Journal of Chromatography B, 960 (2014) 222–229

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A rapid and precise method for quantification of fatty acids in human serum cholesteryl esters by liquid chromatography and tandem mass spectrometry

Songlin Yu[a,b], Jun Dong[b], Weiyan Zhou[c], Ruiyue Yang[b], Hongxia Li[b], Haijian Zhao[c], Tianjiao Zhang[c], Hanbang Guo[b], Shu Wang[b], Chuanbao Zhang[c], Wenxiang Chen[b,c,*]

[a] Department of Clinical Laboratory, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Beijing 100730, China
[b] The Key Laboratory of Geriatrics, Beijing Hospital Institute of Geriatrics, Ministry of Health, Beijing 100730, China
[c] Beijing Hospital National Center for Clinical Laboratories, Ministry of Health, Beijing 100730, China

ARTICLE INFO

Article history:
Received 18 January 2014
Accepted 19 April 2014
Available online 29 April 2014

Keywords:
Fatty acids
Cholesteryl ester
Cardiovascular diseases
Liquid chromatography and tandem mass spectrometry

ABSTRACT

We described a rapid and precise method for simultaneous quantification of eleven fatty acids in human serum cholesteryl esters (CEFAs) by liquid chromatography and tandem mass spectrometry (LC–MS/MS). After extraction of serum lipids with isopropanol, CEFAs were separated on reversed phase liquid chromatography and detected by mass spectrometry in positive ion mode with multiple reaction monitor. Individual CEFA was quantified by peak area normalization method and expressed as molar percent of total CEFAs. The run time was less than 5 min and detection limits were from 0.31 to 14.50 $\times 10^{-5}$ mmol/L. Recoveries of the CEFAs ranged from 91.85% to 104.83% with a mean of 99.12%. The intra and total CVs for the measurement of CEFAs were 0.87–7.70% and 1.02–7.65%, respectively. This LC–MS/MS method required no internal standards, eliminated natural isotope interferences, and provided reproducible and reliable results for 11 major CEFAs in human serum. This method can be used in monitoring and evaluating dietary fatty acid intake. Additional studies are needed to evaluate the associations between serum CEFAs and cardiovascular disease risk factors.

1. Introduction

Dietary fatty acids (FAs) are related to the development and progress of cardiovascular diseases (CVD) [1,2]. Numerous researches have shown that polyunsaturated fatty acids (PUFAs), such as omega-3 FAs (especially eicosapentaenoic acid, EPA and docosahexaenoic acid, DHA), are beneficial in protecting individuals from CVD and saturated fatty acids (SFAs) are linked to increased CVD risks [1,3]. Consequently, serum FA, SFA, PUFA and monounsaturated fatty acid (MUFA) profiles in various biological specimens have been investigated as biomarkers of dietary fat intake as well as CVD risks [4–6]. It is considered that these laboratory biomarkers provide more objective assessment than questionnaires in nutrition surveys and epidemiological studies, especially when nutrients of interest vary widely within foods and food composition data are incomplete [7].

The biological specimens analyzed include triglycerides (TG), phospholipids (PL), cholesterol esters (CE) in human serum or plasma, erythrocyte membranes and specific adipose tissues from various sites [4–6,8]. The drawback of analyzing FAs in TG or PL is the large variations because compositions of FA in TG and PL are easily influenced by everyday food intake. FAs in erythrocyte membrane are stable and can reflect dietary fat intake over the past 1–2 months [8]. However, sample preparation of erythrocyte membranes requires rinse of red blood cells and separation of lipid components, which are quite time consuming. Alternatively, FAs in CE (CEFAs) are relatively more stable than those in TG and PL and are suitable for the assessment of dietary FA intake of the preceding weeks [4,8,9]. In addition, each CE molecule contains only one single FA molecule, which makes it appropriate to be detected directly by multiple reaction monitor (MRM) and quantified by normalization method without hydrolysis and derivatization.

Various methodologies have been described for quantitative analysis of CEFAs including high performance liquid chromatography (HPLC) [10] and gas chromatography (GC) [6,7]. Due to

* Corresponding author at: Beijing Hospital National Center for Clinical Laboratories, No. 1 Dahua Road, Dongcheng District, Beijing 100730, PR China.
Tel.: +86 1058115049; fax: +86 1065132968.
E-mail address: wchen@bjhmoh.cn (W. Chen).

http://dx.doi.org/10.1016/j.jchromb.2014.04.040
1570-0232/

the similarities of serum TG, PL and CE in structure and polarity, complete separation of CEFAs by HPLC is difficult and usually contaminated by other lipid components. GC is the most widely used method for the measurement of CEFAs due to its separation capacities. However, this method involves derivatization of FAs and a long separation time. In recent years, investigators have developed tandem mass spectrometry (MS/MS) methods using ammonium, sodiated or lithiated adducts due to the fact that all the CEFAs generate a fragment ion of *m*/*z* 369 upon collision-induced fragmentation [11–13]. However, most of the reported MS/MS methods are based on direct infusion without chromatographic separation, in which overlaps of CEFAs with their natural occurring isotopes and contamination of other lipid species may not be avoided.

In this study, we investigated the separation, detection and quantification of CEFAs, and established a LC–MS/MS method that required minimum sample preparation, eliminated natural isotope interferences and matrix effects, and used area normalization for quantification. This method was simple and precise and may be useful in monitoring dietary fatty acid intake and in evaluating CVD risk factors.

2. Materials and methods

2.1. Chemicals

CEFA standards including cholesterol myristate (CE14:0), cholesterol palmitate (CE16:0), cholesterol palmitoleate (CE16:1), cholesterol stearate (CE18:0), cholesterol oleate(CE18:1), cholesterol linoleate (CE18:2), cholesterol alpha linolenate (CE18:3), cholesterol homo gamma linolenate (CE20:3), cholesterol arachidonate (CE20:4), cholesterol eicosapentaenoate (CE20:5), and cholesterol docosahexaenoate (CE22:6) were purchased from Nu-Chek Prep (Elysian, MN, USA). Organic solvents isopropanol, hexane, aceonitrile (all HPLC-grade) were products of Fisher Scientific (Pittsburgh, PA, USA). Ammonium formate was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Assay Kits for total cholesterol (TC), triglyceride (TG), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), and high sensitivity C-reactive protein (hsCRP) were products of Sekisui Medical Technologies (Osaka, Japan).

2.2. Serum samples

For methodological studies, fresh serum samples were collected from the leftovers of patient samples in the department of laboratory medicine of Beijing Hospital. Serum was pooled and re-aliquoted and stored at −80 °C until analysis.

For analysis of CEFAs in healthy volunteers, 566 apparently healthy subjects (317 males and 249 females) were randomly selected from the participants for physical examination in Beijing Hospital during September to December, 2012. Fasting blood samples were taken by venipuncture into tubes containing clot-activator. Serum was isolated, frozen, and stored in 1 mL aliquots at −80 °C until analysis.

This study had been reviewed and approved by the Ethics Committee of Beijing Hospital. All studied individuals were informed in writing of the intended use of their samples and each provided written consent.

2.3. Preparation of calibrators

A mixed CEFAs calibrator was prepared, in which concentrations of each CEFA and their ratios were in accordance with the previously reported CEFA compositions [14]. Standards of CE14:0, CE16:0, CE16:1, CE18:0, CE18:1, CE18:2, CE18:3, CE20:3, CE20:4, CE20:5 and CE22:6 were accurately weighed and transferred into a Class A 25 mL volumetric flask, and then isopropanol: n-hexane(3:1) was added to the scale to make the above CEFAs with final concentrations of approximately 0.04, 0.60, 0.04, 0.08, 0.80, 2.00, 0.12, 0.04, 0.20, 0.04 and 0.04 mmol/L and percentages of 1.0, 15.0, 1.0, 2.0, 20.0, 50.0, 3.0, 1.0, 5.0, 1.0 and 1.0% respectively. The mixed calibrator was aliquoted and stored in sealed glass ampoules at −80 °C until analyses.

2.4. Preparation of samples

The calibrators and serum samples were thawed, mixed and equilibrated at room temperature and precisely transferred by an automatic diluter (Hamilton, Reno, NV, USA). Serum CEFAs were extracted by isopropanol [15]. The automatic dilutor was set to dilution mode and aliquots of 20 μL mixed calibrators or serum samples were delivered with 1 mL isopropanol into 2 mL vials respectively. The vials were screw capped and shaken on a mechanical shaker for 15 min, and then centrifuged at 2342 × *g* for 10 min. Subsequently, 0.1 mL of the supernatant was transferred to another 2 mL vial and diluted 8 times with mobile phase, resulting in a 400-fold dilution corresponding to the initial serum volume.

2.5. LC–MS/MS analysis and quantification

LC–MS/MS analysis was performed using an Agilent 1200 Series LC system (Agilent Technologies, Santa Clara, CA, USA) coupled with API 5000 system (Applied Biosystems, Foster City, CA, USA). Aliquots of 1 μL of the reconstituted calibrators or serum samples were injected into a Waters Nova-Pak C18 column (4.6 mm × 150 mm, 4 μm) and eluted with a mobile phase of 95% A (isopropanol:acetonitrile:n-hexane, 55:45:15) and 5% B (methanol containing 0.1 mol/L ammonium formate) at a flow rate of 0.3 mL/min. The MS detection was performed with electrospray ionization (ESI) in the positive ion mode with multiple reaction monitor (MRM). The transitions of CE14:0, CE16:0, CE16:1, CE18:0, CE18:1, CE18:2, CE18:3, CE20:3, CE20:4, CE20:5 and CE22:6 were monitored at *m*/*z* 614.6→369.4, 642.6→369.4, 640.6→369.4, 670.6→369.4, 668.6→369.4, 666.6→369.4, 664.6→369.4, 692.6→369.4, 690.6→369.4, 688.6→369.4 and 714.6→369.4, respectively. Nitrogen was used as the collision gas, curtain gas, ion source gas 1 and ion source gas 2 at settings of 5, 15, 50 and 50 psi, respectively. Ionspray voltage was set at 5500 and the source temperature at 150 °C.

Quantification was achieved by peak area normalization. Each CEFA was calculated using normalization factor (*F*) and expressed as a percent of total CEFAs. The normalization equation was as follows:

$$F_i = \frac{P_i}{\left(A_i / \sum A_i\right)}$$

$$P_S = \frac{A_S / \sum A_S * F_i}{\sum \left(A_S / \sum A_S * F_i\right)}$$

F_i: normalizing factor of individual CEFA; P_i: molar percent of CEFA in mixed calibrators; A_i: area of CEFA in mixed calibrators; A_s: area of CEFA in serum samples; P_s: molar percent of CEFA in serum samples.

2.6. Determination of CEFAs in 566 healthy volunteers

The LC–MS/MS method was applied to 566 apparently healthy volunteers, 317 males and 249 females, from age 19 to 97 y. Height, weight and blood pressure were measured. Baseline demographics and eating habits including frequency of fish consumption were