A sensitive method for the quantification of the mass of inositol phosphates using gas chromatography–mass spectrometry

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Abstract A sensitive method to directly measure the mass of inositol phosphates from biologic samples is described. The procedure uses ammonium sulfate gradient elution anion exchange column chromatography to isolate inositol monophosphate, bisphosphate, trisphosphate, and tetrakisphosphate. The isolated fractions are dephosphorylated and subsequently desalted by a novel approach using solid barium hydroxide in a 1:1 stoichiometric ratio to the amount of ammonium sulfate present in the dephosphorylated sample. The myo-inositol derived from each inositol phosphate species was quantified by stable isotope dilution gas chromatography–mass spectrometry of the hexakis(trimethylsilyl) derivative using hexadeutero-myo-inositol as the internal standard. The applicability and sensitivity of this method are illustrated by measuring the mass of individual inositol phosphates in isolated adult canine cardiac myocytes. — **DaTorre, S. D., P. B. Corr, and M. H. Creer.** A sensitive method for the quantification of the mass of inositol phosphates using gas chromatography–mass spectrometry. *J. Lipid Res.* 1990. **31:** 1925–1934.

Supplementary key words adrenergic receptors • mass spectrometry • myocytes • affinity chromatogrphay

Membrane-bound receptors linked to the catabolism of phosphoinositides as intracellular second messengers have been demonstrated in multiple tissue types. Phosphodiester cleavage of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) by phospholipase-C yields sn-1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP$_3$) (1). IP$_3$ acts as an intracellular second messenger by releasing calcium sequestered within intracellular stores which then serves to mediate diverse metabolic and physiologic responses induced by agonist stimulation of the receptor (2). Available information on the cellular production of IP$_3$ in response to a variety of agonists has relied heavily on measurements obtained after cells were incubated with radiolabeled precursors ([³H]myo-inositol or $^{32}PO_4^{3-}$) with subsequent measurement of label in individual inositol phosphate fractions after anion exchange chromatography. One major disadvantage of this approach is that quantitative assessment of the mass of IP$_3$ cannot be obtained because equilibrium labeling of PIP$_2$ is difficult to achieve and in most studies is usually not verified by direct measurement of the mass of PIP$_2$. Furthermore, the use of lithium chloride (LiCl) to inhibit catabolism of inositol phosphates, with subsequent measurement of accumulation of radiolabel, precludes analysis of the physiologic time course of IP$_3$ production after agonist stimulation. Likewise, direct measurement of inositol phosphates in whole tissue is extremely difficult using radiolabeled precursors due to the inefficiency of incorporation of the radiolabel.

The problems associated with the use of radiolabeled precursors have led to the development of methods to measure directly the mass of inositol phosphates in biological samples (3–7). Rittenhouse and Sasson (3) developed a column chromatographic approach using ammonium formate in formic acid gradients to isolate individual inositol phosphates. This method required a subsequent ion exchange step and elution with LiCl to recover inositol phosphates in a form that could be readily dephosphorylated prior to derivatization and quantification by gas chromatography. Large and variable losses of product occurred during the removal of excess LiCl by repeated extractions with 100% ethanol. Our laboratory simplified this method by using sodium sulfate gradients during anion exchange chromatography to enhance separation and recovery of inositol phosphates (4). However, desalting the inositol phosphate-derived myo-inositol after dephosphorylation in the presence of sodium sulfate is labor-intensive and is the major site of sample loss, reducing overall recovery. A method providing quantitative separation of the various in-

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Abbreviations: IP$_1$, inositol monophosphate; IP$_2$, inositol bisphosphate; IP$_3$, inositol trisphosphate; IP$_4$, inositol tetrakisphosphate; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; DAG, sn-1,2-diacylglycerol; BSTFA, bis(trimethylsilyl)trifluoroacetamide; TMCS, trimethylchlorosilane; d$_6$-Ins, hexadeutero-myo-inositol; GC–MS, gas chromatography–mass spectrometry; TMS, trimethylsilyl; d$_0$-Ins, myo-inositol; EI, electron impact; SIM, selected ion monitoring.

ositol phosphate species followed by rapid desalting would enhance recovery, reduce variability, and improve sample processing time. Additionally, to measure the mass of inositol phosphates in isolated cell preparations or in tissue samples obtainable in only small quantities from studies performed in vivo, a more sensitive method for the quantification of inositol phosphate-derived myo-inositol would be advantageous.

The present study describes a method that uses ammonium sulfate gradients during anion exchange chromatography permitting rapid removal of the sulfate while allowing excellent separation and recovery of individual species of inositol phosphates. A novel approach for desalting by addition of barium hydroxide in a 1:1 stoichiometric ratio to the amount of ammonium sulfate present in the dephosphorylated sample is described. Sensitivity is enhanced by using stable isotope dilution gas chromatography–mass spectrometry (GC–MS) of the hexakis(trimethylsilyl) (TMS$_6$) derivative of the inositol phosphate-derived myo-inositol (d$_0$-Ins) and using hexadeutero-myo-inositol (d$_6$-Ins) as the internal standard. Overall applicability and sensitivity of this method are illustrated by measuring the mass of inositol phosphate levels in isolated adult canine cardiac myocytes.

MATERIALS AND METHODS

Materials

Myo-(2-[^{3}H])-inositol (14.3 Ci/mmol), D-myo-(2-[^{3}H])-inositol-1-phosphate (1.0 Ci/mmol), D-myo-(2-[^{3}H])-inositol 1,4-bisphosphate (1.0 Ci/mmol), D-myo-(2-[^{3}H])-inositol 1,4,5-trisphosphate (1.0 Ci/mmol), and D-myo-(2-[^{3}H])-inositol 1,3,4,5-tetrakisphosphate (1.0 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). Alkaline phosphatase (Type VII S) was purchased from Sigma Chemical Co. (St. Louis, MO) and pyridine was from Aldrich Chemical Co. (Milwaukee, WI). Bis-(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchorosilane (TMCS) was purchased from Pierce Chemical Co. (Rockford, IL). Anion exchange resin (AG1-X8, 200–400 mesh formate form) was obtained from Bio-Rad (Richmond, CA) and Rexyn mixed-bed ion-exchange resin (R208-500) was from Fisher Scientific (Fair Lawn, NJ). Anhydrous barium hydroxide was purchased from Mallinckrodt Chemical (St. Louis, MO) and ammonium sulfate was from Fisher Scientific. Hexadeutero-myo-inositol (d$_6$-Ins) was obtained from MSD Isotopes (Pointe-Claire Dorval, Quebec, Canada). Myo-inositol (d$_0$-Ins) was purchased from Sigma Chemical Co. The gas chromatograph (model number 5890-A) and mass spectrometer (model number 5970) were purchased from Hewlett-Packard (Palo Alto, CA); a 15-meter DB-17 fused-silica capillary gas chromatographic column was obtained from J & W Scientific (Folsom, CA).

The Teknivent Vector-1 computer data system was purchased from Teknivent Corporation (St. Louis, MO). Distilled water was obtained from a Milli-Q Water System (Millipore Corp., Bedford, MA).

Isolation of adult canine myocytes

Intact, viable myocytes were obtained using a perfusion technique modified from the method of Haworth and colleagues (8). Adult mongrel dogs of either sex were heparinized (3000 U, i.v.) and after 15 min anesthetized with thiopental (30–35 mg/kg, i.v.). A left thoracotomy was performed, the heart was rapidly excised and placed into cooled (4°C) oxygenated bicarbonate buffer solution containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl$_2$, 11 mM glucose, 1.2 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, and 1.2 mM CaCl$_2$ (all reagents Sigma Cell Culture grade) saturated with 95% O$_2$/5% CO$_2$ and maintained at pH 7.4. A diagonal branch of the left anterior descending coronary artery or the obtuse marginal branch of the circumflex artery was cannulated using PE-60 tubing. Perfusion with a nominally calcium-free oxygenated bicarbonate buffer solution (same composition as above without added calcium) was initiated immediately and the catheter was sutured in place. The perfused region was excised along the blanched borders of the tissue and the tissue piece was placed on top of a plastic sieve that fit inside a collection funnel in a prewarmed (35–37°C) Napco incubator. Subsequent perfusion was introduced from a height of 60 cm via gravity flow and passed through a glass heating coil/bubble trap that was prewarmed to 36°C by a Haake water circulator/heater. The tissue piece was covered with a glass dome to maintain a humidified atmosphere. A 15-min washout period was begun with a solution containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl$_2$, 11 mM glucose, 1.2 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, 0.01 mg/ml superoxide dismutase (Sigma brand), 0.01 mg of 100 × basal Eagle medium amino acids (Flow Laboratories, Inc., McLean, VA) per ml and 30 μM EGTA (Fluka Chemical Corp., Ronkonoma, NY). Enzymatic digestion was initiated by 30 min of perfusion with washout solution to which 35 μM CaCl$_2$ and 0.1% (w/v) collagenase (Worthington Biochemical Corp., Freehold, NY or Boehringer Mannheim Biochemical, Indianapolis, IN) had been added.

Upon completion of the perfusion steps, the tissue was minced and placed into two Erlenmeyer flasks, each containing 20 ml of the enzyme solution and 0.015 mg/ml trypsin (Sigma). The flasks were then shaken in a Dubnoff metabolic shaker at a rate of 100/min at 37°C for 15 min, with 95% O$_2$/5% CO$_2$ blowing into each flask. At the end of this interval, 0.03 mg/ml trypsin inhibitor (Sigma) was added to each flask and the contents were filtered through 350 μm nylon mesh (Small Parts, Inc. Miami, FL). The

cell suspension was washed in a HEPES buffered solution containing 134 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl$_2$, 1.2 mM KH$_2$PO$_4$, 10 mM HEPES, 10 mM glucose, and 50 μM CaCl$_2$, pH 7.2. Extracellular [Ca^{2+}] was incrementally adjusted to 500 μM by addition of appropriate amounts of 10 mM calcium-HEPES buffer to attain 100 μM, 200 μM, 350 μM, and 500 μM [Ca^{2+}] each at 30-min intervals.

This procedure yielded 15–40 million cells from approximately 10–20 g piece of left ventricular muscle. When the percentage of elongated viable myocytes fell below 50–60%, Percoll (Sigma) gradients were used to separate rounded, nonviable cells from intact rectangular myocytes, using a method adopted from Wittenberg and Robinson (9) and Heathers and colleagues (10). Each tube contained a discontinuous gradient consisting of 6 ml of 5.5 parts of Percoll in 0.9% NaCl to 4.5 parts of HEPES-buffered solution without glucose on top, and 2 ml of 7 parts of Percoll in 0.9% NaCl to 3 parts of HEPES-buffered solution without glucose below. The HEPES-buffered solution consisted of 138 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl$_2$, 1.2 mM KH$_2$PO$_4$, 10 mM HEPES, 500 μM CaCl$_2$, and pH was adjusted to 7.2 with 1 N NaOH. The two different Percoll/NaCl plus calcium-HEPES buffered solutions were each adjusted to pH 7.2 with 1 N acetic acid. After gently layering 2 ml of cell suspension (250,000 cell/ml) on top of each gradient, the tubes were spun in an IEC table top centrifuge at 1/2 speed (120 g) for 5 min. The top buffer and gradient layers were aspirated off removing the rounded, damaged cells. Elongated cells that sedimented to the interface were collected, pooled, and washed in 500 μM calcium-HEPES and spun at 3/8 speed (45 g) for 2 min. Final cell yields consisted of 80–85% elongated myocytes when counted in a hemacytometer.

The viability and functional integrity of the adult canine cardiac myocytes isolated in our laboratory have been extensively characterized (10) using light microscopy, exclusion of trypan blue (0.05%), measurement of cytosolic enzyme release (lactate dehydrogenase, creatine kinase), levels of high-energy phosphates (adenosine 5'-trisphosphate [ATP], creatine phosphate) and measurement of electrophysiologic parameters including normal resting membrane potentials, action potential duration at 95% of full repolarization, and $\dot{V}_{max}$. Additionally, transmission and scanning electron microscopy of these cells reveals intact membranes and no cell surface blebs (11, 12). Recent experiments in our laboratory using these myocytes demonstrate a rapid second messenger (cAMP) response to β-adrenergic receptor stimulation (13) and normal diastolic Ca^{2+} levels with expected paced Ca^{2+} transients using indo-1 (Yamada, K. A., and P. B. Corr, unpublished results) illustrating their functional integrity.

Extraction of inositol phosphates

Isolated adult canine ventricular myocytes (approximately 800,000 cells per sample) were incubated in 2 ml HEPES buffer (115 mM NaCl, 5 mM KCl, 5 mM MgCl$_2$, 500 μM Ca^{2+}, 35 mM sucrose, 10 mM glucose, 10 mM HEPES, 4 mM taurine, pH 7.2). In selected experiments, approximately 100,000 dpm of [^{3}H]IP$_3$ was added to each sample to assess recovery. Incubations of cell suspensions were terminated by addition of an equal volume of ice-cold 15% (w/v) trichloroacetic acid (TCA), samples were vortexed for 15 sec and placed on ice for 10 min. Proteins were sedimented by centrifugation at 2000 g in an IEC-Centra-7R refrigerated centrifuge at 4°C for 15 min. The supernatant was decanted and placed into silanized 16 × 125 mm glass tubes. Protein precipitate was resuspended in 1 ml distilled water and an equal volume of ice-cold 15% TCA was added; the tubes were vortexed and centrifuged to sediment the pellet. Supernatants were decanted and combined with the corresponding supernatant from the first step. TCA was removed by sequential extraction with 4 ml of H$_2$O-saturated diethyl ether until the pH of the myocyte extract (lower phase) was 4.0 or greater. Residual ether was removed by lyophilizing the samples to dryness in a Savant Speed-Vac centrifugal evaporator.

Ion exchange chromatography

Lyophilized samples were resuspended in 2 ml Tris buffer (0.01 M, pH 8.5) and titrated to pH 8.5 with 1 M NaHCO$_3$. Anion exchange resin (Bio-Rad AG1-X8, formate form, 200–400 mesh) was prepared as a slurry (resin-Tris buffer 1:2, w/v) and loaded onto 10-cm columns, such that 2 ml of slurry was applied to each column. The resin was washed with 15 ml of Tris buffer, pH 8.5, before the samples were loaded. After loading the samples, the resin was washed with another 10 ml of Tris buffer, pH 8.5, to remove endogenous free myo-inositol. Approximately 100,000 dpm [^{3}H]myo-inositol was added to samples to validate complete removal of free myo-inositol during this 10-ml Tris buffer wash at the resin bed volume used. Water-soluble inositol phosphates were separated and eluted by discontinuous salt gradients from these columns. Elution of inositol monophosphate (IP$_1$), inositol bisphosphate (IP$_2$), inositol trisphosphate (IP$_3$), and inositol tetrakisphosphate (IP$_4$) was accomplished by sequentially applying 12 ml of 0.05 M (NH$_4$)$_2$SO$_4$, 0.075 M (NH$_4$)$_2$SO$_4$, 0.15 M (NH$_4$)$_2$SO$_4$, and 0.19 M (NH$_4$)$_2$SO$_4$ in Tris buffer, pH 8.5. Each sample was collected in silanized 15-ml Corex glass tubes. Validation of recovery of each inositol phosphate species was assessed by adding approximately 100,000 dpm of [^{3}H]myo-inositol 1-phosphate, [^{3}H]-1,4-bisphosphate, [^{3}H]-1,4,5-trisphosphate, and [^{3}H]-1,3,4,5-tetrakisphosphate to individual samples and

determining the amount of radiolabel from each species to elute from the column in each of the ammonium sulfate fractions. The sample load and subsequent 10-ml Tris buffer rinse were collected and counted to assess potential loss of individual inositol phosphate species during these steps. Column blanks were prepared for each inositol phosphate species by collecting 12 ml of the salt gradient required to elute that particular species from columns to which no myocyte extract had been added.

Dephosphorylation and desalting of inositol phosphates

After anion exchange chromatography, each sample received 200 pmol hexadeutero-myo-inositol (d_6-Ins) as the internal standard. Samples were lyophilized to dryness and resuspended in 2 ml distilled water. To dephosphorylate, 40 μl of 5 mM $MgCl_2$ and 40 μl alkaline phosphatase (Sigma Chemical Co., type VIIS, approximately 70 units) were added to each tube. The samples were adjusted to pH 8.5 with 14.5 N NH_4OH and sealed to prevent loss of the volatile buffer maintaining pH constant throughout the incubation period (37°C, overnight).

A stoichiometric amount of solid $Ba(OH)_2$ was then added to each sample resulting in formation of a white precipitate ($BaSO_4$). The precipitate was sedimented by centrifugation at 12,000 g for 10 min in a Sorvall centrifuge at 4°C. The supernatant was removed after centrifugation using a Pasteur pipette and transferred to a silanized 13 × 100 glass test tube. The precipitate was washed four times by adding 2 ml distilled water, resuspending, and recentrifuging. The supernatant and washes were combined and lyophilized to dryness in the Savant centrifugal evaporator to remove NH_4OH. For all tubes, a small amount of residual salt remained after lyophilization. The residual salt was removed by resuspending the residue in 1 ml of distilled water and applying it to 750 mg mixed-bed ion-exchange resin (Rexyn, Fisher Scientific) packed in serum separator tubes (Fisher) inserted into 13 × 100 mm silanized glass tubes. Inositol was eluted with six successive aliquots (1 ml) of distilled water by partially withdrawing the serum separator tube. Desalting was complete under these conditions as indicated by incomplete color change of the indicator resin and absence of salt in the tubes after repeat lyophilization to dryness.

The extent of dephosphorylation and recovery after $Ba(OH)_2$ treatment were assessed by spiking individual samples with [3H]myo-inositol 1-phosphate, [3H]-1,4-bisphosphate, [3H]-1,4,5-trisphosphate, or [3H]-1,3,4,5-tetrakisphosphate which contained 12 ml of 0.05 M $(NH_4)_2SO_4$, 0.075 M $(NH_4)_2SO_4$, 0.15 M $(NH_4)SO_4$, or 0.19 M $(NH_4)_2SO_4$, respectively. The samples were lyophilized, dephosphorylated, desalted by addition of $Ba(OH)_2$, and the amount of 3H radioactivity in the original supernatant and from four successive washes of the precipitate was determined to permit assessment of recovery.

Derivatization and gas chromatography-mass spectrometry

After the complete removal of salt, samples were lyophilized to dryness, resuspended in 1 ml distilled H_2O, and transferred to 1-ml plastic Sarstedt tubes. The 1-ml sample volumes were again lyophilized to dryness leaving a small amount of dry residue at the bottom of each tube. Preparation of the TMS_6 derivative was accomplished by adding 25 μl of anhydrous pyridine and 25 μl of BSTFA containing 1% TMCS to each dried residue. Samples were vortexed and allowed to sit at room temperature (20°C) overnight.

Standards were obtained by first preparing 1 mM stock solutions of d_0-Ins and d_6-Ins in water. From each stock solution, a 1/100 dilution was made with water achieving a final concentration of 10 pmol/μl. To each 1-ml plastic Sarstedt tube, 200 pmol of d_6-Ins was added and a variable amount (range 10–1600 pmol) of d_0-Ins was then added. The samples were concentrated to dryness under nitrogen and subsequently derivatized to the TMS_6 derivative as described above. Separate samples containing 200 pmol d_0-Ins without d_6-Ins and 200 pmol d_6-Ins without d_0-Ins were also prepared.

Standards and cell samples were analyzed on a Hewlett-Packard 5890-A gas chromatograph interfaced with a Hewlett-Packard 5970-series mass spectrometer. Data were acquired, stored, and displayed on a Teknivent Vector-1 computer system. The derivatized product (1.0 μl) was subjected to electron impact (EI) gas chromatographic–mass spectrometric selected ion monitoring (SIM) analysis by injection onto a 15-meter DB-17 fused-silica capillary column using helium as the carrier gas. Injections were made in the split mode with a head pressure of 10 psi, split flow of 10 ml/min, and purge flow of 0.5 ml/min. Gas chromatographic conditions were: injection temperature 220°C, column temperature isothermal at 180°C with the distal end of the column threaded directly to the ion source (interface temperature 210°C). The indicated ions were monitored for 10 ms dwell times at an electron multiplier voltage setting of 2200 or 2400 volts in the EI mode as described previously (14).

RESULTS

Validation of methodology

The recovery of added [3H]-IP_3 (approximately 100,000 dpm) from four cell suspensions after extraction with TCA was 97 ± 4.5% (mean ± SEM). It is critical that the TCA be removed from the samples prior to anion exchange chromatography. This is accomplished by sequential extraction with H_2O-saturated diethyl ether. Incomplete removal of TCA interferes with binding of the lesser phosphorylated inositol species (particularly IP_1) to the

anion exchange resin during sample loading. An alternative method in which inositol phosphates were extracted with perchloric acid which can be removed in a single step by neutralization with 10 N KOH to form a $KClO_4$ precipitate was also evaluated. Recovery of $[^3H]\text{-}IP_3$ and $[^3H]\text{-}IP_4$ was incomplete and variable with this approach due to sequestration of the sample within the precipitate. Multiple washes of the precipitate were necessary to recover greater than 90% of radiolabeled IP_3 and IP_4. This method proved far more labor-intensive than TCA extraction and tended to contribute additional salt to the samples following the extensive washing steps due to partial solubilization of the $KClO_4$ precipitate.

After TCA extraction and lyophilization to remove residual ether, water-soluble inositol phosphates were separated and eluted by discontinuous ammonium sulfate gradients (**Fig. 1**). Elution of IP_1, IP_2, IP_3, and IP_4 was accomplished with discontinuous ammonium sulfate gradients in Tris buffer applied in a stepwise fashion. There was excellent separation of individual inositol phosphate species with virtually no crossover between fractions. Additionally, this method was more efficient at eluting highly phosphorylated inositol species (IP_3 and IP_4) than could be obtained using sodium sulfate (4) or ammonium formate (3) gradients as previously reported. The recoveries of added $[^3H]$myo-inositol phosphates after anion exchange chromatography are shown in **Table 1**. Greater than 90% recovery was seen for all inositol phosphates after their respective elution with ammonium sulfate gradients in Tris buffer except for IP_4 (84%), similar to the recoveries reported previously (4). IP_4 may bind so avidly to the resin that more complete elution is difficult without adding an exorbitant amount of $(NH_4)_2SO_4$. Silanized glassware was used throughout the procedure to minimize absorptive loss of inositol phosphates to underivatized glass surfaces. We have also found that other cellular anionic species compete with inositol phosphates for binding to the anion exchange resin in a mass-dependent fashion. Therefore, it is important to establish the proper bed volume of resin necessary to bind all inositol phosphate species (particularly IP_1) in the study system selected and to characterize the elution profiles of inositol phosphate species at that particular bed volume.

Individual samples eluted from the columns were concentrated in the Savant centrifugal evaporator to reduce the requirement for alkaline phosphatase. Recovery of $[^3H]$inositol phosphates during this step was 100%. Subsequently, samples were dephosphorylated by addition of alkaline phosphatase at 37°C, pH 8.5, overnight. To assess completeness of dephosphorylation, $[^3H]$inositol phosphates were added to samples prior to the dephosphorylation process. To simultaneously remove $(NH_4)_2SO_4$, a novel approach was used whereby a stoichiometric amount of $Ba(OH)_2$ was added to the
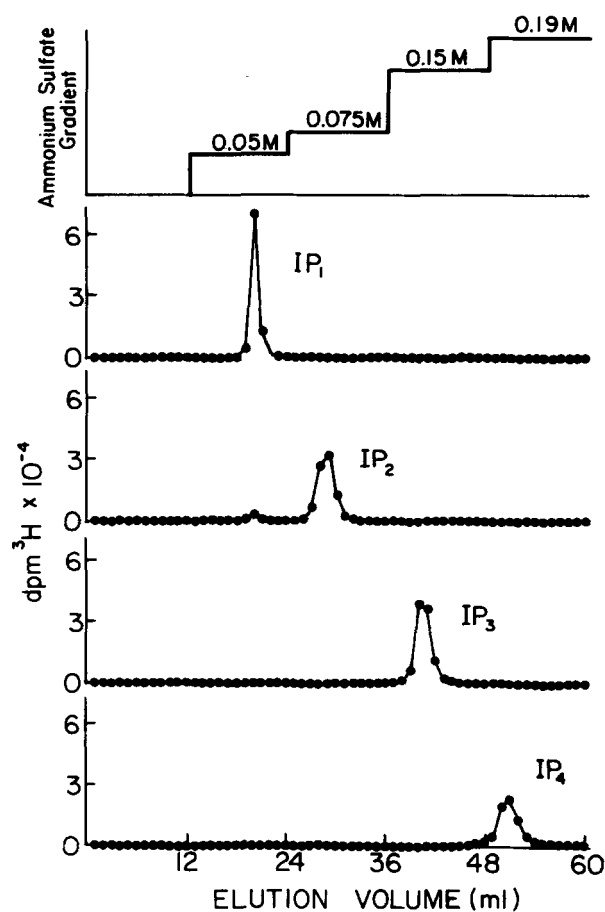

Fig. 1. Elution profiles of $[^3H]$inositol monophosphate (IP_1), bisphosphate (IP_2), trisphosphate (IP_3), and tetrakisphosphate (IP_4) applied to AG1-X8 anion exchange resin and eluted with ammonium sulfate in a discontinuous, stepwise fashion. Approximately 1×10^5 dpm of ^3H-labeled inositol-phosphate species was added separately to TCA extracts of isolated cardiac myocytes (800,000 cells). After application, columns were rinsed with 12 ml Tris buffer and inositol-phosphate species were eluted sequentially with 12-ml aliquots of increasing concentrations of ammonium sulfate as illustrated in the top panel. One-ml fractions were collected and ^3H radioactivity was quantified by liquid scintillation spectrometry. Values shown represent the mean of four separate experiments.

dephosphorylated samples. This results in the formation of a $BaSO_4$ precipitate and NH_4OH which is volatile and easily removed during lyophilization. Assessment of dephosphorylation and recovery for each of the $[^3H]$inositol phosphate species after $Ba(OH)_2$ addition is shown in **Table 2**. Dephosphorylation and recovery of $[^3H]$inositol phosphate-derived $[^3H]$myo-inositol was virtually complete for all species after four washes of the precipitate. The sequential decrease in recovery of $[^3H]$myo-inositol after dephosphorylation of $[^3H]IP_1$, IP_2, IP_3, and IP_4 at the first precipitation and centrifugation step shown in Table 2 is due to the progressive increase in volume of the $BaSO_4$ precipitate in these fractions, respectively, which requires more extensive washing of the precipitate

TABLE 1.   Recovery of [³H]inositol phosphates after separation by anion exchange chromatography using an ammonium sulfate gradient

Ammonium Sulfate Concentration	[³H]I	[³H]IP$_1$	[³H]IP$_2$	[³H]IP$_3$	[³H]IP$_4$
M			%		
0	96 ± 2.1	0.5 ± 0.05	0.4 ± 0.03	0.3 ± 0.03	0.3 ± 0.04
0.05	<0.7	99 ± 0.7	4.0 ± 0.03	0.1 ± 0.02	0.4 ± 0.03
0.075	<0.1	0.4 ± 0.03	92 ± 1.4	0.5 ± 0.03	0.2 ± 0.03
0.15	<0.1	0.4 ± 0.05	0.4 ± 0.03	93 ± 1.3	2.8 ± 0.60
0.19	<0.1	0.3 ± 0.05	0.2 ± 0.03	0.2 ± 0.03	84 ± 1.3

Values are means ± SEM, n = 4, and are expressed as percent recovered in each fraction of the total radiolabel added; I, inositol.

to ensure complete recovery of [³H]myo-inositol. This pattern does not represent incomplete or variable dephosphorylation of the more highly phosphorylated [³H]inositol phosphate species since we found that addition of Ba(OH)$_2$ to a phosphorylated sample results in binding between Ba²⁺ and the phosphate group of the inositol molecule with quantitative precipitation of the [³H]inositol phosphate species which could not be recovered despite extensive washing of the precipitate. Complete dephosphorylation was verified by reloading dephosphorylated samples onto anion-exchange columns with quantitative recovery of radiolabel as [³H]myo-inositol for each inositol phosphate species. Thus, complete dephosphorylation was achieved and is essential to ensure quantitative recovery. Quantitative recovery of [³H]inositol phosphate-derived [³H]myo-inositol applied to the mixed-bed ion-exchange resin to complete desalting was also achieved.

Derivatization of both d$_0$-Ins and d$_6$-Ins was accomplished overnight at room temperature (20°C) as described under Materials and Methods. The derivatized product with the derivatization reagent was subjected to analysis by EI gas chromatography–mass spectrometry with SIM at m/z 305 and m/z 307. The ion pair at m/z 305 and 307 has previously been described for stable isotope dilution gas chromatographic–mass spectrometric measurement of TMS$_6$-d$_0$-Ins and TMS$_6$-d$_6$-Ins derivatives, respectively (14). In addition, the ion pair at m/z 318 and

321 (corresponding to TMS$_6$-d$_0$-Ins and TMS$_6$-d$_6$-Ins, respectively) can be used for this purpose but provides a less intense signal.

As illustrated in **Fig. 2**, when a constant amount of d$_6$-Ins (200 pmol) and varied amounts of d$_0$-Ins (10–1600 pmol) were added to a series of 1-ml Sarstedt tubes, converted to the TMS$_6$ derivative, and analyzed by EI gas chromatography–mass spectrometry SIM, the ratio of the integrated ion current for m/z 305 to that for m/z 307 was a linear function of the amount of d$_0$-Ins added. An on-column injection of 1 μl of a 50 μl solution containing a total of 10 pmol d$_0$-Ins (200 fmol injected) gave a clearly defined signal at m/z 305, demonstrating the excellent sensitivity of the method.
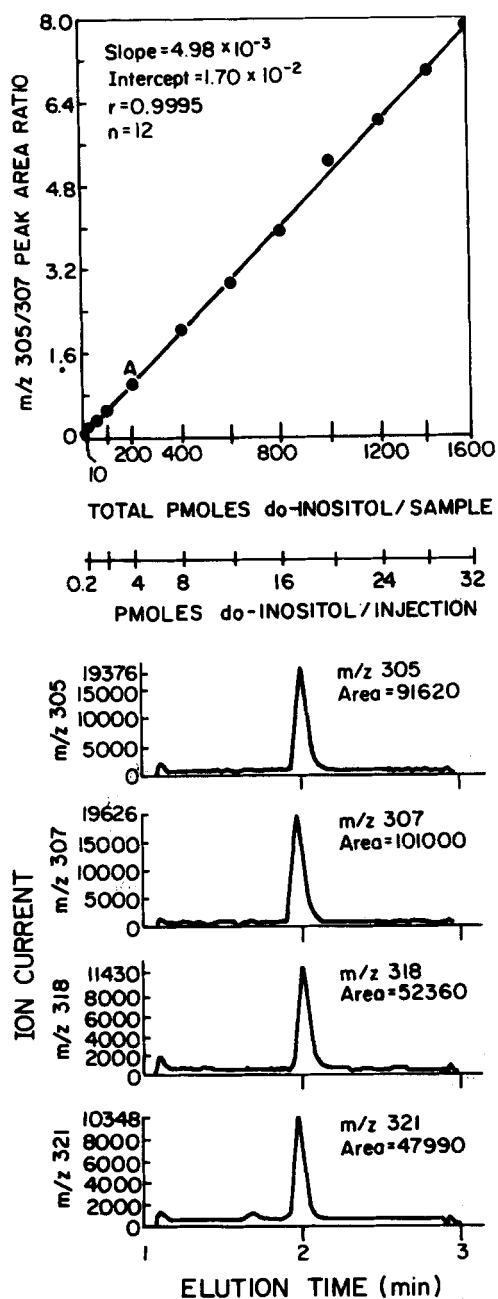
Mass measurements of inositol phosphates in isolated canine myocytes

To illustrate the method used to determine the endogenous inositol phosphate content of isolated canine cardiac myocytes, a representative stable isotope dilution gas chromatographic EI mass spectrometric analysis of IP$_4$-derived myo-inositol obtained from adult canine myocytes is presented in **Fig. 3**. The amount of IP$_4$-derived myo-inositol is determined by comparing the ratio of the integrated ion intensities at m/z 305 (derived from d$_0$-Ins in the sample) and at m/z 307 (derived from the d$_6$-Ins internal standard) with the same ratio obtained from standards.
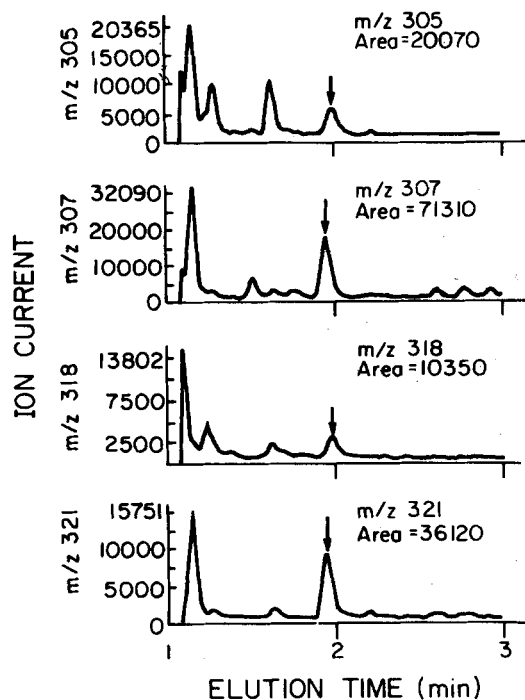
TABLE 2.   Assessment of dephosphorylation and recovery after addition of barium hydroxide

	[³H]IP$_1$	[³H]IP$_2$	[³H]-IP$_3$	[³H]IP$_4$
Precipitation, centrifugation	82 ± 1	79 ± 3	62 ± 2	53 ± 1
Wash precipitate × 1	14 ± 0.3	17 ± 1	24 ± 1	30 ± 1
Wash precipitate × 2	2 ± 0.2	4 ± 0.3	7 ± 1	9 ± 1
Wash precipitate × 3	1 ± 0.1	1 ± 0.3	2 ± 1	3 ± 0.1
Wash precipitate × 4	1 ± 0.1	0.4 ± 0.2	1 ± 0.1	1 ± 0.2
Total recovery	100 ± 1	100 ± 3	96 ± 2	96 ± 2

Values are means ± SEM, n = 3, and are expressed as percent of total added radiolabel recovered at each step of the procedure.

Slope = 4.98 × 10⁻³
Slope = 4.98×10^{-3}
Intercept = 1.70×10^{-2}
r = 0.9995
n = 12

m/z 305/307 PEAK AREA RATIO

8.0
6.4
4.8
3.2
1.6
0

A

200 400 800 1200 1600
10

TOTAL PMOLES do-INOSITOL / SAMPLE

0.2 4 8 16 24 32
PMOLES do-INOSITOL / INJECTION

ION CURRENT

19376 15000 10000 5000 0 — m/z 305 Area = 91620
19626 15000 10000 5000 0 — m/z 307 Area = 101000
11430 8000 6000 4000 2000 0 — m/z 318 Area = 52360
10348 8000 6000 4000 2000 0 — m/z 321 Area = 47990

1 2 3
ELUTION TIME (min)

Fig. 2. Stable isotope dilution analysis of myo-inositol by electron impact (EI) gas chromatography–mass spectrometry with selected ion monitoring (SIM). To 1-ml Sarstedt tubes, 200 pmol d_6-Ins and 10–1600 pmol d_0-Ins were added as solutions in water. Samples were dried under nitrogen; 25 μl of pyridine and 25 μl of BSTFA with 1% TMCS were then added, and samples were allowed to stand overnight at room temperature (20°C). A 1-μl aliquot from each vial was subjected to gas chromatographic–mass spectrometric SIM analysis following injection onto a 15-meter DB-17 capillary column. Gas chromatography was performed as described under Materials and Methods. SIM was begun 1 min after injection and continued for 2 min. Ions at m/z 305, 307, 318, and 321 were monitored for 10 msec dwell times at an electron multiplier voltage setting of 2200 or 2400 volts in the EI mode. Peak area ratios were calculated as the ratio of the integrated ion current at m/z 305 to that at m/z 307 after correction for the heavy isotope contribution of the fragment ion at m/z 305 to the observed ion current at m/z 307 as described in detail under Results. The results of linear regression analysis of the data are shown in the top portion of the figure. The lower tracings represent the SIM data corresponding to point A on the regression line.

ION CURRENT

m/z 305 20365 15000 10000 5000 0 — m/z 305 Area = 20070
m/z 307 32090 20000 10000 0 — m/z 307 Area = 71310
m/z 318 13802 7500 2500 0 — m/z 318 Area = 10350
m/z 321 15751 10000 5000 0 — m/z 321 Area = 36120

1 2 3
ELUTION TIME (min)

Fig. 3. Representative stable isotope dilution gas chromatographic–mass spectrometric analysis of endogenous IP_4 synthesized by isolated canine cardiac myocytes. Isolated myocytes (800,000 cells) were prepared and extracted, and the IP_4 fraction was isolated by gradient elution column chromatography. The TMS_6-d_0-Ins derivative was prepared and analyzed by EI gas chromatography–mass spectrometry with SIM as described under Materials and Methods and in the legend of Fig. 2. Arrows in the SIM scans at m/z 305 and 318 correspond to fragment ions derived from TMS_6-d_0-Ins. Arrows in the SIM scans at m/z 307 and 321 correspond to fragment ions derived from TMS_6-d_6-Ins.

Calculated ion intensities were adjusted to correct for the heavy isotope contribution of the ion at m/z 305 to the integrated ion current (intensity) at m/z 307 (forward contribution, R_F) and the contribution of light isotopes present in the d_6-Ins to the integrated ion intensity at m/z 305 (backward contribution, R_B) as previously described by Biemann (15). Separate analysis of samples containing 200 pmol d_0-Ins alone and 200 pmol d_6-Ins alone yielded a forward contribution (R_F) of 13.6 ± 0.6% (mean ± SEM, n=5) and a backward contribution (R_B) that was negligible (0.5 ± 0.2%, n=5). The calculation used is shown below:

$Area_c$ (305) = corrected ion intensity at m/z 305

$Area_c$ (307) = corrected ion intensity at m/z 307

$Area_0$ (305) = observed ion intensity at m/z 305

$Area_0$ (307) = observed ion intensity at m/z 307

PAR_c = corrected peak area ratio (ratio of integrated ion current (intensity) of the m/z 305 ion to that of the m/z 307 ion)

$$PAR_c = Area_c(305) / Area_c(307) \qquad Eq.\ 1)$$

$$Area_c (305) = Area_0 (305) \text{ (negligible backward}$$

contribution, $R_B < 0.01$ (0.5%)) *Eq. 2*

$$Area_c (307) = Area_0 (307) - R_F \times Area_0$$

(305) (forward contribution (R_F) = 0.136

(13.6%)) *Eq. 3*

Substituting equations 3 and 2 in equation 1 gives:

$$PAR_c = \frac{Area_0 (305)}{Area_0 (307) - R_F \times Area_0 (305)} \quad Eq.\ 4$$

Multiplying both numerator and
denominator of equation 4 by
(1/Area$_0$ (305)) gives:

$$PAR_c = [(Area_0 (307)/Area_0 (305)) - R_F]^{-1} \quad Eq.\ 5$$

Once determined, the PAR$_c$ is used to obtain total pmoles d_0-Ins in the sample by extrapolation from a standard curve. The amount of d_0-Ins present in column blanks, prepared along with endogenous samples for each of the inositol phosphate species, is subtracted from this value.

Two fundamental parameters were used to validate the origin of peaks observed in samples as being derived from d_0-Ins (*m/z* 305 and 318) and d_6-Ins (*m/z* 307 and 321). First, the retention times of the TMS$_6$ derivatives of both the d_6-Ins internal standard and d_0-Ins of cellular origin were compared and co-elution of peaks at *m/z* 307 and *m/z* 305, respectively, were confirmed for each analysis. As described previously, deuterium-labeled compounds elute from the gas chromatograph slightly before the protium forms (deuterium isotope effect) (14). The retention times of TMS$_6$-d_0-Ins and TMS$_6$-d_6-Ins derived from samples and standards were very consistent. A second parameter, referred to as the validation index has also been used for this purpose as previously described (14). This index is based on the observation that the ion intensity ratio of two separate ions in the mass spectrum of a pure compound are unique. It is derived by computing the *m/z* 305/318 ion intensity ratio of samples divided by the ratio obtained from standards of similar inositol content (perfect ratio match, validation index = 1). The validation index computed for the representative sample shown in Fig. 3 was 1.1. The additional peaks in the elution profile possibly represent other endogenous isomers of inositol and were not characterized further in this study.

The mass of individual inositol phosphates in isolated adult canine myocytes under basal nonstimulated conditions is shown in **Table 3**. Total pmol of d_0-Ins in each sample were determined as described above and results expressed per mg protein (85,000 ± 9,000 cells/mg protein, n = 5) as determined by a modification of the Lowry method (16). As illustrated, the mass of IP$_1$ and IP$_2$ is much greater than that of IP$_3$ and IP$_4$ reflecting differences in the rate of synthesis and degradation of individual species under basal conditions.

TABLE 3. Mass of inositol phosphates in isolated adult canine myocytes under nonstimulated conditions

Inositol Phosphate Species	Mass
	pmol/mg protein
IP$_1$ (n = 4)	151 ± 4.2
IP$_2$ (n = 4)	98 ± 7.8
IP$_3$ (n = 4)	10 ± 1.5
IP$_4$ (n = 4)	3.3 ± 1.0

Values are expressed as means ± SEM; n refers to the number of different cell preparations evaluated.

DISCUSSION

These results demonstrate the applicability and excellent sensitivity of the present method to measure directly the mass of inositol phosphates in biological samples. Our method offers a number of significant advantages over previously reported procedures (3, 4). The method is simple, reproducible, and sensitive and better addresses the limitations inherent in previous methods for the measurement of the mass of inositol phosphates. Rittenhouse and Sasson (3) described a technique using ammonium formate gradients in formic acid to separate inositol phosphates. Large and variable losses of inositol phosphates occurred with this procedure in an attempt to remove excess LiCl by repeated extractions with 100% ethanol. We have observed significant crossover of IP$_4$ into the IP$_3$ fraction using the 100 mM formic acid and 1 M ammonium formate gradient to separate IP$_3$. Heathers and colleagues (4) attempted to circumvent these problems by using sodium sulfate gradients to separate and elute inositol phosphates. The use of sulfate offered improved efficiency during chromatographic separation since sulfate possesses greater affinity for the resin than either formate or phosphate. However, a major obstacle using that method was the need to completely remove sodium sulfate to ensure quantitative derivatization of myo-inositol. This desalting step represented the major site of sample loss reducing the overall signal. The present method uses ammonium sulfate as opposed to sodium sulfate gradients during anion exchange chromatography with excellent separation and recovery of inositol phosphates. This approach permits not only the enhanced efficiency of sulfate for separation of inositol phosphate species but permits the use of Ba(OH)$_2$ to form solid BaSO$_4$ and volatile NH$_4$OH, thereby providing a reproducible and rapid desalting procedure. Both of the previous methods used gas chromatographic analysis of the TMS$_6$ derivative of myo-inositol using chiro-inositol as an internal standard to quantitate the mass of inositol. In the present method, mass spectrometry is used which provides several advantages including enhanced sensitivity, increased chemical specificity, and the ability to use heavy isotope-labeled in-

ositol as an internal standard (14). Our method permits accurate detection of as little as 200 fmol during a single injection providing sufficient sensitivity to assess the mass of individual inositol phosphates in isolated cell preparations or in samples obtainable from in vivo sources in only small quantities. The quantification of myo-inositol as its hexakis(trifluoroacetyl) derivative with negative ion chemical ionization (NICI) mass spectrometry has been reported previously by others (17). Although offering greater sensitivity, this method requires considerably more expense and utilizes a much less stable derivative. With our method, we have found that it is possible to store the TMS$_6$ derivative for at least 2 weeks ($-70°C$) without significant loss of signal intensity or other evidence of decomposition of the TMS$_6$ derivative (data not shown). Additionally, two methods are available for the biological assay of 1,4,5-IP$_3$ (18). The first of these is a receptor binding assay in which endogenous 1,4,5-IP$_3$ competes with binding of ^{3}H- or ^{32}P-labeled 1,4,5-IP$_3$ standard. The second approach involves the ability to assay 1,4,5-IP$_3$ by its ability to release ^{45}Ca^{2+} from permeabilized neutrophils. Both of these procedures are relatively easy to perform, sensitive [reported sensitivity of the 1,4,5-IP$_3$ protein binding assay marketed as a kit by Amersham Corp. is 0.2 to 25 pmol/sample (18)], and as capable as our technique for measuring 1,4,5-IP$_3$. However, these biologic methods are limited in being able to detect only biologically active IP$_3$. An obvious advantage of our method, and a major reason for its development, is the ability to measure the mass of each of the individual inositol phosphate species and to assess the relative changes in the mass of each species after agonist stimulation or pathophysiologic perturbations. This may serve to further elucidate the physiologic importance of individual inositol phosphate species and lead to a better understanding of this important system.

Our method offers several advantages over methods using radiolabeled precursors. Utilizing radiolabel, quantitative assessment of the mass of individual inositol phosphates cannot be made readily because equilibrium labeling of membrane inositol-containing lipids is difficult to achieve and in most studies is not verified. The fact that the incorporation of radiolabel increases in the control group progressively with time in some studies suggests further incorporation into membrane pools and non-equilibrium conditions (19). Additionally, poor efficiency of incorporation of the radiolabel has led to the use of LiCl to inhibit catabolism of inositol phosphates with subsequent measurement of accumulation of radiolabeled inositiol monophosphate. The use of LiCl precludes analysis of the physiologic time course of IP$_3$ production following agonist stimulation and assumes that the labeled IP$_1$ which accumulates reflects production and catabolism of IP$_3$ which has not been confirmed. Direct measurement of inositol phosphates from whole tissue is difficult using radiolabeled precursors due to the inefficiency of incorporation of the label under conditions occurring in vivo. Using the procedure described here, the time course of accumulation and catabolism of inositol phosphate species in response to agonist stimulation can be evaluated accurately in tissue derived from studies performed in vitro or in vivo.

The mass of IP$_3$ and IP$_4$ in quiescent adult canine myocytes measured in the present study is very similar to that reported previously from our laboratory. However, the mass of IP$_1$ and IP$_2$ is substantially higher than that reported previously (20). Incomplete removal of TCA and the presence of other cellular anionic species influence binding to the anion exchange resin during sample loading and is most marked for IP$_1$ and IP$_2$. Thus, it is probable that much of the IP$_1$ and IP$_2$ was lost during sample loading using our previous method. The large amount of IP$_1$ and IP$_2$ reported in the present study is an interesting finding since LiCl was not added to the cells to inhibit catabolism of inositol phosphates. The relative contribution of different isomers to the total mass of IP$_1$ and IP$_2$ measured and the etiology of these isomers represent interesting topics for future investigation. Our method also offers a sensitive and reproducible approach to quantitate the mass of individual isomers separated by previously described HPLC procedures. (18.)

In conclusion, we have advanced the ability to accurately measure the mass of inositol phosphates in biologic samples. Our method uses a novel approach for desalting which provides excellent recovery enhancing signal to noise and permits sulfate to be utilized during anion exchange chromatography enhancing efficiency of separation and elution. This method is very sensitive, straightforward, relatively inexpensive and involves a stable derivative which can be analyzed by stable isotope dilution gas chromatography–mass spectrometry.

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