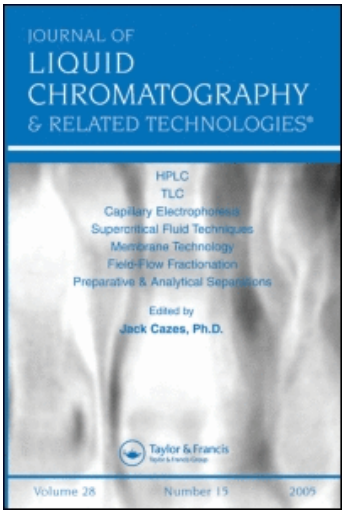

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Fluorimetric Detection of Microsomal Lauric Acid Hydroxylations Using High-Performance Liquid Chromatography After Selective Solvent Partitioning and Esterification with 1-Pyrenyldiazomethane

Steven R. Mason[a]; Leigh C. Ward[a]; Paul E. B. Reilly[a]

[a] Department of Biochemistry Molecular Biosciences Building, University of Queensland, Brisbane, Queensland, Australia

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FLUORIMETRIC DETECTION OF MICROSOMAL LAURIC ACID HYDROXYLATIONS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER SELECTIVE SOLVENT PARTITIONING AND ESTERIFICATION WITH 1-PYRENYLDIAZOMETHANE

STEVEN R. MASON, LEIGH C. WARD, AND PAUL E. B. REILLY
Department of Biochemistry
Molecular Biosciences Building
University of Queensland
Brisbane, Queensland 4072, Australia

ABSTRACT

A novel and simple method for determining microsomal lauric acid hydroxylase activity is presented. Lauric acid and hydroxy-metabolites are separated using differential acid/base solubilities coupled to solvent partitioning. After esterification with 1-pyrenyldiazomethane, metabolites are quantitated using isocratic high-performance liquid chromatography with fluorimetric detection. Column washing and equilibration between samples is not required. The method was verified by measuring the induction, in rats, of microsomal lauric acid hydroxylase activity by clofibrate. The method has clear advantages over published radiochemical procedures for measuring the formation of hydroxylated metabolites of lauric acid.

INTRODUCTION

High-performance liquid chromatography (HPLC) is an analytical method widely used in the determination of the catalytic activities of cytochrome P450-4A family enzymes towards fatty-acid substrates, especially lauric acid. Such methods employ radiochemical detection [1,2] or complex formation with fluorescent probes such as 9-anthryldiazomethane [3,4] or 4-(bromomethyl)-7-methoxycoumarin [5,6]. These methods have been recently reviewed by Jansen and Fluiter [7]. All these methods detect both the 11- and 12-hydroxy-metabolites of lauric acid. Non-HPLC methods also employ radiochemical detection and use thin layer chromatography [8] or solvent partitioning procedures [9]. These methods are useful for the determination only of total hydroxy-metabolite formation.

In this paper, a novel method is described for determining microsomal lauric acid hydroxylase activities. Similar in principal to the solvent partitioning method described by Giera and VAN Lier [9], selective acid/base solubilities coupled with solvent extraction is used. The method is simple and sensitive and employs the economical use of 1-pyrenyldiazomethane (PDAM). This is a highly fluorescent yet virtually overlooked probe for derivatising organic acids. The derivatised hydroxy-metabolites of lauric acid are quantitated using reverse phase HPLC. The method is flexible and allows for both the rapid determination of total hydroxy-metabolite formation from lauric acid and for the determination separately of the 11- and 12-hydroxy-metabolites. The latter analyses require simple procedural modifications. Application of the method is illustrated by the measurement of the induction of cytochrome P450-4A family enzymes by the hypolipidemic agent, clofibrate, in rats.

EXPERIMENTAL

Chemicals

Clofibrate, lauric acid (sodium salt), 12-hydroxy-lauric acid and 16-hydroxy-palmitic acid were purchased from Sigma (St. Louis, MO, USA); 11-hydroxy-lauric acid was a gift from Dr. S. Imaoka, Osaka City University Medical School, Japan. 1-pyrenyldiazomethane (PDAM) was acquired from Molecular Probes (Eugene, OR, USA). NADP (disodium salt), *d*-glucose-6-phosphate (disodium salt) and glucose-6-phosphate dehydrogenase (yeast enzyme, grade 1) were obtained from Boehringer Mannheim (Sydney, Australia). HPLC-grade ethyl acetate and methanol were purchased from Mallinckrodt (Clayton, Australia). All other chemicals and solvents were of reagent grade and deionised water was further purified using an Milli-Q PLUS ultrapure water system (conductance of 0.07 μS/cm).

Animals and treatment

Random outbred male and female Wistar rats (6 weeks old) were obtained from the Central Animal Breeding Unit, University of Queensland, Australia. Animals were maintained on a commercial rodent diet with water *ad libitum*, and at 24 ± 2°C with a 12-hr light/dark cycle. Test animals were administered clofibrate (200 mg/ml in corn oil) for 7 days by intraperitoneal (I.P.) injection at a dose rate of 200 mg/kg/day. Controls were given an I.P. injection of corn oil. Hepatic microsomal suspensions were prepared separately for each animal [11], frozen in liquid N_2 and stored at -80°C. Cytochrome P450 concentrations were determined [12] and protein concentrations were measured by the method of Lowry *et al.*, [13] using bovine serum albumin as standard.

Enzymatic assays

Lauric acid hydroxylase activities were determined in 200 μl reaction mixtures containing 100 mM Tris-HCl pH 7.4, 125 μM sodium laurate, 2.5 mM glucose-6-phosphate, 1.0 mM NADP, 1.0 IU G6PDH, and 0.10 or 0.25 μM cytochrome P450. Laurate was dispensed (25 μl) into monooxygenase assay tubes (1.5 ml microcentrifuge tubes) from a 1.0 mM methanolic stock solution and the samples were evaporated to dryness at room temperature using a gentle stream of dry filtered air. Laurate was then dissolved by sonication at room temperature for 15 min with a Bransonic 2200 bath sonifier, using an aliquot (150 μl) of the monooxygenase reaction buffer. This procedure ensures the exclusion of solvents from the reaction mixtures. Mixtures containing all components except microsomal suspension were preincubated at 37°C for 5.0 min to allow for temperature equilibration and NADPH generation. Monooxygenase reactions were initiated by the addition of microsomal suspension. After 10 min, reactions were terminated by the addition of 100 μl 1.0 M carbonate buffer pH 10.0, vortex mixed and placed on ice. Laurate was extracted using 1.0 ml of ethyl acetate by thorough vortex mixing and centrifuging for 2.0 min (full speed bench centrifuge) at room temperature. The upper solvent phase was aspirated and discarded. Aliquots (250 μl) of the reaction mixture were transfered to 2.0 ml microcentrifuge tubes, 200 μl of 2.0 M HCl were added, followed by 25 μl of tetrahydrofuran containing internal standard (16-hydroxy-palmitic acid, 80 μM) with vortex mixing at each step. Ethyl acetate (1000 μl) was added and the tubes were shaken for 5.0 min, centrifuged for 2.0 min and 750 μl of the upper solvent phase were transfered to 3.0 ml tapered polypropylene centrifuge tubes. A further 750 μl of ethyl acetate were added to the 2.0 ml tubes which were again shaken and centrifuged with 750 μl of the upper phase removed and added to the

first extract. Combined extracts were evaporated using a gentle stream of dry filtered air at room temperature. Samples were reconstituted for HPLC by the addition of 100 μl of a freshly prepared solution consisting of 50% methanol and 50% ethyl acetate containing 0.25 mg/ml PDAM, with thorough vortex mixing. Samples and PDAM were derivatised overnight at 25°C and then loaded onto a automatic sample injector at 25°C or stored at -20°C for subsequent HPLC analyses.

Preparation of standards

Standard solutions for 11- and 12-hydroxy-laurate metabolites were prepared in methanol and stored in glassware treated with 0.2 g/l cetyltrimethylammonium bromide to minimise adsorption. Separate calibration standards for each metabolite (0.5 to 5 nmole) were prepared by adding aliquots of these methanolic solutions and an appropriate aliquot of methanol containing laurate to 1.5 ml microcentrifuge tubes and evaporating to dryness under a gentle stream of dry air. Monooxygenase assay buffer (200μl) was added to the tubes and the samples were dissolved by sonication at room temperature for 15 min. Carbonate buffer was then added to the tubes which were then treated as described in the enzymatic assays. Optimal excitation and emission wavelengths were determined for PDAM derivatives of laurate and hydroxy-metabolites, after removing excess PDAM using SEP-PAK-C18 cartridges [10], in the HPLC mobile phase using an Aminco SPF500 Spectrophotofluorimeter.

Chromatographic procedure

Hydroxy-laurate metabolites, internal standard and residual laurate were separated chromatographically using a Brownlee Spheri-5 reverse

phase C-18 column (100 x 4.6 mm I.D.). For total hydroxy-metabolite determination, the mobile phase consisted of 87% methanol and 13% buffer (0.02% triethylamine, adjusted to pH 5.0 with 1.0 M orthophosphoric acid). HPLC mobile phase was prefiltered through a 0.22 μm membrane (Durapore; Millipore Corp, Bedford, MA), with additional gas being removed with an inline ERMA model ERC3522 membrane degassing unit. A Shimadzu LC-6A liquid chromatography pump was used to deliver the mobile phase at 2.0 ml/min, and 20 μl aliquots of samples were applied to the column using a Shimadzu SIL-9A automatic injector fitted with a 150 μl loop. PDAM-derivatised fatty-acid samples were detected using a Shimadzu RF-535 variable wavelength monitor (set at low sensitivity) with excitation and emission wavelengths set at 345 nm and 395 nm respectively. Fluorescence data were analysed in terms of peak area ratios with reference to the internal standard using a Shimadzu CR4-A Chromatopac recording integrator.

RESULTS and DISCUSSION

As stated previously, monooxygenase reactions were stopped by the addition of carbonate buffer. In the following ethyl acetate extraction, laurate partitions into the organic solvent phase and the hydroxy-metabolites partition into the aqueous phase. Both of these partitions are highly reproducible removing at least 95% of the lauric acid from the aqueous phase, thus greatly reducing the amount of PDAM required for esterification of the hydroxy-metabolites and internal standard. This also allows for greater substrate concentrations to be used in the assays, without complications associated with the substrate elution profile. For total hydroxy-metabolite determinations, chromatographic run times are reduced to 30 min per run with acceptable resolution of all components using the described

chromatographic conditions. Excess acid is then added to an aliquot of the aqueous phase, after the first ethyl acetate extraction, to render the solutions acidic. The use of an internal standard accounts for any extraction variability of the hydroxy-metabolites from the acidic solution. The monooxygenase assay buffer chosen was 100 mM Tris-HCl pH 7.4. When potassium phosphate buffer (100 mM pH 7.4) was used, the hydroxy-metabolites as well as laurate extracted from the carbonate solution. No such extraction occurred when the carbonate step was omitted using the Tris-HCl buffer. The combination of methanol and triethylamine buffer was the most effective for peak resolution, phosphate buffer (10 mM pH 7.4) was less efficient and addition of either acetonitrile or tetrahydrofuran was not beneficial.

A typical HPLC elution profile for the metabolites of lauric acid obtained using clofibrate-treated male rat liver microsomes is shown in Fig. IC. The elution profile for authentic standards is shown in Fig. IB. The peaks observed near the start of the chromatogram are due to PDAM, as seen in the elution profile for PDAM (Fig. IA). The two hydroxy-metabolites for lauric acid elute as a single peak. Since both derivatised hydroxy-metabolites gave identical fluorescent responses, quantification of the combined peak measures total hydroxy-metabolite formation. Esterification between PDAM and the fatty acids was obtained overnight (approx. 16 h.) at 25°C with no noticeable deterioration of compounds. PDAM is commercially available and inexpensive as used in these assays. PDAM is stable when stored at -20°C in solid form (for up to 5 years [10]) and we found it to be stable at room temperature (< 25°) for a few days in 50 % ethyl acetate, 50% methanol, allowing for the use of an automatic sample injector.

A linear relationship was obtained for hydroxy-lauric acid concentration versus the ratio of hydroxy-lauric acid to internal standard

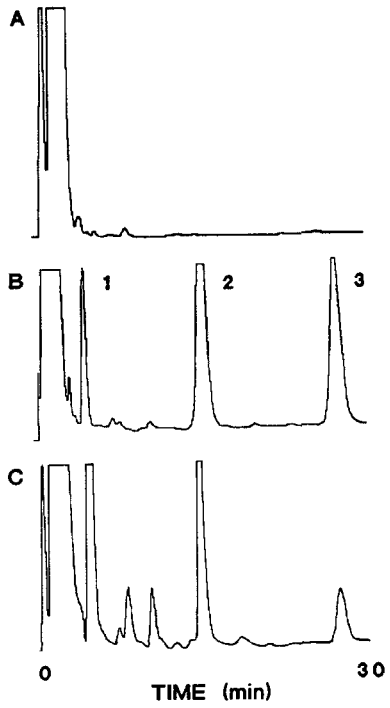

FIGURE 1. Chromatograms obtained with (A) PDAM blank, (B) authentic standards and (C) monooxygenase assays using microsomes prepared from clofibrate treated male Wistar rats. Experimental procedures are described in the text. Peak identities and initial amount are: (1) hydroxylaurate, 0.5 nmol; (2) internal standard, 2.0 nmol and (3) lauric acid, 1.5 nmol. The large peaks associated with the injection front are due to PDAM.

peak areas (Fig. 2), with a coefficient of variation of 0.999. Using the monooxygenase assay conditions described here, total hydroxy-metabolite formation was linear up to 0.15 and 0.30 μM cytochrome P450, for microsomes prepared from control and clofibrate treated male Wistar rats respectively (Fig. 3). Percentage liver to body weight ratios

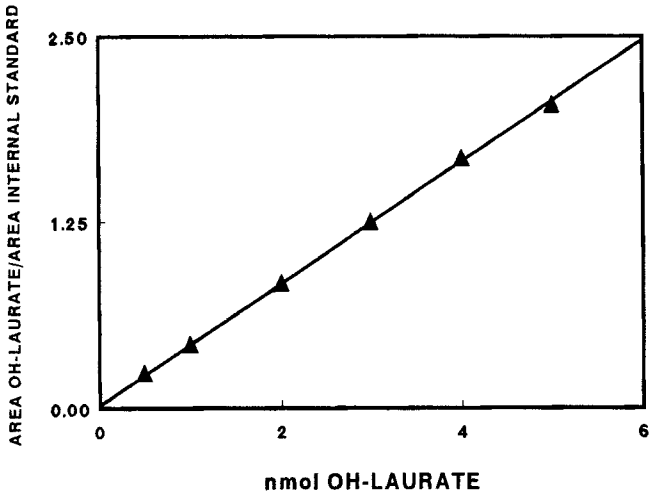

FIGURE 2. Calibration curve for the ratio of peak areas for (11- or 12-) OH-laurate to internal standard (16-OH-palmitate) versus OH-laurate concentration. Experimental procedures are described in the text. Each point represents the mean of 4 values with chromatograms been run over a number of days. Standard deviations are smaller than marker size and less than 3% of the mean in each case. Coefficient of variation is 0.999.

and cytochrome P450 specific contents for clofibrate treated male ($P<0.02$ and $P<0.002$) and female ($P<0.05$ and $P<0.05$) rats were significantly increased compared to the corresponding control animals (Table 1). Monooxygenase activities for lauric acid ω and ω-1 hydroxylases for microsomes prepared from clofibrate treated animals (Table 2) were significantly increased compared to corresponding control animals ($P<0.01$ in each case). These results agree with previously published results [1,2,14] and demonstrate that clofibrate administered by the intraperitoneal route is not as effective as a cytochrome P450 monooxygenase inducer as when given in feed.

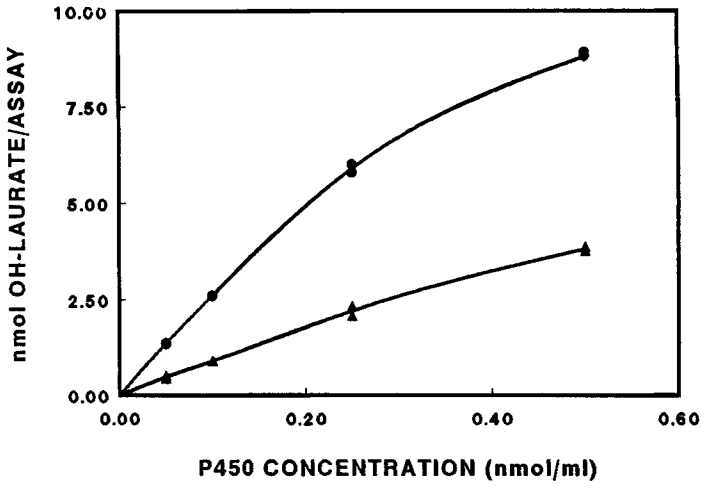

FIGURE 3. Formation of total hydroxylated metabolite from lauric acid using 0.05 to 0.50 μM cytochrome P450. Experimental conditions used are as stated in the text. Duplicate assays for each point are shown. Metabolite formation for microsomes prepared from control and clofibrate treated male Wistar rats were linear up to 0.30 and 0.15 μM cytochrome P450 respectively.

Lauric acid, its hydroxy-metabolites and the internal standard are well separated and resolved with the method described. Chromatograms with low levels of background fluorescence and excellent signal to noise ratios for low metabolite levels were obtained. Quantification of the individual hydroxy-metabolites can be achieved using a recording integrator and the appropriate software. Complete chromatographic separation and quantitation of the lauric acid hydroxy-metabolites requires alternative chromatographic conditions, although run times have to be increased and between run column re-equilibration is needed. With the described method, the following procedural modifications are

TABLE I

EFFECT OF CLOFIBRATE ON LIVER SIZE AND HEPATIC CYTOCHROME P450 SPECIFIC CONTENT IN MALE AND FEMALE WISTAR RATS.
Clofibrate was administered in corn oil for 7 days by I.P. injection at a dose rate of 200 mg/kg/day. Controls received daily I.P. injections of corn oil. Values shown are means ± S.D. (n = 6). Statistical analyses were made with Student's *t*-test.

Animal and treatment	%liver to body weight	P450 specific content nmol/mg protein
male corn oil	3.45 ± 0.24	0.90 ± 0.04
male clofibrate	3.91 ± 0.29[a]	1.05 ± 0.08[b]
female corn oil	3.37 ± 0.29	0.72 ± 0.08
female clofibrate	3.87 ± 0.43[a]	0.83 ± 0.06[a]

a: $P<0.05$ b: $P<0.01$

TABLE 2

EFFECT OF CLOFIBRATE ON HEPATIC MICROSOMAL LAURIC ACID *ω*- AND *ω*-1 HYDROXYLASE ACTIVITIES.
Male and female Wistar rats were administered clofibrate in corn oil for 7 days by I.P. injection at a dose rate of 200 mg/kg/day. Controls received daily I.P. injections of corn oil. Details of analytical procedures are described in the text. Results are expressed as means ± S.D. for triplicate assays of microsomes from 6 animals in each case. Statistical analyses were made with Students *t*-test.

Animal and treatment	Lauric acid hydroxylase activity nmol OH-laurate/min/nmol P450		Total Activity
	11-OH	12-OH	11-OH + 12-OH
male corn oil	2.09 ± 0.13	0.44 ± 0.11	2.53 ± 0.15
male clofibrate	3.40 ± 0.55[c]	4.07 ± 1.38[c]	7.47 ± 1.89[c]
female corn oil	2.47 ± 0.22	0.51 ± 0.09	2.98 ± 0.25
female clofibrate	3.03 ± 0.36[b]	1.24 ± 0.09[c]	4.27 ± 0.43[c]

b: $P<0.01$ c: $P<0.001$

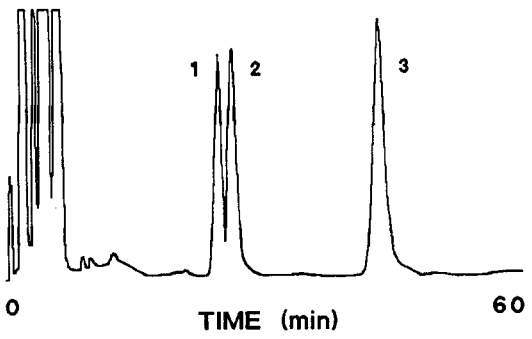

FIGURE 4. Chromatogram obtained for 11- and 12-hydroxy-lauric acids and the internal standard, octanoic acid, using mobile phase consisting of 75% methanol and 25% buffer. Details of analytical procedures are described in the text. Peak identities are : (1) 11-hydroxy-laurate, (2) 12-hydroxy-laurate, and (3) octanoic acid.

required. Octanoic acid is used as an alternative internal standard and is added to the dried residue (from a methanolic stock solution) before the addition of PDAM. The methanol content of the HPLC mobile phase is lowered to 75%. This greatly increases retention times, and after the internal standard has eluted, the column is flushed with HPLC mobile phase (95% methanol) to elute residual laurate, and then equilibrated with the 75% methanol before the next sample is applied to the column. The resultant chromatogram for the 11- and 12-hydroxy-laurate metabolites and octanoic acid is shown in Figure 4.

Although both hydroxy-metabolites of lauric acid may be quantified by this method, it is most suitable for the determination of total hydroxylated metabolite formation due to its simplicity and relatively short chromatographic run times, without the need for radiochemicals, gradient systems, column washing or column equilibration between samples.

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