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Charge-tagging liquid chromatography–mass spectrometry methodology targeting oxysterol diastereoisomers



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ABSTRACT

The introduction of a hydroxy group to the cholesterol skeleton introduces not only the possibility for positional isomers but also diastereoisomers, where two or more isomers have different configurations at one or more of the stereocentres but are not mirror images. The differentiation of diastereoisomers is important as differing isomers can have differing biochemical properties and are formed via different biochemical pathways. Separation of diastereoisomers is not always easy by chromatographic methods. Here we demonstrate, by application of charge-tagging and derivatisation with the Girard P reagent, the separation and detection of biologically relevant diastereoisomers using liquid chromatography – mass spectrometry with multistage fragmentation.

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1. Introduction

The most commonly observed diastereoisomers derived from cholesterol are 7 α -hydroxycholesterol and 7 β -hydroxycholesterol (7 α -HC and 7 β -HC). These can be formed via free radical oxidation by initial abstraction of the C-7 hydrogen followed by reaction with oxygen to form a peroxy radical, hydrogen abstraction to form a peroxide and ultimately reduction to 7 α - or 7 β -HC (Murphy and Johnson, 2008) (Fig. 1). These reactions may proceed in air during sample handling or in the biological systems, e.g. in the lysosomal storage disease Niemann Pick type C (NPC) (Alvelius et al., 2001). 7 α -HC is also formed enzymatically from cholesterol by the enzyme cytochrome p450 7A1 (CYP7A1) in the first step of the neutral pathway of bile acid biosynthesis (Russell, 2003), while 7 β -HC can be formed from 7-oxocholesterol (7-OC) in a reaction

catalysed by hydroxysteroid dehydrogenase 11B1 (HSD11B1) (Hult et al., 2004; Larsson et al., 2007; Mitic et al., 2013). 7-OC can be formed from the cholesterol precursor 7-dehydrocholesterol (7-DHC) in a reaction also catalysed by CYP7A1 (Shinkyo et al., 2011) and also by a free radical oxidation mechanism from cholesterol (Fig. 1). Both 7-OC and 7 β -HC are abundant in the disease Smith Lemli Opitz Syndrome (SLOS) where 7-DHC, the CYP7A1 substrate, is present at elevated concentrations (Griffiths et al., 2016). To understand the origin of 7 α -HC and 7 β -HC it is necessary to differentiate between them. While the epimers 7 α -HC and 7 β -HC can be resolved by gas chromatography (GC) (Dzeletovic et al., 1995) this is not always the case with liquid chromatography (LC) (McDonald et al., 2012).

Less commonly considered diastereoisomers are 24S- and 24R-hydroxycholesterols (24S-HC and 24R-HC). 24S-HC is formed from

Abbreviations: ACOX2, acyl-coenzyme A oxidase 2; AMACR, aliphatic acyl-CoA racemase; CTX, cerebrotendinous xanthomatosis; CYP, cytochrome P450; EADSA, enzyme-assisted derivatisation for steroid analysis; ESI, electrospray ionisation; GC, gas chromatography; GP, Girard P reagent; HSD, hydroxysteroid dehydrogenase; LC, liquid chromatography; LIT, linear ion trap; MRM, multiple reaction monitoring; MS, mass spectrometry; MSⁿ, MS with multistage fragmentation; NPC, Niemann Pick type C; RIC, reconstructed ion chromatogram; SLOS, Smith-Lemli-Opitz syndrome; SRM, standard reference material; 3 β ,7 α -diHCA(25R), 3 β ,7 α -dihydroxycholest-5-en-(25R)26-oic acid; 3 β ,7 α -diHCA(25S), 3 β ,7 α -dihydroxycholest-5-en-(25S)26-oic acid; 3 β ,7 α -diH- Δ^5 -BA, 3 β ,7 α -dihydroxychol-5-enoic acid; 3 β ,7 β -diHCA(25R), 3 β ,7 β -dihydroxycholest-5-en-(25R)26-oic acid; 3 β ,7 β -diHCA(25S), 3 β ,7 β -dihydroxycholest-5-en-(25S)26-oic acid; 3 β ,7 β -diH- Δ^5 -BA, 3 β ,7 β -dihydroxychol-5-enoic acid; 7-OC, 7-oxocholesterol; 7 α -HC, 7 α -hydroxycholesterol; 7 α -HCO, 7 α -hydroxycholest-4-en-3-one; 7 α H,3O-CA(25R), 7 α -hydroxy-3-oxocholest-4-en-(25R)26-oic acid; 7 α H,3O-CA(25S), 7 α -hydroxy-3-oxocholest-4-en-(25S)26-oic acid; 7 α H,3O- Δ^4 -BA, 7 α -hydroxy-3-oxocholest-4-enoic acid; 7 β -HC, 7 β -hydroxycholesterol; 24R-HC, 24R-hydroxycholesterol; 24S-HC, 24S-hydroxycholesterol; (25R)26-HC, (25R)26-hydroxycholesterol; 25-HC, 25-hydroxycholesterol.

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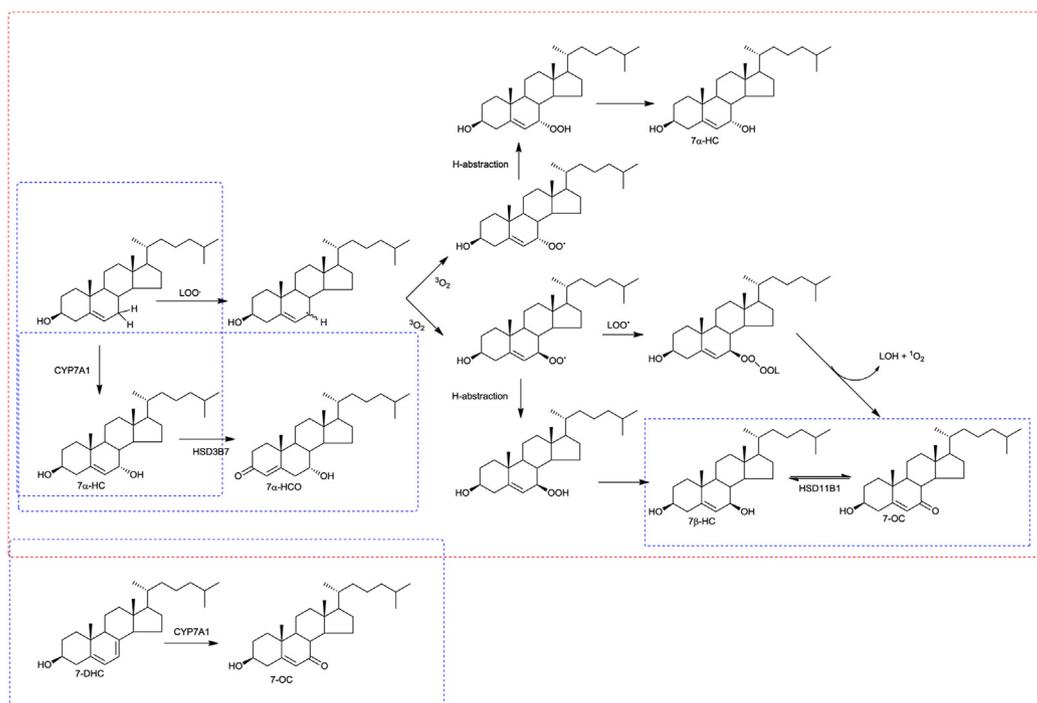


Fig. 1. Formation of 7-hydroxycholesterol (7-HC) and 7-oxocholesterol (7-OC) by radical initiated and enzymatic reactions. LOO[•] corresponds to a lipid hydroperoxy radical and LOH to a hydroxy lipid. Enzyme catalysed reactions are shown in the blue boxes and radical initiated pathways in the red box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

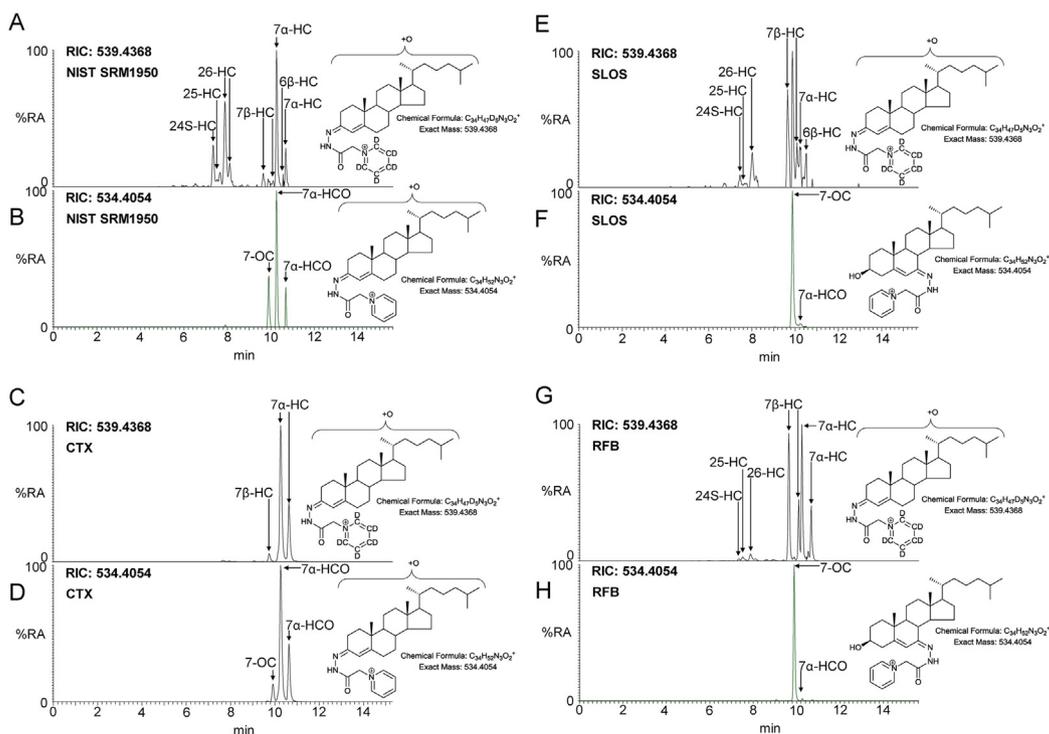


Fig. 2. Reconstructed-ion chromatograms (RICs) displaying how 7α-HC, 7β-HC, 7α-HCO and 7-OC vary in plasma in health and disease and according to sample storage. (A) RIC from NIST SRM1950 plasma treated with cholesterol oxidase and (B) in the absence of cholesterol oxidase. (C) RIC from a typical CTX plasma sample treated with cholesterol oxidase and (D) in the absence of cholesterol oxidase. (E) RIC from a typical SLOS plasma sample treated with cholesterol oxidase and (F) in the absence of cholesterol oxidase. (G) RIC from a dried plasma sample, transported to Swansea from Germany without precaution being taken against oxidation in air, treated with cholesterol oxidase and (H) in the absence of cholesterol oxidase. Aliquots of sample prepared in the presence of cholesterol oxidase were derivatised with [²H₅]GP and those prepared in the absence of cholesterol oxidase with [²H₀]GP. Analyte concentrations are given in supplementary Table S1 (Crick et al., 2015b; Griffiths et al., 2016, 2013a; Theofilopoulos et al., 2014).

cholesterol by the enzyme CYP46A1, predominantly expressed in neurons in brain, however, the origin of 24R-HC is less well established (Russell, 2003). Most GC-mass spectrometry (MS) methods are not optimised to differentiate between these epimers and it is generally assumed that all 24-HC detected is the 24S-epimer. This may not necessarily be the case.

A further stereocentre of biological importance is at C-25. During bile acid biosynthesis side-chain shortening in the peroxisome the stereochemistry at C-25 of the C_{27} acid is changed from R to S by the enzyme alphas-methylacyl-CoA racemase (AMACR). This is necessary for the next step in the pathway, catalysed by the peroxisomal enzyme acyl-coenzyme A oxidase 2 (ACOX2), which introduces a double bond between C-24 and C-25, to proceed and ultimately lead to a C_{24} acid (Russell, 2003). When found in plasma, cholestenic acids are usually assumed to have R stereochemistry at C-25.

Here we describe how we have optimised the charge-tagging technique christened enzyme-assisted derivatisation for steroid analysis (EADSA) (Griffiths et al., 2013b) to separate and identify oxysterol, cholestenic and cholenoic acid diastereoisomers.

2. Materials and methods

2.1. Materials

Human serum and plasma samples were from waste material from previous studies in Swansea (Abdel-Khalik et al., 2017) or supplied by the Reference Institute for Bioanalytics, Bonn, Germany as a reference material for interlaboratory comparison of oxysterol measurements (Lütjohann et al., 2014). Written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. The study was conducted with institutional review board approval. The NIST standard reference material (SRM1950) was prepared by NIST from plasma samples from 100 individuals between 40 and 50 years of age, whose ethnicity was representative of the US population and that included an equal number of men and women (Gaithersburg, MD) (Quehenberger et al., 2010).

All reagents were from sources documented in (Crick et al., 2015a, 2015b). Oxysterol, cholestenic and cholenoic acid authentic standard were from Avanti Polar Lipids Inc (Alabaster, AL) or from kind donations by Professor Jan Sjövall, Karolinska Institute, Stockholm.

2.2. Methods

2.2.1. EADSA

To enhance sensitivity, solubility in solvents used in reversed-phase chromatography and to enrich structure-informative fragmentation we employed EADSA. In brief, after separation of oxysterols and C_{24} and C_{27} acids from cholesterol and other hydrophobic sterols, an aliquot (A) of the oxysterol fraction containing the acids was oxidised with cholesterol oxidase from *Streptomyces* sp. (Sigma-Aldrich, Dorset, UK) to convert 3 β -hydroxy-5-ene structures to 3-oxo-4-enes. The oxo groups were then reacted with the Girard P reagent (GP) to generate GP-hydrazones which were separated from excess reagents on an OASIS HLB reversed phase solid phase extraction column (Waters, Elstree, UK). As some oxysterols naturally contain a 3-oxo-4-ene group e.g. 7 α -hydroxycholest-4-en-3-one (7 α -HCO), to avoid confusion with those oxidised by cholesterol oxidase to contain this function a separate aliquot of sample (B) was prepared in an identical fashion but in the absence of cholesterol oxidase. To allow the simultaneous analysis of aliquots (A) and (B), [2H_5]GP was used to derivatise aliquots (A) and [2H_0]GP to derivatise aliquots (B). The two aliquots were combined immediately before LC-MS analysis. Oxysterols derivatised with

[2H_5]GP will have a mass 5.0314 Da heavier than those derivatised with [2H_0]GP. Note an alkaline hydrolysis step was not performed so free were oxysterols analysed not total oxysterols.

2.2.2. LC-MSⁿ

Derivatised oxysterols and C_{24} and C_{27} acids were separated on a Hypersil Gold reversed phase column (Thermo-Fisher Scientific, Hemel Hempstead, UK) as described in (Crick et al., 2015b) and

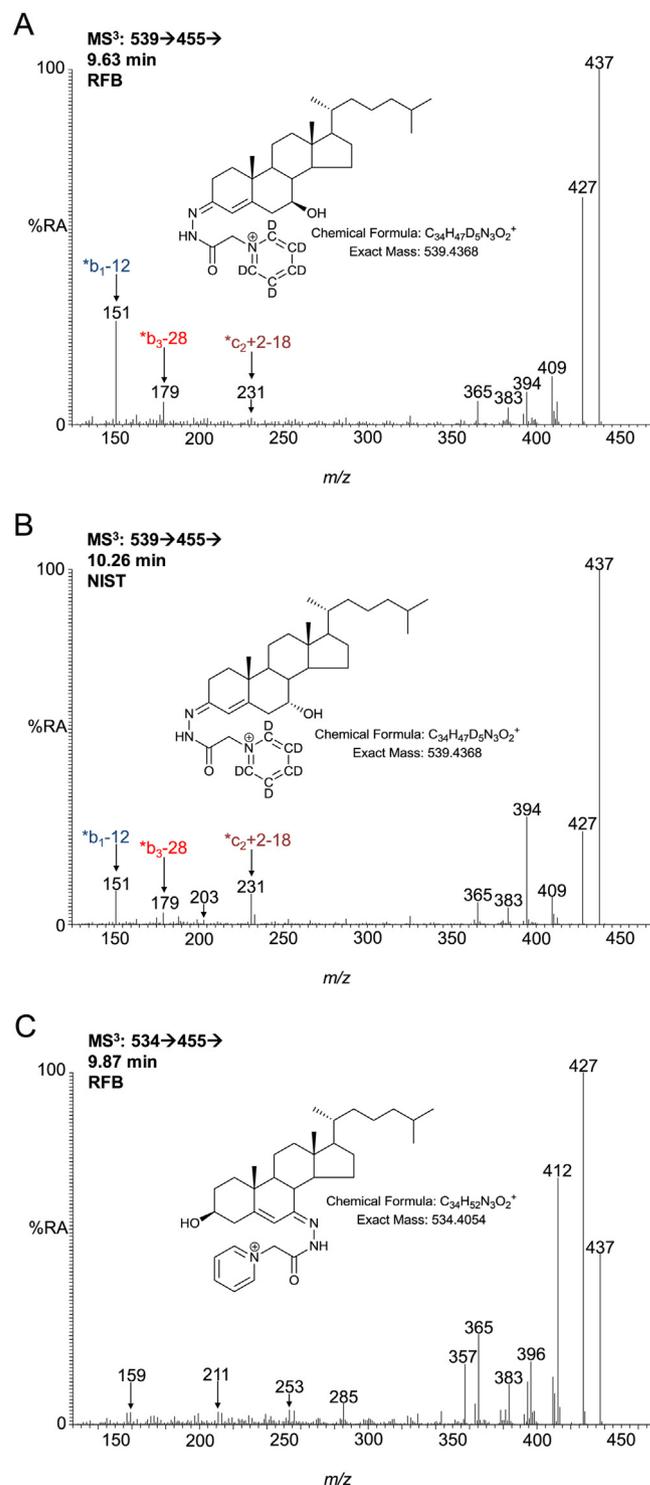


Fig. 3. MS³ ($[M^+ \rightarrow [M-Py]^+ \rightarrow$) spectra of (A) 7 β -HC, (B) 7 α -HC and (C) 7-OC. Aliquots of sample prepared in the presence of cholesterol oxidase were derivatised with [2H_5]GP and those prepared in the absence of cholesterol oxidase with [2H_0]GP.

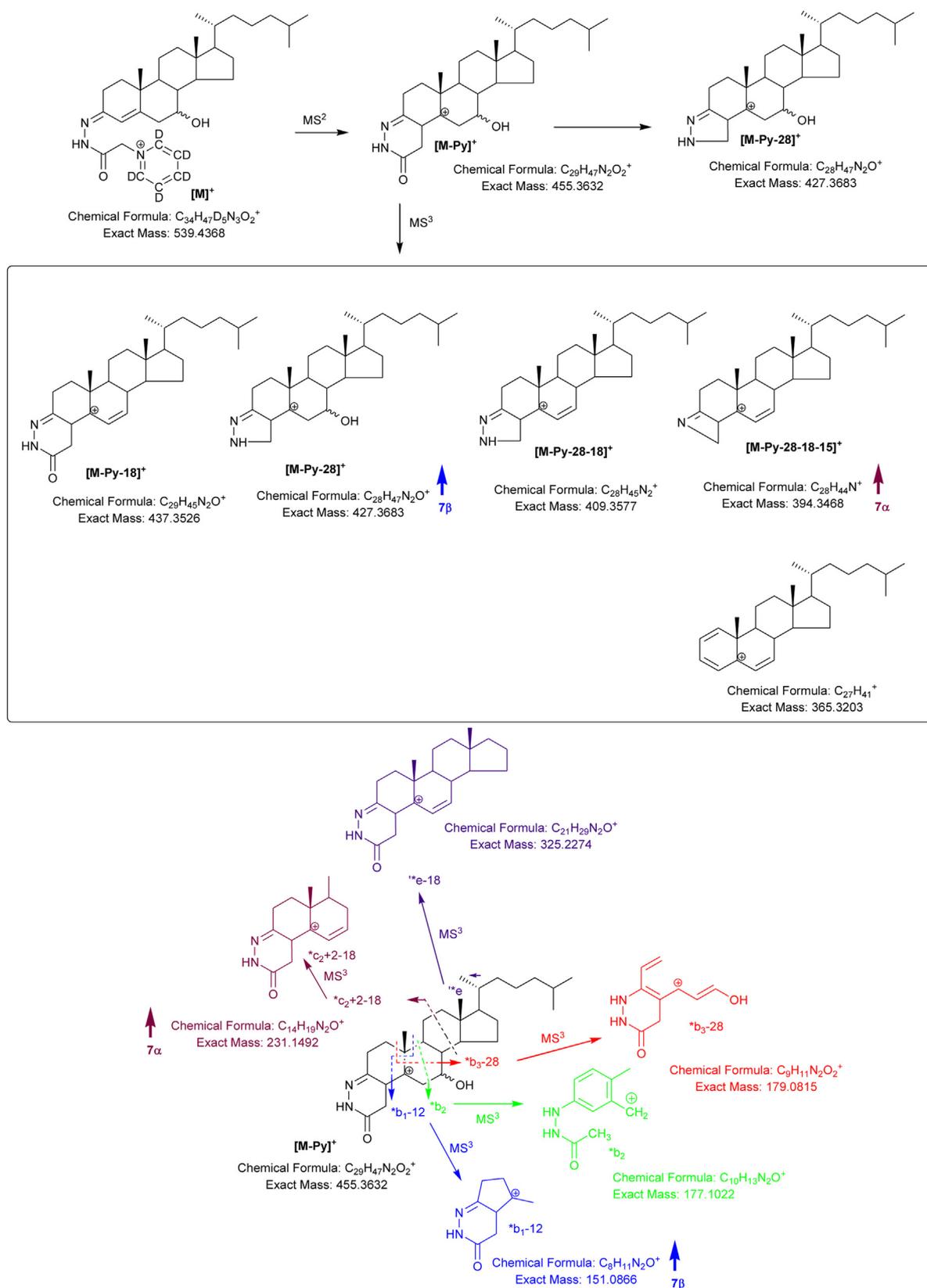


Fig. 4. Fragmentation of 7-hydroxycholesterols. Ions of elevated relative abundance in the spectra of 7α- and 7β- epimers are indicated by arrows.

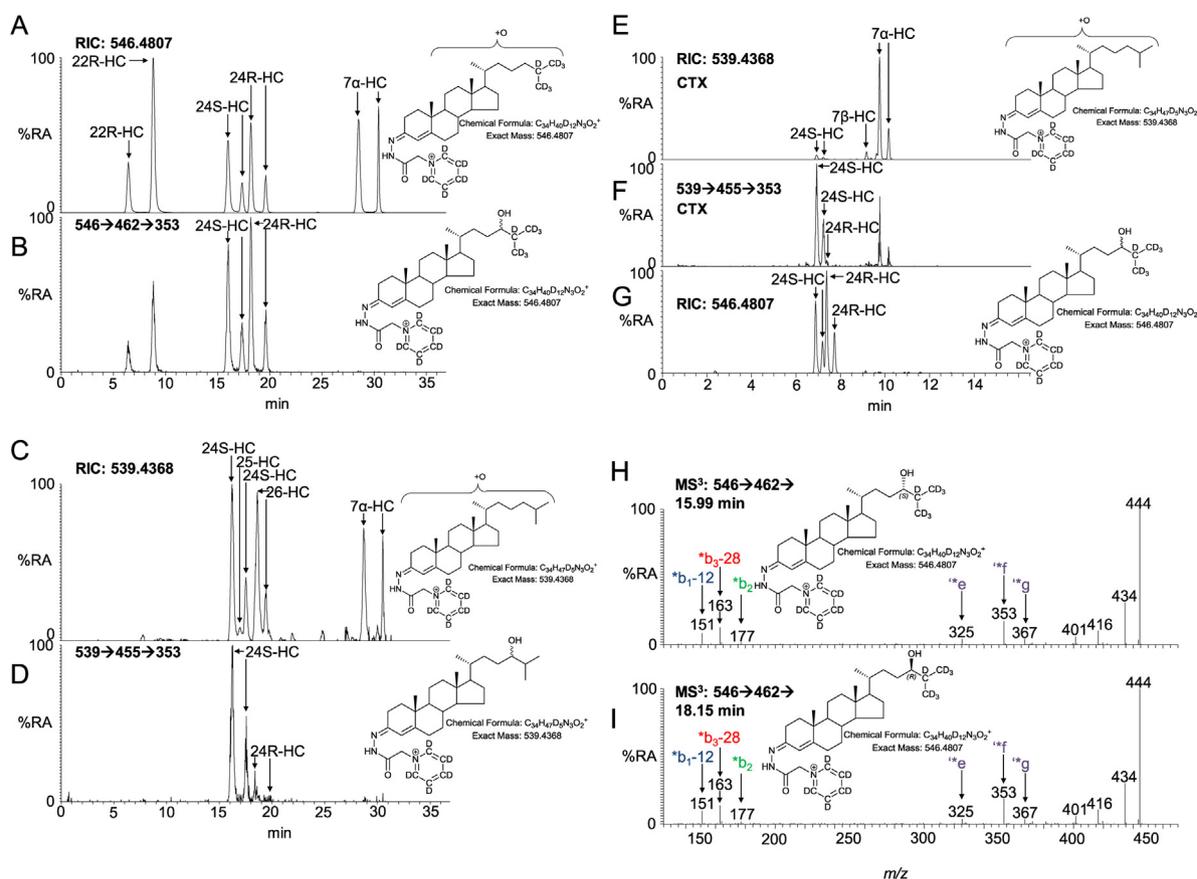


Fig. 5. Separation and fragmentation of 24S-HC and 24R-HC. (A) RIC for 24S-HC and 24R-HC using a 37 min gradient. Other peaks correspond to 22R-HC and 7 α -HC. The hydroxycholesterols (HCs) are heavy isotope labelled with [25,26,26,26,27,27,27-²H₇] and derivatised with [²H₅]GP. (B) MRM chromatogram for the [M]⁺ → [M-Py]⁺ → 353.3 ± 0.3 transition. 22R-HC also shows a fragment ion at *m/z* 353.3 (see Supplementary Fig. S1A). (C) RIC for monohydroxycholesterols in a typical human serum sample and (D) MRM chromatogram for the [M]⁺ → [M-Py]⁺ → 353.3 ± 0.3 transition, highlighting 24-HC, in the same sample. (E) RIC for HCs in a CTX plasma sample using a 17 min gradient. (F) MRM chromatogram for the [M]⁺ → [M-Py]⁺ → 353.3 ± 0.3 transition from the CTX patient using the 17 min gradient (Crick et al., 2015b). (G) RIC for [²H₇] 24S-HC and [²H₇]24R-HC using the 17 min gradient. (H) MS³ ([M⁺ → [M-Py]⁺ →) spectra of [²H₇]24S-HC and (I) [²H₇]24R-HC. Note in the spectra of the [²H₇]24R-HC molecules fragment ions are observed at *m/z* 444, 434, 416 and 401 which are 7 Da heavier than equivalent fragment ions in the [²H₀]24R-HC native molecules (see Supplementary Fig. S1C & S1F and Fig. 6). Analyte concentrations are given in supplementary Table S1.

analysed by electrospray ionisation (ESI) – MS and –MS with multistage fragmentation (MSⁿ) on an Orbitrap Elite hybrid linear ion trap (LIT) – Orbitrap mass spectrometer (Thermo-Fisher Scientific, Hemel Hempstead, UK). While the Orbitrap performed *m/z* scans at high resolution (120,000 full-width at half height definition) the LIT simultaneously performed MS³ scans on preselected precursor and product-ions.

3. Results

3.1. LC separation and MSⁿ fragmentation

As discussed previously (Crick et al., 2015b), GP-derivatised oxysterols fragment in the first MS/MS event (MS²) with the loss of the pyridine ring to give the [M-Py]⁺ fragment, [M-84]⁺ for [²H₅]GP and [M-79]⁺ for [²H₀]GP. [M-Py]⁺ ions then fragment in an MS³ (MS/MS/MS) event to give structurally informative fragment-ions showing cleavage in the ring-system, side-chain and with loss of H₂O and CO and combinations thereof.

3.1.1. 7 α -HC, 7 β -HC and 7 α -HCO

7 α -HC and 7 β -HC are chromatographically resolved in our system (Fig. 2A). The introduction of a polar hydroxyl group to the non-polar β -face of the sterol ring system reduces its hydrophobicity and retention on a C₁₈ reversed-phase column. A

reconstructed ion chromatogram (RIC, ± 5 ppm) from a typical serum sample is shown in Fig. 2A. Both 7 β -HC and 7 α -HC give double peaks as a consequence of *syn* and *anti* conformers of the GP-derivative. The RIC in Fig. 2A is for the aliquot treated with cholesterol oxidase and derivatised with [²H₅]GP. Fig. 2B shows the chromatogram generated from aliquot B in the absence of cholesterol oxidase and derivatised with [²H₀]GP. This chromatogram reveals compounds with a *natural* oxo group, i.e. 7 α -HCO and 7-OC. While 7 α -HCO can be formed *in vivo* from 7 α -HC by HSD3B7 (Fig. 1), 7 β -HCO is not formed from 7 β -HC by this enzyme (Furster et al., 1996) and is thus absent. This, beside chromatographic resolution and differing MS³ spectra (see below), provides further evidence for the identity of the peaks assigned to the 7 β -hydroxy epimer in chromatogram 2A. Absolute confirmation of identity was made by comparison with authentic standards. As peaks in aliquot (A) correspond to the sum of both 3 β -hydroxy-5-ene and 3-oxo-4-ene compounds, while those in the (B) aliquot just to oxo compounds, quantification of 3 β -hydroxy-5-ene compounds is easily performed by subtracting concentrations determined for a peak in (B) from those determined for the equivalent peak in (A). Concentration determined for peaks in (B) are those for the *natural* oxo compounds.

The MS³ spectra of 7 β -HC and 7 α -HC/7 α -HCO are similar but not identical (Fig. 3). The 7 α -hydroxy group is more labile with the result that the [M-Py-18]⁺ fragment-ion (*m/z* 437, Fig. 4) dominates

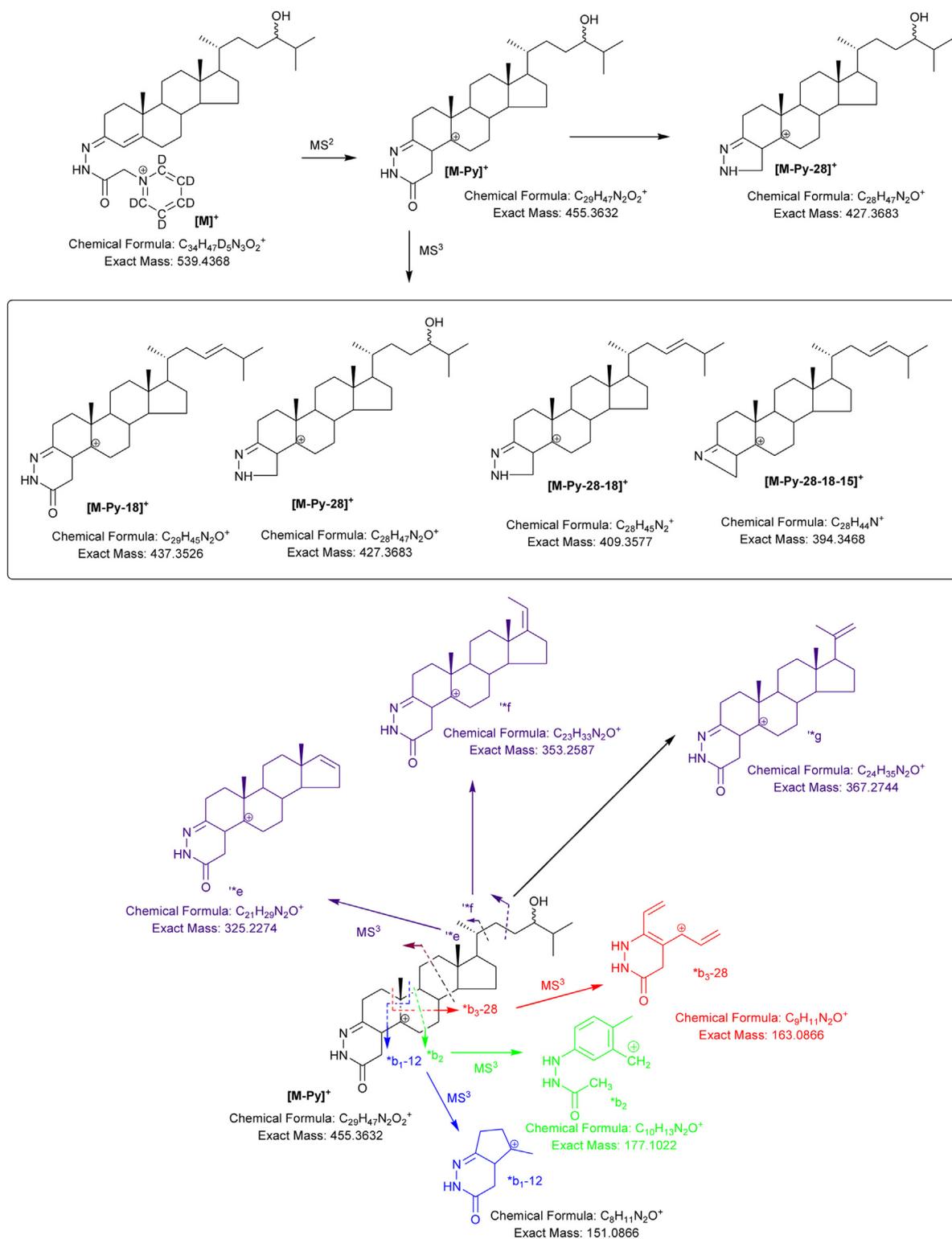


Fig. 6. Fragmentation of 24-hydroxycholesterols.

the MS³ spectrum and the [M-Py-28-18-15]⁺ (*m/z* 394) and *C₂+2-18 (*m/z* 231) fragment-ions are of greater relative abundance than in the MS³ spectrum of 7β-HC. On the other hand, the [M-Py-28]⁺ (*m/z* 427) and *b₁-12 (*m/z* 151) fragment-ions are of higher relative

abundance in the spectrum of 7β-HC than 7α-HC (Figs. 3A and B & 4).

The abundance of 7α-HC and 7β-HC are of diagnostic value. One example is cerebrotendinous xanthomatosis (CTX) where the

enzyme CYP27A1 is deficient resulting in reduced levels of its product (25R)26-hydroxycholesterol ((25R)26-HC) and greatly elevated levels of 7 α -HC and 7 α -HCO in plasma (Fig. 2C and D) (DeBarber et al., 2011). In SLOS where both 7-DHC and 7-OC are elevated in plasma so is 7 β -HC formed from 7-OC by HSD11B1 (Fig. 2E and F). A complication when using 7-HC epimers or 7-OC in disease diagnosis is that each can be formed in air by reaction with oxygen (Fig. 2G and H) (Schroepfer, 2000).

3.1.2. 24S-HC and 24R-HC

24S-HC elutes before 24R-HC (Fig. 5A–D & E–G showing two different gradients). They give an essentially identical MS³ spectrum where the fragment ion **f at m/z 353 is prominent (Figs. 5 H and I & 6). This ion is very minor in closely eluting 25-hydroxycholesterol (25-HC) and (25R)26-HC (see Supplementary Fig. S1). By performing a RIC for m/z 353 in the MS³ chromatogram to give a multiple reaction monitoring (MRM) chromatogram for transitions $[M]^+ \rightarrow [M-Py]^+ \rightarrow 353$ the 24-HC epimers are highlighted. This is demonstrated for $[25,26,26,26,27,27,27\text{-}^2\text{H}_7]$ 24S-HC and $[25,26,26,26,27,27,27\text{-}^2\text{H}_7]$ 24R-HC authentic standards in Fig. 5B (MS³ spectra shown in Fig. 5H and I) and in a typical human serum sample in Fig. 5D and in a CTX plasma sample in Fig. 5F. The fragmentation pattern for the endogenous molecule is described by Fig. 6 and its MS³ spectrum shown in supplementary Fig. S1. Quantification can be performed using the MRM chromatograms for the authentic standard and endogenous molecules.

3.1.3. 3,7-Dihydroxycholest-5-en-26-oic and 7-Hydroxy-3-oxocholest-4-en-26-oic acids

Both 3 β ,7 α -dihydroxycholest-5-en-(25R)26-oic (3 β ,7 α -diHCA(25R)) and 7 α -hydroxy-3-oxocholest-4-en-(25R)-26-oic (7 α H,3O-CA(25R)) acids are found in plasma (Fig. 7A and B). They can be formed via both the neutral and acidic pathways of bile acid biosynthesis (Russell, 2003). Once a 7 α -hydroxy group is present on the B-ring, the sterol becomes a substrate for HSD3B7 and the 3 β -hydroxy-5-ene is converted to a 3-oxo-4-ene. Both acids when derivatised give double peaks corresponding to *syn* and *anti* conformers of the GP-hydrazone. Surprisingly, in plasma a quartet rather than a doublet of peaks is observed in the appropriate RIC, each giving an identical MS³ spectrum (Fig. 7D and E). Custom synthesis of [²H₂]-labelled 25R and 25S epimers (supplementary Fig. S2) showed that the smaller peaks of the quartet correspond to the 25S epimers, 3 β ,7 α -diHCA(25S) and 7 α H,3O-CA(25S), while the larger peaks correspond to the 25R epimers.

Further possible diastereomers are 3 β ,7 β -dihydroxycholest-5-en-(25R)26-oic (3 β ,7 β -diHCA(25R)) and 3 β ,7 β -dihydroxycholest-5-en-(25S)26-oic (3 β ,7 β -diHCA(25S)). As HSD3B7 is not active on sterols with a 7 β -hydroxy group the 7 β -hydroxy-3-oxo-4-ene acids are not expected to present endogenously (Furster et al., 1996). Close scrutiny of the RIC shown in Fig. 7A reveals a peak with retention time and giving an MS³ spectrum (Fig. 7C) identical to the 3 β ,7 β -diHCA(25R) authentic standard. This peak is absent in Fig. 7B where the sample was prepared in the absence of cholesterol oxidase. In keeping with the MS³ spectra of 7 α -HC and 7- β HC, the $[M-Py-18]^+$ ion at m/z 467 dominates the spectrum of the 7 α -epimers, with the $[M-Py-28-18-15]^+$ (m/z 424) and $^*c_2+2-18$ (m/z 231) being of greater relative abundance than in the spectrum of the 7 β -epimer (Fig. 8). On the other hand the fragment ions $[M-Py-28]^+$ (m/z 457) and $^*b_1-12$ (m/z 151) are of greater relative abundance in the spectrum of the 7 β -epimer. An early eluting shoulder can be observed on the chromatographic peak assigned to the 3 β ,7 β -diHCA(25R) diastereomer. This

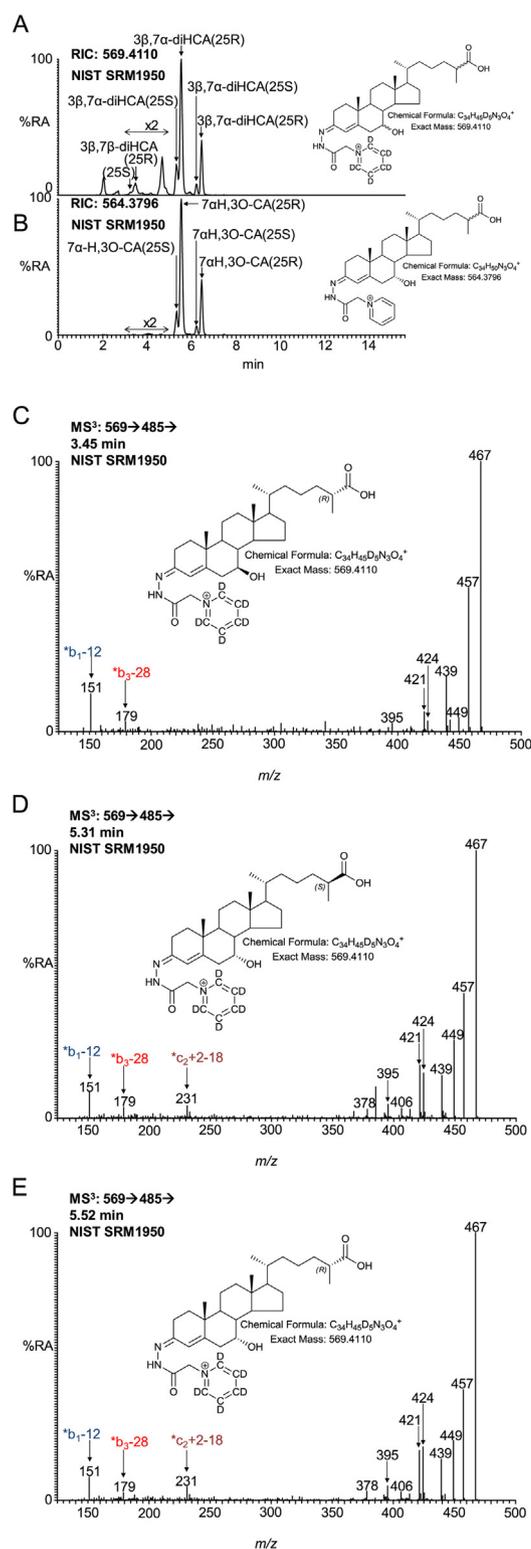


Fig. 7. Separation and fragmentation of diastereoisomers of 3,7-dihydroxycholest-5-en-26-oic and 7-hydroxy-3-oxocholest-4-en-26-oic acids. (A) RIC from NIST SRM1950 plasma showing the separation of 3 β ,7 β -diHCA(25S), 3 β ,7 β -diHCA(25R), 3 β ,7 α -diHCA(25S) and 3 β ,7 α -diHCA(25R). Peak intensities in the time window 3–5 min have been multiplied by 2. (B) The 3 β ,7 β -diHCA epimers are not substrates for HSD3B7 so do not give 7 β H,3O-CA compounds, while 3 β ,7 α -diHCA are and give 7 α H,3O-CA epimers. (C) MS³ ($[M]^+ \rightarrow [M-Py]^+ \rightarrow$) spectra of 3 β ,7 β -diHCA(25R), (D) 3 β ,7 α -diHCA(25S) and (E) 3 β ,7 α -diHCA(25R). Aliquots of sample prepared in the presence of cholesterol oxidase were derivatised with [²H₅]GP and those prepared in the absence of cholesterol oxidase with [²H₀]GP. Analyte concentrations are given in supplementary Table S1.

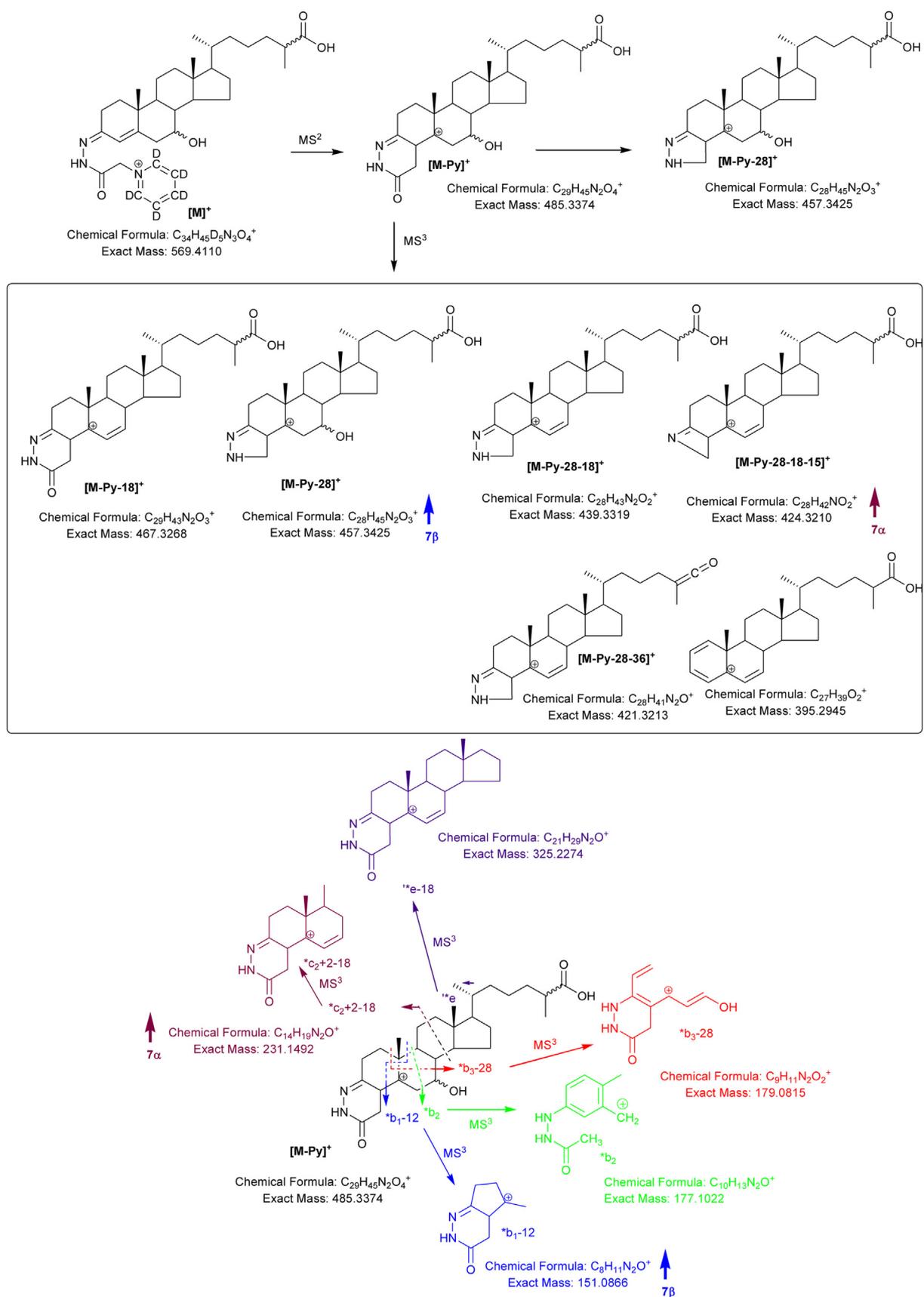


Fig. 8. Fragmentation of 3β,7α-diHCA and 3β,7β-diHCA. Ions of elevated relative abundance in the spectra of 7α- and 7β- epimers are indicated by arrows.

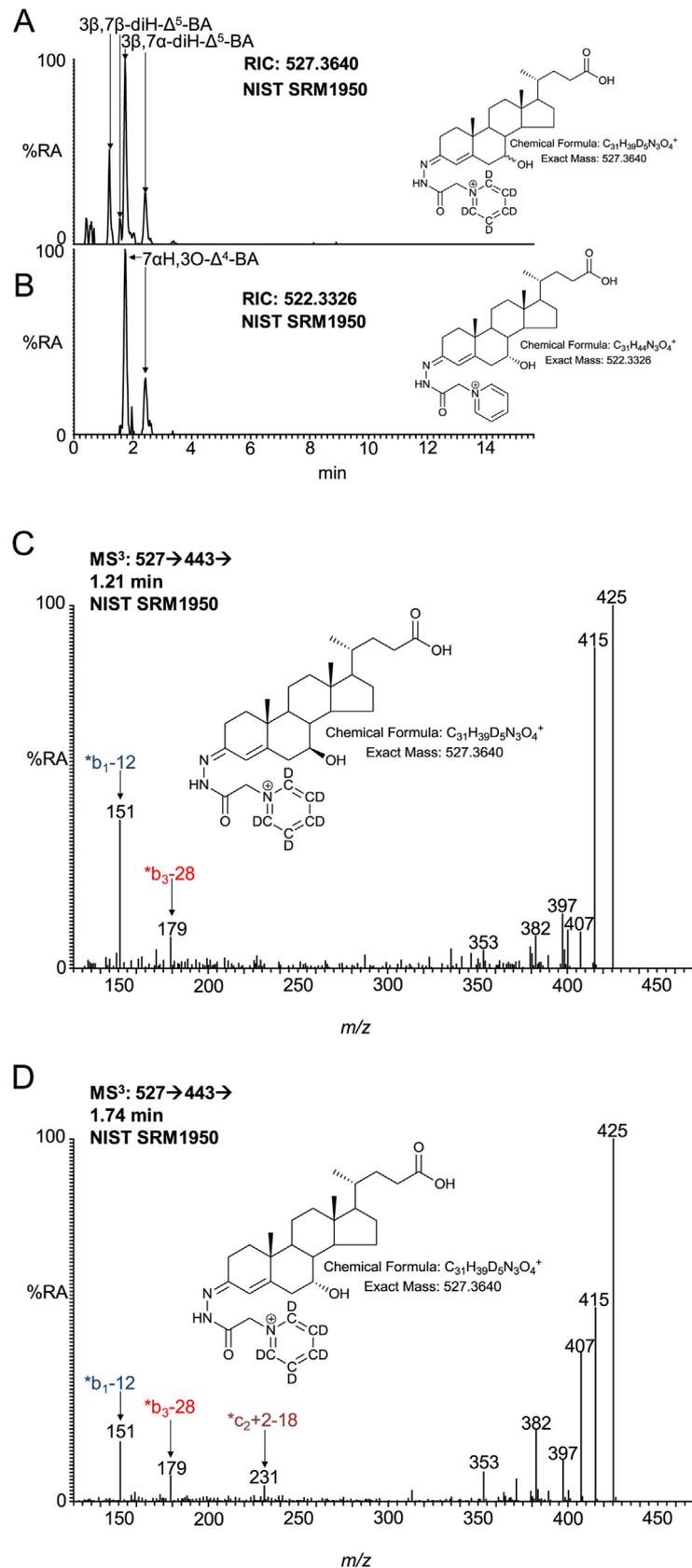


Fig. 9. Separation and fragmentation of diastereoisomers of 3,7-dihydroxycholesterol-5-en-24-oiic and 7-hydroxy-3-oxochol-4-en-24-oiic acids. (A) RIC from NIST SRM1950 plasma showing the separation of 3 β ,7 β -diH- Δ^5 -BA and 3 β ,7 α -diH- Δ^5 -BA. (B) The 3 β ,7 β -diH- Δ^5 -BA is not a substrate for HSD3B7 so does not give 7 β H,3O- Δ^4 -BA, while 3 β ,7 α -diH- Δ^5 -BA is and gives 7 α H,3O- Δ^4 -BA. MS³ ([M]⁺ → [M-Py]⁺ → ...) spectra of (C) 3 β ,7 β -diH- Δ^5 -BA and (D) 3 β ,7 α -diH- Δ^5 -BA. Aliquots of sample prepared in the presence of cholesterol oxidase were derivatised with [²H₅]GP and those prepared in the absence of cholesterol oxidase with [²H₀]GP. Analyte concentrations are given in supplementary Table S1.

shoulder is absent in a chromatogram of the authentic 3 β ,7 β -diHCA(25R) standard, but gives an identical MS³ spectrum. It is thus assigned to 3 β ,7 β -diHCA(25S).

3.1.4. 3,7-Dihydroxychol-5-en-24-oic and 7-Hydroxy-3-oxochol-4-en-24-oic acids

After side-chain shortening in the peroxisome C₂₇ 7-hydroxy acids will be converted to their C₂₄ equivalents, we thus screened for the presence of 3 β ,7 α -dihydroxychol-5-en-24-oic (3 β ,7 α -diH- Δ^5 -BA), 7 α -hydroxy-3-oxochol-4-en-24-oic (7 α H,3O- Δ^4 -BA) and 3 β ,7 β -dihydroxychol-5-en-24-oic (3 β ,7 β - Δ^5 -BA) acids in plasma. 7 β -Hydroxy-3-oxochol-4-en-24-oic acid should not be formed via HSD3B7 oxidation due to the presence of a 7 β - rather than a 7 α -hydroxy group (Furster et al., 1996). Shown in Fig. 9A and B is the appropriate RIC for 3 β ,7-diH- Δ^5 -BA and 7 α H,3O- Δ^4 -BA. Comparison with authentic standards reveals that the first pair of peaks in Fig. 9A correspond to 3 β ,7 β -diH- Δ^5 -BA (*syn* and *anti* GP conformers) while the latter correspond to 3 β ,7 α -diH- Δ^5 -BA. As expected only the latter pair of peaks corresponding to 7 α H,3O- Δ^4 -BA are observed in Fig. 9B recorded on the sample prepared in the absence of cholesterol oxidase. MS³ spectra of the 3 β ,7 β -diH- Δ^5 -BA and 3 β ,7 α -diH- Δ^5 -BA epimers are shown in Fig. 9C and D and as illustrated in Fig. 10 show distinctive features of the respective 7 β - and 7 α -epimers.

4. Discussion

It has previously been shown that 7 β -HC can be formed not only *ex vivo* through autoxidation during sample handling (Schroepfer, 2000), but also *in vivo* enzymatically from 7-OC by HSD11B1 (Hult et al., 2004; Larsson et al., 2007; Mitic et al., 2013) and perhaps also by free radical reactions from cholesterol (Murphy and Johnson, 2008) (Fig. 1). In fact, 7 β -HC is elevated in plasma from people with SLOS who also show elevated 7-OC (Griffiths et al., 2016) and also in patients suffering from NPC disease who similarly show elevated 7-OC in plasma (data not shown). Interestingly, Alvelius et al. showed that bile acids with a 3 β ,7 β -diH- Δ^5 -BA core structure were formed by a patient with NPC disease (Alvelius et al., 2001), while Evans and Natowicz also found bile acids probably with the same structure formed by a patient with SLOS (Natowicz and Evans, 1994). 7 β -HC may represent an early metabolite in the biosynthesis of these acids. Using our optimised EADSA approach we also find comparatively (to the 7 α -epimers) low levels of 7 β -HC, 3 β ,7 β -diHCA and 3 β ,7 β -diH- Δ^5 -BA in plasma from healthy individuals, see Supplementary Table S1 (Abdel-Khalik et al., 2017).

In healthy individuals, we identify four diastereoisomers for 3 β ,7-diHCA. Besides the 25R epimers of 3 β ,7 α -diHCA and 3 β ,7 β -diHCA we also identify the corresponding 25S epimers. The interconversion of 25R and 25S epimers as CoA thioesters is catalysed by AMACR, the next enzyme in the bile acid biosynthesis pathway ACOX2 requiring 25S stereochemistry of the substrate (Russell, 2003). It has recently been reported that a defect in ACOX2 leads to liver fibrosis, mild ataxia, and cognitive impairment (Vilarinho et al., 2016). A blockage in the bile acid biosynthesis pathway at this point would be expected to lead to elevated levels of both epimers of 3 β ,7 α -diHCA and 3 β ,7 β -diHCA in plasma which could easily be determined by the EADSA method.

24S-HC is the dominant oxysterol in brain. It is formed by CYP46A1 mediated oxidation of cholesterol (Russell, 2003). The origin of the 24R-epimer is less clear. Interestingly, 24R-HC is present in brain of the CYP46A1 knockout mouse indicating a source other than a reaction catalysed by CYP46A1 (Meljon et al., 2014).

5. Conclusions

We describe here how the EADSA method has been optimised to reveal diastereoisomers of oxysterols, cholestenic and choleonic acids. Our observations highlight the extreme complexity of the plasma sterolome.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chemphyslip.2017.04.004>.

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