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ASSAY OF CHOLESTEROL 7α-HYDROXYLASE UTILIZING A SILICA CARTRIDGE COLUMN AND 5α-CHOLESTANE-3β,7β-DIOL AS AN INTERNAL STANDARD

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SUMMARY

A simplified and accurate assay of cholesterol 7α-hydroxylase activity using 5α-cholestane-3β,7β-diol as an internal standard is described Endogenous microsomal cholesterol was used as the substrate Following incubation and addition of the internal standard, lipids extracted from the incubation mixture were applied to Bond-Elut silica cartridge columns 7α-Hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol and the internal standard were quantitatively recovered by eluting the column with 6 ml of benzene–ethyl acetate (2 3, v/v) after removal of cholesterol with 6 ml of benzene–ethyl acetate (9 1, v/v) After trimethylsilylation, the mass of 7α-hydroxycholesterol was determined by capillary gas chromatography with selected-ion monitoring The method permits a faster, easier and more sensitive determination of the activity of cholesterol 7α-hydroxylase in small samples

INTRODUCTION

7α-Hydroxylation of cholesterol by hepatic microsomal cholesterol 7α-hydroxylase is generally considered to be the rate-limiting step in the major pathway of conversion of cholesterol to bile acids [1]. Since the measurement of the activity of liver microsomal 7α-hydroxylase was first described in 1950, several modifications and newer methods have been reported In general, there are two major approaches: to measure the conversion rate of added radiolabelled cholesterol into 7α-hydroxycholesterol [2], or to determine the actual amount of 7α-hydroxycholesterol formed directly by a double labelling tech-

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nique [3], high-performance liquid chromatography [4] or gas chromatography with selected ion monitoring (GC–SIM) [5,6].

Although the former is widely used, it is less sensitive and less accurate. Because the 7α-hydroxylation of cholesterol is the rate-limiting step of bile acid biosynthesis, the conversion rate is very low. This poses a great difficulty, especially in microscale analysis in which microsomes are obtained from small amounts of samples such as human liver biopsy specimens [7,8]. The increment of radioactivity at the R_F value of 7α-hydroxycholesterol must be determined by subtracting a considerable background radioactivity In our experience, the radioisotopic assay was unsatisfactory: the signal suffered considerable interference from the background noise and in some instances calculation even resulted in negative values. In addition, there is the possibility of incomplete equilibration between the added labelled cholesterol and the endogenous cholesterol pool.

The latter approach was developed to overcome the above disadvantages. Because of its superior sensitivity and accuracy, GC–SIM is considered to be the best method; it monitors only a few selected ions and therefore suffers little interference from the background noise arising from the impurities present after the chromatographic separation [5,6] Preliminary purification of 7α-hydroxycholesterol seems to be necessary in order to obtain a higher signal-to-noise ratio and to avoid contamination of the GC column and the mass detectors. Formerly preparative thin-layer chromatography (TLC) was usually utilized to semi-purify the sterols, but it is time-consuming and inconvenient for many samples.

The crucial point in developing an accurate chromatographic method is the availability of a suitable internal standard. The use of deuterated 7α-hydroxycholesterol [5] and 5α-cholestane [6] has been reported. Theoretically, the former was considered to be the better internal standard, but the method has not been widely adopted, probably because it is difficult to synthesize deuterated 7α-hydroxycholesterol. In contrast, 5α-cholestane is commercially available. However, there is a great difference in the polarities of this compound and 7α-hydroxycholesterol. The former has no hydroxy group whereas the latter has two hydroxy groups in the molecule. Therefore, they might behave differently during the extraction and sample preparation steps.

This paper describes a method for the determination of the mass of 7α-hydroxycholesterol by GC–SIM A Bond-Elut silica cartridge column was used for easy purification of steroids from the crude lipids extracted from the incubation mixture. As an internal standard we employed 5α-cholestane-3β,7β-diol, which behaved very similarly to 7α- and 7β-hydroxycholesterol and 7-ketocholesterol on the silica gel cartridge column.

EXPERIMENTAL

Chemicals and reagents

All solvents used were of analytical-reagent grade and distilled prior to use TMSI-H (hexamethyldisilazane–trimethylchlorosilane–pyridine, 2 1 10) was obtained from Gasukuro Kogyo (Tokyo, Japan), NADPH from Kojin (Tokyo, Japan), dithiothreitol (DTT) and cholesterol from Sigma (St Louis, MO, U.S.A.) and silica gel G from Merck (Darmstadt, F.R.G.). Bond-Elut silica cartridges (500 mg of silica, Lot No. 011941) were obtained from Analytichem International (Harbor City, CA, U.S.A).

Equipment

A Shimadzu GC-15A gas chromatograph, equipped with a flame ionization detector, a Van den Berg's solventless injector and a data processing system (Chromatopac C-R3A; Shimadzu, Kyoto, Japan), and a Shimadzu Auto GC–MS 9020DF system, equipped with a data processing system (SCAP 1123), were employed. They were fitted with a fused-silica capillary column (15 m × 0 2 mm I.D.) coated with a non-polar stationary phase (HiCap CBP1; Shimadzu). The GC and GC–mass spectrometric (MS) conditions were the same as described previously [9,10].

Synthesis of 7α- and 7β-hydroxycholesterol

7-Ketocholesterol benzoate was prepared from cholesterol as described previously [11]. 7-Ketocholesterol benzoate (2.4 g) in 100 ml of anhydrous diethyl ether was reduced with lithium aluminium hydride (2 5 g) at room temperature overnight. The reaction mixture was poured into a large volume of water, carefully acidified with dilute hydrochloric acid and extracted three times with diethyl ether. The organic phases were combined, washed with water, dried over anhydrous sodium sulphate and evaporated in vacuo The residue was dissolved in diethyl ether and applied to a column of silica gel G (100 g) and eluted with diethyl ether. Fractions containing less polar spots on silica gel TLC using diethyl ether as the eluent, i.e., 7β-hydroxycholesterol, were combined and evaporated under reduced pressure Crystallization from methanol–water afforded 1 g of 7β-hydroxycholesterol, m.p. 177–178°C (lit. [12] m.p., 177–178.5°C). Fractions containing more polar spots TLC (7α-hydroxycholesterol) were also combined and evaporated. Recrystallization from methanol gave 300 mg of 7α-hydroxycholesterol, m.p. 186–188°C (lit [13] m p , 186–187°C).

Synthesis of 5α-cholestane-3β,7β-diol[a]

A solution of 7β-hydroxycholesterol (700 mg) in 50 ml of methanol to which were added 300 mg of platinum dioxide was hydrogenated with hydrogen at

[a]The internal standard, 5α-cholestane-3β,7β-diol, is available from the authors on request

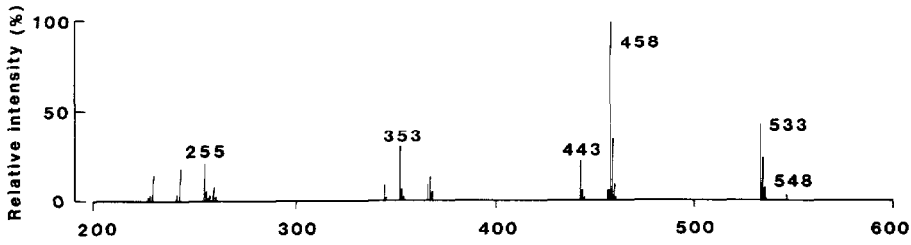

Fig 1 Mass spectrum of the TMS ether derivative of 5α-cholestane-3β,7β-diol

40°C for 20 h [14]. The reaction mixture was filtered and the solvent evaporated in vacuo. The residue was dissolved in 50 ml of methanol, a solution of 0.2 ml of concentrated hydrochloric acid in 10 ml of methanol was added and the mixture was heated at 60°C for 2 h to destroy remaining 7β-hydroxycholesterol, because 5α-cholestane-3β,7β-diol and trace amounts of 7β-hydroxycholesterol form a mixed crystal and therefore recrystallization could not resolve the two compounds. The reaction mixture was concentrated and poured into a large volume of water and extracted three times with diethyl ether The ethereal extract was washed with water, dried over anhydrous sodium sulphate, filtered and evaporated under reduced pressure. Crystallization from benzene and then methanol–water gave crystals of 5α-cholestane-3β,7β-diol (397 mg), m.p. 161.5–162.5°C {lit. [13] m.p., 156–159°C (ethanol–water), 169–170°C (benzene)}. Nuclear magnetic resonance (NMR) spectra were measured at 400 MHz on a JEOL JMN-GX-400 spectrometer NMR (δ ppm): 0.708 (3H, s, 18−CH_3), 0.850 (3H, s, 19−CH_3), 0.876 (3H, d, J=6.6 Hz, 27−CH_3), 0.880 (3H, d, J=6 6 Hz, 26–CH_3), 0.937 (3H, d, J=6.6 Hz, 21−CH_3), 3 234 (1H, m, 7α−H), 3.503 (1H, m, 3α−H). The mass spectra of the trimethylsilyl (TMS) ether derivative of 5α-cholestane-3β,7β-diol is shown in Fig 1.

The purity of the synthesized sterols was checked by TLC using diethyl ether and benzene–ethyl acetate (3 2, v/v) as eluents and by GC. The purities of the sterols used in the study were better than 99% The TLC R_F values of 5α-cholestane-3β,7β-diol using above eluents were almost the same as those of 7α-hydroxycholesterol

Separation of sterols on a Bond-Elut silica cartridge column

All steps in the Bond-Elut silica cartridge column fractionation were carried out at room temperature with elution by gravity flow. For a recovery experiment, 2 μg each of 7α- and 7β-hydroxycholesterol, 7-ketocholesterol and 5α-cholestane-3β,7β-diol were added to crude lipid extracts from freshly prepared microsomal solution. The solvents were evaporated to dryness under nitrogen The residue was dissolved in 100 μl of benzene–ethyl acetate (9 1, v.v) and applied to the silica cartridge column. The column was eluted three times (fractions 1–3) with 2 ml each of benzene–ethyl acetate (9 1, v v) and then

three times (fractions 4–6) with 2 ml each of benzene–ethyl acetate (2 3, v/v). The column was finally washed with 2 ml of ethyl acetate (fraction 7). Each fraction was collected separately, the solvents were evaporated to dryness and the residue was treated with 100 μl of TMSI-H [15]. The resulting TMS ether derivatives were analysed by capillary GC. Coprostanol was used as a recovery standard.

Animals

Male Sprague–Dawley rats (Charles River Japan) were acclimated to the vivarium for at least two weeks with the temperature at 23°C and light periods from 8 a m to 8 p.m. The animals had free access to water and Standard Powder Chow (Oriental Yeast, Tokyo, Japan). The animals were fasted for 12 h prior to the study. The abdomen was opened under diethyl ether anaesthesia. The liver was excised, rinsed with ice-cold homogenizing solution (see below for composition), chilled on ice and weighed.

Preparation of liver microsomes

The microsomal fraction was prepared according to Bjorkhem and Danielsson [5] Liver homogenate (10%, w/v) was prepared in 50 m*M* Tris–HCl buffer (pH 7.4) containing 0 3 *M* sucrose, 10 m*M* DTT and 10 m*M* EDTA in a Potter–Elvehjem homogenizer with a loosely fitting pestle. The homogenate was centrifuged at 20 000 *g* for 15 min. The microsomal fraction was obtained by centrifugation of 20 000 *g* supernatant fluid at 100 000 *g* for 60 min. The microsomal pellet was washed once with homogenizing medium lacking DTT and recentrifuged at 100 000 *g* for 60 min. The resulting microsomal pellet was resuspended in 0.1 *M* phosphate buffer (pH 7.4) containing 1 m*M* EDTA with a loosely fitting pestle in a volume corresponding to the original homogenate. A small aliquot was used for protein determination according to Lowry et al. [16].

Measurement of microsomal cholesterol 7α-hydroxylase activity

The enzymatic conversion of cholesterol to 7α-hydroxycholesterol was measured as follows. The standard assay system consisted of the microsomal preparation corresponding to 0.5–1.0 mg of protein and 0.1 *M* phosphate buffer (pH 7.4) containing 1 m*M* EDTA and 1 m*M* NADPH in a total volume of 1.0 ml. The enzyme assay was carried out in duplicate for 15 min at 37°C in air Care was taken to avoid unnecessary exposure to light. The reaction was terminated by the addition of 10 ml of chloroform–methanol (2 1, v/v). 5α-Cholestane-3β,7β-diol (300 pmol) dissolved in 50 μl of ethanol was added as an internal standard

Sample purification and derivatization

The organic phase was evaporated to dryness under nitrogen. The residue was dissolved in 100 μl of benzene–ethyl acetate (9 1, v/v) and applied to a Bond-Elut silica gel cartridge column. The glassware was rinsed once with 100 μl of the same solvent and the rinsings were added to the column. In order to avoid contamination from the cartridge itself, it was cleaned in advance by elution with 10 ml each of methanol, ethyl acetate, benzene–ethyl acetate (2 3, v/v) and benzene–ethyl acetate (9 1, v/v) in sequence The column was eluted with 6 ml of benzene–ethyl acetate (9 1, v/v) to remove most of the cholesterol and then with 6 ml of benzene–ethyl acetate (2 3, v/v) to recover 7α- and 7β-hydroxycholesterols, 7-ketocholesterol and 5α-cholestane-3β,7β-diol. The solvent of the latter fraction was evaporated and the sterols were converted into TMS ether derivatives [15] and analysed by capillary GC–SIM

GC–MS analysis

On the SIM mode, the ion at *m/z* 456 (M−90) was scanned for TMS ether derivatives of 7α- and 7β-hydroxycholesterols, *m/z* 458 (M−90) for the internal standard and *m/z* 472 (M) for 7-ketocholesterol. Calibration graphs were prepared by SIM analyses of 5α-cholestane-3β,7β-diol (1 μg) with 7α- and 7β-hydroxycholesterol and 7-ketocholesterol (0.1–3 μg) The mass of each sterol was calculated from the peak areas The activity of cholesterol 7α-hydroxylase was calculated by subtracting the amount present in the zero-time assay from that at the end of the incubations and expressed as pmol/mg of protein per min Amounts of 7β-hydroxycholesterol and 7-ketocholesterol were also measured as an index of the so-called autoxidation during the experimental procedure.

Reproducibility and recovery studies

Reproducibility was investigated by analysing five samples in triplicate by SIM. The results were treated according to the one-way layout [17], in which the analytical errors were divided into two sources of sample preparation and measurement of SIM For the recovery experiment, to four groups of duplicate samples known amounts of 7α-hydroxycholesterol were added and the mass of 7α-hydroxycholesterol was determined following the standard work-up. SIM was carried out in triplicate for each sample. The recovery was calculated by dividing the amount recovered by that of 7α-hydroxycholesterol added The calculated values and their confidence limits were obtained using the orthogonal polynomial equation [17].

RESULTS

Recovery of sterols from the Bond-Elut silica cartridge column

Recoveries of sterols from the Bond-Elut silica cartridge column are summarized in Table I. Cholesterol was recovered in fractions II and III The re-

TABLE I

RECOVERY OF STEROLS FROM CHROMATOGRAPHY ON A BOND-ELUT SILICA CARTRIDGE

Solvent systems for fractions 1–3 benzene–ethyl acetate (9 1, v/v), 2 ml each Solvent system for fractions 4–6 benzene–ethyl acetate (2 3, v/v), 2 ml each Solvent system for fraction 7 ethyl acetate, 2 ml

Sterol	Recovery (mean ± S D , n=6) (%)						
	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7
Cholesterol	–	97 8±0 9	2 1±0 9	–	–	–	–
7α-Hydroxycholesterol	–	–	–	1 6±0 1	96 8±2 8	1 6±2 9	–
7β-Hydroxycholesterol	–	–	–	6 2±2 2	93 8±2 2	–	–
7-Ketocholesterol	–	–	–	99 1±2 0	1 0±2 0	–	–
5α-Cholestane-3β,7β-diol	–	–	–	–	95 4±4 7	4 6±4 7	–

TABLE II

GAS CHROMATOGRAPHIC AND MASS SPECTRAL DATA FOR THE TMS ETHER DERIVATIVES OF STEROLS

The values in parentheses are realtive intensities (%)

Sterol	MU[a]	Characteristic ions (m/z)				Base ion/ total ion[c]
		$[M]^{+}$[b]	$[M-15]^{+}$	$[M-90]^{+}$	Other ions	
Cholesterol	31 6	458 (34)[c]	443 (10)	368 (78)	353 (32), 329 (100)	0 12
7α-Hydroxycholesterol	31 4	546 (2)	531 (0)	456 (100)	366 (2)	0 42
7β-Hydroxycholesterol	32 6	546 (2)	531 (0)	456 (100)	366 (2)	0 44
7-Ketocholesterol	34 1	472 (100)	457 (11)	382 (34)	367 (38)	0 12
5α-Cholestane-3β,7β-diol	32 9	548 (2)	533 (43)	458 (100)	443 (21), 353 (32)	0 13

[a]Methylene units (MU) were determined using C_{28}–C_{38} n-alkanes The absolute retention time of the TMS ether derivative of 7α-hydroxycholesterol was 5 8 min under the standard conditions

[b]Molecular ion of the derivatives

[c]Ratios of the base ions to total ions (from m/z 100 to 700 collected by GC–MS)

coveries of 7α- and 7β-hydroxycholesterol, 5α-cholestane-3β,7β-diol and 7-ketocholesterol were greater than 99% in fractions 4–6 in all instances. In order to purify the sterols, the eluate obtained using 6 ml of benzene–ethyl acetate (2 3, v/v) was collected after removal of most of the cholesterol by elution with 6 ml of benzene–ethyl acetate (9 1, v/v).

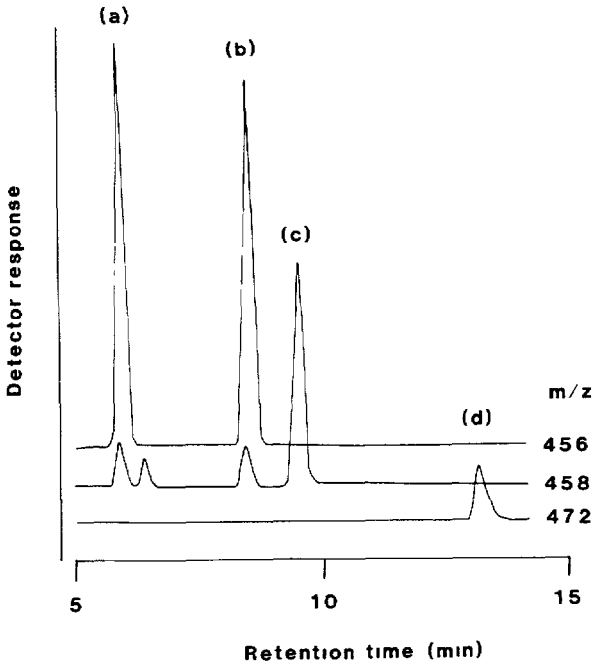

Fig 2 Selected-ion recording of an authentic mixture of the TMS ether derivatives of (a) 7α-hydroxycholesterol, (b) 7β-hydroxycholesterol, (c) 5α-cholestane-3β,7β-diol and (d) 7-ketocholesterol

GC–MS analysis

The mass spectrometric data for the TMS ether derivatives of the sterols are listed in Table II. Representative selected-ion recordings of TMS ether derivatives of a mixture of the authentic compounds are illustrated in Fig. 2. TMS ether derivatives of all these sterols were clearly separated by capillary GC. Fig 3 shows the calibration graphs for 7α- and 7β-hydroxycholesterol and 7-ketocholesterol. The linearity was good in the ranges 20–600 pg for 7α- and 7β-hydroxycholesterol and 0.2–6 ng for 7-ketocholesterol (correlation coefficient > 0.999).

Microsomal cholesterol 7α-hydroxylase activity

Results of SIM obtained in analyses of the incubation mixtures at zero time and at 15 min are shown in Fig. 4 The formation of 7α-hydroxycholesterol increased with time up to 30 min (Fig. 5) and microsomal protein up to at least 1 5 mg (Fig 6). The effect of NADPH concentration on the rate of the reaction is shown in Fig. 7. We chose a reaction time of 15 min, 0.5–1.0 mg of microsomal protein and 1 m*M* NADPH in 1.0 ml of the incubation mixture as the standard assay conditions. 7β-Hydroxycholesterol and 7-ketocholesterol, which are

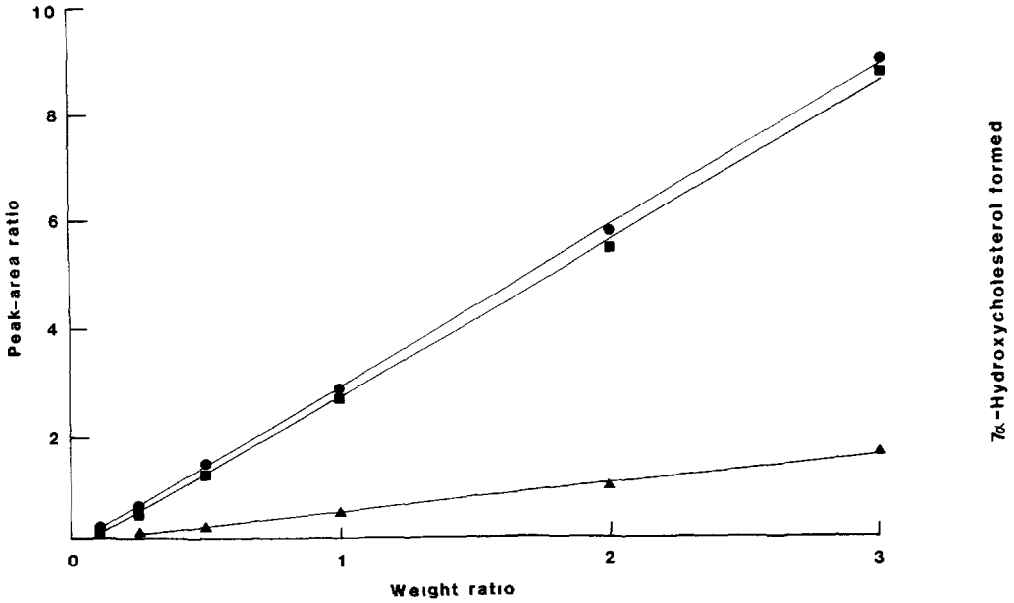

Fig 3 Calibration graphs for TMS ether derivatives of (●) 7α-hydroxycholesterol, (■) 7β-hydroxycholesterol and (▲) 7-ketocholesterol obtained by measuring the peak-area ratio of the component to the internal standard for different weight ratios of the component to the internal standard

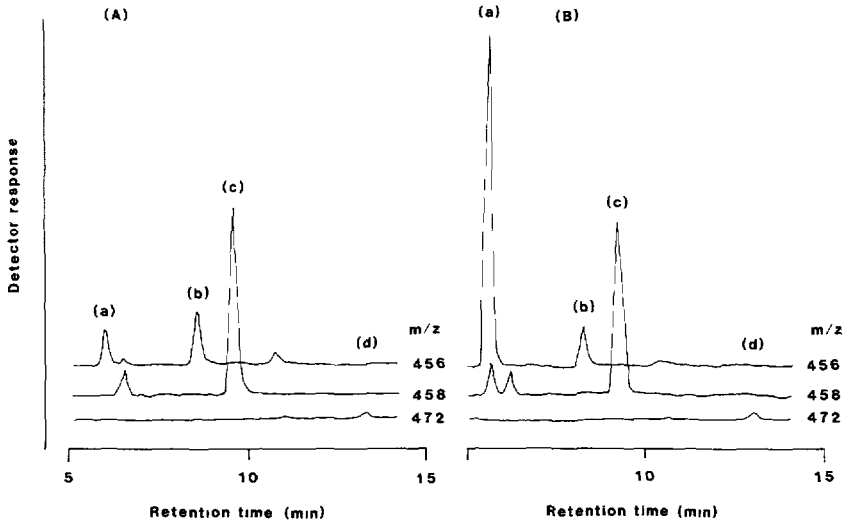

Fig 4 Selected-ion monitoring of the ions at m/z 456, 458 and 472 of the TMS ether derivatives of the extract of (A) a zero-time incubation and (B) a 15-min incubation of rat liver microsomes (a) 7α-hydroxycholesterol, (b) 7β-hydroxycholesterol, (c) 5α-cholestane-3β,7β-diol, (d) 7-ketocholesterol 7β-Hydroxycholesterol and 7-ketocholesterol, which are thought to be the autoxidation products of cholesterol, were detected at zero time and did not increase during the incubation

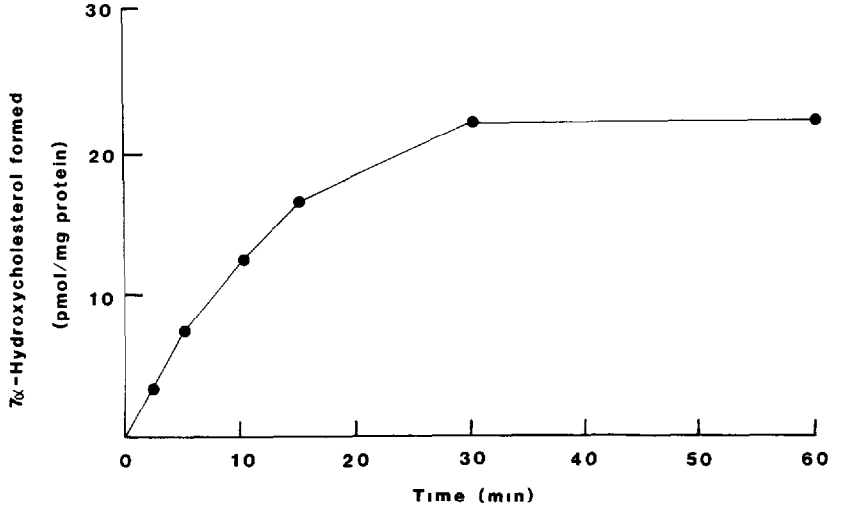

Fig 5 Time course of cholesterol 7α-hydroxylation Standard assay conditions were used, except that incubation time varied from 2 to 60 min

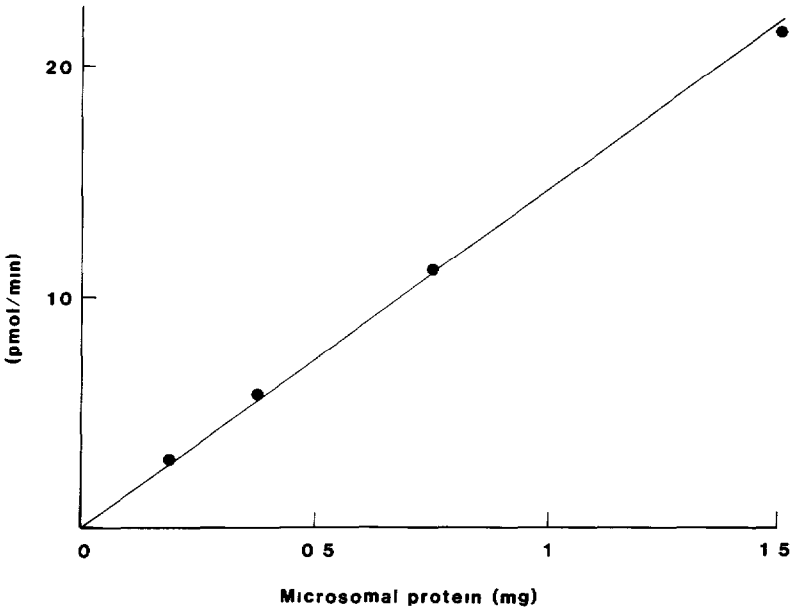

Fig 6 Dependence of cholesterol 7α-hydroxylase activity on the amount of microsomal protein Standard assay conditions were used

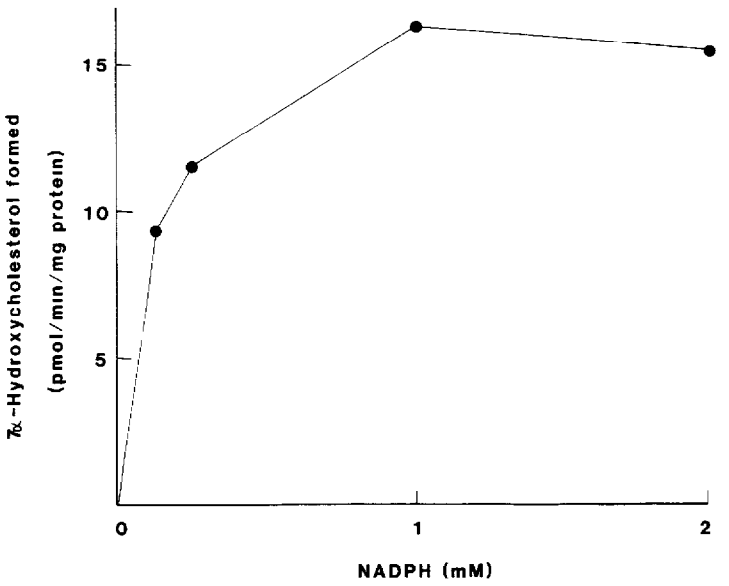

Fig 7 Effect of NADPH concentration on cholesterol 7α-hydroxylase activity Standard assay conditions were used, with the NADPH concentration being varied over the indicated ranges in 1 ml of incubation mixture

TABLE III

RECOVERY OF 7α-HYDROXYCHOLESTEROL

Sample	Amount[a] (X_0+na) ($n=0, 1, 2, 3$)	Amount added (pmol/mg microsomal protein)	Amount found (pmol/mg microsomal protein)	Estimated amount (X_0) ± confidence limit (95%) (pmol/mg microsomal protein)	Recovery[b] (mean ± S D) (%)
A	X_0	0	88 8 93 0 91 0	87 6 ± 2 7	
B	X_0	0	89 8 91 2 90 1		
C	X_0+a	71 3	148 3 150 7 147 8		92 1 ± 5 2
D	X_0+a	71 3	159 7 155 0 150 4		
E	X_0+2a	142 6	233 2 226 6 223 7		98 3 ± 6 6
F	X_0+2a	142 6	233 3 234 8 220 9		
G	X_0+3a	213 9	306 6 287 8 297 9		99 3 ± 8 9
H	X_0+3a	213 9	308 7 305 8 298 8		

[a] X_0 = estimated amount calculated by orthogonal polynomial equation [13], na = amount of 7α-hydroxycholesterol added to the incubation mixture

[b] Recovery (%) = [(amount found − amount added) / estimated amount] 100

TABLE IV

REPRODUCIBILITY OF CHOLESTEROL 7α-HYDROXYLASE ACTIVITY IN THE RAT LIVER MICROSOME

A ANALYTICAL DATA

Sample	Found (pmol/mg per min)	
	Individual values	Mean ± S D
A	11 42, 11 13, 12 49	11 68 ± 0 72
B	12 31, 12 28, 12 35	12 31 ± 0 03
C	11 83, 11 79, 11 32	11 64 ± 0 28
D	12 16, 11 83, 12 69	12 22 ± 0 43
E	12 05, 11 20, 12 14	11 80 ± 0 52
Mean ± S D		11 9 ± 0 4

B ANALYSIS OF VARIANCE[a]

Source	S	f	V	F_0
Sample preparation	1 19	4	0 30	1 25
Error (SIM)	2 37	10	0 24	
Total	3 58	14		
	$F(4,10,0\ 05) = 3\ 478$			

[a] S = residual sum of squares, f = number of degrees of freedom, $f_1 = f$ (sample preparation), $f_2 = f$ (error), V = unbiased variance, F_0 = observed value following F distribution variance ratio ($V_{\text{sample preparation}}/V_{\text{error}}$), $F(f_1,f_2,\alpha)$ = density function of F distribution with f_1 and f_2 degrees of freedom

thought to be the autoxidation products of cholesterol, were detected at zero time and did not increase significantly up to 60 min under the conditions employed.

Statistical analysis of accuracy and precision of the method

Table III shows the recovery of 7α-hydroxycholesterol added to the standard incubation mixture, the calculated value of 7α-hydroxycholesterol and 95% confidence limits The recovery of added 7α-hydroxycholesterol ranged from 84 to 108% with a mean of 97%. The reproducibility of the method is shown in Table IV. The coefficient of variation for sample preparation was about 4 6% and that originating from the SIM determination was about 3.8% Satisfactory precision and reproducibility were obtained.

DISCUSSION

In order to develop a satisfactory method for the determination of hepatic microsomal 7α-hydroxylase activity, we reasoned that the following condi-

tions should be met. (a) endogenous cholesterol is used as the substrate, (b) the internal standard should be synthesized with relative ease or be commercially available and should behave similarly to 7α-hydroxycholesterol, (c) the internal standard and the steroids to be quantified can be purified with relative ease and (d) the actual mass of the steroids can be determined by GC–SIM.

Various compounds were screened as possible internal standards As the internal standard should behave as similarly as 7α-hydroxycholesterol, cholestanediols were the natural candidates. Such a compound should not be present in the ordinal microsomal preparation and should be separable from 7α-and 7β-hydroxycholesterol and 7-ketocholesterol by GC It ought to be synthesized easily and be stable. The final choice fell on 5α-cholestane-3β,7β-diol. For the synthesis of 7α-hydroxycholesterol from 7-ketocholesterol, 7β-hydroxycholesterol was obtained as a by-product and in a sufficient amount A one-step reaction (hydrogenation) of 7β-hydroxycholesterol gave 5α-cholestane-3β,7β-diol in nearly 100%. It could be purified easily and was found to be very stable during storage and sample preparation because it does not contain double bonds It behaves in the same way as the sample steroids during the sample preparation as described below, is separated clearly from these steroids by GC and gives a high-intensity fragment ion at *m/z* 458 on GC–SIM.

At the beginning of the experiments, purification of 7α-hydroxycholesterol was carried out by TLC as described by Bjorkhem and Danielsson [5]. On silica gel TLC with diethyl ether as the eluent, 5α-cholestane-3β,7β-diol and 7α-hydroxycholesterol had similar R_F values and therefore the former seemed to be suitable as an internal standard. However, the amounts of the lipid extract applicable to the TLC plates were very limited and it was troublesome and time-consuming to scrape the silica gel off the plates and to extract the purified sterols from the silica gel The most significant disadvantage of this method was the inherent increase in impurities derived from the silica gel itself. The use of a Bond-Elut silica cartridge containing carefully graded microparticulate silica circumvented this difficulty Almost all of the lipids extracted from the incubation mixture could be applied directly to the column and hence the purification step was greatly simplified. This is of great advantage, especially when it is necessary to determine the enzyme activity in small samples such as human liver biopsy specimens.

With the introduction of the new internal standard and purification procedure, the measurement of cholesterol 7α-hydroxylase was significantly improved. Lipids extracted from the incubation mixture were quantitatively applied to the silica cartridge column Cholesterol was efficiently removed and the internal standard and the steroids to be analysed were recovered quantitatively from the column, as shown in Table I Following derivatization, the 7α-hydroxycholesterol and 5α-cholestane-3β,7β-diol TMS ethers gave prominent fragment ions at *m/z* 456 and 458, respectively. Owing to the simple purification procedure and the use of high-resolution GC–SIM, a higher signal-

to-noise ratio was obtained (Fig 4). Hence the method permitted the sensitive, accurate and reproducible quantification of 7α-hydroxycholesterol (Tables III and IV)

In conclusion, we have succeeded in developing a satisfactory method for the assay of the activity of hepatic microsomal cholesterol 7α-hydroxylase by GC–SIM that is applicable to small amounts of samples such as human liver biopsy specimens by using a new internal standard and a simple pre-clean-up procedure This method is simple, sensitive and accurate, and should obtain wide acceptance

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