Automated isocratic high-performance liquid chromatography of inositol phosphate isomers

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Isomers of inositol phosphates from biological samples can be analysed by anion-exchange h.p.l.c., by using isocratic elution with phosphate buffers. The method involves the preliminary processing of the extracted samples with conventional soft-gel anion-exchange resins, including the commonly used Dowex resins, followed by direct analysis with h.p.l.c. of a portion of relevant fractions. Run times (up to 20 min) and collected fraction numbers (up to 24) are minimal, so that if the method is used in conjunction with automated h.p.l.c. injection a high throughput of samples is achieved.

INTRODUCTION

The current picture of the inositide cell-signalling system reveals the biochemistry of the metabolism of inositol phosphates to be very complex (for reviews see Irvine *et al.*, 1988; Shears, 1989). The existence of almost a dozen different isomeric forms of inositol phosphates which are known to be present in eukaryotic cells has posed a formidable problem to be overcome in analytical studies of cellular responses.

The current analytical approaches centre on the use of radiolabelled precursors, and have used anion exchange as the basis for separation of the compounds of interest (but see also Shayman & BeMent, 1988). Most analyses of radiolabelled inositol phosphates provide an 'all-purpose' separation of a sample into all of its labelled components, and this can be an elaborate process (e.g. Dean & Moyer, 1987; Cunha-Melo *et al.*, 1987; Balla *et al.*, 1988). These various methods of analysis therefore require a large investment of time and cost, and, furthermore, it is only after that investment that it can be seen whether an experiment has been successful. These considerations to some degree limit the nature of the biological questions that can be asked.

An alternative approach is to break up the analytical problem into stages, each with the capacity for automation. The first stage (stage 1) is to fractionate a radiolabelled cell extract into major groups of inositol phosphates based on the number of phosphate groups (e.g. InsP_1 , InsP_2 , InsP_3 etc.) (Berridge *et al.*, 1983; Batty *et al.*, 1985; Wreggett & Irvine, 1987). An advantage of this is that one is not generally concerned with all inositol phosphate isomers in every experiment. For instance, at early times during cell stimulation, very little of InsP_1 and InsP_2 accumulate, and analysis of isomers of InsP_3 only may be all that is necessary. Another practical feature of this approach is that it decreases the time and resources needed to identify interesting samples for a more detailed investigation; i.e. among other advantages, the opportunity is provided to ensure that an experiment has worked (for example, detectable incorporated radioactivity has been obtained, with good replication, before analysis of the isomers).

The second stage of the analysis is then based on the original serendipitous result that InsP_3 isomers in fractions from stage 1 processing can be rapidly separated by isocratic anion-exchange h.p.l.c. with formate buffers titrated to pH 3.7 with H_3PO_4 (Wreggett & Irvine, 1987). The present paper fully describes the expansion and refinement of this initial result to include the resolution of InsP_1 and InsP_2 isomers, by using a buffer system based on the method of Dean & Moyer (1987).

METHODS AND MATERIALS

All reagents were from previously described sources (Wreggett & Irvine, 1987). ACCELL QMA SEP-PAKs (lot nos. P5270A1 and P7147A1) were from Waters Associates (U.K.), and Amprep SAX cartridges (100 mg; batch no. 182040) were provided by Amersham International (U.K.).

Preparation of [^{32}P]Ins(1,4,5) P_3

This was prepared from [5- ^{32}P]PtdIns(4,5) P_2 as described in Irvine *et al.* (1985). The radiolabelled lipid was prepared by incubation of PtdIns(4) P with [γ - ^{32}P]ATP and a partially purified preparation of rat brain PtdIns(4) P 5-kinase (Smith, 1989).

Preparation of [^3H]Ins(1,3,4) P_3

This was obtained from [^3H]Ins(1,3,4,5) P_4 (Amersham International) as described in Batty *et al.* (1985). After purification by h.p.l.c., the compound was desalted by a 10-fold dilution in water and then loading on to the formate form of a QMA SEP-PAK (lot no. P7147A1), followed by washing first with 10 μl of 0.1 M-ammonium formate (pH 4.75 at room temperature) and then 20 ml of water, and elution in 10 ml of 1 M-triethylamine bicarbonate. The [^3H]Ins(1,3,4) P_3 was dried *in vacuo* and then resuspended in water.

Preparation of ^3H -labelled standards of InsP isomers

An alkaline hydrolysate of [^3H]Ins(1,3,4) P_3 to give a mixture of ^3H -labelled Ins(1,3) P_2 , Ins(1,4) P_2 and Ins-(3,4) P_2 was obtained by incubation of the InsP_3 with

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conc. ammonia in a sealed glass tube at 100 °C for 18 h. The mixture was dried *in vacuo* and resuspended in water. [^3H]Ins(3,4) P_2 was prepared from [^3H]Ins(1,3,4) P_3 by partial dephosphorylation with alkaline phosphatase (see Grado & Ballou, 1961; Irvine *et al.*, 1984).

Preparation of radiolabelled Ins P_1 isomers

An alkaline hydrolysate of [^3H]Ins(1,4) P_2 (Amersham International) was prepared as described in the preceding section, to give a mixture of ^3H -labelled Ins(1) P_1 and Ins(4) P_1 . ^{14}C -labelled isomers of Ins P_1 were prepared from [^{14}C]Ins(3) P_1 (Dupont) by incubation with 1 M-HCl in a stoppered tube at 80 °C for 4 h to give a mixture of ^{14}C -labelled Ins(3) P_1 , Ins(1) P_1 and Ins(2) P_1 (Pizer & Ballou, 1959); a small percentage of *trans*-migration of the phosphate can occur under these conditions to give also [^{14}C]Ins(4) P_1 . The mixture was dried and washed *in vacuo* and resuspended in water.

Preparation of samples

Standards of inositol phosphates were diluted into ammonium formate buffers of concentrations appropriate to the conditions for elution from pre-packed anion-exchangers.

Inositol phosphates from cultured human umbilical-vein endothelial cells which had been labelled with [^3H]inositol essentially as described previously (Pollock *et al.*, 1988) were extracted with HClO_4 and neutralized as previously described (Wreggett & Irvine, 1987), after inclusion of phytate hydrolysate (25 μg of P) (Wreggett *et al.*, 1987). Samples were then processed by using pre-packed anion-exchangers, either QMA SEP-PAKs (lot no. P5270A1; Wreggett & Irvine, 1987) or Amprep SAX cartridges (K. A. Wreggett, D. J. Lander & R. F. Irvine, unpublished work), to obtain fractions containing Ins P_1 , Ins P_2 or Ins P_3 in ammonium formate buffers. These fractions were then either stored frozen at -20 °C or kept at 4 °C for as much as 1 month until used for the analysis of isomers.

High-performance liquid chromatography

All chromatographic equipment was obtained from Waters Associates (U.K.). All solutions, previously filtered (0.45 μm pore size; type GVWP, Millipore) and degassed under vacuum, were delivered at 1.5 ml/min by model 510 pumps with an automated gradient controller. The mobile phase was a sodium phosphate buffer (pH 3.8 at room temperature). An adenine nucleotide was included in each sample to serve as an internal marker; generally, enough was dissolved in a stock solution of water so that 10 μl gave a peak measurement of about 1 A_{254} unit. The samples were loaded, with disposable plastic syringes (Monoject) connected to disposable filter units (0.45 μm ; Acro LC 13, Gelman Sciences), through a Rheodyne injector equipped with a 2 ml loop. A_{254} (model 441 detector) was recorded (BBC, model SE 120) to monitor each run. The column was a Partisil 10 SAX (25 cm $\times$ 4.6 mm; Technicol, Stockport, U.K.). Fractions (0.25 ml) were collected into scintillation vials (LKB; 15 mm $\times$ 53 mm) with a FRAC-100 (Pharmacia); a 3.5 ml mixture of scintillation fluid (Ready-Solve, Beckman)/methanol/water (3:2:1, by vol.) was added to each fraction to quantify eluted radioactivity with an LS3801 scintillation counter (Beckman Instruments) operating at an efficiency of 95 % for ^{32}P , 30 % for ^3H and 18 % for ^{14}C .

RESULTS AND DISCUSSION

Isocratic resolution of the isomers of each group of inositol phosphates was designed for a sample volume of up to 2 ml. Loading of larger volumes tended to result in peak spreading, indicating that the sample needed to be concentrated on the head of the column before beginning elution. This was accomplished in each case by loading up to 6 ml of the sample as a series of 2 ml injections every 2 min while running the column at a lower concentration of phosphate buffer, and then immediately switching to the running condition upon injection of the last 2 ml. Successful concentration and elution by using this technique was readily demonstrated by (a) the elution of a single peak of the adenine nucleotide, as monitored by the A_{254} (Figs. 1a–1c, bottom row), (b) the faithful resolution of radiolabelled standards of inositol phosphates (results not shown) and (c) the elution of a single peak of the radiolabelled inositol phosphate standard in samples from cell-culture experiments (Figs. 1a–1c, top row).

Resolution of Ins P_1 isomers

Isocratic separation of Ins(1) P_1 , Ins(2) P_1 and Ins(4) P_1 was accomplished by running the column at 40 mM- NaH_2PO_4 (Fig. 1a, middle panel); larger volumes were loaded on to the column running in water (Fig. 1a, top panel). Under these conditions the total run-time was 12 min; when larger volumes were processed the composition of the mobile phase was returned to the loading condition after 11 min, and the column was left to re-equilibrate for 5 min at the loading condition before injection of the next sample. Collection of fractions was started 6 min after the final injection, and was 5 min in duration; AMP was included as an internal standard, and was used to monitor elution times and to determine collection windows (Fig. 1a, bottom panel).

Resolution of Ins P_2 isomers

Isocratic separation of Ins(1,3) P_2 , Ins(1,4) P_2 and Ins(3,4) P_2 was accomplished by running the column at 240 mM- NaH_2PO_4 (Fig. 1b, middle panel); larger volumes were loaded on to the column running at 100 mM- NaH_2PO_4 (Fig. 1b, top panel). Under these conditions the total run-time was 12 min; the composition of the mobile phase was returned to the loading condition after 11 min, and the column was left to re-equilibrate for 5 min before injection of the next sample. Collection of fractions lasted for 5 min, and was started either 7 min (for sample volumes up to 2 ml) or 8 min (for more than 2 ml) after the final injection; ADP (with some AMP) was included as an internal standard, and was used to monitor elution times and to determine collection windows (Fig. 1b, bottom panel).

Resolution of Ins P_3 isomers

Isocratic separation of Ins(1,3,4) P_3 and Ins(1,4,5) P_3 was accomplished by running the column at 550 mM- NaH_2PO_4 (Fig. 1c, middle panel); larger volumes were loaded on to the column running at 350 mM- NaH_2PO_4 (Fig. 1c, top panel). Under these conditions the total run time was 13 min; the composition of the mobile phase was returned to the loading condition after 11 min and the column was left to equilibrate for 10 min before injection of the next sample. Collection of fractions was started 8 min after the last injection, and was 5 min in

Fig. 1. Isocratic elution of inositol phosphates on Partisil 10 SAX

Radiolabelled isomers of InsP₁ (column a), InsP₂ (column b) or InsP₃ (column c) with appropriate internal standards of adenine nucleotides were loaded and eluted as described in the text. The elution of adenine nucleotides, upon injection of either 2 ml (solid lines) or 4 ml (broken lines), was monitored at 254 nm (bottom row of panels); the arrows indicate the times at which injections of 2 ml were made. Resolution of radiolabelled inositol phosphate standards is shown for a single injection of 2 ml (middle row of panels); the ● and ○ symbols represent elution of ³H label and either ¹⁴C or ³²P label respectively for an isomeric mixture, where appropriate, of [³H]Ins(1)P₁, [³H]- and [¹⁴C]-Ins(4)P₁, [¹⁴C]Ins(3)P₁, [¹⁴C]Ins(2)P₁, [³H]Ins(1,3)P₂, [³H]- and [³²P]-Ins(1,4)P₂, [³H]Ins(3,4)P₂, [³H]Ins(1,3,4)P₃ and [³²P]Ins(1,4,5)P₃. Ins(1/3)P₁ refers to the enantiomeric pair of Ins(1)P₁ and Ins(3)P₁, which cannot be resolved by this method. The top row of panels shows elution of radiolabelled inositol phosphates upon injection of 4 ml (2 × 2 ml) of the relevant fraction from stage-1 processing, by Ampreps (see the text), of a neutralized acid extract from primary cultures of human endothelial cells, labelled with [³H]inositol; also indicated in these panels is the peak of elution of an internal radiolabelled standard of an appropriate inositol phosphate, to facilitate identification of eluted peaks of ³H. The collection windows for the upper two rows are represented in the lower row as shaded boxes; the earlier window corresponds to the case for a single injection.

duration; ATP (with some ADP and AMP) was included as an internal standard, and was used to monitor elution times and to determine collection windows (Fig. 1c, bottom panel).

Conclusions

Isocratic elution of samples can be successfully applied to the analysis of the metabolism of inositol phosphates (Fig. 1, upper row of panels). The method has also been applied to simple questions of metabolism, e.g. when a radiolabelled inositol phosphate has been injected into an intact *Xenopus* oocyte; there the small volume of acid-extracted sample can be injected directly on to the h.p.l.c. without previous processing (M. J. Berridge, R. F. Irvine & K. A. Wreggett, unpublished work). The method need not be limited to samples eluted from pre-packed anion-exchangers, since eluates from Dowex, at much higher concentrations of ammonium formate (Berridge *et al.*, 1983; Batty *et al.*, 1985), can be treated identically; only for InsP₁ isomers was dilution (5-fold) in water necessary to achieve resolution by this technique (results not

shown). For example, elution of InsP₃ from Dowex columns frequently uses 10 ml of 1 M-ammonium formate, pH 4.75 (Berridge *et al.*, 1983). Baseline separation of Ins(1,3,4)P₃ and Ins(1,4,5)P₃ was achieved by injecting 6 ml (as 3 × 2 ml injections) of these InsPs in 1.0 M-ammonium formate on to the h.p.l.c. column. In all cases, the loading conditions were first checked for the retention times of the relevant nucleotide marker to determine the proper collection window; samples loaded in higher salt (i.e. from Dowex columns) were eluted in a different position from that presented in Fig. 1.

The two-stage approach has greatly streamlined the analysis of inositol phosphates. Depending on the conditions of the experiment, not every isomer group needs to be analysed, and unless the data are essential for statistics on the production of different isomers it is not necessary to perform a detailed analysis of every sample in a replicate. Stage-2 processing can then be limited to selected samples, so that the minimum of time and cost will be expended. A further advantage of this method is that the minimum of h.p.l.c. equipment is required (one

pump, a detector and a fraction collector, with an optional solvent-switching valve), whereas at the other extreme we have adopted the use of automated injection (i.e. Waters WISP with Waters 600E Powerline system) so that samples can be left alone to run while other experiments are performed. The small number of fractions required to be collected makes it feasible to process many samples without using on-line detection of radioactivity, which can be expensive in terms of the large amount of radioactive isotopes and scintillation fluid required.

The use of isocratic elution offers many advantages. This particular application uses the property of anion exchange, but other isocratic methods have been described, such as ion-pair chromatography (e.g. Shayman & BeMent, 1988), and this may open the door for the future development of detection methods which require isocratic elution.

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