

## Note

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### Separation of basic, hydrophilic peptides by reversed-phase ion-pair chromatography

#### IV. Preparative applications related to purification of two substrates of cyclic AMP-dependent protein kinase

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Recently we developed an analytical high-performance liquid chromatography (HPLC) system for basic, hydrophilic peptides of synthetic origin, related to the phosphorylatable site of pyruvate kinase and used in substrate specificity studies of cyclic AMP-dependent protein kinase<sup>1,2</sup>. This system also proved to be useful in the analysis of the corresponding phosphopeptides<sup>3</sup>. The basic idea was quite simple, namely to chromatograph the peptides as ion-pairs and simultaneously increase their affinities for reversed-phase column supports by selecting hydrophobic counter-ions. Thus, it turned out that pentane sulphonate and hexane sulphonate could be used for this purpose. So far we have used a non-volatile phosphate buffer with an alcohol, generally ethanol, as organic modifier, and UV detection. This system worked well in some biochemical applications<sup>4</sup>.

Soderling and Walsh<sup>5</sup> recently reported the separation of two peptides from the corresponding phosphopeptides as well as from dehydroalanine peptides. One of their peptides is related to the ones we have studied.

From the outset we have been interested in applying ion-pair HPLC on a preparative scale for purification of our synthetic protein kinase substrates. Therefore we investigated various counter-ions and volatile buffers for their applicability in this context. This paper reports some results from preparative experiments with related crude peptides on a 10-mg scale.

#### MATERIALS AND METHODS

##### *Peptides*

The octapeptide used, Val-Leu-Arg-Arg-Ala-Ser-Val-Ala, was made by a (2 + 6) fragment condensation. The corresponding protected peptide could not be completely purified from its hexapeptide precursor. So the free peptide contained some C-terminal hexapeptide<sup>6</sup>. The heptapeptide, Leu-Arg-Arg-Ala-Ser-Val-Ala, was from a new batch, made by the solid-phase method<sup>7</sup> and prepared as described previously<sup>8</sup>. However, amino acid analysis in this case indicated only 90–92% incorporation of leucine.

### Chemicals

Most chemicals were the same as described previously<sup>1,3</sup>. The volatile buffers were made up from formic acid and triethylamine (TEA) to an ionic strength of 0.1 *M*. *d*-10-Camphorsulphonic acid, 99%, was obtained from Chemical Dynamics (South Plainfield, NJ, U.S.A.). It was recrystallized from glacial acetic acid before use. The strong anion-exchanger, Amberlite resin IRA-400 (Cl), was obtained from BDH (Poole, U.K.). It was regenerated with acetic acid and washed with glass-distilled water to pH 5. Sephadex G-15 was obtained from Pharmacia (Uppsala, Sweden).

### Equipment

The liquid chromatography system consisted of a Model 6000 A solvent-delivery system, a Model 450 variable-wavelength detector (Waters Assoc., Milford, MA, U.S.A.) and a syringe-loading sample injector (Rheodyne, Cotati, CA, U.S.A.).

The separation columns (150 × 4.5 mm I.D.) were, for analytical purposes, packed with Spherisorb C<sub>18</sub> (5 μm) reversed-phase support and the preparative column (250 × 10 mm I.D.) was packed by Jones Chromatography (Llanbradach, U.K.) with the same material. All columns were always preceded by a short guard column containing Bondapak C<sub>18</sub>/Corasil, 37–50 μm (Waters). The Spherisorb support was obtained from Phase Separations (Queensferry, U.K.).

### Procedures

The preparative separations, ion-exchange and gel filtration procedures, were performed at room temperature (*ca.* 22°C). The analytical HPLC columns were thermostatted at 25.0 ± 0.1°C using home-made equipment. The pumps were run isocratically at a nominal flow-rate of 1–4 ml/min. The injector loop was loaded with up to 300 μl of solution, corresponding to *ca.* 10 mg of peptide. The volatile buffers were removed by lyophilization. Acetate was exchanged for the remaining counterions with Amberlite, processed as described above. Final desalting was performed on a Sephadex G-15 column (1100 × 16 mm I.D.) in 10% acetic acid.

All chemicals and solvents were of analytical or reagent grade when not otherwise stated.

### RESULTS

For our first preparative experiment we chose a sample of Val-Leu-Arg-Arg-Ala-Ser-Val-Ala containing some Arg-Arg-Ala-Ser-Val-Ala as impurity, which we knew we could separate on an analytical scale<sup>1</sup>. Fig. 1a demonstrates an analytical separation under our present standard conditions. (In all cases, detailed experimental data are shown in the corresponding legends.) In order to proceed preparatively and thus recover products from the separated fractions, a volatile buffer system was required. A triethylammonium formate buffer replaced phosphate. Most parameters were identical in the two experiments (column, pH, counter-ion, nature, and even the proportions of organic modifier), but the counter-ion concentration was increased, resulting in a higher capacity, and the detector wavelength had to be changed slightly because the intrinsic absorption of the formate buffer was too high. As shown in Fig. 1b, this volatile system seemed quite satisfactory for our octapeptide.

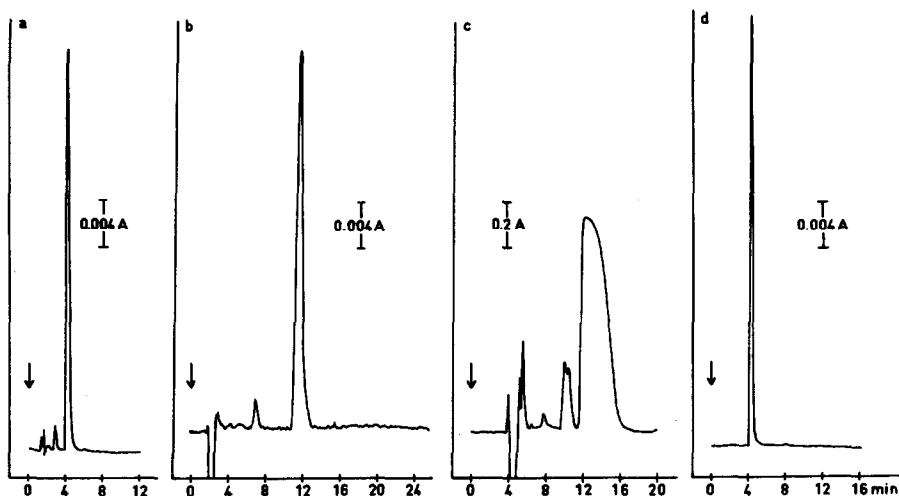


Fig. 1. Separation of the octapeptide, Val-Leu-Arg-Arg-Ala-Ser-Val-Ala, from impurities with pentane sulphonate as counter-ion. (a) and (d) Analytical separations, before and after purification, in phosphate buffer (pH 3.0)–ethanol (72:28); flow-rate 1.0 ml/min; counter-ion, pentane sulphonate (0.015 *M*); detection wavelength ( $\lambda$ ), 210 nm; column, Spherisorb  $C_{18}$  (5  $\mu$ m, 150  $\times$  4.6 mm I.D.). (b) Analytical separation on the crude peptide using volatile buffer [triethylamine–0.1 *M* formic acid (pH 3.0)–ethanol (72:28)]; counter-ion, pentane sulphonate (0.05 *M*);  $\lambda$  = 224 nm; column and flow-rate as for (a). (c) Preparative separation (ca. 10 mg) in the same system as for (b); all parameters were as for (b) except the column (250  $\times$  10 mm I.D.) and the flow-rate (3.0 ml/min). Arrows indicate injection.

In the preparative experiment (Fig. 1c) a column 10-mm wide was used, containing the same reversed-phase material as the analytical column. As shown, the applied sample, 10 mg, was completely resolved. The material from five runs was pooled and lyophilized, and then the counter-ion, pentane sulphonate, was removed by passing the solution through an anion-exchange column, regenerated and equilibrated as mentioned above, followed by another lyophilization. After final desalting and two additional lyophilizations, the octapeptide was obtained in pure form (Fig. 1d) as shown by analysis under the same conditions as used in Fig. 1a. The total yield over all three steps, preparative HPLC, ion-exchange and desalting, was 64% as determined by amino acid analysis after acid hydrolysis of aliquots of crude and purified materials.

Fig. 2b demonstrates an experiment identical with that in Fig. 1b, except that *d*-10-camphorsulphonate was used instead of pentane sulphonate. The corresponding preparative experiment (Fig. 2c) in this case gave even better resolution than with pentane sulphonate. The further processing of the purified fraction was as described in the preceding section. In this case the yield was 70%. The product was as pure as in the previous case (Fig. 2d).

We also applied the new procedure to the purification of a peptide made by the solid-phase procedure<sup>7</sup>. Fig. 3a shows an analytical chromatogram of crude Leu-Arg-Arg-Ala-Ser-Val-Ala in the phosphate system with pentane sulphonate as counter-ion. The preparative experiment was in this case performed with only *d*-10-camphorsulphonate as counter ion. Although in neither the analytical system (Fig. 3b) nor the preparative experiment (Fig. 3c) with this counter-ion was the separation

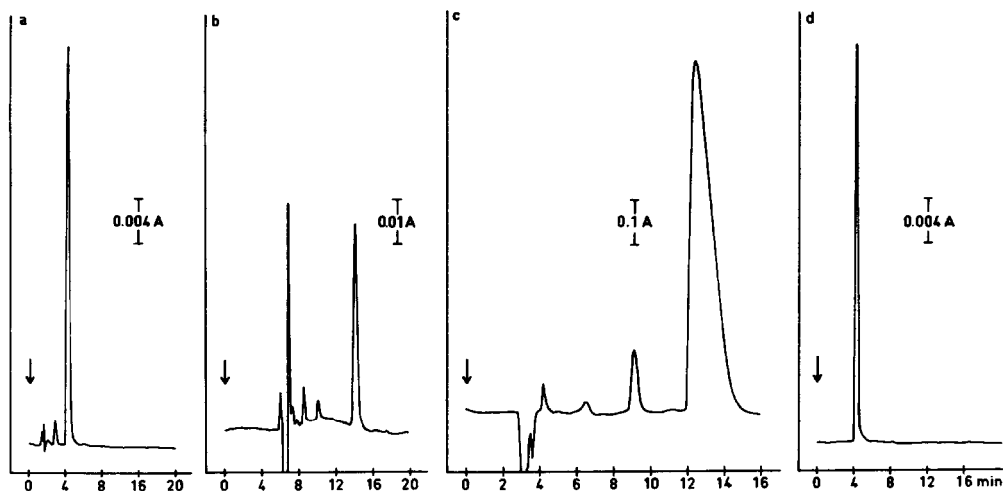


Fig. 2. Separation of the octapeptide from impurities with *D*-10-camphorsulphonate as counter-ion. (a) and (d) As in Fig. 1. (b) Analytical separation in volatile buffer [triethylamine-0.1 *M* formic acid (pH 3.0)-ethanol (65:35)]; counter-ion, *D*-10-camphorsulphonate (0.05 *M*); flow-rate, 2.0 ml/min;  $\lambda = 224$  nm; column, 250  $\times$  10 mm I.D. (c) Preparative separation (ca. 4 mg) in the same system as for (b), flow-rate, 4.0 ml/min.

quite as good as for the octapeptide, after the corresponding two additional ion-exchange and desalting steps the product was perfectly pure (Fig. 3d). The yield was above 90% for the heptapeptide.

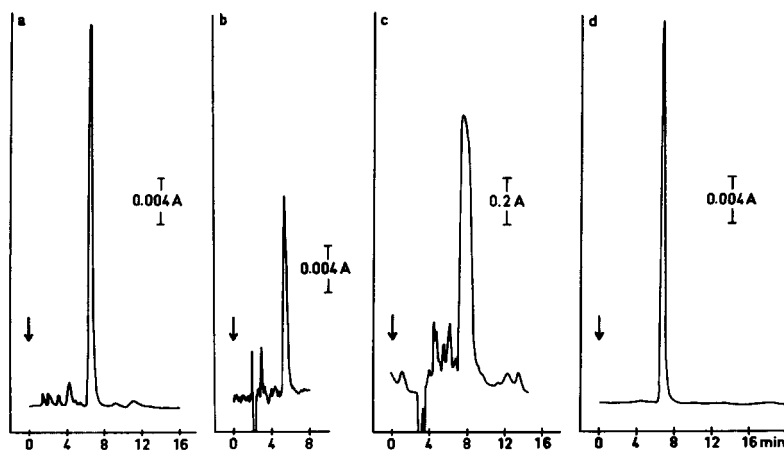


Fig. 3. Separation of the heptapeptide, Leu-Arg-Arg-Ala-Ser-Val-Ala, from impurities with *D*-10-camphorsulphonate as counter-ion. (a) and (d) Analytical separations, before and after purification, in phosphate buffer (pH 3.0)-ethanol (79:21); all parameters as in Fig. 1. (b) Analytical separation on the crude peptide using volatile buffer; all parameters as in Fig. 1 except the counter-ion, which was *D*-10-camphorsulphonate as in Fig. 2. (c) Preparative separation (ca. 10 mg) in volatile buffer [triethylamine-0.1 *M* formic acid (pH 3.0)-ethanol (72:28)]; all other parameters were the same as in the previous preparative experiment.

## DISCUSSION

Our results demonstrate that the previously reported analytical procedure for protein kinase substrates, derived from the phosphorylatable site of pyruvate kinase, can, after slight modification, also be applied to the purification of such substances of synthetic origin. Such peptides are rather small and can nowadays be prepared quite rapidly and very efficiently<sup>7</sup>. Rapid and efficient procedures are therefore also required for their analysis and purification. HPLC is faster than most other current techniques, therefore, our purification procedure has the advantage of speed and convenience and profits from the general merits of this analytical technique.

As pointed out already, the required modification of the earlier procedure was to eliminate the phosphate buffer and introduce instead a volatile buffer that would allow the buffer salts, apart from the sulphonate, to be removed simultaneously with the aqueous organic solvent by evaporation and/or lyophilization afterwards. A pH of *ca.* 3 had earlier been found advantageous for the separation of peptides of this type<sup>1</sup>. Of various buffers available<sup>9</sup>, we chose one containing triethylammonium formate, because of its UV-transparency in the region of the peptide bond absorption below 250 nm. When buffer solutions containing pentane sulphonate or the less expensive *d*-10-camphorsulphonic acid were used with ethanol as chromatographic eluent, separation of the various components was indeed accomplished (Figs. 1b, 2b, and 3b). Similar patterns were obtained in the preparative experiments (Figs. 1c, 2c, and 3c).

After lyophilization the products contained substantial amounts of non-volatile sulphonate. This was removed by simple filtration through an anion-exchange column. After another lyophilization, sodium ions from the pentane sulphonate still remained and had to be removed by desalting on a Sephadex G-15 column. The last step was also included when *d*-10-camphorsulphonic acid was used.

To summarize, the procedure described above allows 10-mg amounts of basic peptides to be purified rapidly and efficiently with a minimum of manipulations afterwards. Only inexpensive chemicals and solvents are needed. As larger columns are becoming available at reasonable prices, purification of basic peptides by ion-pair chromatography on a larger scale might become attractive.

## ACKNOWLEDGEMENTS

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