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Separation of inositol phosphates by capillary electrophoresis

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ABSTRACT

The feasibility of determining inositol phosphates by capillary electrophoresis at levels present in physiological samples was investigated. Methods previously reported for the determination of inositol phosphates are limited, either in their ability to separate structural isomers, the need for pre- or post-column derivatization, or by analysis time. Using capillary electrophoresis with indirect photometric detection, separation of a standard mixture containing mono-, bis-, tris-, and hexakisinositol phosphates was achieved in less than 10 min. The potential for separation of inositol phosphate isomers using capillary electrophoresis was demonstrated by the separation of 1- and 2-inositol monophosphate. Under the conditions used, the minimum detection limit for inositol 2-phosphate in an electrolyte containing phthalate was in the vicinity of 200 ng/ml.

INTRODUCTION

The mechanism by which cells respond to extracellular signals is a topic of considerable research activity [1]. In particular, the role of second messenger molecules in regulating cellular processes is receiving a great deal of attention [2–5]. Inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3], is of particular importance since it releases Ca^{2+} from intracellular stores which in turn triggers a wide variety of cellular processes. Other inositol phosphates are implicated in secondary messenger functions but in most cases, their exact role is not clearly understood. In order to study these cellular processes, a rapid, sensitive method is needed for the determination of inositol phosphates at levels present in physiological samples. A variety of approaches have been used. These usually involve derivatization and radiolabelling detection techniques coupled with various separation techniques including open column anion-exchange chromatography [6], gas–liquid chromatography [7], ion pair chromatography [8], and high-voltage electrophoresis [9]. Most recently, high-performance anion-exchange chromatography with chemically suppressed conductivity detection was applied to the separation of inositol mono- through pentakisphosphates, and inositol phosphate isomers [10]. Each of these approaches has limitations, either in the need for pre- or post-column derivatization, their inability to separate structural isomers, or in analysis time.

Capillary electrophoresis (CE) [11–16] is attractive for the determination of inositol phosphates for the following reasons: (1) only a few nanoliters of sample are used in each analysis; (2) there is the potential for concurrent separation of mono- through hexakisphosphate species in the same analysis; and (3) run times are usually short due to the intrinsically high efficiency of the technique.

Recently, capillary electrophoresis has been applied to the determination of high-mobility inorganic ions and low-molecular-mass organic acids [17–22]. A similar approach was selected for inositol phosphates since they are also non-chromophoric anions. For anion applications, a cationic surfactant or amine is incorporated into the electrolyte to cause the necessary reversal of the normal electroosmotic flow [22]. Indirect photometric detection

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[23,24] is often used to allow detection of non-chromophoric ions. In applying this technique to detect ions [19–21], a background absorbance is created by incorporating a highly absorbing ionic species in the buffer or electrolyte. The detection principle is based on the fact that the non-chromophoric analyte ions displace chromophoric ions as they pass through the detector causing a reduction in absorbance. This can be recorded as a negative peak, or as a positive peak if the detector output polarity is reversed. In order to use indirect photometric detection with CE, the chromophoric ion must also have a similar electrophoretic mobility ot the analytes in order to prevent peak distortion [25].

Based on the ionization characteristics of phytic acid ($InsP_6$) [26] and calculated charge to mass ratios for various degrees of ionization, electrolytes which had previously been used for CE of phosphate were selected as a starting point for CE of inositol phosphates. Two electrolyte systems were used in this initial investigation. They each contain a chromophoric ion (chromate and phthalate, respectively), with similar electrophoretic mobility to phosphate. They also offer different selectivity since the pH range from 5.8 to 7.3 is in the region of the pK_a values for removal of second protons from the phosphate groups on the inositol phosphates [27].

EXPERIMENTAL

Reagents and standards

All reagents were of analytical-reagent or ACS grade. Potassium hydrogenphthalate and tetradecyltrimethyl ammonium bromide (TTAB) were obtained from Aldrich (Milwaukee, WI, USA). Sodium tetraborate, boric acid, and sodium chromate were obtained from Fisher Scientific (Pittsburgh, PA, USA).

Inositol phosphates used in this study were obtained from Sigma (St. Louis, MO, USA). These were of varying purity, as follows:

DL-*myo*-Inositol-1-monophosphate, Ins(1)P, as the cyclohexylammonium salt (Purity *ca.* 75%. Balance primarily 2-monophosphate isomer).

DL-*myo*-Inositol-2-monophosphate, Ins(2)P, as the di(cyclohexylammonium) salt (purity *ca.* 95%).

DL-*myo*-Inositol-1,4-bisphosphate, $Ins(1,4)P_2$, as the potassium salt (purity *ca.* 98% by thin-layer chromatography).

DL-*myo*-Inositol-1,4,5-trisphosphate, $Ins(1,4,5)P_3$, as the potassium salt, (86% 1,4,5-isomer and 14% 2,4,5-isomer).

Phytic acid (*myo*-inositol hexakisphosphate), $InsP_6$, as the dodecasodium salt.

With the exception of phytic acid, inositol phosphates were stored in the dark at −20°C immediately on receipt.

Preparation of inositol phosphate standards

Stock solutions of phytic acid and Ins(2)P were prepared in the conventional manner by weighing and volumetric dilution. Stock solutions of Ins(1)P, $Ins(1,4)P_2$, $Ins(1,4,5)P_3$ were prepared directly in the sample vials (each containing approximately 0.1 mg) using 18-MΩ deionized water, then refrozen. Immediately prior to capillary electrophoresis runs, these stock solutions were allowed to warm to room temperature in a closed box (to minimize photodecomposition), and aliquots removed for dilution immediately prior to analysis by CE.

Electrolyte preparation

Electrolytes used in this study were as follows: (1) 2.5 m*M* K_2CrO_4, 0.5 m*M* tetradecyltrimethyl ammonium bromide (TTAB), 5.0 m*M* H_3BO_3, pH 7.3; and (2) 5.0 m*M* potassium hydrogenphthalate, 0.5 m*M* TTAB, 2.0 m*M* $Na_2B_4O_7$, pH 5.9. All electrolytes were filtered through a 0.45-μm filter and vacuum degassed for 5 min prior to use.

Equipment

CE was performed on a Dionex CES I system with the negative polarity power suplly. Untreated silica capillaries (55 cm × 50 μm I.D. × 375 μm O.D.) from Polymicro Technologies (Phoenix AZ, USA) were used throughout in this work. Prior to use, they were flushed successively with 0.1 *M* phosphoric acid, deionized water, 0.5 *M* NaOH, deionized water, and finally with the operating electrolyte. A Dionex AI-450 chromatography workstation was used for data acquisition and processing. The variable-wavelength UV detector in the CES I was used for indirect photometric detection of the inositol phosphates with the signal switch polarity reversed to give positive peaks. Wavelength settings were 270 nm for the chromate electrolyte and 254 nm for the phthalate electrolyte. Output range was 0.005 AUFS.

RESULTS AND DISCUSSION

Standard runs with individual inositol phosphates in both the chromate and phthalate electrolyte confirmed that it is possible to separate these compounds concurrently in less than 10 min. This is illustrated by the separation of a mixture of inositol phosphates with widely different numbers of phosphate groups using the chromate electrolyte at pH 7.3 (Fig. 1). As shown in Fig. 2, the selectivity is quite different with the phthalate electrolyte at pH 5.9. Separation of all the inositol phosphates was not achieved in this case since the Ins(1,4,5)P_3 and $InsP_6$ are poorly resolved and exhibit fronting.

Although separation of all species was obtained at pH 7.3 with the chromate electrolyte, the development of a routine method for the concurrent separation of Ins(1)P through $InsP_6$ using this particular electrolyte is questionable. The entire separation of the inositol phosphates of interest takes place within 0.7 min, hence, small shifts in migration time could conceivably lead to misidentification. Repeatability of migration times usually were found to agree within ± 0.01 min for consecutive runs, however, significant shifts in migration times occurred from day-to-day and with different capillaries. Buffering of the electrolyte should alleviate this problem, and will very likely be necessary when analyzing physiological samples. The pH 7.3 chromate electrolyte does have potential utility for the analysis of individual inositol phosphate pairs such as mono- from bi- and tri- phosphate species since they are well resolved. For example, Ins(1)P and Ins(1,4)P_2 are separated by almost 1 min. The pH 5.9 phthalate electrolyte does not appear to be useful for concurrent separation of all the inositol phosphates, however, it exhibits excellent selectivity for the mono- and diphosphate species. Ins(1)P_1 and Ins(1,4)P_2 are separated by 1.38 min. In addition, the two monophosphate position isomers, Ins-

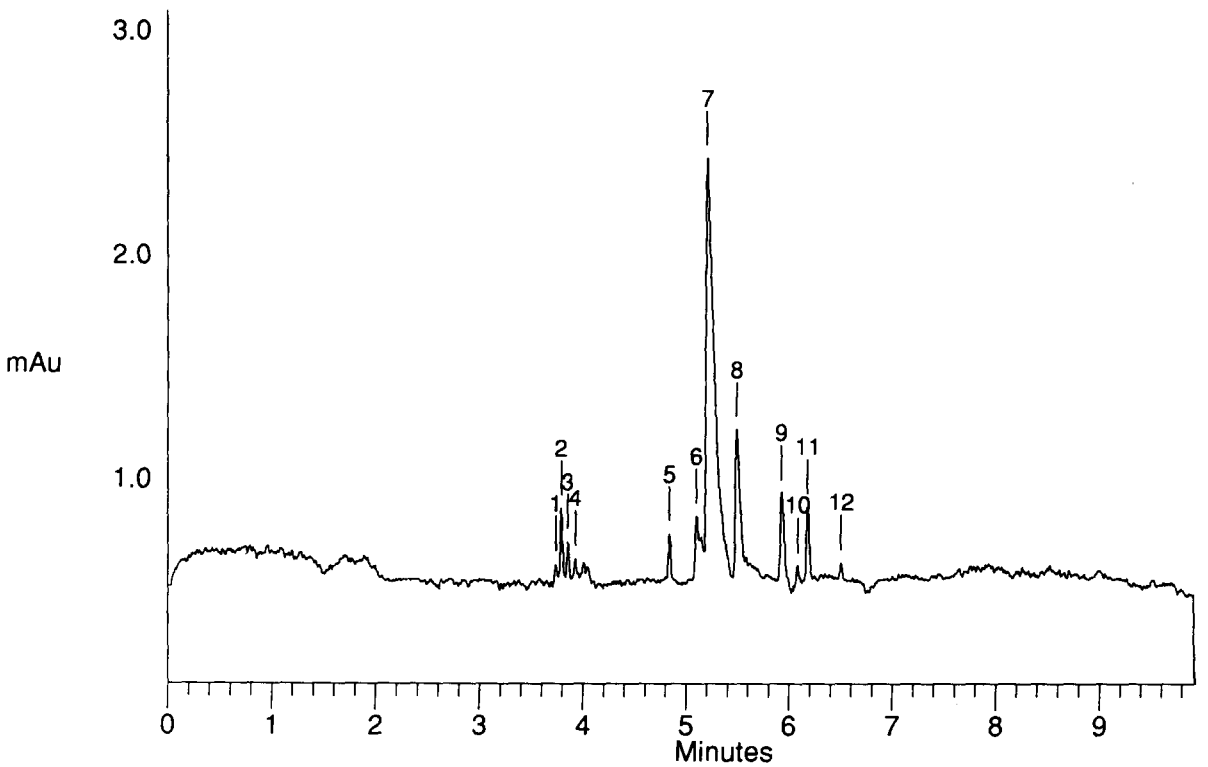

Fig. 1. Separation of inositol phosphate mixture by capillary electrophoresis. Electrolyte: 2.5 m*M* K_2CrO_4, 0.5 m*M* tetradecyltrimethyl ammonium bromide, 5.0 m*M* H_3BO_3, pH 7.3; operating voltage: 15 kV; electroinjection: 5 kV for 2 s; indirect photometric detection at 270 nm. Peaks: 1–4 = inorganic ions; 5 = Ins(1,4)P_2; 6 = $InsP_6$; 7 = Ins(1,4,5)P_3; 8 = Ins(1)P; 9–12 = unknowns.

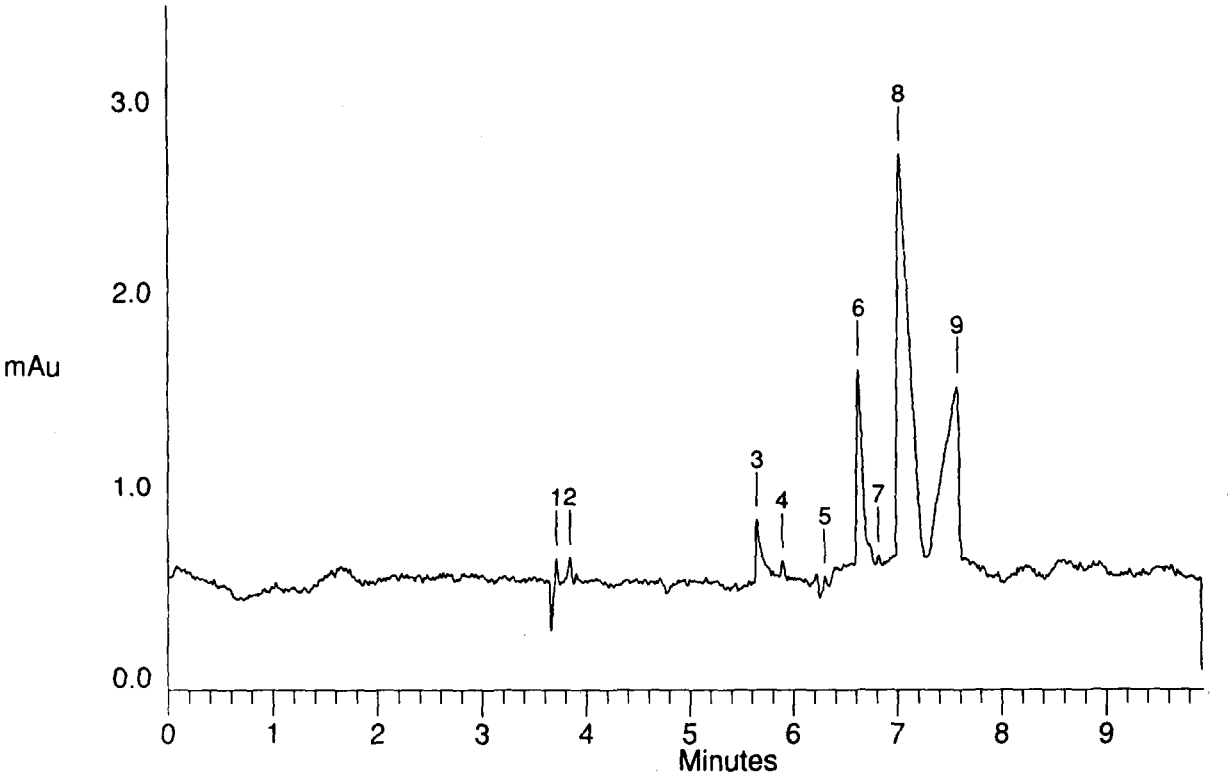

Fig. 2. Separation of inositol phosphates mixture by capillary electrophoresis. Electrolyte: 5.0 m*M* potassium hydrogenphthalate, 0.5 m*M* tetradecyltrimethyl ammonium bromide, 2.0 m*M* $Na_2B_4O_7$, pH 5.9; operating voltage: 15 kV; electroinjection; 5 kV for 2 s; indirect photometric detection at 254 nm. Peaks: 1, 2 = inorganic ions; 3 = Ins(1,4)P_2; 4,5 = unknowns; 6 = Ins(1)P; 7 = unknown; 8 = Ins(1)P; 9 = Ins(1,4,5)P_3 + InsP_6.

(1)P and Ins(2)P, are also well separated. The assignment of the major impurity peak in the Ins(1)P electropherogram to Ins(2)P is supported by the good agreement in migration times for the impurity peak and Ins(2)P, as shown in Fig. 3a and b, and by spiking with Ins(2)P as shown in Fig. 3c. These runs were carried out at a later date with a different capillary and batch of electrolyte than the runs shown in Figs. 1 and 2, hence migration times between the two sets of runs are not directly comparable. Good correlation between peak areas for the amount of the spike was obtained. A further indication that this peak is due to the Ins(2)P impurity known to be present in this sample of Ins(1)P, is that the area ($7.35 \cdot 10^5$ area units) is *approximately* 25% of the main peak ($28.43 \cdot 10^5$ area units). This is in agreement with the Sigma estimate of approximately 25% Ins(2)P impurity by thin-layer chromatographic analysis. Although electromigration injection was used in these runs, concentration bias problems reported by others [28] would be expected to be minimal in this case, since the electrophoretic mobilities of the two ions in question [Ins(1)P and Ins(2)P] are similar.

Detection limits

The results of a brief study to assess the linearity of response, and detection limits for inositol phosphates using indirect photometric detection with the phthalate electrolyte, are shown in Fig. 4. Six dilutions of an accurately weighed Ins(2)P standard in the range from 0.5 to 25 μg/ml (as the free acid) were electro-injected (7.5 kV for 5 s) and yielded a close to linear calibration plot (correlation coefficient = 0.9946) at concentrations in the range from 0 to 17 μg/ml. Detection limits under these conditions appear to be in the vicinity of 200 ng/ml for a signal-to-noise ratio of 3:1.

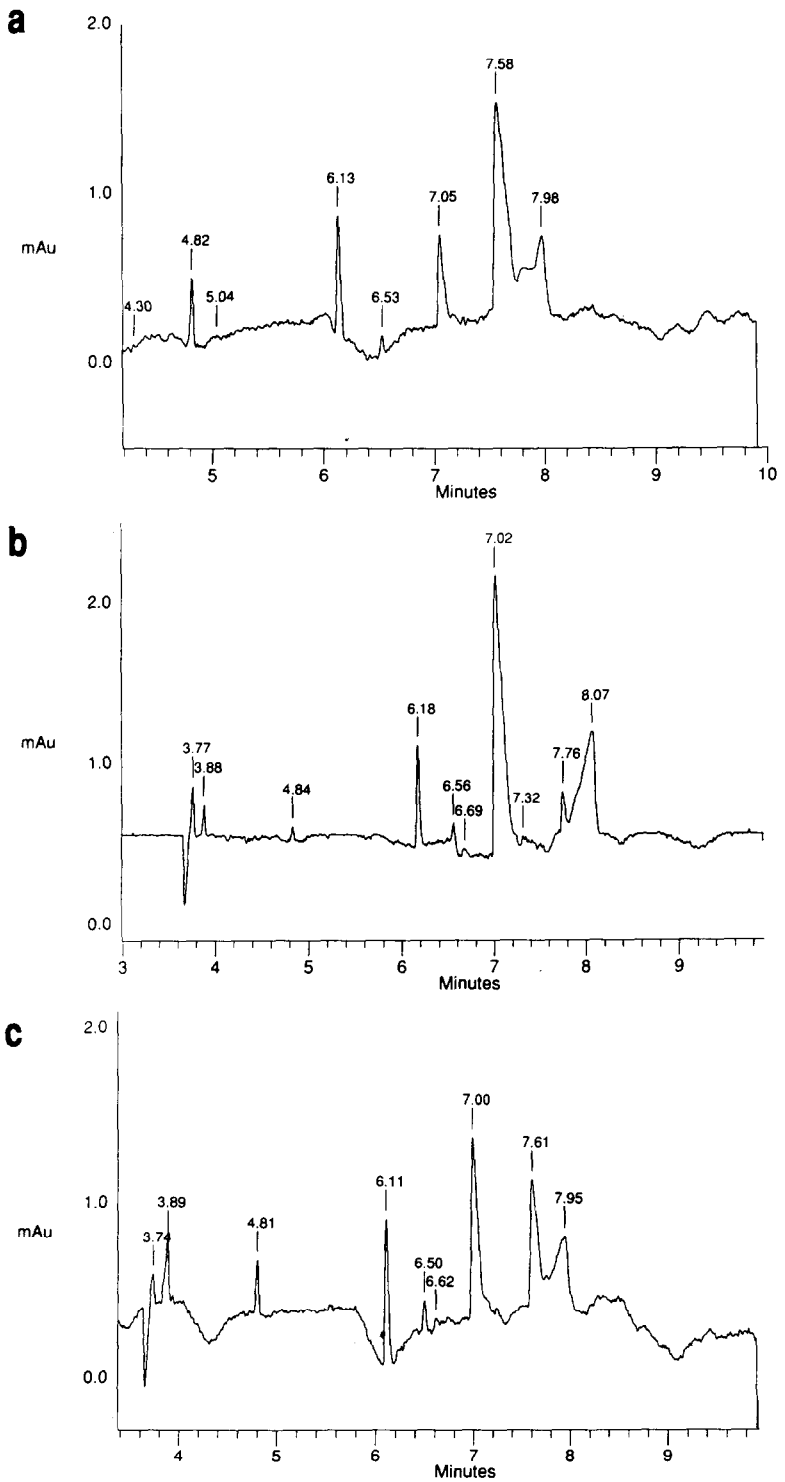

Fig. 3. Comparison of electropherograms for (a) DL-*myo*-inositol-1-monophosphate, (b) DL-*myo*-inositol-2-monophosphate, (c) a mixture of the 1- and 2-isomers. Conditions as for Fig. 2. Peak labels are migration times in minutes.

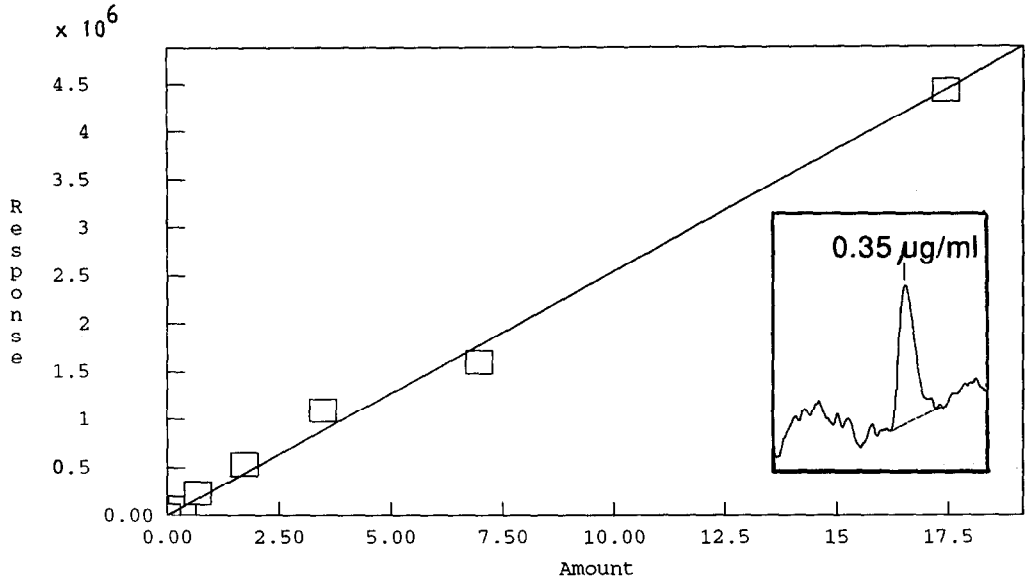

Fig. 4. Calibration plot for Ins(2)P in phthalate electrolyte. Conditions as for Fig. 2 except that the electroinjection was 7.5 kV for 2 s. Inset indicates signal to noise for 0.35 µg/ml level. r^2 = 0.994602. *x*-axis: amount in µg/ml.

Typical levels of inositol phosphates in rat brain ranging from 0.005 µmol/g for Ins(1,4,5)P_3 to 0.040 µmol/g for Ins(4)P have been reported [29,30]. These levels translate to *ca.* 2 µg/g wet mass of tissue for Ins(1,4,5)P_3 and 10.4 µg/g wet mass for Ins (4)P. In studies of this type, therefore, it appears that indirect photometric detection may provide adequate detection sensitivity.

CONCLUSIONS

On the basis of this study, CE shows great promise for the concurrent analysis of inositol phosphates with 1 to 6 phosphate groups, at levels encountered in physiological samples. Separations can be achieved in less than 10 min, and only a few nanoliters of sample are required for each analysis. Selectivity of the inositol phosphates separation can be controlled by varying the electrolyte pH in the vicinity of the pK_a values for ionization of the second proton from the phosphate groups (pH 5–10.5) [26]. The position isomers Ins(1)P and Ins(2)P are easily separated in the phthalate electrolyte system demonstrating the potential for separating other inositol phosphate isomers.

Optimization of the pH for the separation may allow a mapping procedure to be developed for inositol phosphates. With this objective, a more extensive study is now underway to investigate the separation of inositol phosphates in several buffered electrolyte systems over a wider range of pH. The results of this study will be reported at a later date.

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