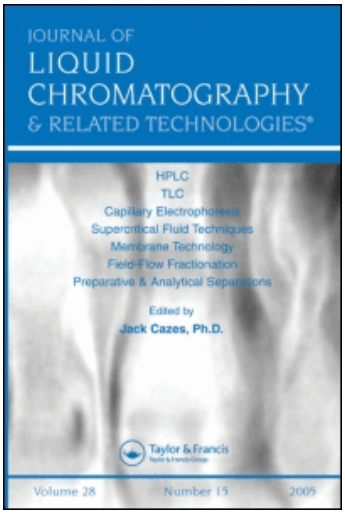

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HPLC Determination of Hepatic Cholesterol 7α-Hydroxylase Activity Using Immobilized Cholesterol Oxidase and Chemiluminescence Detection

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Original Article

HPLC DETERMINATION OF HEPATIC CHOLESTEROL 7α-HYDROXYLASE ACTIVITY USING IMMOBILIZED CHOLESTEROL OXIDASE AND CHEMILUMINESCENCE DETECTION

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ABSTRACT

This paper describes an HPLC method for the determination of cholesterol 7α-hydroxylase activity, at high or low activity levels, that is sensitive and specific for 7α-hydroxycholesterol. The method relies on the generation of hydrogen peroxide by oxidation of 7α-hydroxycholesterol using the enzyme cholesterol oxidase which has been immobilized on porous glass beads. The hydrogen peroxide is subsequently detected by chemiluminescence generated by reaction of peroxide with bis-(2,4,6-trichlorophenyl)-oxalate (TCPO), a commonly used chemiluminescence reagent specific for peroxides. In the procedure, sample preparation is limited to extraction of the incubation mixture and injection of the concentrated extract.

INTRODUCTION

It is now well accepted that cholesterol plays a major role in the development of some of the most common human diseases,

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atherosclerosis and gallstones. We have postulated that the rate of cholesterol disposal significantly influences cholesterol homeostasis and a slow rate of disposal may be an important factor in the accumulation of cholesterol and the development of these diseases. The enzyme responsible for the disposal of cholesterol, cholesterol 7α-hydroxylase, is a cytochrome P-450 isoenzyme. 7α-hydroxylation is the rate limiting and committed step of conversion of cholesterol to bile acids. The enzyme is present in the endoplasmic reticulum of liver cells (1).

In order to elucidate the role of this enzyme in these disease processes, we have developed a method which is specific and sensitive enough to be applied to the small amount of tissue available from liver biopsies.

An array of methods have been devised to measure the end-product of the enzyme reaction, 7α-hydroxycholesterol (7α-HC). These methods include TLC using ^{14}C labeled cholesterol as a substrate, gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS), and high performance liquid chromatography (HPLC).

The use of radiolabeled cholesterol as an exogenous substrate with subsequent separation and quantitation of labeled 7α-HC using TLC (2) is questionable since recent data (3) indicates that the primary substrate is endogenous cholesterol, while exogenous cholesterol is poorly utilized.

Analysis of 7α-HC by GC (4) from biological samples requires a pre-purification step by TLC and a derivitization step for the formation of volitile derivatives. These derivatives are sensitive to moisture and can easily decompose. Combination of GC with selected ion monitoring (5, 6) increases the specificity of the method but does not eliminate the need for pre-purification and derivitization.

HPLC methods might seem ideal for overcoming the necessity of derivatization since 7α-HC is readily soluble in solvents commonly used for HPLC. However, 7α-HC does not contain a highly

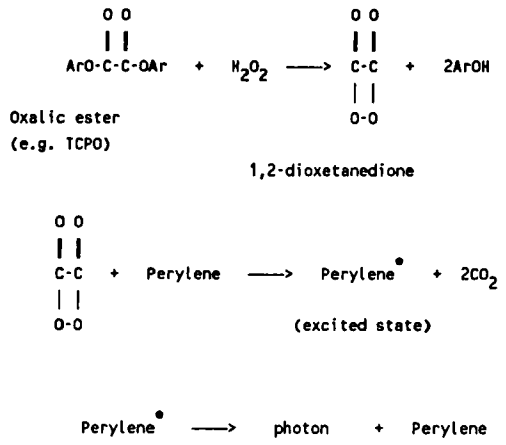
absorbing chromophore, nor is it natively fluorescent or electrochemically active. It is thus not amenable to ready, sensitive detection using HPLC.

There have been HPLC methods devised which rely on the UV absorbance of 7α-HC (7) but they are, in general, insensitive. 7α-HC may be oxidized using cholesterol oxidase to a compound that contains a chromophore that absorbs strongly at 240 nm (8) and can be readily detected with improved sensitivity. Another recent approach is the formation of the fluorescent anthroyl-1-carbonitrile derivative of 7α-HC (9). Both methods are useful, but introduce several extra steps into the analytical procedures. Additionally, in biological systems the presence of chemical species other than the analytes of interest may cause peak overlap which interferes with peak identification and/or quantitation.

This paper describes a sensitive and specific HPLC method for the determination of cholesterol 7α-hydroxylase activity in rat liver microsomes. In the oxidation of 7α-HC, as catalyzed by the enzyme cholesterol oxidase, 7α-hydroxycholest-5-ene-3-one and hydrogen peroxide are formed in a 1:1 stoichiometric ratio. To utilize this reaction analytically, cholesterol oxidase immobilized on porous glass beads was packed into a column and installed in line on an HPLC system. 7α-HC eluting from the HPLC column was oxidized to yield hydrogen peroxide, which was easily detectable at low concentrations using chemiluminescence (CL) methodology.

Bis-(2,4,6-trichlorophenyl)-oxalate (TCPO) is a commonly used CL reagent that reacts specifically with peroxides as shown below.

In the reaction, an energetic intermediate, 1,2-dioxetanedione, is formed and subsequently passes its energy on to a fluorescent compound (perylene) present in the reaction mixture causing photon emission. The reaction between TCPO and hydrogen peroxide is catalyzed by an organic base such as triethylamine (TEA) (10).



MATERIALS AND METHODS

ANIMALS

Microsomes were obtained from the livers of Sprague-Dawley or Zucker Rats which were fed laboratory rat chow. The animals were housed individually and placed on a reversed light cycle. They were sacrificed under ether anesthesia at the midpoint of the dark cycle, where the activity of cholesterol 7α-hydroxylase is at its peak of diurnal variation.

REAGENTS

All reagents were of analytical grade or the highest grade available. All solvents were HPLC grade. Cholesterol oxidase (Streptomyces species) was obtained from Calbiochem Inc.. Bis-(2,4,6-trichlorophenyl)-oxalate (TCPO) was synthesized according to the procedure of Mohan and Turro (11). Reagents for the synthesis of TCPO were obtained from Aldrich Chemical Co. Isocitrate dehydrogenase, sodium isocitrate, $NADP^+$, and controlled pore glass (80 to 120 mesh, 500 Angstrom pore size), were obtained from Sigma Chemical Co. 1,4-Dithiothreitol was obtained from Research Organics, Inc. 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), reagents for protein determinations,

and Triton-X-100, 10% solution, specially purified for removal of peroxides, were obtained from Pierce Chemical Co. 7α-HC and 20α-hydroxycholesterol (20α-HC) were obtained from Steraloids Inc.

CHROMATOGRAPHIC SYSTEM

The chromatographic system was a Model 338 Beckman Gradient HPLC system consisting of two model 110B pumps, a system controller, and a model 210A injector with a 250μL loop. The chromatographic column was a Beckman Ultrasphere, 5 μm, 4.6mm X 150mm octyl bonded phase. A Beckman Model 171 Radioisotope detector was used to monitor the CL signal. A Beckman Model 110B and an SSI Model 300 were used as reagent pumps. Data was collected and analyzed using Beckman Chromatographics software designed specifically for the Model 171 detector.

Figure 1 shows the organization of the system, including identification of the pumps, reagents and flow rates. The 171 detector was designed as a flow detector for radioisotopes, but was also found to effectively monitor CL signals. The detector used a flow cell cartridge which could be fitted with various lengths of Teflon tubing (0.125 inch O.D. X 0.0625 inch I.D.) to allow selectable flow cell volumes ranging from 200μL to 1000μL. The flow cell was inserted into the instrument and held in position between two photomultiplier tubes. In this application, it was necessary to limit the volume of the flow cell to a maximum of 600 μL. Above 600 μL, the background level of CL resulted in failure of the graphics software to autoscale.

Beckman ChromatoGraphics Software allowed efficient monitoring of CL by permitting data collection in an out of coincidence mode. This was necessary because CL phenomena, unlike scintillations from radioisotopes, are single photon events.

To allow detection of the CL signal detector parameters required adjustment. A "new isotope" was programmed that opened the energy window to its full range and the detector was set for operation with a liquid flow cell. To allow collection of data at one point per second, other detector parameters were adjusted as:

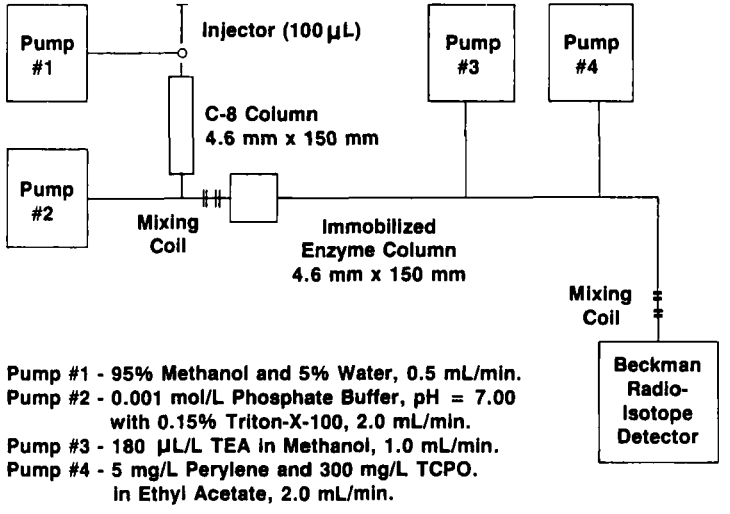


FIGURE 1. Schematic of chemiluminescence detection system.

flow cell volume, 200 µL; scintillation reagent flow rate (this was the flow rate for the CL reagent containing perylene and TCPO in ethyl acetate), 2.0 mL/min; and sample flow rate (flow rate from the chromatographic column), 2.0 mL/min. These parameters were used for all data collection.

IMMOBILIZED CHOLESTEROL OXIDASE

Cholesterol oxidase was immobilized onto controlled pore glass beads using the glutaraldehyde method (12). The enzyme was immobilized at a concentration of 250 U cholesterol oxidase per gram of controlled pore glass (CPG). About 0.5 grams of CPG-immobilized enzyme was slurry packed into a 4.6mm X 50mm stainless steel HPLC column, which was water jacketed and maintained at 37° C during use.

For short term storage, the column was left in a mixture of 20% methanol and 80% 0.001 mol/L phosphate buffer, pH = 7.00. For longer term storage, it was necessary to flush with at least 10 column volumes of 0.001 mol/L phosphate buffer. Leaving the

column stored in buffer containing Triton-X-100 overnight resulted in significant loss of enzyme activity on the column. The flushed column was stored at 4°C to prevent bacterial growth.

DETERMINATION OF CHOLESTEROL OXIDASE ACTIVITY

The activity of cholesterol oxidase when exposed to various methanol concentrations was determined by monitoring the increase in absorbance of the incubation mixture at 240 nm due to formation of cholest-4-ene-3-one from cholesterol. The analysis procedure was as outlined by Allain et al (13).

MICROSOME PREPARATION

Microsomes were prepared from homogenized liver by either ultracentrifugation at 100,000g (3) or by Ca^{++} precipitation and centrifugation at 1500g as outlined by Kamath et al (14). Proteins were determined using the bicinchoninic acid method of Smith et al (15).

CHOLESTEROL 7A-HYDROXYLASE ACTIVITY DETERMINATION

For the determination of enzyme activity, the incubation system contained 100 mmol/L phosphate buffer, pH 7.00, 2.0 mmol/L 1,4-dithiothreitol, 1.0 mmol/L EDTA, 50 mmol/L sodium fluoride and 0.23 mmol/L CHAPS. The incubation was initiated by addition of an NADPH generation system containing 4.0 mmol/L NADPH, 26 mmol/L $NADP^{+}$, 10.0 mmol/L magnesium chloride, 32 mmol/L sodium D,L-isocitrate, and 6 units of isocitrate dehydrogenase. The total volume of 3.0 mL contained 6.0 mg of microsomal protein. The incubation was carried out at 37°C for 20 minutes, and the reaction stopped by addition of 6 mL of ethanol. A zero time incubation served as control. Here, ethanol was added before addition of the NADPH generation system.

After stopping the reaction, 2.5 μg of 20α-HC dissolved in methanol was added as an internal standard. The mixture was extracted three times with 9 mL (per extraction) of petroleum ether, which was evaporated to dryness under a stream of dry

nitrogen at a temperature not exceeding 40°C. The dried material was mixed with 120 μL of methanol, placed into an ultrasonic bath for 15 to 30 seconds to insure dissolution of the analytes, and centrifuged. 100 μL was injected into the HPLC for analysis. 7α-Hydroxylase activities were expressed as pmole of generated 7α-HC per mg of protein in the microsomes per min of incubation (pmole/mg/min).

RESULTS AND DISCUSSION

EFFECT OF METHANOL AND SURFACTANT

The column eluent was primarily methanol and required dilution with buffer to be used with the immobilized cholesterol oxidase. It was necessary to determine a working concentration of methanol which was not detrimental to the enzyme activity. Soluble, rather than immobilized, enzyme was tested on the assumption that immobilization would enhance rather than detract from the enzyme stability.

Figure. 2 shows the effect of increasing methanol concentrations on the enzyme activity. Activity was constant up to about 30% methanol. At 40% and 50% methanol the enzyme began to show signs of slowed kinetics. Above 40% methanol, the enzyme appeared to be denatured almost immediately. Based on this data, the methanol concentration for the immobilized enzyme column was fixed at or below 20% for the duration of the investigation.

Since 7α-HC is highly insoluble in solutions containing large amounts of water, Triton-X-100 was added to the flow stream to facilitate the enzyme-substrate interaction. However, large amounts of peroxide were present in normally available Triton-X-100, in agreement with earlier observations (16,17), resulting high background CL. To lower background CL and maintain low detection limits, purified, peroxide free Triton X-100 was obtained from Pierce Chemical Co.. This resulted in a significantly lowered background CL and improved detection limits.

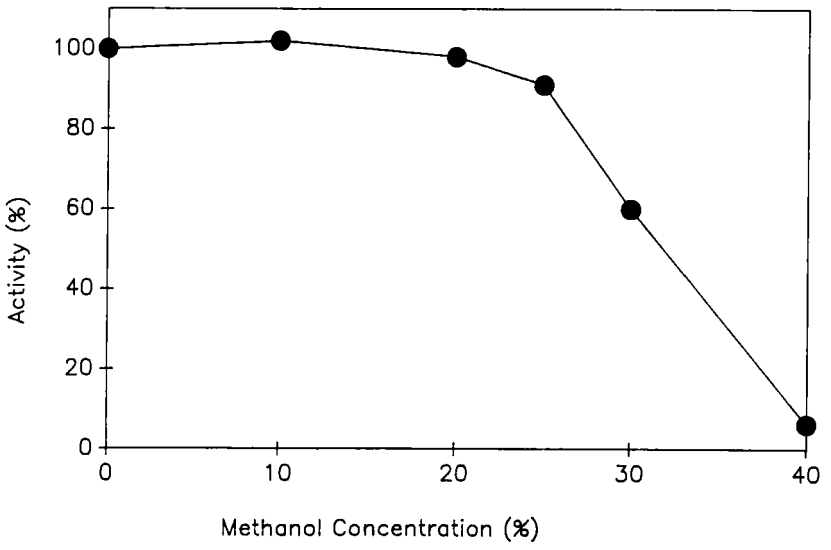


FIGURE 2. Methanol tolerance of soluble cholesterol oxidase. Activities are relative to 0% methanol. 83μg/mL cholesterol in phosphate buffer, pH=7.00, containing 0.25% Triton-X-100 and 0.133 U cholesterol oxidase. 240 nm, 37°C, 3 min preincubation, and 5 min incubation.

OPTIMIZATION OF DETECTION SYSTEM

To minimize band spreading, connections were made as short as possible using low dead volume fittings and 1/16 inch O.D. X 0.01 inch I.D. stainless steel tubing. Mixing coil connections were maintained with a minimum number of smooth bends. The final mixing coil was constructed from a piece of 1/16 inch O.D. x 0.01 inch I.D. black Teflon tubing, 20 cm in length.

Initial trials with the reagents demonstrated that a mixture of solvents rather than a single solvent was required in the reaction stream. Methanol could be used in the column effluent, but could not be used as a solvent for TCPO in the reagent stream since TCPO has demonstrated only short term stability in methanol (17). Dissolving the reactants in either acetonitrile or acetone, in which TCPO has had demonstrated long term stability (18), resulted in formation of an insoluble precipitate. Ethyl acetate,

a good solvent for TCPO (11), did not mix well with the aqueous column effluent until mixed with methanol in the reagent stream. In the final system, TCPO and perylene were dissolved in ethyl acetate as one reagent and TEA was dissolved in methanol as another. These two reagents were pumped separately to keep TCPO from mixing with methanol until just before entering the final mixing coil.

For determination of proper reagent mixing ratios, the analytical and enzyme columns were removed and the CL resulting from injection of 20 μL of 0.10 mmol/L (2 nmole) of hydrogen peroxide was monitored as the mixing ratio was changed systematically. The mobile phase and buffer flows were fixed at 0.5 mL/min and 2.0 mL/min, respectively, to simulate the enzyme column eluent. The best (highest) signal was obtained with a mixing ratio of 1.0/2.0/2.5 mL/min methanol/ethyl acetate/enzyme column eluent.

Proper reagent concentrations were determined on the basis of highest signal/noise (S/N). Individual reagent concentrations were systematically varied while holding all other concentrations constant. 500 ng of 7α-HC was injected as the test compound. Various concentrations of TCPO, perylene, TEA, and Triton-X-100 were investigated.

It was found for TCPO and perylene that increasing their individual concentrations increased both the signal and the noise significantly. With TCPO the increase in noise was higher at each increment than the increase in signal, so that TCPO showed a decreasing S/N from low to high concentration (Figure 3). 300 mg/L TCPO in ethyl acetate was the lowest concentration that gave a good S/N.

Perylene showed an increase in both signal and noise as the concentration was increased (Figure 4), but unlike the variations in TCPO concentration, showed a maximum in the S/N, occurring at a concentration of 5 mg/L.

Variations of Triton-X-100 and TEA concentrations also affected S/N. Increasing concentrations of Triton-X-100 in buffer

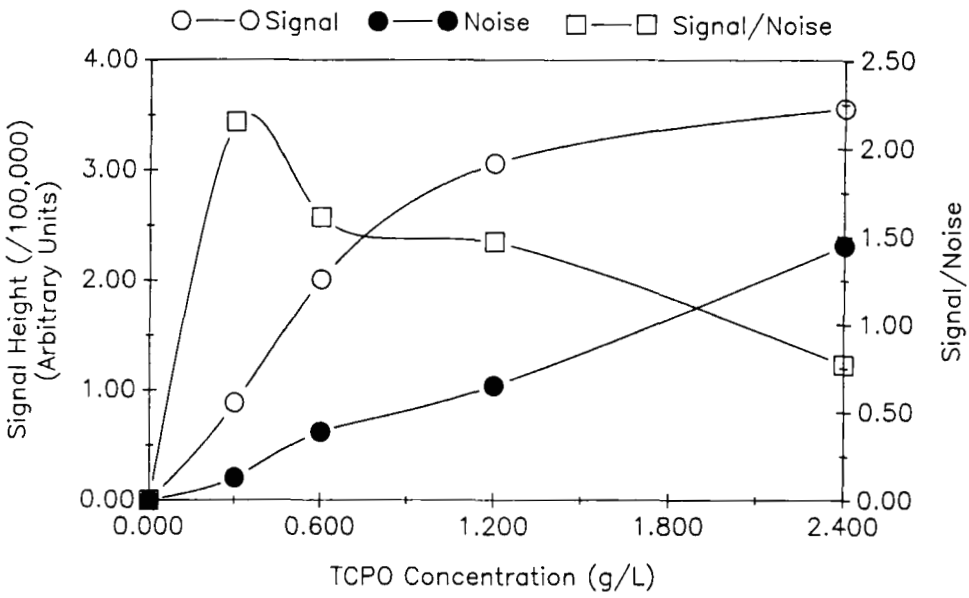


FIGURE 3. Optimization of TCPO concentration. 500 ng 7α-HC per 20 μL injection; Pump 1, 95/5 methanol/water; Pump 2, 0.001 mol/L phosphate buffer, pH = 7.00 and 0.3% Triton-X-100; Pump 3, 200 μL/L TEA in methanol; Pump 4, 5 mg/L perylene and 0.60 g/L TCPO in ethyl acetate.

improved S/N, but only up to a concentration of 0.15% after which the improvement leveled off, perhaps due to complete partitioning of the substrate into the Triton-X-100 micelles.

Variations of TEA concentration in methanol showed a level S/N from 100μL/L to 250μL/L. Above 250μL/L the S/N to dropped off significantly. Since TEA catalyzed the CL reaction, increasing the concentration of TEA increased the rate of light emission (i.e. intensity per unit time). Since the length of the mixing coil between the reagent mixing point and the flow cell was fixed, variation in the TEA concentration represented movement of the position of the signal maximum. A mid-range concentration of 180 μL/L was chosen to allow for variations in pumping rates and temperature which might occur during normal operation of the system.

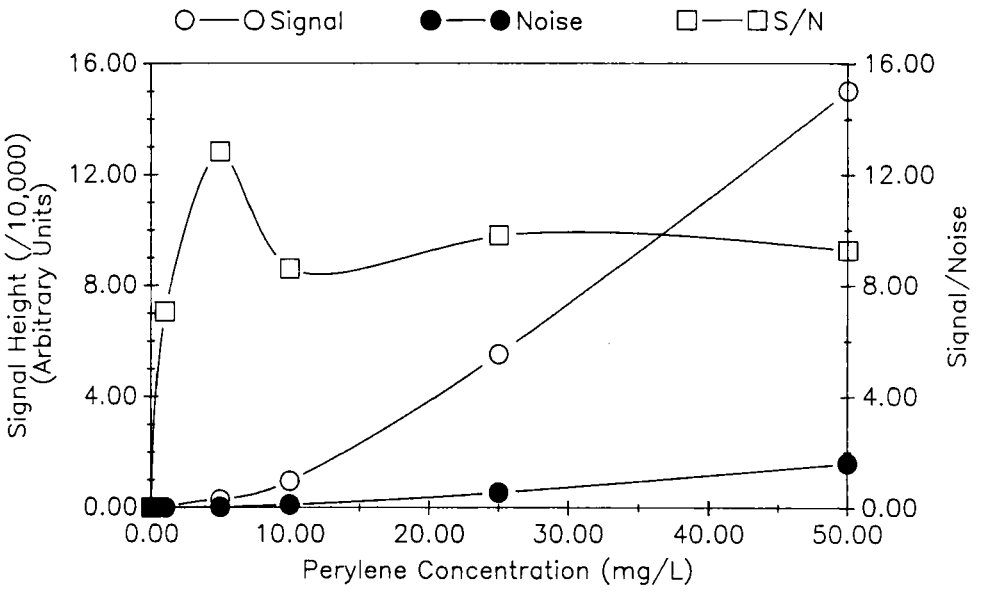


FIGURE 4. Optimization of perylene concentration. 500 ng 7α-HC per 20 μL injection; Pump 1, 95/5 methanol/water; Pump 2, 0.001 mol/L phosphate buffer, pH = 7.00 and 0.3% Triton-X-100; Pump 3, 200 μL/L TEA in methanol; Pump 4, 5 mg/L perylene and 0.30 g/L TCPO in ethyl acetate.

Variations in the pH and operating temperature of the enzyme column were also examined. Variation of pH from 6.0 to 8.0 had little effect on S/N but a small maximum at pH 7.00 was shown. Variation of column temperature showed a decrease in S/N from 37°C to 42°C. At 44°C the S/N increased to a level above that at 37°C. Sustained operation of the column at this temperature caused rapid loss of activity on the column, however.

STANDARDIZATION OF THE METHOD

The analytical system demonstrated good reproducibility. Table 1 shows results obtained for 5 replicate injections of 1 nmole of 7α-HC.

To minimize errors from sample handling and incomplete recovery of the analyte, 20α-HC was chosen as an internal

TABLE 1

Peak Areas	Statistics
512543	
536567	MEAN = 511762
501171	S.D. = 14813
499951	C.V. = 2.89%
508579	

standard. It was not a normal metabolite of liver microsomes and was expected to behave, in all respects, identically to 7α-HC, both in recovery and with the immobilized cholesterol oxidase. 20α-HC eluted earlier than 7α-HC at the conditions used for the separation and was easily resolved in the chromatograms. A typical standard chromatogram is shown in Figure 5.

To examine the matrix effects on recovery of the analytes, peak areas for standard solutions were analyzed and compared to microsome incubation mixtures containing spiked amounts of standard. These mixtures were extracted as outlined in the procedure and 7α-HC and 20α-HC recoveries were calculated from individual samples. The data are presented in Table 2.

Although neither analyte was completely recovered from the incubation mixtures, their recoveries were nearly the same, demonstrating their analytical similarity. If 7α-HC recovery calculations were performed using internal standardization, full recovery of 7α-HC was demonstrated.

Examination of the final analytical system demonstrated a nearly linear increase in yield of 7α-HC with incubation time (Figure 6). The yield began to drop off at longer incubation intervals, perhaps due to substrate exhaustion or product inhibition. A 20 minute incubation was used for subsequent studies as a compromise between a reasonable incubation time and yield of 7α-HC.

A linear increase in yield of 7α-HC was observed with increasing protein concentration (Figure 7) in the incubation

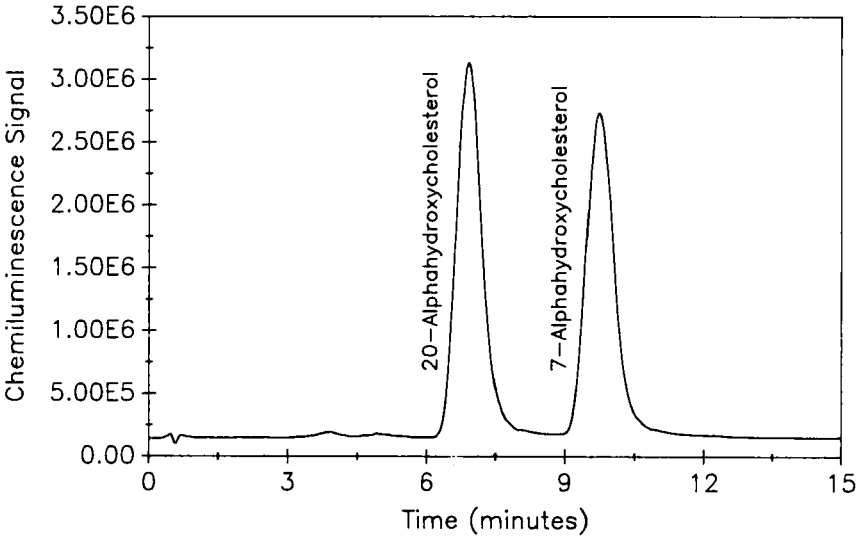


FIGURE 5. Chromatogram of standard mixture of 2500 ng each of 7α-HC and 20α-HC per 100 μL injection. Optimized reagent conditions as stated in the text.

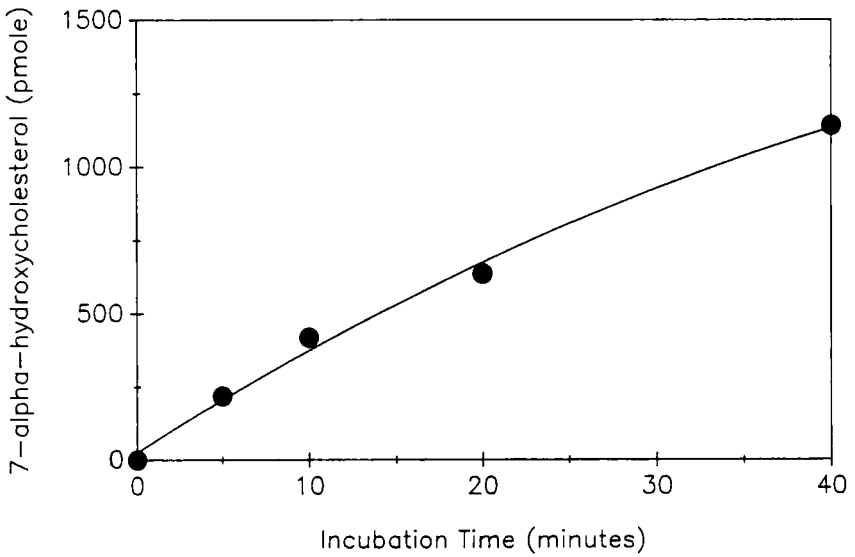


FIGURE 6. Yield of 7α-HC with incubation time. Sprague-Dawley liver microsomes isolated by ultracentrifugation. 1.0 mg/mL protein.

TABLE 2

STANDARD DATA: 2500 ng 20α-HC per sample

Sample	7α-HC (ng)	20α-HC area	7α-HC area
1	1000	40401120	11998143
2	1500	40379044	21326194
3	2000	41504764	29618274
4	2500	41344132	38379008

SAMPLE DATA: 2500 ng 20α-HC per sample

Sample	7α-HC (ng)	20α-HC area	7α-HC area
1	1000	31171574	9361818
2	1500	31196416	16996450
3	2000	31095321	22819750
4	2500	30803503	29528710

RECOVERY DATA

Sample	20α-HC	7α-HC	7α-HC w/IS*
1	77.16%	78.03%	101.1%
2	77.01%	79.70%	103.5%
3	74.92%	77.05%	102.8%
4	74.51%	76.94%	103.3%
Average	75.90%	77.93%	102.7%

*Calculation based upon internal standardization

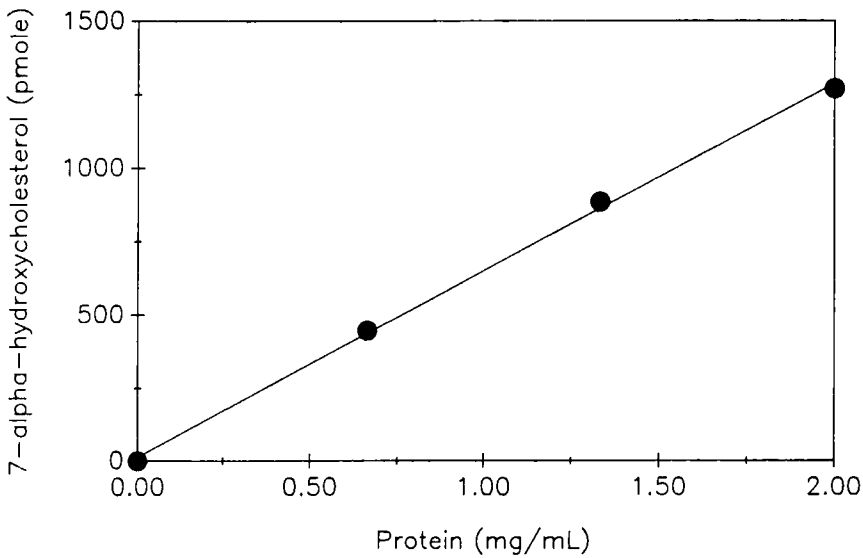


FIGURE 7. Yield of 7α-HC with protein concentration. Sprague-Dawley liver microsomes isolated by ultracentrifugation. 20 minute incubation.

TABLE 3

Activity	Statistics
31.4	
33.3	MEAN = 31.7
31.0	S.D. = 1.50
33.2	C.V. = 4.73%
29.8	

mixture up to a concentration of 2 mg/mL. Above 2 mg/mL, the yield of 7α-HC began to level off. A protein concentration of 2 mg/mL was used in subsequent experiments to maximize yield of 7α-HC in the incubations.

To examine reproducibility of the incubation system, 5 replicate activity determinations from the same liver sample were performed. Results are presented in Table 3.
Reproducibility for the final method was very good and was similar to that observed for replicate injection of the standards (Table 1).

CL DETECTION VS. UV DETECTION

Figure 8 shows a comparison of UV and CL detection for a microsome incubation sample using serial detection with the UV detector placed first. There was approximately a 1 min delay between the two detectors. Using CL detection, 7α-HC and 20α-HC, which eluted at 7 and 9 minutes, respectively, were distinctly removed from one another and from all else in the chromatogram, making identification and quantitation a simple task.

With UV detection, however, there were several peaks, large and small, which eluted in the vicinity of the two analytes and at several other places in the chromatogram. These peaks were from strongly UV absorbing interferences present in the samples. Two peaks on the chromatogram which appeared at approximately the same retention times as 7α-HC and 20α-HC were not 7α-HC and 20α-HC but were an interference of unknown composition. Both peaks appeared

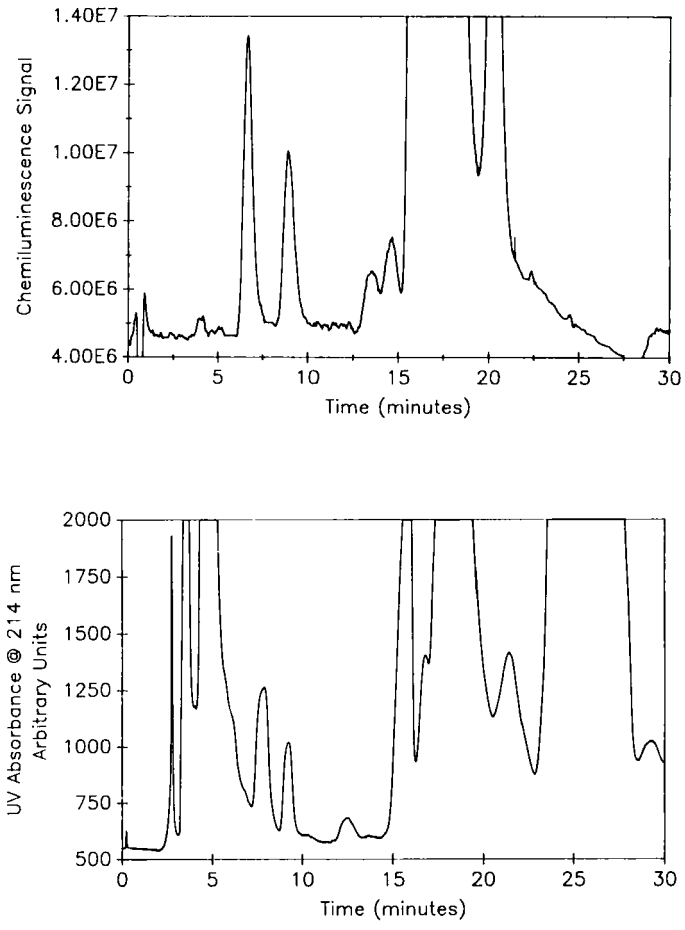


FIGURE 8. Chromatogram of incubation mixture. Serial detectors; Top: CL detection; Bottom: UV detection at 214 nm. Sprague-Dawley liver microsomes isolated by Ca^{++} isolation, 20 minute incubation, 2500 ng 20α-HC added as internal standard.

in the chromatograms regardless of the presence or absence of 7α-HC and 20α-HC in the analysis mixture. Close examination showed 20α-HC as a barely visible shoulder at about 6.5 minutes on the very large peak which eluted at about 5.5 minutes and 7α-HC was buried in the first of the two small peaks appearing at about 7.5 minutes.

The large peak which appeared at about 18 minutes in both chromatograms was due to cholesterol. The large peak after cholesterol which appears at about 25 minutes on the UV chromatogram but does not appear on the CL chromatogram, as well as the peaks which co-eluted with 20α-HC and 7α-HC, effectively demonstrates the specificity of the method for only those solutes which are substrates for cholesterol oxidase. This specificity yielded greatly simplified chromatograms, increased the certainty and reliability of peak identification, and made quantitation easier.

The sensitivity of this CL method was greater than that observed with UV detection, both at 214 nm for native 7α-HC and at 240 nm for oxidized 7α-HC (6). Figure 9 shows standard curves for each mode of detection. The signals were adjusted to give identical integration units and noise levels. Absolute detection limits (for amount injected), calculated as twice S/N, were estimated as 40 pmole for CL detection, 200 pmole for UV detection of oxidized 7α-HC at 240 nm and 5000 pmole for UV detection of native 7α-HC at 214 nm.

METHODS OF MICROSOME ISOLATION AND ACTIVITIES

A comparison of cholesterol 7α-hydroxylase activities from microsomes isolated using ultracentrifugation, which is the standard method of isolation, and those isolated using Ca^{++} precipitation has not been previously reported. In a comparison, little difference was found in the 7α-hydroxylase activities between the two methods. The mean activity obtained in four determinations for the ultracentrifugation method was 41.6 pmole/mg/min (S.D. 10.3) and that for the Ca^{++} method was 42.0 pmole/mg/min (S.D. = 13.5). In each case, fresh Sprague-Dawley rat livers were divided equally and carried through the individual isolation and analytical procedures in parallel.

The cholesterol 7α-hydroxylase activities of 14 individual Sprague-Dawley rats was determined using microsomes isolated by Ca^{++} precipitation. The results showed a mean cholesterol 7α-

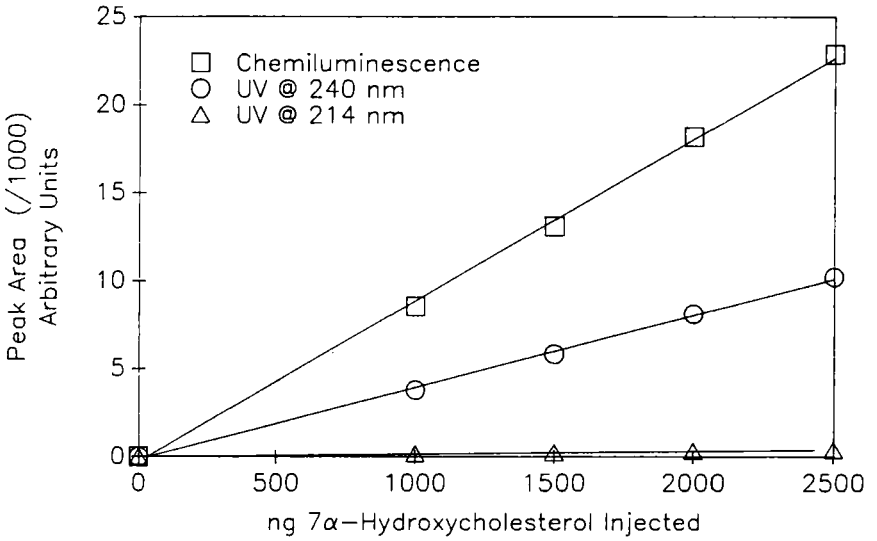


FIGURE 9. Comparison of detection methods. All data normalized to equivalent integration units and noise values.

hydroxylase activity of 39.9 ± 5.41 pmole/mg/min (P = 0.05; N = 14).

CONCLUSION

A new method for the determination of cholesterol 7α-hydroxylase activity using HPLC has been devised utilizing immobilized enzyme technology. The method is more specific and shows improved sensitivity over other existing HPLC methods. Sample preparation is relatively simple, being limited to extraction of the microsomal incubation mixture and injection for analysis after evaporation and reconstitution. There are no extensive derivatization steps or expensive reagents. The method has been successfully applied to analysis of cholesterol 7α-hydroxylase activities in rat liver and should be applicable to other systems as well. The analytical method should prove useful for analysis of other cholesterol metabolic systems and other cholesterol metabolites.

ACKNOWLEDGMENTS

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