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Rapid liquid chromatography-tandem mass spectrometry to determine very-long-chain fatty acids in human and to establish reference intervals for the Chinese population

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ABSTRACT

Very-long-chain fatty acids (VLCFAs), including hexacosanoic, tetracosanoic, and docosanoic acids, are peroxisomal disease markers, whose abnormal accumulation warrants prompt detection for timely, effective treatment. This study aimed to establish and validate a robust liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based method to simultaneously quantify VLCFAs and provide reference intervals among Chinese individuals, quantify VLCFAs in pregnancy, and explore potential associations between plasma and amniotic fluid. Analytes were extracted via water-bath incubation with HCl and liquid-liquid extraction. Method linearity, limit of detection/quantitation, precision, carryover, and recovery were evaluated according to Clinical and Laboratory Standard Institute (CLSI) guidelines. VLCFAs showed good reproducibility based on low within-run coefficient variations (CVs) and total CVs, and correlation coefficients of linearity were > 0.99. The reference interval of C22:0, C24:0, and C26:0 were 32.0–73.4 μmol/L, 30.3–72.0 μmol/L, and 0.20–0.71 μmol/L, respectively; C24:0/C22:0 and C26:0/C22:0 ratios were 0.75–1.28 and 0.005–0.0139, respectively. Plasma and amniotic fluid of the same pregnant women displayed no significant correlation in the second trimester. This study presents the simple, efficient, accurate, and robust LC-MS/MS method to simultaneously detect C22:0, C24:0, and C26:0 without derivatization; it can be used to establish reference intervals among Chinese individuals and has diagnostic and other clinical applications.

1. Introduction

Peroxisomal disorders are a heterogeneous group of genetic metabolic disorders, including Zellweger spectrum disorders, rhizomelic chondrodysplasia punctata spectrum disorders, X-linked adrenoleukodystrophy (X-ALD), and multiple single-enzyme deficiencies, resulting from defects in peroxisome biogenesis or deficiency of a single peroxisomal enzyme leading to various cellular biochemical anomalies [1,2]. X-ALD is the most frequent peroxisomal disorder, being a progressive neurodegenerative disease caused by mutations in *ABCD1*, causing the accumulation of VLCFAs in all tissues [3]. In particular, the discovery of the accumulation of VLCFAs in the adrenal glands of patients with cerebral X-ALD not only expanded the clinical spectrum of X-ALD but also led to its classification as a metabolic disorder [3]. VLCFAs include hexacosanoic, tetracosanoic, and docosanoic acids, comprising a hydrocarbon chain containing 22 or more carbon atoms, and are catabolized via beta-oxidation in the peroxisomes [4]. Patients with X-ALD cannot catabolize VLCFAs effectively, thus leading to their accumulation in the tissue, serum, or plasma [5]. Thus, VLCFAs are considered biochemical diagnostic hallmarks for peroxisomal diseases [6,7]. In men clinically suspected with X-ALD, the diagnosis can be confirmed by quantifying plasma C26:0 levels and the C26:0/C22:0 ratio [8]. X-ALD is clinically characterized by two primary phenotypes: adrenomyeloneuropathy and inflammatory cerebral ALD that manifests either in

Abbreviations: ACN, acetonitrile; CLSI, Clinical and Laboratory Standards Institute; CV, coefficient of variation; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitor; VLCFA, very-long-chain fatty acid; X-ALD, X-linked adrenoleukodystrophy; GC–MS, gas chromatography–mass spectrometry

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children or less frequently in adults [7]. Patients with X-ALD are asymptomatic at birth, and most cases progress to adrenal insufficiency during childhood, although the age of onset is highly variable [2]; this narrow therapeutic window is thus often missed [9]. Owing to the wide heterogeneity of X-ALD, a more sensitive biochemical marker is needed to improve diagnosis along with an accurate, reliable, and rapid diagnostic method to simultaneously evaluate VLCFAs. In particular, because of its low incidence, screening for X-ALD is not routinely conducted in pregnant women. Therefore, evaluation of whether VLCFAs levels in the amniotic fluid of patients with X-ALD are higher than those of healthy pregnant women was not feasible. Since X-ALD is a genetic disease, we considered early neonatal screening for X-ALD via detection of VLCFAs levels in the amniotic fluid; hence, we hypothesized that VLCFAs levels are potentially associated between amniotic fluid and plasma in pregnant women.

VLCFAs are conventionally quantified using gas chromatography–mass spectrometry (GC–MS), although some studies have used LC-MS/MS to detect VLCFAs [10–12]. The (GC–MS) method detects $C_{14–22}$ fatty acids, which are normally abundant in human tissues, with high sensitivity; however, detection of relatively rare $C_{>24}$ VLCFAs is less efficient. Furthermore MS, especially MS/MS, has long been an important tool to develop methods to investigate biomolecules and biomarkers in complex mixtures [13–15]. Moreover, LC-MS/MS has been widely used in China, some diagnostic centers having obtained the medical device registration certificate; thus, clinical assessment based on VLCFAs is feasible. Furthermore, X-ALD patients have higher C24:0/C22:0 and C26:0/C22:0 ratios, which are higher than those in healthy individuals; hence, to accurately diagnose X-ALD, it is better to ensure high sensitivity of methods of detecting C22:0, C24:0, and C26:0 using LC-MS/MS.

Accordingly, this study primarily aimed to establish a robust LC-MS/MS method to simultaneously determine the C22:0, C24:0, and C26:0 levels without derivatization. The secondary aim of the study was to establish reference intervals of C22:0, C24:0, C26:0, C24:0/C22:0, and C26:0/C22:0 in the Chinese population. Furthermore, LC-MS/MS was used to quantify serum VLCFAs levels in women in the first trimester and those in the amniotic fluid and plasma in women in their second trimester to explore the association between plasma and amniotic fluid.

2. Material and methods

2.1. Chemicals and reagents

Behenic acid (22:0)/docosanoic acid (Nu-chekprep, INC, USA), lignocericacid (24:0)/tetracosanoic acid (Dr. Ehrensorfer, INC, Germany) and cerotic acid (26:0)/hexacosanoic acid (Dr. Ehrensorfer, INC, Germany) were purchased (Supplemental Fig. S1). Internal standards were docosanoic acid-12,12,13,13-d_4(C22:0-d_4) (C/D/N isotopes, INC, Canada), tetracosanoic acid-9,9,10,10-d_4(C24:0-d_4) (ISOscience, INC, USA), and hexacosanoic acid-d_4(C26:0-d_4) (Toronto Research Chemicals INC, Canada). Methanol, n-propanol, and acetonitrile (ACN) were purchased from Fisher Scientific (Far Lawn, NJ, USA). Ammonium acetate was purchased from CNW (USA). All solvents used in this study were of high-performance liquid chromatography (HPLC)-grade purity. Water was prepared in the laboratory with a MilliQ Advantage ultrapure water system (Millipore, MA, USA).

2.2. LC and MS conditions

The LC-MS/MS system was a TripleQuad 4500MD mass spectrometer (AB SCIEX, Foster City, CA, USA). Chromatographic separation was achieved using an ACQUITY UPLC BEH C8 column (2.1 × 100 mm, 1.7 μm; Waters). Mobile phase A contained HPLC-grade H_2O-ACN 40/60 (*v/v*) with 5 mmol/L ammonium formate, and mobile B was n-propanol-ACN 20/80 (v/v). Gradient elution was performed at a flow rate of 0.3 ml/min. The initial gradient conditions were 50% B held for 2 min, then increased to 80% B for 2.5 min, and then increased to 95% B for 1.5 min, and finally returned to 50% B at 6.1 min; the total run time per sample was 7 min.

Negative electrospray ionization (ESI) mode and multiple reaction monitor (MRM) mode were used for LC-MS/MS. The MRM channels are described in Supplemental Table 1 and Supplemental Fig. S2. The nebulizer gas (Gas1), heater gas (Gas2), and curtain gas were set at 55, 55, and 30 psi, respectively; collision gas, 8 psi. The ion spray voltage was −4500 V. The optimal probe temperature was determined to be 550 °C, and the column oven temperature was set to 40 °C.

2.3. Preparation of calibration materials

A mixed standard stock solution comprising C22:0, C24:0, and C26:0 was prepared to generate calibration curves. Calibrators were serially diluted with isopropanol at concentrations shown in Supplemental Table 2. All calibrators were stored at 4–8 °C.

2.4. Sample preparation

10μl of the internal standard mix solution was added to 50 μl of samples or quality control (QC) sample (pooled human serum sample). Samples were hydrolyzed via incubation at 70 °C for 1 h with 200 μl ACN and 50 μl hydrochloric acid (12 mol/L). After cooling to ambient temperature, samples were extracted with 1 ml hexane, and then vortexed for 10 s and centrifuged at 3000 rpm for 10 min. The upper solution was transferred to 96-well plates and dried under a nitrogen stream for 15 min. The dried residue was reconstituted in 200 μl methanol, and 5 μl aliquots of the reconstitutes were loaded onto the LC–MS/MS system for analysis.

2.5. Method validation

The method was validated in accordance with the evaluation protocol C62-A approved by CLSI for its final application to quantify serum samples in clinical laboratories. Calibration curves were generated by plotting the peak area ratio (analyte/internal standard) versus the theoretical concentrations and calculation via quadratic regression with 1/x weighting. Based on the guidelines, the accuracy of each concentration point should be within 10% deviation from the theoretical value. Serum samples for precision verification were prepared by spiking the mixed standard stock solution into a pooled human serum sample ($QC_{1–4}$). In accordance with CLSI 15A-3, within-run CVs and total CVs were calculated in five replicates of four concentrations every day for five consecutive days. The matrix effect of serum constituents over the ionization of C22:0, C24:0, and C26:0 was evaluated via the post-extraction spike method [16]. To investigate the efficacy of sample preparation, analyte recovery was determined by comparing the peak areas between two types of samples: 1) serum spiked before sample preparation with a known amount of analyte (pre-extraction) after deduction of the endogenous analyte content, and 2) a pure standard analyte solution. The peak area ratio between samples 1 and 2 reflects the extent of analyte recovery. Recovery samples were prepared by spiking pooled human serum with the mixed standard solution; 10 μl of mixed standard solutions at two different concentrations were added to 50 μl serum, and the two levels of samples were measured. Sample recovery was determined by dividing the measured and expected concentrations. Carryover was measured in triplicate by testing the blank after measuring the highest level of the calibrator. The carryover was considered to have “passed” when no obvious peak in the blank was observed after a high VLCFAs concentration sample was acceptable.

2.6. Clinical application of the method

In total, 187 residual fasting serum samples from apparently healthy

individuals were collected at Peking Union Medical College Hospital (PUMCH) for establishing the reference interval of VLCFAs in the Chinese population. In accordance with CLSI C28-A2, a 2.5% confidence interval (CI) and 97.5% CI were considered as the lower and upper limits, respectively. Residual serum samples from 154 women in the first trimester were collected. The residual plasma samples and amniotic fluid samples of 58 women in the second trimester were also obtained; more importantly, plasma and amniotic fluid samples were collected from the same women at PUMCH in 2018. This study was approved by the Ethics Committee of Peking Union Medical College Hospital of the Chinese Academy of Medical Sciences (S-K663 and S-K665).

2.7. Statistical analysis

Data were analyzed using Microsoft Excel 2010 (Microsoft Corporation, USA) and SPSS 20.0 (SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnow test was used to evaluate the distribution of VLCFAs concentrations. Spearman's correlation analysis was performed to evaluate the association of VLCFAs between plasma and ammonic fluid. A P-value $< .05$ was considered statistically significant.

3. Results and discussion

3.1. MS parameters optimization

Fatty acids were typically analyzed in negative-ion ESI mode. Analyte and internal standard solutions were directly infused into the mass spectrometer to automatically optimize corresponding parameters (DP and CE). Other parameters, such as ion-spray voltage, curtain gas, and source temperature, were optimized by injecting standard mixtures through the LC system into the mass spectrometer, owing to their association with flow rate and mobile phase composition. The adduct ion [M-H] was the base peak for the analytes and internal standards. The VLCFAs are structurally not prone to fragmentation; however, few stable fragments were obtained during the tuning procedure. Finally, the MRM mode with the same Q1 and Q3 ion was used as quantitative transitions to enhance sensitivity and reproducibility.

3.2. LC parameter optimization

A C18 column was initially used for chromatographic separation. A high resolution was achieved between analytes and endogenous interfering substances. However, peak tailing and prominent carry-over effects were observed after injecting hundreds of samples, since the VLCFAs are strongly hydrophobic. Thereafter, we tested a phenyl and a C8 column. Finally, the C8 column was selected for achieving better resolution and peak shapes. A mixed-mobile phase was used to enhance the elution and shorten the runtime. Ammonium formate (5 mM) was added to mobile phase A to enhance the sensitivity and peak shape.

3.3. Optimization of sample preparation

The sample preparation method was optimized in accordance with previous reports [17,18]. In vivo, partial fatty acids combine with glycerol to form lipids. Thus, to measure total fatty acid content, conventional methods including addition of HCl and water-bath incubation were not necessary. We also tested a one-step protein precipitation method with ACN or isopropanol without incubation, although the absolute recovery was much lower and interfering substances were detected at higher amounts. Different HCl concentrations (6 and 12 mol/L), different incubation periods (1, 2, 3, 4, and 5 h) and incubation temperatures (70 °C and 100 °C) were considered for optimization, and the final conditions (12 mol/L HCl and incubation at 70 °C for 1 h) were confirmed on the basis of absolute recovery of the analytes and ease of operation.

3.4. Linearity, limit of detection (LOD), and limit of quantification (LOQ)

Linearity was evaluated via polynomial regression of the calibrator concentrations on the theoretical peak area ratios of the target analyte and its internal standard. Quadratic polynomial regression with 1/x weighting for the analyte were calculated. The correlation coefficients (r) of linearity for C22:0, C24:0, and C26:0 were > 0.99 (Supplemental Fig. S3).

The LOD was determined through serial dilution of the calibrator solutions for each analyte and were extracted using a method similar to that of sample preparation. The LOD was defined as the lowest concentration with a signal-to-noise ratio (S/N) of at least 3. The LOQ was defined by preparing calibrator solutions with decreasing concentrations of each analyte and was set at the lowest concentration with a S/N of at least 10. The LODs of C22:0, C24:0, and C26:0 were 0.48, 0.42, and 0.02 μmol/L; LOQs, 0.96, 0.85, and 0.04 μmol/L, respectively.

3.5. Precision

Precisions of VLCFAs detection, including C26:0, C24:0, and C22:0, were evaluated using different concentrations of mixed standards added to serum to obtain four concentrations of VLCFAs. The within-run CVs for C22:0, C24:0, and C26:0 were 4.5%–8.3%, 6.1%–8.0%, and 6.4%–8.3%; total CVs, 7.7%–9.1%, 7.0%–7.6%, and 8.4%–9.3%, respectively (Table 1).

3.6. Recovery and carryover

Recovery was assessed at two different concentrations for each analyte through its addition at a desired concentration to the pooled serum. As shown in Table 2, the recovery rate ranged 90–110%. The matrix effect was between 90% to 110%. The VLCFAs peaks were undetectable in the blank samples after measuring the highest concentrations of standards, even after multiple repeats of the experiment, and no residual materials were detected, indicating that the carryover was “passed” (Supplemental Fig. S4).

For ensuring accurate recovery, each sample was processed twice in parallel. To completely evaluate the recovery of VLCFAs, two different levels (high and low) of serum was used.

3.7. Application of the method

The Kolmogorov-Smirnow tests revealed that serum C22:0, C24:0, and C26:0 levels were non-normally distributed. Serum chromatograms of C22:0, C24:0, C26:0, C22:0-*d4*, C24:0-*d4*, and C26:0-*d4* in apparently healthy individuals are shown in Fig. 1. The median serum levels of

Table 1
Precision of the analysis of very-long-chain fatty acids using the rapid liquid chromatography-tandem mass spectrometry method.

	C22:0	C24:0	C26:0
QC_1			
Mean (μmol/L)	45.2	44.8	0.28
Repeatability (%)	5.5	6.1	8.3
Total CV (%)	7.7	7.6	8.4
QC_2			
Mean (μmol/L)	51.3	50.3	0.55
Repeatability (%)	6.5	6.3	6.4
Total CV (%)	9.1	7.0	8.8
QC_3			
Mean (μmol/L)	70.6	71.8	2.61
Repeatability CV (%)	4.5	6.1	7.8
Total run CV (%)	8.2	7.0	9.3
QC_4			
Mean (μmol/L)	121.8	149.7	8.77
Repeatability (%)	8.3	8.0	8.3
Total CV (%)	9.1	7.4	9.3

Table 2
Recovery rates in the liquid chromatography-tandem mass spectrometry assay.

Analyte	Serum concentration (μmol/L)	CV %	Recovery (%)
C22:0	76.0	4.22	94
	104.0	5.93	95
C24:0	76.4	1.27	92
	96.0	6.38	91
C26:0	0.60	7.08	106
	0.94	5.18	108

C22:0, C24:0, and C26:0 were 49.2, 46.8, and 0.34 μmol/L, respectively. The median of ratio between C24:0 and C22:0 was 0.9713 and that between C26:0 and C22:0 was 0.007. For comparison, the reference intervals determined herein and those provided by the Mayo clinic are shown in Table 3. The reference interval of C22:0, C24:0, and C26:0 were 32.0–73.4, 30.3–72.0, and 0.20–0.71 μmol/L, respectively, and the ratio of C24:0/C22:0 and C26:0/C22:0 were 0.75–1.28 and 0.005–0.0139, respectively. Spearman's correlation analysis indicated no significant correlations in VLCFAs concentrations, including C22:0, C24:0, and C26:0, between the plasma and ammonic fluid (all $p > .05$) (Fig. 2).

Our laboratory has already been carrying out VLCFA-based clinical assessments since December 2018. In total, 40 clinical samples were sent to our laboratory for assessment until February 2019. We used the reference intervals of the Mayo clinic and established a diagnostic method for X-ALDin our laboratory. Among 40 clinical samples, 4 samples displayed higher values than the reference interval, irrespective of whether the present reference range or that of the Mayo Clinic was used, concurrent with the clinical diagnosis.

Table 3
Reference intervals comparisons of very-long-chain fatty acids in the present study and the Mayo clinic data.

Analyte	This study (μmol/L)		Mayo clinic (μmol/L)
	$P_{2.5}$–$P_{97.5}$	≤ P_{99}	
C22:0	32.0–73.4	≤81.6	≤96.3
C24:0	30.3–72.0	≤74.9	≤91.4
C26:0	0.20–0.71	≤0.88	≤1.3
C24:0/C22:0	0.75–1.28	≤1.31	≤1.39
C26:0/C22:0	0.005–0.0139	≤0.017	≤0.023

Overall, using our rapid LC-MS/MS method, we established the reference interval of VLCFAs in the Chinese population, along with a preliminary distribution of VLCFAs in the first trimester. However, in contrast with our hypothesis, the results revealed no correlation in VLCFAs levels between the plasma and amniotic fluid of the same individuals in the second trimester. One potential reason contributing to

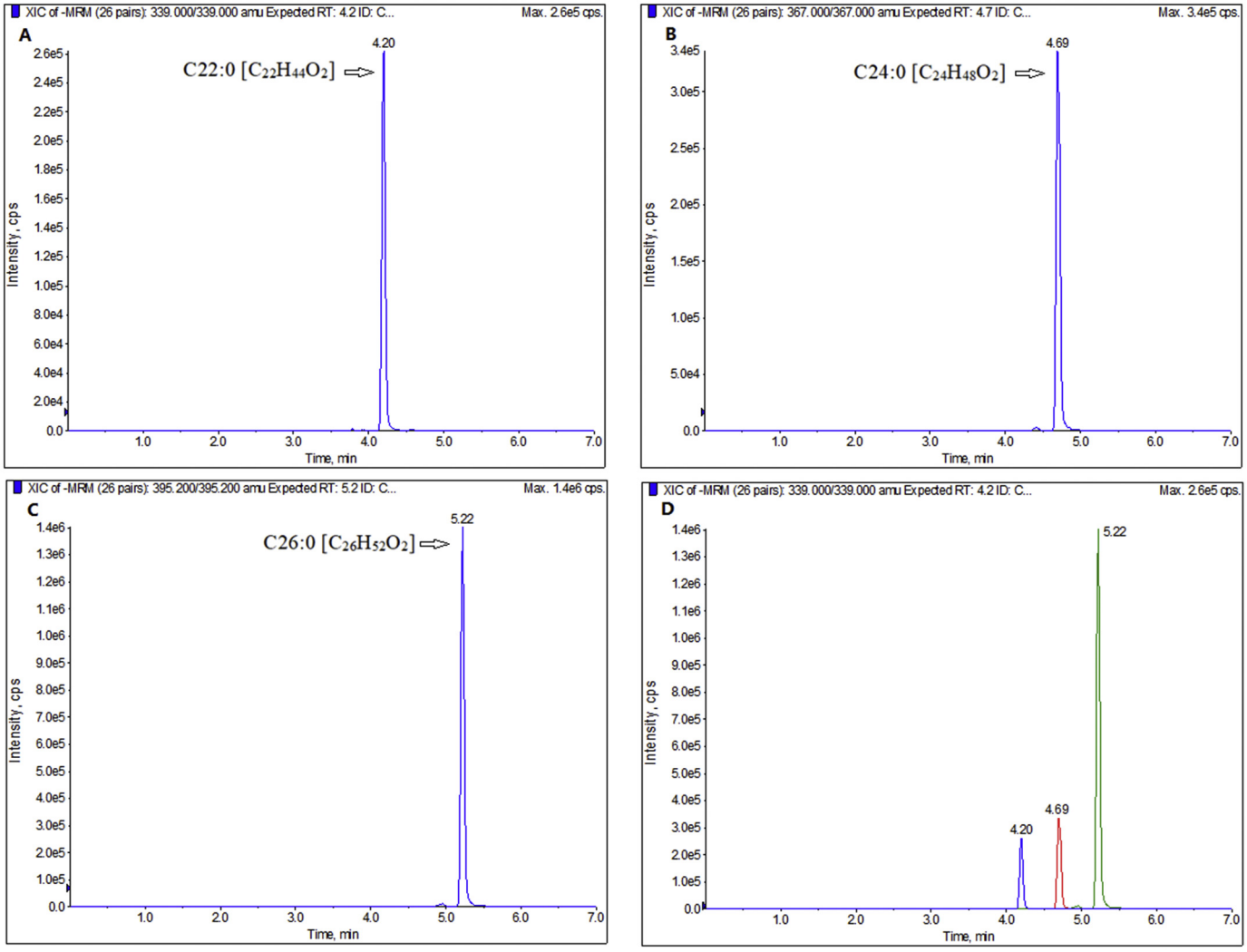

Fig. 1. Representative chromatography of the LC–MS/MS method for VLCFAs. A to C represent the chromatography of C22:0, C24:0, and C26:0, respectively, and D represents the inter standards chromatography of C22:0, C24:0, and C26:0.

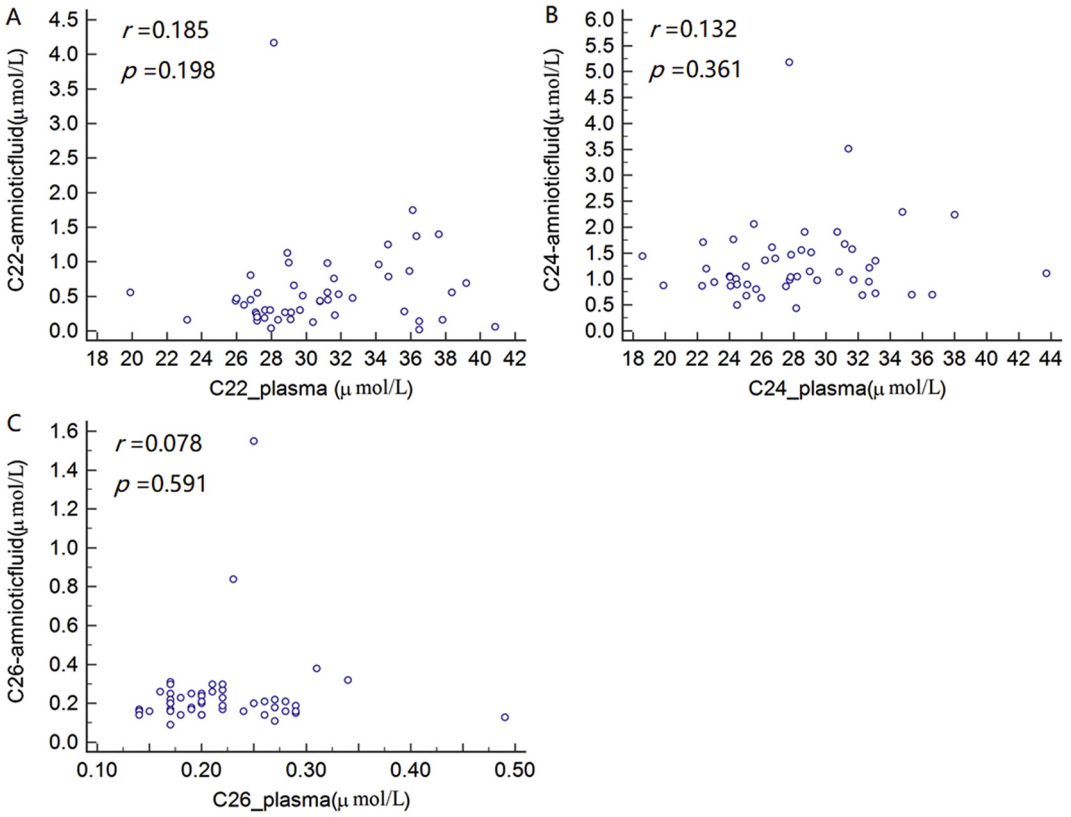

Fig. 2. Scatter plots of serum and amniotic fluid very-long-chain fatty acids in late pregnant women. A to C present the distribution of C22:0, C24:0, and C26:0, respectively, between plasma and amniotic fluid. *r* represents the correlation coefficient.

this lack of correlation could be that in the second trimester, the primary component of the amniotic fluid is fetal urine and its excretions; thus, since relatively low amounts of VLCFAs would be excreted through the urine, thus resulting in lower VLCFAs levels in the ammonic fluid. Alternatively, the plasma sample was maternal and not fetal; thus, this correlation analysis was not ideal. However, there is a limitation in this aspect of the study. It is better to identify mothers of X-ALD fetuses, mostly as carriers of defective *ABCD1* and with elevated VLCFAs. However, the prevalence of X-ALD is relatively low, and it is difficult to sample pregnant women with X-ALD. Thus, we did not determine the genotype of pregnant women in the present study. However, if feasible, we shall undertake further meaningful studies.

However, as opposed to other studies, the present study has some advantages. First, our study estimated the three columns including C18, phenyl, and C8 columns, reporting that the C18 column yielded marked carryover. Furthermore, it is difficult to completely separate the analyte and interference, using the phenyl column. Second, we evaluated the effect of incubation time and temperature and different HCl concentrations. Third, this study also included water and oil bath-based evaluations, finally revealing that the use of a 100 °C oil bath is unsuitable. Most importantly, this method was used to assess clinical samples at PUMCH, thereby yielding a robust clinical diagnostic method.

4. Conclusion

This study is the first to successfully establish a simple, efficient, accurate, and sensitive LC-MS/MS method to simultaneously determine C22:0, C24:0, and C26:0 levels and moreover establish their clinical reference intervals in the Chinese population. Moreover, this method was used to assess VLCFAs levels in the second trimester in pregnant women, demonstrating a lack of correlation between the plasma and ammonic fluid of the same individuals. Because X-ALD is a rare disease and limited information is available regarding the reference intervals of VLCFAs in Chinese population, the present study may provide an auxiliary diagnostic method. Simultaneously, the PUMCH is China's most prominent diagnostic and treatment center for difficult-to-treat and rare diseases, thus increasing the possibility of sampling more X-ALD cases to establish cut-offs of VLCFAs in the future.

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Competing interests

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.04.058.

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