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Short Communication

Quantitation of phenacyl esters of retinal fatty acids by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method for the separation and quantitation of retinal fatty acids containing long-chain polyunsaturated fatty acids is described. Fatty acids from frog retinal lipids were converted to the corresponding phenacyl derivatives which were separated on a C_{18} reversed-phase column and detected at 242 nm. Molar absorptivities (peak area units/nmol) of up to seventeen fatty acid phenacyl derivatives were determined and used for quantitation of fatty acids separated by HPLC. Compared with gas chromatography, the HPLC method gave a similar molar percent distribution of the fatty acids and was twenty to fifty times more sensitive. This HPLC method provides a useful means for the study of chemistry and metabolism of long-chain polyunsaturated fatty acids in retina and other tissues where amounts of material may be limited or recovery of individual components desirable.

INTRODUCTION

The retina and other neural tissues are highly enriched in long-chain polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid (22:6, $n-3$) [1,2]. Docosatetraenoic acid (22:4, $n-6$), docosapentaenoic acid (22:5, $n-3$ and 22:5, $n-6$), and C-24 PUFAs are also present in retinal lipids [1,3]. Recent studies by Voss *et al.* [4] indicate that these C-22 and C-24 PUFAs are metabolically linked. Therefore, separation and quantitation of these and other PUFAs are essential for the study of their synthesis and turnover. Gas chromatography (GC) is used widely for fatty acid analysis and has the advantage of providing high resolution. However, GC detection destroys fatty acids and does not allow for on-line measurement of radioactivity or recovery of material for further analysis. Various high-performance liquid chromatographic (HPLC) techniques with less resolution but higher sensitivity (compared to GC) have been developed for fatty acid analysis [5]. Fatty acids derivatized with ultraviolet-absorbing or fluorescent chromophores can

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be quantified by absorption spectroscopy [5–10]. However, none has been used to separate and quantify fatty acids derived from a tissue containing large amounts of long-chain PUFAs.

In the present report, we describe an HPLC method adapted from that of Wood and Lee [11] for separation and quantitation of phenacyl derivitives of retinal fatty acids, which contain high levels of long-chain PUFAs. The molar absorptivities of individual fatty acid phenacyl esters (FAPEs) were determined, which enabled the conversion of the detector response (peak areas) to the mass units of fatty acids.

EXPERIMENTAL

Reagents

Unless stated otherwise, all the organic solvents were HPLC grade and obtained from Fisher (Pittsburgh, PA, USA).

Preparation of free fatty acids

Retinas and retinal pigment epithelial cells from *Rana pipiens* (J. M. Hazen, Alburg, VT, USA) were isolated under dim red light. Total lipids were extracted with chloroform–methanol (2:1, v/v) two times as described by Bligh and Dyer [12] and washed once by the method of Folch *et al.* [13]. An aliquot of the extracted lipids was dried under nitrogen, immediately suspended in 2 ml of 2.0% potassium hydroxide in ethanol, and saponified at 100°C for 30 min. After cooling to room temperature, 1 ml of water and 100 μl of concentrated hydrochloric acid were added. The released free fatty acids were extracted twice with 2 ml of hexane.

Preparation of FAPEs

Phenacyl esters were prepared as described by Wood and Lee [11]. The isolated free fatty acids (about 3 μmol) were dried under nitrogen and resuspended in 100 μl of 2-bromoacetophenone (10 mg/ml in acetone, Sigma, St. Louis, MO, USA). After vortex-mixing 100 μl of triethylamine (10 mg/ml in acetone, Sigma) were added. The mixture was sealed immediately in a PTFE-lined screw-capped glass tube and heated for 5 min in a boiling water bath. After cooling, 160 μl of acetic acid (2 mg/ml in acetone) were added and the tube was heated for an additional 5 min. The resulting FAPEs were filtered through a 0.45-μm microfilter tube (Rainin, Woburn, MA, USA), dried under nitrogen, and redissolved in 120 μl of methanol for HPLC injection.

HPLC analysis of FAPEs

FAPE analysis was carried out using a Spectra-Physics SP8800 high-performance liquid chromatograph (San Jose, CA, USA) attached to a Waters 715 Ultra WISP sample processor (Waters Assoc., Milford, MA, USA) and a Kratos Spectroflow 773 absorbance detector (Westwood, NJ, USA). Separation was achieved at room temperature on an LC-18 Supelco reversed-phase column (particle size 5 μm; 25 cm × 4.6 mm I.D.) coupled with a Pelliguard LC-18 2 cm × 4.6 mm I.D. guard column (Supelco, Bellefonte, PA, USA). The FAPEs were eluted at a flow-rate of 2 ml/min with a linear gradient for 50 min starting with acetonitrile–water (80:20, v/v) to acetonitrile–water (92:8, v/v) and a 10-min recovery to the starting solvent. The absorbance of the FAPEs was measured at 242 nm and peak areas were integrated using a Spectra-Physics 4270 integrator equipped with the WINner/286 software (San Jose, CA, USA).

Fractions from the HPLC column were collected during each run for identification and quantitation of peaks. FAPEs in HPLC fractions were extracted twice with one volume of hexane and evaporated to dryness under nitrogen for GC analysis.

GC analysis of fatty acid methyl esters (FAMEs)

Methyl esters of fatty acids from individual HPLC fractions and total retinal FAPEs were prepared by heating at 100°C for 2 h in 1 ml of anhydrous methanol containing 2.5% of H_2SO_4 (v/v). Known amounts of the internal standards heptadecanoic acid (17:0) and heneicosanoic acid (21:0) were added prior to methylation. The resulting FAMEs were extracted twice with 2 ml of hexane. The combined hexane extracts were washed once with water, dried under nitrogen,

and dissolved in a small volume of nonane (Aldrich, Milwaukee, WI, USA) for GC analysis.

GC analysis was performed using a Varian 3500 gas–liquid chromatograph (Walnut Creek, CA, USA) equipped with a DB-225 capillary column (30 m × 0.25 mm I.D.; J. & W. Scientific, Folsom, CA, USA) and a 8100 autosampler. The column temperature was programmed to raise from 130 to 160°C at a rate of 10°C/min, from 160 to 220°C at a rate of 1.0°C/min, and hold at 220°C for 2 min. Helium carrier gas flowed at a linear velocity of 70 cm/s. Hydrogen flame detector and injector post-injection temperatures were maintained at 270 and 250°C, respectively. Methyl ester samples in nonane were automatically introduced on-column at 130°C. The chromatographic peaks were integrated as area units by a Spectra-Physics 4270 integrator and further processed with the WINner/286 software. FAMEs were identified by comparison with authentic standards and quantitated by comparison of the relative area units with 17:0 and 21:0 as internal standards.

RESULTS AND DISCUSSION

Fatty acids from frog retinal tissues were converted to phenacyl derivatives and separated by a reversed-phase HPLC column with acetonitrile–water as mobile phase. The identity of each peak was determined by collecting each one and analyzing the fatty acids released after methanolysis by GC. A representative elution profile of retinal FAPEs is presented in Fig. 1. Some of the peaks were completely resolved and had a single fatty acid component. The elution sequence of retinal FAPEs was similar to that of methyl esters from mouse brain phospholipids [14], which was dependent on the molecular structure of fatty acids including chain length, saturation, position of double bond, and isomeric configuration (*cis* or *trans*) as demonstrated previously [5–11]. Fatty acids with shorter chain length or more double bonds had the shortest elution times. Pairs like 16:1, $n-7$ and 16:1, $n-9$ (peaks 6 and 7), 22:5, $n-3$ and 22:5, $n-6$ peaks 8 and 10), and 24:5, $n-3$ and 24:5, $n-6$ (minor components of peaks

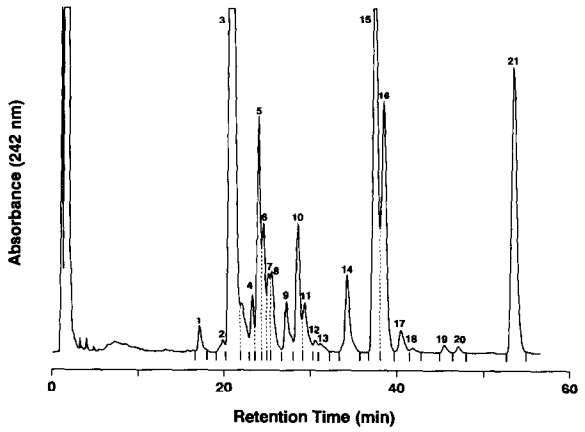

Fig. 1. HPLC separation profile of fatty acid phenacyl esters from retinal tissues. Frog retinal lipids were saponified and the resulting free fatty acids were phenacyl-derivatized. About 100 nmol of FAPEs were injected into the reversed-phase column and eluted with a gradient of acetonitrile–water at a flow-rate of 2 ml/min. Absorbance was monitored at 242 nm. Peaks: 1 = 20:5, $n-3$; 2 = 18:3; 3 = 22:6, $n-3$; 4 = 14:0; 5 = 20:4, $n-6$; 6 = 16:1, $n-7$; 7 = 16:1, $n-9$; 8 = 22:5, $n-3$; 9 = 18:2, $n-6$; 10 = 22:5, $n-6$; 11 = 24:6, $n-3$; 12 = 20:3, $n-6$; 13 = unknown; 14 = 22:4, $n-6$; 15 = 16:0 and 24:5, $n-3$; 16 = 18:1-*cis* and 24:5, $n-6$; 17 = 18:1-*trans*; 18 and 19 = unknown; 20 = 24:4, $n-6$; 21 = 18:0. Fatty acid nomenclature used in the text: the first number denotes the carbon chain length, the second denotes the number of double bonds, and the number after the *n* specifies the position of the first double bond from the methyl end.

15 and 16, respectively) also eluted at different times. In addition, the *cis*-isomer of 18:1 (peak 16) eluted before the corresponding *trans*-isomer (peak 17). Although some of the peaks were not baseline-resolved, this did not prevent their quantitation.

The relationship between the amounts of individual FAPEs and corresponding detector response (area units) was determined by injecting various amounts of retinal FAPEs (total 25, 50, 100, 200, 400, 800 nmol). Each HPLC peak during each run was collected and the fatty acids were quantitated by GC analysis. The molar absorptivities (peak area units/nmol) were calculated by linear regression analysis, either computing the *y*-intercept or forcing it to zero (Table I). Both gave the same degree of linearity indicated by the correlation coefficients (r^2) and the same molar absorptivities over the concentration rang-

TABLE I

MOLAR ABSORPTIVITIES OF VARIOUS FATTY ACID PHENACYL ESTERS FOR HPLC QUANTITATION

Peak No.	Fatty acid[a]	Absorptivity[b] (AU/nmol × 10^{-5})	r^2 [c]	Range (nmol)	Distribution[d] (%)	n[e]
1	20:5, $n-3$	11.34 ± 0.16	0.998	1.1–5.6	100 ± 0	3
2	18:3	12.56 ± 0.42	0.990	0.2–2.3	77 ± 6	4
3	22:6, $n-3$	10.47 ± 0.04	1.000	0.1–20.4	100 ± 0	4
4	14:0	7.70 ± 0.25	0.989	0.4–14.3	93 ± 1	6
5	20:4, $n-6$	7.68 ± 0.17	0.996	4.5–75.0	90 ± 4	5
6	16:1, $n-7$	7.97 ± 0.20	0.994	0.7–17.4	81 ± 7	6
7	16:1, $n-9$	6.01 ± 0.15	0.994	0.7–21.3	84 ± 4	6
8	22:5, $n-3$	12.69 ± 0.15	0.999	0.4–17.4	67 ± 2	6
9	18:2, $n-6$	9.42 ± 0.12	0.998	0.4–16.4	99 ± 1	6
10	22:5, $n-6$	8.70 ± 0.19	0.996	0.9–40.1	98 ± 1	6
11	24:6, $n-3$	19.75 ± 0.24	0.999	0.2–8.3	81 ± 5	6
12	20:3, $n-6$	10.00 ± 0.28	0.993	0.1–3.3	85 ± 9	6
14	22:4, $n-6$	9.25 ± 0.09	0.999	0.6–27.1	96 ± 2	6
15	16:0 (98%) 24:5, $n-3$ (2%)	6.34 ± 0.10	0.997	5.1–191	94 ± 3	6
16	18:1 (97%) 24:5, $n-6$ (3%)	6.81 ± 0.06	0.999	3.0–128	95 ± 4	6
20	24:4, $n-6$	8.19 ± 0.11	0.999	0.1–2.2	100 ± 0	5
21	18:0	6.14 ± 0.04	1.000	4.3–39.1	100 ± 0	4

[a] Fatty acids were characterized by capillary GC analysis.

[b] Molar absorptivities (peak area units/nmol) were determined by injecting discrete amounts of retinal FAPEs. The amounts of fatty acids were quantified by GC analysis of the collected peaks. Molar absorptivities of FAPEs were calculated using linear regression with the y-intercept forcing to zero. In the calculation, the small amounts of fatty acids eluted before or after the main peaks were added to the major ones. The ultraviolet detector (Kratos Spectroflow 773) was set at 242 nm and the absorption range was at 0.01. Data are expressed as mean ± S.D.

[c] Linear regression correlation coefficient over the range given in the table.

[d] Percent distribution of fatty acids in the peaks. Data are expressed as mean ± S.D.

[e] Number of observations.

es investigated (up to 191 nmol for 16:0). Shorter-chain saturated fatty acids, which eluted earliest, had higher molar absorptivities than the longer-chain saturated fatty acids, as illustrated by 14:0 (peak 4), 16:0 (peak 15), and 18:0 (peak 21). The presence of double bonds in the hydrocarbon chain increased the molar absorptivities for C-18 fatty acids: 18:0 (peak 21), 18:1 (peak 16), 18:2, $n-6$ (peak 9), and 18:3 (peak 2). The relationship between the molar absorptivities and the number of double bonds of these C-18 fatty acids was linear ($r^2 = 0.997$) with an average of $2.88 \cdot 10^5$ area units/nmol change per double bond. However, this was not evident for C-20 and C-22 PUFAs, since 20:3, $n-6$ (peak 12) had a higher molar absorptivity than 20:4, $n-6$ (peak 5), and 22:4, $n-6$ (peak 14) was higher than 22:5, $n-6$ (peak 10). The positions of the double bonds ($n-7$ *versus* $n-9$, $n-3$ *versus* $n-6$) also affected the molar absorptivities. Clearly, the molar absorptivities of FAPEs are determined not only by the nature of derivatives but also by the structure of fatty acyl chains, which might implicate a direct interaction between the two moieties. This finding is consistent with the early report that FAPEs respond differently to ultraviolet light [15]. Phospholipid molecular species derivatized with ultraviolet-absorbing chromophores such as benzoate [16] and dinitrobenzene and trinitrophenyl [17] do not have this property.

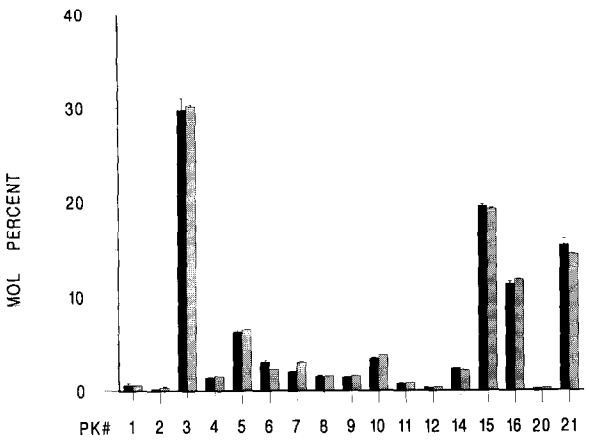

Fig. 2. Comparison of frog retinal fatty acid compositions (mol percentages) determined by HPLC (■) and GC (▩). For HPLC analysis, fatty acids represented by peak number (*x*-axis) were calculated by converting the peak areas to the amounts of fatty acids using the determined molar absorptivities (Table I). For GC analysis, retinal fatty acids were quantitated by comparison of relative area units with the internal standards (17:0 and 21:0). Data shown are means ± S.D. of three independent determinations. Refer to Fig. 1 or Table I for identification of the peaks.

Using the determined molar absorptivities, retinal fatty acids were quantitated as phenacyl esters separated by HPLC. Peak area units were integrated from one peak marker to another as illustrated in Fig. 1. Fused peaks such as peaks 7 and 8 were separated at the valley and integrated. Molar percentages of up to seventeen fatty acids were calculated by converting the peak area units to the corresponding amounts of fatty acids. The results were compared with those from the GC analysis. As seen in Fig. 2, fatty acid compositions calculated from HPLC and GC analysis were similar. Although some of the FAPEs were not well resolved on the HPLC column, this did not affect their quantitation. These results indicate that the HPLC method has a satisfactory precision for the determination of fatty acids in tissues that contain large amounts of long-chain PUFAs.

The sensitivity of HPLC, according to our estimate, is twenty to fifty times greater than that of GC. The limit of detection for most of the fatty acids was in the range 0.8–2 pmol, which is close to the reported value [10]. Reproducibilities of the HPLC method evaluated from multiple injections of double amounts of retinal FAPEs show a variation of less than 5%. In addition, the recoveries of total fatty acids in these runs were doubled, with variation less than 3%.

The present method is simple and rapid, involving only saponification, hexane extraction, and phenacyl derivatization. Any loss of material during these steps can be corrected by using an internal standard [6–9]. This method is sensitive and allows the analysis of small amounts of materials. Also, as pointed out previously [10,14], the resolved fatty acids are not structurally destroyed during the measurement, which allows on-line detection of radioactivity or the recovery of the fatty acids for other analyses. This is especially important for the study on synthesis and metabolism of long-chain PUFAs where determination of specific radioactivities is desired.

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