Journal of Chromatography, 523 (1990) 201–216
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 747

High-performance reversed-phase ion-pair chromatographic study of myo-inositol phosphates

Separation of myo-inositol phosphates, some common nucleotides and sugar phosphates

MIKLÓS PATTHY*
Institute for Drug Research, Szabadságharcosok útja 47–49, 1045 Budapest (Hungary)
TAMÁS BALLA
Department of Physiology, Semmelweis University Medical School, 1088 Budapest (Hungary)
and
PÉTER ARÁNYI
Institute for Drug Research, Szabadságharcosok útja 47–49, 1045 Budapest (Hungary)
(First received April 4th, 1990; revised manuscript received July 20th, 1990)

ABSTRACT

A detailed study of all the major chromatographic variables affecting the retention behaviour and separation of myo-inositol phosphates in reversed-phase ion-pair chromatographic systems was carried out. The parameters studied included the eluent concentration of the pairing ion, the eluent concentration of the organic modifier and the buffer salt, the pH of the eluent, the minimum column plate count necessary for the separation of the inositol trisphosphate isomers and isocratic and gradient modes of separation. The retention behaviour of some common nucleotides and sugar phosphates was also investigated as these phosphates present chromatographic interference problems in biochemical studies based on the cellular incorporation of $[^{32}P]P_i$. The separation methods developed appear to be superior to established anion-exchange separation techniques in terms of separation speed and "mildness" of the chromatographic conditions.

INTRODUCTION

Considerable interest in the cellular production and metabolism of myo-inositol phosphates has developed since the demonstration that activation of some plasma-membrane receptors leads to the production of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], a second messenger that promotes the mobilization of intracellular calcium stores. It is now clear that the metabolism of Ins(1,4,5)P_3 is complex, proceeding by two main routes, resulting in several closely related myo-inositol phosphates. (For a detailed review of the biosynthesis and degradation of the myo-inositol phosphates, see ref. 1.)

0021-9673/90/$03.50

Currently, several methods exist for the separation of myo-inositol phosphates, such as open-bed anion exchange [2], anion exchange in cartridges [3], gas–liquid chromatography [4], high-voltage paper electrophoresis [5], high-performance liquid chromatography (HPLC) using strong anion-exchange packing materials [6,7] and a combination of anion exchange and gas chromatography [8].

However, gas chromatographic methods involve laborious sample preparation (usually with more than one derivate), and open-bed and cartridge separation techniques and high-voltage paper electrophoresis lack the separation efficiency necessary for the analysis of complex samples. High-performance anion-exchange methods, although frequently used, may cause difficulties because polyvalent ions (*e.g.*, polyphosphoinositols) adsorb very strongly on ion exchangers and, as a result, long elution times (1–2 h) and high buffer salt concentrations (1–2 *M*) are needed for the separation, putting a great strain on the whole of the chromatographic system.

High-performance reversed-phase ion-pair chromatography (RP-IPC) has been successfully used in the separation of various ionic compounds [9–11]. The separation speed, efficiency and "mildness" of the chromatographic conditions (*e.g.*, lower buffer salt concentrations and column back-pressures) make this approach an attractive alternative to established anion-exchange HPLC methods.

Two laboratories have used RP-IPC for the separation of myo-inositol phosphates. They developed isocratic methods for the separation of Ins(1,4)P_2 and Ins (1,4,5)P_3 [12], Ins(1,4)P_2 (eluted near the front), InsP_3 isomers and Ins(1,3,4,5)P_4 [13] and also some adenine nucleotides, 2-3-bisphosphoglycerate, D-fructose-1,6-diphosphate (FDP), InsP_3 isomers and Ins(1,3,4,5)P_4 [14]. All these separations were made after studying some characteristics of the RP-IPC systems used.

In order to exploit the full separation potential of RP-IPC (isocratic and gradient), we carried out a detailed study of all the major chromatographic variables that affect the retention (and separation) of some of the more common inositol phosphates Ins(1)P_1, 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. The data obtained can be best used in experiments involving the cellular incorporation of [^{3}H]inositol but, as ample reference has also been made to the retention behaviour of some prominent nucleotides and sugar phosphates in various RP-IPC systems, they can also be used in biochemical studies based on the incorporation of [^{32}P]P_i.

EXPERIMENTAL

Chemicals

Materials and their sources were as follows. Tetrabutylammonium hydrogensulphate (TBAHS) was obtained from Sigma (St. Louis, MO, U.S.A.) and potassium dihydrogenphosphate (LiChropur) and acetonitrile (LiChrosolv) from Merck (Darmstadt, F.R.G.). The Nucleosil 100-5 C_{18} (5 μm) column packing material was the product of Macherey, Nagel & Co. (Düren, F.R.G.). D-Myo-[2-^{3}H]inositol 1-phosphate, D-myo-[2-^{3}H]inositol 1,4-bisphosphate, D-myo-[2-^{3}H]inositol 1,4,5-trisphosphate, D-myo-[2-^{3}H]inositol 1,3,4,5-tetrakisphosphate (all potassium salts) and myo-[2-^{3}H]inositol were supplied by Amersham International (Amersham, U.K.). D-Myo-[2-^{3}H]inositol 1,3,4-trisphosphate was from New England Nuclear (Boston, MA, U.S.A.). Adenosine-5'-monophosphoric acid (AMP), adenosine-5'-diphosphate (ADP), adenosine-5'-triphosphate (ATP), adenosine-3',5'-cyclic monophosphate (c-

AMP), guanosine-5'-diphosphate (GDP) and guanosine-5'-triphosphate (GTP) were obtained from Calbiochem (La Jolla, CA, U.S.A.). Glucose-6-phosphate (G6P), FDP and aldolase (from rabbit muscle) were purchased from Boehringer (Mannheim, F.R.G.). The glucose–hexokinase reagent kit was supplied by Baker Instruments (Allentown, PA, U.S.A.). All other chemicals were of analytical-reagent grade.

Chromatography

For isocratic separation of the compounds studied, the HPLC system included a Varian 8500 pulse-free pump equipped with a stop-flow septumless injector (Varian, Palo Alto, CA, U.S.A.). For gradient separations two Varian 8500 pumps were used. The chromatographic support (Nucleosil 100-5 C_{18}, 5 μm) was packed in stainless-steel columns by Bio-Separation Technologies (Budapest, Hungary). The column dimensions and the composition of the mobile phases are specified in the figure legends. The flow-rate was 1 ml/min and fractions of 0.33 or 0.50 ml were collected. All separations were carried out at ambient temperature. The radioactivity of the eluted fractions was determined with a liquid scintillation counter (Wallac 1410; Pharmacia–LKB, Turku, Finland). Detection of FDP [15] and G6P [16] in the eluted fractions was performed by established enzymatic methods.

The elution of the nucleotides was monitored with a Variscan (Varian) absorbance monitor (at 254 nm) and a Kipp and Zonen (Delft, The Netherlands) BD 40 recorder. Inositol phosphates were injected as obtained from the supplier. The activity of the individual phosphates placed on the column varied between $1 \cdot 10^4$ and $2 \cdot 10^4$ dpm. The nucleotides (0.1 mg/ml solutions) and the sugar phosphates (100 mg/ml solutions) were dissolved in the mobile phase. The amount of the individual nucleotides placed on the column varied between 2 and 5 μg. The amount of FDP or G6P injected was 2 mg. The UV absorbances obtained after the enzymatic procedures with an aliquot of the eluted fraction were measured with a CentrifiChem System 400 spectrophotometer (Union Carbide, Rye, NY, U.S.A.) at 240 nm (FDP) and 340 nm (G6P).

RESULTS AND DISCUSSION

Dependence of k' on the concentration of the pairing ion in the eluent

The capacity factor (k') was calculated using the equation $k' = (t_R - t_0)/t_0$, where t_0 and t_R are the retention times of an unretained solute (the first baseline disturbance observed after the injection of 100 μl of distilled water) and the solute in question, respectively. Of various quaternary amines, TBAHS proved to be the most efficient as the pairing ion for the separation of inositol phosphates [13]. From eluents containing no TBAHS, inositol phosphates (IPs) and other organic phosphates are very poorly retained or not retained at all.

All the k' *vs.* TBAHS concentration plots in Fig. 1. have a similar convex shape. We found that the position of the retention maxima for both IPs and nucleotides was primarily determined by the nature and concentration of the organic modifier in the eluent, and that changes in salt concentration and pH had hardly any effect on the position of the maxima observed. When identical acetonitrile concentrations (*e.g.*, 15%) were used for IPs and nucleotides, the maxima appeared at the same TBAHS concentration (10–15 m*M*). With higher acetonitrile concentrations (20%) the maxi-

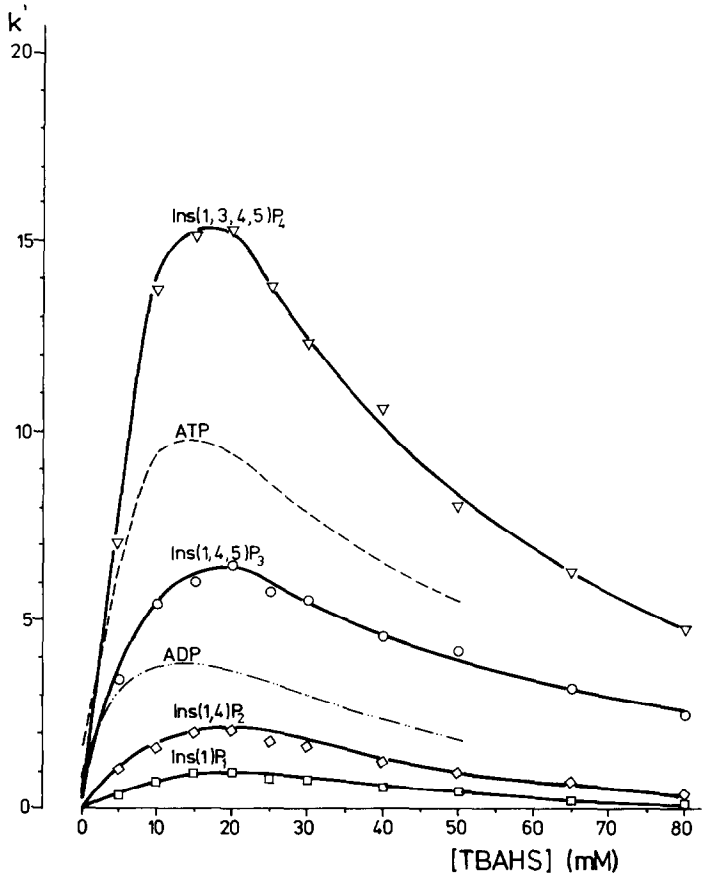

Fig. 1. Dependence of k' on the concentration of TBAHS in the eluent. Column: Nucleosil C_{18} (5 μm), 150 × 4.0 mm I.D., with a 20 × 4.0 mm I.D. precolumn (t_0 = 1.32 min). The eluent for IPs consisted of 20% (v/v) acetonitrile, 25 m*M* KH_2PO_4 and 0–80 m*M* TBAHS, the pH of the aqueous part being adjusted to 7.0 using 20% (w/v) KOH solution. The eluent for adenine nucleotides consisted of 15% acetonitrile, 20 m*M* KH_2PO_4 and 0–80 m*M* TBAHS, the pH of the aqueous part being adjusted to 5.2 using 20% (w/v) KOH solution.

ma appeared at higher TBAHS concentrations (15–20 m*M*), irrespective of pH and/or salt concentration changes in the eluent (see the IP curves in Fig. 1). In aqueous buffers with a very low (1%) concentration of methanol, adenine nucleotides exhibited retention maxima at 0.3 m*M* TBAHS [17].

Thus, the eluent concentration of the organic modifier and the TBAHS concentration at which retention maxima are obtained run parallel with each other (zero concentrations excluded). As limited changes in the eluent pH and salt concentration do not affect the amount of the pairing ion adsorbed [18], maximum retention of IPs (and/or nucleotides) can be achieved at any practical TBAHS concentration in the eluent (*e.g.*, in the range 0.3–30 m*M*), depending on the concentration of the organic modifier used (which influences the adsorption of TBAHS on the surface).

For our experiments a TBAHS concentration of 10 m*M* was preferred as RP-

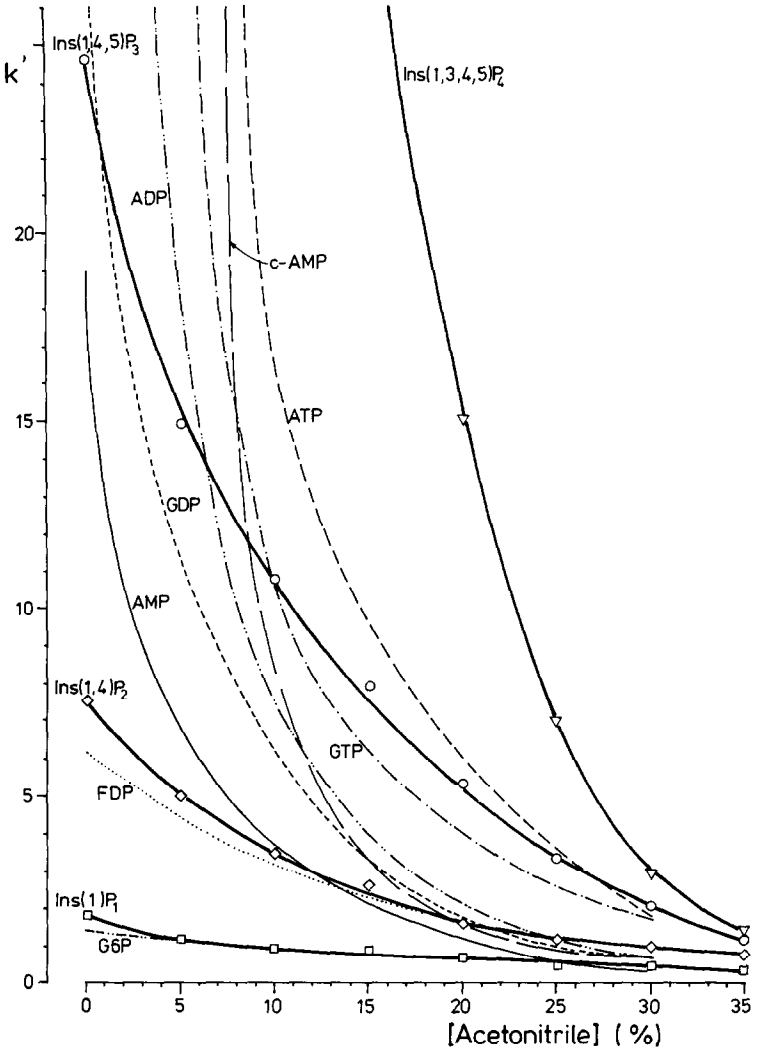

Fig. 2. Dependence of k' on acetonitrile volume percentage in the eluent. Column as in Fig. 1. The eluent consisted of 25 m*M* KH_2PO_4, 10 m*M* TBAHS and 0–35% (v/v) acetonitrile. The pH of the aqueous part was adjusted to 5.0.

IPC systems with much lower pairing ion concentrations are more sensitive to temperature changes and require long re-equilibration times [11,17], which is a disadvantage especially when gradient elution is used. These TBAHS concentrations are too low for micelle formation to be considered as an explanation for the falling side of the k' *vs.* TBAHS concentration curves [10]. The explanation may be related to an increased coverage of the adsorbent surface by the pairing ion [9] and an increased competition by the counter ion of the pairing ion with the sample anions for retention on the surface [19].

Dependence of k' on acetonitrile admixture

An increase in the acetonitrile content in the eluent [in the range 5–30% (at constant pH, TBAHS and phosphate concentrations)] decreases the retention of nucleotides to a greater extent than that of IPs (the retention of c-AMP is affected most, see Fig. 2).These selectivity changes indicate that, although the dominant mechanism of the RP-IPC process with TBAHS as pairing ion is mostly ion exchange [18] or ion interaction [20], non-specific (apolar or hydrophobic) interactions also have an important role.

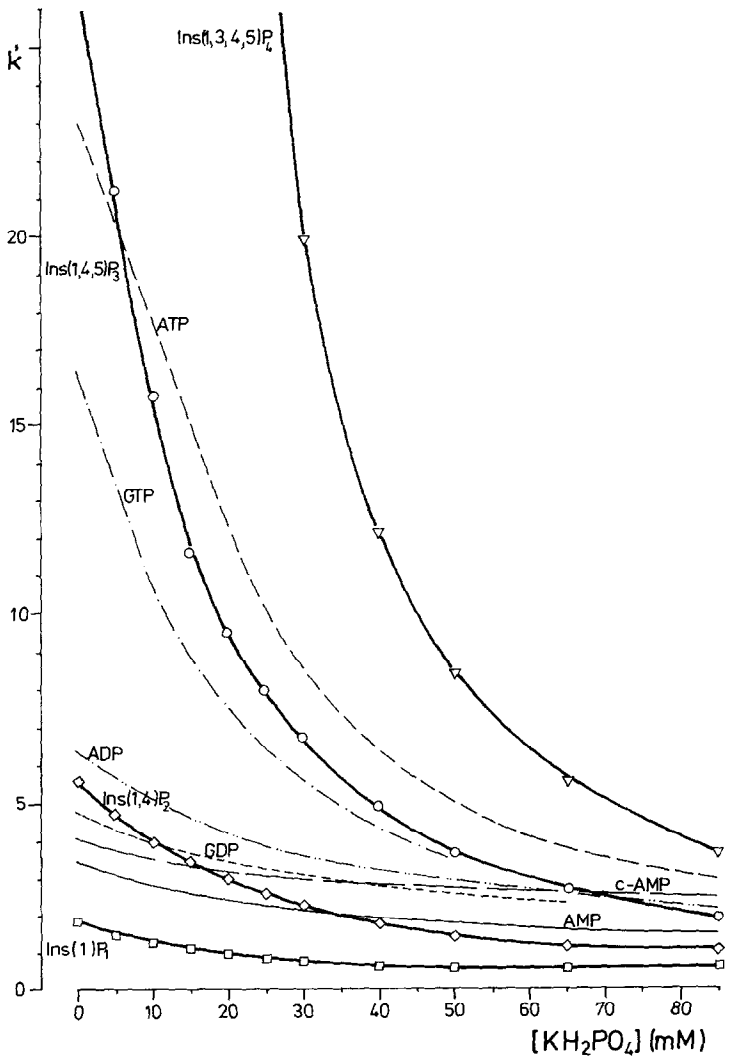

Fig. 3. Dependence of k' on phosphate concentration in the eluent. Column as in Fig. 1. The eluent consisted of 15% acetonitrile, 10 m*M* TBAHS and 0–85 m*M* KH_2PO_4. The pH of the aqueous part was adjusted to 5.0.

Owing to the heterocyclic ring system in their molecules, non-specific interactions are stronger with nucleotides and become significant with eluents containing less than about 10% of acetonitrile. With c-AMP, which has the smallest phosphate moiety of the nucleotides, non-specific interactions appear to be especially strong in eluents containing less than 7% acetonitrile, but their significance is greatly reduced in eluents with over 20% of acetonitrile.

With eluents containing more than 35% of acetonitrile, ionic interactions also approach zero, as acetonitrile concentrations higher than 35% seem to flatten the adsorption isotherm of the pairing ion to such an extent that a 10 m*M* eluent concentration of TBAHS (in our case) cannot provide the surface concentration of the pairing ion necessary for a reasonable retention of the ion pairs formed.

As shown in Fig. 2, sugar phosphates behave very similarly to their inositol phosphate counterparts. The separation of G6P from Ins(1)P_1 is only possible with eluents having a low phosphate concentration and containing no acetonitrile at all. Myo-inositol was invariably eluted with or very near the front.

Dependence of k' on the phosphate concentration of the eluent

As shown in Fig. 2, the retention of IPs is governed mostly by ionic interactions, whereas that of the nucleotides is determined by both ionic and non-specific interactions. An increase in the eluent phosphate concentration, therefore, will cause a more pronounced reduction in the retention of IPs (as it would in ion-exchange systems, in general).

When the phosphate concentration in the eluent is increased in the range 0–50 m*M*, the retention of c-AMP is hardly influenced, whereas the *k'* values of IPs decrease considerably (see Fig. 3). The effective phosphate concentration range is pushed downward in eluents containing more than 15% of acetonitrile, and extended to higher phosphate (or counter ion) concentrations if less than 15% of acetonitrile is used in the eluent.

The eluent strength in most RP-IPC systems is determined by both the volume percentage of the organic modifier and the salt concentration in the mobile phase (at a rough estimate, a 1% change in the eluent acetonitrile concentration equals, in our case, a 5 m*M* change in eluent phosphate concentration). However, eluents of the same average strength may exhibit different selectivity characteristics, depending on the acetonitrile to phosphate concentration ratio in the eluent (see the discussion on Figs. 4 and 5).

It should also be mentioned that phosphate concentrations below 15 m*M* resulted in much poorer than expected column efficiencies (especially when eluent pH was below 4.0). The apparent efficiency improves with increasing phosphate concentration in the range 0–40 m*M*, but at higher concentrations the effect is negligible. In addition, phosphate concentrations higher than 30–40 m*M* caused substantial "quenching" by reducing the solubility of scintillants {a scintillant to eluent ratio of 5:1 was used for the detection of IPs; the cocktail consisted of 6g of 2,5-diphenyloxazole, 0.6 g of 1,4-di[2-(5-phenyl)oxazoyl]benzene and 150 g of naphthalene dissolved in 1800 ml of dioxane and 150 ml of toluene}. Therefore, unless special selectivity effects require differently, the practical phosphate concentration range in RP-IPC eluents for the separation of radiolabeled IPs and nucleotides appears to be 15–40 m*M*.

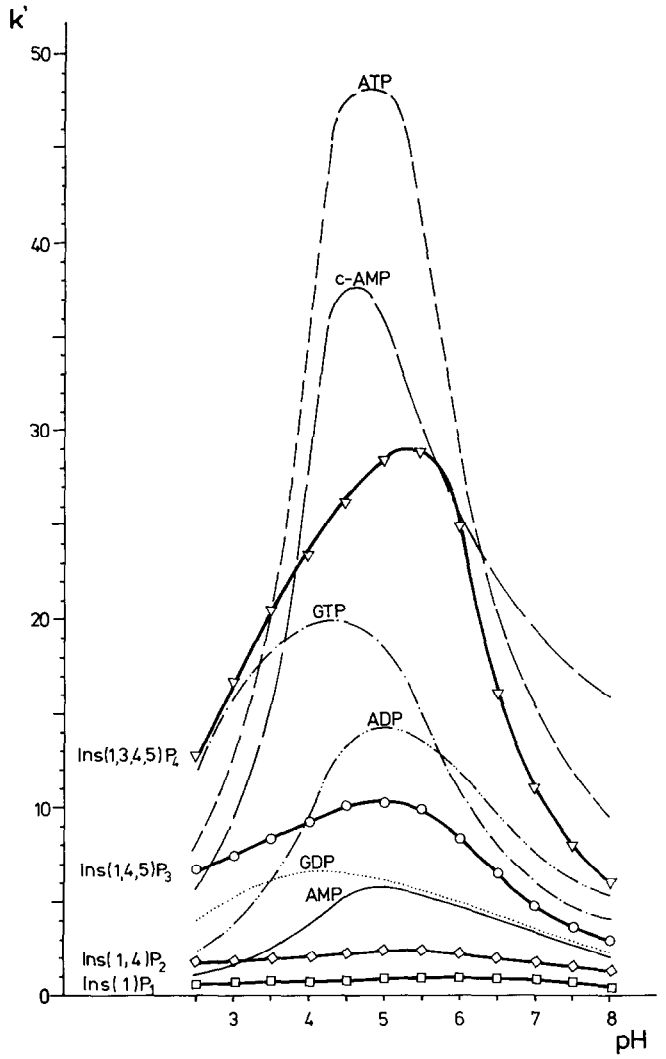

Fig. 4. Dependence of k' on mobile phase pH. Column as in Fig. 1. The eluent consisted of 3% acetonitrile, 10 mM TBAHS and 100 mM KH_2PO_4. The pH of the aqueous part varied between 2.5 and 8.0.

Dependence of k' on mobile phase pH

Ins(1,4,5)P_3-like phosphates have a polyphosphate–polyester character, whereas ATP-like phosphates have a polyphosphate–monoester type group with $pK_a \approx 6.5$ [21] in their molecules. Conflicting data have been published on the pH dependence of the retention of both IPs and adenine nucleotides in RP-IPC systems. Shayman and BeMent [13] reported a narrow pH optimum (maximum retention) for IPs and ATP at pH 3.5. Sulpice *et al.* [14], however, reported an increase in retention for Ins(1,4,5)P_3 in the pH range 3.5–8.0, with pH 8.0 resulting in the highest retention. For adenine nucleotides the maximum retention was reported to be at *ca.* pH 6.5. On the other

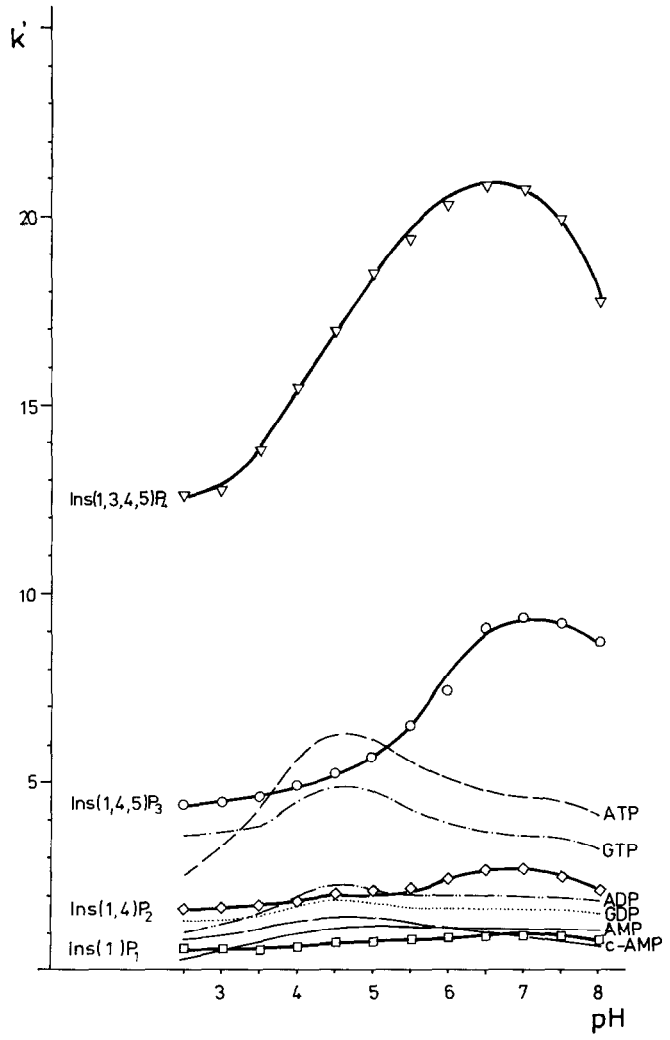

Fig. 5. Dependence of k' on mobile phase pH. Column as in Fig. 1. The eluent consisted of 20% acetonitrile, 10 mM TBAHS and 15 mM KH_2PO_4. The pH of the aqueous part varied between 2.5 and 8.0.

hand, Ingebretsen *et al.* [17] found maxima in the k' *vs.* pH plots of adenine nucleotides at pH 5.0.

All three groups of workers used tetrabutylammonium as pairing ion in phosphate buffer and acetonitrile [13, 14] as organic modifier. There were, however, substantial differences in the concentrations of the organic modifier and the buffer salt in the eluents used by Ingebretsen *et al.* and Sulpice *et al.* (the latter eluent components influence ionic equilibria in aqueous–organic mixtures as both of these modify the pK_a of the solute of interest [22], especially when the eluent pH is established near the pK_a of the solute). In an attempt to explain the discrepancies in the results published by the above workers, we used two eluents with different acetonitrile to phosphate

concentration ratios for studying the pH dependence of the retention of the compounds concerned.

Fig. 4 shows the k' *vs.* pH plots when an eluent with a relatively high phosphate concentration (100 m*M*) and low acetonitrile concentration (3%, v/v) was used, and Fig. 5 presents the k' *vs.* pH curves of the same compounds obtained with an eluent of moderate phosphate and acetonitrile concentrations (15 m*M* and 20%, respectively). In each k' *vs.* pH study the TBAHS, phosphate and acetonitrile concentrations were held constant. The pH of the KH_2PO_4 buffer (which at first decreased on addition of TBAHS) was adjusted using 20% (w/v) potassium hydroxide solution.

Inspection of the data in Fig. 4 shows that, using an organic solvent to counter ion concentration ratio similar to that reported by Ingebretsen *et al.*, we confirmed a maximum retention for adenine nucleotides at about pH 5, although for acids with $pK_a \approx 6.5$ the retention maxima were expected to be at a higher pH.

The chromatographic conditions under which the data in Fig. 5 were obtained were similar to those used by Sulpice *et al.* (there was some difference in the TBAHS concentrations, but these only affect the absolute and not the relative k' values). The k' *vs.* pH plots for the IPs in Fig. 5 are similar to that reported for Ins(1,3,4)P_3 by Sulpice *et al.*, but we failed to confirm the retention maxima (at pH 6.5) of adenine nucleotides which they reported. In our hands, the k' *vs.* pH plots for the nucleotides showed maxima at pH 4.5. We confirmed, however, that the k' *vs.* pH curves of IPs and nucleotides show opposing tendencies in the pH range 5.5–7.0 under such conditions.

Our k' *vs.* pH data (for IPs and ATP) and the results published by Shayman and BeMent [13] are, on the other hand, difficult to reconcile, irrespective of the chromatographic conditions used. The pK_a values of these phosphates make the retention maxima (reported at pH 3.5) very unlikely.

When the respective plots in Figs. 4 and 5 are compared, it is notable that the position of the maxima of the nucleotide curves is much less influenced by the organic solvent to counter ion concentration ratio in the eluent than the position of the maxima of the IP curves.

If we accept $k' = 20$ as a practical maximum for the retention of the compound eluted last in an isocratic system, we have pH 3 and pH 7 (in Figs. 4 and 5) to compare the retention behaviours of the compounds studied in eluents of similar average elution strength. The influence of pH (alone) on retention can be studied when elution orders obtained at pH 3 and 7 in the same figure (Fig. 4 or Fig. 5) are compared.

When we compare the elution orders obtained at the same pH, but in different figures (Figs. 4 and 5; *i.e.*, at different acetonitrile to phosphate concentration ratios in the eluent), we see that at both pH 3 and 7 the retentions of c-AMP and some IPs [Ins(1,4)P_2 and Ins(1,4,5)P_3] are affected most, as was found earlier (at pH 5.0) on studying the plots in Figs. 2 and 3.

The combined effect of the eluent pH and acetonitrile to phosphate concentration ratio on RP-IPC selectivity in the separation of IPs and nucleotides can be studied by comparing the elution order at pH 3 in Fig. 4 with that at pH 7 in Fig. 5, and similarly the elution order at pH 7 in Fig. 4 with that at pH 3 in Fig. 5. In the latter instance the relative retentions of eight solutes out of ten are affected considerably, demonstrating the combined power of these eluent parameters to influence the selectivity in RP-IPC.

Separation of myo-inositol phosphates and some isomers

One problem in the isocratic RP-IPC separation of the IPs mentioned comes from the fact that Ins(1)P_1 is poorly retained (or not retained at all) from solvents that elute Ins(1,3,4,5)P_4 in a reasonable length of time (at $k' \leqslant 15$). On the other hand, if Ins(1)P_1 has a satisfactory retention ($k' \approx 1.5$–2.0), Ins(1,3,4,5)P_4 does not elute from the column in the solvent used.

Another problem is the separation of isomers. Whereas the RP-IPC separation of the IPs presented in Figs. 1–5 was relatively easy, the Ins(1,3,4)P_3 and Ins(1,4,5)P_3 isomers showed very close similarities in chromatographic behaviour throughout the retention studies (Figs. 1–5). The same applies to inositol bisphosphate isomers [although authentic isomers were not available, our Ins(1,4)P_2 probably contained a mixture of positional isomers]. In other words, the sclectivity factor, α ($\alpha = k'_2/k'_1$, where k'_1 and k'_2 are the k' values of peaks 1 and 2, with peak 2 eluting later), for the isomers is low in the RP-IPC systems studied.

When the α values for peaks 1 and 2 (*e.g.*, the inositol trisphosphate isomers) are in the range 1.105–1.109 (which was typical in most of the RP-IPC systems studied), and we have a tolerably high retention for peak 2 ($10 < k'_2 < 15$), a plate count of at least 4500 is needed for a baseline separation of these peaks (we pretested the efficiency of the RP-IPC columns using AMP and ATP as test compounds in the eluent employed, and an average plate count of 4500 was considered to be the necessary minimum).

When the k' values are below the range suggested, columns with even higher plate counts are necessary for the separation of the isomers. In this work, we used columns with an average plate count of about 7500 (for AMP and ATP). Such col-

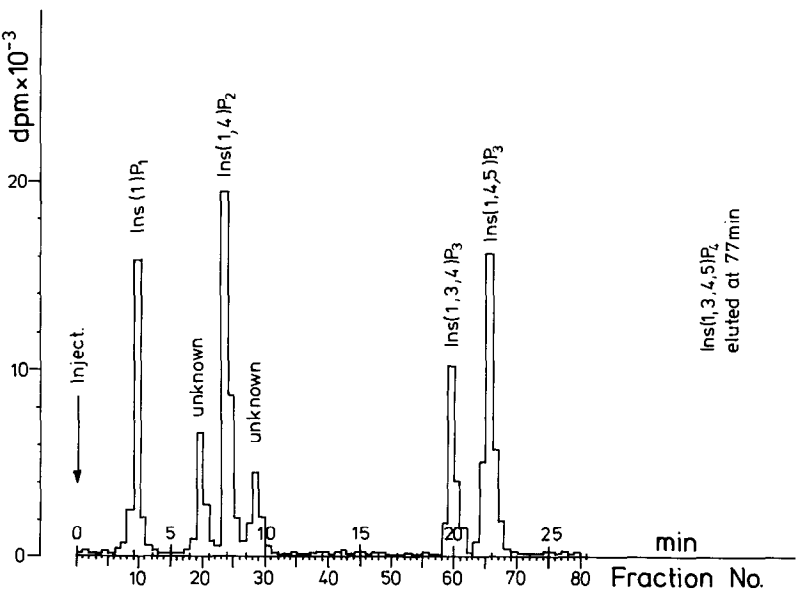

Fig. 6. Separation of myo-inositol phosphates in an isocratic RP-IPC system. Column: Nucleosil C_{18} (5 μm), 200 × 4.0 mm I.D., with a 20 × 4.0 mm I.D. precolumn ($t_0 = 1.66$ min). The eluent consisted of 12% acetonitrile, 10 m*M* TBAHS and 15 m*M* KH_2PO_4. The pH of the aqueous part was adjusted to 5.0.

umns also facilitated the separation of what we assume to be inositol bisphosphate isomers [we could not even speculate, however, whether our systems can separate Ins(1)P_1 and Ins(4)P_1, a major inositol phosphate in cells, as the former eluted in a single peak and the latter was not available]. In order to maintain the required column efficiency, columns should be checked for plate counts every 2–3 days during a series of analyses and, if necessary, the precolumn should be replaced.

A representative chromatogram of [^{3}H]IPs obtained in an isocratic system is shown in Fig. 6. A poor retention for Ins(1)P_1 and a very strong retention for Ins (1,3,4,5)P_4 are the weak points of the isocratic RP-IPC system. The separation of the isomers, however, is excellent. An obvious answer to the problem presented in Fig. 6 is a gradient mode of elution.

Gradient elution is not very common with RP-IPC systems as RP-IPC ionic equilibria ("disturbed" by a gradual increase in the volume percentage of the organic modifier) are time consuming to restore. In the RP-IPC separation of IPs and nucleotides, however, the eluent strength can also be increased by the use of salts (see Fig. 3) and a "mixed gradient" (with increasing acetonitrile and phosphate concentrations by the stronger eluent, eluent B) can be accomplished in a shorter span of acetonitrile concentrations than a "normal" gradient in which eluent strength is increased by acetonitrile alone. Also, a smaller change in acetonitrile concentrations disturbs the basic equilibrium in an RP-IPC process (the partition of the pairing ion between the stationary and the mobile phases) to a lesser extent, as was discussed in relation to

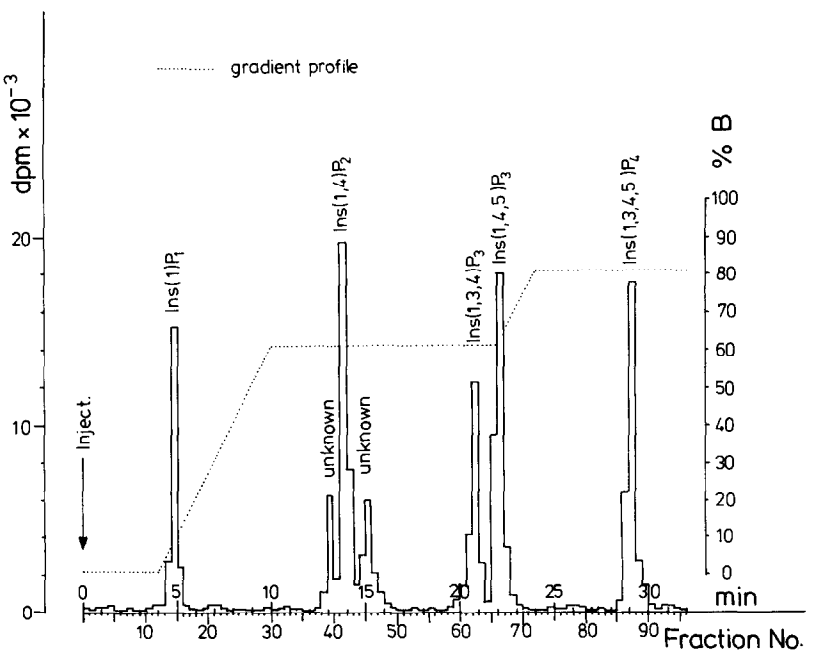

Fig. 7. Separation of myo-inositol phosphates in a gradient RP-IPC system. Column as in Fig. 6. Eluent A consisted of 10 m*M* TBAHS and 15 m*M* KH_2PO_4, the pH being adjusted to 5.0. Eluent B consisted of 30% acetonitrile, 10 m*M* TBAHS and 35 m*M* KH_2PO_4, the pH of the aqueous part being 5.0. The gradient was 1% B (4 min), 1–61% B (6 min), hold (12 min), 61–81% B (2 min), hold (8 min).

Fig. 1. With a mixed gradient (and a relatively high pairing ion concentration), a re-equilibration period of 20 min between chromatographic runs was sufficient and permitted reproducible gradient RP-IPC separations. Day-to-day and run-to-run system reproducibilities (gradient runs) were checked through the retention of AMP, ATP and Ins(1,4,5)P_3 and were, on average, within 2% and 0.2%, respectively.

A representative gradient separation of all the [^{3}H]IPs studied is shown in Fig. 7. The peaks marked "unknown" are thought to be inositol bisphosphate isomers. For the separation of the isomers we chose isocratic conditions (actually "near-isocratic" conditions as there is always a delay in eluent strength during gradient elution). As shown, the retention of Ins(1)P_1 is satisfactory, the isomers are well resolved and the last peak is eluted within 30 min. [^{3}H]Inositol leaves the column unretained and is eluted in the first 4 min even if a relatively large amount of activity is placed on the column. The chromatographic conditions presented in Fig. 7 are likely to provide satisfactory separations of [^{3}H]inositol phosphates also in biological samples.

Separation of myo-inositol phosphates, some common nucleotides and sugar phosphates

The technique of ^{32}P labeling is said to be less expensive and more efficient than ^{3}H labeling, and can be used with all types of cells without permeabilization treatments [14]. However, these advantages are considerably diminished by the fact that ^{32}P labeling leads to a large number of highly labeled phosphorylated compounds in cell or tissue extracts (nucleotides, dinucleotides, sugar phosphates, etc.), among

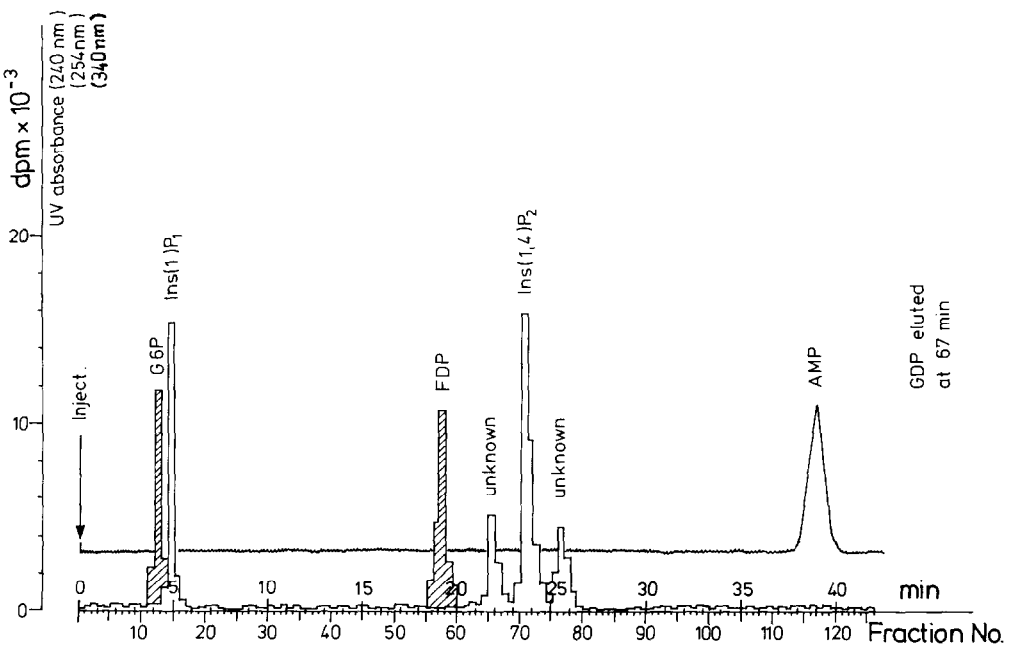

Fig. 8. Separation of some early eluting IPs, nucleotides and sugar phosphates in an isocratic RP-IPC system. Column as in Fig. 6. The eluent consisted of 10 m*M* TBAHS and 15 m*M* KH_2PO_4. The pH of the eluent was adjusted to 5.0.

which IPs represent only a few percent of the total radioactivity. In such instances, the separation of IPs from the interfering compounds is a challenging task even if efficient extraction and concentration steps are combined with efforts to reduce selectively the amount of nucleotides in the extracts [14,23,24]. The retention and separation studies in this work provide some means for controlling elution orders and peak spacing in order to cope with such interference problems.

An isocratic RP-IPC system for the separation of some early eluting phosphates is presented in Fig. 8. Ins(1)P_1 and G6P are partially resolved, but Ins(1,4)P_2 is well separated from both FDP and the nucleotides. This isocratic system was used as the initial step in the gradient separation of all the compounds studied (see Fig. 9). Between the peaks of Ins(1)P_1 and FDP, ample space is left for some (unknown) early eluting phosphorylated compounds in a biological sample. Another function of the relatively long (12 min) initial step is to "preserve" the elution order shown in Fig. 8 during the steep gradient step, by which Ins(1,4)P_2 can be eluted before a group of (otherwise interfering) nucleotides. This objective is also helped by a substantial delay in elution strength (the actual elution strength appearing on the surface of the adsorbent is well below that indicated by the gradient profile for a given moment of the separation). While maintaining a satisfactory resolution for all the compounds of

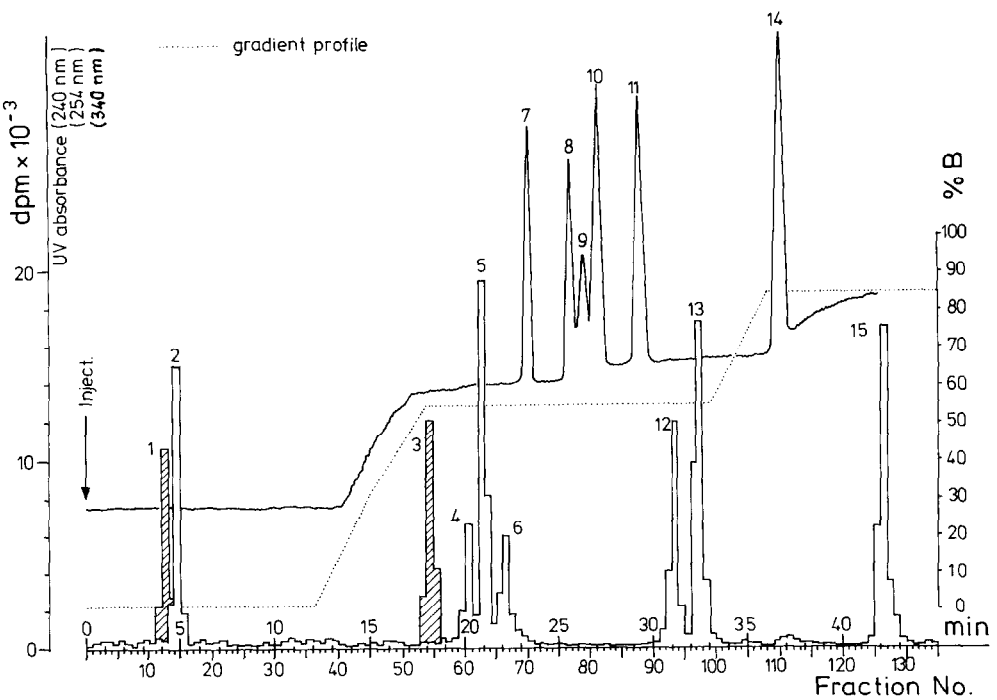

Fig. 9. Separation of myo-inositol phosphates, some common nucleotides and sugar phosphates in a gradient RP-IPC system. Column as in Fig. 6. Eluents A and B as in Fig. 7. The gradient was 1% B (12 min), 1–31% B (3 min), 31–55% B (3 min), hold (15 min), 55–85% B (3 min), hold (6 min). The compounds separated were (1) G6P, (2) Ins(1)P_1, (3) FDP, (4 and 6) unknowns, (5) Ins(1,4)P_2, (7) AMP, (8) GDP,(9) c-AMP, (10) ADP, (11) GTP, (12) Ins(1,3,4)P_3, (13) Ins(1,4,5)P_3, (14) ATP and (15) Ins(1,3,4,5)P_4.

interest, the whole RP-IPC gradient run takes less than 45 min at a flow-rate of 1 ml/min, providing a faster separation of these compounds than the anion exchange HPLC methods published so far.

CONCLUSIONS

The results of this study provide further evidence of the usefulness of the RP-IPC approach in the separation of myo-inositol phosphates, nucleotides and sugar phosphates. The RP-IPC system selectivity, which is very good for Ins(1)P_1, Ins(1,4)P_2, Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and all the nucleotides examined, can be influenced considerably by changing the pH and the organic modifier to buffer salt concentration ratio in the eluent. For the separation of inositol trisphosphate isomers, columns with plate counts of at least 4500 are needed.

Isocratic RP-IPC systems are useful only if two to five closely related IPs are to be separated. However, under proper chromatographic circumstances, gradient RP-IPC separations can also be carried out reproducibly, followed by a 20-min re-equilibration period. Complex gradient separations can be accomplished in less than 45 min under mild chromatographic conditions (using less than 50 m*M* phosphate in the eluent), so RP-IPC is likely to become a useful alternative to established anion-exchange HPLC separation techniques in the determination of myo-inositol phosphates and other organic phosphates in biological samples.

ACKNOWLEDGEMENTS

The authors thank Mrs. E. Blazsek for excellent technical assistance and Mrs. Á. Csikós for typing the manuscript. They also thank S. Juhász of B.S.T. (Budapest, Hungary) for packing the chromatographic columns.

REFERENCES

1 P. W. Majerus, T. M. Connolly, V. S. Bansal, R. C. Inhorn, T. D. Ross and D. L. Lips, *J. Biol. Chem.*, 263 (1988) 3051–3054.
2 M. J. Berridge, *Biochem. J.*, 212 (1983) 849–858.
3 K. A. Wreggett and R. F. Irvine, *Biochem. J.*, 245 (1987) 655–660.
4 A. L. Leavitt and W. R. Sherman, *Methods Enzymol.*, 89 (1982) 9–18.
5 B. W. Agranoff, P. Murthy and E. B. Seguin, *J. Biol. Chem.*, 258 (1983) 2076–2078.
6 R. F. Irvine, E. E. Anggard, A. J. Letcher and C. P. Downes, *Biochem. J.*, 229 (1985) 505–511.
7 N. M. Dean and J. D. Moyer, *Biochem. J.*, 242 (1987) 361–366.
8 G. P. Heathers, T. Juchne, L. J. Rubin, P. B. Corr and A. S. Evers, *Anal. Biochem.*, 176 (1989) 109–116.
9 C. T. Hung and R. B. Taylor, *J. Chromatogr.*, 202 (1980) 333–345.
10 R. S. Deelder and J. H. M. van den Berg, *J. Chromatogr.*, 218 (1981) 327–339.
11 M. Patthy and R. Gyenge, *J. Chromatogr.*, 449 (1988) 191–205.
12 J. Poggioly, J.-C. Sulpice and G. Vassort, *FEBS Lett.*, 206 (1986) 292–297.
13 J. A. Shayman and D. M. BeMent, *Biochem. Biophys. Res. Commun.*, 151 (1988) 114–122.
14 J.-C. Sulpice, P. Gascard, E. Journet, F. Rendu, D. Renard, J. Poggioly and F. Giraud, *Anal. Biochem.*, 179 (1989) 90–97.
15 V. Jagannathan, K. Singh and M. Damodaran, *Biochem. J.*, 63 (1956) 94–105.
16 R. J. L. Bondar and D. C. Mead, *Clin. Chem.*, 20 (1974) 586–590.
17 O. C. Ingebretsen, A. M. Bakken, L. Segadal and M. Farstad, *J. Chromatogr.*, 242 (1982) 119–126.
18 J. C. Kraak, K. M. Jonker and J. F. K. Huber, *J. Chromatogr.*, 142 (1977) 671–688.

19 Q. Xianren and W. Baeyens, *J. Chromatogr.*, 456 (1988) 267–285.
20 B. A. Bidlingmeyer, *J. Chromatogr. Sci.*, 18 (1980) 525-539.
21 H. Sigel, K. Becker and D. B. McCormick, *Biochim. Biophys. Acta*, 148 (1967) 655–664.
22 J. L. M. van de Venne, J. L. H. M. Hendrikx and R. S. Deelder, *J. Chromatogr.*, 167 (1978) 1–16.
23 J. L. Meek, *Proc. Natl. Acad. Sci. U.S.A.*, 83 (1986) 4162–4166.
24 G. W. Mayr, *Biochem. J.*, 254 (1988) 585–591.