

Liquid chromatography-mass spectrometry utilizing multi-stage fragmentation for the identification of oxysterols[§]

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Abstract In humans, the brain accounts for about 20% of the body's free cholesterol, most of which is synthesized de novo in brain. To maintain cholesterol balance throughout life, cholesterol becomes metabolized to 24S-hydroxycholesterol, principally in neurons. In mouse, rat, and probably human, metabolism to 24S-hydroxycholesterol accounts for about 50% of cholesterol turnover; however, the route by which the remainder is turned over has yet to be elucidated. Here, we describe a novel liquid chromatography (LC) multi-stage fragmentation mass spectrometry (MSⁿ) methodology for the identification, with high sensitivity (low pg), of cholesterol metabolites in rat brain. The methodology includes derivatization to enhance ionization, exact mass analysis at high resolution to identify potential metabolites, and LC-MSⁿ (n=3) to allow their characterization. ■ 24S-hydroxycholesterol was confirmed as a major oxysterol in rat brain, and other oxysterols identified for the first time in brain included 24,25-, 24,27-, 25,27-, 6,24-, 7 α ,25-, and 7 α ,27-dihydroxycholesterols. In addition, 3 β -hydroxy-5-oxo-5,6-secocholestan-6-al and its aldol, two molecules linked to amyloidogenesis of proteins, were characterized in rat brain.—Karu, K., M. Hornshaw, G. Woffendin, K. Bodin, M. Hamberg, G. Alvelius, J. Sjövall, J. Turton, Y. Wang, and W. J. Griffiths. **Liquid chromatography-mass spectrometry utilizing multi-stage fragmentation for the identification of oxysterols.** *J. Lipid Res.* 2007. 48: 976–987.

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Cholesterol in brain is synthesized de novo throughout life (1, 2). The majority of experimental evidence suggests that it cannot cross the blood–brain barrier (BBB) (2), and to maintain the cholesterol balance in the brain, it is converted to 24S-hydroxycholesterol, a transport form of cho-

lesterol that can pass the BBB (3). In mouse, rat, and probably human, metabolism of cholesterol to 24S-hydroxycholesterol accounts for about 50% of cholesterol turnover (2, 4, 5); however, the mechanism by which the remaining cholesterol is turned over has yet to be elucidated. 24S-hydroxycholesterol is a member of a class of oxidized cholesterol metabolites collectively known as oxysterols. It not only acts as a transport form of cholesterol, but it is a biologically active molecule, activating the liver X receptors (LXRs) (6). Interestingly, cholesterol 24-hydroxylase (CYP46A1), the enzyme responsible for hydroxylation of cholesterol at C-24 (7), and LXR β show similar patterns of expression in brain (8). Other oxysterols have also been identified in brain (9), including cholest-4-en-3-one (10), and 3 β -hydroxy-5-oxo-5,6-secocholestan-6-al (11) (5,6-seco-sterol). Cholest-4-en-3-one has been shown to be formed in an oxidation reaction catalyzed by an amyloid β (A β) peptide–Cu²⁺ complex, whereas it is suggested that 5,6-seco-sterol is formed as a result of cholesterol ozonolysis, and is proposed to covalently modify A β and initiate amyloidogenesis. This suggestion is at present under debate (12).

Oxysterol analysis by mass spectrometry (MS) has traditionally been performed in combination with gas chromatography (GC) following derivatization (13), i.e., by GC-MS. However, the identification of unknown metabolites is hampered by the elimination of derivatizing groups in the ion source (14), making molecular weight determination difficult, if not impossible. As an alternative, atmospheric pressure ionization (API) methods are used.

Abbreviations: API, atmospheric pressure ionization; ES, electrospray; GP, Girard P; LC-MS, liquid chromatography-mass spectrometry; LIT, linear ion trap; MS/MS, tandem mass spectrometry; RA, relative abundance; RIC, reconstructed ion chromatogram.

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Oxysterol analysis by electrospray (ES) suffers from the poor ionization cross-section of oxysterols, and atmospheric pressure chemical ionization and atmospheric pressure photoionization both lead to dehydrated protonated molecules and an absence of molecular weight information (15–17). To improve the analysis of oxysterols by ES, we have introduced a derivatization method which involves *i*) conversion of the 3 β -hydroxy-5-ene (or 3 β -hydroxy) to a 3-oxo-4-ene (or a 3-oxo group) and *ii*) the reaction of the resultant oxo group with the Girard P (GP) hydrazine reagent to give a GP hydrazone (Fig. 1A). Because the GP hydrazone contains a quaternary nitrogen, the derivatized oxysterol gives a high ion yield in the ES ion source (18).

An important consideration for the MS analysis of oxysterols extracted from brain is their low abundance

among a matrix of other lipids, including phospholipids and cholesterol itself, which is present in 10³- to 10⁵-fold excess (2). To overcome this problem, oxysterols are enriched using straight-phase chromatography, derivatized specifically, and separated in their derivatized form by reversed-phase liquid chromatography (LC) prior to MS analysis. Only compounds that have an oxo group, or that have been oxidized to include one, will become derivatized with the GP reagent, which provides preferential ionization. Further, GP hydrazones give a characteristic neutral loss of 79 Da, i.e., loss of the pyridine group, which can be targeted in neutral-loss-type scans or utilized in multiple reaction monitoring (MRM) experiments, which add an extra dimension of specificity to oxysterol analysis (Fig. 1B). For the analysis of possible oxysterols in brain, advantage can be taken of their predicted elemental com-

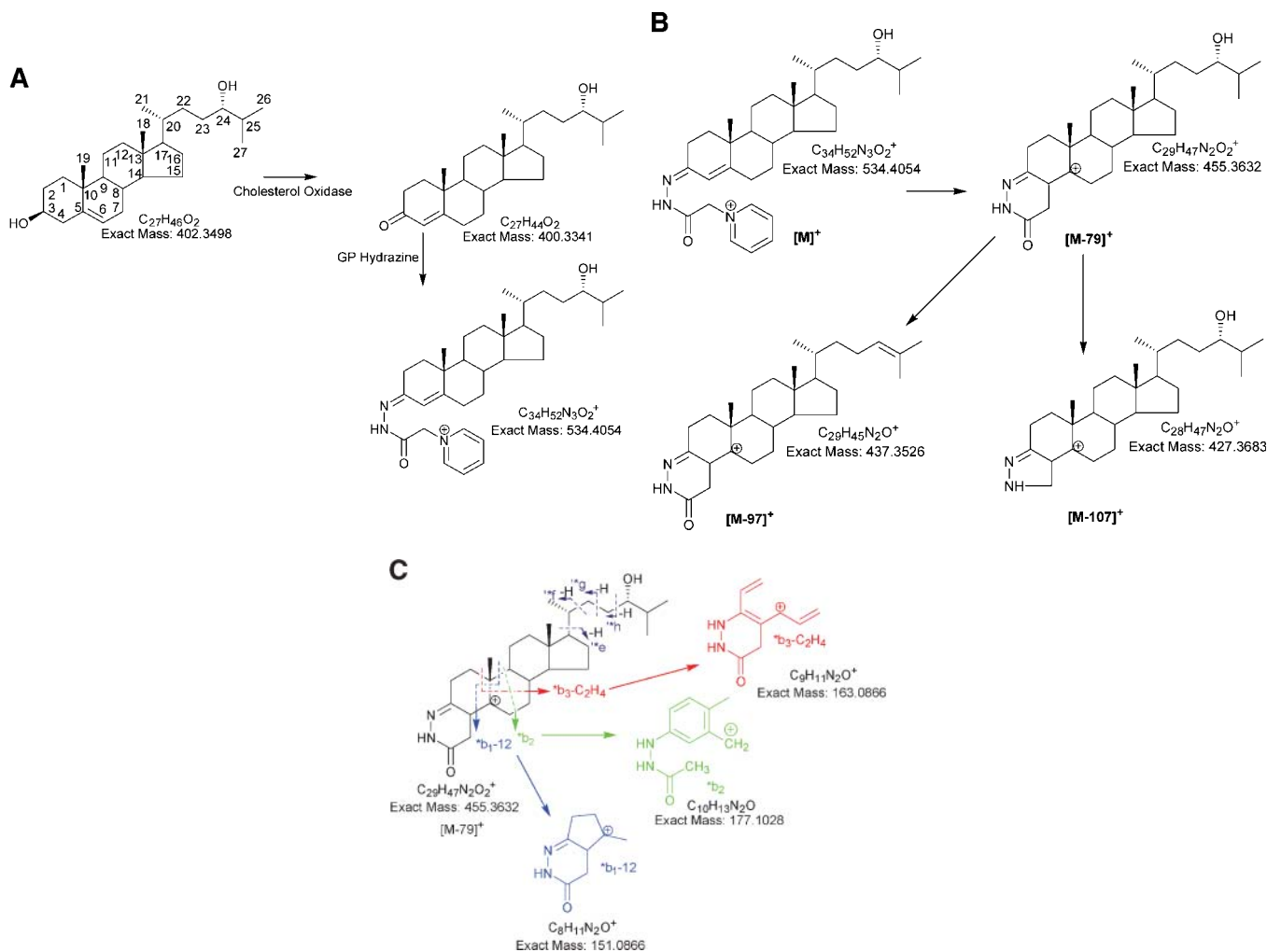


Fig. 1. Oxidation and derivatization of sterols to give Girard P (GP) hydrazones (A), MS² fragmentation of sterol GP hydrazones (B), and MS³ ([M]⁺→[M-79]⁺) fragmentation of 3-oxo-4-ene GP hydrazones (C). In A, B, and C, 24S-hydroxycholesterol is used as an example. Elemental compositions were confirmed by exact mass measurements. An asterisk preceding a fragment ion-describing letter (e.g., *b₁-12) indicates that the fragment ion has lost the pyridine moiety from the derivatizing group. A prime preceding a fragment ion-describing letter (e.g., 'b₃) indicates that cleavage proceeds with the transfer of a hydrogen atom from the ion to the neutral fragment. A prime to the right of the fragment-describing letter indicates that cleavage proceeds with hydrogen atom transfer to the fragment ion. Some confusion can arise concerning the nomenclature used to describe 27-hydroxycholesterol, which was previously denoted as 26-hydroxycholesterol. According to the rules of priority of numbering, the correct description of 27-hydroxycholesterol is 25R,26-hydroxycholesterol. However, because the medical community uses the name 27-hydroxycholesterol, we have used this name in this article.

position (oxysterols are cholesterol metabolites with additional oxygen functionalities and/or changed carbon skeleton), and use can be made of a knowledge of their exact mass to profile LC-MS ion chromatograms for potential compounds.

The benefit provided by the abundant neutral loss of 79 Da for the detection of GP derivatives proves to be a disadvantage for their structural determination. To identify novel oxysterols in brain, it will be necessary to maximize the structural information provided by tandem mass spectrometry (MS/MS), and when fragmentation proceeds predominantly through one or just a few reaction channels, this is difficult to achieve. Multi-stage fragmentation (MS^n) can offer a benefit in this regard. By performing an MS^3 experiment in which the precursor ion, $[M]^+$, fragments by the loss of 79 Da to give an $[M-79]^+$ ion in a first fragmentation event (MS^2), followed by activation of the $[M-79]^+$ ion and recording of its fragmentation pattern, i.e., MS^3 , considerable structural information can be forthcoming. The MS^3 scan also adds specificity to the conventional MS/MS experiment, in which fragmentation pathways can be recorded for precursor ions specifically giving an $[M-79]^+$ "intermediate." Other ions that coincidentally have the same nominal mass as derivatized oxysterols and are co-selected in the MS^2 event will not give an $[M-79]^+$ intermediate, and hence their fragment ions will not be selected further for the MS^3 scan and thus will not contaminate the final fragment ion MS^3 spectrum. This is in contrast to the situation in a simple two-stage MS/MS experiment, where, for low abundance analytes, contamination of MS/MS spectra may be significant (18).

Recent developments in MS instrumentation can take advantage of the properties of derivatized oxysterols. Although tandem quadrupole instruments provide the "gold standard" for recording neutral loss (e.g., the loss of 79 Da), and MRM scans and quadrupole time-of-flight instruments offer the possibility of exact mass and MS/MS analysis, ion trap instruments have the advantage of being able to generate MS^n spectra. A new type of hybrid mass spectrometer, the LTQ-Orbitrap from Thermo Electron Corp. (San Jose, CA), combines many of the merits of the above (19), and we demonstrate here that this instrument is ideal for the identification of cholesterol metabolites in brain.

Even when employing a high-performance mass spectrometer such as the LTQ-Orbitrap, it is desirable to incorporate on-line chromatographic separation when analyzing complex biological mixtures. With respect to oxysterols, there is evidence for cholesterol 24-, 25-, and 27-hydroxylase activity in mammalian brain (2, 5, 7, 14, 20, 21). These enzymes generate 24S-, 25-, and 27-hydroxycholesterols, respectively, isomers differing only by the location of the hydroxyl group on the C-17 side chain. Unless separated, their presence in a mixture will result in a composite MS^3 spectrum, which is difficult to interpret. Oxysterols are hydrophobic molecules with limited solubility in aqueous solvents. However, derivatization to GP hydrazones makes them more hydrophilic and increases the diversity of mobile-phase compositions compatible with analysis by reversed-phase chromatography.

Here we describe a novel LC- MS^n methodology for the identification, with high sensitivity (low pg), of oxysterols in brain. The methodology includes derivatization to add selectivity and enhance ionization, exact mass analysis at high resolution to identify potential metabolites, and LC- MS^3 to allow their characterization.

EXPERIMENTAL METHODS

Materials

Sterols were from Steraloids, Inc. Ltd (London, UK), Sigma-Aldrich Ltd (Poole, Dorset, UK), or from previous studies (see supplementary Table I). GP reagent [1-(carboxymethyl)pyridinium chloride hydrazide] and cholesterol oxidase from *Streptomyces sp.* were also from Sigma-Aldrich Ltd. Unisil (activated silicic acid, 200–325 mesh) was from Clarkson Chromatography Products, Inc. (South Williamsport, PA), and SepPak C_{18} was from Waters Corp. (Milford, MA).

Synthesis of 3 β -hydroxy-5-oxo-5,6-*seco*cholestan-6-al and 3 β ,5 β -dihydroxy-B-norcholestane-6 β -carboxaldehyde

Synthesis of 5,6-*seco*-sterol and its aldol from cholesterol was accomplished essentially as described by Wentworth et al. (22).

*3 β -Hydroxy-5-oxo-5,6-*seco*cholestan-6-al.* Cholesterol (500 mg) in chloroform-methanol (9:1, v/v; 50 ml) was treated with an excess of ozone at -78°C for 5 min. The solvents were evaporated, and the residue was treated with 325 mg of zinc powder suspended in 90% aqueous acetic acid (25 ml) at 23°C for 3 h. Material extracted with dichloromethane was subjected to silicic acid open column chromatography. Elution with diethyl ether-hexane (1:1; v/v) afforded the title compound (468 mg; yield, 86%). An aliquot was treated with *O*-methyl hydroxylamine in pyridine at 23°C for 2 h and subsequently trimethylsilylated. Analysis by GC-MS showed two peaks of comparable magnitude due to the *syn/anti* isomers of the aldoxime-TMS derivative (the C-5 keto group was not derivatized). The mass spectrum showed prominent ions at m/z 488 [$M^+ - 31$], 398 [$M^+ - (31+90)$], and 320 [$M^+ - 199$] (loss of the A-ring). The purity was in excess of 95%.

3 β ,5 β -Dihydroxy-B-norcholestane-6 β -carboxaldehyde. 3 β -Hydroxy-5-oxo-5,6-*seco*cholestan-6-al (400 mg) in acetonitrile-water (20:1, v/v; 50 ml) was treated with 118 mg of L-proline at 23°C for 2 h. The product obtained by extraction with ethyl acetate was subjected to silicic acid open column chromatography. Elution with diethyl ether-hexane (6:4; v/v) afforded the title compound (316 mg; yield, 79%). Treatment of an aliquot with *O*-methyl hydroxylamine and trimethylchlorosilane afforded the aldoxime-mono-TMS derivative (the C-5 tertiary alcohol was not derivatized), which appeared as a single but broadened peak on GC-MS. The mass spectrum showed prominent ions at m/z 488 [$M^+ - 31$], 398 [$M^+ - (31+90)$], 343, and 278. The purity was in excess of 95%.

Extraction of oxysterols from brain

In brief, oxysterols were extracted from three 100 mg portions of rat brain (Sprague Dawley, female, 306 g, 15 weeks; Harlan UK) as follows: 100 mg of brain was homogenized in ethanol (1 ml), the ethanol extract was centrifuged, and the supernatant was retained. The precipitate was ultrasonicated in 1 ml of methanol-dichloromethane (1:1; v/v) and centrifuged, and the supernatant was added to that retained previously. The superna-

tants were evaporated, and the residue was redissolved in 2 ml of hexane-dichloromethane (2:8; v/v). The three brain extracts were then pooled, and one-third of the total extract was applied to a Unisil column (8 × 0.8 cm, 200–325 mesh, activated silicic acid) prepared in hexane, and washed with 20 ml of hexane-dichloromethane (2:8; v/v) prior to sample application. Following sample application, the column was washed with 80 ml of hexane-dichloromethane (2:8; v/v, fraction 1) and eluted with 10 ml of ethyl acetate (fraction 2). The eluate was dried and redissolved in 50 μ l isopropanol. For brain samples found to contain a significant amount of cholesterol in the ethyl acetate eluate (fraction 2), the eluate was dried and reconstituted in 2 ml of hexane-dichloromethane (2:8; v/v), reapplied to a fresh Unisil column, and chromatographed as before (see supplementary Fig. 1). Note that in this initial study, the oxysterols were extracted from whole brain. The extraction, identification, and quantification of oxysterols in defined brain regions is a topic for further study.

Oxidation of 3 β -hydroxy-5-ene sterols to 3-oxo-4-ene sterols

The 3 β -hydroxy-5-ene sterols [both reference sterols (5 μ g) and those extracted from brain (above)] were oxidized with cholesterol oxidase essentially as described by Brooks et al. (23). The 3 β -hydroxy-5-ene sterols were dissolved in 50 μ l of isopropanol, and 2 μ l of cholesterol oxidase from *Streptomyces sp.* (2 mg/ml, 44 U/mg protein) in 1 ml of buffer (50 mM KH₂PO₄, pH 7) was added. The mixture was incubated at 37°C for 60 min and subsequently used as the starting solution for reaction with the GP reagent as described below (Fig. 1A).

Derivatization of 3-oxo-4-ene sterols

The derivatization of oxysterols to GP hydrazones was carried out essentially as described by Shackleton et al. (24) (Fig. 1A). The reaction mixture from the oxidation step above was used directly after incubation with enzyme. The oxidation mixture, 1 ml (50 mM phosphate buffer, 5% isopropanol, 4 μ g enzyme, and reference oxysterols or those extracted from brain) was diluted with 2 ml of methanol to give an ~70% methanol solution, and 150 mg of GP hydrazine and 150 μ l of glacial acetic acid were added. The mixture was left at room temperature overnight.

Following overnight incubation, the GP reaction mixture (~3 ml 70% methanol) was directly applied to a SepPak C₁₈ bed (1 cm × 0.8 cm in a glass column) followed by 1 ml of 70% methanol and 1 ml of 35% methanol. The combined effluent (now 5 ml) was diluted with 4 ml of water. The resulting mixture (now 9 ml in 35% methanol) was again applied to the column, followed by a wash with 1 ml of 17% methanol. To the combined effluent, 9 ml of water was added. The sample was then in 19 ml of about 17.5% methanol. This was again applied to the column, followed by a wash with 10 ml of 10% methanol. At this point, all the GP derivatives had been extracted by the column. They were then eluted with two portions of 1 ml of methanol, followed by 1 ml of chloroform-methanol (1:1; v/v). The three fractions were analyzed separately and in combination by LC-ES-MSⁿ. The GP derivatives were to be found predominantly in the second milliliter of methanol. The derivatization protocol has been applied to mixtures of oxysteroids on the μ g–ng level and is suitable for low-level (pg) derivatization of neutral steroids extracted from tissue (18).

LC-MSⁿ

Chromatographic separation of oxidized/derivatized oxysterols was performed on a Finnigan Surveyor HPLC system utilizing a Hypersil GOLD reversed-phase column (1.9 μ m particles,

50 × 2.1 mm) from Thermo Electron Corp. Mobile-phase A consisted of 50% methanol containing 0.1% formic acid, and mobile-phase B consisted of 95% methanol 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 reequilibration for a further 3 min 54 s, giving a total run time of 17 min. The flow rate was maintained at 200 μ l/min, and eluent was directed to the API source of an LTQ-Orbitrap mass spectrometer (19).

MS analysis was performed on a Finnigan LTQ-Orbitrap (Thermo Electron Corp.) hybrid linear ion trap (LIT)–Orbitrap analyser (19). Ions from the API source, operated in the positive ES mode, are initially stored in the LIT and analyzed in either MS or MSⁿ modes. Ions can be detected at the LIT detector, or ejected axially and trapped in an intermediate C-trap, from which they are “squeezed” into the Orbitrap. Trapped ions in the Orbitrap assume circular trajectories around the central electrode and perform axial oscillation. The oscillating ions induce an image current into the two halves of the Orbitrap, which can be detected. The axial oscillation frequency of an ion (ω) is proportional to the square root of the inverse of m/z [$\omega = (k/m/z)^{1/2}$], and the frequencies of complex signals derived from many ions can be determined using a Fourier transformation.

For the analysis of reference compounds (16 pg/ μ l) infused at 10 μ l/min, the Orbitrap was operated at 60,000 resolution (full width at half maximum height). MS and MSⁿ spectra were recorded using both the LIT detector and the Orbitrap as a detector. The MS² isolation width was set at 2 so as to allow the selection of monoisotopic precursor ions. The Orbitrap was calibrated externally prior to each analytical session, and mass accuracy was in all cases better than 5 ppm. For LC-MS and LC-MSⁿ analysis of reference compounds, each sample (30 pg/ μ l in 60% methanol) was injected (10 μ l) onto the reversed-phase column and eluted into the LTQ-Orbitrap at a flow rate of 200 μ l/min. The Orbitrap was operated at 30,000 resolution.

For the analysis of oxidized/derivatized oxysterols extracted from rat brain, 5 μ l of the methanol eluent (2 × 1 ml) from the SepPak C₁₈ bed (equivalent to 0.25 mg of brain, assuming all the oxysterols elute in the methanol fractions) was diluted with 45 μ l of 60% methanol, and 10 μ l was injected onto the LC column (equivalent to 0.05 mg brain). MS, MS², and MS³ spectra were recorded in a data-dependent mode, with the incorporation of an inclusion list for expected oxidized/derivatized oxysterols. MS³ was programmed to be performed on ions resulting from a neutral loss of 79 Da in the MS² scan. For the acquisition of both MS² and MS³ spectra, the collision energy setting was 30–35.

RESULTS AND DISCUSSION

The experimental method involves homogenization and extraction of oxysterols from brain tissue with ethanol. Ethanol is a good solvent for oxysterols and readily penetrates cell membranes. Oxysterols are present in brain at levels of 10³–10⁵ lower than cholesterol, and to avoid autoxidation artifacts, cholesterol was separated from oxysterols by straight-phase chromatography on a Unisil column immediately after extraction. Because oxysterols possess a 3 β -hydroxy-5-ene or 3 β -hydroxy functionality (or a carbonyl group at C-3 or elsewhere), advantage was taken of the specificity of cholesterol oxidase from *Streptomyces sp.*, which was used to convert oxysterols to 3-oxo-4-ene or 3-oxo analogs (see Fig. 1A). The oxo group was then derivatized with GP reagent to give GP hydrazones, which

possess a charged quaternary nitrogen and give an improvement in ES signal of two to three orders of magnitude (18). This oxidation/derivatization approach adds both specificity and sensitivity to the analysis of oxysterols. It should be pointed out, however, that in its present form, the method does not distinguish between a 3 β -hydroxy-5-ene sterol and its corresponding 3-oxo-4-ene analog. The latter can be separately determined by deletion of the cholesterol oxidase reaction from the analytical protocol.

Oxidation and derivatization

We have demonstrated previously that the process of oxidizing 3 β -hydroxyl groups to 3-oxo groups followed by derivatization to GP hydrazones enhances the oxysterol ion current by at least two orders of magnitude (18). However, this method, like all derivatization procedures, has its drawbacks, one of which is variation in the susceptibility of the 3 β -hydroxyl group to oxidation by cholesterol oxidase, depending on the overall sterol structure and the enzyme employed. A second consideration is the reactivity of other oxo groups present on the sterol skeleton to the GP reagent, which varies according to their location. We have optimized the methodology with brain oxysterols in mind, in that we have used cholesterol and 24S-hydroxycholesterol as the test substrates. By incubating these sterols for 60 min at 37°C with ~ 0.2 U of cholesterol oxidase, conversion to 3-oxo-4-ene sterols is maximized and the formation of oxidation artifacts is avoided. In the current study, cholesterol oxidase from *Streptomyces sp.* has been used. Cholesterol oxidases from different bacterial sources have varying activity to different 3 β -hydroxysteroids and are also likely to generate different profiles of artifacts (23, 25, 26). Test experiments were performed with 5 μ g of cholesterol and 24S-hydroxycholesterol, the sterols were supplied as 99% pure, and this level of purity was maintained throughout the oxidation/derivatization process. However, cholesterol is likely to be 10³-fold more abundant than any oxysterol in brain, and even as little as 0.1% formation of autoxidation products in the oxidation/derivatization process would lead to significant artifacts when analyzed as part of an oxysterols study. To minimize this eventuality, cholesterol is removed in the first stage of the sample purification by a single or double passage through Unisil columns.

To simulate closely the analytical procedure for the analysis of oxysterols extracted from brain tissue, 1 mg of cholesterol, which approximates the cholesterol content of 0.1 g of brain, was subjected to the entire sample purification, oxidation, and derivatization protocol. A single passage of cholesterol through the Unisil column results in 95% elution in fraction 1 and 5% elution in fraction 2 (see supplementary Fig. I). Following treatment of fraction 2 with cholesterol oxidase and derivatization with GP, desmosterol (m/z 516.3948) was found at 0.5–1.5% of the level of cholesterol [relative abundance (RA), 100%, m/z 518.4105] in fraction 2. A minor amount of 6-oxocholestenone (RA 0.3%, m/z 532.3898) was also identified, as were cholesterol epoxides (RA 0.2%, m/z 532.3898). Minor components with m/z 550.4003 were observed

(RA 1%, 2%), both of which gave MS² or MS³ spectra compatible with dihydroxycholesterols with at least one of the added hydroxyl groups in the A or B ring. A second separation of the material collected in fraction 2 after removal of solvents on a fresh Unisil column essentially eliminates all cholesterol and its subsequent autoxidation products.

MS

The reference oxysterols (supplementary Table I) were analyzed after oxidation/derivatization by direct infusion MS, MSⁿ, LC-MS, and LC-MSⁿ.

MS² spectra of most oxysterols are dominated by an [M-79]⁺ fragment ion, accompanied by lower abundance [M-97]⁺ and [M-107]⁺ ions. Exact mass analysis of these fragments performed at high resolution (60,000) confirms that they result from the loss of pyridine (C₅H₅N), pyridine plus water (C₅H₇NO), and pyridine plus carbon monoxide (C₆H₅NO), respectively (Fig. 1B). Dihydroxycholesterols additionally give [M-115]⁺ and [M-125]⁺ ions, which result from the loss of pyridine and two molecules of water (C₅H₉NO₂), and pyridine, carbon monoxide, and water (C₆H₇NO₂). Although not providing a wealth of structural information, MS² spectra complement MS³ spectra, giving information on the lability of the modified cholesterol skeleton. For example, for most oxidized/derivatized monohydroxycholesterols (m/z 534.4054), the MS² spectra show [M-97]⁺ ions with RA of $\sim 1\%$. However, for 25-hydroxycholesterol, the lability of the hydroxyl group on tertiary C-25 results in an [M-97]⁺ ion with RA of $\sim 15\%$ (see supplementary Table II and supplementary Figs. II–VII). In contrast to MS² spectra, MS³ spectra of oxidized/derivatized sterols provide a wealth of structural information, in which different functional groups provide different fragmentation patterns according to their location.

3-oxo-4-ene sterols

Cholesterol oxidase converts 3 β -hydroxy-5-ene sterols to their 3-oxo-4-ene analogs. The simplest sterol to undergo this conversion is cholesterol, which is oxidized to cholest-4-en-3-one. The GP derivative of cholest-4-en-3-one, and other 3-oxo-4-ene sterols, which are unmodified by additional substituents in the A and B rings, give informative MS³ spectra containing a triad of fragment ions at m/z 151, 163, and 177. These fragment ions are formed by cleavage in the B ring and are described as *b₁-12, *b₃-C₂H₄ (or *b₂-CH₂), and *b₂ ions, respectively (18) (Fig. 1C, and see supplementary Fig. IIB) Modification of the B ring by the introduction of a hydroxyl group at C-7 changes the pattern of B-ring fragment ions. The comparative abundance of the *b₁-12 ion at m/z 151 is enhanced, and the *b₃-C₂H₄ fragment is shifted in m/z from 163 to 179 (see supplementary Fig. IIIB). Introduction of a 6-hydroxyl group attenuates the *b₁-12 and *b₃-C₂H₄ fragment ions, leaving an ion at m/z 177. The observation of an ion at m/z 177 rather than 193 indicates that the C-6 hydroxyl is no longer present in the *b₂ ion (see supplementary

Table II). The presence of an oxo group at C-6 eliminates the pattern of B-ring fragment ions, although a low-intensity $*b_2$ ion is observed at m/z 191 (a carbonyl group at C-6 may indicate that the fragment ion at m/z 191 has a structure different from $*b_2$). An oxo group at C-7 attenuates the $*b_1-12$ fragmentation and shifts $*b_3-C_2H_4$ up in mass to m/z 177, leaving a $*b_2-CH_2$ ion at m/z 163. A detailed description of how the location of additional hydroxyl and oxo groups to the steroid skeleton and C-17 side-chain are determined is given in supplementary Results and supplementary Figs. II–XI.

Oxysterols in rat brain

Following oxidation/derivatization, oxysterols extracted from brain were separated by reversed-phase chromatography and analyzed on an LTQ-Orbitrap hybrid tandem mass spectrometer. In an initial run, the instrument was used to record mass spectra at high resolution in the Orbitrap. Reconstructed exact mass (5 ppm) ion chromatograms (RICs) were plotted for all potential oxysterols (Table 1). In a second LC-MS run, MS^2 and MS^3 spectra were recorded in a data-dependent mode with the incorporation of an inclusion list for all potential oxysterols suggested to be present from the initial LC-MS run. MS^3 was programmed to be performed on ions resulting from a neutral loss of 79 Da in the MS^2 scan, i.e., $[M]^+ \rightarrow [M-79]^+ \rightarrow$. The MS^3 scans provide added specificity to the method, because this final product ion spectrum is only generated from precursor ions of the selected mass giving an $[M-79]^+$ intermediate. The exact mass capability of the LTQ-Orbitrap instrument adds further specificity to the method, in that only precursor ions with an exact mass and chromatographic retention time compatible with an oxysterol will be selected for MS^3 .

Passing the brain extract twice through the Unisil column effectively removes all the cholesterol and desmosterol from the final oxysterol fraction. The RA of cholesterol and desmosterol in these samples amount to $<2\%$ and $<0.1\%$ of the major oxysterol in the fraction. The major sterol in the oxysterol fraction has an m/z of 534.4054 (RA 100%) and appears with retention time (7.35 and 7.65 min, *syn/anti* forms of the GP hydrazone) identical to that of oxidized and derivatized 24S-hydroxycholesterol (supplementary Table I). Both the MS^2 and MS^3 spectra are identical to those from the authentic compound, thereby confirming 24S-hydroxycholesterol to be the major oxysterol in rat brain (2) (Fig. 2). With the use of an internal standard the level of 24S-hydroxycholesterol in rat brain was found to vary between 15 and 25 $\mu\text{g/g}$ (data not shown), in good agreement with values for other mammals (9, 27). Evidence from experiments with recombinant CYP46A1 and HEC 293 cells transfected with CYP46A1, suggest that this enzyme can, in addition to 24S-hydroxylation of cholesterol, further metabolize the resulting oxysterol to 24,25- and 24,27-dihydroxycholesterols (14). Additional products of the incubation of 24S-hydroxycholesterol with CYP46A1-transfected HEC 293 cells were partially characterized as 24,X-dihydroxycholesterol, 24,25,X-trihydroxycholesterol and 24,27,X-trihydroxycholesterol, where the

extra hydroxyl group as signified by X is in the steroid nucleus (14). 24-Oxcholesterol with an extra hydroxyl group in the steroid nucleus, which is presumably formed via 24-oxocholesterol, was also identified (14). Exact mass RICs for the m/z values of these oxysterols were generated (Table 1). Although its RA was $<2\%$ that of 24S-hydroxycholesterol (RAs of all oxysterols are measured relative to 24S-hydroxycholesterol), the major component with m/z 532.3898 appeared at a retention time of 7.60 min and gave MS^2 and MS^3 spectra identical to those of authentic oxidized/derivatized 24-oxocholesterol (see supplementary Table II). The additionally hydroxylated version of this compound has an m/z of 548.3847, and gave a peak (RA $<0.5\%$) that appears in the RIC with a retention time of 5.49 min and gives MS^2 and MS^3 spectra compatible with those of an oxidized/derivatized oxohydroxycholesterol.

The MS^2 and MS^3 spectra of oxidized/derivatized dihydroxycholesterols (m/z 550.4003) from brain gave informative fragmentation patterns. As can be seen in the RIC (Fig. 3A), nine distinct peaks are observed, but compared with that of 24S-hydroxycholesterol, all are of low abundance (RA $<1\%$). The MS^2 and MS^3 spectra of the first eluting component at 4.31 min indicate a dihydroxy derivative of a 3-oxo-4-ene sterol. In the MS^3 spectrum (Fig. 3B) the pattern of fragment ions at m/z 151, 163, and 177 confirms the 3-oxo-4-ene structure (derived from cholesterol oxidase treatment of the 3 β -hydroxy-5-ene sterol), and the significant fragment ion at m/z 353 ($*f$, see Figs. 1C, 2B) defines the location of one hydroxyl group at C-24 with the second necessarily on C-25 or C-27. Based on the ratio of $[M-97]^+$, $[M-107]^+$, $[M-115]^+$, and $[M-125]^+$ (m/z 453, 443, 435, 425) fragment ions in both the MS^2 (1.5:10.0:0.5: <0.5) (data not shown) and MS^3 (10.0:3.5:1.0:2.0) spectra (Fig. 3B), and the greater stability of the 27-hydroxyl group in comparison to the 25-hydroxyl group, the second hydroxyl group is located to C-27. The chromatographic peak eluting at 4.82 min gave MS^2 and MS^3 spectra similar to that eluting at 4.31 min, and it is concluded that these are *syn* and *anti* isomers of the GP derivative of 24,27-dihydroxycholesterol. The chromatographic peak at m/z 4.47 min also gave MS^2 and MS^3 spectra compatible with an oxidized/derivatized dihydroxycholesterol where one of the additional hydroxyl groups is on C-24 and the second is on C-25 or C-27. A characteristic of MS^3 spectra of oxidized/derivatized oxysterols with a 25-hydroxyl group is that the $[M-97]^+$ ion dominates the spectrum at the expense of all other fragment ions (see supplementary Table II). This is also evident in the MS^3 spectrum of the peak eluting at 4.47 min (Fig. 3C), with the consequence that the fragment ions at m/z 151, 163, 177, and 353 are of lower abundance than in the spectrum of the 24,27-dihydroxy isomer (Fig. 3B). The abundance of the $[M-107]^+$ fragment is also reduced, but not the abundance of $[M-115]^+$ or $[M-125]^+$ fragments, which is reflected in the ratio of ions of m/z 453, 443, 435, and 425 of 10.0:1.5:1.0:1.5. Further evidence for the location of the second hydroxyl group at C-25 comes from the abundance ratio of this series of ions in the MS^2 spectrum, i.e., 2.5:10.0:0.5:0.5 (data not shown), which is compatible with the enhanced lability of the C-25 hydroxyl

TABLE 1. Potential oxysterols and those identified in brain

Sterol/Oxysterol ^{a,b}	Structure after Oxidation with Cholesterol Oxidase	Formula of GP	Mass of GP	Identified in Current Work (Concentration Wet Brain)	Identified by Others in Brain (Concentration Wet Brain)
C ^{5,x} -3β-ol	C ^{4,x} -3-one	C ₃₄ H ₅₀ N ₃ O ⁺	516.3948	Desmosterol ^c (not quantified)	Rat (0.1% of Total Sterol) ^d
C ⁵ -3β-ol	C ⁴ -3-one	C ₃₄ H ₅₂ N ₃ O ⁺	518.4105	Cholesterol ^c (not quantified)	Mouse (50 ng/mg) ^e Human (7–8 μg/mg) ^f Mouse (20 μg/mg) ^e
5α-C-3β-ol	5α-C-3-one	C ₃₄ H ₅₄ N ₃ O ⁺	520.4261	Not detected	
C ⁵ -3β-ol-x-one	C ⁴ -3,x-dione	C ₃₄ H ₅₀ N ₃ O ₂ ⁺	532.3898	24-Oxcholesterol (~0.5 ng/mg)	
/C ^{5,x} -3β,y-diol	/C ^{4,x} -y-ol-3-one			not detected	
C ⁵ -3β,x-diol	C ⁴ -x-ol-3-one	C ₃₄ H ₅₂ N ₃ O ₂ ⁺	534.4054	24S-Hydroxycholesterol ^g (15–25 ng/mg)	Human (5–15 ng/mg) ^{f,h} Mouse (40–50 ng/mg) ^{e,i}
5α-C-3β,x-diol	5α-C-x-ol-3-one	C ₃₄ H ₅₄ N ₃ O ₂ ⁺	536.4211	Not detected	
C ⁵ -3β-ol-x,y-dione/C ^{5,x} -3β,y-diol-z-one	C ⁴ -3,x,y-trione/C ^{4,x} -y-ol-3,z-dione	C ₃₄ H ₄₈ N ₃ O ₃ ⁺	546.3690	Not detected	
C ⁵ -3β,x-diol-y-one	C ⁴ -x-ol-3,y-dione	C ₃₄ H ₅₀ N ₃ O ₃ ⁺	548.3847	24-Oxohydroxycholesterol (~125 pg/mg)	CYP46A1 ^j
/C ^{5,x} -3β,y,z-triol/CA ⁵ -3β-ol	/C ^{4,x} -y,z-diol-3-one/CA ⁴ -3-one			not detected	Astrocytes ^k
C ⁵ -3β,x,y-triol	C ⁴ -x,y-diol-3-one	C ₃₄ H ₅₂ N ₃ O ₃ ⁺	550.4003	24,25-Dihydroxycholesterol (~125 pg/mg)	CYP46A1 ^j
				24,27-Dihydroxycholesterol (~30 pg/mg)	Astrocytes ^k /CYP46A1 ^j
				25,27-Dihydroxycholesterol (~75 pg/mg)	CYP46A1 ^j
				6,24-Dihydroxycholesterol (~150 pg/mg)	Astrocytes ^k
				7α,25-Dihydroxycholesterol (~30 pg/mg)	CYP46A1 ^j
				7α,27-Dihydroxycholesterol (~30 pg/mg)	Astrocytes ^k
5α-C-3β,x,y-triol	C-x,y-diol-3-one	C ₃₄ H ₅₄ N ₃ O ₃ ⁺	552.4160 ^l	Not detected	
/5,6- <i>Seco</i> -sterol ^l /Aldol ^l	/5,6- <i>Seco</i> -sterol ^l /aldol ^l			5,6- <i>seco</i> -sterol (~100 pg/mg)/Aldol (~300 pg/mg)	Human (150 pg/mg) ^m
C ⁵ -3β-ol-x,y,z-trione/C ^{5,w} -3β,x-diol-y,z-dione/	C ⁴ -3,x,y,z-tetraone/C ^{4,w} -x-ol-3,y,z-trione	C ₃₄ H ₄₆ N ₃ O ₄ ⁺	560.3483	Not detected	
C ⁵ -3β,x-diol-y,z-dione/C ^{5,w} -3β,x,y-triol-z-one	C ⁴ -x-ol-3,y,z-trione/C ^{4,w} -x,y-diol-3,z-dione	C ₃₄ H ₄₈ N ₃ O ₄ ⁺	562.3639	Not detected	
C ⁵ -3β,x,y-triol-z-one/C ^{5,w} -3β,x,y,z-tetraol/	C ⁴ -x,y-diol-3,z-dione/C ^{4,w} -x,y,z-triol-3-one/	C ₃₄ H ₅₀ N ₃ O ₄ ⁺	564.3796	Detected, not characterized	
CA ⁵ -3β,x-diol	CA ⁴ -x-ol-3-one				Astrocytes ^k
C ⁵ -3β,x,y,z-tetraol	C ⁴ -x,y,z-triol-3-one	C ₃₄ H ₅₂ N ₃ O ₄ ⁺	566.3952	Detected, not characterized	CYP46A1 ^j /astrocytes ^k
5α-C-3β,x,y,z-tetraol	5α-C-x,y,z-triol-3-one	C ₃₄ H ₅₄ N ₃ O ₄ ⁺	568.41088	Detected, not characterized	

C, cholestane; CA, cholestanic acid, 3β-hydroxy-5-oxo-5,6-*seco*cholestan-6-al (5,6-*seco*-sterol), 3,5-dihydroxy-B-norcholestan-6-carboxyaldehyde (aldol); GP, Girard P.

^a See below for structures. Superscripts indicate location of double bonds.

^b Oxysterols with more than four oxygen atoms were not detected.

^c Cholesterol and its precursors, not oxysterols. Found in fraction 1 from Unisil column.

^d Other precursors of cholesterol, besides desmosterol, have been identified in rat brain (33, 34). Desmosterol concentration from ref. (33).

^e From ref. (35).

^f From ref. (36).

^g Only 24S-hydroxycholesterol was identified, although chromatographic performance was sufficient to resolve it from closely eluting isomers, i.e., 25-hydroxycholesterol and 27-hydroxycholesterol.

^h Other oxysterols identified in human brain include 27-hydroxycholesterol (1–3 ng/mg) (35), 22R-hydroxycholesterol (45–90 pg/mg) (37), 7-hydroxycholesterol (234–745 ng/mg, cerebellar cortex) (38), and 7-oxocholesterol (170–640 ng/mg cerebellar cortex) (38).

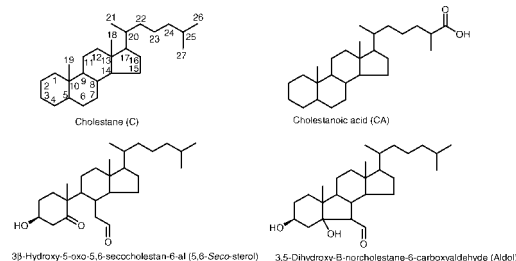
ⁱ Other oxysterols identified in mouse or rat brain include 27-hydroxycholesterol (5 ng/mg mouse) (35), 20S-hydroxycholesterol (50 pg/mg rat) (39).

^j Recombinant CYP46A1 shows 25- and 27-hydroxylase activity when incubated with 24S-hydroxycholesterol. HEK293 cells transfected with CYP46A1 can also hydroxylate the steroid nucleus (14).

^k Astrocytes show 25-hydroxylase activity when incubated with 27- and 24-hydroxycholesterol, and 7α-hydroxylase activity when incubated with 25- and 27-hydroxycholesterol (21).

^l 5,6-*Seco*-sterol and its adol are not oxidized by cholesterol oxidase.

^m Concentration of combined 5,6-*seco*-sterol and its aldol (11).



Supplemental Material can be found at: <http://www.jlr.org/content/suppl/2007/08/10.M600497-JLR200.DC1.html>

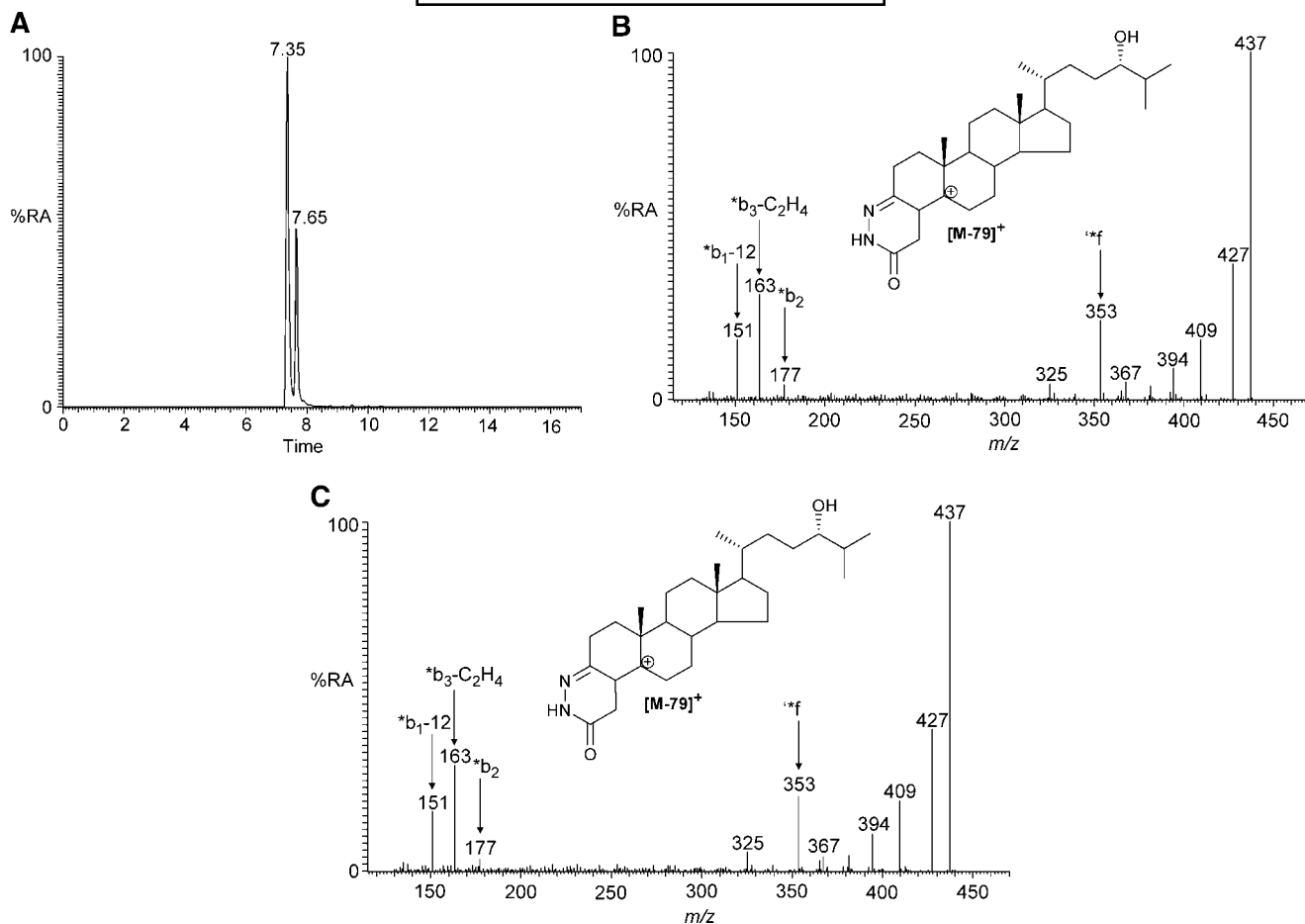


Fig. 2. Reconstructed ion chromatogram (RIC) for m/z 534.4054 (± 5 ppm) corresponding to oxidized/derivatized monohydroxycholesterols extracted from rat brain (A), MS^3 ($[M]^+ \rightarrow [M-79]^+ \rightarrow$) spectrum of the peak eluting at 7.35 min (B), and authentic oxidized/derivatized 24S-hydroxycholesterol with a similar retention time (C). The peaks eluting at 7.35 and 7.65 min in (A) give identical MS^2 and MS^3 spectra and correspond to *syn* and *anti* isomers of the derivative. The RIC was recorded by Fourier transformation (FT) in the Orbitrap analyzer, and MS^3 spectra were recorded by the LTQ detector.

group as compared with that at C-27. These data lead to the conclusion that the peaks eluting at 4.31 and 4.82 min correspond to oxidized/derivatized 24,27-dihydroxycholesterol and that at 4.47 min to 24,25-dihydroxycholesterol. The MS^2 and MS^3 spectra of the peak eluting at 5.06 min again indicate an oxidized/derivatized dihydroxycholesterol. In the MS^3 spectrum (Fig. 3D), the presence of a peak at m/z 325 (*e), and the absence of one at 353 (*f) indicate that both hydroxyl groups are on the C-17 side-chain, but not at C-24. The spectrum does not show fragment ions characteristic of hydroxylation at C-20, -22, and -24 (see supplementary Table II), but the ratio of ions of m/z 453, 443, 435, and 425 in the MS^2 and MS^3 spectra of 2.5:10.0:<0.5:0.5 (data not shown) and 10.0:1.5:1.5:1.5 does strongly suggest a 25,27-diol. 25,27-Dihydroxycholesterol has been identified by Zhang et al. (21) in incubations of 27-hydroxycholesterol with rat brain astrocytes, and has been suggested to be an intermediate in cholesterol metabolism in astrocytes. It should be noted that microchemical reactions, such as treatment with periodate, or the Jones oxidation, could be attempted to confirm the structures of the above oxysterols. However, their

presence at low abundance (ng/g) and as part of a mixture has precluded such a study as part of the present work.

The presence of a 6-hydroxyl group on the cholesterol skeleton alters the MS^3 spectrum of the oxidized derivative, in that instead of a triad of fragment ions at m/z 151, 163, and 177, only the ion at 177 is preserved. Also, in the MS^3 spectrum of oxidized/derivatized 6-hydroxycholesterol, the normal arrangement of $[M-97]^+$ ions as more abundant than $[M-107]^+$ ions is reversed, and an intense ion is observed at m/z 383 corresponding to $[M-79-C_4H_8O]$ (see supplementary Table II). The MS^3 spectrum of the peak eluting at 5.45 min gives a fragment ion at m/z 177, and the $[M-107]^+$ fragment is more abundant than the $[M-97]^+$ ion, signifying hydroxylation at C-6 (Fig. 3E). Further evidence is provided by the presence of a fragment ion at 381 corresponding to $[M-97-C_4H_8O]^+$. A fragment ion at m/z 341 (*e) indicates that the second hydroxyl group is on the C-17 side-chain, and a fragment ion at m/z 369 (*f) locates it to C-24. Thus, the spectrum is compatible with that of oxidized/derivatized 6,24-dihydroxycholesterol. Significantly, when Mast et al. (14) incubated 24-hydroxycholesterol with HEK293 cells transfected with

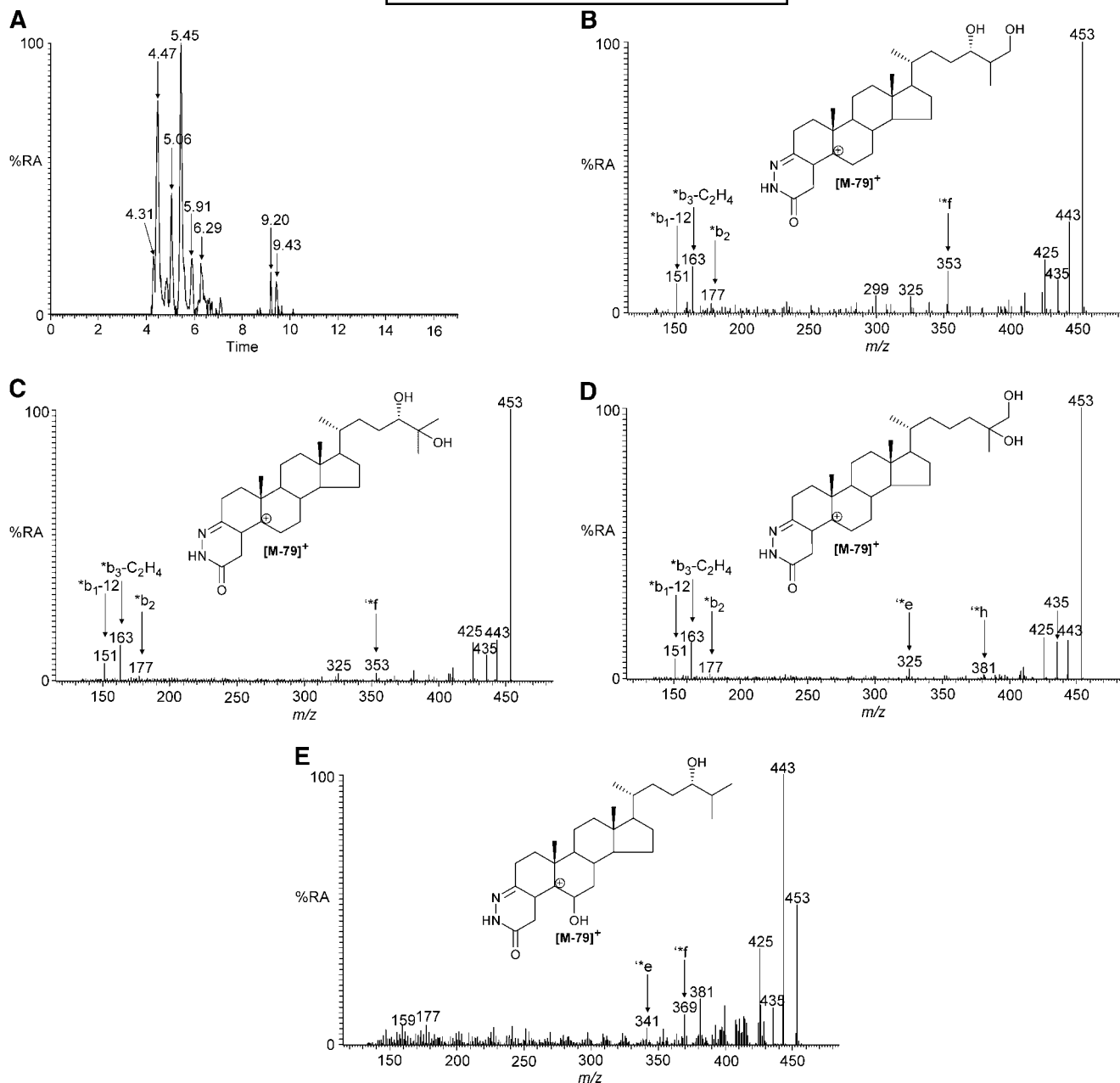


Fig. 3. RIC for m/z 550.4003 corresponding to oxidized/derivatized dihydroxycholesterols extracted from brain (A), MS^3 ($[M]^+ \rightarrow [M-79]^+ \rightarrow$) spectra of chromatographic peaks eluting at 4.31 min corresponding to 24,27-dihydroxycholesterol (B), 4.47 min corresponding to 24,25-dihydroxycholesterol (C), 5.06 min corresponding to 25,27-dihydroxycholesterol (D), and 5.45 min corresponding to 6,24-dihydroxycholesterol (E). The RIC was recorded by FT in the Orbitrap analyser and the MS^3 spectra were recorded by the LTQ detector.

CYP46A1, a dihydroxycholesterol with one hydroxyl group at C-24 and the second in the steroid nucleus was identified. Furthermore, CYP7B1, an enzyme with 6-hydroxylase activity, is expressed in brain (28, 29).

The chromatographic peaks at 5.91 and 6.29 give MS^2 and MS^3 spectra identical to those of authentic standards of oxidized/derivatized $7\alpha,25$ - and $7\alpha,27$ -dihydroxycholesterol. The 7-hydroxyl groups are characterized by fragment ions at m/z 151 and 179 in the MS^3 spectra, and their α -positioning is indicated by the heightened abundance of fragment ions at m/z 231 (see supplementary Fig. XII). The latter eluting peak shows enhanced intensity of ions at

m/z 443 $[M-107]^+$ as a consequence of the stability of the 27-hydroxyl group. Both $7\alpha,25$ - and $7\alpha,27$ -dihydroxycholesterols have been suggested as intermediates in the cholesterol metabolic pathways in rat brain (21). Thus, rat astrocyte cultures convert them by oxidation with 3β -hydroxy- Δ^5 - C_{27} -steroid oxidoreductase/isomerase (HSD3B7, specific for 7α -hydroxylated sterols and bile acids) into the 3-oxo- Δ^4 analogs (included in the chromatographic peaks at 5.91 and 6.29), and 27-hydroxysterols can be converted by astrocytes into cholestenic acids (21). The latter are intermediates in the biosynthesis of C_{24} bile acids, which have recently been identified in rat brain (30). The

enzyme with 7α -hydroxylase activity in brain, i.e., CYP7B1, will 7α -hydroxylate both 25- and 27-hydroxycholesterols, but has much lower activity against 24S-hydroxycholesterol (28, 29). Significantly, 24-hydroxycholesterol was not 7α -hydroxylated by rat astrocyte, Schwann cell, or neuron cultures (21), and $7\alpha,24$ -dihydroxycholesterol was not observed in the present study.

The recent interest in *seco* sterols inspired us to search for 5,6-*seco*-sterol and its aldol condensation product, 3,5-dihydroxy-B-norcholestane-6-carboxyaldehyde, which have been previously suggested to be present in human

brain (11, 31). The RIC for m/z 552.4160 revealed two components, with retention times of 9.23 and 10.03 min. The MS^2 spectra were rich in fragment ions and gave spectra identical to those of the GP hydrazones of 5,6-*seco*-sterol and its aldol, respectively (Fig. 4). The abundance of these isomers was less than 2% that of 24S-hydroxycholesterol.

The analytical method described offers sensitivity and selectivity for the determination of oxysterols in brain. On-column detection limits were of the order of 1.5 pg (data not shown), and at this level, MS^2 and MS^3 spectra were sufficiently informative to allow structure determination.

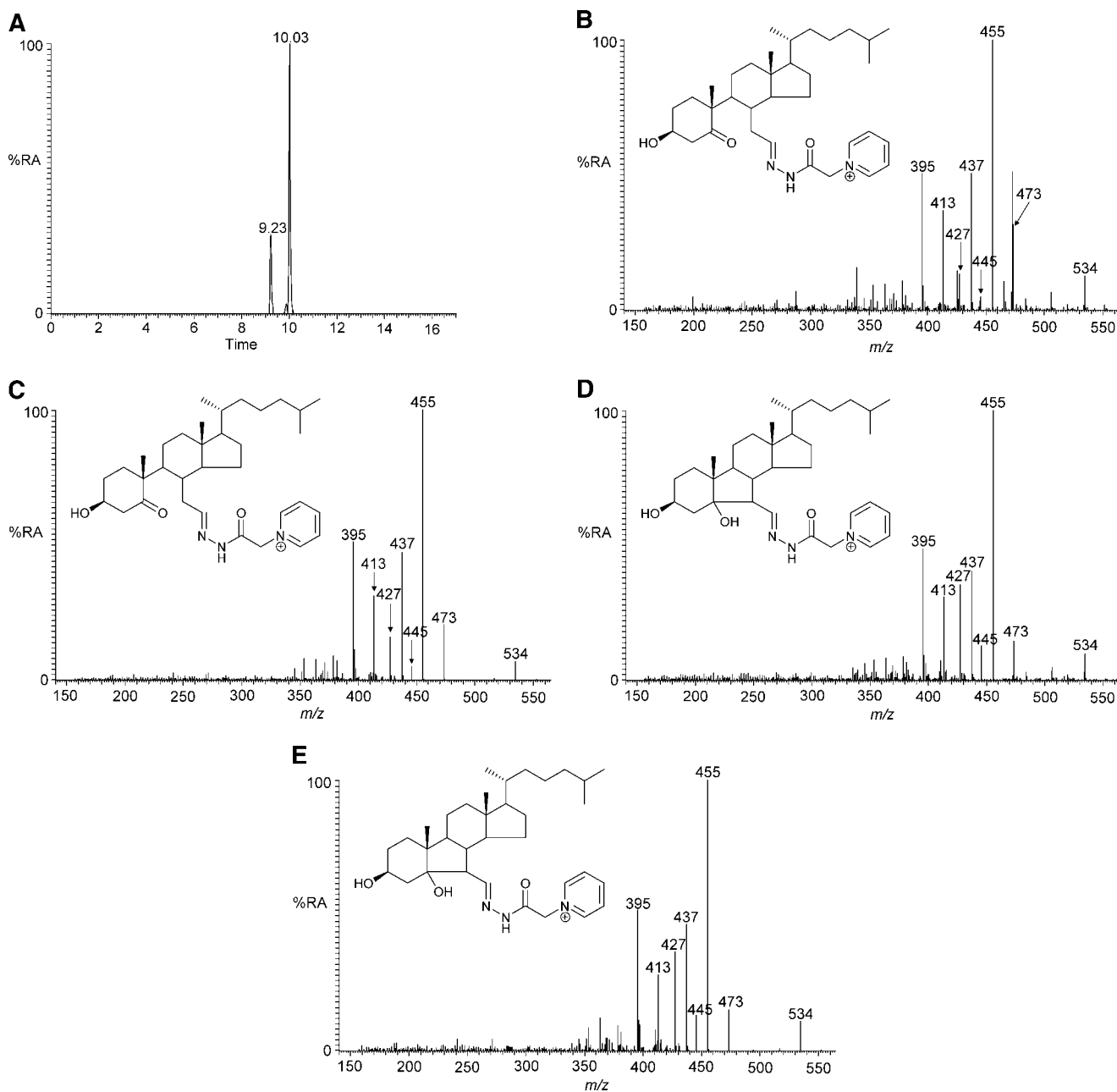



Fig. 4. RIC for m/z 552.4160 corresponding to derivatized 3β -hydroxy-5-oxo-5,6-*seco*cholestan-6-al and 3,5-dihydroxy-B-norcholestan-6-carboxyaldehyde extracted from brain (A), the MS^2 spectra of chromatographic peaks eluting at 9.23 min (B), authentic 3β -hydroxy-5-oxo-5,6-*seco*cholestan-6-al (C), 10.03 min (D), and authentic 3,5-dihydroxy-B-norcholestan-6-carboxyaldehyde (E). The RIC was recorded by FT in the Orbitrap analyzer, and MS^2 spectra were recorded by the LTQ detector.

At this level of sensitivity, it was possible to identify novel oxysterols in brain from only 50 μg of brain injected on-column, opening up avenues of investigation that will allow oxysterol analysis from distinct brain regions. The informative fragmentation, particularly in MS³, of oxidized and GP-derivatized sterols contrasts with their untreated analogs (15–17), and has allowed the identification of novel oxysterols in rat brain. Although previously observed in incubations of 24S-hydroxycholesterol with recombinant CYP46A1 or transfected cells (14), this study provides the first identification and approximate quantification of 24,25-, 24,27-, and 6,24-dihydroxycholesterols in rat brain (Table 1). Furthermore, although 24,25-, 25,27-, 7 α ,25-, and 7 α ,27- dihydroxycholesterols have been observed in incubations of 24-, 27-, 25- and 27-hydroxycholesterols with astrocytes (21), the current study is the first to identify these oxysterols in brain. Of particular interest is the high RA of 24,25-dihydroxycholesterol, which is consistent with the importance of 25-hydroxylation in astrocyte cultures (21). Additionally, here we have detected 5,6-*seco*-sterol and its aldol in rat brain. The presence of these two oxysterols in humans has been under debate (11, 12, 31), but their association with neurodegenerative disease (11, 31) highlights the importance of their analysis. With this in mind, the analytical methodology described here can easily be used for oxysterol analysis in other tissues, as well as plasma or cerebrospinal fluid (CSF). The identification of novel oxysterols in brain raises questions as to their function. Their low abundance (<2%) compared with 24S-hydroxycholesterol does not suggest that they act as transport forms of cholesterol from brain to liver; however, their turnover in brain or abundance in plasma or CSF has yet to be studied. Alternatively, like other oxysterols (6, 32), they may behave as ligands to nuclear receptors, e.g., LXR β , which, like CYP46A1, is expressed in neurons (7, 8) 

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