Rapid and quantitative analysis of unconjugated C_{27} bile acids in plasma and blood samples by tandem mass spectrometry

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Abstract **A subgroup of peroxisomal disorders, peroxisome biogenesis defects (PBD), can be differentiated by elevated levels of C_{27} bile acids in plasma and bile. Patients with peroxisomal disorders, who lack the ability to chain-shorten the C_{27} bile acid intermediates into C_{24} bile acids, show elevated levels of C_{27} bile acids, notably 3α,7α-dihydroxy-5β-cholest-26-oic acid and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid. C_{27} bile acids are normally estimated against other bile acid standards, by time-consuming gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry methods, in plasma (minimum of 50 μl). In this article we describe the quantitation of unconjugated di- and trihydroxy C_{27} bile acids in 5-μl plasma samples and 3-mm blood spots, using deuterium-labeled internal standards. The synthesis of $^2H_3$-labeled di- and trihydroxycoprostanic acids is described. The sample preparation and analysis by electrospray tandem mass spectrometry (ES-MS/MS) takes less than 1 h and features dimethylaminoethyl ester derivatives. The levels of the di- and trihydroxy bile acids are significantly higher in PBD patients than in age-matched control subjects for both plasma and blood spots collected at birth (some stored for up to 18 years). Excellent correlation is observed between the $C_{26:0}/C_{22:0}$ very long chain fatty acid (VLCFA) ratio and the levels of trihydroxy C_{27} bile acids in plasma from PBD patients. The ES-MS/MS method can be used to rapidly screen for PBD patients in plasma samples with elevated $C_{26:0}/C_{22:0}$ VLCFA ratios and in archived collections of neonatal blood spots.**—Johnson, D. W., H. J. ten Brink, R. C. Schuit, and C. Jakobs. **Rapid and quantitive analysis of unconjugated C_{27} bile acids in plasma and blood samples by tandem mass spectrometry.** ***J. Lipid Res.*** **2001.** 42: **9–16.**

Supplementary key words peroxisome • ES-MS/MS • blood spots

The conversion of cholesterol into the primary bile acids chenodeoxycholic acid and cholic acid involves hydroxylation of the steroid nucleus and oxidative shortening of the side chain. Intermediates in this process are the C_{27} bile acid intermediates 3α,7α-dihydroxy-5β-cholest-26-oic acid (DHCA) and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA) (1–4). The major metabolic pathway for DHCA and THCA is chain-shortening via a series of peroxisomal β-oxidation reactions catalyzed by a set of enzymes that act specifically on 2-methyl-substituted fatty acids. Patients with defective peroxisomal β-oxidation caused by either a peroxisome biogenesis defect or a specifically impaired bile acid β-oxidation enzyme (5) accumulate DHCA and THCA in blood and bile. Consequently, measurement of DHCA and THCA in body fluids is an important marker for the diagnosis of peroxisomal disorders, both postnatal and prenatal. Concentrations of C_{27} bile acids have been measured by tedious gas chromatography-mass spectrometry (GC-MS) methods requiring large volumes of plasma or bile (2, 6, 7). More recently a faster high performance liquid chromatography (HPLC) electrospray tandem mass spectrometric (ES-MS/MS) method, requiring 50 μl of plasma, was developed (8). Deuterium-labeled taurine and glycine conjugates of C_{24} bile acids, chenodeoxycholic and cholic acids, were used to quantify the C_{24} bile acids by a procedure previously used for their rapid analysis in blood spots (9). DHCA and THCA, for which no deuterium-labeled standards were available, were measured relative to the respective di- and trihydroxy C_{24} bile acids.

To augment a screening procedure for the diagnosis of peroxisomal disorders (10) in blood spots and plasma, methods to rapidly subclassify these disorders were sought. One obvious solution was to develop a stable isotope dilution tandem mass spectrometric method to quantitate DHCA and THCA in a 3-mm blood spot or an equivalent amount of plasma (5 μl). Neither DHCA, THCA, nor sta-

Abbreviations: DHCA, 3α,7α-dihydroxy-5β-cholest-26-oic acid; ES-MS/MS, electrospray tandem mass spectrometry; GC-MS, gas chromatography-mass spectrometry; MOM, methoxymethyl; MRM, multiple reaction monitoring; THCA, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid.

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ble isotope-labeled analogs are commercially available. We report the chemical synthesis of both unlabeled DHCA and THCA, and [27,27,27-$^2H_3$]DHCA and [27,27,27-$^2H_3$]-THCA from readily available chenodeoxycholic acid and cholic acid, respectively. A scheme based on the synthesis of deuterium-labeled pristanic and phytanic acids was used (11). Our next task was to improve the ES-MS/MS analysis method. The C_{27} bile acids are mostly unconjugated (2), yet the HPLC ES-MS/MS analysis (8) was performed on the minor, conjugated bile acids, because they contained charged groups that facilitated analysis by ES-MS/MS. Very long chain fatty acids (VLCFA), released from lipids, have been successfully analyzed by ES-MS/MS as dimethylaminoethyl (DMAE) esters (12). The tertiary nitrogen of the ester is protonated in acid solution and the resultant ion is detectable at low concentration by ES-MS. Free hydroxyl groups, present in bile acids, interfere with the analysis of DMAE esters of the carboxylic acid group and must be protected (12). Accordingly, acetyl DMAE esters of unconjugated C_{27} bile acids were prepared and shown to be suitable for ES-MS/MS analysis. A simple method for the ES-MS/MS quantitation of DHCA and THCA, in positive ion mode, was subsequently developed. Samples obtained from peroxisomal biogenesis defect (PBD) patients were analyzed for DHCA, THCA, and the $C_{26:0}/C_{22:0}$ VLCFA ratio and compared with those of control subjects.

MATERIALS AND METHODS

Patient samples

This research was approved by the Human Ethics Committee of the Women's and Children's Hospital, North Adelaide. Consent was obtained for the use of the plasma and blood spot samples analyzed in this study.

Plasma samples of PBD patients were referred to our laboratory for investigation and diagnosed by biochemical and enzymatic methods. Control plasma samples were from children, aged <10 years, referred to our laboratory and shown to have no metabolic disorder. Blood spot samples were obtained from Guthrie cards collected for a neonatal blood spot program and kept in sealed containers in a cool, dry place. Permission was obtained for use of these blood spots for tandem mass spectrometric analysis.

Materials

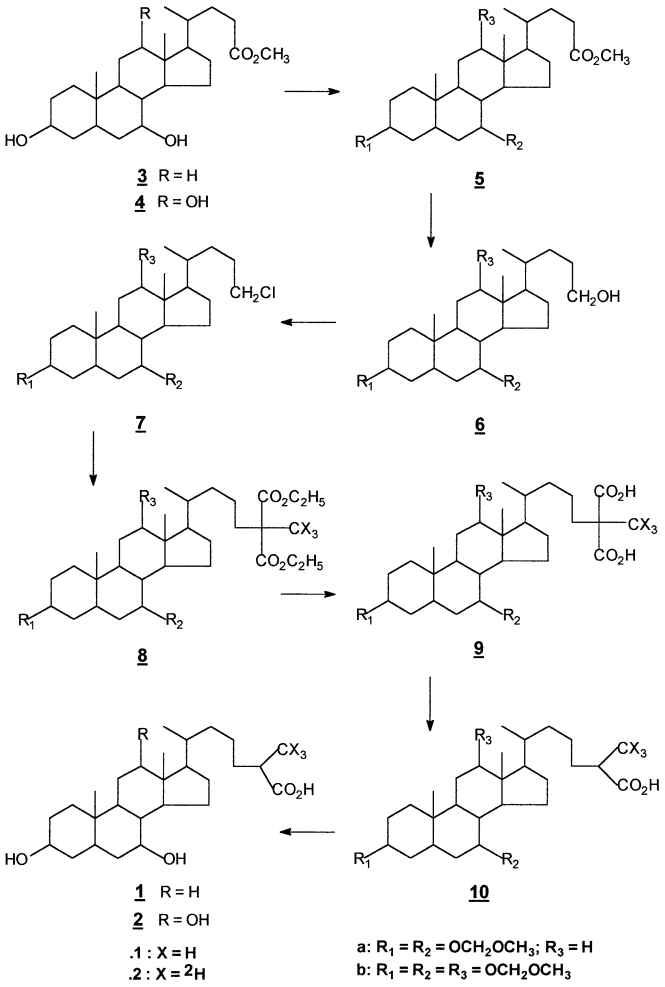
[$^2H_4$]chenodeoxycholic acid (98% ^2H) and [$^2H_4$]cholic acid (98% ^2H) were purchased from C/D/N Isotopes (Montreal, Canada). Iodo[$^2H_3$]methane (99.5% ^2H) was purchased from Aldrich (Milwaukee, WI). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) in the highest purity available. All solvents were HPLC grade.

Synthesis of reference compounds

Reactions were carried out in oven-dried glassware under a nitrogen atmosphere. Analysis of reaction products was performed on a Carlo Erba (Milan, Italy) 4160 gas chromatograph with flame ionization detector and a 25-m, 0.22-mm i.d. CPSil 19 capillary column (Chrompack; Varian U.S.A., Walnut Creek, CA) kept at 300°C with helium (1-ml/min flow rate) as carrier gas. GC-MS was performed on a Hewlett-Packard (Palo Alto, CA) Engine 5989B coupled to a Hewlett-Packard 5890 gas chromatograph, using the same capillary column and conditions described above. Mass spectra were recorded in the positive chemical ionization mode with ammonia as reactant gas.

The preparation of THCA, illustrative for all four standards (see **Scheme 1**), is described below.

24-Chloro-3α,7α,12α-tri(methylenoxymethoxy)-5β cholane (7.b). A solution of methyl cholate **4** (25 g) in chloroform (200 ml) was slowly added to a suspension of phosphorus pentoxide (100 g) in dimethoxymethane (250 ml) and chloroform (100 ml). After stirring at room temperature for 16 h, the solution was decanted, and poured into aqueous saturated sodium hydrogen carbonate solution (500 ml). The remaining solid was washed with diethyl ether (2 × 250 ml). The decanted mixture was extracted with the ether washing, and again with diethyl ether (2 liters total). The combined ethereal extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and evaporated to afford methoxymethyl (MOM)-protected methyl cholate **5.b** as a brown, viscous oil. Crude ester **5.b**, dissolved in dry tetrahydrofuran (200 ml), was slowly added to a suspension of lithium aluminium hydride (2.5 g) in dry tetrahydrofuran (100 ml). The mixture was boiled under reflux for 4 h, cooled in an ice bath, quenched slowly with water (150 ml), and extracted with ethyl acetate (5 × 150 ml). The combined extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and evaporated to afford MOM-protected alcohol **6.b**



Scheme 1. Schematic outline for the syntheses of DHCA, THCA, [27,27,27-$^2H_3$]DHCA, and [27,27,27-$^2H_3$]THCA.

(52.8 g from two preparations) as a pale yellow solid. Crude alcohol **6.b** (45 g) was dissolved in carbon tetrachloride–dichloromethane 1:1 (v/v, 400 ml), and dry triphenylphosphine (45 g) was added. The mixture was stirred at room temperature for 16 h, and then the solvents were evaporated. Treatment of the residue with ethyl acetate separated some remaining triphenylphosphine and the triphenylphosphine oxide by-product, which were removed by filtration over Celite. The filtrate was concentrated, and the residue was repeatedly treated with ethyl acetate in the above-described manner until the weight of the residue after evaporation was constant at 48 g. Crude chloride **7.b** was purified over silica (1.4 kg). Remaining triphenylphosphine and triphenylphosphine oxide were eluted with 5% ethyl acetate in petroleum ether (40–60) and the chloride was eluted with 20% ethyl acetate in petroleum ether (40–60). Fractions containing pure product were combined and evaporated, yielding chloride **7.b** (14.2 g, 31% based on **4**) as a white solid.

THCA (2.1). To a suspension of sodium hydride (60% dispersion in mineral oil, 2.5 g) in dry dimethylformamide (50 ml) was slowly added a solution of diethyl methylmalonate (13.4 g) in dry dimethylformamide (50 ml). The mixture was stirred at room temperature for 4 h. A solution of chloride **7.b** (7.0 g) in dry dimethylformamide (150 ml) was added, and the mixture was stirred and heated at 120°C overnight. After cooling, water was added (500 ml), followed by extraction with ethyl acetate (6 × 100 ml). The combined extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and evaporated. The crude product was purified over silica (120 g) by elution with 15% ethyl acetate in petroleum ether (40–60), yielding ester **8.b.1** (6.8 g) as a colorless oil. Ester **8.b.1** (6.8 g) was saponified in 5% potassium hydroxide in ethanol (150 ml) kept at 60°C overnight. After cooling, water (300 ml) was added and the mixture was washed with ethyl acetate (2 × 300 ml). The aqueous mixture was quickly (to avoid deprotection of hydroxy groups) acidified to pH 1 and extracted with ethyl acetate (4 × 300 ml). The combined extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and evaporated to give crude malonic acid **9.b.1** as a pale yellow oil. This oil, dissolved in ethyl acetate (5 ml), was poured into petroleum ether (40–60) (200 ml), from which the pure product separated as a white solid (5.5 g).

Malonic acid **9.b.1** (5.5 g) was decarboxylated by heating it in xylene (mixture of isomers) at 120°C for 72 h. The solvent was evaporated. For deprotection of the hydroxy groups, the residue was dissolved in methanol (100 ml) containing 10 drops of concentrated hydrochloric acid, and stirred at 60°C overnight. After cooling, water (200 ml) was added and the mixture, acidified with hydrochloric acid to pH 1, was extracted with ethyl acetate (5 × 75 ml). The combined extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and evaporated to give THCA methyl ester (4.2 g) as a yellow oil. This ester was saponified in 5% potassium hydroxide in ethanol (100 ml) kept at 60°C overnight. After cooling, water was added (100 ml) and the mixture was acidified to pH 1, followed by extraction with ethyl acetate (4 × 75 ml). The combined extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and evaporated to give crude THCA as a yellow oil. The crude product dissolved in ethyl acetate (5 ml) was poured into petroleum ether (40–60) (200 ml). The solid was filtered, washed with petroleum ether (40–60), and finally recrystallized from acetone-petroleum ether (40–60) to yield THCA (2.0 g) as white crystals. A sample was converted into its methyl ester by treatment with diazomethane, and then acetylated with acetic anhydride–pyridine 1:1 (v/v) for 3 h at 70°C. GC analysis revealed a purity of >98%.

Diethyl [$^2H_3$]methylmalonate. To a suspension of sodium hydride (60% dispersion in mineral oil, 4 g) in dry dimethylformamide (50 ml) was slowly added diethyl malonate (14.7 g), and the mixture was stirred at room temperature for 4 h. Iodo[$^2H_3$]methane (20 g) was slowly added, and the mixture was stirred at 60°C overnight. After cooling, water was added (60 ml), followed by extraction with petroleum ether (40–60) (4 × 75 ml). The combined extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and evaporated. The crude residue was applied to a column of silica (400 g) and rinsed with petroleum ether (40–60). Elution with 3% ethyl acetate in petroleum ether (40–60) gave overlapping fractions of diethyl di-[$^2H_3$]methylmalonate, diethyl [$^2H_3$]methylmalonate, and diethyl malonate, respectively. After evaporation of the relevant fractions a total amount of 6.6 g of diethyl [$^2H_3$]methylmalonate (80% pure) was isolated, contaminated with diethyl di-[$^2H_3$]methylmalonate (20%). This impurity does not interfere in the subsequent coupling reaction with chloride **7**.

DHCA (1.1), [27,27,27-$^2H_3$]DHCA (1.2), and [27,27,27-$^2H_3$]THCA (2.2). In an analogous manner to the synthesis of THCA (**2.1**), [27,27,27-$^2H_3$]THCA (**2.2**; 1.9 g yield) was prepared from methyl cholate. Methyl chenodeoxycholate served as the precursor for DHCA (**1.1**; 2.0 g yield) and [27,27,27-$^2H_3$]DHCA (**1.2**; 2.0 g yield). Chemical purity for all three was >98% and isotopic purity was >98% $^2H_3$.

Preparation of dimethylaminoethyl esters of bile acids from plasma samples

To a 100 × 13 mm glass tube was added internal standard mixture (5 μl, 2.5 μM each of [$^2H_3$]DHCA, [$^2H_3$]THCA, [$^2H_4$]cholic acid, and [$^2H_4$]chenodeoxycholic acid in methanol), plasma (5 μl), and acetonitrile (400 μl). The solvent was evaporated in a stream of nitrogen, acetyl chloride (200 μl) was added, the tube was sealed, and the mixture was heated at 50°C for 5 min. The mixture was evaporated in a stream of nitrogen, oxalyl chloride (100 μl) was added, and the tube was sealed and heated at 50°C for 5 min. The volatile material was removed in a stream of nitrogen and the residue was suspended in hexane (1 ml). The hexane solution was filtered through a glass wool plug in a Pasteur pipette and evaporated in a stream of nitrogen. The residue was treated with dimethylaminoethanol (60 μl) at 20°C for 5 min. The volatile material was removed in a stream of nitrogen and the residue was dissolved in 100 μl of acetonitrile–water–formic acid 50:50:0.25 (v/v/v).

Preparation of dimethylaminoethyl esters of bile acids from blood and plasma spots

To a 100 × 13 mm glass tube was added internal standard mixture (5 μl, 2.5 μM each of [$^2H_3$]DHCA, [$^2H_3$]THCA, [$^2H_4$]cholic acid, and [$^2H_4$]chenodeoxycholic acid in methanol), a 3-mm blood spot (containing 3.6 μl of blood) or two 3-mm plasma spots (containing 4.3 μl of plasma), and methanol (400 μl). The mixture was vortexed for 1 min, heated at 50°C for 20 min, and vortexed for 1 min. The methanol solution was transferred to another 100 × 13 mm glass tube and the solvent was evaporated in a stream of nitrogen. The residue was treated with acetyl chloride and the subsequent steps applied were as described above for plasma samples.

ES-MS/MS analysis

ES-MS/MS analysis was performed on a Perkin-Elmer (Norwalk, CT) SCIEX API365 instrument equipped with an ion spray assembly, a Hewlett-Packard 1100 HPLC, and a Gilson (Middleton, WI) 215 autosampler. The HPLC flow rate was 35 μl/min and the autosampler injection volume was 20 μl. The mobile phase was acetonitrile–water–formic acid 50:50:0.25 (v/v/v). The API365 was operated in multiple reaction monitoring (MRM) mode and monitored the following ion pairs: 548.5/488.5 (chenodeoxycholic acid), 552.5.5/492.5 ([$^2H_4$]chenodeoxycholic acid), 590.5/530.5

(DHCA), 593.5/533.5 ([$^2H_3$]DHCA), 606.5/546.5 (cholic acid), 610.5/550.5 ([$^2H_4$]cholic acid), 648.5/588.5 (THCA), and 651.5/591.5 ([$^2H_3$]THCA).

RESULTS

Syntheses of unlabeled and trideuterated DHCA and THCA

The scheme for synthesis of unlabeled THCA **2.1**, described in Materials and Methods and outlined in Scheme 1, is common to the preparation of all four standards. The steroidal hydroxy groups of the inexpensive starting materials chenodeoxycholic ester **3** and cholic ester **4** were protected as MOM ethers. The methyl ester side chains of the protected esters **5** were converted to chlorides **7** and coupled with methylmalonic ester. Diethyl [$^2H_3$]methylmalonate, used in the synthesis of the deuterated standards **1.2** and **2.2**, was prepared by methylation of diethyl malonate with iodo[$^2H_3$]methane. Decarboxylation of the resulting malonates **8** and removal of the protective groups afforded the desired coprostanic acids.

ES-MS/MS analyses of DHCA and THCA

The acetyl DMAE derivatives of DHCA and THCA were prepared. Previous studies (12) have shown that the hydroxyl group of hydroxy acids must be protected before DMAE ester formation. The DHCA and THCA derivatives produced strong protonated molecular ions (m/z 590 and 648, respectively) in their electrospray mass spectra (**Fig. 1**). The product ion spectra of the protonated molecular ions were dominated by an ion from neutral loss of 60 Da (acetic acid). This is in contrast to the neutral loss of a 45-Da product ion of long-chain fatty acids (10, 12). A plasma sample from a PBD patient was dried, treated with acetyl chloride, and extracted with hexane. The dried extract was derivatized with the reagents for DMAE ester formation. ES-MS/MS analysis with a neutral loss of 60 Da experiment revealed substantial amounts of DHCA and THCA.

Mixtures of labeled and unlabeled (acetyl DMAE derivatives of) DHCA and THCA were analyzed by ES-MS/MS with four MRM experiments. In each experiment the intensity of the $MH^+/(MH\text{-}60)^+$ ion pair was measured. A 5-point standard curve, constructed over the range expected for plasma and blood spot samples, afforded an R^2 of 0.999 for both DHCA and THCA. The slopes were both 1.25. A normal plasma sample was spiked with DHCA and THCA (5.0 μM each) and replicate analysis (n = 5, mean ± SD) gave 5.04 ± 0.24 μM DHCA and 4.43 ± 0.38 μM THCA. Replicate analysis (n = 6, mean ± SD) of 3-mm spots (containing 2.16 μl of plasma) of the above spiked plasma absorbed on filter paper gave 5.14 ± 0.47 μM

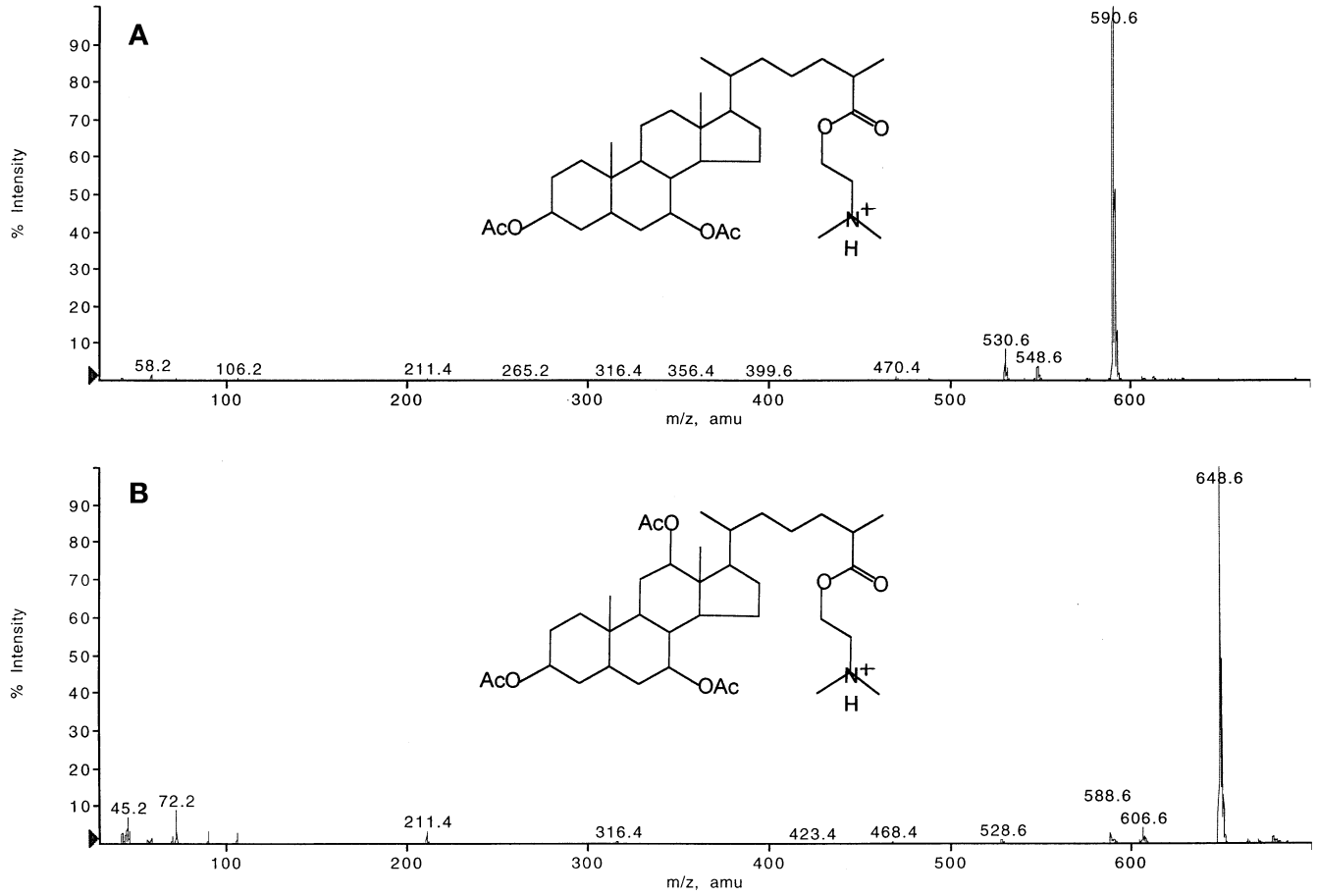


Fig. 1. Electrospray mass spectra of the protonated acetyl dimethylaminoethyl derivatives of (A) DHCA and (B) THCA.

TABLE 1. Analyses for C_{27} bile acids and $C_{26:0}/C_{22:0}$ VLCFA ratios in 5-μl plasma samples from peroxisomal biogenesis defect patients compared with control subjects

	$C_{26:0}/C_{22:0}$	Di-OH-C_{27}	Tri-OH-C_{27}	Tetra-OH-C_{27}
		μM	μM	
PBD patient				
1	0.10	0.44	0.33	
2	0.12	0.17	0.18	
3	0.14	0.50	0.58	
4	0.17	3.46	1.61	x[a]
5	0.26	4.11	1.68	x
6	0.35	15.26	8.40	x
7	0.36	0.53	7.98	x
8	0.40	1.54	9.57	x
9[b]	0.54	4.94	14.24	x
9[c]	0.68	8.63	12.56	x
Control subjects (n = 18, mean ± SD)		0.035 ± 0.01	0.14 ± 0.07	
Blanks (n = 6, mean ± SD)		0.013 ± 0.002	0.036 ± 0.016	

[a] x, Present.
[b] Day 4 after birth.
[c] day 13 after birth.

DHCA and 4.86 ± 0.58 μM THCA. Plasma samples (5 μl) from 18 normal infants were analyzed for dihydroxy and trihydroxy C_{27} bile acids after the addition of d_3-DHCA and d_3-THCA (2.5 μM each). The levels were approximately triple the mean of blank measurements (**Table 1**), obtained by performing the analysis with distilled water instead of plasma. Replicate analysis of a normal plasma sample afforded intra-assay variations (n = 6) of 11.0% (DHCA) and

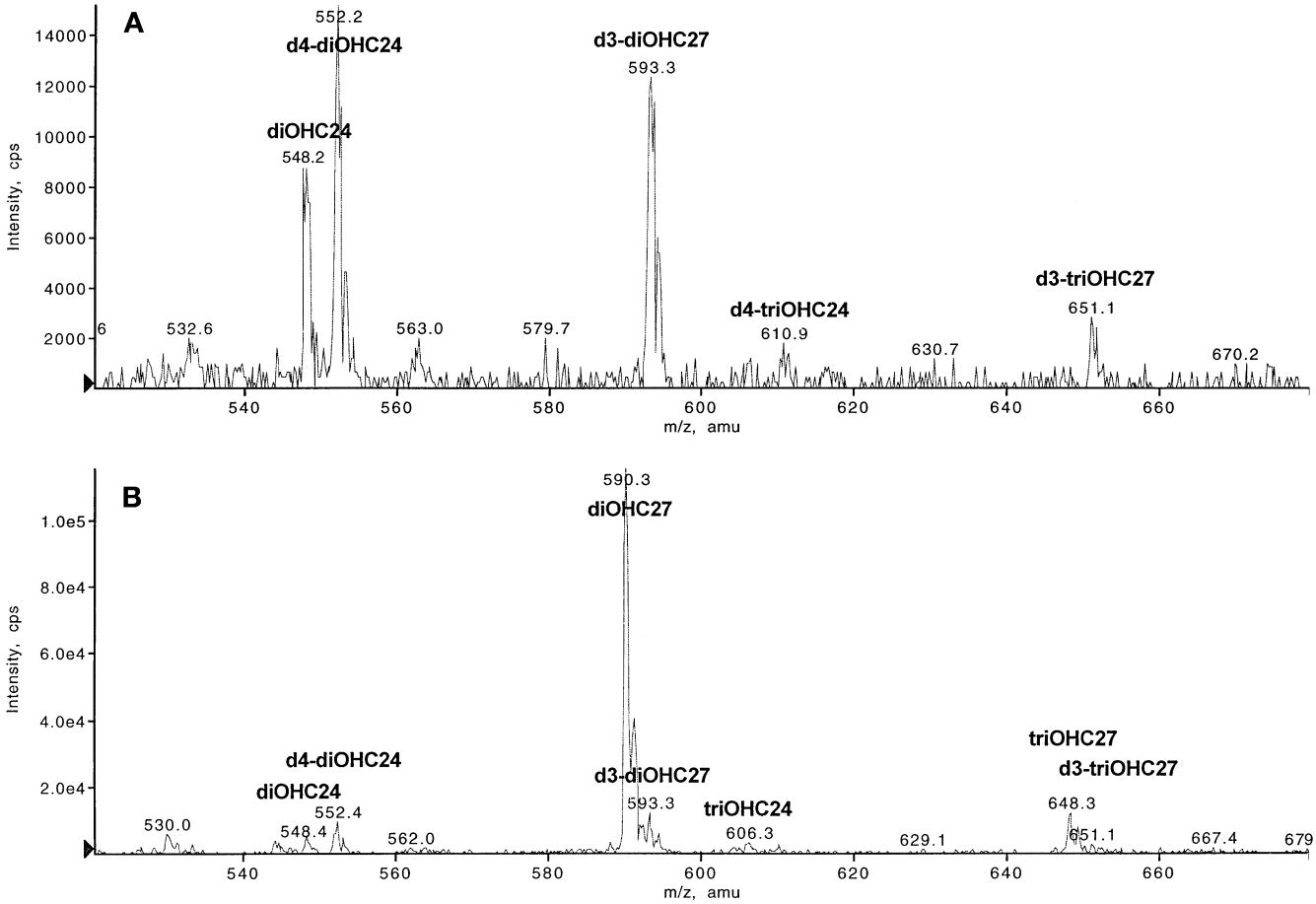


Fig. 2. ES-MS/MS analysis of the acetyl dimethylaminoethyl esters extracted from 5-μl plasma samples. A neutral loss of 60 Da scan is shown for (A) control plasma and (B) peroxisome biogenesis defect patient plasma (patient 6 in Table 1). The x axis shows the molecular weights of the protonated molecular ions that lose acetic acid.

TABLE 2. Analyses for C_{27} bile acids in 3-mm blood spots from Guthrie cards of newborns diagnosed with peroxisomal biogenesis defects and of control subjects

	Di-OH-C_{27}	Tri-OH-C_{27}	Tetra-OH-C_{27}
	μM	μM	
PBD patient			
10[a]	0.83	8.77	x[b]
11[c]	0.42	4.06	x
12[d]	0.83	2.15	x
Control subjects (n = 20, mean ± SD)	0.13 ± 0.04	0.33 ± 0.09	
Blanks (n = 6, mean ± SD)	0.053 ± 0.017	0.12 ± 0.06	

[a] Zellweger syndrome variant, stored 6 years.
[b] x, Present.
[c] Unclassified variant, stored 6 months.
[d] Infantile Refsum's disease variant, stored 18 years.

8.7% (THCA) and interassay variations (n = 4) of 11.4% (DHCA) and 14.2% (THCA). Five-microliter plasma samples of nine PBD patients were then analyzed for dihydroxy and trihydroxy C_{27} bile acids and their $C_{26:0}/C_{22:0}$ VLCFA ratio (Table 1). A high correlation (R^2 = 0.89) was observed between the severity of the PBD, measured by the $C_{26:0}/C_{22:0}$ VLCFA ratio, and the concentrations of the trihydroxy C_{27} bile acids. Significant amounts of tetrahydroxy C_{27} bile acids, for which no labeled standards were available, were also observed in severe PBD patient plasma samples. By way of graphical illustration, neutral loss of 60 Da scans from ES-MS/MS analysis of acetyl DMAE derivatives of bile acids obtained from 5-μl plasma samples of a normal infant (**Fig. 2A**) and a PBD infant (Fig. 2B) are shown.

Minor modifications to the method were made for the analysis of 3-mm blood spots. The unconjugated bile acids were first extracted from the blood spots with methanol and 3.5 μM deuterated internal standards were added. The levels of dihydroxy and trihydroxy C_{27} bile acids in normal blood spot samples were higher than those found in normal plasma and approximately 2.5 times the mean of blank measurements (**Table 2**). The blank measurements were obtained by performing the analysis with blank Guthrie card spots. Three blood spots were available, collected from infants at birth, and subsequently diagnosed with a PBD. One blood spot had been stored for 18 years. The levels of dihydroxy and trihydroxy C_{27} bile acids were at least three times those found in blood spots of unaffected infants (Table 2). Neutral loss of 60 Da scans from ES-MS/MS analysis of derivatized bile acids extracted from 3-mm blood spots obtained from a normal infant (**Fig. 3A**) and a PBD infant (Zellweger syndrome variant; Fig. 3B) are shown.

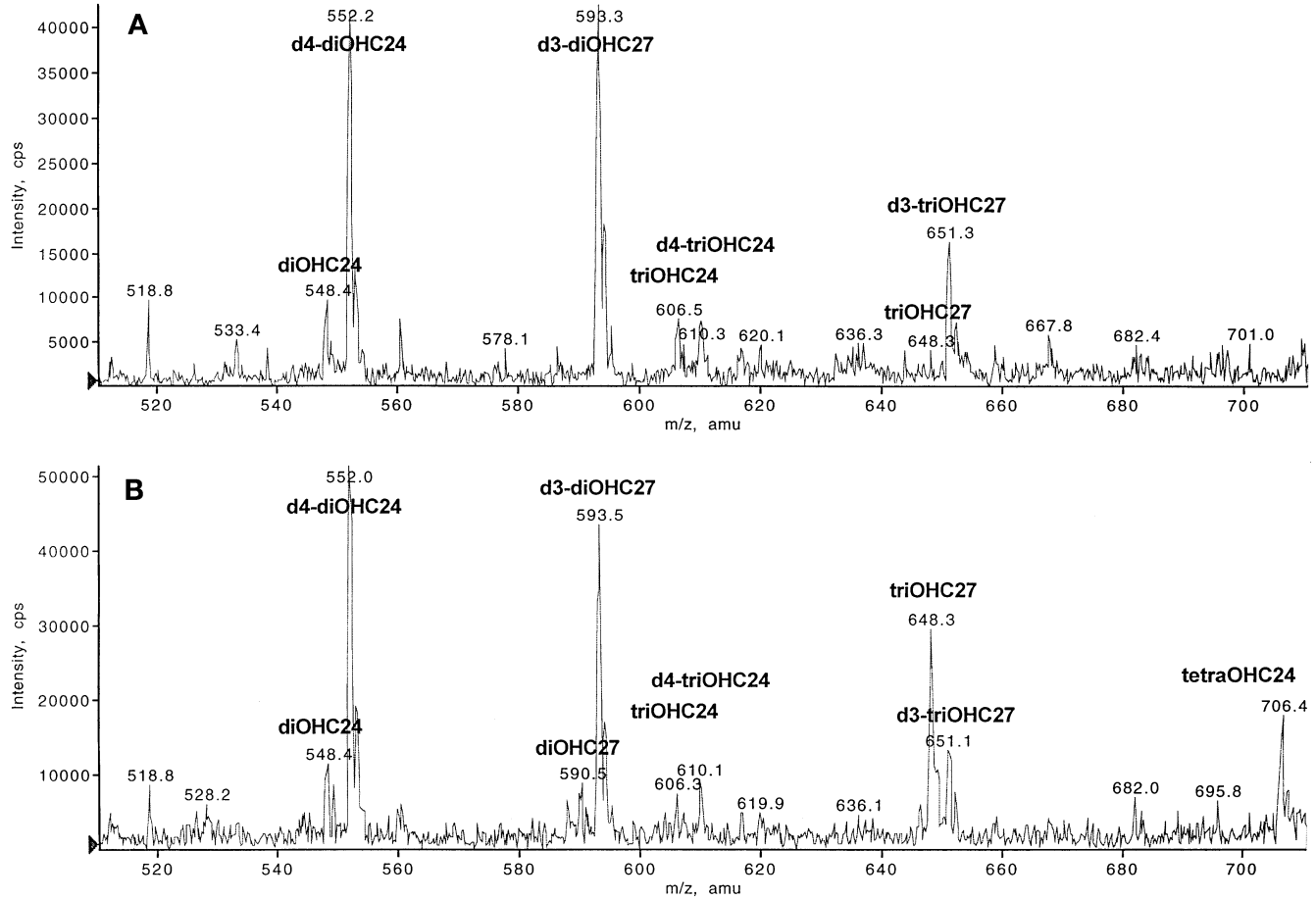


Fig. 3. ES-MS/MS analysis of the acetyl dimethylaminoethyl esters extracted from 3-mm dried blood spots. A neutral loss of 60 Da scan is shown for (A) a control blood spot and (B) a blood spot from a patient with a peroxisome biogenesis defect (Zellweger syndrome variant; patient 10 in Table 2). The x axis shows the molecular weights of the protonated molecular ions that lose acetic acid.

DISCUSSION

Synthesis of unlabeled and trideuterated DHCA and THCA

The hydroxy groups of chenodeoxycholic and cholic acids are reactive to the reagents used for homologation reactions. Their protective derivatization as MOM ethers, which are stable to the basic conditions of malonic acid synthesis, is used for two reasons. First, the introduction of low molecular weight MOM substituents, in contrast to the commonly used tetrahydropyranyl ethers, keeps the synthetic intermediates sufficiently volatile to check their purity by gas chromatography. Second, the ease of removal under mild acidic conditions renders MOM substituents superior to methyl ethers, used in an earlier attempt to synthesize THCA. Although the methyl ethers are stable, volatile intermediates, vigorous conditions are needed for their deprotection. Rupture of the steroid structure, observed by others (13), results in a low yield of end product.

The choice of malonic ester synthesis, as a means for three-carbon homologation, to build up the coprostanic acid chain structure of acids **1.1** and **2.1**, is based on the successful synthesis of 2-methyl-substituted fatty acids (11). It is ideally suited to the introduction of isotope labels as exemplified by the preparation of acids **1.2** and **2.2** containing deuterium at the stable α-methyl position. Theoretically, two diastereoisomers at C-25 of each acid are obtained; however, these are not differentiated by the tandem mass spectrometric analysis described here.

ES-MS/MS analysis of DHCA and THCA

Several potential sources of error are possible in a method such as this, with no purification or separation steps. Other unconjugated hydroxy acids with molecular weights similar to those of C_{27} bile acids in plasma or blood could generate interfering ions. Hydroxylated VLCFA are found in tissues such as brain and reproductive organs but they are mostly monohydroxylated, and incorporated within lipid species (14). No significant, unexplained ions have been observed in control samples. This method is unable to distinguish between isomeric dihydroxy or trihydroxy C_{27} bile acids. At least one isomeric trihydroxy C_{27} bile acid (25-hydroxy-DHCA) has been described, as well as *R* and *S* forms (2). There is no evidence that measuring the total dihydroxy and trihydroxy C_{27} bile acids is less diagnostic for distinguishing a PBD from other peroxisomal disorders than measuring single isomeric forms.

Measurement of the trihydroxy C_{27} bile acids in plasma samples showed excellent correlation with the $C_{26:0}/C_{22:0}$ VLCFA ratio, which reflects the severity of the disorder. Measurement of the dihydroxy C_{27} bile acids, however, was the more discriminating for mildly affected patients. There is an apparent anomaly in the results from PBD patient 7 (Table 1) where the dihydroxy C_{27} bile acids were lower than the trihydroxy C_{27} bile acids. The tetrahydroxy C_{27} bile acids, in this case, were considerably elevated. The pattern of hydroxylation thus appears to differ among the PBD variants. This suggests that summation of the di-, tri-, and tetrahydroxy C_{27} bile acids would be a better overall indicator. Lack of an internal standard for quantitating tetrahydroxy C_{27} bile acids currently prevents this.

The levels of dihydroxy and trihydroxy C_{27} bile acids, measured in blood spots from PBD patients collected at birth, were similar in those collected more recently and those stored for long periods. Retrospective screening for PBD patients in archived collections of Guthrie cards is consequently possible. Unlike VLCFA (10), bile acids are not constituents of Guthrie card filter paper and blank spots do not have to be analyzed and subtracted. The concentrations of DHCA and THCA in normal blood spots are higher than the concentrations of DHCA and THCA in normal plasma. The concentrations of VLCFA are also higher in normal blood spots compared with normal plasma. This is because there are higher levels of VLCFA in the red blood cells, especially erythrocytes (15), and it is thought that the same situation may apply with C_{27} bile acids.

There are additional potential uses for this method. No significant difference was observed in the results of the analyses of DHCA and THCA in 3-mm spots of dried filter paper spotted with plasma and the same volume of plasma. These plasma spots thus provide an inexpensive way of transporting the samples for analysis. Total bile acid measurement in a sample, after overnight enzyme cleavage of conjugates, such as with cholylglycine hydrolase (16), can be performed. Unconjugated C_{24} bile acids can also be simultaneously quantitated. Deuterium-labeled chenodeoxycholic and cholic acid standards were added to the samples that were analyzed for C_{27} bile acids. Unconjugated dihydroxy and trihydroxy C_{24} bile acids were quantitated by using another set of four MRM experiments. Their measurements were for another study using the same control samples. The levels of C_{24} bile acids provide no additional assistance to the subclassification of peroxisomal disorders.

In summary, the ES-MS/MS method for measuring C_{27} bile acids as acetyl DMAE derivatives has been demonstrated to be faster and more accurate, and requires substantially less sample than the LC/ES-MS/MS (8) and GC-MS (6, 7) methods previously described. A typical analysis takes 1 h, quantitation is performed by the "gold standard" isotope dilution method, and acceptable precision is achievable with 5 μl of plasma or a single 3-mm blood spot.

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