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Analysis of inositol phosphates and derivatives using capillary zone electrophoresis–mass spectrometry

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Abstract

Capillary zone electrophoresis (CZE) has been combined with mass spectrometric detection for the separation and determination of inositol phosphates (IPs). Apart from IP1 through IP6 (inositol mono- through hexakisphosphate), an IP3 derivative has been analyzed and identified. The detection limits achieved are in the low micromolar range corresponding to an injected amount of ca. 900 fmol. In addition, an IP3 spiked plasma sample was analyzed after sample pretreatment using ultrafiltration.

1. Introduction

Inositol phosphates (IPs) are important compounds in biochemistry (1,4,5-inositol trisphosphate) [1], agriculture (phytic acid) [2] and pharmaceutical science (1,2,6-inositol trisphosphate) [3]. From their chemical structures (Fig. 1A) it can be concluded that IPs are negatively charged and contain neither chromophores nor fluorophores. For many years, separation of IPs has been performed using liquid (ion-pair, ion-exchange) [4] and gas chromatography (after derivatization) [5]. However, capillary zone electrophoresis (CZE) appeared to be more appropriate and faster for the separation of IPs [6,7]. So far, CZE of IPs using indirect UV detection

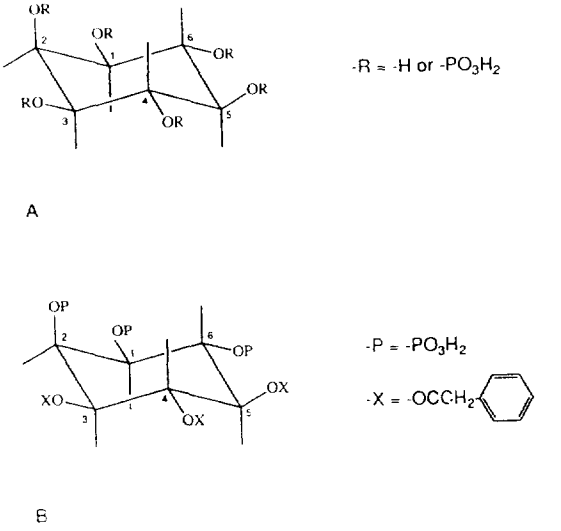

Fig. 1. Chemical structure of inositol phosphates: inositol mono- (M_r 260), bis- (M_r 340), tris- (M_r 420), tetrakis- (M_r 500) and hexakisphosphate (M_r 660) (A) and phenylacetate-IP3 (PIP3) (M_r 774) (B) in the hydrated form.

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has been described. Indirect detection techniques, based on either UV absorbance, fluorescence or amperometric detection, have the disadvantage of increased noise levels and therefore relatively high detection limits. Next to CZE separation of IPs, capillary isotachophoresis (CITP) with conductivity detection has been described [8]. In CITP quantitation of low concentrations is rather limited because zone length instead of peak area or peak height of a compound is related to the amount in the sample volume injected. Sensitive detection of IPs is still problematic. Therefore, alternative detection methods have to be developed for their determination.

Until now, mass spectrometric detection of IPs has been performed using either continuous-flow fast atom bombardment (CF-FAB) or gas chromatography–electron impact ionization mass spectrometry (GC–EI-MS) after per-trimethylsilyl derivatization of the analytes [9]. The detection limits were ca. 10 nmol for 1,4,5-inositol trisphosphate and 1,4-inositol bisphosphate (CF-FAB) and 0.1 pmol for 1-inositol monophosphate (GC–EI-MS).

On-line capillary electrophoresis–mass spectrometry (CE–MS) has been described first in 1987 by Olivares et al. [10]. Since then, quite a number of publications on CE–MS have appeared. Thus far, three types of interfaces have been used for on-line CE–MS, i.e. CF-FAB [11], electrospray [12–16] and ionspray [17]. In addition, CE has been coupled with matrix-assisted laser desorption ionization (MALDI)-MS in the off-line mode [18].

This paper describes the mass spectrometric detection of IPs in the negative ionization mode after CZE separation, without the need of analyte derivatization. The developed method is also applicable for a synthesized IP3 derivative and its impurities with respect to both structure confirmation/elucidation and determination of the synthesis yield. A custom-made electrospray interface was used for the introduction of the column effluent into the mass spectrometer. In order to suppress electroosmotic flow, the capillary wall was coated with polyacrylamide [19].

2. Experimental

2.1. Chemicals

Acetic acid p.a. and methanol were obtained from J.T. Baker (Deventer, Netherlands). Ammonium acetate p.a. was purchased from Merck (Darmstadt, Germany). All IPs, e.g. inositol monophosphate (2-IP1) as dicyclohexylammonium salt and inositol bis- (1,2-IP2), tris- (1,2,6-IP3), tetrakis- (1,2,5,6-IP4), hexakisphosphate (IP6) and phenylacetate-IP3 (PIP3) (Fig. 1B) as sodium salts, were supplied by Perstorp Pharma (Perstorp, Sweden). For the preparation of the stock solutions of the analytes and buffer solutions, deionized water was used (Milli-Q system, Millipore, Bedford, MA, USA). For the polyacrylamide coating, 3-(trimethoxysilyl)-propylmethacrylate, 98% (Janssen, Beerse, Belgium), tetramethylethylenediamine (TEMED) and ammonium persulphate (Bio-Rad, Richmond, CA, USA) and acrylamide (Merck–Schuchardt, Hohenbrunn, Germany) were used.

2.2. Procedures

Sample pretreatment

An amount of 500 μl of blank plasma, spiked to concentrations of either 20 or 200 μM IP3 was applied to AMICON sets (Amicon, Danvers, USA), consisting of a donor and acceptor compartment separated by filter with cut-off of M_r 30 000 . Ultrafiltration was performed using an ultracentrifuge type JA-20 (Beckman, Fullerton, CA, USA) with fixed angle rotor (34°) at 2000 g for 30 min. The ultrafiltrate was injected into the CZE capillary.

Capillary coating

Before the fused-silica capillaries (BGB, Rothenfluh, Switzerland) were coated, they were rinsed for 5 min with 0.1 M sodium hydroxide p.a. (Merck), Milli-Q water and ethanol p.a. (Merck). Subsequently, the polyacrylamide coating procedure according to Hjerten [19] was performed.

2.3. Capillary zone electrophoresis

The experiments were performed using a programmable injection system and power supply (Prince, Lauerlabs, Emmen, Netherlands). The electrophoresis buffer was prepared freshly every day and consisted of ammonium acetate (10 m*M*, pH 5)–methanol (90:10, v/v). The length of the fused-silica capillaries (100 μm I.D. and 170 μm O.D., unless stated otherwise) was 0.85 m. At the capillary inlet, a voltage of −28 kV and in conjunction, a pressure of 10 mbar was applied. Before each run, the capillary was rinsed with electrophoresis buffer for 2 min. Pressurized (200 mbar) sample injection was applied for 0.10 min, corresponding to 250 nl (75 μm I.D.) or 450 nl (100 μm I.D.)

2.4. Electrospray mass spectrometry

All experiments were carried out on a triple quadrupole mass spectrometer (Finnigan MAT TSQ-70) equipped with a custom-made electrospray interface that fitted in the thermospray source [20]. Most of the experiments were done in the negative ionization mode: the electrospray (ES) needle was kept at −3.5 kV with respect to the grounded heated sampling capillary. When operated in the positive ion mode, the ES needle was set at +3.5 kV. The sampling capillary and the ion source were kept at 175 respectively 150°C. A slightly negative voltage was applied on the repeller for signal optimization of all ions. After removal of the polyimide layer at the capillary tip, the outlet of the fused-silica capillary was inserted into the stainless-steel needle assembly, slightly ahead of the needle tip. The sheath liquid consisted of ammonium acetate (100 m*M*, pH 5)–methanol (10:90, v/v) delivered at a flow-rate of 1–2 μl/min by a Model 2400 syringe pump (Harvard Apparatus, Edinbridge, UK).

2.5. Nuclear magnetic resonance

The ^{31}P NMR spectrum of 2 mg phenylacetate-IP3, dissolved in 0.5 ml $^{2}H_2O$, was obtained on a Bruker DMX-600 spectrometer (Karlsruhe, Germany).

3. Results and discussion

3.1. Capillary zone electrophoresis

Depending on the number of phosphate groups, IPs (Fig. 1) have multiple negative charges resulting in high electrophoretic mobilities in the direction of the anode. As the electroosmotic flow (EOF) is in the cathodic direction, net electrophoretic velocities are rather low, implying unacceptably long migration times. Therefore, the EOF must be suppressed using capillaries with either a static or dynamic coating of the wall. In first instance, hydroxypropylmethylcellulose (HPMC), a neutral hydrophilic polymer, was added to the electrophoresis buffer. However, this compound appeared to be incompatible with mass spectrometric detection because of contamination of the ion source. Moreover, the HPMC coating was destroyed when using an organic modifier, e.g. methanol or acetonitrile, as additive in the electrophoresis buffer. The polyacrylamide coating described by Hjerten [19] appeared to be a good alternative. This coating is static, which reduces the risk of ion-source contamination. Furthermore, the coating is compatible with the use of organic modifiers in the electrophoresis buffer and the EOF is substantially reduced. As a consequence of EOF suppression, CZE of IPs has been performed with reversed polarity: the capillary inlet is at −28 kV and the outlet (ES needle) at −3.5 kV.

For the coupling of CZE and MS via an electrospray interface, a buffer must be chosen which is a compromise between aqueous (favourable for CZE of IPs) and non-aqueous (favourable for ES-MS). As CZE buffer, ammonium acetate was chosen, which was appropriate regarding the separation of IPs as well as the required volatility for ES-MS. In order to increase the buffer volatility even more, both methanol and isopropanol were examined as

additives. At comparable modifier content, isopropanol gave longer migration times of the IPs than methanol, caused by its higher viscosity. Therefore, methanol was chosen as modifier.

Resuming, both the not completely suppressed EOF in the cathodic direction (capillary inlet) and the organic modifier as buffer additive lead to an increase of the migration times of the IPs. To compensate for this effect, pressure-assisted CZE can be performed. Compared with conventional CZE, this may lead to decreased efficiencies, caused by the hydrodynamic flow profile. Nevertheless, during all experiments a slight pressure was applied in addition to the high voltage.

3.2. Electrospray-mass spectrometry

In order to investigate the electrospray performance and signal intensity as a function of the sheath liquid composition continuous-infusion–ES-MS experiments of PIP3 were carried out in the negative and positive ionization mode. The electrospray technique is most stable at high organic modifier content, whereas the CZE separation of IPs is optimal without any organic modifier in the buffer at all. The highest organic modifier content in the CZE buffer which was still acceptable in terms of migration times and peak shape appeared to be 30% methanol. Therefore, an initial sheath liquid of ammonium acetate (10 m*M*, pH 5)–methanol (70:30, v/v) was chosen for the continuous infusion of PIP3 in the negative ionization mode. Although the spray performance appeared to be satisfactory, the IP3 derivative could not be detected at all. In the positive ionization mode with the same sheath liquid composition, however, a mass spectrum of PIP3 could be obtained. The spectrum mainly consisted of the $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ peaks (not shown). In the negative ionization mode, a mass spectrum could not be acquired, unless the sheath liquid contained at least 90% organic modifier. The spectrum principally showed the $[M-H]^-$ peak of PIP3 and some impurities. Therefore, all experiments have been performed in the negative ionization mode using a sheath liquid consisting of ammonium acetate (100 m*M*, pH 5)–methanol (10:90, v/v). In addition to the ES-MS experiments, ES-MS–MS of PIP3 in the negative ionization mode was investigated. The observed loss of 98 u corresponds to the cleavage of one H_3PO_4 from the IP3 derivative.

Besides for PIP3, a mass spectrum for 50 μM IP3 has also been obtained in the continuous-infusion mode (Fig. 2). The main peak observed is $[M-H]^-$ (m/z 419). ES-MS–MS experiments of IP3 gave results comparable to those for PIP3: the loss of 98 u, corresponding with H_3PO_4. Based on the results obtained during the continuous-infusion experiments the initial conditions in the CZE–ES-MS experiments could be readily chosen.

3.3. Capillary zone electrophoresis–electrospray mass spectrometry

When the outlet of the CZE capillary was inserted in the electrospray needle and a high voltage of −28 kV was applied, the voltage on the needle tip increased from −3.5 to ca. −4.2 kV. This phenomenon has also been observed by Perkins and Tomer [21], who explained it as a result of conductivity through the column. This increase of the ES voltage was even more pronounced when samples with high conductivity (e.g. plasma) were analyzed. In that case, a total breakdown of the electrospray was observed which necessitated the use of lower voltages, e.g. −20 kV instead of −28 kV.

Based on the results obtained with the continuous-infusion ES-MS experiments of PIP3 a multiple-ion detection (MID) procedure was designed. CZE–ES-MS of a concentrated (2 m*M*) and a 1:10 diluted solution of PIP3 was carried out. Fig. 3 shows that in the concentrated sample (Fig. 3A) more impurities than in the 1:10 diluted sample (Fig. 3B) can be detected. The impurity with m/z 575 has come below the limit of detection in Fig. 3B. By injecting the IP3 derivative and impurities at lower concentrations, the peak shape of the analytes was substantially improved (Fig. 3B). Next to the impurities with m/z 809 and 851, a PIP3 adduct has been detected. The ratios of the impurities and

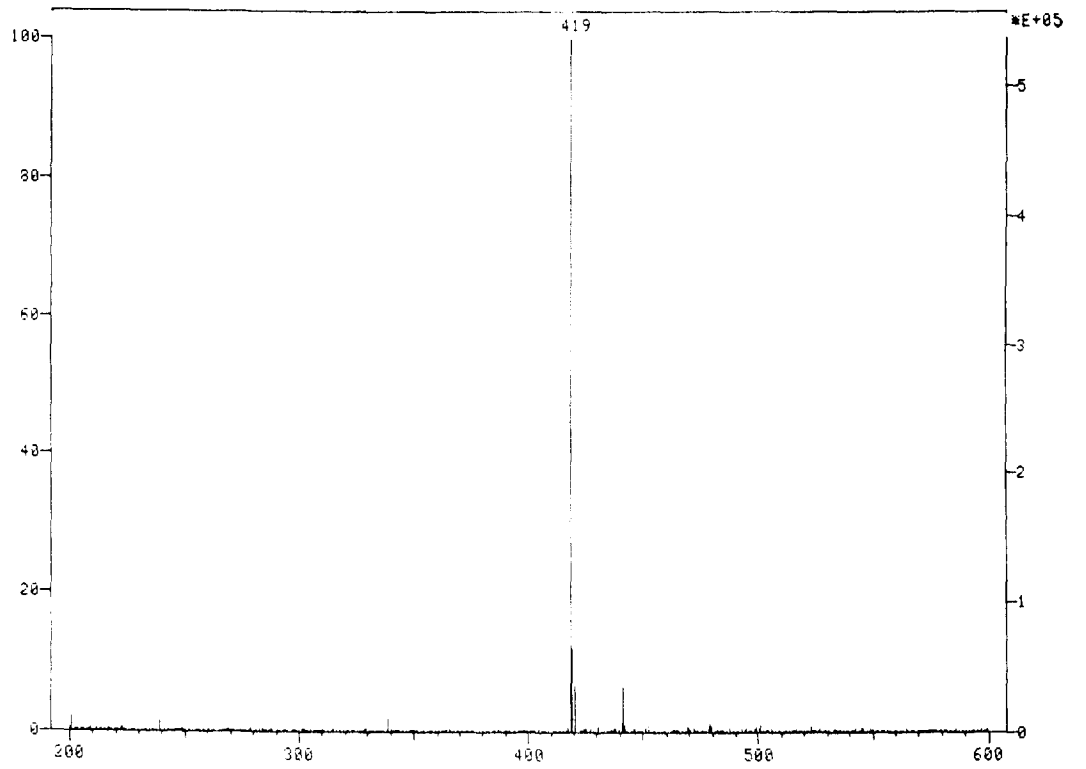

Fig. 2. Electrospray mass spectrum of 1,2,6-inositol trisphosphate in the continuous-infusion mode. Conditions: sheath liquid, ammonium acetate (100 m*M*, pH 5)–methanol (10:90, v/v), 2 μl min; concentration IP3, 50 μM.

PIP3 adduct appear to differ depending on the concentration injected. Probably, the ionization characteristics are dependent on the local conductivities. So far, the masses of the IP3 derivative and the impurities could be determined using CZE–ES-MS. In addition, CZE–ES-MS–MS of PIP3 and some impurities has been performed for further structure elucidation. Fig. 4 shows possible structures for some of the impurities. Structure proposals are based on (i) the mass determination (MS), (ii) the presence of H_3PO_4 in the molecules (MS–MS) and (iii) on the migration times (electrophoretic mobilities) in the mass electropherogram.

For the determination of the synthesis yield, the peak area of PIP3 was compared with the peak areas of the impurities. Using this parameter, the synthesis yield was 74–85%, depending on the injected analyte concentration. One has to realize, however, that the IP3 derivative and the impurities do not have exactly the same response factors. Unfortunately, these factors cannot be determined as the analytes as such are not available. Nevertheless, assuming equal response factors the purity of PIP3 can be estimated. A complementary technique, NMR, has been performed to confirm the estimated purity. The NMR integrals of the main compound were compared with those of the (P-containing) impurities: the calculated purity was 80%. This is in good agreement with the CZE–MS results, which makes this value quite reliable.

A mixture of IP1, IP2, IP3, IP4 and IP6, all at a concentration of ca. 20 μM, was analyzed using CZE–ES-MS. IP5, which is the first degradation product of IP6, was not present in the mixture because, in contrast to the other IPs, IP5 is rather unstable. The result is depicted in Fig. 5. The $[M-H]^-$ ions of IP1 to IP4 and IP6 have been detected. Most of the peaks are symmetrical. At higher concentrations, however, e.g. 200 μM, fronting (IP3–IP6) and tailing (IP1) peaks

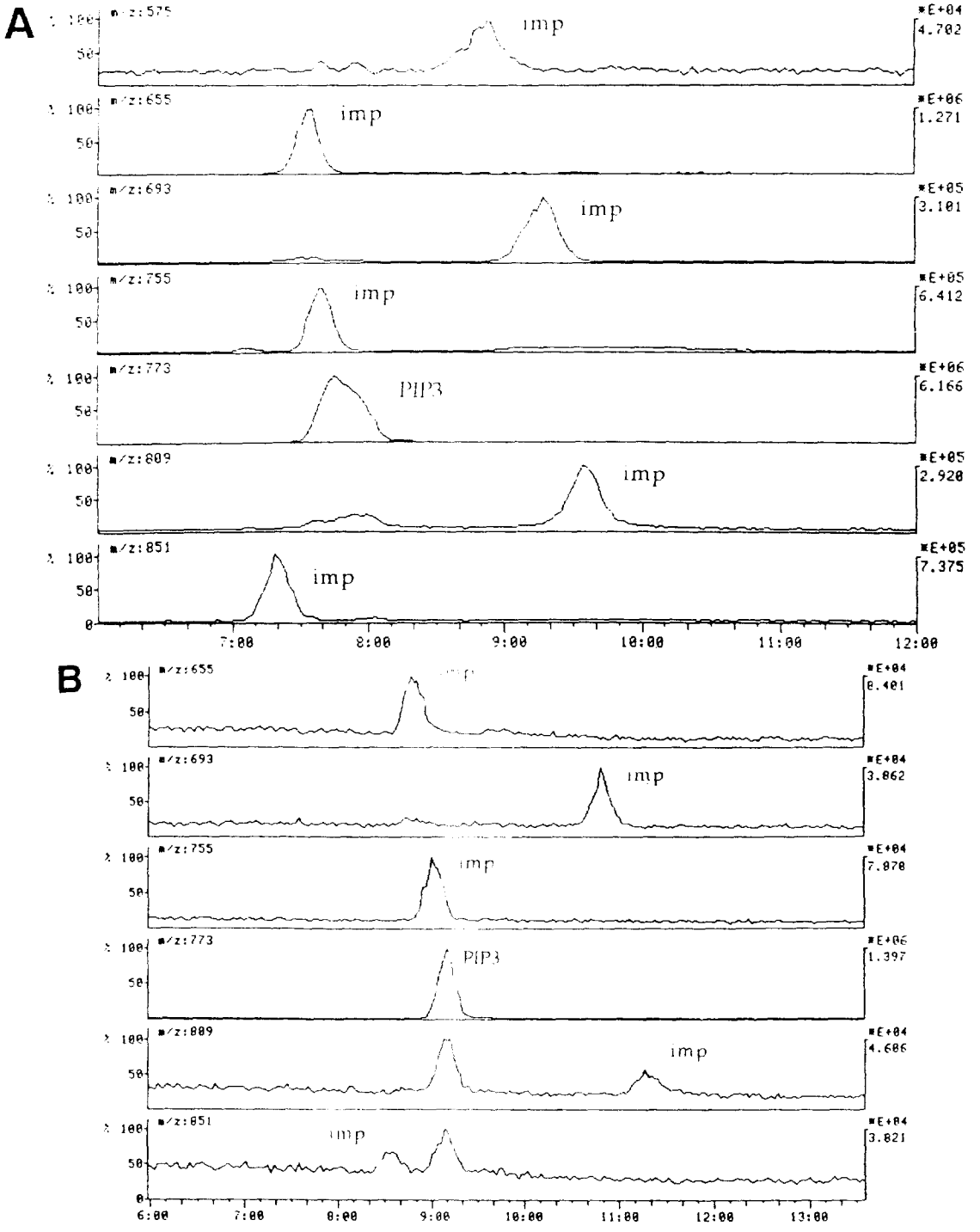

Fig. 3. Mass electropherogram of PIP3 and its impurities. Conditions: polyacrylamide-coated fused-silica capillary, 75 μm I.D., 150 μm O.D.; high-voltage capillary inlet, −28 kV; pressure, 25 mbar; height difference between capillary inlet and ES needle, 5 cm; concentration IP3 derivative, 2 mM (A), respectively 200 μM (B).

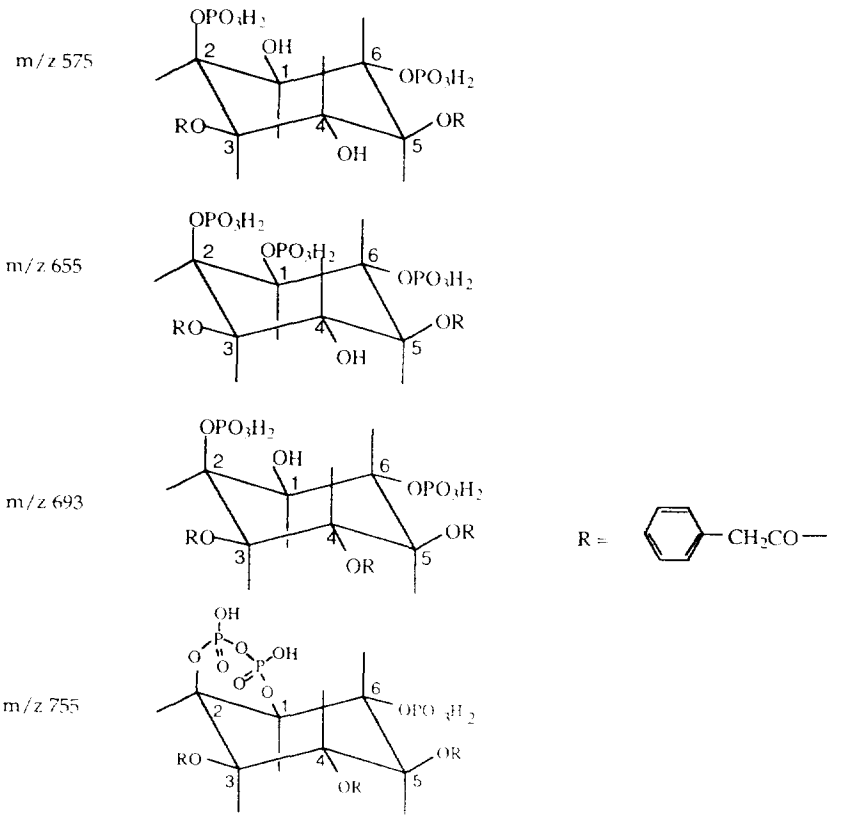

Fig. 4. Structure elucidation of impurities of PIP3.

were more pronounced, which is the result of electromigration dispersion. Only IP2, with an electrophoretic mobility similar to the background electrolyte, had a symmetrical peak shape.

Qualitatively, the developed method appeared to be reproducible: during several days the same mass electropherogram has been obtained. Regarding the quantitative aspects, however, there are some deficiencies. Inter-day reproducibility with respect to the sensitivity is not completely satisfying, which can be overcome by using an internal standard. Furthermore, sensitivity differences between the IPs were observed: at a comparable concentration, the signal-to-noise ratios of the IPs differed substantially, implying different detection limits for the different IPs. Possibly, this is the consequence of working under MID conditions, considering the singly charged ions only. For IP2 and IP3, a concentration of 2 μM (absolute amount 0.9 pmol) could still be detected, whereas the detection limits for IP1, IP4 and IP6 are between 2 and 20 μM.

3.4. Bioanalytical aspects

Eventually, IP3 and its derivatives have to be determined in plasma and urine samples, requiring a sensitive determination method. Preliminary results show that the developed method can be used for the analysis of IP3 in plasma. After the plasma sample was spiked with IP3 to a concentration of 200 μM and pretreated by ultrafiltration, the ultrafiltrate was injected into the CZE capillary. Although the free fraction of IP3 in plasma is below 10%, a mass electropherogram of the ultrafiltrate could be obtained by using CZE–MS–MS (Fig. 6). A loss of 98 and

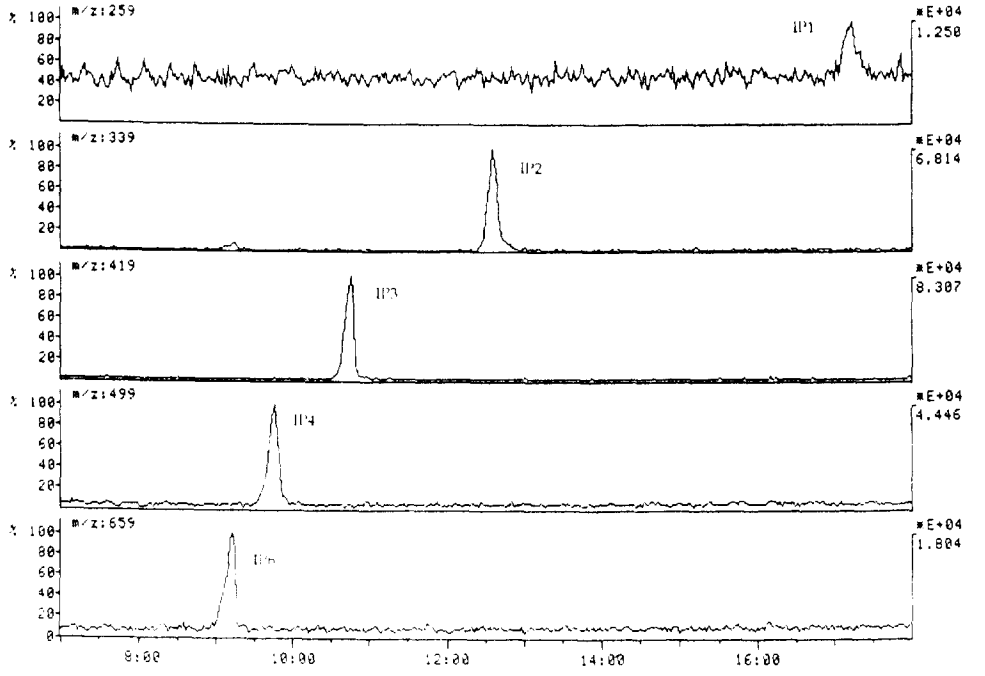

Fig. 5. Mass electropherogram of IP1, IP2, IP3, IP4 and IP6. Conditions: high-voltage capillary inlet, −28 kV; pressure, 10 mbar; concentrations inositol phosphates, 20 μM.

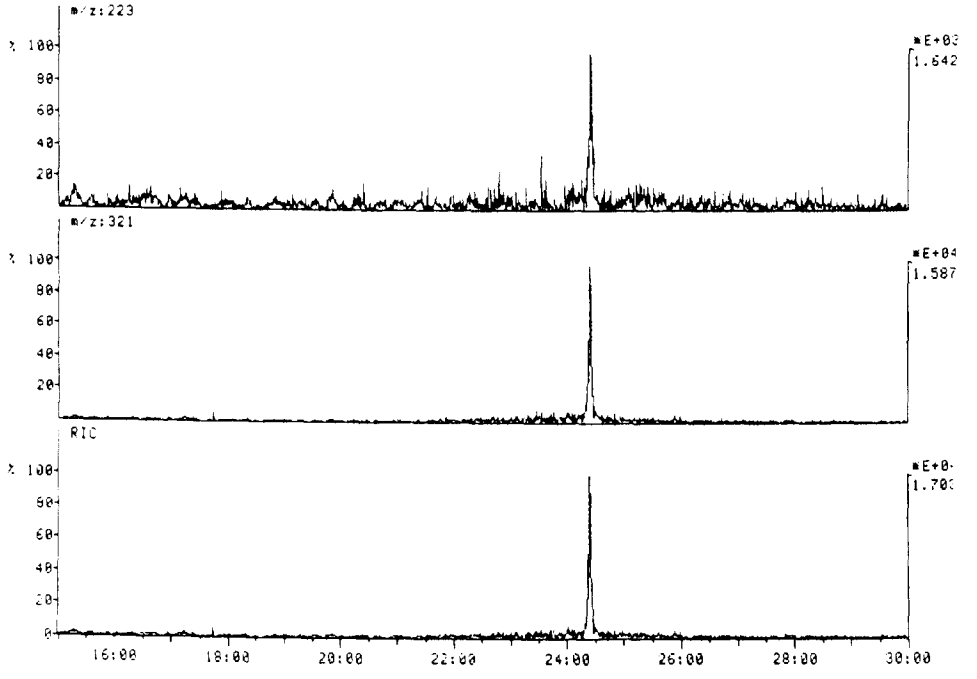

Fig. 6. CZE–ES-MS–MS of IP3 in plasma, pretreated with ultrafiltration. Conditions: high-voltage capillary inlet, −20 kV; pressure, 10 mbar; concentration of IP3 in plasma, 200 μM.

196 u has been observed, which correlates with a subsequent loss of H_3PO_4 (twice).

The developed method can be applied for the determination of IP3 in plasma, but IP3 concentrations in real-life samples will be in the nanomolar range. In order to improve the method for bioanalysis, the protein binding of IP3 must be decreased substantially to increase the recovery of the sample pretreatment. A second approach will be the application of a concentrating technique prior to CZE. For this purpose, isotachophoresis (ITP) can be combined with CZE [13,22–25].

4. Conclusions

The developed method, using CZE–ES-MS (–MS), appears to be applicable for the determination of IPs and analogues without the need of derivatization. The structures of a synthesized IP3 derivative and its impurities have been confirmed and elucidated. The yield of the IP3 synthesis could be well estimated.

Preliminary results show that the determination of IP3 in plasma can be performed after a sample pretreatment consisting of ultrafiltration. For the bioanalysis of real-life samples, however, the sensitivity must be improved. Therefore, future research will be devoted to improvement of the recovery of the sample pretreatment by breaking the plasma protein–analyte bond. Furthermore, on-line concentrating techniques will be considered.

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