Rapid Analysis of Lysophosphatidic Acid and Lysophosphatidyl Choline in Human Samples by Fast Atom Bombardment Tandem Mass Spectrometry

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Phospholipids are found in abundance in the membranes of living organisms. They have been used as biomarkers for the detection and identification of bacteria and algae, and the determination of general membrane function in living matter. Among phosphoglycerides, lysophospholipids such as lysophosphatidic acid (LPA) and lysophosphatidyl choline (LPC) are now of intense interest because of their significant functions in transmembrane signaling.[1~2] LPA produces several cellular responses, such as activation of phosphatidylinositol 4,5-bisphosphate hydrolysis, increase of cytosolic Ca^{+2}, and activation of mitogen-activated protein kinase.[3~5] LPA has also been known to inhibit adenylate cyclase,[3] activate phosphatidyl choline (PC)-hydrolyzing phospholipase D,[2,6] and induce mitogenesis.[7] LPC, produced by the action of PC-hydrolyzing phospholipase A_2 (LPA_2), has been reported to be important in sudden cardiac death by causing electrophysiological derangements[1] and in gastric ulceration.[8] However, most of studies have been done with lysophospholipids synthesized or produced by hydrolyzing phospholipids with PLA_2.

There have been lots of analytical effort to determine the changes of lysophospholipids after treating cells with agonists.[1,9] Several thin layer chromatography (TLC) systems have been developed to measure the amounts of LPA and LPC, but in most of cases, time-consuming two-dimensional TLC systems were used.[6,9] Recently, high performance liquid chromatography (HPLC) was used to separate LPC from other phospholipids, but [3H]acetic anhydride was required to quantify.[1] It is very difficult to analyze the lysophospholipids with conventional methods because the lipids have very complicated physicochemical characteristics and the amount is very small in biological membranes. Therefore, development of new method to easily separate LPA and LPC may be able to contribute to the studies of transmembrane signalling and diagnosis of human disease such as gastric ulceration and stomach cancer.

For the final structure analysis of phospholipids extracted from human organs, we have used gas chromatography-mass spectrometry (GC-MS),[10] which involves enzymatic or chemical cleavages of polar head groups, and derivatization of fatty acyl groups prior to mass analysis. However, this method was time-consuming and caused sample loss during cleavage and derivatization steps. Since the soft ionization technique, fast atom bombardment (FAB), allows the nonvolatile phospholipids to be analyzed without prior derivatization and the tandem mass spectrometric technique (MS/MS)[11] allows phospholipid mixtures to be fully characterized without prior separation we applied the FAB-MS/MS method to analyze LPA and LPC, which are important minor phospholipids in the study of cellular signalling.

Analysis of LPA and LPC by positive FAB-MS has been carried out with crude lipid extracts from gastric juice of adult males. The lipids were extracted according to the procedures of Bligh and Dyer[12] with a modification, in which 0.1 N HCl was used to neutralize negative charged phospholipids and to full down to the chloroform layer.[13] The FAB-MS results show that $[M+H]^+$ ions are abundantly formed so that this is a facile means to detect desired species. Here, molecular weight information is sufficient to define molecular species by total carbon acyl groups and number of double bonds. There can be ambiguity between subclasses, but these subclasses could be further confirmed by tandem mass spectra. Tandem mass spectrometry with precursor ion scanning permitted identification of molecular species that contain a specific fatty acid such as oleic acid or arachidonic acid, even in this complex mixture of molecular species.[14]

The positive ion FAB mass spectrum for a chloroform/methanol extract of male gastric juice is reproduced in Figure 1. Three prominent peaks at m/z 496, 524, and 621, accompanied by several minor peaks, are present in the glycerophospholipid region of the mass spectra. The peaks at m/z 496 and 524 are believed to be LPCs and the former has side acyl group of 16 : 0 and the latter has acyl group of 18 : 0. The spectrum also shows that 1,2-diacylglycerol (1,2-DAG) is the major component which is shown at m/z 621. The protonated molecular mass of LPA is not shown at m/z 459 under the positive mode of FAB-MS. The ions of m/z 460 are believed to be derived from the 3-nitrobenzyl alcohol (NBA) matrix. The presence of LPA could be confirmed from the negative ion FAB-MS because of its different nature of ionic structure from LPC (Figure 2).

During experiments, phospholipid species at low concentrations were often buried in the high chemical background associated with FAB mass spectrometry. The usefulness of FAB-MS in ultratrace phospholipid analysis should depend on the ability to detect and identify the phospholipids in complex matrices and mixtures. As we well understand

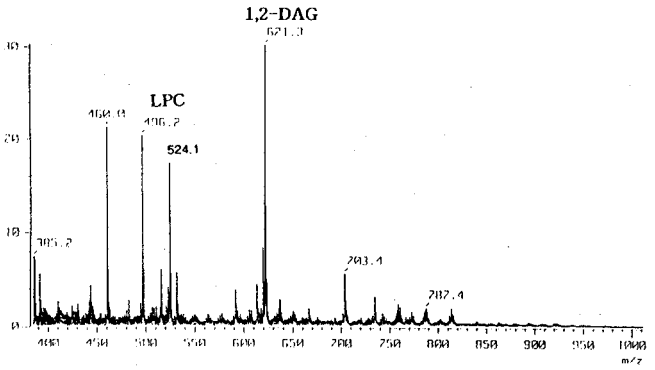

Figure 1. The positive ion FAB mass spectrum of gastric juice extract.

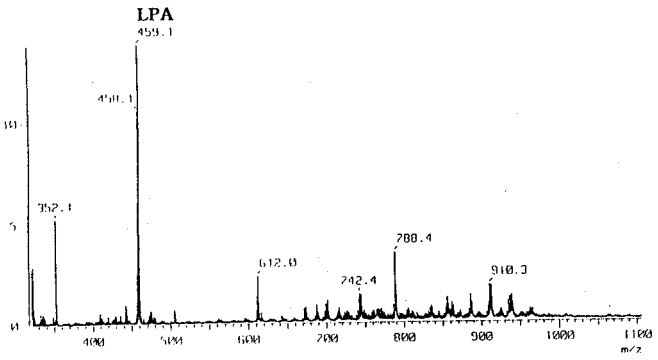

Figure 2. The negative ion FAB mass spectrum of gastric juice extract.

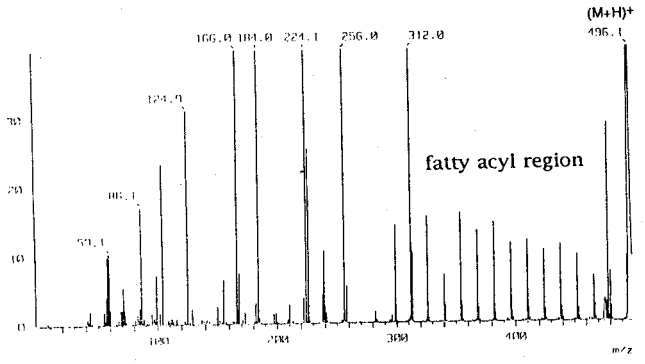

Figure 3. The tandem MS/MS spectrum of m/z 496, LPC.

the ionic character of phospholipids and their behavior in FAB-MS, FAB-MS/MS could more efficiently be used for the analysis of phospholipids, such as LPA and LPC. In these studies, because of high hydrophobic character of fatty acyl moieties, nitrobenzyl alcohol has yielded higher sensitivity than any other matrices during FAB ionization of these molecules. Besides, mass scanning from m/z 400 also is recommended for eliminating chemical background and for enhancing sensitivity of ion detection in the positive ion FAB-MS. However, in the negative ion spectrum, two product carboxylate anions at m/z 283 and 303 are useful to confirm the presence of phosphatidyl choline.[15]

The MS/MS product ion spectrum for the molecular ions of m/z 496 in Figure 2 shows typical fragmentation patterns of LPC (Figure 3). The spectrum contains a prominent ion series of fatty acyl group between m/z 312 and $(M+H)^+$: the charge remote decompositions[13] involve parallel losses of C_nH_{2n+2} groups differing by one carbon (14 u). Therefore, the number of peaks between m/z 312 and $(M+H)^+$ represents the carbon chain length in the fatty acyl moieties of molecule. Other characteristic ions are present at m/z 312, 256, 224, 184, 86, and 59, which provide additional evidence that the compound is LPC (Figure 4). As expected, the MS/MS spectrum for the ions at m/z 621 (1,2-DAG) shows different fragmentation pattern when compared with that of m/z 496 (Figure 5).[16]

The excellent parent ion resolusion of the four-sector mass spectrometer permits diagnostic daughter ion spectra to be required for components differing by two mass units (e.g.,

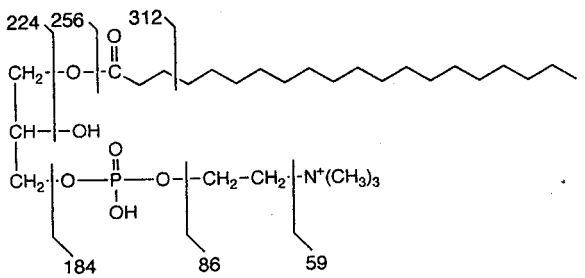

Figure 4. The structure of LPC, indicating fragmentation exhibited in the spectrum of Figure 3.

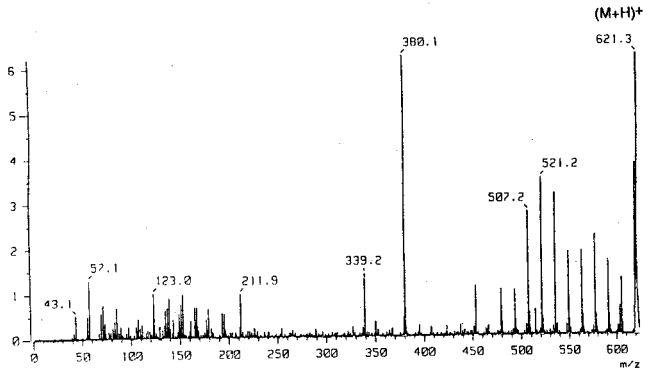

Figure 5. The tandem MS/MS spectrum of m/z 621, 1,2-DAG.

16 : 1 *vs.* 16 : 0); this is not possible using linked scanning at constant B/E in a two-sector instrument. The ambiguity from the peaks between m/z 459 (LPA) and m/z 460 (NBA matrix) in Figure 1 also was clearly explained by this specific ion selection and their distinct MS/MS spectra. The ability to unambiguously identify fatty acids is an advantage of high energy collision mass spectral analysis. Under low-energy collision conditions in triple quadrupole mass spectrometry, carboxylate anions hardly undergo charge-remote-fragmentations that allow for specific structural characterization of the acid chain.

In conclusion, it has proven that analysis of minor phospholipids such as LPA and LPC could be performed most easily by fast atom bombardment mass spectrometry (FAB-MS) and tandem mass spectrometry (MS/MS). Precise information concerning the fatty acyl and polar head groups present in most cases could be obtained. The crude extracts of human organs can be analyzed without prior separation, and reasonable estimates of specific class and molecular species content can both be obtained. Thus, FAB-MS offers exciting possibilities to detect these species in complex mixtures and important information is generated during collision induced decomposition in tandem mass spectrometry. Clearly, the results presented has shown that this method could be applied to the rapid diagnosis of human disease. On the basis of these preliminary results, the application of this procedure to quantify each components by using isotope enriched references is currently under investigation.

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16. The MS/MS spectra of LPA, LPC, and 1,2-DAG from gastric juice extract were compared and well matched with those of reference compounds obtained from commercial sources. The FAB-MS and MS/MS experiments were carried out on a JEOL HX110A/HX110A high resolution tandem mass spectrometer. The accelerating voltage was 10 kV with a mass resolution of 1 : 1000 (10% valley). The JEOL Cs^+ ion gun was operated at 25 kV. The collision cell potential was held at 3.0 kV and the ion collision energies were 7.0 kV. Helium collision gas was introduced into the collision chamber at a pressure sufficient to reduce the precursor ion signal by 50%.

Photoaddition Reactions of *o*-Benzoquinones to 1,4-Diphenylbut-1-en-3-yne : Formation of Phenanthrenes and Dihydrodioxins

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Quinones are an important class of compounds in organic synthesis, in industry, and in nature.[1] Due to their various spectroscopic properties and reactivities, the photochemistry of *o*-quinones has been a subject of interest in many areas.[2~6] Recently, we have found that *o*-benzoquinones can be utilized to synthesize 1-phenylphenanthrenes.[6] In connection with our investigation of the scope of these reactions, we examined the photochemical reactions of *o*-benzoquinones such as tetrachloro-1,2-benzoquinone (TCBQ) **1a** and tetrabromo-1,2-benzoquinone (TBBQ) **1b** with 1,4-diphenylbut-1-en-3-yne (BEY) **2**, having one carbon-carbon double bond and one carbon-carbon triple bond. In this paper, we provide another evidence for the utility of *o*-benzoquinones as starting materials for the synthesis of 9-phenylphenanthrenes.

Preparative photochemical reactions were conducted in dry nitrogen atmosphere in a photochemical reactor composed of a water-cooled system and a pyrex reaction vessel with 300 nm UV lamps (Rayonet Photochemical Reactor, Model RPR-208).

Irradiation (300 nm) of TCBQ **1a** (X=Cl, 246 mg, 1.0 mmol) and BEY **2** (204 mg, 1.0 mmol) in dichloromethane (50 mL) for 24 h afforded 1,3-cyclohexadienes **3a** (X=Cl, 8%)[7] and their oxidized product **5a** (X=Cl, 5%),[6] as well as dihydrodioxins **4a** (X=Cl, 55%).[8] The photoproducts were isolated by flash column chromatography (silica gel, 230-400 mesh) using *n*-hexane and ethyl acetate (9 : 1, v/v) as the eluents.The starting material **2** (28%) was recovered first. Dihydrodioxins **4a** were then obtained as a mixture of two isomers (*cis/trans*) with a ratio of 1.0 : 1.4, as revealed by ^{1}H NMR. Two methine protons of *cis* isomer **4a** (*cis*) were observed at δ 5.15 (d, J=7.3 Hz) and 5.02 (d, J=7.3 Hz), whereas those of *trans* isomer **4a** (*trans*) were found at δ 5.50 (bs) and 5.36 (bs). The product could be further recrystallized from *n*-hexane to yield *cis* isomer **4a** (*cis*) in pure form (23%). The photoproduct **5a** was obtained as a mixture containing a small amount (8% of the two) of 9-phenylphenanthrene **6a** (X=Cl).[9] Rechromatography of the mixture over silica gel with *n*-hexane and ethyl acetate (from 97 : 3 to 9 : 1, v/v, gradient elution) afforded good separation of **5a** from **6a**. The latest fraction gave cyclohexadienes **3a**, which were also obtained as a mixture of two isomers (*cis/trans*) with a ratio of 1.3 : 1.0, as revealed by ^{1}H NMR. Two methine protons of **3a** (*cis*) were observed at δ 4.94 (d, J=7.3 Hz) and 4.87 (d, J=7.3 Hz), whereas those of **3a** (*trans*) were