HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FATTY ACIDS
IN PHOSPHOLIPID FRACTION OF HUMAN PLATELETS AND PLASMA AFTER
ADMINISTRATION OF EICOSAPENTAENOIC ACID

Mitsuo IKEDA^{®*}, Kenji SHIMADA*, and Ushiho MATSUMOTO**

*Niigata College of Pharmacy, 5829 Kamishin'ei-cho, Niigata-shi,
Niigata, 950-21, Japan **Tokyo College of Pharmacy, 1432-1
Horinouchi, Hachioji-shi, Tokyo, 192-03, Japan

An assay method for eicosapentaenoic acid (EPA) and arachidonic acid (AR) in plasma and platelet phospholipids was developed by employing 9-aminophenanthrene (9-AP) as a fluorescence labeling agent by high performance liquid chromatography. EPA was orally administered to a healthy man for 7 consecutive days, and the incorporation of EPA into platelet phospholipids and changes in the level of individual fatty acids including AR were examined. The level of EPA in platelet phospholipid was increased about 2.3 times (in terms of a % molar ratio) by the administration of EPA, indicating the incorporation of the acid into platelet phospholipids. Saturated fatty acids such as C_{14:0} and C_{16:0} in the phospholipid were decreased by EPA administration, while C_{18:1} was increased.

It is known that phospholipid occupies about 76.9% of the total lipid of the platelet¹⁾. Arachidonic acid (AR) taken up into this phospholipid is released into the cytoplasm by some stimulations, and converted to prostaglandins (PG)²⁾ and thromboxane (TX)³⁾, the substances known to play important roles in the platelet agglutination mechanism. It is also known that PG and TX are produced also from eicosapentaenoic acid (EPA) in the living body⁴⁾. Both PGH₃ and TXA₃ which are formed from EPA, however, are devoid of a platelet agglutinating ability, but have been reported by Needleman et al.⁵⁾ to suppress platelet agglutination. This suppressive action has been attracting attention in recent years, and the oral administration of EPA has been clinically utilized to prevent arterioscleroses such as myocardial infarction and cerebral infarction.

Unlike erythrocytes or leukocytes, platelets are able to synthesize fatty acids (FA) and phospholipids de novo. However, since platelets can effectively take up free fatty acids (FFA) from plasma, the composition of FA in platelet phospholipids has been said to be determined by this uptake mechanism⁶⁾. In order to confirm whether ingested EPA, e.g. in the form of foods, clearly shows an antithrombotic or antiarteriosclerotic action, it is necessary to examine the transfer of orally ingested EPA to plasma and the incorporation of EPA into platelet phospholipid. Since the amount of AR or EPA incorporated into platelet phospholipids is small, a sensitive assay method is required to obtain accurate

results. In the past, gas chromatography (GC) has been used for this purpose⁷⁾⁸⁾. Ozawa et al.⁸⁾ and Folch et al.⁹⁾ extracted lipids from human platelets by means of a solvent extraction technique, and phospholipid fractions isolated by thin layer chromatography (TLC) were interesterified with a boron trifluoride-methanol reagent, then the FA content was determined by GC.

Recently, high performance liquid chromatography (HPLC) has been introduced to the assay of FA. The application of HPLC to the assay of phospholipids in cells or cell membranes, however, has been attempted by a few investigators. In order to assay a small amount of FA in a biological specimen, such as AR and EPA in the phospholipid of platelets, by HPLC, a labeling process is certainly required to achieve high sensitivity. We have utilized 9-aminophenanthrene (9-AP) as a fluorescence labeling agent for FA, this compound being that reported previously by us¹⁰⁾¹¹⁾. A HPLC microassay method for phospholipids in human platelet specimens, and AR and EPA in human plasma were developed using 9-AP as a labeling agent.

EXPERIMENTAL

Reagents

Myristic acid (C_{14:0}, MT), palmitic acid (C_{16:0}, PT), palmitoleic acid (C_{16:1}, PL), stearic acid (C_{18:0}, ST), linoleic acid (C_{18:2}, Ll), arachidonic acid (C_{20:4}, AR) were purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Oleic acid (C_{18:1}, OL) and tetramethylammonium hydroxide (TMAH) were purchased from Sigma Chemicals (St. Louis, U.S.A.). Margaric acid (C_{17:0}) and eicosapentaenoic acid (C_{20:5}, EPA) were from Nakarai Chemicals (Kyoto, Japan). 9-Aminophenanthrene (9-AP) was from Aldrich (Milwaukee, WI, U.S.A.). Triethylamine (TEA) was from Tokyo Kasei (Tokyo, Japan). Oxalyl chloride was from Wako Pure Chemical (Osaka, Japan). Extrelut[®] (large pore kieselguhr of granular structure and high pore volume) was from E. Merck (Darmstadt, G.F.R.). Tris-saline buffer solution was prepared with follows: 5 mM D-glucose, 0.134 M sodium chloride (NaCl), and 15 mM Tris buffer adjusted to pH 7.4 with 1 N hydrochloric acid (HCl). EPA capsules were gifted from Nissui Seiyaku (Tokyo, Japan).

Apparatus

A Hitachi high performance liquid chromatograph Model 635A equipped with a Shimadzu RF-530 spectrofluorometer was used.

HPLC conditions

Column: μ Bondapak C₁₈ (300 mm X 4 mm I.D., particle size 8-10 μ m).
Fluorescence detector: the excitation wavelength was set at 303 nm and the emission wavelength at 376 nm. Mobile phase: methanol-acetonitrile-water (48:35:17).
Flow-rate: 2.0 ml/min. Column temperature: 40°C.

Quantitative determination of FA with using 9-AP as a fluorescence labeling agent (9-AP method)

The procedure in the present study was according to the quantitative determination method previously reported by the present authors¹⁰⁾.

Administration of EPA capsule and the scheme for blood collection

Three EPA capsules (containing 225 mg of EPA) were given orally to the healthy male adult 3 times a day for 7 days. Twelve hours after the final administration, the 8th morning at 8 a.m., blood collection was performed at the fasting condition from antecubital veins with using citric acid as an anticoagulant. After the rest period for a month, the same experiment was repeated 3 times with the same volunteers. The volunteer avoided the administration of aspirin or other drugs from 2 weeks before the start of the present experiment and they also avoided from taking hyperlipidemic foods as far as possible during the period of EPA administration.

Preparation of washed blood platelets

Washed blood platelets were prepared according to the method of Bills et al.¹²⁾ Immediately after collecting 5 ml of blood, it was transferred into polycarbonate centrifuge tube containing 0.5 ml of 3.8% sodium citrate, and the mixture was shaken gently; thereafter the centrifugation at 900 rpm was made at room temperature for 10 min, and the platelet rich plasma (PRP) was obtained. After the PRP was cooled to 4°C, 0.1 M ethylenediaminetetraacetic acid (EDTA) was added to a final concentration of 1 mM, and the mixture was centrifuged at 3000 rpm for 20 min at 4°C. The supernatant was removed and the platelet pellet was resuspended in a solution containing 1 mM EDTA, 5 mM D-glucose, 0.134 M NaCl, and 15 mM Tris buffer adjusted to pH 7.4 with 1 N HCl. The platelet suspensions were centrifuged at 3000 rpm for 20 min at 4°C, finally resuspended in a solution containing 5 mM D-glucose, 0.134 M NaCl, and 15 mM Tris buffer adjusted to pH 7.4 with 1 N HCl. The number of platelets was adjusted at $2.5 \times 10^5/\mu\text{l}$, which was served as samples for analysis.

Extraction of total lipids from platelets¹²⁾

One ml of the washed platelet suspension was transferred into 30-ml silicon-treated centrifuge tube with a ground stopper, and 10 ml of chloroform-methanol (1:2) solution was added, and the mixture was shaken in ice-cold water for 30 min. After centrifugal separation at 2000 rpm for 5 min, the aqueous layer was taken and it was acidified by adding formic acid. Thereafter, 20 ml of chloroform-methanol (5:1) was added and the extraction was made again. The organic layer obtained by the centrifugal separation was mixed with the previously extracted organic layer, and the solvent was distilled off under reduced pressure; thus, the total lipids were obtained.

Isolation of the phospholipid fraction with column chromatography¹²⁾

After dissolving the total lipids in 1 ml of chloroform, the solution was poured onto the glass column (80 mm X 7 mm I.D.) filled with 0.5 g silica gel (40/100 mesh). Thereafter, 10 ml of chloroform and 10 ml of 5% methanol in chloroform were poured onto the column in sequence, and thereby, the neutral lipids and PGs were removed. Finally, by eluting with 6 ml of methanol and 4 ml of 99% methanol, phospholipids were isolated.

Quantitative determination of FA in phospholipids

Phospholipid fraction obtained by the previously described procedure was hydrolyzed according to the method of Haan et al.¹³⁾. After dissolving the phospholipid fraction in 0.2 ml of methanol, 0.2 ml of 24% TMAH was added, and the reaction was allowed to take place at 70°C for 10 min. In the next step, released FA was isolated by the column extraction method which was previously reported by the present authors¹⁴⁾. Briefly, after the hydrolysis, the reaction solution allowed to cool down to the room temperature was neutralized with 1 N HCl, and 10 μ l of methanol solution containing 5 μ g margaric acid as internal standard was added. In addition, 1.6 ml of M/15 phosphate buffer solution (pH 7.0) was added and the solution was shaken thoroughly; then, the solution was poured onto glass column (45 mm X 12 mm I.D.) filled with 1 g of Extrelut[®]. After 20 min, FA fraction was eluted with 10 ml of chloroform. Chloroform was distilled off under reduced pressure, and the obtained FA was dried sufficiently in the desiccator containing silica gel. Thus, the amount of each FA was determined by 9-AP method.

Quantitative determination of plasma free fatty acids (FFA)

With using 3.8% citric acid as an anticoagulant, the plasma sample was prepared according to the conventional method. To 0.9 ml of M/15 phosphate buffer (pH 7.0) was added 0.1 ml of this plasma, and 10 μ l of methanol solution containing 5 μ g margaric acid as an internal standard was added; then, the solution was shaken thoroughly. This solution was poured into the glass column (80 mm X 7 mm I.D.) filled with 0.5 g of Extrelut[®]. After adsorption for 20 min, FFA was eluted with 5 ml of chloroform¹⁴⁾. After distilling off the chloroform under reduced pressure, the amount of FFA was determined by 9-AP method¹⁰⁾.

RESULTS AND DISCUSSION

In the GC assay method for FA in phospholipids, FA are easily converted to methylesters by interesterification¹⁵⁾¹⁶⁾ without a hydrolysis process. However, since the rate of interesterification is different for each lipid, the condition of interesterification has to be investigated for individual biological specimens¹⁷⁾. The result of basic investigations on the HPLC assay method using 9-AP as a fluorescence prelabeling agent for AR and EPA was reported in our previous paper¹¹⁾. As described in the report, the values of AR and EPA assayed by the 9-AP method were well correlated with those by a conventional FA assay method. Moreover, the recovery of AR or EPA from human plasma was found to be satisfactory. Based on these observations, it was confirmed that the 9-AP method is applicable to the assay of AR and EPA in biological specimens¹¹⁾. In the present study, eight kinds of FFA including AR and EPA were selected as major FFA in human plasma, and they were labeled with 9-AP, then 10 ng for each was injected onto HPLC. Since it was found that the EPA peak could not be separated from the MT (C_{14:0}) peak when a methanol-acetonitrile-water (53:27:20) mixture¹⁰⁾ was used as a mobile phase, the solvent composition was modified in the present study. As shown in Fig. 1, the solvent system consisting of methanol-acetonitrile-water (48:35:17) was found to

show good resolution of each FA. The detection limit of FA, assuming a signal-to-noise ratio of 3, was about 17 pmol for either AR or EPA. This detection limit is about 10 times as sensitive as that of the GC method in which FA methyl ester is detected with use of a FID¹⁸⁾.

AR and EPA contained in actual biological samples were then assayed by the present method. HPLC patterns of 9-AP-labeled FA are shown in Fig. 2. As shown in the chromatograms, there occurred the increase of EPA administration. Major FA detected from platelet phospholipids were as follows: MT ($C_{14:0}$), PT ($C_{16:0}$), ST ($C_{18:0}$), OL ($C_{18:1}$), L1 ($C_{18:2}$), AR ($C_{20:4}$) and EPA ($C_{20:5}$). The amount of each

Fig. 1 HPLC of 9-AP-labeled free fatty acids

Fig. 2 HPLC of fatty acids in human phospholipids before (A) and after (B) oral administration of EPA

Column: μ Bondapak C_{18} (300 mm X 4 mm I.D.), Mobile phase: $CH_3OH-CH_3CN-H_2O$ (48:35:17), Flow rate: 2.0 ml/min, Detector: Fluorometer (Ex 303 nm, Em 376 nm)

a: 9-AP, 1: $C_{20:5}$ (EPA), 2: $C_{14:0}$, 3: $C_{16:1}$, 4: $C_{20:4}$ (AR), 5: $C_{18:2}$
6: $C_{16:0}$, 7: $C_{18:1}$, 8: $C_{17:0}$, 9: $C_{18:0}$

FA estimated from the chromatogram is shown in Table 1. After 7 days administration of EPA, the EPA level in platelet phospholipids was increased about 2.3 times. Among unsaturated FA, OL was increased, while L1 and AR were slightly affected. On the other hand, saturated FA such as MT and PT showed a decreasing tendency. Although the influence of foods must be taken into consideration, observed changes in the level of each FA seemed to be produced primarily by the administration of EPA, because 3 repetitive experiments showed similar changes in the level of each FA.

Table 1 Effect of the oral administration*1 of EPA on fatty acid composition in human platelet*2 phospholipids

Fatty acid	Fatty acid composition in phospholipid(mol%)			
	before admin.	s	after 1 week	s
14:0	10.0	0.4	4.5	0.5 *3
16:0	33.9	0.4	21.7	0.5 *3
18:0	13.1	0.5	15.3	3.4
18:1	6.4	0.3	15.0	2.9 *3
18:2	8.8	0.5	9.8	1.4
20:4 (AR)	25.7	0.7	28.9	1.2
20:5 (EPA)	2.0	0.2	4.5	0.6 *3

*1 three times administration of each 225 mg of EPA per day for one week

*2 human male adult platelets: $2.5 \times 10^5/\mu\text{l}$ s: standard deviation

*3 $p < 0.05$, compared with value of before administration

Variations in the FFA level in plasma before and after EPA administration are shown in Table 2. The administration of EPA decreased the MT ($\text{C}_{14:0}$) level in plasma, and increased the OL ($\text{C}_{18:1}$) level. These changes in FA levels were in good agreement with changes in the FA composition of platelet phospholipids. The results shown in Table 2 also support the findings of Spector et al.¹⁹⁾ who demonstrated that ^{14}C -labeled PT ($\text{C}_{16:0}$), ST ($\text{C}_{18:0}$), OL ($\text{C}_{18:1}$) and L1 ($\text{C}_{18:2}$) were incorporated into platelet phospholipids. Deykin et al.²⁰⁾ studied the incorporation of ^{14}C -acetate into platelet phospholipids, and reported that FA composing the phospholipid of platelets were released back to plasma. Based on these observations, it may be thought that FA in platelet phospholipids and FFA in plasma are related with each other through repetitive uptake and release processes. In spite of EPA administration, no EPA was detected in plasma in the present study. Since blood samples were collected 12 h after the final EPA administration, it is conceivable that EPA transferred to plasma was either metabolized or taken up by platelets. According to the result obtained by Spector et al.¹⁹⁾, about 35% of ^{14}C -PT was oxidized to carbon dioxide, 36% was incorporated into phospholipids, and 25% was remained as FFA, after ^{14}C -labeling FA was incubated with platelets for 1 h. These findings indicate that FA are rapidly metabolized in the presence of platelets or taken up by platelets. AR, which is the most abundant FA composing platelet phospholipids after EPA administration, was not detected in plasma, but was present only in platelets.

As described above, orally administered EPA was apparently incorporated into the phospholipid of platelets, and it was metabolized at a fairly high rate. Therefore, the content of EPA in platelet phospholipids did not exceed that of AR under the present experimental condition for the dose and frequency of EPA administration. In this report, we examined the applicability of the 9-AP method to the level of FA in platelet phospholipids. As the above experimental results show, satisfactory concentration of EPA was not necessarily achieved in the platelet when 9 EPA capsules per day administered. Sanders et al.⁷⁾ reported that high doses

Table 2 Effect of the oral administration*1 of EPA on fatty acid composition in human plasma

Fatty acid	Fatty acid composition in plasma(mol%)			
	before admin.	s	after 1 week	s
14:0	10.7	0.3	5.0	0.9 *2
16:0	32.0	3.3	28.4	2.2
18:0	12.9	1.4	12.5	2.2
18:1	15.2	1.5	26.7	3.8 *2
18:2	25.2	3.4	24.5	2.6
20:4 (AR)	N.D.		N.D.	
20:5 (EPA)	N.D.		N.D.	

*1 three times administration of each 225 mg of EPA per day for one week

*2 $p < 0.05$, compared with value of before administration

N.D.: not detectable s: standard deviation

and longer duration of EPA administration might be required for achieve the anti-thrombotic and antiarteriosclerotic action of EPA. The authors also examined the dose response relationship of EPA.

CONCLUSION

In the assay method reported here for FA composing human platelet phospholipids, the use of TMAH, as a hydrolysis agent, achieved a short (about 10 min) hydrolysis time, and the use of a chromatographic column filled with Extrelut[®] realized the easy separation of FA fraction¹⁴⁾. Thus, the introduction of a labeling agent became easier, and the HPLC method, which has not been utilized frequently for the assay of FA in phospholipids²¹⁾²²⁾, could be utilized. Using the HPLC method with 9-AP as a fluorescence labeling agent, we carried out the assay of FA such as AR and EPA in platelet phospholipids, and obtained satisfactory sensitivity and accuracy. The level of EPA in platelet phospholipids was increased about 2.3 times (in terms of a % molar ratio) by the administration of EPA, indicating the incorporation into platelet phospholipids. Saturated FA such as C_{14:0} and C_{16:0} in the phospholipid were decreased by EPA administration, while C_{18:1} was increased. Unsaturated FA such as C_{18:2} and C_{20:4}(AR) were not noticeably changed by EPA administration, the latter (C_{20:4}) being not detected in plasma. In the plasma after EPA administration, C_{14:0} and C_{18:1} were decreased and increased, respectively in a similar manner as demonstrated in platelets, indicating the existence of exchange processes between FA in plasma and those in platelet phospholipids. The assay method reported has a high sensitivity for detecting FA. Therefore, it is suitable for the assay of FA in a biological specimen which can be collected only in small amount. Changes in the FA level in plasma and platelets at the time of platelet agglutination are being investigated by using the present assay method, and the results will be reported elsewhere.

The authors are grateful to Nissui Seiyaku Co. Ltd. for kind gift of EPA. capsules. They are deeply indebted to Shimadzu Seisakusho Ltd. to serve the convenience of the fluorometric measurements.

A part of this study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

REFERENCES

- 1) A.J. Marcus: Ann. N.Y. Acad. Sci., 201, 102 (1972).
- 2) M. Hamberg, B. Samuelsson: Proc. Nat. Acad. Sci. USA, 71, 3400 (1974).
- 3) M. Hamberg, J. Svensson, B. Samuelsson: Proc. Nat. Acad. Sci. USA, 72, 2994 (1975).
- 4) J. Dyerberg, H.O. Bang, E. Stoffersen, S. Moncada, J.R. Vane: Lancet, ii, 117 (1978).
- 5) P. Needleman, H. Sprecher, M.O. Whitaker, A. Wyche: "Adv. Prostaglandin Tromboxane Res.", Edited by B. Samuelsson, P.W. Ramwell, R. Paoletti, p. 142 (1980), (Raven Press, New York).
- 6) M. Katori: "Readings on Platelets", Edited by M. Yamanaka, Y. Ueda, p. 81 (1982), (Ishiyaku Publishers, Tokyo).
- 7) T.B.A. Sanders, D.J. Naismith, A.P. Haines, M. Vickers: Lancet, ii, 1189 (1980).
- 8) A. Ozawa, E. Nakamura, H. Jimbo, T. Fujita, A. Hirai, T. Terano, T. Hamazaki, Y. Tamura, A. Kumagai: Bunseki Kagaku, 32, 174 (1983).
- 9) J. Folch, M. Lees, G.A. Sloane-Stanley: J. Biol. Chem., 226, 497 (1957).
- 10) M. Ikeda, K. Shimada, T. Sakaguchi, U. Matsumoto: J. Chromatogr., 305, 261 (1984).
- 11) M. Ikeda, K. Shimada, U. Matsumoto: Bunseki kagaku, 33, 122 (1984).
- 12) T. Bills, J.B. Smith, M.J. Silver: Biochim. Biophys. Acta, 424, 303 (1976).
- 13) G.J. Haan, S. van der Heide, B.G. Wolthers: J. Chromatogr., 162, 261 (1979).
- 14) M. Ikeda, K. Shimada, T. Sakaguchi: Bunseki Kagaku, 31, E199 (1982).
- 15) Y. Kishimoto, N.S. Radin: J. Lipid Res., 6, 435 (1965).
- 16) J.T. Dodge, G.B. Phillips: J. Lipid Res., 8, 667 (1967).
- 17) Y. Takahashi, K. Tanaka, T. Fukasawa: Tanpakushitsu Kakusan Koso, Bessatsu 7, 30 (1967).
- 18) W. Höckel, W. Dünge, A. Holzer, P. Brockerhoff, G.H. Rathgen: J. Chromatogr., 221, 205 (1980).
- 19) A.A. Spector, J.C. Hoak, E.D. Warner, G.L. Fry: J. Clin. Invest., 49, 1489 (1970).
- 20) D. Deykin, R.K. Desser: J. Clin. Invest., 47, 1590 (1968).
- 21) M.S. Manku: J. Chromatogr. Sci., 21, 367 (1983).
- 22) V.P. Agrawal, E. Schulte: Anal. Biochem., 131, 356 (1983).

Keyword phrases

determination of fatty acids by high performance liquid chromatography;
incorporation of eicosapentaenoic acid into human platelet phospholipids;
fluorescence labeling of free fatty acids with 9-aminophenanthrene.

(Received February 20, 1984)