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Accelerated Solvent Extraction for Quantitative Measurement of Fatty Acids in Plasma and Erythrocytes

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ABSTRACT: Consumption of fish rich in n-3 highly unsaturated FAs (i.e., EPA and DHA) has been suggested to decrease the risk of lifestyle-related diseases such as coronary heart disease, cancer, diabetes, and dementia. Blood levels of those FA are known appropriate biomarkers of both the corresponding dietary FA intakes and fish consumption. In place of traditional handwork methods for extracting FA, we performed an accelerated solvent extraction (ASE) for at least 13 selected FA in plasma and erythrocytes to measure them by GLC. The FA levels (concentrations and compositions) in 35–50 μL of plasma or erythrocytes were extracted by ASE and measured by GLC. Intra- and interassay coefficients of variation were ≤6.0% for both blood materials, except with a minor group of FA (≤1.0% of total FA). When ASE was compared with two traditional handwork methods, FA levels in plasma from 18 healthy subjects were all coincident with very high Pearson's correlation coefficients for the three sets of the same 18 samples ($r \geq 0.85$ to 0.95, $P < 0.0001$), except for 18:0 ($r = 0.59$, $P < 0.01$). Using ASE and GLC, we have developed a new method for determination the levels of FA in plasma and erythrocytes as biomarkers for dietary intake of fish, fat, and FA. This new method makes it feasible to measure small volumes of samples, automatically, quantitatively, routinely, easily, rapidly and cheaply, with acceptable precision and accuracy.

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A high-fat diet, especially one rich in animal fat or saturated FA, is suggested to increase the likelihood of obesity and the development of so-called lifestyle-related diseases, such as hyperlipidemia, coronary heart disease, cancers in several sites (colorectum, breast, and prostate), diabetes, dementia, and allergies (1–7). Furthermore, n-6 PUFA such as 20:4n-6 (arachidonic acid) are converted into leukotrienes, prostaglandins, and thromboxanes, and those eicosanoids are suggested to cause arthritis, asthma, cell proliferation, thrombosis, vasospasm, and inflammatory disorders (7–9). On the other hand, consumption of fish and n-3 highly unsaturated FA (HUFA = 20:5n-3 [EPA] + 22:5n-3 + 22:6n-3 [DHA]) exhibits inverse relationships with such diseases (10–19). In contrast to findings based on only dietary assessment, we have demonstrated that increased and decreased risks of colorectal cancer are linked to high compositions of palmitic acid and DHA, respectively, in erythrocyte membranes (20). For accurate assessment of impact on risk, therefore, we think it essential to measure FA in biomaterials (2,19,21–23).

Blood levels (concentrations [mg/dL or mmol/L] and compositions [wt% or mol%]) of EPA, DHA, and n-3 HUFA are known as appropriate biomarkers of dietary intake of FA from fish (24–31). Traditional handwork methods (THM) for measuring FA in biomaterials have gradually been improved (32–38), but require the following common multiple steps (Fig. 1): (1) pretreatment of biomaterial samples; (2) extraction of lipids (triglycerides, cholesterol esters, and phospholipids) and free FA (32–34); (3) conversion from lipids to FA, and then to methyl esters (trans-methylation) (35,36); (4) extraction of methyl esters; and (5) measurement by GLC (37,38). In the first step, lipids and free FA can be separated by silica gel TLC (33,34). The precision for measuring FA is determined primarily by the second and fourth steps, and a total of three extractions with each organic solvent at each of the steps is required to maximize recovery. It takes several hours to perform the extraction procedure by hand with considerable physical effort. Hitherto, two different automatic extraction methods for FA from small numbers of samples of plasma and foodstuffs such as meat have been reported

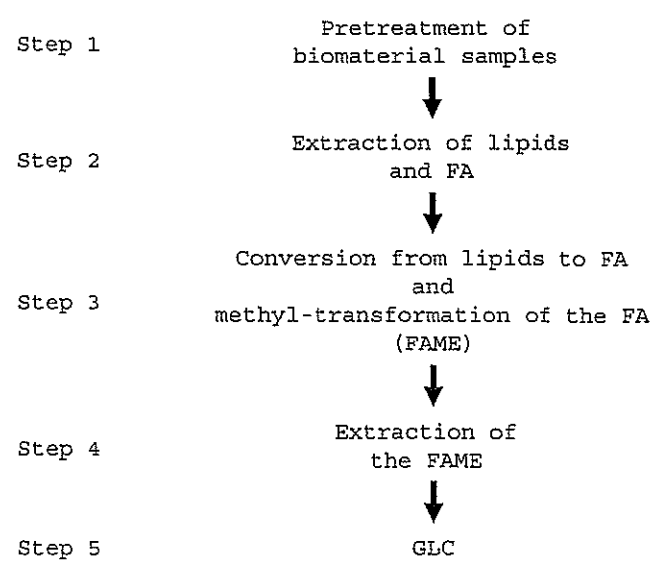

FIG. 1. Procedure for a representative traditional handwork method for measuring FA.

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Abbreviations: ASE, accelerated solvent extraction; HUFA, highly unsaturated FA; THM, traditional handwork method.

(39,40), but we do not have enough information about such methods for biomaterials such as plasma and erythrocytes for clinical use or epidemiological investigations.

To improve the methodology for measuring FA in biomaterials, especially at the second and the fourth steps (Fig. 1), we have established an automatic extraction method featuring accelerated solvent extraction (ASE). Compared with two THM, we here demonstrate high precision and accuracy for quantitative measurement of FA levels (concentrations and compositions) in small volumes of plasma and erythrocytes by using this method.

MATERIALS AND METHODS

Samples and standards were systematically protected from contamination with both natural existing FA and detergents and from auto-oxidation by direct light and oxygen in air during sample processing. Most manipulations and storage were performed in glass tubes or bottles and handling was with glass pipettes (Pasteur pipettes) for organic solvents, to avoid the influence of plasticizers. A 10.0 μL quantity of an internal standard was pipetted with a micro glass syringe (SGE, Victoria, Australia), and the variation in accuracy (CV, %) for a series of 10 samples was ≤5%.

Reagents. We used the following reagents: petroleum ether (containing 20–30% of *n*-hexane), decane (Wako, Osaka, Japan), chloroform, methanol (Kanto Kagaku, Tokyo, Japan), sodium carbonate, disodium hydrogenphosphate dodecahydrate, and sodium dihydrogenphosphate dehydrate (Sigma, Tokyo, Japan). As standard chemicals, the following FA were obtained from Sigma: 14:0 (myristic acid), 16:0 (palmitic acid), 16:1n-7 (palmitoleic acid), 18:0 (stearic acid), 18:1n-9 (oleic acid), 18:2n-6 (linoleic acid), 18:3n-6 (γ-linolenic acid), 18:3n-3 (α-linolenic acid), 20:3n-6 (dihomo-γ-linolenic acid), 20:4n-6, EPA, 22:5n-3 (docosapentaenoic acid), and DHA. 17:0 (*n*-Heptadecanoic acid), BHT, and hydrogen chloride methanol reagent 10 (10–20% hydrochloride) were used as an internal standard, an antioxidant reagent, and a methyltransformation reagent (Tokyo Kasei Kogyo, Tokyo, Japan), respectively. Stock amounts of the internal standard and other standard chemicals were weighed on an analytical balance (with 0.01 mg precision), dissolved in decane containing BHT, then stored in Teflon-lined, screw-capped test tubes at 4°C. Stock solutions of BHT and decane were also made and stored at 4°C.

Blood materials. The Ethics Committee of each relevant institute approved the following three studies. First, in the framework of the Dietary Intervention to Polypectomized Patients (DIPP) study (41) and the Japanese Dietitians' Epidemiologic (JADE) study (29,30,42,43), we collected and stocked pooled plasma as a control, as described elsewhere (44). Plasma from anonymous patients with nonspecific medical conditions in Nagoya City University Hospital was gathered in one glass beaker on ice, mixed, and then poured into 1.5- to 5-mL stock tubes and stored at –80°C. We here used the pooled plasma for establishing measurement conditions for FA levels (mmol/L and mol%) in plasma according to the ASE.

Second, in the framework of the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC) (45), we routinely collect blood materials from patients in Aichi Cancer Center Hospital (20). Blood was collected using EDTA-2Na tubes and centrifuged at 2,000 *g* for 15 minutes at 4°C. Plasma and erythrocytes were prepared and stored at –80°C until analysis of individual FA by GLC. They were provided with an explanatory document and all gave their written informed consent for participation in the study. We here used erythrocytes from three noncancer controls for establishing the measurement conditions for FA composition (mol%) of erythrocytes according to ASE.

Third, in the framework of the Japanese Dietitians' Epidemiologic (JADE) study (29,30,42,43), we collected overnight fasting blood from 106 middle-aged Japanese dietitians (21 men and 85 women). Likewise, plasma and erythrocytes were simultaneously prepared in tubes including EDTA-2Na and stored at –80 °C until analysis of FA by GLC. We collected informed consent for participation in this study from all subjects. We here used plasma from nine male and nine female dietitians for comparison with two THM for measuring FA levels (mmol/L and mol%) in plasma as described herein. The 18 subjects were randomly selected and had no bias, and the samples were of sufficient volume to accomplish our objectives.

Instrumentation. For our ASE system, we used an Accelerated Solvent Extractor (ASE®) 200 (Nippon Dionex, Osaka, Japan), approved for use by the U.S. Environmental Protection Agency SW-846 Method 3545A for Pressurized Fluid Extraction, and accepted under the Contract Laboratory Program, Statement of Work OLMO 4.2. The system is computerized and comprises a cell oven, a cell tray for applying samples, a vial tray for recovering extracted solutions, a solvent sending pump, connected by a bound flexible line to solvent reserved bottles, and a nitrogen gas cylinder. To reduce solvent consumption, the line was changed from the standard 1.0 mm i.d. to 0.5 mm i.d. As optional equipment, an ASE® Solvent Controller allows for automatic mixing and delivery of different solvents. A small-size reciprocal air compressor, Toscon SLP5D-2SV (Toshiba, Tokyo, Japan), was included as part of the driving power to achieve safety for highly inflammable volatile solvents. The ASE 200 also features a series of cells (1 mL, 5 mL, and so on) that are stainless steel vessels for extracting target compounds from samples. A cylindrical cell body and two caps for the upper and bottom ends comprise one cell.

The system is usually employed with the following manipulations for each sample: (1) placement of a filter paper (Nippon Dionex, Osaka, Japan) in the bottom of a cell; (2) introduction of the sample into the cell; (3) movement of the cell to a cell oven; (4) introduction of the extracting solvent into the cell; (5) maintenance of an elevated temperature and pressure; (6) transfer of extracted solution to a glass vial and purging the residue from the cell with nitrogen gas when the extraction is completed. The filtered extracts are then taken

away from the extracted solution. This equipment automatically extracts, one by one, a maximum of 26 samples in minutes, by using conventional solvents at elevated temperatures and pressures, and reduces solvent consumption compared with general THM.

The equipment for the GLC system was as follows: a Shimadzu GC-2010 with a flame ionization detector (FID-2010), an auto-injector AOC-20i, and an auto-sampler AOC-20s and a hydrogen generator OPGU-2100S (Shimadzu, Kyoto, Japan). Shimadzu GC workstation software, GC solution version 2 (Shimadzu, Kyoto, Japan), provides multisystem PC control and advanced sample tracking. An air line bypasses from the line of the extraction system. A silica capillary column (30 m × 0.25 mm i.d.; DB-225 coating thickness 0.25 μm) (J&W Scientific, Folsom, CA) was employed (29,37).

GLC analysis. The GLC analysis has been detailed elsewhere (29,37), but was slightly modified according to the version of our GLC system. In brief, the oven temperature was programmed from 140°C to 180°C at a rate of 20°C/min and then from 180°C to 240°C at 3°C/min, with holding of the final temperature for 23.5 min. Total run time for one sample was 45.5 min. At about 42.5 min, the fraction of cholesterol esters and its derivatives were eluted. The nitrogen carrier flow rate was 10.2 mL/min (pressure, 200.0 kPa as a control mode) and for the nitrogen makeup gas was 28.0 mL/min. The column flow rate was 2.41 mL/min (line speed, 53.0 cm/s; purge flow rate, 3.0 mL/min). For the flame ionization detector, flow rates of hydrogen and air were 40.0 and 400.0 mL/min, respectively. The temperatures of the injection port and detector were 250°C and 260°C, respectively. The auto-injector was programmed to rinse a 10-μL syringe, once for each sample, pump three times to remove air bubbles, and then inject 3.0 μL of sample with a split-less method (sampling time, 1.0 min). The syringe was then rinsed five times with solvent. Integration and calculation of peak areas were performed with GC solution version 2 software, and the chromatographs and data were electronically stored.

Evaluation of FA levels in biomaterials. The identity of individual FA peaks was ascertained by comparison of the peak retention times with those of authentic standards. A single-point calibration was performed with an aliquot of an internal standard stock solution and the linearity of the method was checked by additional calibrators with a series of an internal standard concentration and appropriate dilutions of the stock solution. With integration of areas under the individual peaks adjusted for that of an internal standard, each FA was quantified as an absolute concentration (mmol/L) in plasma and compositions (molar weight percentage [mol%]) of total FA in plasma and erythrocytes. The following 13 FA were referenced to previous reports (29,30,43,46), which were investigated for associations between dietary intake of fish, fat, and FA, serum levels of triglycerides, total and HDL cholesterols, and the blood levels of FA: 14:0, 16:0, 16:1n-7, 18:0, 18:1n-9, 18:2n-6, 18:3n-6, 18:3n-3, 20:3n-6, 20:4n-6, EPA, 22:5n-3, and DHA.

Compared with THM-A as described herein, recovery rates (%) for the same 13 standard chemicals (a series of 10 samples) were calculated after step 2, step 4, and all procedures of the new method featuring ASE (Fig. 1). Intra-assay CV were based on the analysis of a series of 10 samples, derived from one sample/subject of pooled plasma or erythrocytes, and then all extracted and analyzed within a day. Interassay CV were based on replicate analyses of pooled plasma over a period of 10 d, and a total of 100 samples (10 samples/d × 10 d) were measured. Interassay reproducibility (CV), moreover, was checked according to two replicate measurements of the same pooled plasma per day for 20 d (total 40 samples = 2 samples/d × 20 d). With erythrocytes, samples from three subjects were used as each control, because a large volume of erythrocytes could not be collected from a single subject.

Comparison of methodologies. The new method featuring ASE was compared with two different THM for measuring FA levels (mmol/L and mol%) using 18 plasma samples from one part of the Japanese Dietitians' Epidemiologic (JADE) study (42) as described here previously. The three methods are distinctly different regarding extraction, trans-methylation, and GLC analyses of FA, respectively. Using the following THM-A (29), we have demonstrated relationships between dietary intakes of fish, fat, and FA and the corresponding FA levels in plasma in the study (29,30,43). The same samples were also measured by the following THM-B (47) at a clinical laboratory testing service (SRL Inc., Tokyo, Japan). We have no conflict of interest or collaborative agreement with the service company. The samples, therefore, were independently measured for us.

As THM-A (29), measurement was with the same reagents and procedures, except for the following: (1) the required sample volume was 100.0 μL; (2) the proportion of chloroform in the extracting solvent differed at the second step (Fig. 1); (3) each organic layer at the second and fourth steps was extracted from water-mixed phases by centrifugation. In brief, after 100.0 μL of plasma was transferred into a glass tube with a Teflon-lined screw-cap, 10.0 μL of an internal standard and 10 μL of BHT were added. Then 700 μL of 0.9 wt/vol% potassium chloride aqueous solution and 3 mL of Folch's solvent (chloroform/methanol, 2:1, vol/vol) (32) were added followed by vigorous mixing. After standing for 10 min at room temperature, 1 mL of water and 1 mL of chloroform were added with vigorous mixing, and then the lipids and free FA were extracted by centrifugation (2,000 *g*, 20°C, 5 min). The bottom chloroform layer was collected by using a glass pipette, and then the extraction was repeated a total of three times to maximize recovery with 2 mL of chloroform. The extracted solutions were pooled and evaporated to dryness under nitrogen gas, and then converted to FAME by 1 mL of hydrogen chloride methanol reagent 10 (10–20% hydrochloride), at 100°C for 60 min (29). At the fourth step, likewise, a total of three extractions with 2 mL of petroleum ether were performed. The GLC equipment was a Shimadzu GC-17A with a hydrogen gas cylinder and a Chromatopac Integrator C-R7A (Shimadzu, Kyoto, Japan). Measurement condition of GLC has been reported elsewhere (29,37). Re-

garding precision of the FA measurements in plasma, intra- and interassay CV were distributed from 1.8% to 4.8% and from 2.5% to 7.2%, respectively (29).

For THM-B (47), the measurement principles and the steps (Fig. 1) were basically the same as THM-A (29). The equipment was also the same as THM-A, except for a hydrogen generator, HGE-1A (Shimsadzu, Kyoto, Japan). However, the lipids and free FA were first extracted with Folch's solvent (32), and FA were then treated with 100% chloroform after acid hydrolysis by 0.5 M hydrochloride and acetonitrile/water (9:1, vol/vol). Then, 0.4 N potassium methoxide (Kanto Kagaku, Tokyo, Japan) and 14.0 wt% of boron trifluoride–methanol (GL Sciences, Tokyo, Japan) were used for transmethylation of FA at the third step (30,35). The FAME were extracted with Wako-gel C-200, *n*-hexane (Wako, Osaka, Japan), and water at the fourth step. The conditions of centrifugation were 2,000 *g* at 20 °C for 5 min. A fused silica capillary column (30 m × 0.25 mm i.d.; Omegawax 250 coating thickness 0.25 μm) (Supelco, Bellefonte, PA) was employed. For FA measurements in plasma, intra- and interassay CV were distributed from 2.1% to 8.1% and from 5.5% to 8.7%, respectively.

Statistics. Statistical analyses were performed with PC-SAS version 9.1 (SAS Institute Inc., Cary, NC). The data are given as mean ± SD. Pearson's correlation coefficients between the values (mmol/L and mol%) for plasma FA were calculated. All tests were two-sided and a P value of 0.05 or less was taken as significant.

RESULTS

Sample preparation for ASE. When plasma samples were directly introduced into a 1-mL cell for extraction with organic solvent on ASE 200, the extraction at the second step (Fig. 1) needed to be discontinued because very small particles of aggregated soluble proteins in plasma clogged the small exit of the cell bottom cap. As the first step, therefore, a membrane filter (OMMIPORE™, 0.2 μm, JG) (Millipore, Cork, Ireland) was firmly fixed between a cell body and the cell bottom cap to prevent clogging, and then two paper filters were laid on the membrane filter. This manipulation is not described in the manual for using ASE 200 and was our original idea. To prevent elution or contamination with FA, moreover, we washed the paper filters in the extracting solvent before use. The procedure for applying one plasma sample was as follows: (1) 10.0 μL of the internal standard (3.5 mmol/L) and 10 μL of BHT (0.45 mmol/L) were added into the cell; (2) 35.0 μL of plasma and 150 μL of extracting solvent (chloroform/methanol, 1:2, vol/vol) were vigorously mixed in a 1.5-mL micro tube, and then the solution was transferred into the cell; (3) 50 μL of extracting solvent was rinsed in the micro tube and then the residue was also added to the cell. Although we examined the range 25–50 μL of plasma sample for measurement, the optimal volume was 35.0 μL (data not shown). As described above, the extraction step from more than 50 μL of plasma may abort because of being clogged in the ASE 200 line due to aggregated soluble proteins. Two pieces of paper filter were used to absorb the internal standard and BHT, and the pretreated plasma sample, in that order.

To remove hemoglobin and prevent oxidative degradation of lipids and FA caused by iron of hemoglobin, erythrocytes were prepared to erythrocyte membranes (white ghosts) with 5 mM sodium phosphate buffer (pH 7.4) (48). The white ghosts from one sample were directly applied into a 1-mL cell put on one paper filter to extract. In this case, a membrane filter was not needed. As with plasma samples, 10.0 μL of the internal standard (1.0 mmol/L), 10 μL of BHT (0.45 mmol/L), and the white ghosts were added into the cell, in that order. The optimal volume of erythrocytes sample for measuring was 50.0 μL (data not shown). Less than 25 μL of erythrocytes could not be stably measured under our current conditions, while more than 100 μL required much increased rinsing time with sodium phosphate buffer to make white ghosts. It should be noted that FA contents in whole blood are not equivalent to those in erythrocyte membranes.

ASE for lipids, FA, and FAME extraction. After systematic changing in the conditions for ASE 200 at the second step (Fig. 1), the optimized conditions for extracting lipids and FA from blood materials were achieved as summarized in Table 1. There were the same for plasma and erythrocytes. Chloroform/methanol, 1:2 vol/vol, was used for the extraction. We extracted all 13 selected FA in blood materials at one time because the residual material was limited, as determined by repeated extraction tests. For one sample, the volume of the extracted solution

TABLE 1
Optimized Conditions for Extracting Lipids and FA at the Second Step and FAME at the Fourth Step with an Accelerated Solvent Extractor (ASE® 200)

	Lipids and FA (at the second step)	FAME (at the fourth step)
Extraction cell (mL)	1	5
Heating time (min)	0	0
Static time (min)	5	0
Temperature (°C)	75	50
Pressure (psi)	1,500	1,500
Extracting solvent	Chloroform/methanol (1:2, vol/vol)	Petroleum ether
Flash (%)	10% of extracting solvent	10% of extracting solvent
Purge (s)	30	30

and the extracting time were about 5.5–6 mL and 8.5 min, respectively. The time included rinsing of the line for ASE 200. The extracted solution was evaporated under a stream of nitrogen gas at 37°C.

All samples at step 3 (Fig. 1) were transferred into glass tubes with Teflon-lined screw-caps and then treated with 1 mL of hydrochloride-methanol reagent for FA conversion from lipids and subsequent methyl-transformation. The glass tubes were tightly capped and placed in a block heater (100°C), and then withdrawn and cooled after 60 minutes of heating. To prevent erosion of the stainless steel cells by the potent hydrochloric acid, 0.8–2 mL of 0.4 M sodium carbonate aqueous was added into the tubes and the solution was prepared to pH 7. The volume of aqueous sodium carbonate corresponded to 5–15% concentrations (depending on lot numbers of the products) of hydrochloride. In GLC analyses, alkaline hydrolysis of FAME caused by the addition of sodium carbonate aqueous was not observed (data not shown).

At the fourth step (Fig. 1), petroleum ether was used as an organic solvent for extracting FAME from the water-methanol layer on ASE 200, but the layer was not directly applied into a 5-mL cell because of leakage through the exit of the bottom cap. To prevent this, therefore, a membrane filter was firmly fixed between the cell body and the cell bottom cap. This manipulation is not described in the manual for using ASE 200 and was our original idea. Paper filters were not used here. First, the entire water-methanol layer was transferred into the cell. Second, the glass tube was rinsed with 500 μL of petroleum ether and then the residue was also added to the cell. After systematic changes, the optimized conditions for extracting methyl esters by ASE 200 are summarized in Table 1. In this step, we also extracted all 13 selected FAME at one time because residues were limited according to repeated extraction tests. For one sample, the volume of extracted solution and the extracting time (including rinsing time) were about 7.5–8 mL and 3.5 min, respectively.

GLC separation of individual FA. The extracted solution at the fourth step (Fig. 1) was evaporated under a stream of nitrogen gas at 37°C for complete removal of the solvent, and then the residue was redissolved in 400.0 μL of petroleum ether for application to GLC. Representative GLC profiles for individual FA in plasma and erythrocytes are shown in Figure 2. We could clearly demonstrate separation of individual FA.

Precision and accuracy. According to the analysis of a series of 10 samples, recovery rates of the internal standard were 86.4% after step 2, 97.6% after step 4, and 81.6% after all procedures with the new method featuring accelerated solvent extraction. For the 13 selected standard chemicals, however, the ranges of recovery rates (%) adjusted for an internal standard were distributed from 95.6% (for 18:3n-6) to 107.9% (18:0), 95.1% (18:0) to 102.1% (18:3n-6), and 94.5% (18:3n-6) to 109.1% (18:2n-6), in that order. Table 2 shows FA levels (mol/L and/or mol%) in blood materials and the intra- and interassay CV according to the new method. For

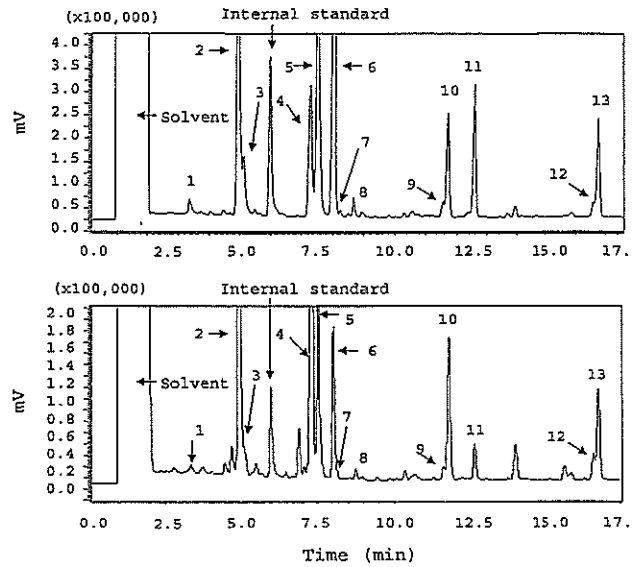

FIG. 2. Representative chromatograms after gas-liquid chromatography for the 13 selected FA in plasma (top) and erythrocytes (bottom). Peaks: internal standard (n-neptadecanoic acid, 17:0); 1, myristic acid (14:0); 2, palmitic acid (16:0); 3, palmitoleic acid (16:1n-7); 4, stearic acid (18:0); 5, oleic acid (18:1n-9); 6, linoleic acid (18:2n-6); 7, γ-linolenic acid (18:3n-6); 8, α-linolenic acid (18:3n-3); 9, dihomo-γ-linolenic acid (20:3n-6); 10, arachidonic acid (20:4n-6); 11, eicosapentaenoic acid (20:5n-3); 12, docosapentaenoic acid (22:5n-3); 13, docosahexaenoic acid (22:6n-3).

plasma, the intra-assay CV were ≤6.0%, except for the concentration and the composition of 14:0 (9.7% and 8.0%, respectively). The interassay CV for mean concentrations and compositions of the total 100 samples (10 samples × 10 d) were <6.0% for all 13 selected FA, and the distribution of the CV over a period of 10 d was <6.0%, except with the concentrations of 14:0, 16:1n-7, 18:3n-6, and 18:3n-3 (8.4, 6.8, 7.0, and 6.8%, respectively) and the composition of 14:0 (6.9%). For the compositions in erythrocytes, the intra-assay CV were also <4.0%, except a minor group of 14:0, 18:3n-6, and 18:3n-3 (≤0.5% of total FA for each). The interassay CV (total 100 samples) were also <4.0%, except for a minor group of 14:0, 18:3n-6, and 18:3n-3. We could not demonstrate CV for mean compositions because we could not collect sufficiently large volumes of erythrocytes from a single subject. Moreover, interassay reproducibility over a period of 20 d (the CV for a total 40 samples [2 samples × 20 d]) was almost the same distribution (data not shown).

Although we lack a gold standard method, the mean levels (mmol/L and mol%) and the SD for each FA were coincident and differences in values among the three different methods were not major (Table 3). For example, plasma concentrations of EPA, which are closely associated with dietary intake, were 0.20, 0.27, and 0.23 for the means and 0.12, 0.16, and 0.14 for the SD, in that order. The compositions were 1.7, 2.0, and 1.9, and 1.0, 1.1, and 1.0, respectively. We demonstrated, furthermore, that most of the Pearson's correlation coefficients between them were ≥0.95 ($P < 0.0001$) and all of them were

TABLE 2
Intra- and Interassay CV for FA Measurements in Plasma and Erythrocytes According to a New Method Featuring Accelerated Solvent Extraction

	Intra-assayCVs (10 samples within a day)		Inter-assay CVs: Total 100 samples (10 samples/d × 10 d)		Inter-assay CVs: Distribution of the CVs (10 d for 100 samples)
	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD
Concentration (mmol/L) in plasma					
14:0	0.10 ± 0.010	9.7	0.10 ± 0.005	4.6	8.4 ± 1.5
16:0	2.67 ± 0.102	3.8	2.63 ± 0.068	2.6	4.4 ± 0.9
16:1n-7	0.35 ± 0.016	4.7	0.35 ± 0.015	4.4	6.8 ± 1.4
18:0	0.77 ± 0.039	5.0	0.77 ± 0.025	3.3	4.7 ± 1.3
18:1n-9	2.50 ± 0.111	4.4	2.46 ± 0.073	3.0	5.6 ± 1.0
18:2n-6	2.84 ± 0.120	4.2	2.79 ± 0.105	3.8	5.5 ± 1.0
18:3n-6	0.03 ± 0.002	6.0	0.03 ± 0.001	5.1	7.0 ± 1.6
18:3n-3	0.08 ± 0.004	4.7	0.08 ± 0.003	4.6	6.8 ± 1.6
20:3n-6	0.09 ± 0.002	2.4	0.09 ± 0.003	3.7	4.4 ± 1.4
20:4n-6	0.51 ± 0.018	3.5	0.51 ± 0.021	4.1	4.4 ± 1.0
20:5n-3	0.16 ± 0.006	4.0	0.16 ± 0.008	5.2	5.0 ± 1.1
22:5n-3	0.04 ± 0.002	4.5	0.04 ± 0.002	5.8	5.0 ± 1.2
22:6n-3	0.32 ± 0.013	4.1	0.33 ± 0.018	5.6	4.4 ± 1.0
Composition (mol%) in plasma					
14:0	1.0 ± 0.08	8.0	1.0 ± 0.05	4.8	6.9 ± 2.0
16:0	25.6 ± 0.21	0.8	25.5 ± 0.19	0.8	0.9 ± 0.2
16:1n-7	3.3 ± 0.09	2.6	3.4 ± 0.08	2.3	2.9 ± 0.8
18:0	7.4 ± 0.35	4.7	7.5 ± 0.12	1.6	3.6 ± 1.3
18:1n-9	23.9 ± 0.31	1.3	23.8 ± 0.17	0.7	1.1 ± 0.2
18:2n-6	27.2 ± 0.26	0.9	27.0 ± 0.26	1.0	0.9 ± 0.1
18:3n-6	0.3 ± 0.01	3.3	0.3 ± 0.01	3.3	3.6 ± 0.9
18:3n-3	0.7 ± 0.03	4.3	0.7 ± 0.03	3.4	5.6 ± 2.4
20:3n-6	0.8 ± 0.02	2.4	0.8 ± 0.02	2.5	2.2 ± 0.7
20:4n-6	4.8 ± 0.06	1.3	4.9 ± 0.09	1.8	1.4 ± 0.2
20:5n-3	1.5 ± 0.02	1.1	1.6 ± 0.04	2.8	1.1 ± 0.3
22:5n-3	0.4 ± 0.01	2.5	0.4 ± 0.02	3.9	3.1 ± 0.7
22:6n-3	3.1 ± 0.06	2.0	3.2 ± 0.11	3.5	2.2 ± 0.3
Composition (mol%) in erythrocytes					
14:0	0.5 ± 0.05	10.4			13.1 ± 7.4
16:0	27.8 ± 0.23	0.8			1.3 ± 0.5
16:1n-7	1.6 ± 0.03	2.0			2.7 ± 1.3
18:0	18.5 ± 0.56	3.0			2.3 ± 0.6
18:1n-9	16.1 ± 0.61	3.8			3.4 ± 0.7
18:2n-6	10.9 ± 0.41	3.8			3.3 ± 1.5
18:3n-6	0.02 ± 0.002	7.7			19.2 ± 8.2
18:3n-3	0.2 ± 0.03	18.1			15.6 ± 5.8
20:3n-6	0.9 ± 0.01	1.4			1.5 ± 0.4
20:4n-6	11.4 ± 0.43	3.8			2.0 ± 0.8
20:5n-3	2.2 ± 0.04	1.8			2.9 ± 1.8
22:5n-3	2.4 ± 0.04	1.6			2.4 ± 0.9
22:6n-3	7.4 ± 0.10	1.3			2.2 ± 0.8

at least ≥0.85 ($P < 0.0001$), except for the composition of 18:0 (Table 4). The reason for the relatively low coefficients in this case was unclear, but the level with the new method featuring ASE significantly correlated with that with THM-A ($r = 0.72$, $P < 0.001$).

DISCUSSION

Some PUFA are particularly useful in assessing the corresponding intakes in the short term in plasma and serum, in the medium term for phospholipids in erythrocytes and platelets, and in the long term for adipose tissue (24,25), because they are not biosynthesized in humans. Especially the levels of EPA, DHA, and n-3 HUFA are appropriate biomarkers of the relative dietary intakes (26–31), but they have not routinely been used due to laborious handwork for measurement, which also means expense in the commercial setting. Using ASE, however, here we could develop a new method featuring ASE for measuring FA in plasma and erythrocytes that demonstrated acceptable precision and accuracy, with many practical advantages.

TABLE 3
Comparison of FA Levels in Plasma from 18 Healthy Subjects Measured by a New Method Featuring Accelerated Solvent Extraction, and Traditional Handwork Methods (THM)-A and -B

	New method Mean ± SD	THM-A1 Mean ± SD	THM-B[a] Mean ± SD
Concentration (mmol/L) in plasma[b]			
14:0	0.11 ± 0.05	0.13 ± 0.08	0.16 ± 0.10
16:0	2.69 ± 0.70	3.12 ± 0.95	3.00 ± 0.96
16:1n-7	0.86 ± 0.11	0.83 ± 0.10	0.73 ± 0.10
18:0	0.91 ± 0.21	0.97 ± 0.28	0.90 ± 0.27
18:1n-9	2.37 ± 0.74	2.70 ± 0.82	2.18 ± 0.79
18:2n-6	3.18 ± 0.76	3.75 ± 0.88	3.54 ± 1.05
18:3n-6	0.03 ± 0.02	0.04 ± 0.02	0.04 ± 0.03
18:3n-3	0.11 ± 0.05	0.13 ± 0.06	0.11 ± 0.05
20:3n-6	0.08 ± 0.03	0.12 ± 0.04	0.10 ± 0.03
20:4n-6	0.56 ± 0.12	0.71 ± 0.18	0.59 ± 0.15
20:5n-3	0.20 ± 0.12	0.27 ± 0.16	0.23 ± 0.14
22:5n-3	0.05 ± 0.02	0.08 ± 0.02	0.07 ± 0.02
22:6n-3	0.37 ± 0.12	0.54 ± 0.17	0.46 ± 0.16
Composition (mol%) in plasma			
14:0	0.9 ± 0.3	0.9 ± 0.4	1.2 ± 0.5
16:0	23.3 ± 2.1	23.1 ± 2.4	24.5 ± 2.6
16:1n-7	7.7 ± 1.6	6.5 ± 1.5	6.4 ± 1.6
18:0	7.9 ± 0.8	7.2 ± 0.7	7.5 ± 0.9
18:1n-9	20.3 ± 2.7	19.9 ± 2.5	17.6 ± 2.5
18:2n-6	27.8 ± 3.6	28.3 ± 3.9	29.5 ± 4.2
18:3n-6	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.2
18:3n-3	0.9 ± 0.2	1.0 ± 0.3	0.9 ± 0.3
20:3n-6	0.7 ± 0.1	0.9 ± 0.2	0.8 ± 0.2
20:4n-6	4.9 ± 0.7	5.3 ± 0.9	5.0 ± 0.9
20:5n-3	1.7 ± 1.0	2.0 ± 1.1	1.9 ± 1.0
22:5n-3	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.2
22:6n-3	3.2 ± 0.9	4.0 ± 1.0	3.8 ± 1.0

[a]THM-A and THM-B are described in text.
[b]Plasma samples from 18 healthy subjects (9 men and 9 women) were measured for each of three different measurement methods. The sample selection is described in text.

TABLE 4
Pearson's Correlation Coefficients Between Levels of FA in Plasma from 18 Healthy Subjects Determined by a New Method Featuring Accelerated Solvent Extraction, and Traditional Handwork Methods (THM)-A and -B

	Between new method and THM-A[a]		Between new method and THM-B[a]		Between THM-A and THM-B	
	r	P	r	P	r	P
Concentration (mmol/L) in plasma[b]						
14:0	0.97	<0.0001	0.99	<0.0001	0.97	<0.0001
16:0	0.96	<0.0001	0.93	<0.0001	0.96	<0.0001
16:1n-7	0.99	<0.0001	0.91	<0.0001	0.93	<0.0001
18:0	0.94	<0.0001	0.93	<0.0001	0.97	<0.0001
18:1n-9	0.96	<0.0001	0.88	<0.0001	0.92	<0.0001
18:2n-6	0.94	<0.0001	0.89	<0.0001	0.91	<0.0001
18:3n-6	0.97	<0.0001	0.97	<0.0001	0.98	<0.0001
18:3n-3	0.97	<0.0001	0.95	<0.0001	0.99	<0.0001
20:3n-6	0.96	<0.0001	0.98	<0.0001	0.97	<0.0001
20:4n-6	0.96	<0.0001	0.95	<0.0001	0.96	<0.0001
20:5n-3	0.99	<0.0001	0.99	<0.0001	0.99	<0.0001
22:5n-3	0.96	<0.0001	0.97	<0.0001	0.98	<0.0001
22:6n-3	0.97	<0.0001	0.98	<0.0001	0.98	<0.0001
Composition (mol%) in plasma						
14:0	0.97	<0.0001	0.99	<0.0001	0.97	<0.0001
16:0	0.97	<0.0001	0.95	<0.0001	0.97	<0.0001
16:1n-7	0.97	<0.0001	0.95	<0.0001	0.97	<0.0001
18:0	0.72	<0.001	0.59	<0.01	0.78	<0.001
18:1n-9	0.98	<0.0001	0.91	<0.0001	0.93	<0.0001
18:2n-6	0.98	<0.0001	0.97	<0.0001	0.96	<0.0001
18:3n-6	0.96	<0.0001	0.96	<0.0001	0.98	<0.0001
18:3n-3	0.97	<0.0001	0.96	<0.0001	0.99	<0.0001
20:3n-6	0.93	<0.0001	0.91	<0.0001	0.85	<0.0001
20:4n-6	0.98	<0.0001	0.89	<0.0001	0.91	<0.0001
20:5n-3	1.00	<0.0001	0.99	<0.0001	1.00	<0.0001
22:5n-3	0.96	<0.0001	0.94	<0.0001	0.97	<0.0001
22:6n-3	0.98	<0.0001	0.96	<0.0001	0.96	<0.0001

For footnotes, see Table 3.

ASE 200 is capable of concentrating small amounts of object chemicals and compounds rapidly, such as herbicides, pesticides, polychlorinated biphenyls (PCB), and dioxins, diluted in environmental media (soil and foods), according to the ASE kinetics made possible by increased temperature while keeping the solvent below its boiling point under pressure. We here demonstrated quantitative extraction of FA from ≤50 μL of blood materials, with acceptable values for accuracy and precision, intra- and interassay CV ≤6.0%, except for a minor group of FA, where most were <10.0%. According to our new method, the mean values of 20:3n-6, 22:5n-3, and DHA in plasma samples were about 20% lower than those with THM-A and B. As well as other 10 FA, however, the recovery rates for the three corresponding standard chemicals were adequately high and were 102.0, 97.9, and 97.1%, in that order. Therefore, we did not clarify the reason. Recovery experiments with repeated extraction also indicated that a single step does not result in large residues. Therefore, we just select the extraction program at the second and the fourth steps (Fig. 1) and push the start button after applying samples to cells of the ASE 200 for extraction of FA and FAME.

Even with our new method there are some limitations to routine measurement of FA in biomaterials. The series of necessary steps cannot be completely automated, and blood materials cannot be applied directly to the ASE system. It takes about 7 h for an extraction as a "day run" and 10 h for analysis as a "night run" to measure 12 samples of biomaterials. However, the method is applicable to homogenates of tissues and silica gel lipid fractions (triglycerides, cholesterol esters, phospholipids, and free FA) separated by TLC. Advantages, similarities, and disadvantages between the new method featuring ASE, THM-A, and B are summarized in Table 5. Compared with THM-A and B, our new method is performed easily without major physical effort, according to the automatic extraction of FA with ASE 200. Although total run time for 12 samples does not differ among the three different methods, our new method has an excellent time performance. However, the speed for extracting lipids or methyl esters, one by one, is a limitation with ASE 200. In the future, therefore, we still hope to make further improvements. The cost for a series of ASE 200 instruments is large, but there are no differences in the running costs for disposables and reagents

TABLE 5
Summary of the Differences for Measuring FA in Plasma and Erythrocytes According to a New Method Featuring Accelerated Solvent Extraction, THM-A, and THM-B

	New method	THM-A[a]	THM-B[a,d]
Instruments			
GLC for analyzing FA	Use	Use	Use
ASE 200 for extracting FA	Use	No use	No use
Centrifugal machines for			
pretreatment of plasma and erythrocytes	Use	Use	Use
1st and 2nd extraction	No use	Use	Use
Sample volume (mL)			
Plasma	35	100	100
Erythrocytes	50	50	(50)
Centrifugation (times)[b]			
Pretreatment of plasma and erythrocytes			
Plasma	12	0	0
Erythrocytes	36	36	36
Extraction steps			
1st extraction	0	36	36
2nd extraction	0	36	36
Manipulation[c]			
Pretreatment of plasma	–	+	+
Pretreatment of erythrocytes	±	±	±
1st extraction	++	–	–
Trans-methylation	+	+	–
(Number of processes)	(1 process)	(1 process)	(2 processes)
2nd extraction	++	–	–
GLC analysis	±	±	±
Run time (min)[b,c,d,e]			
Pretreatment of plasma	30	0	0
Pretreatment of erythrocytes	90	90	90
1st extraction	100	120	(120)
Methyl-transformation	60	60	(90)
2nd extraction	60	90	(90)
GLC analysis	±	±	±
Summary[c]			
Manipulation	++	–	–
Automatic extraction of FA	++	–	–
Non-considerable physical effort	++	–	–
Total run timee	±	±	±
Time performance	++	–	–
Treated sample number per day	±	±	±
Running costs	±	±	±
Cost for instruments	–	±	±

[a]THM-A and THM-B are described in detail in text.
[b]The figures for measuring FA of 12 samples are shown.
[c]Advantages (2 categories), similarities, and disadvantages between a new method, THM-A, and THM-B are here defined as ++ and +, ±, and –, in that order.
[d]When FA in plasma and erythrocytes were measured by THM-B in our laboratory, but not a clinical laboratory company (see the text), the estimated time is shown in parentheses.
[e]Run time is not included for some procedures, such as administration of an internal standard, an antioxidant chemical, and blood samples, and evaporation of the extracted solvents after the two extraction steps.

among the three methods. The commercial laboratory fee is very expensive.

In conclusion, using ASE and GLC, we have developed a new method for determining both the concentrations and the compositions of FA in plasma and erythrocytes as biomarkers for dietary intake of fish, fat, and FA. The advantages of our new method featuring ASE are that it is feasible to measure small volumes of multiple samples, automatically, quantitatively, routinely, easily, rapidly, and cheaply, with acceptable precision and accuracy. As a part of the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC) research, it will be a useful aid for the large numbers of blood samples available for Aichi Fatty Acid (AiFat) Research (20) necessitating routine measurement to clarify associations with cancers in different sites.

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Risks and benefits of omega-3 fatty acids on cancer risk

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Send response to journal:
Re: Risks and benefits of omega-3 fatty acids on cancer risk

Risks and benefits of omega-3 fatty acids on cancer risk

Editor - Hooper et al1 systematically reviewed cohort studies and randomized controlled trials to examine whether intake of omega-3 fatty acids (FAs) or n-3 polyunsaturated fatty acids (PUFAs) is beneficial for prevention of cardiovascular disease and cancer, and concluded that n-3 PUFAs have little effects for reducing the risk of cancer.

We here would like to discuss most frequently probed associations between consumption of n-3 PUFAs and risk of colorectal cancer. Seven articles cited as negative effects, except one, of n-3 PUFAs on colorectal carcinogenesis were reported from the USA (3 articles), Norway (2 articles), Sweden (1 article) and the Netherlands (1 article).1

Consumption of total FAs and saturated FAs seemed greater in those people than that in Japanese, but no differences for the intake of arachidonic acid (AA) and alpha-linolenic acid. Intake of highly unsaturated FAs (n-3 HUFAs) or fish FAs in Japanese, on the other hand, was far greater than those people, indicating that the consumption of n-3 HUFAs by those people is only approximately 1/10th of Japanese. Because n- 3 PUFAs compete with n-6 PUFAs (or AA) in various metabolic processes, the absolute intake of n-3 PUFAs (or n-3 HUFAs) may be crucial for colorectal carcinogenesis.2 Consumption of n-3 HUFAs in those people appears insufficient to exert pharmacologic influence.

The ratio of n-3 PUFAs/n-6 PUFAs (or specifically n-3 HUFAs/AA) may also be critical. The ratios in those people appear to be far less than those in Japanese. Plasma concentration of phospholipids in those people would be expected to be highly saturated with n-6 PUFAs, linoleic acid and AA, in particular, and the concentrations of n-3 PUFAs and n-3 HUFAs might not effectively compete in the arachidonate cascade.3

As seen in our observational study4 and randomized controlled trial,5 omega-3 FAs, n-3 PUFAs and/or n-3 HUFAs may indeed be favorable for the prevention of colorectal adenomas/tumors in populations, including Japanese, who consume appreciable amounts of fish and marine foods. (317 words)

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Having your fish and eating it

27 April 2006

RESEARCH COMMUNICATION

Risk of Endometrial Cancer Mortality by Ever-use of Sex Hormones and Other Factors in Japan

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Abstract

Objectives: To examine associations of ever-use of sex hormones (EUSH) and other factors with endometrial cancer (EC) mortality through a nation-wide Japan Collaborative Cohort Study. Methods: A total of 63,541 women aged 40-79 years, enrolled in 1988-90 from 45 municipalities of Japan, were followed until 2003 to record their vital status. Using baseline data, the Cox proportional hazard model (age adjusted and multivariate) was used to estimate the hazard ratio (HR) and 95% confidence interval (CI) for EC mortality by selected factors, including EUSH. Bivariate analysis was also conducted to establish associations between EUSH and other factors. Results: The mortality rate from EC was 2.6 per 100,000 person-years during the mean follow-up period of 13.3 years. Prevalence rate of EUSH was 5.2%. Significantly increased risk of EC mortality was found for EUSH with both age adjusted (HR=6.43, 95%CI=2.10-19.67) and multivariate (HR=5.33; 95%CI=1.51-18.82) analyses. Bivariate analysis indicated that history of diabetes mellitus, smoking, drinking, and age at first delivery were positively associated with EUSH, whereas age, number of delivery, number of pregnancy, and age at menarche demonstrated inverse links. Conclusions: Our results imply that EUSH may increase the risk of EC mortality among Japanese women. However, further studies with more deaths are needed to validate the results.

Key Words: Endometrial cancer mortality - cohort study - ever use of sex hormones - hormone replacement therapy - Japan

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Introduction

Endometrial cancer (EC) accounted for 198,783 new cases (3.9% of all new cancer cases in women) worldwide in 2002, making this the 7th most common cancer in the world. Because of its favorable prognosis, the number of deaths from this disease was only one-fourth (50,327 deaths, 1.7% of all cancer deaths in women). It is therefore the 13th most common cause of female cancer death worldwide (Parkin et al., 2005). International data indicate that this disease is more prominent in developed than developing countries. Among the developed countries, the incidence rate is relatively low in Japan as compared to the countries of north America and Europe (Parkin et al., 2005; Persson and Adami, 2002; IARC, 2002). International variation in diets, body size, body fat distribution, and exogenous estrogen use may contribute to the observed global differences in the incidence of these hormone-dependent malignancies. For instance, lower prevalence of hormone replacement therapy (HRT) in Japanese community as compared to other countries such as United States (Nagata et al., 1996), and consumption of plant based cuisines which are low in fat and high in fiber that typify the Japanese diets may be some of the reasons of the lower incidence of EC in Japan (Goodman et al., 1997).

Although incidence rates are still low in Japan, several reports (IARC, 2002; Persson and Adami, 2002; IARC 1997; IARC, 1987) have indicated that EC has been increasing gradually over the last few decades. Increasing availability of estrogen to the estrogen sensitive endometrium may increase the risk of EC (Parslov et al., 2000). In Japan, the production of conjugated estrogen, often used for HRT, has increased 2-fold between 1992 and 1993. Pharmaceuticals companies and women's magazines are actively advertising the importance of HRT especially for menopause women (Nagata et al., 1996). Increased life expectancy, a reduction

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in parity, and decreasing trend of breastfeeding practices might be some of the factors that contribute to the increasing trend of EC (Salazar-Martinez et al., 1999; Inoue et al., 1994). Westernization of lifestyle including a change in diet, notably increased fat and animal meat intake, may also be involved in the recent increase in EC in Japan (Inoue et al., 1994).

Various studies reported that uses of unopposed estrogen (Lacey et al., 2005; Emons et al., 2004; Persson and Adami, 2002; Pukkala et al., 2001; Parslov et al., 2000; Persson et al., 1999; Weiderpass et al., 1999; Cushing et al., 1998; Shapiro et al., 1998; Beresford et al., 1997; Pike et al., 1997; Grady et al., 1995; Brinton and Hoover, 1993) are associated with significantly increased risk of EC. Exposure to excessive estrogens, entailing (causing) continued stimulation of the endometrium, appears to be the key mechanism in endometrial carcinogenesis (Persson and Adami, 2002). Some other potential risk factors for EC may include increased BMI, hypertension, diabetes mellitus, nulliparity, non-use of oral contraceptives, decreasing physical activity, and non-smoking (Xu et al., 2004; Persson and Adami, 2002; Jain et al., 2000; Parslov et al., 2000; Salazar-Martinez et al., 1999; Goodman et al., 1997; Inoue et al., 1994; Shu et al., 1993; La Vecchia et al., 1986).

Although various studies, mostly of case-control type, have addressed the associations of EC with hormone use and reported significantly increased risk (Lacey et al., 2005; Emons et al., 2004; Persson and Adami, 2002; Pukkala et al., 2001; Parslov et al., 2000; Persson et al., 1999; Weiderpass et al., 1999; Cushing et al., 1998; Shapiro et al., 1998; Beresford et al., 1997; Pike et al., 1997; Grady et al., 1995; Brinton and Hoover, 1993), except one case-control Japanese study which showed insignificant association (Mizunuma et al., 2001), no cohort study has been conducted in Japan till today and hence these associations are still uncertain from cohort study among Japanese women. To our knowledge, present study is the first prospective cohort study that addressed the associations of EC mortality with ever use of sex hormone (EUSH) and some other potential factors by using the data from the nation-wide Japan Collaborative Cohort (JACC) Study.

Materials and Methods

Study Cohort

The JACC Study for Evaluation of Cancer Risk (sponsored by the Ministry of Education, Culture, Sports, Science and Technology of Japan) is a large and nation-wide multicenter prospective cohort study which enrolled 127,477 apparently healthy subjects (male=54,032 female=73,445) aged from 16 to 112 years (<40 years=12,295, 40-79 years=110,792, and ≥80 years=3,760) at the time of baseline survey (through questionnaire) during 1988-1990. These subjects were recruited from 45 municipal areas (6 cities, 34 towns and 5 villages) located in 7 districts (out of 8) of Japan, either from general population or at the time of participants' general health check up which was periodically provided by these municipalities. Informed consent from participant was obtained using two strategies either by signing the cover page of the questionnaire (at the individual level which covered majority of the participants) or by explaining the aim of the study and confidentiality of the data (at the group level) to the community leader. Nagoya University School of Medicine approved this study. Details of the study design are described elsewhere (Ohno et al., 2001). Excluding 786 women with past medical history of cancer from 64,327 aged 40-79 years at baseline, present study analyzed a total of 63,541 women.

Baseline survey and questionnaire

At the time of enrollment, the subjects completed a self-administered questionnaire which included demographic characteristics such as age, sex, education, marital status, height and weight; lifestyles factors such as smoking, drinking, physical activity; dietary habits; past medical history of several diseases such as diabetes mellitus, and cancer. Present study, however, analyzed only a partial list of the variables such as age, BMI (<18.5, 18.5-25.0, ≥25.0), smoking (non-smoker, ever smoker), drinking (non-drinker, ever drinker), past history of diabetes mellitus (yes, no), marital status (single, else), number of pregnancies (≤2 versus ≥3), number of deliveries (≤2 versus ≥3), EUSH (yes, no), sports/physical activity (Seldom, ≥1-2 hours/wk), and systolic blood pressure (<130 mmHG, ≥130 mmHG) (shown in Table 1 and 2). The categories of the variables smoking, drinking, pregnancy, and delivery were made in such a way to confirm at least some deaths from EC in each category (for estimation purpose). Here ever smoker (drinker) meant current and ex-smoker (drinker). Although most of the studies used the category of null pregnancy and null delivery, we did not use such category because there was no death from EC in null category.

Follow-up

Follow-up survey was conducted annually until the end of 1999 and 2003 in 3 and 42 areas respectively to determine the vital status of the subjects using resident registration records available in the respective municipalities. For deceased subjects, cause of death was identified from the death certificate, available at each municipality, using International Classification of Disease version 10 (ICD-10). C54 code of ICD-10 was used for EC. For the present analysis, all other subjects (except EC deaths) who alive until the follow-up period or who moved out the study areas or lost to follow-up were considered as censored cases during analysis.

Statistical analysis

Persons-years of follow-up were calculated for each subject from the starting point of the study until the date of death, date of move out of study area, date of lost to follow-up or until the end of follow-up, whichever occurred first. The outcome variable of interest was the EC death. We first used Cox proportional hazard model (PHREG procedure)

to estimate the age adjusted hazard ratio (HR) of EC death including 95% confidence interval (CI) by EUSH and other selected variables. Multivariate Cox model was also used to estimate the HR of EC mortality by some selected variables including EUSH. All the computations were performed using the Statistical Analysis System (SAS) software package version 9.1 (SAS Institute Inc., Cary, NC).

Results

During the mean follow-up period of 13.3 years (standard deviation, SD=2.9 years) based on 63,541 women, the total number of deaths from all causes and move-outs were 7,172 (11.3%) and 2,977 (4.7%) respectively. Twenty two deaths were classified as EC among the total deaths, which provided the mortality rate of 2.6 per 100,000 person-years. The mean age at mortality was 69.0 years with a SD=9.2 years for 22 subjects of EC death.

Table 1 presents the distribution of women, person-years, number of deaths, and HR of EC mortality including 95% CI by some of the potential variables. The prevalence rate of the EUSH was 5.2% among Japanese women. According to univariate analysis for age through Cox model, higher age categories had higher EC mortality (HR=3.6 for age group 50-59; HR=3.2 for 60-69, and HR=4.5 for age group 70-79) compared with reference age (40-49) category. Age adjusted HR showed significant association of EC mortality with single marital status (HR=7.1; 95% CI=1.7-30.8) and EUSH (HR=6.4, 95% CI=2.1-19.7). All other variables such as diabetes mellitus, BMI, physical activity, smoking, drinking, and number of delivery were insignificantly associated with EC mortality.

Table 2 shows the distribution of the rate of EUSH by the selected variables. Significantly inverse association (P<0.0001) between age and the rate of EUSH was found. Lowest age group (40-49) indicated highest rate of EUSH (6.2%) and highest age group (70-79) indicated lowest rate (2.8%). History of DM (P=0.0047), smoking (P<0.0001), and drinking (P<0.0001) were positively associated with EUSH. Lower number of delivery (P<0.0001) and pregnancy

Table 1. Person-years, Number of Deaths, Age adjusted Relative Risk (RR) of Endometrial Cancer including 95% Confidence Interval (CI) due to Selected Variables, JACC Study, 1988-2003

Variables	Category	n*	Person-years	Noof deaths	RR	95% CI	P value
Age group	40-49	15391	217332	2	1.00		
(in years)†	50-59	19720	272028	9	3.64	0.79-16.84	0.0983
	60-69	19391	250328	7	3.17	0.66-15.28	0.1498
	70-79	9039	103825	4	4.52	0.83-24.73	0.0816
						P for trend=	0.1172
Past history of	No	52688	708528	19	1.00		
DM	Yes	2363	27987	1	1.64	0.17-9.60	0.8160
Sport activity	Seldom	38376	502391	17	1.00		
(hour/week)	≥1-2	12032	156505	5	1.16	0.41-3.28	0.7769
Body mass	<18.5	3728	46815	1	1.00		
Index (kg/m²)	18.5-25.0	42143	563614	16	1.42	0.19-10.72	0.7398
	≥25.0	13545	180835	3	0.79	0.08- 7.70	0.8418
Marital status	Else	53529	711458	18	1.00		
	Single	874	11650	2	7.14	1.66-30.80	0.0084
Number of	<3	17865	240191	7	1.00		
pregnancies	≥3	39104	515653	10	0.61	0.17- 1.42	0.3252
Number of	<3	27207	364668	10	1.00		
deliveries	≥3	28924	379066	6	0.49	0.13- 7.33	0.1894
Systolic blood	<130	18678	245803	3	1.00		
pressure (mmHG)	≥130	23740	300337	9	1.89	0.50- 7.15	0.3487
Ever use of sex	No	45310	591603	14	1.00		
hormone	Yes	2359	30556	4	6.43	2.10-19.67	0.0011
Age at first	<25	26284	343096	6	1.00		
delivery (years)	≥25	26299	352015	11	1.74	0.64-4.70	0.2772
Age at menarche	<15	24385	325980	5	1.00		
(in years)	≥15	32922	435703	13	1.56	0.54- 4.54	0.4161
Smoking	Non-smoker	50914	679863	18	1.00		
	Ever smoker‡	4013	52153	1	0.77	0.10- 5.76	0.7924
Drinking	Non-drinker	42442	561384	13	1.00		
	Ever drinker‡	15044	202275	6	1.45	0.55- 3.86	0.4464

*Total subjects vary for missing observations; †unadjusted RR shown for age; ‡ ever smokers (drinkers)= current + ex-smokers (drinkers)

Table 2. Ever Use of Sex Hormone by Selected Variables, JACC Study, 1988-2003

Variables	Category	n*	% Users	P value
Age group (in years)†	40-44	5851	6.36	<0.001
	45-49	6046	6.02	
	50-54	6908	5.14	
	55-59	8227	4.79	
	60-64	8354	4.92	
	65-69	6209	4.72	
	70-74	3709	2.94	
	75-79	2365	2.58	
Past history of DM	No	40245	4.78	0.0047
	Yes	1777	6.25	
Sport activity (hour/week)	Seldom	34173	4.81	0.0020
	≥1-2	10538	5.47	
Body mass Index (kg/m^2)	<18.5	2807	5.42	0.1369
	18.5-25.0	32317	4.86	
	≥25.0	10388	5.27	
Marital status	Else	43368	4.98	0.3019
	Single	526	3.99	
Number of pregnancies	<3	14166	5.25	0.0147
	≥3	31276	4.72	
Number of deliveries	<3	22112	5.88	<0.001
	≥3	22888	3.88	
Systolic blood pressure (mmHG)	<130	16400	5.73	<0.001
	≥130	20701	4.74	
Age at first delivery (years)	<25	20716	4.31	0.0042
	≥25	21201	4.89	
Age at menarche (in years)	<15	19983	5.54	<0.001
	≥15	25838	4.42	
Smoker	Non	39536	4.76	<0.001
	Ever‡	2809	8.37	
Drinker	Non	33324	4.46	<0.001
	Ever ‡	11001	6.59	

*Total subjects vary for missing observations; †unadjusted RR shown for age; ‡ ever smokers (drinkers)= current + ex-smokers (drinkers)

Table 3. Multivariate Analysis for Estimating the HR and 95% Confidence Intervals for Endometrial Cancer Mortality, JACC Study, 1988-2003

Variables	Category	HR	95% CI
Age group (in years)†	40-49	1.00	
	50-59	4.08	0.56 - 41.18
	60-69	8.56	1.05 - 69.84
	70-79	11.99	1.23 -116.61
Body mass Index (kg/m2)	<18.5	1.00	
	18.5-25.0	1.42	0.18 - 11.02
	≥25.0	0.65	0.06 - 7.31
Marital status	Else	1.00	
	Single	13.22	2.99 - 58.48
Ever use of sex hormone	No	1.00	
	Yes	5.33	1.51 - 18.82

(P=0.0147), lower level of systolic blood pressure (P<0.0001), and late age at first delivery (P=0.0042) showed higher rate of EUSH. Multivariate Cox model which included age, BMI, marital status, and EUSH (Table 3) also showed that EUSH (HR=5.3, 95% CI= 1.5-18.8), single marital status, and higher age groups were significantly associated with EC mortality.

Discussion

The prevalence of EUSH was 5.2% among Japanese women aged 40-79 years (based on 1988-90), which was found to be lower as compared to the prevalence of another Japanese study (conducted in 1992) based on women aged 45-64 (Nagata et al., 1996). Our study clearly demonstrated that ever use of hormone was a risk factor for EC among Japanese women. This result is consistent with the findings of many other studies (Lacey et al., 2005; Emons et al., 2004; Persson and Adami, 2002; Pukkala et al., 2001; Parslov et al., 2000; Persson et al., 1999; Weiderpass et al., 1999; Cushing et al., 1998; Shapiro et al., 1998; Beresford et al., 1997; Pike et al., 1997; Grady et al., 1995; Brinton and Hoover, 1993). However, this result contradicted with the result of one Japanese case-control study, which reported the odd ratio (OR)=0.92 (95% CI=0.62-1.35) (Mizunuma et al., 2001). Unopposed estrogen enhances the proliferation of the endometrial tissues which leads to a more frequent occurrence of spontaneous mutations and increase the risk of developing cancer in these tissues (Preston-Martin et al., 1997).

As the estrogen related increased risk can be at least partially prevented by adding progestin to estrogen (Persson and Adami, 2002; Weiderpass et al., 1999; Shapiro et al., 1998; Grady et al., 1995; Voigt et al., 1991), recently progestin has been added to estrogen replacement therapy (ERT) for 5-15 days sequentially or continuously with each ERT (Pike et al., 1997). It is suggested that added progestin counteract estrogenic effects through several mechanisms, including reduction in the estrogen receptor levels, enhancement of estradiol metabolism, regulation of several growth factors, decreased DNA synthesis, and endometrial shedding (Weiderpass et al., 1999; Graham and Clarke, 1997). However, the results regarding the associations between combined estrogen-progestin and EC are inconsistent (Lacey et al., 2005; Hill et al., 2000; Persson et al., 1999; Weiderpass et al., 1999; Shapiro et al., 1998; Beresford et al., 1997; Pike et al., 1997; Grady et al., 1995; Voigt et al., 1991), because evidences showed significantly decreased risk for continuous progestin use with each dose of estrogen (Hill et al., 2000; Weiderpass et al., 1999), sometimes significantly increased risk mainly for fewer days of progestin use (Lacey et al., 2005; Weiderpass et al., 1999; Shapiro et al., 1998; Beresford et al., 1997; Pike et al., 1997) and sometimes no significant association (Persson et al., 1999; Shapiro et al., 1998; Beresford et al., 1997; Pike et al., 1997; Voigt et al., 1991). Although inconsistent, previous studies indicated that the larger the duration of days of addition of progestin to

estrogen in the monthly HRT regimens (in sequential manner), the lower the risk of developing EC (Emons et al., 2004; Persson and Adami, 2002; Pukkala et al., 2001; Parslov et al., 2000; Weiderpass et al., 1999; Beresford et al., 1997; Pike et al., 1997). Comparative findings indicated that continuous addition of progestin is found to more effective than sequential addition of progestin (Emons et al., 2004). Compared with use of unopposed estrogen only, combined therapy was associated with at least 50% reduction in the risk of EC (Shapiro et al., 1998), which is also supported by other studies (Persson and Adami, 2002; Beresford et al., 1997). However, long-term combined therapy (5 years or more) is associated with increased risk of EC (Shapiro et al., 1998; Beresford et al., 1997)

The present study indicated that EUSH was higher among the cohort of younger women than the older women, which may mean that the use of sex hormone among the younger women is more popular than the older women. Liu et al. (2005) also reported that HRT was not a common practice in Japan until the last few decades. This pattern also indicates that hormone use is increasing in Japan, which is supported by Nagata et al. (1996). The reasons are yet to be known. However, one of the reasons may be related to the declining fertility of Japanese women (Inoue et al., 1994). Estrogen levels are generally low during delivery and breastfeeding because of removal of epithelial cells from endometrium during delivery and decreasing endogenic estrogens during breastfeeding, i.e., lower number of deliveries means having higher estrogen levels, which might increase the EC risk (Salazar-Martinez et al., 1999; Inoue et al., 1994). Our data may support this argument because women with higher number of deliveries used significantly less sex hormone ($P<0.0001$) than the women with lower number of deliveries. Secondly, it is a cancer of postmenopausal women, as 91% of cases occur in women aged 50 and older worldwide (Parkin et al., 2005; Parslov et al., 2000). In postmenopausal women, the concentration of plasma estrogen is reduced by 70-80% with respect to pre-menopausal women (Parslov et al., 2000). At menopausal stage, women generally face many menopausal problems such as hot flashes, sweating, vaginal dryness and bladder problems, which are reported to be very common among Japanese women (Anderson et al., 2004). The protective effects of the use of sex hormone for managing the unpleasant menopausal symptoms (Weiderpass et al., 1999; Shapiro et al., 1998; Nagata et al., 1996; Daly et al., 1993) might be another reason. Reducing the risk of diseases such as osteoporosis and cardiovascular diseases through the increasing use of estrogen as well as combinations of estrogen-progestin (McPherson and Mant, 2005; Weiderpass et al., 1999; Beresford et al., 1997; Nagata et al., 1996) including Japan where deaths from heart and cardiovascular diseases are very common (Health and Welfare Statistics Association, 2002) and greater interest of health professionals toward HRT mainly based on American studies (Nagata et al., 1996) may also increase the EUSH.

Although one Japanese case-control study reported that Japanese women have the same risk factors for EC such as nulliparity, obesity, hypertension, diabetes mellitus like the women of western countries (Inoue et al., 1994), which are also supported by many other studies (Xu et al., 2004; Persson and Adami, 2002; Jain et al., 2000; Parslov et al., 2000; Salazar-Martinez et al., 1999; Goodman et al., 1997; Inoue et al., 1994; Shu et al., 1993; La Vecchia et al., 1986), our study failed to show any meaningful association regarding the associations between reproductive factors and EC. However, the direction of associations of EC with diabetes mellitus, pregnancy, delivery, systolic blood pressure, and smoking were similar with other studies. The reasons for such discrepancies are yet to be known. However, differences in life-styles between Japanese and western countries (Goodman et al., 1997), small number of EC deaths, and differences between cohort study (present one) and case-control studies (mentioned above) may produce such inconsistent and insignificant results.

The main advantage of the present study lies in its prospective design with a large cohort size. Naturally cohort study is free from recall bias, which is one of the main limitations of case-control studies. Although the use of hormone in Japan has been increasing particularly as a method of symptom management and protection against osteoporosis and cardiovascular diseases (Nagata et al., 1996), unfortunately the consequences of HRT on EC mortality in Japan has not been investigated till today through any cohort study. To our knowledge, this is the first cohort study in Japan that has investigated such objectives. Thus, the result of the present study may carry some importance and should be taken into account before prescribing this therapy by medical professionals.

Several limitations should be discussed briefly. First, small number of deaths from EC is one of the main limitations, which may limit the statistical power of the study. We did not examine the association between EUSH and EC mortality by deleting the women for which the follow up period was very short, although it is sometimes recommended. Second, the information about the type of used hormone (whether estrogen therapy only, or combined estrogen-progestin), current or past users, and duration of use were not available at baseline. According to previous information, separate analyses are sometimes useful by such categories as the associations differed by these categories. Third, oral contraceptives are generally associated with lower risk of EC (Xu et al., 2004; Jain et al., 2000). However, in Japan the influence of oral contraceptives may not be a factor, because these are rarely used in Japan (Liu et al., 2005). One recent survey indicated that only 7% of the female aged 16-49 would use the low-dose pill if it is approved nationally (Kitamura, 1999). Fourth, a lot of missing information regarding the EUSH may also limit the study findings, particularly if the characteristics differed significantly between the women who provided the information and who did not.

In conclusion, EUSH is a risk factor for EC mortality for Japanese women. Therefore, the women who need HRT

should select lower dose of estrogen. As the continuously combined estrogen-progestin replacement seems to be the safest HRT regimen for the endometrium (Emons et al., 2004; Persson and Adami, 2002), health professionals should recommend continuous use of combined estrogen and progestin throughout the treatment cycle, or recommend sufficient dose of progestin for at least 10-15 days per month. Moreover, health professionals should discuss the associated risk of the use of sex hormone before prescribing as well as should provide information regarding the potential risk factors.

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Original article

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Validation study of fatty acid consumption assessed with a short food frequency questionnaire against plasma concentration in middle-aged Japanese people

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Abstract

Objective: To assess the relative validity of data for consumption of fatty acids (FAs) measured with a short food frequency questionnaire (FFQ) in comparison with plasma concentration of FAs.

Design: In this cross-sectional study, completed FFQs were secured from 177 (92 male and 85 female) employees working for a company in August 2001. Intake of FAs was assessed with the FFQ, and the values were validated against FA concentration in plasma in overnight-fasting blood.

Results: Mean $\pm$SD daily intakes of total fatty acids (TFAs) were 44.4 $\pm$ 8.0 g day^{-1} for men and 42.9 $\pm$ 7.2 g day^{-1} for women. Plasma concentration of TFAs were 12.73 $\pm$ 3.78 mmol l^{-1} for men and 10.54 $\pm$ 1.75 mmol l^{-1} for women. Spearman's rank correlation coefficients, unadjusted and energy-adjusted by the energy-density method and residual method, for n-3 highly unsaturated fatty acids (HUFAs) were 0.37 (p <0.001), 0.38 (p <0.001) and 0.40 (p <0.001) for men, and 0.41 (p <0.001), 0.26 (p <0.01) and 0.29 (p <0.01) for women, respectively.

Conclusions: Relative validity values of data for intake of n-3 polyunsaturated fatty acids (PUFAs) for women and n-3 HUFAs in both genders, assessed with the FFQ compared with FA concentration in plasma, were moderate, but no significant associations were found for saturated fatty acids, monounsaturated fatty acids or n-6 PUFAs.

Keywords: *fatty acids*; *food frequency questionnaire*; *plasma concentration*; *relative validity*

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Introduction

Morbidity and mortality associated with chronic diseases such as cancer, cerebrovascular disorders and heart disease are major public health concerns not only in developed countries but also in the developing world (1, 2). These are related to our daily lifestyle, including dietary habits, smoking, alcohol drinking, physical exercise and stress. Smoking appears to be the most potent single factor, the association with disease being unequivocal. Food consumption also seems to play a significant role, but observations are inconsistent, so further research on the relationships between consumption of particular foods/nutrients and health/disease is required.

Controversial findings may depend on the fact that information on dietary intake is not necessarily valid or reproducible owing to several factors

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