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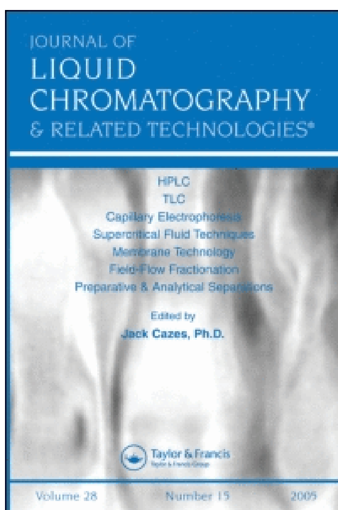

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DETERMINATION OF LAURIC ACID METABOLITES IN PEROXISOME PROLIFERATION AFTER DERIVATIZATION AND HPLC ANALYSIS WITH FLUORIMETRIC DETECTION

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ABSTRACT

A derivatization procedure is described for the long chain fatty acid lauric acid and metabolites using the fluorescent probe 4-(bromomethyl)-7-methoxycoumarin. The derivatives can be separated in an isocratic high-performance liquid chromatographic system and detected using a fluorescence detector. The derivatization is rapid, simple and gives a good quantitation by the use of an internal standard. The procedure has been applied on samples rat liver homogenates from a toxicity experiment with the plasticizer di(2-ethylhexyl)phthalate. The activity of cytochrome P-450 IVA1 or lauric acid hydroxylase (LAH) in liver homogenates can be determined by the determination of the 11- and 12-hydroxylated metabolites of lauric acid. The method turned out to be very sensitive and suitable to replace similar assays using radioactive compounds.

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INTRODUCTION

Peroxisome proliferation in the liver of rodents can be induced by several agents like plasticizers, hypolipidaemic agents, etc [1]. The proliferation of peroxisomes is frequently associated with the development of hepatocellular carcinomas [2]. Although the extend of peroxisome proliferation can be detected very sensitively by the induction of several enzyme systems, such as carnitine acetyl transferase and enzymes involved in the beta-oxidation [3], the early biological response is supposed to be the induction of microsomal cytochrome P-450 IVA1 (or cytochrome P-452) [4]. This enzyme system is involved in the hydroxylation of fatty acids. The present assays for cytochrome P-452 are based on the hydroxylation of radioactive labelled lauric acid [5,6,7].

Another way of detection of long chain carboxylic acids is via the derivatization of the carboxylic group. A number of derivatization methods have been published [8,9,10,11,12]. The most promising methods appeared to be the derivatization with fluorescent coumarin derivatives in the presence of crown ethers [13,14,15,16].

In the present study a method has been described in which the detection of lauric acid and its hydroxylated analogues is performed by derivatization of the carboxylic group by a fluorescent probe, 4-(bromomethyl)-7-methoxycoumarin (BrmmC) followed by subsequent analysis by high-performance liquid chromatography (HPLC). The method has been applied to liver homogenates of rats which were treated with several doses of the plasticizer di(2-ethylhexyl)phthalate (DEHP).

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MATERIALS AND METHODS

CHEMICALS

Lauric acid, potassium carbonate, diethylether, ethanol, ethylenediamine tetraacetic acid (EDTA) and magnesium chloride were obtained from Merck. 4-(Bromomethyl)-7-methoxycoumarin was obtained from Janssen Chimica. Phenantrene, 12-hydroxylauric acid and 18-crown-6 were obtained from Aldrich-Chemie. Acetonitrile and methanol (both HPLC quality) were obtained from Westburg. The acetonitrile was dried on potassium carbonate. Octanoic acid was obtained from Chem Service. Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Boerhringer Mannheim.

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EQUIPMENT

The HPLC equipment has been composed of the following components. An autoinjector (model 231, Gilson), two solvent delivery systems (model 2150, LKB) operated by a controller (model 2152, LKB). The HPLC column system consisted of a holder containing a cartridge with Chromspher C18 (250 x 4.6 mm; Chrompack) and a gard column (10 x 3.0 mm; Chrompack C18). The column and pre-column were thermostatted at 30 C with a column heater (model 2155, LKB). The eluent was monitored with a luminescence spectrometer (model LS5, Perkin Elmer) using an exitation wavelength of 330 nm (slit 15 nm) and an emission wavelength of 396 nm (slit 20 nm). The data were collected and analyzed with a software package of Axxiom (Analytica, Maasdijk, The Netherlands).

ENZYME ASSAY

The incubation mixture was composed as follows: 1 ml NADP solution (10 mM), 1 ml glucose-6-phosphate solution (50 mM), 1 ml glucose-6-phosphate dehydrogenase solution (10 U/ml), 1 ml magnesium chloride (6.1 mg/ml water), 200 ul lauric acid solution (2 mg/ml ethanol) and 5.8 ml phosphate buffer (pH 7.4) containing 1 mM EDTA. After pre-heating 0.970 ml of the incubation mixture at 37 C in a waterbath, 30 ul liver homogenate was added. The incubation was stopped after 10 min by the addition of 150 ul hydrochloric acid (3 M).

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DERIVATIZATION

The incubation mixture was extracted twice with 5 ml diethyl ether. The organic layers were separated by centrifugation (5 min, 2800 rpm). The ether of the combined fractions was evaporated under nitrogen. The residue was dissolved in 2 ml acetonitrile.

The derivatization was performed directly in the HPLC vial. To the HPLC vial was added: 550 ul of the acetonitrile residue, 1.0 mg potassium carbonate, 200 ul BrmmC solution (0.85 mg/ml), 100 ul 18-crown-6 solution (0.33 mg/ml) and 50 ul octanoic acid solution (10 ug/ml) as internal standard. The reaction was performed at 80 C for 40 min.

HPLC ANALYSIS

From the derivatization solution 20 ul was injected onto the HPLC column (Chromspher C18). The flow was 1 ml/min (pressure 90 bar). The column was eluted isocratically with a mixture of methanol/water (67/33, v/v) from 0-44 min, followed by elution with 97% methanol to clean the column from tightly bound components.

ANIMAL EXPERIMENTS

The animal experiments (nr. TOX-TOX 90/41A) will be described in detail elsewhere [17]. Shortly six animals (male rat, RIV-TOX) received 0, 60 or 200 mg DEHP/kg diet for 2 weeks, which corresponds to 0, 5 and 15 mg DEHP/kg body weight per day, respectively. After 2 weeks the animals were killed and the livers were homogenized after perfundation. The data of the lauric acid hydroxylation assay (the production of 11-, 12-hydroxylauric acid and the ratio of both metabolites) were statistically analyzed with the STATA software package by one-way analysis of variance (after logarithmic transformation of the data) using the Bartlett's test of equal variances (according to Scheffe).

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RESULTS AND DISCUSSION

DERIVATIZATION

The derivatization of the long chain fatty acids with BrmmC has been performed as described by Dirven et al. [16]. A number of practical improvements have been made such as the introduction of an internal standard (octanoic acid) and a faster and more simple procedure by avoiding an extraction step. The derivatization reaction is carried out off-line in the HPLC injection vial in the presence of a crown ether and potassium carbonate in acetonitrile at a temperature of 80 C.

The time dependance of the derivatization reaction was investigated with lauric acid and 12-hydroxylauric acid. Because of its fluorescence property, phenantrene was taken as internal HPLC standard. The time dependance is shown in Figure 1. It appeared that already after 15 min the derivatization was

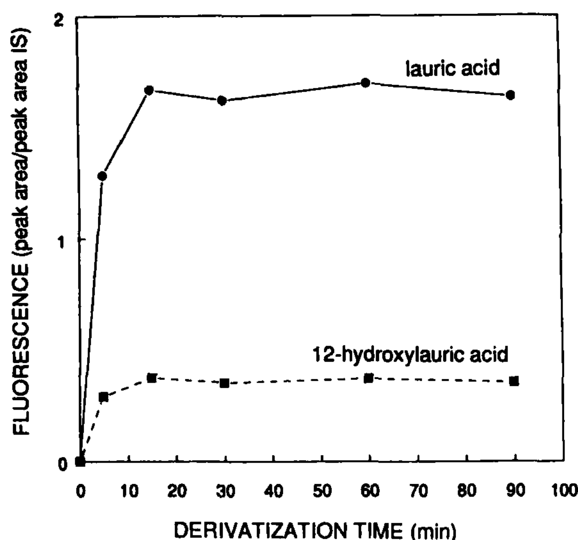

FIGURE 1. Time dependence study of the derivatization of lauric acid and 12-hydroxylauric acid with BrmmC. Phenantrene was used as internal standard.

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complete. The reaction was followed until 90 min. It appeared that no degradation of the fluorescent conjugates occurred. In practice a derivatization time of 40 min was taken tentatively.

The linearity of various amounts of lauric acid and 12-hydroxylauric acid as function of the fluorescence signal after derivatization with BrmmC was investigated with phenantrene as internal HPLC standard. In Figure 2 it is shown that a perfect linear relationship for lauric acid exists between 2.0 and 100 nmol/ml and for 12-hydroxylauric acid between 0.5 and 25 nmol/ml (injection volume was 20 ul). Correlation coefficients were found to be 0.9991 and 0.9997 for lauric acid and 12-hydroxylauric acid, respectively.

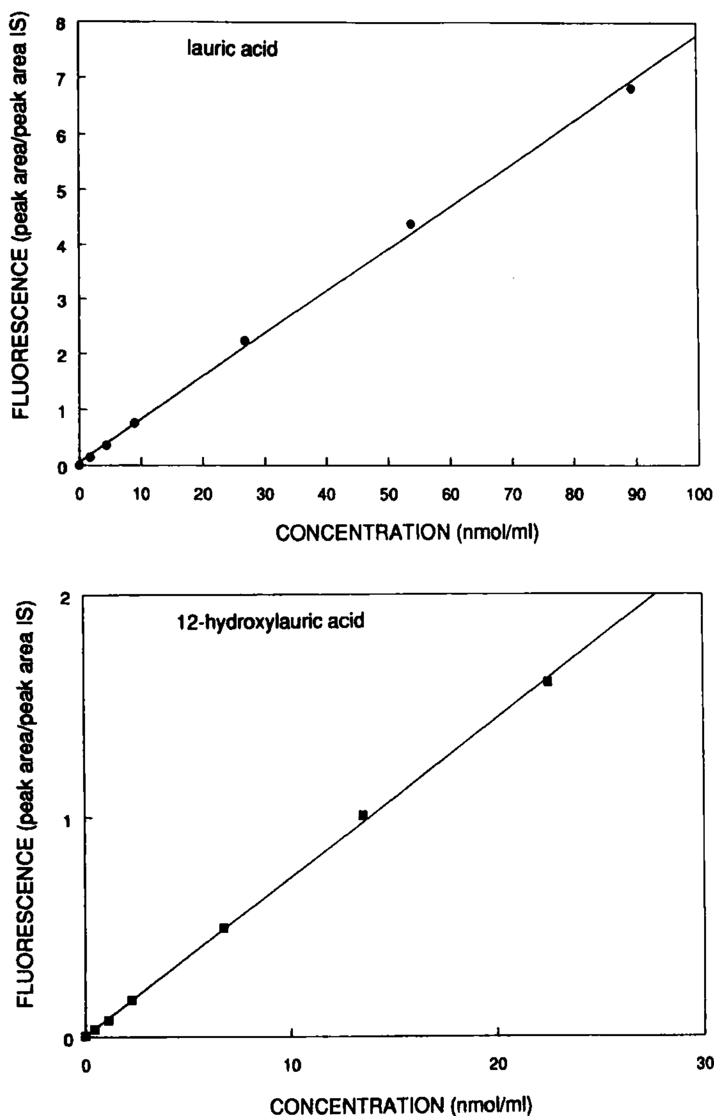

FIGURE 2. Linearity of the derivatization of lauric acid and 12-hydroxylauric acid as function of the amount of fatty acid. Phenantrene was used as internal standard.

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CHARACTERIZATION OF DERIVATIVES

The excitation and emission spectra of both lauric acid and 12-hydroxylauric acid showed the same characteristics: an exitation maximum at 330 nm and an emission maximum at 396 nm. With the relatively large Stoke shift of 66 nm no interferences occurred using fluorescence detection following HPLC separation.

The stability of the BrmmC derivatives at room temperature was tested in methanol and acetonitrile which are commonly used as organic modifier in HPLC analysis. It appeared that the BrmmC derivatives of both lauric acid and 12-hydroxylauric acid are rather stable in acetonitrile for longer times (20 h) (Figure 3). In methanol the stability is much less, especially for the lauric acid derivative. This is in contrast to the procedure described by Dirven et al. [16] who obtained very stable derivatives with the dimethoxy analogue of BrmmC.

The limit of detection was determined for the BrmmC derivative of 12-hydroxylauric acid. Using fluorescence detection (ex 330, em 396) the limit of detection was 0.25 pmol per injection with a signal-to-noise ratio of 5. The ultra violet detection at 254 nm showed a limit of detection of 50 pmol per injection.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

To separate 11- and the 12-hydroxylauric acid on a reversed phase column, isocratic elution was required in combination with a relatively low percentage of organic modifier. This resulted in long retention times of 34 and 36 min., respectively. A typical chromatogram from the analysis of a rat liver homogenate (as described in the next section) is shown in Figure 4. Both metabolites are baseline separated and can be easily quantitated. Octanoic acid

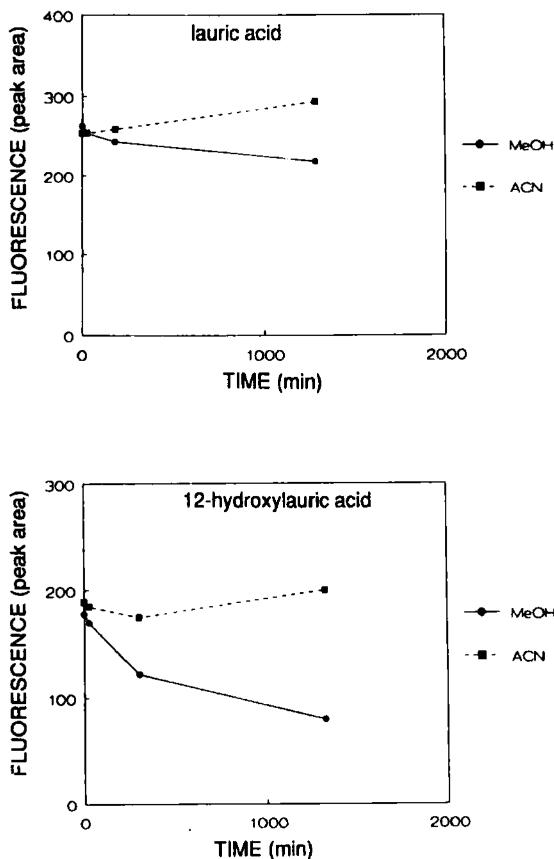

FIGURE 3. Stability in methanol and acetonitrile of BrmmC derivatives of lauric acid and 12-hydroxylauric acid as function of time.

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was found to be the best internal standard based on its retention time (about 45 min) in the chromatogram.

ENZYME ASSAY

The incubation of rat liver homogenates containing the cytochrome P-450 system including cytochrome P-450 IVA1 with lauric acid has been followed by

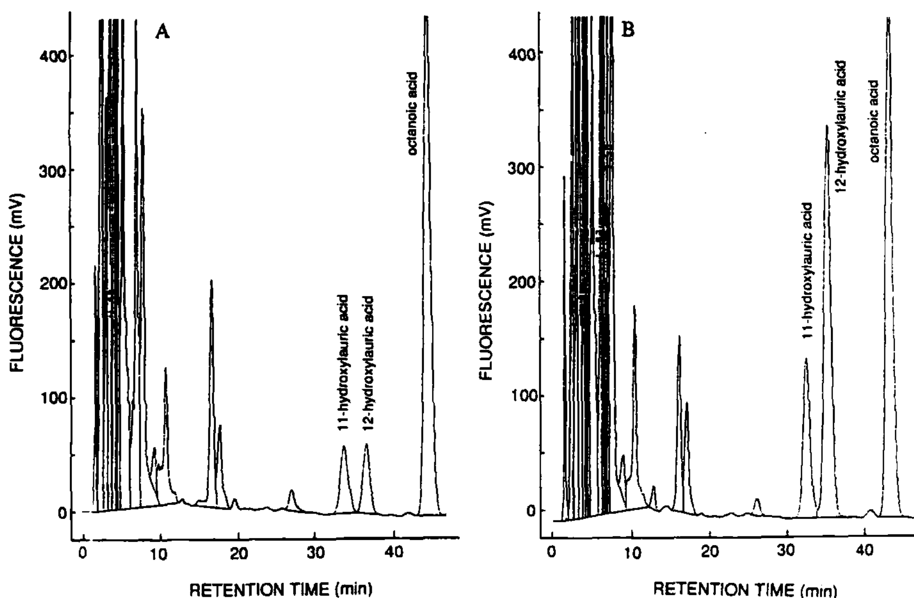

FIGURE 4. HPLC chromatograms of the lauric acid hydroxylation assay by cytochrome P-450 IVA1. A. Liver homogenate of a rat (nr. 1217) from the control group. B. Liver homogenate of a rat (nr. 1242) from the group receiving 200 mg DEHP/kg diet.

the production of 11- and 12-hydroxylauric acid as function of time. A NADPH generating system was added for an optimal enzymatic action of cytochrome P-450. The results are shown in Figure 5. As can be concluded from this Figure a linear relationship is obtained from 0 to about 15 min at 37 C. The ratio protein to lauric acid is 15 to 1 (w/w) in this assay. In this experiment a liver sample was used originating from a rat in which the lauric acid hydroxylase activity was maximally induced by DEHP.

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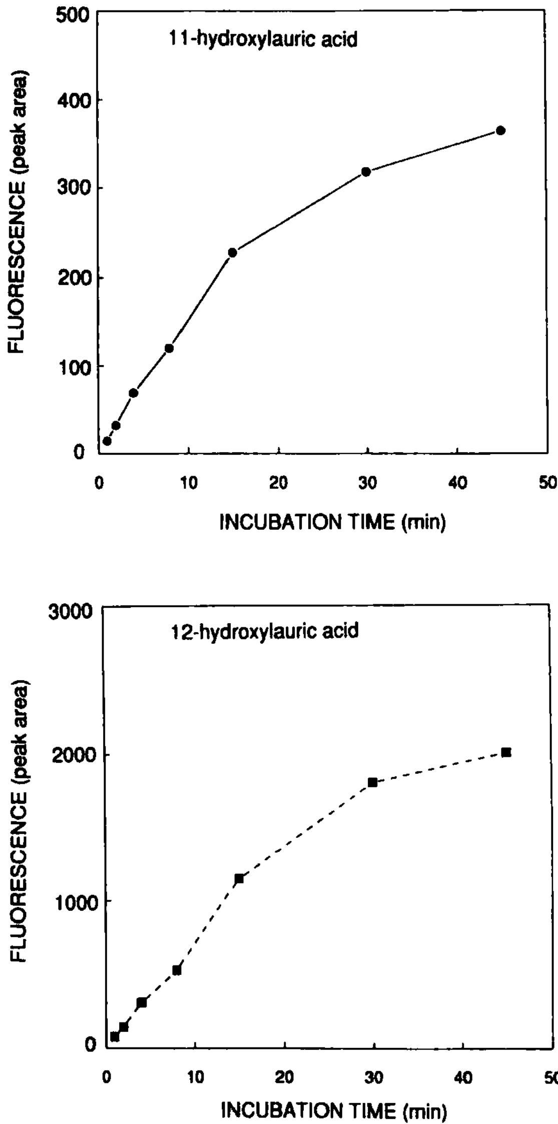

FIGURE 5. Linearity of the total lauric acid hydroxylase assay (enzyme assay and HPLC derivatization) as function of the incubation time. Shown are the production of 11- and 12-hydroxylauric acid.

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TABLE 1

Results (mean ± s.d.; n=6) of the lauric acid hydroxylase assay in liver homogenates of rats homogenates of rats treated with di(2-ethylhexyl)phthalate (DEHP).

group	dose (mg DEHP/kg diet)	activity (ng/ug protein)		ratio 12OH/11OH
		11OH	12OH	
1	0	0.703 ± 0.092	0.727 ± 0.105	1.059 ± 0.258
2	60	0.825 ± 0.139	1.102 ± 0.211*	1.344 ± 0.169
3	200	1.100 ± 0.317*	2.361 ± 0.796***	2.140 ± 0.220***

* $p < 0.05$; *** $p < 0.001$.

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ANIMAL SAMPLES

The application of this derivatization method on rat liver homogenates was performed using octanoic acid as internal standard. The time dependence and linearity of the derivatization of octanoic acid was essentially similar to that of lauric acid. In this assay the enzymatic hydroxylation of lauric acid to 11- and 12-hydroxylauric acid was determined. The intensity (surface area) of both components was corrected for the internal standard and for the amount of protein present in the original sample. From this animal experiment only the control group (6 animals) and the groups receiving the lowest doses of 60 and 200 mg DEHP/kg diet (6 animals in both groups) were analyzed. The results of the amount formed of both hydroxylated metabolites and the ratio 12- to 11-hydroxylauric acid were determined and listed in Table 1. Statistical analysis showed that the ratio (12-OH/11-OH) and the 11-hydroxy metabolite were

significantly different from the control group in the group which received 200 mg. For the 12-hydroxylated metabolite a statistically significant difference was observed with the lowest dosed group which received 60 mg DEHP/kg diet. This result means that based on the activity of cytochrome P-452 the no-effect-level can not be obtained from this experiment, but is lower than 60 mg/kg diet which corresponds with 5.0 mg/kg body weight per day.

It can be concluded that the method presented here to determine small amounts of long chain fatty acids by derivatization with BrmmC followed by HPLC analysis with fluorescence detection is reliable, easy and reproducible to perform. The method can be applied to determine the activity of cytochrome P-450 IVA1 in liver homogenates of rats treated with the plasticizer DEHP. Very low effect levels were found which corresponds with those determined using other enzyme activities as parameter, such as carnitine acetyl transferase [17].

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