Development of a simultaneous quantitation for short-, medium-, long-, and very long-chain fatty acids in human plasma by 2-nitrophenylhydrazine-derivatization and liquid chromatography–tandem mass spectrometry

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Abstract

Fatty acids (FA) have been important in clinical diagnosis for long, which makes the increasing need for a fast, reliable, and economic approach to determine FA of short-, medium-, long-, and very long-chain by widely available equipment and with high-throughput capacity. In the present work, 2-nitrophenylhydrazine derivatization coupling with LC-MS/MS detection was utilized to simultaneously quantitate 18 FAs ranging from C4 to C26 in human plasma. The sample preparation protocol was optimized and extracting with diethyl ether-potassium phosphate buffer twice was found as the highest efficiency along with economic feasibility. Under the optimized conditions, all the FA showed excellent linearity ($R^2 > 0.999$ for each), sufficient sensitivity (LOD 0.2−330 fmol and LOQ 2.3−660 fmol for all), favorable accuracy (recovery ranged from 98.1 ± 3.6% to 104.9 ± 5.5% with coefficient of variation no more than 8.6% for all), and negligible matrix effect. In the clinical application on 30 healthy subjects, compared with the previous HPLC-UV method, the developed method showed high reliability, as well as reduced time and reagent costs. The established method showed the potential to apply to not only diagnostic practice, but also nutritional and epidemiological studies.

Keywords

Fatty acid, LC-MS/MS, 2-nitrophenylhydrazine, derivatization, plasma

1. Introduction

Fatty acid (FA) profiling in the plasma is strongly associated with its metabolism and provides valuable information on various human nutritional and diseases status. As FAs reflect dietary fat intake and lipid metabolism, their amount and composition in plasma lipids are valuable parameters in clinical diagnosis. FAs are highly diverse based on the length of carbon chain and the number of double bonds. Among them, long-chain polyunsaturated FAs, such as eicosapentaenoic acid (FA 20:5 n-3) and arachidonic acid (FA 20:4 n-6), play crucial roles in human physiology and pathophysiology of some major diseases, including infant development, cardiovascular health, neurodegenerative diseases, immune defenses, and even cancers [1,2]. In recent years, short-chain FAs (SCFAs) have been known as important physiological relevant metabolites, involved in local gut microbiota, immunology, and metabolic states [3]. For medium-chain FAs (MCFAs), especially their triacyl-glycerides, there have been studies on the beneficial effects of prevention against obesity, alcohol-induced liver injury, non-alcoholic fatty liver diseases, and insulin resistance, as well as improving serum lipid profiles [4]. The very long-chain FAs (VLCFAs) also showed potential in clinical diagnosis for X-linked adrenoleukodystrophy and peroxisomal disorders, such as Zellweger syndrome, Refsum disease, and neonatal adrenoleukodystrophy [5–7]. Since FAs have already been considered to introduce into the screening panel for the detection of inherited metabolic disorders and other diseases [8], there is an increasing necessity for their overall profiling and measurement, covering short- to very long-

chain FAs, with widely available equipment and high-throughput feasibility.

Most of the studies on FA determination relied on gas chromatography (GC) coupled to flame ionization detector (FID) or mass spectrometry (MS) for detection [9–14]. Among these studies, researchers mainly focus on SCFAs or LCFAs (especially n-3 polyunsaturated FAs). However, for the profiling of whole FAs from short to very-long chain, it is not easy to handle all of them by GC. For instance, LCFAs and VLCFAs are usually derivatized to increase the volatility or thermal stability before GC separation. But SCFAs are volatile and not suitable for common derivatizing methods (methylation, trimethylsilylation, etc.). Instead, high-performance liquid chromatography (HPLC) is a much better separation technology than GC for polar thermolabile molecules [15], which allows lower running temperatures than GC as a definite advantage [16,17]. However, because the biological matrix contains complex components with different concentrations, it could be problematic to resolve and detect the targeted FAs by HPLC coupled to non-selective detectors. Utilizing MS could be a favorable way to deal with this problem, which provides structural information, thus enables the additional purification and resolution [18]. In the past twenty years, HPLC coupled to MS has been adopted in clinical laboratories and gradually become an important approach to quantitate various human metabolites and even metabolome profiling [8].

However, there has been a widely known issue about the ionization efficiency in measuring FA: the best chromatographic resolution with reversed-phase columns is achieved at acidic pH, but ionization of carboxyl groups in acidic solution is suppressed

[19]. An effective approach is to derivatize carboxyl group, changing its polarity and ionization characteristics [20], to enhance the separation and sensitivity [2]. An ingenious derivatization will not only help to enhance the sensitivity of the ionization process, but also ease control of the retention time and *m/*z to avoid potential suppression effects [21], especially for SCFAs that have high polarity and low molecular weights. Volpato et al. developed an accurate and reproducible liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for FA quantitation in cultured cells and human plasma by derivatization with 4-[2-(*N*,*N*-dimethylamino) ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE), in which the analytes were from FA 16:0 to FA 22:6 [2]. But according to that protocol, samples need to be extracted and dried to obtain the FA extract prior to derivatization, and the reaction with DAABD-AE costs 24 h, which should be laborious and thus unsuitable for clinical application dealing with batch samples. There were other designs reported, such as dimethylaminoethyl ester, trimethylaminoethyl ester, 3-picolinyl ester, 3-picolylamide, and O-benzylhydroxylamine hydrochloride ester [3,22]. Another derivatization method using acidic 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl) labeling was firstly described by Miwa et al. [23], who performed FA measurement biological materials by HPLC detection. This method has been applied in our previous works for determining MCFA and LCFA in human blood and commercial milk samples [4,24,25]. Although this derivatization strategy has been proved simple, rapid, and reliable, its combination with MS detection had been yet

applied only for SCFA in human serum [26] and zebrafish plasma and fecal [27]. Therefore, a satisfied method based on NPH-derivatization and LC-MS/MS for the whole FA profile measurement in clinical practice is desirable.

Herein, we describe the development of a practical NPH-derivatized LC-MS/MS method for the simultaneous determination of SCFA, MCFA, LCFA, and VLCFA in human blood sample. The targeted 18 FAs from C4 to C26 were firstly derivatized to FA-NPH and optimized for MS/MS detection. And the sample preparation protocol was improved to ensure simple and economic feasibility for high-throughput analysis, followed by the validation of the whole method. Furthermore, the developed method was applied on human plasma and compared with the previous well-established HPLC-UV protocol [24].

2. Materials and Methods

2.1. Chemicals and standard solutions

2-Nitrophenylhydrazine hydrochloride (2-NPH·HCl), *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC·HCl), and potassium phosphate buffer (pH 4.6) were provided by YMC Co., Ltd. (Kyoto, Japan). The mobile phase additive ammonium acetate and the antioxidant butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich (St. Louis, MO). All the other chemicals and reagents were of LC/MS grade and purchased from Wako Pure Chemical (Osaka, Japan) unless specified.

All the authentic FA standards, namely butyric acid (FA 4:0), caproic acid (FA 6:0),

heptanoic acid (FA 7:0), caprylic acid (FA 8:0), capric acid (FA 10:0), undecanoic acid (FA 11:0), lauric acid (FA 12:0), myristic acid (FA 14:0), palmitic acid (FA 16:0), heptadecanoic acid (FA 17:0), stearic acid (FA 18:0), oleic acid (FA 18:1 n-9), linoleic acid (FA 18:2 n-6), α-linolenic acid (FA 18:3 n-3), nonadecanoic acid (FA 19:0), arachidic acid (FA 20:0), arachidonic acid (FA 20:4 n-6), eicosapentaenoic acid (FA 20:5 n-3), behenic acid (FA 22:0), docosahexaenoic acid (FA 22:6 n-3), tricosanoic acid (FA 23:0), lignoceric acid (FA 24:0), and cerotic acid (FA 26:0), were purchased from Sigma-Aldrich (St. Louis, MO), with the purities no less than 99%. Their 2-nitrophenyl hydrazide derivatives (FA-NPH) were previously synthesized in our laboratory, of which the structures were fully elucidated, and the purities were validated more than 95% [24]. Stock solutions of each FA were prepared in MeOH (with 0.05% BHT) by measuring their dry weight on an ultrasensitive electro-balance (Cubis® ultra micro balance, Sartorius Inc., Göttingen, Germany). All the solutions were stored at −40 °C before using within two weeks.

2.2. Specimens and plasma collection

Ethical approvals for this study were obtained as Faculty of Health Sciences, Hokkaido University (No. 14−17) and as Oita University Faculty of Medicine (No. 715), and informed consent was obtained from all individuals. Non-fasting blood sample was collected from 30 volunteers (18 males, mean age ± SD: 21.3 ± 0.8 years; 12 females, mean age ± SD: 21.2 ± 0.9 years) regardless of dietary intake. All the plasma was separated by centrifugation within 30 min of collection, and then stored at

−80°C until analysis.

2.3. Sample preparation

In principles, the plasma samples were firstly saponified and then derivatized, which was the same as our previous studies on FA-NPH preparation [24,26]. However, in the present work, the whole operation was done in a reduced-scale. There were five internal standards (ISTD) used for quantitating different FAs, and the concentration for each ISTD was depended on both the levels of target analytes and economic costs. Specifically, 10 μL of plasma was spiked with 10 μL of mixed ISTD (48.4 pmol for FA 7:0, 53.7 pmol for FA 11:0, 159.5 pmol for FA 17:0, 18.3 pmol for FA 19:0, 17.9 pmol for FA 23:0, with 0.05% BHT) in a 1.5 mL Eppendorf tube. For saponification, the mixed sample was added with 25 μL of 0.3 M KOH-EtOH and kept in a water bath at 80°C for 45 min. Next, 50 μL of 20 nM 2-NPH·HCl in ethanol and 50 μL of 0.25 M EDC·HCl in EtOH/pyridine 97:3 (v/v) were added into the tube and incubated at 60°C for 20 min. The step of adding 10% KOH in MeOH/Water 50:50 (v/v) for eliminating interfering peaks in previous study [24] had been skipped, because the MS/MS detection system is devoid of such interferences. Then, 500 μL of potassium phosphate buffer and 500 μL of diethyl ether were added followed by intensive vortex, partition by centrifugation (680 × g, 5 min, 4°C), and vacuum dryness of the organic phase. Finally, the obtained FA-NPH derivatives were dissolved in 100 μL of MeOH and stored at −80 °C until injection.

2.4. LC-MS/MS analysis

LC-MS/MS was performed using a Thermo Finnigan Surveyor HPLC-TSQ Quantum Quadrupole mass spectrometer system (Thermo Fisher Scientific Inc., Waltham, MA), equipped with an Ascentis® Express Phenyl-Hexyl column (5 cm × 2.1 mm I.D., 2.7 μm, Supelco, Inc., Bellefonte, PA) for separation. The multiple-reaction monitoring (MRM) under electrospray ionization (ESI) negative mode was utilized for MS detection, and the main parameters were set according to our previous report [26]. In brief, the spray voltage was set at 3000 V, the vaporizer temperature and capillary temperature were set at 350 °C and 200 °C, respectively, and the pressure of sheath gas (nitrogen), auxiliary gas (nitrogen), and collision gas (argon) were set at 50 psi, 10 psi, and 1.8 mTorr, respectively. The mobile phase consisted of 5 mM aqueous ammonium acetate (A), isopropanol (B), and methanol (C) at a flow rate of 200 μL/min. The following gradient elution was applied: 0.0−0.5 min 75% A and 25% C; 0.5–1.5 min 35% A, 15% B, 50% C; 1.5−4.5 min 5% A, 30% B, 65% C; 4.5–7.5 min 30% B, 70% C; this ratio was kept to 11.0 min; 11.0–12.0 min returned to initial gradient and kept to 13.0 min to allow the column to re-establish equilibrium. The column oven temperature and sample tray temperature were set at 40 °C and 4 °C, respectively, and the injection volume was set at 5.0 μL. Quantification was conducted by the workstation Thermo Xcalibur 2.1 software (Thermo Fisher Scientific, Inc.).

2.5. Method validation

Selectivity and specificity were evaluated by analyzing blank samples of the human plasma matrix, while matrix effects were evaluated by the comparison of the

responses (areas) obtained for the mixed FA-NPH standards dissolved in MeOH and same standards dissolved in the blank plasma [28], as well as the retention times. And as the FA-free human blood is hardly available, the plasma sample pretreated without adding 2-NPH·HCl was utilized as the blank plasma matrix. To determine linearity, a series of the mixed standard solutions with varying concentration amounts for each FA were prepared. And each standard solution contained the ISTD mixture of FA 7:0, FA 11:0, FA 17:0, FA 19:0, FA 23:0 with the concentration of 4.84, 5.37, 15.95, 1.83, 1.79 μmol/L, respectively. The integration of the peak area and the plotting of each calibration curve were performed by Xcalibur, and linear regression of the tested FA was implemented by plotting the peak area ratio of the FA-NPH and its corresponded ISTD (y axis) vs. the FA amount (x axis, in pmol). The limit of detection (LOD) and limit of quantification (LOQ) were defined as the level of signal-to-noise ratio ≥ 3 and ratio ≥ 10, respectively. Both reproducibility and recovery assays were tested in 6 replicated analyses for the independently prepared serum samples. Recovery was calculated with the following formula:

Recovery (%) = [(amount found − amount original)/amount spiked] × 100%.

2.6. Statistics

All the shown data are presented as the means ± standard deviation (SD). Statistical analyses were conducted using the unpaired two-tailed *t*-test and one-way ANOVA (using the Tukey *post-hoc* test), and $P < 0.05$ was considered to be statistically

significant. Pearson's correlation coefficient analysis was performed by Prism 7.0 (GraphPad Software, Inc., La Jolla, CA).

3. Results and Discussion

3.1. Selectivity, specificity, and matrix effect evaluation

The selected Ascentis® Express Phenyl-Hexyl column under the current eluting condition provided appropriate retention for each FA-NPH species and maintained satisfied peak shapes for separation. To minimize the quantitation bias caused by matrix effect, a series of odd-chain FA were chosen as ISTD for different targeted FAs depending on their carbon lengths. The MRM conditions for each FA-NPH were optimized individually by Xcalibur and are listed in **Table 1**, and the chromatograms of the blank plasma matrix, the FA-NPH standards in MeOH solution, as well as FA-NPH in plasma sample are shown in **Figure S1 of Supplemental Material**. All these analytes showed identical peaks in both MeOH and plasma, indicating acceptable selectivity and specificity. Besides, by this method, the total run time per injection (with the elution for all the FAs and re-establish equilibrium) could be finished within 13 minutes. Therefore, our method would be considered fast, economical, and suitable for large scale of samples in clinical application.

The mixed FA-NPH solution was spiked into the prepared plasma matrix for the comparation with that spiked to MeOH, and the experiments were repeated 4 times. The average peak area ratio (FA-NPH area in plasma matrix to FA-NPH area in MeOH,

P/M ratio) were obtained and are listed in **Table 2**. For evaluation, the P/M ratio more than 1 means the enhancement effect, while less than 1 means the suppression effect [28]. Most of the FA-NPH showed the P/M ratio within the range 0.90−1.06, except for FA 18:3 (0.895), FA 20:0 (0.881), and FA 24:0 (0.878). All the CV of P/M ratio were no more than 5.65%. Moreover, all the FA-NPH showed very similar retention time (no longer than 0.04 min). These data suggested negligible interfere by matrix effect.

3.2. Optimization of preparation method

Considering the target FAs varies from short- to very long-chain, with some of them are of negligible amount in the plasma, and the sample volume employed in this method is as low as 10 μL, it is necessary to improve the extraction efficiency with simple operation and economic feasibility. Initially, the effects of extraction solvents in organic layer were compared, including n-hexane, diethyl ether, and ethyl acetate as candidates, and the results were evaluated on the basis of the relative intensities for all target FAs. Although extraction efficiency of n-hexane, which was used in previous studies [24,26], showed significantly higher in case of all the very long-chain saturated FAs (FA 20:0−FA 26:0, $P < 0.05$ for each), which might be due to the high nonpolarity of these analytes, diethyl ether showed the best efficiencies for most of the FAs (**Figure 1A**). In terms of aqueous layer, we attempted to replace potassium phosphate buffer with the more economical solvent, water, but failed. The results showed that after the replacement the extract efficiency for all the FAs were dramatically decreased ($P < 0.01$ for FA 12:0 and $P < 0.001$ for all the others), resulted in the loss ranged from

19.9% (FA 26:0) to 59.2% (FA 22:0) (**Figure 1B**). Thus, the use of potassium phosphate buffer as aqueous layer in this study was necessary, which was in accordance with previously published protocol [23]. In order to ensure the FA-NPH were fully extracted, the extraction times were also investigated. It was indicated that extracting twice generally increased the FA-NPH extraction efficiency compared to extracting once, showing an elevating average of 8.0 ± 0.9% ($P < 0.05$ for all but FA 26:0). While by extracting three times it increased only by 1% ($P > 0.05$ for all but FA 6:0), suggesting that extracting twice could be enough (**Figure 1C**). These results were also in agreement with the empirical rule of 9, which is that 90% of the analytes come out of the first extraction, 9% of the second, and 0.9% of the third [29]. With these optimizations, the present method was suitable for even trace-level FA in minor plasma amount and with relatively low cost.

3.3. Linearity and sensitivity

The area ratio of each FA-NPH to its corresponded ISTD was calculated, and the obtained six-point calibration curves exhibited excellent linearity ($R^2 > 0.999$ for all) in the wide ranges (shown in **Figure S1 of Supplemental Material**). Their linear ranges were considered suitable for clinical investigation into human blood [30]. The LOD were from 0.001 pmol (FA 12:0, 14:0, and 20:0) to 0.330 pmol (FA 22:6), while the LOQ were from 0.012 pmol (FA 20:0) to 0.660 pmol (FA 22:6), suggesting sufficient sensitivity (shown in **Table 3**).

3.4. Repeatability and accuracy

The reproducibility and recovery of the present method were determined by analyzing the human plasma with the certain concentration of each FA spiked in 6 replicates. The precision and accuracy of our method were listed in **Table 4**. For all the tested FAs, the coefficient of variation (CV) was no more than 8.0%, suggesting the favorable precision. The average recoveries achieved for all the tested FAs ranged from 98.1 ± 3.6% (FA 16:0) to 104.9 ± 5.5% (FA 26:0), with all the CV no more than 8.6%, which fit the recommendations and acceptance criteria for bioanalytical method validation according to U.S. Food and Drug Administration [31].

3.5. Method application to human plasma samples

The validated LC-MS/MS method was applied to determine the concentration of all the FAs in plasma samples from human volunteers. The summarized results were listed in **Table 5**, and the detailed data were shown in **Table S1 of Supplemental Material**. Among the 18 targeted FAs, FA 16:0, FA 18:1, and FA 18:2 were the major species, accounting for 2486.6 ± 615.4 μmol/L, 2020.4 ± 866.7 μmol/L, and 2350.7 ± 675.1 μmol/L, respectively. In the SCFAs, FA 4:0 was dominant over FA 6:0 (135.3 ± 90.0 μmol/L vs. 11.4 ± 8.4 μmol/L), which was similar to our previous study [26]. In the MCFAs, the concentration of FA 14:0 was the highest (51.1 ± 30.6 μmol/L), followed by FA 12:0 (22.0 ± 19.2 μmol/L), while FA 8:0 and FA 10:0 were at quite low levels (0.3 ± 0.7 μmol/L and 0.4 ± 0.5 μmol/L, respectively). Such low concentrations of FA 8:0 and FA 10:0 were even lower than 1/1000 of FA 16:0 and thus always made them negligible, which was consistent with other researches [24,32,33]. The VLCFAs

also exhibited low levels in plasma, with the concentration from 10.3 ± 3.9 μmol/L to 39.0 ± 19.4 μmol/L. Besides, all these FAs showed no significant difference between male and female ($P > 0.05$ for all). There was a study reported that the LCFAs in plasma, such as FA 20:4 and FA 20:5, were significantly lower concentration in male than in female [34]. In our results, the same trends were observed (for FA 20:4, male 203.2 ± 113.9 μmol/L vs. female 232.6 ± 149.4 μmol/L; and for FA 20:5, male 496.7 ± 304.2 μmol/L vs. female 594.0 ± 278.7 μmol/L), but no statistical significance ($P = 0.5449$ and 0.3828, respectively), which might be explained as the variation of individual differences. The tested results on these human plasma samples indicated that our method could obtain reasonable results for clinical determination.

In addition, in order to further validate the reliability of our method, FAs in the same plasma samples were also determined by HPLC-UV using the previously developed method [24]. Five comparable FAs were selected, including FA 10:0, FA 14:0, FA 16:0, FA 18:0, and FA 18:1, and the correlationship for each FA between HPLC-UV method and the current LC-MS/MS method was calculated, and the results were shown in **Figure 2**. The developed LC-MS/MS method and the HPLC-UV approach suggested satisfied consistency, of which the strong correlations between them were observed: The *r* values for these FAs were 0.999 (FA 10:0) as the highest and 0.955 (FA 16:0) as the lowest, and *P* values were less than 0.001 for all FAs. These results implied the high reliability in clinical usage.

The improvement of sensitivity could be attributable to both NPH derivatization and

MS/MS detection. Using the same MS spectrometer, we selected 5 representative underivatized FAs, namely FA 4:0, FA 10:0, FA 16:0, FA 20:5, and FA 24:0, optimized their SRM parameters (the parameters are shown in **Table S2 of Supplemental Material**), and run the LC-MS/MS injection under the same eluting system. As the results, the LOD for FA 10:0 was 100 pmol (vs. its NPH 9.8 fmol), for FA 16:0 was 25 pmol (vs. its NPH 1.6 pmol), for FA 20:5 was 1 pmol (vs. its NPH 70.7 fmol), and for FA 24:0 was 1 pmol (vs. its NPH 9.8 fmol), suggesting that derivatization really greatly enhanced the sensitivity for FA quantitation. While in terms of FA 4:0, the optimized SRM parameters were quite different from other FAs, which might be due to its polarity, acidity, and volatility. That made the simultaneous determination impracticable, suggesting the necessity of derivatization.

Moreover, with the same derivatization method, we compared the detection by LC-MS/MS and by HPLC-UV (**Table 6**). The LOD by LC-MS/MS reduced to approximately one-thousandth level of previous HPLC-UV method, which enabled the micro-sampling of 10 μL plasma and saved all the pretreatment reagents by 75%. Besides, thanks to the simultaneous measuring of all the FA in one injection, a single preparation of samples can be enough for analysis, thus decreasing the mobile phase and instrumental running time by approximately 90% (**Table 6**).

Furthermore, the present work was also compared with other protocols which combine derivatization with HPLC analysis for FA determination. The DAABD-AE derivatization by Volpato et al was only applied on LCFAs [2], and another

derivatization using O-benzylhydroxylamine by Zeng et al focused on SCFAs and their isomers [3]. While our protocol showed the advantage of enabling the determination of FA profile from C4 to C26 simultaneously. For other derivatization methods using dimethylaminoethanol, 3-picolylamine, or 3-pyridylcarbinol, the LOD of common FA derivatives determined by high-resolution Exactive Orbitrap MS reached 4−25 fmol, 11−112 fmol, and 13−135 fmol, respectively, which are comparable with our data (LOD: 0.2−70.2 fmol, except for FA 22:6 as 330.4 fmol). But when those methods were applied on the triple quadrupole TSQ MS and under SRM mode (which was the same as ours), the LOD increased to 28−280 fmol, 34−176 fmol, and 23−190 fmol, respectively [22]. Considering the practicability and MS instrument cost, the developed method suggested sufficient sensitivity and economy.

It should be noted that, one shortcoming of this method was the inability to separate the double bond position isomers (e.g. FA 18:1 n-9 and FA 18:1 n-7) and cis-trans isomers (e.g. FA 18:1 *cis*-9 and FA 18:1 *trans*-9). This drawback limited the method applying to food chemistry in which the FA isomers are widely existed and considered as healthy concern on diet [35,36]. Also, this method was considered not suitable for microbiological studies or dietary analyses, which often deal with odd- or branched-chain FAs [37,38]. Nevertheless, for a rapid and overall determination of human blood in clinical investigation, the developed LC-MS/MS method provided an ideal alternative approach.

4. Conclusion

In the present work, a fast and reliable method for simultaneous determination of short-, medium-, long-, and very long-chain FAs by LC-MS/MS was established. The optimized protocol was suitable for large scale of samples in clinical application, and the methodology validation proved its sufficient accuracy and reproducibility. Moreover, the current method showed satisfied consistency with previously developed HPLC-UV method, emphasizing its clinical practicability. For the future study, the whole FA profile, especially short- and medium-chain FAs, will be comprehensively studied with clinical index of subjects, to reveal the association with noninfectious chronic diseases as well as lifestyle-related disorders.

Abbreviations

EDC·HCl, *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride; FA, fatty acid; GC, gas chromatography; HPLC, high-performance liquid chromatography; ISTD, internal standard; LOD, limit of detection; LOQ, limit of quantification; LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; MRM, multiple-reaction monitoring; MS, mass spectrometry; NPH, 2-nitrophenyl hydrazide; SCFA, short-chain fatty acid; SD, standard deviation; VLCFA, very long-chain fatty acid.

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Declaration of conflicting interests

The authors declare no conflict of interests.

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Figure captions

Figure 1. Evaluation of the FA-NPH extraction efficiency affected by the following factors: **A**) Comparison of n-hexane, diether ether, and ethyl acetate as the extraction solvents in organic layer. Bars without a common letter represented significantly different at the 0.05 probability level. **B**) Comparison of potassium phosphate buffer and water as the extraction solvents in aqueous layer. ** $P < 0.01$, *** $P < 0.001$ between potassium phosphate buffer and water groups. **C**) Comparison of the extraction times. Bars without a common letter represented significantly different at the 0.05 probability level.

Figure 2. Correlation between the FA concentration determined by HPLC-UV method and LC-MS/MS method.

Tables

Table 1. LC-MS/MS data and MRM condition of each FA-NPH

FA-NPH	Formula	MRM transitions (*m/z*)		Collision energy (V)	Tube lens (V)	Referred IS
		Precursor	Product			
FA 4:0	$C_{10}H_{13}N_3O_3$	222.0	191.5	14	71	FA 7:0
FA 6:0	$C_{12}H_{17}N_3O_3$	250.1	219.3	15	87	FA 7:0
FA 7:0 (IS)	$C_{13}H_{19}N_3O_3$	264.1	233.4	18	71	N/A
FA 8:0	$C_{14}H_{21}N_3O_3$	278.1	247.8	19	80	FA 7:0
FA 10:0	$C_{16}H_{25}N_3O_3$	305.8	275.3	17	94	FA 11:0
FA 11:0 (IS)	$C_{17}H_{27}N_3O_3$	319.7	289.4	18	81	N/A
FA 12:0	$C_{18}H_{29}N_3O_3$	333.7	304.0	18	82	FA 11:0
FA 14:0	$C_{20}H_{33}N_3O_3$	361.7	331.9	19	93	FA 11:0
FA 16:0	$C_{22}H_{37}N_3O_3$	389.7	359.4	21	89	FA 17:0
FA 17:0 (IS)	$C_{23}H_{39}N_3O_3$	403.8	373.9	20	97	N/A
FA 18:0	$C_{24}H_{41}N_3O_3$	417.9	388.1	22	79	FA 17:0
FA 18:1	$C_{24}H_{39}N_3O_3$	416.1	385.4	21	104	FA 17:0
FA 18:2	$C_{24}H_{37}N_3O_3$	413.7	383.2	20	128	FA 17:0
FA 18:3	$C_{24}H_{35}N_3O_3$	411.6	381.3	21	99	FA 17:0
FA 19:0 (IS)	$C_{25}H_{43}N_3O_3$	432.5	402.5	20	80	N/A
FA 20:0	$C_{26}H_{45}N_3O_3$	446.0	415.4	23	99	FA 19:0
FA 20:4	$C_{26}H_{37}N_3O_3$	437.8	407.3	18	97	FA 19:0
FA 20:5	$C_{26}H_{35}N_3O_3$	435.8	405.3	19	88	FA 19:0
FA 22:0	$C_{28}H_{49}N_3O_3$	473.9	443.4	25	118	FA 23:0
FA 22:6	$C_{28}H_{37}N_3O_3$	461.4	431.2	20	84	FA 23:0
FA 23:0 (IS)	$C_{29}H_{51}N_3O_3$	488.0	457.2	23	112	N/A
FA 24:0	$C_{30}H_{53}N_3O_3$	502.0	471.3	26	127	FA 23:0
FA 26:0	$C_{32}H_{57}N_3O_3$	530.0	500.1	30	91	FA 23:0

N/A: Not available.

Table 2. Evaluation of matrix effect for FA-NPH (n = 4)

FA-NPH	RT (min)		Absolute intensity response (as peak area)	
	In MeOH	In plasma matrix	P/M Ratio	CV, %
FA 4:0	1.38	1.38	1.016 ± 0.030	2.93
FA 6:0	2.80	2.76	1.060 ± 0.050	4.69
FA 7:0 (IS)	3.14	3.10	1.036 ± 0.036	3.50
FA 8:0	3.35	3.35	0.936 ± 0.048	5.11
FA 10:0	3.73	3.73	0.985 ± 0.046	4.68
FA 11:0 (IS)	3.90	3.90	1.015 ± 0.049	4.84
FA 12:0	4.07	4.11	0.969 ± 0.048	4.93
FA 14:0	4.40	4.40	0.948 ± 0.052	5.43
FA 16:0	4.74	4.74	0.961 ± 0.044	4.56
FA 17:0 (IS)	4.91	4.95	0.952 ± 0.048	5.05
FA 18:0	5.08	5.12	0.988 ± 0.023	2.33
FA 18:1	4.91	4.95	0.917 ± 0.072	7.89
FA 18:2	4.74	4.78	0.941 ± 0.042	4.47
FA 18:3	4.62	4.62	0.895 ± 0.049	5.44
FA 19:0 (IS)	5.29	5.33	0.945 ± 0.048	5.05
FA 20:0	5.50	5.51	0.881 ± 0.042	4.81
FA 20:4	4.96	4.98	0.942 ± 0.023	2.44
FA 20:5	4.83	4.81	0.913 ± 0.035	3.79
FA 22:0	5.97	6.01	0.910 ± 0.050	5.51
FA 22:6	5.09	5.02	0.901 ± 0.009	0.96
FA 23:0 (IS)	6.26	6.26	0.907 ± 0.028	3.12
FA 24:0	6.56	6.60	0.878 ± 0.050	5.65
FA 26:0	7.27	7.31	0.934 ± 0.037	3.98

Table 3. Linearity, LOD, and LOQ for each tested FAs

FA	Equation	Range		R^2	LOD (fmol)	LOQ (fmol)
		Amount in the tested sample (pmol)	Concentration in human plasma (μmol/L)			
FA 4:0	y = 0.0379x + 0.0422	0.147−300	0.294−600	0.9998	73.2	146.5
FA 6:0	y = 0.0860x + 0.0139	0.024−25	0.048−50	0.9997	12.2	24.4
FA 8:0	y = 0.0683x + 0.1052	0.048−100	0.096−200	0.9994	9.7	48.8
FA 10:0	y = 0.0356x + 0.0171	0.058−100	0.116−200	0.9998	9.8	58.6
FA 12:0	y = 0.0097x + 0.0789	0.029−500	0.058−1000	0.9995	0.2	29.5
FA 14:0	y = 0.0606x + 0.5077	0.002−463	0.004−926	0.9992	0.2	2.3
FA 16:0	y = 0.0581x + 2.7804	0.015−3260	0.030−6520	0.9995	1.6	15.9
FA 18:0	y = 0.1092x + 0.1828	0.056−1730	0.112−3460	0.9998	5.6	56.2
FA 18:1	y = 0.0625x + 4.6226	0.020−8000	0.040−16000	0.9998	2.0	20.4
FA 18:2	y = 0.0112x + 0.8497	0.180−6730	0.360−13460	0.9996	16.4	180.8
FA 18:3	y = 0.0725x − 0.1268	0.360−1480	0.720−2960	0.9995	36.0	360.4
FA 20:0	y = 0.0147x + 0.0060	0.012−25	0.024−50	0.9994	1.2	12.2
FA 20:4	y = 0.0468x − 0.1278	0.374−770	0.748−1540	0.9997	37.4	374.0
FA 20:5	y = 0.0209x + 0.1110	0.388−724	0.776−1448	0.9993	70.7	388.8
FA 22:0	y = 0.0483x + 0.0038	0.024−25	0.048−50	0.9995	10.7	24.4
FA 22:6	y = 0.0041x + 0.0119	0.660−677	1.320−1354	0.9997	330.4	660.8
FA 24:0	y = 0.0102x + 0.0022	0.024−100	0.048−200	0.9995	9.8	24.4
FA 26:0	y = 0.0109x + 0.0004	0.026−25	0.052−50	0.9991	12.2	26.9

Table 4. Repeatability and accuracy of all the tested FAs in human plasma.

FA	Repeatability (n = 6)		Recovery (n = 6)				
	Expected amount (pmol)	Precision (CV, %)	Spiked amount (pmol)	Tested amount (pmol)	Precision (CV, %)	Recovery (%)	Recovery range (%)
FA 4:0	46.09 ± 3.17	6.9	100	102.28 ± 8.02	7.8	102.3 ± 8.0	93.8–114.8
FA 6:0	5.22 ± 0.34	6.5	12	12.39 ± 0.60	5.0	103.3 ± 5.0	97.1–111.9
FA 8:0	25.31 ± 1.41	5.6	50	51.55 ± 4.45	8.6	103.1 ± 8.9	94.9–117.7
FA 10:0	18.86 ± 1.02	5.4	55	54.28 ± 3.79	7.0	98.7 ± 6.9	87.1–108.5
FA 12:0	188.15 ± 4.89	2.6	550	559.27 ± 19.23	3.4	101.7 ± 3.5	95.1–104.4
FA 14:0	297.99 ± 12.64	4.2	800	840.28 ± 33.43	4.0	105.0 ± 4.2	99.5–111.2
FA 16:0	761.73 ± 6.60	0.9	900	882.97 ± 32.06	3.6	98.1 ± 3.6	92.5–102.3
FA 18:0	198.22 ± 8.69	4.4	200	206.40 ± 8.07	3.9	103.2 ± 4.0	95.5–106.5
FA 18:1	501.75 ± 14.58	2.9	570	563.00 ± 4.69	0.8	98.7 ± 0.8	97.9–100.2
FA 18:2	496.87 ± 17.00	3.4	600	628.39 ± 20.71	3.3	104.7 ± 3.5	101.8–109.5
FA 18:3	18.47 ± 0.17	0.9	20	20.54 ± 0.64	3.1	102.7 ± 3.2	97.5–105.8
FA 20:0	142.88 ± 5.68	4.0	250	247.64 ± 12.32	5.0	99.1 ± 4.9	90.2–103.7
FA 20:4	677.08 ± 45.17	6.7	1200	1202.00 ± 49.11	4.1	100.2 ± 4.1	95.8–105.2
FA 20:5	241.71 ± 15.86	6.6	450	449.35 ± 20.65	4.6	99.9 ± 4.6	91.7–105.5
FA 22:0	22.99 ± 0.89	3.9	55	54.85 ± 1.26	2.3	99.7 ± 2.3	96.6–103.6
FA 22:6	91.77 ± 1.98	2.2	100	98.60 ± 2.00	2.0	98.6 ± 2.0	95.9–101.2
FA 24:0	58.89 ± 2.33	4.0	120	123.11 ± 2.62	2.1	102.6 ± 2.2	99.9–105.2
FA 26:0	3.54 ± 0.28	8.0	10	10.49 ± 0.55	5.2	104.9 ± 5.5	98.2–113.1

Table 5. FA concentration in human plasma by LC-MS/MS

FA	Concentration (μmol/L)			*P* value
	Total (n = 30)	Male (n = 18)	Female (n = 12)	
FA 4:0	135.3 ± 90.0	130.0 ± 69.7	143.2 ± 117.2	0.70
FA 6:0	11.4 ± 8.4	11.3 ± 7.9	11.5 ± 9.4	0.96
FA 8:0	0.3 ± 0.7	0.4 ± 0.8	0.3 ± 0.6	0.73
FA 10:0	0.4 ± 0.5	0.3 ± 0.4	0.6 ± 0.6	0.14
FA 12:0	22.0 ± 19.2	18.9 ± 20.9	26.7 ± 16.0	0.28
FA 14:0	51.1 ± 30.6	46.2 ± 33.4	58.6 ± 25.4	0.28
FA 16:0	2486.6 ± 615.4	2450.0 ± 727.3	2541.4 ± 418.8	0.70
FA 18:0	699.5 ± 135.7	687.6 ± 152.0	717.3 ± 110.8	0.57
FA 18:1	2020.4 ± 866.7	1936.0 ± 1003.9	2147.1 ± 627.4	0.52
FA 18:2	2350.7 ± 675.1	2189.9 ± 755.3	2591.9 ± 462.8	0.11
FA 18:3	174.9 ± 145.2	148.6 ± 121.3	214.5 ± 173.3	0.23
FA 20:0	929.2 ± 355.3	853.3 ± 367.9	1043.1 ± 316.3	0.16
FA 20:4	214.9 ± 127.6	203.2 ± 113.9	232.6 ± 149.4	0.54
FA 20:5	535.6 ± 293.4	496.7 ± 304.2	594.0 ± 278.7	0.38
FA 22:0	13.6 ± 6.1	12.8 ± 5.9	14.8 ± 6.3	0.38
FA 22:6	10.3 ± 3.9	10.1 ± 4.1	10.7 ± 3.8	0.70
FA 24:0	39.0 ± 19.4	38.1 ± 22.7	40.5 ± 13.8	0.75
FA 26:0	12.0 ± 5.7	12.2 ± 6.9	11.7 ± 3.6	0.82

Table 6. Comparison of current LC-MS/MS and the previous HPLC-UV method

Method comparison		LC-MS/MS	HPLC-UV
Adaptability			
	Target FA	FA 4:0−FA 26:0	FA 8:0−FA 26:0
Sensitivity			
	LOD	FA 10:0, 9.8 fmol	FA 10:0, 2 pmol
		FA 14:0, 0.2 fmol	FA 14:0, 2 pmol
		FA 16:0, 1.6 fmol	FA 16:0, 2 pmol
		FA 18:0, 5.6 fmol	FA 18:0, 1 pmol
		FA 18:1, 2.0 fmol	FA 18:1, 1 pmol
	LOQ	FA 10:0, 58.6 fmol	FA 10:0, 3 pmol
		FA 14:0, 2.3 fmol	FA 14:0, 3 pmol
		FA 16:0, 15.9 fmol	FA 16:0, 3 pmol
		FA 18:0, 56.2 fmol	FA 18:0, 3 pmol
		FA 18:1, 20.4 fmol	FA 18:1, 3 pmol
Sample cost			
	Volume of plasma needed	10 μL	Totally 60 μL
Reagent cost			
	2-NPH·HCl	50 μL	Totally 200 μL
	1-EDC·HCl	50 μL	Totally 200 μL
	KOH solution	25 μL	Totally 100 μL
	Potassium phosphate buffer	500 μL	Totally 8 mL
Time cost			
	Instrument running time	13 min	Totally 2 hours