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Inhibitors of sterol synthesis. Chromatography of acetate derivatives of oxygenated sterols

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Abstract The separation of the acetate derivatives of a number of oxygenated sterols was achieved by medium pressure liquid chromatography on silica gel columns and by normal and reversed phase high performance liquid chromatography. We have explored the application of these chromatographic systems for the analysis of oxygenated sterols of plasma samples from two normal human subjects. The addition of highly purified [^{14}C]cholesterol to plasma permitted the detection and quantitation of oxygenated sterols formed by autoxidation of cholesterol during processing of the samples. Special attempts to suppress autoxidation of cholesterol included the use of an all-glass closed system for saponification and extraction under argon followed by rapid removal of cholesterol from the polar sterols by reversed phase medium pressure liquid chromatography. Chromatographic analyses of the [^{3}H]acetate derivatives of the polar sterols provided a sensitive approach for the detection and quantitation of the individual oxygenated sterols. Oxygenated sterols detected in plasma included cholest-5-ene-3β,26-diol, (24S)-cholest-5-ene-3β,24-diol, and cholest-5-ene-3β,7α-diol. After correction for their formation by autoxidation of cholesterol during processing of the samples, very little or none of the following sterols were observed: cholest-5-ene-3β,7β-diol, 5α,6α-epoxy-cholestan-3β-ol, 5β,6β-epoxy-cholestan-3β-ol, and cholestane-3β,5α,6β-triol, and the 25-hydroxy, 22R-hydroxy, 21-hydroxy, 20α-hydroxy, and 19-hydroxy derivatives of cholesterol. —**Kudo, K., G. T. Emmons, E. W. Casserly, D. P. Via, L. C. Smith, J. St. Pyrek, and G. J. Schroepfer, Jr.** Inhibitors of sterol synthesis. Chromatography of acetate derivatives of oxygenated sterols. *J. Lipid Res.* 1989. **30:** 1097–1111.

Supplementary key words HPLC • MPLC

The regulation of the biosynthesis of cholesterol and of other products of the metabolism of mevalonic acid is a matter of considerable importance in biology and medicine. In 1973 Kandutsch and Chen (1) made the important discovery that certain oxygenated derivatives of cholesterol were potent inhibitors of sterol synthesis in cultured mammalian cells and lowered the levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity in these cells. Highly purified cholesterol had little or no effect under the same conditions. Since the initial discovery by Kandutsch and Chen (1), a large number of oxygenated sterols have been found to be potent suppressors of sterol synthesis and of the levels of HMG-CoA reductase activity (for reviews see references 2–4). Among the oxygenated sterols found to be active in the suppression of sterol synthesis and HMG-CoA reductase activity are a number of known metabolites of cholesterol. Some of the same sterols and others are known products of the autoxidation of cholesterol (5–7). Others include oxygenated sterols that are known precursors of cholesterol and possible or probable intermediates in the biosynthesis of cholesterol. The very high potency of a number of oxygenated sterols in the regulation of sterol synthesis provides very strong stimuli for investigations of the natural occurrence of oxygenated sterols. Further impetus for these investigations derives from the possible role of oxygenated sterols, by virtue of their effects on the formation of sterols and other isoprenoids, as natural regulators of cellular replication and differentiation (2, 4).

The actual analysis of the occurrence and quantitation of the various oxygenated sterols in biological samples is a nontrivial matter, requiring not only sensitive methods for the detection of the oxygenated sterols but also efficient methods for the separation of the concerned compounds. In addition, since cholesterol and its esters are present in relatively high concentrations in plasma and tissues, considerable attention must be directed towards the suppression of autoxidation of cholesterol present in

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HPLC, high performance liquid chromatography; MPLC, medium pressure liquid chromatography; GLC, gas-liquid chromatography; TLC, thin-layer chromatography; MTBE, methyl *t*-butyl ether; NSL, nonsaponifiable lipids.

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the sample and an estimation of the amount of oxygenated sterols formed by autoxidation of cholesterol during the procedures utilized for the isolation and quantitation of the oxygenated sterols. Consideration of this important matter is critical for studies of the natural occurrence of oxygenated sterols in blood, tissues, and in other materials such as natural or derived food substances.

Described herein is an approach which is based upon the chromatographic separation of acetate derivatives of the various sterols, coupled with careful attempts to suppress autoxidation of cholesterol in the sample, for the investigation of the natural occurrence and quantitation of oxygenated sterols in plasma of human subjects. The use of acetate derivatives for the chromatography of the oxygenated sterols represents an extension of our previous use of acetate derivatives of monohydroxysterols for their separation by chromatography on columns of alumina and silica gel impregnated with $AgNO_3$ (8-16), by high performance liquid chromatography (HPLC) (17), by medium pressure liquid chromatography (MPLC) on columns of alumina-$AgNO_3$ (18-22), and by gas-liquid chromatography (GLC) (8-12). Moreover, the use of ^3H-labeled acetic anhydride for the formation of the acetate derivatives provided not only a simple method to follow the separation of the various oxygenated sterols but also a sensitive approach for the quantitation of the various sterols. Detection and estimation of the amounts of oxygenated sterols formed by autoxidation of cholesterol during procedures used for the isolation of the oxygenated sterols were made by the addition of a known amount of very highly purified [^{14}C]cholesterol prior to any processing of the sample.

MATERIALS AND METHODS

General

Melting points (mp) were recorded as described previously (23). Colorimetric analyses of steryl acetates were made with the Liebermann-Burchard reagent described by Abell et al. (24). Thin layer chromatography (TLC) was carried out on plates (20 cm × 5 cm) of silica gel G (250 μm in thickness) which were purchased from Analtech, Inc. (Newark, DE). Components on the plates were visualized after spraying with molybdic acid (25). Radio-TLC analyses were made as previously described (26). Radioactivity was assayed with Beckman LS-9000, Beckman LS 9800, and Packard Tri-Carb 4640 liquid scintillation spectrometers. The scintillation fluid was 2,5-diphenyloxazole (0.4%) and 1,4-bis[2-(5-phenyl-1,3-oxazolyl)]-benzene (0.05%) in toluene (SintillAR grade; Mallinckrodt, Inc., Los Angeles, CA) or 2,5-diphenyloxazole (0.4%) in toluene-ethanol 2:1. Silica gel (60-200 mesh) for column chromatography was obtained from J. T. Baker Chemical Company (Phillipsburg, NJ). Silica gel (32-63 μm) for MPLC (100 cm × 1.5 cm) was purchased from Universal Scientific, Inc. (Atlanta, GA). Alumina-$AgNO_3$ (16.7% $AgNO_3$ by weight) for MPLC was prepared as described previously (18). Reversed phase MPLC was performed with two LiChroprep RP-8 columns (240 mm × 10 mm), purchased from EM Science (Darmstadt, West Germany), connected in tandem. The following Burdick and Jackson solvents, purchased from American Scientific Products (McGraw, IL) were deoxygenated with argon prior to use: hexane, ethyl acetate, benzene, methyl *t*-butyl ether (MTBE), isopropyl alcohol, and methanol. Pyridine (Mallinckrodt; Los Angeles, CA) was distilled, under nitrogen, from barium oxide prior to use. Argon (99.9995% minimum purity) was obtained from Matheson (Secaucus, NJ) and further deoxygenated with a Matheson gas purifier (model 6406) prior to use.

HPLC analyses were made with one of two combinations of equipment. System 1 was composed of a Waters 6000 pump, U6K injector, and an ALC/GPC 501 differential refractometer. System 2 was composed of a Waters 501 pump, U6K injector, automated gradient controller, and a Lambda-Max Model 481 ultraviolet detector. The individual components were purchased from Waters Associates, Inc. (Milford, MA). Unless specified otherwise, HPLC analyses were made with system 1. The HPLC columns used were 5 μm Spherisorb silica gel (250 mm × 4.6 mm), semipreparative 5 μm Spherisorb silica gel (250 mm × 9.2 mm), 5 μm Spherisorb ODS-II (250 mm × 4.6 mm), 3 μm Spherisorb ODS-II (150 mm × 6.2 mm), and a 5 μm semi-preparative Spherisorb ODS-II (250 mm × 9.2 mm), all of which were purchased from Custom LC, Inc. (Houston, TX). Also used was an 8 μm Dynamax C_{18} column (250 mm × 10 mm) which was purchased from Rainin Instrument Co., Inc. (Woburn, MA). Unless specified otherwise, all normal phase analytical HPLC and reversed phase analytical HPLC utilized columns (250 mm × 4.6 mm) of 5 μm Spherisorb silica gel or 5 μm Spherisorb ODS-II, respectively. GLC analyses were made on a Hewlett-Packard Model 5730 equipped with a flame ionization detector and a 0.1 μm DB-5 fused silica capillary column (0.25 mm × 30 m; J. & W. Scientific; Rancho Cordova, CA). Combined GLC-mass spectrometry (GLC-MS) analyses were performed with a Shimadzu QP-1000 unit (Shimadzu Corporation; Kyoto, Japan) equipped with a Shimadzu GC9A chromatograph containing a 0.1 μm DB-5 fused silica capillary column (0.25 mm × 30 m; J. & W. Scientific). Mass spectra were measured under electron impact conditions at 20 eV.

[1-^{14}C]Acetic anhydride (25.4 mCi/mmol) and [^3H]acetic anhydride (50 mCi/mmol) were purchased from Amersham (Arlington Heights, IL) and New England Nuclear (Boston, MA), respectively. [4-^{14}C]Cholesterol

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(56 mCi/mmol), [1α,2α-^{3}H]cholesterol (44 Ci/mmol), and [^{3}H]sodium borohydride (6.9 Ci/mmol) were purchased from Amersham. For the studies described herein, highly purified [4-^{14}C]cholesterol was required. Accordingly, the labeled cholesterol (200–680 μCi) was diluted with unlabeled cholesterol (3.2–10.1 mg) and subjected to HPLC. Two semi-preparative normal phase or two semi-preparative reversed phase purifications were used. The resulting purified [^{14}C]cholesterol had a radiopurity in excess of 99.9% as judged by radio-TLC analyses (solvent system, 50% ethyl acetate in hexane containing butylated hydroxytoluene (0.1%)). The total amounts of ^{14}C detected in seven 1-cm scrapings with mobilities less than that of [^{14}C]cholesterol ($\sim 5.0 \times 10^5$ dpm) were less than 85 dpm. The highly purified [4-^{14}C] cholesterol was used immediately in the analytical studies of plasma described herein. [1α,2α-^{3}H]Cholesterol was purified by silica gel column chromatography (solvent, toluene) prior to use.

Cholesterol was purified by way of its dibromide derivative (27) and recrystallization from acetone–water. This material showed a single component (>99%) on normal phase HPLC (solvent, 25% ethyl acetate in hexane) and on reversed phase HPLC (solvent, methanol). 5α-Cholestan-3β-ol (mp 142.5–143.0°C [lit. 140–141°C (28)]), was prepared by catalytic reduction of cholesteryl acetate (28) followed by saponification with 0.4 N KOH in 95% ethanol and two recrystallizations from methanol. The purified material showed a single component (>99%) on TLC (solvent, 50% ethyl acetate in hexane), on normal phase HPLC (solvent, 25% ethyl acetate in hexane), and on reversed phase HPLC (solvent, methanol). 5α-Cholestan-3β-ol was further recrystallized from hexane prior to use to avoid formation of the hydrate of the free sterol. 3β-Acetoxy-cholest-5-en-7-one was purchased from Aldrich Chemical Company (Milwaukee, WI) and purified twice by silica gel column chromatography using mixtures of ethyl acetate in benzene (1:30 and 1:40) as the eluting solvent. The purified material, after recrystallization from $CHCl_3$, melted at 162–163°C (lit. 163–164°C (29)) and showed a single component (>99%) on TLC (solvent, 5% ethyl acetate in benzene), on normal phase HPLC (solvent, 7% ethyl acetate in hexane), and on reversed phase HPLC (solvent, methanol). Cholest-5-ene-3β,7α-diol (mp 183–184°C [lit. 183–184°C (30)]) and cholest-5-ene-3β,7β-diol (mp 180.5–181.0°C [lit. 172–175°C (31) and 176–182°C (32)]) were prepared by reduction of 3β-hydroxy-cholest-5-en-7-one with sodium borohydride (31) followed by resolution of the two 7-hydroxy epimers by silica gel column chromatography (solvent, hexane–ethyl acetate 3:2) and recrystallization of the purified sterols from methanol. Each of the 7-hydroxysterols showed a single component (>99%) on TLC (solvent, 50% ethyl acetate in hexane) and on reversed phase HPLC (solvent, 2% water in methanol). 3β-Acetoxy-5α,6α-epoxy-cholestane (mp 97–98°C, clearing point, 117°C [lit. 101°C (33)]) and 3β-acetoxy-5β,6β-epoxy-cholestane (mp 112–113°C [lit. 113°C (33)]) were prepared by epoxidation of cholesterol (34), acetylation of the resulting product with a mixture of acetic anhydride and pyridine, resolution of the two epoxysteryl acetates by MPLC on an alumina-$AgNO_3$ column (18) using 3% ethyl acetate in hexane as the eluting solvent, and recrystallization from methanol. Each of the epoxysteryl acetates showed a single component (>99%) on TLC (solvent, 1% acetone in benzene), on normal phase HPLC (solvent, 5% ethyl acetate in hexane), and on reversed phase HPLC (solvent, methanol).

A mixture of (24R)-cholest-5-ene-3β,24-diol and (24S)-cholest-5-ene-3β,24-diol was prepared from fucosterol (35) as follows. Fucosterol (80 mg; Sigma Chemical Company; St. Louis, MO) was acetylated with a mixture of acetic anhydride and pyridine and, after purification by silica gel column chromatography (solvent, hexane–$CHCl_3$ 3:1), gave fucosteryl acetate (61 mg) which showed a single component on TLC (solvent, hexane–ether 2:1). The fucosteryl acetate in CH_2Cl_2 was subjected to ozonolysis at −78°C and the ozonide was reduced with sodium borohydride in methanol to give (24RS)-3β-acetoxy-cholest-5-en-24-ol (9.7 mg). Saponification (1 N KOH in 95% ethanol at room temperature) gave, after silica gel column chromatography (solvent, 20% ethyl acetate in hexane), (24RS)-cholest-5-ene-3β,24-diol (7.1 mg) which showed a single component (>99%) on TLC (solvent, 50% ethyl acetate in hexane) with the same mobility as an authentic sample of (24S)-cholest-5-ene-3β,24-diol. (24S)-Cholest-5-ene-3β,24-diol and (24R)-cholest-5-ene-3β,24-diol were not separable on TLC or on reversed phase HPLC (solvent, 2% water in methanol). The corresponding diacetates were also not resolved on TLC (solvent, 10% ethyl acetate in hexane) or on normal phase HPLC (solvent, 3.5% ethyl acetate in hexane). A partial resolution of the diacetate derivatives of the two epimeric diols was achieved on reversed phase HPLC (solvent, methanol). (24R)-3β-Acetoxy-24,25-epoxy-cholest-5-ene (mp 110.5–112.5°C) and (24S)-3β-acetoxy-24,25-epoxy-cholest-5-ene (mp 114.5–116.5°C) were prepared as described previously (36). Each epimer showed a single component (>99%) on normal phase HPLC (solvent, 5% ethyl acetate in hexane) and reversed phase HPLC (solvent, 2% water in methanol). (24R)-3β-Acetoxy-24,25-epoxy-lanost-8-ene (mp 194.0–195.5°C [lit. 194–197°C (37)]) and (24S)-3β-acetoxy-24,25-epoxy-lanost-8-ene (mp 140.0–141.5°C, [lit. 137–139°C, remelted at 143.5–144.5°C (37)]) were prepared from commercial lanosterol (Mann Research Lab., Inc., New York, NY) by a published procedure (38) and purified as described

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previously (36). (20S)-Cholest-5-ene-3β,21-diol (single component (>99%) on TLC; solvent, 50% ethyl acetate in hexane), (20S)-3β,21-diacetoxy-cholest-5-ene (single component (>99%) on TLC; solvent, 10% ethyl acetate in hexane, on normal phase HPLC; solvent, 5% ethyl acetate in hexane, and on reverse phase HPLC; solvent, methanol), and a mixture of (20S)-3β,21-diacetoxy-cholest-5-ene and (20R)-3β,21-diacetoxy-cholest-5-ene were prepared according to the procedure of Wicha and Bal (39).

Samples of cholest-5-ene-3β,20α-diol (single component (>99%) on TLC; solvent, 50% ethyl acetate in hexane) were obtained from Sigma Chemical Company and Steraloids, Inc. (Wilton, NH). Cholest-5-ene-3β,19-diol (Sigma Chemical Company) showed a single component (>99%) on reversed phase HPLC. (22R)-Cholest-5-ene-3β,22-diol (single component on TLC; solvent, 50% ethyl acetate in hexane) was obtained from Sigma Chemical Company. Cholestane-3β,5α,6β-triol, purchased from Steraloids, showed a single component (>99%) on TLC (solvent, 50% ethyl acetate in hexane) and on reversed phase HPLC (solvent, 2% water in methanol). (25R)-Cholest-5-ene-3β,26-diol, a generous gift from Syntex, Inc. (Palo Alto, CA), showed a single component (>99%) on TLC (solvent, 50% ethyl acetate in hexane) and on reversed phase HPLC (solvent, 2% water in methanol). (24S)-Cholest-5-ene-3β,24-diol, a generous gift from Professor N. Ikekawa (Tokyo, Japan), showed a single component (>99%) on reversed phase HPLC (solvent, 2% water in methanol).

Acetate derivatives of the free sterols listed above were prepared by treatment with acetic anhydride and pyridine and purified by silica gel column chromatography and/or recrystallization. The purity of each of the acetylated sterols was in excess of 99% as judged by normal phase HPLC (solvent, 1 to 10% ethyl acetate in hexane) and reversed phase HPLC (solvent, methanol or 2% water in methanol, or 10% isopropyl alcohol in methanol). Labeled (^{3}H or ^{14}C) acetate derivatives of authentic samples of the various sterols were prepared by treatment of the free sterols with [^{3}H]acetic anhydride or [1-^{14}C]acetic anhydride in pyridine and purified by silica gel column chromatography and/or recrystallization. The identity of the labeled acetylated derivatives of the sterols was confirmed by their comigration with the corresponding authentic unlabeled steryl acetate derivatives. The radiopurity of each labeled acetylated derivative of the sterols was checked by normal phase HPLC and reversed phase HPLC prior to use. The following ^{3}H-labeled steryl acetate derivatives (specific activity ~8-42 mCi/mmol) were prepared: 3β-acetoxy-cholest-5-ene (I), 3β-acetoxy-5α-cholestane (II), (22R)-3β,22-diacetoxy-cholest-5-ene (III), (24RS)-3β,24-diacetoxy-cholest-5-ene (IV), (25R)-3β,26-diacetoxy-cholest-5-ene (V), 3β-acetoxy-5β,6β-epoxy-cholestane (VI), 3β-acetoxy-5α,6α-epoxy-cholestane (VII), 3β,7α-diacetoxy-cholest-5-ene (VIII), 3β,7β-diacetoxy-cholest-5-ene (IX), 3β-acetoxy-cholest-5-en-20α-ol (X), 3β-acetoxy-cholest-5-en-7-one (XI), 3β-acetoxy-cholest-5-en-25-ol (XII), and 3β,6β-diacetoxy-cholestan-5α-ol (XIII) (see Fig. 1). The following ^{14}C-labeled steryl acetate derivatives (specific activity ~0.2-1.0 μCi per mCi/mmol) corresponding to III, IV, V, VII, VIII, IX, X, XI, XII, and XIII were prepared. In addition, authentic (24S)-3β,24-[1-^{14}C]diacetoxy-cholest-5-ene was prepared.

3β-Acetoxy-[1α,2α-^{3}H]cholest-5-en-7-one was prepared as follows. [1α,2α-^{3}H]Cholesterol (0.99 mCi/mmol) was acetylated with acetic anhydride-pyridine 1:1. The resulting acetate was recrystallized from ethyl acetate and showed a single component (>99%) on TLC with the same mobility as that of authentic cholesteryl acetate (solvent system, hexane-ethyl acetate 9:1). Oxidation of the steryl acetate with 3,5-dimethylpyrazole-chromium trioxide complex (40) gave 3β-acetoxy-[1α,2α-^{3}H]cholest-5-en-7-one which, after purification by silica gel column chromatography (solvent system, increasing concentrations (0-10%) of ethyl acetate in hexane) and reversed phase HPLC on a Dynamax C_{18} column (solvent, methanol), showed a single labeled component (>99%) on HPLC on a 5 μm Spherisorb column (solvent, 8% ethyl acetate in hexane). 3β-Acetoxy-5α,6α-epoxy-[1α,2α-^{3}H]cholestane and 3β-acetoxy-5β,6β-epoxy-[1α,2α-^{3}H]-cholestane (300 mCi/mmol) were prepared from [1α,2α-^{3}H]cholesterol as described above for the cases of the unlabeled epoxysterols. The two labeled epoxysteryl acetates had the same mobilities as the corresponding authentic samples of the 5α,6α- and 5β,6β-epoxysteryl acetates and had radiopurities in excess of 99% on HPLC analyses on a 5 μm Spherisorb ODS-II column (solvent, methanol). 3β,7α-Diacetoxy-[7β-^{3}H]cholest-5-ene and 3β,7β-diacetoxy-[7α-^{3}H]cholest-5-ene were prepared by reduction of 3β-acetoxy-cholest-5-en-7-one (7.8 mg) with an excess of [^{3}H]$NaBH_4$ followed by acetylation and separation of the resulting 3β,7α- and 3β,7β-diacetates by alumina-$AgNO_3$ column chromatography (vide infra). The isolated labeled diacetates had the same mobilities as the corresponding authentic samples of the 3β,7α- and 3β,7β-diacetates and had radiopurities in excess of 99% on HPLC analyses on a 5 μm Spherisorb ODS-II column (solvent, 98% methanol in water). Each epimer was diluted with the appropriate authentic unlabeled sample to give a specific activity of 310 mCi/mmol.

Plasma samples were collected from healthy human volunteers through the Methodist Hospital Blood Donor Center under protocols approved by the Human Experimentation Committees of Methodist Hospital, the Baylor College of Medicine, and Rice University. The samples were stored overnight at -70°C prior to analysis.

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Chromatography of acetate derivatives of oxygenated sterols

The availability of the acetate derivatives of the various oxygenated sterols in unlabeled form and as [^{3}H]- and/or [^{14}C]acetate derivatives greatly facilitated the exploration of various chromatographic columns and solvent systems.

The separation of the acetate derivatives of authentic samples of the various oxygenated sterols by MPLC on a column (100 cm × 1.5 cm) of silica gel is shown in **Fig.1.** The chromatogram shows that the use of this MPLC system permitted the resolution of cholesteryl acetate (I) from the acetate derivatives of the oxygenated sterols. Moreover, this system provided excellent separations of most of the acetate derivatives of the oxygenated sterols. However, an incomplete separation of the diacetate derivative of the Δ^5-3β,26-diol (V) and the acetate derivative of the 5β, 6β-epoxysterol (VI) was observed. In addition, no separation of the diacetate derivatives of the Δ^5-3β,7α-diol (VIII) and the Δ^5-3β,7β-diol (IX) was achieved under these conditions.

MPLC on columns of alumina-$AgNO_3$ was also explored for the separation of the acetate derivatives of selected oxygenated sterols. In contrast to the case of MPLC on silica gel, MPLC on a column (1.5 cm × 100 cm; solvent, 7% ethyl acetate in hexane) of alumina-$AgNO_3$ provided a striking resolution of the diacetate derivatives of cholest-5-ene-3β,7α-diol (VIII) and cholest-5-ene-3β,7β-diol (IX) (**Fig. 2**). MPLC on an alumina-$AgNO_3$ column (1.5 cm × 100 cm; solvent, 3% ethyl acetate in hexane) also gave a very marked separation of the acetate derivatives of 5α,6α-epoxy-cholestan-3β-ol (VII) and 5β,6β-epoxy-cholestan-3β-ol (VI) (Fig. 2). Aringer and Eneroth (41) previously described the separation of the acetate derivatives of the 5α,6α- and 5β,6β-epoxides of cholesterol by TLC on plates of silica gel pretreated with $AgNO_3$ in concentrated NH_4OH.

Separations of the acetate derivatives of the various oxygenated sterols by normal phase HPLC (solvent, 5% ethyl acetate in hexane) on a 5 μm Spherisorb column (4.6 m × 250 mm) are summarized in **Table 1**. Under these conditions, cholesteryl acetate (I) and 3β-acetoxy-5α-cholestane (II) had the same retention time and only a slight separation of the diacetate derivatives of the Δ^5-3α,7α-diol (VIII) and the Δ^5-3β,7β-diol (IX) was achieved. No resolution of the R and S epimers of 24-hydroxycholesteryl diacetate (IV) was observed.

Reversed phase HPLC (solvent, methanol) on a 5 μm Spherisorb ODS-II column (4.6 mm × 250 mm) provided separations of the acetate derivatives of the various oxygenated sterols which were complimentary and, in some cases, superior to those achieved by normal phase HPLC (Table 1). Especially notable was the resolution of the diacetate derivatives of the Δ^5-3β,7α-diol (VIII) and the Δ^5-3β,7β-diol (IX). Under these conditions, only a slight separation of the R and S epimers of 24-hydroxycholesteryl diacetate (IV) was observed. It should be noted that using a 3 μm Spherisorb ODS-II column, very

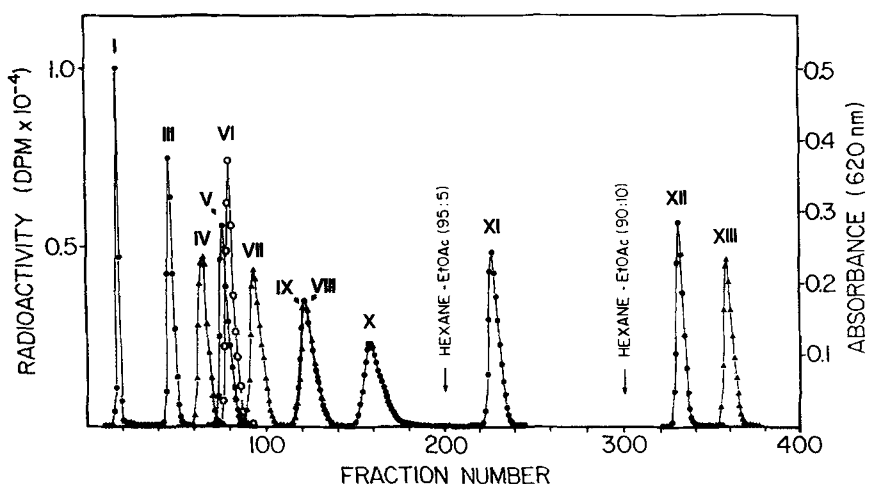

Fig. 1. MPLC of acetate derivatives of oxygenated sterols on column (100 cm × 1.5 cm) of silica gel. Using 3% ethyl acetate in hexane as the initial eluting solvent, fractions 15 ml in volume (4.3 ml/min) were collected. At fractions 200 and 300, the eluting solvent was changed to 5% ethyl acetate in hexane and 10% ethyl acetate in hexane, respectively. The individual steryl acetates were (in order of elution) 3β-[^{3}H]acetoxy-cholest-5-ene (I) (22R)-3β,22-[^{3}H]diacetoxy-cholest-5-ene (III), (24S)-3β-[1-^{14}C]diacetoxy-cholest-5-ene (IV), (25R)-3β,26-[^{3}H]diacetoxy-cholest-5-ene (V), 3β-acetoxy-5β,6β-epoxy-cholestane (VI), 3β-acetoxy-[1-^{14}C]5α,6α-epoxy-cholestane (VII), 3β,7α-[^{3}H]diacetoxy-cholest-5-ene (VIII), 3β,7β-[^{3}H]diacetoxy-cholest-5-ene (IX), 3β-[^{3}H]acetoxy-cholest-5-en-20α-ol (X), 3β-[^{3}H]acetoxy-cholest-5-en-7-one (XI), 3β-[^{3}H]acetoxy-cholest-5-en-25-ol (XII), and 3β,6β-[1-^{14}C]diacetoxy-cholestan-5α-ol (XIII). The amounts applied were: VI, 7mg; I, 5 × 10^4 dpm; and ~10^5 dpm for each of III, IV, V, VII, VIII, IX, X, XI, XII, and XIII; (●), ^{3}H; (△), ^{14}C; (○), steryl acetate measured colorimetrically.

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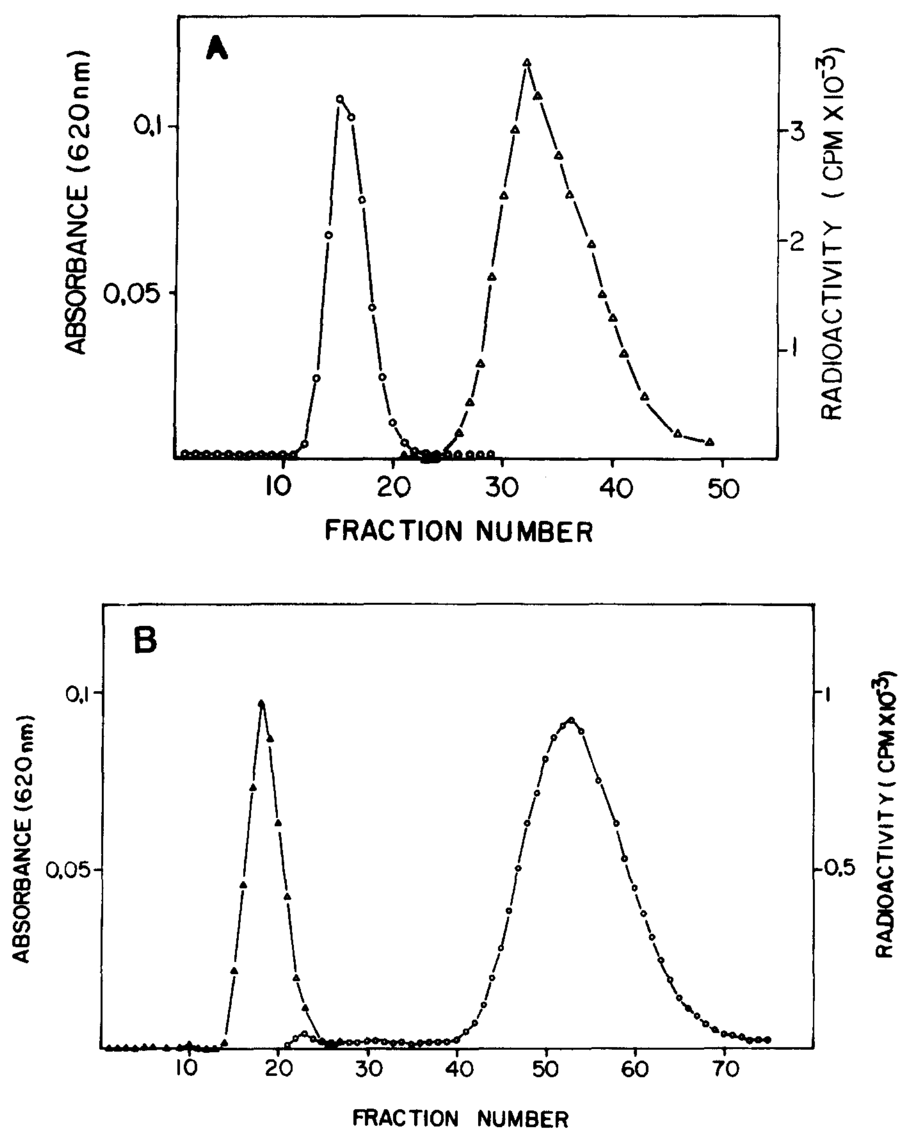

Fig. 2. MPLC of acetate derivatives of oxygenated sterols on a column (100 cm × 1.5 cm) of alumina-$AgNO_3$. A (above): separation of 3β,7α-[^{3}H]diacetoxy-cholest-5-ene (3.5 × 10^5 cpm) and 3β,7β-diacetoxy-cholest-5-ene (~7 mg). Using 7% ethyl acetate in hexane as the eluting solvent, fractions were collected every 13 min at a flow rate of 2.45 ml/min. B (below): separation of 3β-acetoxy-5β,6β-epoxy-cholestane (~5 mg) and 3β-[^{3}H]acetoxy-5α,6α-epoxy-cholestane (3.5 × 10^5 cpm). Using 3% ethyl acetate in hexane as the eluting solvent, fractions were collected every 13 min at a flow rate of 2.45 ml/min; (○), radioactivity; (△), steryl acetate measured colorimetrically.

useful (albeit not complete) separation of the derivatives of the epimers of 24-hydroxycholesterol can be achieved (vide infra).

Analysis of oxygenated sterols in plasma by chromatography of acetate derivatives

The results presented above demonstrate that the separation of the acetate derivatives of a large number of oxygenated sterols can be achieved by chromatography. Preparation of ^{3}H-labeled acetate derivatives of the various oxygenated sterols, using [^{3}H]acetic anhydride of known specific activity, could be used not only to follow the separation of the oxygenated sterols but also to provide a sensitive approach for the quantitation of the individual oxygenated sterols. Since plasma contains substantial amounts of cholesterol and cholesteryl esters, measures were required not only to suppress autoxidation but also to detect and estimate the amounts of oxygenated sterols formed by autoxidation of cholesterol during the procedures used for the isolation and identification of the oxygenated sterols. The addition of a known amount of very highly purified [^{14}C]cholesterol to plasma prior to any processing of the sample provided an internal control

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TABLE 1. Retention times of acetate derivatives of oxygenated sterols upon HPLC

Designation	Name	Retention Time	
		Normal Phase[a]	Reversed Phase[b]
I	3β-Acetoxy-cholest-5-ene	5.81	37.42
II	3β-Acetoxy-5α-cholestane	5.81	42.26
III	(22R)-3β,22-Diacetoxy-cholest-5-ene	9.03	12.58
IV	(24S)-3β,24-Diacetoxy-cholest-5-ene	11.13	14.84
V	(25R)-3β,26-Diacetoxy-cholest-5-ene	12.26	18.06
VI	3β-Acetoxy-5β,6β-epoxy-cholestane	14.84	15.97
VII	3β-Acetoxy-5α,6α-epoxy-cholestane	16.29	18.06
VIII	3β,7α-Diacetoxy-cholest-5-ene	18.20	13.39
IX	3β,7β-Diacetoxy-cholest-5-ene	17.90	14.03
X	3β-Acetoxy-cholest-5-en-20α-ol	20.48	8.71
XI	3β-Acetoxy-cholest-5-en-7-one	34.19	11.61
XII	3β-Acetoxy-cholest-5-en-25-ol	N.D.[c]	10.61
XIII	3β,6β-Diacetoxy-cholestan-5α-ol	N.D.	10.17
XIV	3β,19-Diacetoxy-cholest-5-ene	16.06	N.D.
XV	(20S)-3β,21-Diacetoxy-cholest-5-ene	14.20	N.D.
XVI	(24RS)-3β-Acetoxy-24,25-epoxy-cholest-5-ene	N.D.	13.75
XVII	(24RS)-3β-Acetoxy-24,25-epoxy-lanost-8-ene	N.D.	15.00

[a]Column, 5 μm Spherisorb (4.6 mm × 250 mm); elution solvent, 5% ethyl acetate in hexane at flow rate of 0.8 ml/min.
[b]Column, 5 μm Spherisorb ODS-II (4.6 mm × 250 mm); elution solvent, methanol at flow rate of 0.8 ml/min.
[c]Not determined.

whereby the amounts of oxygenated sterols formed by autoxidation of cholesterol during the processing of the sample could be estimated. The use of this approach also greatly facilitated the development of procedures for the analysis of oxygenated sterols in plasma. The results of a number of experiments indicated that, despite standard attempts to suppress autoxidation, substantial oxidation of the [^{14}C]cholesterol occurred during the processing (extraction, saponification, acetylation, etc.) of the plasma samples. On the basis of the results of these studies, a number of major modifications of standard procedures or approaches were adopted. An all-glass closed system was constructed which permitted saponification and extraction of the nonsaponifiable lipids (NSL) under anaerobic conditions (**Fig. 3**; vide infra). It was also recognized that removal of as much of the cholesterol as possible at a very early stage in the processing of the sample would markedly reduce the formation of oxygenated sterols by autoxidation of cholesterol. Accordingly, reversed phase MPLC of the NSL was used to isolate the polar sterols of plasma. This approach not only reduced the possibility of the formation of oxygenated sterols during subsequent processing of the sample but also greatly facilitated subsequent HPLC analyses. Prior to acetylation of the polar sterols with [^{3}H]acetic anhydride, a known amount of 5α-cholestan-3β-ol was added and the resulting labeled 3β-acetoxy-5α-cholestane not only served as a chromatographic marker in subsequent HPLC but also a compound for which the specific activity of the labeled acetic anhydride could be determined.

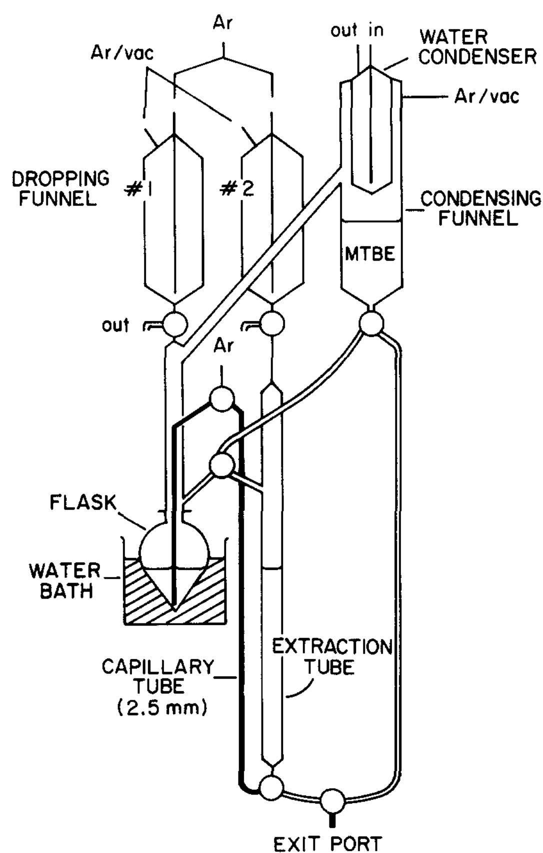

Fig. 3. Diagram of all-glass closed system for the saponification and extraction of NSL under an atmosphere of argon. Not shown is the complex valving and manifold system for argon (Ar) and Vacuum (vac) lines.

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Experiment A

Freshly purified [4-^{14}C]cholesterol (265 μCi; 12.3 mg) of high radiopurity (see above) in absolute ethanol (1 ml) was added to a sample (15 ml) of plasma from a healthy 26-yr-old male subject (total plasma cholesterol, 136 mg/dl). The plasma sample was transferred to an all-glass system (Fig. 3) which permitted distillation of solvents, hydrolysis of samples, extraction of NSL, and evaporation of solvents under a constant positive argon pressure. All solvents and solutions were carefully deoxygenated in the dropping funnels prior to introduction into the system. The system was alternately degassed under reduced pressure and then filled with argon three times. After the addition of a solution ($\sim$30 ml) of 10% KOH in 95% ethanol to the flask from dropping funnel #1, the flask was heated at 80–85°C in a water bath for 15 min (under a constant flow of argon). After cooling to room temperature, water ($\sim$15 ml) was added via dropping funnel #1 and the solution was transferred to the extraction tube. The flask was rinsed twice with small quantities ($\sim$4 ml) of previously distilled MTBE which were introduced from the condensing funnel and the resulting ether washes were transferred to the extraction tube. The extraction tube was isolated by closing the appropriate stopcocks, the flask was replaced, and the system was then degassed and purged with argon. Continuous extraction of the solution in the extraction tube was achieved by flowing MTBE from the condensing funnel through the solution ($\sim$120 drops per min), collection of the organic layer in the flask, and redistillation of the solvent. The extraction was continued for 2 h. After stopping the flow of MTBE through the solution, the lower layer in the extraction tube was removed through the exit port. The remaining organic layer was transferred to the flask and the volume of the MTBE extract in the flask was reduced to $\sim$20–30 ml by distillation. At the same time, a 0.4 N KOH solution was introduced into the dropping funnel #2 and degassed with argon. After cooling to room temperature, the MTBE solution was transferred from the flask to the extraction tube and washed with the 0.4 N KOH solution by dropwise addition from dropping funnel #2 ($\sim$6 drops/min). The water layer was continuously discarded through the exit port. The MTBE solution was then washed with deoxygenated water in the same manner. A clean 500-ml pear-shaped flask was provided and, after evacuation and flushing of this portion of the system with argon, the MTBE solution in the extraction tube was transferred to the flask as described above. The extraction tube was rinsed with a small amount of MTBE from the condensing funnel and this was also transferred to the flask. The solvent was removed under reduced pressure and the resulting residue was dissolved in methanol ($\sim$2 ml), introduced from dropping funnel #1, and removed from the apparatus for immediate reversed phase MPLC and assay of ^{14}C. Recovery of ^{14}C in the NSL was 97.5%.

Reversed phase MPLC was carried out to remove cholesterol and other nonpolar components from the oxygenated sterols. Over 99.8% of the recovered ^{14}C corresponded to the mobility of cholesterol. The contents of early fractions (containing less than 0.04% of the ^{14}C recovered from the column) were, after the addition of 5α-cholestan-3β-ol (0.5 mg), treated with [^{3}H]acetic anhydride (25 mCi; $\sim$500-fold excess relative to 5α-cholestan-3β-ol) in pyridine (1 ml) under argon in a sealed vial. After standing overnight at room temperature, the mixture was heated for 10 min at 60°C. Additional unlabeled acetic anhydride (1 ml) was added, and the resulting mixture was again heated at 60°C for 3 h. After cooling to room temperature, ice water (15 ml) was added and the resulting mixture was extracted 3 times with MTBE (10-ml portions). The ether solution was washed 3 times with water (10-ml portions) and evaporated to dryness under reduced pressure. The resulting acetylated material ($\sim$132 μCi ^{3}H and $\sim$0.18 μCi ^{14}C) was, after the addition of unlabeled samples of the acetate derivatives of the oxygenated sterols, subjected to reversed phase HPLC (5 μm Spherisorb ODS-II; solvent, methanol at a flow rate of 0.8 ml/min) which provided a separation of the 3β-acetoxy-5α-cholestane (II) (retention time $\sim$42 min) from the acetate derivatives of the oxygenated sterols. Fractions corresponding to the mobilities of the various unlabeled steryl acetates on reversed phase HPLC were then subjected to normal phase HPLC (5μm Spherisorb; solvent, 5% or 8% ethyl acetate in hexane at a flow rate of 0.8 ml/min). For example, **Fig. 4** shows the normal phase HPLC of the ^{3}H-labeled material with the chromatographic mobility of 3β,26-diacetoxy-cholest-5-ene on reversed phase HPLC. In some cases, fractions derived from the normal phase HPLC were then analyzed by reversed phase HPLC (system 2) (3 μm Spherisorb ODS-II; solvent, methanol at a flow rate of 0.8 ml/min).

Experiment B

Highly purified [4^{14}C]cholesterol (257 μCi; 5 mg) was added to a sample (30 ml) of plasma from a healthy 29-yr-old male subject (total plasma cholesterol, 193 mg/dl). The sample was processed as described above for experiment A and the NSL (96% recovery of ^{14}C) was subjected to reversed phase MPLC to remove cholesterol (98.3% of recovered ^{14}C). The contents of early fractions (containing $\sim$0.02% of the recovered ^{14}C) were pooled and one half of this material was, after the addition of 5α-cholestan-3β-ol (171 μg), acetylated with [^{3}H]acetic anhydride as described above. The resulting acetylated material ($\sim$164 μCi ^{3}H; 0.62 μCi ^{14}C) was, after the addition of appropriate unlabeled steryl acetate standards, then

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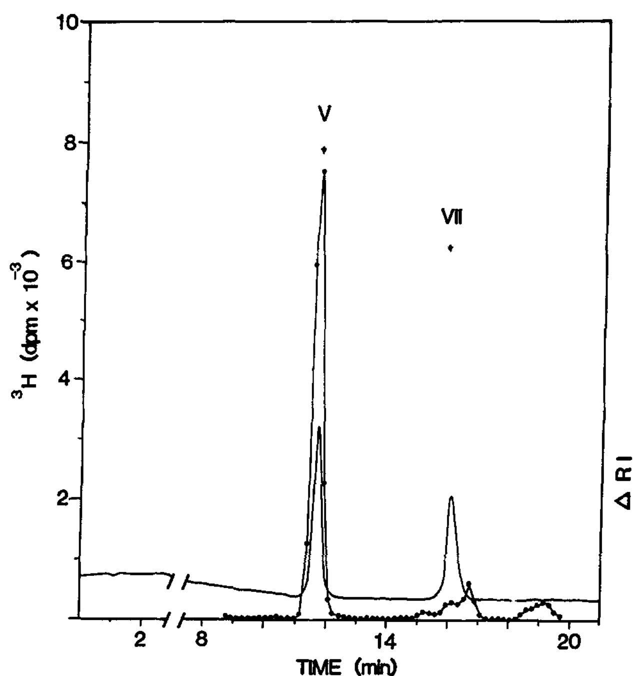

Fig. 4. Normal phase HPLC analysis of ^{3}H-labeled material with the chromatographic behavior of 3β,26-diacetoxy-cholest-5-ene (V) and 3β-acetoxy-5α,6α-epoxy-cholestane (VII) obtained from plasma after saponification, extraction, reversed phase MPLC of the NSL on an RP-8 column, acetylation with [^{3}H]acetic anhydride, and reversed phase HPLC on a Spherisorb ODS-II column. The eluting solvent was 5% ethyl acetate in hexane (0.8 ml/min); (●), ^{3}H; added unlabeled standards were monitored by refractive index.

subjected to reversed phase HPLC and normal phase HPLC analyses.

Since the two epimers (at C-24) of 3β,24-diacetoxy-cholest-5-ene (IV) could be resolved by reversed phase HPLC on a 3 μm Spherisorb ODS-II column, we studied the stereochemical purity of the ^{3}H-labeled sample of IV which had been isolated from a plasma sample by a modification of the method described above, i.e., reversed phase MPLC followed by MPLC on a silica gel column and normal phase HPLC. The [^{3}H]-IV was, after the addition of a mixture of unlabeled 24R-IV and 24S-IV, subjected to reversed phase HPLC (system 2) on a 3 μm Spherisorb ODS-II column (6.4 mm × 150 mm) at 0°C using 20% isopropyl alcohol in methanol as the eluting solvent at a flow rate of 0.8 ml/min. The resulting chromatogram (**Fig. 5**) showed good resolution, albeit not complete, of the 24R and 24S epimers of IV. (The identity of individual epimers was established by the cochromatography of the more retained epimer with that of an authentic sample of (24S)-3β,24-[1-^{14}C]-diacetoxy-cholest-5-ene.) As shown in Fig. 5, almost all of the label of [^{3}H]-IV had the mobility of the 24S epimer. Small amounts of ^{3}H had the chromatographic mobility of the 24R epimer.

Control experiments were also made with regard to the possible lability of various oxysterols to the conditions used in the alkaline hydrolysis of the steryl esters in plasma. 3β-Acetoxy-[1α,2α-^{3}H]cholest-5-en-7-one (1.1 μCi) was added to plasma (5 ml) and the NSL was ob-

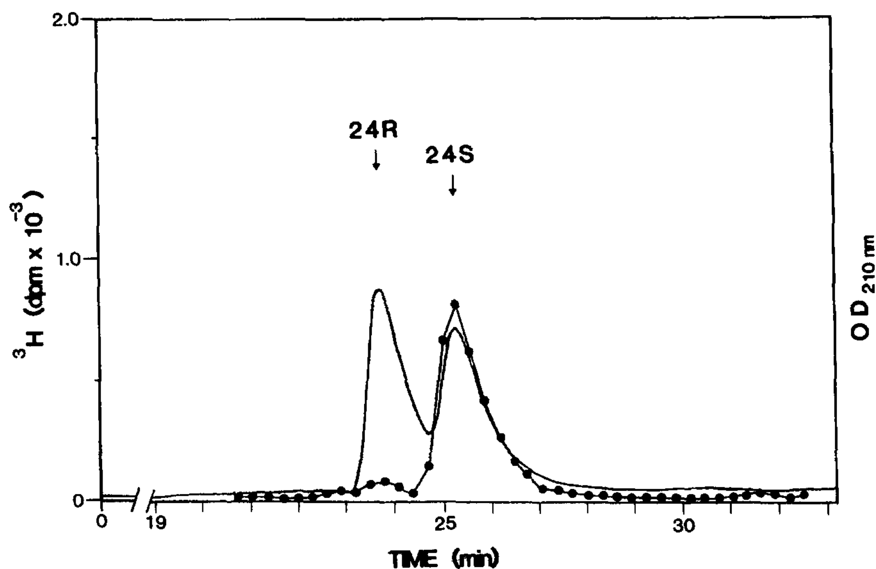

Fig. 5. Reversed phase HPLC (3 μm Spherisorb ODS-II) analysis of ^{3}H-labeled material with the chromatographic behavior of (24RS)-3β,24-diacetoxy-cholest-5-ene (IV) obtained from plasma after saponification, extraction, reversed phase MPLC of the NSL, acetylation with [^{3}H]acetic anhydride, MPLC on a silica gel column, and normal phase HPLC. The eluting solvent was 20% isopropyl alcohol in methanol (0.8 ml/min) and the column was maintained at 0°C; (●), ^{3}H; added standards of the 24R and 24S epimers of 3β,24-diacetoxy-cholest-5-ene were monitored at 210 nm.

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tained as described above. Whereas most (>98%) of the ^{3}H was recovered in NSL, reversed phase MPLC analysis showed that only 5.6% of the ^{3}H had the chromatographic mobility of polar oxysterols. Further analysis of this latter material on a reversed phase 5 μm Spherisorb ODS-II column showed that ~60% corresponded in mobility to authentic 7-ketocholesterol. Thus, only 3.4% of the ^{3}H of the labeled 7-ketocholesteryl acetate added to plasma was, after saponification, recovered as 7-ketocholesterol. This finding is in accord with previous reports of the alkali lability of 7-ketocholesterol (42-44).

Varying quantities of 3β,7α-diacetoxy-[7β-^{3}H]cholest-5-ene and of 3β-acetoxy-5α,6α-epoxy-[1α,2α-^{3}H]cholestane were saponified in 10% KOH in 95% ethanol (2 ml) for 30 min at 70°C. Essentially quantitative recoveries of the ^{3}H in NSL were observed for both labeled steryl acetates (mean recoveries for the 3β,7α-diol and the 5α,6α-epoxide were 108% and 105%, respectively). HPLC analyses of the labeled NSL were made on a 5 μm Spherisorb ODS-II column after the addition of authentic unlabeled standards. The recoveries of ^{3}H in material with the same chromatographic mobilities as cholest-5-ene-3β,7α-diol and 5α,6α-epoxy-cholestan-3β-ol on HPLC analyses are presented in **Table 2**. In both cases, the average recoveries of ^{3}H were in excess of 95%, indicating stability of the Δ^5-3β,7α-diol and the 5α,6α-epoxysterol under the conditions of the alkaline hydrolysis.

RESULTS AND DISCUSSION

The experiments described above demonstrate that MPLC on silica gel and alumina-$AgNO_3$ columns, normal phase HPLC, and reversed phase HPLC provide very useful separations of a number of oxygenated sterols in the form of their acetate derivatives. These chromatographic methods supplement previously described approaches that include TLC of free sterols (45, 46), column chromatography on lipophilic Sephadex gels (46) and Glycophase G (47), HPLC of free sterols (48, 49), GLC of free sterols or their trimethylsilyl (TMS) ether derivatives (46-48, 50-54), and GLC of the TMS ethers of the 3-keto derivatives of oxygenated sterols (55). The methodology described here provides several positive features. In some cases, the separations achieved were considerably superior to those obtained using some of the methods listed above. Moreover, MPLC on silica gel columns provides very high capacity (relative to GLC or analytical HPLC) for the separation of the concerned compounds on a preparative scale. MPLC on alumina-$AgNO_3$ columns also offers promise for the preparative-scale separation of the acetate derivatives of selected oxygenated sterols. In the present study we have observed striking separations of the diacetate derivatives of 7α- and 7β-hydroxycholesterol and of the acetate derivatives of the 5α,6α- and 5β,6β-epoxides of cholesterol. In addition to the results presented here, we have also had notable success in the preparative scale resolution of the R and S epimers of the acetate derivatives of 24,25-epoxy-cholest-8-en-3β-ol and 24,25-epoxy-lanost-8-en-3β-ol by HPLC (36). A very significant advantage of the use of acetate derivatives of the oxygenated derivatives is the possibility of the preparation of labeled acetate derivatives, thereby facilitating the detection and quantitation of the various oxygenated sterols. Moreover, chromatography of the acetate derivatives of the oxygenated sterols provides a readily applicable extension of existing chromatographic methodology for the separation of the acetate derivatives of monohydroxysterols (8-22).

The presence of oxygenated sterols in plasma (or serum) has been reported previously. These reports include 5,6-epoxy-cholestan-3β-ol (50, 56), cholest-5-ene-3β,7α-diol (48, 53, 54), cholest-5-ene-3β, 7β-diol (48, 53, 54), 3β-hydroxy-cholest-5-en-7-one (52, 57), cholest-5-ene-3β,24-diol (48), cholest-5-ene-3β,25-diol (48), and cholest-5-ene-3β,26-diol (47, 48, 52, 54).

The presence of 5α,6α-epoxy-cholestan-3β-ol in serum was reported by Gray, Lawrie, and Brooks (50). The epoxysterol was detected in both the free sterol and steryl

TABLE 2. Recoveries of ^{3}H-labeled cholest-5-ene-3β,7α-diol and 5α,6α-epoxy-cholestane after alkaline hydrolysis of 3β,7α-diacetoxy-[7β-^{3}H]cholest-5-ene and of 3β-acetoxy-5α,6α-epoxy-[1α,2α-^{3}H]cholestane

	Steryl Acetate Added	Free Sterol Recovered[a]
	ng	%
Cholest-5-ene-3β,7α-diol	3.9	98.6
	9.8	93.4
	19.5	94.4
	39.0	98.4
5α,6α-Epoxy-cholestane-3β-ol	2.9	95.6
	5.8	97.3
	14.5	93.9

[a]After HPLC analysis of NSL obtained after saponification with 10% KOH in 95% ethanol for 30 min at 70°C.

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ester fractions, although the amounts in each fraction were not presented. The sterol was characterized by GLC as the free sterol and its TMS ether, trifluoroacetate, and acetate derivatives and by MS of the free sterol and its TMS derivative. Additionally, the epoxide was reduced with $LiAlH_4$ to give a product that was similarly characterized as cholestane-3β,5α-diol. The epoxysterol was detected in 9 of 11 individuals (from trace to 32.5 μg/ml of serum) and at a level of 45.5 μg/ml in a pooled sample of hypercholesterolemic sera. The authors noted that higher levels of the epoxysterol were observed in patients with hypercholesterolemia. It is important to note that details regarding the timing of analyses (relative to blood acquisition) and conditions of storage of samples were not provided. Sample processing involved lipid extraction, preparative TLC, and saponification of steryl esters. No special precautions to exclude oxygen during processing were reported. In a more recent study, Björkhem et al. (56) investigated the levels of unesterified cholesterol-5,6-epoxide (sum of the 5α,6α- and 5β,6β-epimers) in human serum. Methodology involved lipid extraction, preparative TLC, and GLC–mass fragmentography. The levels of the mixture of the 5,6-epoxides in serum samples from 9 young (23–35 years of age) subjects ranged from 67 to 293 ng/ml (mean, 131 ng/ml). In contrast to the very high levels of 5α,6α- epoxide (unesterified plus esters) in serum samples from hypercholesterolemic subjects reported by Gray et al. (50) (2,500, 15,000, and 32,500 ng/ml in 3 individuals and 45,500 ng/ml in a pooled sample), Björkhem et al. (56) observed that the levels of unesterified cholesterol-5,6-epoxide in serum samples from patients with familial hypercholesterolemia (type IIa) were not elevated (mean, 69 ± 14 ng/ml; n = 12) but, in fact, appeared to be lower than those of normal subjects. In the present study, when fresh plasma samples were processed under the rigorous conditions described above, very low levels of 5α,6α- epoxysterol were detected (2.4 ng/ml and 12.1 ng/ml) and, in the latter case, all of the epoxysterol could be ascribed to autoxidation during the processing of the sample (**Table 3**). It should be noted that in analyses of a serum sample processed using standard sterol isolation conditions, a much higher level (137 ng/ml) was observed, all of which could be ascribed to autoxidation of cholesterol arising during the processing of the sample. Moreover, in the same sample analysis, an even higher level of the 5β,6β-epoxycholesterol was observed (376 ng/ml), all of which could be ascribed to autoxidation during sample processing. It is also noteworthy that little (1.7 ng/ml) or no (<2.5 ng/ml) cholestane-3β,5α,6β-triol, a known metabolite of 5α,6α-epoxy-cholestan-3β-ol and 5β,6β-epoxy-cholestan-3β-ol (41, 58, 59), was detected in plasma in the present study. In the former case, all of the triol detected could, on the basis of its ^{14}C content, be attributed to autoxidation of cholesterol during the processing of the sample. Björkhem et al. (56) have reported that the levels of unesterified cholestane-3β,5α,6β-triol in serum were near or below the detection limit (10 ng/ml) under the conditions used.

Smith et al. (48) studied the sterol moieties of the ester fraction of oxygenated sterols of fresh and aged plasma samples. Methodology included lipid extraction, silica gel

TABLE 3. Concentrations of oxygenated sterols in plasma as determined by analysis of their acetate derivatives

Component		Concentration					
		Experiment A			Experiment B		
		Total	$^{14}C^a$	Net	Total	$^{14}C^a$	Net
		nmol/ml					
V	(Δ^5-3β,26-diol)	0.158	<0.001	0.158	0.252	0.006	0.246
IV	(Δ^5-3β,24-diol)	0.074	<0.001	0.074	0.110	0.003	0.107
VIII	(Δ^5-3β,7α-diol)	0.017	0.007	0.010	0.047	0.004	0.043
IX	(Δ^5-3β,7β-diol)	0.009	0.007	0.002	0.007	0.007	0.000
XI	(Δ^5-3β-ol-7-one)	0.016	0.009	0.007	0.010	0.009	0.001
VI	(5β,6β-epoxy-Δ^0-3β-ol)	0.018	0.012	0.006	0.079	0.112	0.000
VII	(5α,6α-epoxy-Δ^0-3β-ol)	0.006	n.d.[b]	0.006	0.030	0.030	0.000
XIII	(Δ^0-3β,5α,6β-triol)	<0.006	n.d.	<0.006	0.004	0.004	0.000
III	(Δ^5-3β,22-diol)	<0.003	n.d.	<0.003	<0.002	n.d.	<0.002
XII	(Δ^5-3β,25-diol)	<0.008	n.d.	<0.008	<0.001	n.d.	<0.001
X	(Δ^5-3β,20α-diol)	<0.005	n.d.	<0.005	<0.002	n.d.	<0.002
XV	(Δ^5-3β,21-diol)	<0.004	n.d.	<0.004	<0.003	n.d.	<0.003
XIV	(Δ^5-3β,19-diol)	<0.003	n.d.	<0.003	<0.003	n.d.	<0.003

[a]Concentration of sterol arising from autoxidation of cholesterol as estimated from ^{14}C after addition of [^{14}C]cholesterol to plasma prior to any processing of the sample.

[b]No radioactivity detected.

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column chromatography, saponification of the steryl esters, HPLC of the free sterols, and chemical ionization MS. The levels of the following oxygenated sterols were reported for fresh and aged samples of plasma: cholest-5-ene-3β,26-diol (106 ng/ml and undetected), cholest-5-ene-3β,25-diol (5 ng/ml and 20 ng/ml), cholest-5-ene-3β,24-diol (7 ng/ml and undetected), cholest-5-ene-3β,7α-diol (19 ng/ml and 800 ng/ml), cholest-5-ene-3β,7β-diol (11 ng/ml and 1000 ng/ml), and 3β-hydroxy-cholest-5-en-7-one (6 ng/ml and 500 ng/ml).

Javitt et al. (47, 52) reported the presence of cholest-5-ene-3β,26-diol in human serum. Methodology included saponification, chromatography on a Glycophase G column, and GLC-MS or GLC-mass fragmentography of the di-TMS derivative. Levels of total 26-hydroxycholesterol in serum in 8 normal individuals varied from 92 to 286 ng/ml (52). In 3 individuals, ~30% of the sterol was in the unesterified form. In a study (47) by the same investigators, the levels of total 26-hydroxycholesterol in 6 normal individuals varied from 43 to 130 ng/ml. As noted above, Smith et al. (48) reported the presence (106 ng/ml) of cholest-5-ene-3β,26-diol in the ester fraction of oxygenated sterols of fresh plasma. The 3β,26-diol was not detected in an aged sample of plasma, a finding for which an explanation was not provided. Koopman et al. (54) also studied the levels of cholest-5-ene-3β,26-diol in serum samples from 40 normal subjects. Methodology included saponification, reversed phase HPLC to isolate the polar sterols, and capillary GLC of the TMS derivative. Observed values ranged from 30 to 129 ng/ml. In the present study, the levels of 26-hydroxycholesterol observed in plasma were 64 and 101 ng/ml. Essentially none of this material could be ascribed to autoxidation of cholesterol during the processing of the sample. Similar levels (116 and 88 ng/ml) were observed in two preliminary independent experiments in which the rigorous conditions to suppress autoxidation utilized in this study were not maintained. Even in these cases only 5% and 0.5% of the 26-hydroxycholesterol could, on the basis of ^{14}C content, be attributed to autoxidation of cholesterol during the processing of the sample. Thus, the results of five studies in four laboratories have indicated the presence of substantial amounts of cholest-5-ene-3β,26-diol in plasma (or serum) from human subjects.

Smith et al. (48) reported the presence of cholest-5-ene-3β,25-diol in the ester fraction in fresh (5 ng/ml) and aged (20 ng/ml) samples of human plasma. In the present study, 25-hydroxycholesterol was not detected in plasma samples of two human subjects (with limits of detection of less than 3.2 and 0.4 ng/ml in the two cases). In the course of their studies of the levels of 26-hydroxycholesterol in plasma, Javitt et al. (47) and Koopman et al. (54) also noted the absence of detectable 25-hydroxycholesterol in serum. Kou and Holmes (60) were unable to detect (< 10 ng/ml) 25-hydroxycholesterol in normal rat plasma.

In the present study, cholest-5-ene-3β,24-diol was observed at levels of 30 and 44 ng/ml. Essentially none of this material could be ascribed to autoxidation of cholesterol during the processing of the sample. Similar levels (47 and 43 ng/ml) were observed in two preliminary independent experiments in which the rigorous conditions to suppress autoxidation used in this study were not maintained. Even in these cases, very little (5.1% and 1.9%) of this material could be ascribed to autoxidation of cholesterol during the processing of the sample. The 24-hydroxycholesterol observed in plasma was shown to correspond almost exclusively to the 24S-epimer. As noted above, Smith et al. (48) reported the presence of 24-hydroxycholesterol (7 ng/ml) in the ester fraction of oxygenated sterols of fresh plasma.

Cholest-5-ene-3β,7α-diol is both a known product of the autoxidation of cholesterol (5-7) and an intermediate in the overall enzymatic conversion of cholesterol to a number of major bile acids (61). Smith et al. (48) reported the occurrence of 7α-hydroxycholesterol in the ester fraction of oxygenated sterols of human plasma. Markedly different levels were reported for fresh plasma (19 ng/ml) and aged plasma (800 ng/ml). Cholest-5-ene-3β,7β-diol is a known product of the autoxidation of cholesterol (5-7). Smith et al. (48) reported levels of 7β-hydroxycholesterol of 11 ng/ml in the ester fraction of oxygenated sterols of fresh plasma and 1000 ng/ml in aged plasma. Björkhem et al. (53) reported a mean serum level of 23 ± 4 (SEM) ng/ml for unesterified 7α-hydroxycholesterol in eight healthy subjects. Methodology involved extraction, TLC, and GLC-mass fragmentography of the di-TMS derivative. The authors reported that, in most analyses, the levels of 7α-hydroxycholesterol were 2 to 4 times higher than that of 7β-hydroxycholesterol. In contrast to the results of Smith et al. (48), evidence was presented "indicating that no significant part of 7α-hydroxycholesterol in serum is esterified under normal conditions." The levels of unesterified 7α-hydroxycholesterol in serum were reported to increase after administration of cholestyramine or after removal of portions of the terminal portion of the ileum (53). Björkhem et al. (53) also observed a sigificant correlation between the levels of 7α-hydroxycholesterol in serum and the levels of cholesterol 7α-hydroxylase in liver. Koopman et al. (54) reported that the levels of 7α-hydroxycholesterol and 7β-hydroxycholesterol in the sera of 40 healthy individuals ranged from 20 to 165 ng/ml and from 12 to 265 ng/ml, respectively. Methodology involved saponification, extraction of nonsaponifiable lipids followed by reversed phase HPLC to remove cholesterol, and capillary GLC of the di-TMS derivatives. In the present study, the observed levels of 7α-hydroxycholesterol and 7β-hydroxycholesterol were much lower. After correction for autoxidation of cholesterol during processing of the samples, the observed levels of the Δ^5-3β,7α-diol were 4 ng/ml and 17 ng/ml. Essentially all of

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the 7β-hydroxycholesterol detected could be ascribed to autoxidation during processing of the sample.

Smith et al. (48) reported the presence of 3β-hydroxycholest-5-en-7-one in the ester fraction of oxygenated sterols of plasma, with values of 6 ng/ml and 500 ng/ml for fresh and aged plasma, respectively. Björkhem (57) reported levels of 60, 41, 31, and 24 ng/ml of unesterified 7-ketocholesterol in fresh plasma. Methodology involved extraction and GLC-mass fragmentography of the TMS derivative. In the present study, levels of total 7-ketocholesterol in plasma were very low (< 3 ng/ml after correction for the amounts attributable to autoxidation of the cholesterol during sample processing). However, it should be noted that 7-ketocholesterol is unstable under conditions of alkaline hydrolysis (42-44 and as described herein) and therefore our method, as described above, should not be applied to the determination of this compound in plasma. Since the studies of Smith et al. (48) involved alkaline hydrolysis of the esters of oxygenated sterols in plasma, their results presented above should be evaluated within this context.

In the present study we were unable to detect the presence of a number of other oxygenated sterols in plasma. These include cholest-5-ene-3β,19-diol, cholest-5-ene-3β,20α-diol, (20S)-cholest-5-ene-3β,21-diol, and (22R)-cholest-5-ene-3β,22-diol, 24,25-epoxy-cholest-5-en-3β-ol, and 24,25-epoxy-lanost-8-en-3β-ol.

The approach presented here for the analysis of oxygenated sterols in plasma involves careful exclusion of oxygen during saponification and subsequent extraction of the NSL to provide for the suppression of autoxidation of cholesterol of plasma during these steps and permits analysis of total oxygenated sterol (free plus esterified). This feature is of importance since, at least in some cases, a very substantial portion of the individual oxygenated sterols in plasma appears to be esterified to fatty acids. The approach presented here also provides for the detection and estimation of the amounts of a given oxygenated sterol formed by autoxidation of cholesterol during processing of the sample. Another positive feature of this approach is the capacity to simultaneously assay a large number of oxygenated sterols. The methodology, as described herein, is not applicable to the case of 7-ketocholesterol due to its lability under the conditions of alkaline hydrolysis of the steryl esters. Another limitation is the requirement for [^{14}C]cholesterol of very high purity. Despite these limitations, the approach permits the simultaneous determination of a large number of oxygenated sterols with the critical provision of estimation of the amounts of a given oxygenated sterol formed artifactually during sample processing.

The results of this study indicate the presence of significant amounts of cholest-5-ene-3β,26-diol and (24S)-cholest-5-ene-3β,24-diol in plasma samples from human subjects and that these sterols did not arise artifactually by autoxidation of cholesterol during the processing of the plasma samples. These compounds, identified by the radiochemical methodology described herein, were also detected by capillary GLC and capillary GLC-MS. The stereochemistry at C-25 of the 3β,26-dihydroxysterol was not studied in the present study. It is important to note that (24RS)-cholest-5-ene-3β,24-diol (62), (25R)-cholest-5-ene-3β,26-diol (63, 64), and (25S)-cholest-5-ene-3β,26-diol (64) have been shown to be highly active in the suppression of the levels of HMG-CoA reductase activity in cultured mammalian cells. The (24RS)-Δ^5-3β,24-diol caused a 50% inhibition of HMG-CoA reductase activity in mouse L cells at 0.3 μM (62) and the 25R and 25S epimers of the Δ^5-3β-26-diol caused a comparable lowering of HMG-CoA reductase activity at 0.26 μM and 0.16 μM, respectively in the L cells (64). The (25R) epimer of the Δ^5-3β,26-diol, at 0.25 μM, caused a 40% lowering of HMG-CoA reductase activity in Chinese hamster ovary cells (63). The results of the present study and of previous studies of others (47, 48, 52, 54) indicate that the levels of the Δ^5-3β,26-diol in plasma (or serum) are comparable to those shown to cause a substantial reduction in the levels of HMG-CoA reductase activity in cultured cells.

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