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Liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry of ω- and (ω-1)-hydroxylated metabolites of elaidic and oleic acids in human and rat liver microsomes

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Abstract

In order to characterize the nature of the active site of cytochrome P450 2E1, the metabolism of various fatty acids with *cis*/*trans* geometric configurations has been investigated. A system coupling atmospheric pressure chemical ionization-mass spectrometry detection with HPLC separation was developed as an alternative method for the characterization of hydroxylated metabolites of oleic and elaidic acids in rat and human liver microsomes. Oxidation of oleic and elaidic acids led to the formation of two main metabolites which were identified by LC–MS and GC–MS as ω and (ω-1)-hydroxylated (or 17-OH and 18-OH) fatty acids, on the basis of their pseudo-molecular mass and their fragmentation. The assay was accurate and reproducible, with a detection limit of 25 ng per injection, a linear range from 25 to 1128 ng per injection, no recorded interference, intra-day and inter-day precision with variation coefficients <14%. This LC–MS method was validated with oleic acid by using both radiometric and mass spectrometric detections. A significant correlation was found between the two methods in human ($r=0.86$ and 0.94 with $P<0.05$ and 0.01) and rat liver microsomes ($r=0.90$ and 0.85 with $P<0.01$ and 0.05) for 17-OH and 18-OH metabolites, respectively. HPLC coupled to mass spectrometry for the analysis of hydroxylated metabolites of elaidic acid offers considerable advantages since the method does not require use of a radioactive molecule, completely separates the two hydroxymetabolites, confirms the identification of each metabolite, and is as sensitive as the radiometric analysis method. This method allowed the comparative study of oleic and elaidic acid hydroxylations by both human and rat liver microsomal preparations.

Keywords: Elaidic acid; Oleic acids; Fatty acid

1. Introduction

Cytochromes P450 [1] are a superfamily of monooxygenases that catalyze the oxidation of various lipophilic substrates, including hormones, fatty acids, therapeutic agents and environmental chemicals. The ethanol-inducible isoform P450 2E1 metabolizes and bioactivates a large number of low-molecular weight compounds, such as drugs and carcinogens [2]. It has been described as being involved in the (ω-1)-hydroxylation of fatty acids such as lauric [3–5] and oleic [6] acids in rat and human liver microsomal enzymatic preparations. In order to better understand the mechanism of this minor metabolic pathway and

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to characterize the geometry of the substrate access channel of the active site of P450 2E1 [7], the metabolism of various saturated and unsaturated fatty acids with *cis*/*trans* configurations has been investigated. One of these, elaidic acid-(*E*)-9-octadecenoic acid-(*trans* C18:1 Δ9), was assayed in rat and human liver microsomes, and its microsomal hydroxylation was compared to that of a *cis* (or *Z*) fatty acid such as oleic acid-(*Z*)-9-octadecenoic acid

Numerous reversed-phase HPLC methods have been developed for the separation of the ω and (ω-1)-hydroxylated metabolites of fatty acids [8–10]. Saturated fatty acids cannot be easily detected by UV, fluorescence or electrochemistry, as they barely absorb UV radiation [11]. On the other hand, fatty acids containing one or more double bonds are detectable by UV, but they have varying responses according to the number of double bonds, since they absorb light only within a small wavelength range [11]. Non-esterified fatty acids can be detected using refractive index [11–13] or mass scattering [14,15] detectors. Linear saturated and unsaturated free fatty acids can also be separated by capillary zone electrophoresis with indirect UV detection [16]. Another possibility is the derivatization of the carboxylic group with fluorescent probes prior to HPLC analysis [10,17]. However, some derivatization reactions can lead to the formation of interferent ghost compounds due to the opening of double bonds during heating of the reaction mixture. An alternative method consists of using radioactive substrates [6,17]. However, as radiolabelled elaidic acid is not commercially available, it could be prepared from radio-labelled oleic acid by isomerization using *p*-toluenesulfinic acid [18] or aerobic bacteria [19]. Additionaly, these methods of elaidic acid synthesis through oleic acid isomerization need tedious purifications of both isomers. Another alternative detection method is the use of mass spectrometry coupled to LC or GC.

The aim of the present study was to develop and validate a reproducible and sensitive method for the determination of elaidic and oleic acid hydroxylase activities in microsomal preparations from rat and human liver, by using HPLC coupled to mass spectrometry. This detection method provides accurate structural information on hydroxymetabolites. In addition, it allows precise quantitative measurement. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) associated with HPLC have been applied, during this study, to the qualitative and quantitative analysis of hydroxylated metabolites of oleic and elaidic acids.

2. Experimental

2.1. Chemicals and solvents

Oleic and elaidic acids were purchased from Fluka (Buchs, Switzerland), while [1-^{14}C]-oleic acid (50 mCi/mmol) was from Amersham (Amersham, UK). NADPH, *N*,*O*-bis-trimethylsilyl-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were from Sigma (Saint Quentin Fallavier, France). HPLC-grade acetonitrile was supplied by SDS (Solvants Documents Synthèse, Peypin, France). All other chemicals and solvents were of the highest purity obtainable from Merck (Darmstadt, Germany) or Sigma (Saint Quentin Fallavier, France). Deionized water and acetonitrile were further filtered (0.45 μm, Millipore, France) before use.

2.2. Preparation of rat and human liver microsomes

Human liver samples were obtained from subjects who died following traffic accidents. In accordance with French law, local ethical committee (CHU, Brest, France) approval was obtained prior to this study.

Male Wistar rats (155–170 g) were housed four per cage and maintained on water and a standard diet with laboratory chow pellets (Extra-Labo, Pietrement, France). They were subjected either to ethanol vapour inhalation for one month [20] or to clofibrate treatment [3] in order to induce specific P450 2E1 and 4A isoforms, respectively.

Rat and human liver samples were frozen immediately after removal and the microsomal fraction was prepared according to a previously described method [21], and stored at −80°C until use. Microsomal protein content was determined using the Biorad protein assay (Biorad, Munich, Germany) based on the Bradford dye-binding procedure, using bovine serum albumin as standard [22]. Human microsomal contents, in terms of specific P450 and various

monooxygenase enzymatic activities, have been reported previously [23].

2.3. Assay of hydroxylated products of oleic and elaidic acids

The ω and (ω-1)-hydroxylations of oleic and elaidic acids were measured by incubating microsomes (0.3 mg of protein) in a reaction mixture containing substrate (0.1 m*M*) in 0.12 *M* potassium phosphate buffer pH 7.4 and 5 m*M* $MgCl_2$. The enzymatic reaction was initiated by the addition of 1 m*M* NADPH. After 30 min, the reaction was stopped by addition of 0.8 ml of a 10% H_2SO_4 solution. The metabolites and residual substrate were extracted twice with 5 ml of diethylether. The organic phase was dried under a stream of nitrogen, the residue was dissolved in 100 μl acetonitrile and 20 μl were injected for HPLC analysis.

For the determination of the kinetic parameters, elaidic acid was added to the reaction mixture in the range 25–400 μ*M*. All kinetic studies were performed under linear conditions with respect to time and protein concentrations.

2.4. HPLC–MS analysis

The elaidic acid hydroxymetabolites were separated and analyzed by RP-HPLC using an Ultraspher C18 column (250×4.6 mm I.D.; 5 μm; specific area, 300 m^2/g; pore size, 120 Å; carbon yield, 13%, Beckman, France). The mobile phase (0.2% acetic acid in water/acetonitrile) began isocratically with a 40:60 mixture (v/v) for 45 min at a flow-rate of 1 ml/min, followed by a 5-min linear gradient to 5:95 (v/v) mixture for 25 min (Pump Spectra System P1500, TSP, Les Ulis, France) in order to elute the residual substrate before returning to the initial conditions.

The oleic acid hydroxymetabolites and residual substrate were separated by HPLC and analyzed as described previously [6].

Both oleic and elaidic acids and their respective hydroxylated metabolites were separated on the same HPLC running a modification of the above conditions. The column, the mobile phase (water–acetonitrile) and the flow-rate were the same as for oleic acid. Only the proportion of water/acetonitrile in the mobile phase, and the time of each step were modified. The mobile phase began isocratically with a 55:45 mixture (v/v) for 120 min, followed by a 5-min linear gradient to 5:95 (v/v) mixture for 35 min before returning to the initial conditions.

The chromatograph was coupled to a mass spectrometer (Navigator from Finnigan, Manchester, UK) equipped with either an APCI- or an ESI-source, both running on a negative ion mode.

Peak areas were calculated using either radiometric (for oleic acid) or mass spectrometric detections (for both oleic and elaidic acids) from the percentage of metabolite area to the total product area. Data were expressed as pmol/min/mg of protein (mean ± SD). Correlation coefficients were calculated using an ANOVA table by the least-squares regression analysis from the raw data.

2.5. GC–MS analysis

The metabolite fractions from human samples were collected after HPLC separation and freeze-dried. Hydroxylated metabolites were then subjected to GC–EI-MS with 70 eV energy. Analysis was performed after derivatization of the hydroxylated and carboxylic groups with a mixture of BSTFA–TMCS–pyridine (80:10:10, v/v) for 60 min at 60°C in order to obtain trimethylsilyl (TMS) derivatives. The samples were dried under a nitrogen stream, resuspended in 50 μl of pentane and 1 μl was injected onto a CP-Sil 5CB capillary column (30 m×0.32 mm I.D.; film thickness=0.25 μm; phase ratio=320; Chrompack, Middelburg, The Netherlands). The column temperature was programmed from 140°C to 290°C at 4°C/min (Fractovap 4160, Carlo Erba, Milano, Italy). The column was coupled to a Nermag R10-10 mass spectrometer (Ribermag, France).

3. Results and discussion

3.1. Identification of hydroxymetabolites of elaidic acid by LC–MS

In Fig. 1 the HPLC profiles obtained from liver microsomes of human (A), or control (B), ethanol-treated (C) and clofibrate-treated (D) rats are shown.

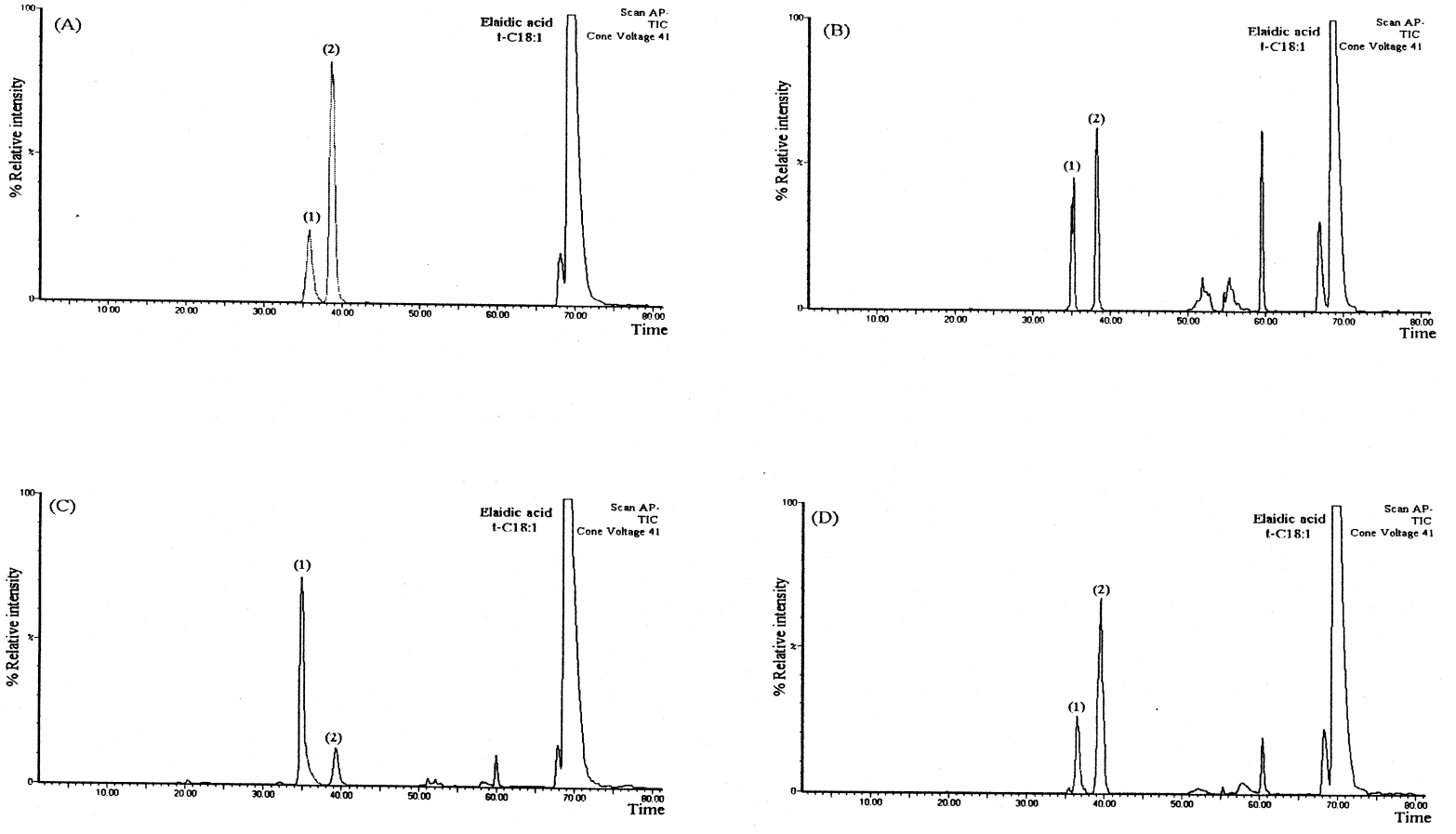

Fig. 1. RP-HPLC profiles of elaidic acid (0.1 m*M*) hydroxymetabolites produced by liver microsomes of human (A), or control (B), ethanol-treated (C) and clofibrate-treated (D) rats as detected by negative ion APCI-mass spectrometry. The two main metabolites (HPLC peaks 1 and 2) were collected for further identification. See experimental section for HPLC conditions.

All analytes were detected by negative ion APCI-mass spectrometry. The two main metabolites (peaks 1 and 2) formed in the presence of NADPH were clearly baseline-separated during the isocratic step of the gradient elution. The retention times of these two major hydroxymetabolites and residual substrate were 35, 39 and 70 min, respectively. The generation of these two main metabolites had an absolute requirement for molecular oxygen and NADPH as a source of electrons (data not shown), suggesting the involvement of P450 enzymes.

The corresponding mass spectra of these two peaks (1 and 2) and the residual substrate are shown in Fig. 2. The two peaks were characterized by a deprotonated molecule/$[M-H]^-$ ion at m/z 297, corresponding to an elemental composition of $C_{18}H_{33}O_3$, a hydroxy-derivative of elaidic acid. The residual substrate exhibited a deprotonated molecule/$[M-H]^-$ ion at m/z of 281.

In Fig. 3A the HPLC chromatogram of elaidic acid hydroxymetabolites (peaks 1 and 2) from human liver microsomes, with ESI mode detection is shown. In Fig. 3B the mass spectra of these metabolites and substrate using electrospray detection are presented. The respective mass of the metabolites and substrate is clearly characterized. However, this detection method could be used only as a qualitative method, since the area of the residual substrate, elaidic acid, does not represent its real value. The mechanism of ion generation is different between ESI and APCI modes. Electrospray ionization operates by the process of emission of ions before evaporation, while under APCI mode, an aerosol is formed before chemical ionization by corona discharge. So, APCI detection is more universal and sensitive than ESI, allowing detection of all HPLC eluted compounds according to their relative concentrations (Fig. 1). On the other hand, ESI detection is more specific than APCI mode because ionization depends upon the relative basicities of compounds, allowing a better response from hydroxy-derivatives of elaidic acid than from the substrate itself (Fig. 3).

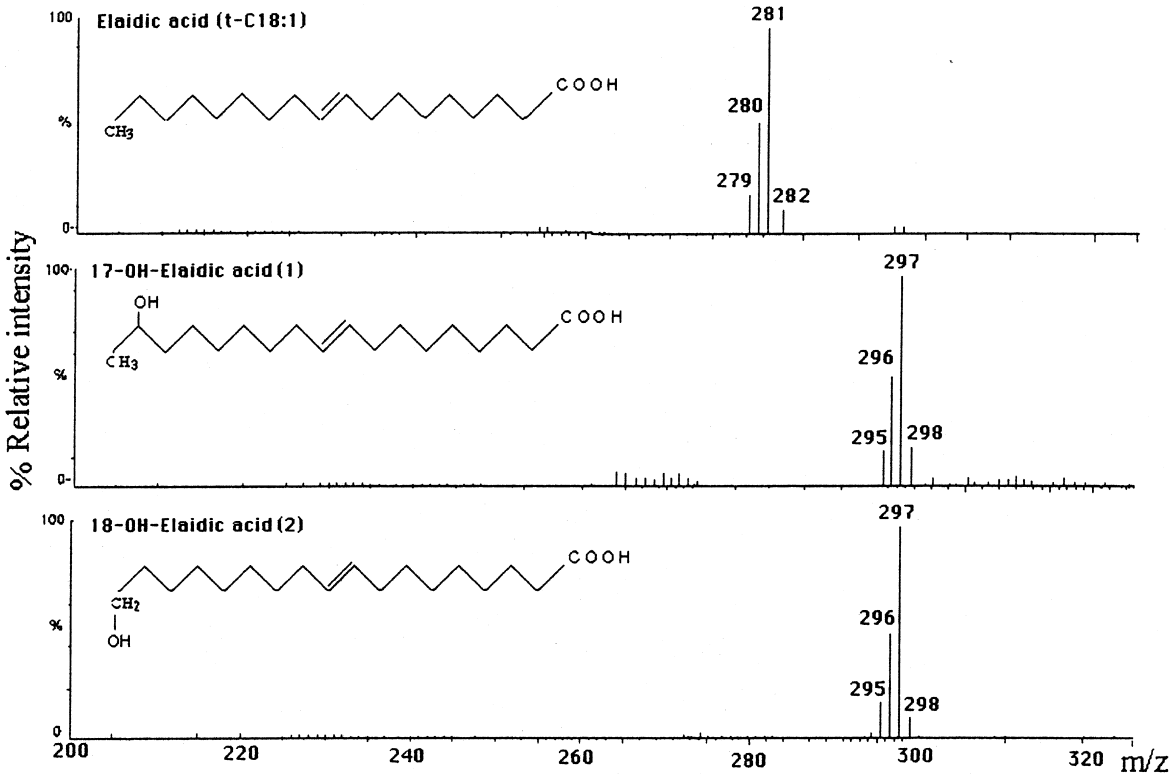

Fig. 2. APCI-mass spectra (negative ion mode) of elaidic acid and its hydroxylated metabolites after HPLC separation.

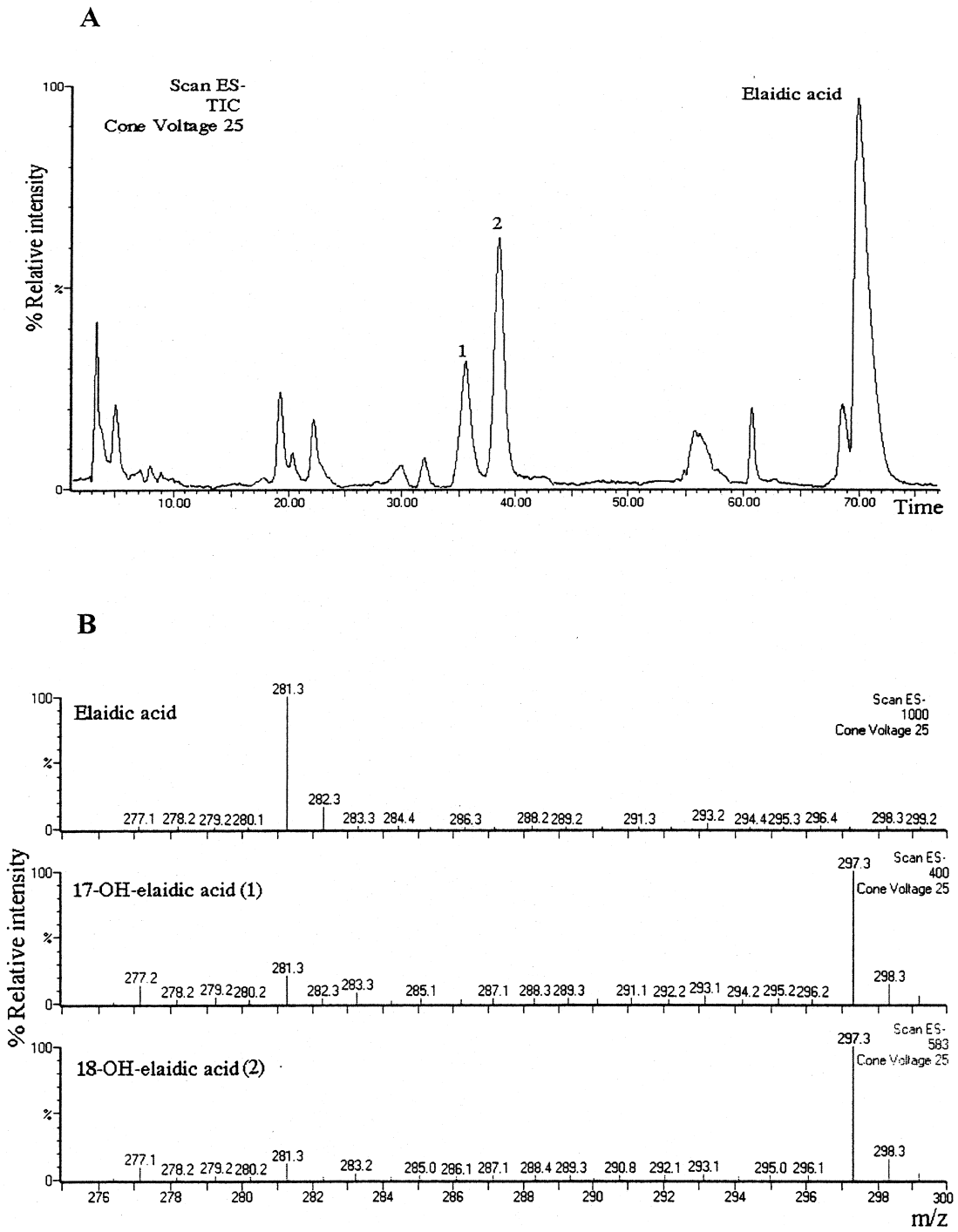

Fig. 3. HPLC separation of elaidic acid hydroxymetabolites (A) and mass spectra (B) of the metabolites using an electrospray ion source.

In Fig. 4 the HPLC separation of the two monounsaturated fatty acids, oleic and elaidic acids, according to their *Z*/*E* configuration is shown. Their metabolic profiles were quite similar, giving two main metabolites which were ω and (ω-1)-hydroxylated by microsomal P450 enzymes.

3.2. Identification of the hydroxymetabolites by GC–MS

In order to ascertain the structure of elaidic acid metabolites, HPLC peaks 1 and 2 were collected separately after HPLC analysis of human liver microsomes and freeze-dried. Each fraction was derivatized and analyzed by GC–EI-MS, as described in the experimental section. As shown in Fig. 5, the mass spectra of the TMS derivatives showed characteristic fragmentation patterns according to their expected structure.

The ion fragment *m*/*z* 117 represented the major fragment in the mass spectrum for the TMS derivatives of metabolite 1. This ion resulted from the fragment [CH_3–CHO–Si–Me_3^+] which is specific to (ω-1)-hydroxylated fatty acids, as previously described [24]. This ion fragment was also observed in the mass spectrum of the TMS derivative of metabolite 2, which is due to the fragment [COO–Si–Me_3^+] and is characteristic of the TMS derivative of a carboxylic group. However, this fragment represented only 20% of the base peak intensity. On the other hand, an abundant ion fragment *m*/*z* 103 was detected in this second spectrum, and was due to the fragment [CH_2–O–Si–Me_3^+] which is characteristic of the ω-hydroxylated fatty acid TMS derivatives. Moreover, TMS derivatives of ω-hydroxylated metabolites produced a rearrangement ion at *m*/*z* 147 [Me_3–Si–O–Si–Me_2^+] which is common to all ω-hydroxylated compounds such as oleic acid [6].

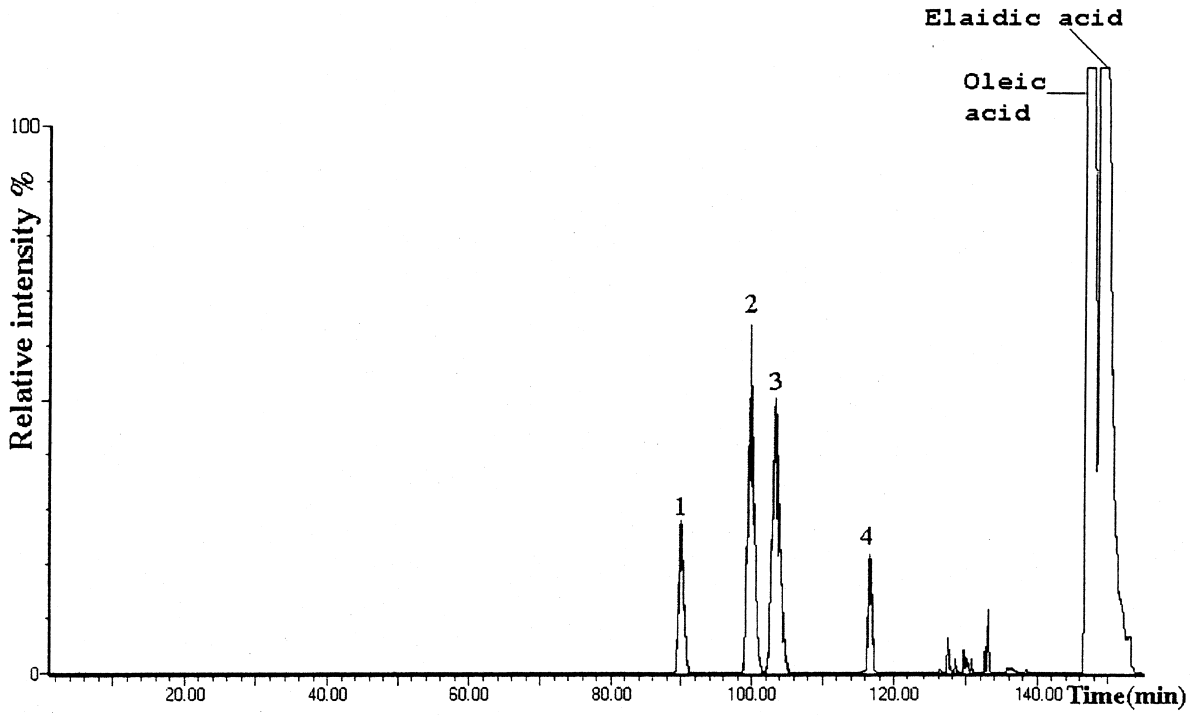

Fig. 4. HPLC separation and mass spectrometric detection (negative ion APCI-mass spectrometry) of oleic and elaidic acids and their (ω-1) and ω metabolites. 0.3 mg of liver microsomal protein from ethanol-treated rats were incubated in the presence of both oleic (0.1 m*M*) and elaidic (0.1 m*M*) acids at 37°C for 30 min. Peaks 1, 2, 3 and 4 were identified as (ω-1)-OH-oleic, (ω-1)-OH-elaidic, ω-OH-oleic and ω-OH-elaidic acids, respectively. See experimental section for HPLC conditions.

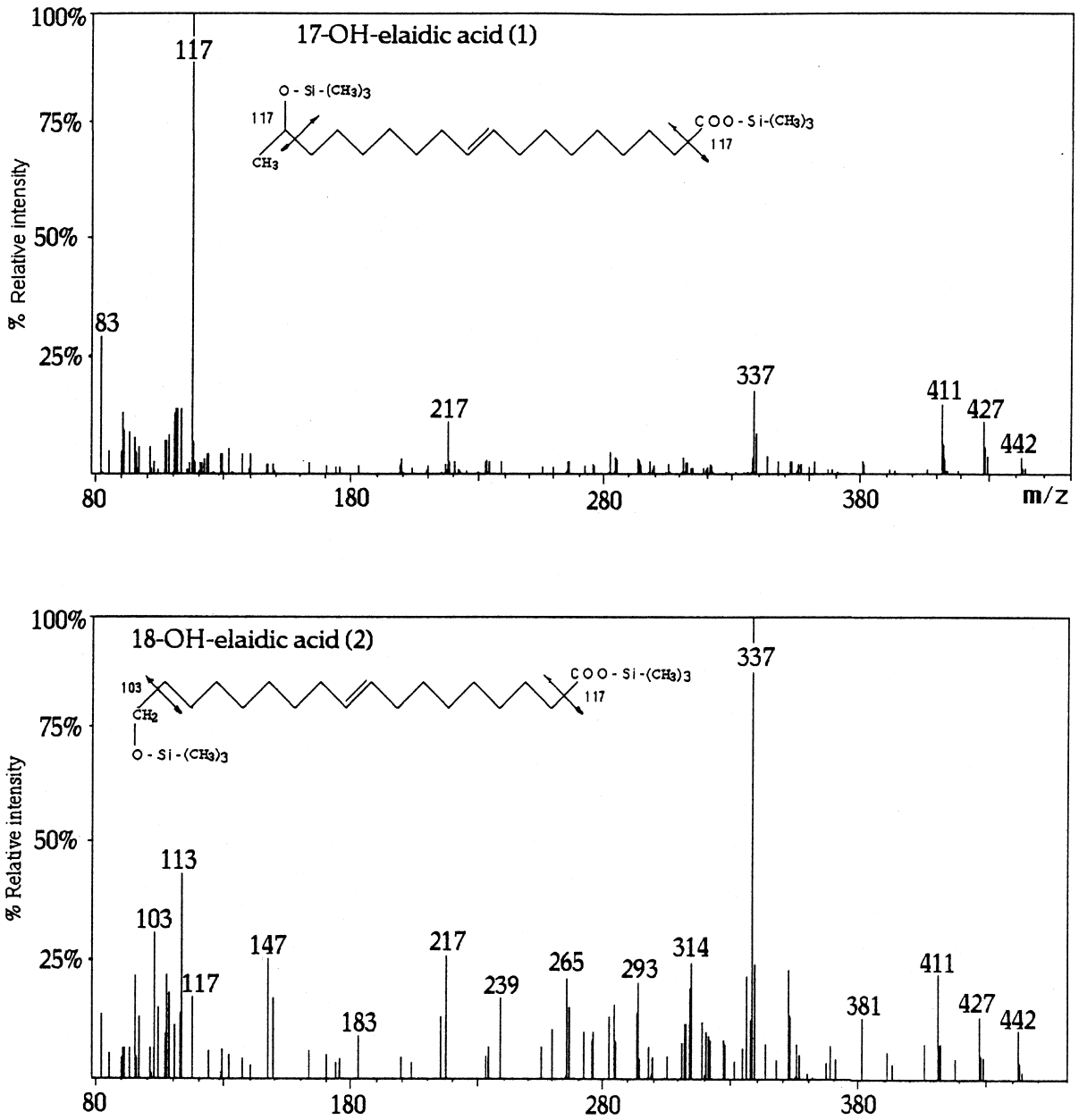

Fig. 5. Mass spectra of the TMS-ester derivatives of elaidic acid hydroxymetabolites, according to the procedure previously described in the Experimental section. The structure and major cleavage sites of the derivatives are shown by arrows. Peak 1 was identified as (ω-1)-OH-elaidic acid and peak 2 as ω-OH-elaidic acid.

Furthermore, the two metabolites had common fragments at m/z 442 [M^+], m/z 427 [M^+-15], m/z 411 [M^+-31] and m/z 337 [$M^+-90-15$], which is caused by loss of methyl and methoxy groups, respectively. The same pattern of fragmentation was observed with the TMS derivatives of oleic acid

Table 1
Correlation coefficients (r) between metabolic rates measured by radiometric and APCI-mass spectrometric detections of ω and (ω-1) hydroxylated metabolites of oleic acid after HPLC separation in both human and rat liver microsomes

	(ω-1)-OH-oleic acid	ω-OH-oleic acid
Rat liver microsomes ($n=6$)	$r=0.90$	$r=0.85$
	$P<0.01$	$P<0.05$
Human liver microsomes ($n=6$)	$r=0.86$	$r=0.94$
	$P<0.05$	$P<0.01$

metabolites [6]. Assuming that the double bond remained at its original position, and when compared to previous spectra obtained for oleic acid in rat and in plants [6,25], the two major metabolites of elaidic acid (1 and 2) were identified as (ω-1) and ω-hydroxylated metabolites, respectively. Moreover, their order of elution in GC, as well as in LC, i.e. the (ω-1) before the ω-hydroxylated derivative, was in agreement with that described for the other fatty acids, such as laurate [3,4], oleate [6] or arachidonate [26].

3.3. Validation of the method

In order to validate our detection method using mass spectrometry, a comparative study was carried out with oleic acid as substrate, using both radiometric and mass spectrometric detections. Incubations with oleic acid (0.1 m*M*; 1 μCi) were carried out in six human and six rat (control, ethanol-treated and clofibrate-treated) liver microsomes. The correlation coefficents based on the least squares method, were calculated for the two metabolites and are reported in Table 1. Although radiometric detection is the reference method for measuring metabolite rates, mass spectrometric detection was found to be similar in several respects. The two procedures used gave quite similar results with significant correlation coefficients between the two techniques in human and rat liver microsomes.

A calibration plot using elaidic acid detected by APCI-MS gave excellent linearity with a correlation coefficient of 0.99 over the injected concentration range of 25 ng (88 pmol) to 1128 ng (4 nmol). The limit of detection for elaidic acid (signal-to-noise ratio of 3) was estimated to be 25 ng per injection, and this value was not as low as that obtained with fluorimetric detection [13].

The intra-day and inter-day precision of mass spectrometric detection was evaluated by analyzing the enzymatic activities of human and rat liver microsomes. The samples were injected five times on the same day, and daily for 5 days, respectively (Table 2). Results showed that the reproducibility was nearly identical for the two metabolites, with coefficients of variation (C.V.) between 7 and 13.6%. The method of detection based upon the APCI-mass spectrometry coupled with HPLC appears to be sensitive and specific. It makes the assay reliable for the study of fatty acid metabolism.

3.4. Kinetic parameters

In Fig. 6 the kinetic parameters of 17- and 18-

Table 2
Intra-day and inter-day precision (C.V. %) of the mass spectrometry detection of elaidic acid metabolism in human and rat liver microsomes

	Human liver microsomes		Rat liver microsomes	
	(ω-1)-OH	ω-OH	(ω-1)-OH	ω-OH
Intra-day precision ($n=5$)	7.1%	8.75%	9.7%	11.4%
Inter-day precision ($n=5$)	12.5%	13.1%	12.9%	13.0%

The samples were analyzed five times on the same day (intra-day) and five times daily for 5 days (inter-day).

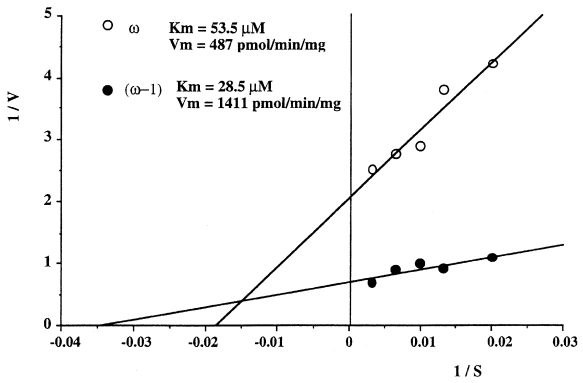

Fig. 6. Kinetic parameters (K_m and V_m) of ω and (ω-1)-elaidic acid hydroxylations in ethanol-treated rat liver microsomes. 0.3 mg of microsomal protein were incubated at 37°C for 30 min with increasing concentrations of elaidic acid ranging from 25 to 400 μ*M*.

hydroxyelaidic acid formation from elaidic acid by rat liver microsomes are shown. Elaidic (ω-1) and ω-hydroxylations displayed single enzyme kinetic properties.

Table 3
Comparison of the kinetic parameters of elaidic and oleic acids in rat liver microsomes

	Elaidic acid		Oleic acid[a]	
	(ω-1)-OH	ω-OH	(ω-1)-OH	ω-OH
K_m (μ*M*)	28.5	53.5	59.8	28.7
V_m (pmol/min/mg)	1411	487	1500	900

The kinetic parameters of elaidic and oleic acids were measured in microsomes of ethanol-treated rats.
[a] Results from Ref. [6].

An apparent K_m of 28.5 μ*M* and a V_m of 1411 pmol/min/mg was determined for the formation of (ω-1)-hydroxyelaidic acid, while a K_m of 53.5 μ*M* with a V_m of 487 pmol/min/mg was found for the formation of ω-hydroxyelaidic acid. These V_m values were not very different from those obtained with oleic acid (Table 3) [6].

3.5. Elaidic hydroxylase activities in rat and human liver microsomes

The elaidic acid hydroxylase activities were measured in rat and human liver microsomes and compared to oleic acid hydroxylases (Table 4).

In three human liver microsomes (namely FH3, Br039 and Br048 samples, see [23]), the elaidic acid hydroxylations presented a large individual variation due to the heterogeneous origin of the liver donors. The values ranged from 289 to 924 pmol/min/mg (mean±SD=614±351) for (ω-)-hydroxylation, and ranged from 229 to 755 pmol/min/mg (mean±SD= 492±282) for ω-hydroxylation. The same individual variation was observed when oleic acid was used as substrate. However, the relative ratio ω/ω-1 of the two hydroxylated metabolites was different between these two fatty acids. It was found to be 5.2±2.6 and 1.3±1.1 for oleic and elaidic acids, respectively.

In contrast, the distribution of oleic and elaidic acid hydroxylations was found to be quite similar in liver microsomes from control rats, suggesting that the geometry of the double bond did not modify the ability of the fatty acid to bind to the substrate access channel of cytochrome P450 2E1 active site. The relative ratio ω/ω-1 was found to be 1.14±0.1 with

Table 4
ω- and (ω-1)-hydroxylase activities of elaidic and oleic acids in microsomes from human or control, ethanol-treated and clofibrate-treated rat livers

	Elaidic acid			Oleic acid[a]		
	(ω-1)	ω	ω/(ω-1)	(ω-1)	ω	ω/(ω-1)
Human						
(*n*=3)	614±351	492±282	1.32±1.1	305±162	1357±378	5.2±2.6
Rat						
Control	302±32.7	345±47	1.14±0.1	755±242.5	926±302.6	1.2±0.01
Ethanol	1383±262	310±37	0.23±0.09	1017±99.6	687±158.3	0.71±0.12
Clofibrate	349.5±30.4	816.5±118	2.36±0.54	554.5±72.8	1839.5±4.9	3.1±0.06

Results are expressed as pmol/min/mg of microsomal protein (mean±SD).
[a] Results are from ref [6].

elaidic acid, approximately the same value as when oleic acid was used as substrate, namely 1.2±0.01. The (ω-1)-hydroxylation of elaidic acid was inducible by ethanol (4.6-fold) while the ω-hydroxylation was enhanced by clofibrate (2.4-fold). These inductions were more important than those obtained when oleic acid was used as substrate (1.3 and 1.9-fold with ethanol and clofibrate respectively).

4. Conclusion

An alternative detection method has been developed for hydroxylated fatty acids which are not easily detectable by UV, fluoresence or electrochemistry. Moreover, when the use of radiolabeled substrate is not possible (or is prohibited), HPLC coupled to mass spectrometry can be used and gives quite similar results. MS detection has the advantage of avoiding the use of radioisotopes or scintillation cocktails which are necessary for radiometric HPLC analysis. When compared to GC–MS, the described method presents advantages such as the fact that no derivatization is required and therefore there are no clean-up steps. The chromatographic analysis does not take any longer, and more importantly, the hydroxymetabolites are well separated, and each metabolite's identity can be verified by its mass spectra on any particular run.

5. Abbreviations

Cytochrome	P450 (EC 1.14.14.1);
Oleic acid	(*Z*)-9-octadecenoic acid;
17-OH-oleic acid	17-hydroxyoleic acid or (ω-1)-hydroxyoleic acid;
18-OH-oleic acid	18-hydroxyoleic acid or ω-hydroxyoleic acid;
Elaidic acid	(*E*)-9-octadecenoic acid;
17-OH-elaidic acid	17-hydroxyelaidic acid or (ω-1)-hydroxyelaidic acid;
18-OH-elaidic acid	18-hydroxyelaidic acid or ω-hydroxyelaidic acid;
APCI	Atmospheric pressure chemical ionization;
ESI	Electrospray ionization;
LC–MS	Liquid chromatography–mass spectrometry;
GC–EI-MS	Gas chromatography–electron ionization mass spectrometry;
BSTFA	*N*, *O*-bis-trimethylsilyl-trifluoroacetamide;
TMCS	Trimethylchlorosilane;
TMS	Trimethylsilyl derivative;
K_m	Michaelis constant;
V_m	maximal velocity according to Michaelis kinetics.

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