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Non-radioactive, isomer-specific inositol phosphate mass determinations: high-performance liquid chromatography–micro-metal–dye detection strongly improves speed and sensitivity of analyses from cells and micro-enzyme assays

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Abstract

A microbore high-performance liquid chromatographic (HPLC) method is presented allowing rapid and sensitive mass analysis of inositol phosphates from cells and tissues. An analysis starting from inorganic phosphate up to inositol hexakisphosphate displaying a similar isomer selectivity as compared to the standard metal–dye detection system takes about 15 min. The detection sensitivity was about 15 pmol for inositol trisphosphate, about 10 pmol for inositol tetrakisphosphate, about 5 pmol for inositol pentakisphosphate and less than 5 pmol for inositol hexakisphosphate. The method was validated regarding day-to-day variations and variations at the same day of retention times and peak areas of standard inositol phosphates. Standard deviations of retention times ranged from 0.25 to 0.62% (same day) and from 0.64 to 1.61% (day-to-day variations). Ranges of standard deviations of peak areas were between 2.24% and 3.91% (same day) and 6.13% and 13.8% (day-to-day variations). Linearity of the post-column complexometric metal–dye detection system was demonstrated in the range of a few picomoles and at least 800 pmol. The method was applied to the analysis of inositol phosphates in Jurkat T-lymphocytes and assays from minute amounts of enzymes interconverting inositol phosphates. While measurements of inositol phosphates from cell extracts are now possible using significantly reduced cell numbers, micro-enzyme assays are feasible in reasonable repeated analysis times and with sufficient isomer selectivity. In conclusion, a substantial improvement towards speed of analysis and detection sensitivity of inositol phosphate mass analysis was achieved by microbore metal–dye detection HPLC.

1. Introduction

Inositol phosphates (InsP) have been found in all eukaryotic cell types so far investigated. Among these molecules at least inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] is of particular interest since it plays a central role in Ca^{2+}-signalling in many different cells and tissues [1]. Other InsP have been postulated also to play a role in cellular signalling [1]; however, the biological function of most of these compounds remains much more unclear.

Analysis of InsP has evolved from paper

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chromatography [2] to liquid and gas chromatography (GC) [2,3] and subsequently to HPLC [4]. The major problem in instrumental analysis of non-radioactive InsP is their on-line detection since the physico-chemical properties of these compounds do not allow detection at UV or visible light wavelengths. Likewise, the synthesis of volatile derivatives for gas chromatography is adequately possible only for inositol monophosphate ($InsP_1$) and inositol bisphosphate ($InsP_2$) [3,5]. To overcome these problems numerous investigators have prelabelled intact cells or tissue samples with myo-[^{3}H]inositol or [^{32}P]PO_4^{3-} and detected the ^{3}H- or ^{32}P-labelled InsP in high- or low-performance liquid chromatography. This methodology is very sensitive and in case of separation by HPLC selective towards different InsP isomers. However, in spite of the very broad usage and the success of these techniques they have a number of drawbacks, namely: (i) labelling to isotopic equilibrium is often difficult to achieve; (ii) separation efficiency of chromatographic methods is often limited due to. fraction collecting for liquid scintillation counting; (iii) low sensitivity if on-line radioactivity detectors are used; (iv) non-specificity for InsP and precursor pool heterogeneity when [^{32}P]PO_4^{3-} is employed; (v) huge amounts of radioactive waste in the case of [^{3}H]inositol prelabelling and off-line scintillation counting; and (vi) time-consuming prelabelling and off-line analysis work. These reasons prompted some investigators to develop detection methods not requiring radioactive labelling. Among these, a very sensitive gas chromatographic–mass spectrometric (GC–MS) method has been published [5]. However, a major problem was that inositol trisphosphate ($InsP_3$) and more polar InsP could not be derivatized for GC. Combined methods using high- or low-performance liquid chromatography of InsP followed by desalting and phosphatase treatment and subsequent analysis of inositol hexatrimethysilylderivatives by GC have been introduced [6,7]. These methods are also very sensitive. However, disadvantages are the extensive instrumentation (HPLC *and* GC) necessary and the time consumption for off-line analysis. Recently, capillary zone electrophoresis with indirect UV detection has been reported to be a very sensitive and fast method for InsP analysis [8]. However, despite its high sensitivity, resolution of higher phosphorylated InsP was poor and separation of isomers obviously was impossible by this method [8]. Because of these disadvantages, including the fact that only very small sample volumes can be applied to capillary electrophoresis (nanoliter range), the method does not seem to be applicable for the analysis of InsP from cells or tissues.

A different principle, namely post-column derivatization, was used by Meek and Nicoletti [9]. They coupled an anion-exchange HPLC system to a post-column reactor loaded with immobilized alkaline phosphatase. Free phosphate was then monitored on-line by mixing the reactor eluate with ammonium molybdate reagent [9,10]. Although this first development of an on-line HPLC detection system made some headway, the low sensitivity of the method and unpublished problems with incompletely dephosphorylated InsP in the phosphomolybdate assay made it hardly useful for many applications.

A complexometric dye based post-column system with much higher sensitivity was introduced in 1988 and called metal–dye detection (MDD)–HPLC [11]. This method utilizes separation of InsP by strong anion-exchange HPLC. Subsequent on-line detection of individual compounds is done by competition of an anionic dye and InsP for binding to Y^{3+}. This method has been described in detail elsewhere [12,13] and has been applied in a number of studies on the analysis of InsP in living cells and tissues [14–20]. Although the MDD system was much more sensitive than other HPLC methods for mass determination (see above), we intended to improve speed and sensitivity of the system. Since miniaturization of the existing metal–dye detection method appeared the most promising possibility to achieve such goals, miniaturization to a 3-μm particle MiniQ column and a micro-HPLC system (Smart, Pharmacia) was carried out. The successfully miniaturized microbore MDD system revealed an about ten-fold increase in sensitivity as well as a four-fold reduction of the time needed for a complete analysis.

2. Experimental

2.1. Materials

InsP isomers were prepared by acidic hydrolysis of inositol hexakisphosphate ($InsP_6$) and purified either on Q-Sepharose or MonoQ as detailed earlier [16,21]. Characterization of these standard compounds was done by nuclear magnetic resonance spectroscopy analysis as described [22]. $YCl_3 \cdot 6H_2O$ was purchased from Janssen (Nettetal, Germany) and 2-(4-pyridylazo)resorcinol (PAR) was from Serva (Heidelberg, Germany). Triethanolamine (pro analysi) and HCl (pro analysi) were products of Merck (Darmstadt, Germany). Bidistilled water or MilliQ water (Millipore-Waters) were used throughout all experiments. The anti-CD3 (CD = cluster of differentiation) monoclonal antibody OKT3 was purified from hybridoma supernatant on protein G-Sepharose (Pharmacia, Freiburg, Germany). In brief, about 650 ml of OKT3-hybridoma supernatant were loaded onto a protein G-Sepharose column (50 ml gel volume) at 3 ml/min. The column was washed with 0.1 *M* Na_2PO_4, pH 7.0, at 3 ml/min until the detector signal (280 nm) had reached the baseline again. Then the antibody was eluted from the column with 0.1 *M* glycine · HCl, pH 2.7, at 2 ml/min. The acidic solution was immediately brought to neutral pH by addition of Tris base. The purity of the antibody preparation was checked by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). All other chemicals were of the highest purity available.

2.2. Cell culture

The human T-lymphocyte cell line Jurkat was cultured as described in earlier publications [18,20].

2.3. D/L-Inositol 1,2,3,4,5-pentakisphosphate ($Ins(1,2,3,4,5)P_5$) 5-phosphatase assay

The standard incubation mixture contained 100 μg of protein extracted from fetal bovine thymus and 1–2 nmol of D/L-$Ins(1,2,3,4,5)P_5$ isomer in a volume of 100 μl. For a typical time course, 440 μg of protein and 4.4 nmol of D/L-$Ins(1,2,3,4,5)P_5$ in a volume of 440 μl were incubated at 37°C. Also, a mixture of protease inhibitors [final concentrations: 50 μ*M* leupeptin, 1 μ*M* pepstatin, 50 μ*M* antipain and 1 m*M* 4-(2-aminoethyl)benzolsulfonylfluoride (AEBSF)] was included. At 0, 30, 60 and 90 min, 100 μl of the mixture were taken and the reaction was stopped by addition of 150 μl of ice-cold 10% (v/v) perchloric acid and 50 μl of 0.2 *M* EDTA solution. The latter was added to prevent co-precipitation of metal–InsP complexes when adding KOH (see below). The perchloric acid extract was left on ice for 30 min to extract the soluble InsP. Then, precipitated protein was removed by centrifugation (8800 *g*, 10 min) and the supernatant titrated to pH 4–5 by addition of KOH. After standing for 30 min on ice, the samples were again centrifuged (8800 *g*, 10 min) to remove the $KClO_4$ precipitate.

2.4. Extraction of InsP from Jurkat cells

The InsP from stimulated and unstimulated Jurkat cells were extracted using the perchloric acid procedure and subsequent sample processing as described in detail by Mayr [11] and by Guse and Emmrich [16,23]. The quantitation of InsP isomers in biological samples generally requires efficient extraction procedures. Such procedures including the usage of internal standards have been described in detail elsewhere [11,12,16,23] and will not be repeated here.

2.5. Sample preparation for HPLC

Nucleotides interfering with InsP detection by MDD were removed from the samples by extraction with charcoal as described earlier [14].

Separation parameters such as retention time and resolution on the small MiniQ column is sensitive to salts and ionic compounds [12] being present either in perchloric or trichloroacetic acid (TCA)-quenched samples from cells or tissues. Before analysis, these ionic contaminations were removed by solid-phase extraction on

Q-Sepharose as follows. Disposable columns were filled with Q-Sepharose FF (Pharmacia Biotech) which had been brought to the acetate form previously. Samples were diluted with water to 40 ml (conductivity <2 mS) and applied to the column. For samples <100 mg wet weight, columns containing 0.5 ml of wet packed gel were used, whereas samples having wet weights >100 mg were applied to columns containing 1 ml of wet packed Q-Sepharose. The columns were washed twice with 2.5 ml of 2 m*M* HCl. Then the samples were eluted with 1.5 *M* ammonium acetate (two-fold addition of 2.5 ml when using 0.5 ml of gel, two-fold addition of 5 ml when using 1 ml of gel). The samples were then diluted with water to approximately 20 ml and lyophilized, re-dissolved in 20 ml of water and again lyophilized to remove the remaining ammonium acetate and stored at −20°C. Samples were redissolved in 250 μl of buffer containing 1 m*M* NaF, 5 m*M* sodium acetate, pH 6.0, and then filtered through disposable 0.45-μm filters directly before injection. The Q-Sepharose FF gel was regenerated by addition of 50 volumes of 0.5 *M* HCl, then by washing with water to pH >5, and then brought back to the acetate form via NaOH.

This procedure did not induce migration of phosphate groups as described for the acidic solid-phase extraction protocol [14].

2.6. HPLC conditions

The micro-HPLC instrumentation consists of a Smart system (Pharmacia Biotech) equipped with an additional two-piston HPLC pump (LC-10AD, Shimadzu, Duisburg, Germany). The system set-up in principle is based on the version described [11]. Alterations and additional features are shown in Fig. 1. We used a MiniQ PC 3.2/3 column (3-μm particles made of a divinylbenzene–polystyrene co-polymer, 30×3.2 mm; Pharmacia Biotech) that could be placed directly into the Smart column holder. Instead of directly connecting the UV-M monitor to the column holder, an appropriate T-junction was placed below the column holder (Fig. 1). Thereby, the dye solution could be mixed with the column eluate. The reaction was then completed in a 125-μl knitted reaction coil (0.5 mm I.D.) before passing through the UV-M monitor (dead volume 10 μl) equipped with a 546-nm optical filter (Fig. 1).

When setting up the method, a main problem was baseline noise arising from mechanical

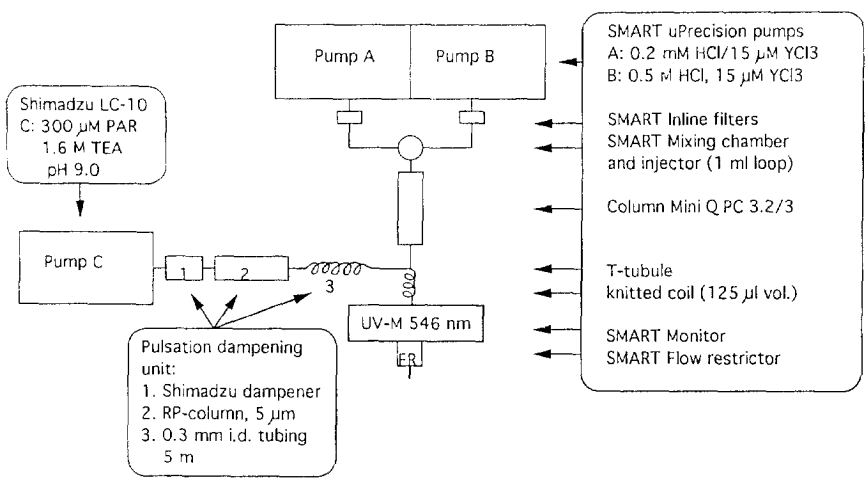

Fig. 1. System set-up of the micro-MDD analysis. Schematically shown are the different parts of the micro-MDD system. As central part a Smart system (Pharmacia) was used. Note the pulsation dampening unit shown in between pump C and the T-tubule. We now have only the reversed-phase column routinely in use.

pulses from pump C, thereby leading to lower sensitivity than theoretically expected. We tried to minimize these pulses by placing (i) a commercially available pulse dampener, (ii) a reversed-phase HPLC guard column (LiChrosorb, 4 cm long, 5-μm particles, Merck) and (iii) a 5-m piece of 0.3 mm I.D. HPLC PTFE tubing, either alone or in combinations between pump C and the T-junction. Initially, we used a combination of all three components in line. However, pump C is in use continuously for several months now and the baseline is nearly as smooth as without any pulse dampening. Apparently, gas bubbles in the LC-10AD pump are the major source of stronger pulsation. In our system, we are now routinely using the reversed-phase guard column to eliminate remaining pulses originating from pump C. The effect of this column appears to be mainly due to the higher operating pressure, which reduces the time until small gas bubbles are resorbed.

Flow-rates for pumps A, B and C and the volume of the reaction coil have been stepwise optimized using selectivity, peak broadness and peak symmetry, sensitivity and speed of separation as parameters. The optimal flow-rates are for pumps A and B 500 μl/min and for pump C 300 μl/min. This 5:3 ratio resulted in a somewhat improved sensitivity compared to the 2:1 ratio originally described [11]. The gradients used for the different applications have been established for the standard MDD system (10 or 20 cm MonoQ column) and were scaled-down for the miniaturized system. Thereby, selectivities of isomer separation comparable to 5-mm-diameter MonoQ columns of about 10 cm length were obtained.

For the separation of InsP on MiniQ columns the following buffers were used: A, 0.2 m*M* HCl, 15 μM YCl_3, B, 0.45 *M* HCl, 15 μM YCl_3. Gradient 1 was routinely used for the separation of samples from cells, tissues or enzyme assays at a flow-rate of 500 μl/min: 0 min, 4% B; 4.5 min, 4% B; 7 min, 5% B; 9 min, 10% B; 11 min, 20% B; 13.5 min, 35% B; 16 min, 60% B; 17 min, 80% B; 17.5 min, 100% B; 19.5 min, 100% B; 20.5 min, 0% B; 23.5 min, 0% B. The sample volume was usually 1 ml, and the sample was injected between 0.5 and 2.5 min. The dye solution C consisted of 300 μM PAR, 1.6 *M* triethanolamine, pH 9.0, and was pumped at 300 μl/min. Pump C was switched on at 0 min and was switched off at 18.5 min. This is important since buffer B is then flowing without dye reagent through the system for one further minute, thereby cleaning the knitted coil as well as the detector flow cell from dye remnants. For the D/L-Ins(1,2,3,4,5)P_5 5-phosphatase assay a different gradient with improved selectivity for $InsP_4$ isomers had to be developed. We succeeded with the following gradient (gradient 2) using the same buffers and flow-rates as above: 0 min, 14% B; 2.5 min, 14% B; 11.5 min, 30% B; 15 min, 100% B; 16.5 min, 100% B; 17 min, 14% B; 18.5 min, 14% B. The sample volume for this gradient was 500 μl and injection was done between 0.5 and 1.5 min.

2.7. Cleaning procedures for the Smart system and MiniQ column

The Smart system, although made of biocompatible inert material, must be rinsed thoroughly with bidistilled water after use with the acidic buffers described above. In particular pump B is prone to corrosion of the piston. Therefore, the piston should be checked routinely. The MiniQ column, in comparison to the MonoQ columns previously used for MDD analysis, are more susceptible to particulate contaminations (due to the smaller particle size of the column material) as well as to protein contaminations adsorbing unspecifically to the polymer beads (small quantities of protein from the cell and tissue samples are left unprecipitated by both the acidic extraction procedures). Such contaminations must be circumvented by (i) micro-filtration of all elution buffers and all samples directly before injection, and (ii) routine cleaning procedures of the column as described by the manufacturer. For example, when assaying samples from muscle tissue, the column was daily cleaned from contaminating protein by incubation with pepsin and subsequent removal of peptides by 1.75 *M* KOH.

Table 1
Day-to-day reproducibility of retention times and peak areas

Compound	Amount injected (pmol)	Retention time (mean ± S.D.) (min)	Retention time (range) (min)	Peak area (mean ± S.D.) (min mA.U.)	Peak area (range) (min mA.U.)
$InsP_3$	416	10.35 ± 0.07	10.29–10.49	18.129 ± 1.977	15.46–22.47
$Ins(1,4,5,6)P_4$	109	13.41 ± 0.22	13.25–14.05	6.649 ± 0.918	5.271–7.938
$Ins(1,2,3,4,5)P_5$	76	14.41 ± 0.21	14.24–15.05	8.655 ± 0.929	7.229–9.746
$InsP_6$	235	16.68 ± 0.18	16.54–17.22	26.836 ± 1.644	24.197–29.007

A standard mixture of InsP was injected at fourteen different days over a period of about three months using identical HPLC conditions. Samples (volume of 1 ml) were analyzed on MiniQ PC 3.2/3 (Pharmacia Biotech) using gradient 1 with flow-rates of 500 μl/min (pumps A and B) and 300 μl/min (pump C) (see also Section 2). Data of retention time and peak area are given as mean ± S.D. ($n = 14$). Also, the highest and lowest values are given to demonstrate the range of retention time and peak-area data.

3. Results and discussion

3.1. Micro-HPLC analysis of inositol phosphates—principles and method validation

The chemistry used for detection is identical to that of the original method [11,12] and will not be repeated here. Our idea was to reduce the analysis time and increase the detection sensitivity by scaling down the whole analytical set-up, i.e. decreasing the column volume, flow-rates and dead volume of the system. The recent development of excellent micro-columns based on divinylbenzene–polystyrene co-polymers with bead diameters of 3 μm and a total column volume of 240 μl was extremely helpful in the realization of this aim. As detailed in Section 2, a Smart system (Pharmacia) with an additional LC-10AD pump (Shimadzu) was employed.

The method was validated with respect to variations of retention times and peak areas either on the same day or over a period of three months (Tables 1 and 2). The standard deviations for the retention times of some selected standards ranged between 0.25 and 0.62% when analyzed at the same day ($n = 8$), and between 0.64 and 1.61% when analyzed at fourteen different days over a period of three months (Tables 1 and 2).

Peak areas measured at fourteen different days over a period of three months showed relatively high standard deviations in the range 6.13–13.8%

Table 2
Reproducibility of retention times and peak areas on the same day

Compound	Amount injected (pmol)	Retention time (mean ± S.D.) (min)	Retention time (range) (min)	Peak area (mean ± S.D.) (min mA.U.)	Peak area (range) (min mA.U.)
$InsP_3$	416	10.41 ± 0.04	10.37–10.47	17.985 ± 0.402	17.587–18.543
$Ins(1,4,5,6)P_4$	109	13.45 ± 0.07	13.39–13.55	6.498 ± 0.254	6.375–6.872
$Ins(1,2,3,4,5)P_5$	76	14.44 ± 0.04	14.40–14.47	8.399 ± 0.329	8.004–8.834
$InsP_6$	235	16.66 ± 0.10	16.55–16.80	24.823 ± 0.819	23.629–25.878

A standard mixture of InsP was analyzed eight times at the same day using identical HPLC conditions. Samples (volume of 1 ml) were analyzed on MiniQ PC 3.2/3 (Pharmacia Biotech) using gradient 1 with flow-rates of 500 μl/min (pumps A and B) and 300 μl/min (pump C) (see also Section 2). Data of retention time and peak area are given as mean ± S.D. ($n = 8$). Also, the highest and lowest values are given to demonstrate the range of retention times and peak-area data.

(Table 1). In contrast, standard compounds analyzed at the same day resulted in a significantly lower range of standard deviations (2.24–3.91%; Table 2). This is mainly due to the fact that the PAR solution in triethanolamine has been loosing some "detection sensitivity" by an unknown mechanism. Microscopic aggregation or slow denaturation are the most likely possibilities. Adsorption of small amounts of PAR to the knitted coil and other wetted surfaces, e.g. in the detector cell, were apparent further reasons. Therefore, the flow cell usually was cleaned every seven to ten days by flushing it with nitric acid (10%, v/v). After such cleaning procedures an immediate increase in sensitivity was observed which then slowly decreased again over the next seven to ten days. These procedures explain the relatively high long-term standard deviations described above and imply that the system has to be calibrated daily.

Linearity was demonstrated plotting peak areas obtained after injections of different dilutions of selected standard InsP (Fig. 2). As expected from the results obtained with the standard MDD system [11,23], the slope of the linear calibration curve depends on the number of phosphate groups (Fig. 2). Quasi-linear calibration curves were obtained in the ranges 15–850 pmol for $InsP_3$, 10–800 pmol for $InsP_4$, 5–600 pmol for $InsP_5$, and 3–500 pmol for $InsP_6$ (Fig. 2). These results show that quantitation of different InsP isomers by microbore MDD is possible as has been shown for the standard MDD technique [11,23].

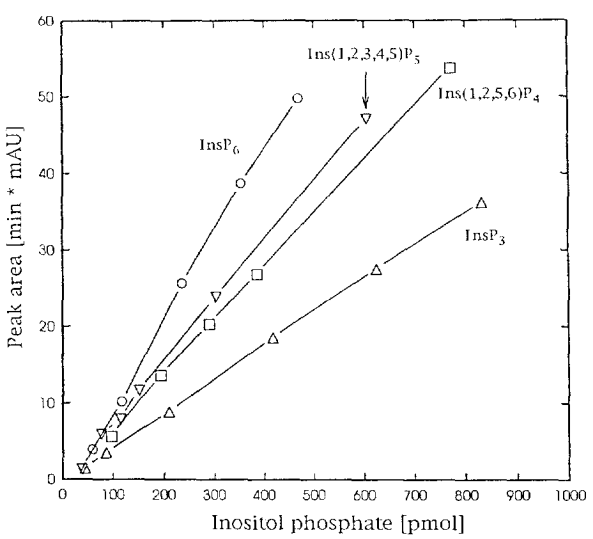

Fig. 2. Linearity of the microbore MDD system. Different amounts of $InsP_3$, Ins(1,2,5,6)P_4, D/L-Ins(1,2,3,4,5)P_5 and $InsP_6$ were analyzed on MiniQ PC 3.2/3 (Pharmacia Biotech) using gradient 1 with flow-rates of 500 μl/min (pumps A and B) and 300 μl/min (pump C). The peak areas were plotted against the amount of sample. Data from one representative experiment out of three are shown.

The two main improvements of the microbead-based separation method are strongly improved sensitivity and speed of analysis. As can be seen from Fig. 3A an analysis starting with inorganic phosphate to $InsP_6$ takes about 15 min. The detection limits (defined at a 3:1 signal-to-noise ratio) are: for $InsP_3$ 15 pmol, inositol tetrakisphosphate ($InsP_4$) 10 pmol, inositol pentakisphosphate ($InsP_5$) 5 pmol and $InsP_6$ 1–3 pmol. These low detection limits allow to analyze samples from biological material not available in large quantities, e.g. primary cell cultures, body fluids, tissue samples, etc.

3.2. Micro-HPLC analysis of inositol phosphates—applications

In Fig. 3 the method is applied to the analysis of InsP in Jurkat T-lymphocytes either unstimulated (Fig. 3B) or stimulated for 3 or 5 min with the anti-CD3 monoclonal antibody OKT3 (Fig. 3C and D). The amount of cells used per sample was $5 \cdot 10^7$. This cell number, about ten-fold less as compared to our earlier reports using standard MDD analysis of InsP in mononuclear cell lines [16,18,20], was highly sufficient to measure the content of inositol 1,3,4,6-tetrakisphosphate (Ins(1,3,4,6)P_4), inositol 1,3,4,5-tetrakis-phosphate (Ins(1,3,4,5)P_4), D/L-inositol 1,4,5,6-tetrakisphosphate (D/L-Ins(1,4,5,6)P_4), as well as inositol 1,2,3,4,6-pentakisphosphate (Ins(1,2,3,4,6)P_5), D/L-Ins(1,2,3,4,5)P_5, D/L-inositol 1,2,4,5,6-pentakisphosphate (D/L-Ins(1,2,4,5,6)P_5), inositol 1,3,4,5,6-pentakisphosphate (Ins(1,3,4,5,6)P_5) and $InsP_6$ under resting conditions (Fig. 3B). Upon stimulation with OKT3 (10 μg/ml) synthesis of $InsP_3$ as well as formation of

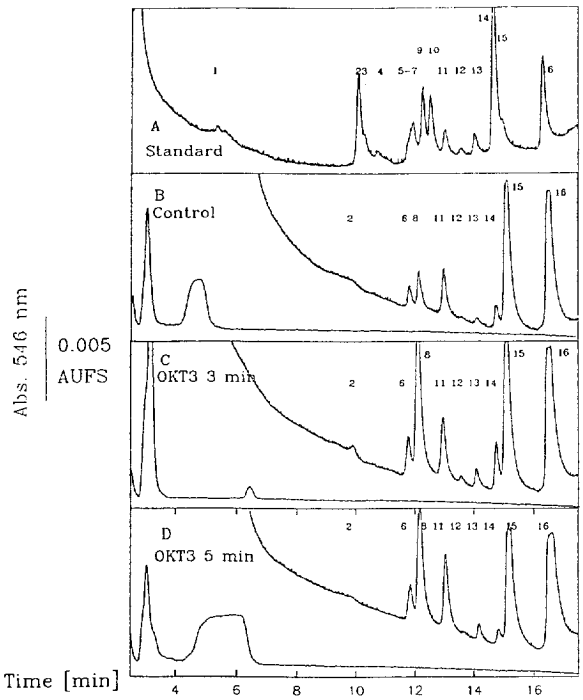

Fig. 3. Analysis of InsP in Jurkat T-lymphocytes. InsP were extracted from Jurkat T cells ($5 \cdot 10^7$) and processed as described in Section 2. Samples (volume of 1 ml) were analyzed on MiniQ PC 3.2/3 (Pharmacia Biotech) using gradient 1 with flow-rates of 500 μl/min (pumps A and B) and 300 μl/min (pump C) (see also Section 2). In (A) separation of a standard mixture of InsP is shown. Standard InsP isomers in their order of elution (injected pmol): 1 = $InsP_2$, 1065 pmol; 2 = Ins(1,3,4)P_3 + Ins(1,4,5)P_3, 416 pmol; 3 = Ins(1,5,6)P_3, 41 pmol; 4 = Ins(4,5,6)P_3, 33 pmol; 5 = Ins(1,2,3,5)P_4 + Ins(1,2,4,6)P_4, 63 pmol; 6 = Ins(1,2,3,4)P_4 + Ins(1,3,4,6)P_4, 58 pmol; 7 = Ins(1,2,4,5)P_4, 65 pmol; 8 = Ins(1,3,4,5)P_4, 0 pmol; 9 = Ins(1,2,5,6)P_4, 193 pmol; 10 = Ins(2,4,5,6)P_4, 159 pmol; 11 = D/L-Ins(1,4,5,6)P_4, 109 pmol; 12 = Ins(1,2,3,4,6)P_5, 28 pmol; 13 = D/L-Ins(1,2,3,4,5)P_5, 76 pmol; 14 = D/L-Ins(1,2,4,5,6)P_5, 493 pmol; 15 = Ins(1,3,4,5,6)P_5, 39 pmol; 16 = $InsP_6$, 376 pmol. In (B) a sample from unstimulated Jurkat cells is displayed, whereas (C) and (D) represent samples from Jurkat cells stimulated for 3 and 5 min with OKT3 (10 μg/ml). The following amounts of InsP were detected: $InsP_3$ < 15 pmol (B), 31 pmol (C), < 15 pmol (D); Ins(1,3,4,6)P_4 97 pmol (B), 134 pmol (C), 121 pmol (D); Ins(1,3,4,5)P_4 161 pmol (B), 900 pmol (C), 812 pmol (D); D/L-Ins(1,4,5,6)P_4 187 pmol (B), 320 pmol (C), 397 pmol (D); Ins(1,2,3,4,6)P_4 6 pmol (B), 31 pmol (C), 14 pmol (D); D/L-Ins(1,2,3,4,5)P_5 22 pmol (B), 74 pmol (C), 61 pmol (D); D/L-Ins(1,2,4,5,6)P_5 86 pmol (B), 173 pmol (C), 59 pmol (D). Ins(1,3,4,5,6)P_5 and $InsP_6$ were out of the linear calibration range. In (B), (C) and (D) the upper tracings represent microbore MDD–HPLC analysis, whereas the lower tracings were obtained at 254 nm without MDD to monitor nucleotides.

Ins(1,3,4,5)P_4 can be observed (Fig. 3C and D). Furthermore, increases in Ins(1,3,4,6)P_4, D/L-Ins(1,4,5,6)P_4, Ins(1,2,3,4,6)P_5, D/L-Ins(1,2,3,4,5)P_5 and D/L-Ins(1,2,4,5,6)P_5 were detected (Fig. 3C and D). Although the gradient employed is highly selective for the separation of $InsP_4$ and $InsP_5$ isomers, $InsP_3$ isomers are not very well separated (Fig. 3A). However, better separation of $InsP_3$ isomers requires longer columns in case of the acidic elution protocol, or can be overcome be using a neutral buffer system as described [12]. In the Jurkat cell line nearly all of the Ins(1,4,5)P_3 formed is rapidly metabolized into Ins(1,3,4,5)P_4 by Ins(1,4,5)P_3 3-kinase. Since Ins(1,3,4,5)P_4 is apparently metabolized much slower, its content is a much more sensitive parameter of activation of these cells. A CD3-mediated increase of Ins(1,3,4,5)P_4 was still detectable when samples corresponding to 10^7 cells were applied (data not shown) allowing to use a forty-fold smaller cell number as described for the standard MDD system [16]. To be sure that none of the peaks detected were due to contamination by nucleotides, samples were split and analysed without the post-column derivatization at a detector setting of 254 nm (Fig. 3B–D, lower tracings). All nucleotides eluted before 7.5 min, excluding interference with InsP detection. The different amounts of material detected at 254 nm in the three samples, e.g. nucleotides (Fig. 3B–D) reflect differences in the effectiveness of removal of these compounds by charcoal extraction.

The method was also applied to the analysis of InsP in other cell lines, tissues and body fluids (data not shown), displaying similar results regarding the improvements in sensitivity and speed as described above.

Another application is the analysis of micromolar concentrations of reaction products derived from small-volume enzyme assays (Fig. 4). This is necessary for many InsP-metabolizing enzyme purifications since only low amounts of enzyme with low specific activity are present in many tissues and cells and since many of these enzymes have their kinetic working optima at micromolar substrate concentrations. Up to now, it was mostly necessary to employ radioactive

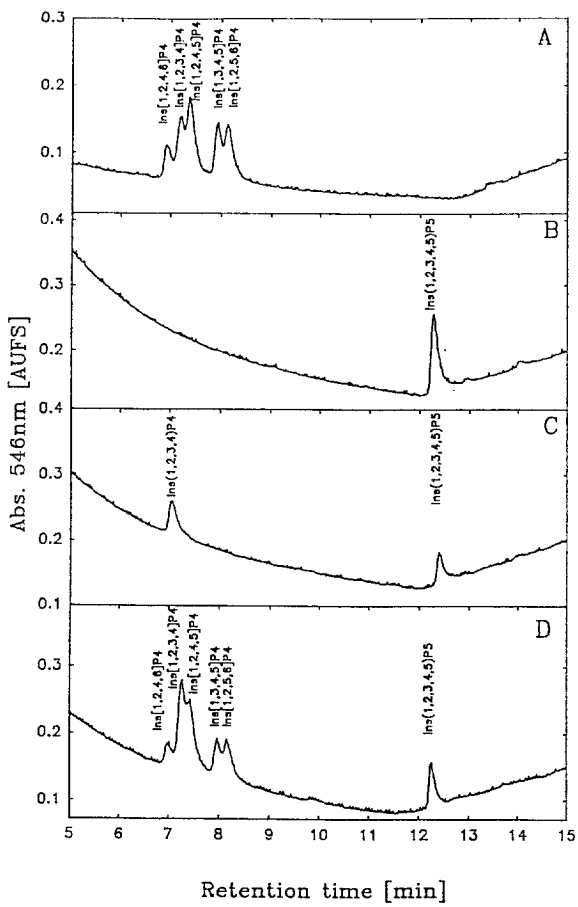

Fig. 4. Characterization of the reaction specificity of a D/L-Ins(1,2,3,4,5)P_5 phosphatase activity from fetal calf thymus. A soluble D/L-Ins(1,2,3,4,5)P_5 phosphatase activity was prepared from fetal calf thymus and the phosphatase assay was carried out as detailed in Section 2. In (A) the separation of standard Ins(1,2,4,6)P_4, Ins(1,2,3,4)P_4, Ins(1,2,4,5)P_4, Ins(1,3,4,5)P_4 and Ins(1,2,5,6)P_4 on MiniQ PC 3.2/3 (Pharmacia Biotech) by gradient 2 at flow-rates of 500 μl/min (pumps A and B) and 300 μl/min (pump C) is demonstrated. Note the improved selectivity for $InsP_4$ isomers as compared to gradient 1 shown in Fig. 3A. Analysis of reaction products from the phosphatase assay at time point 0 (B) and 60 min (C) shows pure D/L-Ins(1,2,3,4,5)P_5 (500 pmol) and the product Ins(1,2,3,4)P_4. In (D) the identity of the product was confirmed by co-chromatography with the standard shown in (A). Compare the amounts of Ins(1,2,3,4)P_4 in (A) and (D). Amounts of compounds injected were (in pmol): (A) Ins(1,2,4,6)P_4 180, Ins(1,2,3,4)P_4 230, Ins(1,2,4,5)P_4 263, Ins(1,3,4,5)P_4 226, Ins(1,2,5,6)P_4 219; (B) D/L-Ins(1,2,3,4,5)P_5 450; (C) D/L-Ins(1,2,3,4,5)P_5 131, Ins(1,2,3,4)P_4 307; (D) mixture (1:1) of samples from (A) and (C).

substrates and laborious product separation procedures to make such assays feasible. We show here that these restrictions can also be overcome by microbore MDD–HPLC without any radioactivity necessary in the substrate. Fig. 4 shows the characterization of the reaction specificity of a thymic Ins(1,2,3,4,5)P_5 5-phosphatase activity. Incubation of a thymic cytosolic extract with purified D/L-Ins(1,2,3,4,5)P_5 for 0 and 60 min resulted in production of an $InsP_4$ isomer (Fig. 4B and C). This isomer co-eluted with standard D/L-Ins(1,2,3,4)P_4 in subsequent runs (compare Fig. 4A and C). Since under such conditions sometimes small differences in retention times occurred, co-chromatography of the standard $InsP_4$ mixture with a 60-min incubation mixture confirmed that the reaction product behaved like D/L-Ins(1,2,3,4)P_4 (Fig. 4C). Other possible reaction products were excluded as follows: Ins(1,2,3,5)P_4 (product of a potential 4-phosphatase activity) co-eluted with Ins(1,2,4,6)P_4 [12], D/L-Ins(1,2,4,5)P_4, D/L-Ins(1,3,4,5)P_4 and finally D/L-Ins(2,3,4,5)P_4 (or D/L-Ins(1,2,5,6)P_4) are shown as separate peaks in Fig. 4A. These data demonstrate the high selectivity towards $InsP_4$ isomers.

4. Conclusion

Micro-MDD analysis represents a novel and precise tool to analyze simultaneously a large spectrum of InsP without any radioactive labelling necessary by a 20-min HPLC run with sensitivity in the 1–15 pmol range. This is demonstrated here showing the fast and sensitive analysis of InsP spectra from cells ($1–5 \cdot 10^7$ cells needed per sample), or from small-volume enzyme assays with substrate/product concentrations in the low micromolar range.

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