Assay of unesterified cholesterol-5,6-epoxide in human serum by isotope dilution mass spectrometry. Levels in the healthy state and in hyperlipoproteinemia

Ingemar Björkhem, Olof Breuer, Bo Angelin, and Sten-Åke Wikström

Department of Clinical Chemistry, Metabolism Unit, Department of Medicine, and Research Center, Karolinska Institutet, at Huddinge University Hospital, Huddinge, Sweden

Abstract Accurate methods based on isotope dilution-mass spectrometry were developed for assay of free cholesterol-5,6-epoxide (sum of 5α,6α- and 5β,6β-epimer) and cholestan-3β,5α,6β-triol in human serum. In all serum samples tested, the level of cholesterol epoxide was well above the detection limit (about 10 ng/ml) whereas the level of cholestan-3β,5α,6β-triol was below or near the detection limit in most cases. Immediate addition of antioxidant was found to be necessary in order to obtain reproducible results in the serum analyses, and prolonged storage of frozen samples had to be avoided. The level of cholesterol epoxide in healthy subjects 23–35 years of age ranged from 67 ng/ml to 293 ng/ml (mean 131 ng/ml, n = 9). There was a tendency to higher levels with increasing age, but there was no correlation to serum cholesterol. In marked contrast to results previously reported with a less accurate method, patients with various forms of hyperlipoproteinemia did not have increased levels of cholesterol epoxide. On the contrary, many of these patients had levels lower than normal. — Björkhem, I., O. Breuer, B. Angelin, and S.-Å. Wikström. Assay of unesterified cholesterol-5,6-epoxide in human serum by isotope dilution mass spectrometry. Levels in the healthy state and in hyperlipoproteinemia. J. Lipid Res. 1988. 29: 1031–1038.

Supplementary key words cholestan-3β,5α,6β-triol

The biological role of autoxidation products of cholesterol is controversial. These products are potent inhibitors of the rate-limiting enzymes in cholesterol and bile acid biosynthesis, HMG-CoA reductase and cholesterol 7α-hydroxylase, respectively (for a general review, see ref. 1). In addition, they possess carcinogenic and atherogenic properties (1). These compounds are formed from cholesterol in connection with lipid peroxidation or by direct exposure of cholesterol to different oxygen species.

The possibility that autoxidation products are of regulatory importance for cholesterol synthesis and degradation cannot be excluded at the present state of knowledge, but direct evidence for this is lacking. Thus, the levels of these compounds in tissues and blood are low and difficult to measure. It should be pointed out that the concentration of cholesterol is 10³–10⁴ times higher than that of autoxidation products in blood and tissues. Since cholesterol is easily autoxidized during extractions and chromatographic procedures, it is evident that the techniques for collection of the material, extraction, and workup may be crucial. This may be the explanation for the varying concentrations of autoxidation products that have been reported by different groups (1–5).

Among the major autoxidation products in human blood are the two isomers cholesterol-5α,6α-epoxide and cholesterol-5β,6β-epoxide (1–3, 5). In most studies, cholesterol-5α,6α-epoxide has been reported to dominate over cholesterol-5β,6β-epoxide (3, 5), but the opposite has been found in one study (4). Patients with Wolman’s disease were found to accumulate about equal amounts of cholesterol-5α,6α-epoxide and cholesterol-5β,6β-epoxide in the liver (6). In a study by Gray, Lawrie, and Brooks (3), the serum concentration of cholesterol-5α,6α-epoxide was found to be lower in healthy subjects (below 50 ng/ml) but up to 1,000-fold higher in patients with hypercholesterolemia. In addition, there seemed to be a correlation between the degree of atherosclerosis and the level of cholesterol-5α,6α-epoxide in serum. The number of subjects studied was very limited, and the mode of isolation of the steroid fraction may be criticized. Thus, no antioxidant was added before the workup procedure. On the other hand, one would expect that a general autoxidation of cholesterol during the workup would lead to increased levels also of other autoxidation products of cholesterol. The increase seemed, however, to be limited to the cholesterol-5α,6α-epoxide.

Abbreviations: GLC, gas-liquid chromatography; TLC, thin-layer chromatography; MS, mass spectrometry.

Journal of Lipid Research Volume 29, 1988 1031
Isotope dilution–mass spectrometry is the method of choice when high accuracy is required (7). In the present work, we have developed isotope dilution methods for assay of cholesterol epoxides (sum of 5α,6α- and 5β,6β-epimer) and its hydrolysis product cholestane-3β,5α,6α-triol in serum. This technique has been used for assay of these compounds in samples from healthy subjects and from patients with two forms of genetic hyperlipoproteinemia known to be associated with premature atherosclerosis, familial hypercholesterolemia, and familial combined hyperlipidemia.

MATERIALS AND METHODS

Materials

Cholesterol-5α,6α-epoxide and cholestane-3β,5α,6α-triol were obtained from Sigma Chemical Co. (St. Louis, MO). The cholesterol-5α,6α-preparation contained less than 5% of the 5β,6β-isomer. Cholesterol-5β,6β-epoxide was synthesized from the triacetate of cholestane-3β,5α,6α-triol by the method of Davis and Petrow (8). The product was purified by preparative thin-layer chromatography and had the same retention time as cholesterol-5α,6α-epoxide under the thin-layer and gas-liquid chromatographic conditions used below. The acetate of the cholesterol-5β,6β-epoxide could, however, be separated from the acetate of cholesterol-5α,6α-epoxide by gas–liquid chromatography when using the conditions described previously (9). 2H2-Labeled cholesterol-5α,6α-epoxide was prepared from 2H2-labeled cholesterol (with 2H in the 25-, 26-, and 27-positions, obtained from Applied Science Laboratories Inc., State College, PA) by treatment with monoperphtallic acid in chloroform (10) for 24 hr. The product was isolated by preparative TLC (cf. below). The material appeared pure as judged by TLC and GLC (as trimethylsilyl ether, see below). It was shown with the method above, however, that the material contained about 18% of the 5β,6β-epoxide. The isotopic purity was better than 95% as judged by combined GLC-MS and the material contained less than 3% unlabeled molecules. 3H2-Labeled cholestane-3β,5α,6β-triol was prepared from the above labeled epoxide by acid hydrolysis (reflux in 1 M methanolic sulfuric acid for 2 hr). The product was purified by preparative TLC as below. The material obtained was pure as judged by TLC and GLC of the trimethylsilyl ether. The isotopic composition was the same as that of the labeled epoxide.

Subjects

Serum was obtained from healthy medical students (age 23–35), and from patients with minor illnesses (herna, hemorrhoids, etc.) that did not affect their general state of health. Sera were also obtained from patients with familial hyperlipoproteinemia (5 females, 14 males; age 20–68). All blood samples were taken in the morning after an overnight fast.

The patients with hyperlipoproteinemia had been, without medical treatment for at least 2 months before the collection of the blood sample. They had no clinical or laboratory evidence of secondary hyperlipoproteinemia, and they had all been adequately classified with regard to the genetic origin of their hyperlipoproteinemia (11). Altogether, 12 patients had heterozygous familial hypercholesterolemia and 7 had familial combined hyperlipidemia. According to WHO criteria (12), all patients with familial hypercholesterolemia displayed type IIA phenotype (isolated elevation of low density lipoprotein levels). Of the patients with familial combined hyperlipidemia, 4 had a type IIB (combined elevation of low density and very low density lipoproteins) and 3 had a type IV phenotype (isolated elevation of very low density lipoproteins). The serum cholesterol levels were 9.5 ± 0.4 mmol/l, 9.5 ± 0.5 mmol/l, and 7.1 ± 0.6 mmol/l in the patients with type IIA, type IIB, and type IV hyperlipidemia, respectively. The serum triglyceride levels were 1.3 ± 0.1 mmol/l, 5.3 ± 1.0 mmol/l, and 8.6 ± 1.3 mmol/l, respectively, in the three groups of patients.

Collection of blood samples and isolation of autoxidation products

In the general procedure, 500 μg of BHT (butylated hydroxytoluene) was added to each ml of blood immediately after its collection. After clotting, the material was either extracted immediately or frozen and extracted after storage at −20°C for a maximum of 1 week. The internal standard (2H2-labeled cholesterol-5α,6α-epoxide or cholestane-3β,5α,6β-triol), 1 μg, was added to the serum sample, 1 ml, and the mixture was extracted with chloroform-methanol 2:1 (v/v) after addition of 1 ml of saline (0.9%, w/v). The material in the chloroform phase was subjected to preparative TLC, using toluene-ethyl acetate 3:7 (v/v) (cholesterol epoxides) or ethyl acetate (cholestanetriol, 3β,5α,6β-triol) as mobile phase. Under these chromatographic conditions the cholesterol epoxides had an RF between 0.56 and 0.58, whereas cholestane-3β,5α,6β-triol had an RF between 0.34 and 0.36 in the two solvent systems, respectively. The added BHT separated from the steroids in these TLC systems. The appropriate chromatographic zone containing the epoxides or the triol was scraped off and eluted with methanol immediately after development.

Analysis by combined gas–liquid chromatography–mass spectrometry

The isolated mixture of unlabeled and 2H2-labeled cholesterol epoxide was converted into trimethylsilyl ethers (13). Under these conditions there was no cleavage of the
epoxide and the silylation was complete. The material was analyzed by combined GLC-MS using an LKB 9000 instrument equipped with a 1.5% SE-30 column. The carrier gas was helium and the flow rate was 30 ml/min. The temperature of the column was 270–290°C and the temperature of the flash heater and ion source was about 30°C higher. The electron energy was set to 20 eV and the trap current to 60 μA. The first channel of the multiple ion detector unit was focused on the ion at m/z 474 and the second channel on the ion at m/z 481. Peak heights of the recordings were measured as this was found to give more reproducible results than measurements of peak area. In the calculation of the level of cholestero1-5a,6tr-epoxide in serum, a standard curve was used. This standard curve was obtained by analysis of mixtures of 2H7-labeled cholesterol-5α,6α-epoxide (1 μg) with various amounts of unlabeled cholesterol-5α,6α-epoxide (0–1000 ng). In some cases the standard mixtures were carried through the same procedures as for serum before the analysis. Such treatment had no effect on the standard curve.

The isolated mixture of unlabeled and 2H7-labeled cholestane-3β,5α,6α-triol was analyzed by combined GLC-MS as above, with the exception that the channels of the multiple ion detector were focused on the ion at m/z 403 and m/z 410, respectively. A standard curve was obtained by analysis of mixtures of 2H7-labeled cholestane-3β,5α,6α-triol with unlabeled cholestane-3β,5α,6α-triol. Mass spectra of trimethylsilyl ethers of reference compounds were obtained by analysis with an LKB 2091 instrument, using essentially the same conditions for GLC as above.

Analysis of serum cholesterol and triglycerides

Serum cholesterol and triglycerides were assayed with fully enzymatic methods as described previously (14). The accuracy of these methods has been established (14).

RESULTS

Isotope dilution–mass spectrometry of cholesterol-5,6-epoxide and cholestane-3β,5α,6α-triol

Fig. 1 shows the partial mass spectrum of the trimethylsilyl derivative of unlabeled and 2H7-labeled cholesterol-5α,6α-epoxide. The mass spectrum of the derivative of unlabeled cholesterol-5α,6α-epoxide was essentially identical to previously published mass spectra of this compound (3, 14) and had prominent peaks at m/z 474 (M), m/z 459 (M–15), and m/z 366. As expected the mass spectrum of the derivative of 2H7-labeled cholesterol-5α,6α-epoxide had prominent peaks at m/z 481 (M), m/z 466 (M–15), and m/z 391 (M–90). In accordance with previous work (9, 15), the mass spectrum of the trimethylsilyl derivative of unlabeled cholesterol-5β,6β-epoxide was identical to that of the trimethylsilyl derivative of cholesterol-5α,6α-epoxide, and both epimers had the same GLC retention time under the conditions employed (cf. Materials and Methods).

Fig. 2 shows the partial mass spectrum of the trimethylsilyl derivative of unlabeled and 2H7-labeled cholestane-3β,5α,6α-triol. The mass spectrum of the unlabeled derivative was essentially identical to previously published mass spectra of this compound (9), and had prominent peaks at m/z 548 (M–90), m/z 458 (M–2 × 90), m/z 403, and m/z 321. The structure of the fragments at m/z 403 and m/z 321 could not be established. As expected, the mass spectrum of the 2H7-labeled compound had prominent peaks at m/z 555 (M–90), m/z 465 (M–2 × 90), m/z 410, and m/z 328.

For quantitation of cholesterol-5,6-epoxide with 2H7-labeled cholesterol-5α,6α-epoxide as internal standard, the ions at m/z 474 and m/z 481 were used. A standard curve for the quantitations was obtained by analyzing
Fig. 2. Partial mass spectrum of trimethylsilyl derivative of unlabeled (A) and $^3$H$_7$-labeled (B) cholestane-3β,5α,6β-triol. Peaks with an intensity less than 4% of the base peak are omitted.

Various amounts of unlabeled cholesterol-5α,6α-epoxide together with a fixed amount (1 μg) of $^3$H$_7$-labeled cholesterol-5α,6α-epoxide. There was a linear relationship between the amount of unlabeled cholesterol-5α,6α-epoxide and the ratio between the peak heights at m/z 474 and m/z 481. An essentially identical standard curve was obtained when the unlabeled cholesterol-5α,6α-epoxide in the above procedure was substituted for freshly prepared cholesterol-5β,6β-epoxide. The 5β,6β-epoxide, in contrast to the 5α,6α-epoxide was labile, and decomposed to unknown products after a few weeks in solution at 4°C. The lability of the cholesterol-5β,6β-epoxide has been noted also by others (Aringer, L., unpublished observation).

For quantitation of cholestane-3β,5α,6β-triol with use of $^3$H$_7$-labeled cholestane-3β,5α,6β-triol, the ions at m/z 403 and m/z 410 were used. These ions are highly characteristic for the compound. A standard curve for the quantitations was obtained by analyzing various amounts of unlabeled cholestane-3β,5α,6β-triol together with a fixed amount (1 μg) of $^3$H$_7$-labeled cholestane-3β,5α,6β-triol. A linear relationship was observed between the amount of unlabeled cholestane-3β,5α,6β-triol and the ratio between the peak heights at m/z 403 and m/z 410.

Assay of cholesterol-5,6-epoxides in serum

Fig. 3 shows a typical mass fragmentographic recording of the derivative of a purified serum extract from a patient, to which a fixed amount (1 μg) of $^3$H$_7$-labeled cholesterol-5α,6α-epoxide had been added. The concentration of unlabeled cholesterol-5,6-epoxides (sum of 5α,6α-epoxide and 5β,6β-epoxide) could be calculated with use of a standard curve. The detection limit of this assay was about 10 ng/ml serum under the conditions employed. The coefficient of variation of the assay was relatively high: 10–15% in the range 10–100 ng of cholesterol-5,6-epoxides/ml serum and about 10% at concentrations above 100 ng/ml. The apparent imprecision
may, in part, be due to autoxidation of serum cholesterol during the workup procedure, in spite of the addition of the antioxidant. Addition of an antioxidant was found to be necessary, otherwise falsely high levels of cholesterol-5,6-epoxide were sometimes (but not always) obtained. Analyses of a fresh serum sample and of the same serum after storage for a maximum of 2 weeks at -20°C gave essentially identical results. After storage for a longer period of time, however, higher values were sometimes obtained. In spite of all the precautions taken, a falsely high concentration of cholesterol-5,6-epoxides was occasionally obtained in one out of several replicates of the same serum sample. All analyses were therefore done in triplicate.

In order to evaluate the accuracy of the method, serum samples to which known amounts of cholesterol-5α,6α-epoxide had been added were assayed. In one such experiment, addition of cholesterol-5α,6α-epoxide, 500 ng/ml, increased the measured concentration of cholesterol-5,6-epoxide from 79 ± 12 ng/ml to 570 ± 23 ng/ml (n = 4). The difference between calculated and found values was thus 2%. In another experiment, addition of the same amount of cholesterol-5α,6α-epoxide increased the measured concentration of cholesterol-5,6-epoxide from 247 ± 18 ng/ml to 794 ± 83 ng/ml (n = 4). The difference between calculated and found values was thus 6%.

The above assay only measures underivatized cholesterol-5,6-epoxides in serum, and esters of this compound are not included. Several attempts were therefore made to assay cholesterol-5,6-epoxides in serum after mild alkaline hydrolysis (3). In one set of experiments, levels of cholesterol-5,6-epoxides were obtained similar to those in unhydrolyzed serum. In some other experiments both higher and slightly lower values were obtained. It should be pointed out that, in the above experiments, the 2H7-labeled standard was added before the hydrolysis step. Theoretically, a possible degradation of the unlabeled cholesterol-5α,6α-epoxide during the hydrolysis should be the same as that affecting the 2H7-labeled cholesterol-5α,6α-epoxide. The cholesterol-5β,6β-epoxide in serum may, however, be degraded to a higher extent, leading to falsely lower levels of measured cholesterol-5,6-epoxide. The hydrolysis may also lead to increased autoxidation of the serum cholesterol, which would counteract the above effect. In view of the uncertainties and the lack of reproducibility, no further attempts were made to analyze the total concentration of cholesterol-5,6-epoxides in serum (free plus esterified).

Serum levels of underivatized cholesterol-5,6-epoxides in healthy subjects and in patients with hyperlipoproteinemia

Table 1 summarizes the results of the measurements of the serum levels of underivatized cholesterol-5,6-epoxides in healthy subjects and in patients with hyperlipoproteinemia. Healthy medical students, 23–35 years of age, had levels of cholesterol-5,6-epoxides varying from 67 to 293 ng/ml with a mean of 131 ng/ml (n = 8). In a study of healthy subjects and outpatients (25–73 years of age) with minor nonmedical diseases, there was a tendency to higher levels of cholesterol-5,6-epoxides with increasing age. Thus, subjects 20–40 years of age had a lower level (108 ± 16 ng/ml) than subjects 61–80 years of age (193 ± 18 ng/ml). This difference was statistically significant (P < 0.05, Student’s t-test). There was no sex difference and no correlation between the serum levels of cholesterol-5,6-epoxide and cholesterol (r = 0.1 for all subjects studied).

Patients with familial hypercholesterolemia (phenotype IIa) had lower levels of cholesterol-5,6-epoxides than the healthy subjects and the patients with minor nonmedical diseases. Also in this case, there was no correlation between the serum level of cholesterol-5,6-epoxides and cholesterol. The patients with familial combined hyperlipidemia had levels of cholesterol-5,6-epoxides similar to those of the healthy subjects. The individual variation was high in this group of patients, but there was no difference between patients displaying the type IIb or the type IV phenotype. Furthermore, there were no differences between
### TABLE 1. Concentration of cholesterol 5,6-epoxide in different subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Age</th>
<th>Number</th>
<th>Concentration of Cholesterol 5,6-epoxide (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy medical students</td>
<td>22–35</td>
<td>8</td>
<td>131 ± 29*</td>
</tr>
<tr>
<td>Healthy subjects and patients with minor non-medical diseases</td>
<td>20–40</td>
<td>5</td>
<td>108 ± 16</td>
</tr>
<tr>
<td></td>
<td>41–60</td>
<td>4</td>
<td>172 ± 31</td>
</tr>
<tr>
<td></td>
<td>61–80</td>
<td>10</td>
<td>193 ± 18</td>
</tr>
<tr>
<td>Patients with familial hypercholesterolemia (phenotype IIA)</td>
<td>20–66</td>
<td>12</td>
<td>69 ± 14</td>
</tr>
<tr>
<td>Patients with familial combined hyperlipidemia (phenotype IIB)</td>
<td>41–56</td>
<td>4</td>
<td>105 ± 58</td>
</tr>
<tr>
<td>Patients with familial combined hyperlipidemia (phenotype IV)</td>
<td>46–68</td>
<td>3</td>
<td>93 ± 64</td>
</tr>
</tbody>
</table>

*Mean ± SEM.

the 7 patients with clinically overt coronary heart disease and the remaining 12 patients.

**Assay of cholestane-3\(\beta\),5\(\alpha\),6\(\beta\)-triol in serum**

**Fig. 4** shows a typical mass fragmentographic recording of the derivative of a purified serum extract from a patient, to which a fixed amount (1 \(\mu\)g) of \(^2\)H\(_7\)-labeled cholestane-3\(\beta\),5\(\alpha\),6\(\beta\)-triol had been added. The concentration of unlabeled cholestane-3\(\beta\),5\(\alpha\),6\(\beta\)-triol was calculated with use of a standard curve. The detection limit of the assay was about 10 ng/ml serum under the conditions employed. The coefficient of variation was about 10%. As above, the apparent imprecision may, in part, be due to autoxidation of serum cholesterol during the workup procedure. Also in this case, addition of antioxidant was found to be necessary; otherwise, falsely high levels of cholestane-3\(\beta\),5\(\alpha\),6\(\beta\)-triol were obtained.

In order to test the accuracy of the method, serum samples were assayed to which known amounts of cholestane-3\(\beta\),5\(\alpha\),6\(\beta\)-triol had been added. In one such experiment addition of cholestane-3\(\beta\),5\(\alpha\),6\(\beta\)-triol, 500 ng/ml, increased the measured concentration of cholestane-3\(\beta\),5\(\alpha\),6\(\beta\)-triol from 73 ± 10 ng/ml to 603 ± 4 ng/ml. The difference between expected and found values was thus 5%. The concentration of cholestane-3\(\beta\),5\(\alpha\),6\(\beta\)-triol in the above serum sample was relatively high due to storage at 4°C for some days before the analysis.

In all the subjects tested, the level of cholestane-3\(\beta\),5\(\alpha\),6\(\beta\)-triol was near or below the detection limit (10 ng/ml) when the serum had been collected under the conditions described in Materials and Methods. In view of this, no further measurements of cholestane-3\(\beta\),5\(\alpha\),6\(\beta\)-triol were made.

![Fig. 4](https://www.jlr.org)  
**Fig. 4.** Multiple ion recording of trimethylsilyl ether derivative of material isolated from serum of a healthy subject to which \(^2\)H\(_7\)-labeled cholestane-3\(\beta\),5\(\alpha\),6\(\beta\)-triol had been added.
Mild alkaline hydrolysis of serum prior to the assay of cholestane-3β,5α,6β-triol did not, in general, increase the measured concentration of cholestane-3β,5α,6β-triol. Acid hydrolysis can be expected to convert cholesterol-5,6-epoxides into cholestane-3β,5α,6β-triol. Such hydrolysis yielded higher concentrations of cholestane-3β,5α,6β-triol, in some cases considerably higher than those of cholesterol-3,6-epoxide measured in the same serum sample. The variations in the experiments with acid as well as alkaline hydrolysis were however unacceptably high, possibly due to uncontrolled autoxidation of cholesterol during the hydrolysis step.

**DISCUSSION**

According to current concepts, autoxidation and hydroperoxide-mediated oxidation yield predominantly the β-isomer of cholesterol-5,6-epoxide (1, 16), whereas cytochrome P-450-dependent oxidation yields predominantly the α-isomer (17). The possibility has been discussed that circulating cholesterol-5,6-epoxide may be used as a marker for the effects of free radicals in the body formed by influence of both exogenous and endogenous factors (4). Theoretically, the 5β-isomer should be most suited as such a marker.

The present method for assay of cholesterol-5,6-epoxides should be more accurate than previous methods based on GLC only (3-5). No extensive attempts were made to separate the two isomers in individual serum samples. It is possible to separate the 5α- and 5β-epoxides as the acetate by TLC or GLC (9) and in a recent study a separation of the two underivatized isomers was achieved by high performance liquid chromatography (18). The primary goal of the present work was, however, to try to confirm the finding by Gray et al. (3) that cholesterol-5,6-epoxide is markedly elevated in patients with hyperlipidemia. This goal could be achievable with the present method with no separation of the two isomers. The analytical variation was relatively high, with a coefficient of variation of 10-15%. With the technique used, the analytical variation in the assay could be expected to be less than 5% (7). The reason for the relatively high imprecision is most probably a small uncontrolled autoxidation of cholesterol occurring during the workup procedure in spite of the precautions that were taken. Attempts are in progress to further minimize such autoxidation by introducing a more rapid procedure than that used here for separation of cholesterol from its autoxidation products.

After completion of the present study, a publication appeared reporting that isotope dilution-mass spectrometry was used for assay of cholesterol-5,6-epoxides and cholestane-3β,5α,6β-triol in nipple aspirates of human breast fluid (19). That method should have the same analytical merits as the present method.

In similarity with cholesterol, some part of the cholesterol epoxides present in serum is most likely esterified. Several attempts were therefore made to measure the total amounts of cholesterol-5,6-epoxides in serum by introducing a mild hydrolysis step. Less reproducible results were obtained, however, possibly due to uncontrolled autoxidation occurring during the hydrolysis. As a consequence, only free cholesterol epoxides could be analyzed with sufficient accuracy. It may be mentioned that a hydrolysis step was included in the isotope dilution method used by Gruenke et al. (19). According to these authors, the autoxidation occurring during the hydrolysis could be controlled by addition of ascorbate.

Under in vivo and different in vitro conditions, both epimers of cholesterol-5,6-epoxide are hydrolyzed to yield cholestane-3β,5α,6β-triol (1). In accordance with the work by Gray et al. (3) only very low or undetectable levels of this compound were found in serum. The rapid metabolism of cholestane-3β,5α,6β-triol is well established (1, 20). In theory, acid hydrolysis of serum would lead to conversion of all unesterified cholesterol-5,6-epoxide. Less reproducible results were obtained by this method, however, possibly due to varying degrees of conversion. In view of the low levels, no further attempts were made to quantitate cholestane-3β,5α,6β-triol in all individual serum samples.

The levels of cholesterol-5,6-epoxides found in serum of healthy subjects were somewhat higher than those reported by Aringer (4) (30-80 ng/ml) but similar to those reported by Kukais et al. (5) (100 ng/ml). The higher levels obtained in the present work may be due to the use of an ideal internal standard, which makes the assay insensitive to losses during the workup procedure. No sex difference could be demonstrated. There was a tendency to higher levels of cholesterol-5,6-epoxide with increasing age. This was probably not due to the age-dependent increase in serum cholesterol level since no correlation was found between serum cholesterol and serum concentration of cholesterol-5,6-epoxides.

Using the present method for assay of cholesterol-5,6-epoxides, the previous finding by Gray et al. (3) that patients with hyperlipidemia had markedly higher levels of cholesterol-5α,6α-epoxide than healthy subjects could not be confirmed. On the contrary, patients with type IIA hyperlipoproteinemia had lower levels of this compound in serum than normal subjects. In view of the great individual variation, the significance of this difference is difficult to evaluate. The reason for the different results is not known, but the fact that Gray et al. did not add antioxidant to their serum samples may be of some importance. The possibility cannot be excluded that lipid peroxidation and autoxidation may occur more easily in serum from hyperlipidemic patients. The fact that more substrate for autoxidation of cholesterol is available may also be of some importance. In the publication by Gray et al.
(3) it was stated that there seemed to be a correlation between the degree of atherosclerosis and the level of cholesterol-5α,6α-epoxide. Although both familial hypercholesterolemia and familial combined hyperlipidemia are associated with premature atherosclerosis, we did not observe any influence of clinically overt coronary heart disease. Preliminary experiments with geriatric atherosclerotic patients also do not show markedly elevated levels of cholesterol-5,6-epoxides when using the present method (Björkhem, I., K. Einarsson, and P. Henriksson, unpublished observations).  

The skillful technical assistance of Manfred Held is gratefully acknowledged. The authors are grateful to Dr. Leif Aringer for helpful discussions. This work was supported by the Swedish Medical Research Council (Projects 03X-9141 and 03X-7137) and the King Gustaf V and Queen Victoria Foundation.

Manuscript received 22 October 1987 and in revised form 23 February 1988.

REFERENCES