DIONEX

Application Note 65

Analysis of Inositol Phosphates

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INTRODUCTION

Information transmitted between cells is carried by compounds that are often impermeable to the cell membrane. These compounds bind to cell surface receptors, thereby activating reaction pathways that terminate in the release of a compound into the cytoplasm that carries the original information. This compound is called the second messenger and it can act either directly or indirectly. Second messengers transmit information regulating a variety of physiological and biochemical processes, including cell growth. Inositol 1,4,5-triphosphate (Figure 1) is an important second messenger. In response to extracellular signals, the membrane phospholipid, phosphatidylinositol bisphosphate [Ptd-(4,5) P2] is enzymatically cleaved into diacylglycerol and inositol 1,4,5-triphosphate [Ins(1,4,5)P3], both of which are second messengers. This reaction is believed to be one of the first after receptor activation. Ins(1,4,5)P3 causes the release of Ca^{2+} from intracellular stores, which triggers a wide variety of cellular processes. Ins(1,4,5)P3 can be converted to other inositol phos-

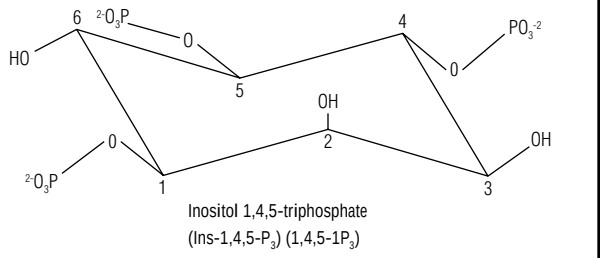
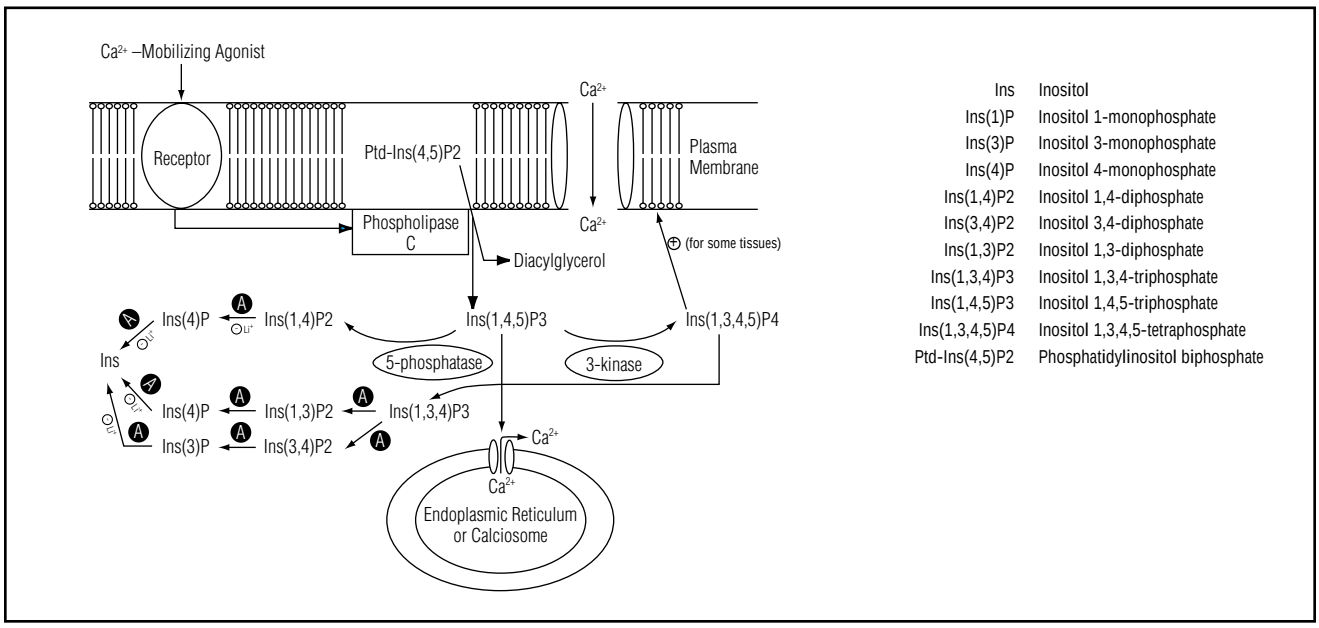


Figure 1 *Structure of inositol 1,4,5-triphosphate.*



Ins	Inositol
Ins(1)P	Inositol 1-monophosphate
Ins(3)P	Inositol 3-monophosphate
Ins(4)P	Inositol 4-monophosphate
Ins(1,4)P2	Inositol 1,4-diphosphate
Ins(3,4)P2	Inositol 3,4-diphosphate
Ins(1,3)P2	Inositol 1,3-diphosphate
Ins(1,3,4)P3	Inositol 1,3,4-triphosphate
Ins(1,4,5)P3	Inositol 1,4,5-triphosphate
Ins(1,3,4,5)P4	Inositol 1,3,4,5-tetraphosphate
Ptd-Ins(4,5)P2	Phosphatidylinositol biphosphate

phates (tetra-, tri-, di-,and mono-phosphorylated inositols), the functions of which are yet to be understood. It has been proposed that inositol 1,3,4,5-tetraphosphate acts to increase the intracellular calcium concentration when the calcium-releasing factor, Ins(1,4,5)P3, is present.[1] The basic metabolism of the inositol phosphates is depicted in Figure 2. A more detailed description of inositol phosphate metabolism can be found in a review by Berridge and Irvine.[1]

Traditionally, inositol phosphates have been radiolabeled or derivatized in another manner for detection and separation by a variety of techniques, including open bed anion-exchange chromatography,[2] gas-liquid chromatography,[3] high-voltage electrophoresis,[4] and ion-pair chromatography.[5] These techniques are all limited either in their ability to separate structural isomers, the need for pre- or postcolumn derivatization, or by analysis time.

This report describes the use of solvent-compatible high-performance anion-exchange chromatography with chemically suppressed conductivity detection to determine inositol phosphate isomers.

RECOMMENDED EQUIPMENT

Dionex DX 500 BioLC® system consisting of:

GP40 Gradient Pump

CD20 Conductivity Detector or
ED40 Electrochemical Detector

LC20 Chromatography Enclosure

AS3500 Autosampler

PeakNet Chromatography Workstation

CONDITIONS

Column:	Omnipac® PAX-100 (4 x 250 mm) and PAX-100 Guard (4 x 50 mm)
Eluent A:	Deionized water (18 MΩ-cm)
Eluent B:	200 mM NaOH
Eluent C:	50% Isopropyl alcohol (aqueous)
Suppressor:	Anion Trap Column, ATC-1 Self-Regenerating Suppressor, ASRS, external water mode

Note: Eluent B should be prepared from 50% liquid NaOH rather than pellets. This limits the amount of carbonate in the eluent, which can reduce the effective column capacity. The OmniPac column requires a minimum of 1% organic solvent at all times. To ensure that this condition is met, it may be prudent to have 1% isopropyl alcohol in eluent A. If the background conductivity becomes too high, the ATC probably needs to be cleaned (see ATC product insert) or replaced. All eluents should be sparged with and pressurized under helium.

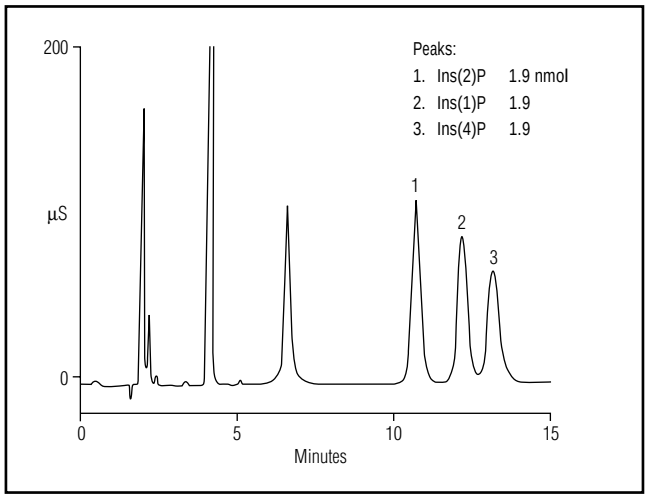


Figure 3 *Separation of inositol monophosphates.*

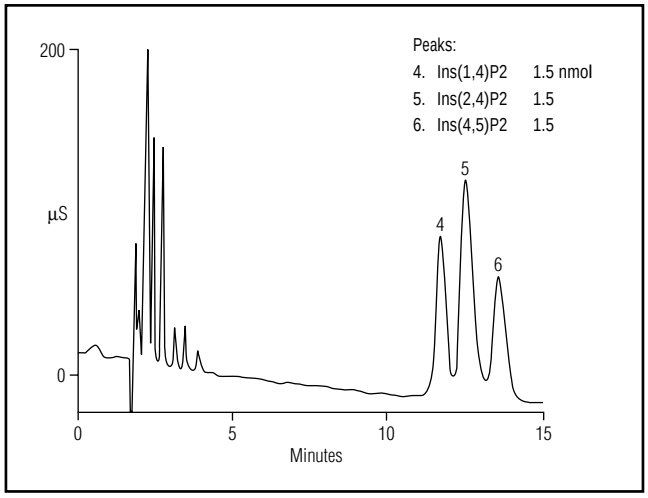


Figure 4 *Separation of inositol diphosphates.*

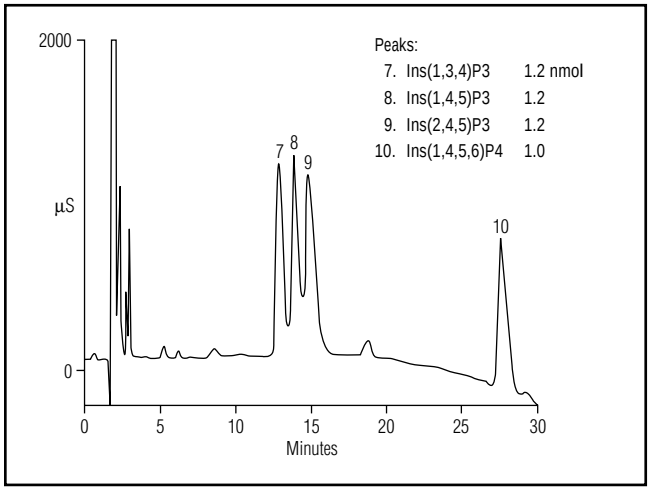


Figure 5 *Separation of inositol triphosphates and an inositol tetraphosphate.*

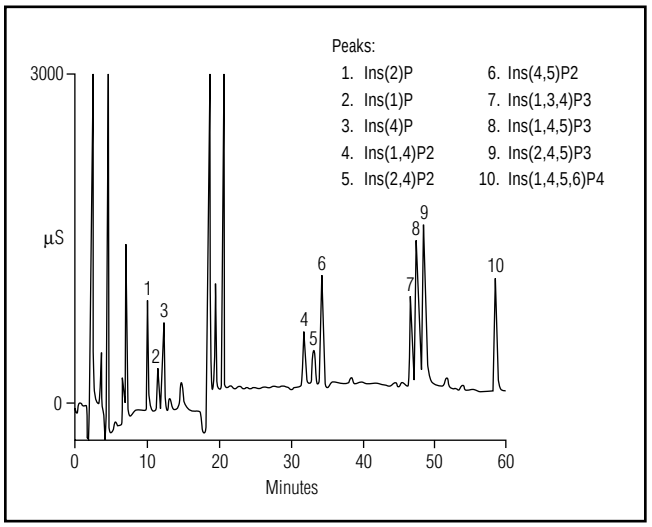
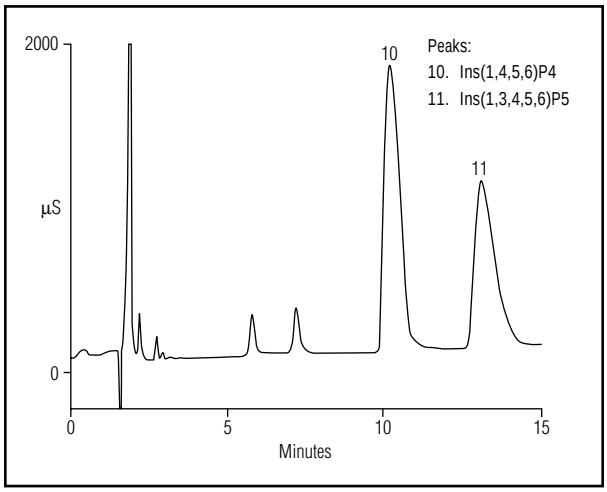


Figure 6 *Separation of inositol mono-, di-, tri- and tetraphosphates in one injection.*



GRADIENT PUMP OPERATION PROGRAMS

Analysis of Inositol Monophosphates

Time (min)	Flow (mL/min)	Percentage A	B	C	Comment
0.0	1.0	82	6	12	Start
0.1	1.0	82	6	12	Injection
15.0	1.0	82	6	12	End

Analysis of Inositol Diphosphates

Time (min)	Flow (mL/min)	Percentage A	B	C	Comment
0.0	1.0	68	30	2	Start
0.1	1.0	68	30	2	Injection
15.0	1.0	68	30	2	End

Analysis of Inositol Triphosphates

Time (min)	Flow (mL/min)	Percentage A	B	C	Comment
0.0	1.0	42	56	2	Start
0.1	1.0	42	56	2	Injection
10.0	1.0	42	56	2	End Isocratic
25.0	1.0	31	61	8	Gradient
30.0	1.0	31	61	8	End

Combined Analysis of Inositol Mono-, Di-, and Triphosphates

Time (min)	Flow (mL/min)	Percentage A	B	C	Comment
0.0	1.0	82	6	12	Start
0.1	1.0	82	6	12	Injection
13.0	1.0	82	6	12	End 1st Step
13.1	1.0	68	30	2	Start 2nd Step
30.0	1.0	68	30	2	End 2nd Step
30.1	1.0	42	56	2	Start 3rd Step
43.0	1.0	42	56	2	End 3rd Step
55.0	1.0	31	61	8	Gradient
65.0	1.0	31	61	8	End

POSSIBLE INTERFERENCES

ADP and ATP do not interfere with the determination of any of the inositol phosphates. Both elute early in the inositol triphosphate method. Phosphate, sulfate, fructose 1-phosphate, and fructose 1,6-diphosphate all elute before the inositol monophosphates. Glucose 1-phosphate elutes between Ins(2)P and Ins(1)P. Glucose 6-phosphate elutes early in the inositol diphosphate method. Fructose 2,6-diphosphate elutes early in the inositol triphosphate method.

SAMPLE PREPARATION

There are a variety of ways to extract inositol phosphates from tissues. These methods are tailored to the tissue of interest, but all methods are designed to remove proteins and organics. Protein can be removed by precipitation with trichloroacetic acid. Trichloroacetic acid and organics are then removed by extraction [chloroform:methanol (2:1)[6] and diethylether[7,8] have been used]. The aqueous phase can then be passed through a Dionex OnGuard Ag sample treatment cartridge (P/N 39637) to remove chloride ion. The OnGuard H cartridge (P/N 39596) is also useful for removing metal ions. Nucleotides can be removed by charcoal adsorption.[7] A report has also appeared investigating sample preparation methods for rat brain tissue. It was determined that homogenization with 10 volumes of deionized water and extraction with 10 volumes of chloroform:methanol (2:1) gave the best recovery of inositol monophosphates.

RESULTS

Figures 3, and 4, and 5 demonstrate the success of separating the inositol monophosphates, inositol diphosphates, and inositol triphosphates with the methods described in the Conditions section. In addition, inositol 1,4,5,6-tetraphosphate can be determined simultaneously with the inositol triphosphates. The tetraphosphate can be eluted earlier if more isopropyl alcohol is added, but there is some baseline drop. All three methods can be combined if a simultaneous determination of mono-, di-, and triphosphates is required. If one chooses to string the three methods together, a few minutes must be added to the final two methods to account for the fact that the column is not pre-equilibrated at those conditions. A chromatogram of this type is shown in Figure 6. The chromatogram in Figure 6 requires baseline subtraction. An inositol tetraphosphate can also be isocratically separated from an inositol pentaphosphate in less than 15 minutes (Figure 7). This separation uses the final eluent conditions of the inositol triphosphate separation.

Detection was linear for inositol 1-monophosphate between 200 picomoles and 200 nanomoles. Values outside of this range were not investigated. As little as 70 picomoles of this standard was detectable. More highly phosphorylated inositols give better response with the suppressed conductivity detector.

CONCLUSION

The use of solvent-compatible anion-exchange chromatography with chemically suppressed conductivity detection affords rapid separation of inositol phosphate isomers without pre- or postcolumn derivatization.

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