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Application of liquid chromatography–thermospray mass spectrometry in the analysis of glycerophospholipid molecular species

FRANS A. KUYPERS*, PETER BÜTIKOFER and CEDRIC H. L. SHACKLETON

Children's Hospital Oakland Research Institute, 747 52nd Street, Oakland, CA 94609 (U.S.A.)

ABSTRACT

We report the application of high-performance liquid chromatographic (HPLC) separation with ultraviolet detection and direct, on-line, structural analyses by mass spectrometry of glycerobenzoate derivatives from complex mixtures of phospholipid molecular species. Individual phospholipids were resolved from total lipid extracts by thin-layer chromatography (TLC). Diradylglycerols were released from phospholipids by phospholipase-C treatment, converted to diradyl glycerobenzoates and subsequently separated by TLC into subclasses (alk-1-enylacyl, alkylacyl and diacyl types). The molecular species within each subclass were resolved by HPLC with an octadecyl reversed-phase column in acetonitrile–isopropanol (80:20, v/v). Individual peaks were quantitated at the picomole level by measuring absorbance at 230 nm. After post-column addition of methanol–0.2 *M* ammonium acetate (50:50, v/v), peaks were introduced through the thermospray interface into a VG Masslab 30-250 quadrupole mass spectrometer. Molecular species showed as base peaks the salt adducts of the molecular ion which permitted easy deduction of the overall fatty acyl composition. In addition, the diglyceride fragment of each species was found at $[MH-122]^+$ and two fragments formed by the loss of the fatty acyl groups (R) in the *sn*-1 or *sn*-2 position were found at $[M-R_1]^+$ and $[M-R_2]^+$, respectively. Since preferential release of either fatty acyl group was observed in positional isomers, the ratio of the intensity of these fragments gave information on the position of the fatty acyl groups in the individual HPLC peaks. We show that the use of on-line mass spectrometry, however, provides easy identification of all molecular species present in a complex phospholipid mixture, even when more than one molecular species is contained in an HPLC peak.

INTRODUCTION

Analysis of phospholipid molecular species is of interest in elucidating the specific physiological functions of phospholipids. Biological membranes contain complex mixtures of phospholipid molecular species [1–5] in which the fatty acyl length, degree of unsaturation, the type of linkage to, as well as the position on the glycerol backbone are very important determinants of membrane physical properties [6], and precise control appears to be important for membrane integrity [7,8]. Molecular species analysis has been approached by a variety of techniques, including argentation thin-layer chromatography (TLC) and gas chromatograhpy (GC) [9–14]. Major developments in chromatographic techniques, in particular high-performance liquid chromatography (HPLC) [15–19], as well as

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mass spectrometry (MS) [20–25] during the last decade have made analysis of the glycerophospholipid molecular species composition more feasible on a routine basis. HPLC of diradylglycerol derivatives of phospholipids has proven to be a powerful tool for separation and quantification of phospholipid molecular species [26,27]. Furthermore, benzoate derivatives of diradylglycerols can be readily separated and quantified into the three subclasses (alk-1-enylacyl, alkylacyl and diacyl types). However, in complex mixtures several problems arise. Certain molecular species are not separated from each other by HPLC and identification of individual molecular species based on retention time, enzyme degradation and fatty acid analysis may not be satisfactory. Furthermore, no separation or identification is accomplished of positional isomers of molecular species in which the fatty acyl groups in the *sn*-1 and *sn*-2 positions are exchanged.

MS has proven to be useful for phospholipid structural analysis. In particular, techniques such as thermospray liquid chromatography–mass spectrometry (LC–MS) have shown great potential for the on-line analysis of lipids separated by HPLC [19–23]. In the present study, we applied this technique to the analysis of diradyl glycerobenzoates of synthetic phospholipid standards as well as of molecular species of human red cell membrane glycerophospholipids. This approach combines established TLC and HPLC separation and quantitation techniques [27] with powerful MS identification.

EXPERIMENTAL

Materials

Synthetic phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Human erythrocytes were prepared from fresh human venous blood collected in heparinized tubes after obtaining informed consent from laboratory volunteers. Erythrocytes were pelleted by centrifuging for 5 min. at 1000 *g* and washed three times with a ten-fold excess of buffer (5 m*M* phosphate, 140 m*M* NaCl, pH 7.4). The buffy coat was removed carefully by aspiration after each wash. Lipids were extracted according to Rose and Oklander [28] and stored under argon at −20°C.

Methods

Individual erythrocyte glycerophospholipid classes were separated by TLC on Silica Gel HL (Analtech, Newark, DE, U.S.A.) in a solvent system of chloroform–methanol–acetic acid–0.9% saline (100:50:16:5) and visualized with diphenylhexatriene (Sigma, St. Louis, MO, U.S.A.). The lipids were eluted from silica in chloroform–methanol–acetic acid–water (100:78:2:20) and washed with 4 m*M* NH_4OH. Erythrocyte phospholipids and phospholipid standards were hydrolyzed for 1 h with *Bacillus cereus* phospholipase C (Sigma) in a mixture of buffer (100 m*M* Tris, 5 m*M* $CaCl_2$, 1 m*M* $ZnCl_2$, pH 7.4) and diethyl ether, with vigorous mixing at 37°C. Hydrolysis of the phospholipids to diradyglycerols was

monitored by analysis of an aliquot of the ether layer by TLC in a solvent system of diethyl ether–hexane (85:15). The ether layer was removed and evaporated with a stream of nitrogen. Benzoate derivatives of diradylglycerols were prepared and the subclasses were separated according to Blank *et al.* [27].

Separation of molecular species within each subclass was performed on a Bio-Rad Bio-Sil ODS 5S column (5-μm C_{18}, 15 cm × 4 mm I.D., Bio-Rad, Richmond, CA, U.S.A.) in a solvent system of acetonitrile–isopropanol (80:20) at a flow-rate of 1 ml/min. Separated components were quantitatively measured at 230 nm with a Kratos Spectroflow 757 UV detector and an HP 3390A integrator. After UV detection, a mixture of methanol–0.2 *M* ammonium acetate in water (50:50, v/v) was added at a flow-rate of 0.2 ml/min and the mixture was delivered into the thermospray interface of a VG Masslab 30-250 quadrupole mass spectrometer (VG Masslab, Altrincham, U.K.).

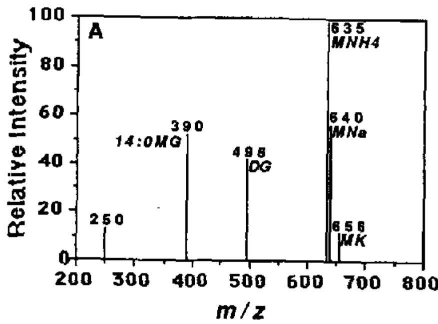
In order to optimize the tuning conditions, dimyristoyl glycerobenzoate was injected post-column and parameters of the vaporizer were adjusted. These parameters were used for subsequent analysis of the other diradyl glycerobenzoates. The optimum temperature for the capillary vaporizer was in the range 150–170°C and varied with the vaporizer used. The temperature of the downstream source was 245°C. The electrode voltage was kept at 350 V unless stated otherwise to ensure sufficient ionization and fragmentation of the compounds. The analyzer pressure was maintained at approximately $1.5 \cdot 10^{-5}$ Torr with a mobile phase flow-rate of 1.2 ml/min.

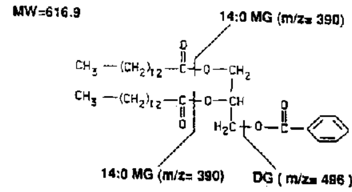
RESULTS AND DISCUSSION

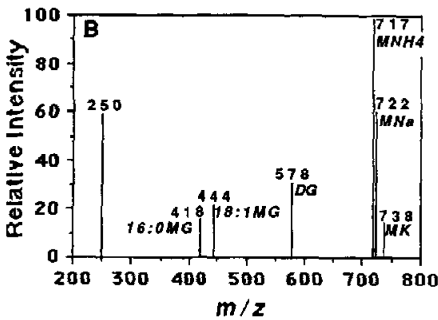
Nature of the mass spectra

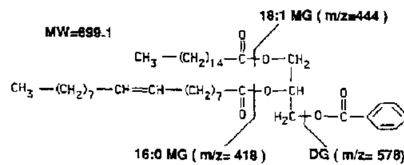
The positive-ion spectra of the glycerobenzoate derivatives of phospholipids showed a simple fragmentation pattern containing information on the total fatty acyl composition as well as position of the fatty acyl groups on the glycerol backbone (*sn*-1 or *sn*-2). Fig. 1 shows the mass spectra of three standard diradylglycerols: dimyristoyl glycerobenzoate (14:0 14:0, MW = 616.9), 1-palmitoyl-2-oleoyl glycerobenzoate (16:0 18:1, MW = 699.1) and 1-oleoyl-2-palmitoyl glycerobenzoate (18:1 16:0, MW = 699.1). The spectrum contains insignificant amounts of the protonated molecular ion. The major ions are the sodium ($[M+Na]^+$), ammonium ($[M+NH_4]^+$) and potassium ($[M+K]^+$) adducts of the molecular ion at m/z = M + 23, M + 18 and M + 39, respectively. The abundance of both the sodium and ammonium adduct was always higher than that of the potassium adduct. Tuning conditions strongly affected the relative abundance of the sodium and ammonium adducts. The post-column addition of the mixture of methanol–ammonium acetate greatly improved the ionization and sensitivity of detection.

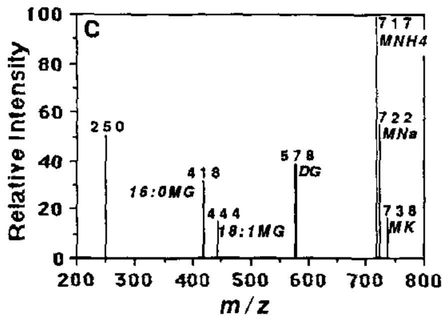
The chemical structure of the three compounds is shown, with the proposed structure of the major fragments (Fig. 1D), the diacylglyceride ($[DG]^+$) fragment

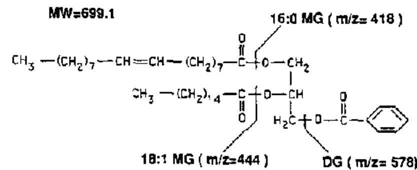












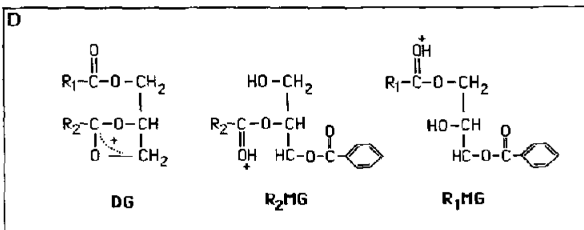


Fig. 1. Positive-ion spectrum obtained from thermospray LC–MS analysis of (A) dimyristoyl glycerobenzoate, (B) 1-palmitoyl-2-oleoyl glycerobenzoate and (C) 1-oleoyl-2-palmitoyl glycerobenzoate. (D) Proposed structures of the fragment ions. The chemical structure of the three compounds is shown, as well as the proposed fragmentation. See *Methods* for mass spectral conditions. Abbreviations: MNH_4, MNa and MK, the ammonium, sodium and potassium adducts of the molecular ion, respectively; DG, diacylglyceride fragment; R_1MG and R_2MG, monoacylglyceride ions; 14:0 MG, 16:0 MG and 18:1 MG, monoacylglyceride ions, containing the fatty acyl group indicated by the total number of carbon atoms and double bonds.

at $m/z = MH - 122$, and the monoacylglyceride ions $[(R_2MG]^+$ and $[R_1MG]^+)$ formed by the loss of the fatty acyl groups in the *sn*-1 or *sn*-2 position, respectively. As an example, $[16{:}0\ MG]^+$ represents the monoglyceride fragment containing palmitic acid (16:0) in either the *sn*-1 or *sn*-2 position of the glycerol backbone. A different relative abundance of the 16:0 MG and 18:1 MG fragments was observed in the positional isomers 16:0 18:1 and 18:1 16:0 indicating a preferential release of either fatty acyl group.

Quantitation and calibration

Our measurements of UV absorbance of diradyl glycerobenzoates confirmed reported data [27] that molar absorptivities as measured in acetonitrile–isopropanol at 230 nm are virtually identical for the different molecular species of the glycerobenzoates. Furthermore, a linear correlation exists between the amounts of diradyl glycerobenzoates injected into the HPLC column and the integration units from the HP3390A recorder (correlation coefficient = 0.98 for eight in-

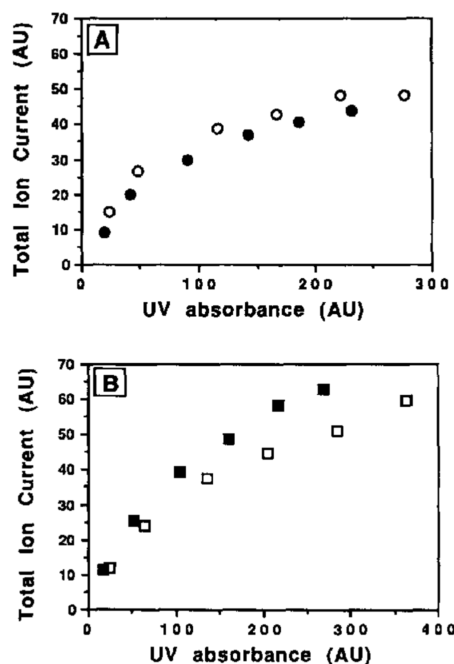


Fig. 2. Calibration curve for the quantitation of (A) 1-palmitoyl-2-oleoyl glycerobenzoate (○) and 1-oleoyl-2-palmitoyl glycerobenzoate (●) and (B) 1-palmitoyl-2-stearoyl glycerobenzoate (□) and 1-stearoyl-2-palmitoyl glycerobenzoate (■). Increasing amounts of the glycerobenzoates (0–2 nmol) were injected. The molar amount of each component was determined by integration of the UV peaks at 230 nm and was compared to the calculated total ion current of the major peaks (the three salt adducts of the molecular ion, diglyceride and monoglyceride fragments as indicated in Fig. 1) collected in SIR on the mass spectrometer (expressed as arbitrary units, AU).

jections from 0.1 to 2 nmol). The absolute (or relative) molar amount of each molecular species can therefore be determined from the HPLC–UV integration units. Since the compound is transferred directly, on-line, from the UV detector into the mass spectrometer, the correlation of UV absorbance units with ion current gives an excellent opportunity for calibration of the thermospray interface. In Fig. 2, the calibration curves of four diradyl glycerobenzoates are shown. The total ion current as calculated from the major peaks [the three salt adducts of the molecular ion, diglyceride and monoglyceride fragments as indicated in Fig. 1, collected in selected-ion recording (SIR) on the mass spectrometer] did not show a linear relationship with the UV absorbance or range of molar amounts (0 to 2 nmol) injected (Fig. 2A and B). This was probably caused by saturation effects in the ion source of the thermospray interface at high sample concentrations. A linear relationship was found between the logarithm of the UV absorbance and the total ion current for all standards tested with correlation coefficients better than 0.96 ($n=6$). This logarithmic relationship can be used for quantitation with the mass spectrometer.

We observed that different molecular species, even positional isomers, can ionize differently under identical source conditions. Fig. 2 shows that while 1-palmitoyl-2-oleoyl glycerobenzoate and 1-palmitoyl-2-stearoyl glycerobenzoate ionize at similar levels, 1-stearoyl-2-palmitoyl glycerobenzoate gave a higher level of ionization. These findings indicate that, in order to use thermospray MS for quantitation of molecular species, a wide range of standards is necessary. In addition, the source conditions strongly affected the ionization in thermospray. The instrument was tuned daily on a diradyl glycerobenzoate standard, such as dimyristoyl glycerobenzoate. The optimal source conditions changed slightly with the vaporizer used, likely as a result of a variation in the spray when a

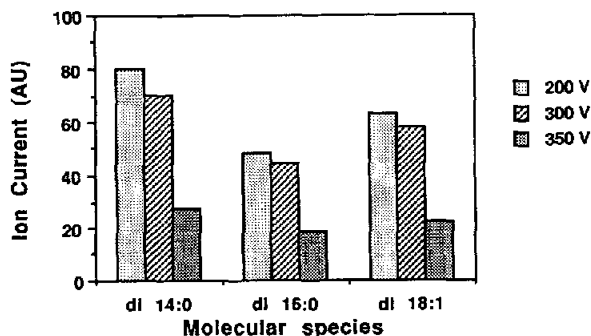


Fig. 3. Effect of source condition on the ion current. A mixture containing equimolar amounts of three molecular species, dimyristoyl (di 14:0), dipalmitoyl (di 16:0) and dioleoyl (di 18:1) glycerobenzoate, was analyzed by LC–MS in three consecutive runs with source voltages of 200, 300 and 350 V. The ion current was calculated from the SIRs of the salt adducts of the molecular ions and expressed as arbitrary units (AU).

different (new) vaporizer was mounted in the interface. Fig. 3 shows the effect of changing the source voltage on the detection and fragmentation patterns of the glycerobenzoates. An increase in voltage led to a lower ion current of the salt adducts of the molecular ion, but a change from 200 to 300 V led to a drastically higher fragmentation level. Further increase lowered the abundance of fragments. Furthermore, the relative amount of the sodium or ammonium adduct was strongly affected by these conditions. We optimized tuning on a good response of the salt adduct as well as a reasonably good signal for the monoglyceride fragments. All glycerobenzoates tested were affected similarly by the change in source conditions. Although the total ionization and fragmentation was affected, the relative intensity of the monoglyceride peaks was not significantly affected by the source voltage.

Fragmentation

In species with identical fatty acyl groups, such as shown in Fig. 3, no distinction can be made between the monoglyceride formed by the loss of the fatty acyl group at the *sn*-1 or *sn*-2 position. In mixed species, preferential release of either fatty acyl group gave information on the position of the fatty acyl groups of individual molecular species. We documented the fragmentation of a series of standards as shown in Table I. The abundance of the monoglyceride ions relative to the sodium adduct is given as well as the ratio of the monoglyceride fragments with the fatty acyl chain at the *sn*-1 position (FA1 MG) relative to the monoglyceride fragment with the fatty acyl chain at the *sn*-2 position (FA2 MG). In

TABLE I

FRAGMENTATION OF PHOSPHOLIPID STANDARDS

Data were collected in SIR. The total abundance of the monoglyceride ions relative to the sodium adduct is given as well as the ratio of the monoglyceride fragment with the fatty acyl at the *sn*-1 position (FA_1 MG) relative to the monoglyceride fragment with the fatty acyl at the *sn*-2 position (FA_2 MG). Average and standard deviation are given for six determinations in which various amounts were analyzed (0.1–1.6 nmol injected). N.D. = not detected.

Molecular species	MNa/MG	FA_1 MG/FA_2 MG
16:0 18:1	0.81 ± 0.05	1.82 ± 0.03
18:1 16:0	0.80 ± 0.04	0.71 ± 0.01
16:0 18:0	0.80 ± 0.06	0.83 ± 0.01
18:0 16:0	0.89 ± 0.03	1.30 ± 0.04
16:0 18:2	1.01 ± 0.05	1.70 ± 0.03
18:2 16:0	1.02 ± 0.06	0.80 ± 0.03
14:0 14:0	0.42 ± 0.06	N.D.
16:0 16:0	1.01 ± 0.11	N.D.
18:1 18:1	0.88 ± 0.04	N.D.

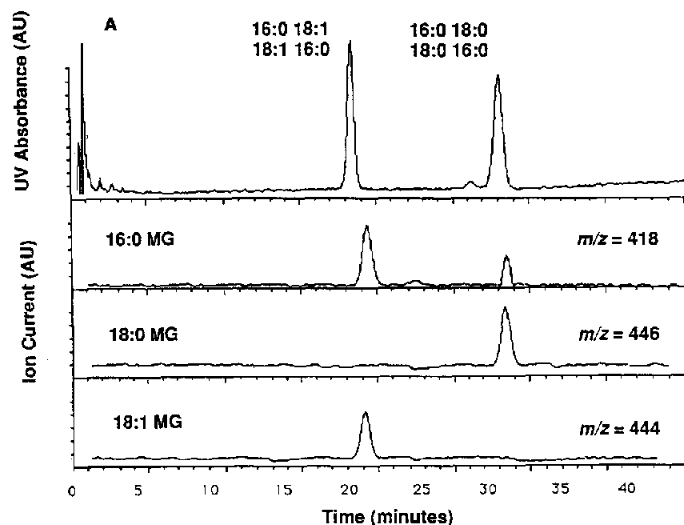
mixed species such as those shown in Table I, the fatty acyl groups at the *sn*-1 or *sn*-2 position are not lost at the same rate. The abundance of the monoglyceride peak depends on the number of carbon atoms, degree of unsaturation and the positional distribution of the fatty acyl groups as indicated by the ratios of the monoglyceride intensities. While 18:1 was released at a 1.82 higher rate from the *sn*-2 position in 16:0 18:1, this rate is only 1.4 times faster when the same fatty acyl group was released from the *sn*-1 position of the positional isomer (as determined by the inverse of 0.71). Similar calculations were made for the two other sets of positional isomers. While 16:0 is released at a 1.30 higher rate from the *sn*-2 position in 18:0 16:0, this rate is only 1.2 times higher when the same fatty acyl group is released from the *sn*-1 position. A similar pattern is found for the release of 18:2 from 16:0 18:2 and 18:2 16:0 glycerobenzoate as for 18:1 from 16:0 18:1 and 18:1 16:0 glycerobenzoate. Thus, in all these species the fatty acyl group in the *sn*-2 position is lost at a higher rate than the fatty acid in the *sn*-1 position. The characteristics of the fatty acyl chains are also important; however, since only a limited selection of pure phospholipid positional isomers is (commercially) available, a complete and exact comparison of the release rates of different fatty acyl chains is difficult to obtain. Our results, however, are consistent with the following relative order of release of the fatty acyl groups from the same *sn* position of the diacyl glycerobenzoates thus far examined: 14:0 > 18:2 ≅ 18:1 > 16:0 > 18:0.

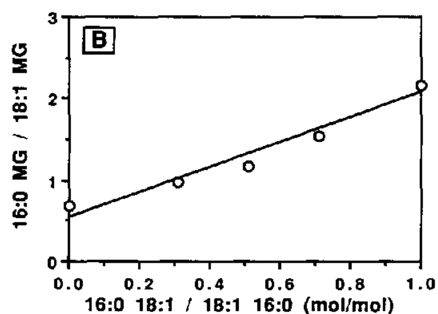
Determination of positional isomers

The clear difference in fragmentation in positional isomers made the determination of their relative amount in a mixture possible. This was shown on a set of five mixtures of four molecular species as shown in Fig. 4. The UV trace of the HPLC separation gave two peaks and SIR was used to scan for the monoglyceride fragments (Fig. 4A). The ratio of the intensities of the monoglyceride fragments in each peak showed a linear relationship with the mole fraction of the positional isomer present. These data indicate that the relative amounts of positional isomers in a peak can indeed be calculated from the intensities of the monoglyceride fragments.

Analysis of complex mixtures

We tested the applicability of our method to analyze the molecular species composition of complex mixtures on a relatively simple mixture of six glycerobenzoate derivaties of synthetic phosphatidylcholine molecular species (Fig. 5). Detection by UV absorbance at 230 nm is shown in the top panels as well as SIR of the sodium adduct of the molecular ions (A) and the monoacylglyceride fragments (B). Monitoring of the sodium adducts of the molecular ions permitted easy deduction of the overall fatty acyl composition. In addition, the monoglyceride fragments confirmed the presence of the expected fragments in each peak. The 16:0 MG (*m*/*z* 418) was found in three peaks, the 18:1 MG (*m*/*z* 444) in





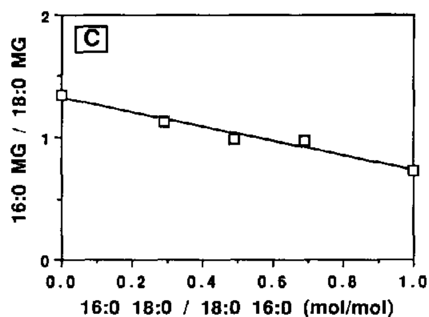


Fig. 4. (A) HPLC separation and analysis by MS of a typical mixture of 1-palmitoyl-2-oleoyl glycerobenzoate (16:0 18:1), 1-oleoyl-2-palmitoyl glycerobenzoate (18:1 16:0), 1-palmitoyl-2-stearoyl glycerobenzoate (16:0 18:0) and 1-stearoyl-2-palmitoyl glycerobenzoate (18:0 16:0) in relative amounts of 31:13:39:17 (mol/mol). (B, C) Ratio of monoacylglyceride fragments as a function of the mol fraction of positional isomers in mixtures of 16:0 18:1–18:1 16:0 and 16:0 18:0–18:0 16:0.

two, whereas the 14:0 MG (*m*/*z* 390) and 18:2 MG (*m*/*z* 442) were found in one peak.

The composition of the mixture of which the separation is shown in Fig. 5 is shown in Table II. The relative molar amount of the species was calculated based on the UV tracing. In addition, from the ratio of the abundance of the 16:0 MG and 18:1 MG fragments, it could be calculated that the peak at 18.5 min contained a mixture of 70% 16:0 18:1 and 30% 18:1 16:0. The ion current of the sodium adduct of the molecular ion in SIR after HPLC separation is shown in Table IIA. The reproducibility, as indicated by the standard deviation, in four runs with a four-fold difference in injected amount was good as measured in UV as well as in SIR. The differences between UV and SIR are due to differences in

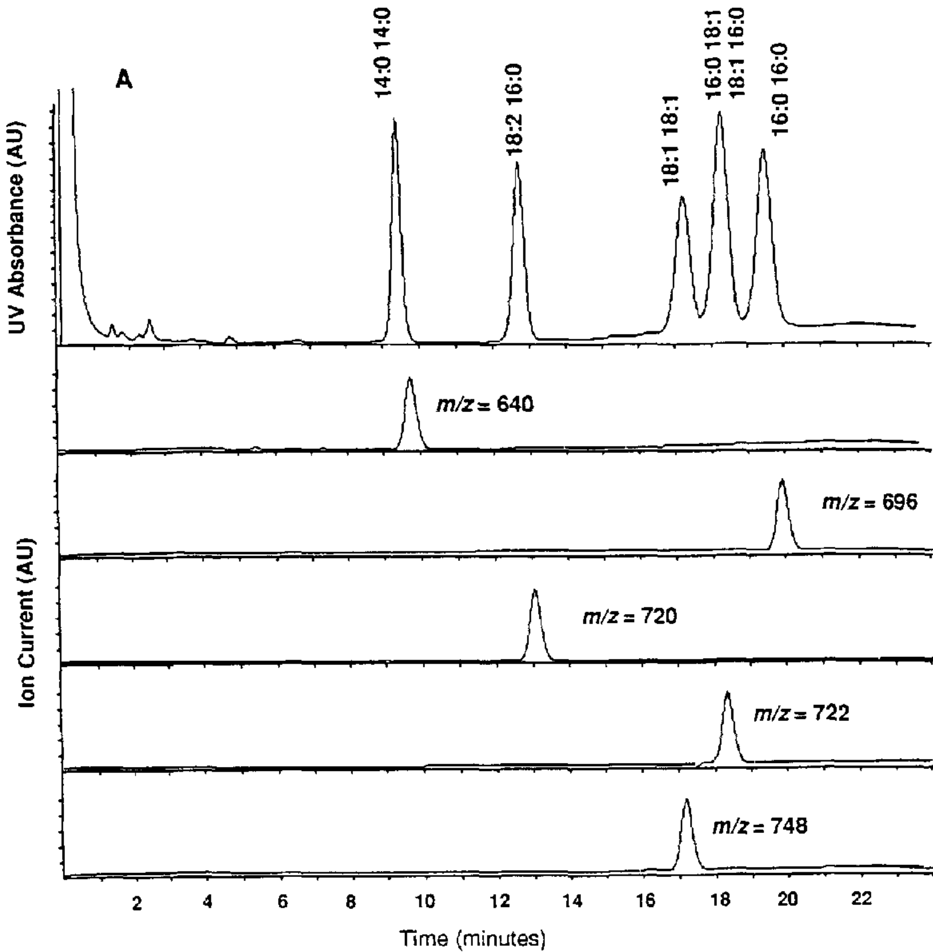


Fig. 5.

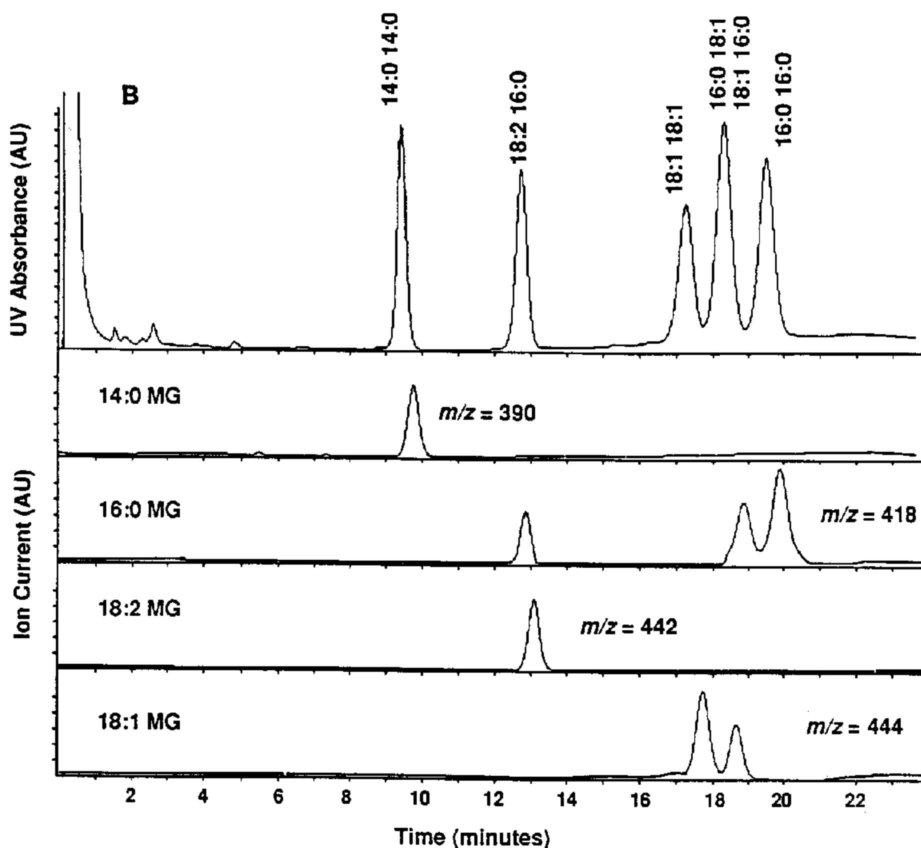


Fig. 5. HPLC separation and analysis by MS of a mixture of six glycerobenzoate derivatives of synthetic phosphatidylcholine molecular species: dimyristoyl (14:0 14:0), 1-linoleoyl-2-palmitoyl (16:0 18:2), dioleoyl (18:1 18:1), 1-palmitoyl-2-oleoyl (16:0 18:1), 1-oleoyl-2-palmitoyl (18:1 16:0) and dipalmitoyl (16:0 16:0) glycerobenzoate. Detection by UV absorbance at 230 nm is shown (top panels) as well as SIR of (A) the sodium adduct of the molecular ions and (B) the monoacylglyceride fragments.

ionization of the molecular species tested. When corrected by a standard curve, similar results were found for the relative percentage in UV as in SIR.

When the same mixture was directly, without HPLC separation, introduced into the mass spectrometer, the relative amount of each glycerobenzoate could also be calculated from SIR (Table IIB). In the mixture tested, especially dimyristoyl glycerobenzoate seems to be affected by the presence of other molecular species, leading to an underestimation of its molar fraction in the mixture. The underlying reason for the difference between Table IIA and B could be the result of competition for reagent ion by the analyte in the source and be related to the chemical characteristics of the different molecular species. These competition and mutual suppression effects were dependent on the sample concentration (not

TABLE II

ANALYSIS OF A STANDARD MIXTURE OF SIX GLYCEROBENZOATE DERIVATIVES OF SYNTHETIC PC SPECIES

The mixture was separated by HPLC (A) or directly introduced into the mass spectrometer (B).

Molecular species	Mol fraction (%)	Ion current (MNa^+) (%)	
		A	B
14:0 14:0	16.7 ± 0.2	9.1 ± 0.3	4.9 ± 0.5
18:2 16:0	16.8 ± 0.3	25.8 ± 0.6	24.7 ± 0.7
18:1 18:1	16.7 ± 0.3	18.8 ± 0.3	21.6 ± 0.9
18:1 16:0	8.3 ± 0.5	26.3 ± 0.9	31.6 ± 0.3
16:0 18:1	19.4 ± 0.2		
16:0 16:0	22.2 ± 1.1	20.0 ± 1.6	17.0 ± 1.2

shown). Despite these differences, we feel that even in the case of a peak which contains a complex mixture of six molecular species, an estimation can be made with respect to the relative amount of each individual component. Moreover, it has to be noted that after HPLC separation, it is unlikely that more than a few molecular species will be present in a particular HPLC peak. More accurate results can be obtained when standard curves are used of mixtures of molecular species as described for positional isomers in Fig. 4 or by the addition of a stable isotope as internal standard.

In order to test our method on a complex mixture of molecular species from a biological source, we determined the phospholipid molecular species composition of red cell membranes and compared our results with recently reported data [29]. The majority of the molecular species in the red cell membrane is found in the diacyl subclasses of phosphatidylcholine (PC), -ethanolamine (PE), and -serine (PS), and in the alk-1-enylacyl subclass of PE. Using the technique described, more than 100 different molecular species were identified in these phospholipid (sub)classes of red cell membranes. In Fig. 6, the reversed-phase HPLC separation of the molecular species of the diacyl and alk-1-enylacyl PE subclasses from erythrocytes are illustrated. The top panels give the UV trace while the lower panels show SIR traces for six (A) or five (B) masses, respectively. The recorded masses for the sodium adduct of the molecular ions of these species give a relatively easy confirmation of the total number and carbon atoms and double bonds in each peak. This approach is very helpful since some UV peaks may contain more than one molecular species. A good example is the peak at a retention time of 11–12 min which contains 16:0 18:1 (m/z 722) as well as 18:0 18:2 (m/z 748) glycerobenzoate. The combined molar amount of these two species could be determined by the UV recording. In addition, since the mass spectrometer cali-

bration data were determined for these species, their relative amounts could be calculated.

Reversed-phase HPLC separation of these compounds is related to the length and unsaturation of the fatty acyl chains, and a prediction scheme for retention times for phospholipids has been developed by Patton *et al.* [15] and applied for glycerobenzoates by Blank *et al.* [27]. Because of the multiplicity of peaks obtained by HPLC analysis of diradyl glycerobenzoates and identical molecular

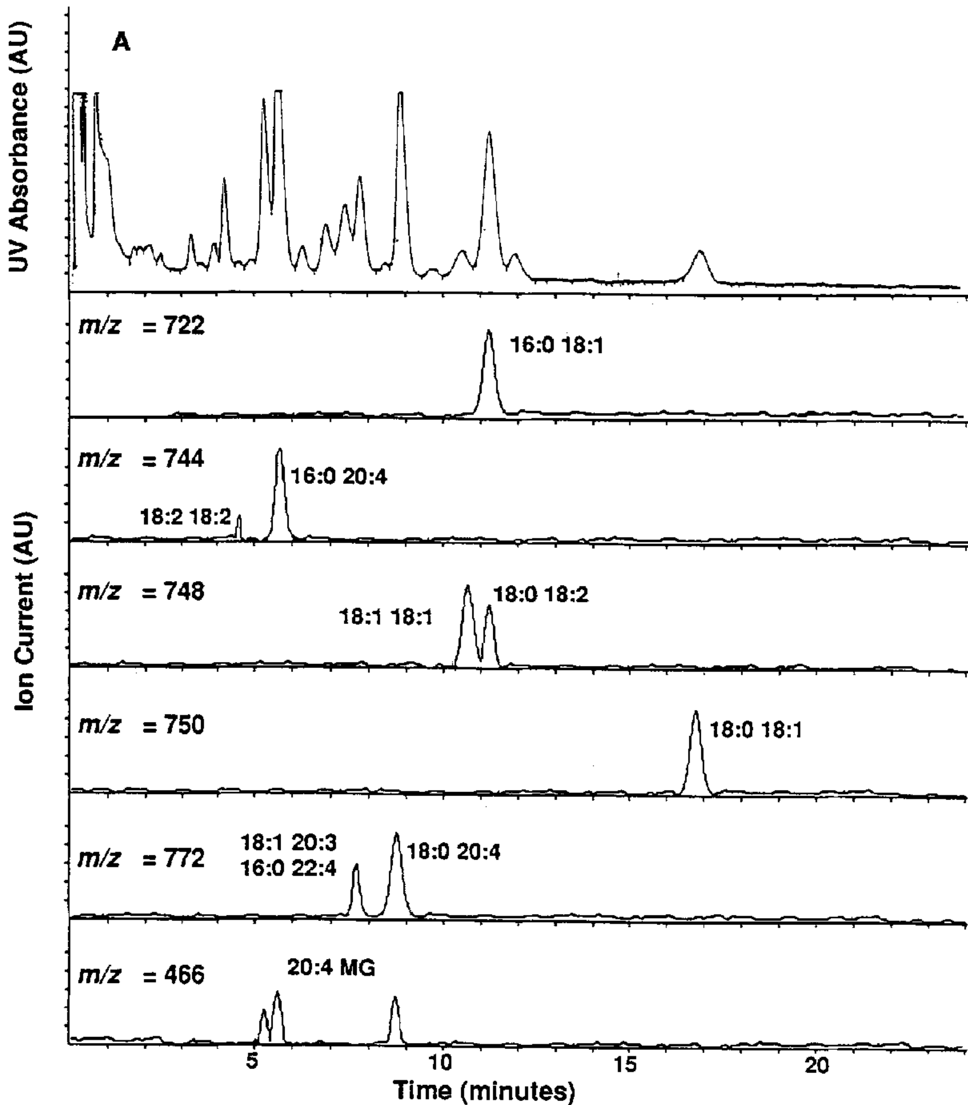


Fig. 6.

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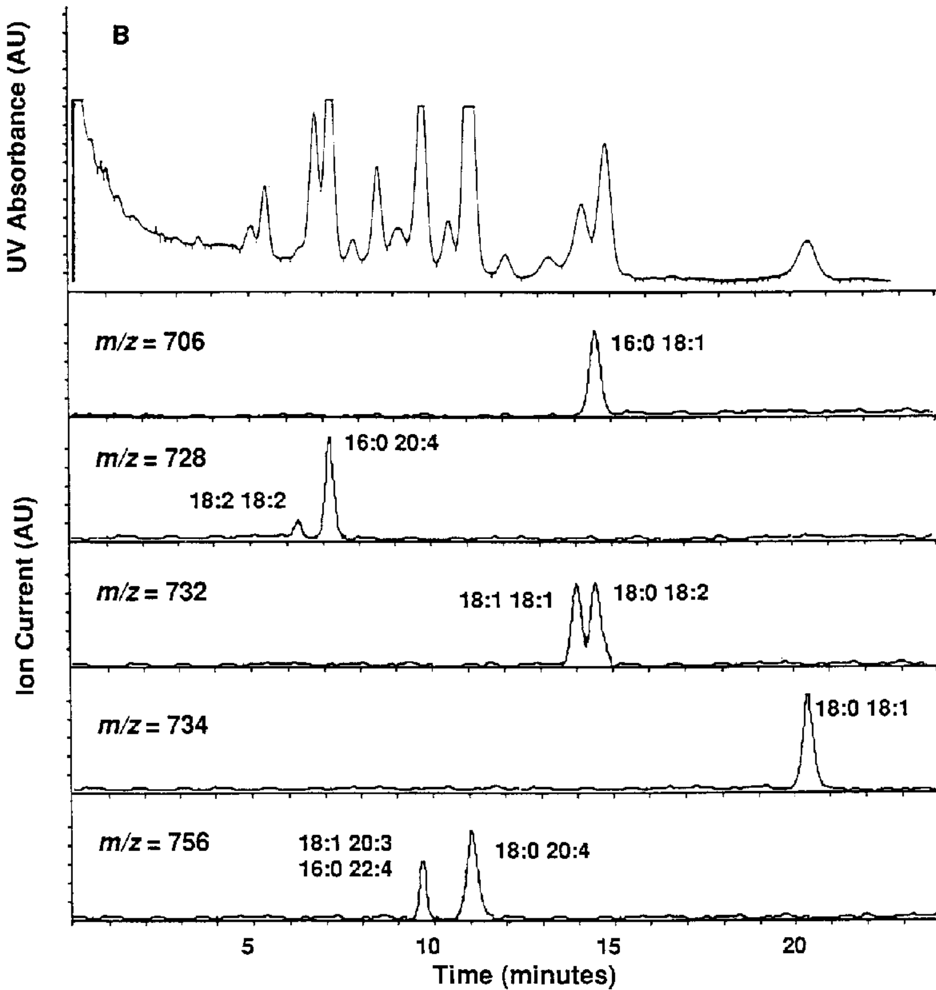

Fig. 6. HPLC separation of glycerobenzoate derivatives of (A) erythrocyte diacylphosphatidylethanolamine and (B) erythrocyte 1-alkenyl-2-acylphosphatidylethanolamine. Detection by UV absorbance at 230 nm is shown as well as SIR of the sodium adduct of major molecular species and the arachidonoyl monoacylglyceride fragment (20:4 MG).

masses for a number of species, the use of a prediction scheme is useful as a first step in identification. An example is illustrated by the two peaks at *m/z* 748. According to the prediction scheme, the retention time of 18:1 18:1 is expected to be shorter than that of 18:0 18:2. This identification could be confirmed by detection of monoglyceride peaks which showed that the first peak contained only 18:1 MG while the second peak contained 18:0 MG and 18:2 MG. This identification by the monoglyceride fragments is especially helpful if compounds have an identical mass and are not separated by HPLC such as 18:1 20:3 and 16:0 22:4, both

with a sodium adduct mass of *m/z* 772. The monoglyceride fragments (16:0 MG, 18:1 MG, 20:3 MG and 22:4 MG) identified the presence of these two species. The total amount of these two species was readily determined by their UV absorbance, but only an estimate on the relative amount of each individual species could be made since no standards were available for calibration.

The 20:4 MG is shown as an example for a monoglyceride tracing. Diacyl PE contains three major species with arachidonic acid (20:4): 18:1 20:4 (only shown in UV trace), 16:0 20:4 (*m/z* 744), and 18:0 20:4 (*m/z* 772).

The monoglyceride peaks can also be used to identify the positional isomers as decribed above. This can only be accomplished, however, if a standard curve as described in Fig. 4 can be made. We were only able to do so for a limited number of species such as 16:0 18:1 and 16:0 18:0.

The retention time of the diacyl glycerobenzoates was significantly shorter than that of the alkenylacyl glycerobenzoates (compare the UV traces of Fig. 6A and B). Although most of the species found in one subclass can also be found in the other, the relative amount can vary significantly [29]. A problem for the quantitation of the alk-1-enylacyl and alkylacyl compounds with the mass spectrometer is the unavailability of these types of phospholipid standards for calibration. Our results on the molecular species composition of the red cell membrane was in good agreement with data recently reported by Myher *et al.* [29], although several minor quantitative differences were noted (not shown).

Taken together, our investigation shows that the use of thermospray MS can greatly facilitate the identification of individual molecular species of complex mixtures of diradyl glycerobenzoates. Furthermore, when appropriate standards are available, different molecular species as well as positional isomers of individual molecular species can be quantified even if they are not separated by HPLC.

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