Identification and quantitation of cholest-5-ene-3β,4β-diol in rat liver and human plasma

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Abstract: Cholest-5-ene-3β,4β-diol (4β-hydroxycholesterol) was identified in human plasma and rat liver by gas–liquid chromatography–mass spectrometry. An assay based on isotope dilution–mass spectrometry with a deuterium-labeled internal standard was developed for quantitation of this compound. The concentration of cholest-5-ene-3β,4β-diol in plasma from healthy subjects was 36 ± 4.3 ng/ml (mean ± SD, n = 8). The concentration in rat liver was 0.62 ± 0.19 μg/g wet weight (mean ± SD, n = 6). These levels are of the same order of magnitude as other common oxysterols.—**Breuer, O.** Identification and quantitation of cholest-5-ene-3β,4β-diol in rat liver and human plasma. *J. Lipid Res.* 1995. **36:** 2275–2281.

Supplementary key words cholestenediols • hydroxycholesterols • oxysterols • isotope dilution–gas-liquid chromatography–mass spectrometry • deuterium-labeled internal standard

Cholesterol oxidation products (oxysterols) are formed in living tissue either as a consequence of enzymatic cholesterol metabolism or by cholesterol autoxidation (for reviews see refs. 1 and 2). The latter may be due to attack by free radicals generated during enzymatic lipid metabolism (such as fatty acid peroxidation) or due to the presence of metal ions that catalyze oxidative reactions (1). Oxysterols have been shown to exert a wide range of biological effects (3), including inhibition (4) or stimulation (5, 6) of important enzymes in cholesterol homeostasis and mutagenic effects (7). Also, oxysterols have been isolated from human plasma (8–11) and aortal plaques (12, 13). It has been shown that these compounds may cause endothelial cell damage (14).

Many oxysterols are easily formed in vitro during sample processing unless antioxidative precautions are taken. The relative contribution of enzymatic sterol metabolism, in vivo autoxidation, and in vitro oxidation to the measured levels of various oxysterols is still controversial. The compounds that most frequently have been reported to occur in animal and human tissues are the α- and β-epimers of 5,6-epoxycholestan-3β-ol, the 5,6-epoxide hydrolysis product 5α-cholestane-3β,5,6β-triol, 3β-hydroxycholest-5-en-7-one, and cholesterol species monohydroxylated at positions C-7, C-20, C-22, C-24, C-25, or C-27 (1, 2). Presented here is the result of a search for other, less known, oxysterols in living tissues. It is shown that the concentration of cholest-5-ene-3β,4β-diol (4β-hydroxycholesterol) in human plasma and rat liver is of the same order of magnitude as other common oxysterols. This compound should therefore be included in future studies on oxysterol metabolism.

MATERIALS AND METHODS

[26,26,26,27,27,27-$^2H_6$]cholesterol was obtained from Medical Isotopes Inc., Concord, NH.

Synthesis of cholest-5-ene-3β,4β-diol

Cholesterol was oxidized with selenium dioxide as previously described (15), with the exception that toluene was used instead of benzene in the reaction mixture. After the last recrystallization step, the product obtained, i.e., cholest-5-ene-3β,4β-diol, was dissolved in toluene and purified on celite/charcoal. The yield was approximately 30% at a purity of >99%, as judged by gas–liquid chromatography (GLC) of the trimethylsilyl (TMSi) ether derivative. The melting point was 175.0–175.5°C, which is close to previously published

Abbreviations: BHT, butylated hydroxytoluene; GLC–MS, gas–liquid chromatography–mass spectrometry; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; TBDMSi, *tert*-butyldimethylsilyl; TMSi, trimethylsilyl.

values (176–177°C, ref. 15). ^{1}H-NMR (Bruker AM-400 instrument at 400 MHz, $CDCl_3$, $(CH_3)_4Si$ as internal standard) confirmed the configuration at C-4: δ 3.56 (m, H–3α, 1H); 4.13 (d, H–4α, 1H, J = 3 Hz), irradiation at δ 3.56 transformed the H–4α doublet into a singlet.

Synthesis of [26,26,26,27,27,27-$^2H_6$]cholest-5-ene-3β,4β-diol

[26,26,26,27,27,27-$^2H_6$]cholesterol was used for synthesis as described above. As the desired product was obtained in small quantities (a few milligrams), the final recrystallization step was excluded and purification was done by HPLC. The purity was >99%, as judged by gas–liquid chromatography of the TMSi ether derivative.

Synthesis of cholest-5-ene-3β,4α-diol

Cholest-5-en-3-one was prepared from cholest-4-en-3-one (50 mg) as described previously (16). After evaporation under a stream of argon, the compound was immediately dissolved in 200 µl toluene and oxidized with lead tetraacetate (17) to yield 4α-hydroxycholest-5-en-3-one acetate. The crude reaction mixture was extracted with diethyl ether. The extract was washed to neutrality with water, evaporated under a stream of argon, and dissolved in 1 ml tetrahydrofuran. After addition of 50 mg lithium aluminum hydride, the mixture was left stirring for 1 h at room temperature. Ethyl acetate, ethanol, and hydrochloric acid were added. After extraction with diethyl ether and washing to neutrality with water, the solvent was evaporated under a stream of argon. Conversion to TMSi derivatives and gas–liquid chromatography–mass spectrometry (GLC–MS) was performed as described below. Several peaks were observed in the total ion chromatogram (not shown), one of which had a retention time that was 1.2 min longer than that obtained for cholest-5-ene-3β,4β-diol. The mass spectrum of this peak was identical with that of cholest-5-ene-3β,4β-diol. The yield was less than 5%.

Animal experiment

Male rats (weight 210 g) of an outbred Sprague-Dawley strain were used. The animals had free access to water and a standard chow supplemented with 10% (weight/weight) peanut oil. The animals were anesthetized with diethyl ether and killed by cervical dislocation. The livers were removed, chilled on ice, and homogenized in 0.1 M potassium phosphate buffer (containing 2 mM EDTA and 11 µg/ml butylated hydroxytoluene [BHT], pH 7.4, 9 ml buffer/g liver) using a Potter-Elvehjem type homogenizer. The homogenates were stored for 20 days at -20°C. The experiment was approved by the local Ethics Committee for Animal Experiments.

Preparation of chow samples

Rat chow, 10 g, supplemented with 10% (weight/weight) peanut oil was mixed with 100 ml chloroform and shaken vigorously for 1 min. From this slurry 10 ml was taken and added to a mixture of 100 µg BHT and 100 ng deuterium-labeled internal standard (dissolved in 50 µl methanol). After washing with water, the extract was evaporated under a stream of argon and the residue was dissolved in 1 ml methanol.

Subjects and sample collection

Subjects (three females and five males) were healthy volunteers from laboratory staff, 28–49 years of age. Blood samples, 10 ml, were taken in the morning after an overnight fast. Evacuated vials (10 ml, Vacutainer®, Becton Dickinson) containing 0.12 ml 0.34 M potassium EDTA were used. Plasma was obtained by low speed centrifugation in the cold. It was stored for 15–20 days at -20°C.

Hydrolysis and extraction

The procedures for alkaline hydrolysis and solid-phase extraction of oxysterols in rat liver homogenate (3 ml), rat chow extract (1 ml), or human plasma (1 ml) were as described previously (11).

Conditions for gas–liquid chromatography–mass spectrometry

After removal of solvent under a stream of argon, oxysterols were converted to TMSi ethers by treatment with 100 µl pyridine–hexamethyldisilazane–trimethylchlorosilane 3:2:1 (vol/vol/vol) at 60°C for 30 min. Alternatively, *tert*-butyldimethylsilyl (TBDMSi) ethers were prepared by treatment with 100 µl *tert*-butyldimethylsilylimidazole–dimethylformamide (Supelco, Inc., Bellefonte, PA) at 22°C overnight, followed by addition of 1 ml water and extraction twice with ethyl acetate. After the derivatization and removal of solvent under a stream of argon, the samples were dissolved in 50 µl hexane.

Combined gas–liquid chromatography–mass spectrometry (GLC–MS) was performed on an HP 5890 gas–liquid chromatograph equipped with a 25 m HP Ultra-1 fused silica column (0.2 mm inner diameter, 0.33 µm film thickness) coupled to an HP 5970 quadrupole type mass spectrometer (Hewlett-Packard, Palo Alto, CA). Helium was used as carrier gas with a column pressure of 75 kPa. Samples (2 µl) dissolved in hexane were splitless injected. Electron impact ionization at 70 eV was applied. The column temperature was kept at 180°C for 1 min, subsequently raised at a rate of

35°C/min to 270°C, and finally increased 20°C/min to 300°C (TMSi-derivatized compounds) or 310°C (TBDMSi-derivatized compounds). Mass spectra were obtained by scanning the quadrupole between 100 and 560 *m/z* at a rate of 1.3 scans/sec. The cycling rate in selective ion monitoring mode was 2.3 cycles/sec.

Quantitation of cholest-5-ene-3β,7α-diol, cholest-5-ene-3β,7β-diol, cholest-5-ene-3β,24-diol, cholest-5-ene-3β,25-diol, and cholest-5-ene-3β,27-diol

The plasma concentrations of common cholest-5-ene-diols, except cholest-5-ene-3β,4β-diol, were determined by GLC–MS as described previously (11).

RESULTS

Identification of cholest-5-ene-3,4-diols in rat liver homogenate and human serum

Samples from rat liver homogenates were prepared and subjected to GLC–MS after TMSi derivatization as described above. As shown in **Fig. 1,** several prominent peaks were observed in the ion chromatogram, one of which had a retention time of 16.6 min. Its mass spectrum (Fig. 1) had a molecular ion at *m/z* 546 which suggested a cholestenediol structure. This is further supported by fragment ions at *m/z* 253 (loss of two trimethylsiloxy groups and the side chain, M–2 × 90–113), *m/z* 456 (M–90) and *m/z* 366 (M–2 × 90).

In mass spectrometry of TMSi-derivatized 3-hydroxy-Δ^5 steroids, cleavage of the A-ring of the steroid nucleus often yields fragment ions with *m/z* 129 and 327 (18). The presence of fragment ions at *m/z* 417 (M–129), *m/z* 327 (M–90–129), and *m/z* 129 (Fig. 1) therefore suggests that fragmentation might be similar in this compound, with or without prior loss of a trimethylsiloxy group.

A significant feature of the mass spectrum in Fig. 1 is a prominent fragment ion of unknown origin at *m/z* 147. In a previous report, fragment ions at *m/z* 147 were observed in a number of synthetic 4-hydroxy bile acid analogues (19). This suggests that a hydroxy group might be present at the C-4 position also in this compound. The retention time and mass spectrum were identical with those of authentic cholest-5-ene-3β,4β-diol (**Fig. 2**), synthesized as described in Materials and Methods. Furthermore, the presence of cholest-5-ene-3β,4β-diol in human plasma was established by comparison of mass spectra and GLC retention times with those obtained from rat liver preparations and authentic material. These comparisons were successfully repeated with a different GLC column (type HP-5MS) as well as with TBDMSi-derivatized oxysterols.

Attempts were made to identify the 4α-epimer, cholest-5-ene-3β,4α-diol, in samples from human plasma and rat liver homogenates. The retention time was known by analysis of authentic cholest-5-ene-3β,4α-diol. Indeed, a tiny peak was observed on the GLC chromatogram at a retention time similar to cholest-5-ene-3β,4α-diol. However, the low abundance did not permit further identification of this peak.

Assay for quantitation of cholest-5-ene-3β,4β-diol

A method based on isotope dilution–mass spectrometry was developed. The internal standard, [26,26,26,27,27,27-$^2H_6$]cholest-5-ene-3β,4β-diol, was synthesized as described in Materials and Methods. Its identity was confirmed by comparing the retention time and the mass spectrum with those obtained from unlabeled cholest-5-ene-3β,4β-diol (Fig. 2). As shown in the spectrum of the TMSi-derivatized internal standard, there is a shift of six mass units for almost all peaks above *m/z* 253 due to the presence of six deuterium atoms in the side chain. Analyzing TBDMSi ether derivatives seemed to improve the sensitivity of the assay as compared to TMSi ether derivatives. This was probably due to a lower degree of fragmentation and more efficient GLC separation from interfering compounds present at low concentrations. However, the use of TBDMSi derivatives resulted in considerably longer retention times. The final GLC oven temperature was therefore increased to 310°C, which yielded a retention time of 27.6 min for the cholest-5-ene-3β,4β-di-TBDMSi ether.

The mass spectrum of TBDMSi-derivatized cholest-5-ene-3β,4β-diol (not shown) consists of three major fragment ions: *m/z* 573 (loss of one *tert*-butyl group, M–57), *m/z* 367 (loss of two *tert*-butyldimethylsiloxy groups, M–132–131), and *m/z* 147.

Internal standard, 100 ng dissolved in 40 µl methanol, was added to 1 ml plasma or 3 ml liver homogenate immediately after thawing. After subsequent hydrolysis, extraction and TBDMSi derivatization, samples were subjected to GLC–MS with selective ion monitoring at *m/z* 573 and 579. Known mixtures of deuterium-labeled internal standard and unlabeled reference were analyzed, a standard curve was constructed, and the concentrations of cholest-5-ene-3β,4β-diol in plasma and liver homogenate samples were calculated.

A recovery experiment was carried out in order to estimate the accuracy and precision of the assay. The concentration of cholest-5-ene-3β,4β-diol in a plasma sample was measured as two quintuplicates, each of 5 × 1 ml volume. Unlabeled cholest-5-ene-3β,4β-diol, 5 × 25 ng, was added to one of the quintuplicates. Measured concentrations were 46 ± 2.3 and 22 ± 1.9 ng/ml (mean ± SD, n = 5, with versus without addition of 25 ng cholest-5-ene-3β,4β-diol). The calculated difference was 24 ± 3.0 ng/ml (mean ± SD, n = 5). Thus, the coefficients

of variation were about 5% and 9% at levels of 46 and 22 ng/ml, respectively.

Quantitation of cholest-5-ene-diols in human plasma, rat liver, and chow

The results from determinations of common cholest-5-ene-diols, including cholest-5-ene-3β,4β-diol, in plasma from eight healthy volunteers are shown in **Table 1.** The content of cholest-5-ene-3β,4β-diol in rat livers was 0.62 μg/g wet weight (range 0.48–0.93, SD 0.19, n = 6). The concentration of cholest-5-ene-3β,4β-diol in rat chow was 27 ng/g dry weight (n = 1).

DISCUSSION

Identification and quantitation of cholest-5-ene-3β,4β-diol in animal and human tissues

There are few previous reports on the occurrence of cholest-5-ene-3β,4β-diol in animal or human tissue. This compound has been detected in heated butter (20), salted and dried beef (21), spray-dried egg yolk (22), and air-aged cholesterol (23). To our knowledge, there is only one report on the occurrence of cholest-5-ene-3β,4β-diol in native tissue (24). According to that report, cholest-5-ene-3β,4β-diol has been detected in some, though not all, human sera. Virtually nothing is known about the occurrence of this compound in other living tissues.

In the present investigation, an assay based on isotope dilution–mass spectrometry was developed in order to accurately measure cholest-5-ene-3β,4β-diol in various tissues. With this method, cholest-5-ene-3β,4β-diol was detected in all preparations from human plasma and rat liver. The observed plasma concentration, 36 ng/ml mean value, is comparable with the serum levels of other major cholesterol oxidation products (refs. 9–11 and Table 1). Also, the concentration in rat liver is in the same order of magnitude as reported for other major oxysterols in murine liver (25).

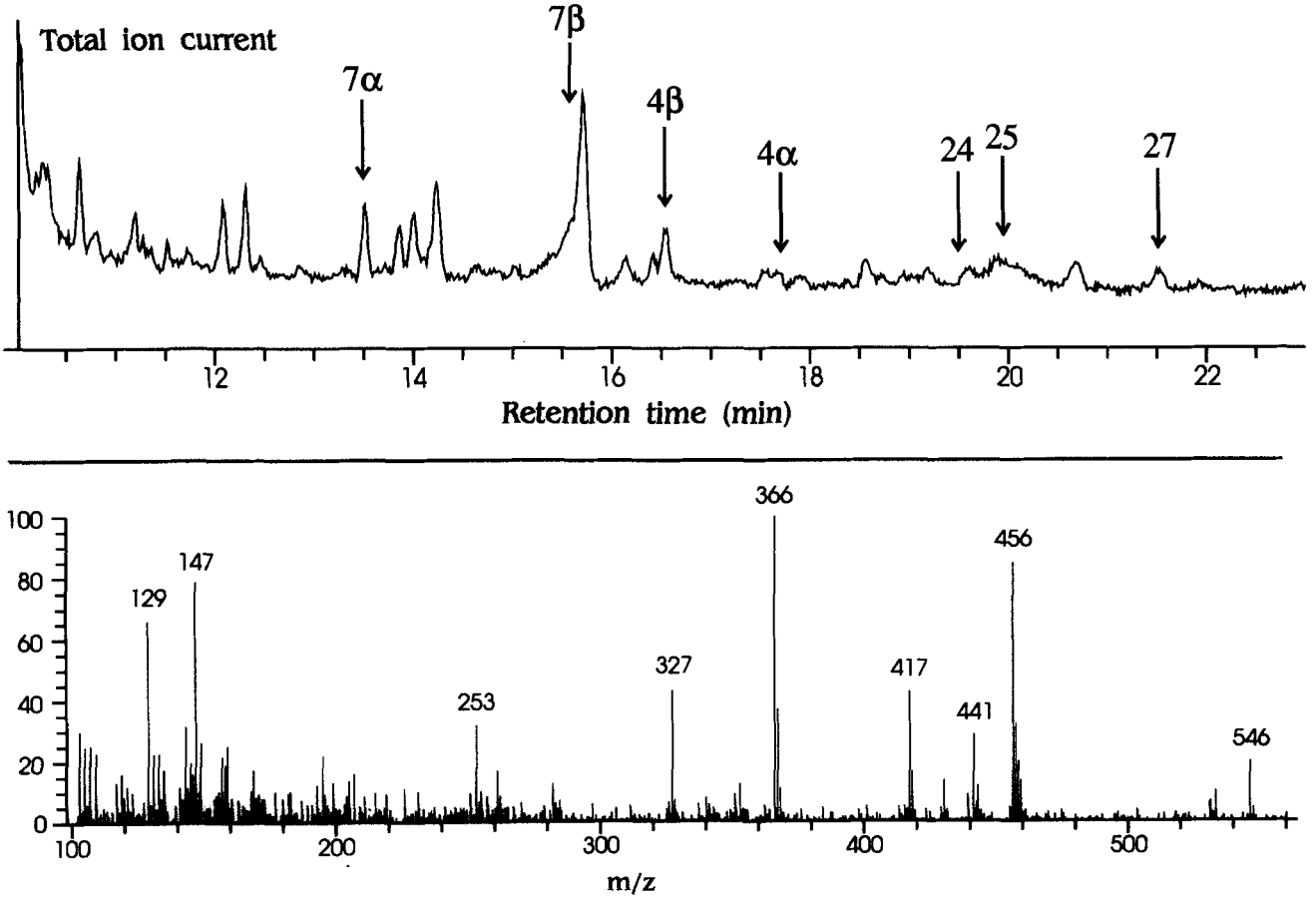

Fig. 1. Total ion current chromatogram (upper panel) obtained by GLC–MS analysis of TMSi-derivatized oxysterols that were isolated from rat liver. Arrows indicate the retention times of cholest-5-ene-3β,4α-diol (4α), cholest-5-ene-3β,4β-diol (4β), cholest-5-ene-3α,7α-diol (7α), cholest-5-ene-3β,7β-diol (7β), cholest-5-ene-3β,24-diol (24), cholest-5-ene-3β,25-diol (25), and cholest-5-ene-3β,27-diol (27) as determined by analysis of authentic material. The electron impact (70 eV) mass spectrum obtained for the peak at 16.6 min retention time (4β) is shown in the lower panel.

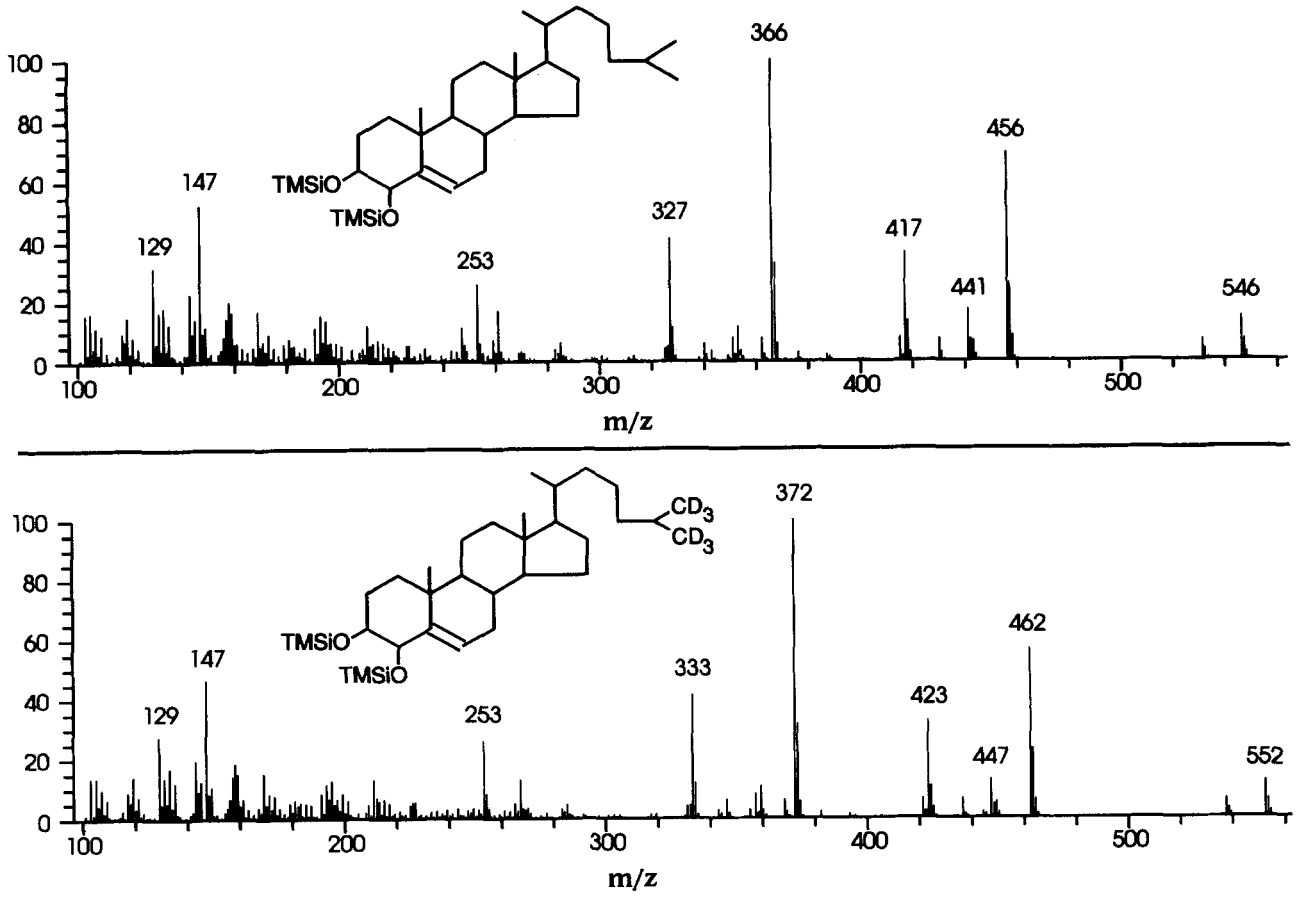

Fig. 2. Electron impact (70 eV) mass spectra obtained for TMSi-derivatized cholest-5-ene-3β,4β-diol (upper panel) and [26,26,26,27,27,27-$^2H_6$]cholest-5-ene-3β,4β-diol (lower panel).

In vitro autoxidation, a possible cause of artifacts

In all quantitative analyses of oxysterols, one has to consider the risk of artifacts due to cholesterol oxidation during sample processing. The awareness of this potential error source has increased during the last decade and, as a result, antioxidative procedures are routinely being used nowadays. This might also explain why the observed blood concentrations of common cholesterol autoxidation products, such as 5,6α-epoxy-5α-cholestan-3β-ol, are much lower in recent studies (9–11) than in early investigations (8).

It would be possible to quantitate and correct for in vitro cholesterol oxidation by adding pure radiolabeled cholesterol to fresh samples and eventually measuring the formation of labeled oxysterols. However, such techniques call for an exceptional purity of the radiolabeled cholesterol. Furthermore, it is difficult to exclude the possibility that purified labeled cholesterol might be oxidized before it is added to the samples.

In a carefully conducted study by Kudo et al. (9), the addition of [^{14}C]cholesterol to fresh samples and further processing was performed under an inert atmosphere by means of specially designed glassware. According to Kudo et al. (9), cholest-5-ene-3β,27-diol, (24S)-cholest-5-ene-3β,24-diol, and cholest-5-ene-3β,7α-diol were detected in plasma, whereas very little or none of other oxysterols, including cholest-5-ene-3β,7β-diol, cholest-5-ene-3β,25-diol, 5,6α-epoxy-5α-cholestan-3β-ol, and 5,6β-epoxy-5β-cholestan-3β-ol, were observed. Cholest-5-ene-3β,4β-diol was not analyzed in the study by Kudo et al. (9).

In the present investigation, several precautions were taken to avoid autoxidation during sample processing, e.g., alkaline hydrolysis was performed under an inert atmosphere and EDTA was added to quench metal ions which might initialize autoxidation. Although the possibility of in vitro cholesterol oxidation can never be excluded with any method, the low variances observed in the recovery experiment indicate that there was no

TABLE 1. Concentrations of cholest-5-ene-3β,4β-diol and other common cholest-5-ene-diols in human plasma from eight subjects

Compound	Concentration	Range	SD
	ng/ml	ng/ml	
Cholest-5-ene-3β,4β-diol	36	28–41	4
Cholest-5-ene-3β,7α-diol	37	8–66	21
Cholest-5-ene-3β,7β-diol	<0.3		
Cholest-5-ene-3β,24-diol	71	37–119	24
Cholest-5-ene-3β,25-diol	0.8	0–3	1
Cholest-5-ene-3β,27-diol	165	98–216	43

appreciable random error due to in vitro autoxidation under the conditions used.

Origin and metabolic fate of cholest-5-ene-3β,4β-diol

A possible mechanism for the autoxidative 4-hydroxylation of cholesterol is via formation of 4-hydroperoxides and subsequent decomposition into 4α- and 4β-hydroxysteroids. As none of 4α-hydroperoxycholest-5-en-3β-ol, 4β-hydroperoxycholest-5-en-3β-ol, and cholest-5-ene-3β,4α-diol have been detected in animal tissues (2), one would instead suspect a metabolic origin of cholest-5-ene-3β,4β-diol. On the other hand, in an investigation of air-aged cholesterol by Ansari et al. (23), no cholest-5-ene-3β,4α-diol was found, whereas cholest-5-ene-3β,4β-diol was detected and isolated. It is therefore questionable whether the absence of cholest-5-ene-3β,4α-diol implies a metabolic origin of cholest-5-ene-3β,4β-diol.

The measured content of cholest-5-ene-3β,4β-diol in rat chow, 27 ng/g, corresponds to an approximate intake of 0.6 μg per day. From our observations, the estimated total content of cholest-5-ene-3β,4β-diol in the liver is, at least, in the order of 5 μg, i.e., eight times the daily intake.

In a recent $^{18}O_2$ inhalation study in our laboratory, in vivo formation of cholest-5-ene-3β,4β-diol was observed in rats (26). However, the relative contribution from dietary sources versus in vivo formation of this sterol remains to be clarified.

Interestingly, the formation of 4β-hydroxylated bile acids has been observed in fetuses and newborn infants (19). It is therefore tempting to suggest that the metabolism of these bile acids might involve formation of cholest-5-ene-3β,4β-diol.

Very few reports on biotransformation of cholest-5-ene-3β,4β-diol are available. The binding of this compound to steroid-free purified bovine cytochrome $P450_{scc}$ has been described (27). This adrenocortical mitochondrial enzyme is involved in the cholesterol side-chain cleavage reaction. Metabolism of cholest-5-ene-3β,4β-diol by this cytochrome has also been suggested (27). However, more investigations on the metabolic fate of cholest-5-ene-3β,4β-diol are needed.

Biological effects

It has been demonstrated in vitro that cholest-5-ene-3β,4β-diol induces echinocytic transformation of human erythrocytes (28). Further, the osmotic fragility of red blood cells is diminished after treatment with cholest-5-ene-3β,4β-diol (29). The physiological relevance of these findings is uncertain, however. In a study on human polymorphonuclear leukocytes, cholest-5-ene-3β,4β-diol did not inhibit chemotaxis, in contrast to certain other oxysterols (30). Some degree of inhibition of sterol synthesis was observed in concanavalin A-stimulated human lymphocytes (28), whereas no inhibitory effects on rates of sterol synthesis from acetate were found in murine liver cells or fibroblasts (4). Effects of cholest-5-ene-3β,4β-diol on other important events in cholesterol homeostasis, such as cholesterol esterification or transformation to bile acids, are not known at present.

To summarize, cholest-5-ene-3β,4β-diol was isolated from human plasma and rat livers. This compound appears to be one of the predominating oxysterols in human plasma and should therefore be included in future studies on oxysterol metabolism. The possible metabolic role of cholest-5-ene-3β,4β-diol remains to be elucidated.

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