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Simultaneous assay of the activities of two key enzymes in cholesterol metabolism by gas chromatography–mass spectrometry

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ABSTRACT

A very sensitive and specific method for the simultaneous assay of the activities of two key regulatory enzymes in cholesterol metabolism, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.34), and cholesterol 7α-hydroxylase (EC 1.14.13.7), is described. The assay is based on the measurement of [$^2H_3$]mevalonolactone and 7α-hydroxycholesterol produced by the incubation of [$^2H_3$]HMG-CoA and endogenous cholesterol with hamster liver microsomes using isotope dilution mass spectrometry. The incubation mixture was purified by means of solid extraction cartridges, and the extract was treated with benzylamine followed by dimethylethylsilyl imidazole. The resulting ether derivatives of the mevalonylbenzylamide and 7α-hydroxycholesterol were quantified by gas chromatography–mass spectrometry with selected-ion monitoring in a high resolution mode. The method made it possible to assay simultaneously the activities of HMG-CoA reductase and cholesterol 7α-hydroxylase in hamster liver microsomes with high sensitivity and accuracy.

INTRODUCTION

Cholesterol homeotasis in the body is preserved mainly through the modulation of the activities of two key enzymes: 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.34), the major regulatory enzyme in

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cholesterol biosynthesis [1,2], and cholesterol 7α-hydroxylase (EC 1.14.13.7), a key regulatory enzyme in the conversion of cholesterol into bile acids [3–6]. Many efforts have been made to develop rapid and accurate methods for the simultaneous assay of these activities.

The activity of HMG-CoA reductase has been assayed extensively by measuring the radioactivity in [3-^{14}C]mevalonolactone (MVL) produced from [3-^{14}C]HMG-CoA [7–12]. However, this method has the disadvantage of low sensitivity in the detection of the radioactivity. In a previous paper we reported a method for the assay of hepatic microsomal HMG-CoA reductase activity by gas chromatography–mass spectrometry (GC–MS) [13], because isotope dilution MS is one of the most sensitive and specific methods. This method was very sensitive but required a tedious maintainance procedure for the use of chemical ionization (CI) mode. Cighetti and co-workers [14,15] and Hoffmann *et al.* [16] have developed a method for assay of the activity by GC–MS with selected-ion monitoring (SIM) in the electron ionization (EI) mode. However, the sensitivity of their method was little better than that of the conventional radioisotopic methods because of appearance of interfering peaks in the analysis.

On the other hand, the activity of cholesterol 7α-hydroxylase has generally been assayed by measuring the radioactivity of 7α-hydroxycholesterol produced from [^{14}C]cholesterol by incubation with liver microsomes [17]. However, since the extent of equilibration of exogenous labelled cholesterol with the endogenous cholesterol pool under different conditions still remains to be clarified, the measurement of 7α-hydroxycholesterol formed from endogenous cholesterol in microsomes by the incubation is thought to be a more accurate index of cholesterol 7α-hydroxylase activity, as reported by Sanghvi *et al.* [18]. For this purpose several methods have been reported: the double-isotope technique [19,20], including some modifications [21,22], high-performance liquid chromatographic (HPLC) procedures [23–26], and GC–MS [12,15,18,27–33]. In HPLC and GC–MS, endogenous microsomal cholesterol has been used as the substrate for the enzyme. Of these methods, GC–MS, especially isotope dilution MS [12,27,28,30–32] has been considered to be the best method owing to its superior sensitivity and specificity.

This paper describes a method for simultaneous assay of the activities of HMG-CoA reductase and cholesterol 7α-hydroxylase by GC–MS with SIM, using [$^{2}H_3$]HMG-CoA and endogenous microsomal cholesterol as the substrates, and [$^{2}H_7$]MVL and [$^{2}H_7$]7α-hydroxycholesterol as the internal standards. In addition, the method was applied to assay the activities of both enzymes in the hamster, which has a capacity for cholesterol biosynthesis [34] and a composition of bile acids [35] similar to those of humans.

EXPERIMENTAL

Chemicals

Mevalonolactone was purchased from Sigma (St. Louis, MO, U.S.A.), 7α-

hydroxycholesterol from Steraloids (Wilton, NH, U.S.A.) and [2-^{14}C]MVL (2.1 GBq/mmol) and [2-^{14}C]mevalonic acid dibenzylethylenediamine (DBED) salt (1.8 GBq/mmol) from Du Pont (Wilmington, DE, U.S.A.). 3-Hydroxy-[3-$^{2}H_3$]methylglutaric acid (HMG-d_3), 3,5-dihydroxy-[3-$^{2}H_3$]methylvalerolactone (MVL-d_3), 3,5-dihydroxy-[3-$^{2}H_3$]methyl-[4,4,5,5-$^{2}H_4$]valerolactone (MVL-d_7) and [25,26,26,26,27,27,27-$^{2}H_7$]cholesterol (cholesterol-d_7) were obtained from Merck Frosst Canada, (Montreal, Canada). HMG-d_3-CoA was synthesized in our laboratory. 7α-Hydroxycholesterol-d_7 was synthesized by Tohma *et al.* according to the method of Nickon and Bagli [36] and Tohma *et al.* [37]. 7α,12α-Dihydroxy-4-cholesten-3-one was kindly supplied by Professor T. Hoshita and Dr. K. Kihira (Pharmaceutical Institute, Hiroshima University, Hiroshima, Japan). The purities were checked by HPLC, and each of them gave only a single peak on the chromatograms.

Glucose-6-phosphate (G-6-P), G-6-P dehydrogenase (EC 1.1.1.49) and NADPH were purchased from Oriental Yeast (Tokyo, Japan), dithiothreitol (DTT) from Wako (Osaka, Japan) and EDTA from Sigma. Solvents and other reagents were of analytical grade. Bond Elut CN (cyanopropyl, 500 mg), SAX (quaternary amine, 500 mg), C_{18} (octadecyl, 1000 mg) and SI (unbonded silica, 500 mg) cartridges were obtained from Analytichem International (Harbor City, CA, U.S.A.), Sephadex LH-20 from Pharmacia (Uppsala, Sweden) and dimethylethylsilyl imidazole (DMESI) [38] and N,O-bis(diethylhydrogensilyl)trifluoroacetamide (DEHS-BSTFA) [39] from Tokyo Kasei Kogyo (Tokyo, Japan).

Thin-layer chromatography (TLC) was carried out using plates precoated with Kieselgel 60 (Merck, Darmstadt, Germany). The solvent system was benzene–acetone (1:1, v/v) [7] for MVL and mevalonic acid (MVA).

The radioactivities of [^{14}C]MVA and [^{14}C]MVL were determined by liquid scintillation counting (LS9000, Beckman, U.S.A.). The yields of the opening of the lactone ring of MVL, the lactonization of MVA and the conversion into the corresponding benzylamide were checked by scanning the radioactivities on the plate using a TLC scanner (Aloka, Tokyo, Japan).

HPLC for quantitation of 7α-hydroxycholesterol was carried out according to the method described by Noshiro *et al.* [23] with some modifications: a Finepak SIL column (250 mm × 4.6 mm I.D., JASCO, Tokyo, Japan) using *n*-hexane–2-propanol (82:18, v/v) as mobile phase at a flow-rate of 1.5 ml/min, and 7α,12α-dihydroxy-4-cholesten-3-one as an internal standard (I.S.).

Gas chromatography–mass spectrometry

GC–MS was performed using a JMS-SX102 instrument equipped with a data-processing system JMA DA-6000 (JEOL, Tokyo, Japan) and a ZAB-SE instrument equipped with a data-processing system Dig Disital 5000 (VG Analytical). The column used was an Ultra Performance capillary (25 m × 0.32 mm I.D.), coated with methylsilicone (Hewlett-Packard, Palo Alto, CA, U.S.A.). The flow-rate of the carrier gas (helium) was 1.0 ml/min. The column oven was pro-

grammed from 70 to 180°C at 50°C/min and from 180 to 320°C at 20°C/min, after a 1-min delay from the start time. The accelerating voltage was 10 kV, the separator and ion source temperature 230°C, the ionization energy 70 eV, and the trap current 600 μA. The reconstructed ion profile was obtained by repetitive scanning of the mass range *m/z* 50–600. High-resolution SIM was carried out with aid of the data system. The mass spectral resolution was *ca.* 10 000.

Animals

Male Golden Syrian hamsters, five weeks old with a mean weight of 91 ± 3 g, were purchased from Charles River Canada (St. Constant, Canada) and divided into two groups. They were fed *ad libitum* with Standard Rat Chow (Oriental Yeast) with or without 5% (w/w) Cholestyramine resin (Sigma), and were maintained at 23°C and light periods from 8:00 a.m. to 8:00 p.m. for one week.

Preparation of liver microsomes

Animals were used between 9:00 and 10:00 a.m. after overnight fasting. Under diethyl ether anaesthesia the abdomen was opened, and the liver was immediately excised. Each liver (*ca.* 2 g) was washed in ice-cold normal saline solution. The liver was blotted dry, weighed, and suspended in 5 ml of fresh cold 50 m*M* Tris–HCl buffer (pH 7.4) containing 0.3 *M* sucrose, 10 m*M* DTT, 10 m*M* EDTA, and 50 m*M* NaF, to inhibit the activity of endogenous protein phosphatase [10,11,40,41], per gram of tissue. The livers were minced with scissors, then homogenized with a loose-fitting (1-mm clearance) Teflon pestle. The homogenate was centrifuged at 20 000 *g* for 15 min. The supernatant solution was centrifuged at 100 000 *g* for 60 min. The resulting microsomal fraction was suspended in a homogenizing medium and recentrifuged at 100 000 *g* for 60 min. The resulting microsomal fraction was suspended in 1 ml of fresh cold 0.1 *M* potassium phosphate buffer (pH 7.4) containing 5 m*M* DTT and 1 m*M* EDTA per gram of the first-used tissue, and frozen separately in liquid nitrogen. Fractions can be stored for several months at −80°C without significant loss of activity.

Protein concentration

The microsomal content of protein was determined by the method of Bradford [42].

Standard assay system

The standard assay was carried out in a volume of 250 μl using an incubation mixture of 0.1 *M* potassium phosphate buffer (pH 7.4) containing 5 m*M* DTT and 1 m*M* EDTA, G-6-P (12 m*M*), G-6-P dehydrogenase (1 unit), microsomes (50–100 μg of protein), HMG-d_3-CoA (0.1 m*M*), and NADPH (3 m*M*). The mixture was placed in a 10-ml culture tube and shaken at 37°C for 5 min. The reaction was stopped by the addition of 50 μl of 1 *M* NaOH.

Clean-up procedure

A mixture of 4 ng of MVL-d_7 and 8 ng of 7α-hydroxycholesterol-d_7 in 200 μl of acetonitrile was added to the above incubation mixture as internal standards. After ultrasonification for 15 min, the sample was applied to a Bond Elut CN cartridge (prewashed with 10 ml of chloroform, ethanol and distilled water) and washed with 0.7 ml of acetonitrile–distilled water (2:3, v/v) (washed-out fraction), and eluted with 5 ml of ethanol (eluted fraction). Each fraction was collected and purified separately.

Washed-out fraction. After addition of 0.5 ml of distilled water to this fraction, the mixture (*ca.* 2 ml) was applied to a Bond Elut SAX cartridge (prewashed with 3 ml of 0.6 *M* HCl and 20 ml of distilled water) and eluted with 1 ml of 0.6 *M* HCl. This eluate was kept at room temperature for 15 min to lactonize the resulting MVA. After lactonization, this fraction was applied to a Bond Elut C_{18} (1000 mg) cartridge (prewashed with 10 ml of chloroform, ethanol and distilled water) and eluted with 3 ml of acetonitrile. The eluate was evaporated to dryness under reduced pressure, and the resulting residue was dissolved in 2 ml of benzene. The benzene solution was applied to a Bond Elut CN cartridge (prewashed with 3 ml of dichloromethane and benzene) and washed with 3 ml each of benzene and benzene–dichloromethane (1:1, v/v). MVL was eluted with 5 ml of dichloromethane.

Eluted fraction. The fraction containing 7α-hydroxycholesterol was evaporated to dryness under reduced pressure. The residue was dissolved in 0.3 ml of *n*-hexane–2-propanol (95:5, v/v), and applied to a Bond Elut SI cartridge (prewashed with 3 ml of the same solvent). After washing with 3 ml of the same solvent, 7α-hydroxycholesterol was eluted with 3 ml of *n*-hexane–2-propanol (8:2, v/v).

The resulting eluates of both fractions were mixed and evaporated to dryness.

Derivatization

A 50-μl volume of benzene and 20 μl of benzylamine were added to the residue and kept at room temperature for 30 min. After evaporation under reduced pressure, the residue was treated with 30 μl of distilled pyridine and 20 μl of DEHS-BSTFA [39,44,45], or 100 μl of distilled pyridine and 20 μl of DMESI [38,43]. The former was kept at room temperature for 30 min and analysed without purification. The latter was heated at 80°C for 60 min, and excess silylating reagent was removed on a Sephadex LH-20 column (60 mm × 6 mm I.D.) equilibrated with *n*-hexane–chloroform (1:1, v/v). The DMES ether derivative of mevalonylbenzylamide and 7α-hydroxycholesterol were recovered in the first 3 ml of effluent. After evaporation under reduced pressure, the residue was redissolved in 50 μl of *n*-hexane.

RESULTS AND DISCUSSION

Clean-up procedure

The recoveries of MVL and 7α-hydroxycholesterol from the extraction and purification procedures were checked by addition of ^{14}C-labelled MVL (130 ng, 2.1 kBq) or cold 7α-hydroxycholesterol (1 μg), which was measured by HPLC using 7α,12α-dihydroxy-4-cholesten-3-one as an internal standard. Table I shows the absolute recoveries of MVL and 7α-hydroxycholesterol in each step of the solid extraction. The overall recoveries were found to be 74.5 ± 9.7% for MVL and 90.1 ± 4.2% for 7α-hydroxycholesterol. The completeness of the opening of the lactone ring of MVL and the lactonization of MVA were examined by addition of ^{14}C-labelled MVL or ^{14}C-labelled MVA DBED salt to each step of the purification, the yields were 99.6 ± 0.3% for the former (n = 7) and 97.3 ± 0.2% for the latter (n = 7).

In alkaline medium, the lactone ring of MVL was converted quantitatively into the open form, suggesting that MVL-d_7 could be used as a convenient internal standard for the quantitation of MVA-d_3 produced by HMG-CoA reductase. In order to extract MVA [8,9,13–15] and 7α-hydroxycholesterol [15,18,27–33] from biological specimens, tedious liquid–liquid extraction procedures have conventionally been used. We attempted to use solid–solid extraction for the extraction and purification. The separation of 7α-hydroxycholesterol and MVA was achieved perfectly by the use of a Bond Elut CN cartridge in the reversed-phase mode. MVA, which was washed out with acetonitrile–distilled water (2:3, v/v) from a Bond Elut CN cartridge, was purified with a Bond Elut SAX, in the anion-exchange mode. MVA was eluted as the lactone form with 0.6 *M* HCl. After extraction with a Bond Elut C_{18} cartridge, MVL was further purified on another Bond Elut CN cartridge in the normal-phase mode. 7α-Hydroxycholesterol, which was eluted from a Bond Elut CN cartridge, was further purified on a Bond Elut SI cartridge in normal phase mode.

TABLE I

ABSOLUTE RECOVERIES OF MEVALONOLACTONE AND 7α-HYDROXYCHOLESTEROL IN EACH STEP OF SOLID PHASE EXTRACTION

Values are mean ± S.D.; n = 7.

Mevalonolactone		7α-Hydroxycholesterol	
Column	Recovery (%)	Column	Recovery (%)
Bond-Elut CN	97.9 ± 3.2	Bond-Elut CN	100.8 ± 3.4
Bond-Elut SAX	93.3 ± 1.4	Bond-Elut SI	89.4 ± 2.5
Bond-Elut C_{18}	91.6 ± 7.3		
Bond-Elut CN	89.0 ± 5.3		
Overall	74.5 ± 9.7		90.1 ± 4.2

Derivatization

After evaporation of the solvent from the resulting extract for MVL and 7α-hydroxycholesterol, the derivatization was performed simultaneously. MVL was easily converted into the corresponding alkylamides by addition of alkylamines such as *n*-propylamine and benzylamine (Fig. 1). The yields of the amidation were found to be 99.8 ± 0.04% for *n*-propylamide and 99.5 ± 0.2% for benzylamide. The benzylamide derivative had better GC–MS properties than the propylamide. The benzylamide and 7α-hydroxycholesterol were converted into cyclic diethylsilylene (DES) and diethylhydrogensilyl (DEHS) or DMES ethers with DEHS-BSTFA or DMESI. The DES ether derivative of the mevalonylbenzylamide had excellent GC–MS properties, as described previously [39,44,45]. 7α-Hydroxycholesterol gave the DEHS ether derivative owing to lack of the vicinal diol group in the molecule. This is less stable than the cyclic DES ether derivative, so the DMES ether was chosen as the best derivative for this analysis because of its

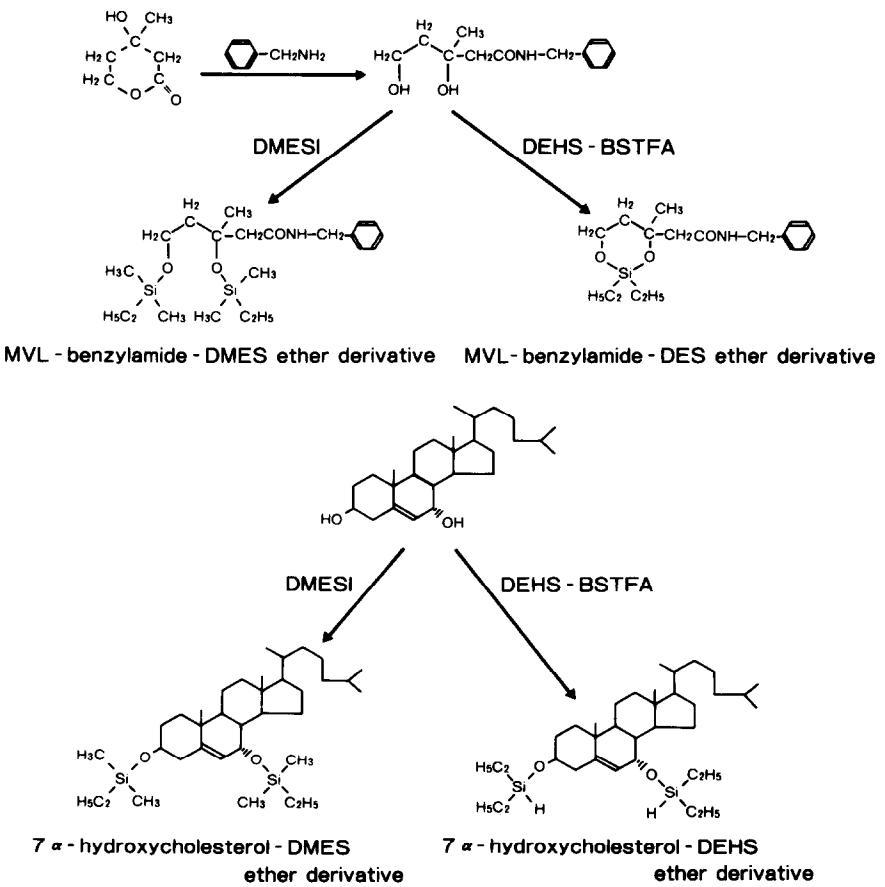

Fig. 1. Derivatization of MVL and 7α-hydroxycholesterol for GC–MS analysis.

excellent GC–MS properties, better than those of the DES and DEHS ether derivatives of both incubation products.

Gas chromatography–mass spectrometry

Under standard conditions, the retention times of the DMES ether derivatives

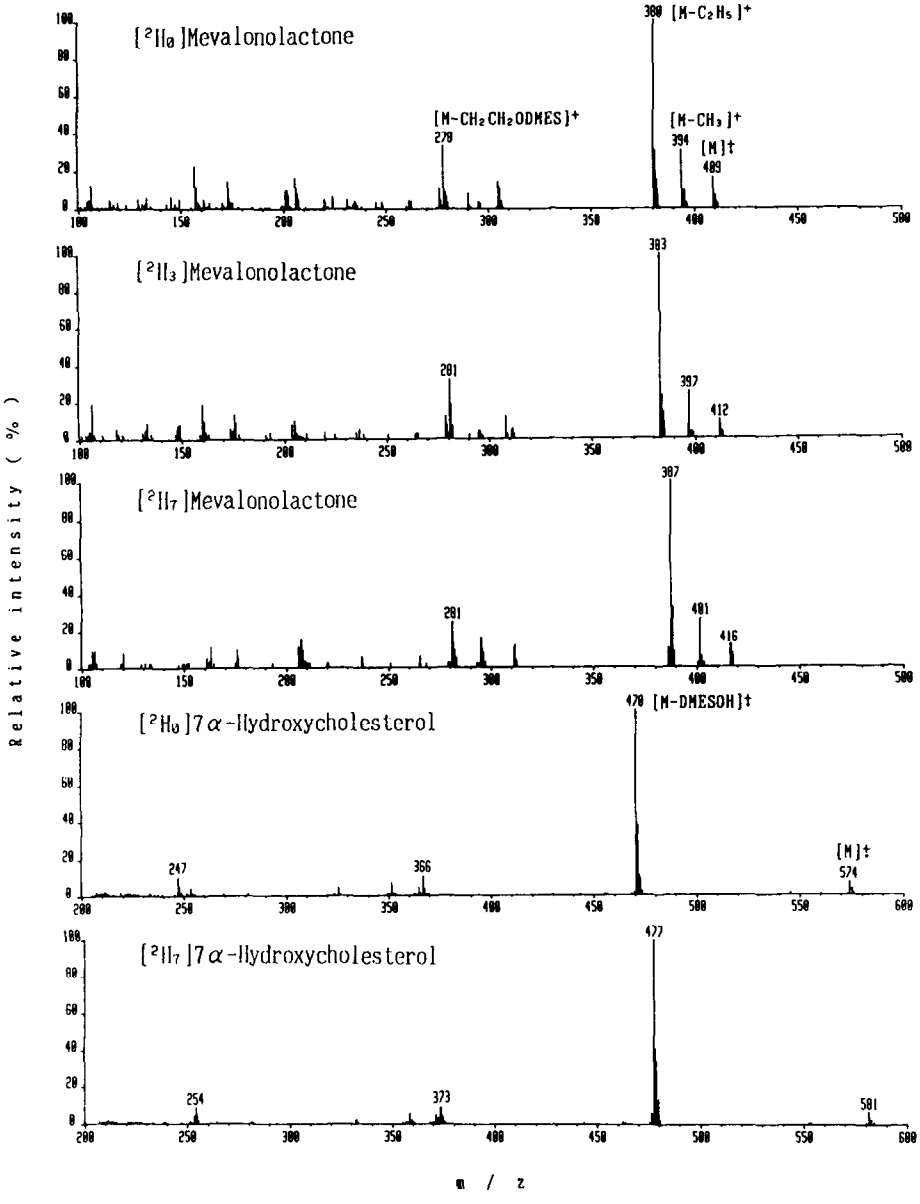

Fig. 2. Mass spectra of the benzylamide-DMES ether derivatives of MVL-d_0, MVL-d_3 and MVL-d_7, and the DMES ether derivatives of 7α-hydroxycholesterol-d_0 and -d_7.

of the MVL-benzylamide and 7α-hydroxycholesterol were 7.4 and 10.8 min, and the methylene unit (MU) values [46] were 24.2 and 33.5, respectively. On the other hand, the MU values of the DES and DEHS ether derivatives of them were 22.0 and 34.8, respectively. Fig. 2 shows the mass spectra of the DMES ether derivatives obtained by GC–MS in the EI mode. The MVL derivatives gave the characteristic fragment ion of $[M - 29]^+$ formed by the elimination of the ethyl group from the DMES moiety. The 7α-hydroxycholesterol DMES ether derivative gave the $[M - 104]^+$ fragment ion, formed by the elimination of the dimethylethylsilanol as a prominent peak. As described previously, the TMS ether derivative of MVL-d_0 gave a prominent ion at *m/z* 145 [14,15,47], whereas the DMES derivative gave a prominent peak at *m/z* 380. The appearance of a prominent ion in the high mass region is a great advantage in the quantitation of both products in the incubation mixture by GC–MS with SIM, because it makes it possible to avoid interfering peaks.

Calibration curves

Calibration curves were established for MVL and 7α-hydroxycholesterol. Good linearities were obtained in the ranges 0.8–20 pg for MVL and 1.6–40 pg for 7α-hydroxycholesterol.

Representative SIM

The influence of impurities that have the same retention times as MVL and 7α-hydroxycholesterol was completely eliminated by the combined use of the above-mentioned purification and high-resolution mass spectrometry. Fig. 3 illustrates the representative high-resolution selected-ion recordings of a mixture of the authentic compounds. The multiple ion detector was focused on *m/z* 383.2266/387.2517 for MVL and *m/z* 470.3944/477.4383 for 7α-hydroxycholesterol. The peaks of MVL-d_3 and 7α-hydroxycholesterol-d_0 that appeared on select-

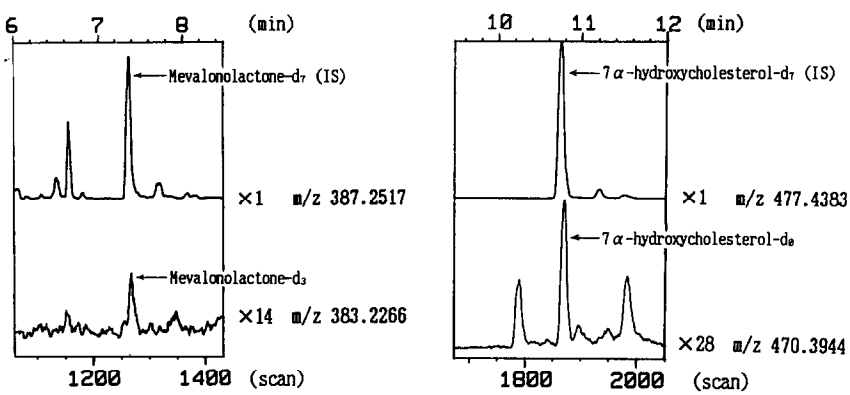

Fig. 3. High-resolution selected-ion recordings of an authentic mixture of the benzylamide-DMES ether derivative of MVL and the DMES ether derivative of 7α-hydroxycholesterol ($M/\Delta M$ = 10 000).

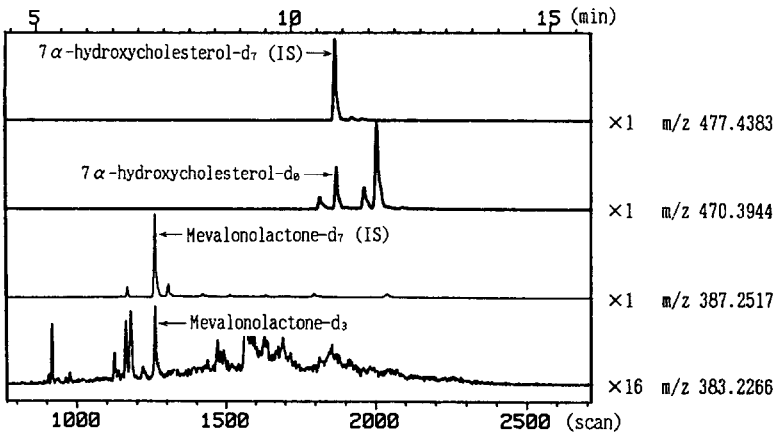

Fig. 4. High-resolution selected-ion recording of the benzylamide-DMES ether derivative of MVL and the DMES ether derivative of 7α-hydroxycholesterol in extracts from the standard incubated mixture (sample A, Table IV).

ed-ion recordings corresponded to *ca.* 0.8 and 1.6 pg, respectively, indicating that the present method is very sensitive and specific. Fig. 4 shows a representative selected-ion recording obtained by analysis of a standard incubation mixture from a cholestyramine-untreated hamster. The peaks of MVL-d_3 and 7α-hydroxycholesterol-d_0 in the selected-ion recording corresponded to *ca.* 4.4 pg (1.1 pmol/mg protein per min) and 89.4 pg (7.4 pmol/mg protein per min), respectively. The peak at *m/z* 470.3944, corresponding to 7α-hydroxycholesterol-d_0, was observed in the extract obtained at zero time, as described previously [15,30,33]. The amount of 7α-hydroxycholesterol formed during incubation was estimated by subtracting the amount present in the zero-time assay from the amount present at the end of incubation.

Properties of the microsomal assay systems

Cholestyramine-treated hamsters were used in the following experiments, because this treatment enhances the activity of these enyzmes in the microsomes. Fig. 5 illustrates the relationship between the reaction rate and the amounts of enzymes as represented by microsomal protein. Proportionality was observed when the amount of microsomal protein ranged from 0 to at least 200 μg for both enzymes. In the standard assay system containing 50–100 μg of microsomal protein, the rate of formation of MVL was linear with time for at least 30 min (Fig. 6). The formation of 7α-hydroxycholesterol increased with time up to at least 30 min, but good linearity was observed only for 5 min. Therefore, we chose a reaction time of 5 min for the standard simultaneous assay.

Statistical analysis of the accuracy and precision of the method

The accuracy and precision of the present method were investigated by use of

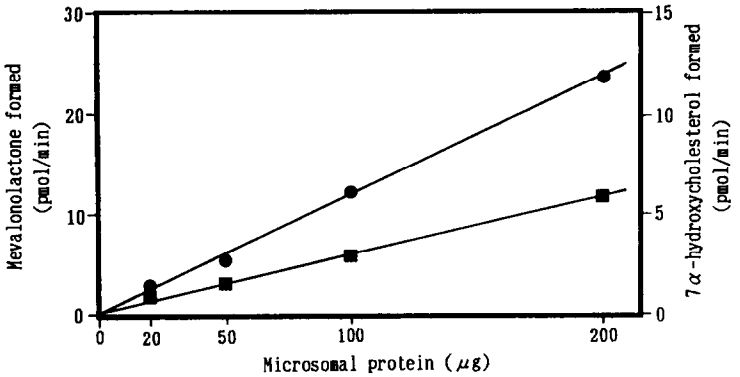

Fig. 5. Effect of increasing amounts of microsomes on the rates of reduction of HMG-CoA (●) and 7α-hydroxylation of cholesterol (■) (cholestyramine-treated hamster).

cholestyramine-untreated hamsters. Reproducibilities were investigated by analysing four samples in triplicate by SIM. The results were analysed according to a one-way layout [48], where the analytical errors were divided into two sources of sample preparation and measurement of SIM. Tables II and III show the analytical data and the analysis of variances in MVL and 7α-hydroxycholesterol. The latter variances were thought to be attributable to the SIM measurement, because the errors during sample preparation were negligible.

For the recovery experiment, known amounts of MVL and 7α-hydroxycholesterol were added to four groups of duplicate samples. These samples were subjected to the clean-up procedure, and SIM was carried out in triplicate for each sample. Table IV shows analytical data, recoveries of MVL and 7α-hydroxycholesterol, estimated amounts and 95% confidence limits for MVL and 7α-hydroxycholesterol in the standard incubation mixture. The recoveries were cal-

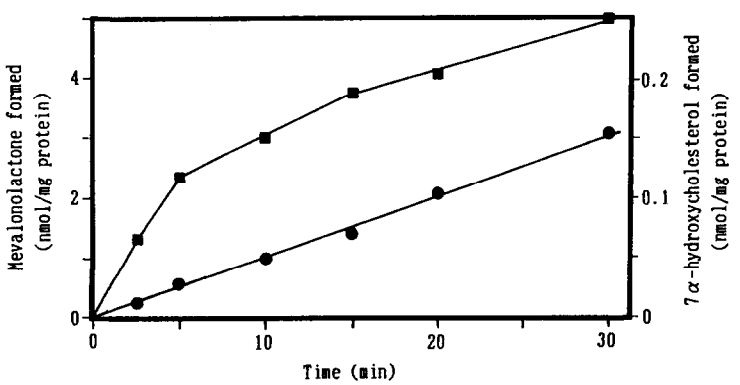

Fig. 6. Time course of enzymic reduction of HMG-CoA (●) and 7α-hydroxylation of cholesterol (■) (cholestyramine-treated hamster).

TABLE II

REPRODUCIBILITIES OF HMG-CoA REDUCTASE AND CHOLESTEROL 7α-HYDROXYLASE ACTIVITIES IN HAMSTER LIVER MICROSOMES: ANALYTICAL DATA

Sample	Mevalonolactone (pmol/mg per min)				7α-Hydroxycholesterol (pmol/mg per min)			
	Individual values			Mean ± S.D.	Individual values			Mean ± S.D.
A	1.21	1.06	1.16	1.14 ± 0.08	4.67	3.52	3.81	4.00 ± 0.60
B	1.06	1.06	1.00	1.04 ± 0.04	3.70	3.76	3.69	3.72 ± 0.03
C	1.12	1.06	1.18	1.12 ± 0.06	3.91	4.14	4.19	4.08 ± 0.15
D	1.07	1.07	1.03	1.06 ± 0.02	3.91	4.01	4.06	3.99 ± 0.08
Mean + S.D.				1.09 + 0.06				3.95 + 0.30

culated by dividing the amount recovered by that of MVL and 7α-hydroxycholesterol added. The estimated values and their confidence limits calculated by the orthogonal polynomial equation [48] constituted indices for precision and accuracy of the present method. The recoveries of added MVL ranged from 96 to 100%, with a mean of 98%, and those of added 7α-hydroxycholesterol ranged from 94 to 102%, with a mean of 97%. These values indicate that the present method provides analytical results of high reliability and reproducibility.

TABLE III

REPRODUCIBILITIES OF HMG-CoA REDUCTASE AND CHOLESTEROL 7α-HYDROXYLASE ACTIVITIES IN HAMSTER LIVER MICROSOMES: ANALYSIS OF VARIANCE

S = residual sum of squares; f = number of freedom, $f_1 = f_{\text{sample preparation}}$, $f_2 = f_{\text{error}}$; 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; C. V. = coefficient of variation.

Source	Mevalonolactone					7α-Hydroxycholesterol				
	S	f	V	F_0	C.V. (%)	S	f	V	F_0	C.V. (%)
Sample preparation	0.0220	3	0.00732	2.56	7.8	0.229	3	0.0763	0.786	7.0
Error (SIM)	0.0229	8	0.00286		4.9	0.779	8	0.0974		7.9
Total	0.0448	11				1.008	11			
		$F(3,8,0.05) = 4.066$								

TABLE IV

RECOVERIES OF MEVALONOLACTONE AND 7α-HYDROXYCHOLESTEROL FROM MICROSOMAL FRACTIONS

Sample ($X_0 + na$) ($n=0,1,2,3$)	Amount added (pmol/mg microsomal protein)	Amount found (pmol/mg microsomal protein)			Recovery[a] (mean ± S.D.) (%)	Estimated amount ± 95% confidence limit (pmol/mg microsomal protein)
Mevalonolactone						
A X_0	0	6.3	5.8	6.4		
B X_0	0	6.2	5.9	6.1	[$\bar{X}_0$ = 6.12 ± 0.23]	5.8 ± 1.4
C X_0 + a	15.0	20.9	20.2	20.8		
D X_0 + a	15.0	21.1	19.8	20.5	96.1 ± 3.3	
E X_0 + 2a	30.0	35.8	35.6	34.8		
F X_0 + 2a	30.0	34.8	35.3	35.5	97.1 ± 1.3	
G X_0 + 3a	45.0	51.2	49.7	50.2		
H X_0 + 3a	45.0	51.3	51.5	51.9	99.5 ± 1.9	
7α-Hydroxycholesterol						
A X_0	0	38.9	39.8	38.7		
B X_0	0	35.8	33.0	31.1	[$\bar{X}_0$ = 36.22 ± 3.55]	36.8 ± 8.2
C X_0 + a	29.0	59.2	67.2	66.2		
D X_0 + a	29.0	63.3	66.5	59.4	94.7 ± 12.4	
E X_0 + 2a	57.9	95.0	97.8	96.0		
F X_0 + 2a	57.9	92.4	96.1	95.1	102.1 ± 3.1	
G X_0 + 3a	86.9	116.7	115.9	123.7		
H X_0 + 3a	86.9	120.1	116.5	114.9	94.1 ± 3.8	

[a] Recovery (%) = [(amount found − $\bar{X}_0$)/amount added] · 100.

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