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Journal of Chromatography A, 667 (1994) 119–124

JOURNAL OF CHROMATOGRAPHY A

Effect of mobile phase composition on the separation of thyrotropin-releasing hormone and some metabolites by reversed-phase ion-pair chromatography

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(Received November 26th, 1993)

Abstract

Sodium dodecyl sulphate was used for the separation of thyrotropin-releasing hormone (TRH) and the related compounds deamido-TRH (TRHOH), histidylprolinediketopiperazine, proline and prolineamide by reversed-phase ion-pair chromatography. The effects of mobile phase composition on retention and selectivity were determined. The parameters studied included acetonitrile, pairing ion and salt concentrations, salt type and pH. The results show that the separation of TRH and its analogue TRHOH can be easily adjusted by small modifications of the pH in the vicinity of pH 2. A remarkable improvement of peak width and peak shape was observed for some analytes when a potassium salt was added to the mobile phase.

1. Introduction

Reversed-phase ion-pair chromatography (RP-IPC) has become a popular technique for the separation of peptides. Hydrophilic and hydrophobic pairing ions have been used and their effects on peptide retention, selectivity and peak shape have been widely described [1–6]. Small polar peptides, such as thyrotropin-releasing hormone (TRH) and other di- and tripeptides, have been separated by the addition of hydrophobic alkyl sulphonates or alkyl sulphates, from C_5 to C_{12}, to the mobile phase [4–6]. The separation and elution time were improved by varying the percentage of organic modifier in the eluent and the chain length of the pairing ion.

In previous studies [7,8] we have shown that other parameters, such as pairing ion concentration, salt concentration, salt type and pH, can also be used to manipulate retention times and selectivity in RP-IPC with hydrophobic pairing ions. However, the multiple possibilities provided by this technique have not been sufficiently exploited in the case of peptides.

One purpose of this paper is to show the effect of each mobile phase component on the retention of small peptides and amino acids in RP-IPC. The other is the application of these effects to improve the separation of the important tripeptide TRH and some metabolites.

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0021-9673/94/$07.00
SSDI 0021-9673(93)E1305-J

2. Experimental

2.1. Apparatus and materials

Chromatographic experiments were carried out on a Waters (Milford, MA, USA) HPLC system consisting of two piston pumps, Models 45 and 590, an RCM-100 radial compression module, a Model 481 UV spectrophotometer and a U6K valve injector. Radial compression cartridges, Resolve C_{18} from Waters (10 cm × 8 mm I.D., particle size 10 μm, containing 4 g of packing), were used throughout. Fixed conditions for all experiments were isocratic elution, flow-rate 1.3 ml/min, ambient temperature and detection wavelength 215 nm.

HPLC-grade acetonitrile (density 0.78 kg/l) from Merck (Darmstadt, Germany) and purified water obtained from a Milli-Q system (Millipore, Bedford, MA, USA) were used to prepare sample solutions and mobile phases. Sodium dodecyl sulphate (SDS) from Bio-Rad Labs. (Richmond, CA, USA) was used as the pairing ion. Salts added to the mobile phase, lithium, sodium and potassium dihydrogenphosphate, were obtained from J.T. Baker (Phillipsburg, NJ, USA). The pH of the eluent was adjusted with orthophosphoric acid (J.T. Baker). Sample solutes, pyroglutamylhistidylprolineamide (TRH), proline (PRO) and prolineamide ($PRONH_2$), were purchased from Sigma (St. Louis, MO, USA), and pyroglutamylhistidylproline (TRHOH) and histidylproline-diketopiperazine (DKP) from Peninsula Labs. (San Carlos, CA, USA).

2.2. Methods

For each experiment, the pH of an aqueous solution containing the required amounts of SDS and one of the inorganic salts was adjusted with 10% orthophosphoric acid solution. Mobile phases were prepared in 500-ml volumetric flasks, adding the above solution, a weighed amount of acetonitrile and the necessary volume of water.

The volume of mobile phase needed to equilibrate the column was evaluated for each experiment. The following equation, relating adsorption of alkyl sulphates on reversed phases to mobile phase composition, was used [7]:

$$\log[C]_{st} = -0.25 - 0.66\varphi_{org} + 0.06N_c + \log[C]_m[0.72 + 1.68\varphi_{org} - 0.05N_c] \quad (1)$$

where $[C]_{st}$ is the concentration of pairing ion in the stationary phase at equilibrium (mmol/g), $[C]_m$ is the concentration of this species in the eluent (mmol/ml), φ_{org} is the volume fraction of acetonitrile in the mobile phase (w/v fraction/density) and N_c is the number of carbon atoms in the alkyl sulphate molecule.

The volume of eluent that must pass through the column to reach equilibrium (V_{eq}) was calculated from $[C]_{st}$ using the relationship

$$V_{eq} = \frac{[C]_{st}M_{st}}{[C]_m} + V_m \quad (2)$$

where M_{st} is the mass of stationary phase in the column and V_m is the void volume.

For dodecyl sulphate and the different mobile phase compositions used in this work, the calculated volume of eluent to equilibrate the Resolve C_{18} cartridge varied from 37 to 78 ml. Eq. 1 was deduced for a different reversed-phase packing (LiChrosorb RP-8 from Merck), and therefore equilibrium was always tested by repetitive injections of a solute before injection of the sample.

Hold-up times were determined by injection of $NaNO_3$ solution. Measurements were made for each mobile phase composition, but without a pairing ion present.

After each experiment, the column was washed with 30 ml of acetonitrile–water (1:1, v/v) to remove all SDS from the stationary phase.

All peptides and amino acids were taken up in 0.01% acetic acid and, prior to injection, made up in the mobile phase. The amount of each compound in 5 μl of injected sample varied from 0.25 to 3 μg. Fig. 1 shows the structures of the analytes used in this work.

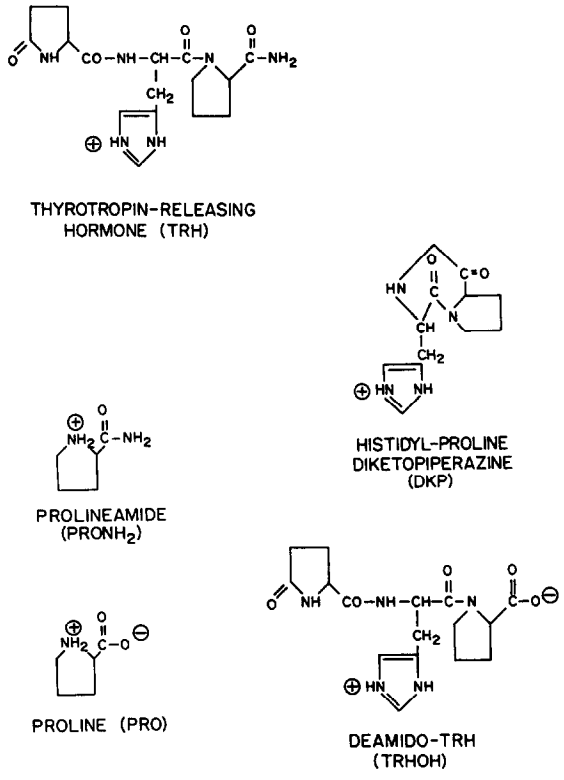

Fig. 1. Structures of TRH and some metabolites.

3. Results and discussion

The percentage of acetonitrile and the concentration of SDS in the mobile phase have a strong effect on solute retention, as illustrated in Figs. 2 and 3. For all solutes, the capacity factor, k', decreases almost exponentially when the concentration of acetonitrile in the eluent increases. On the other hand, k' increases with increasing pairing ion concentration but tends to a constant value at high concentrations. Both effects are characteristic of RP-IPC with hydrophobic pairing ions and have been observed with many different solutes [7–10].

Figs. 2 and 3 also show that the selectivity slightly increases with increase in retention, but TRH and its analogue TRHOH cannot be baseline separated, even at the highest k'. However, the separation of these compounds can be dramatically improved by a small change in the pH of the mobile phase, from 2.15 to 2.5 (Fig. 4). This is possible because TRHOH has an ionizable carboxylic acid group (Fig. 1) with a pK_a value of *ca.* 2. Small pH changes near this value induce large variations in the solute net

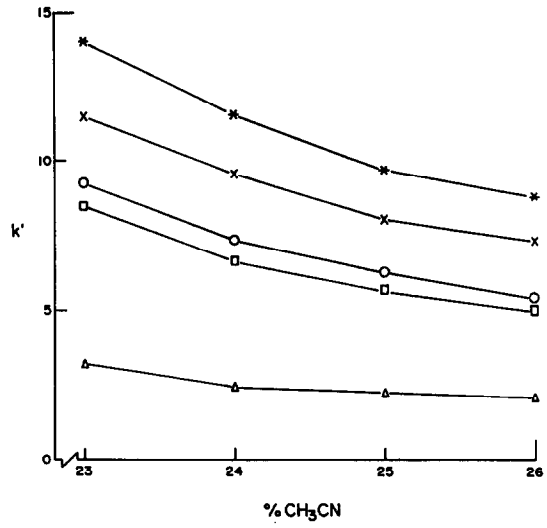

Fig. 2. Effect of acetonitrile concentration (in %, w/v) on the retention of small peptides and amino acids. Mobile phase, acetonitrile–water containing 5.19 m*M* SDS and 15 m*M* LiH_2PO_4 (pH 2.15). △ = PRO; □ = TRHOH; ○ = TRH; × = $PRONH_2$; * = DKP.

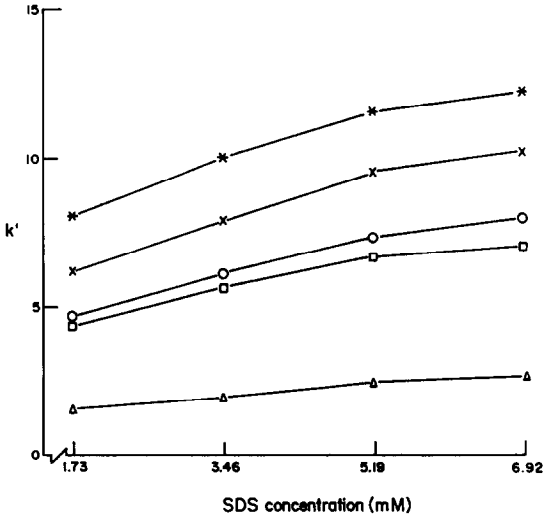

Fig. 3. Effect of pairing ion concentration on the retention of TRH and metabolites. Mobile phase, acetonitrile (24%, w/v)–water containing SDS and 15 m*M* LiH_2PO_4 (pH 2.15). Symbols as in Fig. 2.

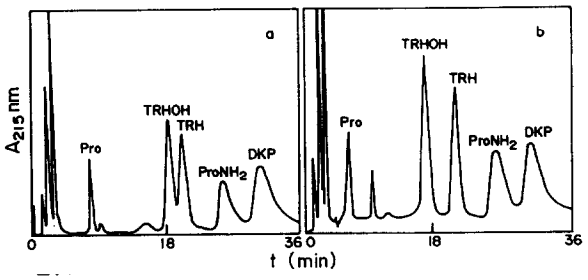

Fig. 4. Influence of pH on the separation of TRH and TRHOH. Mobile phase, acetonitrile (24%, w/v)–water containing 5.19 m*M* SDS and 15 m*M* LiH_2PO_4. (a) pH 2.15; (b) pH 2.5.

charge. As the pH and ionization of the carboxylic group increase, the interaction of TRHOH with the negatively charged dodecyl sulphate becomes weaker and retention progressively decreases. The same effect is observed for the amino acid proline.

In previous work [7,8], we demonstrated that an ion-exchange mechanism is, in great part, responsible for solute retention in RP-IPC with hydrophobic pairing ions. Therefore, another parameter that can be used to control the separation and analysis time is the salt concentration in the eluent and, in particular, the concentration of ions of the same charge as the solute (co-ions). Fig. 5 shows the effect of Li^+ concentration on retention and selectivity. It can be seen that retention decreases in a hyperbolic manner with increase in Li^+ concentration and, because this effect is stronger for the most retained solutes, selectivity is also affected. Complete separation of TRH and TRHOH can be achieved, even at pH 2.15, by using low salt concentrations, but at the expense of longer analysis times and more asymmetric peaks for $PRONH_2$ and DKP (Fig. 6).

Co-ions compete with solutes for association with the adsorbed pairing ion. Different co-ions have different affinities for the ionic stationary phase. Therefore, the solute retention also depends on the type of co-ion present in the mobile phase. Fig. 7 shows the separation of the five solutes using an eluent of pH 2.85 with a Li^+, Na^+ or K^+ salt. The retentions of TRH, $PRONH_2$ and DKP decrease in the order $Li^+ <$

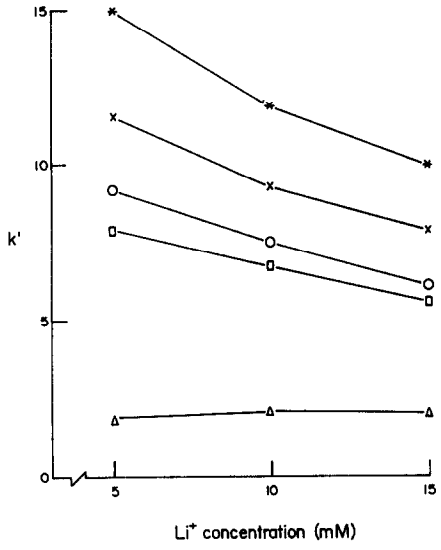

Fig. 5. Effect of salt concentration on the retention of TRH and its metabolites. Mobile phase, acetonitrile (24%, w/v)–water containing 5.19 m*M* SDS and LiH_2PO_4 (pH 2.15). Symbols as in Fig. 2.

$Na^+ < K^+$, meaning that this is the affinity order of the three cations in the RP-IPC system. In fact, the same affinity order is found in ion-exchange chromatography.

There are two interesting observations in Fig. 7. First, PRO is almost unretained and TRHOH behaves ambiguously, probably because at pH 2.85 their net charge approaches zero and the effects of pairing ion and co-ion are negligible. Second, the wide peaks of $PRONH_2$ and DKP become narrower and more symmetrical when K^+ is present in the mobile phase. To our knowledge, this effect of co-ion type has not been reported before in RP-IPC.

4. Conclusions

RP-IPC with hydrophobic pairing ions is a powerful technique for the separation of small peptides. The retention can be adjusted by variation of at least four parameters of the mobile phase: acetonitrile, pairing ion and salt concentrations and salt type. Changes in the proportion of organic solvent in the eluent have the strongest effect on solute retention. There-

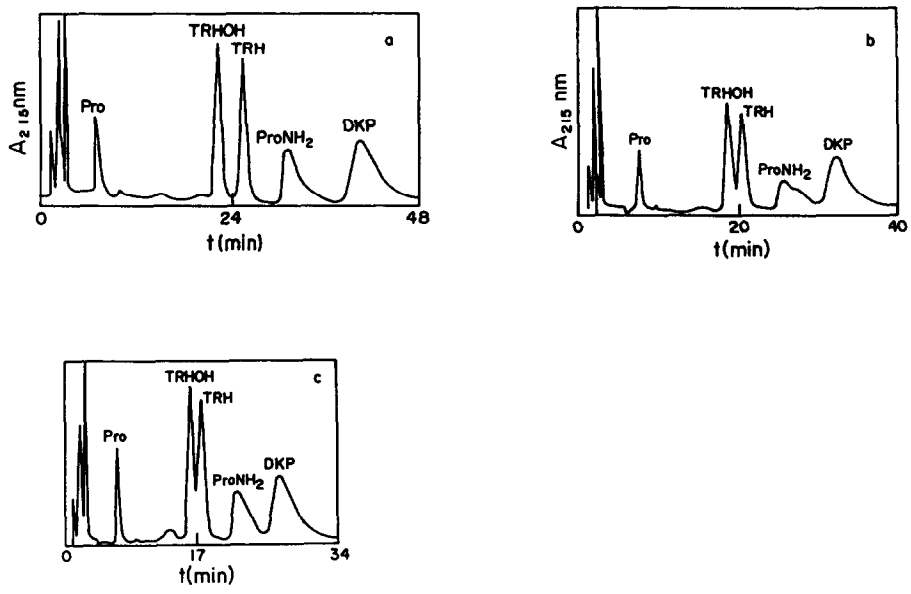

Fig. 6. Separation of TRH and some metabolites at various concentrations of LiH_2PO_4: (a) 5; (b) 10; (c) 15 m*M*. Other conditions as in Fig. 5.

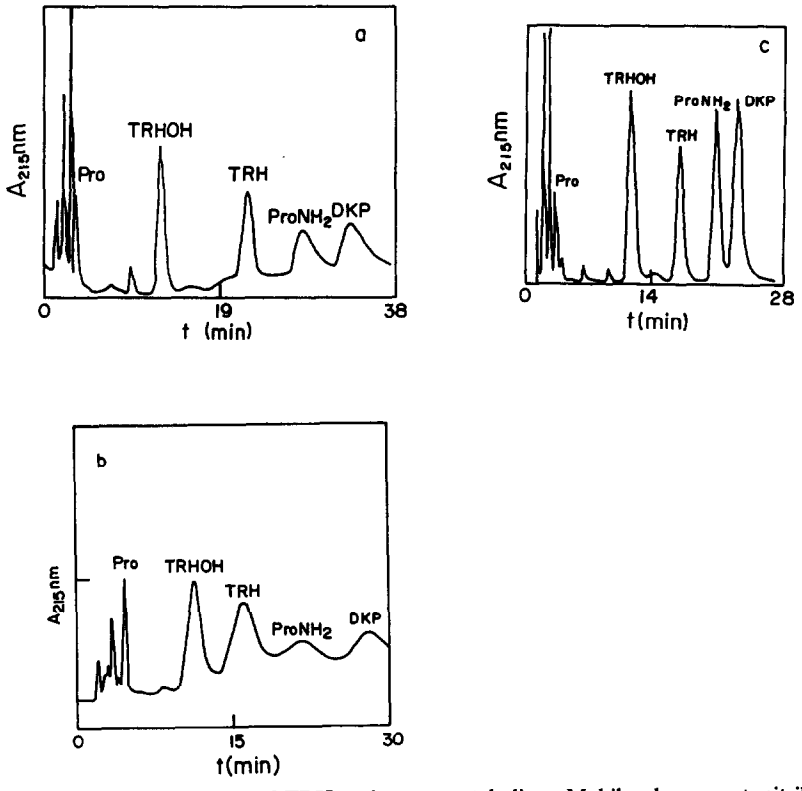

Fig. 7. Influence of salt type on the separation of TRH and some metabolites. Mobile phase, acetonitrile (24%, w/v)–water containing 5.19 m*M* SDS and 15 m*M* phosphate salt (pH 2.85). (a) LiH_2PO_4; (b) NaH_2PO_4; (c) KH_2PO_4.

fore, to obtain reproducible results, this parameter must be carefully controlled. On the other hand, the parameter of choice for improving selectivity is pH, but only if the analytes have functional acid–base groups with different pK_a values. Finally, the results of this work indicate that the peak width and peak shape of some solutes can be dramatically modified by a change of co-ion, even if the new ion is a member of the same chemical family. This interesting effect is being studied.

5. Acknowledgements

The authors are indebted to Dr. Jean Louis Charlie (Biotechnological Institute, National University of Mexico) for providing the peptides, amino acids and solvents used in this work and to Leopoldo Güereca and Salvador Antonio Pérez for technical assistance.

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