paper on methodology

Isotopic exchange during derivatization of platelet activating factor for gas chromatography–mass spectrometry

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Abstract One approach to the quantitative analysis of platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycerol-3-phosphocholine; also referred to as AGEPC, alkyl glyceryl ether phosphocholine) is hydrolytic removal of the phosphocholine group and conversion to an electron-capturing derivative for gas chromatography–negative ion mass spectrometry. [$^2H_3$]Acetyl-AGEPC has been commonly employed as an internal standard. When 1-hexadecyl-2-[$^2H_3$]acetyl glycerol (obtained by enzymatic hydrolysis of [$^2H_3$]-C16:0 AGEPC) is treated with pentafluorobenzoyl chloride at 120°C, the resulting 3-pentafluorobenzoate derivative shows extensive loss of the deuterium label. This exchange is evidently acid-catalyzed since derivatization of 1-hexadecyl-2-acetyl glycerol under the same conditions in the presence of a trace of ^{2}HCl results in the incorporation of up to three deuterium atoms. Isotope exchange can be avoided if the reaction is carried out at low temperature in the presence of base. Direct derivatization of [$^2H_3$]-C16:0 AGEPC by treatment with pentafluorobenzoyl chloride or heptafluorobutyric anhydride also results in loss of the deuterium label. The use of [$^{13}C_2$]-C16:0 AGEPC as an internal standard is recommended for rigorous quantitative analysis. —**Haroldsen, P. E., S. J. Gaskell, S. T. Weintraub, and R. N. Pinckard.** Isotopic exchange during derivatization of platelet activating factor for gas chromatography–mass spectrometry. *J. Lipid Res.* 1991. **32:** 723–729.

Supplementary key words deuterium exchange • electron capture mass spectrometry • quantitative analysis • carbon-13 labeling

Platelet activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycerol-3-phosphocholine) is a lipid mediator implicated in inflammation and related physiological and pathological processes. (PAF is also known as AGEPC, acetyl glyceryl ether phosphocholine, and this name is used in this paper when the chemical structure, rather than the biological properties, is of primary importance.) Improved understanding of the biological roles of PAF is dependent on the availability of accurate and precise means for its quantification. Hitherto, determination of PAF production has most commonly been achieved by bioassay (1–3) or by measurement of [^{3}H]acetate incorporation (4, 5). The specificity of these methods is in doubt, however, and they necessarily disregard the molecular heterogeneity of PAF (C_{15}–C_{22} alkyl chain lengths at the 1-position have been reported: see reference 6 for a review). Analyses for molecular species of PAF/AGEPC have been described using gas chromatography-mass spectrometry (GC-MS) after dephosphorylation and derivatization (7–11). Optimal sensitivities of detection have been obtained using negative ion MS detection of strongly electron capturing derivatives, such as the heptafluorobutyrate (HFB) (10) and pentafluorobenzoate (PFB) (9, 11). Rigorous quantification has been reported using a [$^2H_3$]acetyl-analogue as internal standard; this is readily prepared by deuteroacetylation of 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine.

Recent work in our laboratories by Haroldsen and Gaskell (12) has included the development of a new approach to the quantitative analysis of AGEPC, using fast atom bombardment/tandem MS. High sensitivities and selectivities of detection were observed. As a component of the validation of this new procedure, analyses using the GC-electron capture MS method were also performed to permit a comparison of the quantitative results obtained by the two methods. When the originally published procedure (9) for PFB derivatization was used, we observed unexpected and poorly reproducible response ratios during GC-MS of derivatives of standard mixtures of C16:0-AGEPC and the [$^2H_3$]acetyl analogue.

Abbreviations: PAF, platelet-activating factor; AGEPC, alkyl glyceryl ether phosphocholine; GC-MS, gas chromatography–mass spectrometry; PFB, pentafluorobenzoyl; HFB, heptafluorobutyroyl; TLC, thin-layer chromatography; CI, chemical ionization; SIM, selected ion monitoring.

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Parallel findings have been made in the San Antonio laboratory, in which a new, direct method for the derivatization of AGEPC has been developed (13, 14). Treatment of AGEPC with pentafluorobenzoyl chloride in a one-step reaction yielded the 1-*O*-alkyl-2-acetyl-3-*O*-pentafluorobenzoyl-*sn*-glycerol required for GC-MS analysis. When the reaction was performed using [$^2H_3$]acetyl-AGEP, unexpected isotopic compositions of the derivative were observed.

Here we describe additional experiments to clarify these findings and to develop strategies to avoid systematic quantitative errors in the quantification of AGEPC using isotope dilution and GC-MS.

MATERIALS AND METHODS

Materials

1-*O*-Hexadecyl-2-acetyl-*sn*-glycerol-3-phosphocholine (C16:0-AGEPC), 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine, Tris base, phospholipase C (*Bacillus cereus*, Type XIII) and ^{2}HCl (35% solution in ^2H_2O) were from Sigma (St. Louis, MO). 1-*O*-[^{3}H]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine ([^{3}H-C16:0-AGEPC; 45 Ci/mmol) was obtained from New England Nuclear (Boston, MA). [$^2H_3$]acetyl-C16:0-AGEPC was prepared from 1-*O*-hexadecyl-*sn*-glycerol-3-phosphocholine as previously described (12). [$^{13}C_2$-acetyl]-C16:0-AGEPC was prepared in an analogous manner using [$^{13}C_4$]acetic anhydride (Isotec, Miamisburg, OH). Pentafluorobenzoyl chloride, heptafluorobutyric anhydride, pyridine, triethylamine, dimethylaminopyridine and diisopropylamine were from Aldrich (Milwaukee, WI). Thin-layer chromatography (TLC) plates (silica gel GHL, 250 μm thickness) were purchased from Analtech (Newark, DE) and were heat-activated prior to use (100°C, > 2 h).

Two-stage hydrolysis and derivatization of C16:0-AGEPC

Hydrolysis and derivatization procedures used 0.5–10 μg of C16:0-AGEPC, incorporating 10^5 dpm of the ^{3}H-labeled analogue. Standard curves typical of those required for quantitative analyses (12) used mixtures of 0–312 ng of C16:0-AGEPC and 100 ng of [$^2H_3$]C16:0-AGEPC. C16:0-AGEPC was converted to 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol as previously described (15). Thus, to the dry sample were added 1 ml of 0.1 M Tris-HCl buffer (pH 7.5), 10 units of phospholipase C, and 2 ml of diethyl ether. The two-phase mixture was shaken for 2 h, after which the ether layer was removed and taken to dryness under a stream of nitrogen.

Two procedures were evaluated for the conversion of dephosphorylated C16:0-AGEPC to the pentafluorobenzoyl derivative.

Method A (9). The sample was dissolved in 200 μl of pentafluorobenzoyl chloride in a screw-capped tube and heated to 120°C for 45 min. The excess reagent was removed under a stream of nitrogen and the residue was dissolved in hexane for GC–MS analysis. In one experiment, 2 μl of aqueous ^{2}HCl was added to the derivatization reaction to assess the incorporation of deuterium.

Method B. The sample was dissolved in 100 μl of ice-cold hexane–pyridine 73:27 (v/v); alternatively, molar equivalents of triethylamine, dimethylaminopyridine, or diisopropylethylamine were substituted for pyridine. To this was added 100 μl ice-cold hexane–pentafluorobenzoyl chloride 50:50 (v/v), with vigorous shaking of the sample as it solidified. The sample was allowed to warm to room temperature and was mixed with 3 ml of water and 1.5 ml of hexane. The hexane layer was recovered and placed at < −25°C for 30 min. The solution was removed from precipitated material and placed in a fresh tube on ice. Pyridine (0.3 ml) and water (3 ml) were added with vortex mixing. After centrifugation, the hexane layer was recovered and washed with 1.0 ml 0.5 M sodium carbonate buffer (pH 9.0). The solution was taken to dryness and redissolved in hexane for GC–MS analysis.

Yields of the pentafluorobenzoyl derivative were assessed by TLC of the reaction products after derivatization of [^{3}H]C16:0-AGEPC. The solvent system was benzene–hexane–diethyl ether 50:40:8 (v/v/v). Underivatized 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol remained at the origin; the pentafluorobenzoyl derivative appeared at R_f 0.45–0.6. One-centimeter bands of silica were removed to scintillation vials; methanol (0.5 ml) and scintillant were added prior to scintillation counting.

Direct derivatization of C16:0-AGEPC

Direct derivatization was performed as described previously (13, 14). For samples of 0–20 μg, a chloroform solution of the AGEPC homologue or analogue was dried under a stream of nitrogen in a 10-ml tube, and 100 μl of pentafluorobenzoyl chloride was added. The tube was sealed with a Teflon-lined screw cap and incubated at 125°C for 4 h. The volatile reactants were then evaporated by a stream of nitrogen at 60°C, and the residue was partitioned between 400 μl of hexane and 400 μl of water. The water layer was washed twice with 200 μl of hexane. The organic phases were combined, dried under a stream of nitrogen, and redissolved in hexane for GC–MS analysis.

Formation of the heptafluorobutyroyl (HFB) derivative using heptafluorobutyric anhydride was accomplished in an analogous manner to the PFB reaction, with the following exceptions: incubation was at 40°C; removal of the residual reagent was accomplished by a nitrogen stream at room temperature; and the combined organic phase was washed twice with 200 μl of water.

Mass spectrometry

Baylor laboratory. Fast atom bombardment/tandem MS analyses were performed using a VG ZAB SEQ hybrid instrument (VG Analytical, Manchester, U.K.) as previously described (12). GC–MS analyses were performed using a HP-5890 gas chromatograph (Hewlett-Packard, Avondale, PA) coupled to a VG TS-250 mass spectrometer (VG Analytical, Manchester, U.K.). Separations were made on an open-tubular fused silica capillary column (7 m × 0.25 mm, i.d.) of the DB-5 bonded phase type (J & W Scientific, Rancho Cordova, CA), with temperature programming, 200–300°C, 4°C/min, or 240–300°C, 6°C/min. Helium was the carrier gas. Sample introduction used an all-glass falling needle injector (Allen Scientific, Boulder, CO). Ionization was by electron capture, using methane as moderator gas. The ion source temperature was 160°C. Data acquisition was under the control of the VG 11-250 data system.

Syntex laboratory. GC–MS analyses were performed using a HP-5890A gas chromatograph coupled to a TSQ-70 triple quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA). Separations were made on an open-tubular fused silica DB-5 capillary column (30 m × 0.25 mm, i.d.), with temperature programming, 90–290°C, 40°C/min. Helium was the carrier gas. Sample introduction was by splitless injection. Analyses were performed in the electron capture mode, using methane as moderator gas. The ion source temperature was 160°C.

San Antonio laboratory. GC–MS analyses were performed using a Finnigan MAT model 4615 mass spectrometer in combination with an INCOS data system (Finnigan MAT, San Jose, CA). Gas chromatographic separations were accomplished using a BP-1 fused silica column (12 m × 0.32 mm, i.d.; Scientific Glass Engineering, Austin, TX), which was connected directly to the mass spectrometer via a short length of 0.25 mm deactivated fused silica tubing. The linear velocity of the helium carrier gas was approximately 80 cm/sec. The column temperature was initially maintained at 180°C for 1 min, and was then increased to 250°C at a rate of 30°C/min. The injector temperature was 250°C; split or splitless modes of injection were used, according to sample size. Analyses were performed in the electron capture mode, using methane as the moderator gas, or in the positive chemical ionization (CI) mode with methane serving as the reagent gas. The ion source temperature was 100°C.

RESULTS

In agreement with earlier reports (9, 11), GC–negative ion MS analysis of the PFB derivative of 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol (obtained from C16:0-AGEPC) yielded a spectrum in which the molecular anion, $M^{-\cdot}$, was the single prominent feature. No change in the appearance of the spectrum was observed when the ion source temperature was varied between 100°C and 200°C. Initial quantitative analyses in the Baylor laboratory involved dephosphorylation of standard mixtures of C16:0-AGEPC and [$^2H_3$]C16:0-AGEPC and derivatization using the procedure of Ramesha and Pickett (9). Thus, samples were dissolved in pentafluorobenzoyl chloride and heated to 120°C for 45 min. **Fig. 1A** shows the dual selected ion monitoring analysis of a standard mixture with an expected molar ratio of approximately 1.7/1 for C16:0-AGEPC/[$^2H_3$]C16:0-AGEPC. The ions monitored correspond to the respective $M^{-\cdot}$ species. Each SIM trace shows two peaks, corresponding to partial isomerization of the natural 1,2-configuration to the earlier eluting 1,3-form. The response ratio, C16:0-AGEPC/[$^2H_3$]C16:0-AGEPC, was determined after summation for each analyte of the areas correponding to the 1,2- and 1,3-isomers. The observed value of 19.2 is approximately an order of magnitude higher than expected. When an aliquot of the same standard mixture was derivatized at 0°C in the presence of pyridine (see Experimental section), subsequent GC–MS analysis (Fig. 1B) gave a response ratio corresponding to the expected molar ratio. In addition, the milder reaction conditions promoted a lesser conversion to the 1,3-isomer. The yield of the derivative, as judged by scintillation counting of the appropriate TLC fraction after hydrolysis and derivatization of [^{3}H]C16:0-AGEPC, was similar using both procedures (approximately 90% overall). Equivalent results were obtained using alternative bases as components of the derivatization mixture (dimethylaminopyridine, triethylamine, diisopropylethylamine); pyridine was selected for routine use.

A more detailed study of the effect of derivatization conditions on the analysis of authentic mixtures was performed with the generation of standard curves. **Table 1** shows the results of linear regression of response ratios (analyte/internal standard) on quantity of analyte, using analytical data obtained after two of the derivatization procedures. Also shown are the equivalent data recorded by FAB/tandem MS analyses (12) of the standard mixtures prior to dephosphorylation and derivatization. Good agreement was observed between the latter data and those obtained by GC–MS analyses of the standards derivatized at low temperature in the presence of base. The data obtained after derivatization at 120°C in pure reagent were again significantly different; high values were observed for both slope and intercept, and the low correlation coefficient suggested poor reproducibility (using the same batch of reagent).

These results suggest that exchange of the deuterium label in [$^2H_3$]AGEPC can occur under the conditions of PFB formation originally proposed for this quantitative method (9). We assessed the possibility that the exchange may be catalyzed by traces of acid present in the acylation

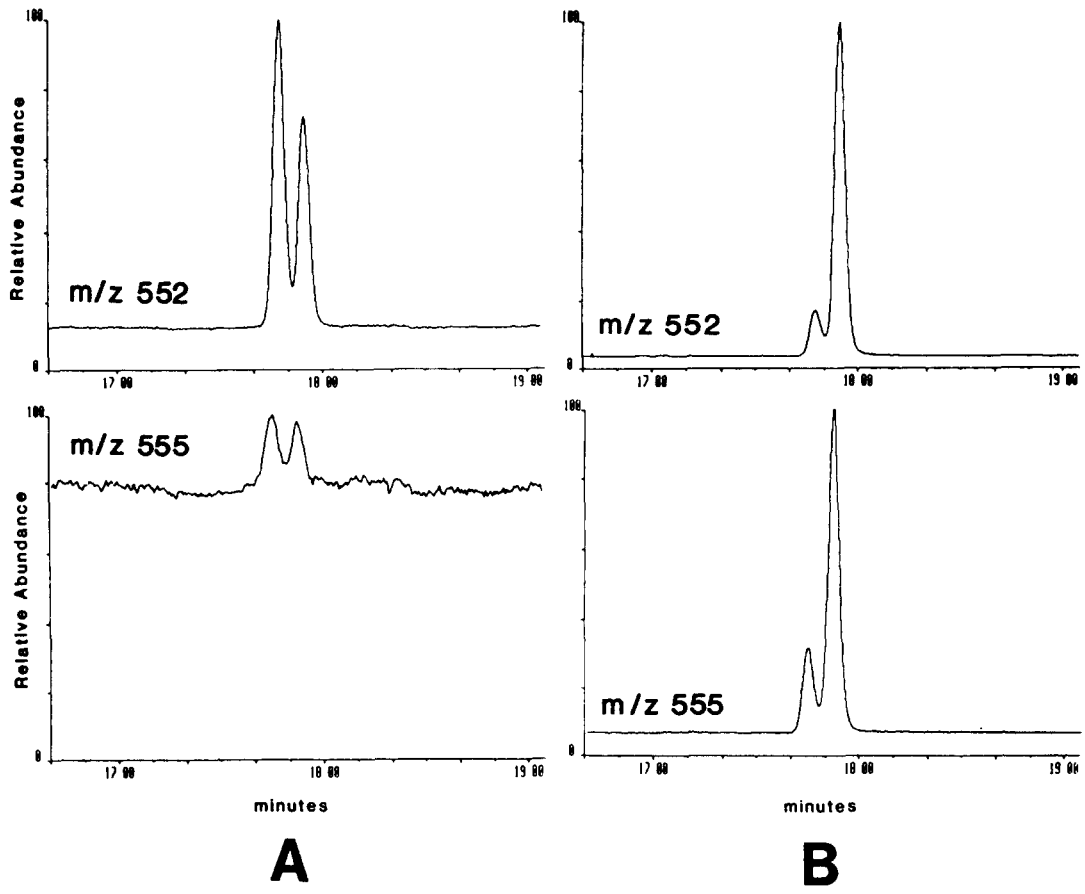

Fig. 1. GC-MS/dual selected ion monitoring (SIM) analyses of the PFB derivative of the product of enzymatic hydrolysis of a mixture of C16:0-AGEPC and the [$^2H_3$]analogue (molar ratio, 1.7:1). $M^{-\cdot}$ ions were monitored. In each case, the SIM traces are individually normalized. A, derivatization with pentafluorobenzoyl chloride at 120°C; response ratio for *m/z* 552:*m/z* 555 = 19.2:1. B, derivatization with pentafluorobenzoyl chloride-pyridine-hexane 2:1:4 (by volume) at 0°C; response ratio for *m/z* 552:*m/z* 555 = 1.7:1. (Data obtained in the Baylor laboratory; see Materials and Methods section for details).

reagent by derivatizing 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol (obtained by enzymic hydrolysis of C16:0-AGEPC) at 120°C in pentafluorobenzoyl chloride containing a trace of ^{2}HCl. **Fig. 2** shows the $M^{-\cdot}$ region of the negative ion mass spectrum recorded during GC-MS analysis of the derivatized product; the isotopic distribution observed for the $M^{-\cdot}$ ion clearly indicates extensive isotope exchange involving three (and only three) hydrogens.

Experiments in the San Antonio laboratory have indicated that loss of deuterium from [$^2H_3$]AGEPC also takes place under conditions employed for the direct conversion of AGEPC to the PFB derivative of 1-*O*-alkyl-2-

TABLE 1. Standard curve data obtained by FAB/tandem MS of authentic mixtures of C16:0-AGEPC (0-312 ng) and [$^2H_3$]-C16:0-AGEPC (100 ng) and by GC-MS of the derivatized mixtures[a]

	Standard Curve[b]		
Analytical Method	100 × Slope	Intercept	Correlation Coefficient (*r*)
FAB/tandem MS	0.68	0.0842	0.992
GC-MS (method A)[c]	4.71	5.433	0.858
GC-MS (method B)[d]	0.73	− 0.0029	0.996

[a]Data obtained in the Baylor laboratory; see Materials and Methods section for details.
[b]Linear regression of response ratio (analyte/internal standard) on quantity of analyte.
[c]GC-MS analysis following enzymatic hydrolysis and derivatization with pentafluorobenzoyl chloride at 120°C.
[d]GC-MS analysis following enzymatic hydrolysis and derivatization with pentafluorobenzoyl chloride-pyridine-hexane (2:1:4, by volume), 0°C.

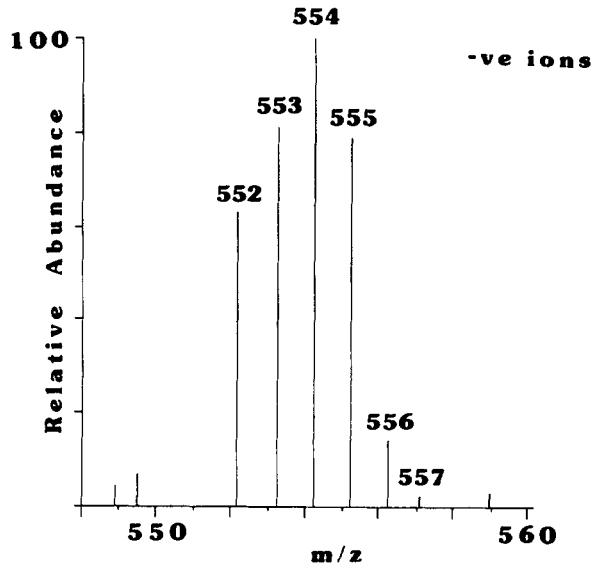

Fig. 2. Partial mass spectrum showing the $M^{-\cdot}$ region of the PFB derivative prepared by treatment at 120°C of 1-hexadecyl-2-acetyl-glycerol with pentafluorobenzoyl chloride containing a trace of ^2HCl. (Data obtained in the Syntex laboratory; see Materials and Methods section for details.)

acetyl glycerol. **Fig. 3** shows the GC-MS analysis of the PFB derivative of [$^2H_3$]C16:0-AGEPC prepared in this way. The reconstructed ion chromatograms (Fig. 3A) for *m/z* 552, 553, 554, and 55 indicate exchange of 1-3 hydrogens. It is noteworthy also that the direct derivatization procedure results in negligible isomerization of the 1-*O*-alkyl-2-acetyl-precursor to the 1-*O*-alkyl-3-*O*-acetyl-form, confirming earlier findings (13). The relative proportions of the labeled species after derivatization are more readily apparent from the summed spectrum (Fig. 3B) covering the $M^{-\cdot}$ region.

As reported previously (13, 14), direct conversion of AGEPC to the HFB derivative of 1-*O*-alkyl-2-acetyl glycerol may be achieved by heating with heptafluorobutyric anhydride in a manner analogous to formation of the PFB derivative. Experiments with [$^2H_3$]C16:0 AGEPC have indicated that isotopic exchange is extensive under these derivatization conditions. Electron capture negative ion MS of the HFB derivative yields no detectable $M^{-\cdot}$ ion but positive ion chemical ionization (CI) gives an abundant MH^+ ion (13). GC-CIMS analysis of the HFB derivative of [$^2H_3$]C16:0AGEPC (**Fig. 4**) provided little evidence of the original isotopic labeling of the precursor, indicating essentially complete exchange in this instance.

Finally, the utility of a ^{13}C-labeled analogue as an internal standard is evident from the electron capture MS analysis of the PFB derivative obtained by direct derivatization of [$^{13}C_2$-acetyl]C16:0 AGEPC (**Fig. 5**). The expected isotope pattern is observed.

DISCUSSION

For quantitative analysis of AGEPC based on mass spectrometry, the use of the [$^2H_3$-acetyl]-labeled ana-

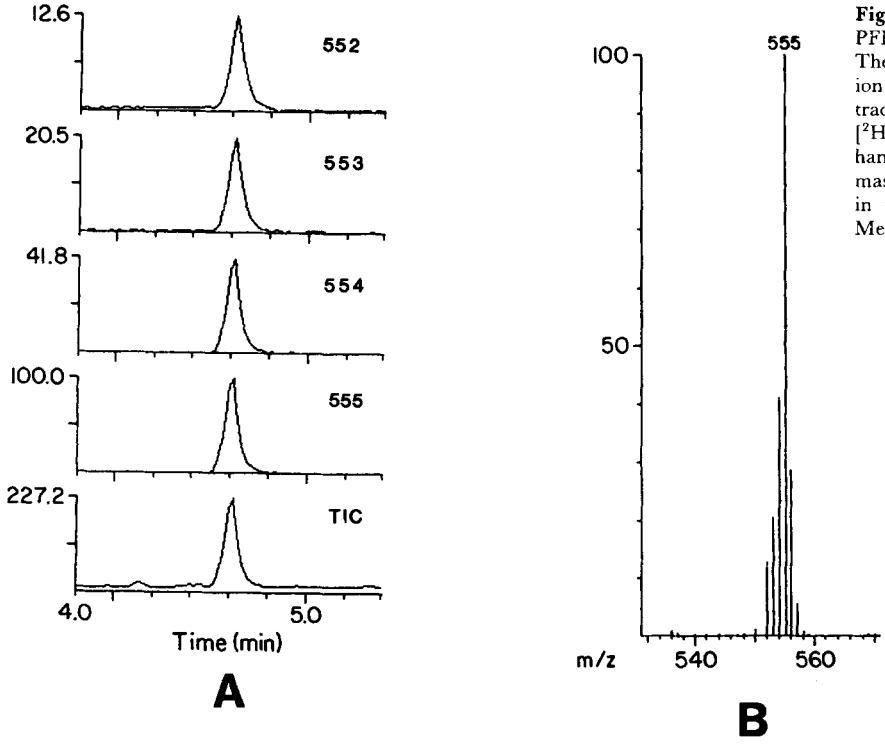

Fig. 3. GC-MS analysis of the product of direct PFB derivatization of [$^2H_3$]C16:0 AGEPC (10 μg). The left-hand portion of the figure (A) shows the total ion current chromatogram and selected ion retrieval traces corresponding to the detection of [$^2H_0$]- to [$^2H_3$]-species (split injection of ca. 800 ng). The right-hand portion (B) shows the corresponding summed mass spectrum of the PFB derivative. (Data obtained in the San Antonio laboratory; see Materials and Methods section for details.)

logues as internal standards is attractive because of the ease and economy of their preparation from lyso-precursors. Our data indicate, however, that serious quantitative inaccuracies can arise as a result of isotopic exchange during the preparation of derivatives commonly employed for GC-MS analyses. Thus, conversion of [$^2H_3$]AGEPC to the PFB derivative, after hydrolysis of the phosphocholine moiety, is accompanied by extensive loss of deuterium label when the reaction is performed in pentafluorobenzoyl chloride at elevated temperatures. Isotopic exchange is apparently catalyzed by the presence of acid; minor amounts of acid may be present in the reagent due to hydrolysis during storage. We observed the extent of exchange to be somewhat variable; it was eliminated in the present study by performing the derivatization at low temperature in the presence of base. In addition to serving as an acid scavenger, the base may be effective as a catalyst of the acylation reaction. A recent analysis for AGEPC in human saliva (11) used the [$^2H_3$-acetyl]C16:0-AGEPC as internal standard and used pentafluorobenzoyl chloride–toluene–pyridine 1:5:2 for derivatization; no isotopic exchange was reported.

Similar problems of isotopic exchange are encountered in the application of the recently introduced (13, 14) direct derivatization of AGEPC, in which conversion to PFB or HFB derivatives of 1-*O*-alkyl-2-acetyl glycerol is achieved by a single-stage treatment with the appropriate acyl chloride or anhydride. In this instance it is clearly preferable to use ^{13}C-labeled internal standards for rigorous quantification.

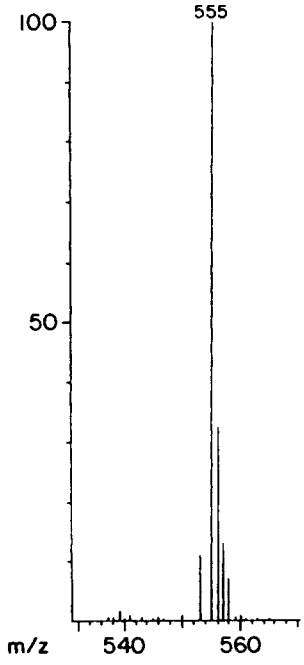

Fig. 4. Partial positive ion chemical ionization mass spectrum of the HFB derivative obtained by direct derivatization of [$^2H_3$]C16:0 AGEPC (15 μg; ca. 350 ng injected). (Data obtained in the San Antonio laboratory; see Materials and Methods section for details.)

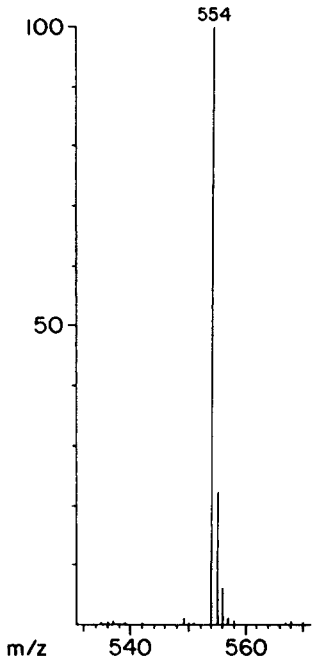

Fig. 5. Partial negative ion electron capture mass spectrum of the PFB derivative obtained by direct derivatization of [$^{13}C_2$]C16:0 AGEPC (5 μg derivatized, ca. 50 ng injected). (Data obtained in the San Antonio laboratory; see Materials and Methods section for details.)

Isotopic exchange of alpha-hydrogens in carboxylic acid esters has been studied extensively (and is reviewed in reference 16); a possible mechanism involves abstraction of the alpha-hydrogen promoted by the presence of acid (17). The discrepancies between the present findings and the success of the original derivatization procedure in quantitative applications (9) may be attributable to a variability in the extent of acid contamination of acylation reagents (as commercially obtained). It is clear, however, that caution is required in the validation of quantitative GC-MS procedures for AGEPC based on the use of [$^2H_3$-acetyl]-analogues as internal standards.

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