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High-performance liquid chromatographic separation of inositol phosphate isomers employing a reversed-phase column and a micellar mobile phase

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ABSTRACT

Surfactants have been employed in high-performance liquid chromatography (HPLC) for the separation of ionic and non-ionic compounds. We have developed a method employing a reversed-phase column and a mobile phase containing a surfactant, hexadecyltrimethylammonium hydroxide ($HDTMA^+OH^-$), for the separation of several inositol phosphate positional isomers. Various parameters were studied for their effect on the chromatographic capacity factor (k'). They included the concentration of $HDTMA^+OH^-$, the pH of the bulk micellar suspension and the addition of inorganic salts to the mobile phase. Resolution of the inositol monophosphates was controlled by a mixed mechanism, where the predominant elements were electrostatic forces and the formation of micelles. The elution of the inositol polyphosphate isomers was obtained by increasing the amount of a non-polar solvent, in agreement with an ion-pairing process. This method represents an alternative to ion-exchange HPLC. If offers a practical advantage when detection of radiolabeled samples by in-line radioactive flow detectors is required, because low-quenching solvents with good miscibility with scintillant fluids are employed. The analysis of various chromatographic conditions, the system reproducibility and its application to the analysis of biological samples are described.

INTRODUCTION

Many hormones, neurotransmitters and growth factors activate inositol phospholipid-specific phospholipase C (PLC) when they bind to their specific receptors on the cell surface [1,2]. Activated PLC hydrolyzes cellular inositol

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phospholipids, with the production of diacylglycerol and inositol phosphates. Among the different inositol phosphate isomers, inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], plays an important role in cell activation by mobilizing calcium from intracellular compartments [1–3]. Ins(1,4,5)P_3 is subsequently metabolized by the action of specific phosphorylases and phosphatases [4]. Biological responses to stimuli which are coupled to PLC activation therefore depend on the metabolic fate of the inositol polyphosphate isomers to a certain extent. Some of these metabolites may possess biological activity, while other are inactive. In particular, inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4], formed from Ins(1,4,5)P_3 by the action of a 3-kinase, may open certain sensitive membrane calcium channels, allowing influx of extracellular calcium [5]. Ins(1,3,4)P_3, produced from Ins(1,3,4,5)P_4 by a 5-phosphatase, and Ins(2,4,5)P_3 may also play a role in calcium mobilization, but appear to be less potent [3].

The study of inositol phosphate metabolism is made difficult by the low concentrations of the compounds involved. The chemical similarity of the different positional isomers requires separation by highly selective and sensitive techniques. Radioisotope-based techniques mandate the use of solvents miscible with hexane-based scintillation fluids and minimal radioactivity quenching.

Recently, surfactants have been introduced into column chromatography analysis. These substances act either as ion-pairing agents or by forming a micellar pseudostationary phase [6,7] and allow better separation of chemically similar compounds. In this paper, we describe a high-performance liquid chromatographic (HPLC) method for the separation of inositol phosphates which uses a reversed-phase column and a mobile phase with a surfactant. This procedure avoids the high salt concentrations generally used in anion-exchange chromatography, shortens analysis time, increases sensitivity and allows more efficient use of in-line radioactivity detection. The system is applicable to analysis of biological samples.

EXPERIMENTAL

Reagents

Radiolabeled phospholipids, phosphatidylinositol (PtdIns), PtdIns(4)P and PtdIns(4,5)P_2, were obtained from NEN (Boston, MA, U.S.A.). Unlabeled phospholipids were obtained from Sigma (St. Louis, MO, U.S.A.). KH_2PO_4, H_3PO_4, methanol and tetrahydrofuran (THF) were all HPLC grade and were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). HPLC-grade 1-propanol was from Aldrich (Milwaukee, WI, U.S.A.). Hexadecyltrimethylammonium hydroxide ($HDTMA^+OH^-$), hexadecyltrimethylammonium bromide and hexadecyltrimethylammonium chloride were purchased from Eastman Kodak (Rochester, NY, U.S.A.). All solutions were

made with high-purity water obtained by processing deionized water through a Darco ultrapure water system (Dracor, Durham, NC, U.S.A.).

Mobile phases were degassed by brief sonication in a bath under house vacuum.

Inositol phosphate standards

Radiolabeled inositol phosphate standards Ins(1)P, Ins(1,4)P_2, Ins(1,4,5)P_3, Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4 were obtained from NEN. Glycerophosphoinositol (GlyPIns), GlyPInsP, and GlyPInsP$_2$ were synthesized by mild methanolysis of PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2, respectively [8]. In addition to commercially available standards, Ins(1)P and Ins(2)P were obtained by alkaline hydrolysis of PtdIns; Ins(4)P, Ins(1,4)P_2 and Ins(2,4)P_2 from PtdIns(4)P; Ins(4,5)P_2, Ins(1,4,5)P_3 and Ins(2,4,5)P_3 from PtdIns(4,5)P_2, respectively [9]. Ins(1)P, Ins(1,4)P_2, and Ins(1,4,5)P_3 thus synthesized were identified by co-elution with commercial standards, using ion-exchange chromatography [9]. By deduction, Ins(2)P, Ins(4)P, Ins(2,4)P_2, Ins(4,5)P_2 and Ins(2,4,5)P_3 were the inositol monophosphate, bisphosphate and trisphosphate isomers which did not co-elute with the commercial standards.

Chromatographic apparatus

The chromatographic hardware consisted of a Waters Millipore system (Waters Assoc., Millford, MA, U.S.A.), equipped with two Model 590 programmable solvent delivery modules, a WISP Model 710B automatic sample processor or a Model U6K manual injector and a Waters 840 control station. Columns used were a 3-μm Supelcosil LC-8-DB (150 mm$\times$4.6 mm I.D.), a 5-μm Supelcosil LC-8 (250 mm$\times$4.6 mm I.D.) and a 5-μm Supelcosil LC-1 (250 mm$\times$4.6 mm I.D.), all from Supelco (Bellefonte, PA, U.S.A.). A 5-μm RP-8 OS-GU pre-column (Brownlee Labs., Santa Clara, CA, U.S.A.) was connected in-line before the injector. An Alltech Assoc. (Deerfield, IL, U.S.A.) 0.5-μm in-line filter was connected before the column. Column temperatures above room temperature (22°C) were maintained with a thermostat. The column eluate was automatically mixed with Tru-Count scintillant fluid (Tru-Lab, Libertyville, IL, U.S.A.), and radioactivity detected by a Flow-one/Beta (Model CR or Model A200) in-line radioactivity flow detector (Radiomatic Instruments, Tampa, FL, U.S.A.) equipped with either a 1-ml or a 2.5-ml liquid flow cell, as indicated.

Preparation of biological samples

Human mononuclear cells were obtained by Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) density gradient centrifugation of buffy coats prepared from healthy volunteers. Monocytes were isolated by counter-current centrifugation elutriation as described previously [10]. Fractions containing more than

88% monocytes (as determined by morphologic criteria after Wright's staining and positive histochemical staining for α-naphtyl-acetate esterase [11] were used. Platelets were absent. Monocytes were washed with cold phosphate-buffered saline (PBS) supplemented with 1 mg/ml bovine serum albumin (BSA) and resuspended at a concentration of 10^7 cells per ml in inositol-free RPMI 1640 medium (GIBCO, Grand Island, NY, U.S.A., special formulation No. 85-0189), supplemented with 1 mg/ml BSA, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 m*M* L-glutamine, 25 m*M* HEPES–Na buffer (pH 7.45) and 15 μCi/ml [^{3}H]myoinositol (Amersham, Arlington Heights, IL, U.S.A., specific activity 56 Ci/mmol). Cells were labeled for 18 h at 37 °C in a humidified incubator in a polypropylene tube maintained under continuous gentle rocking in the presence of 5% carbon dioxide and 95% air. At the end of the incubation, cells were washed three times with PBS supplemented with 1 mg/ml BSA, 300 μ*M* unlabeled myoinositol and 10 m*M* lithium chloride and resuspended at a concentration of $7 \cdot 10^7$–$10 \cdot 10^7$ cells per ml in RPMI 1640 supplemented with BSA, myoinositol and lithium chloride as above. Aliquots of 100 μl (containing $7 \cdot 10^6$–$10 \cdot 10^6$ cells) were distributed into 1.5-ml polypropylene tubes and preopsonized, washed zymosan A (Sigma) was added in 100 μl of medium to a final ratio of 100–200 particles per cell. Incubation was terminated at various times by the direct addition of 0.8 ml of ice cold, 12.5% (w/v) trichloroacetic acid (TCA). The TCA-soluble material was recovered upon centrifugation for 15 min at 4 °C and 10 000 *g*. TCA was removed with a single extraction with 1 ml of a water-saturated mixture of trichlorotrifluoroethane (FREON, Fisher) and tri-*n*-octylamine (Sigma) (74:26, v/v) [12]. Samples were maintained on ice and 100 μl of acid-washed, activated charcoal (400 mesh, 45% slurry, Sigma) were added to the recovered aqueous phase. The tubes were vortex-mixed, then centrifuged within 4 min at 10 000 *g* for 5 min at 4 °C. The residual water-soluble material was evaporated to dryness, reconstituted with 100 μl of water and filtered through a 0.45-μm HV Millex filter (Millipore). An aliquot was counted for radioactivity, and 70–80 μl were injected using a refrigerated automated injector (WISP, Waters Assoc.).

RESULTS AND DISCUSSION

Optimal separation of various inositol phosphate species, including GlyPIns, Ins(1)P, Ins(2)P, Ins(4)P, GlyPInsP, Ins(4,5)P_2, Ins(1,4)P_2, Ins(2,4)P_2, GlyPInsP$_2$, Ins(1,4,5)P_3, Ins(2,4,5)P_3, Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4, was achieved using a 5-μm octyldimethylsilyl 250 mm × 4.6 mm I.D. column (Supelco LC-8) (Fig. 1). Prior to sample injection, the column was equilibrated with 40 ml of $HDTMA^+OH^-$ (20 mmol/l) buffered at pH 5.80 with KH_2PO_4 (20 mmol/l) and containing 2.4% (v/v) methanol (solvent A). Samples were injected in the same solution and eluted with a five-step linear gradient from 0 to 100% of a 1:1 (v/v) mixture of 1-propanol and

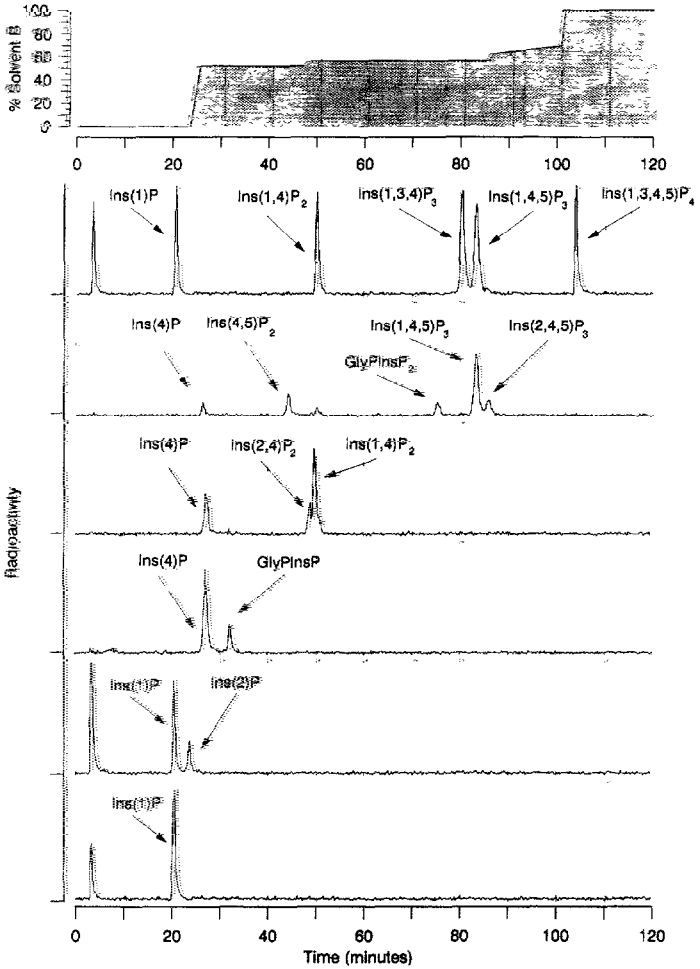

Fig. 1. Gradient elution of inositol phosphate standards using a reversed-phase column and the surfactant, hexadecyltrimethylammonium hydroxide. Column: 5-μm octyldimethylsilyl, 250 mm×4.6 mm I.D. (LC-8, Supelco). Mobile phase: solvent A, $HDTMA^+OH^-$ (20 mmol/l), KH_2PO_4 (20 mmol/l), pH 5.80; solvent B, 1-propanol-KH_2PO_4 (80 mmol/l), pH 4.50, 1:1 (v/v). Flow-rate: 0.9 ml/min. Gradient: see Table I. Temperature: 30°C. Detection: in-line radioactivity flow detector equipped with a 1-ml flow-cell. Scintillant: Tru-Count fluid. Scintillant flow-rate: 2.8 ml/min.

KH_2PO_4, 80 mmol/l, pH 4.50 (solvent B), as shown in Table I. The flow-rate was maintained at 0.9 ml/min and the column temperature was maintained at 30°C. The compounds eluted without broadening and in a reproducible fashion (Table II). Radioactivity quenching was minimal and substantially constant during the entire run, with a constant tritium efficiency of 31%, using Tru-Count scintillant fluid.

TABLE I

GRADIENT TABLE

Solvent gradient selected for optimal separation of several inositol phosphate isomers under conditions as described in Fig. 1. The time indicated refers to the time after the injection.

Time (min)	Total flow-rate (ml/min)	Solvent A (%)	Solvent B (%)
0	0.9	100	0
23	0.9	100	0
25	0.9	48	52
47	0.9	48	52
48	0.9	44	56
85	0.9	44	56
86	0.9	38	62
100	0.9	30	70
101	0.9	0	100

TABLE II

VARIATION OF THE ELUTION TIME FOR THE SEPARATION OF THE MAJOR INOSITOL PHOSPHATE ISOMERS UNDER OPTIMAL CONDITIONS OF ANALYSIS

Experiment conditions as described in Fig. 1. Retention times were determined at the peak apex. Values were obtained from four separate analyses.

Isomer	Retention time (mean ± S.D.) (min)
Ins(1)P	21.05 ± 0.25
Ins(1,4)P_2	49.40 ± 0.37
Ins(1,3,4)P_3	80.40 ± 0.80
Ins(1,4,5)P_3	83.45 ± 0.64
Ins(1,3,4,5)P_4	104.00 ± 0.00

Separation of inositol monophosphate isomers

Initial studies were performed to select for the optimal separation of inositol phosphate isomers. These studies were performed under isocratic conditions.

The role of the stationary phase, surfactant, salt concentration and pH were analyzed in detail. The elution characteristics of the inositol phosphate isomers were expressed as capacity factor (k') defined as $(V_e - V_0)/V_0$, where V_e is the elution volume of the compound of interest (determined at the peak apex) and V_0 is the void volume, determined as the elution volume of an unretained compound (myoinositol).

The effect of different concentrations of $HDTMA^+OH^-$ on the elution of GlyPIns, Ins(1)P, and Ins(2)P was studied using $HDTMA^+OH^-$ buffered at pH 5.2 with 20 mmol/l KH_2PO_4 and employing a 3-μm octyldimethylsilyl column, 150 mm × 4.6 mm I.D. (Supelco LC-8-DB). The surfactant concen-

trations tested were all above the critical micellar concentration (cmc) (cmc for aqueous dispersions of $HDTMA^+OH^- = 2.3\text{-}3.4$ mmol/l [13]). No compound was retained in the absence of surfactant (data not shown). The capacity factor was found to increase linearly with the concentration of $HDTMA^+OH^-$ up to 20 mmol/l, at which point it decreased for Ins(1)P and Ins(2)P (Fig. 2). This effect indicates that interactions between the surfactant and the phosphorylated compounds are essential to their retention.

An interpretation of this phenomenon was given by Armstrong and Nome [14] in their analysis of partitioning behavior of solutes in micellar chromatography. These authors considered three partition coefficients: that between the stationary phase and the solvent, that between the stationary phase and the micelles and that between the micelles and the solvent. In our system, the stationary phase is represented by a "surfactant-conditioned" stationary phase. The degree of conditioning of the stationary phase depends on a process of surfactant adsorption, which was related to the surfactant concentration employed. This was suggested by the observation that, after a column wash out, compound retention depended upon the volume of re-equilibration of the column in surfactant-containing buffers (data not shown). If a solute partitions to the conditioned stationary phase, its elution volume increases with increasing concentrations of surfactant. This will occur until the equilibrium is shifted to the micelles by either saturation of the stationary phase, increased micelle concentration or both. The relationship between the concentrations of $HDTMA^+OH^-$ and k' for Ins(1)P and Ins(2)P is in agreement with this

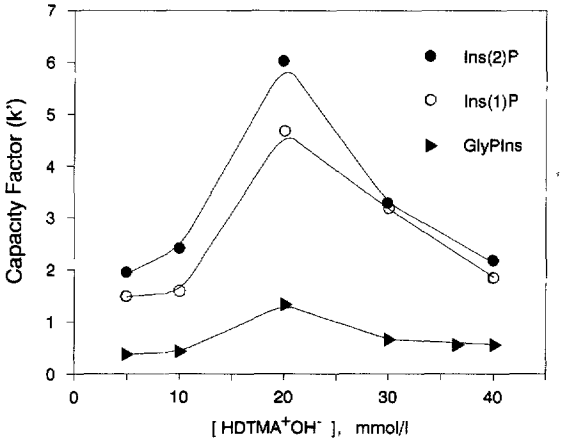

Fig. 2. Effect of varying the $HDTMA^+OH^-$ concentration on the capacity factor (k') for the isocratic elution of inositol monophosphate isomers. Column: 3-μm octyldimethylsilyl, 150 mm × 4.6 mm I.D. (LC-8-DB, Supelco). Mobile phase: $HDTMA^+OH^-$ at the indicated concentrations, buffered with KH_2PO_4 (20 mmol/l), pH 5.20. Flow-rate: 0.7 ml/min. Temperature: 25 °C. Detection: in-line radioactivity flow detector equipped with a 2.5-ml flow-cell. Scintillant: Tru-Count fluid. Scintillant flow-rate: 4.0 ml/min.

hypothesis. GlyPIns was not as efficiently retained as the other inositol monophosphates, and its partition was unaffected at the concentrations of $HDTMA^+OH^-$ tested. Its behavior, relative to the micellar phase, was probably that of a "non-binding" solute, according to Armstrong and Nome's classification [14].

The relationship between the pH of solvent A and k' for GlyPIns, Ins(1)P, Ins(2)P and Ins(4)P was tested using three different stationary phases (Fig. 3). The solvent employed contained $HDTMA^+OH^-$ (20 mmol/l) and KH_2PO_4 (20 mmol/l) buffered at the desired pH with H_3PO_4. Increasing pH in the range 3–6 increased k' for Ins(1)P, Ins(2)P and Ins(4)P. Retention was diminished when the pH was increased to 7 using an octyldimethylsilyl stationary phase, but continued to increase when a trimethylsilyl column was used.

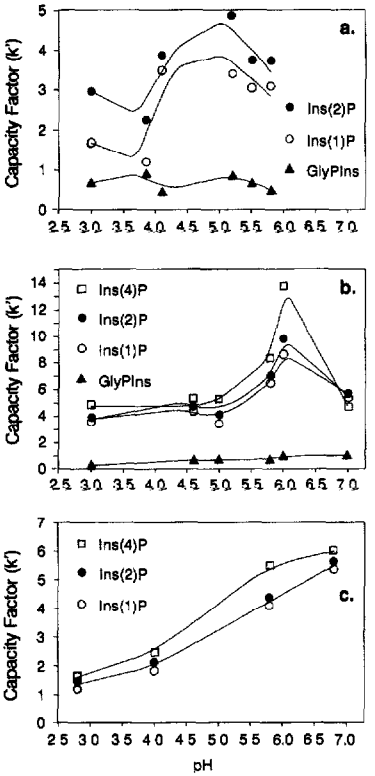

Fig. 3. Effect of pH variation on the capacity factor (k') for the isocratic elution of inositol monophosphate isomers. Columns: (a) 3-μm octyldimethylsilyl, 150 mm × 4.6 mm I.D. (Supelco LC-8-DB); (b) 5-μm octyldimethylsilyl, 250 mm × 4.6 mm I.D. (Supelco LC-8); (c) 5-μm trimethylsilyl, 250 mm × 4.6 mm I.D. (Supelco LC-1). Mobile phase: $HDTMA^+OH^-$ (20 mmol/l), KH_2PO_4 (20 mmol/l), titrated to the indicated pH values with H_3PO_4. Flow-rate: 0.7 ml/min. Temperature: 25°C. Detection: in-line radioactivity flow detector equipped with a 2.5-ml flow-cell. Scintillant: Tru-Count fluid. Scintillant flow-rate: 4.0 ml/min.

Although the predominant effect is likely to be on the charge of the analytes [15], raising the pH may also increase the aggregation number of quaternary compounds by promoting solvation. These data underscore the importance of pH of the bulk micellar suspension in determining the elution behavior of anionic compounds.

The k' value for GlyPIns was pH-independent. The effect of pH variations on undissociated compounds is less pronounced than that on dissociated compounds. If GlyPIns behaves as an undissociated molecule in the pH range tested, the effect of pH variation may be too small to contribute significantly to modifying k'.

The addition of increasing concentrations of KH_2PO_4 decreases the k' value for Ins(1)P and Ins(2)P (Fig. 4). This suggests that electrostatic forces play an important role. Addition of solutes to a micellar dispersion decreases the potential at the micellar/solvent interface, decreasing the elution volume for negatively charged compounds eluting with oppositely charged micelles [15]. Another equilibrium which may be affected by the addition of salts may be ion exchange: dissociated ions may compete with the analytes for their binding to the surfactant adsorbed onto the stationary phase.

In conclusion, several equilibria and competing phenomena are involved in the elution of the inositol monophosphates, although electrostatic forces and the formation of a micellar pseudostationary phase are likely to play a predominant role.

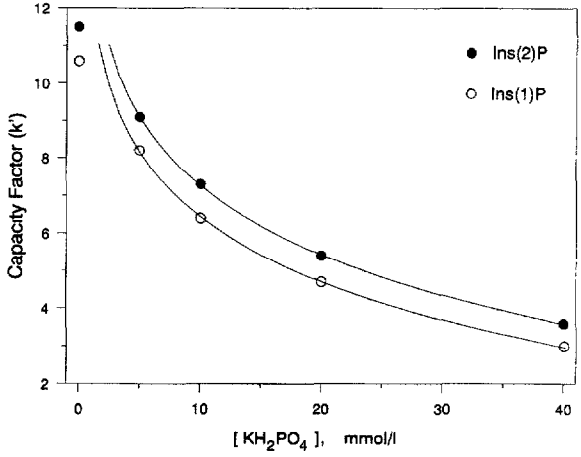

Fig. 4. Effect of varying the concentration of potassium phosphate on the capacity factor (k') for the isocratic elution of inositol monophosphate isomers. Column: 5-μm octyldimethylsilyl, 250 mm × 4.6 mm I.D. (LC-8, Supelco). Mobile phase: $HDTMA^+OH^-$ (20 mmol/l), with the indicated concentrations of KH_2PO_4, titrated to pH 5.8. Flow-rate: 0.7 ml/min. Temperature: 25°C. Detection: in-line radioactivity flow detector equipped with a 2.5-ml flow-cell. Scintillant: Tru-Count fluid. Scintillant flow-rate: 4.0 ml/min.

Separation of the inositol polyphosphate isomers

Several inositol polyphosphate isomers, including Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, eluted with peak broadening under pure micellar conditions (data not shown). These separations were performed by gradient elution employing a concentrated (up to 100 mmol/l) aqueous solution of $HDTMA^+OH^-$ buffered with phosphoric acid or formic acid. By stressing the ion-exchange characteristics with the addition of ammonium formate (1 mol/l, pH 4.0) to the micellar eluting buffer, it was possible to obtain the rapid elution of several compounds (Table III) with good peak shape, but further attempts to improve the selectivity of the separation by acting either on the gradient or on the salt concentration have been unsuccessful (data not shown).

Since the selectivity of chromatographic systems employing a surfactant is modified by the addition of organic modifiers [6,7], we studied the effect of THF and 1-propanol. The relationship between the polarity of the eluting solvent and the retention time of various inositol polyphosphate isomers was evaluated by adding water to THF as the eluting solvent. The retention time for all compounds studied increased with increasing amounts of water (Table IV). The addition of water results in an increased solvent polarity, suggesting that compound elution under these conditions is due to competition of the mobile phase with the stationary phase for the pairing agent–analyte complex, according to an ion-pair mechanism [6].

The concentration of surfactant added to solvent A greatly influenced the elution of Ins(1,4)P_2, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (Fig. 5). If ion pairing were the only process acting in our chromatographic system, the capacity factor of the analytes would be expected to increase along with the concentration of surfactant, as described by Karnes et al. [16]. In the case of Ins(1,4)P_2 this

TABLE III

MICELLAR CHROMATOGRAPHIC ELUTION OF INOSITOL PHOSPHATE ISOMERS IN THE ABSENCE OF ORGANIC MODIFIER

Colum: 3-μm octyldimethylsilyl, 150 mm×4.6 mm I.D. (Supelco LC-8-DB). Mobile phase: solvent A, $HDTMA^+OH^-$ (40 mmol/l), pH 4.00 with formic acid; solvent B, $HDTMA^+OH^-$ (70 mmol/l), ammonium formate (1 mol/l), pH 4 00. Flow-rate: 1.5 ml/min. Gradient: linear from 0 to 100% solvent B in 30 mın. Temperature: 25°C. Detection: in-line radioactivity flow detector equipped with a 2.5-ml flow-cell. Scintillant: Tru-Count fluid. Scintillant flow-rate: 5.0 ml/min

Isomer	Capacity factor (k')
Ins(1)P	2.25
Ins(4)P	2.75
Ins(4,5)P_2	6.75
Ins$(1,4)_2$	7.25
Ins(1,4,5)P_3	12.50
Ins(1,3,4,5)P_4	17.00

TABLE IV

EFFECT OF THE POLARITY OF SOLVENT B ON THE ELUTION OF INOSITOL POLYPHOSPHATE ISOMERS

Column: 3-μm octyldimethylsilyl, 150 mm × 4.6 mm I.D. (Supelco LC-8-DB). Mobile phase: solvent A, $HDTMA^+OH^-$ (40 mmol/l), titrated to pH 5.8 with H_3PO_4; solvent B, THF containing the indicated percentage of water. Flow-rate: 0.7 ml/min. Gradient: linear from 0 to 100% solvent B in 60 min. Temperature: 25 °C. Detection: in-line radioactivity flow detector equipped with a 2.5-ml flow-cell. Scintillant: Tru-Count fluid. Scintillant flow-rate: 4.0 ml/min. Data are expressed as the mean ± S.D. capacity factor of three to five determinations

Isomer	Capacity factor (k')		
	5% Water	20% Water	30% Water
Ins(4,5)P_2	6.88 ± 0.1	6.94 ± 0.3	8.63 ± 0.2
Ins(1,4)P_2	8.94 ± 0.3	9.75 ± 0.0	11.06 ± 0.1
Ins(1,4,5)P_3	10.10 ± 0.2	11.25 ± 0.0	12.67 ± 0.1
Ins(1,3,4,5)P_4	10.75 ± 0.2	11.88 ± 0.1	13.25 ± 0.0

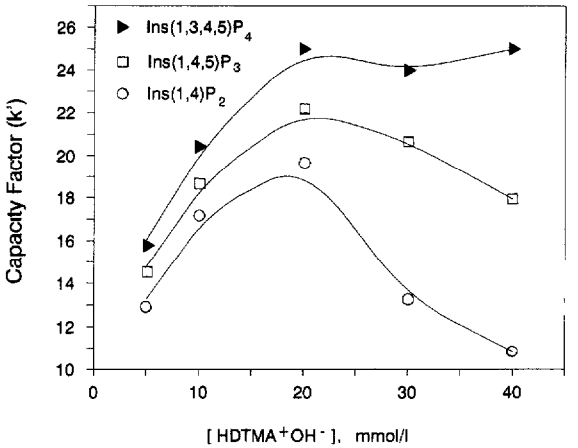

Fig. 5. Effect of varying the $HDTMA^+OH^-$ concentration on the capacity factor (k') for the elution of inositol polyphosphates. Column: 3-μm octyldimethylsilyl, 150 mm × 4.6 mm I.D. (LC-8-DB, Supelco). Mobile phase: solvent A, $HDTMA^+OH^-$ at the indicated concentrations, buffered with KH_2PO_4 (20 mmol/l), pH 5.20; solvent B, THF–water 70:30 (v/v). Flow-rate: 0.7 ml/min. Gradient: isocratic at 100% of solvent A for 15 min, followed by a linear increase from 0 to 60% solvent B in 30 min. Temperature: 25 °C. Detection: in-line radioactivity flow detector equipped with a 2.5-ml flow-cell. Scintillant: Tru-Count fluid. Scintillant flow-rate: 4.0 ml/min.

was only partially true, probably because both micellar elution and ion-pairing interaction occur. In fact, Ins(1,4)P_2 was initially retained longer with increasing concentrations of $HDTMA^+OH^-$. In excess of 20 mmol/l $HDTMA^+OH^-$,

an inverse relationship between Ins(1,4)P_2 elution volume and the surfactant concentration was observed, suggesting an elution mechanism based on a micellar process. In contrast, the k' value for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 increased with the surfactant concentration up to 20 mmol/l, remaining essentially constant thereafter as expected for ion-pairing chromatography. Furthermore, these compounds eluted when the percentage of THF was equal to or greater than 20%. At this percentage of organic modifier, the micelle concentration was probably reduced to a minimum value [17]. A more polar eluting solvent (such as a mixture of water and methanol) increased the elution volume, but did not improve the selectivity of the system (data not shown). Similarly, addition of surfactant to eluent B did not improve the elution characteristics (data not shown).

Ins(1,4,5)P_3 and Ins(2,4,5)P_3 were found to be the most difficult isomers to separate. Optimal selectivity was obtained using a 250-mm-long octyldimethylsilyl column, with particle size of 5 μm and 1-propanol as the organic modifier in eluent B. THF and 1-propanol have very similar polarity: the better results obtained with 1-propanol may be due to its "wetting" characteristic, which improves the interaction with the stationary phase [18].

Variations of the initial pH had only modest effects on the k' values for the inositol polyphosphate isomers (Fig. 6). These compounds are more charged than the inositol monophosphates, and their total charge may not be substantially affected by the pH variation tested.

Role of the stationary phase and a comparison of different surfactants

Under similar conditions, an octyldimethylsilyl column retained the compounds longer than a trimethylsilyl column, probably because the long, 16-carbon chain of the $HDTMA^+OH^-$ could interact better with the stationary phase (Figs. 3 and 6, and data not shown). This observation indirectly supports the hypothesis that the organic modifiers act as competitors for the analyte–pairing agent complex with the stationary phase. An increased elution volume and improved selectivity have been obtained with the trimethylsilyl column by adding 100 m*M* $HDTMA^+OH^-$ to eluent B (data not shown). This may be due to the surfactant countering the action of the organic eluent.

Additional surfactants were tested. Tetramethylammonium hydroxide or tetrabutylammonium hydroxide was found unsatisfactory with regard to retaining the various inositol monophosphates (data not shown). Hexadecyltrimethylammonium bromide, whose lower cmc value ($0.8 \cdot 10^{-3}$ *M* as opposed to $2.3 \cdot 10^{-3}$–$3.4 \cdot 10^{-3}$ *M* for $HDTMA^+OH^-$) [13] makes it preferable for an exclusive micellar-based process, was also tested. Selectivity and reproducibility were rather poor, however (data not shown).

Analysis of biological samples

The optimized separation method was applied to studying inositol phosphate formation of zymosan A-stimulated human monocytes. This stimulus

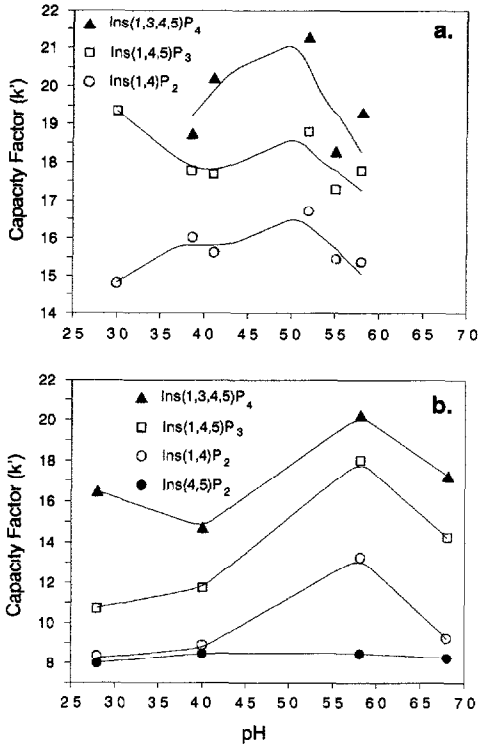

Fig. 6. Effect of pH variation on capacity factor (k') for the elution of inositol polyphosphates. (a) Column: 3-μm octyldimethylsilyl, 150 mm×4.6 mm I.D. (Supelco LC-8-DB). Mobile phase: solvent A, $HDTMA^+OH^-$ (20 mmol/l), KH_2PO_4 (20 mmol/l), titrated to the indicated pH values; solvent B, THF–water, 70:30 (v/v). Gradient: isocratic at 100% of solvent A for 15 min, followed by a linear increase from 0 to 60% solvent B in 30 min. Flow-rate: 0.7 ml/min. Temperature: 25°C. Detection: in-line radioactivity flow detector equipped with a 2.5-ml flow-cell. Scintillant: Tru-Count fluid. Scintillant flow-rate: 4.0 ml/min. (b) Column: 5-μm trimethylsilyl, 250 mm×4.6 mm I.D. (Supelco LC-1). Mobile phase: solvent A, $HDTMA^+OH^-$ (20 mmol/l), KH_2PO_4 (20 mmol/l), titrated to the indicated pH values; solvent B, $HDTMA^+OH^-$ (80 mmol/l), KH_2PO_4 (100 mmol/l), titrated to the indicated pH values. Gradient: isocratic at 100% of solvent A for 20 min, followed by a linear increase from 0 to 50% and 65% solvent B in 10 and 25 min, respectively. Flow-rate: 1.0 ml/min. Temperature: 25°C. Detection: in-line radioactivity flow detector equipped with a 2.5-ml flow-cell. Scintillant: Tru-Count fluid. Scintillant flow-rate: 4.0 ml/min.

has been shown to induce inositol phospholipid hydrolysis in these cells [19]. We chose to terminate the incubation by direct addition of ice-cold TCA. This strategy had the advantage of allowing instantaneous termination of the incubation for performing rapid kinetic assays, in addition to deproteinating the samples. The presence of salts carried over from the incubation medium did not interfere with the elution pattern of the inositol monophosphates if the incubation volume was maintained in the 100–200 μl range (data not shown).

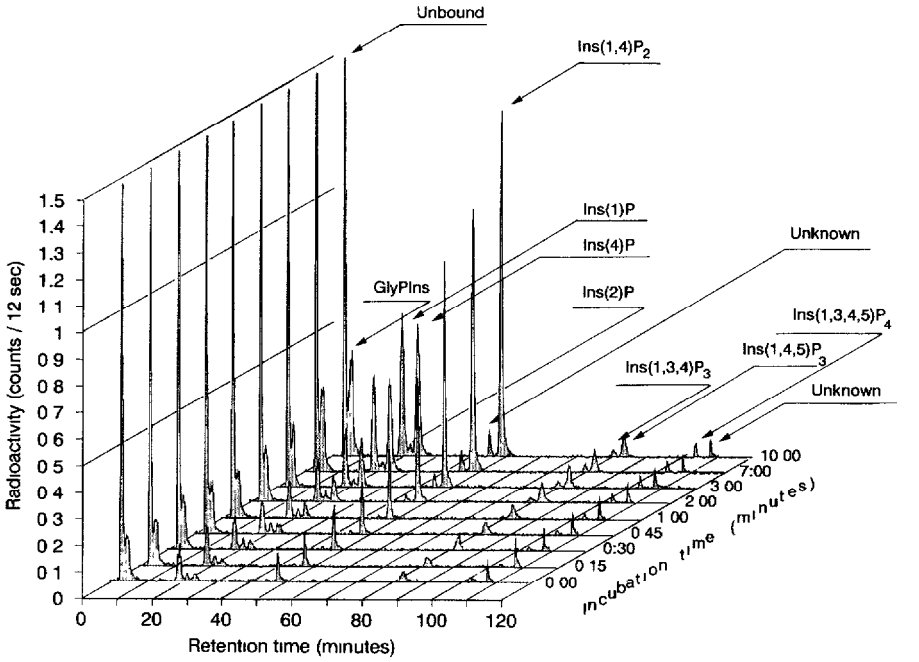

Fig. 7. Elution profile of radiolabeled material extracted from lithium chloride-treated human monocytes stimulated with opsonized zymosan A particles. Human elutriator-purified monocytes were labeled for 18 h with [^{3}H]myoinositol (15 μCi/ml) in inositol-free RPMI 1640 medium, washed and incubated with medium or opsonized zymosan A for the indicated time. The incubation medium contained 300 μM unlabeled myoinositol and 10 mM lithium chloride. The incubation was terminated at the indicated times by the direct addition of ice cold, concentrated TCA as described in Experimental. The TCA was removed and the samples concentrated, reconstituted with water, filtered and injected in a volume of 70 μl. Chromatographic conditions were as described in Fig. 1.

Alternatively, cells may be pelletted by centrifugation, the medium removed and extraction performed after replacing the medium with water.

Charcoal adsorption did not interfere with the recovery and elution profile of the inositol phosphates, as determined by spiking the samples with radiolabeled standards (data not shown). Although not strictly necessary under the experimental conditions used here, this procedure provided a useful means of sample clean up when large amounts of nucleotides were present (i.e. utilization of larger amounts of cells or tissue, or addition of nucleotides to the incubation medium). Sample recovery, determined by spiking unlabeled experimental samples with authentic labeled standards, was better than 93% (data not shown).

The elution profile of radioactive material extracted from medium-treated monocytes was unchanged for the entire length of the incubation (data not

shown) and was virtually identical to that observed at time 0 (Fig. 7). Small amounts of Ins(1)P, Ins(2)P, Ins(4)P, Ins(1,4)P_2 and Ins(1,4,5)P_3 were present. Material extracted from lithium chloride-treated, opsonized zymosan A-stimulated cells contained compounds eluting with the retention time of Ins(1,4,5)P_3 and Ins(1,4)P_2. These metabolites increased 45 s after cell activation and were followed by the appearance of peaks with the retention time of Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3. These isomers are products of the phosphorylation of Ins(1,4,5)P_3 by a 3-kinase and its subsequent catabolism by a 5-monoesterase [4]. Additional radioactive peaks coeluting with Ins(1)P, Ins(4)P and Ins(1,4)P_2 were observed at later stages. One peak in the elution area of the inositol bisphosphate isomers appeared later in the incubation, after the increase in Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4. This peak did not coelute with any available standard. One peak coeluting with Ins(2)P did not change during the time of the study. An additional peak, also present in unstimulated cells, whose retention time was longer than that of Ins(1,3,4,5)P_4, remained unmodified during the time of the analysis. Its identification is currently in progress. The remaining inositol phosphates, including material coeluting with the various inositol bis- and trisphosphate standards and Ins(1,3,4,5)P_4, accumulated over time. This likely reflected the inhibition of inositol phosphate phosphatases by Li^+ present in the incubation medium [20].

Application of this method during the course of several analyses has shown that the reversed-phase columns employed exhibited a long life, with little loss of selectivity and efficiency after multiple runs. Approximately 30 injections of biological samples were performed without column regeneration. When initial performance deterioration was observed, regeneration was easily accomplished by a wash out procedure employing an apolar solvent, such as a mixture of 1-propanol and water (70:30, v/v).

CONCLUSIONS

The method described represents an alternative to ion-exchange methodology in the separation of inositol phosphate positional isomers. The system offers increased flexibility due to the possibility of acting on multiple elements governing the elution process. These include the stationary phase, surfactant, buffer concentration and pH, and the organic modifier. This flexibility makes the system easily adaptable to various analyses. For instance, we have used intermediate isocratic conditions for the rapid analysis of only a set of isomers (i.e. inositol bisphosphates or trisphosphates).

Since the number of theoretical plates is not as crucial as in ion-exchange chromatography, low-porosity columns (i.e. with a particle size of 5 μm) may be used with good efficiency. This characteristic is particularly useful in terms of time saved for column re-equilibration, which may be accomplished at two to three times the elution flow-rate (we typically used 2.0 ml/min for 15–20 min) and occurs without problems related to pressure.

In general, the major limitation to high flow-rates when radioactivity detection by in-line liquid scintillation counting is employed has been the miscibility with scintillant fluids. The utilization of a mixture of a low-quenching organic solvent and a low-molarity buffer as the eluting solvent has alleviated this restriction. This is a marked advantage over ion-exchange chromatography, where the limited miscibility and substantial quenching of the high salt concentrations required to elute the polyphosphorylated inositol isomers greatly compromise sensitivity. The sensitivity of the system described herein makes it of potential interest for the separation of the highly phosphorylated inositol isomers (inositol pentakis- and hexakisphosphates); its suitability for this application is currently under investigation.

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