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Analytical strategies for characterization of oxysterol lipidomes: Liver X receptor ligands in plasma

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ABSTRACT

Bile acids, bile alcohols, and hormonal steroids represent the ultimate biologically active products of cholesterol metabolism in vertebrates. However, intermediates in their formation, including oxysterols and cholestenoic acids, also possess, e.g., as ligands to nuclear and G-protein-coupled receptors, and unknown regulatory activities. The potential diversity of molecules originating from the cholesterol structure is very broad and their abundance in biological materials ranges over several orders of magnitude. Here we describe the application of enzyme-assisted derivatization for sterol analysis (EADSA) in combination with liquid chromatography–electrospray ionization–mass spectrometry to define the oxysterol and cholestenoic acid metabolomes of human plasma. Quantitative profiling of adult plasma using EADSA leads to the detection of over 30 metabolites derived from cholesterol, some of which are ligands to the nuclear receptors LXR, FXR, and pregnane X receptor or the G-protein-coupled receptor Epstein–Barr virus-induced gene 2. The potential of the EADSA technique in screening for inborn errors of cholesterol metabolism and biosynthesis is demonstrated by the unique plasma profile of patients suffering from cerebrotendinous xanthomatosis. The analytical methods described are easily adapted to the analysis of other biological fluids, including cerebrospinal fluid, and also tissues, e.g., brain, in which nuclear and G-protein-coupled receptors may have important regulatory roles.

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Introduction

Oxysterols and their downstream metabolites, including cholestenoic acids, represent important biologically active components of plasma. These molecules are of increasing interest to bioscientists on account of their important signaling roles in the immune system [1–3], as agonists to nuclear receptors [4–7], and as markers of

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oxidative stress [8], atherosclerosis [9,10], and neurodegenerative disease [11]. Oxysterols can be formed from cholesterol and its sterol precursors both enzymatically and nonenzymatically. In vertebrates, the first step of all cholesterol metabolism leads to the formation of an oxysterol. 22R-Hydroxycholesterol and 20R,22R-dihydroxycholesterol are the precursors of pregnenolone and steroid hormones [12], whereas 7α-, 24S-, 25-, and (25R),26-hydroxycholesterols all represent precursors of bile acids [13]. Some of these oxysterols and their downstream metabolites are ligands to nuclear receptors, e.g., liver X receptors (LXRs)[1] [5,6], farnesoid X receptor (FXR) [7], pregnane X receptor [4,14], vitamin D receptor [15], and other receptors involved in lipid homeostasis, e.g., INSIG [16,17], and also G-protein-coupled receptors [2,18,19]. Oxysterols also play a role in the immune response [1,20,21], in which, e.g., 25-hydroxycholesterol is secreted by macrophages in response to Toll-like receptor activation and suppresses immunoglobulin A production, whereas its metabolite 7α,25-dihydroxycholesterol directs B-cell migration [1,3]. Clearly, the presence of these regulatory molecules in biological fluids is of major physiological importance and analytical methods are required to reliably identify and quantify such molecules.

Oxysterols have traditionally been analyzed in plasma by gas chromatography (GC)–mass spectrometry (MS), after saponification and derivatization, utilizing selected ion recording (SIR) to attain the necessary sensitivity [22], although liquid chromatography (LC)–MS methods utilizing SIR or multiple reaction monitoring (MRM) are gaining popularity [23–25]. To improve MS response in LC–electrospray ionization (ESI)–MS studies a number of groups are now exploiting derivatization methods to enhance ionization [26–29]. Cholestenoic acids are usually analyzed by GC–MS in an analysis separate from oxysterols [30], this is on account of different requirements of sample preparation. However, Axelson and colleagues developed a GC–MS assay for C_{27} acids in blood and plasma [31], which was extended to include some oxysterols.

Oxysterols represent just one subgroup of the lipidome, and in recent years lipidomics has become a field of great activity in, and intense interest to, the bioscience community [32–35]. Many of the lipidomic studies performed have been on biofluids, particularly plasma or serum [25]. The dominant technology has been ESI-MS. ESI-MS analysis has been performed linked to LC, i.e., LC-ESI-MS, and also in a stand-alone direct infusion mode. Stand-alone, or shotgun, lipidomics offers the advantage of simplicity, but is unable to differentiate between isobaric metabolites. Interfacing ESI with LC separation can overcome this shortcoming, but introduces some added complexity to the analysis. GC–MS offers an alternative analytical method; however, the requirements of solvolysis and/or hydrolysis followed by derivatization, often of multiple functional groups with freshly prepared reagents and solvents, discourage many would-be analysts [36].

Any lipidomic experiment consists of essentially three key stages: (i) lipid extraction, (ii) lipid analysis, and (iii) lipid quantification. Clearly the extremely different physical properties of many lipids and their presence in cells, tissues, and body fluids at widely different levels make analysis of the global lipidome extremely challenging. An alternative to global lipidomics is to adopt a targeted approach. Here a particular class of lipid is targeted, usually based on physicochemical properties. This is the approach adopted by many lipid scientists [32,33,37]. Whereas glycerolipids, glycerophospholipids, and sphingolipids are often well represented in global ESI-MS-based lipidomic studies, this is not true of members of the other classes [38]. The explanation for this is simple; most ESI-MS methods are biased toward the most abundant and readily ionized compounds. Cholesterol and some of its metabolites are present at high levels in blood and plasma but are barely detectable in a global lipidomics experiment based on the ESI-MS analysis of these fluids. This is on account of difficulties experienced in sample handling and poor ionization characteristics of sterols. Whereas established MS methods exist for analysis of the ultimate products of cholesterol metabolism, i.e., hormonal steroids and bile acids [39], methods for the analysis of intermediates in their biosynthesis, i.e., oxysterols and cholestenoic acids, are less mature, and to date this region of the lipidome has been largely ignored.

Over the past decade we have developed targeted methods for sterol, oxysterol, and cholestenoic acid analysis, which we have exploited in the analysis of brain tissue, cerebrospinal fluid, plasma/serum, cells, and cell media [40–45]. Our methodology is based on separating oxysterols and cholestenoic acids from cholesterol in the first step of the sample preparation process. This avoids the potential problem of cholesterol autoxidation generating oxysterols nonenzymatically with structures similar to those formed endogenously [46] and allows the subsequent storage of oxysterols without the possibility of their formation via cholesterol and air. Oxysterols are then activated to allow subsequent "click chemistry" with a charge-baring group, which enhances their ESI-MS response. In our experience, unprocessed samples can be stored effectively at $-80\,^{\circ}C$

before sample preparation with minimal autoxidation. However, growing peaks corresponding to 7-oxocholesterol, 7β-hydroxycholesterol, 5,6-epoxycholesterol, and cholestane-3β,5α,6β-triol should alert the analyst of potential autoxidation problems.

Note on nomenclature

Here we regard a steroid as a molecule based on the cyclopentanoperhydrophenanthrene ring structure. In vertebrates hormonal steroids usually contain 18, 19, or 21 carbon atoms; bile acids 24 carbon atoms with a carboxylic acid group at C-24; and cholestanoic and cholestenoic acids 27 carbon atoms with an acid group at C-26 or C-27. In cholestenoic acids the stereo center at C-25 is usually 25R. Endogenous sterols are precursors or metabolites of cholesterol and include cholestenoic acids, which, like cholesterol, possess a hydroxy (or oxo) group at C-3 and usually contain 27 carbons. Oxysterols are a category of sterols, mostly derived from cholesterol, containing an additional oxygen function. In this article we have adopted the nomenclature recommended by the Lipid Maps Consortium [37].

Principles

In this article we describe a LC-ESI-MS method for the quantitative profiling of a wide range of oxysterols and their downstream acidic metabolites from microliter quantities of plasma. Our method is based on ethanol extraction, separation of cholesterol metabolites from cholesterol itself by reversed-phase (RP) solid-phase extraction (SPE), followed by enzyme-assisted derivatization for sterol analysis (EADSA). EADSA consists of enzymatic conversion of 3β-hydroxy-5-ene- and 3β-hydroxy-5α-hydrogen-containing sterols to 3-oxo-4-ene and 3-oxo sterols followed by tagging a positively charged quaternary nitrogen group to the resulting oxo group in a "click reaction" (Scheme 1). Analysis and quantification are performed by LC-ESI-MS. Our preference is to perform mass analysis at high mass resolution (30,000, full width at half-maximum height, FWHM) using stable-isotope or structural analogue internal standards for quantification, with compound identification achieved using exact mass measurements (< 5 ppm) and multistage fragmentation (MS^n). We perform these analyses on an LTQ-Orbitrap instrument. Alternative MS formats can be used, such as quadrupole–time-of-flight (Q-TOF), tandem quadrupole, and cylindrical or linear ion trap (LIT), but none of these offers the combination of high-resolution exact mass measurements and MS^n provided by LTQ-Orbitrap or LTQ-FT-ICR instruments. We complement the EADSA LC-ESI-MS method with shotgun ESI-MS, also performed at high resolution with exact mass measurement and MS^2, to characterize oxysterols and their downstream metabolites conjugated with sulfuric and/or glucuronic acids. Shotgun ESI-MS is appropriate for analysis of cholesterol metabolites sulfated at C-3 and thus inaccessible to EADSA. Using shotgun ESI-MS we do not attempt to exactly identify or quantify conjugated oxysterols on account of an absence of authentic standards. Ongoing work is in progress to rectify this situation by the synthesis of appropriate standards.

Materials

High-performance liquid chromatography (HPLC)-grade water, absolute ethanol, and other HPLC-grade solvents were from Fisher Scientific (Loughborough, UK) or Sigma–Aldrich (Dorset, UK). Acetic acid was AnalaR NORMAPUR grade (BDH, VWR, Lutterworth, UK). Authentic sterols, steroids, bile acids, and their precursors were from Avanti Polar Lipids (Alabama, USA), Steraloids, Inc. (Rhode Island, USA), Sigma–Aldrich, or previous studies in our laboratories [41].
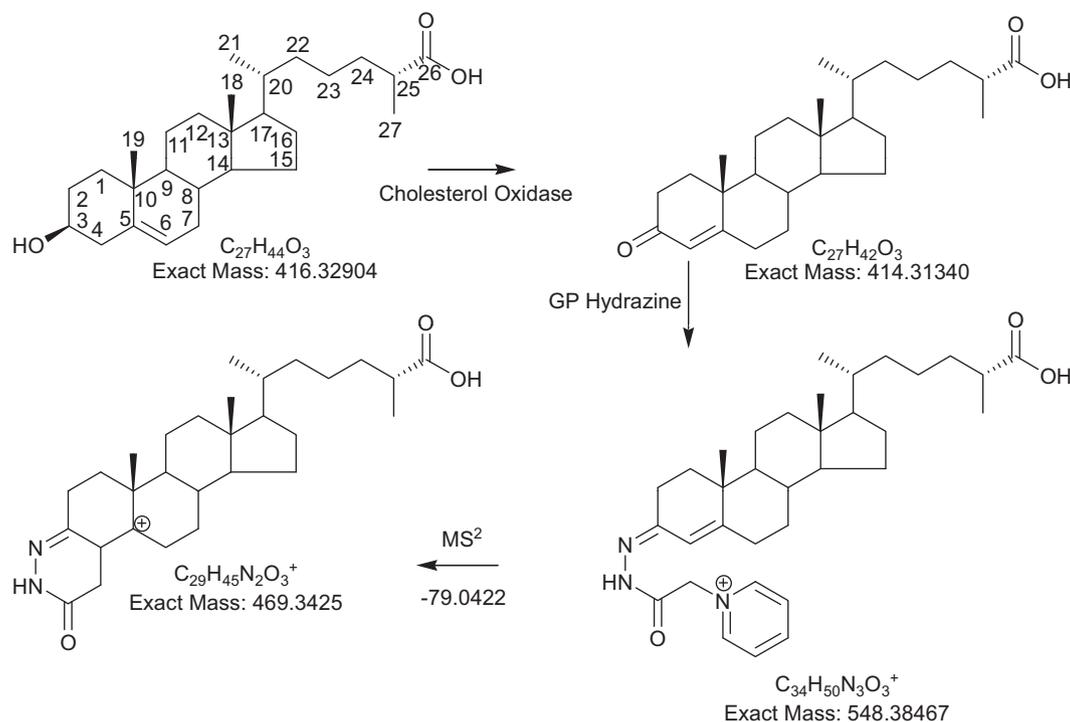
Scheme 1. Enzyme-assisted derivatization for sterol analysis. Sterols possessing a 3β-hydroxy-5-ene function are oxidized with cholesterol oxidase (from *Streptomyces* sp.), and the resulting 3-oxo-4-ene group is then derivatized with Girard P (GP) hydrazine in a "click reaction" [42]. The cholesterol oxidase enzyme is also active toward sterols with a 3β-hydroxy-5α-hydrogen function generating 3-oxo sterols [47]. Sterols naturally containing an oxo group can be derivatized with GP hydrazine in the absence of cholesterol oxidase. Once derivatized, sterols are analyzed by LC-ESI-MS and MS^n. The derivatization procedure is exemplified by 3β-hydroxycholest-(25R)-5-en-26-oic acid. The nomenclature recommended by Lipid Maps has been adopted, by which a hydroxyl group attached to the terminal carbon of the cholestene side chain introducing the R configuration at C-25 is said to be attached to C-26. This nomenclature is also extended to C_{27} acids [37].

Table 1
Suppliers of reagents and materials.

Material	Supplier	Cat. No
24(R/S)-[26,26,26,27,27,27-^{2}H$_6$]Hydroxycholesterol[a]	Avanti Polar Lipids	700049P
24(R/S)-[25,26,26,26,27,27,27-^{2}H$_7$]Hydroxycholesterol[a]	Avanti Polar Lipids	700018P
http://avantilipids.com/index.php?option=com_content&view=article&id=2323&Itemid=305&catnumber=700018		
[25,26,26,26,27,27,27-^{2}H$_7$]Cholesterol	Avanti Polar Lipids	700041P
http://avantilipids.com/index.php?option=com_content&view=article&id=693&Itemid=305&catnumber=700041		
Girard P reagent	TCI Europe	G0030
http://www.tcieurope.eu/en/catalog/G0030.html		
Cholesterol oxidase from *Streptomyces* sp.	Sigma–Aldrich	C8868
http://www.sigmaaldrich.com/catalog/product/sigma/c8649?lang=en&region=GB		
Certified Sep-Pak C$_{18}$ 200-mg, 3-ml, VAC cartridges	Waters	186004618
http://www.waters.com/waters/partDetail.htm?partNumber=186004618		
BD Biosciences Luer-lock syringe	Sigma–Aldrich	Z192120-100EA
http://www.sigmaaldrich.com/catalog/product/aldrich/z192120?lang=en&region=GB		
Corning 15-ml centrifuge tube	Fisher Scientific	CFT-420-031Y
https://extranet.fisher.co.uk/insight2_uk/mainSearch.do		
Corning 50-ml centrifuge tube	Fisher Scientific	CFT-900-031F
https://extranet.fisher.co.uk/insight2_uk/mainSearch.do		
Greiner 12-ml round-bottom tubes, polypropylene	Sigma–Aldrich	Z642975
http://www.sigmaaldrich.com/catalog/product/sigma/z642975?lang=en&region=GB		
Cultubes 4-ml polypropylene tube	Simport Plastics	T415-2A
http://www.bioventures.com/_pdf/Simport-Scientific-Catalog.pdf		
Microcentrifuge tubes	Fisher Scientific	TUL-150-290U
https://extranet.fisher.co.uk/insight2_uk/getProduct.do?productCode=TUL-150-290U&resultSetPosition=26		

[a] 24(R/S)-[26,26,26,27,27,27-^{2}H$_6$]Hydroxycholesterol is no longer commercially available and has been replaced by 24(R/S)-[25,26,26,26,27,27,27-^{2}H$_7$]hydroxycholesterol.

Girard P (GP) reagent (1-(carboxymethyl)pyridinium chloride hydrazide) was from TCI Europe (Oxford, UK) and cholesterol oxidase from *Streptomyces* sp. was from Sigma–Aldrich. Certified Sep-Pak C$_{18}$ 200-mg cartridges were from Waters (Elstree, UK). Luer-lock syringes were from BD Biosciences (Sigma–Aldrich). All plastic materials used were made of polypropylene. Latex-containing material, including gloves, should be avoided. Further supplier details including Web addresses are given in Table 1.

Instrumentation

Sample preparation

The following instruments were used in the sample preparation steps: ultrasonic bath, Grant XB3 (VWR Jencons); multispeed refrigerated centrifuge, ALC, PK12R; vacuum manifold (Agilent Technologies); ScanLaf ScanSpeed vacuum concentrator; vortex mixer.

Sample analysis

Chromatographic separation of derivatized sterols was performed on either an UltiMate 3000 HPLC system or an UltiMate 3000 Binary RSLCnano system, both operated with a conventional flow rate configuration (both from Dionex, Surrey, UK) utilizing a Hypersil GOLD RP column (1.9-μm particles, 50×2.1 mm; Fisher Scientific, Loughborough, UK). MS analysis was performed with the chromatographic eluent directed to the atmospheric pressure ionization (API) source of an LTQ-Orbitrap XL or LTQ-Orbitrap Velos MS (Thermo Fisher, San Jose, CA, USA). These instruments have the hybrid linear ion-trap–Orbitrap analyzer format. The Orbitrap is a Fourier transform (FT) mass analyzer capable of high resolution (up to 100,000 FWHM) and exact mass measurement (< 5 ppm).

Although the work described in this article employed LTQ-Orbitrap instruments the methodology can also be exploited using tandem-quadrupole, Q-TOF, and ion trap instruments [41,44].

Protocol

Extraction of plasma for analysis of sterols

Plasma (100 μl) was added drop-wise into a 2-ml microcentrifuge tube (Eppendorf, Cambridge, UK) containing 1.05 ml of absolute ethanol containing 5 μl of 24(R/S)-[26,26,26,27,27,27-^{2}H$_6$]hydroxycholesterol (Avanti Polar Lipids) in propan-2-ol (4 ng/μl) in an ultrasonic bath and sonicated for 5 min (empirically we have found that drop-wise addition of plasma to ethanol is important to maximize the extraction of sterols from plasma proteins). The resultant solution was diluted to 70% (v/v) ethanol by the addition of 0.35 ml of water, ultrasonicated for a further 5 min, and centrifuged at 14,000g at 4 °C for 30 min. If cholesterol or sterols of similar polarity are the object of the study then 5 μl of [25,26,26,26,27,27,27-^{2}H$_7$]cholesterol (Avanti Polar Lipids) in propan-2-ol (4 μg/μl) is added with 24(R/S)-[26,26,26,27,27,27-^{2}H$_6$]hydroxycholesterol to the ethanol solvent.

A 200-mg Certified Sep-Pak C$_{18}$ cartridge (Waters) was rinsed with 4 ml of absolute ethanol followed by 6 ml of 70% ethanol (v/v). Plasma in 70% ethanol (1.5 ml) was applied to the cartridge and allowed to flow at a rate of ~ 0.25 ml/min (although this flow rate is rather slow, it provides secure extraction of the desired analytes on the SPE column). Flow was aided by application of a slight pressure from a BD Biosciences Luer-lock syringe (Sigma–Aldrich). Alternatively, flow was assisted by the use of a vacuum manifold providing a negative pressure at the column outlet. The flowthrough (1.5 ml) and a column wash of 5.5 ml of 70% ethanol were collected in a 15-ml Corning centrifuge tube (Fisher Scientific) or 12-ml Greiner tube (Sigma–Aldrich) (i.e., SPE-1-Fr-1, 7 ml 70% ethanol; Scheme 2). By testing the method with a solution of cholesterol and 24(R/S)-[26,26,26,27,27,27-^{2}H$_6$]hydroxycholesterol in 70% ethanol, we found that cholesterol was retained on the column even after the 5.5-ml column wash, whereas 24(R/S)-[26,26,26,27,27,27-^{2}H$_6$]hydroxycholesterol elutes in the flowthrough and column wash. After a further column wash with 4 ml of 70% ethanol (SPE-1-Fr-2), cholesterol was eluted from the
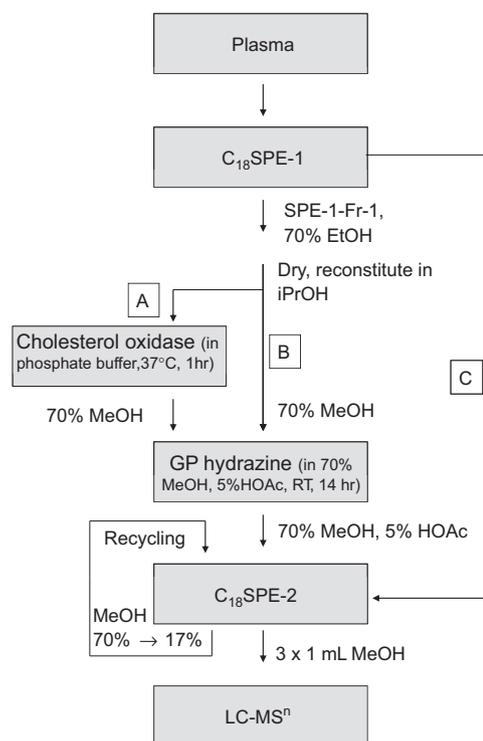
Scheme 2. Sample preparation method for analysis of sterols in plasma.

column in 2 ml of absolute ethanol (SPE-1-Fr-3). The column was further stripped with an additional 2 ml of absolute ethanol to elute more hydrophobic sterols (SPE-1-Fr-4). Each fraction was divided into two subfractions, A and B, and transferred by pipette (using polypropylene tips) into 4-ml polypropylene Cultubes (Simport, Beloeil, QC, Canada) or 12-ml Greiner tubes and dried under reduced pressure using a vacuum concentrator (Scheme 2). The entire procedure was repeated with an additional 50 or 100 μl of plasma, but the final division into A and B subfractions followed by drying down was omitted to leave an intact fraction C.

Enzyme-assisted derivatization

The dried sterol fractions A from above were reconstituted in 100 μl of propan-2-ol and vortex mixed thoroughly (2 min), and 1 ml of 50 mM phosphate buffer (KH$_2$PO$_4$, pH 7; Sigma–Aldrich) containing 3.0 μl of cholesterol oxidase from *Streptomyces* sp. (2 mg/ml in H$_2$O, 44 U/mg protein) was added to each. After a further vortex (2 min), the mixture was incubated at 37 °C for 1 h and then quenched with 2 ml of methanol. Glacial acetic acid (150 μl) was added to the reaction mixture above (now in $\sim 70\%$ methanol, v/v) followed by 150 mg of GP reagent. The mixture was thoroughly vortexed (2 min) and incubated at room temperature overnight in the dark. For the treatment of fractions B cholesterol oxidase was omitted from the procedure.

SPE of derivatized sterols

Even when derivatized with GP reagent, sterols may be difficult to solubilize (or retain in solution) when using a highly aqueous mixture of methanol and water. This can make the extraction of the desired analytes using RP-SPE challenging, as insoluble material will not be extracted by the stationary phase of the column and will be lost from the analysis. To circumvent this problem a recycling procedure was used [41,45].

A 200-mg Certified Sep-Pak C_{18} cartridge (SPE-2) was washed with 6 ml of 100% methanol, 6 ml of 10% methanol (v/v), and conditioned with 4 ml of 70% methanol (v/v). The derivatization mixture from above ($\sim$3 ml of 70% methanol, 5% acetic acid, 3% propanol-2-ol, containing 150 mg of GP reagent and 6 μg of cholesterol oxidase) was applied to the column followed by 1 ml of 70% methanol (used to rinse the reaction tube) and 1 ml of 35% methanol. The combined effluent (5 ml) was collected in a 15-ml Corning polypropylene tube and diluted with water (4 ml) to give 9 ml of $\sim$35% methanol. The resulting solution was again applied to the column followed by a wash with 1 ml of 17% methanol. The combined effluent (10 ml) was added to 9 ml of water in a 50-ml Corning polypropylene tube (or divided into two 5-ml aliquots and decanted into two 12-ml Greiner polypropylene tubes each containing 4.5 ml of water) to give 19 ml of $\sim$17.5% methanol. This solution was again applied to the column followed by a wash with 6 ml of 10% methanol. At this point all the derivatized sterols were extracted by the column and excess derivatization reagent was in the flowthrough and wash. Derivatized sterols were then eluted in three 1-ml portions of 100% methanol and collected in microcentrifuge tubes (SPE-2-Fr-1, -Fr-2, -Fr-3) and combined (either as SPE-2-Fr-1,2 or as SPE-2-Fr-1,2,3); any remaining sterols were eluted with 1 ml of absolute ethanol (SPE-2-Fr-4). LC-MS^n analysis revealed that the derivatized oxysterols were present in the first 2 ml of methanol eluent (SPE-2-Fr-1, SPE-2-Fr-2), whereas derivatized cholesterol also tailed into the third milliliter (SPE-2-Fr-3). Acidic sterols eluted predominantly in the first milliliter of methanol (SPE-2-Fr-1).

To differentiate sterols that naturally possess a 3-oxo function from those oxidized to contain one, samples were analyzed in parallel in the presence (fractions A, e.g., SPE-1-Fr-1A) and absence (fractions B, e.g., SPE-1-Fr-1B) of cholesterol oxidase (see Scheme 2).

SPE of Underivatized sterols

Sterols in fraction C were extracted and recycled on a second SPE column essentially as described for the derivatized sterols. An initial solution of 70% ethanol was applied to the column, eluted, and diluted to 23% ethanol/11% methanol/66% H_2O (v/v/v). This procedure was repeated to give an 11% ethanol/6% methanol/83% H_2O solution at which point all sterols were extracted by the column. After a wash with 10% methanol the sterols were eluted with four 1-ml portions of methanol and combined.

LC-ESI-MSn on the LTQ-Orbitrap

Chromatographic separation of GP-tagged sterols was performed on either an UltiMate 3000 HPLC system or an UltiMate 3000 Binary RSLCnano system (both Dionex) utilizing a Hypersil GOLD RP column (1.9-μm particles, 50 × 2.1 mm; Fisher Scientific). Mobile phase A consisted of 33.3% methanol, 16.7% acetonitrile, containing 0.1% formic acid, and mobile phase B consisted of 63.3% methanol, 31.7% acetonitrile containing 0.1% formic acid. After 1 min at 20% B, the proportion of B was raised to 80% B over the next 7 min and maintained at 80% B for a further 5 min, before returning to 20% B in 6 s and reequilibrating for a further 3 min 54 s, giving a total run time of 17 min. The flow rate was maintained at 200 μl/min and the eluent directed to the API source of an LTQ-Orbitrap XL or LTQ-Orbitrap Velos (both Thermo Fisher, San Jose, CA, USA) MS.

The Orbitrap was calibrated externally before each analytical session. Mass accuracy was in general better than 5 ppm on the XL and better than 1 ppm on the Velos during the entire analytical session. For LC-ESI-MS and LC-ESI-MS^n analysis of reference compounds, sample (1 pg/μl in 60% methanol, 0.1% formic acid)

was injected (20 μl) onto the RP column and eluted into the LTQ-Orbitrap at a flow rate of 200 μl/min. For the analysis of GP-tagged oxysterols and cholestenoic acids from plasma, 12 μl of the combined methanol fractions (2 ml) from the second Sep-Pak C_{18} cartridge (SPE-2-Fr-1,2; equivalent to 0.3 μl of plasma assuming all the relevant sterols elute in the first two methanol fractions) was diluted with 8 μl 0.1% formic acid and 20 μl injected onto the LC column. Two experimental methods were utilized. In the first experimental method (Experiment 1) three scan events were performed: first an FT-MS scan in the Orbitrap analyzer over the m/z range 400–650 (or 300–800) at 30,000 resolution (FWHM) with a maximum ion fill time of 500 ms, followed by data-dependent MS^2 and MS^3 events performed in the LIT with maximum ion fill times of 200 ms on the XL and 100 ms on the Velos. For the MS^2 and MS^3 scans performed on the XL, three microscans were performed, whereas only one microscan was required by the Velos; the precursor-ion isolation width was set at 2 (to select the monoisotopic ion) and the normalized collision energy was at 30 and 35 (instrument settings), respectively. A precursor-ion include list was defined according to the m/z of the $[M]^+$ ions of predicted sterols so that MS^2 was preferentially performed on these ions in the LIT if their intensity exceeded a preset minimum (500 counts). If a fragment ion corresponding to a neutral loss of 79 Da from the precursor ion was observed in the MS^2 event and was above a minimal signal setting (200 counts), MS^3 was performed on this fragment (Scheme 3). To maximize efficiency, the MS^2 and MS^3 events were performed in the LIT at the same time that the high-resolution mass spectrum was being recorded in the Orbitrap. The second experimental method (Experiment 2) involved a targeted MRM-like approach. In event 1 the Orbitrap analyzer was scanned as above, in the second event the MS^3 transition, e.g., 534.4→455.4→, was monitored using collision energies of 30 and 35 for MS^2 and MS^3, respectively. In the third event another MS^3 transition, e.g., 540.4→461.4→, was monitored in a similar manner (e.g., to accommodate the 24(R/S)-[$^2H_6$]hydroxycholesterol internal standard). The precursor-ion include list and MRM transitions utilized for the analysis of plasma are given in Supplementary Table S1. Other fractions from the SPE columns were analyzed in an identical fashion.
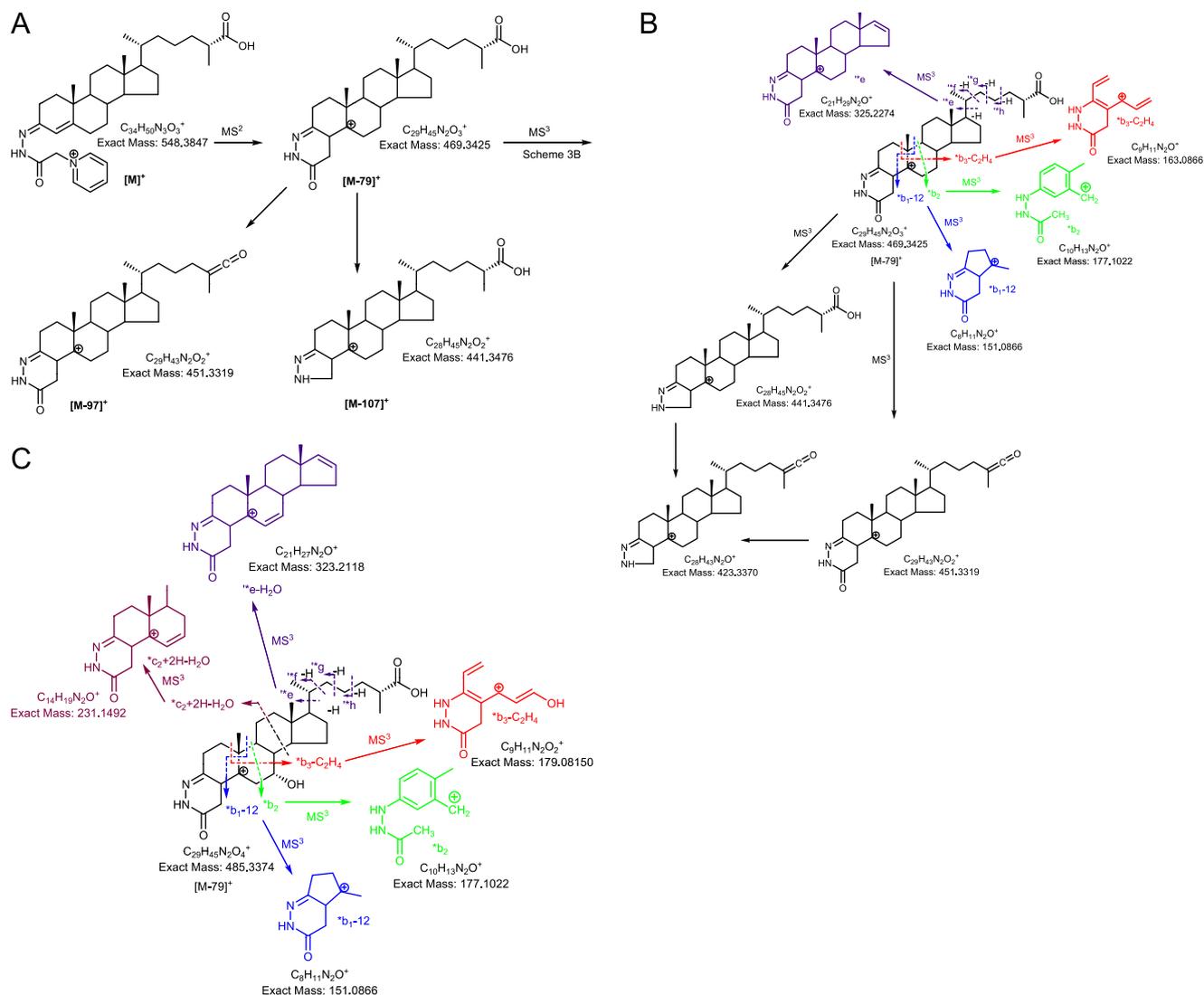
Shotgun ESI-MS on the LTQ-Orbitrap

Underivatized sterols were analyzed by negative-ion ESI-MS on the LTQ-Orbitrap XL. Sample introduction was achieved using an Advion TriVersa NanoMate (Advion BioSciences, USA) ESI chip utilizing nano-ESI nozzles. Accurate mass spectra ($<$5 ppm) were recorded at up to 100,000 (FWHM) resolution over the m/z range 100–1000. Peaks appearing at an m/z appropriate to a C_{24} or C_{27} bile acid or sulfated and/or glucuronidated sterol were subjected to MS^2 when of appropriate intensity. MS^2 spectra were generated in the LIT and recorded using either the LIT or the Orbitrap detector. To avoid the low-mass cutoff inherent to ion traps, MS^2 spectra were also recorded in the "pulsed Q collision induced dissociation" (PQD) mode [48]. In some instances the eluent from the second Sep-Pak C_{18} column was concentrated to 100 μl before analysis to enhance signal intensity.

Results

General considerations related to the EADSA methodology

In this article we describe enzyme-assisted derivatization with charge-tagging using the GP hydrazine reagent (Scheme 1). The advantages provided by this form of derivatization include:

A

$C_{34}H_{50}N_3O_3{}^+$ Exact Mass: 548.3847   [M]$^+$

$\xrightarrow{MS^2}$

$C_{29}H_{45}N_2O_3{}^+$ Exact Mass: 469.3425   [M−79]$^+$

$\xrightarrow[\text{Scheme 3B}]{MS^3}$

$C_{29}H_{43}N_2O_2{}^+$ Exact Mass: 451.3319   [M−97]$^+$

$C_{28}H_{45}N_2O_2{}^+$ Exact Mass: 441.3476   [M−107]$^+$

B

$C_{21}H_{29}N_2O^+$ Exact Mass: 325.2274

$C_{29}H_{45}N_2O_3{}^+$ Exact Mass: 469.3425   [M−79]$^+$

$C_9H_{11}N_2O^+$ Exact Mass: 163.0866   *b$_3$-C$_2$H$_4$

$C_{10}H_{13}N_2O^+$ Exact Mass: 177.1022   *b$_2$

$C_8H_{11}N_2O^+$ Exact Mass: 151.0866   *b$_1$-12

$C_{28}H_{45}N_2O^+$ Exact Mass: 441.3476

$C_{28}H_{43}N_2O^+$ Exact Mass: 423.3370

$C_{29}H_{43}N_2O_2{}^+$ Exact Mass: 451.3319

C

$C_{21}H_{27}N_2O^+$ Exact Mass: 323.2118

$C_{14}H_{19}N_2O^+$ Exact Mass: 231.1492   *c$_2$+2H-H$_2$O

$C_9H_{11}N_2O_2{}^+$ Exact Mass: 179.08150   *b$_3$-C$_2$H$_4$

$C_{10}H_{13}N_2O^+$ Exact Mass: 177.1022   *b$_2$

$C_8H_{11}N_2O^+$ Exact Mass: 151.0866   *b$_1$-12

$C_{29}H_{45}N_2O_4{}^+$ Exact Mass: 485.3374   [M−79]$^+$

Scheme 3. Fragmentation of GP-tagged sterols. (A) MS^2 ([M]$^+$ →) fragmentation and (B) MS^3 ([M]$^+$ →[M−79]$^+$ →) fragmentation illustrated for GP-tagged 3β-hydroxycholest-(25R)-5-en-26-oic acid. (C) The effect of 7-hydroxylation is illustrated for GP-tagged 3β,7α-dihydroxycholest-(25R)-5-en-26-oic acid.

(i) enhanced lipid solubility in solvents used for RP chromatography; (ii) improved gas-phase ion formation via the ESI process; (iii) derivative-specific MS^2 spectra, i.e., prominent loss of 79 Da from the molecular ion; and (iv) structurally informative MS^3 ([M$^+$]→[M−79]$^+$ →) spectra (Scheme 3). A library of MS^2 and MS^3 spectra of authentic standards can be found at http://sterolanalysis.org.uk/. The utilization of the MS^3 scan provides an extra dimension of separation as only ions fragmenting in the MS^2 event by loss of 79 Da are fragmented further in MS^3. A drawback of the charge-tagging methodology is that sterols with a labile 7-hydroxy group, e.g., 7-hydroxy-3-oxocholest-4-en-26-oic acids, can dehydrate to a minor extent during sample preparation to give conjugated dienes. This probably accounts for a proportion of 3-oxocholesta-4,6-dien-26-oic acid found in plasma (Table S2). A further complication is that 24-oxo-26(or 27) acids eliminate CO$_2$ to give 26-*nor*-sterols accounting for the presumptive identification of 7α-hydroxy-26-*nor*-cholest-4-ene-3,24-dione. Similar decarboxylation reactions of 24-oxo-26 acids have been reported by Yuri et al. [49], Bun-ya et al. [50], and Karlaganis et al. [51]. The sample preparation method utilized here has been designed with oxysterols and their acidic metabolites in mind; however, it is also applicable to other steroids possessing an oxo group, e.g., dehydroepiandrosterone (DHEA), testosterone, or their sulfates.

Identification and quantification

Here we regard a sterol to be "identified" when its retention time, exact mass, and MS^n spectra are identical to those of the authentic standard. The term "presumptively identified" is used when the retention time, exact mass, and MS^n spectra are compatible with those of the proposed structure, but there is an absence of authentic standard. Quantification of monohydroxycholesterols was achieved by stable-isotope dilution MS using 24(R/S)-[^{2}H$_6$]hydroxycholesterol as the internal standard [45]. Quantitative estimates were made for other cholesterol metabolites; this is possible using 24(R/S)-[^{2}H$_6$]hydroxycholesterol as the internal standard, as previous studies have shown that GP-tagged 3-oxo-4-ene sterols give a similar mass spectrometric response irrespective of the other functional groups attached to the steroid skeleton [43]. For accurate quantification calibration curves should be generated using authentic standards. In this study the levels of free sterols are measured. Sterols may also be esterified, glucuronidated, and/or sulfated. The last two conjugates are investigated here using shotgun ESI-MS but are not quantified. If sterols esterified to fatty acids are the subject of the study the intact molecules may be analyzed directly by LC-ESI-MS after appropriate extraction and purification [25]. Alternatively, the

free sterols can be analyzed following saponification and EADSA [52].

Whereas fractions SPE-2-Fr1,2A in the sample preparation protocol will contain the GP charge-tagged stable-isotope-labeled standard 24(R/S)-[^{2}H$_6$]hydroxycholesterol, the absence of cholesterol oxidase in the parallel procedure generating SPE-2-Fr-1,2B means that 24(R/S)-[^{2}H$_6$]hydroxycholesterol in this fraction is *not* derivatized and thus does not act as an internal standard for quantification. One solution to this problem is to generate, e.g., a 22R-[^{2}H$_7$]hydroxycholest-4-en-3-one reference standard from 22R-[^{2}H$_7$]hydroxycholesterol (Avanti Polar Lipids). The isotope-labeled 3-oxo-4-ene compound can then be added to ethanol during the initial extraction step and can be used for quantification of sterols in SPE-2-Fr-1,2B. Alternatively, DHEA 3-sulfate, which is abundant in plasma and possesses a 17-oxo group and is not oxidized further by cholesterol oxidase, will appear in both SPE-2-Fr-1,2A and SPE-2-Fr-1,2B fractions and can be used to normalize fraction B to fraction A.

Oxysterols

(25R),26-Hydroxycholesterol is the most abundant oxysterol present in adult plasma (Fig. 1). The level of free (25R),26-hydroxycholesterol determined here (19.12 ± 0.70 ng/ml, mean of 84 adult subjects ± standard error) is in good agreement with values of the free oxysterol determined by GC–MS (13–41 ng/ml), which tend to be about 10% of the combined free and fatty acid ester values [22,53]. In addition to (25R),26-hydroxycholesterol, 7α- (0.82 ± 0.39 ng/ml), 24S- (6.86 ± 0.31 ng/ml), and

25-hydroxycholesterols (4.06 ± 0.22 ng/ml) were found in adult plasma (Fig. 2, Table S2). The levels of free 7α-hydroxycholesterol determined here are somewhat lower than values determined by GC–MS (7–15 ng/ml), which correspond to about 10–20% of the combined free and fatty acid ester values [22,25,27,53]. The downstream metabolite 7α-hydroxycholest-4-en-3-one (2.29 ± 0.25 ng/ml) is also found in plasma [53,54].

It should be noted that the methodology described here is primarily targeted toward side-chain-oxidized sterols (oxysterols and acids) and there may be underestimation of the levels of both 7α-hydroxycholesterol and 7α-hydroxycholest-4-en-3-one, which may not be completely recovered in Fr-1 from SPE-1. If these oxysterols are the target of study SPE-1 may be eluted with a stronger solvent.

With the current method we have identified 25-hydroxycholesterol and its hydroxylated (7α,25-dihydroxycholesterol, < 0.2 ng/ml) and oxidized products (7α,25-dihydroxycholest-4-en-3-one, 1.24 ± 0.20 ng/ml) (see Fig. 3, Table S2). Although GC–MS and LC-MS/MS analysis of plasma regularly identifies 25-hydroxycholesterol, downstream metabolites are not usually detected from healthy subjects because of their low abundance [22,25,27]. Other oxysterols identified in adult plasma include (25R),7α,26-dihydroxycholesterol (1.65 ± 0.39 ng/ml) and 7α,26-dihydroxycholest-(25R)-4-en-3-one (5.55 ± 0.41 ng/ml). Again, these metabolites are not usually investigated in GC–MS or LC-MS/MS analysis of plasma from healthy subjects [27,30,53].

24S,25-Epoxycholesterol is an unusual oxysterol in that it is formed in parallel to cholesterol via a shunt of the mevalonate pathway by the same enzymes that synthesize cholesterol
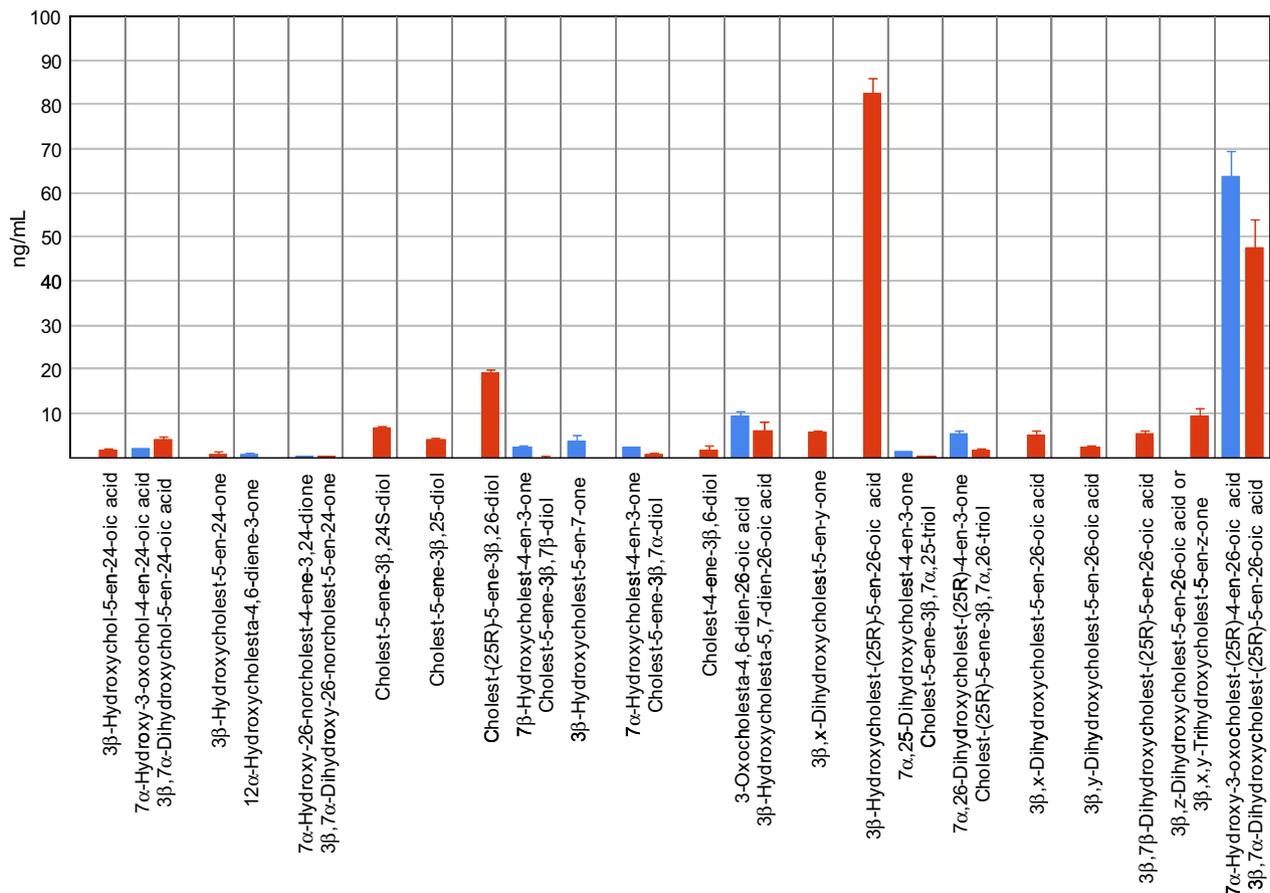

Fig. 1. Levels (ng/ml ± SE) of free oxysterols and cholenoic and cholestenoic acids measured in adult plasma (*n*=84). Blue bars correspond to endogenous molecules with a 3-oxo-4-ene structure and red bars to molecules with a 3β-hydroxy-5-ene function. Systematic names are used. To translate to common names see Supplementary Table S2. Measured values are given in Supplementary Table S2, as are activities as nuclear receptor (e.g., LXR, FXR) agonists.
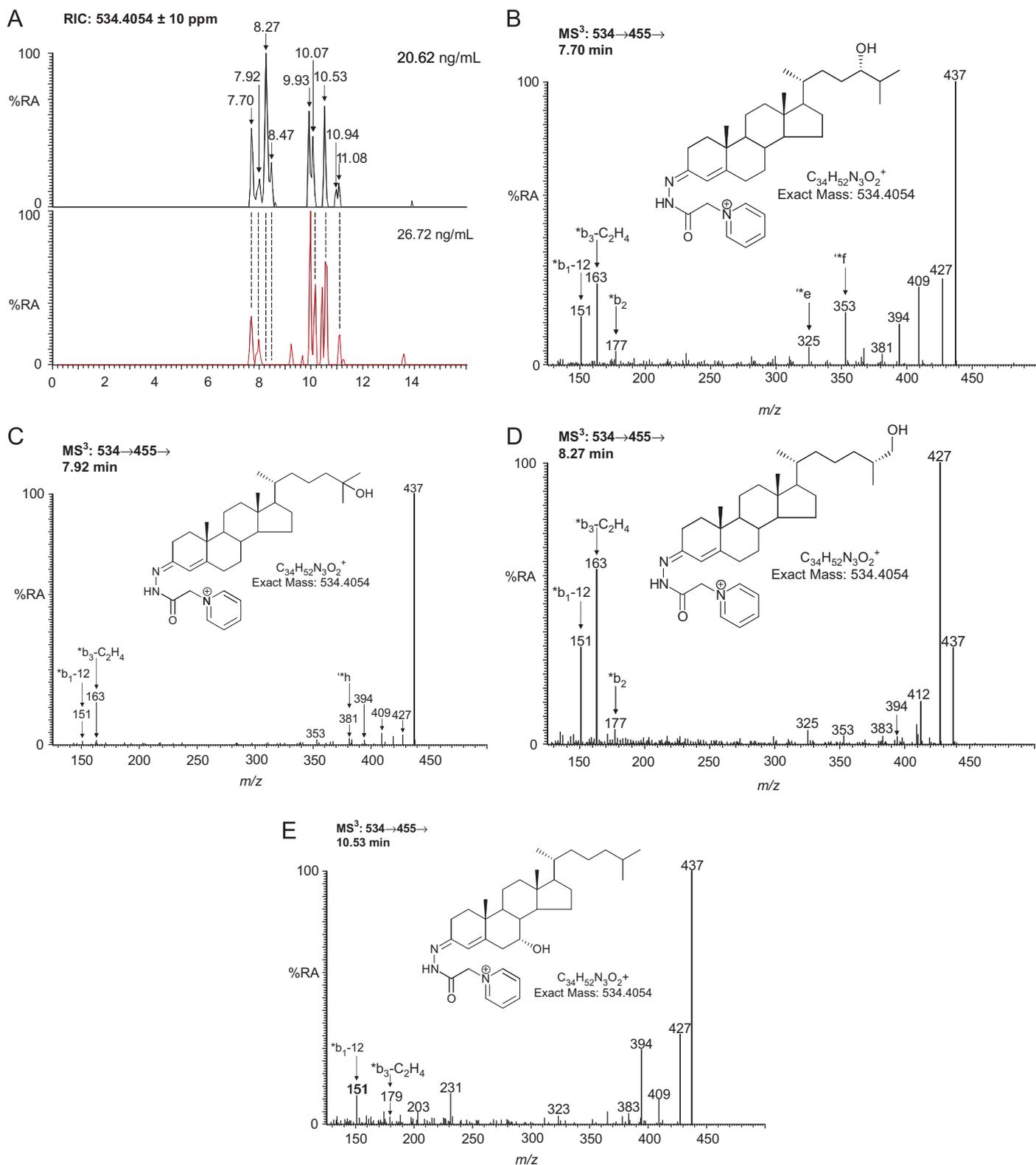
Fig. 2. (A) LC-MS reconstructed ion chromatograms (RICs) of m/z 534.4054 ± 10 ppm corresponding to monohydroxycholesterols modified by EADSA from representative adult (top) and cerebrotendinous xanthomatosis (CTX) (bottom) plasma samples. The CTX patient was on treatment with chenodeoxycholic acid. The samples were oxidized with cholesterol oxidase before GP derivatization to convert 3β-hydroxy-5-ene groups to their 3-oxo-4-ene equivalents. The MS^3 (534.4→455.4→) spectra of components eluting at (B) 7.70 min (24S-hydroxycholesterol), (C) 7.92 min (25-hydroxycholesterol), (D) 8.27 min ((25R),26-hydroxycholesterol), and (E) 10.53 min (7α-hydroxycholesterol and 7α-hydroxycholest-4-en-3-one) are shown. Syn and anti conformers of (25R),26-hydroxycholesterol are observed eluting at 8.27 and 8.47 min, and those of 7α-hydroxycholesterol at 10.53 and 11.08 min. Other peaks in (A) correspond to 7β-hydroxycholesterol, 7-oxocholesterol, and cholest-4-en-3β,6-diol eluting at 9.93, 10.07, and 10.94 min, respectively. It is likely that cholest-4-en-3β,6-diol is derived from 5,6-epoxycholesterol during the derivatization process. It is probable that the concentration of 25-hydroxycholesterol is overestimated as there is tailing of the 24S-hydroxycholesterol peak into that of the 25-hydroxy isomer. Concentrations of the most abundant metabolites are indicated in the chromatograms. Spectra were recorded on the LTQ-Orbitrap XL. %RA, percentage relative abundance.
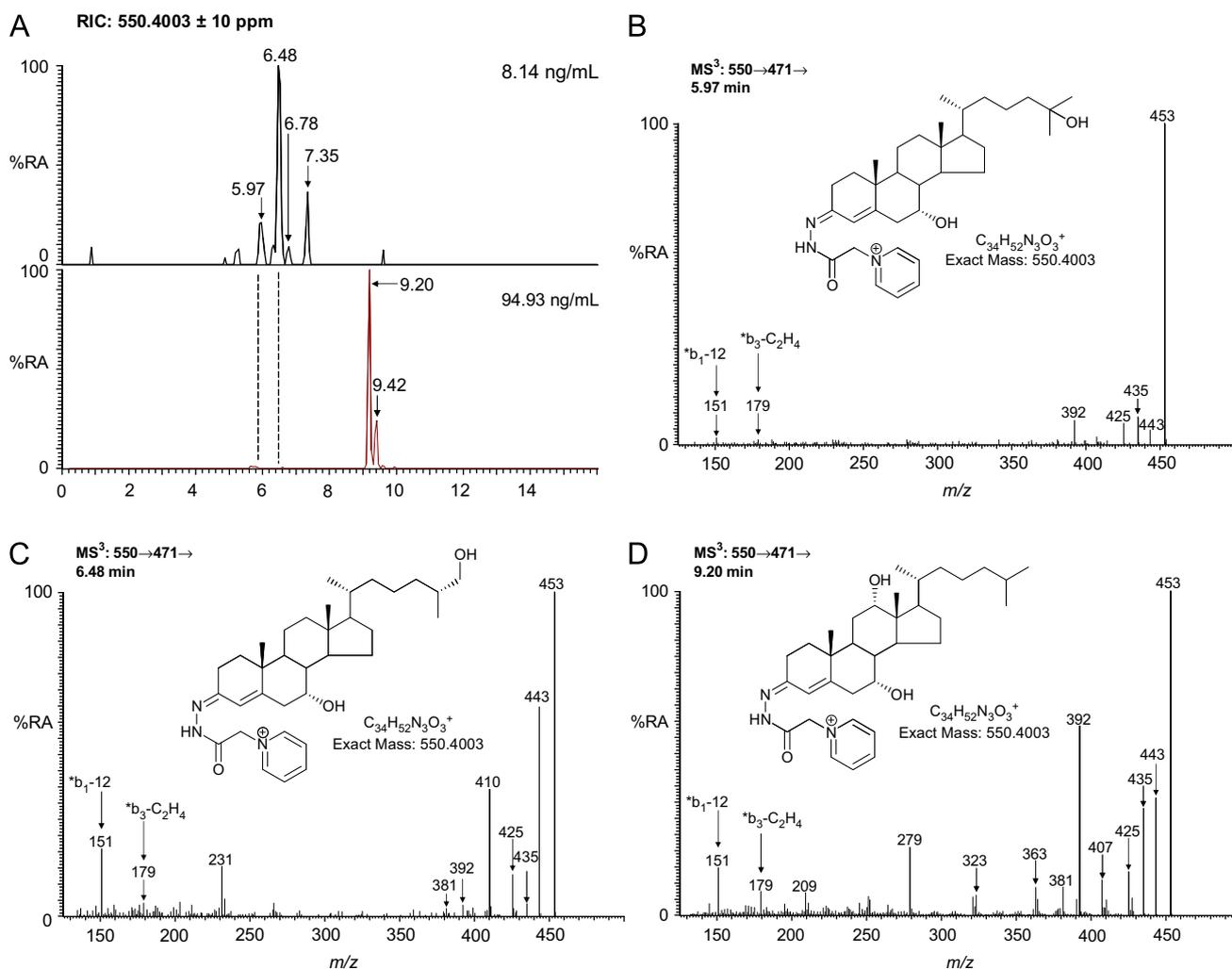
Fig. 3. (A) LC-MS RICs of m/z 550.4003 $\pm$ 10 ppm corresponding to GP-tagged dihydroxycholesterols from representative adult (top) and CTX (bottom) plasma samples. The samples were treated with cholesterol oxidase before GP derivatization to convert 3β-hydroxy-5-ene groups to their 3-oxo-4-ene equivalents. The MS^3 (550.4→471.4→) spectra of components eluting at (B) 5.97 min (7α,25-dihydroxycholesterol and 7α,25-dihydroxycholest-4-en-3-one), (C) 6.48 min ((25R),7α,26-dihydroxycholesterol and 7α,26-dihydroxycholest-(25R)-4-en-3-one)), and (D) 9.20 min (7α,12α-dihydroxycholesterol and 7α,12α-dihydroxycholest-4-en-3-one) are shown. 7α,25-Dihydroxycholesterol/7α,25-dihydroxycholest-4-en-3-one and (25R),7α,26-dihydroxycholesterol/7α,26-dihydroxycholest-(25R)-4-en-3-one and 7α,12α-dihydroxycholesterol/7α,12α-dihydroxycholest-4-en-3-one elute as *syn* and *anti* conformers at 5.97 and 6.78 min, 6.48 and 7.35 min, and 9.20 and 9.42 min, respectively. Spectra in (B) and (C) are from a healthy adult, (D) is from a CTX patient on treatment with CDCA. Concentrations of the most abundant metabolites are indicated in the chromatograms. Spectra were recorded on the LTQ-Orbitrap XL.

[55,56]. 24S,25-Epoxycholesterol has been found previously in plasma at low levels (2 ng/ml) by LC-MS/MS [27]. During our sample preparation we find that 24S,25-epoxycholesterol can isomerize to 24-oxocholesterol [56]. Here we identify 24-oxocholesterol at a level of < 1 ng/ml (0.59 $\pm$ 0.85 ng/ml) in plasma, presumably formed from the 24S,25-epoxide.

Cholenoic and cholestenoic acids

The acidic cholesterol metabolites identified in plasma with a 3β-hydroxy-5-ene or 3-oxo-4-ene structure include unsaturated C_{24} precursors of primary bile acids, i.e., 3β-hydroxychol-5-en-24-oic acid (1.78 $\pm$ 0.25 ng/ml), 3β,7α-dihydroxychol-5-en-24-oic (4.13 $\pm$ 0.77 ng/ml), and 7α-hydroxy-3-oxochol-4-en-24-oic (1.91 $\pm$ 0.26 ng/ml) acids (see Fig. 1). 3β,7α-Dihydroxychol-5-en-24-oic and 7α-hydroxy-3-oxochol-4-en-24-oic acids are potential precursors of chenodeoxycholic acid in bile acid biosynthetic pathways [13,57]. The most abundant members of the bile acid biosynthesis pathways [13] found in plasma are 3β-hydroxycholest-(25R)-5-en-26-oic (82.60 $\pm$ 3.50 ng/ml), 3β,7α-dihydroxycholest-(25R)-5-en-26-oic (47.42 $\pm$ 6.61 ng/ml), and

7α-hydroxy-3-oxocholest-(25R)-4-en-26-oic (63.89 $\pm$ 5.44 ng/ml; Fig. 4 and 5) acids; these data are consistent with findings by others using GC–MS [31,53]. Surprisingly, 3β,7β-dihydroxycholest-(25R)-5-en-26-oic acid (5.36 $\pm$ 0.81 ng/ml) was detected in plasma, probably formed by the hepatic mitochondrial epimerization reaction suggested by Shoda et al. [58].

In addition to 3β,7α- and 3β,7β-dihydroxycholest-(25R)-5-en-26-oic acids in plasma (Fig. 5), two other dihydroxycholest-5-en-26-oic acids were presumptively identified. Their MS^3 spectra suggest the locations of the additional hydroxyl groups to be on the C-17 side chain. Tentative suggestions for the locations of the additional hydroxyl groups are C-25 (5.27 $\pm$ 0.74 ng/ml, Fig. 5B) and C-22 (2.27 $\pm$ 0.34 ng/ml, Fig. 5C). A third compound gave an MS^3 spectrum that could correspond to a dihydroxycholest-5-en-26-oic acid (9.63 $\pm$ 1.46 ng/ml) or alternatively a trihydroxycholest-5-enone, possibly 3β,22,25-trihydroxycholest-5-en-24-one (Fig. 5E). Whereas the major monohydroxycholestenoic acid in adult plasma is 3β-hydroxycholest-(25R)-5-en-26-oic acid (82.60 $\pm$ 3.50 ng/ml), a minor earlier eluting peak in the RIC of m/z 548.3847 suggested the possible presence of the 25S isomer (Fig. 4A). However, comparison of retention time and MS^n spectra
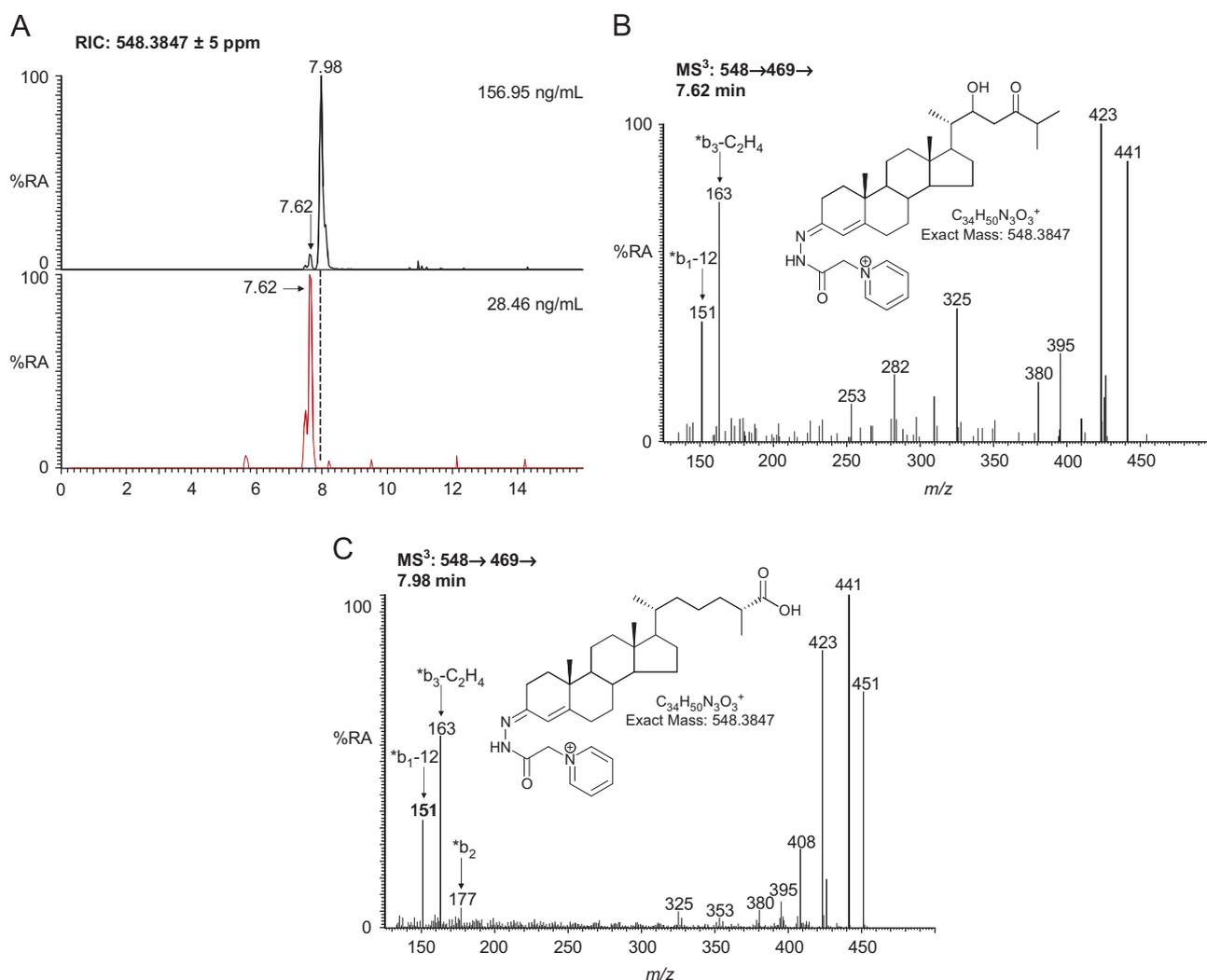
Fig. 4. LC-MS RICs of m/z 548.3847 ± 5 ppm corresponding to 3β,x-dihydroxycholest-5-en-y-one and 3β-hydroxycholest-(25R)-5-en-26-oic acid after EADSA modification and found in plasma from a representative adult (top) and a CTX patient (bottom). The CTX patient was on treatment with CDCA. Chromatograms are normalized to the most abundant peak. The samples were oxidized with cholesterol oxidase before GP derivatization to convert 3β-hydroxy-5-ene groups to their 3-oxo-4-ene equivalents. The MS³ (548.4→469.3 →) spectra of components eluting at (B) 7.62 min (3β,x-dihydroxycholest-5-en-y-one) and (C) 7.98 min (3β-hydroxycholest-(25R)-5-en-26-oic acid) from adult plasma are shown. The peak at 7.62 min in the chromatogram from the CTX patient gave a spectrum essentially identical to that in (B). Concentrations of the most abundant metabolites are indicated in the chromatograms. Spectra were recorded on the LTQ-Orbitrap XL.

with those of the authentic standard ruled out this possibility. A possible alternative structure is 3β,22-dihydroxycholest-5-en-24-one (5.64 ± 0.50 ng/ml, Fig. 4B).

CTX

CTX is characterized by the absence of a functional CYP27A1 enzyme, which precludes the formation of (25R),26-hydroxycholesterol and subsequent cholesten-(25R)-26-oic acids [59–61]. A consequence of this is that hydroxylated cholesterol metabolites become shunted to alternate metabolic pathways. CTX in early infancy can present with cholestatic liver disease, in early childhood with chronic diarrhea and cataracts, in later childhood with tendon xanthomata and learning difficulties, and in adult life with spastic paraparesis, with a fall in IQ or dementia, with ataxia and/or dysarthria, with seizures, or with peripheral neuropathy [61]. Treatment is with CDCA. In Figs. 2–5 representative data are shown for CTX patients on treatment with simvastatin and CDCA at the time of sampling. From profiling the oxysterol content of plasma of CTX patients the following diagnostic features were defined: (1) an absence of unsaturated C_{24} acids; (2) a major reduction in the level of (25R),26-hydroxycholesterol (Fig. 2A); (3) an

elevation in the level of 7α-hydroxycholest-4-en-3-one; (4) an absence of 3β-hydroxycholest-(25R)-5-en-26-oic, 3β,7α-dihydroxycholest-(25R)-5-en-26-oic, and 7α-hydroxy-3-oxocholest-(25R)-4-en-26-oic acids (Fig. 4A and 5A); and (5) a great enhancement in the level of 7α,12α-dihydroxycholest-4-en-3-one (Fig. 3A). These features were independent of treatment with CDCA and simvastatin and are also observed for patients not on treatment. However, the levels of 7α-hydroxycholest-4-en-3-one are lower in patients treated with CDCA than in untreated patients. These diagnostic features, summarized in Table 2, are revealed from the analysis of microliter quantities of plasma (easily obtainable from infants) and in combination are unique to CTX. The absence of a functional CYP27A1 enzyme is directly revealed here by an absence of (or great reduction in) its direct metabolic products. This may be seen as a diagnostic advantage over alternative indirect tests based on an elevated abundance of metabolites shunted into other bile acid/alcohol biosynthetic pathways.

Shotgun analysis

Shotgun ESI-MS in the negative-ion mode is appropriate for the analysis of acidic cholesterol metabolites, particularly sulfates
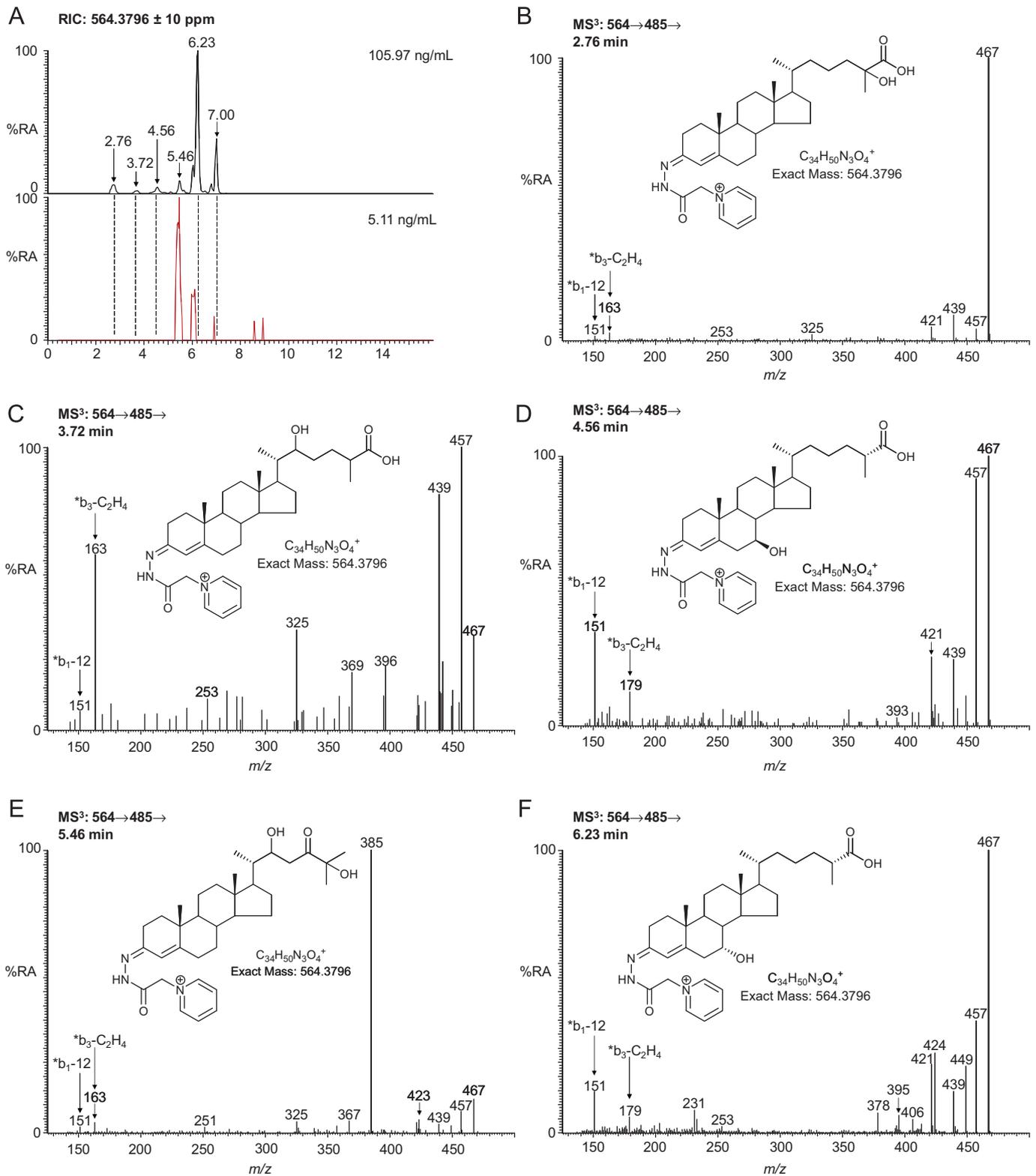
Fig. 5. (A) LC-MS RICs of m/z 564.3796 ± 10 ppm corresponding to dihydroxycholestenoic acids and trihydroxycholestenones after EADSA modification from representative adult (top) and CTX (bottom) plasma samples. The CTX patient was on treatment with CDCA. The samples were oxidized with cholesterol oxidase before GP derivatization to convert 3β-hydroxy-5-ene groups to their 3-oxo-4-ene equivalents. The MS³ (564.4→485.3→) spectra of components eluting at (B) 2.76 min (3β, x-dihydroxycholest-5-en-26-oic acid), (C) 3.72 min (3β,y-dihydroxycholest-5-en-26-oic acid), (D) 4.56 min (3β,7β-dihydroxycholest-(25R)-5-en-26-oic acid), (E) 5.46 min (3β,z-dihydroxycholest-5-en-26-oic acid or 3β,x,y-trihydroxychelest-5-en-z-one), and (F) 6.23 min (3β,7α-dihydroxycholest-(25R)-5-en-26-oic and 7α-hydroxy-3-oxocholest-(25R)-4-en-26-oic acid) are shown. 3β,7α-Dihydroxycholest-(25R)-5-en-26-oic/7α-hydroxy-3-oxocholest-(25R)-4-en-26-oic acid appear as *syn* and *anti* conformers (6.23 and 7.00 min). Spectra in (B–F) were from a healthy adult. Where spectra match those of authentic standards chemical structures are given in the legend. Locations x, y, and z are probably on the side chain of the sterol. Suggested structures of the GP-tagged molecules are shown. Concentrations of the most abundant metabolites are indicated in the chromatograms. Spectra were recorded on the LTQ-Orbitrap XL.

Table 2
Most important diagnostic features of CTX.

EADSA (plasma) Absence of (or major reduction in)	Shotgun ESI-MS (plasma) Abundant glucuronides of	Alternative (ESI-MS urine) Abundant glucuronides of
• (25R),26-Hydroxycholesterol • 3β-Hydroxycholest-(25R)-5-en-26-oic acid • 3β,7α-Dihydroxycholest-(25R)-5-en-26-oic acid • 7α-Hydroxy-3-oxocholest-(25R)-4-en-26-oic acid Elevation in • 7α-Hydroxycholest-4-en-3-one • 7α,12α-Dihydroxycholest-4-en-3-one	• Cholestanetetrols • Cholestanepentols • Cholestanehexols	• Cholestanetetrols • Cholestanepentols • Cholestanehexols

and sulfonates. The method is fast and simple and, when performed at high resolution with exact mass measurements, provides elemental formula information. When complemented by MS^2, structural information is also forthcoming; however, the absence of online chromatography means that structural isomers are not differentiated. Shotgun ESI-MS is highly complementary to EADSA, as it is appropriate for sulfate esters, many of which are located at C-3 and thus invisible to EADSA methodology. In terms of clinical chemistry, shotgun ESI-MS offers two important advantages over LC-MS-based EADSA: (1) it is high-throughput, with MS and MS^2 spectra being recorded in less than a minute, and (2) by utilizing an individual nano-ESI nozzle for each sample, problems of "carryover" are eliminated. Alternatively, a negative-ion LC-ESI-MS approach could be adopted, in which case carry-over between samples can be a problem, and the penalty of chromatographically resolving isomers is paid in analysis time. Further, authentic standards for sterol glucuronides and sulfates, other than for cholesterol sulfate, are not commercially available, making isomer identification essentially impossible.

Adults and infants

Shotgun analysis was performed on the plasma extract delipidated on SPE-1 and desalted on SPE-2. No further chromatography was performed, hence the resulting spectra were complicated by numerous endogenous molecules other than sterols, including free fatty acids, which tend to dominate the negative-ion spectra, e.g., palmitic acid (m/z 255.2334) and stearic acid (m/z 283.2645), and exogenous molecules such as plasticizers from polypropylene storage vessels (Fig. 6A). Despite this, high-resolution accurate mass spectra of healthy infants and adult plasma samples show the presence of sulfates of C_{19} and C_{21} steroids, particularly DHEA (m/z 367.1590); isomers of hydroxyandrostanone, e.g., androsterone, epiandrosterone, and androstenediol (m/z 369.1761); pregnenediol (m/z 397.2029); and pregnenetriol (m/z 413.2005) (Fig. 6A). MS^2 performed in the LIT exploiting PQD allows the confirmation of the presence of the sulfate group by the characteristic fragment ion at 97 (HSO_4^-) (Fig. 6C).

Shotgun analysis of CTX plasma

In comparison to normal plasma, the untreated CTX patient gave a spectrum dramatically different at the high m/z end (m/z 600–650), where peaks characteristic of glucuronides of cholestanetetrols (m/z 611.3798), pentols (m/z 627.3737), and hexols (m/z 643.3680) are evident (cf. Fig. 6A and B) [60,62]. Whereas accurate mass measurements (< 5 ppm) made at high resolution suggest an elemental composition, further structural information is forthcoming by performing MS^2. This is evident in the MS^2 spectra of the three glucuronides, which show neutral losses of

60, 118, 176, and 194 characteristic of the glucuronic acid group [63] (Fig. 6D). From the MS^2 spectra we are unable to define the stereochemistry of the cholestane skeleton; however, detailed studies on glucuronides found in the bile of a CTX patient indicate a predominant 5β-cholestane-3α,7α,12α,25-tetrol skeleton with glucuronidation at the 3α position [64]. Alternative and additional sites of side-chain hydroxylation are at C-22, C-23, and C-24 [60]. In patients on CDCA treatment, $[M-H]^-$ ions corresponding to these glucuronides are absent from the shotgun spectra.

Shotgun analysis of plasma from a patient with suspected hydroxysteroid dehydrogenase 3B7 deficiency

Hydroxysteroid dehydrogenase (HSD) 3B7 (also known as 3β-hydroxy-C_{27}-steroid oxidoreductase or 3β-hydroxysteroid-Δ^5-C_{27}-steroid dehydrogenase) is the enzyme responsible for converting C_{27} sterols with a 3β,7α-dihydroxy-5-ene structure to those with a 7α-hydroxy-3-oxo-4-ene structure in the bile acid biosynthesis pathway. An absence of functional enzyme results in a buildup of sterols and bile acids retaining the 3β-hydroxy-5-ene function [61]. In the circulation these are found sulfated and/or glucuronidated. Shown in Fig. 7 is the shotgun ESI-MS spectrum of a child with suspected HSD3B7 deficiency. It is likely that sulfation is predominantly at position C-3, in which case many of the metabolites observed in this spectrum will be transparent to EADSA. Thus, in this case shotgun ESI-MS provides invaluable information that would be lost by simply performing EADSA. HSD3B7 deficiency may present with neonatal conjugated hyperbilirubinemia, rickets, hepatomegaly, pruritus, and steatorrhea. Provided that liver damage is not too advanced at the time of diagnosis, the condition responds extremely well to bile acid replacement therapy [61].

Caveats

In this article we have extolled the virtues of EADSA in combination with shotgun ESI-MS for the analysis of cholesterol metabolites. The two methods are complementary, as EADSA works with steroids possessing a free 3β-hydroxy or oxo group and shotgun negative-ion ESI-MS works most effectively for steroids conjugated with a strongly acidic group, e.g., sulfuric acid ester, in which the site of modification is often at C-3, making the molecule transparent to EADSA, but readily ionized by negative-ion ESI.

To the best of our knowledge, EADSA offers sensitivity advantages for the analysis of sterols that exceed those of any other methodology. There are, however, a number of disadvantages of EADSA methodology, which should be considered.

1. Steroids with a free 3α-hydroxy group are not oxidized by cholesterol oxidase [47]; thus, e.g., primary bile acids cannot
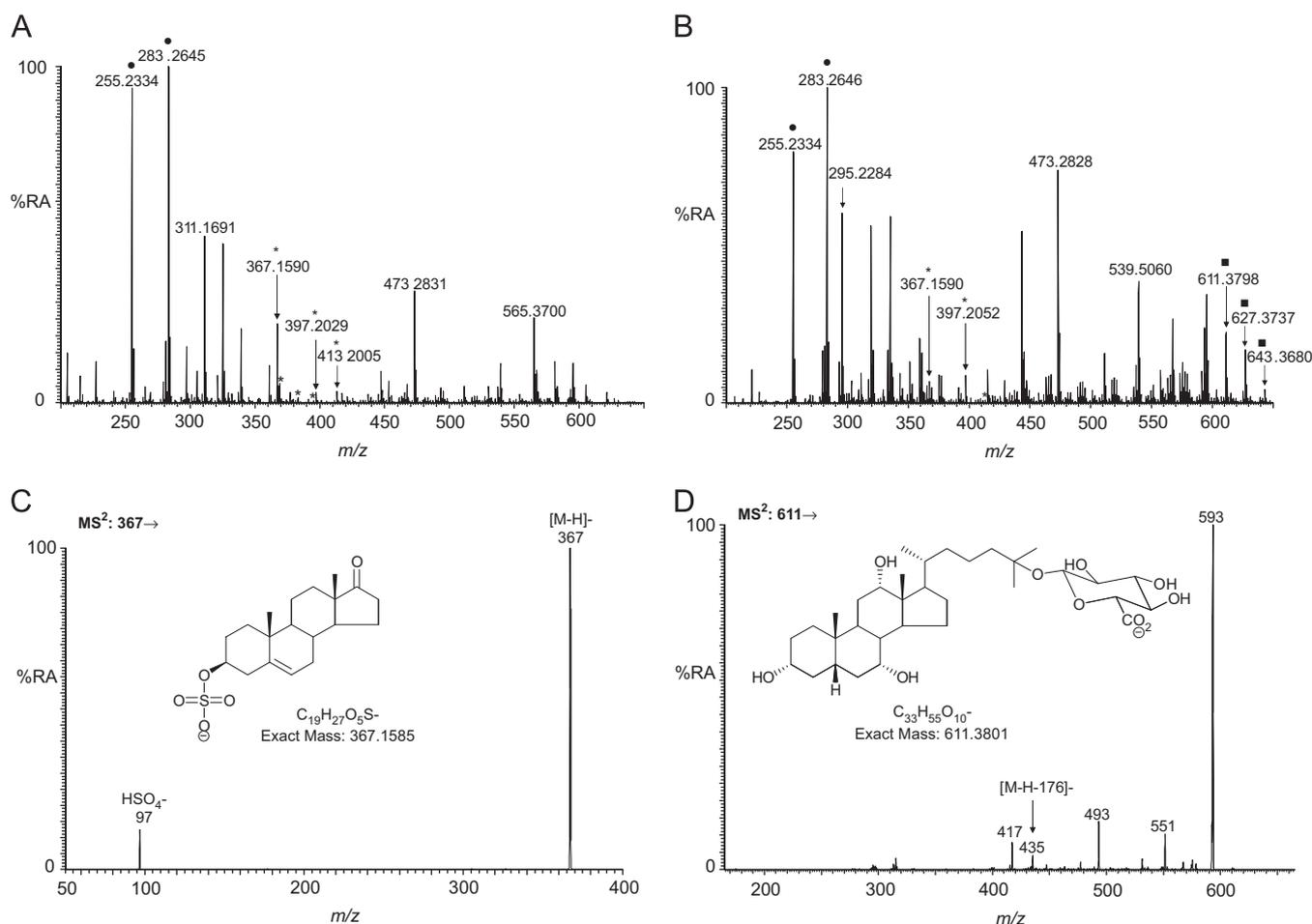
Fig. 6. Negative-ion ESI-MS spectra of plasma samples from (A) a control infant and (B) a boy with CTX not on treatment. (C) ESI-MS^2 spectrum of the ion m/z 367 from the control sample. (D) ESI-MS^2 spectrum of the ion m/z 611 from the boy with CTX. Peaks labeled with an asterisk correspond to C_{19} and C_{21} steroid sulfates, those with a filled square to oxysterol glucuronides, and those with a filled circle to fatty acids. Spectra were recorded on the LTQ-Orbitrap XL. Spectra in (A) and (B) were recorded at high resolution, (C) and (D) utilized the ion-trap detector.

be converted to 3-ketones and subsequently derivatized. However, this problem can be circumvented by the use of 3α-hydroxysteroid dehydrogenase instead of cholesterol oxidase, which generates the 3-ketone suitable for subsequent derivatization.

2. A problem with all steroid derivatization reactions is the prevalence of labile hydroxy groups to be eliminated as water during the derivatization process. This is seen with the method presented here for 7α-hydroxy-3-oxocholest-(25R)-4-en-26-oic acid, which undergoes dehydration to the 4,6-diene despite the reaction being performed at room temperature.

3. Steroids possessing an epoxide group are labile in acidic solvents and we find that 24S,25-epoxycholesterol becomes isomerized to the 24-ketone, hydrolyzed to the 24,25-diol, and methanolyzed to the 24-hydroxy-25-methyl ether (and/or 25-hydroxy-24-methyl ether) during the EADSA process. 5,6-Epoxycholesterol becomes hydrolyzed to the 3β,5,6-triol, which eliminates water to leave the cholest-4-ene-3β,6-diol [56].

4. A disadvantage of the hydrazone derivative is that its formation is reversible in acidic solvents. This precludes the storage of derivatized steroids in acidic solvents and dictates that GP-derivatized steroids are not kept for long periods (> 24 h) in their injection solvent before injection onto the LC system. However, we find that when stored in 100% methanol GP-derivatized steroids are stable.

5. A consequence of derivatization with a hydrazine reagent is the formation of syn and anti hydrazones. These are often

resolved in our chromatography system, highlighting the quality of the chromatography, but complicating the ultimate chromatogram.

6. The chromatographic method described here utilizes a Hypersil Gold RP column. By employing the sample preparation procedure described here columns have an exceedingly long lifetime (all columns purchased in the past 4 years are still in use today with no reduction in performance). However, there is some batch-to-batch variation, which means that with some columns 25-hydroxycholesterol is only partially resolved from the 24S-isomer. This problem can be overcome by lengthening the gradient but at the expense of throughput.

7. In the EADSA methodology reported here quantification is made using 24(R/S)-[^{2}H$_6$]hydroxycholesterol as the internal standard. Cholesterol oxidase from Streptomyces sp. has similar activities toward most sterols with a 3β-hydroxy-5-ene structure [47], and we have found that 3-oxo-4-ene sterols once derivatized with GP-hydrazine give essentially the same ESI response irrespective of other functional groups present [43], allowing good quantitative estimates to be made without the inclusion of additional reference standards other than [^{2}H$_7$]cholesterol for sterols eluting in Fr-3 from SPE-1. However, the sample preparation protocol described here has been designed with side-chain-oxidized sterols and cholestenoic acids in mind, and it is likely that 7α-hydroxycholesterol and other ring-oxidized sterols are not fully eluted from SPE-1 in Fr-1; hence their amount is probably underestimated. This is
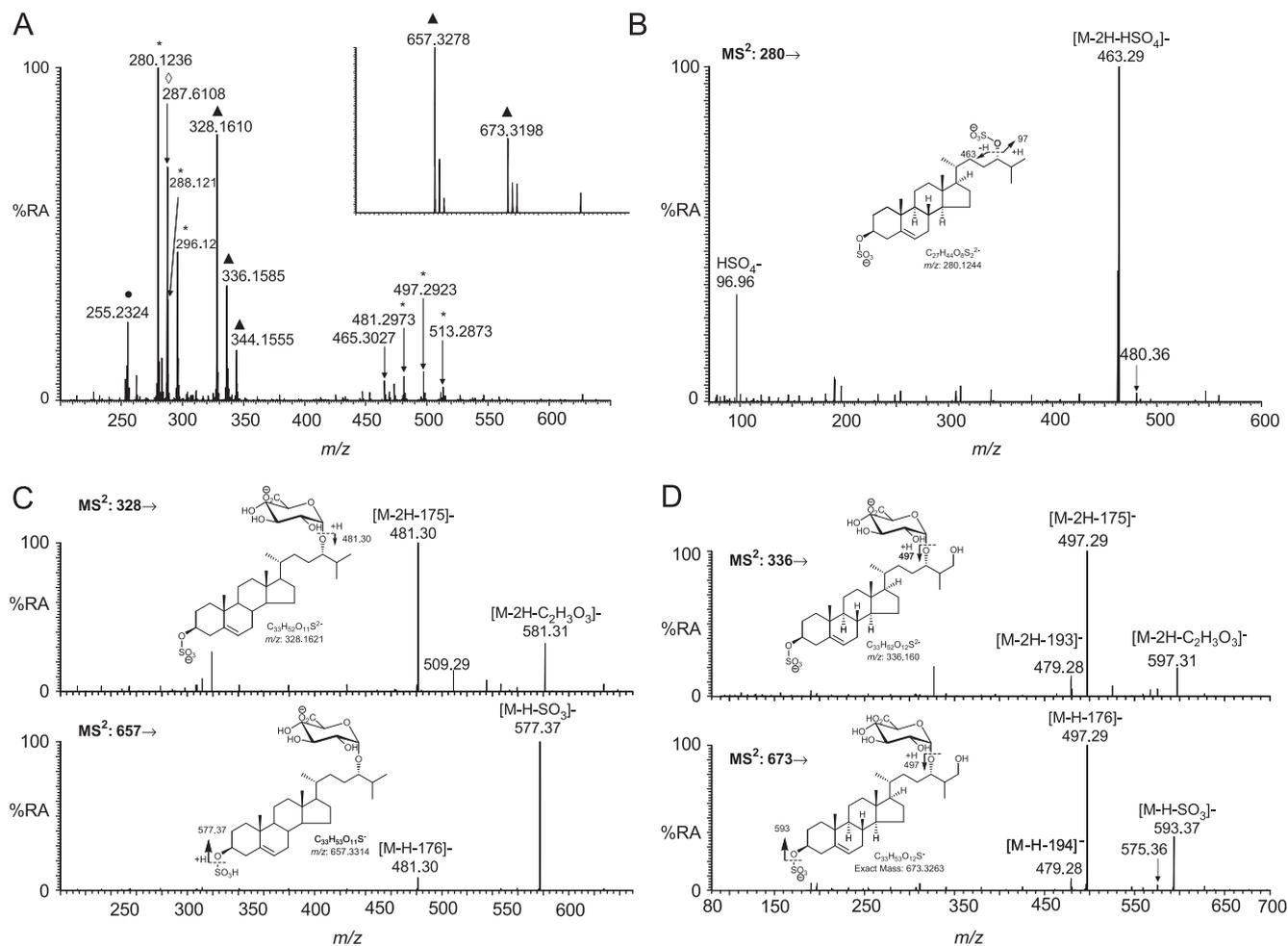
Fig. 7. (A) Negative-ion ESI-MS spectrum of a plasma sample from an infant with suspected HSD3B7 deficiency. The inset shows the high m/z range 640–700. MS^2 spectra ions of (B) m/z 280, (C) m/z 328 and 657, and (D) m/z 336 and 673, corresponding to cholest-5-ene-3β,x-diol disulfate [M $-$ ^{2}H]$^{2-}$, cholest-5-ene-3β,x-diol 3-sulfate and x-glucuronide [M $-$ ^{2}H]$^{2-}$ and [M $-$ H]$^-$, and cholest-5-ene-3β,x,y-triol 3-sulfate and x-glucuronide [M $-$ ^{2}H]$^{2-}$ and [M $-$ H]$^-$ ions, respectively, are shown. Sulfation is assumed at C-3, and x and y are drawn on the side chain. Peaks labeled with an asterisk correspond to oxysterol mono- or disulfates, those with a filled triangle to oxysterols sulfated and glucuronidated, that with a diamond to taurine-conjugated bile acid sulfates, and that with a filled circle to fatty acids. Spectra were recorded on the LTQ-Orbitrap XL and at high resolution.

easily rectified by using a stronger eluent to elute 7α-hydroxycholesterol from SPE-1.

8. A number of sterols can exist naturally in their 3-oxo-4-ene and 3β-hydroxy-5-ene forms; this dictates the analysis of A (with cholesterol oxidase) and B (without cholesterol oxidase) fractions, and the difference A − B equals the amount of free 3β-hydroxy-5-ene metabolite. In most studies we use DHEA 3-sulfate, an abundant steroid present in plasma, not oxidized by cholesterol oxidase, as a natural internal standard to allow the normalization of fraction B to the fraction A equivalents. Alternatively, 22R-[^{2}H$_7$]hydroxycholest-4-en-3-one can be used as an internal standard for B fractions.

Despite these caveats, the advantages of EADSA lie in its simplicity and robustness, offering greatly improved sensitivity for the analysis of steroids and sterols.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2012.07.027.

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