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Application Note

Quantitative Analysis of Four Long-Chain Lysophosphatidylcholines (LPCs) in Dried Blood Spot using Liquid Chromatography Tandem Mass Spectrometry for Clinical Research

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Contact Sales

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Abstract

Described here is a clinical research method for the quantitative analysis of four long-chain LPCs in dried-blood spot (DBS) using a Xevo™ TQ-S micro Mass Spectrometer.

LPCs were extracted using methanol and shaking for 30 min. at room temperature. An ACQUITY™ UPLC™ I-Class PLUS FL System was used to separate C26:0-LPC, C24:0-LPC, C22:0-LPC, and C20:0-LPC on an ACQUITY Premier CSH™ C_{18} Chromatographic Column with detection using multiple reaction monitoring (MRM) in negative electrospray ionization (ESI) on a Xevo TQ-S micro Mass Spectrometer. Method performance characterization indicated no significant carryover from a concentration of 2 µM into a consecutive blank injection; linearity was demonstrated over the range 0.1-10 µM, with a precision of ≤15% CV and bias ≤20%. This method offers a simple extraction procedure and reliable quantification of C20:0, C22:0, C24:0, and C26:0 LPCs in DBS for clinical research.

Benefits

- Good chromatographic resolution of all forms
- Short extraction time compared to traditional methods for lipid extraction
- Short analysis injection-to-injection 7 minutes

Introduction

Historically, very long chain fatty acids have been quantified using direct transesterification and gas chromatography methods which involve time-consuming and labor-intensive sample preparation and analysis. Consequently, simpler LC-MS/MS methods have been reported for the measurement of long-chain LPCs (LPCs generic chemical structure, Figure 1) in DBS. Interferences have been reported using positive ESI, thus researchers have developed methodologies utilizing negative ESI to circumvent isobaric interferences when there is lack of chromatographic resolution. Here a UHPLC-MS/MS method is described using acetate adducts for the reliable analysis of four long-chain LPCs in DBS for clinical research.

A UHPLC-MS/MS method for quantifying a panel of LPCs (C20:0, C22:0, C24:0, and C26:0-LPCs) was developed. The mobile phase composition consisted of a mixture of water, acetonitrile, and isopropanol with 5 mM ammonium acetate as a modifier. The DBS sample extraction used a working solution prepared with the

deuterated LPC internal standards in methanol. The method was developed with an ACQUITY UPLC I-Class PLUS System (Fixed Loop) using an ACQUITY Premier CSH C_{18} Column, which provided baseline resolution of endogenous isobaric interferences for the saturated forms of the targeted LPCs. The detector was a Xevo TQ-S micro Mass Spectrometer operated in negative ESI mode. This methodology resulted in a total analysis time (injection-to-injection) of 7 minutes.

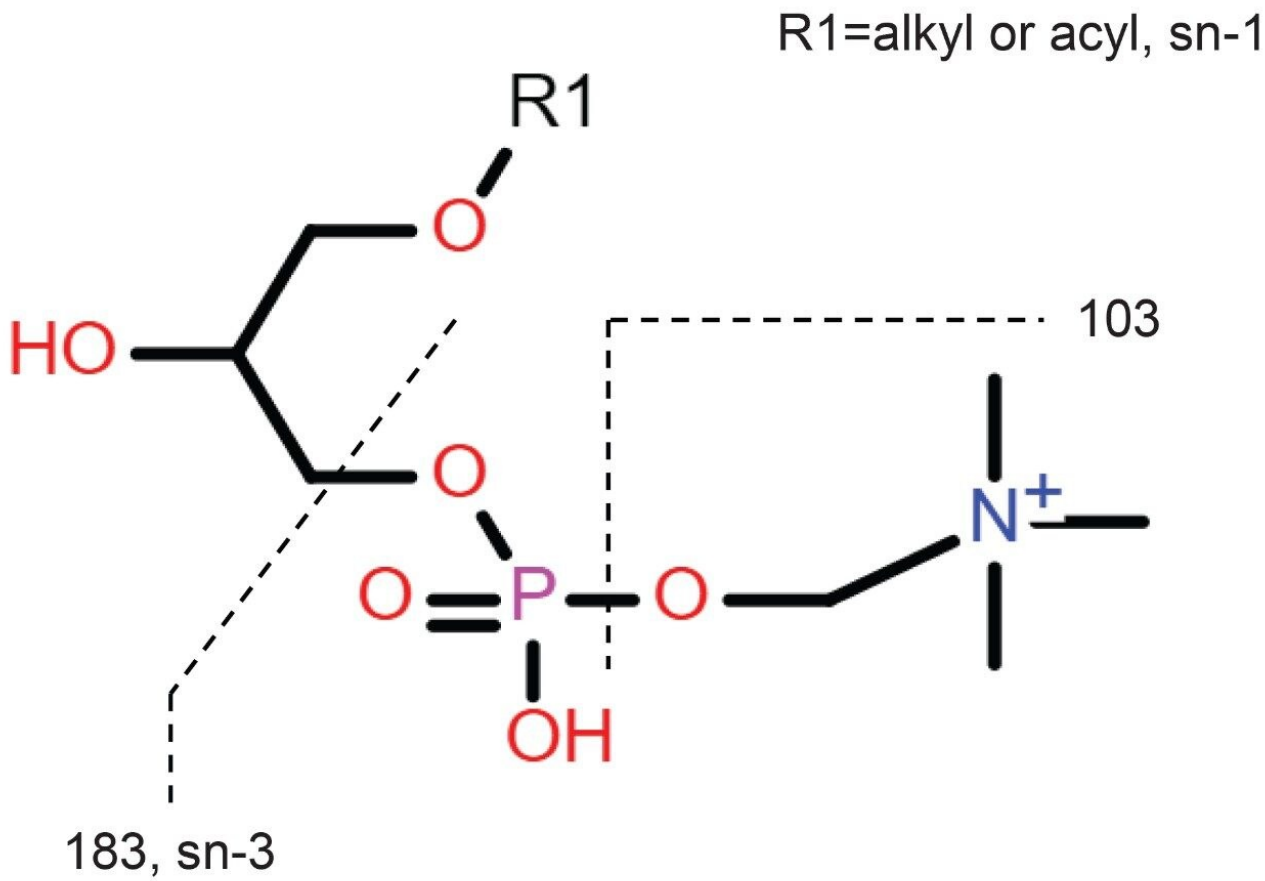

Figure 1. Chemical structure of the backbone of lysophosphatidylcholines.

Experimental

Calibrators and Quality Control (QC) Samples

DBS materials were provided by the Centers for Disease Control and Prevention (CDC). Two independent DBS sample sets were used for this work: Lots 2110 and 2215.

Four samples from Lot 2110 were used as calibrators including A (unenriched), B (spiked at 0.25 µmol/L), C (spiked at 0.5 µmol/L), and D (spiked at 2 µmol/L).

Samples B (spiked at 0.25 µmol/L), C (spiked at 0.5 µmol/L), and D (spiked at 2 µmol/L) from Lot 2215 were used as QC samples: Low, Mid, and High respectively.

An additional set of DBS samples (Lot 2021) were provided covering a range of enrichment concentrations comprising 0.1, 0.25, 0.5, 1.25, 2.5, 5, 7.5, and 10 µmol/L for linearity testing purposes.

Internal Standards Preparation

Three out of the four stable isotopically labelled internal standards were purchased from Cambridge Isotope Laboratories, and C26:0-LPC-d4 was supplied from Avanti Polar Lipids. Stock solutions were prepared in methanol at 341.8 µmol/L, 325.4 µmol/L, 310.5 µmol/L, and 309.4 µmol/L for C20:0-LPC-d4, C22:0-LPC-d4, C24:0-LPC-d4, and C26:0-LPC-d4, respectively.

These stock solutions were dispensed into glass vials, capped and stored at -20°C. An extraction working solution (EWS) at 0.04 µmol/L for each internal standard was prepared by taking an aliquot of each respective internal standard stock into a final volume of 200 mL of methanol. The EWS was used as a system suitability test daily to verify system performance.

Sample Extraction

1. DBS material was removed from -20 °C storage and brought to room temperature.

2. A 3.2 mm punch was placed into a 96-well round bottom plate.

3. 100 µL of EWS was added to each well. The plate was sealed with a foil adhesive cover and mixed on a shaker at 500 rpm for 30 minutes.

4. The plate was removed from the shaker and the liquid extracts were transferred into a clean 96-well plate, sealed with a silicone mat and placed into the sample manager prior to analysis.

LC Conditions

LC system:	ACQUITY UPLC I-Class PLUS FL System
Detection:	Xevo TQ-S micro Mass Spectrometer
Plate:	Waters 96-well Sample Collection Plate, 350 µL
Column(s):	ACQUITY Premier CSH C_{18} Column, 2.1 mm x 50 mm, 1.7 µm (p/n: 186009460)
Column temperature:	55 °C
Sample temperature:	8 °C
Injection volume:	7.5 µL
Injection mode:	Partial Loop with needle-overfill
Needle size:	20 µL
Sample syringe:	100 µL
Sample loop:	20 µL
Flow rate:	Refer to Gradient Table
Mobile phase A:	5 mM ammonium acetate, 60:40 (v/v) acetonitrile:water
Mobile phase B:	5 mM ammonium acetate, 90:10 (v/v) isopropanol:acetonitrile

Weak wash:	Methanol
Strong wash:	0.1% formic acid in 25/25/25/25 (v/v/v/v) acetonitrile/methanol/IPA/water
Run time:	6.32 minutes

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.4	80	20	Initial
3.00	0.4	35	65	6
3.50	0.4	15	85	6
3.51	0.4	1	99	11
4.51	0.4	1	99	11
4.52	0.4	80	20	11
6.32	0.4	80	20	11

MS Conditions

MS system:	Xevo TQ-S micro Mass Spectrometer
Ionization mode:	ESI negative
Capillary voltage:	2.5 kV
Desolvation temperature:	450 °C
Source temperature:	150 °C
Desolvation gas:	1000 L/Hr
Cone gas:	50 L/Hr

MS1 resolution:	Unit (0.7 Da)
MS2 resolution:	Unit (0.7 Da)

Method Events

Time	Event	Action
4.0	Flow state	Waste
4.0	Solvent delay	Begin

Data Management

MS software:	MassLynx™ v4.2 Software SCN 1045
Informatics:	TargetLynx™ XS v4.2 Software SCN1045 and QUAN Review

MRM Parameters

Analyte	Precursor (*m/z*)	Product (*m/z*)	Cone (V)	Collision (V)	Dwell time (seconds)
C20:0-LPC (Quan)	610.6	311.1	20	35	0.015
C20:0-LPC (Qual)	610.6	536.2	20	20	0.015
C20:0-LPC-D4 (Quan)	614.4	315.1	20	35	0.015
C20:0-LPC-D4 (Qual)	614.4	540.5	20	20	0.015
C22:0-LPC (Quan)	638.5	339.2	20	35	0.015
C22:0-LPC (Qual)	638.5	564.5	20	20	0.015
C22:0-LPC-D4 (Quan)	642.5	343.3	20	35	0.015
C22:0-LPC-D4 (Qual)	642.5	568.5	20	20	0.015
C24:0-LPC (Quan)	666.4	367.4	20	35	0.015
C24:0-LPC (Qual)	666.4	592.5	20	20	0.015
C24:0-LPC-D4 (Quan)	670.7	371.4	20	35	0.015
C24:0-LPC-D4 (Qual)	670.7	596.4	20	20	0.015
C26:0-LPC (Quan)	694.5	395.5	20	35	0.015
C26:0-LPC (Qual)	694.5	620.5	20	20	0.015
C26:0-LPC-D4 (Quan)	698.5	399.5	20	35	0.015
C26:0-LPC-D4 (Qual)	698.5	624.5	20	20	0.015

Results and Discussion

During method development, different types of column chemistry, mobile phase composition, and ESI polarity modes were assessed to optimize chromatographic peak shape, analytical sensitivity and manage interferences. In positive mode, ammonium formate favored the formation of *m/z* 184 and *m/z* 104 as daughter ions for all the LPCs. The *m/z* 184 ion lacks specificity because most phospholipids share this moiety, so the ion *m/z* 104 was selected as quantifier. Having evaluated conditions in positive mode for the analysis of the four saturated LPCs, it was observed that some existing interferences were not only affecting the parent masses of the analytes but their corresponding internal standard parent ion masses too. Hence, negative ionization using ammonium acetate as modifier was assessed for the detection of LPCs. Acetate adducts $[M+CH_3COO]^-$ were the most abundant parent ion masses, generated under these conditions for each LPC. The acyl-chain product ions generated in negative ionization reduce the detection of interferences coming from other phosphocholine headgroups. The latter detection conditions were used for the validation of the method presented herein.

The chromatographic resolution and retention time comparison to a matched internal standard allowed

unambiguous identification (Figure 2). The elution order depended on the length of the carbon chain starting with C20:0-LPC and ending with C26:0-LPC. The detection of the 2-acyl isomer could be postulated for the adjacent peak in each LPC form, as these isomers may form readily in a pH dependent fashion. In addition, there might be a contribution on the presence of isomers attributed to the purity of the synthetic internal standards which was reported to be 95% for each LPC.

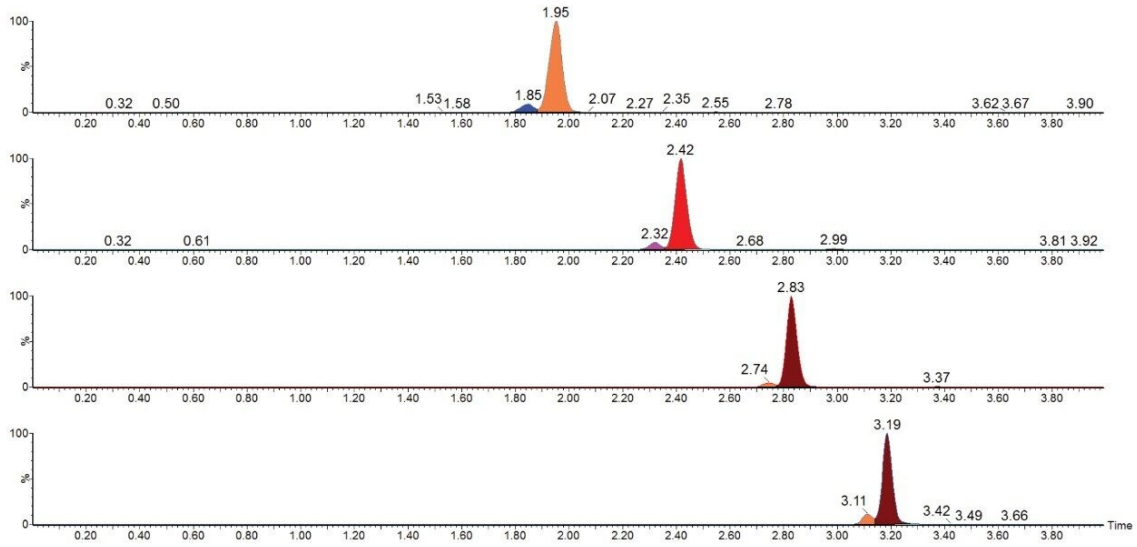

Figure 2. Representative chromatograms for C20:0-LPC eluting at 1.95 min, C22:0-LPC at 2.42 min, C24:0-LPC at 2.83 min, and C26:0-LPC at 3.19 min.

LPCs are compounds present endogenously in blood and as such their levels vary amongst different donor pools. Thus, the measurement of enrichment concentrations lower than 0.1 µmol/L showed higher values of imprecision (> 20%). The LLMI values were then determined by analyzing the lowest concentration of enriched DBS samples (n=2) over five days. LLMI values including endogenous levels were 0.24 µmol/L for C20:0-LPC, 0.16 µmol/L for C22:0-LPC, 0.2 µmol/L for C24:0-LPC and 0.27 µmol/L for C26:0-LPC.

The clinical research method was shown to be linear from 0.1–10 µmol/L, with $r^2 \geq 0.99$ across all five analytical runs. No significant carryover into blank samples was observed after analyzing alternate injections of the QC High level samples.

Matrix effects were evaluated qualitatively by comparing six independent unenriched DBS samples to a solvent

blank using post-column infusion of the target analytes at low (0.25 µmol/L) and high (2 µmol/L) concentrations. No significant matrix effects were observed for any of the LPCs (Figure 3).

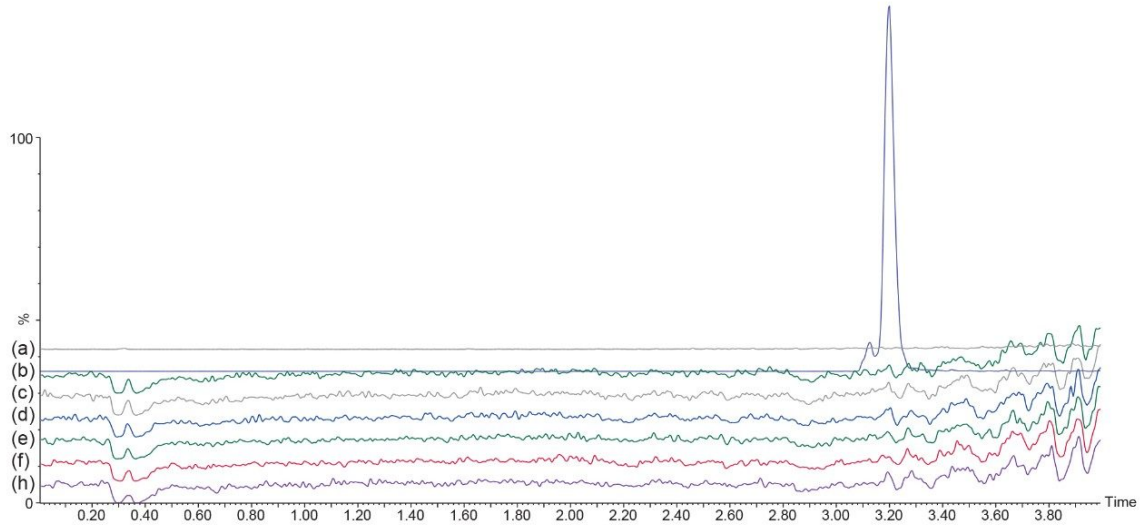

Figure 3. Example chromatogram for C26:0-LPC post-column infusion at 2 µmol/L. Top gray trace corresponds to solvent blank card, analyte standard peak is indicated in top blue trace. The subsequent (bottom) six traces are independent DBS samples without enrichment.

A four-point calibration curve (including unenriched DBS samples) was run each day alongside the QC samples for all LPCs. An example is shown in Figure 4.

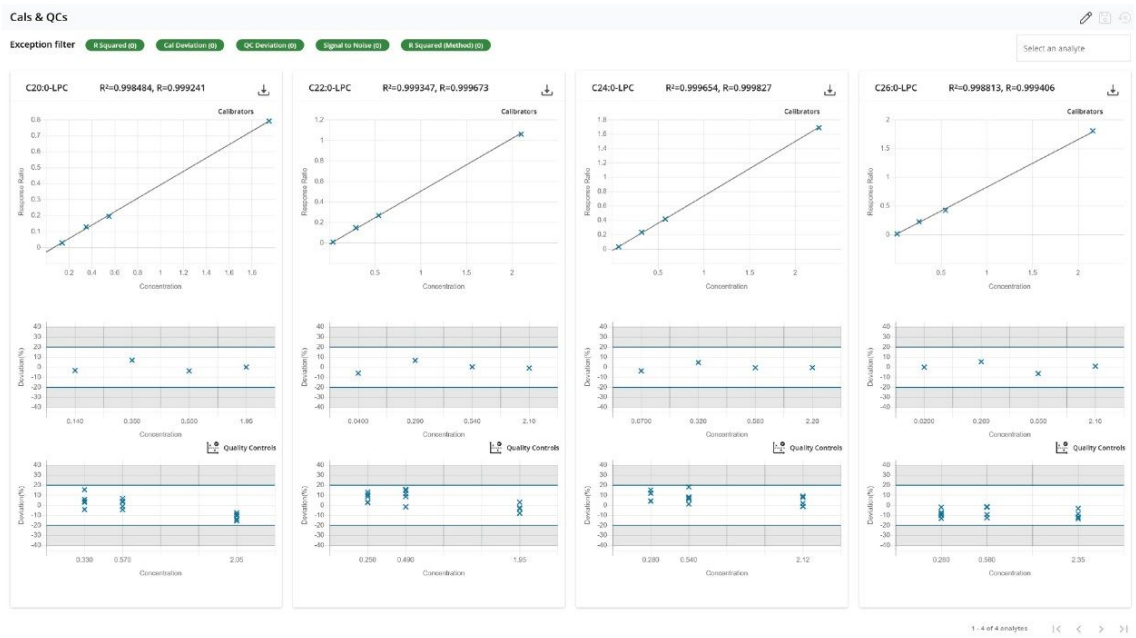

Figure 4. Calibration curve for C20:0-LPC, C22:0-LPC, C24:0-LPC, and C26:0-LPC in Quan Review.

Within-day and total precision were determined over 5 days at 0.25 µmol/L, 0.5 µmol/L, and 2 µmol/L enrichment concentrations (n=5). Both parameters met the acceptance criteria of %CV ≤15 for all LPCs as shown in Table 1. The % bias was calculated for all three QC concentration levels with respect to their assigned values by CDC is also shown on Table 1. The bias for all LPCs was ≤20% within acceptance criteria.

	C20:0-LPC QC conc. (µmol/L)			C22:0-LPC QC conc. (µmol/L)			C24:0-LPC QC conc. (µmol/L)			C26:0-LPC QC conc. (µmol/L)		
Assigned mean	0.33	0.57	2.05	0.25	0.49	1.95	0.28	0.54	2.12	0.28	0.56	2.35
Mean	0.33	0.59	1.91	0.26	0.52	1.9	0.27	0.54	2.03	0.24	0.53	2.12
%Bias	1.3	4.1	-6.6	3.2	6.5	-2.8	-2.6	-0.1	-4.2	-12.9	-5.9	-9.9
Precision												
Within day %CV	5.4	4.6	5.4	5.5	5.4	5.4	6.7	4.1	5.2	6.3	6.3	5.3
Total %CV	7.2	7.9	7.5	5.9	6.7	6.4	8.5	8.7	7.8	6.3	8.9	5.9

Table 1. Within-day and total precision, and QC %Bias summaries.

The analytical data presented in this material are intended to demonstrate the robustness of a Waters research

method. These data in no way substitute for independent method validation required by any applicable legal or laboratory standards.

Conclusion

An LC-MS/MS clinical research method for measuring C20:0, C22:0, C24:0, and C26:0 LPCs with the ACQUITY UPLC I-Class PLUS FL System, and the Xevo TQ-S micro Mass Spectrometer that:

- Demonstrates good linearity, with no significant carryover, or matrix effects
- Offers good precision with a total reproducibility and repeatability ≤10% CV
- Offers reliability in the identification of LPCs from interfering isobaric compounds
- Provides good selectivity and analytical sensitivity in negative mode and is relatively fast as approximately 220 samples can be analyzed in 24 hours

Featured Products

ACQUITY UPLC I-Class PLUS System <https://www.waters.com/nextgen/global/products/chromatography/chromatography-systems/acquity-uplc-i-class-plus-system.html>

Xevo TQ-S micro Triple Quadrupole Mass Spectrometer <https://www.waters.com/nextgen/global/products/mass-spectrometry/mass-spectrometry-systems/xevo-tq-s-micro.html>

MassLynx Mass Spectrometry Software <https://www.waters.com/nextgen/global/products/informatics-and-software/mass-spectrometry-software/masslynx-mass-spectrometry-software.html>

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