Measurement of cholesterol 7α-hydroxylase activity with selected ion monitoring

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Summary Formation of 7α-hydroxycholesterol by rat liver microsomes was quantitated using a gas chromatograph–mass spectrometer operated in selected ion monitoring mode. This procedure measures the mass of 7α-hydroxycholesterol formed and does not require addition of labeled cholesterol to monitor the reaction, since endogenous microsomal cholesterol serves as substrate for the enzyme cholesterol 7α-hydroxylase. The method requires only the extraction of lipids from microsomal incubations and injection of a small fraction of this extract into the apparatus. Microsomes from normal rat livers incubated for different periods were found to yield increased 7α-hydroxycholesterol with time.—**Sanghvi, A., E. Grassi, C. Bartman, R. Lester, M. Galli Kienle, and G. Galli.** Measurement of cholesterol 7α-hydroxylase activity with selected ion monitoring. *J. Lipid Res.* 1981. **22:** 720–724.

Supplementary key words 7α-hydroxycholesterol · gas–liquid chromatograph–mass spectrometry

Cholesterol 7α-hydroxylase (EC 1.14././) has a critical role in both the metabolism of cholesterol and bile acids synthesis. A unique feature of this mixed function oxidase enzyme is that it utilizes endogenous microsomal cholesterol as its substrate. This has complicated attempts to measure its activity accurately (1). Generally, 7α-hydroxylase activity is estimated by measuring the extent of conversion of added radiolabeled cholesterol to labeled 7α-hydroxycholesterol. The radioactive cholesterol is added to the microsomal incubation mixture with the aid of a detergent, most frequently as a Tween 80-buffer emulsion. However, since the extent of equilibration of exogenous cholesterol with the endogenous "substrate pool" is not precisely known under different conditions, measurement of the actual mass of 7α-hydroxycholesterol formed is thought to be a more accurate index of 7α-hydroxylase activity. Several approaches, direct and indirect, are available for measuring the mass of 7α-hydroxycholesterol. These include the double isotope derivative dilution method (2), measurement of tritium as ^3H_2O of the medium-^3H being released from C-7 of specifically labeled [7α-^{3}H]cholesterol (3), and a gas–liquid chromatography–mass spectrometry procedure (4). A most recent study by Nimmannit and Porter (5) describes an apparent solubilization of cholesterol 7α-hydroxylase activity after removal by butanol extraction of more than 90% of endogenous microsomal cholesterol. The butanol-extracted microsomes are then subjected to treatment with a nonionic detergent, Emulgen 911, to achieve solubilization of enzyme activity. This is a significant advance which should allow a proper study of the enzyme itself. For studies concerned with the measurement of relative changes in enzyme activity pursuant to experimental manipulation or changes in physiological environment, the above procedure (5) is similar to others utilizing incorporation of isotope from labeled cholesterol into 7α-hydroxycholesterol and requires extensive manipulation. In an attempt to simplify this task we have used selected ion monitoring (SIM) to measure monohydroxy and monoketone derivatives of cholesterol (6). Here we report the application of this technique in specifically measuring the formation of 7-hydroxycholesterol, which reflects 7α-hydroxylase activity in rat liver microsomes.

EXPERIMENTAL PROCEDURES

Synthesis, separation, and purification of sterols

3β-Acetoxy-cholest-5-en-7-one (Steraloids) was crystallized once from methanol–ether, m. p. 159°C (reported 159°C (7)). 7α-Hydroxy, 7β-hydroxy, and 7-ketocholesterol were prepared from this starting material and purified essentially as described by Björkheim and Danielsson (4). One hundred milligrams of this sterol was dissolved in 10 ml of anhydrous diethyl ether (distilled over $LiAlH_4$). Two hundred milligrams of $LiAlH_4$ was added gradually while the mixture was kept over ice. The reaction was stirred gently and allowed to remain at room temperature for 1 hr. Ethyl acetate (about 15 ml) was added carefully until H_2 evolution had ceased and 10 ml of ice cold water was then added. Ethyl acetate was removed and the water phase was washed twice

Abbreviations: GLC–MS, gas–liquid chromatography–mass spectrometry; SIM, selected ion monitoring; TMS, trimethylsilyl.

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with 10 ml of diethyl ether. Organic phases were combined, washed with water, and kept overnight over Na_2SO_4. After evaporation under N_2 the sterols were dissolved in a small volume of chloroform and purified by repeated preparative thin-layer chromatography on plates of 2 mm Silica gel 60 F-254 (E. Merck, Darmstadt, West Germany). The plates were developed with ethyl acetate–benzene 70:30 (v/v). The R_f values were: cholesterol, 0.71; 7-ketocholesterol, 0.56; 7β-hydroxycholesterol, 0.30; 7α-hydroxycholesterol, 0.19. The zones corresponding to 7α- and 7β-hydroxycholesterol were scraped into a small glass column; sterols were eluted with ethyl acetate and crystallized twice from methanol–ether. The melting points were 7α-hydroxycholesterol 183.5–184°C, reported 184–187°C (2); and 7β-hydroxycholesterol, 170°C, reported 166°C (8). Analyses of homogeneity of these sterols were by GLC and GLC–MS and they were used as standards for SIM work.

Animal studies

Male Sprague-Dawley rats (220–250 g) were used throughout. Animals were kept in artificially lighted quarters under a strict twelve-hour (8 PM to 8 AM light, 8 AM to 8 PM dark) light-dark cycle. Thirty grams of Purina rat chow was allowed each rat per day at 8 AM and the remainder was withdrawn each evening at 6 PM; water was allowed ad libitum. Animals were killed at 2:00 PM by decapitation and the peritoneal cavity was flushed immediately with 10 ml of cold saline. Livers were excised, quickly chilled in 0.25 M sucrose containing 10 mM EDTA (pH 7.1), minced, and homogenized in four volumes of the sucrose solution (9, 10). After removal of cell debris at 500 *g* and mitochondria at 10,000 *g*, microsomes were obtained from the supernatant by centrifugation at 100,000 *g* for 1 hr. The microsomal pellet was washed once with 0.25 M sucrose solution, recentrifuged for 1 hr at 100,000 *g* and suspended in 0.1 M phosphate buffer (pH 7.4) containing 10 mM β-mercaptoethylamine so that 4 ml was equivalent to 1 g of liver. Incubations were performed in air for 30 min at 37°C in a Dubnoff shaker at 120 oscillations per minute. The incubation mixture contained in 0.1 M phosphate buffer, 10 mM β-mercaptoethylamine; 4 mM $MgCl_2$; 20 mM glucose-6-phosphate; 2 units of glucose-6-phosphate dehydrogenase; 2 mM NADP; 200 μl of microsomal suspension; final volume 1.0 ml. Controls were boiled microsomes containing cofactors and incubated as above. Reaction was stopped with 10 ml of chloroform–methanol 2:1 (v/v) (HPLC or pesticide grade, Fisher) and 10 μg of 5α-cholestane was added to each flask. Lipids were extracted in chloroform–methanol; the organic phase was washed with 0.9% saline and evaporated under N_2. Measurement of 7α-hydroxycholesterol formation by SIM analyses was performed using a portion of the lipid extract as described below.

Cholesterol was measured in a Beckman Cholesterol Analyzer with an enzymatic procedure. In this method cholesteryl esters are hydrolyzed by cholesterol esterase and the liberated free cholesterol is oxidized by cholesterol oxidase to cholest-4-ene-3-one with hydrogen peroxide formation. The rate of oxygen consumption during this process is measured by an oxygen electrode. Cholesterol in lipid extracts was also measured by GLC–MS using area ratio response factors for cholesterol:5α-cholestane which were obtained from the standard curves. Protein determinations were made using the method of Lowry et al (11).

GLC–MS analysis

SIM analyses were performed with Hewlett-Packard 5995A and 5986A GLC–MS instruments. Results were the same with both instruments. The dried lipid extract was dissolved in 100 μl of chloroform and 25 μl were taken into a 0.3-ml reaction vial (Supelco). Chloroform was evaporated, 50 μl of Silprep (Applied Science) was added, and trimethylsilyl (TMS) ether derivatives were obtained by heating the mixture for 10 min at 70°C. Routinely, 2–3 μl of the TMS-sterol mixture was injected. The glass GLC column was 0.2 cm i.d. × 1 meter, packed with 3% SP-2250 on 100–120 Supelcoport (Supelco). The operating parameters were: carrier gas, helium, flow rate 30 ml/min; oven, programmed 230–280°C, 10°/min; injector, 265°C; transfer line, 275°C; analyzer, 210°C; ion source, 200°C; electron multiplier, 2400 volts; electron energy 70 ev. The following ions were focussed: m/z 546, the parent ion of monoTMS ether of 7α-, and 7β-hydroxycholesterol; m/z 472 the parent ion of monoTMS ether of 7-ketocholesterol; m/z 456, M^+-90 ion of diTMS ethers of 7α-, and 7β-hydroxycholesterol; m/z 443, M^+-15 ion of TMS-choleserol; and m/z 372, M^+ ion of 5α-cholestane. The retention times, relative to cholestane on the total ion current trace as TMS ethers were: 7α- and 7β-hydroxycholesterol, 1.24, and 1.45, respectively; cholesterol, 1.36; and 7-ketocholesterol, 2.1. The resolution of sterol-TMS ether derivatives on selected ion (m/z 456) current trace was: 1.0 between 7α-hydroxycholesterol and cholesterol; 0.82 between 7β-hydroxycholesterol and cholesterol; and 2.45 between 7α-, and 7β-hydroxycholesterol. The criteria for selection of ions monitored have been described (6). Standard curves were prepared by SIM analyses of 10 μg and 15 μg of 5α-cholestane and cholesterol, respectively, and progressively increasing amounts of 7α-hydroxy, 7β-

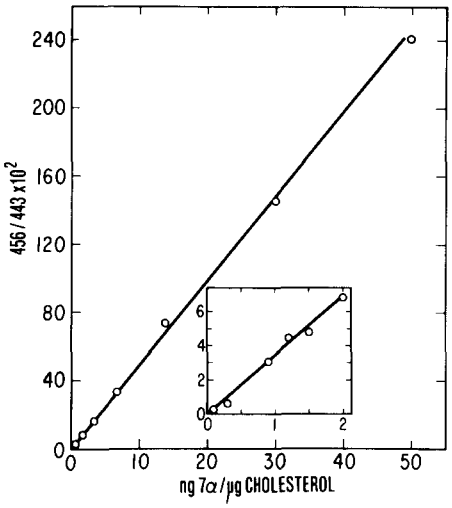

Fig. 1. Standard curve for ditrimethylsilyl ether of 7α-hydroxycholesterol obtained by measuring m/z 456/443.

hydroxy, and 7-ketocholesterol. Peak areas of ions monitored were measured from the selected ion current trace and ratios of peak areas for m/z 546/443, 472/443, and 456/443 were computed for standards and for lipid extract of specimens. These were normalized for recovery of 5α-cholestane as measured from m/z 372 ion abundance. The ratios of peak areas for standards were plotted against known values of ng of oxygenated sterol/μg of cholesterol. Thus for any given specimen, knowing the peak area ratios, the ratio of ng of oxygenated sterol/μg of cholesterol is obtained from the standard curve. Multiplying this number by the amount of cholesterol in the lipid extract gives the total amount of oxygenated sterol in that specimen. Addition of 5α-cholestane serves the dual purpose of correcting for procedural losses throughout as well as acting as a reference for the estimation of cholesterol which is calculated from cholesterol:5α-cholestane area response factor as mentioned earlier.

RESULTS AND DISCUSSION

The ratios of relative intensities of ions m/z 546/443, 456/443, and 472/443 (**Figs. 1 and 2**) as a function of ng oxygenated sterol added to a constant amount of cholesterol were linear; the correlation coefficient varied between 0.98 and 0.99. This was also true when standard curves were prepared by adding known amounts of 7α-, and 7β-hydroxycholesterol to boiled microsomes, extraction with choloroform–methanol, and SIM analyses. The experimentally determined values of these sterols were within 3–5% of the known values for several such preparations. Replicate analyses of microsomal preparations containing known quantities of oxygenated sterols indicated an error of ±3.5%. Furthermore, the amount of 7α-hydroxycholesterol calculated from either m/z 546/443 or m/z 456/443 was the same regardless of which ion pair ratio was used. But m/z 456 ion is much more intense than m/z 546 ion; as a result there is considerable gain in sensitivity which has allowed us to detect routinely about 50 pg and to quantitate accurately 200 pg of 7α-hydroxycholesterol under the operating conditions described in Methods. For this reason we have abandoned the use of m/z 546 ion in preference to m/z 456 to quantitate 7α-hydroxycholesterol content; however, we continue to use m/z 546 for location and identification of TMS-monohydroxyderivatives of cholesterol. TMS-cholesterol also yields m/z 456 ion; however, its abundance is quite low (0.2% of total), and because it is well separated from TMS-7α-, and 7β-hydroxycholesterol, it does not interfere with the analysis.

It appears possible to measure even smaller quantities with the HP 5986A instrument by manually tuning the instrument and appropriately changing the operating parameters. Microsomal cholesterol content measured by Beckman Cholesterol Analyzer and by GLC–MS using cholesterol:5α-cholestane area response factor (m/z 443/372) differed by ±4.8%. The average GLC–MS response factor was 0.15 ± 0.02 (n = 22). The amounts of 7α-hydroxycholesterol we have encountered in microsomes from normal rat livers incubated for different times have ranged from 0.4 (boiled control) to 50 ng of 7α-hydroxycholesterol/μg cholesterol. More typically, however, the amounts obtained are between 1 (boiled controls) and 30 ng/μg cholesterol. The standard curve depicted in Fig. 1 encompasses this range of values.

Fig. 3 illustrates that the formation of 7α-hydroxycholesterol was linear with different concentrations

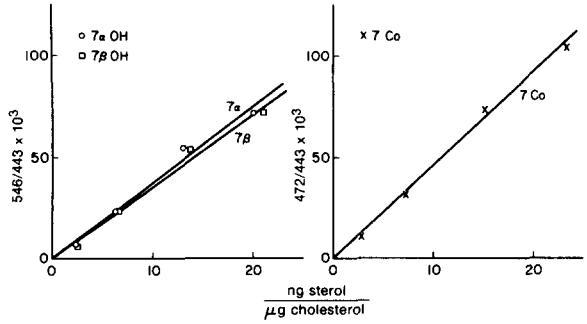

Fig. 2. Standard curves for ditrimethylsilyl ethers of 7α- and 7β-hydroxycholesterol obtained by measuring m/z 546/443 ratios for different concentrations of ng oxygenated sterol/μg cholesterol; standard curve for monotrimethylsilyl ether of 7-ketocholesterol obtained by measuring m/z 472/443.

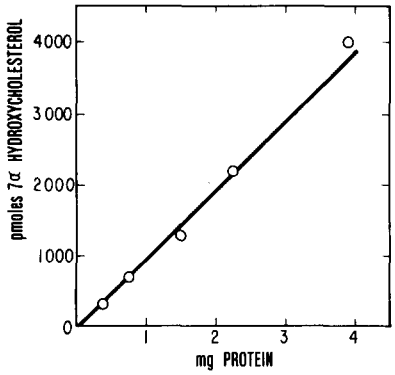

Fig. 3. Formation of 7α-hydroxycholesterol as a function of the amount of microsomal protein.

of microsomal protein tested. These measurements were made after 30-min incubations. The rate of formation of 7α-hydroxycholesterol was similarly linear at 10- and 60-min incubations. The amount of microsomal protein in all other experiments was 1.8 ± 0.1 mg.

Table 1 shows 7α-hydroxycholesterol formation at different times in microsomes from normal rat livers as measured using the SIM technique. The amount of 7α-hydroxycholesterol in boiled controls may represent its in vivo microsomal concentration and is equivalent to 680 ng/g of liver, since 200 μl out of 4 ml (≡1 g liver) of microsomal suspension was utilized for the assay. The amount of 7β-hydroxycholesterol in these studies was found to be quite small but measurable. Its concentration in boiled controls was 10 pmol/mg microsomal protein or 145 ng/g of liver. This amount did not change during the various incubation periods in all experiments. 7-Ketocholesterol in this work was monitored with m/z 472, the M^+ ion of its TMS derivative. This ion is not as intense as the m/z 456 ion used to measure 7α-hydroxy and 7β-hydroxycholesterols; consequently, the sensitivity of detection and quantitation of 7-ketocholesterol is also comparatively less at approximately 300 and 500 pg, respectively. In the present studies 7-ketocholesterol was not detected in any of the experiments, implying that its presence, if any, is less than the above limits of detection. The relative absence of 7β-hydroxycholesterol and 7-ketocholesterol also indicates an absence of the so-called "autooxidation" during the experimental procedures through which these sterols may arise and complicate interpretation of data.

Results in this report may be further viewed in relation to the recent measurements of the activity of cholesterol 7α-hydroxylase by Nimmannit and Porter (5). Similar to their findings, the activity of the enzyme increases linearly with the amount of microsomal protein used (Fig. 3); however, whereas the Nimmannit and Porter method (5) requires butanol extraction of microsomes as a condition to achieve linear increase in enzyme activity with increasing microsomal protein concentration, such a manipulation is unnecessary in the SIM procedure. Moreover, the amount of 7α-hydroxycholesterol formed, 554 pmol/mg protein at 30 min (Table 1), is greater than 316 pmol/mg protein at 25 min reported in Table III of their work (5). In this connection it may be pointed out that to achieve this level of enzyme activity the Nimmannit and Porter method (5) requires *i*) butanol extraction of microsomes and *ii*) assumption of a complete equilibration of exogenous labeled cholesterol with the endogenous cholesterol. The 2.9 nmoles of endogenous cholesterol still remaining in butanol-extracted microsomes may not be considered an insignificant amount in view of the very small amounts, (0.013 nmol/min per mg protein, Table III, (5)) of 7α-hydroxycholesterol formed assuming complete equilibration; the actual amounts measured in fact are even smaller. The variation of the measurement of enzyme activity in this study (Table 1) may be compared with the variation of 13% which may be

TABLE 1. Formation of 7α-hydroxycholesterol in rat liver microsomes

	Minutes					Boiled Control
	2	5	10	30	60	
	pmol 7α-hydroxycholesterol/mg protein[a]					
Mean (n = 13)	100	224	333	554	686	47
SEM	8.2	21.3	15.2	21.8	41.2	8.8
C.V.	2.3%	2.8%	2.6%	2.9%	4.7%	2.6%

[a] Formation of 7α-hydroxycholesterol in normal rat liver microsomes measured by SIM. Results are from four experiments conducted over a period of 4 weeks using combined microsomes from two to five rats in each experiment. Values for boiled controls have been subtracted from all data points. 7α-hydroxycholesterol formation was measured at above times for each experiment. Incubation conditions as described under Methods. Microsomal protein was 1.8 ± 0.1 mg for all experiments.

calculated from their data in Table II (5). In experiments (to be reported)[5] concerned with assessing the effect of several detergents commonly employed in the study of 7α-hydroxylase activity, we have found that Tween 80 and Renex 30 (polyoxyethylene (12) tridecylether), which is similar to Emugen 911, exert an inhibitory effect on enzyme activity proportional to the concentration of detergent used, further complicating interpretation of data, even those obtained after butanol extraction of microsomal cholesterol and solubilization of enzyme activity with a detergent. While it is true that proper study of the characteristics of the enzyme itself would be ideal under conditions of an absence of endogenous substrate, such a removal in this particular instance would have to be complete in view of the very large excess of substrate relative to the amount of product formed. In conditions where information is sought concerning relative changes in enzyme activity, removal of endogenous cholesterol may not be essential regardless of the method used. This is due to the fact that all methods that depend upon introduction of labeled exogenous substrate require the use of a detergent; moreover, one must make assumptions regarding its equilibration with the endogenous substrate. Considerations of changes in the "metabolically active substrate pool" of endogenous cholesterol and its equilibration with the exogenous labeled cholesterol may be different under different experimental conditions and using different detergents (which themselves may modify the enzyme activity) to solubilize the external substrate. These considerations serve merely to highlight the considerable difficulty in the study of this enzyme.

The SIM method reported here measures the actual mass of 7α-hydroxycholesterol formed under any given condition, thus accounting for any changes in the size of the metabolically active "substrate pool" of endogenous cholesterol under different conditions. In this regard it compares with the method of Björkhem and Danielsson (4) but it avoids *i*) synthesis of deuteraed sterols and their purification, and *ii*) separation of sterols in lipid extract by thin-layer chromatography and their extraction from plates. The SIM method, however, is considerably simpler than the method of Mitropoulos and Balasubramaniam (2) in which extracted 7α-hydroxycholesterol is first purified by thin-layer chromatography and the purified sterol is acetylated with [^{3}H]acetic anhydride. The mass of 7α-hydroxycholesterol is then obtained from the ratio of ^{3}H/^{14}C radioactivity in the product and the specific activity of acetic anhydride. The SIM method is at most a two-step procedure requiring lipid extraction and analysis of sterol-TMS derivatives by GLC–MS. Undoubtedly, such factors as simplicity and sensitivity are tempered not only by the initial substantial cost of the instrument but by its proper maintenance as well.

Manuscript received 23 July 1980 and in revised form 15 December 1980.

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