Journal of Chromatography, 442 (1988) 307–315
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 459

DETERMINATION OF [^{32}P]PHOSPHOAMINO ACIDS IN PROTEIN HYDROLYSATES BY ISOCRATIC ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received January 18th, 1988)

SUMMARY

We have developed a simple method for phosphoamino acid analysis of ^{32}P-labeled phosphoproteins using anion-exchange high-performance liquid chromatography (HPLC). Phosphoproteins undergo partial acid hydrolysis and the resulting hydrolysate is injected directly onto a column. The sample is then isocratically eluted from the column by 35 m*M* phosphoric acid at pH 3.0 with collected fractions analyzed by Cerenkov counting. Phosphoamino acid identification is accomplished by the comparison of the retention times of ^{32}P-labeled peaks to retention times of phosphoamino acid standards which had been monitored at 206 nm. This method has greater sensitivity and is more reliable than cellulose thin-layer electrophoresis and the results obtained by high-efficiency Cerenkov counting can be evaluated immediately, instead of waiting days or weeks for autoradiographic development of cellulose plates. This HPLC protocol is an improvement over other published HPLC protocols in that there is no need for pre- or post-column derivatization and the free [^{32}P]phosphate elutes long after the phosphoamino acids. Thus sensitivity is increased as there is no interference from the free phosphate. Selection of an HPLC anion-exchange column is critical for this separation. Only two of the four columns that we tested performed well. We present data from several phosphoproteins including calcium–calmodulin dependent protein kinase, the β-subunit of the insulin receptor, and phosphorylated calmodulin to demonstrate the utility of this procedure.

INTRODUCTION

Biological phosphorylation is an important process by which cells regulate various enzymatic or hormonal processes[1]. Biological phosphorylation of proteins is mainly studied in terms of O-monoester linkages with the hydroxyl groups of

serine, threonine, and tyrosine. Other amino acids such as arginine and histidine can be biologically phosphorylated[2,3] but these phospho derivatives are unstable under the conditions of standard protein purification procedures. Phosphorylation is most commonly found on serine and threonine yet tyrosine phosphorylation has recently generated much interest in the fields of viral transformation and growth factor mechanisms[4]. Phosphotyrosine identification can be difficult to establish as normal cells contain only 0.02–0.06% of their total phosphoamino acid content as phosphotyrosine and transformed or growth factor-stimulated cells yet still only contain 0.10–0.50% phosphotyrosine as part of their total phosphoamino acid content[4].

Phospho-monoester analysis is usually accomplished by either one- or two-dimensional thin-layer electrophoresis (TLC)[4–6] or by using high-performance liquid chromatography (HPLC) with fluorscence or radioactivity detection[7–12]. In our experience, thin-layer electrophoresis is less than ideal because (i) the volt-hours required for the total separation of phosphoserine, phosphothreonine, and phosphotyrosine can vary; (ii) the analysis is slow and tedious; and most importantly (iii) small quantities such as 100–200 cpm [^{32}P]phosphotyrosine may require several weeks to visualize on X-ray film via autoradiography. This last characteristic is especially inconvenient in tyrosine kinase research but can be overcome by direct Cerenkov counting of HPLC fractions. We prefer not to utilize pre- or post-column derivatization because (i) there is a high degree of uncertainty and sample loss; (ii) pre- or post-column derivatization may require dedicated instrumentation thus restricting those instruments from multiple functions; and (iii) when manipulations are performed on a small amount of sample the results tend to lack reproducibility. Therefore we wished to develop a simple and direct method for the detection and quantitation of [^{32}P]phosphoamino acids present in protein samples.

This paper describes our routine isocratic anion-exchange HPLC method for phosphoamino acid analysis for [^{32}P]phosphoproteins labeled either *in vitro* or *in vivo*. Examples are given for the analysis of calmodulin, calcium–calmodulin dependent protein kinase, and the β-subunit of the insulin receptor. Column choice, pH effect, and buffer concentration are discussed in terms of phosphoamino acid and free phosphate retention times for optimal separation.

MATERIALS AND METHODS

O-Phosphothreonine (L-2-amino-3-hydroxybutanoic acid 3-phosphate), O-phosphoserine (L-2-amino-3-hydroxypropanoic acid 3-phosphate), and O-phosphotyrosine [L-3-(4-hydroxyphenyl)-alanine 4′-phosphate] were purchased from Sigma (St. Louis, MO, U.S.A.). Adenosine 5′-[γ-^{32}P]triphosphate (ATP) triethylammonium salt was obtained from Amersham (Arlington Heights, IL, U.S.A.). Ribose 5′-phosphate, adenosine 3′-monophosphate (3′-AMP), guanosine 3′-monophosphate (3′-GMP), and uridine 3′-monophosphate (3′-UMP) were purchased from United States Biochemical (Cleveland, OH, U.S.A.). Autophosphorylated calcium/calmodulin dependent protein kinase was used as an example of a protein phosphorylated on serine and threonine residues[13]. The β-subunit of the insulin receptor and phosphorylated calmodulin were used as examples of proteins containing phosphotyrosine[13]. Calcium–calmodulin dependent protein kinase isolated from radiation-induced insulinoma cells was a gift from M. Landt (Washington University, St. Louis, MO,

U.S.A.). The kinase was allowed to undergo autophosphorylation in the presence of calcium, calmodulin, and [γ-^{32}P]ATP. ^{32}P-β-Subunit of rat adipocyte insulin receptor autophosphorylated on tyrosine was a gift from J. M. McDonald (Washington University) who had prepared it by the method of Graves *et al.*[14]. [^{32}P]Calmodulin phosphorylated on tyrosine 99[15] by the insulin receptor tyrosinekinase[14] was also obtained from J. M. McDonald.

Preparation of [^{32}P]phosphoamino acids

[^{32}P]Phosphoproteins were precipitated with 10–20% trichloroacetic acid (TCA) and were allowed to set at 4°C for 1 h prior to centrifugation. The supernatant was discarded and the resultant pellet was resuspended in 1 ml of double-distilled water and evaporated to dryness in a rotary evaporator (Savant Instruments, Farmingdale, NY, U.S.A.) to remove residual TCA. Volumes of 200 μl of 6 *M* hydrochloric acid and 10 μl of phenol were added to the dried precipitate and the mixture was heated at 110°C for 1 h under vacuum in a Pico-Tag workstation (Waters Assoc., Milford, MA, U.S.A.). The phosphoester bond on phosphotyrosine is very acid labile. It has been shown that all phosphotyrosines present in phosphoproteins are destroyed after 4 h of acid hydrolysis at 110°C in 6 *M* hydrochloric acid. Therefore we chose a hydrolysis time of 1 h in an attempt to preserve the integrity of the phosphotyrosines present in the sample. This consideration is especially important if one is analyzing a small amount of material. The resulting hydrolysate was mixed with 1 ml of double-distilled water and redried in the rotary evaporator. Samples were dissolved in 50 μl of the appropriate mobile phase and injected directly onto the column.

HPLC

HPLC analysis was performed using a Varian Model 5000 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) equipped with a UV-100 detector containing an 8-μl flow cell and a Valco 6-port injection valve (Anspec, Ann Arbor, MI, U.S.A.) fitted with a 50-μl sample injection loop. Chromatograms were recorded on either a Varian 402 data station or a Kipp & Zonen dual channel recorder (Anspec, Ann Arbor, MI, U.S.A.).

Four different anion-exchange columns were used in the study; Column A, an experimental quaternary amine 250 × 4.6 mm I.D. column containing 5 μm Vydac silica with 300 Å pores, but not commercially available, was obtained from The Separations Group (Hesperia, CA, U.S.A.); column B, a 250 × 4.6 mm I.D. Vydac oligonucleotide column (No. 304OL54) based on a DEAE-type weak pellicular ion-exchange coating; column C, a 250 × 4.6 mm I.D. Vydac quaternary amine strong-anion exchanger (No. 301TP104); and column D, a 250 × 4.6 mm I.D. ProteoPac polystyrene based quaternary amine anion-exchanger obtained from Dionex (Sunnydale, CA, U.S.A.), recommended by the manufacturer for protein separations only. All columns employed in the study were run at a flow-rate of 0.6 ml/minute.

[^{32}P]Phosphoamino acid analysis was performed at ambient temperature by isocratic elution of the protein hydrolysates using 35 m*M* phosphoric acid, pH 3.0, as the mobile phase. The pH was adjusted by addition of 10 *M* potassium hydroxide. The pH was measured with a Corning 130 pH meter with a GK2321C Radiometer-Copenhagen combination electrode (Radiometer America, Westlake, OH, U.S.A.).

Non-radioactive phosphoamino acid standards were detected by monitoring the column eluent at 206 nm. Fractions of 0.3 ml were collected for the entire period of the HPLC run starting from the time of injection. Radioactivity in the fractions was assessed by Cerenkov counting on a Packard Tricarb 4640 scintillation counter (Packard Instruments, Downers Grove, IL, U.S.A.).

RESULTS AND DISCUSSION

An example of the complete separation of phosphothreonine, phosphoserine, phosphotyrosine and free [^{32}P]phosphate on column A is shown in Fig. 1a. This separation was achieved utilizing 35 m*M* phosphoric acid, pH 3.0, as the mobile phase. Fig. 2a–d illustrates the effect of pH on the retention times of the phosphoamino acids and free [^{32}P]phosphate separated on the various anion-exchange columns used in this study. Columns A and B gave the best resolution of the phosphoamino acids and the free [^{32}P]phosphate but these separations were also the most pH sensitive. In the case of column A in Fig. 2a, the resolution between phosphoserine and phosphotyrosine was obscured when the pH was lower than 2.8, when the pH was raised above 3.3 the [^{32}P]phosphate peak interfered with the identification of phosphotyrosine. The advantage of this anion-exchange technique over cation-exchange methods[9,11] is that [^{32}P]phosphate is the last rather than the first component eluted in the analysis. This is an important point as phosphoprotein hydrolysis generates relatively large amounts of free [^{32}P]phosphate which if eluted early could interfere with the detection of small amounts of later eluting phosphoamino acids. This characteristic is especially helpful when trace amounts of [^{32}P]phosphotyrosine need to be distinguished from large amounts of free [^{32}P]phosphate, such as the case for the β-subunit of the insulin receptor shown in Fig. 1c. Separation of all of the phosphoamino acids and free [^{32}P]phosphate also occurred at pH 2.0 on column A but the late eluting free [^{32}P]phosphate significantly increases the analysis time. However, separation at pH 2.0 can serve as a second dimensional confirmation of trace phosphorylation as the elution order between phosphoserine and phosphotyrosine is reversed. The elution order on column A at pH 3.5 is the same as that demonstrated by Robert *et al.*[12] on a strong anion-exchange Partisil 10-SAX column at pH 4.0, but column A gave increased resolution between phosphotyrosine and free [^{32}P]phosphate. Phosphate of 35 m*M* gave the best overall resolution in our initial survey of buffer concentrations on column A (data not shown). Since Robert *et al.*[12] also found 35 m*M* phosphate to be an optimal buffer concentration, we decided to perform the pH optimum studies shown in Fig. 2 at this fixed concentration. Column C eluted all of the phosphoamino acids and free [^{32}P]phosphate within 10 min as shown in Fig. 2c. It is known that decreasing phosphate concentration would increase elution time[12], so column C was tested using 15 m*M* phosphate in an attempt to increase resolution. Results showed that although retention times were increased, the resolution was not significantly enhanced (data not shown).

Three well-characterized phosphoproteins were analyzed in this study utilizing column A to test the analysis technique. [^{32}P]Autophosphorylated calcium–calmodulin dependent protein kinse was used as an example of a protein phosphorylated on serine and threonine[13]. Results shown in Fig. 1b demonstrate the detection of [^{32}P]phosphoserine and [^{32}P]phosphothreonine. The β-subunit of the rat adipocyte

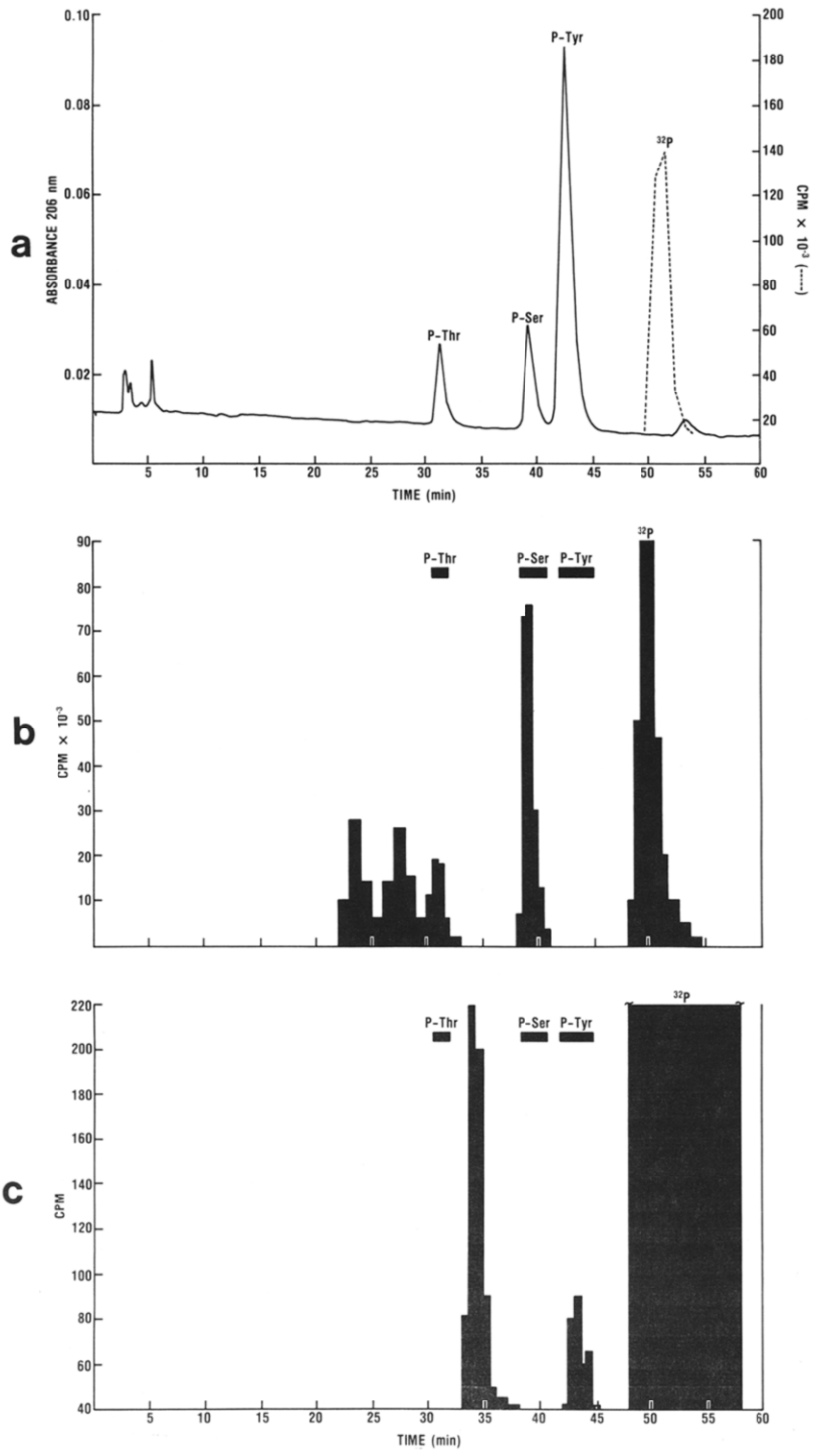

Fig. 1. (a) Isocratic elution of phosphothreonine (57 μg), phosphoserine (112 μg), phosphotyrosine (10 μg), and $^{32}P_i$ from a quaternary amine anion exchanger (column A in Materials and methods section). (b) Phosphoamino acid analysis from acid hydrolysis of autophosphorylated calcium–calmodulin dependent protein kinase. (c) Analysis of autophosphorylated insulin receptor β-subunit. Mobile phase, 35 m*M* phosphoric acid, pH 3.0; Flow-rate, 0.6 ml/min; UV wavelength, 206 nm, a.u.f.s.

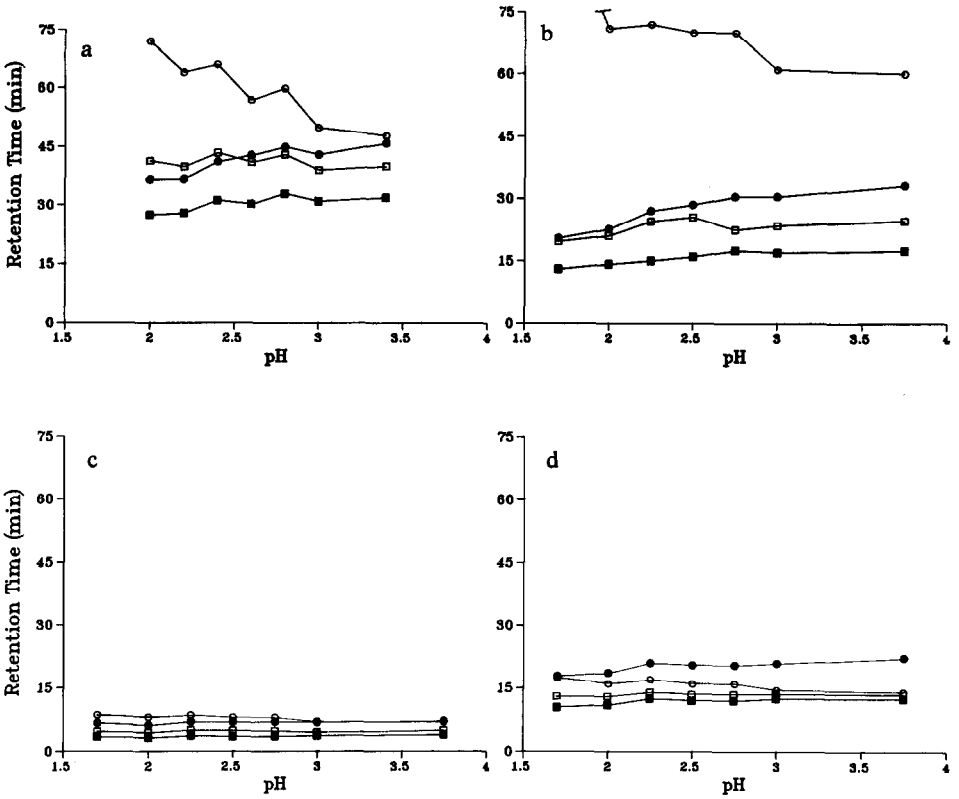

Fig. 2. Retention time as a function of pH for phosphoamino acids and free phosphate on four anion-exchange columns. a–d Correspond to columns A, B, C, and D in Materials and methods section. The buffer concentration was kept constant at 35 m*M* phosphoric acid. (■) phosphothreonine; (□) phosphoserine, (●) phosphotyrosine, and (○) [^{32}P]phosphate.

insulin receptor was used as an example of a protein phosphorylated on tyrosine[13,16,17]. As can be seen in Fig. 1c, the recovery of [^{32}P]phosphotyrosine was very low yet it was still separated, detected, and identified by HPLC in a matter of a few hours. Another example of [^{32}P]phosphotyrosine identification is shown in Fig. 3b for the *in vivo* phosphorylation of calmodulin by the tyrosine kinase present on the insulin receptor[14].

HPLC analysis for [^{32}P]phosphoamino acid determination is about 60 min on either column A or column B. However, this time can be reduced by increasing the flow-rate from 0.6 ml/min to 1.5–2.0 ml/min or by increasing the buffer concentration[12], but we prefer to allow extra time between [^{32}P]phosphoamino acid peaks because of ^{32}P-labeled contaminants which may interfere with the analysis[4,5,12]. For example, the sample may be contaminated with unhydrolyzed [^{32}P]phosphopeptides as observed by Robert *et al.*[12] in the case of [^{32}P]phosphohistone. It is also important to note that ^{32}P-labeled nucleotides, especially 3′-UMP, are known to be major side products of *in vivo* phosphorylation experiments. This has been observed in two-dimensional electrophoretic analysis experiments[5]. Therefore an important aspect of

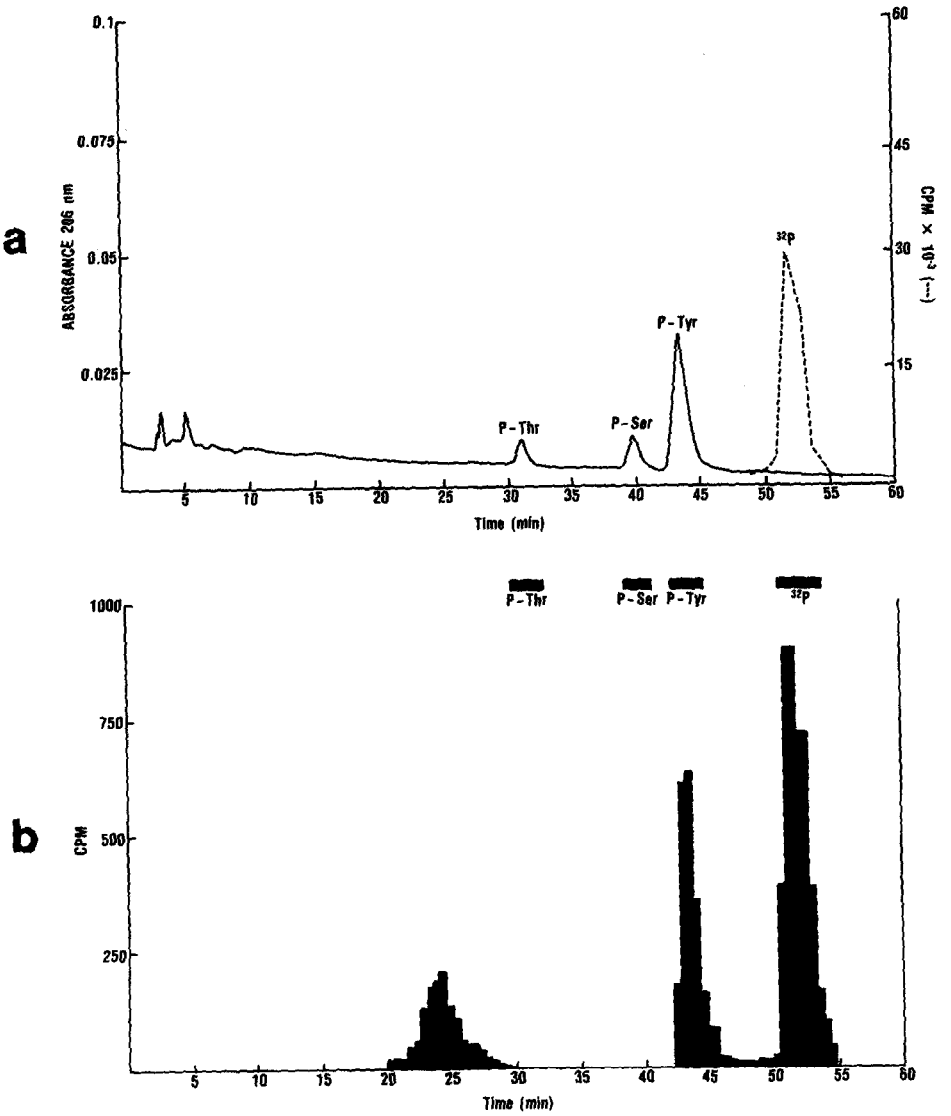

Fig. 3. (a) Phosphoamino acid standards, same elution conditions and column as in Fig. 1 except for injected mass of standards; phosphothreonine (10 μg), phosphoserine (20 μg), and phosphotyrosine (2 μg). (b) Analysis of calmodulin phosphorylated by the insulin receptor tyrosine kinase. Elution conditions and column same as in Fig. 1.

our HPLC method is that possible radiolabeled 3'-UMP, 3'-CMP and ribose-5'-phosphate contaminants do not co-elute with the [32P]phosphoamino acids (Fig. 4).

In summary we report the complete resolution of [32P]phosphothreonine, [32P]phosphoserine, [32P]phosphotyrosine, and free [32P]phosphate resulting from [32P]phosphoprotein acid hydrolysates by isocratic anion-exchange HPLC. Total HPLC analysis time was 60 min on either column A or B. Resolution was not satis-

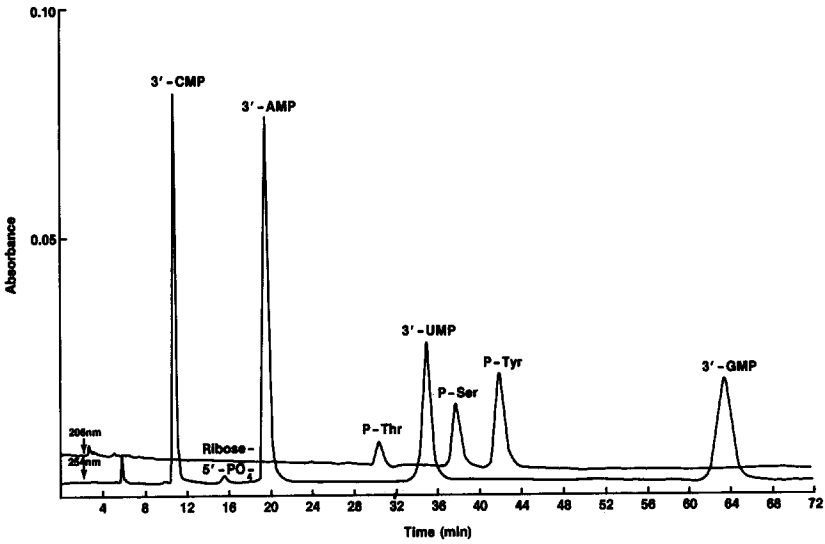

Fig. 4. Isocratic elution of mononucleotides (7 μg each), ribose-5′-phosphate (7 μg), phosphothreonine (35 μg), phosphoserine (20 μg), and phosphotyrosine (2.5 μg) from quaternary amine anion-exchange column A. Same conditions as Fig. 1 with the addition of 254 nm UV detection for mononucleotides and the ribose-5′-phosphate.

factory on either column C or D. Column B, the Vydac DEAE-type pellicular exchanger is recommended for routine use because of its commercial availability. [^{32}P]Phosphoamino acids were detected by Cerenkov counting with as little as 100 cpm of the [^{32}P]phosphoamino acid present in the sample. Cerenkov or even liquid scintillation counting negates the need for pre- or post-column derivatization. The results of the analysis after the hydrolysis step can be established in a matter of hours as opposed to days or even weeks as in the case of thin-layer electrophoresis with autoradiography.

ACKNOWLEDGEMENTS

We thank Drs. Martin Deibel and Ferenc Kezdy for their helpful comments and Mrs. Paula Lupina for assisting in the preparation of this manuscript.

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