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Note

High-performance liquid chromatographic separation of tachykinins using Tris–phosphate as the buffer component of the mobile phase

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Increasing interest has been focused on the determination of neuropeptides, such as the tachykinins, in various biological samples. Their carboxy-terminal region is characterized by the amino acid sequence Phe-X-Gly-Leu-Met, where X represents the only variable within the whole family. During the past decade the originally used immunological detection methods, mainly the radioimmunoassay (RIA) technique, have been supplemented by high-performance liquid chromatographic (HPLC) methods. However, the very low amounts (pg/ml range) found in samples of serum, plasma, cerebrospinal fluid (CSF) and perfusion liquids were still detected by RIA either by direct measurement of the peptides in the biological sample (provided that a specific antibody without significant cross-reactions with structurally related peptides is available) or after prior HPLC separation of the sample. The tachykinin family, and especially substance P (SP 1–11) and its derivatives or metabolic fragments, play an important role in nociception and immunological and pharmacological pathways [1–5].

Many different procedures have been used for the HPLC separation of neuropeptides. The majority of them made use of columns consisting of octadecylsilyl residues covalently linked to a silica matrix, and the mobile phase was generally trifluoroacetic acid (TFA)–acetonitrile. Both isocratic and gradient elution systems were used [6–15]. Other mobile phases also successfully used for the purification of neuropeptides were triethylammonium phosphate (TEAP)–acetonitrile [16], triethylammonium formate (TEAF)–1-propanol [17], TEAF–

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acetonitrile [18,19], TEAF–methanol [20], sodium acetate and triethylamine (TEA)–acetonitrile [21], sodium dihydrogenphosphate–acetonitrile [22,23] and phosphoric acid–acetonitrile [24]. Either isocratic or gradient elution was performed in these cases.

We have studied the use of TEAP–acetonitrile for either isocratic or gradient elution of somatostatin-14 (SS-14) and related peptides [*e.g.* SS-28, [Tyr^0]-SS-14 and SS-28 (1–12)] and other neuropeptides such as bombesin and cholecystokinin. The mobile phases containing TFA or sodium dihydrogenphosphate proved to be less suitable in our cases. For use in HPLC–RIA coupling experiments, volatile buffer systems consisting of TEAF–acetonitrile or triethylammonium acetate (TEAA)–acetonitrile generally provided sufficient separation. Several stationary phases and mobile phases were studied for the separation of tachykinins. The results show that the inclusion of Tris–phosphate in mobile phases significantly improved the peptide separation.

EXPERIMENTAL

Reagents and chemicals

SP 1–11, SP 2–11, SP 4–11, SP 5–11, pGlu-SP 5–11 (pyroglutamyl SP 5–11), SP-COOH (SP free acid), [Tyr^8]-SP 1–11, eledoisin and physalaemin were purchased from Peninsula Labs. (Belmont, CA, U.S.A.) and were HPLC-grade according to the supplier's specifications. Tris–HCl was from Serva (Heidelberg, F.R.G.). Orthophosphoric acid (85%), formic acid, acetic acid and triethylamine (of analytical purity) for the preparation of the TEAP, TEAF and TEAA solutions were from Merck (Darmstadt, F.R.G.), and acetonitrile (HPLC grade S) was from Rathburn (Walkerburn, U.K.). Water for HPLC was purified by use of a Milli-Q reagent water system (Millipore-Waters, Eschborn, F.R.G.).

Chromatography

Chromatographic separations were performed isocratically by means of a 2150 HPLC pump (LKB, Bromma, Sweden) and a Type 7125 injection valve equipped with a 20-μl sample loop (Rheodyne, Cotati, CA, U.S.A.). Detection was performed at 210 nm (0.5 a.u.f.s.) by means of an LC–UV detector (Pye Unicam, Cambridge, U.K.) and the signals were recorded with an RDK Model 104/102 recorder (Rikadenki, Freiburg, F.R.G.). The different mobile phases consisted of 80:20 (v/v) mixtures of 0.1 *M* TEAP, TEAF, TEAA and Tris–phosphate and acetonitrile. Only in the case of a phenyl column was the percentage of acetonitrile decreased to 15%. The TEAP–acetonitrile and Tris–HCl–acetonitrile mixtures were adjusted to an apparent pH of 2.8 by dropwise addition of 85% orthophosphoric acid, whereas the TEAF–acetonitrile and TEAA–acetonitrile mixtures were adjusted with formic and acetic acid to pH 3.0 and 3.5, respectively. The following columns were tested: Hypersil ODS, 125 mm × 4.6 mm I.D., 5 μm (Shandon, Frankfurt, F.R.G.); Spherisorb ODS II, 125 mm × 4.6

mm I D., 5 μm (Gynkotek, Germering, F.R.G.); Spherisorb 5C8 (octyl), 125 mm × 4.6 mm I.D., 5 μm (Kontron, Eching, F R.G.); C_{18} "Resolve", 125 mm × 4.6 mm I.D., 5 μm (Waters, Eschborn, F.R.G.); Zorbax ODS, 250 mm × 4.6 mm I.D., 5 μm (Du Pont, Bad Homburg, F.R.G.); Phenyl, 250 mm × 4.6 mm I.D., 5 μm (Vydac Phenyl, Gynkotek).

RESULTS AND DISCUSSION

In some cases SP 1–11 and the closely related physalaemin eluted as a single

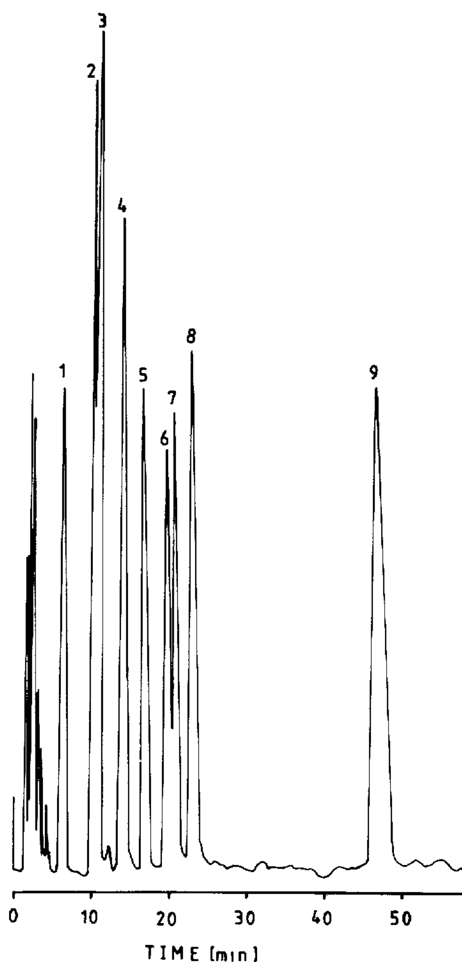

Fig 1 HPLC of a mixture of tachykinin-like peptides on a Spherisorb ODS II column (125 mm × 4 6 mm I D , 5-μm particles) The mobile phase consisted of a 80 20 (v/v) mixture of 0 1 *M* TEAP in acetonitrile adjusted to an apparent pH of 2.8 Detection wavelength, 210 nm Peaks 1 = [Tyr⁸]-SP 1–11, 2 = physalaemin, 3 = SP 1–11, 4 = SP-COOH (free acid), 5 = eledoisin; 6 = SP 2–11, 7 = SP 4–11, 8 = SP 5–11, 9 = pGlu SP 5–11

peak or at least insufficiently resolved, if TEAP, TEAF or TEAA were used as the buffer components of the mobile phases. However, the use of a Hypersil ODS or, to a minor extent, a C_{18} "Resolve" column, provided adequate separation of the two peptides. Good separation of SP 1–11 and physalaemin was also obtained by means of a phenyl column, but resolution of fragments of SP 1–11 (*e.g.* SP 2–11, SP 4–11, SP 5–11) was decreased. The Zorbax ODS column did not provide sufficient separation because the basic SP 1–11 exhibited substantial peak tailing, presumably owing to an excess of uncapped (free) silanol groups on the silica matrix. When Tris–phosphate was used as the buffer component, the separation improved with all the columns that were chosen for the test. The best increase of peak resolution was observed with a Spherisorb 5C8 (octyl) and a Spherisorb

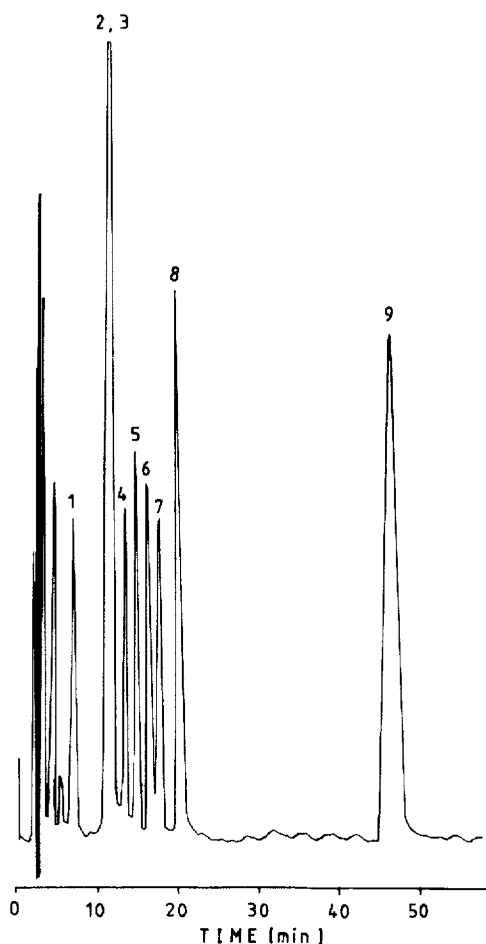

Fig 2 HPLC of a mixture of tachykinin-like peptides on a Spherisorb 5C8 column (125 mm × 4 6 mm I D., 5-μm particles) The mobile phase consisted of a 80 20 (v/v) mixture of 0 1 *M* TEAP in acetonitrile adjusted to an apparent pH of 2 8 Detection wavelength, 210 nm. Peaks as in Fig 1.

ODS II column. SP 1–11 and physalaemin were eluted as a single peak (Δt = 0 min) in the case of a Spherisorb octyl column, and they were only partially resolved (Δt = 0.5 min) by a Spherisorb ODS II column if TEAP, TEAF and TEAA were used as the buffer salts. However, when Tris–phosphate was used as the buffer component, the differences in retention times increased to 3 3 and 4.0 min, respectively.

Figs. 1 and 2 show the HPLC patterns of nine tachykinin-like peptides, separated with TEAP–acetonitrile on a Spherisorb ODS II and a Spherisorb 5C8 column, respectively. Figs. 3 and 4 show the chromatograms of the peptide mixture on the same columns obtained using a Tris–phosphate–acetonitrile mobile phase. The differences in retention times were less marked but nevertheless significant if a Hypersil ODS column (4 0 *versus* 6 min) or a C_{18} "Resolve" column (1.8

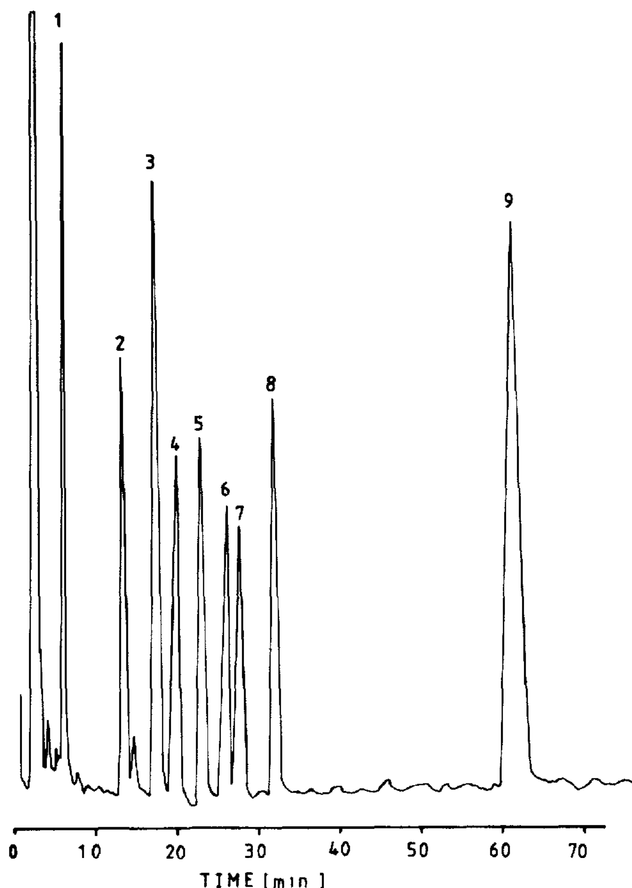

Fig 3. HPLC of a mixture of tachykinin-like peptides on a Spherisorb ODS II column (125 mm × 4.6 mm I D., 5-μm particles) The mobile phase consisted of a 80 20 (v/v) mixture of 0.1 *M* Tris–phosphate in acetonitrile adjusted to an apparent pH of 2 8 Detection wavelength, 210 nm Peaks as in Fig. 1

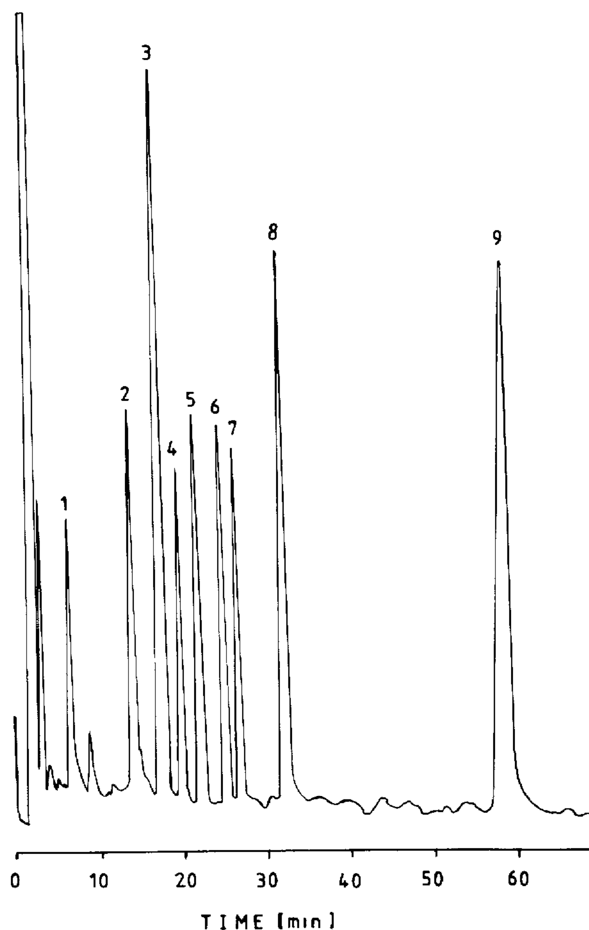

Fig 4. HPLC of a mixture of tachykinin-like peptides on a Spherisorb 5C8 column (125 mm × 4 6 mm I D , 5-μm particles) The mobile phase consisted of a 80 20 (v/v) mixture of 0 1 *M* Tris–phosphate in acetonitrile adjusted to an apparent pH of 2 8 Detection wavelength, 210 nm Peaks as in Fig 1

versus 4.0 min) were used. Additionally, separation of all other peptides tested was improved, although the differences were less substantial than in the case of the separation of SP 1–11 and physalaemin. The chromatograms obtained using TEAF and TEAA are similar to those obtained with TEAP (results not shown).

The use of octyl and phenyl columns may open new ways in peptide separation in the future. The octyl column in particular proved to be very suitable owing to the marked effect on the separation of peptides during the change of the buffer salt of the mobile phase, as demonstrated by our investigations. The effects of these minor changes to the experimental conditions on the separation may increase the number of possible applications and thus facilitate chromatographic separation of peptides and enable fine tuning of separation conditions to specific

requirements. If, in addition, variations of mobile phase composition and/or of gradient systems could be achieved, a phenyl column may represent a valuable alternative to the octadecylsilyl (ODS) columns.

With all the columns and mobile phases used for the separation, pGlu-SP 5–11 was the late-eluting component. Its retention time was markedly decreased by application of a steep mobile phase gradient, started after 25 min (results not shown). The long retention time of this peak can be explained by an increase in hydrophobicity caused by intramolecular cyclization, which additionally amplifies the non-polar character of this C-terminal fragment of SP 1–11. It should be mentioned that commercial preparations of SP 5–11 contain various amounts of pGlu-SP 5–11, and it is evident that an equilibrium between the two components is attained in solution [25], but this phenomenon was not further investigated.

At present, we cannot explain the mechanism of the improved separation of tachykinins when Tris–phosphate is used in the mobile phases. The use of TEAP and TEAA with a common cation did not improve the separation. Therefore, an interaction of the polar hydroxymethyl substituents in the Tris cation with polar segments of the tachykinins could play an important role, by additionally intensifying the ionic interaction elicited by the positively charged amino group. In contrast, ionic interactions are preponderantly possible if TEAP or TEAA are used as the buffer salts.

In summary, the addition of Tris–phosphate to the mobile phase markedly improved the separation of tachykinin-like peptides, and presumably also of other peptides of neuronal or gastrointestinal origin, compared with the hitherto used elution media. Separation would presumably be further improved by using gradient systems; these, however, should be adapted to individual problems.

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