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Short Communication

Determination of the cytochrome P-450 IV marker, ω-hydroxylauric acid, by high-performance liquid chromatography and fluorimetric detection

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ABSTRACT

The formation of ω-hydroxylauric acid from lauric acid is an indicator of the activity of cytochrome P-450 IV family proteins. The two main metabolites of lauric acid, (ω − 1)- and ω-hydroxylauric acid, have been completely separated by reversed-phase high-performance liquid chromatography. Measurement of lauric acid hydroxylase activity in microsomal liver samples, based on derivatization of the substrate and metabolites with the fluorescent agent 4-bromomethyl-6,7-dimethoxycoumarin, is a precise method (coefficient of variation = 7.6 and 10% for ω and (ω − 1) metabolites, respectively) with good sensitivity (signal-to-noise ratio in microsomal samples of untreated rats >20). In microsomal fractions from livers of rats treated with di-(2-ethylhexyl)phthalate the extent of ω-hydroxylation of lauric acid increased dose-dependently (*ca.* ten-fold). The (ω − 1)-hydroxylase activity was not altered. A strong correlation between immunochemically determined cytochrome P-450 IVA1 and lauric acid ω-hydroxylase activity was found (r = 0.94, n = 30).

INTRODUCTION

The formation of ω-hydroxylauric acid (12-hydroxylauric acid) from lauric acid is widely acknowledged to be a marker of the activity of the cytochrome P-450 IVA1 isoenzyme [1]. After treatment of rats with hypolipideamic, peroxisome-proliferating compounds, such as clofibrate, nafenopin and di-(2-ethylhexyl)phthalate (DEHP), ω-hydroxylase activity is induced in liver as well as in kidney [2,3].

Since the group of hypolipidaemic, peroxisome-proliferating compounds is classified as a unique class of epigenetic hepatocarcinogens [4], much attention has been paid to these responses. Recently, Sharma *et al.* [2] postulated that a perturbation of lipid metabolism in the liver results in a proliferation of peroxisomes. Disturbances in lipid metabolism are due to enhanced ω-hydroxylation of fatty acid by cytochrome P-450 IVA1.

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The formation of ω- and ($\omega - 1$)-hydroxylauric acid (11-hydroxylauric acid) is usually determined by gas chromatography (GC) [5] or high-performance liquid chromatography (HPLC) [6]. Since lauric acid and its metabolites have no UV absorption or fluorescence characteristics, detection in the HPLC method is usually achieved by radiochemistry, which necessitates the use of radiolabelled lauric acid substrate. Recently, two reports were published in which non-radioactive lauric acid was used as the substrate. In one study, lauric acid and its metabolites were converted into a UV-absorbing ester derivative before HPLC separation. However, co-eluting UV-absorbing peaks in organic solvent extracts of microsomes were reported [7]. Imaoka *et al.* [8] determined lauric acid and its metabolites by HPLC with fluorimetric detection, following derivatization with 9-antryldiazomethane. However, no data on the sensitivity and reproducibility of this method were presented.

The aim of the present study was to develop a reproducible and sensitive method for the determination of the hydroxylase activities towards lauric acid in microsomal preparations without the necessity for radiolabelled substrates. Since 4-bromomethyl-6,7-dimethoxycoumarin (Br-mdmc) can be used as a fluorimetric derivatization agent for carboxylic acid [9], we applied this compound in the determination of the ω- and ($\omega - 1$)-hydroxy metabolites of lauric acid in microsomal fractions.

EXPERIMENTAL

Reagents and chemicals

Lauric acid, ω-hydroxylauric acid and NADPH were obtained from Sigma (St. Louis, MO, U.S.A). ($\omega - 1$)-Hydroxylauric acid was a generous gift from Dr. S. Imaoka (Osaka City University Medical school, Osaka, Japan). 18-Crown-6 ether and 4-(bromomethyl)-6,7-dimethoxycoumarin were obtained from Aldrich (Milwaukee, WI, U.S.A.). DEHP was obtained from Janssen Chimica (Beerse, Belgium). Antibodies against cytochrome P-450 IVA1 were a generous gift from Dr. G. Gordon Gibson (University of Surrey, Guildford, U.K.). All other chemicals were of the highest purity obtainable.

Chromatography

The analyses were performed using the HPLC gradient system Model 8800 from Spectra Physics. A 20 μl portion was injected on to a 150 × 4.6 mm I.D. Nucleosil C_{18} (5.0 μm) column (Macherey-Nagel, Düren, Germany) (column temperature, 30°C; flow-rate, 1.0 ml/min). The solvent programme started isocratically with 67% (v/v) methanol and 33% water for 25 min, followed by a 10-min linear gradient to 97% (v/v) methanol and 3% water, and a hold for 5 min. The chromatograph was equipped with a fluorescence spectrophotometer (Shimadzu RF-530), interfaced with an Apple II computer. The excitation wave-

length was 340 nm and the emission wavelength 420 nm. Peak areas were calculated using a Chromatochart program (Interactive Microware).

The rate of the hydroxylase reaction was calculated from the fractional conversion of lauric acid into ($\omega-1$)- and ω-hydroxylauric metabolites. Concentration of the hydroxylated metabolites formed were determined from their relative peak areas of the total area of the parent compound and metabolites. Since the methanol percentage increases during the run, and methanol increases the fluorescence intensity of the Br-mdmc derivatizing agent [9], peak areas of ($\omega-1$)- and ω-hydroxylauric acid were corrected for reduced fluorescence.

Animals and treatment

Male random-bred Wistar rats (150–200 g) were pretreated by gastric intubation once a day with DEHP for three consecutive days at the following dose levels: 50, 100, 250, 500 and 1000 mg/kg body weight per day. Five or six animals were used in each group. Olive oil was used as a vehicle for DEHP administration. Control animals were given olive oil at 5 ml/kg body weight per day. Animals were killed by decapitation 24 h after the last dose. Livers were perfused with 0.9% NaCl (w/w) for 10 min. Whole liver homogenates (20%, w/v) were prepared in 0.25 *M* sucrose–2 m*M* EDTA and 10 m*M* Tris–HCl (pH 7.4). Microsomal fractions were prepared by a standard centrifugation method [10].

Assay of ω- and ($\omega-1$)-hydroxylase activities towards lauric acid

The activities of the microsomal fraction (1 mg of protein) towards lauric acid was measured in a reaction mixture containing 200 nmol of lauric acid in 2 ml of 50 m*M* Tris–HCl (pH 7.5). The test-tubes were incubated for 5 min at 37°C, prior to the addition of 50 μl of 40 m*M* NADPH to initiate the reaction. After 10 min the reaction was stopped by the addition of 300 μl of 3 *M* HCl. The reaction mixture was extracted twice with 5 ml of diethyl ether. The extracts were evaporated to dryness under a gentle flow of nitrogen at 35°C.

A 1-ml volume of a solution of 18-crown-6-ether in acetone (250 μg/ml) was added to the dried extract. After the addition of 1 mg of dried potassium carbonate and 0.5 ml of acetone with Br-mdmc (2 mg/ml), the derivatization was performed at 70°C for 45 min in a sealed test-tube. The mixture was evaporated to dryness under a gentle flow of nitrogen at 40°C. the residue was dissolved in a fixed volume of methanol, and an aliquot was analysed by HPLC.

Other methods

Total cytochrome P-450 was determined from the CO difference spectrum [11]. Cytochrome P-450 IVA1 was determined immunochemically by an enzyme-linked immunosorbent assay (ELISA) method described by Sharma *et al.* [2]. Protein concentrations were determined by the method of Bradford [12].

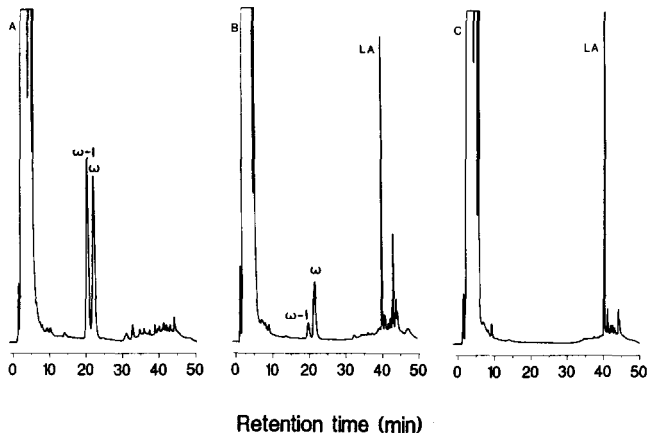

Fig. 1. Chromatograms of diethyl ether extracts of microsomal fractions of rat liver after derivatization with 4-bromomethyl-6,7-dimethoxycoumarin (Br-mdmc). Peaks: $\omega - 1$ = ($\omega - 1$)-hydroxylauric acid; ω = ω-hydroxylauric acid; LA = lauric acid. Gradient elution as described in Experimental. (A) Chromatogram of authentic ω- and ($\omega - 1$)-hydroxylauric acid standards. (B) Chromatogram of lauric acid metabolites generated by a microsomal fraction of a DEHP-treated rat (1000 mg/kg body weight), after a 10-min incubation of 1 mg of protein at 37°C with NADPH. (C) As B, but without NADPH in the incubation mix.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms obtained from authentic compounds (Fig. 1A), and lauric acid metabolites generated by a microsomal fraction of the liver with (Fig. 1B) and without (Fig. 1C) NADPH, after derivatization with a fluorescent compound. The retention times of the esters of ($\omega - 1$)-hydroxylauric acid, ω-hydroxylauric acid and lauric acid were 19.5, 22.0 and 40.0 min, respec-

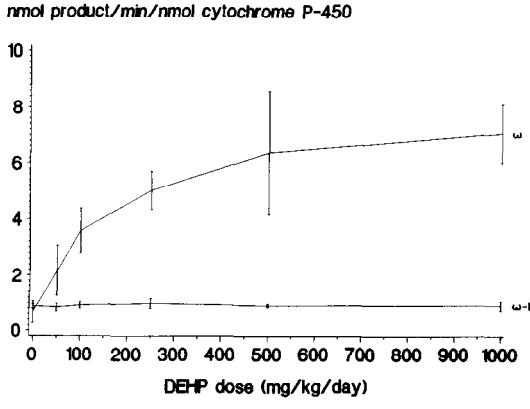

Fig. 2. Effect of DEHP on ($\omega - 1$)- and ω-hydroxylase activity towards lauric acid in rat liver microsomes. Mean ± S.D. values are presented. Each group consisted of five or six rats.

tively. The ($\omega-1$)- and ω-hydroxylauric acid formed in the presence of NADPH by a microsomal fraction of the liver are clearly baseline-separated and are not detected in the absence of NADPH. Interferences from fatty acids extracted from the microsomal fractions were not observed.

The ω- and ($\omega-1$)-hydroxylase activities towards lauric acid in microsomal fractions isolated from the livers of rats treated with DEHP (doses: 0, 50, 100, 250, 500 and 1000 mg/kg body weight per day) are shown in Fig. 2. A dose-dependent increase in the ω-hydroxylase activity was observed, but no alteration in the ($\omega-1$)-hydroxylase activity was found. Similar results have been reported by Sharma *et al.* [13].

The formation of ω-hydroxylauric acid from lauric acid is recognized as a marker of the activity of cytochrome P-450 IVA1 [1]. Cytochrome P-450 IVA1 concentrations in the microsomal fractions have been determined with ELISA. The immunochemically determined cytochrome P-450 IVA1 concentration correlated strongly with the ω-hydroxylase activity towards lauric acid in these samples ($r = 0.94$, $p = 0.0001$, $n = 30$) (Fig. 3) and did not correlate with the ($\omega-1$)-hydroxylase activity ($r = 0.17$, $p = 0.36$, $n = 30$). Sharma *et al.* [13] used the same ELISA procedure and applied a hydroxylase assay with radiolabelled lauric acid as substrate and found identical results. As the determination of lauric acid metabolites was possible in microsomal liver samples of untreated rats, the sensitivity of the method is sufficient (signal-to-noise ratio for ω- and ($\omega-1$)-hydroxylauric acid in samples of the untreated rats is > 20). The coefficient of variation (C.V.) based on duplicate incubations is 7.6% for ω-hydroxylauric acid ($n = 40$) and 10% for ($\omega-1$)-hydroxylauric acid ($n = 40$). The Br-mdmc esters of lauric acid and ($\omega-1$)- and ω-hydroxylauric acid in methanol are stable for at least two months when stored in the dark at ambient temperature.

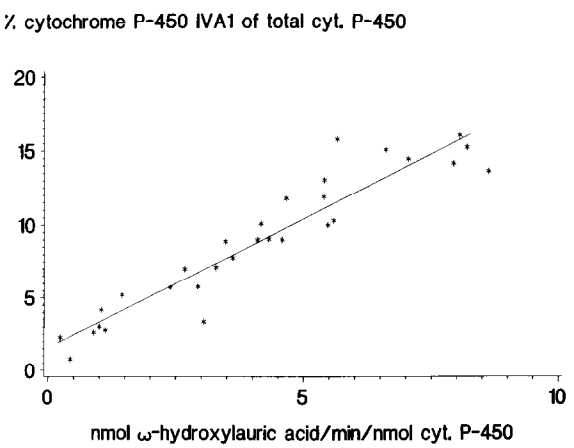

Fig. 3. Plot of immunochemically determined cytochrome P-450 IVA1 concentrations and ω-hydroxylase activities towards lauric acid in microsomal fractions from rats treated with DEHP ($y = 1.6 + 1.8x$, $r = 0.94$, $p = 0.0001$, $n = 30$).

Measurement of lauric acid hydroxylase activity, based on derivatization of the substrate and metabolites with Br-mdmc, provides a precise and sensitive method for the determination of lauric acid hydroxylase activities without the need for radiolabelled substrates.

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