HPLC with Inductively Coupled Plasma Optical Emission Spectrometric Detection for the Analysis of Inositol Phosphates

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Abstract

The use of inductively coupled plasma optimal emission spectroscopy as a detector for the high-performance liquid chromatographic analysis of inositol phosphates is studied. It is found that separation of different inositol phosphates with a mobile phase consisting of tetraethylammonium (0.14%, w/v), methanol (5%, v/v), and formic acid (0.18%, w/v) may be obtained on a PRP-1 column with an analysis time of 18 min. In addition, high specificity and sensitivity of the detection system used permits detection of the inositol phosphates from bi- to hexaphosphate free from interference of other chromatographic peaks, which could be from the sample or mobile phase. Additionally, it is possible to use less sample because of the high sensitivity of the detection system.

Introduction

Phytic acid (inositol hexaphosphate, IP6) and other inositols of lesser phosphate groups are found in many alimentary grains and legumes. Some studies have shown that phytic acid may interfere with the bioavailability of iron, calcium, and zinc (1) and may produce nutritional problems should the consumer maintain a diet of high phytic acid content and few minerals. During the feeding and digestion process, IP6 may be degraded to inositol penta (IP5), tetra- (IP4), tri- (IP3), bi- (IP2), and monophosphate (IP). These degradation products show important physiological functions in humans, specially the IP3 isomers (2,3). Likewise, positive aspects have been attributed to phytic acid because of its antioxidant, anti-inflammatory, energy-storing, and anticarcinogenic properties, which may permit this component to decrease the cholesterol and triglyceride content in the blood, in addition to the possible prevention of heart disease, kidney stones, and certain types of cancer such as colon cancer (3). This has motivated the search for analysis techniques to enable separation and quantitation of the different inositol phosphate species. One of the most current is high-performance liquid chromatography (HPLC) with refractive index (RI) detection; however, rigorous control of the chromatographic separation conditions is required to prevent the solvent band from interfering with that of the inositol triphosphate (4–6). This inconvenience may also be found using conductivity detectors (7,8). On the other hand, the total phytic acid content has been quantitated in grain and urine samples with inductively coupled plasma (ICP) optical emission spectroscopy (OES) systems, by separating the phytic acid from the sample first and then quantitating the total phosphorus content (9,10). In addition, in 1979, Gast et al. (11) showed that ICP-OES may be used as a detector in line with liquid chromatography (LC), offering elemental specificity, low detection limits for a large number of elements, and a wide linear dynamic range. In this paper, HPLC coupling is accomplished with ICP-OES for the separation, detection, and quantitation of inositol hexa-, penta-, tetra-, tri-, and biphosphate.

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Experimental

Materials

Sodium phytate (79%, w/v) (dodecasodium salt hydrate) was determined by elemental ICP-OES phosphorus analysis and was provided by Aldrich Chemical Company (Milwaukee, WI). Tetraethyl ammonium (TEA) hydroxide (40%, w/w in aqueous solution) and tetrabutyl ammonium (TBA) hydroxide (1.0M in methanol solution) were also from Aldrich. AG 50W-X4 of H^+ ion-exchange resin (50–100 mesh) was from the Bio-Rad company (Richmond, CA). High-purity methanol (99.8%) was from the Riedel-de Han AG Company (Seelze, Hannover). Formic acid (90%, w/w purity) was from the BDH Laboratory Reagents Company (Poole, U.K.) and and 18ΩM/cm deionized water was from a Barnstead NANOpure apparatus.

Instrumentation

HPLC analysis was carried out using the INERT Model 9012 Varian pump equipped with Rheodyne 7725i injection valve with 20- and 100-L loops and a PRP-15 µm 150- × 4.1-mm macroporous polymer HPLC column from Hamilton (Reno, NV), which

was constructed in polyetheretherketone (PEEK) material. Connections between the injection valves, injection loop, pump, and chromatographic column were accomplished with a PEEK-material pipe (0.24-mm i.d.). The detection system was a JY-24 Jobin Yvon ICP-OES. ICP operation conditions used 1.2-kW power, 40-MHz frequency, 16-L/min plasmogenous gas flow, 0.2-L/min intermediate gas flow, 0.6-L/min haulage gas, and 213.715-nm wavelengths. The analog output from the detector was connected with the A/D converter model SS420, and the data was processed in EZCHROM software, which was provided by Scientific Software (Pleasanton, CA). A high-pressure hydraulic nebulizer (HHPN) with a cooled sedimentation chamber was used as an interface between the HPLC and ICP-OES techniques. The nebulizer outlet diameter was 15 µm and the impact bed separation distance was 4 cm (12).

Phytic acid hydrolysis

The mixture of the inositol phosphate from bi- to hexaphosphate was obtained by phytic acid hydrolysis. To achieve this, sodium phytate salt (0.5316 g) was dissolved in 20 mL of water, and 2 g of cationic exchange resin (3 × 10 cm, AG 50W-X4) was added to this solution. This solution was filtered and collected in a 100-mL volumetric flask. At this point, 20-mL aliquots were taken from the solution and heated with reflux to 95°C for 10 and 16 h, respectively. The hydrolyzed products were transferred to a 100-mL volumetric flask and diluted to the mark.

Mobile phase

In this study, three mobile phase types were evaluated, as presented in Table I.

Results and Discussions

To achieve separation of the inositols from tri- to hexaphosphate, in 1994, Lehrfeld (6) proposed the use of reversed-phase chromatography with RI detection. This author studied two mobile phase types to perform this separation, the compositions of which are presented in Table II.

Table I. Types of Mobile Phase Used

Mobile phase identification	TBA (%, w/v)	TEA (%, w/v)	Methanol (%, v/v)	Formic acid (%, w/v)
A	0.30	–	5.0	0.18
B	–	0.30	5.0	0.18
C	–	0.14	5.0	0.18

Table II. Mobile Phases Studied in Lehrfeld's Work of 1994

Mobile phase identification	Counterion (%, w/w)	Methanol (%, w/w)	Acetonitrile (%, w/w)	Formic acid (molar)
A'	TBA (0.40)	56	–	0.015
B'	TBA (0.40)	–	43	0.015

Although the best results were obtained with acetonitrile in the mobile phase (B'), this author recommends the use of methanol because of the higher toxicity of acetonitrile. However, none of the two mobile phases managed to detect inositol biphosphate because of the interference of the solvent bands. In this manner, with 56% methanol in the mobile phase (A'), a total analysis time of 18 min was required. It is important to note the high content of organic component used by this author.

In this work, some of the recommendations presented in the mentioned article of Lehrfeld were followed in order to perform the separation of the inositol phosphate mixture.

As the first part of our study, coupling between the HPLC and ICP-OES systems was performed by developing in our laboratories (12–14) a nebulizer with similar characteristics to

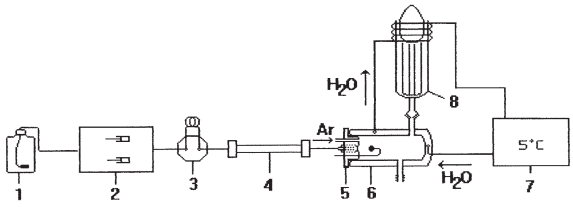

Figure 1. HPLC–HHPN–ICP-OES system. (1) Reservoir with the mobile phase, (2) HPLC pump, (3) injection valve, (4) column, (5) HHP nebulizer, (6) sedimentation chamber with coolant, (7) cooled water recirculation system, and (8) ICP torch.

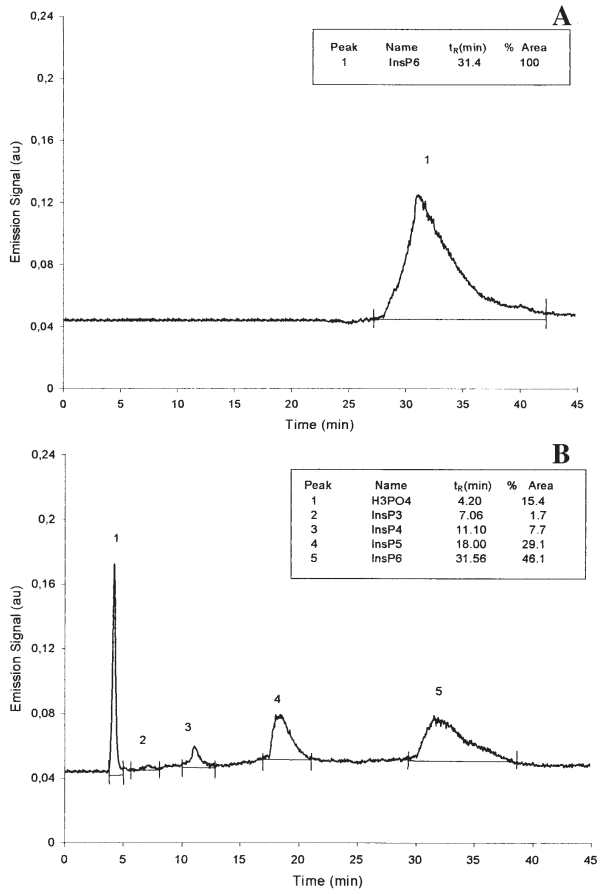

Figure 2. Chromatogram of (A) phytic acid and (B) IP6 hydrolysis products for 10 h at 95°C. Mobile phase A was used with an injection volume of 100 µL.

the HHPN designed by Berndt in 1989 (15). This type of nebulizer was selected because of its high-nebulization performance, which enables greater insertion of the sample to the plasma. In addition, instead of using a desolvation chamber, as recommended by Luo and Berndt in 1998 (16) for this type of nebulizer, a cooled sedimentation chamber was used. Thus, it is possible to decrease the large dead volume that the desolvation chamber may produce. However, because of the low solvent elimination performance of the cooled sedimentation chamber, compared with the desolvation chamber, we decided to decrease the methanol content in the mobile phase in order to prevent any problems arising from the large quantities of organic solvents in the plasma (one of which is the increase in noise and possible extinction of the plasma) (17). Likewise, the operator health hazard is reduced because he or she handles a lesser quantity of solvents. Figure 1 presents a diagram of the coupling system employed in the study.

In this manner, the chromatographic conditions proposed by Lehrfeld in 1994 (6) were adjusted to the chromatographic system under study. All chromatograms were obtained at a constant mobile phase flow of 0.58 mL/min.

Figures 2A and 2B show the chromatograms (with their respective t_R and %A values) obtained from the 100-µL phytic acid injection before and after hydrolysis for 10 h at 95°C, respectively. In chromatogram A, it is possible to observe the presence of a single peak corresponding to the phytic acid. In chromatogram B, 5 peaks are observed; of those, inorganic phosphate has been identified as peak 1; phytic acid as peak 5; and tri-, tetra-, and pentainositol phosphate correspond to peaks 2, 3, and 4, respectively. These chromatograms were performed with mobile phase A. The analysis time was 45 min. This time was considerably greater than that obtained by Lehrfeld in 1994, probably because of the decrease of the methanol content in the mobile phase. Considering that the analysis time was very high, the use of TEA is proposed as a substitute of the TBA because less methanol requirements are suggested with this ionic par forming agent (6).

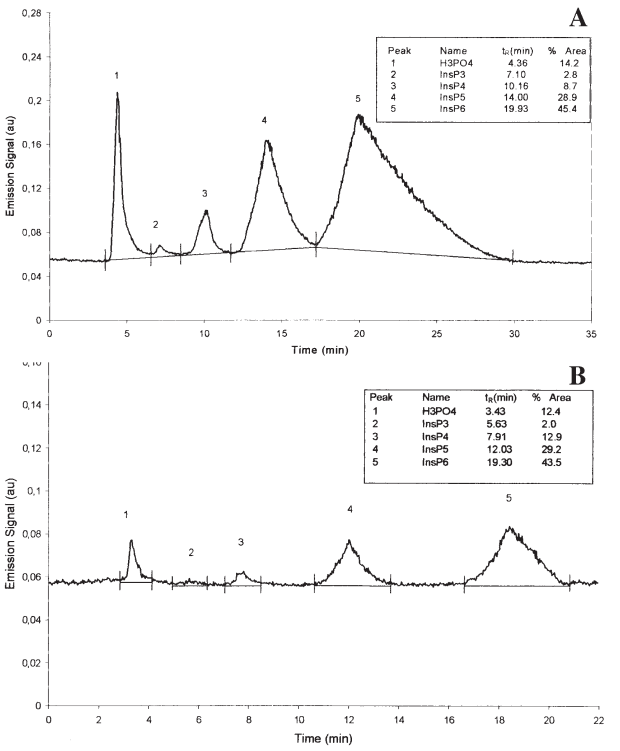

Figure 3. Chromatograms of (A) 100 and (B) 20 µL, of the IP6 hydrolysis products for 10 h at 95°C. Mobile phase B was used.

Counterion nature effect

From the data presented in Figures 2B and 3, it is possible to interpret that the TBA hydroxide change (mobile phase A) to TEA hydroxide (mobile phase B) produced a reduction in retention of over 11 min for the IP6 and 4 min for IP5, (i.e., when using the shortest counterion chain). In the other compounds, the t_R was hardly altered. Area percentages remained practically invariable. A decrease of around 8 min in analysis time was obtained with TEA; however, this continues to be high (30 min) compared with those reported in the literature.

Injection volume change effect

From the data presented in Figures 3A and 3B, it is observed that when reducing the injection volume of 100 to 20 µL, the t_R decreased for all compounds, probably because of the combined effects of reducing sample volume and sample mass overload. In this study, the analysis time was reduced to 22 min, yet continues to be higher than that reported by Leherfeld. The difference in area percent between peaks 1 (H_3PO_4) 2 (IP3), and 3 (IP4) among these chromatograms are assumed to be a product of the difficulty to determine the area of peaks 2 and 3.

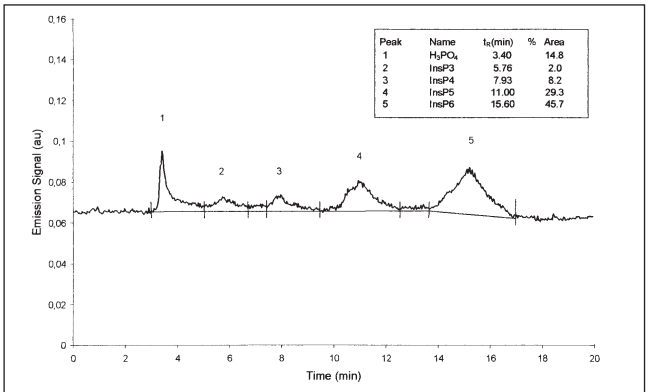

Figure 4. Chromatogram of the IP6 hydrolysis products for 10 h at 95°C. Mobile phase C was used with an injection volume of 20 µL.

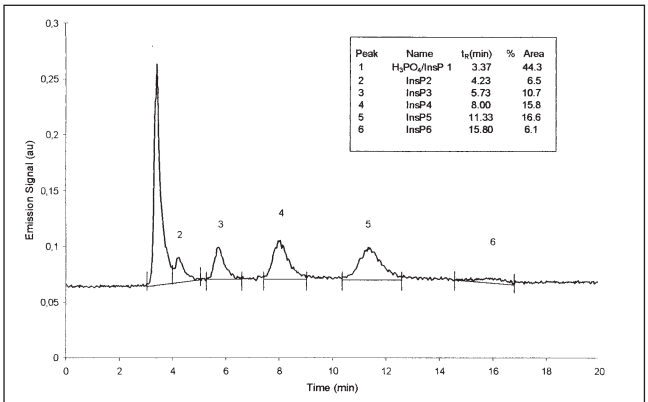

Figure 5. Chromatogram of IP6 hydrolysis products for 16 h at 95°C. Mobile phase C was used with an injection volume of 20 µL.

Effect of TEA concentration presented.

In the analysis of the data present in Figures 3B and 4, it is possible to observe that the affected retention times are those of the inositols of higher molecular weight (IP5 and IP6), as also observed when changing the nature of the counterion. This effect may be interpreted by the competition generated with the amount of existing counterion in the medium, which favors the inositol phosphate of higher molecular weight. It is important to note that, in these results, a similar analysis time (17 min) was obtained to those reported in previous studies.

Effect of hydrolysis time

In this case, when studying Figure 5, it was possible to observe the appearance of inositol biphosphate at 4.23 min because the t_R of the other peaks were generally maintained, this being a new peak, because of the increase in hydrolysis time. This event also explains the increase in the area percentages of the inositols of lower molecular weight at the expense of the reduction in the phytic acid quantity. It should be noted that the large increase in the inorganic phosphate peak was associated with hydrolysis, yet the possible presence of inositol monophosphate was also not to be ruled out. As expected, the analysis time was maintained at 17 min.

Finally, the coupling system sensitivity was studied. The minimum detectable quantity (10s) was found to be 120 mg/L for inositol hexaphosphate, employing a 20-µL injection volume, equivalent to 0.37 µmol/g. This result is interesting because, in 1986, Sandberg et al. reported 6-µmol/g phytic acid contents for the analysis of biological samples (intestine) with the HPLC–RI technique. Because the proposed system may detect lower quantities than this, there is the possibility of studying samples of lesser weight such as biopsies or containing a very low percent of phytic acid.

Conclusion

These preliminary studies show that the coupling of HPLC with ICP-OES may be used for separation and detection of inositols from biphosphate to hexaphosphate. Because ICP-OES is a specific detector for phosphorous, interference of the solvent band usually observed with the RI detector was eliminated, thus permitting specific inositol biphosphate analysis. In addition, the use of TEA as an alternative to TBA reduced the amount of methanol in the mobile phase, thus reducing the operator health hazard. Likewise, it was found that with a 16-h phytic acid hydrolysis time and 95°C temperature, it was possible to obtain a high inositol phosphate content mixture from bi- to tetraphosphate. Finally, the high sensitivity of the proposed system allows samples of lesser weight or low phytic acid content, to be examined.

Acknowledgments

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