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Short Communication

Preparation of samples for high-performance liquid chromatography of inositol phosphates

Elizabeth A. Woodcock and Jennifer K. Tanner

Baker Medical Research Institute, Commercial Road, Prahran 3181 (Australia)

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ABSTRACT

A simple method is described for the removal of extraneous material from tissue extracts prior to anion-exchange high-performance liquid chromatography of inositol phosphates. Samples are prepared by extraction with trichloroacetic acid or perchloric acid followed by removal of the excess acid. The extracts are then passed through small Dowex-50 cation-exchange columns and eluted with water. Dowex-50 pretreatment removes most of the ultraviolet absorbing material and cations from the samples but does not alter the content of inositol phosphates. This treatment results in improved reliability of chromatography, especially with respect to weakly retained molecules such as adenosine 5′-phosphate and the isomers of inositol monophosphate. In addition, sample pretreatment improves the useful lifetime of the analytical anion-exchange columns.

INTRODUCTION

The discovery that the turnover of inositol phospholipids is related to changes in cytosolic calcium and to cellular responses has led to an enormous amount of experimentation on the release and metabolism of inositol phosphates. Stimulation of cells with an appropriate agonist initiates the release of inositol-1,4,5-trisphosphate ($145IP_3$) which, in turn, releases calcium from specific intracellular stores, thereby initiating a wide range of physiological responses [1]. Metabolism of $145IP_3$ is complex involving both direct dephosphorylation to $14IP_1$ and phosphorylation to $1345IP_4$, which *via* complex metabolism is eventually degraded to $1IP_1$ and $3IP_1$ [2]. Study of inositol phosphate metabolism has been made difficult by the complex array of inositol phosphate isomers differing only in the position of the phosphate groups. Identification and quantitation of the range of inositol phosphates requires complex gradient chromatography, is expensive and is time-consuming. Conventional methods for the separation of inositol phosphates involve an anion-exchange column (generally Partisil SAX or Partisphere SAX) and sample elution using complex ammonium formate or phosphate gradients (for a review of these methods see ref. 3). These methods suffer from the different properties of columns from different batches and from deterioration of the columns after a relatively short number of runs. Problems of column deterioration are compounded when

Correspondence to: Dr. E. Woodcock, Baker Medical Research Institute, Commercial Road, Prahran 3181, Australia.

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samples are prepared from tissues such as heart or kidney where acid extracts contain large amounts of extraneous material. This can cause chromatographic problems as well as deterioration of the columns. In addition, samples containing high concentrations of cations (such as Ca^{2+} from culture medium) rapidly reduce the lifetime and the performance of the columns.

In this paper a rapid, simple and inexpensive method is described for preparation of tissue samples prior to inositol phosphate chromatography. The method removes most extraneous material including UV-absorbing material and cations and does not cause any alteration in the observed profile of inositol phosphates.

EXPERIMENTAL

Preparation of heart extract

Hearts from adult male Sprague–Dawley rats were cannulated via the aorta according to the method of Langendorff. Hearts were perfused for 30 min with oxygenated Kreb's medium at 37°C to remove blood. Subsequently, the hearts were homogenised in 5% trichloroacetic acid (TCA) using a Polytron homogeniser followed by sonication. After centrifugation, TCA was removed using Freon and tri-N-octylamine as described elsewhere [4].

Preparation of [^{3}H]inositol-labelled rat adrenal glomerulosa cells

Adrenal glomerulosa cells were prepared as described previously [5]. Cells were labelled by incubating for 24 h in medium 199 containing 2% bovine serum albumin (BSA) and 50 μCi/ml [^{3}H]inositol. Excess radioactivity was removed by washing the cells in medium containing non-radioactive inositol. After stimulation with angiotensin II, inositol phosphates were extracted using TCA as described earlier.

Dowex-50 chromatography

Dowex-50 cation-exchange resin was prepared by treatment successively with 1 *M* NaOH, water, 1 *M* HCl and water and columns containing 1 ml of pretreated resin were prepared in Bio-Rad Econo columns. Alternatively, columns can be prepared in siliconised Pasteur pipettes. Samples were added in 0.5 ml of water and eluted with the indicated volumes of water. Aliquots were taken for liquid scintillation counting and absorbance measurements or were subjected to anion-exchange high-performance liquid chromatography (HPLC) as explained below.

Chromatography of inositol phosphates

Separation and quantitation of the isomers of the inositol phosphates was achieved by the method of Dean and Moyer [6]. The column used was a 10-cm-long Whatman Partisil SAX 10-μm column packed by Waters (Milford, MA, USA) for use in a radial compression module. Inositol phosphates were eluted using a complex gradient of ammonium phosphate buffered to pH 3.8 with phosphoric acid. In this study the gradients used were 0–8 m*M* over 17 min (for IP_1), 200–280 m*M* over 30 min (for IP_2) and 500–520 m*M* over 30 min (for IP_3). IP_4 was eluted using isocratic elution with 2 *M* ammonium phosphate. After all runs the column was maintained in 2 *M* ammonium phosphate for 30 min followed by equilibration in water for 60 min before commencing another run. ^{3}H-Labelled compounds were identified and quantitated using an on-line radioactivity detector (Radiomatic Instruments, Model CR). This provided retention times and integrated peak values for each compound separated. The scintillation fluid used was Flo Scint IV.

Materials

Dowex-50W cation exchange resin (200–400 mesh and 4% cross-linked) was obtained from Sigma (St. Louis, MO, USA). [^{3}H]Inositol, [^{3}H]-inositol-1,4,5-trisphosphate, and [^{3}H]inositol-1,3,4,5-tetrakisphosphate were obtained from the Radiochemical Centre (Amersham, UK). [^{3}H]-Inositol-4-monophosphate was obtained from DuPont (Melbourne, Australia). Flo Scint IV was from Packard Instruments (Melbourne, Australia).

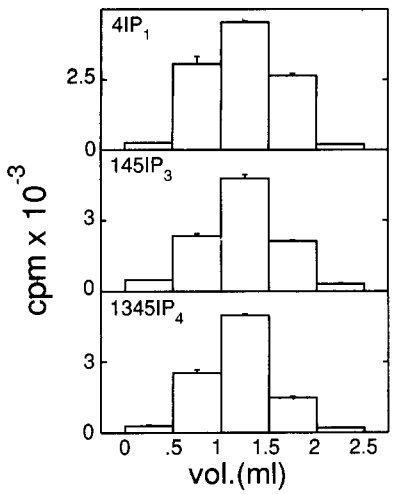

Fig. 1. Recovery of inositol phosphates from columns of Dowex-50 cation-exchange resin. Approximately 10 000 cpm of [^{3}H]inositol-4-monophosphate ($4IP_1$), [^{3}H]inositol-1,4,5-trisphosphate ($145IP_3$) or [^{3}H]inositol-1,3,4,5-tetrakisphosphate ($1345IP_4$) were added to unlabelled heart extract and applied to 1-ml columns of resin. Columns were eluted with the indicated volume of water. The first 0.5-ml volume indicated is the sample addition. Results shown are the average of three experiments. Error bars indicate the standard error of the mean of the measurements (n = 3).

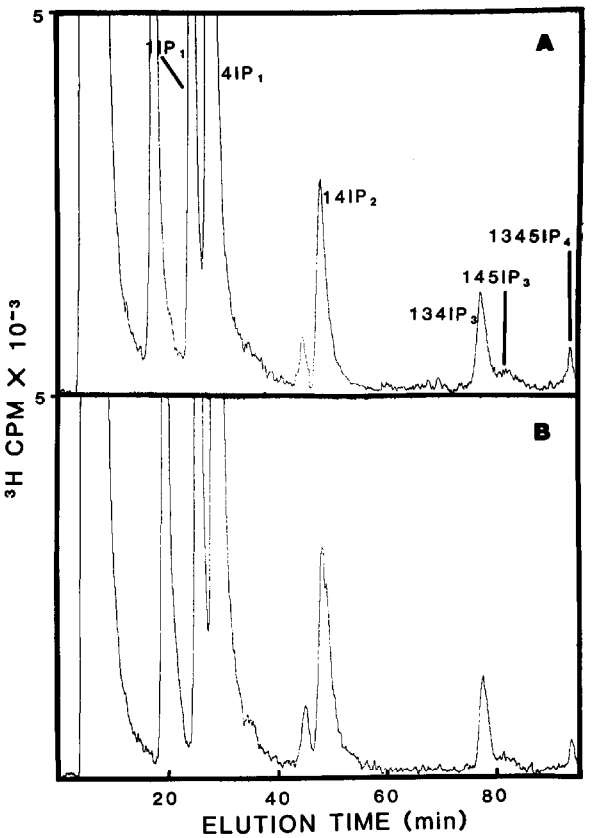

Fig. 2. Effect of Dowex-50 pretreatment on the profile of inositol phosphates in extracts of rat adrenal glomerulosa cells. Elution positions of inositol phosphate (IP) standards are indicated. The experiment was performed three times with similar results. (A) Untreated extract; (B) Dowex-50-treated extract.

RESULTS

Dowex-50 chromatography of inositol phosphate standards

Approximately 10 000 cpm of ^{3}H-labelled $4IP_1$, $145IP_3$ or $1345IP_4$ was added to 0.5 ml of heart extract and applied to 1-ml columns of Dowex-50 cation-exchange resin. Samples were eluted with 0.5-ml volumes of water and samples were taken for ^{3}H counting and absorbance measurements. Profiles of recovery for each of the inositol phosphates tested are shown in Fig. 1.

For all three inositol phosphates, 100% of the applied radioactivity was recovered in the subsequent 1.5-ml fraction. In contrast, UV-absorbing material was markedly reduced by passage through Dowex-50, from 19.6 ± 2.3 to 0.4 ± 0.08 absorbance units (n = 9). Similarly, Dowex-50 treatment reduced the UV absorbance in extracts of adrenal glomerulosa cells and renal papillary cells from by 95 ± 3 and 91 ± 5%, respectively (n = 3 in each instance).

Effect of Dowex-50 treatment on the profile of inositol phosphates in cell extracts

The effect of Dowex-50 treatment on the profile of inositol phosphates was assessed using adrenal glomerulosa cells. [^{3}H]Inositol-labelled cells were incubated with angiotensin II for 5 min to generate a complex mixture of inositol phosphates. The extracts were halved and one portion was subject to pretreatment with Dowex-50 resin prior to anion-exchange HPLC. The other sample was analysed without prior treatment. The sample (0.5 ml) was applied to a 1-ml Dowex-50 column and eluted with 1 ml of water. A comparison of the profiles with treated and untreated samples is shown is shown in Fig. 2. Dowex-50 treatment did not alter the profile of [^{3}H]inositol-

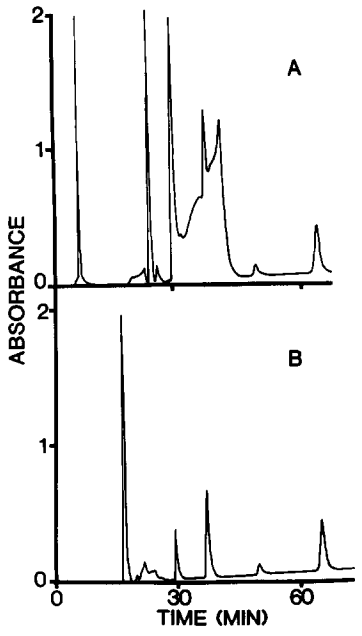

Fig. 3. Effect of Dowex-50 treatment on the UV-absorbing material present in extracts of rat adrenal glomerulosa cells. Shown is the profile of absorbance at 254 nm corresponding to the ^{3}H profile shown in Fig. 2. In profile B, the retention times of AMP, ADP and ATP were 17.5, 37.8 and 65.5 min, respectively. The experiment was performed three times with similar results. (A) Untreated extract; (B) Dowex-50-treated extract.

labelled compounds present in the extracts. In contrast, as shown in Fig. 3, the UV-absorbing material in the sample was markedly reduced. Note that the adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP) standards were added after Dowex-50 chromatography.

Effect of Dowex-50 pretreatment on column life and chromatographic performance

The effect of Dowex-50 pretreatment on column lifetime was evaluated over three years of use. Columns were retired when peak splitting was observed in the UV standards or when peaks were unacceptably broad or tailing. The average number of runs obtained without Dowex-50 pretreatment was 52 ± 27 (mean ± S.D., $n = 9$) compared with 162 ± 22 ($n = 4$) when all tissue extracts were passed through Dowex-50 columns.

The effect of Dowex-50 pretreatment on chromatographic reliability was assessed by examining the variability of the retention times of AMP. AMP was chosen because, being weakly retained by the column, it shows more variability in retention time than the more highly phosphorylated nucleotides. The retention time of AMP averaged over 50 analyses using three different columns was 17.1 ± 1.2 (mean ± S.D.) for Dowex-50-treated samples and 15.9 ± 3.4 for untreated samples.

The effectiveness of the Dowex-50 columns in removing Ca^{2+} ions was assessed by adding 10 000 cpm of $^{45}CaCl_2$ to TCA extracts of heart tissue. After passage through the columns only 0.0004% of the added $^{45}Ca^{2+}$ remained in the extract.

DISCUSSION

A simple, inexpensive and effective method has been developed for the preparation of samples for the determination of inositol phosphates in cell or tissue extracts. The method involves passing the acid extract, after removal of the TCA or perchloric acid, through a small column of Dowex-50 cation-exchange resin. None of the inositol phosphates is retained by the resin. Therefore it is only necessary to pass the sample through the column and elute with a further void volume of water to recover all of the inositol phosphates. In practice, the extracts (generally 2 ml) are passed through a 1-ml column immediately after removal of the TCA and eluted with a further 1 ml of water. Eluates are then lyophylized for storage prior to HPLC. Clearly, where samples contain less extraneous matter, smaller columns and smaller elution volumes can be used.

In our experience, this sample preparation results in improved reproducibility of chromatographic profiles especially with respect to minimally retained components such as AMP and the isomers of IP_1. The retention time of AMP is especially sensitive to fluctuation. Furthermore, with samples prepared from heart or kidney which contain large amounts of UV-absorbing material it can be difficult to estimate the retention times of the nucleotide standards because of

other UV-absorbing material present in the extracts. Using the sample preparation method described here together with adequate column cleaning and re-equilibration, the retention time of AMP varies by less than 1 min.

Another important advantage lies in the increase in column lifetime. In our experience, extracts of tissues such as heart which contain a large amount of UV-absorbing material caused a rapid deterioration of the SAX columns with loss of acceptable chromatography after fifteen to twenty runs. While extracts of adrenal glomerulosa cells are less damaging, the columns required regular, extensive cleaning procedures using 2 *M* ammonium phosphate (or ammonium formate) to remove tightly bound UV-absorbing material and maintain acceptable chromatographic performance.

To date, most detailed studies of inositol phosphate metabolism have been carried out using cultured cells where problems of extraneous material are not generally as acute. It is important that more detailed studies of inositol phosphate metabolism are performed in a greater variety of tissues. The provision of a simple method of sample preparation suitable for use with extracts from tissues should facilitate the progress of such studies.

ACKNOWLEDGEMENTS

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