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Quantitation of the fatty acid composition of phosphatidic acid by capillary gas chromatography electron-capture detection with picomole sensitivity

Christopher Major, Bryan A. Wolf*

Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 217 John Morgan, Philadelphia, PA 19104, USA

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Abstract

We describe a relatively simple and sensitive method to measure femtomole amounts of phosphatidic acid in cells. Phosphatidic acid was extracted from cells in the presence of 1-heptadecanoyl-2-heptadecanoyl-*sn*-glycero-3-phosphate as an internal standard, purified by two-dimensional thin-layer chromatography, and hydrolyzed to its constituent free fatty acids which were then derivatized to the corresponding pentafluorobenzyl esters. Pentafluorobenzyl esters of fatty acids were analyzed by gas chromatography with electron-capture detection. Long-chain fatty acids were resolved with excellent signal-to-noise ratios. Using heptadecanoic acid as an internal standard for quantitation, as little as 1 fmol of pentafluorobenzyl ester of stearic acid was detected with a linear response up to 10 pmol. Linear detector responses were obtained for all major classes of fatty acids. For phosphatidic acid measurement, the detection limit was at least 50 fmol thus achieving a 1000-fold increase in sensitivity compared to the most sensitive of the previously described methods. An example is provided of quantitating phosphatidic acid from minute amounts of biological samples such as islets of Langerhans.

1. Introduction

Phosphatidic acid is an intracellular molecule which has recently been implicated in a number of signal transduction events [1,2]. After agonist stimulation of cells, phosphatidic acid may accumulate by various mechanisms which include phospholipase D activation, de novo synthesis from glucose or glycerol, and diacylglycerol kinase phosphorylation of diacylglycerol (a second messenger released by phospholipase C) [1–4]. In order to implicate phosphatidic acid accumulation in a signal transduction pathway, most investigators will measure agonist-induced phosphatidic acid accumulation. A widely used approach is to label cells with a radioactive precursor such as a fatty acid or phosphate, and quantitate phosphatidic acid accumulation after TLC separation, although radiolabelled pools may not always reflect endogenous pools [3,5–7].

Alternative methods have also been described which measure the mass of phosphatidic acid. Mass measurements have been performed by colorimetric assay [8], by immunoassay with a

* Corresponding author.

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monoclonal antibody to phosphatidic acid [9], by TLC separation and flame-ionization detection using the Iatroscan system [10], by short-bed/continuous-development TLC followed by photodensitometric analysis of charred lipid spots [11], by an enzymatic technique which involves methylamine transacylation of phosphatidic acid to glycerol-3-phosphate measured enzymatically [12], by Coomassie Blue staining and densitometry [13], and by quantitation of the fatty acid composition of phosphatidic acid [14,15]. The most sensitive of these methods can detect 50 pmol of phosphatidic acid [11,12].

Lipid-derived second messengers have been implicated in insulin secretion by the ß cell of the islets of Langerhans [16]. In particular, agonists which stimulate insulin secretion result in phosphatidic acid accumulation as assessed by radiolabeling techniques [17–20]. Mass measurements of endogenous lipid-derived mediators in islets of Langerhans are technically challenging due to the very limited amount of tissue available. Typically, low abundance lipids in these cells have been measured by mass spectrometric techniques to obtain a sufficient degree of sensitivity [21–24]. We now report the development of an assay to measure phosphatidic acid mass and its fatty acid composition with femtomole sensitivity. The principle of the assay is based on extraction of the lipids in the presence of an internal standard for phosphatidic acid (C17:0-PA, 1-heptadecanoyl-2-heptadecanoyl-*sn*-glycero-3-phosphate[2]), separation by two-dimensional TLC, hydrolysis of phosphatidic acid to its constituent unesterified fatty acids, derivatization to the corresponding fatty acyl pentafluorobenzyl esters, and analysis by gas chromatography with electron-capture detection.

2. Experimental

2.1. Chemicals

The internal standard 1-heptadecanoyl-2-heptadecanoyl-*sn*-glycero-3-phosphate (C17:0-PA) and all other phospholipids were purchased from Avanti Polar Lipids (Pelham, AL, USA). Fatty acids were obtained from Nu-Chek-Prep (Elysian, MN, USA). Pentafluorobenzyl bromide was from Regis Chemical Company (Morton Grove, IL, USA) or Sigma Chemical Company (St. Louis, MO, USA). N,N-diisopropylethylamine, Rhodamine G, and butylated hydroxytoluene were from Sigma. Dimethoxyethane, lithium hydroxide, and all solvents (Optima or HPLC grade) were from Fisher Scientific (Pittsburgh, PA, USA). Dimethyldichlorosilane was purchased from Alltech Associates (Deerfield, IL, USA).

2.2. Islet isolation and culture

Islets were isolated aseptically from 4–8 male Sprague–Dawley rats as previously described [25–27]. In brief, the bile duct was cannulated and the pancreas was inflated with approximately 20 ml of Hanks balanced salt solution (Sigma) supplemented with penicillin (25 U/ml) and streptomycin (25 μg/ml). The distended pancreas was then excised. Lymph nodes, fat, blood vessels and bile duct were removed under a stereomicroscope. The tissue was then chopped, rinsed extensively with Hanks' solution and then digested with collagenase P (Boehringer-Mannheim, Indianapolis, IN, USA) (3–6 mg/ml of tissue) at 39°C for 4.5–6.5 min. The digested tissue was then rinsed with Hanks solution and then purified by centrifugation on a discontinuous Ficoll gradient [dialyzed and lyophilized; 4 layers of 27%, 23%, 20.5% and 11% in Hanks' Hepes (25 m*M*) buffer]. The majority of the islets rose to the 11–20.5% interface. Islets were harvested, and washed in "complete" CMRL-1066 (Gibco, Grand Island, NY, USA) culture medium (supplemented with 10% heat-inactivated newborn bovine serum, 2 m*M* L-glutamine, 50 U/ml of penicillin and 50 μg/ml of streptomycin, and containing 5.5 m*M* D-glucose). This procedure typically provided 350–400 islets per rat [25–27]. Islets were then cultured in complete CMRL-1066 (Gibco) at 5.5 or 17 m*M*

glucose for 7 days at 37°C under an atmosphere of 95% air–5% CO_2.

2.3. Phospholipid extraction

Following incubation, cells were quenched with 1.5 ml of ice-cold methanol (supplemented with 0.375 mg/ml of butylated hydroxytoluene) and transferred to silanized disposable round-bottom Pyrex borosilicate 13 × 100 mm screw-cap tubes (Corning catalog No. 99449-13, purchased from Fisher). Tubes were capped with open-top closures (Kimble catalog No. 73806E-13425, Fisher) and PTFE/silicone septa (Kimble catalog No. 73818B-13). (Silanization was performed with fresh 10% dimethyldichlorosilane in toluene, followed by two toluene and two methanol rinses.) Blank tubes were also processed in parallel from this step on. The internal standard (C17:0-PA, 6 nmol in most experiments) was added to each tube. Each tube received chloroform (1 ml) and water (1 ml), was vortex-mixed for 1 min on a Troemmer Multi-Tube Vortexer (1 min), and then sonicated in a water bath sonicator with ice present for 30 min. Tubes were centrifuged for 15 min at 500 *g* at 4°C, and the lower organic phase removed with a silanized Pasteur pipette into a clean silanized Pyrex 13 × 100 mm tube. The remaining aqueous upper phase was back-extracted twice with chloroform. The combined organic phase was washed with water (1 ml) which was discarded after centrifugation, and the organic phase evaporated in a Savant evaporator/concentrator with a −90°C organic trap, reconstituted in chloroform (0.5 ml), vortex-mixed, evaporated, and reconstituted in 15–25 μl of chloroform.

2.4. TLC of phosphatidic acid

Samples were applied to 10 × 10 cm high-performance HP-K silica gel TLC plates (Whatman Biosystems, Clifton, NJ, USA) which had been activated 30 min at 110°C. Plates were developed in the first dimension with chloroform–methanol–ammonium hydroxide (65:35:5.5; v/v) for 20–30 min. Plates were carefully dried (60 min) and then developed in the second dimension with chloroform–methanol–formic acid–water (55:28:5:1; v/v) for 20–30 min [28,29]. During the periods of high humidity of spring and summer, we found it necessary to desiccate the plates under nitrogen at room temperature in between developments to remove any water absorbed onto the plates. Under these conditions, excellent separation of phosphatidic acid from other phospholipids is obtained [20]. The following peaks were typically identified and localized using cold and radioactive commercial standards: phosphatidylcholine (R_{F1} in the first dimension: 0.19, R_{F2} in the second dimension: 0.38), lysophosphatidylcholine (R_{F1}: 0.03, R_{F2}: 0.11), phosphatidylethanolamine (R_{F1}: 0.31, R_{F2}: 0.63), sphingomyelin (R_{F1}: 0.09, R_{F2}: 0.23), phosphatidylserine (R_{F1}: 0.06, R_{F2}: 0.39), phosphatidylinositol (R_{F1}: 0.01, R_{F2}: 0.28), free fatty acid (R_{F1}: 0.28, R_{F2}: 0.85), phosphatidic acid (R_{F1}: 0.08, R_{F2}: 0.63).

2.5. Hydrolysis of phosphatidic acid to unesterified fatty acids

After TLC, each plate was sprayed with Rhodamine G (0.0012% in water), and the phosphatidic spot localized with a hand-held fluorescent light. The corresponding silica spot (0.5 × 0.5 cm) was scraped into a silanized Pyrex borosilicate 13 × 100 mm tube, to which was added dimethoxyethane (0.5 ml) and lithium hydroxide (0.1 ml of a 3 *M* solution in water). Tubes were capped, vortex-mixed (1 min), and incubated 90 min with vigorous shaking at 60°C in a Dubnoff water bath. Tubes were cooled to room temperature, water (3.5 ml) was added, and the pH adjusted to 3.0 with 1 *M* HCl (approximately 280 μl). Methylene chloride (1 ml) was added, the tubes vortex-mixed (1 min), centrifuged (15 min, 500 *g*), and the lower organic phase transferred to a clean silanized Pyrex borosilicate 13 × 100 mm tube. The upper aqueous phase was back-extracted twice with methylene chloride (1 ml). The combined organic phase was evaporated to dryness in the Savant evaporator.

2.6. Derivatization of unesterified fatty acids to pentafluorobenzyl esters

The following steps must be performed in a fume hood. To the dried unesterified fatty acids was added acetonitrile (30 μl and vortex-mixed), 35% pentafluorobenzyl bromide in acetonitrile (10 μl, CAUTION: potent lachrymator), and diisopropylethylamine (10 μl) [30]. The tubes were capped, gently mixed, and incubated 10 min at room temperature in the hood. Samples were then evaporated in the Savant evaporator, and reconstituted in 1 ml of heptane, and vortex-mixed 1 min prior to analysis.

2.7. Gas chromatography–electron-capture detection

The capillary gas chromatography system consisted of a Varian 3400 gas chromatograph with a Varian 1093 septum-equipped programmable (SPI) injector, a Varian 1077 split/splitless capillary injector, a flame ionization detector, and a ^{63}Ni electron-capture detector (Varian Chromatography, Walnut Creek, CA, USA). Data acquisition and analysis was performed on an IBM-compatible computer (486DX2 chip, 50 MHz) running the Varian GC Star Workstation software (version A2). Pentafluorobenzyl esters of the fatty acids samples were analyzed on a 25 m × 0.33 mm I.D., 0.25 μm SGE BPX70 column (Fisher) at a head pressure of 48 kPa using high-purity nitrogen as the carrier gas. On-column injection was performed with the SPI injector equipped with a silanized high-performance insert (Restek Corporation, Bellefonte, PA, USA) and a low-bleed Thermolite septa (Restek). After injection, the injector was programmed from 65°C to 165°C at 150°C/min, and from 165°C to 300°C at 50°C/min. The electron-capture detector was maintained at 300°C. The oven temperature was held at 85°C for 2 min, increased to 165°C at 40°C/min, then to 250°C at 3.5°C/min, and held 1 min at 250°C. Peak identity was assigned by the retention times which were obtained by comparison to standard fatty acid pentafluorobenzyl esters, which were determined systematically before each set of analyses. The fatty acid pentafluorobenzyl esters were quantitated relative to heptadecanoylpentafluorobenzyl ester (C17:0-PFBE). Any signal derived from blank samples was subtracted.

3. Results and discussion

3.1. Fatty acid analysis

Fatty acids were derivatized to their pentafluorobenzyl esters, injected onto the column with a temperature-programmable injector and analyzed by electron-capture detection. Fig. 1 shows a typical chromatogram of the separation of a mix of fatty acids. The major classes including the 18-carbon series were well resolved with excellent signal-to-noise ratio. As illustrated in Fig. 2, when increasing amounts of the pentafluorobenzyl ester of stearic acid were analyzed, the peak area was a linear function of the amount of stearate added. The limit of detection was 1 fmol, with a linear response up to 10 pmol. Although 1 fmol was clearly detected above background, the signal-to-noise ratio was poor. At 10 fmol, however, the peak of stearic acid was several-fold greater than background (Fig. 2). At levels greater than 10 pmol, the electron-capture detector was saturated.

Fig. 3 illustrates a standard curve (50 fmol to 2.5 pmol) of a mix of pentafluorobenzyl esters of fatty acids (C14:0, C16:0, C18:0, C18:1, C20:4) using PFBE-C17:0 as an internal standard with analysis by gas chromatography electron-capture detection. Fatty acids were identified based on their retention time: the area under the peak was expressed as a ratio to that of the internal standard and a standard curve constructed (Fig. 3). The amount of each fatty acid recovered was a linear function of the amount added with a slope close to 1 (myristic acid: slope = 1.15, intercept = −0.01, $r = 0.998$; palmitic acid: slope = 1.09, intercept = 0.08, $r = 0.996$; stearic acid: slope = 0.63, intercept = 0.07, $r = 0.994$; oleic acid: slope = 1.02, intercept = 0.06, $r = 0.995$; arachidonic acid: slope = 0.68, intercept = −0.02, $r = 0.999$).

There have been relatively few reports of fatty

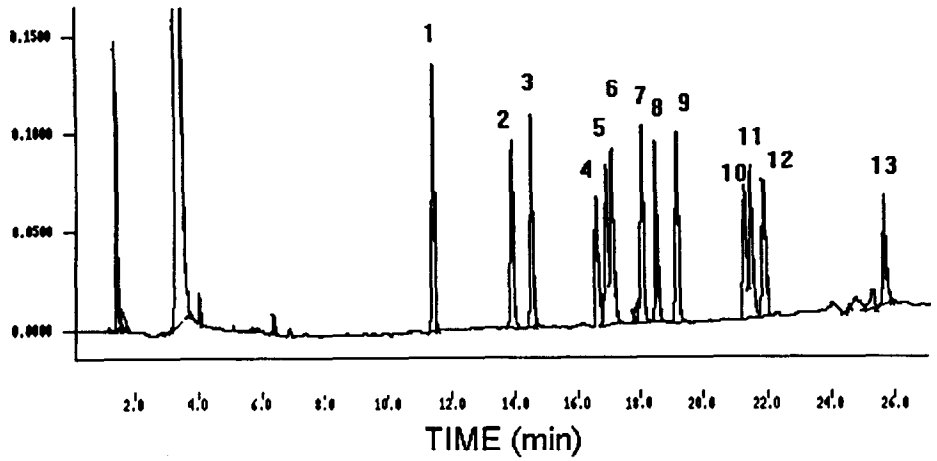

Fig. 1. Chromatogram of a mix of pentafluorobenzyl esters of fatty acids analyzed by gas chromatography with electron-capture detection. A mix of standard fatty acids were derivatized to their pentafluorobenzyl esters and analyzed on a Varian 3400 gas chromatograph with electron-capture detection as described in Experimental. Analysis was performed on an SGE BPX70 column (0.25 μm, 25 m × 0.33 mm I.D.) with a head pressure of 48 kPa using nitrogen as the carrier gas. The temperature-programmable SPI injector was programmed from 65°C to 165°C at 150°C/min, and 165°C to 300°C at 50°C/min. The oven was held at 85°C for 2 min, programmed to 165°C at 40°C/min, then to 250°C at 3.5°C/min, and maintained at 250°C for 1 min. The electron-capture detector was maintained at 300°C. Peaks: 1 = C14:0, myristate; 2 = C16:0, palmitate; 3 = C16:1, palmitoleic acid; 4 = C18:0, stearate; 5 = C18:1, petroselinic acid; 6 = C18:1, oleate; 7 = C18:2, linoleate; 8 = C18:3 ($n-3$), gamma-linolenic acid; 9 = C18:3 ($n-6$), linolenic acid; 10 = C20:3, eicosatrienoic acid; 11 = C20:4, arachidonate; 12 = C20:3, homogamma linolenic acid; 13 = C22:6, docosahexenoic acid. The Y-axis shows the signal in volts.

acid quantitation by gas chromatography with electron-capture detection [31–35]. Most of these reports have focused on the detection of microbial fatty acids for species identification. Ramesha and Taylor have shown that arachidonic acid can be measured by gas chromatography–electron-capture detection with equivalent sensitivity to a radiometric assay [35]. Our study demonstrates the feasibility of measuring fatty acids with femtomole sensitivity using gas chromatography–electron-capture detection. We have used a derivatization method and clean-up technique which results in samples with low background noise by electron-capture detection [30]. In order to maintain low background noise for femtomole sensitivity, we have found it necessary to systematically include blank tubes which are processed identically to monitor background levels. Specifically, the use of disposable, freshly silanized glassware with open closures and Teflon liners, wearing of nitrile gloves (which avoids measuring the skin-derived soaps), high-grade solvents, and low-bleed septa for the gas chromatograph are important factors in maintaining this level of sensitivity. Compared to flame ionization detection, this methodology provides several orders of magnitude greater sensitivity. Pentafluorobenzyl esters of fatty acids can also be quantitated by negative-ion chemical ionization mass spectrometry (typically with a deuterated internal standard) with equivalent sensitivity [30,36]. The advantage of mass spectrometry is the definitive identification of the fatty acid. The disadvantages are the expense and time requirements to acquire and maintain a mass spectrometer.

3.2. Phosphatidic acid analysis

Phosphatidic acid mass may be measured by a variety of techniques which have a range of sensitivity of μmol to approximately 50 pmol [8–15]. We have developed a technique which consists of purification of phosphatidic acid in the presence of C17:0-PA as an internal standard by two-dimensional TLC, hydrolysis of phosphatidic acid to its constituent unesterified fatty acids, derivatization to pentafluorobenzyl esters,

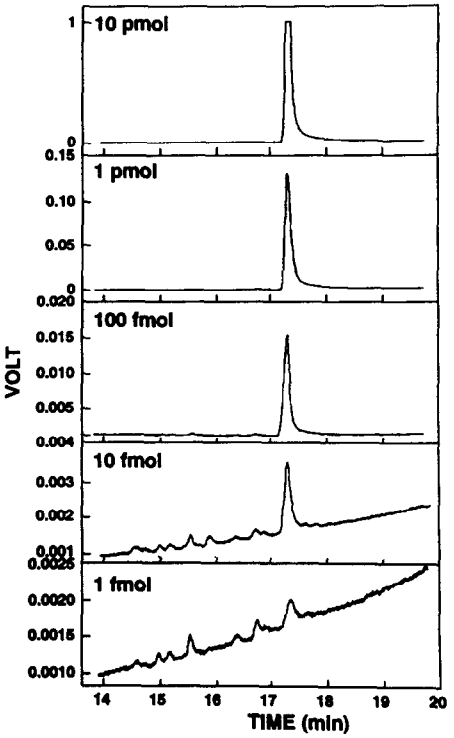

Fig. 2. Response curve of the pentafluorobenzyl ester of stearic acid by gas chromatography with electron-capture detection. Varying amounts of pentafluorobenzyl esters of stearic acid were injected into the gas chromatograph and analyzed by electron-capture detection as described in Experimental. The linear regression line was: counts = $1.1 \cdot 10^{18} \times$ [stearate in moles] − 5530, $r = 0.9999$.

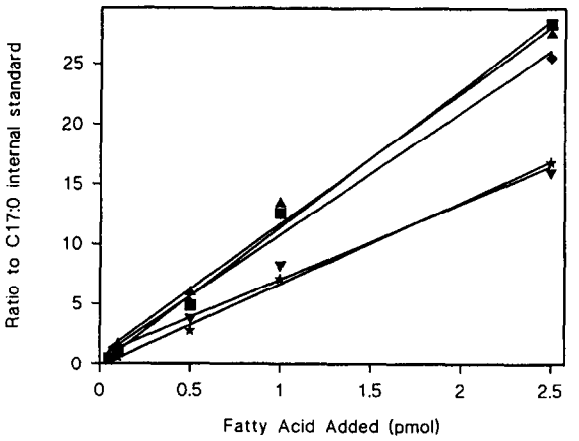

Fig. 3. Standard curve of a mix of pentafluorobenzyl esters of fatty acids analyzed by gas chromatography with electron-capture detection. Various mixes of unesterified long-chain fatty acids (■: myristic acid, ▲: palmitic acid, ▼: stearic acid, ◆: oleic acid, ★: arachidonic acid) which were prepared in the presence of heptadecanoic acid as an internal standard, were derivatized to their corresponding pentafluorobenzyl esters as described in Experimental. Increasing amounts of pentafluorobenzyl esters (from 50 fmol to 2.5 pmol of the various fatty acids with a constant 100 fmol of pentafluorobenzyl ester of heptadecanoic acid as an internal standard) were then injected into the gas chromatograph with electron-capture detection as described in Fig. 1. The area under the peak of the compounds of interest was integrated, and expressed as a ratio to the internal standard. Linear regression analysis was performed for each fatty acid (see the Results and Discussion section for the slope and intercept of the calculated regression line).

and analysis by gas chromatography with electron-capture detection. Phosphatidic acid can be quantitated with femtomole sensitivity. Calibration of the assay with a standard curve of 1-palmitoyl-2-palmitoyl-*sn*-glycerol-3-phosphate which was extracted, purified by two-dimensional TLC, hydrolyzed to unesterified fatty acids, which were then derivatized to their pentafluorobenzyl esters, and analyzed by gas chromatography with electron-capture detection yielded a linear response (ratio to C17:0 internal standard = 3.41 [mass of phosphatidic acid in pmoles] − 0.13, $r = 0.9968$). Fifty fmoles of phosphatidic acid were easily detected with an excellent signal-to-noise ratio. Inter-assay coefficients of variation varied from 10% for saturated fatty acids to a maximum of 31% for unsaturated fatty acids. Table 1 illustrates the quantitation of the fatty acid composition of phosphatidic acid from islets of Langerhans cultured for seven days in high-glucose medium. Previous evidence suggests that prolonged exposure of islets to elevated glucose results in de novo synthesis of diacylglycerol and phosphatidic acid [17,21,27,37]. As expected, glucose caused a doubling in phosphatidic acid levels measured as its fatty acid content.

4. Conclusions

We have described a convenient and relatively simple method based on TLC purification and gas chromatography with electron-capture detec-

Table 1
Fatty acid composition of phosphatidic acid in islets cultured seven days in 5 or 17 m*M* glucose

Glucose level in culture	Fatty acid composition of phosphatidic acid extracted from islets (pmol/islet)							
	Total	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	C20:4
5 m*M*	799	31	70	145	115	172	52	0
17 m*M*	1619	55	140	308	312	279	143	16

Isolated pancreatic islets were cultured 7 days in culture medium containing low (5 m*M*) or high (17 m*M*) glucose as described in Experimental. Phospholipids were extracted in the presence of 6 nmol of 1-heptadecanoyl-2-heptadecanoyl-*sn*-glycero-3-phosphate as an internal standard, and purified by two-dimensional TLC. The phosphatidic spot was localized, scraped, and hydrolyzed to its constituent unesterified fatty acids, which were derivatized to their pentafluorobenzyl esters and analyzed by gas chromatography electron-capture detection as described in Experimental. Results are expressed as pmol of fatty acid in phosphatidic acid per islet from duplicate observations.

tion which allows the quantitation of the fatty acid composition of phosphatidic acid with femtomole sensitivity. Furthermore, this approach can be easily adapted to measure other phospholipids.

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