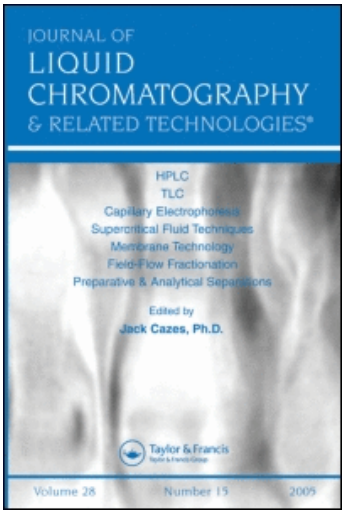

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Clarke, M. J. , Hawke, R. L. and Welch, R. M.(1986) 'Separation of Hydroxylated Metabolites of Fatty Acids (C_{10}-C_{18}) on a µPorasil Silica Column Using an Isocratic HPLC System', Journal of Liquid Chromatography & Related Technologies, 9: 8, 1711 — 1725
To link to this Article: DOI: 10.1080/01483918608076713
URL: http://dx.doi.org/10.1080/01483918608076713

JOURNAL OF LIQUID CHROMATOGRAPHY, 9(8), 1711-1725 (1986)

SEPARATION OF HYDROXYLATED METABOLITES OF FATTY ACIDS (C_{10}-C_{18}) ON A μPORASIL SILICA COLUMN USING AN ISOCRATIC HPLC SYSTEM

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ABSTRACT

A simple, versatile, and rapid normal-phase isocratic HLPC system is described for the analysis of the major (omega and omega-1) metabolites of C_{10}-C_{18} chain length fatty acids formed upon incubation with rat liver microsomes and NADPH. Quantitation was achieved by radiometric detection. Chromatographic separation was performed by elution of the fatty acids and their omega and omega-1 metabolites from a 10μ silica column (μPorasil) with hexane:2-propanol:acetic acid (96.5:2.5:1.0). Retention times for these metabolites ranged from 10 to 13 minutes for stearic acid and from 16 to 21 minutes for capric acid. Recovery of the fatty acids and their metabolites from the column was greater than 95 percent. Relative quantitative conversion of the fatty acid substrates to omega and omega-1 metabolites was in the following order: myristic acid > capric acid = lauric acid = palmitic acid >> stearic acid. The omega products were formed preferentially over the omega-1 products of all the fatty acids except lauric

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acid. The method proved suitable for routine determination of NADPH-dependent fatty acid hydroxylase activities in rat liver microsomes.

INTRODUCTION

Saturated fatty acids serve as substrates for a highly specific form(s) of cytochrome P-450 in microsomes from rat liver and kidney, which yield the omega and omega-1 hydroxylated products as major metabolites (1,2). Current methods employed for analysis of hydroxylated fatty acids consist of TLC procedures that fail to resolve the omega and omega-1 products (3), and radio-gas chromatographic procedures that require derivatization (1). Reverse-phase HPLC procedures, which require methylation of the metabolites or a step-wise gradient system have been described for lauric acid hydroxylation products (4,5). These HPLC methods are hampered by long analysis times and poor resolution. More recently, attempts have been made to derivatize hydroxylated fatty acids with strong UV-absorbing chromatophores (6) with some success, but resolution is still unsatisfactory and reasonable retention times require high flow rates.

Although the biochemical importance of omega and omega-1 hydroxylations are not fully understood, they are thought to be initial oxidation steps (7) in the metabolism of fatty acids and prostaglandins to their final excretory products (8). The

selective induction of a cytochrome P-450 isozyme in rat liver microsomes catalyzing the omega and omega-1 hydroxylation of lauric acid has been demonstrated following exposure of rats to the hypolipidemic drug clofibrate (5). Holm et al. (9) demonstrated the formation of novel omega hydroxylation products of PGE_1 and PGE_2 in rat liver microsomes and showed that their formation was inhibited in the presence of lauric acid, suggesting that fatty acids may compete as alternative substrates for the same PG omega hydroxylases. Omega hydroxylation of leukotriene b_4 is a major route for its degradation by polymorphonuclear leukocytes (10) and rat liver microsomes (11). The dicarboxylic acids of prostaglandins are present in the urine of mammals (12), but dicarboxylic acids of straight chain fatty acids are present only in substantial amounts in mammalian urine during fasting or ketotic states (13). The observation that omega hydroxylation is elevated during fasting has prompted several investigators to study the involvement of omega hydroxylation products of fatty acids in gluconeogenesis (14).

The availability of a rapid and more sensitive analytical procedure for quantitating the omega and omega-1 hydroxylation products of a variety of fatty acids is important to further our understanding of the role of the hepatic monooxygenase system in fatty acid metabolism. This report describes a simple and versatile normal phase isocratic HPLC system for the analysis of omega and omega-1 hydroxylation products of fatty

acids (C_{10}-C_{18}) formed during incubation with rat liver microsomes.

METHODS AND MATERIALS

NADPH and 12-hydroxylauric acid were purchased from Sigma Chem. Co. (St. Louis, Mo.). [1-^{14}C] Lauric, (26 mCi/mmol); palmitic, (58 mCi/mmol); stearic, (57 mCi/mmol); and myristic, (60 mCi/mmol) acids were purchased from Amersham/Searle Corp. (Arlington Heights, Ill.). [1-^{14}C]Capric acid (52 mCi/mmol) was purchased from CEA (Yvette, Fr.). Mobile phase solvents used were pesticide grade hexane, 2-propanol, acetonitrile, and 1,2-dichloroethane purchased from Burdick and Jackson Labs (Muskegon, Mich.). Analytical grade ether was purchased from Mallinckrodt Inc. (Paris, Ky.), and acetic acid was purchased from Aldrich Chemical Co. (Milwaukee, Wis.).

Microsomes were prepared from the livers of male CD rats (300 g) supplied by Charles River Breeding Labs (Wilmington, Mass.). Liver homogenates (20% w/v) were prepared in 0.25 M phosphate buffer (pH 7.4) containing 1.15% KCl. The homogenates were centrifuged at 9000 x g for 20 minutes, and the pellet was discarded. The supernatant was centrifuged at 105,000 x g for one hour and the pellet from 1.0 gram of liver was resuspended in 1.0 ml of 0.1 M phosphate buffer (pH 7.4). Incubation mixtures contained 50 nmoles of each [1-^{14}C] substrate

and microsomes (approximately 0.5 mg protein) in 1.0 ml of 0.1 M phosphate buffer (pH 7.4). The reaction was initiated by the addition of 1.0 umoles of NADPH. After 10 minutes incubation at 37°C, the reaction was stopped by the addition of 0.25 ml of 4.0 N HCl. The hydroxylated products and substrates were extracted with 5.0 ml ether, which was removed and evaporated to dryness under N_2 in an ice bath. By this procedure over 99% of the total radioactivity was recovered in the ether extracts for each of the fatty acids studied.

The dried samples were redissolved in 0.175 ml of the mobile phase (2-propanol:hexane:acetic acid/2.5:96.5:1.0), and 0.05 ml was injected for HPLC analysis. The HPLC analyses were performed on a Waters Model 720 system controller, a Model 710B Autosampler, and two 6000A pumps. The column used in the separation was a 10 micron 3.9 x 30 cm μPorasil column (Waters Associates Inc. Milford, Mass.) at a flow rate of 1 ml/min. Detection of the radiolabelled substrates and products was performed on a Packard Model RAM 7500 radioactivity flow monitor (Downer Grove, Ill.) equipped with a 0.6 ml flow cell. The radioactive products from the HPLC analysis were collected using a Gilson Model 201 fraction collector (Villier Le Bel, Fr.). The trimethylsilyl (TMS) methyl esters of the hydroxylated fatty acids were prepared by first methylating the dried samples with diazomethane dissolved in ether at 80°C for 30 minutes. Next, the ether was evaporated, and the methylated

products were silylated with BSA (N,O-bis(Trimethylsilyl)acetamide; Supelco Inc., Houston, Tx.) at 80 °C for 1.0 hour for gas chromatographic/mass spectroscopic (GC/MS) analysis. The gas chromatograph was a Sichromat 2 Siemens (ES Indus., Marlton, N. J.) equipped with DB-17/DB-5 capillary columns (Supelco Inc., Houston, Tx.). The mass spectrometer was a 5970 Hewlett Packard equipped with a 9836C Hewlett Packard computer (Hewlett Packard Inc., Palo Alto, Calif.). The GC was programmed for a 3 minute hold at 100°C followed by a ramp to 280°C at 25°C per minute on the DB-17 column. At 5.5 minutes the flow was diverted to the DB-5 column for GC/MS analysis generated by electron impact (70eV) fragmentation. The TMS methyl ester of authenic 12-hydroxylaurate was prepared as described above and used as the reference standard.

RESULTS AND DISCUSSION

Incubation of each fatty acid with rat liver microsomes in the presence of NADPH resulted in the formation of two or more polar metabolites. The two most polar metabolites (labelled A and B in Figure 1) formed by the incubation system were selected for further characterization, because previous literature reports on the hydroxylation of fatty acids by microsomes from various sources indicate that the omega and omega-1 hydroxy-

lation products account for most, if not all, of the polar metabolites formed in vitro. The elution pattern for capric acid and its major metabolites A and B is shown in Figure 1. Approximately 11% of the capric acid was metabolized; 74% of the total metabolites formed were represented by metabolites A and B. Clear resolution of the two metabolites was observed with retention times of 16 and 21 minutes, respectively. Metabolites A and B of lauric acid had retention times of 13 and 17 minutes, respectively, and they accounted for 86% of the total radioactive metabolites formed (Figure 1). Interestingly, four metabolite peaks were observed for lauric acid while eight metabolite peaks were observed for capric acid. Extensive conversion of myristic acid to polar metabolites was evident as depicted in the radiochromatogram. Approximately 31% of the myristic acid was metabolized and 53% of the metabolites formed were represented by metabolites A and B with retention times of 11.5 and 15 minutes, respectively. The longer chain fatty acid, palmitic acid, and its metabolites A and B are shown in Figure 1. Approximately 14% of the substrate was metabolized, with metabolites A and B accounting for 71% of total radioactive metabolites formed. The retention times for metabolites A and B of palmitic acid were 11 and 14 minutes, respectively, somewhat shorter than those observed for the shorter chain hydroxylated metabolites. With stearic acid, only 7% of the total substrate was metabolized, and 30% of the products formed

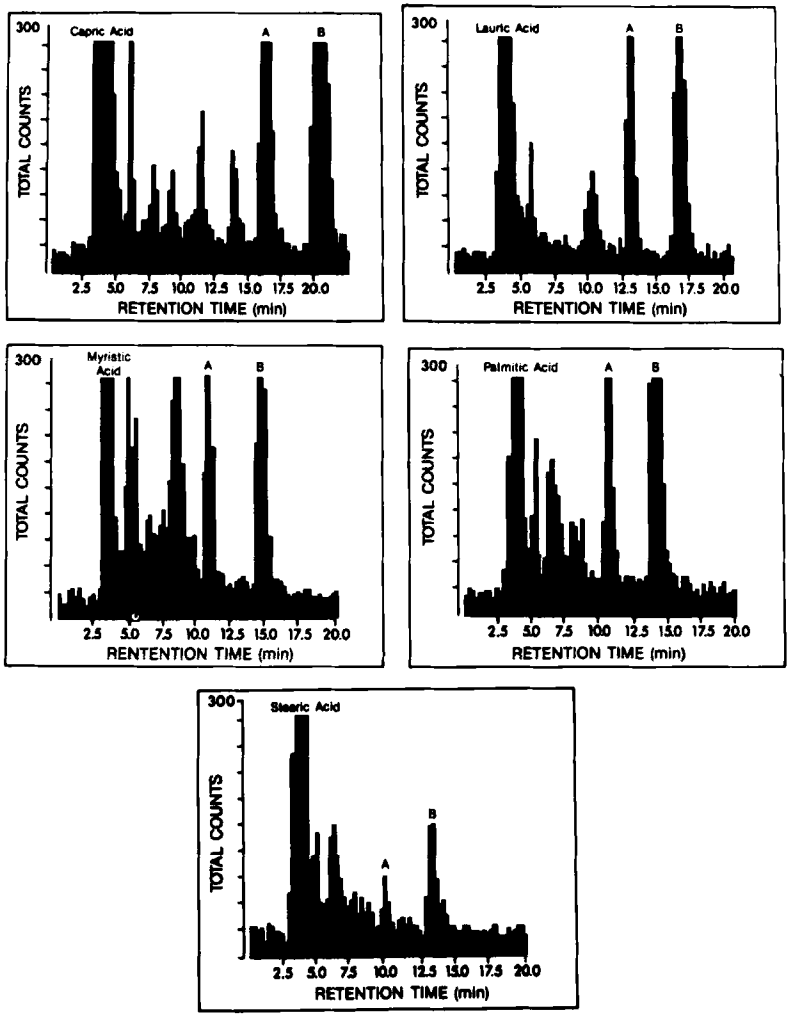

Figure 1. HPLC radiochromatograms of [1-^{14}C] Fatty Acids (C_{10}-C_{18}) and their metabolites formed by rat liver microsomes using conditions stated in the text.

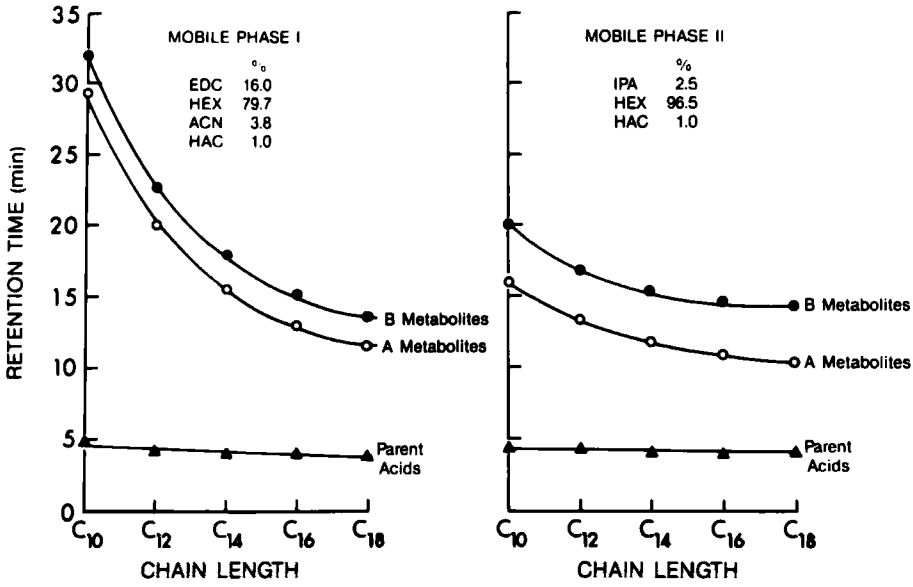

Figure 2. The effects of mobile phase composition on separation of C_{10}-C_{18} Fatty Acid metabolites.

could be accounted for by metabolites A and B, with retention times of 10 and 13.5 minutes, respectively.

During the course of this work two mobile phases were employed in an attempt to increase resolution. The relationship between retention time and chain length for the fatty acid metabolites A and B are illustrated in Figure 2. Mobile phase I resulted in poorer resolution even though retention times increased for both metabolites. Based on this finding, mobile phase II was selected for the separation of the A and B metabolites. Figure 2 also shows that the parent acids were not

selectively retained on the column (all of the fatty acids eluted within 5 minutes).

Metabolites A and B from some of the fatty acids were collected from the HPLC, and their TMS methyl esters were subjected to gas chromatographic and mass-spectral analyses. Their retention times, ion assignments and relative intensities are shown in Table 1. The fragmentation of the 12-hydroxylaurate standard yielded mass fragments at m/z 287, 271, 255 (M-47 and base peak), 159, 146, 103, 95, 89, 75, and 69. Likewise, metabolite B fractions from all of the fatty acids examined

Table 1

Mass Spectral Analysis of the TMS Methyl Ester of the Hydroxylated Products of C_{10}-C_{16} Fatty Acids

Metabolite	R.T.[1] minutes	Relative Abundance		
		M-$[CH_3]^+$	m/z 103	m/z 117
Capric Acid				
B	7.28	50.9	30.9	-
Lauric Acid				
A	8.00	3.4	-	100
B	8.45	37.5	16.1	-
Myristic Acid				
A	8.98	2.5	-	100
B	9.45	30.1	21.0	-
Palmitic Acid				
B	10.4	36.2	22.7	-

[1]Retention time from GC analysis.

yielded a base peak (M-47) as was observed with the standard. The m/z fragment at 103 is indicative of an omega hydroxy group for the TMS derivatives of the hydroxylated fatty acids. This fragmentation product, $[(CH_3)_3SiOCH_2]^+$, is a major ion found in all of the B metabolites as well as the standard. The mass spectra of the A metabolites were similar, with the base peak occurring at 117. The m/z 117 ion, $[(CH_3)_3SiOCHCH_3]^+$, is generally produced from omega-1 hydroxylated fatty acid TMS methyl esters (15,16).

Based on GC/MS data, the major metabolites formed from the C_{10}-C_{18} fatty acids are the omega (B metabolites) and omega-1 (A metabolites) hydroxylated products. Mobile phase II permitted good resolution of these products without the high flow rates as needed during reverse-phase HPLC analysis. In addition, sharp peak were achieved with this system, permitting improved quantitation even with low enzyme activity.

Table 2 shows the rates of omega and omega-1 product formation in the incubation system for C_{10}-C_{18} chain length fatty acids. The highest rate of omega and omega-1 product formation was observed with myristic acid, while comparable rates were observed for capric, lauric, and palmitic acids. Stearic acid was the least favorable substrate of those examined in this system. Interestingly, Ellin and Orrenius made similar observation when rat kidney microsomes served as an enzyme source (2). The product ratios for omega/ omega-1 hydroxylations ranged from

Table 2

Formation of Omega and Omega-1 Hydroxylated Products of Fatty Acids Using Rat Liver Microsomes

Fatty Acid	Chain Length	Sp. Activity (nmoles formed / 10 min x mg protein)		Ratio
		Omega-1	Omega	$\frac{\omega}{\omega-1}$
Capric	C_{10}	2.32 ± .14	6.81 ± .66	2.93
Lauric	C_{12}	3.05 ± .27	4.00 ± .41	1.31
Myristic	C_{14}	9.23 ± .24	15.40 ± 2.46	1.67
Palmitic	C_{16}	2.19 ± .16	5.90 ± .48	2.69
Stearic	C_{18}	-	1.24 + .10	-

Values represent means ± standard error of duplicate or triplicate assays for four animals.

1.31 for lauric acid to 2.93 for capric acid (Table 2). The values are comparable to those reported by Bjorkhem et al. (1).

This method has proved to be suitable for the routine determination of NADPH-dependent fatty acid hydroxylase activity in rat liver microsomes. We have identified major metabolites of C_{10}-C_{16} fatty acids to be omega and omega-1 hydroxylated products. Tamburini et al. (17) suggested that induction of omega hydroxylase activity is linked to the pharmacology and

toxicology of clofibrate and other hypolipidemics. The development of a versatile and rapid HPLC system for the separation of major metabolites of C_{10}-C_{18} fatty acids may assist in establishing the relationship between omega hydroxylation and hypolipidemia.

ACKNOWLEDGEMENT

The authors thank Mr. Thurman Allsup for his technical assistance in performing the GC/MS analysis.

REFERENCES

1. Björkhem, I. and Danielsson, H., Ω- and (Ω-1)-Oxidation of Fatty Acids by Rat Liver Microsomes, Eur. J. Biochem., 17, 450, 1970.

2. Ellin, Å. and Orrenius, S., Fatty Acid Hydroxylation in Rat Kidney Cortex Microsomes, Mol. Cell. Biochem., 8, 69, 1975.

3. Parker, G. L. and Orton, T. C., Biochemistry, Biophysics, and Regulation of Cytochrome P-450, Gustaffson, J. A., Duke, J. C., Mode, A., and Raffer, J., eds., Elsevier/ North Holland Biomedical Press, Amsterdam/New York, 1980, p. 373.

4. Fan, L. L., Masters, B. S. S., and Prough, R. A., Microsomal Lauric Acid 11- and 12-Hydroxylation: A new assay method utilizing high pressure liquid chromatography, Anal. Biochem., 71, 265, 1976.

5. Orton, T. C. and Parker, G. L., The Effect of Hypolipidemic Agents on the Hepatic Microsomal Drug-Metabolizing enzyme system of the Rat: Induction of cytochrome(s) P-450 with specificity toward terminal hydroxylation of lauric acid, Drug Metab. Dispos., 10, 110, 1982.

6. Bandi, Z. L. and Reynolds, E. S., Adsorption and Reverse-Phase High-Performance Liquid Chromatography of p-Nitrobenzyl Esters of Monohydroxy Fatty Acids, J. Chrom., 329, 57, 1985.

7. Israelsson, U. Hamberg, M., and Samuelsson, B., Biosynthesis of 19-Hydroxy-Prostaglandin A_1, Eur. J. Biochem., 11, 390. 1969.

8. Kupfer, D., Navarro, J., Miranda, G. K., Piccolo, D. E., and Theoharides, A., Studies on the Oxidation of Ω-Hydroxy-prostaglandins by an NAD-Dependent Dehydrogenase from Mammalian Liver Cytosol, Arch. Biochem. Biophys., 199, 228, 1980.

9. Holm, K. A., Engell, R. J., and Kupfer, D., Regioselectivity of Hydroxylation of Prostaglandins by Liver Microsomes Supported by NADPH versus H_2O_2 in Methylcholanthrene-Treated and Control Rats: Formation of novel prostaglandin metabolites, Arch. Biochem. Biophys., 237, 477, 1985.

10. Shak, S. and Goldstein, I. M., Carbon Monoxide Inhibits Ω-Oxidation of Leukotriene B_4 by Human Polymorphonuclear Leukocytes: Evidence that catabolism of leukotriene B_4 is mediated by a cytochrome P-450 enzyme, Biochem. Biophys. Res. Commun., 123, 475, 1984.

11. Newton, J. F., Eckardt, R., Bender, P. E., Leonard, T., and Straub, K., Metabolism of Leukotriene B_4 in Hepatic Microsomes, Biochem. Biophys. Res. Commun., 128, 733, 1985.

12. Samuelsson, B., Granstrom, E., Green, K., and Hamberg, M., Metabolism of Prostaglandins, Ann. N. Y. Acad. Sci., 180, 138, 1971.

13. Björkhem, I., On the Mechanism of Regulation of Ω-Oxidation of Fatty Acids, J. Biol. Chem., 251, 5259, 1976.

14. Kam, W., Kumaran, K., and Landau, B. R., Contribution of Ω-Oxidation to Fatty Acid Oxidation by Liver of Rat and Monkey, J. Lipid Res., 19, 591, 1978.

15. Shoun, H., Sudo, Y., and Beppu, T., Subterminal Hydroxylation of Fatty Acids by a Cytochrome P-450-Dependent Enzyme System from a Fungus, Fusarium oxysporum, J. Biochem., 97, 755, 1985.

16. Albro, P. W., Chae, K., Philpot, R., Corbett, J. T., Schroeder, J., and Jordan, S., In Vitro Metabolism of Mono-2-ethylhexyl Phthalate by Microsomal Enzymes: Similarity to Ω- and (Ω-1) oxidation of fatty acids, Drug Metab. Disp., 12, 742, 1984.

17. Tamburini, P. P., Masson, H. A., Bains, S. K., Makowski, R. J., Morris, B., and Gibson, G. G., Multiple Forms of Hepatic Cytochrome P-450: Purification, characterisation and comparison of a novel clofibrate-induced isozyme with other major forms of cytochrome P-450, Eur. J. Biochem., 139, 235, 1984.