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Simultaneous radiometric and fluorimetric detection of lauric acid metabolites using high-performance liquid chromatography following esterification with 4-bromomethyl-6,7-dimethoxycoumarin in human and rat liver microsomes

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Abstract

The formation of (ω-1)-hydroxylauric acid from lauric acid (LA) can be used as an indicator of the activity of cytochrome P450 2E1 (CYP2E1) in rat and human liver microsomes. A high-performance liquid chromatographic (HPLC) method that is capable of identifying and measuring the two main metabolites of lauric acid, (ω-1)- and ω-OH-LA, has been developed and used in the study of rat and human liver microsomes. Measurement of the enzymatic activities, based on the esterification of the metabolites and substrate with the fluorescent agent, 4-bromomethyl-6,7-dimethoxycoumarin, is described using both radiometric and fluorimetric detection methods. Extraction efficiencies of metabolites and residual substrate were calculated using radioactivity and were greater than 85%. The assay is accurate and reproducible and has a detection limit of 75 pg (0.37 pmol). Additionally, a strong correlation between the two techniques was found in both human (r=0.945, n=15, p<0.01) and rat (r=0.949, n=18, p<0.01) livers, for the (ω-1)-hydroxylauric acid.

Keywords: Lauric acid; Hydroxylauric acid; 4-bromomethyl-6,7-dimethoxycoumarin

1. Introduction

Microsomal cytochrome P450s[1] constitute a superfamily of heme-thiolate enzymes which catalyze the monooxidation of a wide variety of lipophilic compounds, such as fatty acids. The various cytochrome P450 isoforms exhibit distinct but overlapping patterns of substrate specificities. In recent years, particular interest has focused on the ethanol-inducible cytochrome P450 2E1 (CYP2E1) which has been shown to be involved in the activation of many low-molecular-mass toxins and carcinogens [1]. CYP2E1 also has the capacity to metabolize other endo- and xenobiotic compounds such as ketone bodies, acetaminophen, chlorzoxazone and ethanol [2]. Because of the importance of CYP2E1 in alcohol-related diseases, a number of compounds have been investigated as substrate probes for this enzyme, such as 4-nitrophenol [3,4] or chlorzoxazone [5,6], which have been used as markers of

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[1]Cytochrome P450 (EC 1.14.14.1): P450 or CYP. The name cytochrome must be abandoned according to the Nomenclature Committee of the International Union of Biochemistry, the appropriate name being 'heme-thiolate protein'.

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CYP2E1 enzymatic activity in a number of animal species.

The NADPH- and oxygen-dependent ω-oxidation of fatty acids, such as lauric acid (LA), has been shown to be catalyzed by the microsomal P450 family and leads to the formation of both ω- and (ω-1)-hydroxylated products. The (ω-1)-hydroxylation of lauric acid, a medium-chain-length fatty acid, was recently described as being due to CYP2E1 in rat and human liver microsomes [7–9]. The ratio between the two metabolites was shown to be dependent on environmental parameters, such as drug administration (hypolipidaemic drugs) and phthalate esters, which enhanced the ω-hydroxylated metabolite, or starvation, diabetes and alcoholism, which increased the (ω-1)-hydroxylated compound.

A number of reversed-phase HPLC methods have been developed for the detection of ω- and (ω-1)-hydroxylated metabolites of fatty acids. In most of these studies [10–13], radioactive lauric acid was used as the substrate in order to obtain sensitive detection. More recently, a number of reports using HPLC in conjunction with non-specific detection methods, mainly pre-column derivatization of the carboxylic group, have been published. These authors used either the diastereoisomeric compound, methoxyfluoromethyl phenylacetate [14], 4-bromomethyl-6,7-dimethoxycoumarin [15,16], the UV-absorbing compound, *p*-bromophenacyl bromide [17], 1-pyrenyldiazomethane (PDAM) [18] or *p*-(9-anthroyloxy)phenacylbromide (panacyl bromide) [19].

The aim of this paper was to describe and compare the formation and the detection of the metabolites of lauric acid using both [1-^{14}C]lauric acid, as the substrate probe, and a pre-column ester derivatization of the carboxylic function with a simultaneous fluorescent marker, 4-bromomethyl-6,7-dimethoxycoumarin. This method gives a fluorimetric and radiometric analysis of lauric acid metabolites.

2. Experimental

2.1. Chemicals

Lauric acid was obtained from Fluka (Buchs, Switzerland), while the metabolites [11- and 12-hydroxylauric acid (11-OH-LA and 12-OH-LA or ω- and (ω-1)-OH-LA)] were given to us by Dr. Salaün (Strasbourg, France). [1-^{14}C]Lauric acid (50 mCi/mmol) was purchased from Amersham (Amersham, UK). 18-Crown-6-ether and 4-bromomethyl-6,7-dimethoxycoumarin were obtained from Aldrich (St. Quentin Fallavier, France) and NADPH (nicotinamide adenine diphosphate hydrogen) was from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from SDS (Peypin, France). All other chemicals and solvents were of reagent grade. Deionised water and acetonitrile were further filtered before use.

2.2. Preparation of rat and human liver microsomes

Human liver samples were obtained in accordance with French legal considerations and approval of the ethical committee.

Male Sprague–Dawley rats were maintained on a water and standard diet ad libitum at 22±2°C with a 12 h light–dark cycle.

Rat and human liver samples were frozen immediately after removal and the microsomal fraction was prepared according to a previously described method [20]. The subcellular fraction, in a 100 m*M* phosphate buffer, pH 7.4, containing 20% glycerol, was stored at −80°C until use. Microsomal protein content was determined using the Bradford method (Biorad, Munich, Germany).

2.3. Assay of lauric acid metabolism

A 2-ml reaction mixture containing microsomal proteins (0.3 mg), lauric acid (1 μCi, 0.1 m*M*) in 0.12 *M* phosphate buffer, pH 7.4, and 5 m*M* $MgCl_2$, was incubated at 37°C for 10 min. The reaction was initiated by the addition of 1 m*M* NADPH. After terminating the reaction with 0.8 ml of 10% H_2SO_4, the metabolites were extracted twice with 6 ml of diethyl ether, dried on Na_2SO_4 and evaporated under a nitrogen stream. Fluorescent derivatives were prepared by adding 0.1 ml of a solution of 18-crown-6-ether in acetonitrile (2.5 mg/ml), 2 mg of dried potassium carbonate and 0.1 ml of a solution of 4-bromomethyl-6,7-dimethoxycoumarin in acetone (10 mg/ml) to the dried residue. Following vigorous shaking, the derivatization was performed at 70°C

for 60 min in darkness. The mixture was then cooled and diluted with acetonitrile before HPLC analysis.

2.4. HPLC analysis and both radiometric and fluorimetric detections

The ω- and (ω-1)-hydroxylauric acids and residual lauric acid were separated by HPLC using a reversed-phase Nucleosil C_{18} column (5 μm, 250× 4.6 mm I.D.; specific area, 300 m^2/g; pore size, 120 Å; carbon yield, 13%, Interchim, Montluçon, France). The mobile phase (1% acetic acid in water–acetonitrile) programme began isocratically with a 45:55 mixture (v/v) for 20 min at a flow-rate of 1.0 ml/min, followed by a 3-min linear gradient to 5:95 (v/v) water and acetonitrile, and a hold for 15 min before returning to the initial conditions.

The chromatograph was equipped with a fluorimeter (300–400 and 417–700 nm excitation and emission wavelengths, respectively; Fluoromonitor III, LDC-Milton-Roy, Riviera Beach, FL, USA), interfaced with a Flo-one Beta radiometric detector (Packard, Meriden, CT, USA). Emission and fluorescence were recorded by a TSP FL 200 spectrofluorometer (Thermo Separation Products, San Jose, CA, USA). Peak areas were calculated, using both radiometric and fluorimetric data, from the percentage of metabolite area to the total product area (metabolite+residual substrate). Data were expressed as nmol/min/mg of protein. Statistical analysis was performed using the Student's *t*-test.

3. Results and discussion

3.1. Elution profiles of lauric acid and its metabolites by HPLC using both fluorimetric and radiometric detections

Fig. 1 shows the lauric acid metabolite HPLC profiles generated by enzymes from a microsomal fraction of rat (A and B) and human (C and D) livers, following derivatization with the fluorescent marker. Detection of the peaks was performed using a simultaneous fluorimetric (A and C) and radiometric (B and D) method.

Fluorescence detection was used because it provides a greater specificity than UV absorbance. Several mobile phases were investigated in order to establish optimum separation and to acheive the highest analytical sensitivity possible for lauric acid metabolites. The best results were obtained under the conditions reported in Section 2.4, using acetonitrile rather than methanol.

The ω- and (ω-1)-hydroxylauric acid metabolites were identified on the basis of their retention times and their radiometric and fluorimetric labelling. The retention times of the esters of 11-OH-LA, 12-OH-LA and LA were 20.5, 22 and 41 min, respectively. The two metabolites formed in the presence of NADPH were clearly baseline-separated during the isocratic step of the gradient elution and were not detected in the absence of NADPH (data not shown). Blank samples (without substrate or without NADPH) from rat and human liver microsomes showed no endogenous peaks interfering with the assays. The peaks shown near the beginning of the chromatogram when using fluorimetric detection were due to the fluorescent marker.

3.2. Excitation and emission spectra from fluorescent compounds

Excitation and emission scan profiles are illustrated in Fig. 2. Each of the three compounds (11-OH-LA, 12-OH-LA and LA) had the same profiles, both in emission and excitation scans (excitation range 300–420 nm, emission range 350–500 nm). The maxima were 340 and 440 nm for excitation and emission respectively and these conditions were used in our experiments.

3.3. Validation of the method

Calibration curves using fluorescent standards gave excellent linearity, the intercepts of the regression lines were close to zero and the correlation coefficients were greater than 0.995. The limit of detection for lauric acid (signal-to-noise ratio of 3) was 75 pg (0.37 pmol) and this value was in accordance with previous data [15], where the authors had a detection limit for fatty acids of about 0.5 pmol.

The extraction efficiency of lauric acid and its metabolites was determined by radioactivity. The extraction solvent, diethyl ether, was chosen as it

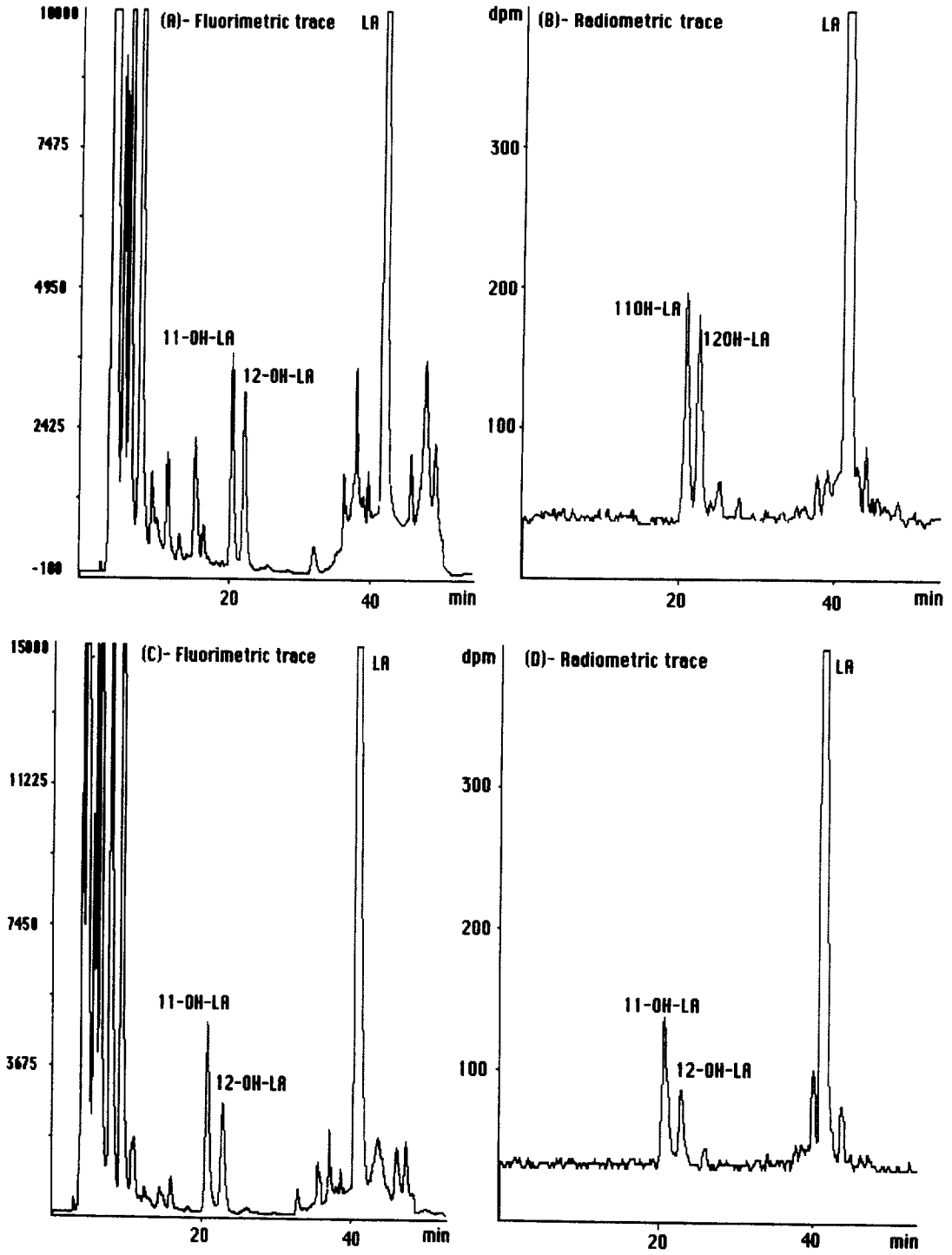

Fig. 1. Lauric acid metabolite HPLC profiles following esterification with 4-bromomethyl-6,7-dimethoxycoumarin: (A) and (B) incubation of 0.3 mg of microsomal protein from control rat liver; (C) and (D) incubation of 0.3 mg of microsomal protein from human liver FH3. Detection was carried out by either fluorimetry (left) or radiometry (right).

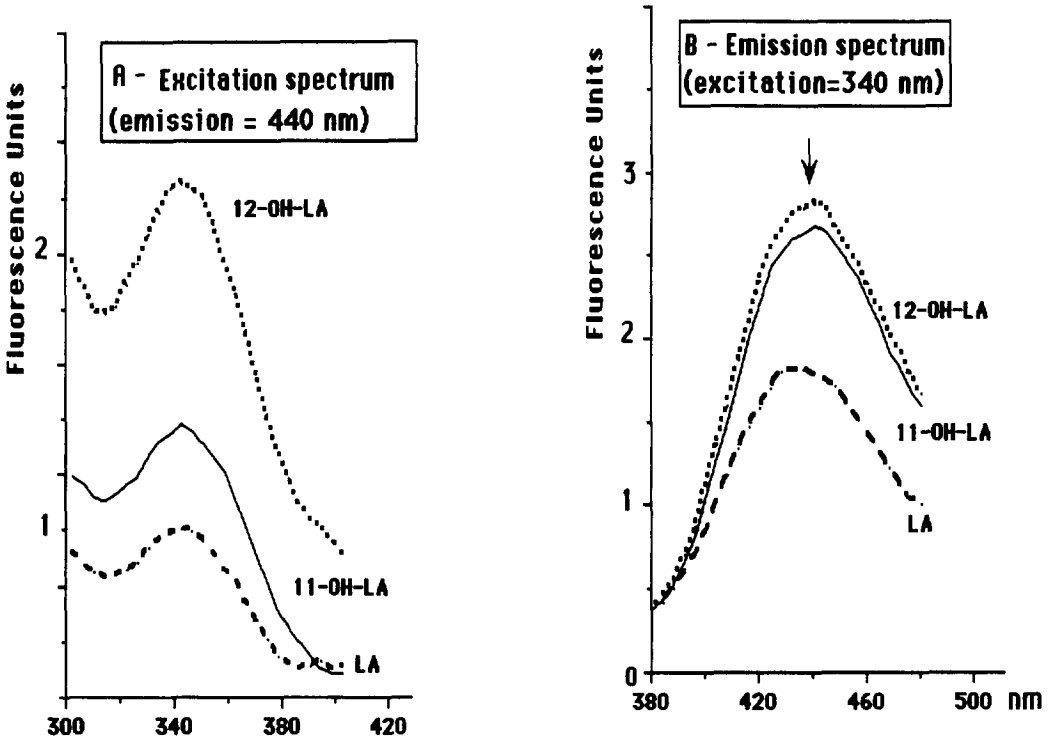

Fig. 2. Excitation (A) and emission (B) spectra of lauric acid metabolites following esterification with 4-bromomethyl-6,7-dimethoxy-coumarin.

produced no potentially interfering peaks and led to an extraction efficiency of 87.5±7.2% (n=12), with suitable reproducibility. Additionally, interferences from fatty acids extracted from the microsomal fractions were not observed. Under these conditions, it was found that the coumarin esters of lauric acid and its metabolites are stable for at least two months when stored in the dark at 4°C and diluted in acetonitrile rather than in methanol.

The reproducibility of the esterification and fluorescence or radiolabelling detections was evaluated by duplicate injections of three separate incubations of rat control microsomal preparations. The reproducibility for the two metabolites was excellent with a coefficient of variation (C.V.) of 5.8±1.9% and 6.2±1.7% for radiometric and fluorimetric detection, respectively.

Calibration of the method was carried out using caproic acid, which had a retention time of 30 min under the above conditions, as the internal standard. A calibration graph was constructed for each compound by linear regression analysis of the metabolite peak concentration versus the ratio of lauric acid metabolite to internal standard peak areas. The results showed good r values (y=0.0508x+0.125, r=0.985 and y=0.0897x+0.140, r=0.958, for 11-OH and 12-OH-LA, respectively), suggesting that this internal standard could be used to calibrate the method and also that it was possible to calculate the enzymatic activities by the area ratio method.

3.4. Comparison of the fluorimetric and radiometric detection of lauric acid metabolism in rat and human liver microsomes

This method of fluorescent derivatization was carried out in fifteen human and eight control rat liver microsomal fractions. The ω- and (ω-1)-hydroxylase activities of lauric acid are shown in Table 1. The rate of metabolite formation was calculated based on the percentage of either the fluorescent compound or the radioactivity of the given metabo-

Table 1
ω- and (ω-1)-Hydroxylase activities of lauric acid from microsomes of human ($n=15$) and control rat ($n=8$) livers

	(ω-1)-OH-LA		ω-OH-LA	
	Fluorimetric	Radiometric	Fluorimetric	Radiometric
Rat	1.51±0.31	1.42±0.28	1.66±0.27	1.42±0.38
Human	1.66±0.80	1.31±0.69	1.07±0.65	0.88±0.30

Detection of the peaks was performed using either fluorimetric or radiometric detection. Results are expressed as nmol/min/mg of microsomal protein±standard deviation.

lite relative to the total fluorescence or radioactivity eluting from the HPLC column. The enzymatic activities were significantly correlated between the two detection methods and are in accordance with results previously described [7,8].

Correlation coefficients (r) were calculated using either eighteen control and CYP2E1-induced rats or fifteen human livers. Detection of the peaks was performed with the fluorimetric and radiometric methods. The results are illustrated in Fig. 3 and the correlation coefficients were approximately 0.95 ($p<0.001$).

3.5. Comparison with other fluorescent derivatives

In order to validate the present method, other fluorescent derivatives were tested and compared to the method described in Section 2, i.e. fluorescent esters obtained with PDAM and panacyl bromide. These fluorescent markers permitted a less sensitive detection than the one described in Section 2.4 and the limit of detection was greater than with 4-bromomethyl-6,7-dimethoxycoumarin (150 and 200 pg for the panacyl bromide and PDAM esters, respectively), results which were only slightly higher than those previously described [18,19].

4. Conclusion

When the use of radiolabelled substrate is not possible or is prohibited, derivatization of fatty acids with fluorescent or UV-labelling probes is necessary and therefore the method must provide great sensitivity, a weak detection limit and suitable reproducibility. In our study, 4-bromomethyl-6,7-di-

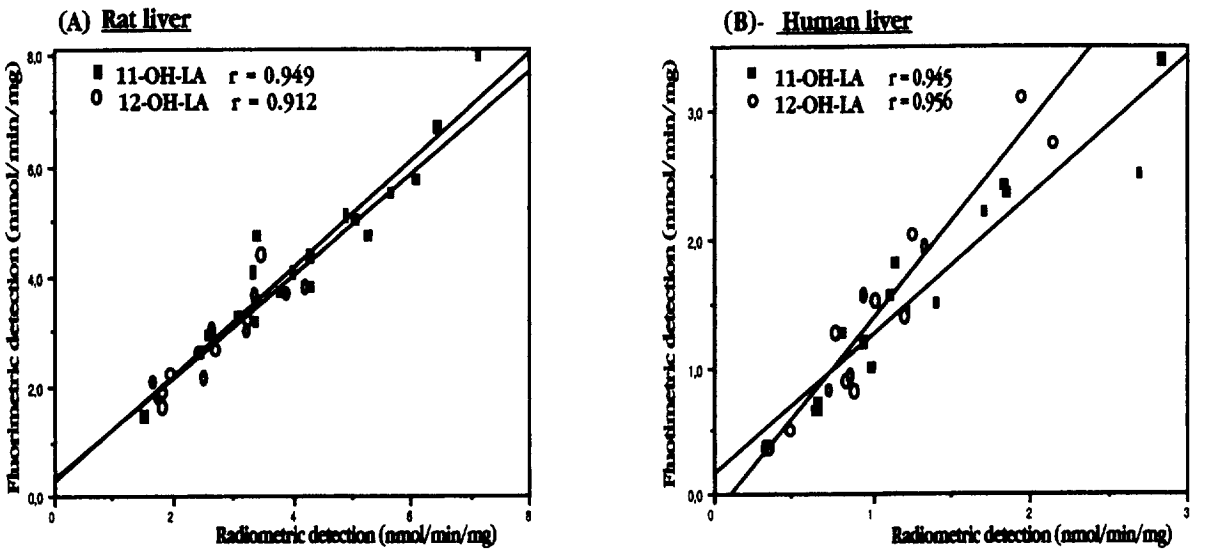

Fig. 3. Correlation plots of radiometric and fluorimetric detections of 11-OH- and 12-OH-LA in human liver microsomes (A; $n=15$) and control or CYP2E1-induced rat liver microsomal samples (B; $n=18$).

methoxycoumarin appears to be a useful fluorescent marker. The derivatization reaction is rapid and provides a good yield. Fluorescence detection is approximately as sensitive as radiometric detection and is more sensitive than UV detection.

In conclusion, these results indicate that 4-bromomethyl-6,7-dimethoxycoumarin is an excellent derivative for the detection of fatty acids and hydroxylated metabolites in biological samples following HPLC analysis.

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