

# A rapid separation method for inositol phosphates and their isomers

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A technique is described using ACCELL QMA anion-exchange SEP-PAKs (Waters Associates) with ammonium formate-based solutions, whereby a sample can be processed within minutes to yield resolution of inositol phosphates. Isomers of inositol trisphosphate can then be separated by using this technique in combination with a rapid (5–6 min) isocratic h.p.l.c. procedure. The use of QMA SEP-PAKs offers a degree of reproducibility comparable with that of h.p.l.c. while maintaining the capacity for automation, allowing large numbers of samples to be processed rapidly.

## INTRODUCTION

Considerable interest in inositol phosphates has developed since the demonstration that activation of some classes of plasma-membrane receptor leads to the production of  $\text{Ins}(1,4,5)P_3$ , which promotes mobilization of intracellular calcium stores (for a review, see Berridge & Irvine, 1984). The further discovery that  $\text{Ins}(1,4,5)P_3$  is phosphorylated to form  $\text{Ins}(1,3,4,5)P_4$  (see, e.g., Batty *et al.*, 1985; Irvine *et al.*, 1986a), with its own potential to serve as an intracellular messenger (Irvine & Moor, 1986) and the recognition of even-more-highly-phosphorylated forms in some cell types (Heslop *et al.*, 1985), has served to extend the list of inositol phosphates which must be identified and studied.

Radiolabelled inositol phosphates are measured in radiolabelled stimulated tissues either by determination of total inositol phosphates formed in the presence of lithium (Berridge *et al.*, 1982) or by separation and identification of the various individual inositol phosphates, including the different isomers, such as  $\text{Ins}(1,3,4)P_3$  and  $\text{Ins}(1,4,5)P_3$  (for a review, see Irvine, 1986). Clearly for rigorous application the use of h.p.l.c. methods is superior (Irvine *et al.*, 1985; Heslop *et al.*, 1985; Meek, 1986), although these limit the practicality of processing large numbers of samples. Although the batch technique of Dowex chromatography (Hübscher & Hawthorne, 1957; Richards *et al.*, 1979; Downes & Michell, 1981; Berridge *et al.*, 1983) is ideally suited for a large sample number, it is not a rapid procedure.

We describe here the application of ACCELL QMA anion-exchange SEP-PAKs (Waters Associates) to the separation of inositol phosphates. This method offers the advantages of high sample recovery combined with rapid sample processing, and excellent reproducibility; a further feature is that it is amenable to automation. A preliminary report of this technique was given at the 619th Meeting of the Biochemical Society (Wreggett *et al.*, 1986).

## MATERIALS AND METHODS

### Inositol phosphates

$[^3\text{H}]\text{GroPIns}$ , prepared as described by Dawson *et al.* (1962), was a gift from Dr. C. P. Downes (Smith, Kline and French, Welwyn, Herts., U.K.).  $[^3\text{H}]\text{Ins}(1,4)P_2$ ,  $[^3\text{H}]\text{Ins}(1,4,5)P_3$ ,  $[^3\text{H}]\text{Ins}(1,3,4)P_3$  and  $[^3\text{H}]\text{Ins}(1,3,4,5)P_4$  were kindly supplied by Amersham International. *myo*- $[^3\text{H}]\text{Inositol}$  was purchased from Amersham.  $[^{32}\text{P}]\text{Ins}(1,4,5)P_3$  was purchased from Du Pont.

$\text{Ins}(1,4)P_2$ ,  $\text{Ins}(1,3,4)P_3$ ,  $\text{Ins}(1,4,5)P_3$  and  $\text{Ins}(1,3,4,5)P_4$  were prepared as described by Irvine *et al.* (1984, 1986b).  $\text{Ins}(2)P$  and phytic acid ( $\text{Ins}P_6$ ) as the sodium salt were purchased from Sigma.  $\text{Ins}(1,3,4,5,6)P_5$  was purchased from Calbiochem and converted into its potassium salt before use.

### Preparation of phytic acid hydrolysate

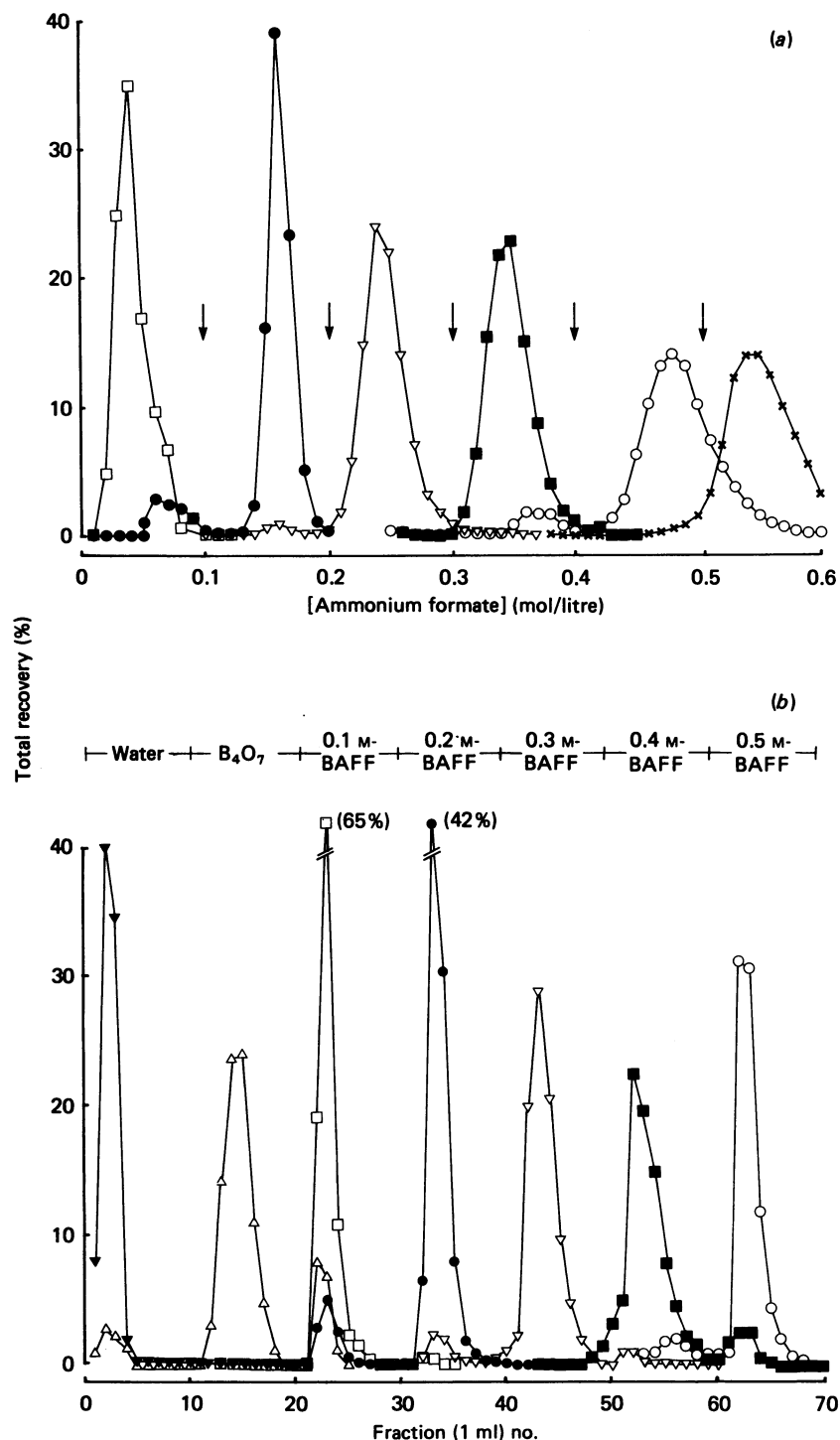
A random mixture of inositol phosphates ( $\text{Ins}P_{1-6}$ ) was prepared by the method of Desjobert & Petek (1956). A 1 g portion of sodium phytate was dissolved in 5 ml of 0.2 M-sodium acetate/acetic acid, pH 4.0, in a glass-stoppered tube and heated for 8 h in a boiling-water bath. After cooling, the solution was desalted by passage through 4 ml of Amberlite IR-120 (H) resin (BDH Chemicals) in a Pasteur pipette. After being dried *in vacuo* the mixture was redissolved in distilled water. The relative composition of the hydrolysate was estimated by phosphorus analysis (Rouser *et al.*, 1969) after fractionation with the SEP-PAK technique described below. In one preparation of this hydrolysate (analysing the equivalent of 0.2 mg of phosphorus), we estimated 14%  $\text{Ins}P_6$ , 24%  $\text{Ins}P_5$ , 18%  $\text{Ins}P_4$ , 9%  $\text{Ins}P_3$ , 4%  $\text{Ins}P_2$ , and 12%  $\text{Ins}P$ , with the remainder probably representing inorganic phosphate, although it is likely that inorganic phosphate also accounts for some of the estimated  $\text{Ins}P$ .

### Sample preparation

All inositol standards were dissolved in a final volume of 0.2 ml of 50 mM-Tris/maleate (pH 8.0 at 37 °C)/

Abbreviations used: BSA, bovine serum albumin; Freon, 1,1,2-trichlorotrifluoroethane; *GroPIns*, glycerophosphoinositol;  $\text{Ins}P$ ,  $\text{Ins}P_2$ ,  $\text{Ins}P_3$ ,  $\text{Ins}P_4$ ,  $\text{Ins}P_5$ , and  $\text{Ins}P_6$ , *myo*-inositol mono-, bis-, tris-, tetrakis-, pentakis- and hexakis-phosphate respectively, with isomeric positioning of phosphate groups as indicated.

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**Fig. 1. (a) Gradient and (b) batch elution of inositol phosphate standards on ACCELL QMA anion-exchange SEP-PAKs**

Standards were prepared as described in the Materials and methods section and loaded on to the SEP-PAK previously converted into the formate form as described in the Materials and methods section. The cartridge was eluted with 2 ml of 10 mM increments of ammonium formate/formic acid (pH 4.75)/5 mM-disodium tetraborate, and the recovery of inositol phosphates was determined. Data are expressed as percentage of total recovery of standards, which in all cases was 90–100%. A slight contamination with other inositol phosphates resulted in multiple peaking of some of the standards (see the text). Standards presented are: □, Ins(2)P; ●, [<sup>3</sup>H]Ins(1,4)P<sub>2</sub>; ▽, [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub>; ■, Ins(1,3,4,5)P<sub>4</sub>; ○, Ins(1,3,4,5,6)P<sub>5</sub>; ×, InsP<sub>6</sub>. The arrows show selected concentrations used for batch elution of inositol phosphates (see b). (b) Standards were loaded as for (a), and the cartridge was washed with 10 ml each of distilled water, followed by 5 mM-disodium tetraborate (B<sub>4</sub>O<sub>7</sub>) and then by concentrations (see a) of ammonium formate/formic acid as indicated (pH 4.75)/5 mM-disodium tetraborate (BAFF), 1 ml fractions being collected. Data are expressed as percentage of total recovery of standards, which in all cases was 90–100%. A slight contamination with other inositol phosphates resulted in multiple peaking of some of the standards (see the text). Standards presented are: ▼, [<sup>3</sup>H]inositol; △, [<sup>3</sup>H]GroPIns; □, Ins(2)P; ●, [<sup>3</sup>H]Ins(1,4)P<sub>2</sub>; ▽, [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub>; ■, Ins(1,3,4,5)P<sub>4</sub>; ○, Ins(1,3,4,5,6)P<sub>5</sub>.

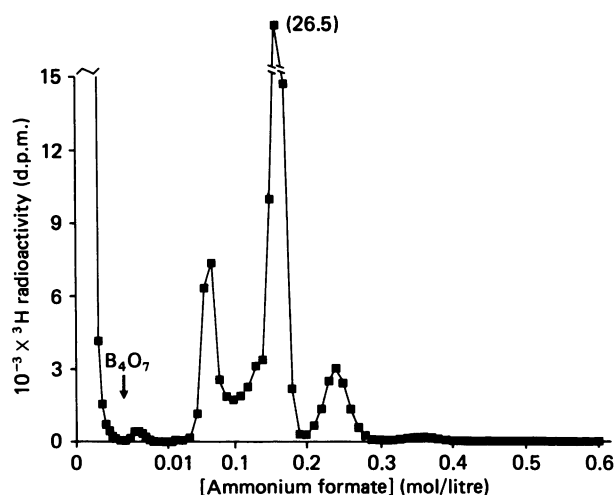


Fig. 2. Gradient elution on ACCELL QMA anion-exchange SEP-PAK of extracted sample from [ $^3\text{H}$ ]inositol-labelled carbachol-stimulated rat parotid-gland slices

Tissue was prepared and treated as described by Batty *et al.* (1985), and water-soluble radioactivity was extracted by using the trichloroacetic acid/diethyl ether-wash procedure. A 2 ml sample was diluted to 5 ml with distilled water, to which was added 2  $\mu\text{l}$  of a phytate hydrolysate (equivalent of 25  $\mu\text{g}$  of phosphorus, prepared as described in the Materials and methods section). The entire amount was loaded on to the SEP-PAK, which had been previously converted into its formate form. The cartridge was eluted with 10 ml each of distilled water, followed by 5 mM-disodium tetraborate ( $\text{B}_4\text{O}_7$ ), 1 ml fractions being collected. Radiolabelled inositol phosphates were then eluted with 2 ml of ammonium formate/formic acid (pH 4.75)/5 mM-disodium tetraborate from 0.01 to 0.6 M at 10 mM increments (see Fig. 1a). Eluted radioactivity in each fraction was measured by liquid-scintillation counting. Measurements of radioactivity for the first five collected fractions were off the scale; these values were 144 170, 636 240, 576 600, 449 100 and 117 700 d.p.m.

20 mM-magnesium acetate in a 1.5 ml Microfuge tube (Eppendorf). These samples were then processed by using the  $\text{HClO}_4$  precipitation and extraction procedure described by Sharpes & McCarl (1982) as adapted by Downes *et al.* (1986). Briefly 0.3 ml of 10% (v/v)  $\text{HClO}_4$  was added to each sample, followed by 0.1 ml of BSA (1 mg/ml) in water. After 5 min on ice the precipitated protein was removed by centrifugation (1 min in an MSE Micro Centaur centrifuge, 2000 g) and the supernatant transferred into another Microfuge tube by means of a disposable plastic pipette. These samples were then neutralized by the addition of 0.75 ml of freshly prepared 1:1 (v/v) Freon/tri-n-octylamine (BDH), followed by 30 s of vigorous vortex-mixing and then 30 s centrifugation (MSE Micro Centaur, 2000 g) to aid phase separation. The upper phase was removed (0.55 ml, > 90% recovery of total sample) and diluted with 2 ml of distilled water in a plastic tube. Some samples (see below and Fig. 2) had been processed by the trichloroacetic acid precipitation procedure (Batty *et al.*, 1985).

In experiments designed to determine the conditions required for sample processing and chromatography,

radiolabelled inositol phosphates were dissolved in water containing various reagents and then processed as described above (see the Results and discussion section).

### Chromatography

ACCELL QMA anion-exchange SEP-PAKs (Waters Associates) were converted into the formate form by washing first with 10 ml of a solution of 1.0 M-ammonium formate in 0.1 M-formic acid, followed by 20 ml of distilled water.

Samples were loaded on to the SEP-PAK and then eluted with various solutions as described in the Results and discussion section. In all manipulations with the cartridges care was taken to remove air bubbles trapped inside and to prevent air from passing into the cartridge. Sample loading and solution delivery were performed manually by using disposable plastic syringes; an approximate flow rate of 10–15 ml/min was typically maintained.

Elution of radiolabelled compounds was determined by quantification of radioactivity as d.p.m. in portions of eluted samples using liquid-scintillation spectrometry (Beckman model LS3801 instrument, operating at around 40% efficiency for  $^3\text{H}$  and at > 95% efficiency for  $^{32}\text{P}$ ). Elution of unlabelled compounds was determined on portions of eluted fractions by phosphorus analysis as described by Rouser *et al.* (1969).

### Inositol phosphates from tissue samples

Samples from stimulated rat parotid and cerebral-cortical slices, prepared as described by Batty *et al.* (1985), were generously provided by Dr. I. R. Batty and Dr. S. R. Nahorski (University of Leicester Medical School, Leicester, U.K.). These samples had been extracted by precipitation with trichloroacetic acid, and the precipitate neutralized by washing with diethyl ether (cf. Batty *et al.*, 1985). Traces of diethyl ether remaining in the aqueous phase were removed by subjecting the sample to a stream of air.

## RESULTS AND DISCUSSION

### Sample preparation

During the development of the application of QMA SEP-PAKs and the choice of sample conditions, we observed some problems with both the recovery and the elution properties of inositol phosphate standards, particularly with the radiolabelled compounds. These problems we have attributed to non-specific binding of high-specific-activity inositol phosphates in the octylamine perchlorate phase (Wreggett *et al.*, 1987) and were circumvented by inclusion of phytate hydrolysate. The elution properties of high-specific-radioactivity inositol phosphates from the SEP-PAK cartridges were also variable initially, but this too could be completely eliminated by the addition of a portion of a phytate hydrolysate (25  $\mu\text{g}$  of phosphorus) to the samples. Presumably in both instances the various inositol phosphates in the phytate hydrolysate act as carrier molecules, so that any trace losses due to non-specific binding represent an insignificant proportion of the total radioactivity.

Provided that the samples are sufficiently diluted in water, there does not appear to be any dependency of elution properties on the initial conditions of the sample;

nevertheless, for batch processing we advocate prior testing by stepwise elution (as in Fig. 2) of a typical sample with an inositol phosphate standard to determine suitable concentrations for batch elution.

#### Chromatography of inositol phosphate standards with SEP-PAKs

All inositol phosphates examined were retained on ACCELL QMA SEP-PAKs and could be eluted with ammonium formate buffers at pH 4.75 (prepared from a stock solution of 1 M-ammonium formate/0.1 M-formic acid by dilution with distilled water) with greater than 95% recovery; this high recovery was found also for carrier-free radiolabelled compounds. An elution profile for six inositol phosphates was initially determined by eluting the cartridge in a stepwise fashion, using 2 ml of buffer with 10 mM increments of ammonium formate (Fig. 1a). Each major group (InsP, InsP<sub>2</sub>, etc.) was eluted distinctly, with the more phosphorylated group being more strongly retained on the cartridge; only for Ins(1,3,4,5,6)P<sub>5</sub> and InsP<sub>6</sub> was a slight overlap observed (Fig. 1a). A few of the standards displayed apparent multiple peaking, but this is due to slight contamination by (an)other inositol phosphate(s) (see below). We demonstrated that we could achieve resolution of each standard by running single samples containing pairs of adjacently eluted inositol phosphates, one labelled and the other not (results not shown). Although not shown, Ins(1,3,4)P<sub>3</sub> was eluted just slightly before Ins(1,4,5)P<sub>3</sub>, and we could not achieve a satisfactory separation of these InsP<sub>3</sub> isomers, although Ins(1,3,4)P<sub>3</sub> did separate completely from [<sup>3</sup>H]Ins(1,4)P<sub>2</sub>. The presence of 5 mM-disodium tetraborate in the ammonium formate buffers was found to improve slightly the elution properties of the inositol phosphate standards by decreasing sample trailing; elution profiles shown in Fig. 1(a) are those obtained in ammonium formate buffers containing 5 mM-disodium tetraborate.

The data in Fig. 1(a) indicate suitable concentrations of ammonium formate to use for batchwise separation; to test the fidelity of the batchwise elution, standards, either alone or with another inositol phosphate [e.g. Ins(2)P with [<sup>3</sup>H]Ins(1,4)P<sub>2</sub>], were eluted with 10 ml each of water, followed by 5 mM-disodium tetraborate, and then with the concentrations of ammonium formate buffer (containing 5 mM disodium tetraborate) indicated in Fig. 1(b), 1 ml fractions being collected. The results clearly show that complete resolution of several different inositol phosphates can be achieved in this fashion; however, some contamination of InsP<sub>5</sub> with InsP<sub>6</sub> (about 10%) will occur with this procedure. We have no information concerning the elution of inositol phosphates other than the isomers presented here.

[<sup>3</sup>H]Inositol was not retained on the QMA SEP-PAK, and, by washing the cartridge with 10 ml of distilled water, the eluted radioactivity was decreased to a 'background' value, even when very high initial amounts of radioactivity (Fig. 1b) had been used.

As in Fig. 1(a), some of the inositol phosphates were found to be contaminated with minor traces of less-polar compounds; these were quantified in Fig. 1(a) (where they were eluted as distinct peaks and were therefore demonstrably contaminants), and in Fig. 1(b) quantitatively identical contaminants were eluted by the stepwise procedure. As well as confirming that the stepwise elution had removed all of an inositol

phosphate within the 10 ml of designated eluant, these results show the ability of this stepwise method to detect faithfully small quantities of minor contaminants. We should note that this also applies to [<sup>3</sup>H]GroPIns; the analysis of this is not shown in Fig. 1(a), but by gradient elution we found that some contamination by [<sup>3</sup>H]inositol and [<sup>3</sup>H]Ins(1)P had occurred, as might be expected (Clarke & Dawson, 1981), which was reproduced quantitatively by the stepwise elution in Fig. 1(b).

We routinely use a single SEP-PAK for at least 10–15 sample runs by simply regenerating the cartridge with first 10 ml of 1 M-ammonium formate/0.1 M-formic acid (where not previously applied), followed by 20 ml of distilled water. Loss of cartridge integrity was usually seen as a decrease in sample recovery; cartridge life can be further extended by keeping flow rates down to about 10 ml/min and by using filtered buffers.

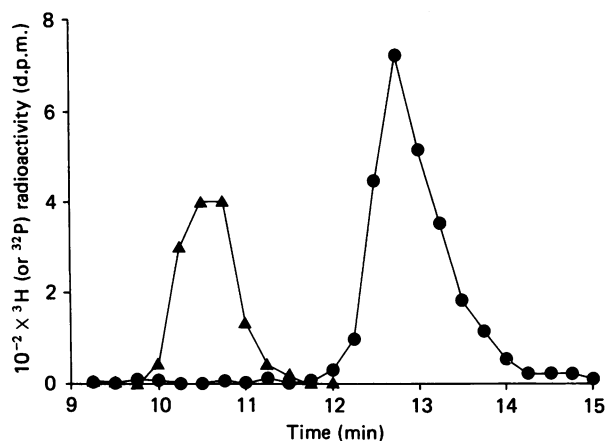
The capacity of these cartridges for inositol phosphates is quite high, as we were able to load at least 2 mg of InsP<sub>6</sub> (about 0.6 mg of phosphorus) with no pass-through (as detected by phosphorus analysis). With large sample mass we found a significant decrease in sample resolution; for example, we were unable to obtain an acceptable fractionation of a phytate hydrolysate by using a sample load of 2 mg of phosphorus.

#### Applicability to biological systems

Inositol phosphates extracted from [<sup>3</sup>H]inositol-labelled parotid slices were loaded on to an ACCELL QMA SEP-PAK and eluted with 10 ml each of distilled water and 5 mM-disodium tetraborate, followed by a step gradient of 2 ml of 10 mM increments of ammonium formate/5 mM-disodium tetraborate. The results from a typical carbachol-stimulated sample (Fig. 3 below) demonstrate that discrete peaks of radioactivity were obtained that are eluted in a pattern identical with that shown by the inositol phosphate standards (see Figs. 1a and 1b). Even the very large amount of radioactivity in the [<sup>3</sup>H]InsP<sub>2</sub> fraction was completely eluted from the SEP-PAK before the [<sup>3</sup>H]InsP<sub>3</sub> fraction began to be eluted (Fig. 2). Extracts from four different samples were processed by the batch procedure, and in all cases we found that the effect of stimulation by carbachol on InsP<sub>3</sub> and InsP<sub>4</sub> (Batty *et al.*, 1985; Hawkins *et al.*, 1986) was as faithfully reported by the SEP-PAK as by the h.p.l.c. technique.

#### Separation of inositol phosphate isomers by isocratic anion-exchange chromatography

The SEP-PAK method does not separate different isomers one from another; this is not normally required, but, in some instances in particular, separation of InsP<sub>3</sub> isomers is necessary to examine, for example, the metabolism of Ins(1,4,5)P<sub>3</sub> (see, e.g., Burgess *et al.*, 1985; Irvine *et al.*, 1985). We found that it is a simple matter to process further fractions eluted from the SEP-PAK. A 2 ml portion of the InsP<sub>3</sub> fraction eluted from the SEP-PAK in 0.3 M-ammonium formate/5 mM-disodium tetraborate was injected directly on to a Partisil 10 SAX anion-exchange h.p.l.c. column running at 1.25 ml/min with a mobile phase of either 0.85 or 0.9 M-ammonium formate/0.1 M-formic acid adjusted to pH 3.7 with phosphoric acid. Collection of 0.25 min fractions was begun 8–9 min after injection and continued for 5–6 min, during which time complete separation of these two InsP<sub>3</sub>



**Fig. 3. Isocratic anion-exchange h.p.l.c. of inositol trisphosphate isomers after batch elution from ACCELL QMA anion-exchange SEP-PAKs**

A mixture of [ $^3\text{H}$ ]Ins(1,3,4) $P_3$  and [ $^{32}\text{P}$ ]Ins(1,4,5) $P_3$  was loaded on to a QMA SEP-PAK and then eluted as an 'Ins $P_3$  fraction' in 10 ml of 0.3 M-ammonium formate/formic acid (pH 4.75)/5 mM-disodium tetraborate (see Fig. 1b). A 2 ml sample of this fraction (containing about 2000 c.p.m. of each isomer) was injected on to a Partisil 10 SAX h.p.l.c. column with a mobile phase of 0.85 M-ammonium formate/formic acid (adjusted to pH 3.75 with phosphoric acid) at a flow rate of 1.25 ml/min. With this column, [ $^3\text{H}$ ]Ins(1,3,4) $P_3$  (▲) had a retention time of 10.5–10.75 min, whereas [ $^{32}\text{P}$ ]Ins(1,4,5) $P_3$  (●) had a retention time of 12.75 min; both isomers were eluted within 6 min.

isomers was achieved (Fig. 3). Load volumes of up to 2 ml have been successfully resolved in this fashion; beyond this, some slight peak spreading occurred, which hampered the separation (results not shown). Larger loading volumes can, however, be achieved with a column mobile phase of 0.65 M-ammonium formate, pH 3.7; this will sufficiently delay Ins $P_3$  elution so that, upon injection of the last of several 2 ml samples, the mobile phase can be switched immediately to either 0.85 or 0.9 M-ammonium formate, pH 3.7, and the collection begun as described above (result not shown). Although the processing time for each sample was a few minutes shorter when elution was with 0.9 M-ammonium formate, we found that better separation of the two isomers could be achieved at 0.85 M-ammonium formate.

Although we have not tried it, we expect that this methodology could be applied to other inositol phosphate isomers.

### Conclusions

The present results, which are representative of many experiments, show that, provided that separation of individual inositol phosphate isomers is not required, then the SEP-PAK separation method for inositol phosphates has a reproducibility comparable with h.p.l.c. With processing of many samples (when, for the Dowex method, many columns can be run at once) some of the advantage of the greater rapidity of the SEP-PAK technique is lost. Facilities exist, however, for processing of multiple SEP-PAKs, and the potential for automation of the SEP-PAK method obviously renders this an extremely rapid and reliable technique.

In those cases where only total inositol phosphate production is of interest, then, by simply washing a loaded sample with 10 ml each of water and then 5 mM-disodium tetraborate, followed by elution of all inositol phosphates with 10 ml of 1 M-ammonium formate/0.1 M-formic acid, the sample processing time is decreased to just a few minutes. If various groups of inositol phosphates are to be identified, then we recommend that a typical sample and an inositol phosphate standard [e.g. Ins(1,4,5) $P_3$ ] be processed by using a stepwise gradient of ammonium formate as described in Figs. 1(a) and 2. All samples can then be either processed in this fashion or else concentrations of ammonium formate can be selected for batch processing.

In conclusion, the speed and accuracy of the SEP-PAK method may prove to be of considerable advantage, particularly when used in combination with the h.p.l.c. isomer-separation method described here, and we recommend its use for the routine analysis and purification of inositol phosphates.

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