Separation and Quantitation of Hydroxy and Epoxy Fatty Acids by High-Performance Liquid Chromatography with an Evaporative Light-Scattering Detector

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A new high-performance liquid chromatography technique with an evaporative light-scattering detector (ELSD) has been developed for the separation and quantitative analysis of hydroxy and epoxy fatty acids. This method employs a gradual binary gradient (hexane/isopropanol) and ELSD detection. The minimum limit of detection is about 1 μg and ratio of mass to signal is essentially linear in the range of 10 to 200 μg. This high-performance liquid chromatography (HPLC) technique is able to separate various positional isomers of monohydroxy and dihydroxy fatty acids and can also discriminate between monohydroxy, epoxy, epoxyhydroxy, dihydroxy and trihydroxy fatty acids.

KEY WORDS: Dihydroxy fatty acids, epoxy fatty acids, evaporative light-scattering detector, fatty acids, lipids, normal phase HPLC, ricinoleic acid, trihydroxy fatty acids.

Hydroxy and epoxy fatty acids are quite abundant in many plants, animals, and microbes. Castor oil is a triacylglycerol with approximately 90% of its fatty acids comprised of ricinoleic acid (1). The seeds of certain other plants have been shown to contain triacylglycerols that contain high levels of epoxy fatty acids (2) or a mixture of epoxy and hydroxy fatty acids (3). Plants and animals contain sphingolipids such as cerebrosides with a high proportion of hydroxylated fatty acids (4). The aerial surfaces of plants are covered with cutin, which is a complex polymer composed mainly of hydroxy and epoxy fatty acids (5). Certain species of bacteria have also been shown to convert oleic acid to monohydroxy and dihydroxy fatty acids (6), a feature being investigated for possible industrial utilization.

Several methods for the separation of hydroxy fatty acids by high-performance liquid chromatography (HPLC) have been published (7–15). Unfortunately, the existing techniques for quantitative analysis of hydroxy fatty acids by HPLC are quite laborious and often not accurate. Gas chromatography has been used, but it usually requires methylation and/or acetylation or trimethylsylilation. Thin-layer chromatography has been used but is primarily a qualitative technique. To improve the sensitivity of detection of hydroxy fatty acids in a reversed-phase HPLC system, some authors have prepared derivatives that are detectable by ultraviolet (UV) (7–9) or fluorescence (10) detectors, but the use of these derivatives requires extensive knowledge of their reactions and can interfere with the HPLC analyses (11).

Refractive index detection (12,13) and UV detection at 210 nm (14) or 205 nm (H.C. Gerard and R.A. Moreau, unpublished data) in a normal-phase HPLC system have been tested but they are not quantitative. Radiolabelled molecules have been used in a reverse-phase HPLC system for the separation and detection of hydroxy fatty acids (15), but the individual peaks were not identified.

In this paper, we describe a method for the separation and quantitation of various monohydroxy, epoxy, dihydroxy and trihydroxy fatty acids by normal-phase HPLC with an evaporative light-scattering detector (ELSD).

MATERIALS AND METHODS

Chemicals. All HPLC-grade solvents (hexane, 2,2,4-trimethylpentane, chloroform, methanol, isopropanol) were obtained from Burdick and Jackson (Muskegon, MI). N-methyl-N′-nitro-N-nitrosoguanidine, octadecanoic acid, hexadecanoic acid, 16-hydroxyhexadecanoic acid, ricinoleic acid, 9,10-epoxyoctadecanoic acid, stearyl alcohol and hexadecanedioic acid were obtained from Sigma Chemical Co. (St. Louis, MO) and 1,16-dihydroxyhexadecane was purchased from Aldrich Chemical Co. (Milwaukee, WI). Monohydroxy fatty acids (2-, 4-, 10-, and 12-hydroxyoctadecanoic acids) and other hydroxy compounds (9,10-dihydroxyeicosane, 9,10-dihydroxyoctadecanoic acid, 9,10,12-trihydroxyoctadecanoic acid) were gifts from Dr. Dan Schwartz, Eastern Regional Research Center, Philadelphia, PA.

Preparation of methyl esters. Methyl esters of hydroxy fatty acids were prepared by removing the solvents with a stream of nitrogen and adding several drops of cold diazomethane (N-methyl-N′-nitro-N-nitrosoguanidine) dissolved in hexane (16). After incubating the samples for 10–15 min at 0–4°C, excess reagent was removed with a stream of nitrogen.

Standard HPLC protocol. Lipid analyses were performed with a Hewlett-Packard Model 1050 HPLC fitted with a gradient programmer and an autosampler. All studies were performed with a Chrompack ChromSep 7-micron LICHROSORB Si 60 silica cartridge system (10 cm × 3.0 mm) (Chrompack, Raritan, NJ) at a flow rate of 0.5 mL/min. Other columns (Chrompack ChromSep 7-micron LICHROSORB DIOL and Chrompack ChromSep 7-micron SPHERISORB CN cartridges, 10 cm × 3.0 mm) were tested and considered inferior for the separation of hydroxy components. A Varex Universal Evaporative Light-Scattering Detector (ELSD) (Rockville, MD) was operated at a temperature of 40°C with nitrogen (25 psi) as a nebulizing gas. Free fatty acids or their corresponding methyl esters were dissolved in 1 mL of chloroform/methanol (85/15) and analyzed with a binary-gradient hexane/acetic acid/isopropanol mixture (Table 1). Samples of various hydroxy and epoxy fatty acids were injected without any further preparation (no esterification or derivatization).

Initial attempts to separate the various hydroxy and epoxy fatty acids employed a ternary gradient (iso-

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TABLE 1

Mobile-Phase Gradient for Separation of Hydroxy Components on a Chrompack Chromsep 7-micron LiCHROSORB Si 60 silica column (10 cm × 3.0 mm)

Time (min)	Composition of mobile phase, % (v/v)		Isopropanol concentration
	Eluant A[a]	Eluant B[b]	
0	99	1	0.70
3	97.5	2.5	0.99
16	80	20	4.40
33	40	60	12.20
39	0	100	20
42	99	1	0.70
50	99	1	0.70

[a]Eluant A: hexane/isopropanol/acetic acid (99.3%:0.5%:0.2%).
[b]Eluant B: hexane/isopropanol/acetic acid (79.8%:20%:0.2%).

octane/isopropanol/water) described by Moreau *et al.* (17), which was similar to that described by Hennion *et al.* (14) for the separation of hydroxy fatty acids by HPLC with a binary gradient. With this gradient (16), most of the various hydroxy and epoxy fatty acids eluted together in a single peak with a retention time of 21 min.

By adding acetic acid to the binary gradient system, and by using conditions similar to those of Veazey (18), separation of monohydroxy, epoxy, and dihydroxy fatty acids was achieved, but trihydroxy fatty acid was not eluted. Trihydroxy fatty acid was eluted when the level of isopropanol at the end of the gradient was increased to 20% (Table 1). By modifying the gradient shape and duration, separation of all of these components was achieved.

RESULTS AND DISCUSSION

After trying different types of columns (Chrompack ChromSep 7-micron LICHROSORB DIOL and Chrompack ChromSep 7-micron SPHERISORB CN), gradient shapes, and various combinations of solvents (*e.g.*, butanol or *iso*-amylalcohol instead of isopropanol, cyclohexane or 2,2,4-trimethylpentane instead of hexane), various acids (*e.g.*, sulfuric acid or formic acid) and acetic acid concentrations (from 0.01% to 0.4%), the gradient system presented in Table 1 was adopted as optimal for this HPLC system.

Hexane can be replaced with either cyclohexane or 2,2,4-trimethylpentane. However, isopropanol was essential; addition of *iso*-amylalcohol or n-butanol worsened the separation. The nature and concentration of the acid were important; acetic acid could be replaced with formic acid but not with sulfuric acid. For optimum separation, the carboxyl group must be in the free acid form. This gradient system allowed separation of all monohydroxy fatty acids tested as well as all classes of epoxy, dihydroxy, and trihydroxy fatty acids with retention times between 3.0 min and 32.5 min (Table 2).

Fatty acids and monohydroxy fatty acids. The retention times of hexadecanoic acid and octadecanoic acid were similar (2.5 and 2.7 min, respectively). Separation of various monohydroxy fatty acids, which differed only in the position of their hydroxyl groups (2-, 4-, 10-, and 12-hydroxyoctadecanoic acid), was also achieved (Fig. 1). Thirteen minutes separated 2-hydroxy- and 12-hydroxyoctadecanoic acids. Figure 2 shows the concentration of isopropanol necessary to elute each hydroxy fatty acid as a function of the position of the hydroxyl group on the octadecanoic acid and indicates a linear relationship. In

TABLE 2

Retention Time of Lipid Standards on a Chrompack ChromSep 7-Micron LICHROSORB Si 60 Silica Column (10 cm × 3.0 mm)

Compound	Retention time (min)	
	Free acids	Methyl esters
Fatty acids		
hexadecanoic acid	2.5	1.1
octadecanoic acid	2.7	
Monohydroxy fatty acids		
2-hydroxyoctadecanoic acid	2.9	
4-hydroxyoctadecanoic acid	8.9	
10-hydroxyoctadecanoic acid	12.3	
12-hydroxyoctadecanoic acid	15.6	6.3
16-hydroxyhexadecanoic acid	16.0	
9Δ-12-hydroxyoctadecenoic acid[a]	9.2	5.9
Epoxy fatty acids		
9,10-epoxyoctadecanoic acid	5.7	2.8
9,10-epoxy-12-hydroxyoctadecanoic acid	12.1	5.9
Di- and trihydroxy fatty acids		
9,10-dihydroxyoctadecanoic acid	21.8	
9,10,12 trihydroxyoctadecanoic acid	32.5	
Other related compounds		
1,16-dihydroxyhexadecane	23.8	
9,10-dihydroxyeicosane	11.6	
stearyl alcohol	7.5	
hexadecanedioic acid	10.0	

[a]Ricinoleic acid.

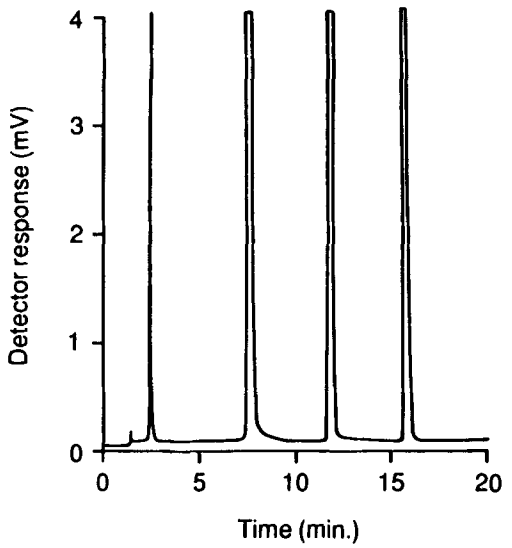

FIG. 1. High-performance liquid chromatogram of a mixture of 10 μg each of 2-hydroxy (2.9 min), 4-hydroxy (8.9 min), 10-hydroxy (12.3 min) and 12-hydroxyoctadecanoic acids (15.6 min). The chromatographic conditions are described in Table 1.

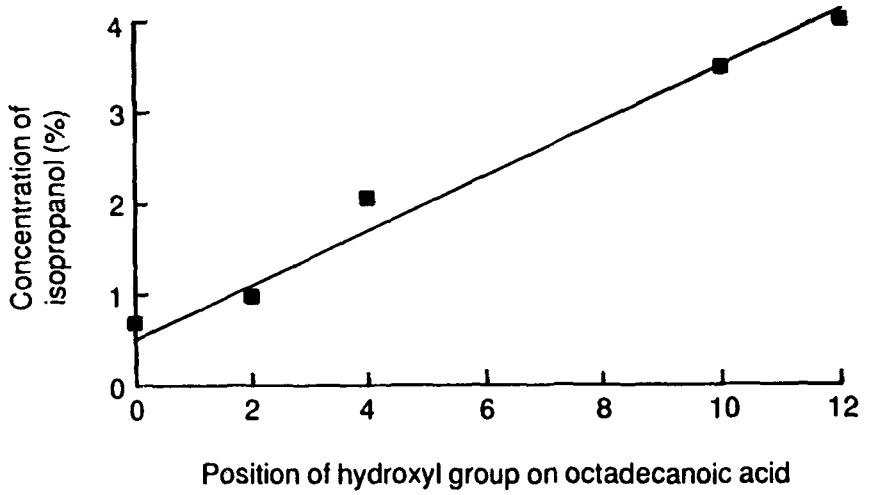

FIG. 2. Relationship between the percentage of isopropanol (at the moment of elution) and the position of the hydroxyl group on octadecanoic acids separated on a Chrompack ChromSep 7-micron LICHROSORB Si 60 silica column (10 cm × 3.0 mm).

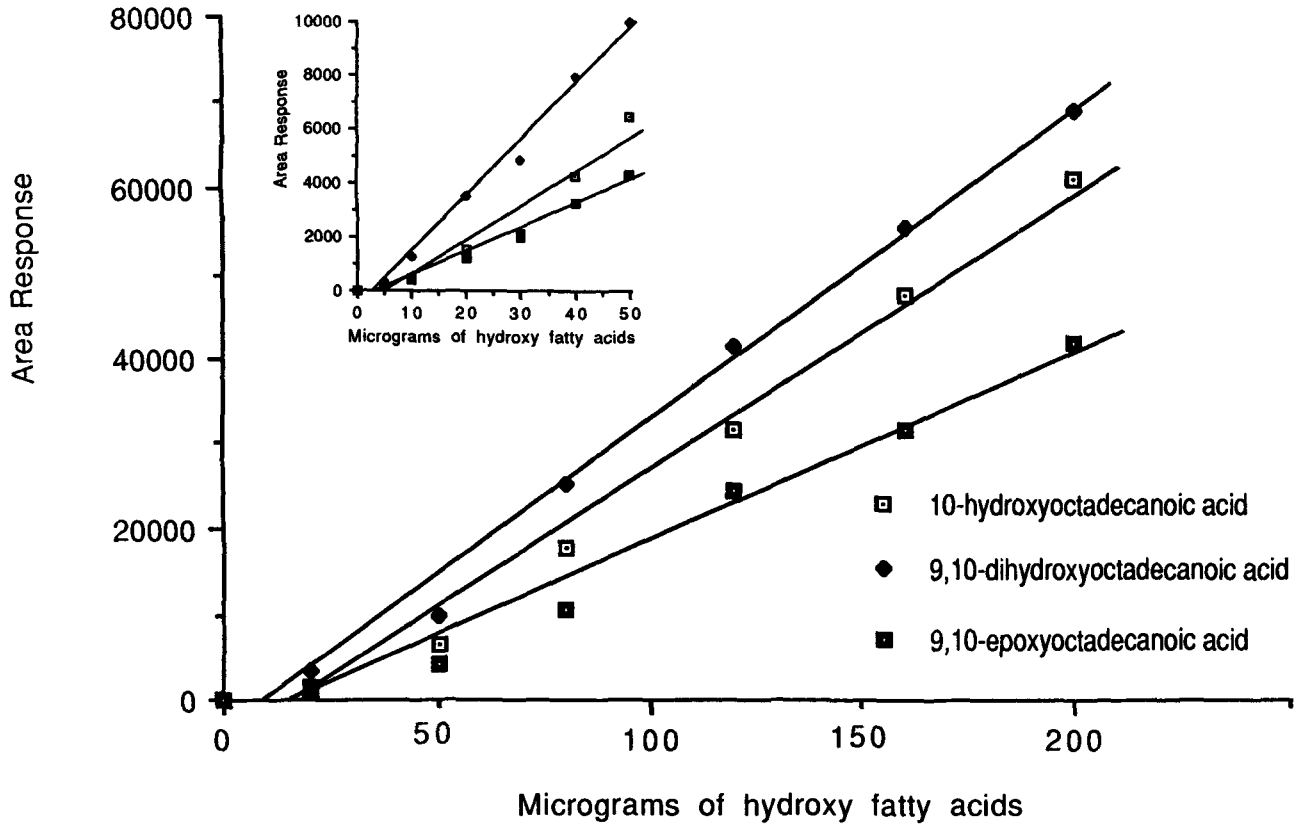

FIG. 3. Response curves for 10-hydroxy-, 9,10-epoxy-, and 9,10-dihydroxyoctadecanoic acid with an ELSD on a Chrompack ChromSep 7 micron LICHROSORB Si 60 silica column (10 cm × 3.0 mm). In the range of 20 μg to 200 μg, correlation coefficients for 10-hydroxy-, 9,10-epoxy-, and 9,10-dihydroxy- were 0.92, 0.95 and 0.96 respectively.

this separation, interactions between silica and hydroxyoctadecanoic acid were stronger the farther the hydroxyl group was from the carboxyl group. It should be possible to predict the retention time of other (unknown) hydroxyoctadecanoic acids along this curve. The retention times of the corresponding methyl esters were each several minutes shorter.

Di-, trihydroxy, and epoxy fatty acids. This gradient system was successful in separating mono-, di- and trihydroxy fatty acids. The separation of 10-hydroxy-, 9,10-dihydroxy- and 9,10,12-trihydroxyoctadecanoic acids was achieved with retention times of 12.3, 21.8 and 32.5 min, respectively. The retention times of two epoxy fatty acids indicate that an epoxy group reduces the Rf by about 50% relative to that of the corresponding hydroxyl group at the same position on the chain (Table 2).

Quantitation of hydroxy fatty acids. In order to use this HPLC method to perform quantitative analysis, it was necessary to construct standard curves showing the relationship between peak area and mass. Data points represent the mean of triplicate determinations for each sample. Standard curves for 10-hydroxy, 9,10-epoxy, and 9,10-dihydroxyoctadecanoic acids were obtained in the range of 5 μg to 200 μg (Fig. 3). The response curves of the dihydroxy and epoxy fatty acids were linear in the range of 10–200 μg, whereas the curve of the monohydroxy fatty acid was less linear. The minimum limits of detection were about 1 μg. From 1–10 μg, the ratio of peak area to mass was nonlinear for all three components. This method of analysis could be useful for the separation and quantitation of various hydroxy fatty acids found in plants, animals and microbes.

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REFERENCES

1. Lide, D.R., in *CRC Handbook of Chemistry and Physics, 71st edition*, CRC Press Inc., Boston, MA, 1990, pp. 7–29.
2. Ayorinde, F.O., B.D. Butler and M.T. Clayton, *J. Am. Oil Chem. Soc. 67*:844 (1990).
3. Chaudhry, A., R. Kleiman and K.D. Carlson, *Ibid. 67*:863 (1990).
4. Cahoon, E.B., and D.V. Lynch, *Plant Physiol. 95*:58 (1991).
5. Kolattukudy, P.E., in *The Biochemistry of Plants, Vol. 4*, edited by P.K. Stumpf, and E.E. Conn, Academic Press, New York, NY, 1980, p. 571.
6. Hou, C.T., M.O. Bagby, R.D. Plattner and S. Koritala, *J. Am. Oil Chem. Soc. 68*:99 (1991).
7. Jordi, H.C., *J. Liquid Chromatogr. 1*:215 (1978).
8. Russel, N.E., and M.A. Miller, *Ibid. 2*:697 (1979).
9. Bandi, Z.L., and A.S., Ansari, *J. Chromatogr. 363*:402 (1986).
10. Lloyd, J.B.F., *Ibid. 178*:249 (1979).
11. Bandi, Z.L., and E.S. Reynolds, *Ibid. 329*:57 (1985).
12. Henke, H., and J. Schubert, *J. High Resolut. Chromatogr. Commun. 3*:69 (1980).
13. Henke, H., and W. Dietrich, *Ibid. 3*:69 (1980).
14. Hennion, M.C., J.C. Thieblemont, R. Rosset, P. Scribe, J.C. Marty and A. Sallot, *J. Chromatogr. 280*:351 (1983).
15. Grechkin, A.N., T.E. Gafarova, O.S. Korolev and R.A. Kuramshin, in *Biological Role of Plant Lipids*, edited by P.A. Biacs, K. Gruiz and T. Kremmer, Plenum Press, New York, NY, and Akademiai Kiado, Budapest, Hungary, 1989, p. 83.
16. Baker, E.A., M.J. Bukovac and G.M. Hunt, in *The Plant Cuticle, Vol. 10*, edited by D.F. Cutler, K.L. Alvin and C.E. Price, Academic Press, New York, NY, 1982, p. 33.
17. Moreau, R.A., P.T. Asmann and H.A. Norman, *Phytochemistry 29*:2461 (1990).
18. Veazey, R.L., *J. Am. Oil Chem. Soc. 63*:1043 (1986).

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