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High-Performance Liquid Chromatographic Determination of Eicosapentaenoic Acid in Serum by a Chemiluminescence Labeling Method

HIDETAKA YUKI, YUTARO AZUMA, NORIO MAEDA, and HIDEKI KAWASAKI*

School of Pharmaceutical Sciences, Toho University, Miyama 2-2-1, Funabashi, Chiba 274, Japan

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A sensitive method for the determination of eicosapentaenoic acid (EPA) in human serum and the detection of some other fatty acids was developed. Fatty acids were labeled with *N*-(4-aminobutyl)-*N*-ethyl-isoluminol (ABEI), and separated by reversed-phase high-performance liquid chromatography. The eluate was mixed with 0.2 μM microperoxidase solution and 90 mM hydrogen peroxide solution successively, and the chemiluminescence was detected in a flow cell placed in front of a photomultiplier. The calibration curve of EPA was linear in the range of 2 pmol to 2 nmol. The recovery of EPA added to human serum was nearly 100%, and the detection limit was 200 fmol. Eleven fatty acids were detected in human serum samples by this method.

Keywords——*N*-(4-aminobutyl)-*N*-ethyl-isoluminol; chemiluminescence; determination; eicosapentaenoic acid; fatty acid; high-performance liquid chromatography; human serum; unsaturated fatty acid

It is known that eicosapentaenoic acid (EPA) and arachidonic acid not only act as precursors of prostaglandins, but also play various important physiological roles themselves. Recently several studies have demonstrated beneficial effects on atherosclerosis.[1] In contrast with the diverse studies on arachidonic acid metabolites, rather few biological studies on EPA have been undertaken, and a method of analysis of EPA has not yet been established either. Although high-performance liquid chromatography (HPLC) with fluorescence detection is an effective method for the determination of trace amounts of biological constituents, and has been applied to the analysis of fatty acids in serum samples,[2,3] this method has limited sensitivity because of the inherent noise that arises from a radiation source. On the other hand, chemiluminescence detection, in which no radiation source is needed, offers an ultrasensitive method for chemical analysis in biochemical and clinical fields.[4] As an application of chemiluminescence for detection of amines and carboxylic acids in HPLC, Kawasaki *et al.*[5] used *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI) as a prelabeling reagent, and detected the oxidation of the isoluminol moiety.

In this report, the authors have applied this sensitive method for the determination of EPA and detection of other saturated and unsaturated higher fatty acids in serum by HPLC.

Materials and Methods

Reagents——Eicosapentaenoic acid and dihomo-γ-linolenic acid were obtained from Funakoshi Pharmaceutical Co. (Tokyo, Japan). *N*-(4-Aminobutyl)-*N*-ethyl-isoluminol and microperoxidase (MP-11) were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). 3,4-Dihydro-2*H*-pyrido[1,2-*a*]pyrimidin-2-one(DPP) was purchased from Tokyo Kasei Co. (Tokyo, Japan). 2-Chloro-1-methylpyridinium iodide (CMPI) and other fatty acids were obtained from Nakarai Chemicals Ltd. (Kyoto, Japan). Methanol used as the mobile phase for HPLC was also of HPLC grade from Nakarai Chemicals Ltd. All other reagents were of guaranteed reagent grade. Appropriate potassium dihydrogen

phosphate and potassium hydrogen phosphate solutions were used for the preparation and adjustment of pH of phosphate buffer solutions.

Procedure for Derivatization with ABEI——A mixture of methanol solutions of 0.1 mM fatty acid (100 μl) and 0.5 mM ABEI (100 μl) was evaporated to dryness under a stream of nitrogen in a reaction vial (5 ml). Next, 100 μl each of 2 mM DPP and 0.4 mM CMPI in acetonitrile were added to the residue, and the vial was screwcapped tightly and heated at 90°C for 30 min. The reaction mixture was evaporated and the residue was redissolved by the addition of 200 μl of methanol. A 10 μl aliquot of this solution was injected into the HPLC column.

HPLC Conditions and Chemiluminescence Detection System——Reversed-phase HPLC was performed with a Shimadzu LC-6A liquid chromatograph equipped with a Rheodyne 7125 sample injector and a NOVA PAK C_{18} column (150 × 3.9 mm i.d., Waters). The mobile phase was 75% methanol-10 mM phosphate buffer solution, pH 6.50, and the flow rate was 0.5 ml/min. The eluate from the column was mixed with 0.2 μM microperoxidase (in 50 mM phosphate buffer solution, pH 8.60) at the flow rate of 0.5 ml/min and 90 mM H_2O_2 (in 10 mM phosphate buffer solution, pH 7.4) at the flow rate of 1.0 ml/min successively with a mixing tee joint, and the chemiluminescence generated was detected in a flow cell (75 μl) placed in front of the photomultiplier of a NITI-ON flow cell unit. The output from the photometer was recorded with a Shimadzu C-R3A plotter-integrator (Fig. 1).

Procedure for Determination of Fatty Acids in Serum——The extraction solvent was prepared by mixing 18 ml of chloroform–methanol (2 : 1) and 250 μl of 0.4 mM margaric acid in methanol as an internal standard. A 100 μl aliquot of serum was mixed with 500 μl of 33 mM phosphate buffer solution, pH 6.40, and 3 ml of the extraction solvent. The mixture was shaken for 5 min at 160 strokes/min and centrifuged for 10 min at 1500 rpm. The upper aqueous layer containing denatured protein was removed by suction, and a 200 μl portion of the remaining organic layer was subjected to the labeling reaction in a reaction vial as mentioned above.

Recovery Tests——EPA or arachidonic acid standard was diluted with the extraction solvent to make concentrations of 1.3 or 0.65 μM. With this solution, the extraction procedure was performed for "spiked" samples. The peak areas of EPA or arachidonic acid in normal serum, spiked serum and standard solutions were quantitated, and the recoveries were calculated from the peak areas corrected for that of the internal standard. These procedures were repeated three times for each sample.

Results and Discussion

Labeling of the fatty acid with ABEI was examined first by the dicyclohexylcarbodiimide (DCC) method, but the result was not satisfactory, so the method with DPP and CMPI was chosen according to Kawasaki *et al.*[5] The reaction conditions were examined by using EPA standard solution. Various reaction temperatures were tested in the range of 40 to 90°C, and various reaction times in the range of 15 min to 5 h. The chemiluminescence peak areas of the labeled compounds exhibited a maximum after 30 min of reaction time at every temperature examined. The peak area increased as the reaction temperature was raised. This suggests that the higher the reaction temperature, the greater the reaction yield. However, prolonged reaction seemed to destroy the isoluminol residue and consequently reduced the strength of chemiluminescence. Thus, the labeling reaction was routinely carried out at 90 °C for 30 min. Although this condensation reaction proceeds in acetonitrile, the solubility of the product, labeled fatty acid, in this solvent seemed to be limited, because the peak area obtained was one-half of that of the samples dissolved in methanol. However, since methanol may form an ester with fatty acids, acetonitrile was selected as the reaction solvent; it was removed after the reaction, and the residue was dissolved in methanol.

Various concentrations of methanol and phosphate buffer solution were examined for separation of ABEI derivatives of standard fatty acids (500 pmol) found in human serum (C_{18}–C_{20}). The best separation of the derivatives was achieved on a reversed-phase C_{18} column by isocratic elution with 75% methanol–10 mM phosphate buffer solution, pH 6.5. Under these conditions, unreacted ABEI was eluted in the hold-up volume and fatty acids were efficiently separated.

A chromatogram of the fatty acids extracted from normal human serum is shown in Fig. 2, and fatty acids detected are listed in the legend. No peak of interfering substance was observed in the chromatogram. The calibration curve for EPA was linear between 2 pmol and 2 nmol (Fig. 3, $r = 0.994$). The reproducibility of the peak areas of chromatograms was within

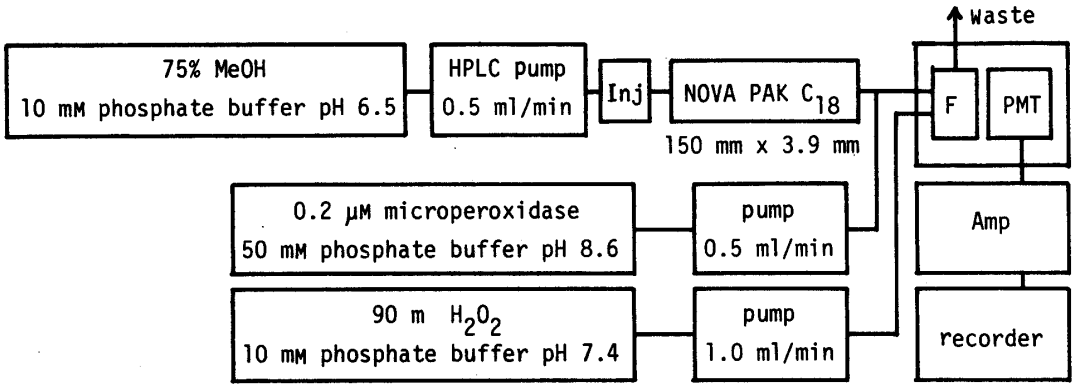

Fig. 1. HPLC System with Chemiluminescence Detector

Amp, amplifier; F, flow cell; Inj, injector; PMT, photomultiplier.

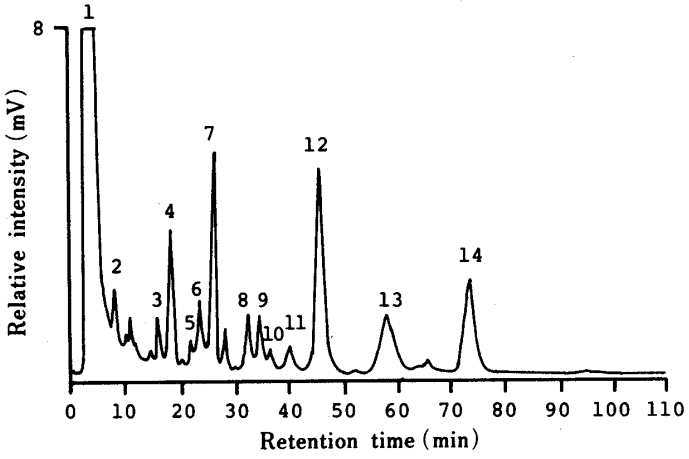

Fig. 2. Chromatogram of Fatty Acids in Human Serum

Chromatographic conditions were as described in the experimental section and Fig. 1.

1, ABEI; 2, lauric acid; 3, myristic acid; 4, linolenic acid; 5, eicosapentaenoic acid; 6, palmitoleic acid; 7, unknown substance; 8, linoleic acid; 9, arachidonic acid; 10, docosahexaenoic acid; 11, dihomo-γ-linoleic acid; 12, palmitic acid; 13, oleic acid; 14, margaric acid.

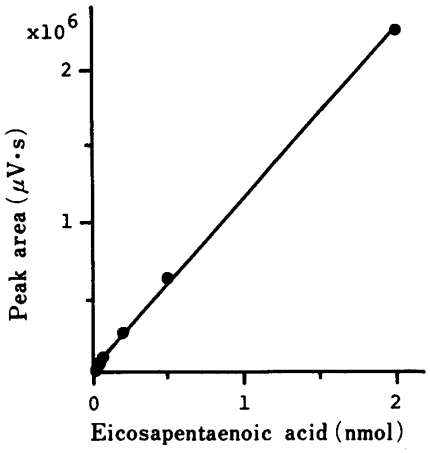

Fig. 3. Calibration Curve of EPA

TABLE I. Recoveries of Eicosapentaenoic Acid and Arachidonic Acid Added to Human Serum (100 μl)

	Added (μg)	Recovery (%)	C.V. (%, $n=3$)
Eicosapentaenoic acid	0.06	100.9	1.7
	0.6	99.5	4.9
	1.2	99.7	2.1
Arachidonic acid	0.6	103.2	3.3
	1.2	101.4	2.4

C.V. = coefficients of variation.

2.96% (C.V., $n=3$), and the detection limit was 200 fmol for EPA (S/N = 2). The results of recovery tests of EPA and arachidonic acid of various concentrations are shown in Table I. The satisfactory recoveries for both compounds suggest that this method may be applicable for other fatty acids. The concentration of EPA in serum determined by this method was 0.5 nmol/ml, which agrees well with the literature value (0.34—0.55 nmol/ml).[6]

In conclusion, the present method is reliable for the determination of EPA and may also be applicable for other higher fatty acids in biological materials. The sensitivity of this method

was equal to or greater than that of the fluorescence method. We are now attempting to improve the sensitivity and to apply this method to physiologically active long-chain unsaturated fatty acids such as prostaglandins.

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