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Flow injection ionization-tandem mass spectrometry-based estimation of a panel of lysophosphatidylcholines in dried blood spots for screening of X-linked adrenoleukodystrophy

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ABSTRACT

Background: Elevated blood C26:0 lysophosphatidylcholine (LPC) is a diagnostic marker for X-linked adrenoleukodystrophy (X-ALD). Our aim was to develop a flow injection ionization-tandem mass spectrometry (FIA-MS/MS) method for estimating a panel of LPCs (C20:0-C26:0-LPCs) in dried blood spots (DBS) and to determine the sensitivity and specificity of this method for high-throughput screening for X-ALD.

Methods: LPCs (C20:0-C26:0) were extracted from 3.2 mm DBS in a 96-well plate, spiked with isotopically-labelled internal standard (C26:0-d4-LPC) and measured by FIA-MS/MS in electrospray ionization (ESI)-positive, multiple reaction monitoring (MRM) mode using a triple quadrupole, tandem mass spectrometer. The sensitivity and specificity of the FIA-MS/MS method for screening of X-ALD was determined. The FIA-MS/MS method was compared with the LC-MS/MS method for estimating LPC concentrations.

Results: Elevated C26:0 and C24:0-LPCs were 100% sensitive for identification of X-ALD. However, specificity was only 78.33% for C26:0 and 98.33% for C24:0-LPCs. Sensitivity for C22:0 and C20:0 LPCs were 89.29%, 78.33% and specificity, 67.86% and 73.33%, respectively. The FIA-MS/MS method showed good concordance with the LC-MS/MS method.

Conclusion: The FIA-MS/MS method for estimating C26:0 and C24:0-LPCs in DBS is suitable for first-tier screening of newborns for X-ALD. Second-tier confirmatory testing is required to screen positive cases.

1. Introduction

X-linked adrenoleukodystrophy (X-ALD), the most common inherited peroxisomal disorder, affects 1:15,000 to 1:17,000 newborns [1,2]. It is caused by mutations in the *ABCD1* gene, that maps to Xq28 and codes for the peroxisomal ATP binding cassette (ABC), subfamily D transporter-adrenoleukodystrophy (ALD) protein [3–6]. This results in impaired breakdown of very-long chain fatty acids (VLCFA) in the peroxisomes and elevation of VLCFA in all tissues, mainly the brain, spinal cord and adrenal glands [3,4,6–9]. X-ALD can present with a wide range of clinical features with phenotypes like the rapidly progressive childhood cerebral ALD (CCALD) along with adult and adolescent cerebral forms, adrenomyeloneuropathy (AMN), Addison-only type and women with ALD [2,6,10–13]. Elevated VLCFAs are the conventional diagnostic markers for X-ALD [5,7,14]. However, recent studies have found that elevated C26:0-lysophosphatidylcholine (LPC) is a sensitive and specific biomarker which could be used for screening of X-ALD and other peroxisomal disorders [15–23]. Consequently, the conventional tedious method of measuring VLCFA in plasma by gas chromatography mass spectrometry (GC–MS) is being replaced by the estimation of C26:0 and C24:0-LPCs in dried blood spots (DBS) by liquid chromatography tandem mass spectrometry (LC-MS/MS) [15,16,24,25].

A flow injection ionization-tandem mass spectrometry (FIA-MS/MS)

Abbreviations: X-ALD, X-linked adrenoleukodystrophy; ALD, Adrenoleukodystrophy; CCALD, Childhood cerebral ALD; AMN, Adrenomyeloneuropathy; CDC, Centers for Disease Control and Prevention; NSQAP, Newborn screening quality assurance programme; VLCFA, Very long chain fatty acid; DBS, Dried blood spot; GC-MS, Gas chromatography–mass spectrometry; ESI, Electrospray ionization; LPC, Lysophosphatidylcholine; FIA-MS/MS, Flow injection ionization tandem mass spectrometry; NBS, Newborn screening; LC-MS/MS, Liquid chromatography tandem mass spectrometry

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method for estimating C26:0, C24:0, C22:0 and C20:0-LPCs in DBS extracts was first established by Turgeon and co-workers and this method was demonstrated to be suitable for high throughput newborn screening for X-ALD [25]. Consequently, the FIA-MS/MS method for first-tier screening of X-ALD using DBS was implemented by American NBS laboratories having expertise in multiplex screening of various disorders by including C26:0-LPC and its tetradeuterated internal standard C26:0-d4 LPC with amino acids, acylcarnitines, succinylacetone, acid α-glucosidase assay (GAA), β-glucosidase (ABG), galactocerebrosidase (GALC), α-galactosidase A (GLA), and α- L-iduronidase (IDUA) analysis without reduction in the sensitivity of the existing analytes measured [26,27].

Newborn screening has not yet been implemented in India as a national health program [28]. However, high-risk screening by FIA-MS/MS for the targeted diagnosis of aminoacidopathies, organic acidemias and fatty acid oxidation defects has been successful in identifying, diagnosing and treating various inborn metabolic disorders [28–30]. This diagnostic method has a great potential for high-throughput screening of newborns, if it is included as a nationwide health programme. Individuals affected with X-ALD are normal at birth and symptoms present only later in life [18,31]. Since X-ALD cases were reported previously from India using conventional VLCFA estimation by GC–MS/MS which is tedious and expensive [11,32–35], we established an LC-MS/MS method for measuring a panel of LPCs in DBS with 6 minutes analysis time [23]. Using this method we identified 21 cases of X-ALD in a period of one year. This high number of cases and the relatively long time for analysing each sample by the LC-MS/MS method emphasized the need for establishing a high-throughput method for larger population screening in India. A faster method would also mean that many patients could be screened and this could be another diagnostic test added to the expanded screening panel for high-risk cases tested by MS/MS. So, our aim was to establish a high-throughput, faster FIA-MS/MS method to estimate a panel of LPCs (C26:0, C24:0, C22:0, C20:0) in DBS extracts and to determine the sensitivity and specificity of this method for first level screening of newborns or high risk cases for X-ALD.

2. Materials and methods

2.1. Ethics

Ethical approval (No.NIMH/DO/105th IEC/2016, dated 15.07.2016) for the study protocol was obtained from the Institutional Ethics Committee of the National Institute of Mental Health and Neuro Sciences (NIMHANS), Bengaluru, India and this work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Subjects were recruited for the study only after informed consent and child assent were taken.

2.2. Materials

Isotope labelled internal standard of C26:0 LPC (C26:0-d4-LPC) and C26:0, C24:0, C22:0 and C20:0-LPC standards were procured from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Solvents and chemicals (LC-MS grade) consisting of methanol, isopropanol, acetonitrile, chloroform, formic acid and ammonium acetate and Whatman S&S 903 (Schleicher & Schuell) filter paper for spotting blood samples were obtained from Merck, Sigma-Aldrich Corp (St. Louis, MO, USA). Quality control materials were received as a part of the Newborn Screening Quality Assurance Program (NSQAP) for X-ALD conducted by the Newborn Screening and Molecular Biology Branch, Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA.

2.2.1. Preparation, storage and calibration of dried blood spots

Calibration set for LPCs was prepared by pooling EDTA blood and mixing it with the aqueous spiking solution mixture of 4 LPC (C26:0 – C20:0) standards in the range of 0.015 to 3.6 μmol/L. 50 μL of each spiked calibrator was spotted on the filter paper cards which were dried for 4 h at room temperature and later stored at −80 °C in a zip-lock pouch containing a desiccant.

2.2.2. 2 Patient samples and controls

Left over DBS samples from two hundred and eighty two (282), apparently normal subjects (aged > 1–60 years) whose sample had been referred to our laboratory for routine testing were used as healthy controls. Twenty eight (28) patients aged (3–40 years) who had presented to the Neurology Outpatient Department at the National Institute of Mental Health and Neuro Sciences, Bengaluru, India, with clinical and cranial MRI features suggestive of X-ALD and confirmed positive for X-ALD, were included in the study. The diagnosis of X-ALD was confirmed by estimating C26:0 and C24:0 LPCs by the LC-MS/MS method and plasma VLCFA analysis by GC–MS.

2.3. Methods

2.3.1. LPC extraction from dried blood spots

Using an automated DBS puncher (Perkin Elmer, Waltham, MA, USA), 3.2 mm dried blood spots were punched into 96 well microtiter plates to which the internal standard, C26:0-d4-LPC solution reconstituted to a concentration 0.39 μmol/L with absolute methanol, was added. After sealing with an aluminium cover, the sample plates were vortexed at 26 °C at 450 rpm for 1 h in order to extract the LPCs from DBS.

2.3.2. FIA-MS/MS/measurement

Post extraction, 10 μL/sample was injected via an Acquity UPLC sample manager into the Xevo TQD MS/MS (Waters Corporation, Milford, MA, USA) by an isocratic flow (Supplementary Table 1) with mobile phase containing a mixture of 80% acetonitrile and methanol [50,50] and 20% 2 mM ammonium acetate in water with 0.1% formic acid. The analysis time was 1.5 min/sample. The analytes were monitored in the electrospray ionization positive (ESI +) ion mode with source temperature, 150 °C and desolvation temperature, 600 °C. The following (*m/z*) mass transitions were monitored in the multiple reaction monitoring (MRM) mode 640.65 > 104.09 (C26:0-d4-LPC), 636.65 > 103.96 (C26:0-LPC), 608 > 104(C24:0-LPC), 580 > 104.06(C22:0-LPC) and 552.41 > 104.06(C20:0-LPC). LPCs were quantified using NeoLynx software of Mass Lynx 4.1.1 from Waters Corporation. C26:0, C24:0, C22:0 and C20:0- LPC concentration in normal controls and patients was expressed in micromoles/litre (μmol/L).

2.4. Statistical analyses

Recovery, accuracy and coefficient of variation (CV) were expressed as percentage and LPC concentration and their ratios for calculating the inter-day and intra-day precision were expressed as mean with standard deviation. The medians along with concentration ranges of LPCs and their ratios expressed as the 1st and 99th percentile were compared between the control and patient samples using Mann-Whitney *U* test where *P* values < 0.05 were considered statistically significant. The cut-off values of C26:0- C20:0-LPCs and their ratios were calculated using receiver operating characteristic (ROC) curves with the maximum value of the sum of specificity and sensitivity. Bland Altman analysis was performed to find out the agreement between the FIA-MS/MS method and the LC-MS/MS methods, where the differences between the two methods are plotted against the average of the two methods. The maximum allowed mean difference or Limits of Agreement (LOA) was fixed between ± 2 standard deviation (SD). IBM SPSS statistical software version 17.0 and Graph Pad Prism 5.1 were used for statistical analyses.

3. Results

3.1. Linearity

A standard curve was plotted for the calibrators which contained a mixture of four LPCs (C26:0- C20:0). Linearity of this method was determined from 0.015 to 3.6 μmol/L and best fit was observed in the range 0.079 to 3.6 μmol/L,. A 6 point calibration curve was plotted with the measured analyte concentrations as a function of the corresponding known spiked standards. First order linear regression was used to get the best fit of the individual standard curve for C26:0, C24:0, C22:0 and C20:0-LPCs and squared correlation coefficients of $R^2 = 0.9893$, $R^2 = 0.9977$, $R^2 = 0.9975$ and $R^2 = 0.9966$, were obtained, respectively. The analytes demonstrated good linearity up to the tested range, as shown in Supplementary Fig. 1 and Supplementary Table 2.

3.2. Recovery

Recovery was > 70% for C26:0- C20:0-LPCs when spiked at different concentrations, as given in Supplementary Table 3. However, recovery was low at 0.079 μmol/L for C26:0-LPC, 0.083 μmol/L for C24:0-LPC, 0.091 μmol/L for C22:0-LPC and 0.086 μmol/L for C20:0-LPC.

3.3. Accuracy, precision and quality control

DBS specimens were spiked with a mixture containing C26:0, C24:0, C22:0 and C20:0 LPCs at different concentrations and the accuracy and precision of the method (Table 1) were calculated using these concentrations. Our laboratory participated in the X-ALD proficiency testing assessment and quality control programme for X-ALD screening conducted by CDC, Atlanta, GA, USA. The proficiency testing assessment for results reported by our method was 100% satisfactory. For quality control, enriched DBS specimens containing C26:0, C24:0, C22:0 and C20:0-LPCs were received from CDC, with the concentration, 0 μmol/L (un-enriched), 1 μmol/L and 5 μmol/L. Using these, the accuracy and precision of the method in measuring LPCs was also determined. Data is represented in Table 2.

3.4. LPC concentration in patient samples and controls

LPC concentrations and their ratios were measured in left-over blood spots of 282 apparently normal subjects (healthy controls) and compared with 28 patients diagnosed with X-ALD. Their medians with concentration range of LPCs and their ratios (Table 3, Fig. 1 and Supplementary Fig. 2) were represented by a Box and Whisker plotted between the 1st and the 99th percentile. The medians were compared using the non-parametric *t*-test (Mann- Whitney *U* test) and were significant with a P value < 0.0001. C26:0-LPC was elevated by 6 folds, C26:0/C20:0-LPC ratio by 3.5 folds, C24:0-LPC ratio by 3 folds, C26:0/C22:0- LPC by 2.5 folds in patients when compared to controls. In contrast, only a slight increase was seen in the concentration of C22:0 and C20:0-LPCs, the ratios of C24:0/C22:0 and C24:0/C20:0-LPCs in patients when compared to controls. ROC curve analysis was performed and cut- off values were determined for LPCs and their ratios based on which the sensitivity and specificity of our method was assessed. Table 4 represents the cut-off values for the LPCs and their ratios with their sensitivity, specificity and area under the ROC curve (AUC) with 95% Confidence Interval (CI).

3.5. Method comparison

LPC concentration and the ratios of 28 X-ALD patients measured by the FIA-MS/MS method and the LC-MS/MS method previously developed by us were compared by Bland Altman analysis [23]. The agreement between the two methods was represented by calculating the bias (mean/average of the difference between the two methods) and the

Table 1
Measured concentration of LPCs in spiked blood spot specimens.

Spiked Standard concentration in μmol/L	Interday concentration (Standard deviation) (μmol/L)(N = 6)	Accuracy (%)	Interday coefficient of variation (%) (N = 6)	Intraday concentration (Standard deviation) (μmol/L) (N = 6)	Intraday coefficient of variation (%) (N = 6)
C26:0-LPC					
0.079	0.042(0.008)	53.84	19.77	0.050(0.005)	10.83
0.157	0.114(0.009)	72.61	8.39	0.120(0.012)	10.21
0.785	0.636(0.045)	81.01	7.15	0.668(0.045)	6.85
1.570	1.470(0.070)	93.63	4.82	1.407(0.100)	7.13
2.355	2.689(0.070)	114.0	2.62	2.788(0.223)	7.98
3.140	3.335(0.178)	106.2	5.36	3.317(0.215)	6.50
C24:0-LPC					
0.083	0.077(0.009)	93.9	12.35	0.070(0.008)	12.78
0.165	0.162(0.003)	98.7	2.37	0.126(0.025)	19.92
0.825	0.624(0.031)	76.09	5.07	0.623(0.045)	7.29
1.650	1.320(0.046)	80.48	3.52	1.258(0.094)	7.51
2.475	2.440(0.121)	99.1	4.95	2.482(0.214)	8.64
3.300	3.025(0.060)	92.22	2.04	2.942(0.181)	6.16
C22:0-LPC					
0.091	0.096(0.016)	106.6	16.60	0.088(0.010)	12.45
0.181	0.180(0.015)	99.44	8.78	0.160(0.017)	11.18
0.905	0.730(0.027)	80.66	3.71	0.748(0.060)	8.12
1.810	1.604(0.069)	88.60	4.34	1.488(0.080)	5.40
2.715	2.870(0.040)	105.90	1.63	2.925(0.257)	8.80
3.620	3.606(0.120)	99.60	3.46	3.543(0.215)	6.09
C20:0-LPC					
0.086	0.074(0.012)	86.0	16.95	0.070(0.005)	6.90
0.173	0.171(0.020)	99.4	12.44	0.153(0.013)	8.52
0.865	0.711(0.018)	82.67	2.53	0.696(0.059)	8.49
1.730	1.509(0.089)	87.7	5.94	1.407(0.086)	6.12
2.595	2.579(0.057)	99.9	2.24	2.570(0.211)	8.21
3.460	3.373(0.092)	98.05	2.75	3.340(0.188)	5.65

LPC- Lysophosphatidylcholine.

Table 2
Measured concentration of LPCs in the quality control samples from CDC, Atlanta, USA, for X-ALD NSQAP programme.

Spiked standard concentration (μmol/L)	Interday concentration (Standard deviation) (μmol/L)(N = 6)	Accuracy (%)	Interday coefficient of variation (%) (N = 6)	Intraday concentration (Standard deviation) (μmol/L)(N = 6)	Intraday coefficient of variation (%) (N = 6)
C26:0-LPC					
Un-enriched	0.225(0.019)	NA	8.79	0.258(0.017)	6.67
1	1.185(0.060)	118.5	5.28	1.543(0.093)	6.09
5	5.469(0.258)	109.38	4.73	6.810(0.554)	8.13
C24:0-LPC					
Un-enriched	0.368(0.040)	NA	10.95	0.471(0.024)	5.09
1	1.253(0.134)	125.3	10.77	1.676(0.116)	6.97
5	5.378(0.427)	107.56	7.94	6.330(0.283)	4.48
C22:0-LPC					
Un-enriched	0.167(0.020)	NA	12.28	0.205(0.013)	6.72
1	0.937(0.094)	93.7	10.08	1.290(0.112)	8.68
5	4.630(0.398)	92.6	8.59	5.404(0.434)	8.03
C20:0-LPC					
Un-enriched	0.350(0.039)	NA	11.16	0.453(0.012)	2.67
1	1.181(0.066)	118.1	5.65	1.668(0.117)	7.03
5	5.120(0.290)	102.4	5.69	5.763(0.225)	3.91

LPC- Lysophosphatidylcholine.

Table 3
Concentration of LPCs in controls and patients.

Analytes measured in DBS	[a] Median and concentration range in putative normal subjects (μmol/L) N = 282	[a] Median and concentration range in patients (μmol/L) N = 28	Significance
C26:0-LPC	0.16 (0.10–0.41)	0.95 (0.44–2.16)	$P < 0.0001$
C24:0-LPC	0.29 (0.15–0.53)	0.97 (0.58–1.68)	$P < 0.0001$
C22:0-LPC	0.17 (0.09–0.30)	0.34 (0.16–1.20)	$P < 0.0001$
C20:0-LPC	0.32 (0.14–0.58)	0.48 (0.31–0.88)	$P < 0.0001$

Analyte ratios measured in DBS	[b] Median and concentration ratio range in putative normal subjects N = 282	[b] Median and concentration ratio range in patients (μmol/L) N = 28	Significance
C26:0/C22:0-LPC	1.08 (0.50–1.73)	2.77 (1.05–5.64)	$P < 0.0001$
C24:0/C22:0-LPC	1.79 (1.04–2.36)	2.69 (1.44–3.86)	$P < 0.0001$
C26:0/C20:0-LPC	0.56 (0.31–1.04)	1.94 (0.73–4.85)	$P < 0.0001$
C24:0/C20:0-LPC	0.90 (0.62–1.37)	1.90 (1.06–3.19)	$P < 0.0001$

[a] Median (Range is expressed between 1st and 99th percentile).
[b] Median of ratios (Range is expressed between 1st and 99th percentile).

95% limits of agreement (± 2 SD, dotted lines) plotted on the X axis with the Y axis corresponding to the difference between the LC-MS/MS method and FIA -MS/MS method. The X-Y plots of all the analytes demonstrated good correlation except for C22:0-LPC and C24:0/C22:-LPC ratio. The concentrations of C26:0, C24:0, C22:0 and C20:0-LPCs were found to be 0.34, 0.27, 0.25 and 0.17 μmol/L higher when measured by the FIA-MS/MS method when compared to the LC-MS/MS method. This data has been represented in Fig. 2, and Supplementary Fig. 3.

4. Discussion

Newborn screening is carried out by many laboratories worldwide in order to identify disorders which can be detected at birth, by collecting the sample in first few hours or days after the child is born [26,36]. Patients with X-ALD are not symptomatic at birth, and early detection at the asymptomatic stage could help in monitoring the affected children before the development of the clinical symptoms. Screening for X-ALD has been added to the existing mandatory newborn screening panel in many states in the U.S.A. Under the NBS programme, C26:0-LPC was identified as sensitive and specific biomarkers for X-ALD screening in dried blood spots. Since reports identified C24:0-LPC also as a similar sensitive and specific marker for the diagnosis of X-ALD, it was also included in the panel [15,16,25]. Various methods of LPC estimation have been developed, right from the ESI positive ion mode LC-MS/MS method by Hubbard and co-workers [15,16] which involved the chromatographic separation of LPC species with a 6–8 minute run time, to the modified and simplified shorter run time methods for LPC estimation which took only 3 min and 2 min in the negative mode and positive ion ESI mode respectively [17,18,24]. LC-MS/MS method in the ESI positive mode helped in the better separation of the LPC species and when combined with the ESI negative ion mode, provided structure specificity, thus enabling it to work well as a sensitive, specific, high throughput screening as well as a second tier testing method for the X-ALD diagnostic screening protocol. Simultaneously, many such LC-MS/MS methods were developed for LPC estimation by various authors for X-ALD diagnosis [19–23]. Later, methods were also developed to include the estimation of C26:0-LPC into the recommended uniform screening panel (RUSP) for state newborn screening(NBS) programs in the United States of America, where amino acids, acyl carnitines, succinylacetone and enzymes for lysosomal storage disorders were measured simultaneously [24,26,27,37]. A panel of LPCs (C20:0, C22:0, C24:0 and C26:0) were also analysed by Turgeon et al [25] by the reliable, simple and fast FIA-MS/MS method, where they identified C26:0-LPC and C24:0-LPC as the most reliable markers for differentiating between patients and controls [25].

X-ALD cases have been identified in India [11,32,38] but dried blood spot-based testing for estimating LPCs have not been reported until we identified 21 cases successfully by the LC-MS/MS method developed by us [23]. Though newborn screening, as a nationwide

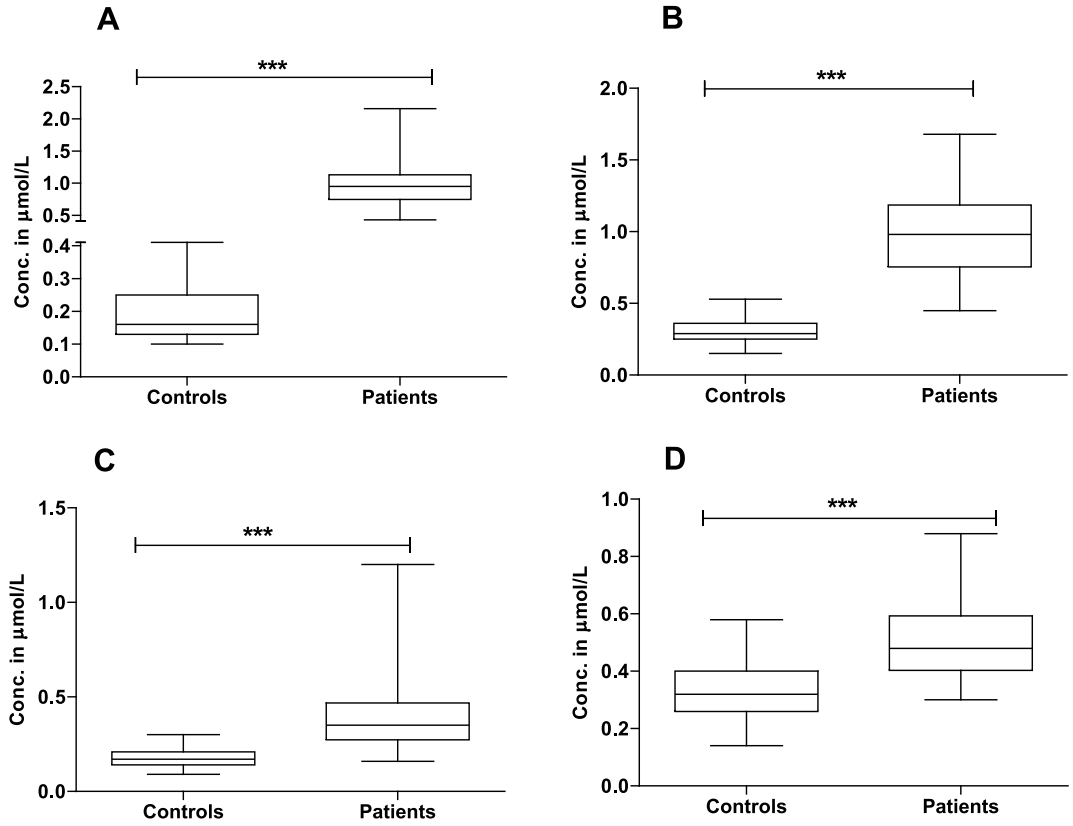

Fig. 1. Box and whisker plot for concentration ranges between the 1st and 99th percentile for comparison of C26:0-LPC (Fig. 1A), C24:0-LPC (Fig. 1B), C22:0-LPC (Fig. 1C) and C20:0-LPC (Fig. 1D) concentrations between normal subjects (N = 282) and patients (N = 28).
Conc – Concentration; LPC – Lysophosphatidylcholine.

Table 4
Sensitivity, specificity and area under the ROC with 95% confidence interval for LPCs and their ratios.

	Cut off (µmol/L)	Sensitivity (%)	Specificity (%)	AUC & (95% CI)
Analytes measured in DBS				
C26:0-LPC	0.42	100	78.33	1 (1–1)
C24:0-LPC	0.55	100	98.33	0.99 (0.99–1.00)
C22:0-LPC	0.25	89.29	78.33	0.94 (0.89–0.99)
C20:0-LPC	0.41	67.86	73.33	0.84 (0.76–0.91)
Analyte ratios measured in DBS				
C26:0/C22:0-LPC	1.47	85.71	33.33	0.96(0.91–1.00)
C24:0/C22:0-LPC	2.09	82.14	81.67	0.89(0.81–0.98)
C26:0/C20:0-LPC	0.81	92.86	46.67	0.98(0.96–1.00)
C24:0/C20:0-LPC	1.18	92.86	71.67	0.98(0.96–1.00)

LPC- Lysophosphatidylcholine; CI- Confidence Interval; AUC- Area Under the Curve.

programme, has not been implemented as yet in India, the FIA-MS/MS method for the diagnosis of aminoacidopathies, organic acidemias and fatty acids oxidation disorders for symptomatic or high-risk cases has existed from almost a decade [28–30]. Since the LC-MS/MS method for estimating LPCs involves the use of column thus adding to cost of the test, a FIA-MS/MS method was developed for estimating a panel of LPCs for high throughput screening, following a similar extraction protocol as done for the estimation by LC-MS/MS method with 1.5 minutes run time in the ESI positive, MRM mode. The linearity, accuracy and precision(intraday and interday) was within acceptable limits and the CDC, NSQAP proficiency testing results of our method for LPC estimation were 100% satisfactory. However, the concentration of the LPCs and their ratios were higher when measured by the FIA-MS/MS method in comparison to the LC-MS/MS method.

The FIA-MS/MS method was able to correctly identify 28 cases of X-ALD, which were confirmed by the LC-MS/MS method and plasma VLCFA analysis by GC–MS. Blood C26:0-LPC, C26:0/C20:0-LPC ratio and C24:0-LPC were elevated significantly by 6, 3.5 and by 3 folds respectively, C22:0-LPC, C20:0-LPC, C24:0/C22:0-LPC and C24:0/C20:0- LPC ratios were also slightly elevated in X-ALD patients when compared to controls. Based on ROC curve analysis, cut-offs were established and we identified C26:0-LPC and C24:0- LPC as 100% sensitive markers for the screening of X-ALD with their specificity being 78.33% and 98.33% respectively. Since recovery was 75-85% for C26:0-LPC at 0.42 µmol/L and 72-75% for C24:0-LPC at 0.55 µmol/L concentations and false positives were reported by the FIA-MS/MS method, the use of second tier LC-MS/MS chromatographic separation technique is essential to find out true positives in cases which tested positive by this method.

This method could be used for high throughput, first level screening of X-ALD in newborns as well as symptomatic cases and those patients with an elevated C26:0 and C24:0-LPC could be further confirmed by the second tier LC-MS/MS analysis of LPCs and their ratios. Introduction of newborn screening for X-ALD to identify affected cases before neurological symptoms develop would help clinicians in initiating early treatment and improve their clinical outcome.

5. Conclusions

For the first time in India, we were able to successfully develop a high-throughput, FIA-MS/MS method for estimating a panel of LPCs in

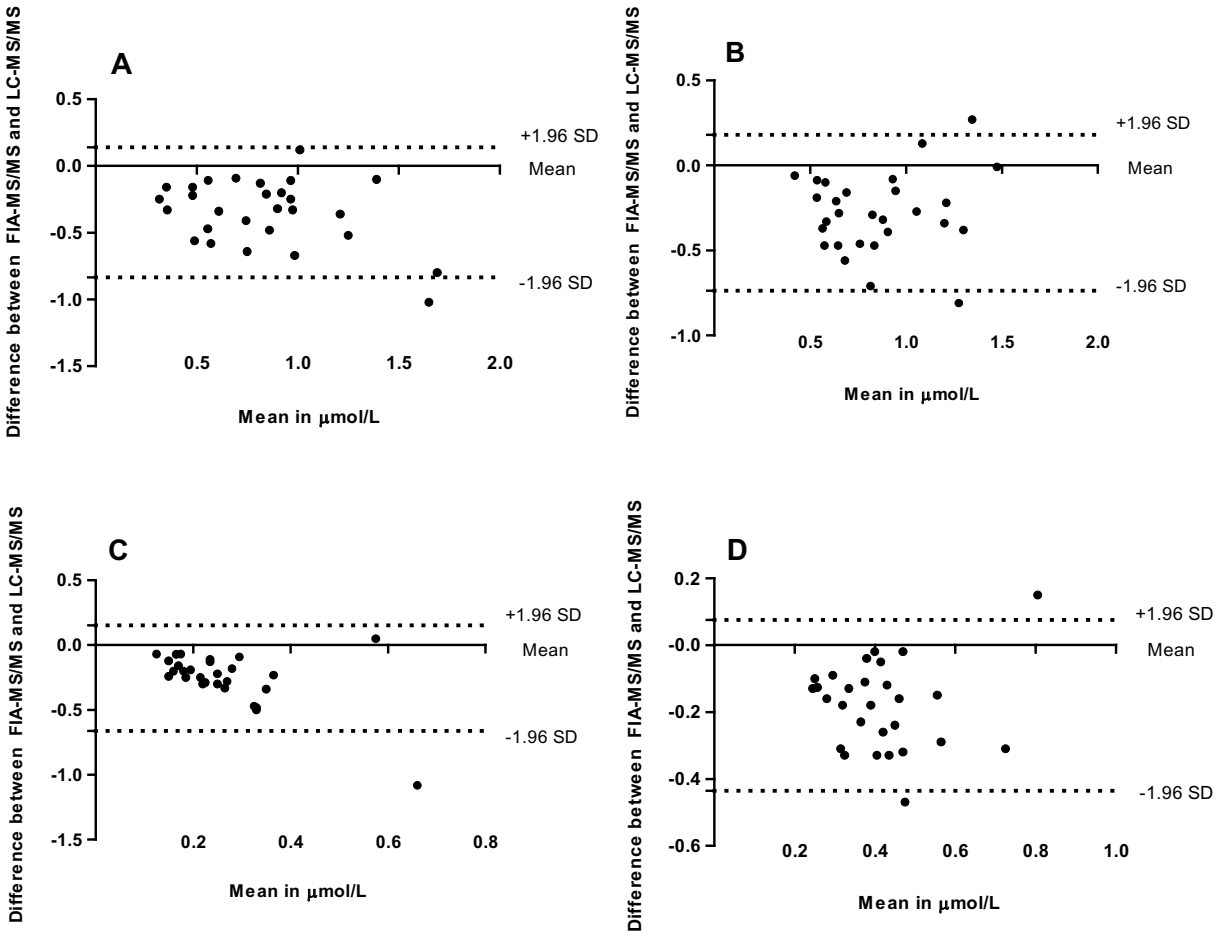

Fig. 2. Bland-Altman plots of the difference (LC-MS/MS minus FIA-MS/MS) vs. the mean of paired LPC values between the LC-MS/MS and FIA-MS/MS methods. The mean difference and the limits of agreement, ± 1.96 SD have been represented as solid and dotted lines respectively for (2A): C26:0-LPC, (2B): C24:0-LPC, (2C): C22:0-LPC and (2D): C20:0-LPC.

DBS with a sample run time of 1.5 min. The results of FIA-MS/MS screening were confirmed with the second tier LC-MS/MS testing method to identify X-ALD in clinically suspected patients. This two tier testing strategy rules out false positives which are likely to be identified by this method. This high-throughput and rapid method can be used for mass screening of newborns or high-risk cases for X-ALD in the Indian population in whom genetic disorders could be highly prevalent as parental consanguinity is common.

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Declaration of interest

None declared.

Author contributions

AN: Performed the experiments analysed data and wrote the paper, NM, MB and SRC: Provided the clinical material and approved the manuscript, RC: Conceived and designed the study and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.04.059.

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