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Electrospray Ionization Mass Spectrometric Method for the Determination of Cannabinoid Precursors: *N*-Acylethanolamine Phospholipids (NAPEs)†

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***N*-Acylethanolamine phospholipids (NAPEs) serve as endogenous precursors of *N*-acylethanolamines (NAEs), e.g. *N*-arachidonoylethanolamine (anandamide) and *N*-palmitoylethanolamine that are endogenous ligands of cannabinoid receptors. Under physiological conditions, NAPE is found in very low concentrations in mammalian tissue (3–12 nmol g^{-1}). However, pathophysiological conditions may increase the endogenous NAPE levels, which again may cause an increase in endocannabinoid concentrations. This paper presents a simple and selective method for the determination of NAPE standards using negative electrospray ionization mass spectrometry (ESI-MS). The procedure provides complete positioning of all acyl and alkenyl groups contained in each NAPE species. The calibration curve for standard NAPE was linear over the range 100 fmol–50 pmol (0.1–50 ng) per injection. The lower limit of detection (signal-to-noise ratio of 3) was 100 fmol, implying that this method is superior to previous methods for the determination of NAPE. These results suggest that this ESI-MS method can be used to identify and quantify NAPE species in mammalian tissues and provide information on the corresponding NAEs to be released from the endogenous NAPE pool.**

KEYWORDS: electrospray ionization mass spectrometry; *N*-acylphosphatidylethanolamine; *N*-acylethanolamine; plasmalogen; anandamide

INTRODUCTION

N-Acylethanolamines (NAEs) comprise a novel group of signaling molecules of lipid nature. *N*-Arachidonoylethanolamine (anandamide) was described as the first endogenous cannabimimetic substance in mammalian tissue, exhibiting high affinity for cannabinoid receptors.[1] The binding to cannabinoid receptors produces a wide range of *in vitro* and *in vivo* pharmacological effects comparable to those of other psychotropic cannabinoid compounds.[2–6] Additionally, other *N*-acylethanolamines have been suggested to exert their effects on cannabinoid receptors, in particular *N*-palmitoylethanolamine.[7–9]

At present, little is known about the physiological relevance of these compounds and their impact on pathophysiological conditions remains to be elucidated. Therefore, in order to gain further insight into the homeostasis of the endocannabinoid system, it is crucial to determine the regulation of endogenous NAE levels. However, the metabolic regulation of the endogenous levels of these compounds is poorly understood. A proposed mechanism for cellular synthesis of NAE is that the *N*-acylethanolamine moiety is released from a corresponding phospholipid precursor, an *N*-acylethanolamine phospholipid (NAPE), through the action of a specific calcium-independent phospholipase D (PLD), as first reported by Schmid and coworkers.[10–12] These results were later confirmed by others.[13–18] This PLD subtype shows no substrate specificity between the different *N*-acyl groups of NAPE species, suggesting that the synthesis of *N*-arachidonoylethanolamine phospholipids by a calcium-dependent *N*-acyltransferase could be the rate-limiting step of anandamide production.[19–21] This enzyme catalyzes a transferase reaction between the *sn*-1 acyl-position of a donor phospholipid and the primary amino group of ethanolamine phospholipids. Consequently, the composition of *O*-acyl, alkenyl and alkyl groups in NAPE are identical with those found in ethanolamine phospholipids from the same tissue.[22] Hence mammalian NAPE is a mixture of *N*-acyl-1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (diacyl-NAPE), *N*-acyl-1-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (alkenylacyl-

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NAPE) and *N*-acyl-1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (alkylacyl-NAPE), with a proportional distribution in mammalian brain tissue of approximately 60, 35 and 5%, respectively.[23–25]

Compared with other phospholipids, the physiological tissue levels of these unusual phospholipids are very low. So far, only two groups have reported the presence of NAPE in mammalian brain tissue under physiological conditions.[16,17] Determining the content of *N*-acylethanolamines after PLD treatment of a purified NAPE fraction, concentrations of NAPE were reported to range from 3 to 12 nmol g^{-1} tissue, corresponding to about 0.1% of the total phospholipid content. Also, the authors reported that the fraction of NAPE species containing an *N*-arachidonoyl moiety accounts for <1% of the total NAPE pool, thereby making the physiological concentration of the anandamide precursor extremely low. The finding of these low brain levels of anandamide is also in accordance with a study using an isotope dilution mass spectrometric method.[26] In contrast, earlier studies using less sensitive methods of quantification failed to find NAPE in lipid extracts from normal brain tissue.[10,23,24] Consequently, to determine delicate changes of *in vivo* NAPE metabolism it is necessary to employ a very sensitive analytical method. Mass spectrometry has proven to be efficient for monitoring biological molecules at very low concentrations, and several mass spectrometric techniques are used extensively for phospholipid determination, in particular electrospray ionization mass spectrometry (ESI-MS), which facilitates the detection of mammalian phospholipids in the picomolar range.[27–30] However, mass spectrometry has only been applied to determine NAPE by one research group, using negative fast atom bombardment ionization mass spectrometry for the identification of plant-derived NAPE species.[31,32]

In this paper, we present a method for the determination of NAPEs in the femtomolar concentration range using negative polarity ion trap ESI-MS. Also, this technique facilitates identification of the position of all acyl groups in standard diacyl-NAPE and alkenylacyl-NAPE samples, suggesting that it will be efficient for the determination of endogenous levels of NAPEs, including the subpopulation of endogenous cannabinoid precursors.

EXPERIMENTAL

Materials

1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine, arachidonic acid, heptadecanoyl anhydride, lauric anhydride, triethylamine, *N*,*N*′-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), ethyl formate, 2,7-dichlorofluorescein, rhodamine B and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical (St Louis, MO, USA). *N*-Palmitoyl-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine was purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1-*O*-1′-Alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (bovine brain ethanolamine plasmalogen, 90% purity) was purchased from Doosan-Serdary Research Laboratories (Englewood Cliffs, NJ, USA). Sodium hydroxide pellets, analytical-grade chloroform, analytical-grade methanol, analytical-grade dichloromethane, molecular sieve beads and thin-layer chromatographic (TLC) plates (20 × 20 cm, silica gel 60, 0.25 mm thick) were purchased from Merck (Darmstadt, Germany) MS-grade methanol (methanol 205) was purchased from Romil (Cambridge, UK).

NAPE synthesis

N-Arachidonoyl-1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (APL-NAPE) was synthesized using the method of Klausner and Bodansky.[33] 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (33 μmol) and arachidonic acid (33 μmol) were dissolved in 5 ml of dichloromethane in a screw-capped tube and kept at 4 C for 30 min. The reaction was then initiated by adding DCC (33 μmol) and incubation was continued for 4 h at room temperature with continuous stirring. The sample was filtered through glass fiber and the filtrate was evaporated under a stream of nitrogen. The residue was dissolved in chloroform–methanol (90 : 10, v/v). The synthesized APL-NAPE standard was purified by silica gel TLC using a solvent system of chloroform–methanol–ammonia solution (80 : 20 : 2, v/v). A commercially available NAPE standard (*N*-palmitoyl-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) was used as a reference compound, as all NAPEs have similar R_f values. After development, separate lanes with the reference standard were sprayed with 0.2% ethanolic 2,7-dichlorofluorescein (with 0.001% rhodamine B) and the spots were visualized under ultraviolet light (365 nm). The band of silica corresponding to the R_f of the reference standard was scraped into a screw-capped tube with 5 ml of chloroform–methanol (1 : 2) and centrifuged at 2440 *g* at 4 °C for 15 min (IEC Centra GP8R centrifuge). After centrifugation, the supernatant was filtered through glass fiber and the silica was re-extracted with an additional 3 ml of the same solvent. The extract was dried gently with a stream of nitrogen, the residue was dissolved in chloroform–methanol (90 : 10, with 0.02% BHT added) at a concentration of 1 mg ml^{-1} and the solution was stored at −20 °C until used.

N-Heptadecanoyl-1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (HPL-NAPE) was prepared using the method by Shangguan *et al.*[34] Briefly, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (7 μmol) was dissolved in 2 ml of chloroform in a screw-capped tube and stirred for 24 h with heptadecanoic anhydride (14 μmol) and triethylamine (77 μmol). The solvent was evaporated to dryness under a stream of nitrogen, the residue was dissolved in chloroform–methanol (90 : 10) and HPL-NAPE was subsequently purified by TLC as above.

In order to obtain an alkenylacyl-NAPE standard with known acyl group composition, *N*-heptadecanoyl-1-*O*-1′-alkenyl-2-lauroyl-*sn*-glycero-3-phosphoethanolamine was semi-synthesized and purified following a three-step procedure, as follows. (i) Bovine brain ethanolamine plasmalogen was *N*-acylated with heptadecanoic anhydride using the method of Shangguan *et al.*[34] and purified by TLC, as described above. (ii) The *sn*-2 position of NAPE was deacylated by mild alkaline hydrolysis using a slightly modified procedure of Somerharju and Renkonen.[35] In

brief, the NAPE product was dissolved in 1 ml of chloroform, 170 μl of methanolic 0.35 M NaOH were added and hydrolysis was performed for 3 h with continuous stirring at room temperature. Then, 100 μl of ethyl formate were added and incubation was continued for 10 min. The incubate was blown to dryness under a stream of nitrogen and the lipids were extracted according to Bligh and Dyer.[36] The lyso-NAPE product was purified by TLC (see above), scraping off the band of silica corresponding to about half the R_f value of the NAPE reference.[11] (iii) Thereafter, lyso-NAPE was acylated at the *sn*-2 position using lauric anhydride according the method by Han *et al*.[37] In brief, lyso-NAPE (10 μmol) and lauric anhydride (10 μmol) were evaporated to dryness under a stream of nitrogen in a screw-capped reaction vial, and the residue was subsequently dissolved in 1 ml of water-free chloroform (chloroform dried using water-absorptive molecular sieve beads). The reaction was initiated by addition of DMAP (12 μmol) and incubation was performed for 24 h with continuous stirring at room temperature. The sample was evaporated to dryness under a stream of nitrogen and the lipids were extracted according to Bligh and Dyer.[36] The semi-synthetic alkenylacyl-NAPE standard was then purified by TLC as above. Before each incubation procedure, a stream of nitrogen was blown over the reaction mixture and the screw-capped test-tube was covered with aluminum foil to minimize exposure to light.

Electrospray ionization mass spectrometry

Aliquots of NAPE standards were dried gently under a stream of nitrogen and the residue was dissolved in MS-grade methanol (with 0.02% BHT added). Addition of BHT did not interfere with the ESI-MS procedure. All experiments were performed using a quadrupole ion-trap LCQ mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an ESI ionization interface. Negative ion polarity was applied. A 32-gauge hypodermic metal needle with blunt tip was inserted in the ESI guide needle and applied a spray voltage and current of −3.75 kV and 0.14 μA. The metal tip exceeded the ESI guide needle by ∼1 mm. The capillary was heated to 230 °C and −16 V were applied. The sheath and auxiliary nitrogen gas flow-rates were set to 50% and 10% (arbitrary units), respectively. Application of the hypodermic metal needle increased the signal intensity by at least twofold and further stabilized baseline interference, as compared with a 0.1 mm i.d. fused-silica electrospray capillary (Polymicro Technologies, Phoenix, AZ, USA) (results not shown). To optimize the sensitivity, the capillary/tube lens voltage and ion optics were tuned before analysis. The mass spectrometer was operated in the full-scan mode. Operating the instrument in the single-ion monitoring mode did not further improve the signal-to-noise ratio (results not shown). Spectra were collected and analyzed using on-line system software (Navigator v. 1.1, Finnigan MAT, San Jose, CA, USA). Spectra were acquired with a repetitive scan cycle time of 1.30 s and averaged after 60 s of acquisition time.

Concentration–response calibration curves for diacyl-NAPE were obtained by injecting samples ranging from 5 pg/μl to 10 ng/μl. Four consecutive injections of each concentration were performed. The sample was directed to the electrospray source using the flow-injection analysis principle, i.e. the sample was injected via a 20 μl loop into an infusion flow of methanol–chloroform–redistilled water (60 : 35 : 5, v/v) at a flow-rate of 200 μl min^{-1}. Precursor ions were monitored in single-stage MS mode in the *m/z* interval 950–1150 and the response was calculated as integrated peak areas of precursor ion base peaks.

Structural elucidation of precursor ions of diacyl-NAPE and alkenylacyl-NAPE was accomplished by fragmentation of precursor ions in the ESI-MS/MS mode using collision-induced dissociation (CID) performed in the ion-trap (mass analyzer) region. The procedure was followed by MS/MS/MS of product ions of interest. Ions were monitored in the *m/z* interval 100–1100 using on-line advanced setting applications in the Navigator software. The precursor ion fragmentation efficiency was optimized with parameters set to approximately activation amplitude 1.50 V and activation *Q* value 0.190 (arbitrary units). CID experiments were performed with helium as collision and damping gas, using a 2 or 3 *m/z* window about the precursor ion to facilitate maximum precursor ion transmission. During the CID procedure, NAPE samples of 5 or 10 ng μl^{-1} were infused directly to the electrospray source via a 0.1 mm i.d. fused-silica transfer line (Polymicro Technologies) using an LCQ-integrated micropump device delivering the sample in a 250 μl syringe (Unimetrics, Shorewood, IL, USA) in a volume of 10 μl min^{-1}.

RESULTS AND DISCUSSION

The NAPE and NAE concentrations in mammalian cells are increased following induction of cell injury, e.g. caused by ischemia and glutamate-induced excitotoxicity, suggesting that these lipids may have pathophysiological roles.[20,22,38] Of special interest is the *N*-arachidonoylphosphoethanolamine subpopulation of the endogenous NAPE pool, since it is the precursor of anandamide. Hence, to establish a principal MS/MS fragmentation pattern employing the ESI-MS technique we synthesized an *N*-arachidonoyl-containing NAPE species. Additionally, we semi-synthesized homogenous alkenylacyl-NAPE species from a source of purified bovine brain ethanolamine plasmalogens.

ESI-MS of diacyl-NAPE

The choice of ion polarity sets the first level of selectivity in phospholipid analysis by ESI-MS, since the possible charge of diacyl-NAPE in solution determines the optimal ion polarity. Accordingly, the negative ion spectrum shows the abundant ion at *m/z* 1001.3 of APL-NAPE (M_r 1001.5), corresponding to deprotonation of the phosphate group, assigned as the anionic $[M\text{-}H]^-$ ion (see Fig. 1(A)). Additionally, HPL-NAPE (M_r 967.5) also appeared as an $[M\text{-}H]^-$ ion of *m/z* 967.4 (see Fig. 1(B)). Using the flow-injection analysis approach, the concentration–response calibration curve was established with a detection limit of 100 fmol (0.1 ng) of APL-NAPE and a linear relationship ranging from 0.1 to 50 ng ($r^2 = 0.99$) with a very low intravariation of response (mean ± standard deviation) (see Fig. 2). Injection of more than 50 ng resulted in a gradually saturated response. For comparison,

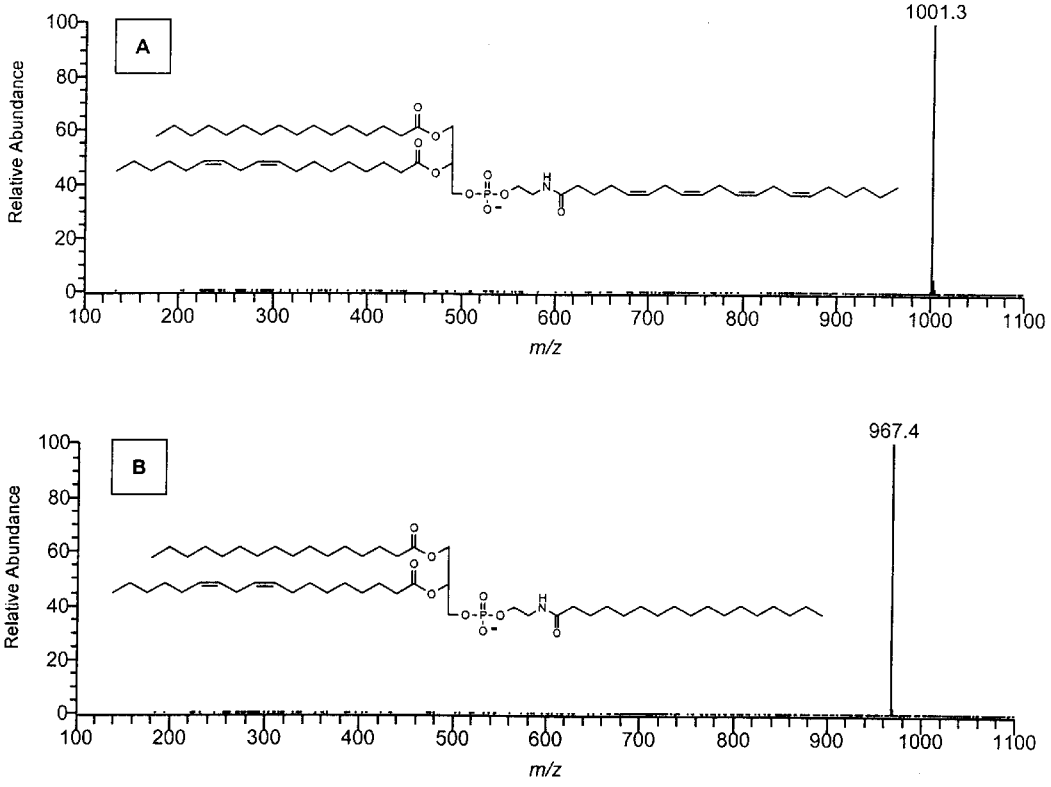

Figure 1. Negative ion ESI-mass spectrum (full scale *m/z* 100–1100) of *N*-arachidonoyl-1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (APL-NAPE, expected *m/z* 1001.5, (A) and *N*-heptadecanoyl-1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (HPL-NAPE, expected *m/z* 967.5, (B). The structures are shown.

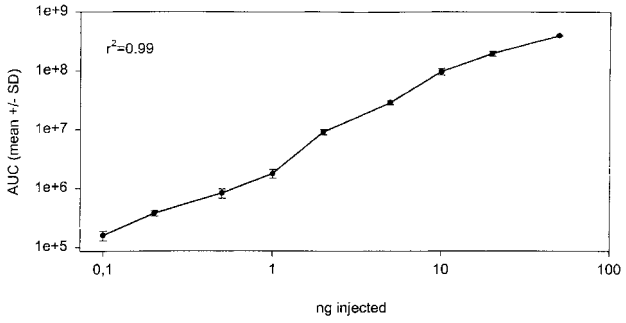

Figure 2. Concentration–response calibration curve for *N*-arachidonoyl-1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (APL-NAPE).

diacyl-NAPE standards were also subjected to negative ion atmospheric pressure chemical ionization mass spectrometry on the same instrument. However, as judged by the signal-to-noise ratio, optimization of this ionization technique was slightly less sensitive compared with ESI-MS (results not shown).

ESI-MS/MS of diacyl-NAPE

A set of eight fragment ions appeared on applying CID to diacyl-NAPE species, as illustrated by the fragmentation of APL-NAPE and HPL-NAPE (Fig. 3); see also Fig. 4(A) for a proposed fragmentation model. Fragments of *m/z* 255 and 279 represents the loss of fatty acid carboxylate anions from the *sn*-1 (palmitate) and *sn*-2 positions (linoleate). The *sn*-2 carboxylate anion was more abundant that the *sn*-1 carboxylate anion, thereby facilitating the positioning of individual acyl groups along the glycerol backbone of diacyl-NAPE. Absolute identification of all other product ions, however, was only possible on performing additional CID on the product ions, i.e. ESI-MS/MS/MS. Interpretation of these spectra further confirmed the MS/MS fragmentation pattern of diacyl-NAPEs (results not shown). As illustrated by APL-NAPE, fragment ions of *m/z* 720.6 indicate the neutral loss of 280 (linoleic acid) and *m/z* 744.4 represents neutral loss of 256 (palmitic acid). Further, the fragment ion at *m/z* 738.5 corresponds to neutral loss of 262 (= dehydrate of linoleic acid), and the fragment ion at *m/z* 762.5 indicates neutral loss of 238 (= dehydrate of palmitic acid). This principal diacyl-NAPE fragmentation pattern was comparable to results obtained by Chapman and co-workers using negative fast atom bombardment mass spectrometry.[31,32] These authors identified a simple fraction of plant-derived diacyl-NAPE species, but the method was of limited applicability owing to a low mass resolution efficiency.[31,32] Also, the principal diacyl-NAPE fragmentation pattern, indicating the dissociation of acyl groups located at the glycerophospho backbone, was in accordance with previously published data from more complex ESI-MS experiments performed on 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine.[27,30,39–41]

Specifically for NAPEs, neutral loss of a fatty acid from the glycerophospho backbone is followed by further fragmentation of the phosphodiester bond, thereby leading to neutral loss of the *N*-arachidonoylethanolamine moiety. Characteristic for this process is the appearance of two fragment ions of *m/z* 391.3 (neutral loss of linoleic

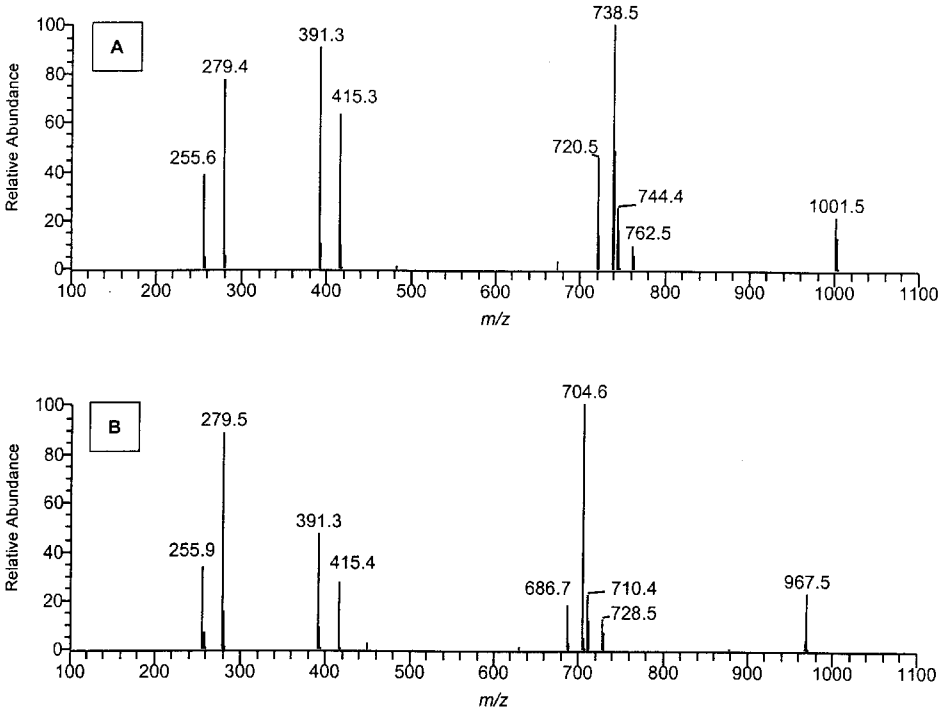

Figure 3. Negative ion ESI tandem mass spectrum of *N*-arachidonoyl-1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (APL-NAPE, (A)) and *N*-heptadecanoyl-1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (HPL-NAPE, (B)), showing a set of eight product ions. See Fig. 4(A) for a proposed fragmentation model of APL-NAPE.

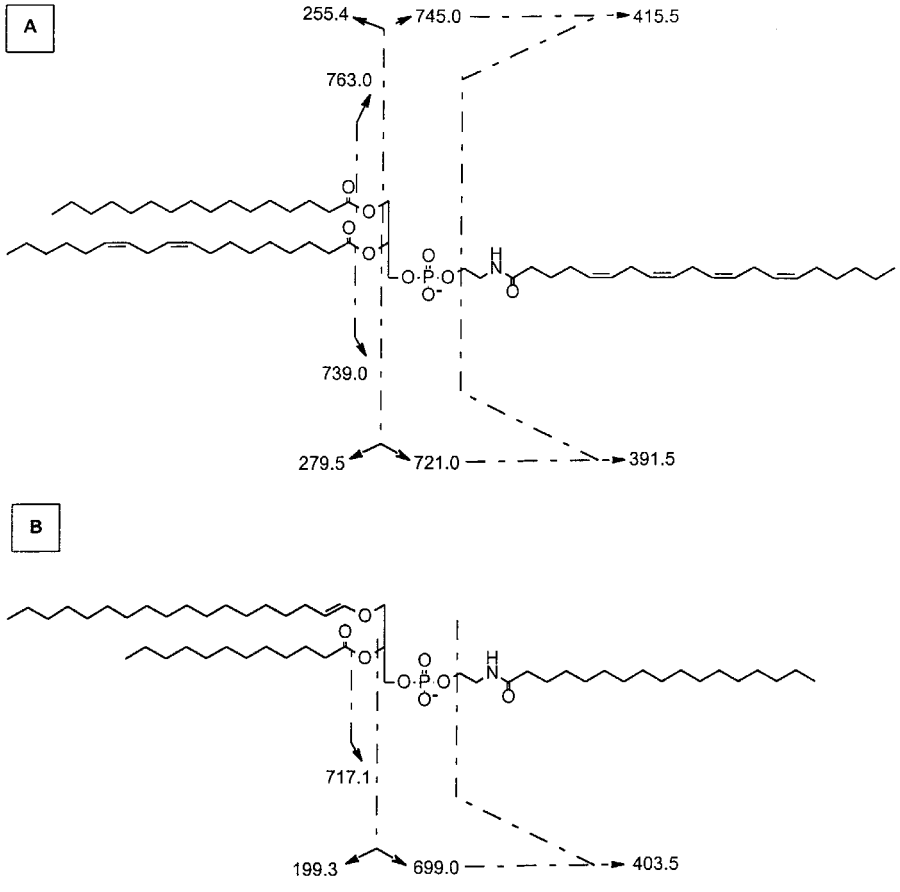

Figure 4. Proposed fragmentation pattern of diacyl-NAPE, illustrated by *N*-arachidonoyl-1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (APL-NAPE, (A)), and alkenylacyl-NAPE, illustrated by *N*-heptadecanoyl-1-*O*-octadec-1′-enyl-2-lauroyl-*sn*-glycero-3-phosphoethanolamine (B). The *m*/*z* values refer to the expected *m*/*z*.

acid from the *sn*-2 position) and *m/z* at 415.3 (neutral loss of palmitic acid from the *sn*-1 position), the product ion at *m/z* 391.3 being more abundant due to the MS/MS regiospecificity of the *sn*-2 acyl moiety in asymmetric diacyl-NAPEs. Consequently, the identity of the acyl group in the *N*-acyl moiety of diacyl-NAPE species can be deduced from the tandem mass spectrum by subtracting the *m/z* values of the two carboxylate anions from the *m/z* value of the precursor ion, followed by subtraction of the molecular mass of the glycerophosphoethanolamine backbone ($C_5H_{10}NO_4P$, M_r 179.0). This can be illustrated by the tandem-mass spectrum of APL-NAPE (M_r 1001.5), containing an arachidonoyl group ($C_{20}H_{31}O$, M_r 287.5) at the *N*-acyl position: 1001.5 (precursor ion) −279.5 (linoleate) −255.5 (palmitate) −179.0 (glycerophosphoethanolamine backbone) = 287.5.

ESI-MS/MS of alkenylacyl-NAPE

Using bovine brain ethanolamine plasmalogens for the semi-synthesis of a homologous alkenylacyl-NAPE standard with known acyl group composition, it is expected that only a few abundant ions would be present in the ESI mass spectrum. This is due to the fact that the 1-*O*-octadec-1′-enyl and 1-*O*-hexadec-1′-enyl chains are found in approximately 50% and 25% of all brain ethanolamine plasmalogens, respectively.[27,39,41–43] Accordingly, the ESI mass spectrum of *N*-heptadecanoyl-1-*O*-1′-alkenyl-2-lauroyl-*sn*-glycero-3-phosphoethanolamine shows two abundant ions, at *m/z* 899.5 and 871.6 (Fig. 5(A)). Following CID on the ion of *m/z* 899.5, four product ions appeared, with laurate at *m/z* 199.3 (Fig. 5(B)); see also Fig. 4(B) for a proposed fragmentation model. Performing MS/MS/MS on ions of *m/z* 403.4, 698.6 and 717.8 confirmed the interpretation of the ESI tandem mass spectrum (results not shown), i.e. the product ions correspond to dissociation of laurate and neutral loss of the *N*-heptadecanoylethanolamine moiety (*m/z* 403.4), *N*-heptadecanoyl-1-*O*-octadec-1′-enyl-2-dehydro-*sn*-glycero-3-phosphoethanolamine (*m/z* 698.6) and *N*-heptadecanoyl-1-*O*-octadec-1′-enyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine. Consequently, this alkenylacyl-NAPE specie was identified as *N*-heptadecanoyl-1-*O*-octadec-1′-enyl-2-lauroyl-*sn*-glycero-3-phosphoethanolamine (M_r 899.4); see the molecular structure in Fig. 5(A). Additionally, the precursor ion at *m/z* 871.6 represents *N*-heptadecanoyl-1-*O*-hexadec-1′-enyl-2-lauroyl-*sn*-glycero-3-phosphoethanolamine (M_r 871.3, results not shown). It then appears that the alkenyl group in alkenylacyl-NAPE is not subjected to CID fragmentation.

This principal difference between diacyl-NAPE and alkenylacyl-NAPE is in accordance with previously published data from ESI-MS experiments performed on 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine and 1-*O*-1′-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine species.[27,30,39–41] This indicates that alkenylacyl-NAPEs can be identified by the absence of a carboxylate anion from the *sn*-1 position in the ESI tandem mass spectra.

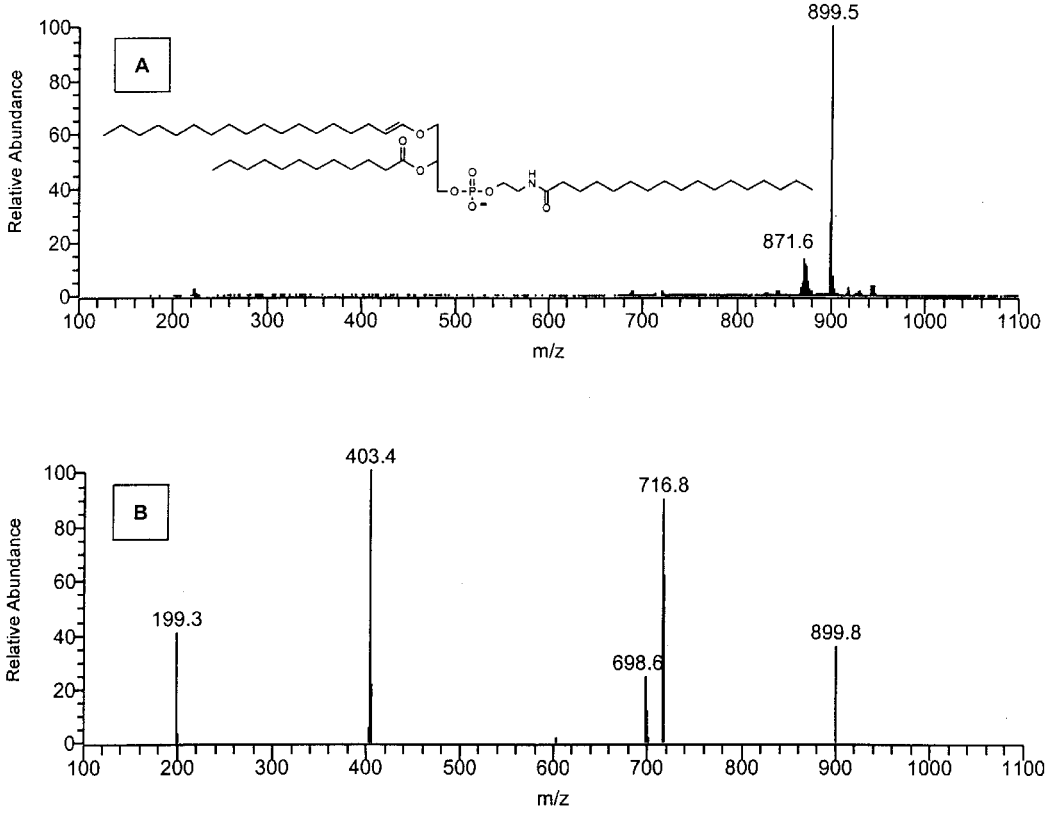

Figure 5. Negative ion ESI-mass spectrum (full scale *m/z* 100–1100) of semi-synthetic alkenylacyl-NAPE species (*N*-heptadecanoyl-1-*O*-1′-alkenyl-2-lauroyl-*sn*-glycero-3-phosphoethanolamine). The structure of *N*-heptadecanoyl-1-*O*-octadec-1′-enyl-2-lauroyl-*sn*-glycero-3-phosphoethanolamine is shown (B). Negative ion ESI tandem mass spectrum of a single alkenylacyl-NAPE standard species, *N*-heptadecanoyl-1-*O*-octadec-1′-enyl-2-lauroyl-*sn*-glycero-3-phosphoethanolamine, showing a set of four product ions. See Fig. 4(B) for a proposed fragmentation model.

Since 1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamines also shows no fragmentation of the *sn*-1 position upon ESI-MS/MS,[27,40] it suggests that alkylacyl-NAPEs can also be identified by this method.

In general, using ESI-MS/MS the characteristic principal fragmentation pattern of NAPEs is similar to that found in ethanolamine glycerophospholipids, showing carboxylate anions after deacylation of the glycerophosphoethanolamine backbone and the corresponding fragments containing the deprotonated phosphorous group. A principal difference, however, is the higher molecular mass of the NAPE precursor ions due to *N*-acylation of the primary amino group. Upon fragmentation this acyl group is not identified in the tandem mass spectrum owing to neutral loss. However, the appearance of abundant signals originating from defragmentation of both an *O*-acyl and *N*-acylethanolamine group helps to identify NAPEs, as this general pattern is not observed in negative ion ESI tandem mass spectra of common mammalian phospholipids, e.g. ethanolamine, choline, serine and inositol glycerophospholipids.[27,39,41]

In conclusion, the ESI-MS method described here facilitates the unequivocal identification of NAPE species with the assignment of each acyl and alkenyl chain position, including identification of the *N*-acyl moiety. Therefore, the method can provide important information on NAEs to be released from the endogenous NAPE pool upon phospholipase D activation.

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