

A gas chromatography–mass spectrometry method for the measurement of fatty acid ω and ω_{-1} hydroxylation kinetics by CYP4A1 using an artificial membrane system

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Abstract

A gas chromatography–mass spectrometry assay method for the analysis of lauric, myristic, and palmitic acids and their ω and ω_{-1} hydroxylated metabolites from in vitro incubations of cytochrome P450 CYP4A1, involving solid-phase extraction and trimethylsilyl derivatization, was developed. The assay was linear, precise, and accurate over the range 0.5 to 50 μ M for all the analytes. It has the advantages of a more rapid analysis time, an improved sensitivity, and a wider range of analytes compared with other methods. An artificial membrane system was optimized for application to purified CYP4A1 enzyme by investigating the molar ratios of cytochrome b_5 and cytochrome P450 reductase present in the incubation mixture. Using this method, the kinetics of ω and ω_{-1} oxidation of lauric, myristic, and palmitic acids by CYP4A enzymes were measured and compared in rat liver microsomes and an artificial membrane system.

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Cytochromes P450 (CYP)¹ are a large group of related hemthiolate monooxygenase proteins important in both endogenous and xenobiotic metabolism. Understanding the structure–activity relationships of P450s is important for applications such as de novo drug design and identification of possible routes of drug or endogenous metabolism, including any potentially adverse drug interactions.

Cytochrome P450 family 4 is part of an ancient and highly conserved P450 clan [1] with representatives from *Caenorhabditis elegans* to humans. The prototypical member of this family is CYP4A1, found in rats [2].

The primary substrate for CYP4A1 is the 12-carbon fatty acid lauric acid. This enzyme is unusual in that it hydroxylates with high specificity at the energetically unfavorable terminal ω carbon (although some metabolism at the ω_{-1} position can be seen). The restricted metabolism and substrate preference of CYP4A1 makes it suitable as a simple model for studying relationships between P450 structure and metabolism, which are relatively poorly characterized for the CYP4 family.

However, to be able to study structure–activity relationships, a method to identify and quantitate the substrates and their metabolites is required. In our studies we needed to measure simultaneously and rapidly (less than 10 min), lauric (12-carbon fatty acid), myristic (14-carbon fatty acid), and palmitic (16-carbon fatty acid) acids and their ω and ω_{-1} hydroxylated metabolites in a single assay compatible with in vitro metabolism studies carried out using a small-scale (12.5 pmol CYP4A) microsomal or artificial membrane system. The assay method was required to monitor potentially very low

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¹ Abbreviations used: CYP, cytochromes P450; SPE, solid-phase extraction; G6P, D-glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; BSTFA, bis(trimethyl)trifluoroacetamide; TMS, trimethylsilane; CPR, cytochrome P450 reductase; MCP, methylclofenapate.

levels of enzyme activity resulting from CYP4A1 enzymes in which specific residues close to the active site were changed. None of the existing published methods were able to provide the sensitivity or speed of analysis nor the ability to simultaneously measure the required range of analytes.

HPLC has commonly been used for the separation and quantitation of fatty acids and metabolites [3]. Fatty acids have no inherent chromophore or fluorophore [4] and hence can be detected directly only in high concentration using refractive index [5] or light-scattering detectors [6]. Derivatization of the carboxylic acid or hydroxyl group is generally used to improve sensitivity [7] in HPLC analysis. However, these methods generally have very low throughput because analysis times are in the region of 1 h per sample, due to problems separating the ω and $\omega - 1$ hydroxylated metabolites [8]. A liquid chromatography–mass spectrometry method was developed by Adas et al. [9] to study the ω and $\omega - 1$ hydroxylated metabolites of elaidic and oleic acids, but this required different methods for each chain length and required further gas chromatography–mass spectrometry (GC–MS) analysis to confirm the structures of metabolites.

GC–MS approaches to fatty acid analysis, though requiring fairly complex sample extraction and derivatization procedures, have proved suitable for in vitro studies of lauric acid metabolism [10]. To date, no validated GC–MS methods suitable for high-throughput monitoring of lauric, myristic, and palmitic acid oxidation in in vitro enzyme incubations have been reported. Therefore, we report a fully validated method for the analysis of lauric, myristic, and palmitic acids and their ω and $\omega - 1$ hydroxylated metabolites using solid-phase extraction (SPE), trimethylsilylation, and GC–MS analysis. The method is applicable to small-volume microsomal incubations (0.25 ml) and small quantities (12.5 pmol) of purified recombinant P450 enzymes in an artificial membrane in vitro system.

Materials and methods

Chemicals

Sodium cholate, glycerol, and sodium chloride were obtained from Fisher Scientific (UK). Dilauroylphosphatidyl choline, catalase, D-glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase type VII from bakers yeast (G6PDH), β -nicotinamide adenine dinucleotide phosphate (NADP), lauric acid, 10-hydroxy decanoic acid, anhydrous acetonitrile, bis(trimethyl)trifluoroacetamide (BSTFA) with 1% trimethylsilane (TMS), 16-hydroxy palmitic acid, myristic acid, palmitic acid, and 12-hydroxy lauric acid were from Sigma–Aldrich (UK). Potassium dihydrogen phosphate was obtained from

May and Baker (UK). All chemicals were HPLC grade or better. Deionized water purified by reversed osmosis was used where required.

The CYP4A1, cytochrome b_5 , and cytochrome P450 reductase (stored at -80°C) were prepared and purified in our laboratory, and the expression of CYP4A1 will be described elsewhere. Briefly, recombinant CYP4A1 with a C-terminal histidine tag was expressed in *Escherichia coli* JM109, harvested by centrifugation, and bacterial lysed by sonication in 10 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{ml}$ aprotinin, and 10% glycerol. The 100,000g pellet was re-suspended in 0.1 M potassium phosphate buffer, 20% glycerol, pH 7.4, followed by solubilization with 0.75% (w/v) Emulgen 913 and 0.6% (w/v) sodium cholate. The solubilized P450 was desalted into the same buffer using a PD-10 column (Pharmacia) and then subjected to nickel-affinity chromatography in 20% glycerol, 0.1% Emulgen 913, 300 mM NaCl, 50 mM sodium phosphate, pH 8.0, containing 10 mM (bind buffer), 20 mM (wash buffer), or 250 mM (elution buffer) imidazole. The P450 was desalted into 20% glycerol, 20 mM potassium phosphate, pH 7.4 for storage at -80°C . Likewise, recombinant human cytochrome b_5 and rat cytochrome P450 reductase were expressed in *E. coli* and then purified using standard techniques [11]. The microsomes were prepared from liver, as described by Ashley et al. [12], of naïve Wistar rats or rats dosed with 25 mg methylclofenapate MCP per kilogram by gavage. P450 content was assayed spectrophotometrically by complexing with carbon monoxide [13], and protein was measured by the Bradford method [14]. The microsomes were then stored in 200- μL fractions at -80°C and thawed only as required. 11-Hydroxy lauric acid was synthesized by Dr. B. Kellam, School of Pharmacy, University of Nottingham.

All glassware was silanized before use with 5% dichlorodimethylsilane (Sigma–Aldrich) in toluene and rinsed with methanol.

In vitro assay system

The in vitro artificial membrane system was based on that described by Hoch et al. [15] with modifications to optimize the system for application to small volumes and the shortest possible incubation time. The linearity of metabolite production with time was investigated for all the analytes. Cytochrome b_5 concentrations from 0 to 35 pmol per 250- μL incubation and cytochrome P450 reductase concentrations from 0 to 250 pmol per 250- μL incubation were examined.

The optimized procedure was as follows. In amber 2.5-mL Eppendorf vials 12.5 pmol of CYP4A1 or mutant enzyme was incubated with cytochrome b_5 (12.5 pmol), P450 reductase (specific activity of 0.32 μM cytochrome c reduced/min), dilauroylphosphatidylcholine (5 μg), sodium cholate (0.05 mg), and catalase

(2.5 µg). After gentle mixing for 10 min at room temperature the required substrate concentration was added. NADP (125 µg) and G6P (0.5 mg) were then added followed by buffer (50 mM KH₂PO₄, pH 7.4, containing 250 mM NaCl, 0.02% sodium cholate, and 10% glycerol) to give a final volume of 225 µL. After gentle mixing the reaction was incubated in a gently shaking water bath at 37 °C for 2 min.

The reaction was initiated with the addition of G6PDH (25 mU) to give a final assay volume of 250 µL and incubated at 37 °C (palmitic acid was incubated for 30 min). The reaction was terminated after 10 min (30 min for palmitic acid) with 25 µL ice-cold 6 M HCl, the vial contents were mixed, and the vessels were immediately placed on ice. Internal standard (10-OH decanoic acid, 25 nmol, in methanol:water) was then added, the samples were mixed again and transferred for solid-phase extraction.

Microsomal incubations

The microsomal assay was similar to that of the reconstituted system described above with the following modifications: 1 nmol of total P450 was used per incubation and a total incubation volume of 1.0 ml was used. The assay was initiated by the addition of the microsomes and terminated with 100 µL ice-cold 6 M HCl.

Solid-phase extraction methodology

A Cerex SPE nitrogen positive-pressure manifold (Varian, UK) was used with Bond-Elut cartridges C18, 200 mg, 3 mL (Varian, UK). Cartridges were conditioned sequentially with 2 mL methanol, ethyl acetate, and water. The samples were then transferred to the cartridges and slowly drawn through. Cartridges were washed with 2 mL water and thoroughly dried for 1 h with nitrogen at 15 psi. Analytes were eluted using two 1-mL aliquots of ethyl acetate into 10 mL silanized glass screw-top tubes with Teflon-lined caps, and the ethyl acetate was evaporated to dryness under nitrogen.

Samples were derivatized to trimethylsilyl derivatives by reconstitution with 100 µL of 15% BTSFA in anhydrous acetonitrile, at 70 °C overnight. Once derivatized, samples were allowed to cool, vortexed, and transferred to sealed autosampler vials.

GC-MS methodology

Analysis was performed using a Thermoquest GC 8000 coupled to a MD 800 positive electron impact single-quadrupole mass spectrometer. The autosampler was a Thermoquest AS200. This system was interfaced with a PC running VG Masslab version 1.3. A 15-m ZB-5 capillary column (5% phenylmethylpolysiloxane) (Phenomenex, UK), 0.25-mm i.d., 0.1-µm film, was used

with helium as the carrier gas and a head pressure of 80 kPa. A 5-m retention gap of deactivated silica capillary was used in front of the main analytical capillary. Injection (0.5 µL) was splitless with the injector temperature at 250 °C using a silanized injection liner. The GC oven ramp was set at 60 °C for 1 min rising to 180 °C at 30 °C/min, then to 185 °C at 3 °C/min, and finally to 250 °C at 30 °C/min. The MS source and interface were maintained at 250 °C, and a solvent delay of 3.5 min was used to avoid excess contamination of the MS detector. MS parameters were a full scan from 35 to 600 mass units, a scan time of 90 s, and an interscan delay of 0.01 s. The emission was set at 70 eV.

Analytes were identified from a total ion chromatogram by investigating their relevant M⁺-15 fragment ion and fragmentation pattern (Table 1). This was not possible for the 14-OH myristic acid or potential ω₋₁ metabolites of myristic and palmitic acids, as no standards were available. In these cases identification was confirmed by monitoring the expected fragmentation ions with elution times being confirmed by comparison with the available standards of hydroxylated fatty acids.

Peaks were integrated and quantified using a ratio of the M⁺-15 analyte peak area to that of the internal standard. For the metabolites where no standards were available a semiquantitative method was used to produce metabolism data. For 14-OH myristic acid an average of the calibration curves obtained for 12-OH lauric and 16-OH palmitic acid (within the same run) was taken, and for 13-OH myristic and 15-OH palmitic acid metabolites an average ratio of the 11-OH to 12-OH lauric acid calibration curves was used.

Assay validation methods

Duplicate six-point calibration curves at concentrations of 0.5, 2, 5, 10, 30, and 50 µM were prepared in a basic reconstitution system for each analyte: lauric, myristic, and palmitic acids, 11-OH and 12-OH lauric acids; and 16-OH palmitic acid. The analyte/internal standard peak area ratios were calculated and used to

Table 1
Selected *m/z* ions used to identify and quantify the analytes by GC-MS and retention times of each analyte

Analyte	Characteristic ion <i>m/z</i> M ⁺ -15	Retention time (min)
Decanoic acid (ISTD)	317/318	4.9
Lauric acid	257	4.2
11-OH Lauric acid	345	5.6
12-OH Lauric acid	345	6.1
Myristic acid	285	5.1
13-OH Myristic acid	373/374	6.8
14-OH Myristic acid	373/374	7.4
Palmitic acid	313	6.4
15-OH Palmitic acid	401/402	8.2
16-OH Palmitic acid	401/402	8.5

construct calibration lines of peak area ratio against analyte concentration using unweighted linear regression analysis. The slope, intercept, and regression coefficient of the calibration lines were determined. Analyte recovery was determined by comparing extracted standards with nonextracted standards at 0.5 μM ($n = 6$) and 50 μM ($n = 5$), respectively. With each run, suitable positive and negative controls and reagent blanks were also prepared. Samples were prepared at fixed concentrations (Table 3) to determine the intraday (6 replicate analyses on a single day) and interday (18 replicate analyses over a period of 5 days) precision and accuracy. The lower limit of quantitation for each analyte was determined from the linearity tests as the lowest concentration of the analyte to give a RSD of 20% or less for intraday precision and accuracy.

Enzyme kinetics

Data were analyzed and fitted to a single-substrate Michaelis–Menten curve using the statistical package Prism (version 3). To check for deviations from this model R^2 values of the fit were examined. A secondary Hanes–Woolfe plot was also used and deviations from linearity were examined.

Results and discussion

We have developed a simple generic method that is able to examine the kinetics of ω and ω_{-1} hydroxylation by cytochrome P450 of a series of fatty acids, lauric, myristic, and palmitic acids, in microsomes and an

artificial membrane system. The reconstitution system is particularly applicable to recombinant CYP4A enzymes, of which we have investigated CYP4A1.

Optimization of assay procedures

The analysis time was minimized by using a GC capillary length of 15 m. A “hinge point” in the temperature gradient was also introduced to maximize separation of the analytes, with a rapid temperature ramp either side to elute the more and less volatile compounds quickly. We also found that peak shape and sensitivity could be improved by using solvent focusing to further concentrate the sample. The total run time with all the analytes being baseline separated was 9.5 min (Fig. 1).

Common to all analytes was a dominating signal at m/z 73 due to the cleaved TMS moiety $[(\text{CH}_3)_3\text{Si}]^+$ and for hydroxylated metabolites m/z 75 $[(\text{CH}_3)_2\text{Si-OH}]^+$ [16]. For all the ω_{-1} hydroxylated metabolites the primary ion seen was at m/z 117, due to an α -cleavage of the O-trimethylsilyl group $[\text{CH}_3\text{CH}=\text{O}-\text{SiMe}_3]^+$, and this was diagnostic for ω_{-1} -hydroxylated n -alkanoic derivatives. Although the m/z 117 ion was seen for all the fatty acids and metabolites, where it is due to a loss of the terminal two carbons and the silyl group with a subsequent McLafferty rearrangement and loss of a methyl group $[\text{CH}_2=\text{C}(\text{OH})-\text{OSiMe}_2]^+$, it was significantly less abundant than the α -cleavage ion [9,10,16]. With the ω hydroxylated metabolites strong ions were seen at M^+-31 due to the loss of a methoxy group and M^+-90 and M^+-15 . Rearrangements of ions common to ω hydroxylated metabolites were also seen at m/z 147

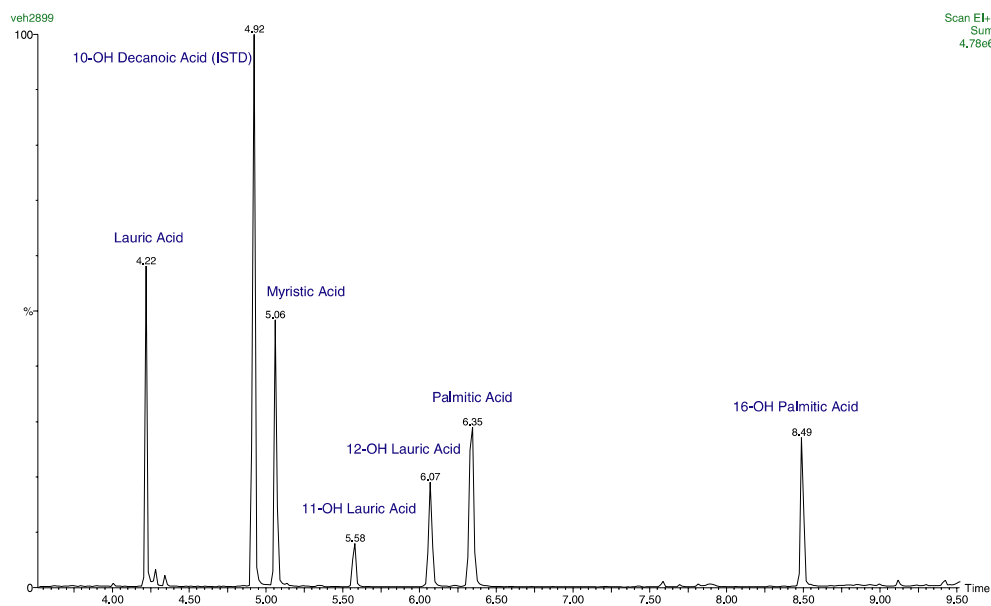


Fig. 1. GC–MS chromatogram of a standard mixture of fatty acids and hydroxylated metabolites (30 μM), showing the retention times of all the available standards and internal standard 10-OH decanoic acid. Peaks shown correspond to a selected ion chromatogram of the m/z M^+-15 ions for each analyte.

$[\text{Me}_3\text{Si-O-SiMe}_2]^+$ and m/z 103 $[\text{CH}_2\text{-O-Si-Me}_3]^+$ [9,10,16].

The MS response to the ω and ω_{-1} metabolite selected ions was quite different, with an average ratio of response for the 11-OH lauric acid/12-OH lauric acid of 0.35. This perhaps relates to the large m/z 117 ion shown by the ω_{-1} metabolites, which reduced the amount of the M^+-15 ion. In previous reports [e.g., 10] ω_{-1} metabolites were quantified from calibration response obtained from the ω metabolite MS response and this approach will have led to underestimation of the production of the ω_{-1} metabolites. This illustrates the importance of using authentic standards of fatty acid metabolites where possible.

Extraction methods

The extraction methodology was optimized to minimize the processing time while maximizing the recovery. The drying time needed for the samples at room temperature on the SPE tubes was found to be optimum

at 1 h to ensure complete removal of water, which was essential prior to sample derivatization. Chaurasia et al. [10] reported loss of 40–90% of the lauric acid during evaporation when removing the ethyl acetate in an evacuated centrifuge at ambient temperature. However, the SPE method developed here did not suffer from this problem and measured recoveries at the SPE stage were greater than 90% for all fatty acids and metabolites. Use of nitrogen as a drying gas under positive pressure eliminated unwanted oxidation of the fatty acids; oxidation was observed when using air in a vacuum-based SPE system (data not shown).

Validation

Table 2 shows the variation in the fitted calibration lines over five separate analyses for all the analytes and confirms the linearity of the assay over the range of concentrations of 0.5–50 μM . The fatty acids and their metabolites showed a reproducible recovery (Table 3) over the assay range, comparable to that

Table 2
Variation of calibration curves

Analyte	$R^2 (\geq)(n = 5)$	Slope (ratio to ISTD \pm SD) ($n = 5$)	Y intercept (ratio to ISTD \pm SD) ($n = 5$)
Lauric acid	0.967	0.0119 \pm 0.0021	0.0214 \pm 0.011
11-OH Lauric acid	0.986	0.00257 \pm 0.00013	-0.00133 \pm 0.00033
12-OH Lauric acid	0.990	0.00843 \pm 0.00042	-0.00458 \pm 0.0017
Myristic acid	0.993	0.0109 \pm 0.0066	0.0037 \pm 0.0012
Palmitic acid	0.986	0.0139 \pm 0.0019	0.0257 \pm 0.0033
16-OH Palmitic acid	0.970	0.0101 \pm 0.00095	-0.00758 \pm 0.0046

Each calibration curve is a six-point duplicate curve prepared and analyzed on separate days.

Table 3
Recovery and intra- and interday assay precision and accuracy of the GC-MS method for the fatty acids and hydroxylated metabolites in an artificial membrane reconstitution system

Analyte	Concentration (μM)	Recovery (% \pm SD)	Intraday		Interday	
			Precision (RSD%)	Accuracy (RSD%)	Precision (RSD%)	Accuracy (RSD%)
Lauric acid	0.5	172 \pm 49	12	n.d.	19	n.d.
	10	n.d.	18	135	21	119
	50	61.2 \pm 5.9	7.7	90.4	9.3	92.7
11-OH Lauric acid	0.5	86.3 \pm 15	24	n.d.	24	n.d.
	10	n.d.	6.5	110	13	102
	50	64.1 \pm 11	13	104	12	102
12-OH Lauric acid	0.5	90.2 \pm 15	6.7	n.d.	18	n.d.
	10	n.d.	6.9	107	15	96.5
	50	76.0 \pm 7.8	16	98.9	13	96.9
Myristic acid	0.5	103 \pm 41	13	n.d.	24	n.d.
	10	n.d.	9.8	170	11	161
	50	64.9 \pm 3.9	13	117	15	120
Palmitic acid	0.5	184 \pm 61	16	n.d.	38	n.d.
	10	n.d.	8.9	137	16	123
	50	61.1 \pm 4.2	17	94.8	14	97.5
16-OH Palmitic acid	0.5	72.1 \pm 16	13	n.d.	19	n.d.
	10	n.d.	8.1	115	24	102
	50	58.1 \pm 8.4	13	108	15	102

seen by Chaurasia et al. [10]. For lauric acid and palmitic acid an apparent recovery of over 100% was measured at the lowest assay concentration (0.5 μM) which may have been due to uncorrected losses of standards at these concentrations. The lower limit of quantitation for all analytes was determined as 0.5 μM (0.625 pmol on-column) in a standard sample volume and this was confirmed by a direct measurement of a signal to noise ratio of greater than 3 to 1 for the extracts.

In addition, Table 3 summarizes the precision and accuracy of the assay method for each analyte. Interfering peaks in extracts of blank microsomal and artificial membrane matrix gave responses of at least one order of magnitude below that of the lowest standard when monitored on each individual analyte selected ion.

Optimization of the microsomal and reconstitution systems

With the artificial membrane reconstitution system small differences in the concentrations of the components can vastly alter the turnover and metabolite ratios. It is known that the ratio of cytochrome b_5 and cytochrome P450 reductase (CPR) to P450 can affect the turnover of the enzyme [25–29]. We investigated different concentrations of cytochrome b_5 and CPR in the assay to see what effect it had on lauric and myristic acid metabolism (Figs. 2 and 3). A small alteration in the concentration of cytochrome b_5 had a major effect on 12-OH lauric acid production but had little effect on 11-OH lauric acid production (Fig. 2). The optimum value selected was therefore 12.5 pmol/250- μL incubation,

which was in the center of a range of concentrations of cytochrome b_5 , which was less sensitive to small changes in conditions.

The effect of CPR concentration on the production of 11-OH and 12-OH metabolites is shown in Fig. 3. The final concentration of reductase to be used in the incubations was therefore decided to be reductase with a specific activity of 0.32 μM cytochrome c reduced/min, which equates to 59.7 pmol when using a molecular weight of 76.5 kDa. This gives a rate of turnover equivalent to 125 pmol/250- μL incubation as used by Hoch et al. [15], while using approximately 50% less enzyme. This is a 4.8:1 ratio of reductase to P450.

It was necessary to determine the maximum initial period of linearity for metabolite production by both the purified enzyme and the microsomes. With microsomes a 30-min incubation time was investigated, this showed a linear portion over 10 min for both 11- and 12-OH lauric acid (data not shown). With recombinant CYP4A1 and the reconstituted system a 10-min incubation was retained as for the microsomes, although linearity was maintained up to 30 min (Fig. 4). In both cases a concentration of 100 μM lauric acid was used. A 10-min linear incubation time was also reported by Chaurasia et al. [10] using 100 μM lauric acid. Linearity of metabolite production up to 30 min was observed for palmitic acid and myristic acid.

Application of the method to lauric acid hydroxylation in rat hepatic microsomes

The methods were used to investigate lauric acid turnover in naïve and MCP-induced rat hepatic

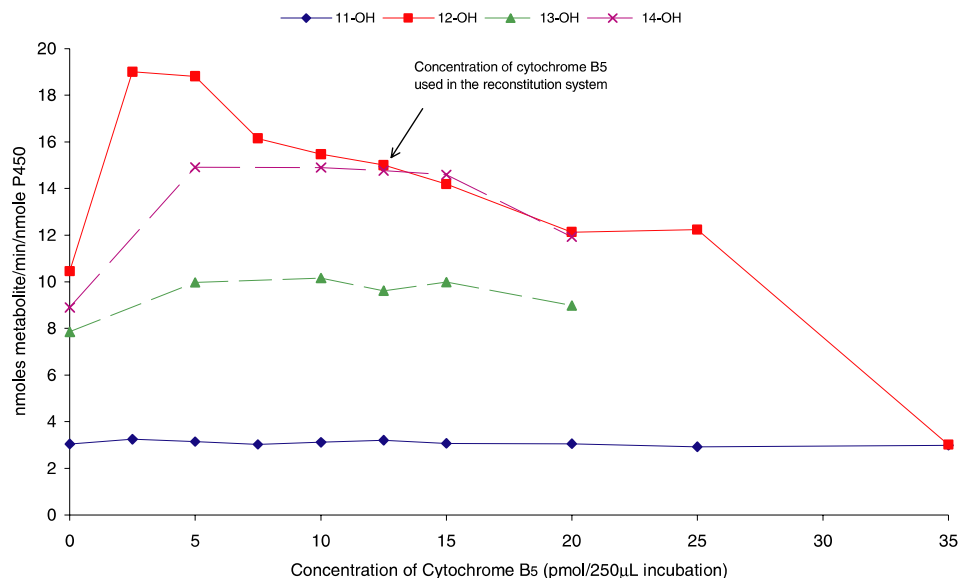


Fig. 2. Effects on the turnover of lauric and myristic acids with different cytochrome B_5 concentrations as described under Materials and methods. An initial concentration of 50 μM lauric or myristic acid was used in the incubation. The standard concentration of b_5 used per assay is 12.5 pmol [1]. 11-OH and 12-OH correspond to 11- and 12-OH lauric acid, respectively, and 13-OH and 14-OH correspond to 13- and 14-OH myristic acid, respectively (13-OH and 14-OH myristic data are semiquantitative). Each point is a single determination.

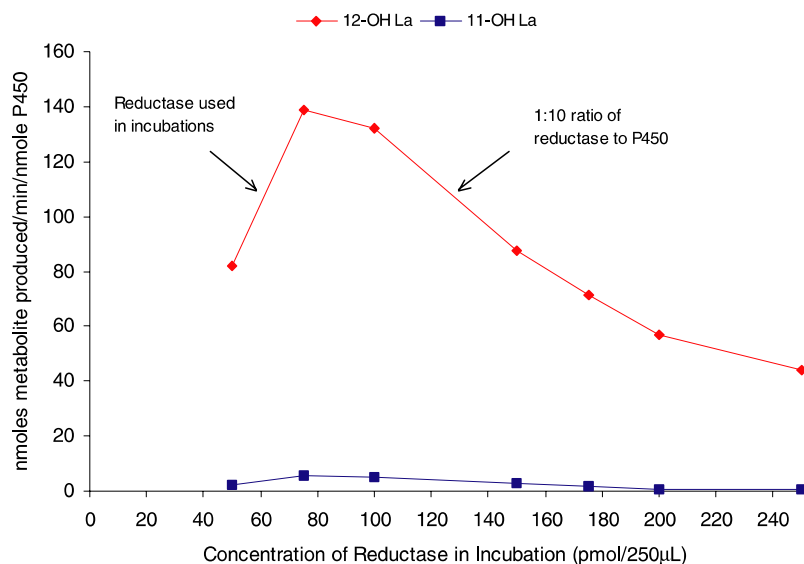


Fig. 3. Effect of reductase concentration in the reconstitution system on the turnover of lauric acid by CYP4A1 as described under Materials and methods. Incubations were performed with an initial lauric acid concentration of 100 μ M. Data are pre-11-OH lauric acid synthesis; therefore 11-OH lauric acid data are semiquantitative based on a calculated ratio to the 12-OH curve of subsequent calibrations. The concentration of reductase used by Hoch et al. [15] was equivalent to 125 pmol per incubation. Each point is a single determination.

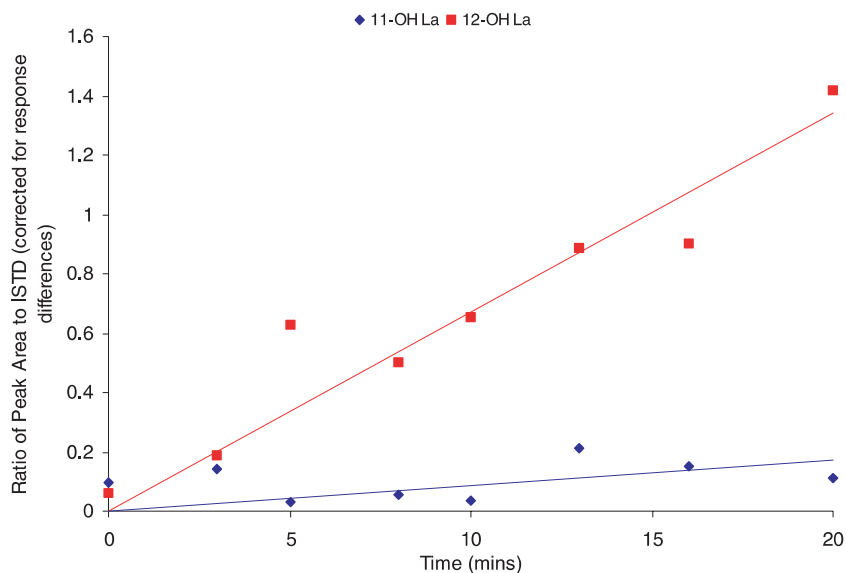


Fig. 4. Linearity of ω and ω_{-1} hydroxylated lauric acid production over time, metabolism by CYP4A1 in the reconstitution system. The standard reconstitution system was used as described under Materials and methods using a lauric acid concentration of 100 μ M. Data were not quantified, but the differences in response of the ω and ω_{-1} metabolites were corrected for. Each point is a single determination.

microsomes. For untreated microsomes (Table 4) K_m^{app} and $V_{\text{max}}^{\text{app}}$ values for 12-OH and 11-OH production were similar to those reported by Chaurasia et al. [10] of K_m^{app} 12.9 μ M and $V_{\text{max}}^{\text{app}}$ 0.7 nmol/min/nmol P450 and K_m^{app} 33.9 μ M and $V_{\text{max}}^{\text{app}}$ 0.5 nmol/min/nmol P450, respectively.

We observed greater 11-OH (ω_{-1}) lauric acid production than 12-OH (ω) lauric acid production in the naïve microsomes. In naïve microsomes there are many CYP isoenzymes producing 11-OH lauric acid, primarily CYP2E1 [17–19] although others are also involved, such

as CYP1A1 [21], CYP2B1 [20], and CYP2C2 [19]. These form a large percentage of the total P450 content compared with CYP4A enzymes which represent only 1–4% of the total P450 content [22]. In the case of the CYP4A-induced microsomes, a much larger turnover of lauric acid to 12-OH lauric would be expected, as CYP4A proteins increase \sim 20-fold in the livers of MCP-treated rats [23]. This was reflected in our results, where we observed the $V_{\text{max}}^{\text{app}}$ for 12-OH lauric acid rise to 47.3 nmol/min/nmol P450, an almost 25-fold increase, compared

Table 4

Kinetic data for microsomes and purified CYP4A1 as determined by curve fitting (Prism) to a Michaelis–Menten single substrate model

Enzyme system	Metabolite				V_{\max} ratio 12/11-OH
	11-OH Lauric acid		12-OH Lauric acid		
	K_m^a (μM)	V_{\max}^a (nmol metabolite/min/nmol P450)	K_m^a (μM)	V_{\max}^a (nmol metabolite/min/nmol P450)	
Naïve microsomes	147.5 \pm 43.0	3.8 \pm 0.7	20.9 \pm 6.5	2.0 \pm 0.8	0.53
Induced microsomes	288.4	12.6 \pm 2.3	229.0	47.3 \pm 15.9	3.8
CYP4A1	1.5 \pm 0.1	2.2 \pm 0.3	7.1 \pm 1.1	30.0 \pm 4.3	13

The ratio is of the V_{\max} values. For microsomal systems $n = 3$, except for the induced microsomes K_m where the data from the most well defined kinetic curve were used ($n = 3$ values were K_m 11-OH 490.4 \pm 558.7 and 12-OH 198.7 \pm 154). Different batches of microsomes were used for kinetic determination. For CYP4A1 12-OH lauric acid $n = 6$ and 11-OH lauric acid $n = 3$.

^a Kinetic values are apparent for microsomal systems.

with the V_{\max}^{app} for 11-OH lauric acid which rose only to 12.6 nmol/min/nmol P450, approximately a 4-fold increase. This is similarly reflected in the increase of the 12/11-OH metabolite ratio. Chaurasia et al. [10] showed lower values of V_{\max}^{app} for 12-OH and 11-OH lauric acid, 9.9 and 4.1 nmol/min/nmol P450, respectively, in clofibrate-induced rat microsomes but this may be explained by a weaker induction of the CYP4A1 in their rat strain. This is not unexpected, as Chaurasia et al. [10] used clofibrate as an inducing agent, which is known to be markedly less potent than MCP (vide supra) Ashby et al. [24].

The \sim 25-fold induction of lauric acid ω hydroxylation is consistent with our previous studies, showing that the CYP4A1 RNA is induced \sim 20-fold over control levels [23].

Application of the method to lauric acid hydroxylation by purified CYP4A1 in an artificial membrane system

Taking into account the limit of fatty acid concentrations that could be used because of nonlinearity in the artificial membrane system, the ω and ω_{-1} hydroxylation kinetics of all the fatty acids fitted well to a Michaelis–Menten equation and to a Hanes–Woolfe transformation. Typical data are shown for lauric acid hydroxylation kinetics in (Fig. 5), in which it can be seen that the 11-OH and 12-OH lauric acid kinetic profiles are well defined. The Hanes–Woolfe plot of lauric acid hydroxylation (Fig. 6) shows good linearity, with an $R^2 = 0.994$ for 12-OH lauric acid and $R^2 = 0.998$ for 11-OH lauric acid, supporting the use of a simple single-binding-site Michaelis–Menten equation. The V_{\max}^{app} value observed for 12-OH lauric acid production in the recombinant CYP4A1 system was similar to that of the MCP-induced microsomes, giving evidence that our reconstitution system provides a good representation of ω hydroxylation.

Chaurasia et al. [10] were not able to quantify the production of 11-OH lauric acid by purified CYP4A1 under their standard assay conditions, but with the improved sensitivity of our method, we were able to detect and quantify kinetics for its production (Tables 4 and 5).

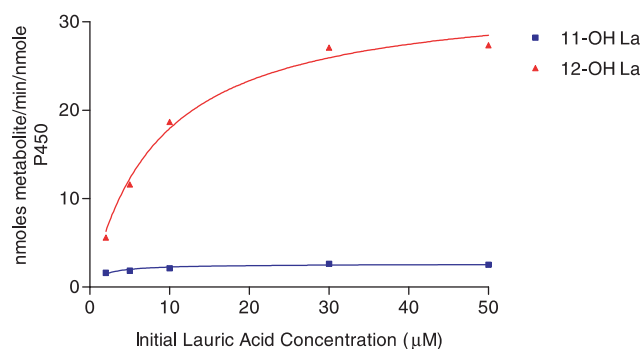


Fig. 5. Example of the metabolism of lauric acid by CYP4A1 in the reconstitution system fitted to a Michaelis–Menten single substrate model. $n = 3$ for 11-OH lauric acid; $n = 6$ for 12-OH lauric acid.

Our results for CYP4A1 metabolism of lauric acid to 12-OH lauric acid (Fig. 7) were very similar to those of Chaurasia et al [10]; K_m of 8.9 μM and V_{\max} of 34.0 nmol metabolite/min/nmol P450. We were also able to detect the hydroxylation of myristic acid and palmitic acid, although the level of turnover was too low to accurately determine K_m data for palmitic acid. These showed the typical result that ω metabolism decreased with increasing chain length. However, they also showed an increase in ω_{-1} production, suggesting that regioselectivity of the CYP4A1 decreases as the alkyl chain length of the fatty acid increases.

Ratios of ω/ω_{-1} production in rat microsomes (Table 3) and by purified CYP4A1 (Table 4) were calculated based on the measured kinetics. Our CYP4A1 was purified after recombinant expression in *E. coli*, and the value of 14:1 cannot be influenced by any other contaminating rat cytochromes P450 and is based on a fully validated assay method using authentic standards of the 11-OH and 12-OH lauric acid metabolites. Initial characterization of CYP4A1 by Tamburini et al. [25] reported a ratio of 6:1 for lauric acid metabolism. Others have reported a range from 17:1, from a rat liver preparation [20], to 11:1 for microsomes from HepG2 cells infected with vaccinia virus containing the gene for

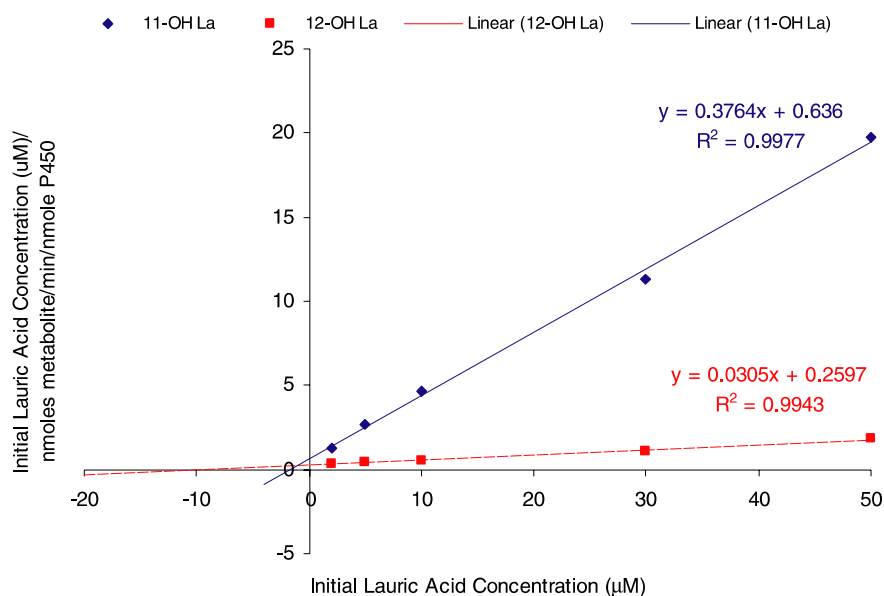


Fig. 6. Secondary Hanes–Woolfe Plot of the turnover of lauric acid by CYP4A1 in the reconstitution system, illustrating that there is a linear relationship between the points and therefore the data fit well to a single-substrate Michaelis–Menten plot. $n = 3$ for 11-OH lauric acid, $n = 6$ for 12-OH lauric acid.

Table 5

Summary of the kinetic data for lauric, myristic, and palmitic acids turnover by CYP4A1 in the reconstitution system

Substrate	Metabolite				V_{\max} ratio ω/ω_{-1}
	ω_{-1}		ω		
	K_m (μM)	V_{\max} (nmol metabolite/ min/nmol P450)	K_m (μM)	V_{\max} (nmol metabolite/ min/nmol P450)	
Lauric acid	1.5 ± 0.1	2.2 ± 0.3	7.1 ± 1.1	30.0 ± 4.3	14
Myristic acid	16.4 ± 7.7	10.0 ± 3.4	11.0 ± 1.7	24.1 ± 8.7	2.4
Palmitic acid	NQ	NQ	NQ	9.1 ± 7.8	NA

NQ, Not quantifiable, NA, not applicable. For 12-OH lauric acid $n = 6$ and 11-OH $n = 3$; 14-OH myristic acid $n = 2$ and 13-OH $n = 3$; palmitic acid $n = 2$ for 16-OH Pa.

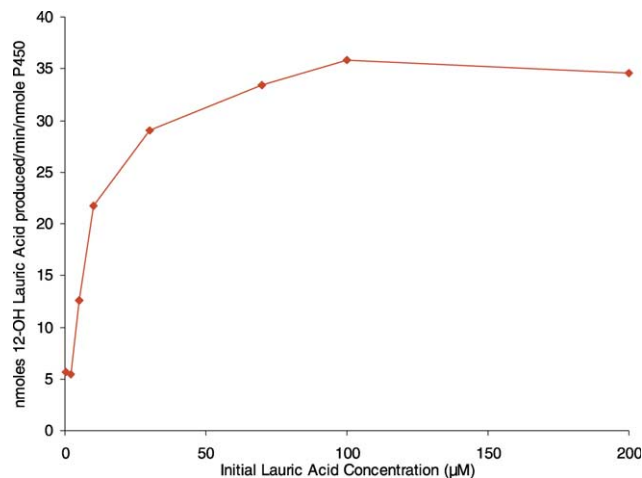


Fig. 7. Effect of increasing the initial lauric acid concentration on the metabolism by CYP4A1 to 12-OH lauric acid. Standard reconstitution assay system conditions were used as described under Materials and methods. Each point is the mean of six determinations.

CYP4A1 [26], and no 11-OH lauric acid at all from a purified enzyme from rat liver treated with diethylhexyl phalate [27]. Dierks et al. [28] reported a 20:1 ratio for lauric acid ω/ω_{-1} metabolite production by recombinant CYP4A1, although their K_m and V_{\max} values are much larger than ours. Hoch et al. [29] reported a ratio of 40:1, much higher than previously reported values but they also reported a much higher turnover value. However, Hoch et al. [29] also reported ω/ω_{-1} ratios for myristic acid turnover of 3:1 and palmitic acid of 1:1 and these are in good agreement with our ratios of 2.4 and 0.85, respectively. The slight differences in ratio could be due to differences in CPR and cytochrome b_5 molar ratios to CYP4A1, which we have shown could have a considerable effect on the metabolite ratios shown. Only the data obtained from recombinantly produced CYP4A1 is likely to be free of contaminating cytochromes P450 which could produce either 11-OH or 12-OH lauric acid. In addition, it is probable that the

11-OH lauric acid has not been measured accurately in some published data because inaccurate concentration may have been obtained due to an authentic standard not being used. Overall, we believe that our assay system enables the accurate measurement of ω and ω_{-1} metabolism of fatty acids in both microsomes and a reconstituted membrane system leading to artifact-free data on enzyme kinetics.

We have developed a validated, sensitive, and specific assay for the analysis of the C12, 14, and 16 fatty acids and their ω and ω_{-1} metabolites which is an improvement over previously published methods. This assay method has subsequently been successfully applied to the study of the effect of a range of site-specific mutations of purified, recombinant CYP4A1 on the kinetics of ω and ω_{-1} metabolism of fatty acids in a reconstituted membrane.

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References

- [1] D.R. Nelson, Cytochrome P450 and the individuality of species, *Arch. Biochem. Biophys.* 369 (1999) 1–10.
- [2] G.G. Gibson, T.C. Orton, P.P. Tamburini, Cytochrome P450 induction by clofibrate. Purification and properties of a hepatic P-450 relatively specific for 12 and 11-hydroxylation of dodecanoic acid (lauric acid), *Biochem. J.* 203 (1982) 161–168.
- [3] E.S. Lima, D.S.P. Abdalla, Review: high-performance liquid chromatography of fatty acids in biological samples, *Anal. Chim. Acta* 465 (2002) 81–91.
- [4] M. Hein, H.D. Isengard, Determination of underivatized fatty acids by HPLC, *Food Res. Technol.* 204 (1997) 420–424.
- [5] L. Svensson, U. Hansson, S. Gronovitz, T. Kingstedt, The relationship between the structure of monoalkyl branched saturated triacylglycerols and some physical properties, *Lipids* 32 (1997) 661–666.
- [6] B. Rehbock, D. Gansser, R.C. Berger, Analysis of oxylipins by high-performance liquid chromatography with evaporative light-scattering detection and particle beam mass spectrometry, *Lipids* 32 (1997) 1003–1010.
- [7] J.M. Rosenfeld, Review: application of analytical derivatisations to the quantitative and qualitative determination of fatty acids, *Anal. Chim. Acta* 465 (2002) 93–100.
- [8] R.T. Okita, J.E. Clark, J.R. Okita, B.S. Masters, ω - and $(\omega - 1)$ -hydroxylation of eicosanoids and fatty acids by high-performance liquid chromatography, *Methods Enzymol.* 206 (1991) 432–441.
- [9] F. Adas, D. Picart, F. Berthou, B. Simon, Y. Amet, Liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry of ω - and $(\omega - 1)$ -hydroxylated metabolites of elaidic and oleic acids in human and rat liver microsomes, *J. Chromatogr. B* 714 (1998) 133–144.
- [10] C.S. Chaurasia, M.A. Alterman, P. Lu, R.P. Hanzlik, Biochemical characterisation of lauric acid ω -hydroxylation by a CYP4A1/NAPDH-cytochrome P450 reductase fusion protein, *Arch. Biochem. Biophys.* 317 (1) (1995) 161–169.
- [11] Fan Ming Qi, Ph.D. thesis, University of Nottingham, 2002.
- [12] C.M. Ashley, M.G. Simpson, D.M. Holdich, D.R. Bell, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is a potent toxin and induces cytochrome P450 in the crayfish *Pacifastacus leniusculus*, *Aquatic Toxicol.* 35 (1996) 157–169.
- [13] G.G. Gibson, P. Skett, *Introduction to Drug Metabolism*, second ed. (1994) reprinted (1999), Stanley Thorne Ltd. pp. 36–49.
- [14] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding, *Anal. Biochem.* 72 (1976) 248–254.
- [15] U. Hoch, J.R. Falck, P.R. Ortiz de Montellano, Molecular basis for the ω -regiospecificity of the CYP4A2 and CYP4A3 fatty acid hydroxylases, *J. Biol. Chem.* 275 (35) (2000) 26952–26958.
- [16] R.A. Clare, S. Huang, M.V. Doig, G.G. Gibson, Gas-chromatographic mass-spectrometric characterization of some novel hydroxyeicosatetraenoic acids formed on incubation of arachidonic-acid with microsomes from induced rat livers, *J. Chromatogr.* 562 (1991) 237–247.
- [17] Y. Imai, T. Uno, M. Nakamura, Characterization of rabbit liver p450IIE1 synthesized in transformed yeast-cells, *J. Biochem.* 108 (1990) 522–524.
- [18] T. Fukuda, Y. Imai, M. Komori, M. Nakamura, E. Kusunose, K. Satouchi, M. Kusunose, Replacement of the Thr-303 of P450 2E1 with serine modifies the regioselectivity of its fatty acid hydroxylase activity, *J. Biochem.* 113 (1993) 7–12.
- [19] T. Fukuda, Y. Imai, M. Komori, M. Nakamura, E. Kusunose, K. Satouchi, M. Kusunose, Different mechanisms of regioselection of fatty-acid hydroxylation by laurate (ω -1)-hydroxylating P450S, P450 2C2 and P450 2E1, *J. Biochem.* 115 (1994) 338–344.
- [20] C.A. CaJacob, W.K. Chan, E. Shephard, P.R. Ortiz de Montellano, The catalytic site of rat hepatic lauric acid ω -hydroxylase. Protein versus prosthetic heme alkylation in the ω -hydroxylation of acetylenic fatty acids, *J. Biol. Chem.* 263 (1988) 18640–18649.
- [21] J.P. Hardwick, B.-J. Song, E. Huberman, F.J. Gonzalez, Isolation, complementary DNA sequence, and regulation of rat hepatic lauric. Identification of a new cytochrome P-450 gene family, *J. Biol. Chem.* 262 (2) (1987) 801–810.
- [22] A.E.C.M. Simpson, Review: the cytochrome P450 4 (CYP4) family, *Gen. Pharmacol.* 28 (1997) 351–359.
- [23] D.R. Bell, R.G. Bars, G.G. Gibson, C.R. Elcombe, Localisation and differential induction of cytochrome P450IVA1 and acyl-CoA oxidase in rat liver, *Biochem. J.* 275 (1991) 247–252.
- [24] J. Ashby, A. Brady, C.R. Elcombe, B.M. Elliott, J. Ishmael, J. Odum, J.D. Tugwood, S. Kettle, I.F.H. Purchase, Mechanistically-Based Human Hazard Assessment of Peroxisome Proliferator-Induced Hepatocarcinogenesis, *Human Exp. Toxicol.* 13 (1994) S1–S117.
- [25] P.P. Tamburini, H.A. Masson, S.K. Bains, R.J. Makowski, G.G. Gibson, Multiple forms of hepatic cytochrome P450. Purification, characterisation and comparison of a novel clofibrate-induced isozyme with other major isoforms of cytochrome P-450, *Eur. J. Biochem.* 139 (1984) 247–252.
- [26] T. Aoyama, J.P. Hardwick, S. Imaoka, Y. Funae, H.V. Gelboin, F.J. Gonzalez, Clofibrate-inducible rat hepatic P450s IVA1 and IVA3 catalyse the ω and $(\omega - 1)$ -hydroxylation of fatty acids and the ω -hydroxylation of prostaglandins E_1 and $F_{2\alpha}$, *J. Lipid Res.* 31 (1990) 1477–1482.
- [27] R.T. Okita, J.R. Okita, Characterisation of a cytochrome P450 from di (2-ethylhexyl) phthalate-treated rats which hydroxylates fatty acids, *Arch. Biochem. Biophys.* 294 (1992) 475–481.
- [28] E.A. Dierks, S.C. Davis, P.R. Ortiz de Montellano, Glu-320 and Asp-323 are determinants of the CYP4A1 hydroxylation regio-specificity and resistance to inactivation by 1-aminobenzotriazole, *Biochemistry* 37 (1998) 1839–1847.
- [29] U. Hoch, Z. Zhang, D.L. Kroetz, P.R. Ortiz de Montellano, Structural determination of the substrate specificities and regioselectivities of the rat and human fatty acid ω -hydroxylases, *Arch. Biochem. Biophys.* 373 (1) (2000) 63–71.