LC-MS of Sterols

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Definition

According to the Lipid Maps classification system sterols are a class of compounds based on the cyclopenatanoperhydrophenanthrene skeleton and include cholesterol and its cyclic precursors, steroid hormones, bile acids and the ring opened *seco*sterol vitamin D_3 (1).

Introduction

As for other components of the "lipidome" the "sterolome" is extremely complex with a potentially huge number of distinct chemical entities. The complexity is magnified by variability of stereochemistry resulting in diastereoisomers with differing biological properties. This chapter will concentrate on sterol molecules found in the mammalian system but similar LC-MS methods will be appropriate for sterols derived from plants and microbes.

In mammals cholesterol is the dominant sterol, it is biosynthesised by cells from acetyl-CoA and taken from the diet via absorption. Its major function is structural but it can also act as a signalling molecule regulating its own biosynthesis and uptake (2) and through binding to the G protein coupled receptor (GPCR) Smoothened regulating the Hedgehog signalling pathway important in embryonic development (3). Cholesterol is predominantly metabolised to C_{24} bile acids but also C_{21}-C_{18} steroids (Figure 1) (4). Cholesterol and bile acids are mostly exerted in the faeces while C_{21}-C_{18} steroids are excreted in urine. This chapter will concentrate on LC-MS of cholesterol-like sterols, oxysterols their oxidised metabolites and C_{27} and C_{24} acids. There will be minimal discussion of LC-MS analysis of C_{21}-C_{18} steroids, and the interested reader is directed to dedicated review chapters found elsewhere (5).

Figure 1 . Abbreviated pathways of cholesterol biosynthesis and metabolism.

Extraction prior to LC-MS

There are many different extraction methods for sterols each tailored to a specific sterol-type and biological matrix. In terms of a global sterolomic analysis, extraction from tissue (or fluids) into absolute ethanol with homogenisation and/or ultra-sonication is recommended (6). Cholesterol and the most non-polar sterols can then be removed/isolated by extraction on a reversed-phase C_{18} solid phase extraction (SPE) column following dilution to and loading in 70% ethanol (6) or alternatively on a straight-phase SPE column after solvent exchange into e.g. toluene or hexane-dichloromethane (2:8; v/v). Cholesterol will elute from a C_{18} column in absolute ethanol (6). Using straight-phase silica SPE sterols loaded in toluene can be fractionated into sterylester-rich, cholesterol-rich and oxysterol-rich fractions by elution with hexane, 0.1% propan-2-ol in hexane and 30% propan-2-ol in hexane, respectively (7, 8), or alternatively on a silicic acid column after loading in hexane-dichloromethane (2:8; v/v) with elution in hexane-dichloromethane (2:8; v/v) followed by ethyl acetate (9). Ethanol is preferred for general sterol extraction as it is a good solvent for sterols, steroids and bile acids. The efficiency of extraction can be studied, however, determination of recoveries of added reference

compounds, radioactive or not, is unsatisfactory as added compounds are not distributed in the sample as endogenous compounds. The only satisfactory method with minimal bias is repeated extractions of the pellet after ethanol extraction.

Saponification

Sterols are present in biological systems both as the free molecules and as conjugates with fatty acids, sulphuric acid, sugars, including glucuronic acid, and the amino acids glycine and taurine. While sterols with a polarity-enhancing conjugating group will be extracted into ethanol, sterols esterified with fatty acids may be better extracted into a mixture of ethanol and propan-2-ol. Potassium hydroxide can then be added for saponification of esters. Following incubation and neutralisation with e.g. glacial acetic acid, sterols can be fractionated by SPE (6). The saponification procedure can lead to loss of labile groups e.g. dehydration of 7α-hydroxy-4-en-3-ones (10) and also introduces the risk of autoxidation (11). It is recommended that the products of saponification are analysed independently from a non-saponified sterol sample.

Global LC-MS Methods for Sterol Analysis

An important consideration for the LC-MS analysis of cholesterol and more hydrophobic sterols is solubility. If not properly solubilised cholesterol will not be efficiently retained on a reversed-phase sorbent, the consequence of which is that it will elute earlier than expected and contaminate proceeding fractions. This is problematic as cholesterol is usually very much more abundant than its precursors or metabolites. Solvents recommended for cholesterol and similar sterols are methanol, ethanol, propan-2-ol and combinations, thereof. Cholesterol is soluble in 70% ethanol, so caution should be taken with the use of more polar solvents.

Depending on the study, it may be beneficial to analyse specific classes of sterols individually, or alternatively take a more global lipidomic approach. If the latter is the aim neutral sterols can be fractionated according to polarity and separated from acidic metabolites by mixed mode anion exchange chromatography, then the most non-polar neutral fraction separated by e.g. isocratic reversed-phase LC with e.g. a 75% methanol 25% propan-2-ol mobile phase. The more polar neutral fraction can then separated by reversed-phase LC using a gradient system (e.g. A: 60% ethanol, 0.1% formic acid, B: propan-2-ol, 0.1% formic acid) (12). Using the same column acidic sterols can be separated using a gradient (e.g. A: 10 mM ammonium acetate, pH 7.2, in 5% methanol, B: 10 mM ammonium acetate, pH 7.2, in 95% methanol).

Targeted LC-MS Methods for Sterol Analysis

Non-Polar Neutral Sterols

Non-polar neutral sterols present a challenge for LC-MS analysis in that mammalian samples tend to be dominated by cholesterol making observation of similarly non-polar minor sterols difficult. A further issue to consider is that non-polar sterols tend to ionise poorly by atmospheric pressure ionisation (API) methods, often suffer in-source dehydration and fragment upon collision induced dissociation (CID) into multiple low mass fragment ions. To overcome these issues, many groups now employ a derivatisation approach; however, this can be laborious and in some cases introduce unwanted side-products, but does offers benefits in terms of sensitivity.

LC-MS without Derivatisation

McDonald et al from Dallas have published a method for comprehensive sterol analysis including the non-polar sterols lanosterol, zymosterol, desmosterol, 7-dehydrocholesterol (7-DHC), cholesterol, vitamin D_3, the plant sterols brassicasterol, campesterol, stigmasterol, sitosterol and the fungal sterols ergosterol and vitamin D_2 (13). Their method is based on reversed-phase LC-MS/MS exploiting multiple reaction monitoring (MRM) on a triple quadrupole instrument. The sensitivity of their method allowed ng/mL detection of sterols in plasma. Sterols injected in 90% methanol were resolved on a C_{18} LC column using 96% methanol containing 0.1% acetic acid as mobile phase A and methanol containing 0.1% acetic acid as mobile phase B. The gradient started at 100% A and increased to 100% B. Sterols were ionised by atmospheric pressure chemical ionisation (APCI) in the positive-ion mode optimised for either $[M+H]^+$ or $[M+H-H_2O]^+$ ions. Others have optimised LC-MS/MS methods for specific sterols, e.g. 7-DHC and cholesterol for the specific identification of the autosomal recessive disorder Smith-Lemli-Opitz syndrome (SLOS) (14). Becker et al injected sample in methanol:propan-2-ol (1:1, v/v) and separated the sterols on a C_{18} LC column using a gradient going from 75% methanol to 100% propan-2-ol and used positive-ion atmospheric pressure photoionisation (APPI) to generate $[M+H-H_2O]^+$ ions ready for MRM analysis on a triple quadruple mass spectrometer (14).

LC-MS with Derivatisation

There are numerous derivatisation methods which have been developed to enhance the analytical properties of non-polar sterols and oxysterols. Liu et al have cleverly exploited the 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) derivative to analyse 7-DHC and its metabolites in the context of diagnosis of SLOS (Figure 2) (15). PTAD has been widely used in the past for derivatisation of vitamins D compounds as it will react via a Diels-Alder cycloaddition with the diene of the opened B-ring in these compounds (16). Similarly, PTAD will react with the 5,7-diene structure in 7-DHC. The multiple heteroatoms in the derivative facilitates ionisation while the derivative stabilises the sterol, removing the reactive 5,7-diene structure. By judicious choice of reaction conditions derivatisation can alternatively be tuned towards an "ene" reaction with the Δ^5 double bond in cholesterol or to the Δ^{24} double bond in desmosterol. The Diels-Alder reaction is favoured by reaction with PTAD in methanol at room temperature for 30 min but under these conditions desmosterol will undergo the "ene" reaction at the Δ^{24} double bond to give the PTAD adduct and its methanolysis product. The presence of chloroform or dichloromethane, from Folch-like extractions, will also facilitate the "ene" reaction at Δ^5 in cholesterol at room temperature. Liu et al found that PTAD derivatisation enhanced the LC-MS/MS sensitivity for 7-DHC analysis by a factor of 1,000 (15). They used a reversed-phase C_{18} column and an isocratic solvent system of methanol : acetic acid (100:0.1, v/v), APCI and MRM exploiting the transition from the $[M+H]^+$ ion of the derivatised sterol to the corresponding fragment having lost PTAD and water. A significant attraction of this method is that the derivatisation mix of PTAD in methanol (1 mg/mL) can be injected onto the LC directly following the 30 min reaction.

An alternative derivativisation strategy is to target the 3-hydroxy group of sterols and form esters with picolinic acid (Figure 2) (17). The nitrogen of the pyridine ring is readily sodiated facilitating ionisation of the derivative. The method pioneered by Honda et al is applicable to cholesterol, its sterol precursors and oxysterol metabolites (see below) (17, 18). The derivatisation reaction is performed by adding a reaction mixture of 2-methyl-6-nitrobenzoic anhydride, 4-(*N,N*-

dimethylamino)pyridine, picolinic acid and tetrahydrofuran and triethylamine to dried sample. The reaction is complete after 30 min at room temperature (17). After solvent exchange to acetonitrile the supernatant is suitable for injection onto an LC-MS/MS system. Honda et al used a reversed-phase column, an acetonitrile, methanol, water gradient going from 40:40:20 (v/v/v) to 45:45:10 (v/v/v) with 0.1% acetic acid as a constant additive, electrospray ionisation (ESI) and MRM on a triple quadrupole instrument (17). The MRM was rather non-specific being the transition $[M+Na+CH_3CN]^+ \rightarrow [M+Na]^+$. Limits of detection were 1 pg on-column allowing analysis of sterols from 1 µL of serum (17).

Figure 2. Derivatisation of sterols to enhance LC-MS/MS analysis. (A) PTAD derivatisation of 7-DHC and desmosterol. (B) Derivatisation of cholesterol to its picolinyl ester. Abbreviations: DMAP, 4-(*N,N*-dimethylamino)pyridine; THF, tetrahydrofuran; TEA, triethylamine.

Polar Neutral Sterols – Oxysterols

As for non-polar sterols, oxysterols have been analysed by LC-MS with and without derivatisation.

LC-MS without Derivatisation

McDonald et al have developed a LC-MS method applicable to a wide range of oxysterols extending from 4β-hydroxycholesterol at one end of the hydrophobicity range to 7α,25-dihydroxycholesterol and 7α(25R)26-dihydroxycholesterol at the other (13). Note, we use here systematic nomenclature to describe side-chain hydroxylated sterols where according to IUPAC rules hydroxylation at the terminal carbon of cholesterol is at C-26 (19). The resulting stereochemistry is assumed to be 25R (Figure 1). Oxysterols injected in 90% methanol were separated on a C_{18} LC column using a mobile phase of A: 70% acetonitrile with 5 mM NH_4OAc, B: 1:1 acetonitrile, propan2-ol (v/v) with 5 mM NH_4OAc and a gradient going from 0% B to 100% B. Upon ESI the most prominent ions were $[M+NH_4]^+$ or $[M+H]^+$ and MRM transitions to fragment ions having lost one or two molecules of water were exploited (13). The sensitivity of the method allowed the detection of oxysterols in plasma at a level of 1 ng/mL with an instrument detection limit of < 50 pg on column. One limitation of the chromatography system used was that 7α- and 7β-hydroxycholesterol were not resolved. The reproducibility of McDonald et al's method was good with relative standard errors mostly below 5%. Quantification was made with the use of isotope labelled standards. Stiles et al, also in Dallas, have used this methodology in a study of 3,230 serum samples measuring > 60 sterols including oxysterols and vitamins D derivatives (20). Oxysterols analysed included 4β-, 7α-, 22R-, 24S-, 25- and (25R)26-hydroxycholesterol, 7-oxocholesterol and 7α,26-dihydroxycholesterol (20). Other LC-MS methods developed for oxysterol analysis come from Rentsch and colleagues in Zurich and Bandaru and Haughey in Baltimore and who both targeted on 24S- and (25R)26-hydroxycholesterol (21-23), DeBarber et al in Portland who separated closely eluting 24S-, 25- and (25R)26-hydroxycholesterol (24) and Björkhem's group in Stockholm who focused on 7α-hydroxycholest-4-en-3-one (25).

LC-MS with Derivatisation

Honda and colleagues exploited their picolinic acid derivavatisation method discussed above for a similar analysis of oxysterols, however, the derivatisation reagent mix was modified slightly to include pyridine rather than tetrahydrofuran and triethylamine was excluded (Figure 3) (18). The reaction mixture was incubated at 80 ^oC for 60 min and derivatised oxysterols extracted into hexane.

The supernatant was blown-down and reconstituted in acetonitrile for LC-MS/MS analysis. Separation of oxysterols was achieved on a reversed-phase column using an acetonitrile : methanol : water mobile phase containing 0.1% acetic acid with a gradient going from 40:40:20 (v/v/v) to 45:45:10 (v/v/v). Ionisation was by ESI and detection using MRM with a triple quadruple instrument. The oxysterols 4β-, 7α-, 22R-, 24S-, 25- and (25R)26-hydroxycholesterol were analysed, each of which gave the dipicoliyl esters while 24S,25-epoxycholesterol was found to give the monopicolinyl ester. All picolinyl esters gave $[M+Na]^+$ ions which fragmented to give abundant $[M+Na\text{-}C_6H_5NO_2]^+$ or $[C_6H_5NO_2+Na]^+$ product-ions suitable for MRM (18). $C_6H_5NO_2$ corresponds to the molecular formula of picolinic acid. Honda et al determined on-column detection limits of 2 – 10 fg and the method was suitable for analysis of oxysterols from only 5 µL of plasma following alkaline hydrolysis. One potential drawback of the derivatisation protocol is the elevated temperature used could encourage reaction with molecular oxygen and lead to autoxidation artefacts (11).

A similar derivativatisation reagent to picolinic acid its isomer nicotinic acid. Sidhu et al have used derivatisation to nicotinyl esters to enhance the analysis of oxysterols found in cerebrospinal fluid (CSF) and plasma (26). Oxysterols were extracted and dried and treated with a reagent mix of *N,N*-diisopropylcarbodiimide, nicotinic acid, 4-(*N,N*-dimethylamino)pyridine in chloroform at 50 ^oC for 1 hr. Following removal of chloroform and reconstitution in methanol the oxysterols were ready for analysis. Derivatised oxysterols were separated on a reversed-phase C_{18} column using mobile phase of A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile:methanol 1:4 (v/v) and a gradient going from 95% to 100% B. The oxysterols 4β-, 7α-, 7β-, 24S-, 25- and (25R)26-hydroxycholesterol were found to give dinicotinyl esters, which with the exception of the 7α- and 7β-epimers separated on the LC column. The dinicotinyl esters give $[M+H]^+$ and $[M+2H]^{2+}$ upon ESI and both generate $[M+H\text{-}C_6H_5NO_2]^+$ and $[C_6H_5NO_2+H]^+$ as major product-ions suitable for MRM analysis. The lower limit of quantification for the method was 1 ng/mL from 50 µL of plasma.

Figure 3. Derivatisation of oxysterols to enhance LC-MS/MS analysis. (A) Derivatisation to picolinyl esters. (B) Derivatisation to nicotinyl ester. (C) Derivatisation to dimethylglycine esters. (D) Derivatisation to dimethylaminobutyrate esters. Abbreviations: DMAP, 4-(*N,N*-dimethylamino)pyridine; Py, pyridine; DIPC, *N,N*-diisopropylcarbodiimide; DMG, dimethylglycine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DMABI, dimethylaminobutyric acid imidazolide.

Another derivatisation method targeting hydroxy groups and using carbodiimide catalysis is that developed by Jiang et al for the oxysterols 7α-, 7β-, 24S-, 25- and (25R)26-hydroxycholesterol (27), in this case derivatisation is to *N,N*-dimethylglycine esters. A reagent mixture of dimethylglycine, 4-(*N*,*N*-dimethylamino)pyridine and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in chloroform is added to dried sample and heated at 50 ^oC overnight. The reaction products are then extracted into diethylether against 0.1N ammonia solution. *Bis*dimethylglycine esters are formed with hydroxycholesterols while 7-oxocholesterol gives the monodimethylglycine ester (Figure 3). Subsequently Jiang et al developed the method further by incorporating LC separation (28). They exploited the *N,N*-dimethylglycine ester derivatisation for the diagnosis of the rare neurodegenerative lysosomal storage disease Niemann-Pick type C1 (NPC1) which shows elevated levels of 7-oxocholesterol and cholestane-3β,5α,6β-triol in plasma. The derivatisation reaction was performed as described above but the temperature was 45 ^oC and the reaction time reduced to 1 hr. The reaction was quenched with methanol, dried under nitrogen and the derivatives reconstituted in

80% methanol for injection. LC separation was on a reversed-phase column using a mobile phase of A: 0.015% trichloroacetic acid, 0.5% acetic acid in water, B: 0.015% trichloroacetic acid, 0.5% acetic acid in acetonitrile. The gradient was from 35% B to 100% B. APCI was used in combination with MRM. Cholestane-3β,5α,6β-triol was found to give a more abundant $[M+H]^+$ than $[M+2H]^{2+}$ ion for the double derivative while 7-oxocholesterol gave the $[M+H]^+$ for the monoderivative. $[M+H]^+$ ions upon CID gave a prominent fragment corresponding to the loss of dimethylglycine and at *m/z* 104 corresponding to protonated dimethylglycine, both appropriate for MRM transitions. This derivatisation is being used by others for the identification of NPC1 (29-31). Interestingly, Klinke et al found that Niemann-Pick type A and B patients also showed elevated levels of cholestane-3β,5α,6β-triol in plasma (30). Pataj et al found that the dimethylglycine derivatisation method was also applicable to the diagnosis of cerebrotendinous exanthomatosis (CTX) an inborn error of cholesterol metabolism where (25R)26-hydroxycholesterol is essentially absent from plasma and tissues (29). Rather than using APCI on a triple quadrupole instrument Pataj et al used ESI on an Orbitrap high resolution instrument (29). Johnson and colleagues in Australia have used derivatisation to dimethylaminobutyric esters to enhance the analysis of cholestane-3β,5α,6β-triol and 7-oxocholesterol in the diagnosis of NPC (32). Following extraction of oxysterols from 50 µL of plasma into ethylacetate and lyophilisation, dimethylaminobutyric acid imidazolide (prepared from dimethylaminobutyric acid and carbonyldiimidazole hydrochloride in methylenechloride and stable for up to one week in a desiccator) was added and the mixture allowed to incubate at 65 ^{o}C for 15 min (Figure 3). The mixture was lyophilised ready for LC-MS/MS analysis on a reversed-phase C_{18} column using a mobile phase of A: 5 mM ammonium formate, pH 3, B: acetonitrile, and a gradient of 40% - 100% B. The sample was injected in a 60/40 (v/v) solution of A:B. Mono derivatives were formed with cholestane-3β,5α,6β-triol and 7-oxocholesterol which both gave $[M+H]^+$ ions upon ESI and fragmented upon CID to give a major product-ion at *m/z* 132 corresponding to protonated dimethylaminobutyric acid. The assay was linear over a concentration range of 1 – 200 ng/mL and showed both intra-day and inter-day coefficients of variation (CV) of below 15%. For control plasma median concentrations of cholestane-3β,5α,6β-triol and 7-oxocholesterol were determined to be 6.4 ng/mL and 26.6 ng/mL and in NPC patients 55.5 ng/mL and 120 ng/mL, respectively. All samples from NPC patients showed abnormal concentration of cholestane-3β,5α,6β-triol and it was concluded that this was the "gold standard" disease biomarker. A danger of basing a diagnosis on cholestane-3β,5α,6β-triol and 7-oxocholesterol concentrations is that the latter is formed from cholesterol, the major sterol in plasma, by reaction with air, as is the former following hydrolysis of the cholesterol autoxidation product 5,6-epoxycholesterol (11).

An alternative LC-MS/MS method targeting oxosterols is derivatisation with the Girard reagents (Figure 4). Shackleton et al pioneered the use of this derivatisation method in the LC-MS/MS analysis of testosterone esters in a doping control environment (33). DeBarber et al further exploited the method in the diagnosis of the autosomal recessive disorder CTX, where CYP27A1, the first enzyme of the acidic pathway of bile acid biosynthesis and a sterol (25R)26-hydroxylase required for side-chain shortening is deficient (34). In the absence of functional CYP27A1, sterols with a 7α-hydroxy-4-en-3-one structure are known to accumulate. These are substrates for reactions with the Girard reagents. In DeBarber et al's method 10 µL of plasma was deivatised with Girard P (GP) reagent in methanol containing 1% acetic acid. The reaction was complete after 2 hr at room temperature. After centrifugation, the supernatant was injected onto a C_{18} trap column, washed with 33% methanol, 17% acetonitrile, 50% water to remove unreacted reagent and back-flushed onto a C_{18}

analytical column. Mobile phase A was 33% methanol, 17% acetonitrile and 50% water, B was 63% methanol, 32% acetonitrile, 5% water both containing 0.1% formic acid and the gradient was from 25% to 100% B. DeBarber et al used an LTQ-Orbitrap instrument which allowed the acquisition of high resolution mass spectra (34). DeBarber at all used ESI where the derivatised molecules are observed as $[M]^+$ ions. Quantification exploited reconstructed-ion chromatograms (RICs, ± 5 ppm) with isotope dilution MS. DeBarber et al found very high concentrations of both 7α-hydroxycholest-4-en-3-one (>3,000 ng/mL) and 7α,12α-dihydroxycholest-4-en-3-one (>2,000 ng/mL) in plasma from CTX patients (34). Importantly, 7α-hydroxycholest-4-en-3-one and 7-oxocholesterol (3β-hydroxycholest-5-en-7-one) are isomers and the analyst must make sure these are adequately resolved to avoid confusion in disease diagnosis.

Figure 4. Derivatisation with Girard reagents to enhance LC-MS/MS analysis of sterols. (A) Derivatisation of oxosterols with GP reagent and the major fragment-ion formed upon CID. (B) Derivatisation of oxosterols with GT reagent and the major fragment-ion formed upon CID. (C) Oxidation of sterols with a 3α-hydroxy-5β-hydrogen structure to 3-oxo equivalents suitable for derivatisation with GP reagent. (D) Differentiation of oxysterols oxidised to contain a 3-oxo group from those naturally containing an oxo group. Abbreviations: GP, Girard P reagent; GT, Girard T reagent; HOAc, acetic acid; HSD, hydroxysteroid dehydrogenase.

The authors of this chapter have also extensively exploited the GP derivatisation methodology for the diagnosis of the rare disease SLOS, CTX, NPC and spastic paraplegia type 5 (35-39). We have also applied the technology to the discovery of potential biomarkers for amyotrophic lateral sclerosis and multiple sclerosis (40, 41). We developed the Girard derivatisation method further by incorporating an enzyme catalysed oxidation step so that sterols with a 3β-hydroxy-5-ene or 3β-hydroxy-5α-hydrogen structure could be converted to 3-oxo-4-enes or 3-oxo sterols, respectively (9, 42). The method was extended to allow sterols with a 3α-hydroxy-5β-hydrogen structure to be converted to 3-oxo equivalents. Cholesterol oxidase from Streptomyces sp was used to oxidise 3β-hydroxy groups, while 3α-hydroxysteroid dehydrogenase from Pseudomonas testosterone was used to oxidise 3α-hydroxy groups (Figure 4). To allow for the differentiation of sterols with a natural 3-oxo group from those oxidised to contain one we utilise isotope labelled versions of the GP reagent, where the $[^2H_5]$GP reagent is used to derivatise an aliquot of sample treated with enzyme while $[^2H_0]$GP is used to derivatise a separate aliquot of sample untreated by enzyme (43) (Figure 4). In brief, our methodology is as follows. Sterols are extracted from fluid or tissue into ethanol. After dilution to 70% ethanol, non-polar sterols, including cholesterol, desmosterol and 7-DHC are extracted on a C_{18} column, while more polar sterols including oxysterols, bile acids and C_{21-18} steroids flow through in 70% ethanol. This provides an "oxysterol fraction" devoid of cholesterol and eliminates the possibility of introducing oxysterol artefacts from cholesterol oxidation during subsequent sample preparation. In most studies we target non-esterified oxysterols, if we are interested in oxysterols esterified with fatty acids, we perform a saponification step during the initial ethanolic extraction, and after neutralisation and dilution, sterols can be fractionated as above. The "oxysterol fraction" from the C_{18} column is then divided into two sub-fractions A and B then lyophilised. The sub-fractions are reconstituted in propan-2-ol. To fraction A enzyme is added in buffer to oxidise 3-hydroxy to 3-oxo groups. In most studies we have used cholesterol oxidase in 50 mM phosphate buffer pH 7 targeting upon 3β-hydroxy containing sterols. After an incubation period of 1 hr at 37 ^{o}C the reaction is quenched by addition of methanol. Fraction B is treated in an identical manner but in the absence of cholesterol oxidase. Derivatisation is completed by the addition of

acetic acid catalyst and [$^2H_5$]GP to sub-fraction A and [$^2H_0$]GP to sub-fraction B and the mixture left overnight in the dark. The next day we use a re-cycling SPE protocol using Oasis HLB columns to remove excess GP reagent and derivatised oxysterols are eluted in methanol (44). Following dilution to 60% methanol oxysterol sub-fractions A and B can be combined and injected on to the LC column. We use a reversed-phase C_{18} column with mobile phase A: 33.3% methanol, 16.7% acetonitrile, 0.1% formic acid, B: 63.3% methanol, 31.7% acetonitrile, 0.1% formic acid and a gradient of 20% to 80% B. The cholesterol-rich fraction is treated in an identical manner. We perform analysis on an LTQ-Orbitrap, exploiting the high-resolution capability of the instrument to generate RIC's for the ions of interest. ESI gives intense $[M]^+$ ions suitable for quantification by isotope-dilution methods. $[M]^+$ ions from fraction B are 5 Da lighter than equivalents from fraction A (Figure 4), but elute with the same retention time. The method is suitable for quantification of oxysterols from plasma at sub-ng/mL levels and from CSF at concentrations of 30 pg/mL and above (40).

A major advantage of the Girard derivatives is that they give informative fragmentation patterns upon CID. On triple quadrupole and Q-TOF type instruments the major fragment-ions corresponds to the loss of the pyridine ring from GP or trimethylamine from Girard T (GT) derivatives providing suitable transitions for high-sensitivity MRM analysis (Figure 4) (42, 45). The resulting $[M\text{-}Py]^+$ and $[M\text{-}TMA]^+$ ions fragment further to give structurally informative MS/MS spectra(42, 45). This can be exploited on ion-trap instruments by isolating $[M\text{-}Py]^+$ or $[M\text{-}TMA]^+$ fragments in the ion-trap and fragmenting them further in a multistage fragmentation (MS^n) event (9). We have found that even an apparently minor change in structure e.g. epimerisation of the 7-hydroxy group, results in a characteristically different spectrum. The major objection to the methodology described above is its laborious nature.

Roberg-Larsen and Wilson and colleagues have enhanced the sensitivity for oxysterol analysis using the GT derivative and miniaturised columns with low-flow rate ESI (46, 47). Similar studies were performed earlier by Karu et al but with GP derivatisation (48). Roberg-Larsen et al exploited on-line C_{18} sample clean-up using an automatic filtration and back-flush set-up prior to the analytical column (46). Using a nano-LC set-up they were able to achieve a lower limit of quantification of about 50 fg on-column and were able to determine fg quantities of oxysterols from 10,000 cells (46). Following treatment with cholesterol oxidase oxysterols were derivatised in a similar manner as described above but with the GT reagent replacing the GP reagent. The derivatised sample solution was then injected onto the LC system in 0.1% formic acid. Cell debris and precipitated matter was trapped on an on-line filter while derivatised oxysterols were trapped on a C_{18} trap-column. Unreacted derivatisation reagent was flushed to waste. By valve-switching the on-line filter was back-flushed to waste using the loading solvent, while the trap column was switched in-series with an analytical C_{18} column (0.1 mm i.d.). Oxysterols were separated with an iscocratic mobile phase of methanol 95% containing 0.1% formic acid at a flow-rate of 0.5 µL/min. Roberg-Larsen et al used an Q-Exactive quadrupole – Orbitrap type instrument and exploited the $[M]^+ \rightarrow [M\text{-}TMA]^+$ transitions for quantification (46). A disadvantage of the nano-scale format, despite the improvement in sensitivity, is the long analysis times as a consequence of operating at sub-µL/min flow-rates. Roberg-Larsen et al subsequently scaled-up to capillary column LC (0.3 mm i.d.) with a flow-rate of 5 µL/min, this allowed both shorter run-times and faster system equilibration while maintaining high sensitivity and allowing increased injection volumes compared to their nano-LC system (47). Using this capillary LC system they were able to determine increased levels of (25R)26-hydroxycholestrerol in exosomes from an ER+ breast cancer cell line compared to an ER- cell line (47).

Bile Acids

There are well described methods for the LC-MS analysis of bile acids from urine and plasma/serum (5). Many exploit reversed-phase SPE followed by reversed-phase LC (49) but others exploit protein precipitation (50) or just dilute the sample in methanol prior to injection on the LC column (51). There are fewer methods described for the LC-MS analysis of bile acids from bile or faeces (52, 53).

Clayton and colleagues in London have developed an LC-MS/MS method suitable for bile acids extracted from urine by dilution in methanol and from blood on screening cards by elution with methanol (51). They separate bile acids on a reversed-phase C_{18} column using mobile phase of A: 0.01% formic acid, B: methanol, and a gradient going from 40% B to 99% B. Negative-ion ESI is used and quantification by MRM. Clayton et al screen for cholic, chenodeoxycholic, deoxycholic, lithocholic, hyocholic and hyodeoxycholic acids in their unconjugated forms and conjugated with glycine or taurine (51). They exploit the MRM transitions $[M\text{-}H]^- \rightarrow m/z$ 74 and $[M\text{-}H]^- \rightarrow m/z$ 80 for glycine and taurine conjugates while selected ion recording of $[M\text{-}H]^-$ ions is used to detect and quantify unconjugated bile acids. Clayton and colleagues have an interest in inborn errors of bile acid biosynthesis and have described unusual bile acids characteristic for different disorders, e.g. 3β-hydroxy-Δ^5-C_{27}-steroid (HSD3B7) deficiency, Δ^4-3-oxo-5β-reductase (AKR1D1) deficiency and NPC. The bile acids characteristic of 3β-hydroxy-Δ^5-C_{27}-steroid deficiency include 3β,7α-dihydroxychol-5-enoic and 3β,7α,12α-trihydroxychol-5-enoic acids conjugated with glycine and or sulphuric acid or with *N*-acetylglucosamine (GlcNAc). Δ^4-3-oxo-5β-reductase deficiency is characterised by 7α-hydroxy-3-oxochol-4-enoic and 7α,12α-dihydroxy-3-oxochol-4-enoic acids conjugated with glycine or taurine, while NPC is characterised by 3β,7β-dihydroxychol-5-enoic acid conjugated with glycine alone or with GlcNAc or as the double conjugates with glycine and GlcNAC, taurine and GlcNAc or sulphuric acid and GlcNAc (51). The transition $[M\text{-}H]^- \rightarrow m/z$ 97 is characteristic for sulphuric acid conjugates. The neutral-loss of 203 Da is characteristic for $[M\text{-}H]^-$ ions of GlcNAc conjugates and was used for the MRM transition $[M\text{-}H]^- \rightarrow [M\text{-}H\text{-}203]^-$ to identify and quantify GlcNAc conjugates. However, a common homozygous polymorphism in the *UGT3A1* gene leads to an absence in activity of UDP *N*-acetylglucosaminyl transferase and an inability to form GlcNAc conjugates, thus not all NPC patients show elevated levels of GlcNAc conjugates. Instead Clayton and colleagues identified a trihydroxy bile acid with a probable structure of 3β,5α,6β-trihydroxycholanoyl-glycine as a better diagnostic marker of NPC in plasma or blood (51).

There is a growing interest in faecal bile acid profiles. This is driven by the realisation of the importance of the gut microbiota which can metabolise bile acids secreted with bile into the intestine. Bioconversions catalysed by microbiota alter the signalling properties of bile acids, but bile acids can also alter the composition of the microbiota (54). Kakiyama et al have developed a simple LC-MS/MS method for faecal analysis of bile acids (53). Powdered lyophilised stool is suspended in cold acetate buffer pH 5.6 then refluxed with ethanol. Following centrifugation the supernatant is diluted with to 15% ethanol and bile acids extracted on a C_{18} SPE column. The column is washed with 25% ethanol and bile acids eluted in absolute ethanol. The eluate is concentrated and re-dissolve in 50% ethanol ready for LC-MS/MS analysis (53). Kakiyama et al separated the bile acids on a C_{18} LC column using a mixture of ethanol, methanol and 5 mM ammonium acetate as mobile phase, bile acids were ionised by negative-ion ESI and identified and quantified by MRM (53, 55). Thirty six C_{24} bile acids were analysed including unconjugated, glycine or taurine conjugated, as well as C-3 sulphated bile acids.

The bile acid concentration in bile is about 10 µg/µL, in which case dilution in 50% methanol with heating to 60 – 80 ^{o}C followed by centrifugation is sufficient sample preparation prior to LC-MS/MS analysis. The LC-MS/MS methods discussed above are then suitable for bile acid analysis.

C_{21-18} Steroids

It is beyond the scope of this chapter to discuss the LC-MS/MS of C_{21-18} steroids in detail. The interested reader is directed to comprehensive chapters in the book Steroid Analysis edited by Makin and Gower (5).

Quantification

The gold standard method for quantification by LC-MS and LC-MS/MS is stable isotope dilution MS where a known amount of an isotope labelled version of the analyte of interest is added as early as possible during the sample preparation procedure. As the target analyte and the isotope labelled standard will have (almost) identical physical and chemical properties any losses of analyte during sample preparation will be corrected for, elution times from the LC column will be (almost) the same and response factors will be equivalent. An isotope labelled version of the target analyte is not always available in which case a close structural analogue, not present in the sample to be analysed, may be used.

Future Directions

To maximise throughput there is a drive towards automation. The use of on-line multidimensional chromatography is an attractive option where sterols can be fractionated according to pKa and polarity on a mixed mode ion exchange column and then separated according to hydrophobicity on a reversed-phase analtical column prior to MS/MS analysis. In an effort to gain maximum sensitivity one option is derivatisation. This may become more popular when coupled with on-line sample clean up e.g. using the filtration back flush SPE method. Alternatively, sensitivity can be enhanced by exploiting nano-LC-MS/MS or capillary-LC-MS/MS with or without derivatisation. One note of caution, in omics-type experiments there is a growing tendency to use the term “identified” when a measured *m/z* is matched to an entry in a database. This may be supported by retention time information. The analyst should be reminded of the extreme diversity of sterol isomers with the exactly same *m/z* and possibly similar retention time. In the absence of a comparison to an authentic standard the term “annotated” may be more appropriate than “identified”.

Acknowledgements

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Conflict of Interest

WJG and YW have made the patent application number WO 2014037725 A1 “Kit and method for the quantification of steroids”.

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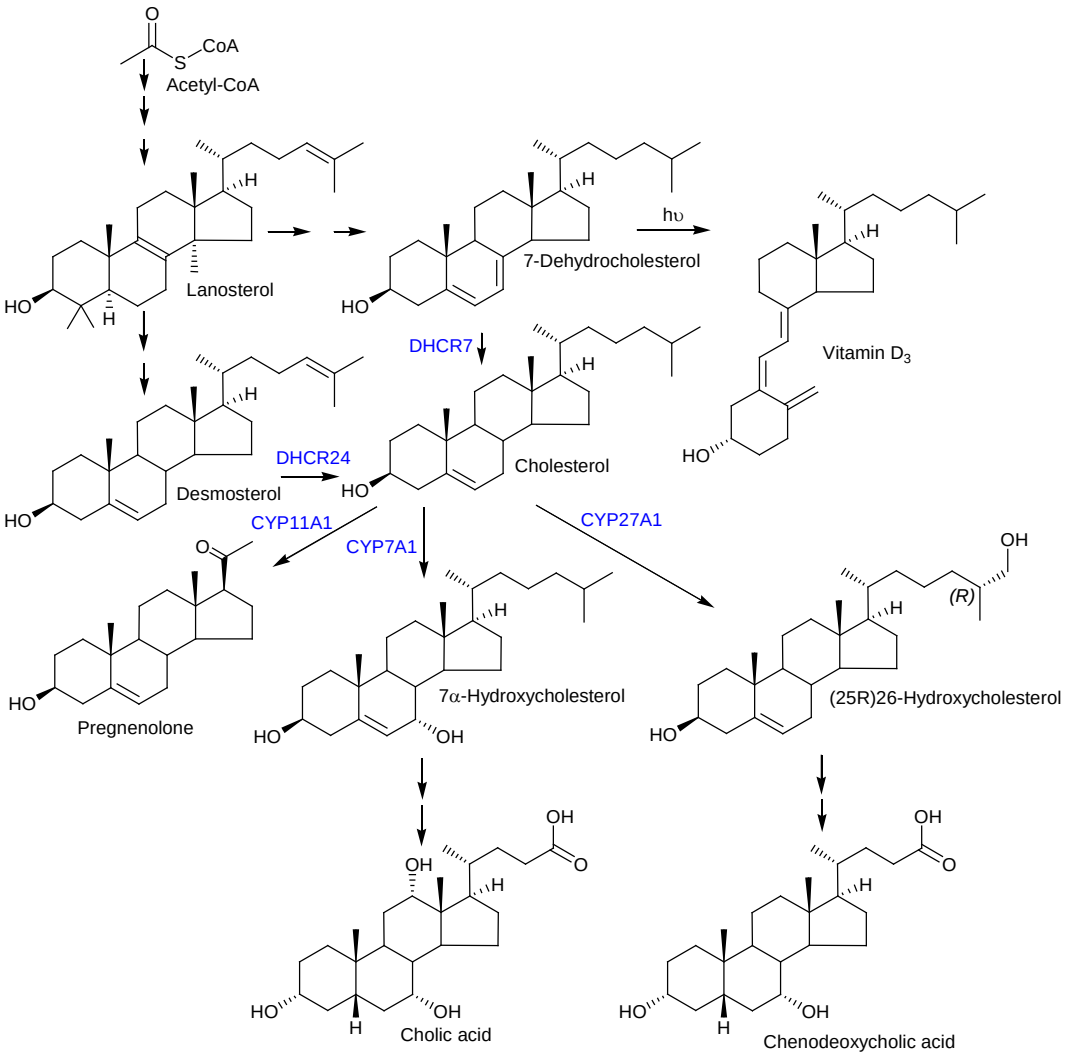
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CYP11A1
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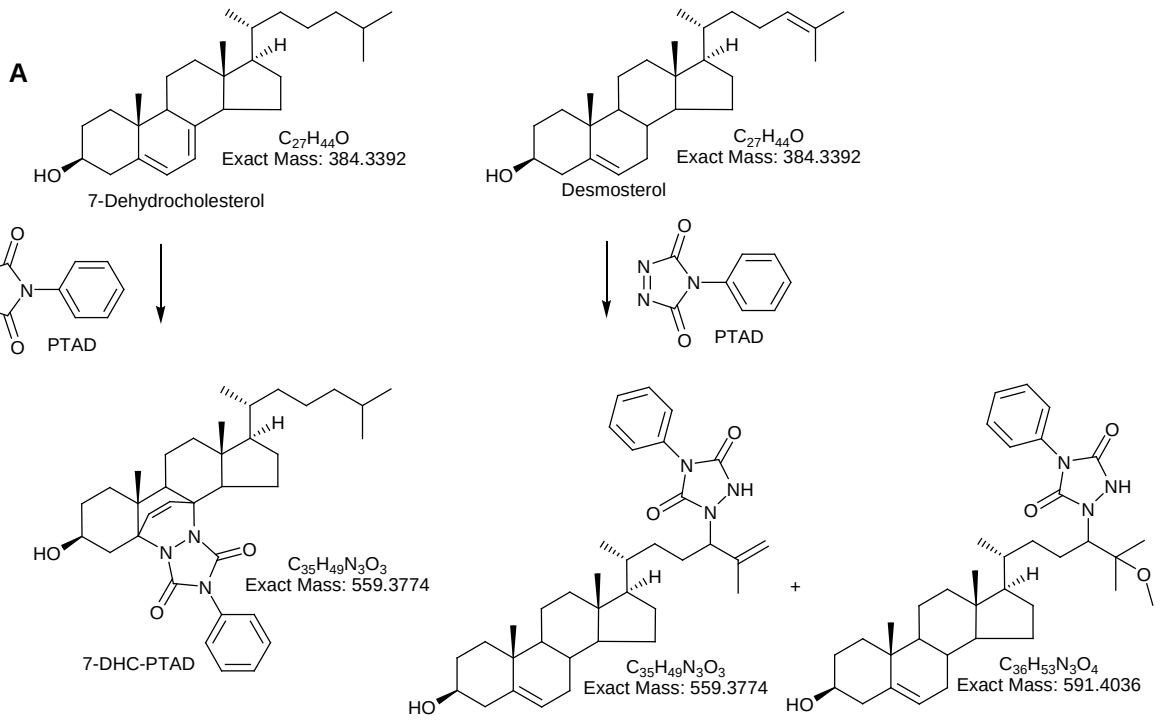
(R)
Cholic acid
Chenodeoxycholic acid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Desmosterol
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PTAD
PTAD
7-DHC-PTAD
$C_{35}H_{49}N_3O_3$
Exact Mass: 559.3774
$C_{35}H_{49}N_3O_3$
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+
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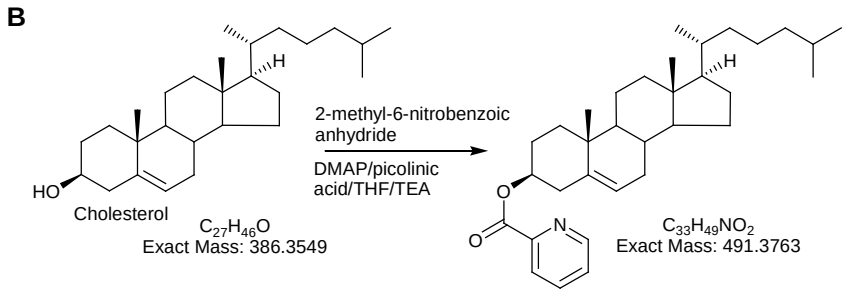
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DMAP/picolinic acid/THF/TEA
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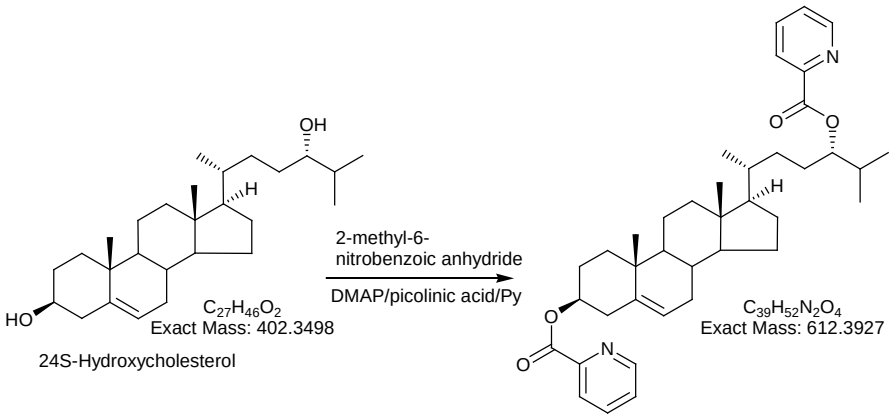
A



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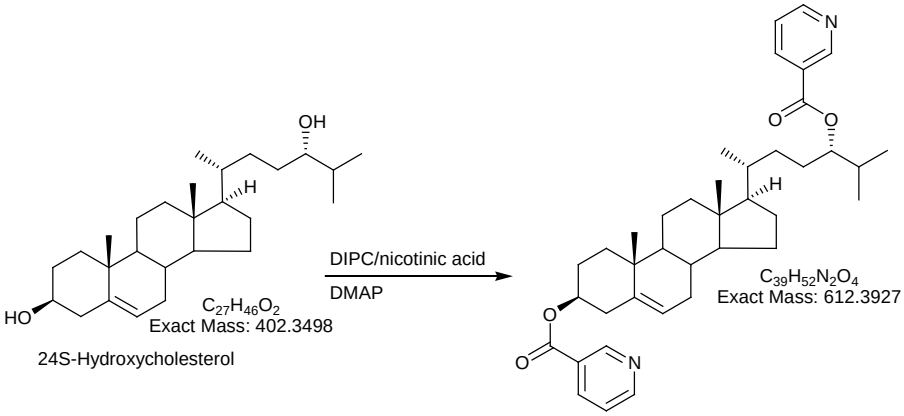
24S-Hydroxycholesterol

$C_{39}H_{52}N_2O_4$
Exact Mass: 612.3927

B



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Exact Mass: 402.3498
24S-Hydroxycholesterol

$C_{39}H_{52}N_2O_4$
Exact Mass: 612.3927

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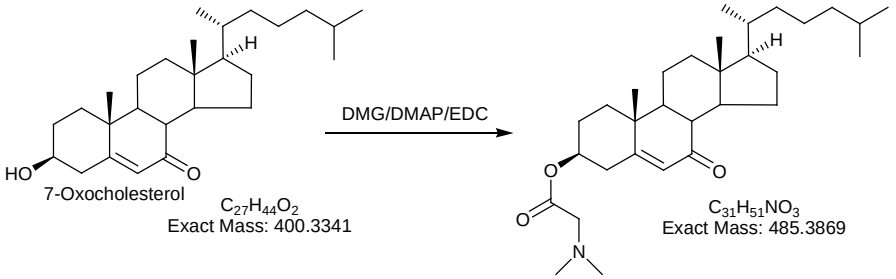

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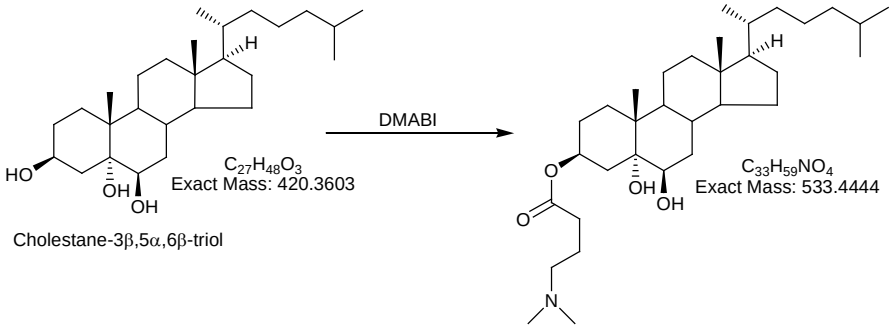

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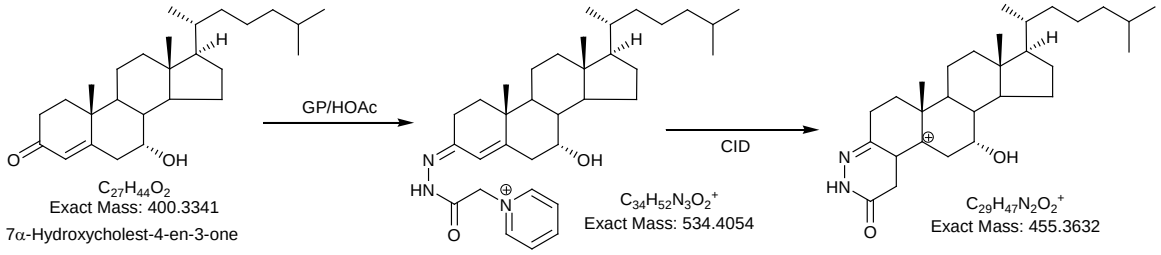
Cholestane-3β,5α,6β-triol

$C_{33}H_{59}NO_4$
Exact Mass: 533.4444

A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CID
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7α-Hydroxycholest-4-en-3-one
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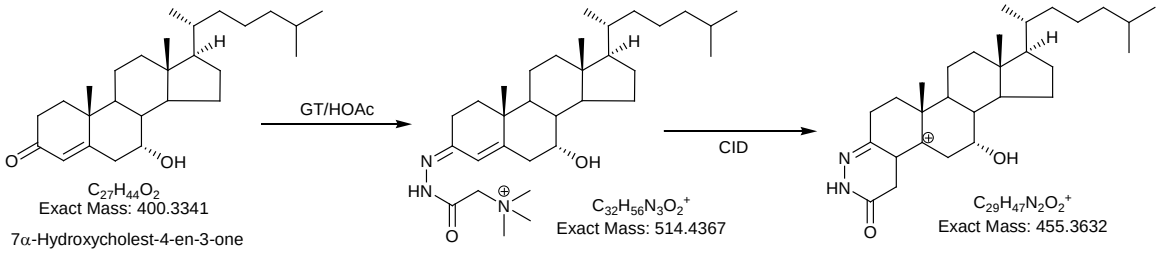
$C_{29}H_{47}N_2O_2^+$
Exact Mass: 455.3632

B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CID
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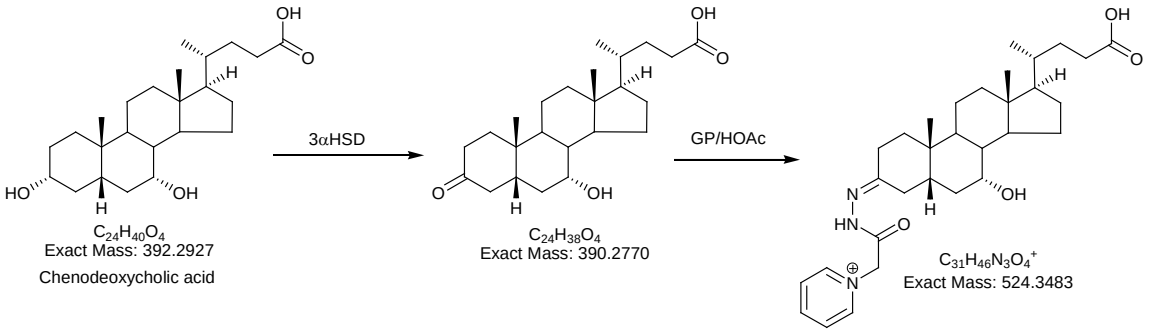
C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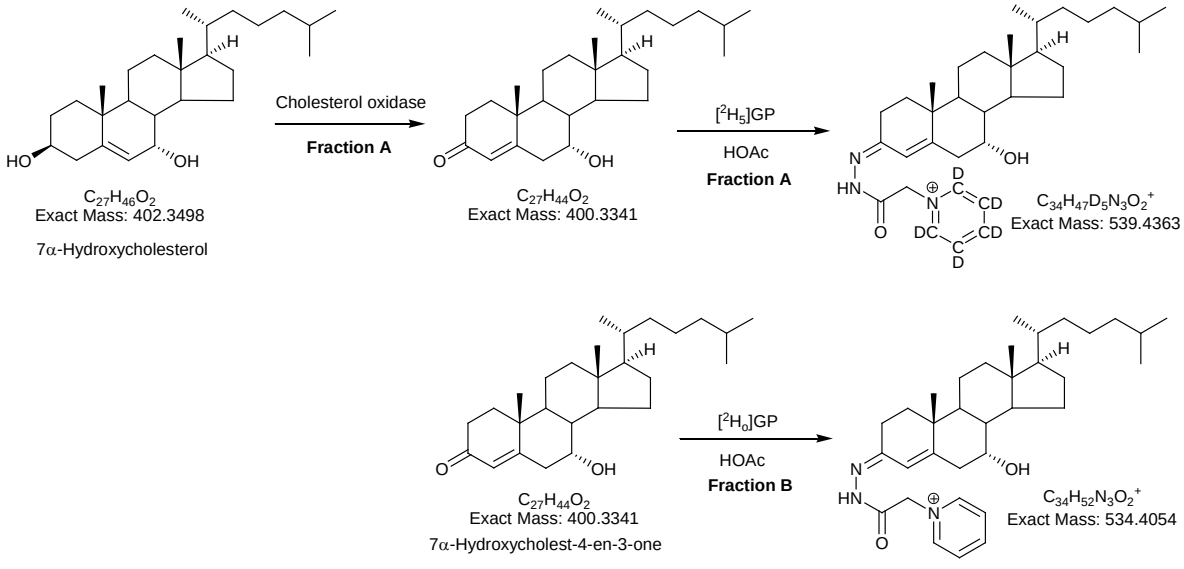
Exact Mass: 524.3483

D


Cholesterol oxidase
Fraction A
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HOAc
Fraction A
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7α-Hydroxycholesterol
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Exact Mass: 400.3341
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Exact Mass: 539.4363
[2H0]GP
HOAc
Fraction B
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Exact Mass: 400.3341
7α-Hydroxycholest-4-en-3-one
$C_{34}H_{52}N_3O_2^+$
Exact Mass: 534.4054