid groups. The CRF patterns from both the mono- and di-taurine conjugates allow the positions of conjugation and the hydroxyl groups to be determined. These spectra will be further discussed elsewhere (W. J. Griffiths, J. Zhang, and J. Sjövall, unpublished results).

The method is useful for derivatization of bile acids in biological extracts with taurine or other aminosulfonic acids (19). The CRF patterns in the FAB/CID spectra of pseudomolecular ions from the taurine derivatives yield structural information that is useful for the identification of many of the bile acids present in biological mixtures. Such studies are currently being carried out in this laboratory.

Finally, it may be mentioned that the method can be used for analysis of other classes of carboxylic acids by FAB/CID. The only requirement is that the compound is soluble in the reaction mixture and that the product is extracted by ODS-silica. For example, when the reaction was applied to the fraction of unconjugated bile acids in a urine sample, a pseudomolecular ion was observed at

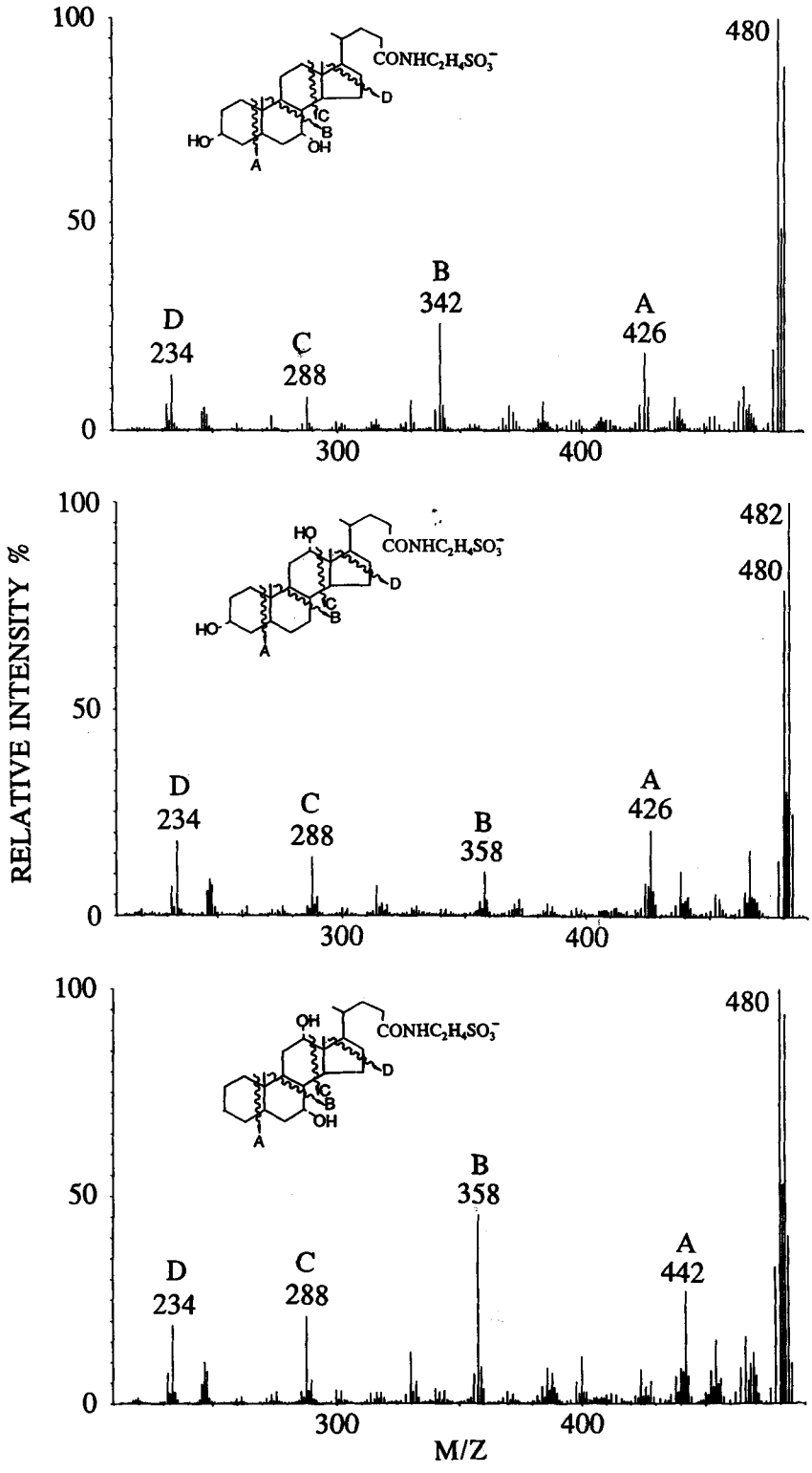

Fig. 2. Spectra of daughter ions produced by CID of the $[M-H]^-$ ions from chenodeoxycholyltaurine (top), deoxycholyltaurine (middle), and 7α,12α-dihydroxycholanoyltaurine (bottom).

m/z 378 which upon CID gave a CRF showing that it was the taurine derivative of 10-hydroxypalmitic acid. Thus, the method should have a wide applicability.

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