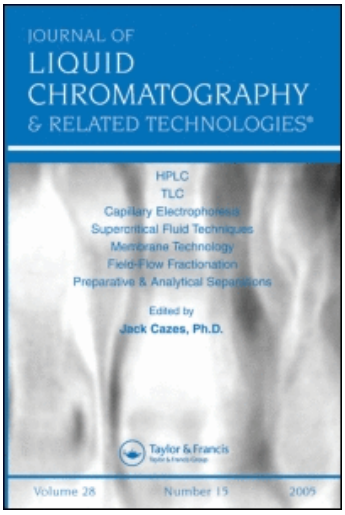

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A MODIFIED REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE MEASUREMENT OF HEPATIC CHOLESTEROL 7α-HYDROXYLASE ACTIVITY WITH A FILTER UV DETECTOR

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ABSTRACT

Hepatic cholesterol 7α-hydroxylase (CH7OH) is the first and rate-determining enzyme for the biosynthesis of bile acids from free cholesterol in the liver cells. It is important in the cholesterol metabolism, and in the formation of cholesterol gallstones in humans. A rapid reversed phase high performance liquid chromatographic (HPLC) procedure for the measurement of hepatic CH7OH was modified from an HPLC-spectrophotometric method (Chiang, J.Y.L., Meth. Enzymol., 206: 483-91,1991) and validated. A shorter column, a one-component mobile phase (100% acetonitrile), a higher flow rate, and a filter UV detector equipped with 254 nm wavelength were used in this modified procedure. The peaks of reaction products of 20α-, 7α- and 7β-hydroxycholesterol were resolved at baseline with retention times of 9, 10, and 11 min respectively. 20α-hydroxycholesterol was used as internal standard. A peak due to reaction product of 7α-hydroxycholesterol was validated by

retention time and spiked test. The linearity of the reaction product of 7α-hydroxycholesterol is up to at least 1250 pmole. Compared to the original procedure, this modified procedure is simpler (single vs binary component mobile phase), faster (22 vs 30 min of running time), and it does not require a variable wavelength UV detector, and it still retains the advantages of the original procedure.

INTRODUCTION

One of the key enzymes of cholesterol metabolism in the liver is cholesterol 7α-hydroxylase (CH7OH), which is the first and rate-determining enzyme for the biosynthesis of bile acids from free cholesterol in the hepatocytes.[1-3] This metabolic pathway is one of the two pathways for cholesterol excretion from liver. CH7OH has been shown to be a cytochrome P450 isozyme and it is down-regulated by bile acids returning to liver via enterohepatic recirculation.[4,5]

Three procedures have been used to analyze hepatic CH7OH activities: isotope incorporation method,[6] GC/MS method,[7] and high performance liquid chromatographic (HPLC)-spectrophotometric method.[8-11] The isotope method involves the use of radioactive material, [^{14}C]cholesterol, and it is interfered by endogenous substrate. GC/MS method is sensitive and specific but it needs an expensive instrument and special technical skills. HPLC-spectrophotometric procedure is a simple and reliable method and without the requirement of radioactive material and expensive instrument. However, it requires a variable wavelength UV detector to monitor response at 240 nm and it takes at least 30 minutes to have a chromatographic run. Here, a modified procedure of Chiang's HPLC-spectrophotometric method[11] was developed. This modified procedure uses a filter UV detector (254 nm), has a shorter running time and a simplified mobile phase.

MATERIALS

All reagents except those specified were obtained either from Sigma Chemical Co.(St. Louis, MO) or from Aldrich Co. (St. Louis, MO). 7α-hydroxycholesterol (7α-HOC) was from Steraloids (Wilton, NH). Cholesterol oxidase was bought from Calbiochem Co. (San Diego, CA). Absolute ethanol was from J.T. Baker Co. (Phillisburg, NJ). Acetonitrile and water were of HPLC grade (CMS, Houston, TX).

METHODS

HPLC System

The HPLC system (Waters) included an U6K injector, an M6000A solvent delivery system, an M400 absorbance detector (254 nm for sample analysis and 254/280 nm for the identification of a contaminant peak), an 820 Maxima work station, a reversed phase C_{18} column (Ultrasphere, 150X4.6 mm, ID. from Beckman Instruments, Fullerton, CA) and a Beckman C_{18} Ultrasphere guard column (45X4.6mm, ID.). Mobile phase is 100% acetonitrile, degassed by vacuum for 30 minutes and pumping at 1.0 mL/min flow rate during the first 12 minutes. The flow was increased to 3.0 mL/min between 12-13 min and then it was maintained until 21 min. It was changed back to 1.0 mL/min between 21 and 22 min for the next analysis. All analyses were performed in ambient temperature.

Preparation of Microsomes and Measurement of Enzyme Activity

Two rat livers were from Sprague-Dawley. Human liver biopsies (n=38) were obtained from subjects undergoing gastric bypass to treat their obesity. Patient consent was obtained and the protocol was approved by the Institutional Review Board of Louisiana State University Medical Center. Microsomal fraction was prepared from homogenized liver tissue by differential ultracentrifugation[10,11] and described briefly as follows. An aliquot of liver (0.5-1 g) was washed in isolation buffer once and minced with scissors to small pieces. Tissue pieces in isolation buffer were homogenized with Polytron and centrifuged at 10,000 g for 10 min twice to obtain supernatants. Microsomal fraction was isolated by centrifuging the collected supernatant at 105,000 g for 2 hours. The pellet was homogenized in resuspension buffer and recentrifuged at 105,000 g for 1 hr. Final pellet was homogenized in isolation buffer. Protein was assayed by Lowry's method[12] with bovine serum albumin as the standard. Enzyme activity was assayed by Chiang's method.[10,11] Microsomal fraction (0.5-1.0 mg protein) was incubated with cholesterol (10 mM) in buffer solution for 20 min at 37°C. Reaction was stopped with sodium cholate and 20α-hydroxycholesterol (20α-HOC) was added as an internal standard (IS). Cholesterol oxidase solution was added to change 7α-HOC and 20α-HOC to 7α-hydroxy-4-cholesten-3-one (7α-HCO) and 20α-hydroxy-4-cholesten-3-one (20α-HCO) respectively under 37°C for 10 min. The reaction mixture was extracted with 6 mL of petroleum ether three times. Petroleum ether extracts were pooled, dried under nitrogen in 37°C water bath and the residue was stored at -70°C until analysis. Residues were reconstituted with 100 uL mobile

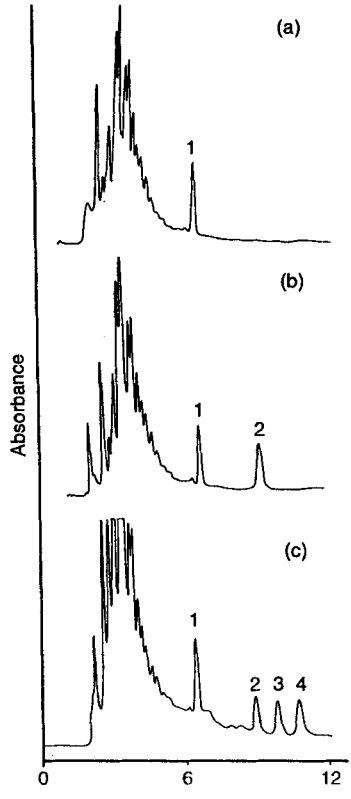

Figure 1. Chromatograms of a) reagent background, b) a sample of internal standard (20α-HOC), and c) a sample with 20α-HOC, 7α-HOC, and 7β-HOC added. Peak identification: 1= contaminant, 2= 20α-hydroxy-4-cholesten-3-one (20α-HCO), 3= 7α-hydroxy-4-cholesten-3-one (7α-HCO), and 4= 7β-hydroxy-4-cholesten-3-one (7β-HCO).

phase and aliquots (20-40 μL) were injected into HPLC for analysis. Both 7α-HCO and 20α-HCO reaction products have maximal absorption near 240 nm. Five calibration standards containing 7α-HOC (0-1250 pmole) with IS (2500 pmole each) added were constructed to evaluate linearity. Detectability of 7α-HCO was determined by injection of 20 uL of different dilutions of a calibrator (250 pmole). Enzyme activity of each sample was corrected with individual sample blanks, which measured the level of 7α-HOC at the 0 time of incubation.

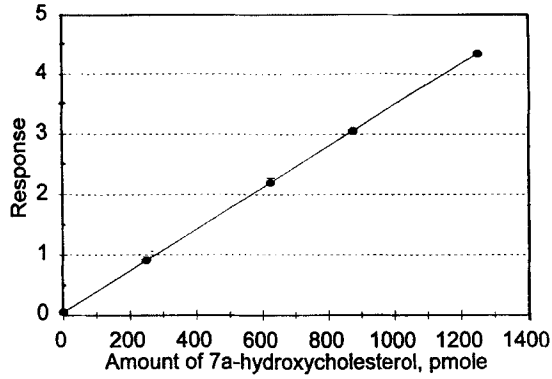

Figure 2. Calibration curve for 7α-hydroxycholesterol

Table 1

Comparison of this Method with Chiang's Method

Items	Chiang's Method	This Method
Column length	250X4.6 mm, ID.	150X4.6 mm, ID.
Mobile Phase	Acetonitrile methanol (70%:30%)	100% Acetonitrile
Flow rate	0.8-2.0 mL/min	1.0-3.0 mL/min
UV Detector/ Wavelength	Variable wavelength 240 nm	Filter 254 nm
Run time	30 min	22 min
Retention time for 20α, 7α, 7β-HCO	9, 12, 13 min	9, 10, 11 min

RESULTS AND DISCUSSION

The summary of this method compared to Chiang's is shown in Table 1. When the mobile phase of Chiang's procedure (acetonitrile:methanol = 7:3) was tested with a shorter column (150x4.6 mm, ID) and a flow rate of 1.0 mL/min, we could not obtain a similar separation. A contaminant peak coeluted with the peak of 20α-HCO. This contaminant peak is completely separated from 20α-HCO peak when 100% acetonitrile is used as mobile phase,

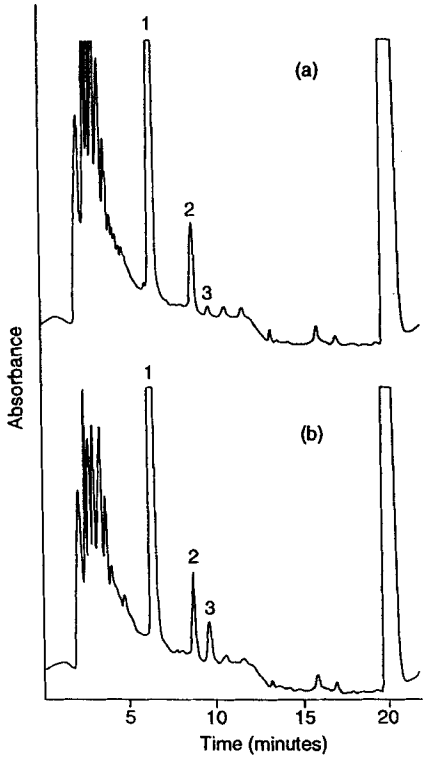

Figure 3. Chromatograms of a) CH7OH activity of a microsomal fraction from human liver sample, b) 7α-HCO spiked sample of a). Peak identification: 1= contaminant, 2= 20α-hydroxy-4-cholesten-3-one (20α-HCO), and 3= 7α-hydroxy-4-cholesten-3-one (7α-HCO).

(Figure 1). The contaminant peak has absorption at both 254 nm and 280 nm but peaks of 20α-HCO and 7α-HCO have absorption at 254 nm but not of 280 nm. This makes it being easily differentiated from 20α-HCO and 7α-HCO peaks. This contaminant is present in every sample including the reagent background and the source of it is not investigated.

The flow programming was optimzed in order to shorten the run time and still keep a baseline separation for 7α-HCO and IS. When the flow rate was of 2.0 mL/min the peaks of 20α-HCO and 7α-HCO were partially overlapped. Therefore, the flow rate of 1.0 mL/min was chosen for the first 12 min of the chromatographic run in this study. Selection of a shorter column and use of

100% acetonitrile as mobile phase also allowed us to increase the flow to 3.0 mL/min after 12 min to elute late peaks before 22 min without having high back pressure (pressure ≤ 3000 psi).

Under these modified conditions, we can have a chromatographic run of 22 min and it still has a baseline separation of peaks of 20α-HCO, 7α-HCO, and 7β-hydroxy-4-cholesten-3-one (7β-HCO) with retention times of about 9, 10, and 11 minutes respectively (Figure 1). 7β-HOC is an alternate IS in Chiang's method. The response of (7α-HCO)/(20α-HCO) is linear up to at least 1250 pmole of 7α-HOC (Figure 2). Detectability of 7α-HCO is 12.5 pmole (at S/N=5) by this procedure. This HPLC procedure was used to measure hepatic CH7OH in rats and humans. We obtained hepatic CH7OH activities of 6.0 and 8.8 pmole/min/mg protein in two rats and activities ranging from 0 to 16.4 pmole/min/mg protein (mean±SEM =4.02±0.59, n=38) in obese humans. These values are comparable to those of other studies.[2,7,11] Chromatograms of a human sample and 7α-HCO spiked human sample are shown in Figure 3. The spiked chromatogram confirms the identity of 7α-HCO peak in the sample.

Therefore, a modified HPLC procedure was developed to measure hepatic CH7OH in rats and humans. Compared to original procedure, this HPLC procedure is simpler (single vs binary component mobile phase), quicker (22 vs 30 minutes of chromatographic run), and it does not require a variable wavelength UV detector and still retains the advantages of the original procedure.

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