Measurement of eicosapolyenoic acids in the serum by gas–liquid chromatography–chemical ionization mass spectrometry

Minoru Suzuki, Masatoyo Nishizawa, Tadashi Miyatake, and Yasuo Kagawa

Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Tokyo, Japan 113, and Departments of Neurology[1] and Biochemistry,[2] Jichi Medical School, Minamikawachi, Tochigi-ken, Japan 329-04

Summary The use of octadeutero eicosatetraenoic acid as an internal standard for the reproducible measurement of serum eicosapolyenoic acids ($C_{20:3}$, $C_{20:4}$, and $C_{20:5}$) by gas-liquid chromatography–chemical ionization mass spectrometry is described. The method has the following advantages. The physicochemical properties of the internal standard and the eicosapolyenoic acids are similar. The acids are easily separated from compounds of similar retention times by means of selected ion monitoring. The measurements can be made more rapidly, (10 min per sample) than with previous techniques.—**Suzuki, M., M. Nishizawa, T. Miyatake, and Y. Kagawa.** Measurement of eicosapolyenoic acids in the serum by gas–liquid chromatography–chemical ionization mass spectrometry. *J. Lipid Res.* 1982. **23:** 363–366.

Supplementary key words eicosapolyenoic acid • eicosapentaenoic acid • arachidonic acid • octadeuteroarachidonic acid • prostaglandins • thromboxanes • eicosatrienoic acid

Eicosapentaenoic acid is the precursor of prostaglandins and thromboxanes of the "3" series (those having three double bonds in their side chains). It is found in the serum of Eskimos (1) and Japanese (2), whose incidence of myocardial infarction is much lower than that of Caucasians. Arachidonic (5,8,11,14-eicosatetraenoic) acid and eicosatrienoic acids are also found in the serum of all ethnic groups and play important physiological roles.

[1] M. Nishizawa and T. Miyatake.
[2] Y. Kagawa.

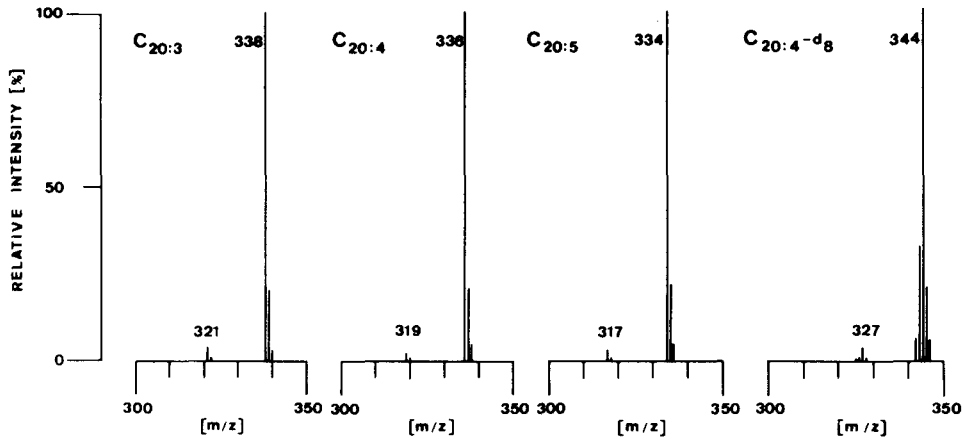

Fig. 1. Ammonia chemical ionization mass spectra of methyl eicosapolyenoates. $C_{20:3}$, methyl eicosatrienoate; $C_{20:4}$, methyl eicosatetraenoate; $C_{20:5}$, methyl eicosapentaenoate.

Accurate determination of these eicosapolyenoic acids in the serum has been hampered by the presence of substances with similar retention times, such as docosaenoic acid ($C_{22:1}$), in serum and marine products (see Fig. 2A and 3A of ref. 3), and by loss of these acids during extraction and methylation. Recently, mass spectrometry was used for analysis of these acids to overcome the problem of peak-overlapping by selected ion monitoring (4). In order to correct for a poor recovery of saturated acids added as internal standards, 8,11,14-eicosatrienoic acid was used instead (5), although the latter procedure was not entirely satisfactory since this compound is also present in serum (5).

Another difficulty is the long time required for fatty acid analysis of serum by gas–liquid chromatography, owing to the presence of docosahexaenoic acid ($C_{22:6}$) and other substances with long retention times.

We therefore developed a rapid and sensitive assay for eicosapolyenoic acids using octadeutero eicosatetraenoic acid as an internal standard by selected ion monitoring, and applied this method to the analysis of many serum samples.

MATERIALS AND METHODS

[5,6,8,9,11,12,14,15 - $^2H_8$]5,8,11,14 - Eicosatetraenoic acid was supplied from Toray Industries, Kamakura, Japan. [1-^{14}C]5,8,11,14-Eicosatetraenoic acid (56.4 mCi/mmol, 99% pure) was purchased from Radiochemical Centre Amersham, England. 8,11,14-Eicosatrienoic acid ($C_{20:3}$), 5,8,11,14-eicosatetraenoic acid ($C_{20:4}$), 5,8,-11,14,17-eicosapentaenoic acid ($C_{20:5}$), and eicosanoic acid ($C_{20:0}$) were purchased from the Applied Science Laboratory, Rockford, IL, USA; purity of the methyl esters of these fatty acids was found by gas–liquid chromatography to be more than 99%, 99%, 97.5%, and 99%, respectively. Soy bean phosphatidylcholine and phosphatidylethanolamine were prepared as described previously (6). Normal serum was obtained from 80 healthy Japanese (total cholesterol 190 ± 34 mg/dl, phospholipids 205 ± 33 mg/dl, and triglyceride 117 ± 55 mg/dl), and kept at −80°C until use.

Solvents contained, 2,6-di-*tert*-butyl-*p*-cresol at a final concentration of 0.005% to prevent oxidation of unsaturated lipids (7). The extraction method was essentially as reported by Bligh and Dyer (8). To 0.5 ml of serum, 1.9 ml of chloroform–methanol 1:2 containing 50 μg of octadeutero eicosatetraenoic acid was added, and the mixture was gently shaken at 20°C for 1 hr and centrifuged at 3,000 rpm for 10 min. The white insoluble material was extracted with 2.9 ml of chloroform–methanol–water 1:2:0.8 and the supernatant fraction and extract were combined. To 1 ml of this combined solution were added 1.3 ml each of chloroform and water, and the resulting mixture was centrifuged at 3,000 rpm for 10 min. The resulting lower layer was mixed with an equal volume of benzene and dried carefully under a stream of nitrogen. The total lipid fraction thus obtained was subjected to methanolysis in 5% HCl in methanol at 80°C for 2 hr under nitrogen in a screw-capped glass tube. The resulting mixture of fatty acid methyl esters was extracted three times with 1-ml volumes of *n*-hexane, washed and concentrated.

The fatty acid methyl esters were analyzed in a Shimadzu LKB Model 9000A gas chromatograph–mass spectrometer equipped with a chemical ionization (CI) source and a data processing system GC-MS-PAC 300 DG, consisting of an Okitac 4300 minicomputer with a 16 K core and a magnetic disc (4, 9). A glass column (1 m × 3 mm internal diameter) filled with 10% diethyleneglycolsuccinate adsorbed on 80–100 mesh Gaschrome Q was used. The temperature of the column, injection port, and ionization chamber were kept at

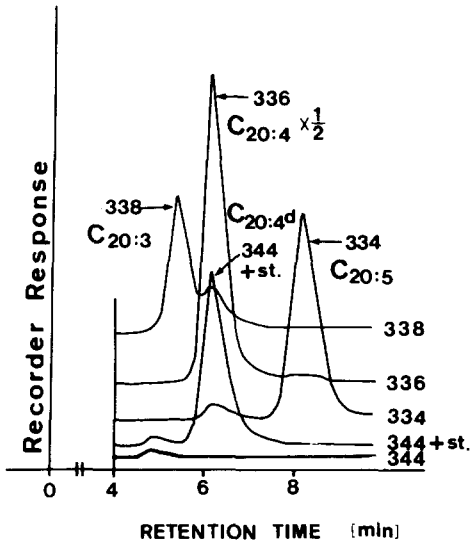

Fig. 2. Mass fragmentograms of methyl eicosatrienoate, methyl eicosatetraenoate, methyl eicosapentaenoate, and octadeuteromethyl eicosatetraenoate prepared from serum as described in the text. The abbreviations for these methyl esters are the same as in Fig. 1. Mass fragmentogram for m/z 344 was taken both in the presence (344 + st.) and absence (344) of added internal standard, octadeuteromethyl eicosatrienoate. Detector sensitivity of the selected ion monitor for methyl eicosatetraenoate was reduced to one-half of that for other methyl esters.

195°C, 210°C, and 230°C, respectively. The flow rate of carrier gas was 30 ml He per minute. CI mass spectra were obtained at an electron energy of 500 eV, an emission current of 500 μA and an accelerating voltage of 3.5 kV. Ammonia was used as a reagent gas at 0.9 Torr.

RESULTS AND DISCUSSION

The quasi-molecular ions $(M + 18)^+$, the ammonium adduct ion $(M + NH_4)^+$, of octadeuteromethyl eicosatetraenoate, methyl eicosatrienoate, methyl eicosatetraenoate, and methyl eicosapentaenoate were observed as base peaks in these ammonia CI mass spectra at m/z of 344, 338, 336, and 334, respectively (**Fig. 1**). The mass patterns of these spectra were very simple; the protonated molecular ions $(M + 1)^+$ were extremely weak (less than 4% of the base peaks) and the fragment ions were barely detectable in any of these mass spectra between 100 and 350 m/z, as is the case with methyl docosanoates (9). Therefore, mass fragmentographic analysis of individual fatty acid methyl esters was performed by tracing the base peak ions at m/z 344, 338, 336, and 334, respectively (**Fig. 2**). Quantitative analyses were made by measuring the ratios of the peak heights of these $(M + 18)^+$ ions of methylated fatty acids to those of octadeuteromethylated eicosatetraenoic acid. The retention times of the methyl esters of eicosatrienoate, eicosatetraenoate, eicosapentanoate, and deuteroeicosatetraenoate were 5.45, 6.27, 8.21, and 6.20 minutes, respectively. There was no interference from overlapping fatty acid fractions, such as docosahexaenoate of the preceding samples or docosaenoate in the same sample, in these mass fragmentograms. As shown in Fig. 2, the mass fragmentogram for m/z 344 from a serum sample to which the internal standard has not been added resulted in a very small peak at 4.7 minutes, perhaps owing to docosanoic acid ($C_{20:0}$), and no other following peak. On the other hand, the mass fragmentogram for m/z 344 from the internal standard showed only a large peak at 6.20 minutes. The calibration curves were practically linear from 50 to 1,500 ng, as shown in **Fig. 3.** The reproducibility of the repeated injection of the standard samples is indicated in Fig. 3 as the standard deviation width. The coefficients of variation of the measurement in the same serum, obtained by repeating the entire procedure, ranged from 0.49 to 0.74%, 0.24 to 0.76%, and 1.05 to 3.00%, for eicosatrienoic, eicosatetraenoic, and eicosapentaenoic acid, respectively (repeated on three different sera). The ratio of the area in the gas–liquid chromatography–mass spectrometry of equal weights of eicosatrienoate, eicosatetraenoate, and eicosapentaenoate

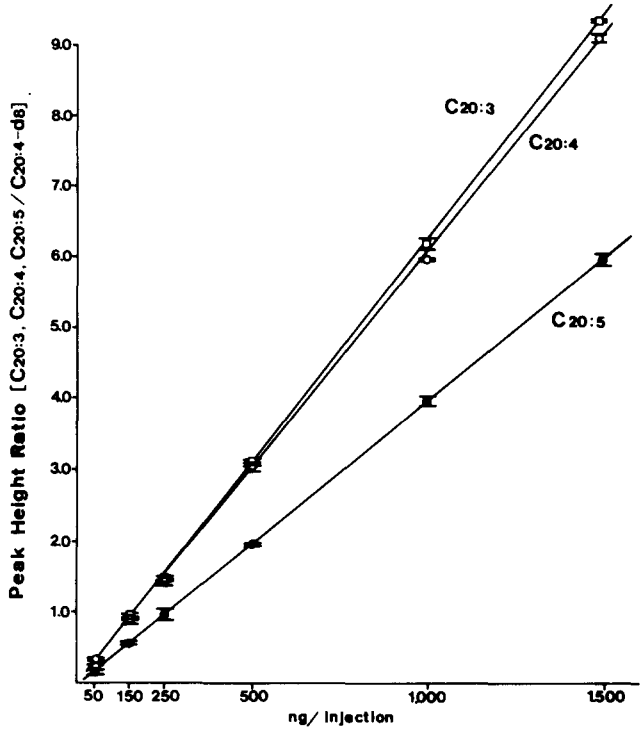

Fig. 3. Calibration curves for methyl eicosapolyenoates. Eicosatrienoic acid, eicosatetraenoic acid, eicosapentaenoic acid, and octadeuteroeicosatetraenoic acid as standards were methylated together in the solution described in the text, and various amounts of the methylated mixture were injected into the gas chromatography–ionization mass spectrometer as described in the text.

were 1.000, 1.030 ± 0.057, and 1.060 ± 0.042, respectively.

The stability of these fatty acids in *n*-hexane containing 0.005% of 2,6-di-*tert*-butyl-*p*-cresol on storage at 20°C for 3 days was more than 96%. The recoveries of eicosatrienoic acid, eicosatetraenoic acid, and eicosapentaenoic acid (100 μg each) added to a serum were 97.5 ± 1.84%, 97.8 ± 1.27%, and 97.5 ± 1.34%, respectively (average of six experiments). The recoveries of phosphatidylcholine and phosphatidylethanolamine extracted by this method were 96.1 ± 1.9% and 97.4 ± 2.5%, respectively, of total phospholipid phosphate added to serum. The recovery of [1-^{14}C]eicosatetraenoic acid (50 μg, 5.5×10^3 cpm.) added to 0.5 ml of serum was 95.7 ± 4.5%. The loss of radioactivity found in the water layer after the addition of 1.3 ml each of chloroform and water to 1 ml of the extract was only 1.8 ± 0.2%. The absolute value of recovery was greatly influenced by the recovery of the lower chloroform layer at this stage, but the use of an internal standard corrected the loss. Methanolysis of serum lipids was optimal at 2 hr, and losses of total eicosapolyenoic acids during 5 and 12 hr of methanolysis were 3.3% and 6.7%, respectively, when eicosanoic acid ($C_{20:0}$) was used as an internal standard.

Thus, 74 samples of eicosapolyenoic acids in 0.5-ml volumes of serum could be analyzed precisely in 1 day. The values of eicosatrienoic acid, eicosatetraenoic acid, and eicosapentaenoic acid were 6.52 ± 1.66, 32.9 ± 6.92, and 6.84 ± 2.47 mg/dl, respectively.

The authors thank Toray Industries for their supply of octadeutero eicosatetraenoic acid.

Manuscript received 1 June 1981 and in revised form 29 September 1981.

REFERENCES

1. Dyerberg, J., H. O. Bang, and H. Hjørne. 1975. Fatty acid composition of the plasma lipids in Greenland Eskimos. *Am. J. Clin. Nutr.* **28:** 958–966.
2. Hirai, A., T. Hamazaki, T. Terano, T. Nishikawa, Y. Tamura, A. Kumagai, and J. Sajiki. 1980. Eicosapentaenoic acid and platelet function in Japanese. *Lancet:* **2:** 1132–1133.
3. Ackman, R. G. 1960. Gas–liquid chromatography of fatty acids and esters. *Methods Enzymol.* **14:** 329–381.
4. Murata, T. 1978. Analysis of fatty acid methyl esters by a gas–liquid chromatography–chemical ionization mass spectrometry computer system. *J. Lipid Res.* **19:** 166–171.
5. Gerber, J. G., J. S. Barnes, and A. S. Nies. 1979. Measurement of arachidonic acid in the plasma by gas–liquid chromatography–flame ionization using dihomo-γ-linolenic acid as an internal standard. *J. Lipid Res.* **20:** 912–914.
6. Kagawa, Y., A. Kandrach, and E. Racker. 1973. Partial resolution of the enzymes catalyzing oxidative phosphorylation. XXVI. Specificity of phospholipid required for energy transfer reactions. *J. Biol. Chem.* **248:** 676–684.
7. Kuksis, A. 1977. Routine chromatography of simple lipids and their constituents. *J. Chromatogr.* **143:** 3–30.
8. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
9. Tsuji, S., M. Suzuki, T. Ariga, M. Sekine, M. Kuriyama, and T. Miyatake. 1981. Abnormality of long-chain fatty acids in erythrocytes membrane sphingomyelin from patients with adrenoleukodystrophy. *J. Neurochem.* **36:** 1046–1049.