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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF 11-HYDROXYLAURIC ACID ENANTIOMERS

APPLICATION TO THE DETERMINATION OF THE STEREOCHEMISTRY OF MICROSOMAL LAURIC ACID ($\omega-1$) HYDROXYLATION

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SUMMARY

Racemic 11-hydroxylauric acid was obtained in a two-step synthesis and derivatized to diastereoisomeric (*S*)-α-methoxy-α-trifluoromethylphenylacetate esters of methyl 11-hydroxylaurate, which were readily resolved by isocratic normal-phase high-performance liquid chromatography (HPLC). The assignment of absolute configuration of each diastereoisomer was carried out using proton NMR spectroscopy and a lanthanide shift reagent. From this result, an analytical method was designed for the determination of the stereochemical outcome of ($\omega-1$) lauric acid hydroxylation mediated by rat liver microsomes. ^{14}C-labelled ω- and ($\omega-1$)-hydroxylated lauric acids were separated by reversed-phase HPLC and quantified by liquid scintillation counting. Enantiomeric ($\omega-1$)-methyl laurates were derivatized into their diastereoisomeric (*S*)-MTPA esters, resolved and quantified by HPLC. Some typical inducers of cytochrome P-450 were used and their effects on lauric acid hydroxylase activity, regioselectivity and stereoselectivity were shown.

INTRODUCTION

The cytochrome P-450-mediated hydroxylation of medium-chain fatty acids has been reported in microsomes from mammals[1–3], fishes[4] and plants[5]. This reaction occurs either at the terminal methyl carbon (ω-hydroxylation) or at the penultimate carbon atom [($\omega-1$)-hydroxylation]. The total fatty acid hydroxylase activity and the ratio of ω to $\omega-1$ products have been found to depend on fatty acid chain length[2,6,7] species[4,8,9], tissues[10] or chronic administration of drugs inducing distinct isozymes of cytochrome P-450[11].

Microsomal lauric acid hydroxylase is one of the useful enzymatic activities allowing cytochrome P-450 characterization, and several methods have been developed to establish overalll metabolism and regioselectivity. The lack of a UV chromophore and the presence of endogenous fatty acids led to procedures requiring derivatization of the medium-chain fatty acid metabolites[12,13] and/or radioactive

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quantification after high-performance liquid chromatographic (HPLC) separation[14,15].

Hydroxylation at the $\omega-1$ position of lauric acid introduces an asymetric centre into the fatty acid molecule and two enantiomers can be formed. HPLC separation on a chiral stationary phase has been used to determine the positional and optical isomers of the lipoxygenase-derived hydroxypolyenoic fatty acids[16]. This procedure requires only methylation of the carboxylic group and can be improved by further derivatization of the free hydroxyl group[17]. Another useful method involved the formation of diastereomeric derivatives of the hydroxypolyenoic fatty acids and separation by gas–liquid chromatography (GLC)[18] or by HPLC[19]. The absolute configuration of the lipoxygenase-derived products was then determined either by comparison with authentic samples or by NMR spectroscopy using a lanthanide shift reagent[20].

This paper describes a nanomole-scale method for the determination of the stereochemistry of lauric acid ($\omega-1$) hydroxylation and compares the metabolic activities of different preparations of rat liver microsomes.

EXPERIMENTAL

Chemicals and reagents

Proton (^{1}H) NMR spectra were recorded on a Brucker 250-MHz NMR spectrometer. The samples were dissolved in C^2HCl_3 and the chemical shifts are reported in ppm relative to tetramethylsilane as an internal standard. The coupling constants (J) are reported in Hz and multiplicity of the signals was noted: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet.

[1-^{14}C] lauric acid (50 mCi/mmol) was obtained from CEA (Gif sur Yvette, France) and was used without further purification. Lauric acid (LA) (sodium salt) and 12-hydroxylauric acid (12-OH-LA) were purchased from Sigma, undecanedioic acid, 3-N,N-dimethylaminopropylamine and clofibrate [ethyl 2-(4-chlorophenoxy)-2-methyl propionate] from Fluka, G6P, G6PDH and NADP from Boehringer, methyl lithium (2.5 M solution in hexane), S-(−)-α-methoxy-α-trifluoromethyl-α-phenylacetic acid (MTPA), 4-N,N-dimethylaminopyridine (DMAP) and β-naphthoflavone (β-NF) from Aldrich, the shift reagent Eu(fod)$_3$ from Janssen and phenobarbital (PB) from Merck. S-(−)-MTPA chloride was obtained by refluxing S-(−)-MTPA with excess of thionyl chloride followed by vacuum distillation[21].

Racemic 11-hydroxylauric acid (11-OH-LA) was prepared by the following procedure. To 4.6 mmol of undecanedioic acid in 30 ml of dry tetrahydrofuran were added, at −75°C, 14.0 mmol of methyllithium. The mixture was kept for 30 min at −50°C, raised to room temperature in 1 h and maintained at 20°C for 2 h before hydrolysis and extraction with diethyl ether. The aqueous phase was acidified and extracted with ethyl acetate. Crude 11-oxododecanoic acid was purified by silicic acid column chromatography [diethyl ether–light petroleum–acetic acid (70:30:1)], giving 2.7 mmol (58%) of 11-oxododecanoic acid: m.p. 64°C (uncorrected); lit.[22] m.p., 61–63°C; ^{1}H NMR, 8.50 (m, 1H, COO*H*), 2.43 (t, 2H, J = 7.0, CH_2CO), 2,36 (t, 2H, J = 7.0, CH_2COOH), 2,15 (s, 3H, CH_3CO), 1.60–1.30 (m, 14H). Reduction of 11-oxododecanoic acid (1.0 mmol) by 1.1 mmol of $NaBH_4$ occurred in 10 ml of ethanol after stirring for 2 h at room temperature. Evaporation of ethanol was followed by

acidification and extraction with ethyl acetate. Crude (±)-11-hydroxylauric acid was purified as for 11-oxododecanoic acid, affording 0.85 mmol of pure product: m.p., 56°C (uncorrected); lit.[7] m.p., 53–54°C; ^{1}H NMR, 7.50 (m, 1H, COO*H*), 3.73 (m, 2H, C*H*OH), 2,27 (t, 2H, J = 6.6, CH_2COOH), 1.60–1.30 (m, 16H), 1.15 (d, 3H, J = 6.3, CH_3CH). The mass spectrum (electron-impact) of the methyl ester trimethylsilyl ether of (±)-11-hydroxylauric acid showed ions of high intensity at m/z 302 (M^+), 287 ($M^+ - CH_3$), 271 ($M^+ - OCH_3$), 258 ($M^+ - CHOCH_3$) and 117 [$CH_3CH{=}O^+SiCH_3)_3$], and was comparable to reported spectra[15,23].

S(−)-MTPA esters of (±)-methyl-11-hydroxylaurate were prepared as follows. To 0.05 mmol of 11-OH-LA in 200 μl of methanol was added etheral diazomethane until a faint yellow colour persisted. After evaporation, the residue was dissolved in 500 μl of dry carbon tetrachloride. Dry pyridine (100 μl), DMAP (1 mg) and *S*-(−)-MTPA chloride (25 μl) were added successively and the mixture was stirred overnight at room temperature. Excess of *S*-(−)-MTPA chloride was removed by adding 50 μl of 3-N,N-dimethylaminopropylamine. After stirring for 10 min, washing twice with 1 *M* hydrochloric acid, then brine, drying and evaporating, the residue was dissolved in *n*-hexane and the diastereoisomeric esters were separated by HPLC system 2. ^{1}H NMR:7.51 (m, 2H, *o*-Ph*H*), 7.37 (m, 3H, other Ph*H*), 5.13 (m, 1H, C*H*O), 3.65 (s, 3H, COOCH_3), 3.53 and 3.55 (2s, total 3H, OCH_3), 2.28 (t, 2H, J=6,6, CH_2COOCH$_3$), 1.60—1.30 (m, 16H), 1.23 and 1.31 (2d, total 3H, J=6.3, CH_3-CH).

All other reagents and solvents were of the highest purity commercially available.

Incubation procedures

Liver microsomes were prepared from adult male Sprague–Dawley rats according to Kremers *et al.*[24]. Protein concentrations were determined by the Lowry's method[25] with bovine serum albumin as standard. The cytochrome P-450 content was determined by the method of Omura and Sato[26]. The animals were pretreated with clofibrate (500 mg/kg, 10% in corn oil, i.p. for 4 days), phenobarbital (50 mg/kg in 0.9% saline, 20 mg/ml, i.p. for 4 days) and β-naphthoflavone (50 mg/kg in corn oil, 10 mg/ml, i.p. for 4 days).

Laurate hydroxylations were performed in phosphate buffer (0.1 *M*, pH 7.4) in a final volume of 500 μl containing sodium laurate (200 μ*M*), [1-^{14}C]lauric acid (0.25 μCi), G6P (20m*M*), NADP (2 m*M*) and microsomes (0.1–0.5 mg of proteins). Samples were preincubated for 2 min at 37°C before addition of 2 units of G6PDH and were incubated for 10 min. The reactions were stopped by addition of 500 μl of methanol containing 1 *M* hydrochloric acid (3:1) and 100 μl of brine. The mixtures were extracted twice with 500 μl of ethyl acetate and the solvent was evaporated under nitrogen. Unlabelled 12-OH- and 11-OH-LA (500 μl of 0.2 mg/ml solution in HPLC system 1 solvent) were added to the residue before HPLC separation (system 1) and liquid scintillation counting. Under these conditions, more than 95% recovery of the initial radioactivity was obtained.

Analytical methods

Reversed-phase HPLC (system 1). Separation of lauric acid, 11-OH-LA and 12-OH-LA was performed by RP-HPLC using an Altex Chromatem 380 solvent-

delivery system equipped with a 5-μm Nucleosil C_{18} column (125 × 4.6 mm I.D.) (Société Française Chromato-Colonne, Paris, France). The UV absorbance was monitored at 230 mm using a Pye-Unicam (Philips) UV spectrophotometer. The mobile phase was a mixture of acetonitrile and 0.1 *M* ammonium acetate buffer (pH 4.6) (35:65, v/v) for 15 min, followed by acetonitrile. The flow-rate was 1 ml/min. The retention times for 11-OH-LA, 12-OH-LA and LA were 8.0, 10.0 and 25.0 min, respectively. Fractions were collected at 30-s intervals in 3-ml polypropylene tubes in a Radirac fraction collector (LKB-Pharmacia); 2 ml of Pico-Fluor (Packard) were added and radioactivity was measured on a Packard Tri-Carb 300 scintillation counter with quench correction. This procedure was used to determine LA hydroxylase activity and regioselectivity (12-OH- to 11-OH-LA ratio). For the determination of ($\omega-1$) stereoselectivity, 11-OH-LA separated from incubation mixtures by the above procedure was collected and the solvent evaporated. The residue was dissolved in methanol (300 μl) and methylated with ethereal diazomethane. After evaporation of the solvent, MTPA esters of methyl 11-OH-LA were prepared as described for authentic (±)-methyl-11-OH-LA and subjected to HPLC (system 2). It was verified that similar treatment of enantiomerically pure (*S*)-11-OH-LA resulted in the exclusive formation of the (*S,S*)-MTPA ester (data not shown).

Normal-phase HPLC (System 2). Separation of diastereoisomeric (*R,S*)- and (*S,S*)-MTPA esters of methyl-11-OH-LA was carried out by normal-phase HPLC using an Ultrasphere SI (5-μm) (Altex) column (250 × 4.6 mm I.D.). The mobile phase was *n*-hexane–ethyl acetate (99 : 1, v/v) at a flow-rate of 2 ml/min. The effluent was monitored at 254 nm. Fractions were collected at 30-s intervals, mixed with 2 ml of toluene scintillator (Packard) and analysed by scintillation counting.

Absolute configuration of 11-OH-LA derivatives

The mixture of (*S*)-MTPA esters prepared from (±)-methyl-11-OH-LA was enriched in the fast-migrating component after HPLC (system 2) separation of each diastereoisomer and mixing to a final ratio of 7:3 (2.4 mg). To a solution of these esters in C^2HCl_3 (0.5 ml) were successively added, in five additions, 0.17–0.7 equiv. of $Eu(fod)_3$ and the ^1H NMR spectra of the solutions were recorded. The main proton chemical shifts corresponding to each diastereoisomer were localized and their lanthanide-induced shifts (LIS) measured after each addition and used for the determination of the absolute configuration of each isomer.

RESULTS AND DISCUSSION

We obtained racemic ($\omega-1$)-hydroxylauric acid (11-OH-LA) by a two-steps synthesis based on the procedure of Tegner[27]. The addition of 3 equiv. of methyllithium to undecanedioic acid gave 11-oxododecanoic acid, which was reduced by sodium borohydride to afford 11-OH-LA in an overall yield of 50%. 11-OH-LA was methylated with etheral diazomethane and diastereoisomeric MTPA esters were obtained by treatment with (*S*)-MTPA chloride in pyridine. Fig. 1 illustrates the overall transformations involved.

The mixture of diastereoisomeric derivatives was then resolved by normal-phase HPLC (system 2) using *n*-hexane–ethyl acetate (99:1) as eluent. Synthetic 11-OH-LA derivatives gave two separated peaks of equal intensities, eluting at 34 and 36 min, as shown in Fig. 2.

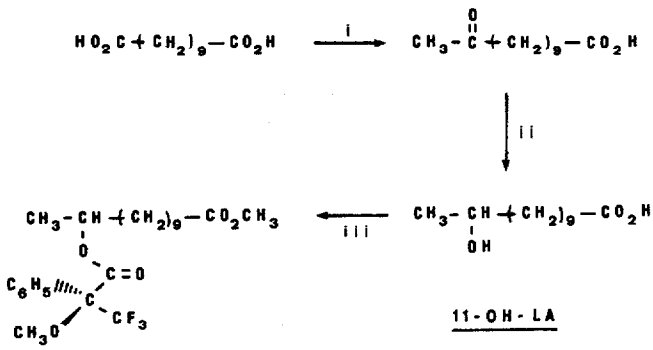

Fig. 1. Synthesis of racemic 11-hydroxylauric acid and diastereomeric MTPA ester derivatives. (i) CH_3Li, 3 equiv.; (ii) $NaBH_4$; (iii) CH_2N_2, then (*S*)-MTPA-chloride.

The absolute configuration of 11-OH-LA derivatives was determined by Yamaguchi *et al.*'s[28] NMR method. The lanthanide-induced shifts of the proton resonance of a mixture of (*S*)-MTPA esters of enantiomeric methyl-11-OH-LA containing an excess (7:3) of the first-eluted diastereoisomer were determined. Before any addition of $Eu(fod)_3$ complex, the OCH_3 resonance of the MTPA ester of the less abundant isomer was at a slightly higher field (3.53 ppm) than that of the most abundant isomer (3.55 ppm). However, after successive additions of Eu^{3+} complex, a higher downfield shift was measured for the OMe group of the less abundant MTPA ester, indicating an *S,S* stereochemistry (Table I). The 12-methyl group resonance was similarly shifted, confirming the stereochemical attribution[29]. Thus, the most abundant isomer, which corresponded to the less retained peak, was the diester of (*R*)-11-OH-LA, while the second peak (more retained) corresponded to the diester of (*S*)-11-OH-LA.

We applied the method to determine the stereochemistry of lauric acid ($\omega-1$)-

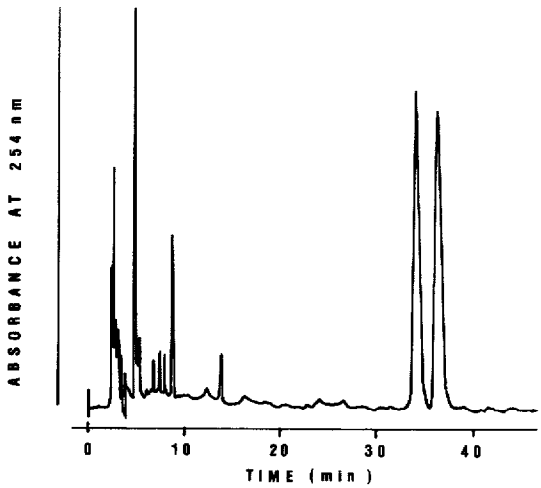

Fig. 2. HPLC separation (system 2) of diastereoisomeric (*S*)-MTPA esters of racemic methyl 11-hydroxylaurate.

TABLE I

LANTHANIDE-INDUCED SHIFT (LIS, IN ppm/Eu^{3+} equiv.) OF SELECTED PROTONS OF THE DIASTEREOISOMERIC (*S*)-MTPA ESTERS OF METHYL 11-HYDROXYLAURATE

A corresponds to the more retained isomer using HPLC system 2 and B to the less retained one.

Proton	*A*	*B*	*LIS = A − B*
OCH_3	+ 4.02	+ 2.90	+ 1.12
o-Ph*H*	+ 2.75	+ 1.96	+ 0.79
Other Ph*H*	+ 0.65	+ 0.46	+ 0.19
12-CH_3	+ 0.51	+ 0.22	+ 0.29
CO_2CH_3	+ 2.76	+ 2.77	− 0.01
11-C*H*O	+ 0.90	+ 0.69	+ 0.21

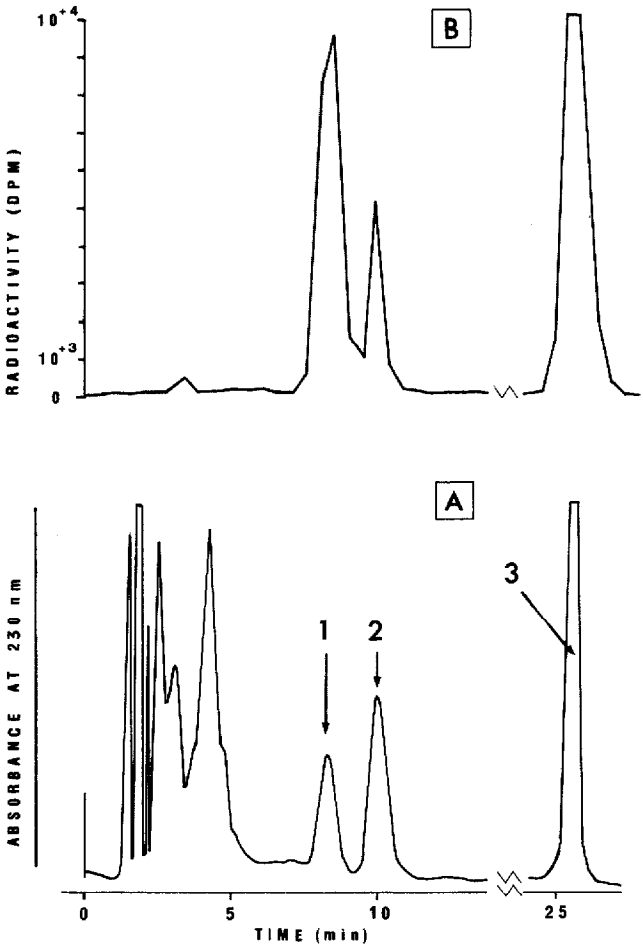

Fig. 3. HPLC separation (system 1) of [1-^{14}C]lauric acid and its 11- and 12-hydroxylated metabolites after incubation with PB-treated rat liver microsomes and treatment as described under Experimental. Fractions were collected every 30 s and counted for radioactive quantitation. (A) Absorbance at 230 nm; peaks were identified as follows: 1 = 11-OH-LA; 2 = 12-OH-LA; 3 = LA. (B) Radioactivity contained in each fraction.

hydroxylation catalysed by rat liver microsomes. Incubations were performed with [1-^{14}C]LA, an NADPH-generating system and 0.2–1.0 mg/ml microsomal proteins from untreated or treated rat. After extraction and mixing with unlabelled 11- and 12-OH-LA, an aliquot of the incubate was analysed by reversed-phase HPLC (system 1), which separated the hydroxylated metabolites of lauric acid. Two major radioactive peaks were observed, coeluting with authentic 11- and 12-OH-LA (Fig. 3). Collecting and counting radioactive fractions determined the two hydroxylated metabolites and untransformed lauric acid. Lauric acid hydroxylase activities and the ω to $\omega-1$ ratios observed with microsomes from different origins are given in Table II.

($\omega-2$)-Hydroxylation of lauric acid may occur in such incubations, but to a small extent[23]. 10-OH-LA obtained after incubation of LA with Jerusalem artichoke microsomes was coinjected with an aliquot of the PB-treated rat liver microsomes incubates using the reversed-phase HPLC system proposed by Salaün *et al.*[30]. No 10-OH-LA could be observed from our rat liver microsomal incubations or, if present, it was less than 5% of the amount of 11-OH-LA.

Acidification of the mixtures at the end of the incubation improved the total radioactive recovery and neither 11-acetoxy- nor 12-acetoxy-LA was observed on the radiochromatograms from reversed-phase HPLC (system 1). These two derivatives may be formed after ethyl acetate extractions of incubates under neutral conditions[31].

11-OH-LA from pooled incubations was obtained by reversed-phase HPLC (system 1), methylated with etheral diazomethane and esterified by treatment with excess of *S*-(−)-MTPA chloride to assess the ($\omega-1$)-lauric acid hydroxylase stereospecificity. The diastereoisomeric MTPA esters were separated by normal-phase HPLC (system 2), fractions being collected and measured using liquid scintillation counting. Before any derivatization procedure, an aliquot of pooled 11-OH-LA was resubjected to reversed-phase HPLC (system 1) to ascertain the absence of 12-OH-LA. If present, the MTPA ester of methyl-12-OH-LA may interfere in the separation and determination of 11-OH-LA derivatives. In some incubates, several non-

TABLE II

LAURIC ACID HYDROXYLATION BY RAT LIVER MICROSOMES:ACTIVITY, REGIOSELECTIVITY AND ($\omega-1$)-STEREOSELECTIVITY, AS MEASURED BY 12-OH-, (*R*)-11- AND (*S*)-11-OH-LA FORMATION FROM [1-^{14}C]LA

Microsomal fraction obtained from untreated or treated rat liver; incubations were performed as described under Experimental using 0.2 nmol of P-450 by incubation (0.1–0.3 mg of protein).

Treatment	*Formation of*[a,b]		*12- to 11-OH-LA ratio*	*(S)- to (R)-11-OH-LA ratio*
	11-OH-LA	*12-OH-LA*		
None (6)[c]	4.0 ± 0.4	2.4 ± 0.2	0.60 ± 0.05	74 : 26
Clofibrate (10)	6.2 ± 1.1	15.3 ± 2.9	2.45 ± 0.45	58 : 42
PB (6)	8.8 ± 0.7	2.6 ± 0.3	0.30 ± 0.04	57 : 43
β-NF (3)	4.8 ± 0.4	1.9 ± 0.2	0.40 ± 0.04	62 : 38

[a] Values represent means ± standard error.
[b] nmol/mg protein · min.
[c] Number of assays in parentheses.

radioactive peaks could be observed on the UV chromatograms (system 2), indicating the presence of endogenous fatty acid derivatives (Fig. 4).

Lauric acid hydroxylase activity, the 12- to 11-OH-LA ratios (regioselectivity) and (*S*)- to (*R*)-11-OH-LA ratios (stereoselectivity) obtained after incubation of LA with liver microsomes from untreated or treated rats are shown in Table II. Untreated rats predominantly formed (*S*)-11-OH-LA [12- to 11-OH-LA ratio = 0.6 and (*S*)- to (*R*)-ratio = 2.8]. Clofibrate, an hypolipidaemic agent, stimulated lauric acid hydroxylase activity 3.5-fold, ω-hydroxylation being greatly enhanced. The ratio of terminal to subterminal oxidation products was found to be 2.5, in agreement with published data[32], and nearly equivalent amounts of (*S*)-11- and (*R*)-11-OH-LA were observed [(*S*)- to (*R*)-ratio = 1.4]. Phenobarbital treatment doubled the ($\omega-1$)-lauric acid hydroxylase activity, compared with untreated rat liver microsomes[33], but had little effect on ω-hydroxylation; the observed regioselectivity was higher (12- to 11-OH-LA ratio = 0.30). Very similar amounts of (*S*)-11- and (*R*)-11-OH-LA were formed [(*S*)- to (*R*)-ratio = 1.3]. β-Naphthoflavone, a known inducer of cytochrome P-450 iso-

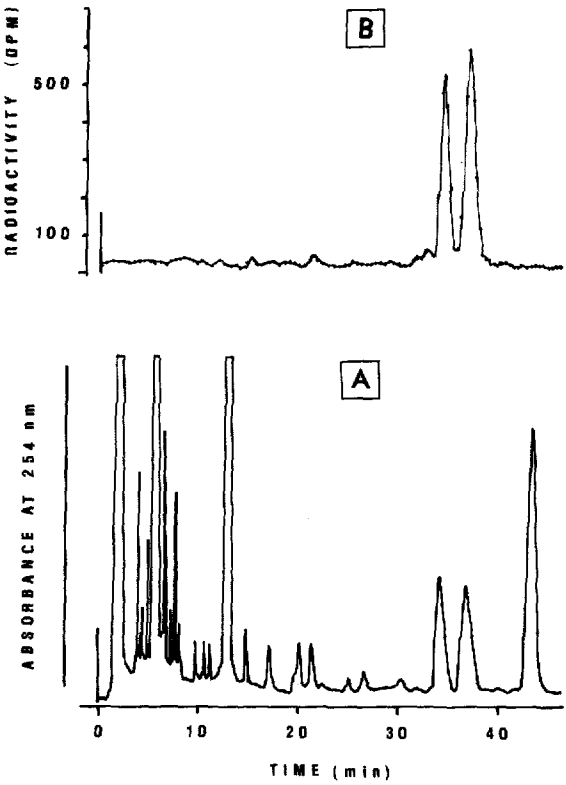

Fig. 4. Normal-phase HPLC separation (system 2) of diastereoisomeric MTPA esters of racemic methyl 11-OH-LA (A) and radiochromatogram of MTPA esters of methyl 11-OH-LA obtained from incubation of [1-^{14}C]LA with PB-treated rat liver microsomes (B). [1-^{14}C]LA was incubated with PB-treated rat liver microsomes, 11-OH-LA was separated by RP-HPLC (system 1), methylated with diazomethane and treated with (*S*)-MTPA chloride as described under Experimental; fractions were collected and analysed using liquid scintillation counting.

zymes not involved in medium-chain fatty acid metabolism[11], had no effect on ω- and ($\omega-1$)-lauric acid hydroxylase activities (12- to 11-OH-LA ratio = 0.40), but a slight loss of stereoselectivity was observed [(*S*)- to (*R*)-ratio = 1.6], compared with control experiments.

We verified that the observed (*S*)-11-to (*R*)-11-OH-LA ratios were not modified by varying the incubation times (up to 40 min), the protein content (up to 3 mg/ml) or the amount of substrate (up to 500 μM) (data not shown).

Resolution of the 11-OH-LA enantiomers showed that treatment of rats with cytochrome P-450 inducers not only modifies the activity and regioselectivity of the lauric acid hydroxylase, but also the stereoselectivity of the ($\omega-1$)-hydroxylation. In all instances, (*S*)-11-OH-LA was formed in larger amounts than (*R*)-11-OH-LA, but all drugs clearly gave rise to a decrease in the *S* selectivity. Untreated rat liver microsomes metabolized lauric acid at the penultimate carbon atom with similar stereoselectivity to that observed by Hamberg and Björkhem[34] for the ($\omega-1$)-hydroxylation of decanoic acid. After derivatization of the metabolites of decanoic acid to their methyl esters and N-(1-phenylethyl)urethanes, GLC separation and comparison with authentic derivatives, they found that the 9-hydroxydecanoic acid was a mixture of (*S*)-9- (about 75%) and (*R*)-9- (about 25%) isomers.

The formation of both isomers might be the result of the action of either one non-stereospecific or several stereospecific isozymes. Phenobarbital treatment is known to reduce the stereoselectivity observed in the hydroxylation of ethylbenzene[35,36] by untreated rat liver microsomes. Much less information is available on the effects of such treatment on the stereoselectivity of the ($\omega-1$)- or ($\omega-2$)-hydroxylation of medium-chain fatty acids, prostaglandins or drugs. More work is needed to interpret such observations.

The proposed procedure could be useful in a systematic study of cytochrome P-450 isozymes, allowing a simultaneous determination of the stereo- and regioselectivities with high sensitivity and a simple work-up.

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