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# DETECTION OF INOSITOL TRISPHOSPHATE AND OTHER ORGANIC PHOSPHATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AN ENZYME-LOADED POST-COLUMN REACTOR

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## SUMMARY

A technique is described which permits detection of inositol bis- and trisphosphates (and other organic phosphates) by high-performance liquid chromatography. After separation of the compounds by anion-exchange chromatography, the column effluent is first passed through a column of immobilized alkaline phosphatase to hydrolyse the phosphate esters. Then, the stream is continuously mixed with a molybdate solution to allow detection of the inorganic phosphate formed. The enzyme can be immobilized simply by adsorbing it to a moderately hydrophobic support. The limit of detection is less than 1 nmol. Separations are made using sodium sulfate gradients at pH 7.4 on polymeric stationary phases. Addition of calcium ions (5 m$M$) to the mobile phase improves separation of positional isomers, and changes retention relative to adenine nucleotides which are among the main interferences in tissues.

## INTRODUCTION

Inositol trisphosphate (IP$_3$) is a compound of great biological interest for which no convenient, sensitive measurement technique exists. Increased formation of IP$_3$ is a consequence of activation of most cells by transmitters, hormones or signals including acetyl choline, angiotensin, serotonin, insulin, glucose and light[1,2]. Studies of IP$_3$ function have usually involved examination of the formation *in vitro* of radio-labelled IP$_3$ in cells or tissue slices incubated with radioactive inositol or inorganic phosphate, or, examination of the effects of addition of IP$_3$ to cell preparations. We are not aware of direct measurements of the content in any tissue of any species of IP$_3$ or its metabolite inositol bisphosphate (IP$_2$). Indirect estimates of IP$_3$ content have been made for isolated liver cells based on guesses of IP$_3$ specific activities after radio-labelling[3]. Development of a technique for IP$_3$ measurement would open up new possibilities for study of the mechanism of action of drugs and hormones

in intact animals. Anion-exchange separations of inositol phosphates have been made, but detection was cumbersome, requiring collection of fractions for off line measurement of radioactivity[4], or for hydrolysis at 160°C followed by colorimetric measurement of released inorganic phosphate[5]. Inositol phosphates are cyclitols, carbohydrates with no chromophores, fluorophores, or electroactive groups which could be used for high-performance liquid chromatography (HPLC) detection. Since inorganic phosphate (Pi) can be rapidly measured with high sensitivity using its reaction with molybdate, we examined whether enzymatic post-column hydrolysis of the inositol phosphates would allow their detection. Enzymatic post-column reactors have been used for analysis of other difficult to detect compounds such as acetylcholine[6], hydroxysteroids[7,8], and cyanogenic glycosides[9]. In this paper, we report that IP_3 can be measured by HPLC using immobilized alkaline phosphatase and an inorganic phosphate detection system.

MATERIALS AND METHODS

Phenoxyacetyl cellulose, cellulose carbonate, inositol-2-monophosphate, inositol trisphosphate, HEPES (hydroxyethylpiperazine sulfonic acid), Triton X-100, ammonium molybdate and calf intestinal alkaline phosphatase type VII-NT were obtained from Sigma, St. Louis, MO, U.S.A. The enzyme solution contained 8.5 mg protein, 5000 units, in 0.43 ml 3 $M$ sodium chloride, where 1 unit will hydrolyse 1 $\mu$mol/min $p$-nitrophenyl phosphate at pH 10.4 and 37°C. An experimental fluoropolymeric support for enzyme adsorption was obtained from Biotechnology Systems Division of Dupont Biomecial Products. A partial hydrolysate of phytic acid (inositol hexakisphosphate) was a gift of W. R. Sherman, Washington Univ., St. Louis, MO, U.S.A. The hydrolysate, which contained inositol bis, and some trisphosphate was prepared by refluxing sodium phytate at pH 9 for 48 h.

The chromatographic system consisted of an HPLC pump with gradient former, an injection valve, a strong anion-exchange column, a second injection valve with a 3 cm × 4.6 mm immobilized enzyme column instead of a loop, a mixing tee for addition of acid molybdate reagent pumped by a second pump, and a fluorometer
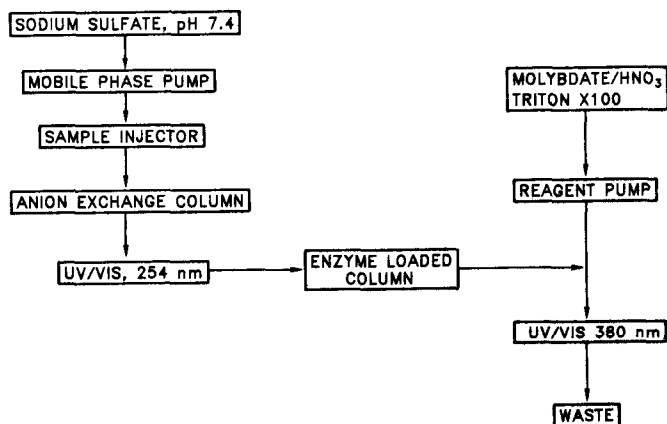


Fig. 1. Block diagram of the apparatus.

or a variable-wavelength detector set at 380 nm. Chromatographic data was collected by a Hewlett-Packard HP1000 laboratory automation system for later plotting. In some experiments, a second UV monitor was added immediately after the column (Fig. 1) to permit selective detection of nucleoside phosphates in the presence of inositol phosphates. The HPLC columns used were: Pharmacia Mono Q HR 5/5, 10 $\mu$m particle size, and Bio-Rad Aminex A-27, 10 cm × 4.6 mm, 15 $\mu$m particle size. The mobile phase gradients were generated from mixtures of a weak eluent A (0.1 m$M$ zinc sulfate containing 5 m$M$ HEPES and adjusted to pH 7.4 with sodium hydroxide), and a strong eluent B (1 $M$ sodium sulfate containing 5 m$M$ HEPES buffer and 0.1 m$M$ $Zn^{2+}$). An acid molybdate stock reagent was prepared by mixing equal volumes of 10% (w/v) ammonium molybdate and 70% nitric acid. In initial experiments, this reagent was diluted 1:10 with water and Triton X-100 was added to give a final concentration of 0.01%. In recent work, the final concentration of Triton X-100 was increased to 1%. The flow-rates of the mobile phase and reagent were 1.0 and 0.3 ml/min, respectively. Good quality phosphate-free deionized or distilled water is required both for preparing all solutions, and for rinsing glassware of traces of phosphate left from detergents. Phosphate was removed from the Triton X-100 by stirring a 10% (v/v) solution with 5% its weight of Dowex 1 X-8 (100–200 mesh) and then filtering off the resin.

*Phosphate detection*

In initial experiments, phosphate was detected turbidimetrically. Phosphate and molybdate form a complex which becomes turbid at low concentrations in the presence of 0.01% Triton X-100[10]. For detection, we used a fluorometer (with the excitation and emission filters replaced by pin-hole slits) or a UV monitor set at 380 nm[11]. A knitted coil capillary[12] (2 m × 0.012 in. PTFE tubing) provided a delay for precipitation to occur. The limit of detection (with no columns) was about 1 nmol for the fluorometer, and <100 pmol for the UV monitor. This system had the disadvantage that the precipitate would gradually coat the detector cell walls, causing the signal to drift after injection of excess phosphate. This coating required daily removal with acetone or methanol. Kyaw et al.[13] recently found that excess Triton X-100 dissolves the phosphomolybdate complex, and increases its absorbance. We find that for continuous monitoring of phosphate, a high concentration of Triton X-100 provides sensitivity similar to that of the turbidimetric mode, without a baseline shift problem or need for a delay coil.

*Enzyme immobilization*

Both covalent and non-covalent immobilizations methods were tested. First, enzyme was coupled via glutaraldehyde cross links[8] to aminopropylsilica. The immobilized enzyme was active. However, phosphates were retained by the support, which is a weak anion exchanger. Coupling of 50 $\mu$l of alkaline phosphatase to cellulose carbonate was also successful. The column was useable for about 2 weeks. However, a more convenient means of immobilization for HPLC is simple adsorption[6]. Moderately hydrophobic packings were chosen which would adsorb the enzyme, but should have low affinity for the phosphorylated substrates or detected product (Pi). Phenoxyacetylcellulose[14] or the polymeric support were packed into the 3-cm column as a slurry in 90% methanol at 2 ml/min. Because of the short

column length and low back pressure, the column can also be packed by hand using a 1-ml syringe and a 2-cm length of 1/4 in. O.D. plastic tubing connected to the empty column via a union. Slurry is added to the column in successive portions until the level rises into the plastic tubing. The column is then washed with mobile phase to remove the methanol before adding the enzyme. Phenoxyacetylcellulose columns last for about a month at room temperature before hydrolysis of the ester groups decreases adsorptive capacity. The inert fluoropolymeric support appears to last much longer. Enzyme can be added as necesssary to the column either via the loop valve, or with the column removed from the system, via a short length of 1/16 in. O.D. tubing as an adapter.

*Preparation of IP$_2$ and IP$_3$*

Examination of separation conditions requires standards. At the beginning of these experiments, IP$_3$ was not commercially available, and the synthesis reported[15] for specific IP$_2$ isomers was complex. Phytate hydrolysates contain both IP$_2$ and IP$_3$, but are contaminated with a large excess of Pi. Pure samples of IP$_2$ and IP$_3$ were prepared by a simple phosphorylation procedure. Inositol (4 g) and 85% phosphoric acid (8 ml) were heated to 120°C for 2 h under vacuum produced by a mechanical pump with dry ice trap. The syrupy residue was dissolved in 40 ml water. A 5-ml aliquot was neutralized with potassium hydroxide and passed over a 30 × 1 cm column of Dowex 1 X-8 in the bicarbonate form, and eluted with a linear gradient of 0–1 $M$ triethylamine bicarbonate. The buffer was prepared by adding 140 ml of triethylamine to 700 ml water, and bubbling carbon dioxide (generated from dry ice pellets) until the 2 phases mix and the pH approaches 7.6, and making up to 1 l. Effluent fractions were tested in the HPLC system for Pi (with no columns) or for inositol phosphates (with only the enzyme column). Peaks were observed for IP$_2$, IP$_3$, and an intermediate peak which might contain inositol pyrophosphates or cyclic phosphates. The fractions containing the IP$_2$ and IP$_3$ were evaporated, converted to the acid form by Dowex 50 (H$^+$), adjusted to pH 10 with potassium hydroxide, and evaporated to give an amorphous crystalline material.

RESULTS

The extent of conversion of substrate to product by the enzyme column was routinely checked with a readily available test substance (fructose-1,6-diphosphate). Conversion was quantitative in the 20-s residence time of the substance in the enzyme loaded column (Fig. 2), *i.e.* the peak height for the diphosphate was twice that of an equimolar amount of Pi. Without the enzyme column, the peak height for Pi was increased slightly due to decreased band spreading, while fructose di phosphate gave only a small signal (probably from Pi contained as an impurity in the preparation). Injection of deionized water gave no signal at all. IP$_3$ (2 nmol) was readily detectable, but the reaction was only about 60% complete with the 50 $\mu$l of enzyme used on the column.

Standards of IP$_2$ and IP$_3$ were not cross contaminated with each other (Fig. 3). IP$_2$ gave only a single peak, although it contains a number of positional isomers. The single IP$_3$ peak was broader, probably due to non-resolved positional isomers. The baseline shift after the IP$_3$ was due to sample overload.

WITH ENZYME                    WITHOUT
                               ENZYME
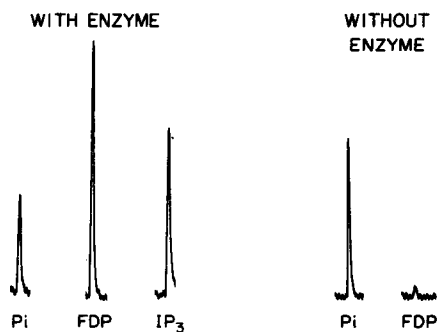
Pi        FDP      IP$_3$              Pi        FDP

Fig. 2. Conversion of phosphate esters to inorganic phosphate in a flow system. Samples (2 nmol each) of inorganic phosphate, fructose diphosphate or inositol trisphosphate (Sigma) were injected, both with and without the enzyme column in the stream. The anion-exchange column was not connected. The effluent was mixed with molybdate–nitric acid–1% Triton X-100 and the absorbance measured at 380 nm (0.1 a.u.f.s.).

The separation of positional isomers can be improved by changing the stationary phase, or by adding divalent ions to the mobile phase. When a phytic acid hydrolysate was chromatographed on a silica based ion exchanger (Whatman SAX), the IP$_2$ peak was separated into two completely resolved components (data not shown). Unfortunately, such silica columns are less stable at pH 7.4 than polymer based columns. With a Mono-Q column, the single IP$_2$ peak in a phytate hydrolysate sample was split into two components by the addition of calcium to the mobile phase (Fig. 4).

The major interfering compounds in tissue extracts are likely to be nucleotides (such as ATP), "dinucleotides" (such as NADP), and for IP$_2$, fructose diphosphate. All of the nucleotides absorb strongly at 254 nm, allowing their possible co-elution with inositol phosphates to be checked if 2 detectors are used in series (Fig. 5). On the Mono-Q column, with no calcium in the mobile phase, IP$_2$ was partly resolved from fructose diphosphate, and completely separated from the adenine nucleotides
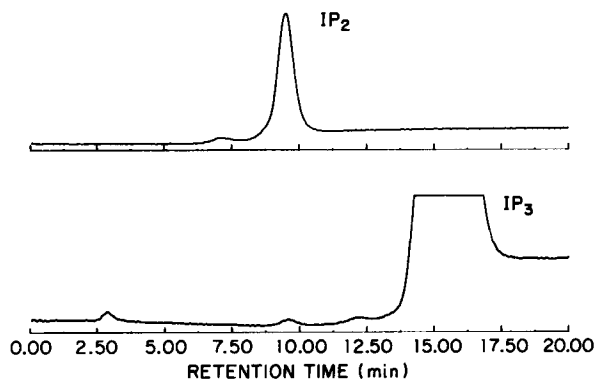
IP$_2$

IP$_3$

0.00   2.50   5.00   7.50   10.00  12.50  15.00  17.50  20.00
          RETENTION TIME (min)

Fig. 3. Purity of standards of IP$_2$ (top) and IP$_3$ (bottom). Conditions: Aminex A-27 column, 10 cm × 4.6 mm, 70°C, 0–1 $M$ sodium sulfate in 20 min at 1 ml/min. Molybdate–nitric acid–0.01% Triton X-100 reagent was pumped at 0.3 ml/min. The mobile phase contained 100 $\mu M$ Zn$^{2+}$, but no Ca$^{2+}$.
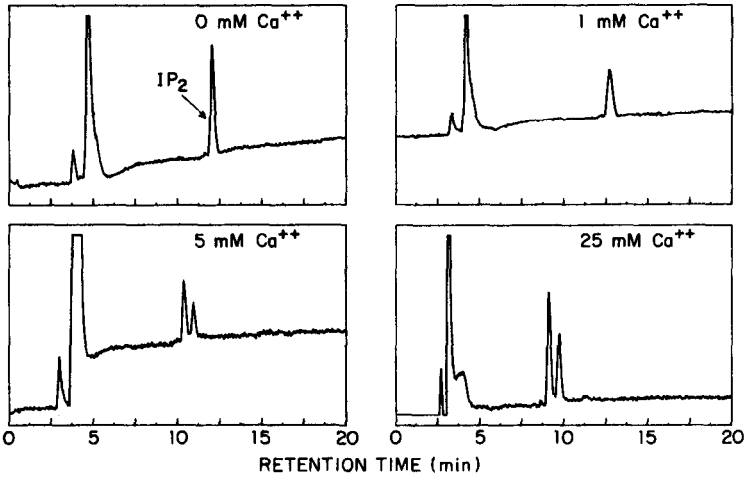
Fig. 4. Effect of $Ca^{2+}$ on the separation of postional isomers of $IP_2$. A phytic acid hydrolysate was injected onto a Mono-Q column at room temperature, with a gradient of 0.01–0.25 $M$ sodium sulfate containing varying amounts of calcium acetate. Phosphate detection was by light scattering in a fluorometer.
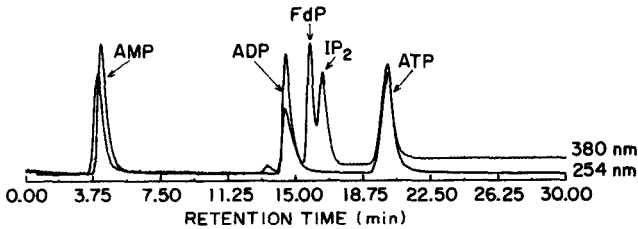


Fig. 5. Separation of standards on a Mono-Q column with a 0–0.2 $M$ sulfate gradient in 30 min. The mobile phase contained 100 $\mu M$ zinc, but no calcium.
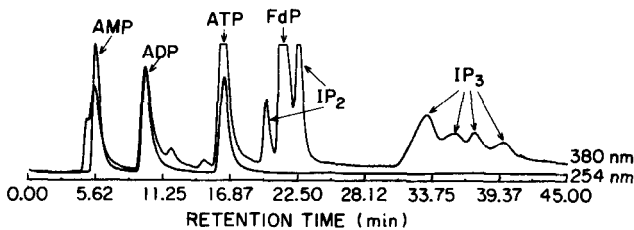


Fig. 6. Separation of $IP_2$ and $IP_3$ from interfering compounds on a Mono-Q column using a 0–0.1 $M$ sulfate gradient in 40 min. The buffers contained 5 m$M$ calcium sulfate.
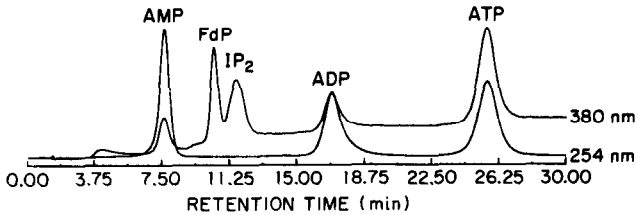
Fig. 7. Separation of standards on an Aminex A-27 column at 70°C with a 0.1–0.6 $M$ sulfate gradient in 30 min. The mobile phase contained no calcium.

(Fig. 5). Addition of calcium to the mobile phase decreases the retention of all compounds, and changes the elution order (Fig. 6). IP$_2$ was split into 4 peaks, 2 of which co-elute with fructose diphosphate. The IP$_3$ peak was split into 4 poorly resolved components.

On Aminex A-27 columns, peak spacing can be controlled by both temperature and ionic effects. At 70°C, with no calcium, IP$_2$ elutes between AMP and ADP; IP$_3$ coelutes with ADP (Fig. 7). At 30°C, IP$_2$ coelutes with AMP, and IP$_3$ elutes between AMP and ADP (data not shown). At 60°C with 5 m$M$ calcium, IP$_2$ and IP$_3$ are split into 2 and 3 peaks respectively, and separated from the nucleotides (Fig. 8).

Linearity and precision were determined using samples chromatographed on a Mono Q column at a flow-rate of 1 ml/min and a reagent flow-rate of 0.3 ml/min. Peak areas were linear with samples sizes from 0.5 to 100 nmol IP$_2$. Regression analysis gave a correlation coefficient of 0.992 and an intercept of 0.023 nmol. The precision of the system was determined by measuring the peak area for repeated injections of 5 nmol samples of IP$_2$ or IP$_3$ within a single run (10 injections) or for single injections on 7 different runs during a 14-day period. The relative standard deviation was 7% for variability within runs, and 14% between runs. The cause of variability on different days include temperature changes (4°C), different preparations of buffers and molybdate reagent as well as instrumental variation. The Mono Q column itself appears quite stable: it has been in occasional use for 9 months.

DISCUSSION

The technique reported here consists of three components (separation, enzymatic hydrolysis and phosphate detection) which are much more interrelated than in conventional HPLC with UV detection. The choice of mobile phase components
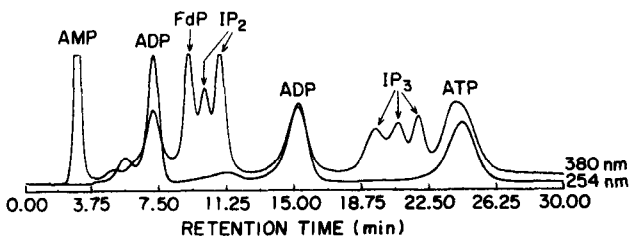


Fig. 8. Separation of IP$_2$ and IP$_3$ from interfering compounds at 60°C on an Aminex A-27 column with a 0–0.4 $M$ sulfate gradient in 30 min with a mobile phase containing 5 m$M$ calcium sulfate.

and pH, for example, is restricted. Strong ions such as sulfate are preferred for the gradient over weak ions such as formate since the effluent must be brought to a very low pH for Pi detection. Chromatograms with chloride and nitrate ions showed similar separations to those seen with sulfate. However, chloride is more corrosive to steel HPLC systems than sulfate containing mobile phases, and nitrate apparently damaged the column stationary phases. Nitric acid was chosen for the molybdate reagent, since it is less corrosive to the pumping system than sulfuric or hydrochloric acids. The pH chosen for the mobile phase (7.4) is likely to be optimal for enzyme stability, even if not for enzyme activity, which is maximal at pH 9–10.5, depending both on buffer and substrate. Separation of inositol monophosphate from Pi is poor at pH 7.4, and is likely to be better at a pH less than 5. However, enzyme activity under such conditions would be too low to be useable.

Adsorptive losses can occur with the phosphates (especially IP$_3$, but even Pi) when dealing with low amounts of substance ($< 10$ nmol). Adsorption occurs primarily when the column packing becomes contaminated, but can also occur on column frits or connecting tubing. In recent experiments, adsorptive losses disappeared when steel connecting tubing and frits were replaced with PTFE.

Alkaline phosphatase requires divalent ions ($Zn^{2+}$ or $Mg^{2+}$) for activity. Use of a chelating buffer (citrate) caused rapid enzyme inactivation, which was reversed by adding some zinc. Although the enzyme will remain active even after prolonged washing with a HEPES buffered mobile phase supposedly free of zinc or magnesium, we routinely add zinc to all buffers ($100 \ \mu M$ final concentration). Addition of ions can, however, have peculiar effects. For example, Pi was retained by the phenoxy-acetylcellulose enzyme column, when a low ionic strength mobile phase was used (5 m$M$ calcium acetate, 5 m$M$ HEPES, and no sodium sulfate). Addition of large amounts of $Mg^{2+}$ or $Zn^{2+}$ can cause total loss of sensitivity due to inhibition of the enzyme or to adsorption of the substrates or products to the anion exchanger or enzyme column.

The retention of enzyme activity on the columns used here is surprisingly stable. When they the columns are left continuously at room temperature, 50 $\mu$l enzyme is normally added only once a week. There was little if any obsesrvable loss of phosphate hydrolysis when a phenoxyacetylcellulose column with 50 $\mu$l enzyme adsorbed was refrigerated overnight during 2 weeks of daily use. The loss of enzyme that does occur might be due to elution, loss of metal ions, physical damage or thermal denaturation due to room temperature operation. The enzyme must of course be protected when used with an anion exchanger at 70°C: the first UV detector acts as a heat sink in addition to aiding in peak identification. This detector should not be placed after the enzyme column even at room temperature operation. Compounds such as ATP are hydrolysed to Pi (which is not retained by the enzyme column), and adenosine (which is slightly retained, and would be observed as a broad peak). The hydrophobic interactions of the nucleotide bases with the matrix of the ion exchangers is probably the cause of the different elution patterns seen with the Mono-Q and Aminex columns. The Mono-Q has a hydrophilic support, while the anion exchanger is polystyrene based. It is probably the hydrophobic interactions that causes the temperature sensitivity of the elution order on Aminex columns.

The use of enzymatic hydrolysis and post column reaction add considerable complexity to an HPLC system. However, the sensitivity and selectivity achieved by

this technique should be sufficient to detect IP$_2$ and IP$_3$ in tissue extracts. Such measurements should be possible when satisfactory extraction and concentration steps are developed.

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