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Quantification of lysophosphatidic acids in rat brain tissue by liquid chromatography–electrospray tandem mass spectrometry

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ABSTRACT

Lysophosphatidic acid (LPA) is a lipid mediator with multiple biological functions. A highly selective and sensitive liquid chromatography–tandem mass spectrometry (LC/MS/MS) method was developed for the determination of LPAs (16:0 LPA, 18:0 LPA, 18:1 LPA, 20:4 LPA) in rat brain cryosections. After partitioning the LPAs from other lipophilic material present in the tissue with a liquid–liquid extraction, a reversed-phase column and ion pair technique was used for separating analytes with a gradient elution. An internal standard (17:0 LPA) was included in the analysis. Detection and quantification of the LPAs were carried out with a triple quadrupole mass spectrometer using negative electrospray ionization (ESI) and multiple reaction monitoring (MRM). The artificial formation of LPAs from lysophosphatidylcholines during the sample preparation procedure and instrumentation was carefully studied during the method development. The method was validated; acceptable selectivity, accuracy, precision, recovery, and stability were obtained for concentrations within the calibration curve range of 0.02–1.0 μM for LPAs. The quantification limit of the assay was 54 fmol injected into column for each LPAs. The method was applied to comparative studies of LPA levels in rat brain cryosections after the various chemical pre-treatments of the sections.

1. Introduction

Lysophosphatidic acid (1-acyl-2-hydroxy-*sn*-glycero-3-phosphate, LPA) is a lipid mediator with multiple biological functions mainly mediated through specific G protein-coupled receptors [1,2]. When detected *in vivo,* the acyl group of LPA is a mixture of species that differ in length, degree of unsaturation, and functional connectivity of the hydrocarbon chain to the glycero-3-phosphate backbone (Fig. 1). LPA is produced through several enzymatic pathways, mostly from lysophospholipids, such as lysophosphatidylcholine (LPC) or from phosphatidic acid [3–5]. LPA is known to mediate several cellular responses, such as cellular proliferation, cell migration, prevention of apoptosis, and platelet aggregation and it is also involved in the development and function of cardiovascular, nervous, immune, and reproductive systems as well as in wound healing and tumor progression [6,7]. Altered function of LPA is associated with common human diseases such as arteriosclerosis and cancer [8]. Recent advances in LPA research have revealed its potential therapeutic and diagnostic usefulness as well as the need for development of selective and highly sensitive analytical methods, for instance in the diagnostics of the very malignant ovarian cancer [9,10].

Several methods have been developed to analyze LPA levels in biological samples. LPA has been detected from plasma, serum and other body fluids as well as from tissue homogenates. In most cases, LPA is first extracted from the biological matrix using modified liquid–liquid extraction methods as described by Folch et al. [11] or Bligh and Dyer [12]. Extraction procedures used together with strong acids [13–15], however, raises the concern about artificial formation of LPA from LPC under highly acidic conditions [16]. There are several described methods for determining the total LPA content, e.g. bioassay [17], immunoassay [18], and radioenzymatic assay [19] but there are some limitations in those methods. Bioassays determine the biological effects elicited by LPA, such as the changes in the calcium-dependent chloride currents in voltage-clamped *Xenopus* oocytes [17]. Although sensitive, bioassays are susceptible to disturbance by interfering compounds present in biological samples. Immunoassay [18] suffers from the poor selec-

Abbreviations: BSA, bovine serum albumin; FWHM, full-width at half-maximum; IS, internal standard; LLOQ, lower limit of quantification; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; QC, quality control; TEA, triethylamine.

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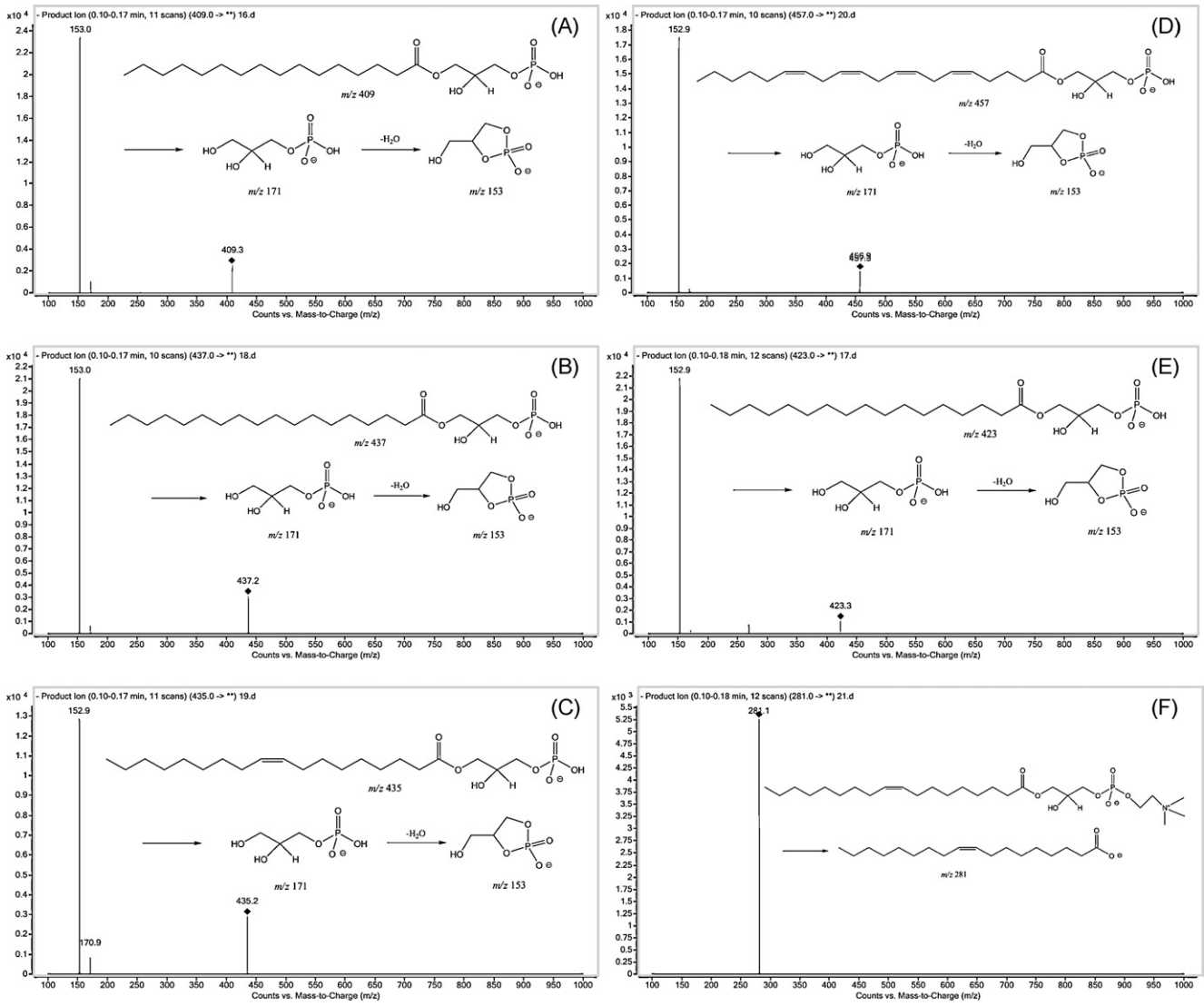

Fig. 1. Product ion mass spectra and molecular structures with proposed fragmentation of (A) 16:0 LPA (*m/z* 409 → 100–1000), (B) 18:0 LPA (*m/z* 437 → 100–1000), (C) 18:1 LPA (*m/z* 435 → 100–1000), (D) 20:4 LPA (*m/z* 457 → 100–1000), (E) 17:0 LPA (*m/z* 423 → 100–1000), and (F) 18:1 LPC (*m/z* 281 → 100–1000). Instrumentation parameters were similar to those described in Section 2.5, apart from following exception: One microliter of standard solution in methanol (1:10 dilution from stock solutions, ~20 μM) was injected directly into the LC/MS/MS using an isocratic mobile phase consisting of 30% of 1% TEA in 50 μM ammonium acetate and 70% of 1% TEA in 90% methanol delivered at 0.3 ml/min.

tivity of the antibody and finally the radioenzymatic assay [19] requires the use of radioactive reagents.

A general approach used to quantify individual LPA species from biological samples has been gas chromatographic (GC) analysis [9,20,21]. Nonetheless, as an indirect method, GC requires thin layer chromatography (TLC) purification as well as hydrolysis and derivatization of non-volatile LPA prior to analysis and thus is extremely laborious and time-consuming. Moreover, some indirect analysis protocols have not included appropriate internal standards in the assay [9]. High-performance liquid chromatography (HPLC) [22] and capillary electrophoresis [23] methods have also been used though light-scattering detection and indirect ultraviolet detection are rather insensitive and unselective.

Higher sensitivity and selectivity is obtained by mass spectrometric (MS) detection. Some authors have reported analysis of phospholipids by flow injection directly coupled to MS [24,25]. Unfortunately, flow injection can be problematic since there is no prior chromatographic separation to avoid ion suppression effects from highly abundant phospholipid species and other matrix components [13] and also artificial conversion of other lysophospholipids to LPA at the ion source has been demonstrated [26]. However, high-throughput shotgun lipidomics provides a powerful tool for untargeted analysis of total lipid extracts, including hundreds of molecular species of glycerophospholipids, glycerolipids, and sphingolipids [27,28]. Preferred methods for targeted quantifying of the levels of individual LPA species from biological samples are liquid chromatography–mass spectrometry (LC/MS) [29] and liquid chromatography tandem mass spectrometry (LC/MS/MS) methods [13–16].

Previously, substantial amounts of LPA species were found in rat brain by GC analysis [20,21]. Here we report the highly selective and sensitive LC/MS/MS method for quantitative measurement of LPA species from rat brain cryosections. As far as we are aware, this is the first time that LPA has been measured from brain tissue by LC/MS/MS. The artificial formation of LPAs from other lysophospholipids during the sample preparation procedure and instrumentation was carefully studied during the method development. The method was validated and proved to be highly selective, accurate and precise. The method was applied to determine the differences in LPA contents of chemical pre-treated rat

brain cryosections under conditions closely mimicking those of functional autoradiography where the neuroanatomical localization of LPA receptor signaling can be studied [30–32]. In this paper, we report preliminary results of brain sections to depict the endogenous content of LPA species in rat brain.

2. Materials and methods

2.1. Chemicals and reagents

16:0 LPA (1-palmitoyl-2-hydroxy-*ns*-glycero-3-phosphate), 18:0 LPA (1-stearoyl-2-hydroxy-*ns*-glycero-3-phosphate), 20:4 LPA (1-arachidonoyl-2-hydroxy-*ns*-glycero-3-phosphate), and 17:0 LPA (1-heptadecanoyl-2-hydroxy-*ns*-glycero-3-phosphate) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 18:1 LPA (1-oleyl-2-hydroxy-*ns*-glycero-3-phosphate), 18:1 LPC (1-oleyl-2-hydroxy-*ns*-glycero-3-phosphocholine), chloroform, and ammonium acetate were from Sigma (St. Louis, MO, USA). Methanol was obtained from J.T. Baker (Deventer, Holland) and triethylamine (TEA) from Fluka (Buchs, Switzerland). De-ionized H_2O was produced using a Milli-Q water purification system from Millipore (Milford, MA, USA). All reagents were of analytical grade, and the solvents were of HPLC grade.

2.2. Preparation of standards

The stock solutions of 16:0 LPA, 18:0 LPA, 18:1 LPA and 20:4 LPA and internal standard (IS) (17:0 LPA) were prepared by dissolving the compounds in methanol. The stock solutions were stored at −20 °C. Standard working solutions were prepared daily in methanol to first obtain a concentration of 2.0 μM and 0.6 μM for IS. The dilutions from calibration standard working solutions were prepared in methanol to give concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, and 1.0 μM. An internal standard was added into each sample to obtain a final concentration of 0.3 μM. Quality control (QC) sample working solutions (0.03, 0.1, 0.3 and 0.8 μM) were prepared in methanol. An internal standard was added into each solution to obtain a final concentration of 0.3 μM. To prepare the calibration and QC samples, 100 μl of each particular standard solution, 80 μl of 50 mM Tris–HCl (pH 7.40), and 220 μl of methanol were mixed to obtain 400 μl of solution. Each calibration and QC sample was then prepared according to Section 2.4 starting from the chloroform addition into the sample.

2.3. Brain tissue samples

Experiments were performed using brain sections of 4-week-old male Wistar rats obtained from the National Laboratory Animal Centre, University of Eastern Finland, Kuopio, Finland. Approval for the animal experiments was obtained from the local ethics committee. The experiments did not involve any *in vivo* treatment. The sections were prepared as previously described [32]. Briefly, the rats were decapitated and within the next 5 min, the whole brain was dissected out, dipped briefly in isopentane (chilled on dry ice) and stored at −80 °C. Horizontal sections (20 μm thick) were cut at −20 °C using a Leica cryostat, thaw-mounted onto Superfrost®Plus slides (Menzel-Gläser, Germany), dried and stored thereafter at −80 °C.

2.4. Sample preparation

The modified extraction method of Bligh and Dyer [12] was applied for the isolation of analytes from the tissue matrix and for removing the majority of the lipophilic material from the sample. Glassware was used throughout the sample preparation procedure. One sample comprised of tissue obtained from eight slides with two horizontal rat brain sections. The brain tissue was scraped manually from the slides with a spatula using the mixture of 50 mM Tris–HCl, pH 7.40 and methanol with a ratio of 1:4 (v/v). The tissue was transferred to a screw capped Pyrex® borosilicate glass test tube. The mixture of 50 mM Tris–HCl, pH 7.40 and methanol (1:4, v/v) was added to the test tube to bring the volume up to 400 μl. Chloroform was added to yield a water/methanol/chloroform ratio of 1:4:2 (v/v/v) and sample was shaken for 1 h with a vertical shaker (Heidolph Multi Reax, Heidolph Instruments GmbH & Co, Schwabach, Germany). 160 μl of chloroform and 160 μl of water were added to achieve the phase separation. After vortexing for 1 min, the sample was centrifuged at 1800 × *g* for 15 min at room temperature. The upper aqueous layer was transferred to an HPLC sample vial.

2.5. Instrumentation

The HPLC system comprised of a Agilent 1200 Series Rapid Resolution LC System (Agilent Technologies, Waldbronn, Germany) consisting of a solvent micro vacuum degasser, a binary pump, a thermostatted column compartment SL, and an autosampler SL. The mass spectrometric analysis was carried out with an Agilent 6410 Triple Quadrupole LC/MS equipped with an electrospray ionization source (Agilent Technologies, Palo Alto, CA, USA). Data were acquired by Agilent MassHunter Workstation Acquisition software (Agilent Technologies, Data Acquisition for Triple Quad., version B.01.03).

Ten microliters of sample solution was injected onto a reversed-phase HPLC column (XBridge™ C8 2.1 × 50 mm, 2.5 μm) (Waters, Ireland) using gradient elution with 50 μM ammonium acetate + 1% TEA (A) and 1% TEA in 90% methanol (B) as follows: 0–6.0 min 20% B → 90% B, 6.0–10.0 min 90% B, 10.0–10.1 min 90% B → 20% B, 10.0–15.0 min 20% B. An in-line filter (RRLC In-line filter, 2 mm, max 600 bar, 0.2 μm, Agilent Technologies) was used for protecting the analytical column. The flow rate was 0.3 ml/min, column temperature was maintained at 40 °C and the autosampler tray temperature was set to 10 °C. The following ionization conditions were used: ESI negative ion mode, drying gas (nitrogen) temperature 300 °C, drying gas flow rate 8 l/min, nebulizer pressure 40 psi and capillary voltage 4000 V.

Analyte detection was performed using multiple reaction monitoring (MRM) with the following transitions: *m/z* 409 → 153 for 16:0 LPA, *m/z* 437 → 153 for 18:0 LPA, *m/z* 435 → 153 for 18:1 LPA, *m/z* 457 → 153 for 20:4 LPA, *m/z* 423 → 153 for 17:0 LPA, and *m/z* 281 → 281 for 18:1 LPC. For LPAs, fragmentor voltage was 160 V and collision energy 20 V except 23 V for 17:0 LPA. For LPC, fragmentor voltage and collision energy were 300 and 0 V, respectively. Dwell time was 100 ms and mass resolutions (peak full-width at half-maximum) for MS1 and MS2 quadrupoles were 1.2 FWHM for LPAs and 0.7 FWHM for 18:1 LPC. The divert valve was programmed to allow eluent flow into the mass spectrometer from 2 to 10 min of each run. An internal standard (17:0 LPA) was used for quantification, and peak area ratios of the analyte to the IS were calculated as a function of the concentration ratios of the analyte to the internal standard using Agilent MassHunter software (Quantitative Analysis Version B.01.03). The protein content of brain tissue was determined by the Pierce BCA Protein Assay Kit with BSA as the standard and the tissue LPA concentrations were expressed as nmol per gram of protein in tissue.

2.6. Assay validation

This method was validated in terms of selectivity, linearity, precision, accuracy, recovery, and stability [33,34]. LPAs are endogenous compounds and a brain matrix with no analytes is not available. Therefore 4% (m/V) bovine serum albumin (BSA) in Tris-buffer was used as a surrogate matrix [34]. Homogenized rat

brain tissue was used as the authentic biological matrix. The whole brain was homogenized in 50 mM Tris–HCl (pH 7.40) buffer using a Soniprep 150 homogenizer (MSE Ultrasonic Disintegrator; MSE Scientific Instruments, Manor Royal, Crawley, Sussex, England) and samples were prepared according to Section 2.4. The volume of the homogenate in each sample corresponded to that of the tissue cryosection samples.

The selectivity of the method was assessed by analyzing reference standards, tissue samples, buffers, and solvents for interfering peaks at the retention times of LPAs. The standard addition method was used to further study the selectivity and matrix effect by spiking two concentrations of standards (0.2 and 0.4 μM) into the brain tissue homogenate to obtain regression curves for each LPA species [34]. The slopes of the curves were compared to the slopes of standard curves prepared without tissue matrix using analysis of covariance (ANCOVA) with GraphPad Prism® 4.03 for Windows (San Diego, CA, USA). Selectivity was also studied by diluting the tissue samples 1:2, 1:5 and 1:10 with methanol before injecting into LC/MS/MS [35] and comparing concentrations of diluted samples and undiluted samples. An in-source fragmentation experiment with 18:1 LPC molecule [36] was performed in order to probe ionization matrix effects by other lipid classes, such as glycerophosphocholines and lysophosphatidylserines. The transition m/z 281 → 281 was utilized to monitor 18:1 fatty acid anion, which is an important fragment in all phospholipids, distinct from LPAs. Furthermore, a post-column infusion experiment was performed to evaluate the ion suppression after the injection of the tissue sample. The infusion setup consisted of a syringe pump and a post-column T-piece as reported elsewhere [37].

The linearity of the assay for each of the analytes was assessed by analyzing the calibration curves from six concentrations of calibration samples in triplicate covering the range of 0.02–1.0 μM corresponding to 36–1790 fmol of LPA injected into the column. The calibration curve included samples without analytes including IS (a blank sample) and excluding IS (a zero sample). Unweighted linear regression analysis was used to make the calibration curve. Correlation coefficients were also calculated. The lower limit of quantification (LLOQ) was determined by calculating precision and accuracy for five LLOQ samples that were independent of the calibration curve. The intra-day precision of the assay was assessed by calculating the relative standard deviation (RSD) for the analysis of QC samples in five replicates, and inter-day precision was determined by the analysis of QC samples on three days. Accuracy was assessed by calculating the deviation of the measured value from the nominal value, which compared the calculated and known concentrations. BSA was used as a surrogate matrix in the above-mentioned experiments [34] and samples were prepared according to Sections 2.2 and 2.4. QC samples including LLOQ were prepared in the presence of BSA and intra-day precision and accuracy were calculated as described above. The recovery of the analytes using brain tissue homogenate where two concentrations of standards (0.2 and 0.4 μM) had been spiked was calculated with the following equation: Recovery (%) = $100 \times (S - U)/C$. In equation S represents the concentration of spiked sample, U represents the concentration of non-spiked sample and C represents the nominal concentration of the analyte.

The stability of the analytes was studied in three replicates using brain tissue homogenate samples. The concentrations of the stability samples were compared to those of freshly prepared samples. The freeze and thaw stability was determined after three freeze–thaw cycles. The short-term temperature stability was investigated by keeping the samples for 4 h at room temperature before sample preparation. The long-term stability was evaluated by analyzing samples which had been stored up to two months at −80 °C. The stock solution stability was investigated by comparing freshly prepared standards to standards prepared from a stock which had been frozen for 30 days and kept at room temperature for 6 h after thawing. The post-preparative stability was assessed by keeping the samples in autosampler at 10 °C for 24 h.

3. Results and discussion

3.1. Method development

During the early method development, several extraction procedures and test tube materials were tested. When modifications of chloroform-based liquid–liquid extraction methods described by Bligh and Dyer [12] and extraction with 1-butanol [14,16,24,29] were compared, the best extraction efficiency and precision were obtained using a modified Bligh and Dyer method (data not shown). The majority of lipophilic material from the tissue matrix was removed by a single liquid–liquid extraction step to the chloroform phase; the LPAs stayed in the water phase. During those experiments, we observed that LPA could be adsorbed easily onto the surface of test tubes, especially when plastic materials were used. The peak areas were approximately 35% lower when extractions were performed in polypropylene microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) compared to Pyrex® borosilicate glass tubes (SciLabware, Stone, Staffordshire, UK). Therefore all the following experiments were performed using glassware. In addition, a significant improvement was found in the precision and accuracy, especially in the case of 18:0 LPA, by washing glass test tubes with hydrochloric acid (6 M) between the experiments.

Several authors have used extraction procedures with strong acids to move the LPAs into the lower organic phase of the Bligh and Dyer extract [13–15,25]. This makes sample preparation laborious with a two-step extraction followed by separation and manipulation of chloroform extract. Moreover, one serious concern about acidification is the artificial formation of LPA by acid-catalyzed hydrolysis of more abundant lysophospholipids. In our hands, treatment of the sample with 0.6 M hydrochloric acid in the presence of 18:1 LPC increased the amount of 18:1 LPA during sample preparation significantly (data not shown). Overall, there were several reasons for choosing to analyze the water phase. First, sample preparation was straightforward and quick to perform. Second, without using any pH adjustment with acid, the artificial formation of LPA from LPC during the sample preparation could be avoided. Finally, the majority of LPC remained in the lower phase, being absent from analysis and MS ion source where the loss of choline from LPC has been reported [26]. Though the majority of LPCs were washed out into the chloroform during sample preparation, about 10% of LPC still remained in the water phase which makes us to conclude that a chromatography step has to be included in the method (Supplementary Figure 1). Glycerophosphocholines, such as LPC, are also reported to cause LC/MS/MS ion suppression during the analysis of biological samples [36].

Different reversed-phase columns and mobile phases were tested in order to obtain the best peak symmetry, selectivity, and resolution between LPAs and other lipid classes such as LPCs. Strongly retentive high resolution sub 2 μm stationary phases containing C8 and C18 were initially tested resulting in narrow and symmetric peaks. Unfortunately, the peaks started to broaden considerably after only a few injections and the column performance deteriorated, probably due to irreversible attachment of LPAs to the stationary phase. Similar results were obtained with bridged ethylsiloxane/silica hybrid technology reversed phase C8 and C18 columns (XBridge, Waters, Ireland). In reversed-phase columns, sample retention depends on three characteristics of the column: type and concentration of bonded phase and column surface area. Very hydrophobic analytes, like LPAs, are strongly retained, and in some cases their elution from a strong column (e.g. narrow bore columns with small internal volumes) may not be possible, even

Table 1
The linear range, calibration curve parameters with standard errors and regression coefficients of 16:0 LPA, 18:0 LPA, 18:1 LPA and 20:4 LPA ($n = 3$). Unweighted linear regression analysis was used to make the calibration curve. LLOQ was determined by calculating precision and accuracy for five LLOQ samples that were independent of the calibration curve. All the samples were prepared according to Sections 2.2 and 2.4.

Compound	Linear range (fmol)	Regression parameters		R^2	LLOQ (fmol)
		Slope ± S.T.D error	Intercept ± S.T.D error		
16:0 LPA	36–1790	1.0007 ± 0.0131	−0.0002 ± 0.0210	0.999	54
18:0 LPA	36–1790	0.6322 ± 0.0110	0.0117 ± 0.0156	0.999	54
18:1 LPA	36–1790	0.7878 ± 0.0158	−0.0177 ± 0.0253	0.999	54
20:4 LPA	36–1790	0.7355 ± 0.0171	−0.0151 ± 0.0273	0.998	54

with mobile phases containing high percentages of organic solvent. We achieved improved chromatographic performance with a C8 column with a larger particle size (3.5 μm) and pore size of 300 Å (Zorbax 300 SB-C8, Agilent Technologies, Palo Alto, CA, USA). In this case, the less strong wide pore column allowed the convenient elution of the sample without loss of column performance. However, retention times of LPAs and LPCs were overlapping with the mobile phase gradients in use.

Initially, the mobile phase consisted of acetate buffer and acetonitrile:isopropanol (5:2, v/v). During method development, several types of gradients were tested, but resolution between LPAs and LPCs could never be achieved. We found a wide pore reversed-phase column (300 Å) and methanol together with acetate buffer to sharpen chromatographic peaks and also give resolution between LPAs and LPCs. Different solvents are enriched on the surface of the bonded phase to different degrees. Among the commonly used reversed-phase organic solvents, methanol is adsorbed less than acetonitrile, making the system less retentive, and more suitable for LPA analysis. To further improve the resolution between LPAs and LPCs, we tested ion pairing reagents such as triethylamine (TEA) and N,N-dimethylhexylamine. Ion-pair chromatography provides an additional opportunity for achieving selectivity. Poor chromatographic peak symmetries were found with 10 mM TEA probably due to the simultaneous retention of LPAs by both reversed-phase and ion-pair processes. Instead, a high concentration (1%) of TEA was found to improve peak symmetries due to retention being determined by the ion-pairing properties, and it also increased the intensity of the MS signal. TEA has been reported to promote the formation of molecular ion and diminish the response of adduct ion [38]. In addition, it was further hypothesized that mobile phase additives with higher proton affinity would aid in formation of negative ions by extracting hydrogen ions in negative mode ESI [38]. A narrow bore column in favour of a wide pore reversed-phase column was chosen to our final ion-pair chromatography method since this further improved column efficiency and selectivity, and therefore also improved resolution between LPAs and LPCs.

It should be noted, however, that a high concentration of TEA may contaminate the instrument to some extent which could be seen especially in the subsequent measurements in the positive ion mode [39]. Therefore careful cleaning of the instrument is needed after the measurements and the use of project-specific capillaries is highly recommended. After the measurements we cleaned the instrument carefully with mixture of water and acetonitrile supplemented with formic acid (1%). Mass spectrometer spray chamber was rinsed carefully with a mixture of isopropanol and water. In addition, after the project the HPLC instrument was cleaned with a mixture of 50% methanol–potassium phosphate buffer (100 mM, pH 5). We also cleaned the capillary with a mixture of isopropanol and water and replaced the electrospray nebulizer needle and the LC filter element (5 μm). By these cleaning procedures, the residual TEA (*m/z* 102) was almost completely removed from the instrument and there was no effect (e.g. reduced sensitivity) of following measurements in the positive ion mode.

Mass spectrometric detection was performed using a highly selective MRM technique in the negative ion mode. Using full-scan MS experiments, the deprotonated molecular ions $[M-H]^-$ for 16:0 LPA, 18:0 LPA, 18:1 LPA, 20:4 LPA and IS (17:0 LPA) were found to be *m/z* 409, *m/z* 437, *m/z* 435, *m/z* 457, and *m/z* 423, respectively. A specific and sensitive assay was developed by monitoring transitions to the most intensive product ions. The following transitions were used: *m/z* 409 → 153 for 16:0 LPA, *m/z* 437 → 153 for 18:0 LPA, *m/z* 435 → 153 for 18:1 LPA, *m/z* 457 → 153 for 20:4 LPA, and *m/z* 423 → 153 for 17:0 LPA (Fig. 1), where an ion at *m/z* 153 is formed by loss of water from the ion at *m/z* 171.

3.2. Selectivity

In order to determine the selectivity of the method, the standards and tissue samples prepared with the sample preparation method were analyzed (Fig. 2). The solvents did not contribute any interfering peaks or background in any of the standard chromatograms. However, in the chromatograms of tissue samples, additional peaks were observed with the same parent-to-daughter ion transitions as the LPA species (Fig. 2), which has previously been reported by Shan et al. [13]. These peaks are likely due to loss of choline from the LPC molecule in the MS ion source [26].

When two concentrations of standards (0.2 and 0.4 μM) were added into the brain tissue homogenate and the slopes of the regression curves were compared to those prepared without tissue matrix, the slopes were found to be statistically equal for other LPA species, except for 18:0 LPA (Fig. 3). This was taken as evidence of the absence of significant matrix effect or interference induced by the brain matrix. In the case of 18:0 LPA, the absorption of the analyte on the surface of test tube may explain the results to some extent. When tissue samples were diluted 1:2, 1:5 and 1:10 with methanol before injecting into LC/MS/MS, the concentrations of diluted samples were found to be constant due to simultaneous dilution of internal standard. This further ensures our observation that there is no matrix effect or interference, which could cause systematic error to the results (data not shown). Furthermore, no significant ion suppression at the retention time of analytes was found in a post-column infusion study where remarkable ion suppression occurred at a retention time of 0.5 min but this was normalized by the retention time of 3.0 min (data not shown). In-source fragmentation of 18:1 LPC gave transition of *m/z* 281 → 281 (Fig. 1) which was used to optimize the resolution between LPA and other lipids like ion suppression causing glycerophosphocholines [36].

3.3. Linearity, precision, accuracy, and recovery

The six point calibration curves were highly linear over the range of 36–1790 fmol of LPA injected into column (0.02–1.0 μM). The calibration curve parameters with standard errors and regression coefficients are summarized in Table 1. The LLOQ with acceptable accuracy (±10%) and precision (≤15% RSD) was 54 fmol of LPA injected into the column. Previously, the detec-

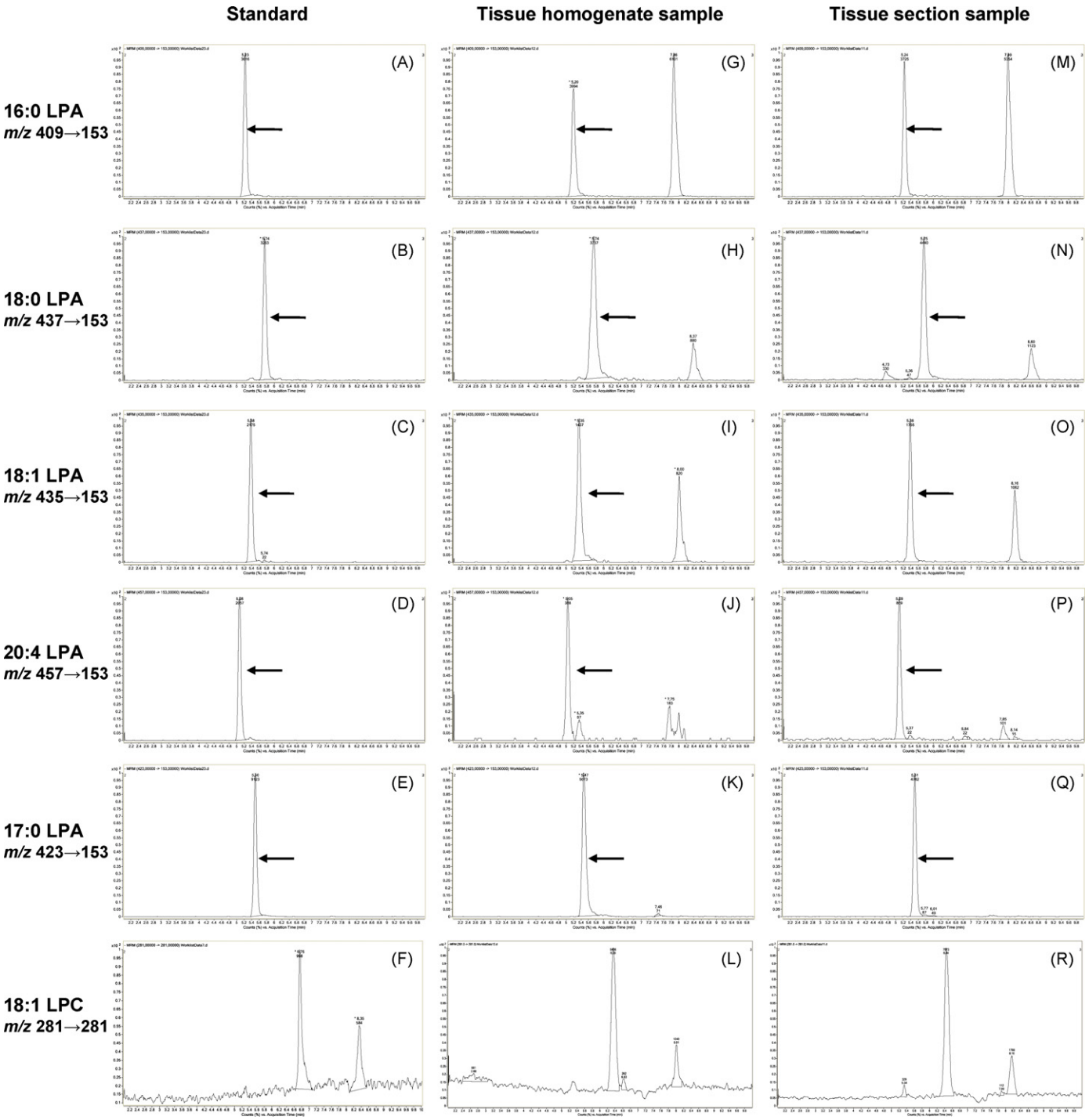

Fig. 2. Representative MRM chromatograms of (A) standard sample of 16:0 LPA (0.1 μM, retention time (RT) 5.23 min), (B) 18:0 LPA (0.1 μM, RT 5.74 min), (C) 18:1 LPA (0.1 μM, RT 5.38 min), (D) 20:4 LPA (0.1 μM, RT 5.08 min), and (E) internal standard (IS) 17:0 LPA (0.3 μM RT 5.50 min). Endogenous LPA content was measured from a rat brain homogenate and brain cryosections. Representative MRM chromatograms of a rat brain homogenate of (G) 16:0 LPA (0.17 μM), (H) 18:0 LPA (0.20 μM), (I) 18:1 LPA (0.11 μM), (J) 20:4 LPA (0.04 μM), and (K) IS (0.3 μM). Representative MRM chromatograms of a rat brain cryosections of (M) 16:0 LPA (0.17 μM), (N) 18:0 LPA (0.19 μM), (O) 18:1 LPA (0.16 μM), (P) 20:4 LPA (0.08 μM), and (Q) IS (0.3 μM). Additional peaks on MRM channels of LPAs in brain homogenate and cryosections were due to artificial formation of LPAs from LPCs in MS ionization chamber [26]. The transition *m/z* 281 → 281 monitored 18:1 fatty acid anion and it was used to optimize the resolution between LPA and ion suppression causing lipids [36]. Representative MRM chromatograms of (F) 18:1 LPC standard (8 μM, RT 6.75 min and 8.35 min), (L) tissue homogenate, and (R) tissue section. All the samples (A–R) have been prepared according to Section 2.4. In the chromatograms the individual LPAs are indicated with the arrow.

tion limits for LPA species achieved with LC/MS/MS analysis have been in the range of 0.01–0.03 μM (200–600 fmol/injection) [10] whereas with flow injection, a detection limit as low as of 0.3–1.0 nM (6–20 fmol/injection) [25] has been reported. However, only Shan et al. [13] have reported a quantification limit for LC/MS/MS method (160–500 fmol/injection). With radioenzymatic assay the detection limit of 0.2 pmol has been reported [19].

The precision and accuracy of all QC samples were within the acceptable range (Table 2). The method was accurate and precise between runs and within individual runs at each QC level for all the LPAs. The method was accurate and precise at all studied levels also

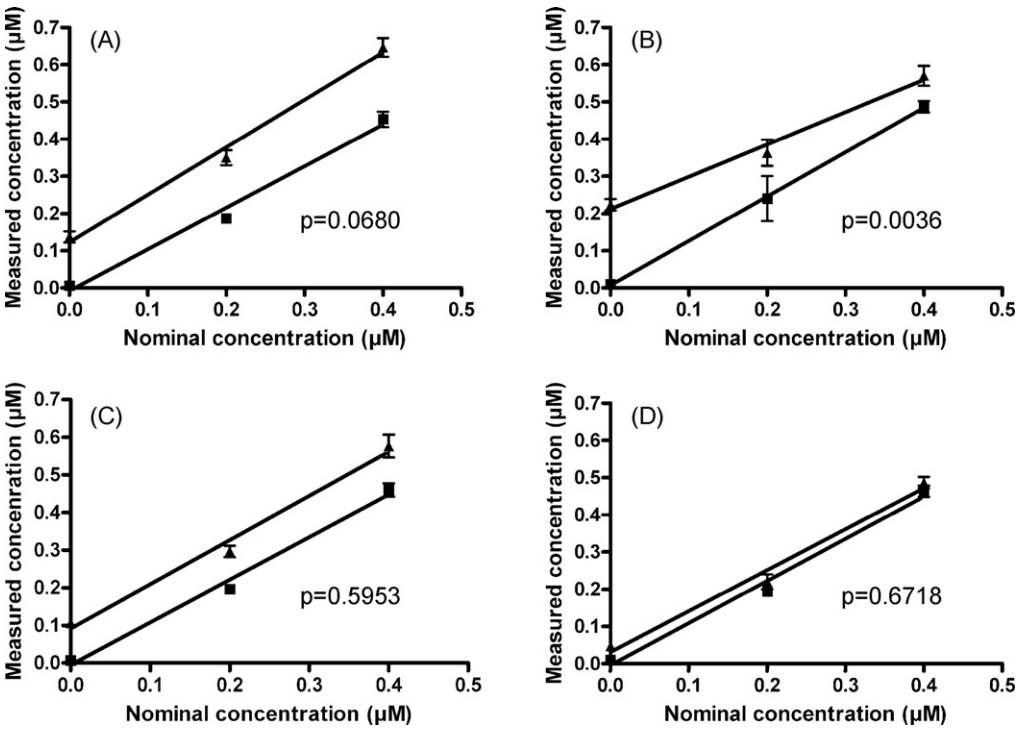

Fig. 3. The matrix effect was studied with the standard addition method. Two concentrations of standards (0.2 and 0.4 μM) were spiked into the brain tissue homogenate to obtain a regression curve (triangles). Standard curve using the same concentrations was prepared without tissue matrix (squares). The standard addition method shows no matrix effect on 16:0 LPA (A), 18:1 LPA (C), and 20:4 LPA (D). Statistically significant difference was found between slopes of homogenate and reference standards of 18:0 LPA (B). This matrix effect is probably due to the adsorption of the analyte on the surface of the test tube. All the samples were prepared according to Section 2.4. The statistical equality of the slopes ($p < 0.05$ considered as statistically significant difference) was determined using analysis of covariance (ANCOVA) with GraphPad Prism® 4.03 software (mean ± SD, $n = 3$).

when QC samples were determined in the presence of the surrogate sample matrix (4% BSA, m/V). No statistically significant differences (one-way ANOVA with Tukey's multiple comparison *post hoc* test with $p < 0.05$ considered as statistically significant) were found between results of QC samples with or without surrogate matrix at any concentration level (data not shown). The recoveries for 16:0 LPA, 18:0 LPA, 18:1 LPA and 20:4 LPA were found to be 104%, 69%, 92%, and 86%, respectively.

3.4. Stability

There was no significant degradation of LPAs after three freeze-thaw cycles (24 h interval between the cycles) in comparison with freshly prepared samples (−10 to +17% for other LPAs and +37% for 18:0 LPA). The short-term temperature stability showed no degradation of LPAs but, instead, increased levels of LPAs 24–101% after 4 h storage at +20 °C. The increasing amount of LPAs after storage

Table 2
Intra-day and inter-day precision and accuracy for 16:0 LPA, 18:0 LPA, 18:1 LPA, and 20:4 LPA and their nominal values at each QC level. All the samples were prepared according to Sections 2.2 and 2.4.

Compound	Nominal conc. (μM)	Intra-day precision and accuracy			Inter-day precision	
		Mean ($n = 5$) (μM)	RSD (%)	Mean accuracy (%)	Mean ($n = 3$ days) (μM)	RSD (%)
16:0 LPA	0.03	0.03	5.0	92	0.03	22
	0.1	0.1	3.6	85	0.1	11
	0.3	0.3	2.3	90	0.3	5.4
	0.8	0.8	1.6	94	0.8	5.2
18:0 LPA	0.03	0.03	14	104	0.03	33
	0.1	0.1	2.8	103	0.1	10
	0.3	0.3	2.4	114	0.3	12
	0.8	1.0	1.2	120	0.9	9.4
18:1 LPA	0.03	0.03	6.5	108	0.03	17
	0.1	0.1	4.0	89	0.1	12
	0.3	0.3	3.0	89	0.3	9.1
	0.8	0.7	1.2	92	0.8	6.9
20:4 LPA	0.03	0.03	4.5	100	0.03	17
	0.1	0.1	2.1	83	0.1	11
	0.3	0.3	3.5	85	0.3	11
	0.8	0.7	1.3	85	0.8	11

at +20 °C has been previously described [16]. LPAs may have been formed enzymatically in the brain tissue when stored at +20 °C before the enzymatic machinery is switched off by addition of methanol and chloroform into the sample. The LPA concentrations in 30 and 60 days of long-term stability samples were within the range of −17 to +10% for other LPAs but up to −42% for 18:0 LPA. The overall divergent results of 18:0 LPA are likely to be due to adsorption of the analyte onto the surface of test tubes. The stock solutions of LPAs in methanol were stable for 30 days when stored at −20 °C and kept at room temperature for 6 h after thawing. Post-preparative stability samples were found to be stable when they were stored for 24 h at 10 °C in the autosampler.

3.5. Application

In preliminary experiments with rat brain cryosections, the following endogenous LPA levels were found (nmol/g protein in tissue, means ± SD of three replicate samples from two individual animals, $n = 6$): 3.2 ± 0.4 for 16:0 LPA, 5.8 ± 1.8 for 18:0 LPA, 2.6 ± 0.6 for 18:1 LPA, and 1.2 ± 0.4 for 20:4 LPA. According to GC analysis [21], the four LPA species followed in our LC/MS/MS method account for 93% of LPA species in rat brain. Previously, in the GC analysis [21] 18:1 LPA was found to be the predominant LPA species in rat brain homogenate. Based on our findings, 18:0 LPA seems to predominate; this may be due to differences in the method and the age of the rats used (we used young 4-week-old rats). In preliminary experiments, the concentrations of LPA species were above the LLOQ of the method and all the measured concentrations were within the range of the method.

The LC/MS/MS method described here was specifically designed for the purpose of comparative studies of the LPA content of pre-treated rat brain sections under conditions closely mimicking those of functional autoradiography where the neuroanatomical localization of LPA receptor signaling can be studied [30–32]. In future experiments, rat brain sections will be first incubated with buffer solution including chemicals of interest, and the results will be used to supplement a larger series of studies concerning the regulation of enzymatic pathways involved in LPA production and degradation.

4. Conclusions

A highly selective and sensitive method using LC/MS/MS was developed for the determination of LPA species in rat brain cryosections. As far as we are aware, this is the first report where the LPA content of brain tissue has been measured using LC/MS/MS. From the analytical point of view, LPAs are a demanding group of compounds since, for example, artificial formation of LPA from LPCs has been demonstrated during sample preparation and within the instrumentation. To prevent the artificial formation of LPA, we used a single step extraction procedure without any strong acid treatment to remove lipophilic material from the water phase, which was analyzed. This sample pre-treatment also removed most of the lipids causing the matrix effect and the method was found to be highly selective. Since residual LPC remained in the water phase after sample preparation, additional peaks were observed at the same parent-to-daughter ion transitions as the LPA species. Therefore, we developed a chromatographic method based on a narrow bore reversed-phase column and ion-pair technique to optimize the efficiency of the column and to achieve resolution between LPAs and LPCs. Mass spectrometric detection was performed using a highly selective MRM technique in the negative ion mode. The method was validated and acceptable accuracy, precision, recovery, and stability were obtained for concentrations within the range of calibration curve for all of the studied LPAs. According to the validation results and data from the preliminary study, we conclude that the LC/MS/MS method described in this paper is applicable for the targeted quantitative analysis of LPA species in rat brain sections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.03.030.

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