Journal of Chromatography, 574 (1992) 150–155
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6165

Short Communication

Rapid and selective isolation of radiolabelled inositol phosphates from cancer cells using solid-phase extraction

Lorraine Anderson, Jeffrey Cummings* and John F. Smyth

Imperial Cancer Research Fund, Medical Oncology Unit, Western General Hospital, Edinburgh EH4 2XU (UK)

(First received May 2nd, 1991; revised manuscript received September 30th, 1991)

ABSTRACT

A method is described for rapid and selective determination of radiolabelled inositol phosphates in cancer cells using solid-phase extraction with Bond Elut strong anion-exchange minicolumns. The inositol phosphates IP1, IP2 and IP3 are selectively eluted with 0.05, 0.3 and 0.8 *M* ammonium formate–0.1 *M* formic acid, respectively. Cancer cells are extracted with 10% perchloric acid which is then neutralised prior to loading samples on to the minicolumns. Recovery is 54.1, 66.6 and 61.3% for IP1, IP2 and IP3 with between-day coefficients of variation of 7.6, 6.8 and 1.9%, respectively. When the method was applied to cancer cells high-performance liquid chromatographic analyses confirmed both the identity of the IP1, IP2 and IP3 fractions and showed that there was no detectable cross contamination of these inositol phosphates with each other.

INTRODUCTION

The cell membrane and cell signalling are receiving increasing attention as possible new targets in cancer chemotherapy [1]. Crucial to a number of signal transduction mechanisms is the phosphatidylinositol (PI) cycle. After receptor activation the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2) is degraded by phospholipase C to the second messengers inositol 1,4,5-trisphosphate (IP3) which is released into the cytoplasm and diacylglycerol (DAG). IP3 mobilises intracellular calcium from the endoplasmic reticulum whilst DAG activates protein kinase C which occupies a central role in mitogenesis and possibly carcinogenesis [2]. IP3 can be dephosphorylated to less complex species such as inositol 4,5-bisphosphate (IP2) and inositol 1-monophosphate (IP1). Alternatively further phosphorylation of IP3 results in the production of inositol 1,3,4,5-tetrakisphosphate (IP4), pentakisphosphate (IP5) and hexakisphosphate (IP6) as well as several isomers. Involved fractionation procedures are therefore required for *in vivo* analysis of the various components of the PI cycle.

Traditionally, the isolation and fractionation of inositol phosphates have been performed using low-pressure anion-exchange chromatography [3], which, although useful, does not lend itself to automation nor can it resolve isomers. High-performance liquid chromatography (HPLC) has the latter advantage but nevertheless complex apparatus and gradients employing long run times are usually required to achieve only modest resolution of isomers [4]. A role therefore exists for a chromatographic technique which is capable of both higher sample throughput and which is not hindered by excessively long elution times. In this report application of solid-phase extraction (SPE) to the analysis of inositol phosphates in cancer cells is presented.

0378-4347/92/$05.00

EXPERIMENTAL

Chemicals and reagents

Ammonium formate, formic acid and methanol were HPLC reagent grade and obtained from Fisons Analytical (Loughborough, UK). *myo*-[^{3}H]Inositol with PT6-271 (1mCi/ml) and an inositol polyphosphate marker set containing 1 μCi each of D-*myo*-[2-^{3}H]inositol 1-monophosphate (IP1), D-*myo*-[2-^{3}H]inositol 1,4-bisphosphate (IP2) and D-*myo*-[2-^{3}H]inositol 1,4,5-trisphosphate (IP3) were obtained from Amersham International (Little Chalfont, UK). All other chemicals and reagents were of the highest grade available commercially; water was deionised and bidistilled in a quartz glass still.

Solid-phase extraction of standard inositol phosphate mixtures

Standards of a [^{3}H]inositol polyphosphate mixture containing equal amounts of IP1, IP2 and IP3 were made up in either water or 10% perchloric acid (PCA). PCA solutions were neutralised with 1 *M* KOH containing 50 m*M* HEPES buffer and 5 m*M* EDTA, and salts formed were removed by centrifugation. Samples (2 ml) were then directly applied to 500 mg Bond Elut strong anion-exchange (SAX), 40 μm silica gel columns with 2.4 ml reservoirs, and inositol phosphates were fractionated using a Vac Elut-10 place column manifold operating under negative pressure (Analytichem International, Cambridge, UK, supplied as a kind gift by Dr. R. Calverly). The SAX minicolumns were first activated with 5 ml of 1 *M* ammonium formate then washed with 15 ml of water prior to loading of sample. Free inositol, glyceroinositol phosphates, IP1, IP2 and IP3 were then sequentially eluted from the columns with 15 ml of water, 5 ml of 60 m*M* ammonium formate–5 m*M* sodium tetraborate, 5 ml of 0.05 *M* ammonium formate–0.1 *M* formic acid, 5 ml of 0.3 *M* ammonium formate–0.1 *M* formic acid for 10% PCA-derived samples but 5 ml of 0.4 *M* ammonium formate–0.1 *M* formic acid for water-derived samples and 5 ml of 0.8 *M* ammonium formate–0.1 *M* formic acid, respectively. From each 5-ml fraction 2 ml were added to 10 ml of Optiphase MP liquid scintillation fluid and radioactivity was counted as dpm in a Packard Model 1900 CA liquid scintillation counter (both from Canberra Packard, Pagbourne, UK). To obtain detailed elution profiles, 1-ml fractions were collected and counted as above.

For comparison, aqueous and 10% PCA solutions were extracted using Dowex anion-exchange resin (Dowex-1,Cl$^-$, 2% cross-linkage, 100–200 mesh, Sigma, Poole, UK) essentially according to the method of Downes and Michell [3].

High-performance liquid chromatography

Fractions eluted from Bond Elut minicolumns were subjected to HPLC to determine their inositol phosphate composition.

Prior to HPLC, fractions were desalted on Dowex H$^+$ resin columns (Sigma) and then passed through a 0.45 μm filter. Samples (100 or 500 μl) were then injected onto the HPLC column. The stationary phase was Partisil SAX, 10 μm silica obtained prepacked in stainless-steel columns measuring 250 mm × 4.6 mm I.D. (Capital HPLC, Bathgate, UK). The mobile phase was delivered at a constant flow-rate of 2.0 ml/min from a Waters 501 solvent delivery system (Waters/Millipore, Northwich, UK). Stepwise gradient elution was employed using a Kontron Model LMV 470 low-pressure switching valve and Kontron systems controller (Kontron, Watford, UK) according to the following programme: 0–10 min, 100% water; 10–20 min, 0.2 *M* ammonium formate; 20–35 min, 0.4 *M* ammonium formate; 35–40 min, 0.8 *M* ammonium formate (all solutions were adjusted to pH 7.6 with PCA). Labelled inositol phosphates were detected using a continuous, on-line HPLC radioactivity monitor (Berthold Model LB503, Hemel Hempstead, UK). The insertion of a "T" piece upstream from the monitor cell meant that eluate from the HPLC column was mixed with scintillation fluid (1:4, v/v) Quickszint Flow 306, Zinsser, supplied by Beveridge (Edinburgh, UK) by means of a low-pressure pumping module prior to its entry into the cell.

Extraction of inositol phosphates from cancer cells

Monolayer cultures of pituitary carcinoma cells (10^7) were incubated for 48 h with 2 μCi of

[^{3}H]inositol in inositol-free DMEM medium containing 0.5% heat-inactivated foetal calf serum. Prior to any drug additions, cells were incubated with lithium chloride (10 m*M* for 15 min).

Incubations were terminated by placing samples on ice and adding 3 ml of ice cold 10% PCA. After 15 min the PCA-containing inositol phosphate was removed and neutralised with 1 *M* KOH containing 50 m*M* HEPES buffer and 5 m*M* EDTA. Salts formed were removed by centrifugation and samples were directly applied onto either Bond Elut SAX or Dowex anion exchange columns for the separation of inositol phosphates.

RESULTS AND DISCUSSION

Fractionation of standard mixtures of inositol phosphates

Fig. 1 illustrates a typical elution profile for inositol phosphates after a standard mixture containing equal amounts of ^{3}H-labelled IP1, IP2 and IP3 was loaded onto a Bond Elut SAX sample preparation column. Superimposed on to this is a typical elution profile from a Dowex anion exchange column. In the case of Bond Elut all counts associated with a particular fraction were recovered in 5 ml of buffer and there was a clear separation of radioactivity into three sharp bands with an efficiency approaching that of HPLC [5]. Previous studies show that much larger elution volumes are required with Dowex (up to 22 ml) and cross-contamination of IP fractions can occur [6]. These findings are borne out by our own results (Fig. 1) where 10-ml elution volumes were employed and it is clear that only partial resolution of the IP2 and IP3 fractions was achieved.

In a previous study Dowex fractions have been analysed by HPLC to determine their inositol phosphate composition [5]. There it was shown that the IP2 fraction was contaminated with 17% IP3 and 3% IP1, and the IP3 fraction with 2% IP1, 10% IP2 and 29% IP4. In the present study fractions collected from Bond Elut SAX were also subjected to HPLC (Fig. 2). These revealed no detectable cross-contamination and show that IP1, IP2 and IP3 (as illustrated in Fig. 1) are selectively removed from Bond Elut.

Another solid-phase sample preparation method has been described for the fractionation of inositol phosphates [7]. Here Accell QMA anion-exchange Sep-Pak cartridges (Waters–Millipore) were employed with an ammonium acetate–formic acid–disodium tetraborate (BAFF) eluting buffer system. Comparable results to those presented in this present work were reported but with evidence of significant cross-contamination of individual fractions.

Extraction efficiency and reproducibility

The extraction efficiency and between-day coefficients of variation for the Bond Elut assay are contained in Table I for both aqueous standards and 10% PCA samples which replicate the treatment of cancer cells. Recovery was close to 100% with aqueous standards but dropped to around 60% for PCA-derived standards but with good reproducibility recorded.

Isolation and fractionation of inositol phosphates from cancer cells

Fig. 3 illustrates a typical Bond Elut SAX elution profile of inositol phosphates from a 10%

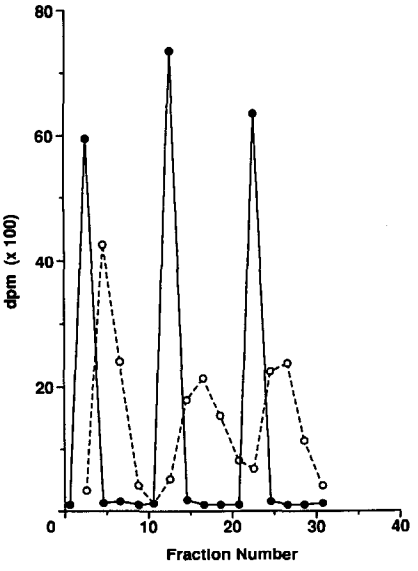

Fig. 1. Elution profiles of recovered radioactivity after application of a ^{3}H-labelled mixture of IP1, IP2 and IP3 standards made up in 10% PCA followed by KOH neutralisation. Fractions of 1 ml were collected. (●) Bond Elut SAX; (○) Dowex 1. Bond Elut fractions 1–5 (0.05 *M* ammonium formate) contained only IP1; fractions 10–15 (0.3 *M* ammonium formate) contained only IP2; fractions 20–25 (0.8 *M* ammonium formate) contained only IP3 with no evidence of cross-contamination (see Fig. 2).

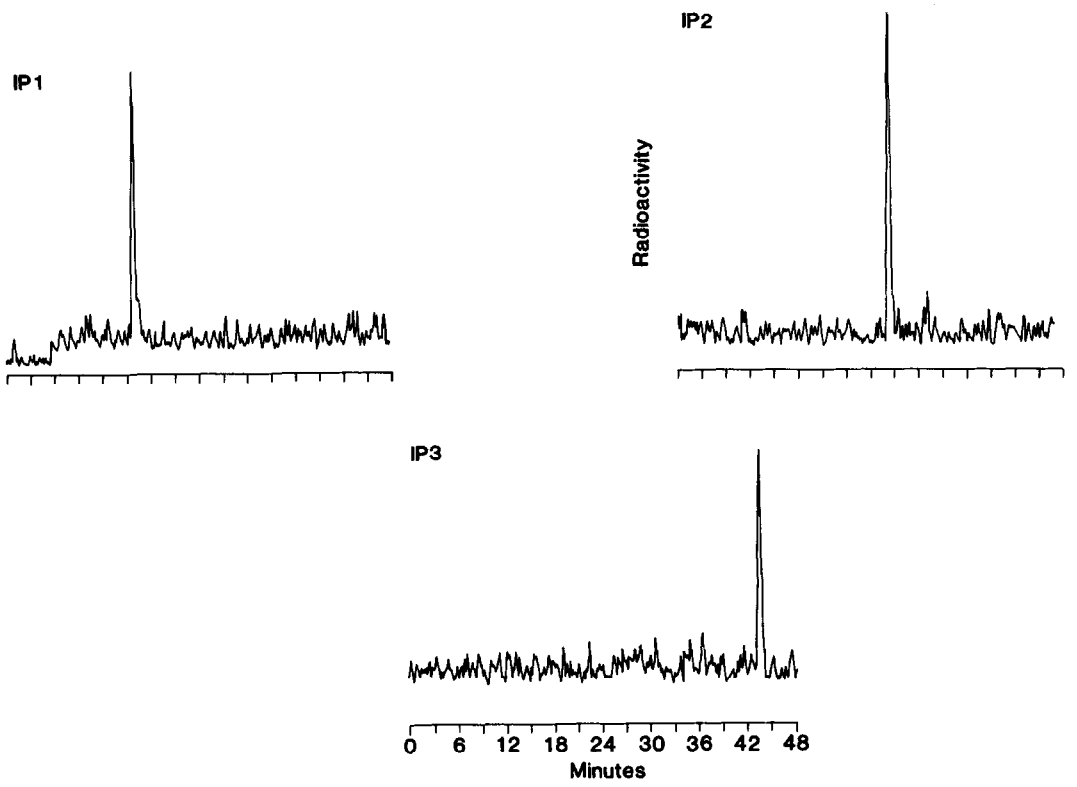

Fig. 2. HPLC analysis of fractions eluted from Bond Elut SAX columns with an ammonium formate–formic acid gradient after the application of a mixture of labelled IP1, IP2 and IP3 (see Fig. 1). Top left trace, fractions 1–5 containing only IP1 (t_R 16 min) with no contamination of IP2 (t_R 25 min) and IP3 (t_R 43 min). Top right trace, fractions 10–15 containing only IP2. Lower trace, fractions 20–25 containing only IP3.

TABLE I

EFFICIENCY AND BETWEEN-DAY REPRODUCIBILITY OF BOND ELUT SAX SOLID-PHASE EXTRACTION OF INOSITOL PHOSPHATES

Standards of an inositol polyphosphate mixture containing equal amounts of ^{3}H-labelled IP1, IP2 and IP3 were made up in either water or 10% PCA followed by KOH neutralisation and were fractionated on Bond Elut SAX as described in Experimental section. C.V. = coefficient of variation.

Medium	IP1		IP2		IP3	
	Efficiency (%)	C.V. (%)	Efficiency (%)	C.V. (%)	Efficiency (%)	C.V. (%)
Aqueous	97.3	15.0	101.6	6.1	80.9	16.9
10% PCA	54.1	7.6	66.6	6.8	61.3	1.9

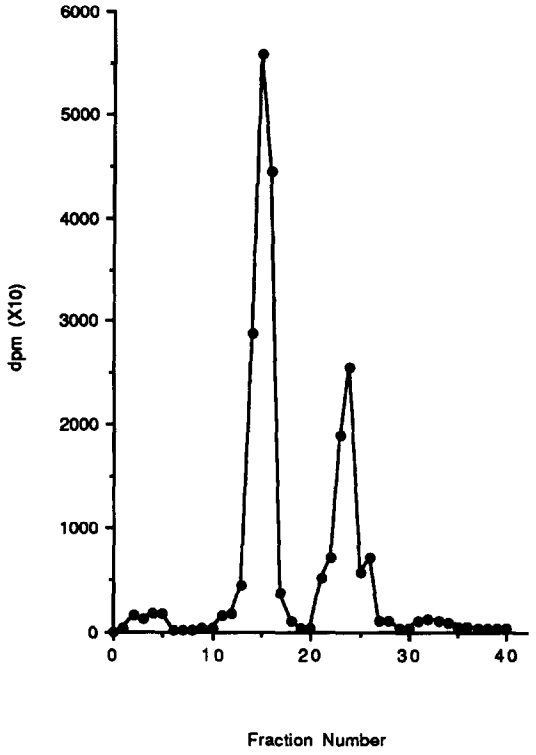

Fig. 3. Elution profile of radioactivity from a PCA cancer cell extract after application to a Bond Elut SAX column. Fractions of 1 ml were collected and analysed by HPLC to confirm inositol phosphate composition. Fractions 1–10 (60 m*M* ammonium formate–sodium tetraborate) are the glyceroinositol phosphates. Fractions 10–20 (0.05 *M* ammonium formate) contained IP1. Fractions 20–30 (0.3 *M* ammonium formate) contained IP2. Fractions 30–40 (0.8 *M* ammonium formate) contained IP3. All are free from cross-contamination with each other (see Fig. 4).

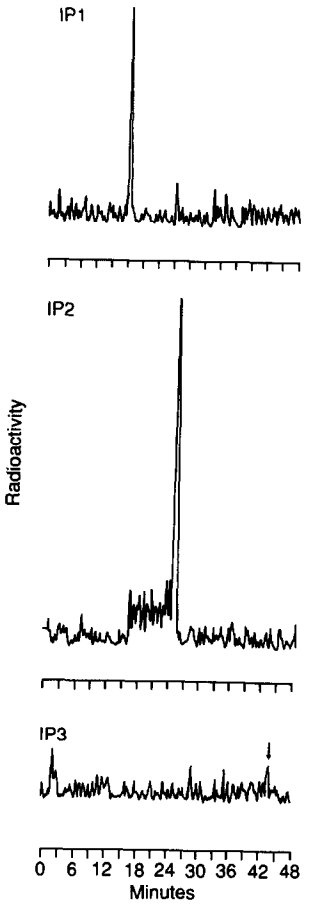

Fig. 4. HPLC analysis of Bond Elut SAX fractions from cancer cell extracts. Cells prelabelled with [^{3}H]inositol were extracted with 10% PCA which was applied to and eluted from Bond Elut columns with an ammonium formate–formic acid gradient and the collected fractions subjected to HPLC. Upper trace, fractions 10–20 contained only IP1 (t_R 16 min, Fig. 2). Middle trace, fractions 20–30 contained only IP2 (t_R 25 min, Fig. 2). Lower trace, fractions 30–40 possibly contained IP3 indicated by the arrow (t_R 43 min, Fig. 2) and was free from contamination with IP1 and IP2.

PCA extract of pituitary cancer cells. Although 10 ml of elution buffer were necessary to collect all the counts associated with a particular fraction, there was still a clear separation of radioactivity into discrete peaks (compare Fig. 3 with Fig. 1). HPLC analysis of these fractions confirmed the identity of IP1 and IP2 with the suggestion of an IP3 peak and most importantly revealed no evidence of detectable cross-contamination (Fig. 4). The lack of a substantial IP3 peak in either HPLC or Bond Elut-analysed fractions can be attributed to its rapid formation and breakdown in cells. Nonetheless these data show that IP1, IP2 and IP3 can be selectively determined in cancer cells using the method described in this work.

Several different sample preparation techniques have been coupled to the chromatographic analysis of inositol phosphates from cells of which acidic extraction with either PCA or trichloroacetic acid (TCA) are preferred [8]. TCA can be removed by repeated washing with water-saturated diethyl ether but this is time-consuming and no more efficient than PCA [6]. Alternatively neutralisation with Freon and tri-*n*-octylamine is suggested although the recovery of IP3 is poor [7]. We have chosen PCA extraction which results

in only a modest reduction in inositol phosphate recovery (Table I).

The Bond Elut SAX method presented offers several advantages over Dowex anion exchange: it is quick and lends itself to automation using 10- or 24-place vacuum manifolds with collection tubes; smaller volumes are required making further sample analysis less tedious and most importantly there is effectively no cross-contamination of IP1, IP2 and IP3 fractions even in biological specimens. On a cautionary note, Bond Elut SAX will not separate positional isomers, HPLC is still required. We envisage the present method being useful as a more rapid and reliable stage 1 isolation of IP prior to the separation of isomers with newer isocratic HPLC methods [9]. It may also be useful in screening programmes for new anticancer drugs, where the isolation of isomers is less important but nonetheless provides a quick means of determining inositol phosphate production in cancer cells.

REFERENCES

1 Meeting Report, *Cancer Res.*, 50 (1990) 2203.
2 S. B. Shears, *Cell Signalling*, 1 (1989) 125.
3 C. P. Downes and R. H. Michell, *Biochem. J.*, 198 (1981) 133.
4 G. M. Burgess, J. S. McKinney, R. F. Irvine and J. W. Putney, Jr., *Biochem. J.*, 232 (1985) 237.
5 G. S. Taylor, J. G. N. Garcia, R. Dukes and D. English, *Anal. Biochem.*, 188 (1990) 118.
6 S. Palmer, P. T. Hawkins, R. H. Michell and C. J. Kirk, *Biochem. J.*, 238 (1986) 491.
7 K. A. Wregget and R. F. Irvine, *Biochem. J.*, 245 (1987) 655.
8 S. Palmer and M. J. O. Wakelam, *Biochim. Biophys. Acta*, 1014 (1989) 239.
9 K. A. Wregget and R. F. Irvine, *Biochem. J.*, 262 (1989) 997.