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# An accelerated mass spectrometric method for measuring *myo*-inositol in phosphatidylinositol in rat brain

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**Abstract**

A fast and efficient chemical ionization mass spectrometric (CI–GC–MS) method for measuring *myo*-inositol in phosphatidylinositol (PtdIns) in rat brain has been developed. Previously, quantitation of PtdIns involved the release of the *myo*-inositol by two enzymatic reactions using phospholipase C and alkaline phosphatase. The hydrolytic action of these enzymes was replaced by using commercially available 48% hydrofluoric acid (HF) at 80 °C for 30 min. The process can be carried out on the crude Folch extract of brain phospholipids without prior thin layer chromatography (TLC) purification, thereby significantly increasing the speed of analysis. For quantification, unlabeled m*yo*-inositol, labeled *myo*- and *neo*-inositol (internal standard) were converted to acetate derivatives and analyzed by CI–GC–MS.


*Keywords:* Rat brain; Folch extraction; Phosphatidylinositol; *myo*-Inositol; CI–GC–MS

## 1. Introduction

In the cellular phosphatidylinositide (PI) cycle, *myo*-inositol (Ins) is a substrate for the synthesis of membrane lipid phosphatidylinositol (PtdIns), which is then phosphorylated on the Ins moiety to form bis- and tris-phosphatidylinositol phosphate (PtdIns(4)P and PtdIns(4,5)$P_2$). PtdIns(4,5)$P_2$ (Fig. 1) is hydrolyzed by phospholipase C (PLC) to release *myo*-inositol trisphosphate, a reaction regulated by a large number of stimuli in biological systems [1–3]. The signaling pathway involving Ins-containing phospholipids is cyclic (PI cycle), requiring continuous incorporation, and release of Ins into the cell membrane [4]. The dynamics of Ins turnover may be altered in certain pathophysiological conditions. These include diabetes, where the Ins pool utilized for PtdIns turnover is depleted in peripheral neurons [5]. Approximately 50% higher than normal concentrations of Ins in brain occur in Down syndrome (trisomy 21) and in animal models of this disorder [6,7]. Magnetic resonance spectroscopy also indicated elevated concentrations of Ins and Ins metabolites in the Alzheimer's disease brain [8]. Certain therapeutic agents like lithium may alter Ins turnover in PtdIns signaling related reactions. For instance, lithium at therapeutic levels used in bipolar disorder [9] inhibits *myo*-inositol monophosphatase, and reduces brain Ins in patients with bipolar disorder [10]. Ins depletion leading to decreased PtdIns turnover has been proposed as the mechanism of action of lithium in such patients [11].

The turnover rate of Ins in PtdIns has been measured in cultured cortical neurons from the fetal mouse, and may be useful to measure neuropathological conditions in vivo [12]. The method that was used involved incubating the neurons in a culture medium where Ins was completely replaced with deuterium-labeled *myo*-inositol (Ins*), giving a specific activity of 1. A mass spectrometric method was used to measure the time–dependent changes in specific activities of *myo*-inositol in the cell cytosol and PtdIns. To measure PtdIns specific activity, phospholipids were isolated from the cortical neurons by Folch extraction. Subsequently, PtdIns was isolated by thin layer chromatography (TLC), Ins phosphate was released from PtdIns by exogenous PLC, de-phosphorylated by alkaline phosphatase, and derivatized for GC/MS quantification [12].

Here, we describe a faster, easier method for measuring PtdIns concentration and specific activity. We achieved the

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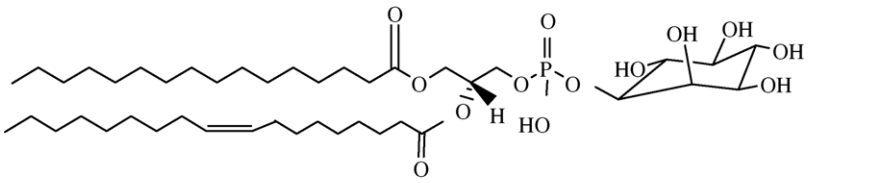
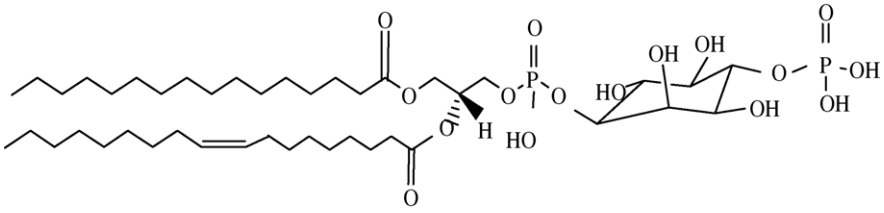
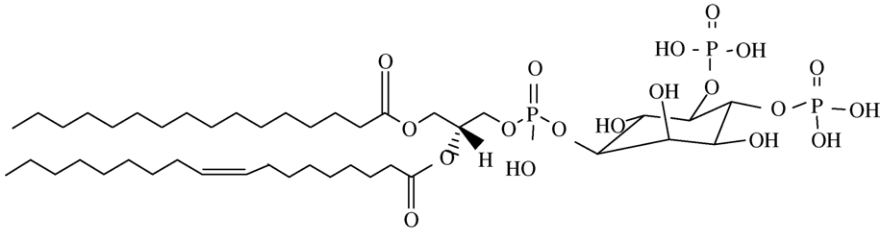


PtdIns



PtdIns(4)P



PtdIns(4,5)$P_2$

Fig. 1. Structure of PtdIns, PtdIns(4)P and PtdIns(4,5)$P_2$.

complete hydrolysis of PtdIns to Ins with a 48% solution of hydrofluoric acid (HF) in 30 min at 80 °C. This one step replaces the two enzymatic reactions using PLC and alkaline phosphatase, dramatically decreasing the time of analysis. Although PtdIns is not the only *myo*-inositol-containing phospholipid or its component in a tissue extract, the same reaction can be used directly on the extract for PtdIns determination, or after further isolation/analytical procedures like thin layer chromatography.

## 2. Experimental

### 2.1. Materials

Phosphatidylinositol (PtdIns), *myo*- and *neo*-inositol internal standards were purchased from Sigma Chemicals (St. Louis, MO). HF solution 48 wt.% in water was from Aldrich (Milwaukee, WI). The tracer, *myo*-[$^2H_6$]inositol, was purchased from C/D/N Isotopes (Pointe-Claire, Que., Canada). Chemical ionization gas chromatographic mass spectrometric (CI–GC–MS) analysis indicated that the product was practically free of unlabeled Ins and contained >98% enriched [$^2H_6$]-species. Acetic anhydride and pyridine were from Altech Associates (Deerfield, IL) and 4-dimethylamino-pyridine was from Fluka Chemical Corp. (New York, NY). Solvents used for extracting and processing analytes were from Burdick & Jackson (Muskegon, NY). The CI–GC–MS was a Varian SATURN 2100T GC/MS/MS (Lake Forest, CA) provided with a chemical ionization ion source with acetonitrile as reagent. The capillary column (WCOT Fused silica 30 m × 0.25 mm i.d.) was obtained from Varian (Lake Forest, CA). TLC plates for phospholipid separation were Kiselgel 60 (20 cm × 20 cm, 0.25 mm thickness), purchased from EM Science (Gibbstown, NJ). The probe sonicator was from Heat Systems-Ultrasonics (Farmingdale, NJ). 1.5 ml Microcentrifuge tubes, Blue Cap, Grad were from Abgene, Inc. (Rochester, NY).

### 2.2. *myo*-[$^2H_6$]inositol infusion study

ALZET pumps (Durect Corporation, Cupertino, CA) were used for subcutaneous delivery of *Myo*-[$^2H_6$]inositol as described [13]. The pump model 2001 (capacity 200 μl; flow 1 μl/h) was used for time points between 12 h and 7 days. Male Fisher 344 rats (3 months old, Charles River Laboratories, Wilmington, MA) were anesthetized with 1–3% halothane in a Plexiglas chamber. Unconscious rats were then secured on a plastic dissecting tray equipped with a constant 1–3% halothane delivery system to maintain anesthesia during surgery. The site for the subcutaneous implantation of the ALZET pump was on the back, slightly posterior to the scapula. At timed intervals, animals were killed by intraperitoneal injection of a lethal dose of pentobarbital (200 mg/kg weight). The head was subjected to

high energy microwaving and the brain was removed from the skull and immediately frozen using cold isopentane (on dry ice). The frozen brain was wrapped in a Teflon sheet (6 cm × 6 cm) and placed in a propylene tube.

### 2.3. Brain phosphatidylinositol

Microwaved, frozen rat brain (∼1.0 g) was homogenized in methanol as previously described [12,13]. Twenty milliliters chloroform was added to the methanol homogenate, mixed (by vortex for 1 min) and 7.5 ml 0.5 M KCl solution added and dispersed. The biphasic solution was centrifuged in a SW rotor centrifuge for 20 min at $1.5 \times 10^3$ rpm. The organic phase containing the lipids (bottom layer) was concentrated at room temperature (Speed Vac, Vapornet, Ont., Canada) and the pellet was solubilized in chloroform:methanol (2:1) mixture (5.0 ml/g brain) and stored at −80 °C.

### 2.4. Analysis of myo-inositol released from phosphatidylinositols

A portion of the organic solution (50 μl) and the *neo*-inositol internal standard (25 μl, 50 ng) were mixed in a 1.5 ml microcentrifuge tube, dried under nitrogen and mixed with 50 μl hydrofluoric acid (HF), 48% in water. The mixture was heated at 80 °C for 30 min with occasional stirring. The HF solution was evaporated under nitrogen, 100 μl pyridine (containing 1% of 4-dimethyl amino-pyridine) and 100 μl acetic anhydride were added, and the solution (total 200 μl) was transferred to a 5 ml glass tube and heated for 30 min at 80 °C. After the sample was cooled, the solvents were evaporated under nitrogen. The residue was dissolved in 3 ml hexane:ethyl acetate (80:20, v/v), washed with 1 ml 5% $NaHCO_3$, and the phases separated. The organic phase was transferred to a clean 5 ml glass tube and dried in a Vapornet (∼15 min at medium temperature). The residue was dissolved in 50 μl ethyl acetate in an auto-sample vial. One microliter was injected into CI–GC–MS for analysis.

### 2.5. CI–GC–MS analysis

Ins, Ins* and *neo*-inositol (internal standard) in the sample were converted to acetate derivatives as described and analyzed by CI–GC–MS. One microliter of this solution was injected into the GC–MS and selected ions obtained by chemical ionization were monitored: *m*/*z* 373 for Ins and *m*/*z* 379 for Ins* (Fig. 2). The percent of tracer (or specific activity) in phosphatidylinositol is the percentage of peak area of *m*/*z* 379 ion compared to the peak areas of *m*/*z* 373 and 379 ions combined.

The samples were injected split mode (split ratio 15) with the injector temperature at 250 °C and the oven at 150 °C. After 0.5 min, the oven temperature was ramped at 25 °C/min to 190 °C, and then continued at 2 °C/min to 230 °C. Finally, the column was heated to 300 °C for 8 min, and returned to initial temperature.

Concentrations of brain PtdIns were calculated from the standard curve.

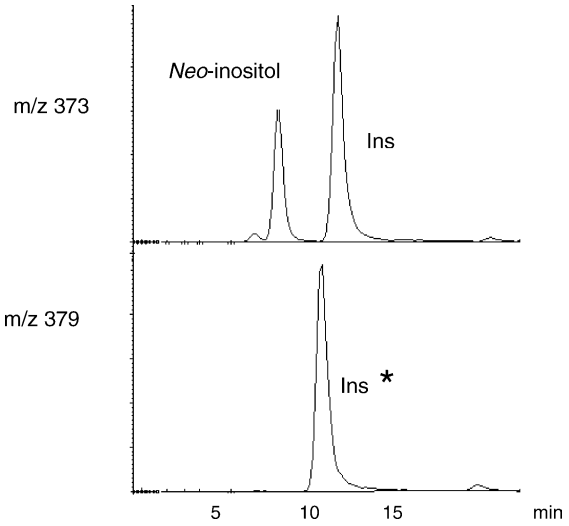



Fig. 2. Selected ion monitoring of Ins, Ins* and *neo*-inositol (internal standard).

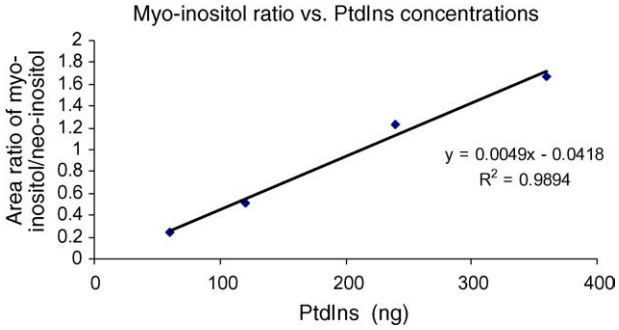



Fig. 3. Standard curve for the quantification of PtdIns.

## 3. Results and discussion

A CI–GC–MS method was developed to measure endogenous PtdIns in rat brain. The method involved cleaving the head group from the phospholipid moiety in the de-phosphorylated form. Previously used PtdIns measurements involved an enzymatic cleavage with PLC, and then treating with alkaline phosphatase. The enzymatic procedure is tedious and not suitable if a large number of samples are involved. We replaced this enzymatic procedure with a one-step hydrolysis reaction with a commercial 48% solution of HF at 80 °C that requires only 30 min. We found that the hydrolysis could be carried out on the crude Folch extract without the purification step on TLC, increasing significantly the speed of the analysis. For the mass spectrometric quantification, *myo*-inositol, labeled *myo*- and *neo*-inositol (internal standard) were converted to acetate derivatives and

Table 1
Comparison of recovery of *myo*-[$^2H_6$]inositol after and before treatment with HF

| Sample | *neo*-Inositol Ng | *myo*-[$^2H_6$]inositol Ng | *myo*-[$^2H_6$]inositol % (recovered)[a] | Reagent |
|---|---|---|---|---|
| 1 | 50 | 50 | 74.94 (average) | HF |
| 2 | 50 | 50 | 92.82 (average) | – |

[a] Three measurements.

Table 2
Comparison of the activity (percentage of incorporation of *Ins in PtdIns, two duplicates) at different time points

| HF de-phosphorylation [Ins*/Ins + Ins*] (%) | | | | | | Enzymatic de-phosphorylation [13] [Ins*/Ins + Ins*] (%) | |
|---|---|---|---|---|---|---|---|
| Day | Sample 1 | Sample 2 | Sample 3 | Mean[a] | S.D. | Mean[a] | S.D. |
| 1 | 0.073<br>0.083 | 0.061<br>0.086 | 0.099<br>0.092 | 0.082 | 0.00014 | 0.195 | 0.00060 |
| 7 | 0.867<br>1.169 | 0.885<br>1.090 | 0.807<br>0.780 | 0.933 | 0.00016 | 1.202 | 0.00268 |

[a] Six measurements.

analyzed by CI–GC–MS. Commercial PtdIns was incubated with HF and gave 90–92% hydrolysis, producing a linear curve (Fig. 3). Even after extensive washing with water, we found that the brain organic extract still contained 1–1.5% free *myo*-inositol, and thus the concentrations of PtdIns measured in the extracts were corrected accordingly.

In order to evaluate the extent of loss of deuterium atoms from *myo*-$^{2}[H_6]$inositol in the process of hydrolysis with HF, a comparison of the results obtained from *myo*-$^{2}[H_6]$inositol, treated and non-treated with HF, and then spiked with *neo*-inositol, was carried out (as described in Section 2.4). 75% of the deuterium-labeled *myo*-inositol was recovered after the HF treatment (Table 1).

The levels of PtdIns found in the control rat brain (1.56–1.78 μmol/g) agreed with published values. Previously, a concentration of approximately 3 μmol/g phosphoinositides and inositol phosphate, combined, was reported [12]. Recently, levels of 1.66–1.89 μmol/g of only PtdIns were found by combining TLC isolation and total phosphorous measurements [13].

The present method can be used to determine concentrations and/or turnover rates of Ins in animal brain tissue when a large number of samples are measured. Quantification of the extraction by the Folch procedure can give variable results due to the extreme charge/hydrophobicity of PtdIns, PtdIns(4)P and even PtdIns(4,5)$P_2$. Interestingly, our results (Table 2) obtained by direct HF hydrolysis of Folch extracts of rat brain treated with Ins* (see Section 2.2) at two time points, are almost identical to results obtained using the two-step enzymatic hydrolytic procedure on TLC purified samples [13]. Comparison of the activity (percentage of incorporation of *Ins in PtdIns, two duplicates) at different time points is shown in the Table 2. We detected an increase from 0.08% incorporation after the first day to 0.93% incorporation after 1 week of infusion.

## 4. Conclusion

In this work, a new quantitative rapid HF-hydrolytic procedure has been shown to successfully replace the previous time-consuming enzymatic release of *myo*-inositol from *myo*-inositol-containing phospholipids, thereby dramatically decreasing the time required for the analysis of *myo*-inositol in these phospholipids. PtdIns and PtdIns-phosphates are not the only *myo*-inositol-containing phospholipids or components in an extract of tissue, and HF hydrolysis can be used either directly on the extract for PtdIns determination or after further isolation/analytical procedures like TLC. This more rapid analytical procedure for PtdIns analysis should be of considerable benefit in further understanding the metabolism of *myo*-inositol-containing phospholipids in mammalian tissue.

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## References

[1] K. Mikoshiba, Trends Pharmacol. Sci. 4 (1993) 86.
[2] J.M. Berridge, Nature 361 (1993) 315.
[3] K.S. Fisher, M.A. Heacock, W.B. Agranoff, J. Neurochem. 58 (1992) 18.
[4] A.D. Fruman, E.R. Mayers, C.L. Cantley, Annu. Rev. Biochem. 67 (1998) 481.
[5] X. Zhu, J. Eichberg, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 9818.
[6] U.H. Shetty, B.M. Shapiro, W.H. Holloway, S.I. Rapoport, J. Clin. Invest. 95 (1995) 542.
[7] U.H. Shetty, W. Huang, B.M. Shapiro, J. Neurochem. 70 (1998) S66C.
[8] K.T. Shonk, A.R. Moats, P. Gifford, T. Michaelis, C.J. Mandigo, J. Izumi, D.B. Ross, Radiology 195 (1995) 65.
[9] M.L. Hallcher, R.W. Sherman, J. Biol. Chem. 255 (1980) 10896.
[10] J.G. Moore, M.J. Bebchuk, K.J. Parrish, W.M. Faulk, L.C. Arfken, J. Strahl-Bevacqua, K.H. Manji, Am. J. Psichiatry 156 (1999) 1902.
[11] J.M. Berridge, P.C. Downes, R.M. Hanley, Cell 59 (1989) 411.
[12] E.G. Chikhale, A. Balbo, Z. Galdzicki, S.I. Rapoport, U.H. Shetty, Biochemistry 40 (2001) 11114.
[13] Shetty et al., to be published.