Journal of Chromatography, 439 (1988) 83–92
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 314

ION CHROMATOGRAPHIC DETERMINATION OF SUGAR PHOSPHATES IN PHYSIOLOGICAL SAMPLES

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SUMMARY

Ion chromatography is shown to be capable of simultaneous determination of biologically important anions. Application of this technique is illustrated for the separation and quantification of the major anions present in rat brain and liver tissues. Sugar phosphates and carboxylic acids are separated on high-performance anion-exchange columns and are detected using chemically suppressed conductivity. Detection limits range from 20 to 100 pmol for the anions tested, including inositol phosphates, lactate, pyruvate, glucuronic acid-1-phosphate, fructose-6-phosphate and glucose-6-phosphate. The coefficient of variation for the determination of most anions was in the range 5–10%. Many of these anions are either difficult to separate with other methods, or require expensive radiochemical techniques for detection. This method should be applicable to other biological studies, from the flow of carbons in photosynthesis to the study of synaptic transmission.

INTRODUCTION

Ion chromatography is a technique that was first described in 1975 by Small *et al.*[1]. Although there have been numerous modifications using different detection methods, the original use of a suppressor column to reduce the conductivity of the eluent and enhance the conductivity of the analytes is still widely used and has been described in recent books and review articles[2–4]. Until recently, biochemists have made little use of this technique in determining a most important class of anions, sugar phosphates. For some analytes, such as ribulose-1,5-bisphosphate, enzyme-coupled assays are often used, and only one anion is determined. For others, such as the

0021-9673/88/$03.50

inositol phosphates, most laboratories use radiolabeled compounds for detection[5]. Recently, one laboratory has described a chromatographic method for the detection of phosphorylated compounds that employs a phosphatase-containing column that is placed after the ion-exchange column[6] for the detection of these compounds. In this procedure, the phosphates released by this column are directed to a stream of molybdate, enabling spectrophotometric detection.

The biochemistry of inositol phosphates is currently a subject of intense research, because of data indicating that inositol-1,4,5-trisphosphate (IP_3) is an intracellular second messenger for numerous hormones, peptide growth factors, neurotransmitters, and other agonists[7–11]. Binding of these agonists to specific receptors is coupled to the hydrolysis of phosphatidylinositol-4,5-bisphosphate catalyzed by phospholipase C. The products of this reaction are diacylglycerol and IP_3, both of which serve as regulators of intracellular events. Hydrolysis of the IP_3 produces other inositol phosphates and subsequently free inositol, which may be reutilized in phosphatidylinositol biosynthesis. The IP_3 also may undergo phosphorylation to IP_4, which stimulates calcium influx into cells. The interest in the inositol phosphates relates, therefore, to the importance of this mechanism in controlling cellular functions. The development of a general procedure for the quantification of the inositol phosphates would contribute to the elucidation of these important receptor-mediated events.

In this study, we describe the use of ion chromatography for the simultaneous determination of inositol phosphates, sugar phosphates and aliphatic organic acids, such as lactate, pyruvate and citrate. The results of this study demonstrate that brain and liver can be analyzed effectively and reproducibly with this method.

EXPERIMENTAL

Reagents

All carboxylic acid and sugar phosphate standards were from Sigma (St. Louis, MO, U.S.A.) except inositol-1,4,5-trisphosphate (IP_3) which was from Calbiochem (Richmond, CA, U.S.A.) and 4-cyanophenol from Aldrich (Milwaukee, WI, U.S.A.). Sodium bicarbonate, sodium carbonate, and sodium hydroxide were from Fisher.

Apparatus

A Dionex (Sunnyvale, CA, U.S.A.) Bio liquid chromatograph, equipped with AS4A (50 cm × 4 mm I.D.) and AS5 (50 cm × 4 mm I.D.) anion-exchange columns, an AG5A Guard column, an anion micromembrane suppressor (AMMS), and a conductivity detector, was used. A 50-μl constant volume injection loop was used throughout. Brain and liver samples were passed through a water-washed CAT-Ag disposable sample treatment cartridge (Dionex) prior to injection.

Mobile phase

The most commonly used mobile phase for anion exchange was 2.4 m*M* $NaHCO_3$–1.92 m*M* Na_2CO_3, flowing at 1.5 ml/min. The AMMS was continually regenerated with 0.0125 *M* sulfuric acid.

Sample preparation

Rat brains were obtained immediately after decapitation, by immersing the heads in liquid nitrogen for 20–25 s to briefly freeze the brain, followed by dissection of cerebral cortices. These were immediately homogenized in ice-cold distilled water and then extracted with two volumes of chloroform–methanol (2:1, v/v). The samples were then centrifuged at 20 000 *g* for 15 min and the aqueous phase was passed through a pre-washed (two times with 5 ml deionized water) disposable cartridge, filled with CAT-Ag, a cation-exchange resin in the silver form. The filtrate was then injected into the liquid chromatograph.

Rat liver samples were prepared by freeze clamping of tissue immediately after removal from the animal and ground with a mortar and pestle with a mixture of ice-cold distilled water containing an equal volume of chloroform–methanol (2:1, v/v). After centrifugation at 20 000 *g* at 4°C for 15 min, the aqueous phase was treated with the CAT-Ag cartridge.

Quantitation

The identity of each peak in the chromatograms was verified by spiking the sample with known standards. Quantitation was performed by analyzing five standards before injecting the sample, then by re-injecting these same five standards after the final sample injection. Peak areas were plotted against concentrations of analytes and the data reduced by calculating the best fit by least squares analysis. Correlation coefficients ranged from 0.996 to 0.999.

RESULTS

As demonstrated previously, biologically relevant anion standards can be separated by ion exchange, using an AS4A or AS5 column[12]. The application of this technique to rat brain and liver samples is demonstrated in Fig. 1A and B. Because most anions in liver and brain have charges of -1 or -2 at pH 10, and because of its previous extensive use, bicarbonate–carbonate was selected as the eluent to be most thoroughly investigated in this study. As shown in the chromatogram, the largest peak is that due to chloride. Using the AS5 column and the carbonate eluent, sugar phosphates elute within 6–10 min. Inositol-1-phosphate, glycolytic intermediates, and glycerol-3-phosphate elute in this region, but are difficult to quantify in the presence of large amounts of chloride. The peak for inorganic phosphate follows that for the sugar phosphates.

The large amounts of chloride and protein in the tissue samples present potential problems in the analysis of trace amounts of anions. To detect anions present in trace amounts, it would be necessary to inject so much sample that the chloride would overload the anion-exchange column. In addition, extraction with chloroform–methanol does not remove all the protein, which will be strongly retained on the guard column. If too much protein is injected in each analysis, the lifetime of the guard column may be shortened significantly. For these reasons, the chloride and protein content of the samples was reduced by passing the sample through a disposable cartridge, CAT-Ag, packed with a cation-exchange resin in the silver form. The protein content in brain homogenate is reduced about eight-fold by this treatment as measured by the decrease in absorbance at 280 nm from about 0.85 to 0.12 (av-

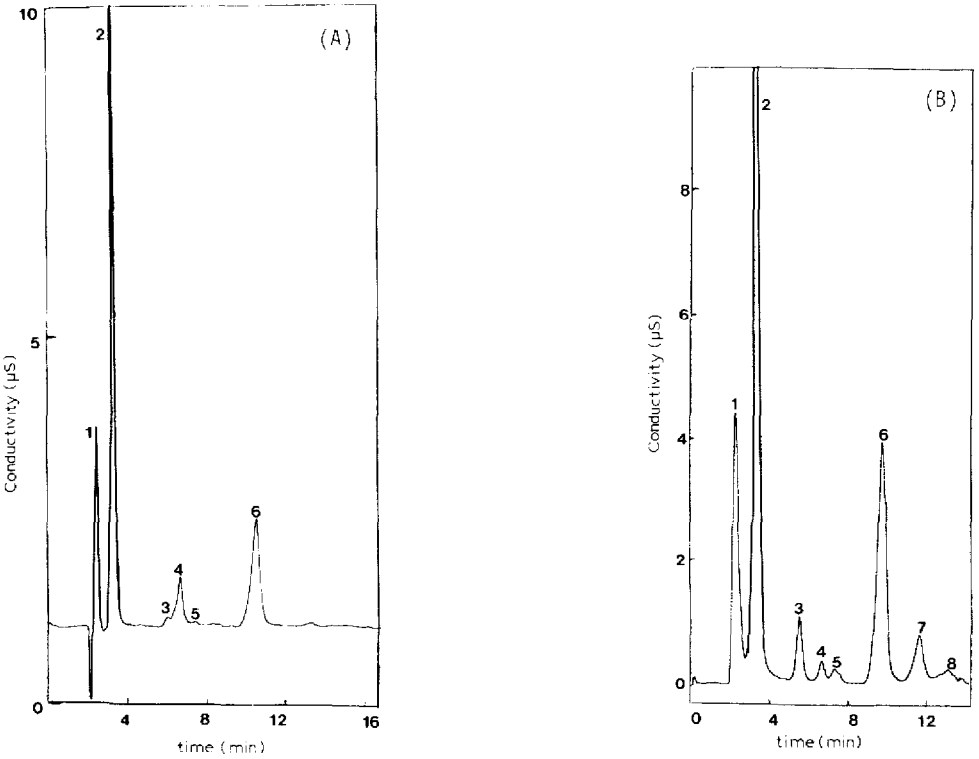

Fig. 1. Elution profile for rat brain and liver samples. (A) Anion chromatogram of rat brain. Column: 2 × AS5, eluent: 2.4 m*M* $NaHCO_3$–1.92 m*M* Na_2CO_3 at 1.5 ml/min. Detection: chemically suppressed conductivity using an AMMS. (B) Anion chromatogram of rat liver. The chromatographic conditions are as described above. Peaks: 1 = lactate; 2 = chloride; 3 = IP; 4 = glucose-6-phosphate region; 5 = glycerol-3-phosphate; 6 = phosphate; 7 = sulfate region; 8 = oxalate region.

erage of seven brains). Furthermore, the samples appeared to be stable following CAT-Ag treatment and the guard column showed little degradation. After twenty injections of brain samples, the capacity factor for sulfate decreased by 18%. Similar results were obtained with samples from liver. As shown in Fig. 2A and B, the chloride peak is almost completely eliminated after treatment with the CAT-Ag cartridge.

The peaks in the chromatograms of brain and liver were identified in two principal ways. First, the retention times of standards reported previously[12] were compared to retention times in the sample, and second, by spiking each sample with the suspect anion. In some cases, identification is based on the known composition of biological samples. For example, the peak with retention time of 2.3 min is assigned to lactate, although analysis of standards show that fluoride, acetate, and formate also elute at this time. As a further aid to the identification of peaks, an anion-exchange column with different selectivity, the AS4A, was used. As shown in Fig. 2C, most of the peaks produced by mono- and divalent brain anions appear in the same order, and are the same size as those obtained using the AS5 column (Fig. 2A).

Peak 5 is due to glucose-6-phosphate and an unknown anion which appears as peak 7 in Fig. 2C. None of the known compounds tested in this study or reported earlier[12] coelute with both peak 5 (Fig. 2A) and peak 7 (Fig. 2C).

In a recent study[12], the effect of eluent concentration on the capacity factors for several anions were determined. From plots of the logarithm of the capacity factor *versus* the logarithm of the eluent concentration (in isocratic elutions), isocratic constants can be calculated[3]. These constants, together with the known charge on the analytes, were used to calculate gradient constants, which relate the expected capacity factors in a gradient elution to the rate of change of eluent concentration. A gradient elution program was calculated from the isocratic and gradient constants. These data indicated that polyvalent anions are retained less on the AS4A than the AS5 column. The application of this technique to the analysis of rat brain samples is shown in Fig. 3A and B, where eleven different peaks are observed. Using a gradient of relatively high 4-cyanophenolate concentrations, mono- and divalent anions, being present in high concentrations, are not well separated. As seen in Fig. 3A, anions with charges of three or more are present at low concentrations in comparison to mono- and divalent anions. An expanded scale is used to visualize these as shown in Fig. 3B. The peak which appears at 6.9 min represents the 6-phosphogluconic acid region. Both 3-phosphoglycerate and 2-phosphoglycerate co-elute at 7.3 min, and citrate elutes at 7.6 min. The peaks at 8.3, 8.6, 8.8 and 9.8 min have not been identified, but are very likely trivalent anions. The peaks at 12.2, 13.2 and 15.2 min are fructose-1,6-bisphosphate, IP_2 and IP_3, respectively.

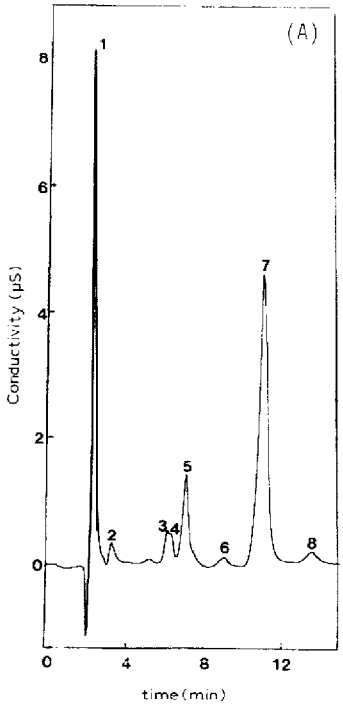

Fig. 2.

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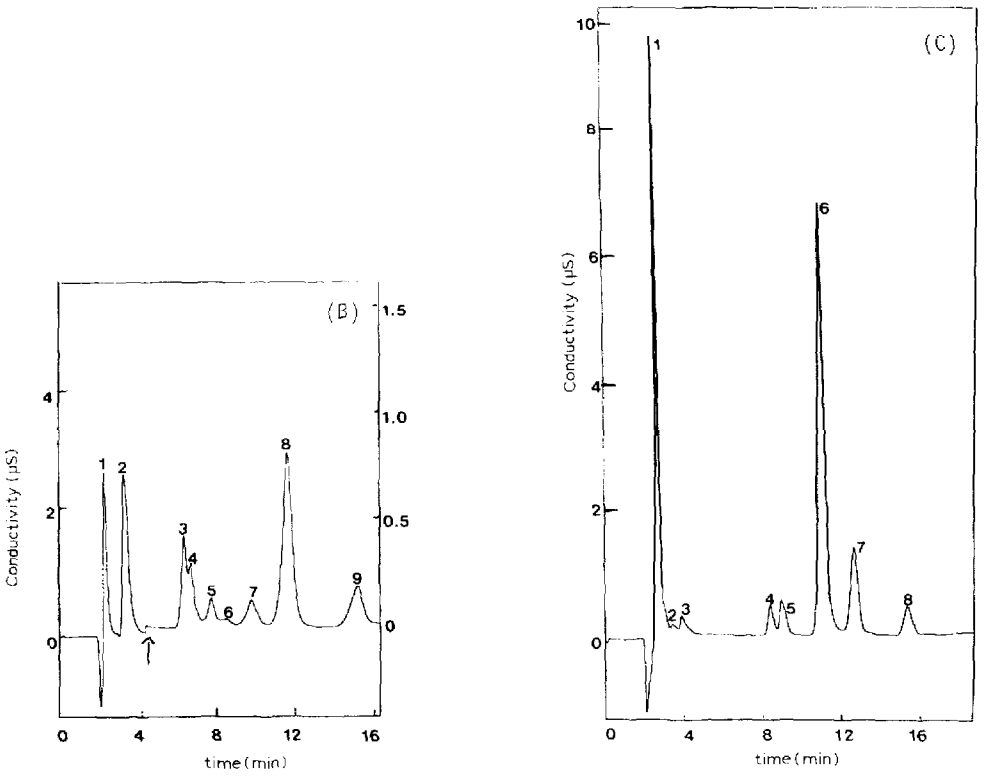

Fig. 2. Elution profile of samples treated with CAT-Ag cartridge. (A) Anion chromatogram of rat brain after treatment with CAT-Ag. The chromatographic conditions are the same as those in Fig. 1. Peaks: 1 = lactate; 2 = chloride; 3 = IP; 4 = glucose-1-phosphate; 5 = glucose-6-phosphate region; 6 = glycerol-3-phosphate; 7 = phosphate; 8 = sulfate. (B) Anion chromatogram of rat liver after treatment with CAT-Ag. The chromatographic conditions are the same as those in Figs. 1 and 2A. Peaks: 1 = lactate; 2 = chloride; 3 = IP; 4 = glucose-1-phosphate; 5 = glucose-6-phosphate; 6 = fructose-6-phosphate; 7 = glycerol-3-phosphate; 8 = phosphate; 9 = sulfate. (C) Chromatographic analysis of rat brain using the AS4A column. The conditions are the same as those in Fig. 2A. Peaks: 1 = lactate; 2 = pyruvate; 3 = chloride; 4 = glucose-1-phosphate; 5 = glucose-6-phosphate; 6 = phosphate region; 7 = unknown; 8 = sulfate. In (B) detection started at 10 μS f.s. At the arrow it was changed to 3 μS f.s.

To better visualize the IP_3 peak, a stronger isocratic eluent, 26.4 m*M* 4-cyanophenolate, was used (Fig. 4). Under these conditions, all anions with charges of -4 or less elute in the void volume. Thus, 26.4 m*M* 4-cyanophenolate was only used as an eluent for determining IP_3.

From calibration curves, the concentrations of the various analytes in brain were determined as presented in Table I. The effects of sample treatment with the CAT-Ag were tested in these studies. The values obtained for lactate, inositol-1-phosphate, phosphate and sulfate were found to be the same with or without sample treatment with the CAT-Ag. However, the use of the sample treatment was important for determinations of polyvalents and trace divalent anions. These anions cannot be easily detected without CAT-Ag pretreatment, whereas they are easily detected when chloride and proteins are removed from the sample. This allows the injection of more

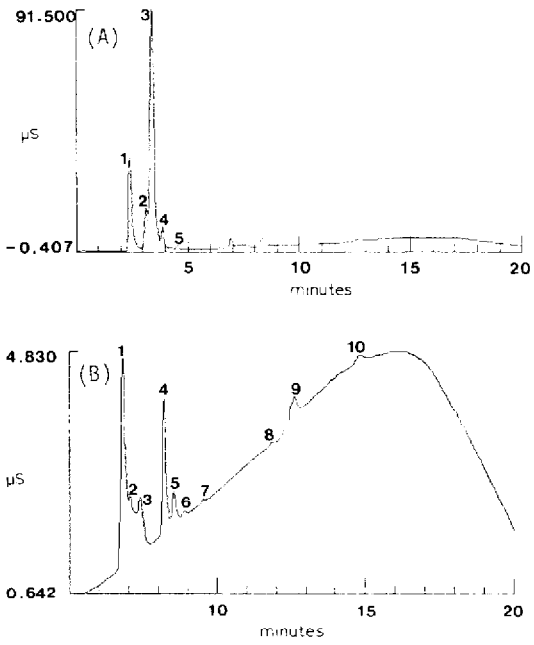

Fig. 3. Polyvalents in rat brain. (A) Column: AS4A. The linear gradient elution program consisted of the following, where A refers to water and B refers to 44 m*M* 4-cyanophenolate: zero time, 85%A and 15%B; 10.0 min, 40%A and 60%B; 12.0 min, 40%A and 60%B; 16.0 min 85%A and 15%B. Eluent flow-rate was 2.0 ml/min. Peaks: 1 = lactate; 2 = IP; 3 = other sugar phosphates and inorganic phosphate; 4 = sulfate; 5 = oxalate region. (B) Region 6–20 min in Fig. 3A. Peaks: 1 = 6-phosphogluconate region; 2 = 2-phosphoglycerate and 3-phosphoglycerate; 3 = citrate; 4 = unknown; 5 = unknown; 6 = unknown; 7 = unknown; 8 = fructose-1,6-bisphosphate; 9 = IP_2; 10 = IP_3.

concentrated homogenates without overloading the analytical column, and thus enabling the detection of trace components that are likely to appear as transiently occurring metabolites.

On the basis of these results, the analyses of the liver samples were performed with CAT-Ag pretreatment. Results of analysis for mono- and divalent anions are summarized in Table II.

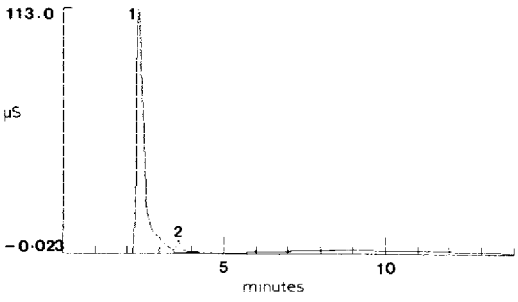

Fig. 4. IP_3 in brain. Column: AS4A. The eluent was 26.4 m*M* 4-cyanophenol used at a flow-rate of 2.0 ml/min. Peak 1 represents the anions of charge −1 to −5 and peak 2 is IP_3.

TABLE I

CONCENTRATIONS OF ANIONS IN RAT BRAIN

Anion	*Concentration (μmol/g)*	*Standard deviation (n = 5)*
Lactate*	3.64	0.02
Inositol-1-phosphate*	0.16	0.03
Glucose-1-phosphate*	0.19	0.03
Glucose-6-phosphate**	0.80	0.05
Phosphate*	2.37	0.05
Sulfate*	0.048	0.003
Oxalate*	0.040	0.003
Citrate***	0.085	0.006
IP_2***	0.021	0.0024
IP_3§	0.015	0.0011

* Chromatographic conditions as in Fig. 2A and B.
** Chromatographic conditions as in Fig. 2C.
*** Chromatographic conditions as in Fig. 3A.
§ Chromatographic conditions as in Fig. 4.

All samples were treated in a manner suitable for automation. To prevent hydrolysis and other enzyme-catalyzed reactions, enzymes were inactivated by a combination of solvent extraction, treatment with the CAT-Ag disposable cartridge, and freezing. The stability of the samples was tested by storing the samples at room temperature for extended periods prior to injection into the liquid chromatograph and comparison of the results with samples that were analyzed immediately after thawing. All peak areas and retention times for the two sets of samples were within 15% of each other. Samples were further analyzed repetitively and found to produce similar results over an 8-h period. The averages and relative standard deviations presented in Table I are from data that did not vary significantly during this period.

TABLE II

CONCENTRATIONS OF ANIONS IN LIVER

Chromatographic conditions as in Fig. 2.

Anion	*Concentration (μmol/g)*	*Standard deviation*
Lactate	3.66	0.11
Inositol-1-phosphate	0.56	0.03
Glucose-1-phosphate	0.41	0.04
Fructose-6-phosphate	0.03	0.004
Glycerol-3-phosphate	0.31	0.03
Phosphate	2.63	0.21
Sulfate	0.16	0.013

DISCUSSION

Ion chromatography has been demonstrated to be an effective method for the analysis of anions in biological samples. Using the AS5 column and chemically suppressed conductivity detection, 9–10 anions have been quantified simultaneously. For detection of inositol phosphates and other polyvalent anions at low concentrations, the sensitivity[3] of chemically suppressed conductivity detection is needed. These compounds do not have strong UV chromophores, and therefore are commonly detected using radiochemical techniques[5] or a phosphatase-loaded post-column reactor[6].

The data presented here demonstrate the repeatability of replicate injections and the effect of CAT-Ag sample treatment. It should be emphasized that the concentrations given in Tables I and II may differ from values *in vivo*, depending on the physiological state of the animal at the time of death and the method of sample handling. Although all samples were prepared by rapidly removing and freezing the tissues, delays in removal or denaturation of enzymes may affect the values obtained. The data reported here indicate, however, that solvent extraction and pre-column clean-up will denature or filter the enzymes in brain and liver homogenates. Of all the anion standards tested previously[12], most were stable for hours in dilute, alkaline solution, and showed no detectable hydrolysis to produce inorganic phosphate over several hours.

To perform detailed metabolic or clinical studies, it is important to analyze samples in short time intervals, requiring multiple samples to be analyzed. Automated liquid chromatography (LC) may have advantages over manual analysis or radiochemical methods for this purpose. Analysis of 100 samples using an autosampler and the procedures described in Figs. 1–3 would require that some samples would have aged approximately 25 h more than the first one analyzed. It is important, therefore, that stability of the samples be assured. This condition is met for the samples once they are treated with the CAT-Ag disposable column. In addition, the guard column (AG5A) shows little degradation after repeated use. The performance of the guard column should be monitored to determine the time for regeneration using well-known procedures[3]. Thus, fully unattended analysis lasting several hours is possible.

Compared to other ion-exchange methods[5,10,11], the described LC procedure has some unique features. First, the suppressor column lowers the pH of the eluent to 4.3, converting the analytes into the corresponding acids. Sample components, such as phosphocreatine, amino acids, and nucleosides are converted into their cationic form, thus, being exchanged for protons in the micromembrane suppressor. Therefore, they will not interfere with the determination of inositol phosphates or other sugar phosphates. Second, no radiolabeled compounds are used in this procedure. All anions were detected as their naturally occurring isotopes. Finally, this LC method enables simultaneous, multi-component determinations. This enables the biochemist to monitor several reactions or metabolic pathways simultaneously.

Although mammalian samples were used in this study, it is anticipated that similar LC procedures may have broad applicability to the analysis of samples from plants, since anions such as ribulose-1,5-bisphosphate can be easily determined. Thus, it is hoped that LC methods will play an increasing role in addressing serious biological and pharmaceutical questions in the future.

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