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COMPLEMENTARY INFORMATION FROM ISOTACHOPHORESIS AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN PEPTIDE ANALYSIS

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SUMMARY

Reversed-phase high-performance liquid chromatography is a valuable analytical technique to support the synthesis, isolation and purification of peptides, as is illustrated by some critical separations. In addition to this technique, capillary isotachophoresis can give useful information on the purity determination of peptides and on the presence of ionic compounds of a non-peptidic nature. With regard to the latter aspect, isotachophoresis proved to be a suitable technique as a check on the effective removal of salts after preparative high-performance liquid chromatography.

INTRODUCTION

Adequate analytical support for complicated multi-step synthetic, isolation and purification procedures is of utmost importance in current peptide chemistry. Moreover, the end-product, *i.e.*, the purified peptide preparation, should meet high quality requirements. A wide range of analytical techniques are now available to support peptide synthesis. Among these, reversed-phase high-performance liquid chromatography (RP-HPLC), mainly performed on alkyl-bonded silica supports, has found wide application[1]. In addition, capillary isotachophoresis (ITP) has also been successfully applied to the analysis of peptide preparations[2–11].

This paper describes applications of both ITP and HPLC and the complementary nature of the information obtained is demonstrated.

EXPERIMENTAL

HPLC

All experiments were performed on an HP 1090 M liquid chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) provided with a ternary solvent delivery system, an auto-injector and autosampler and a diode-array detector. The apparatus was equipped with a computer workstation and printer/plotter facilities.

As supporting material a reversed-phase octadecylsilica (Supelcosil LC-18DB, 3-μm particles) column (150 × 4.6 mm I.D.) (Supelco, Bellefonte, PA, U.S.A.) was

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used. A guard column (20 × 4.6 mm I.D.) filled with the same material preceded the analytical column.

Details of the chromatographic conditions applied in the various separations are given in Table I. Prior to use the mobile phases were filtered and degassed with helium. Peptide samples were dissolved in the initial mobile phase and 100 μl of this solution, corresponding to 10–20 μg of peptide material, were injected. Detection was at 210 nm. The retention times (RT) and the peak areas (%) were recorded.

Uvasol-quality acetonitrile (Merck) and ultra-pure Milli-Q water (Millipore, Bedford, MA, U.S.A.) were used. Type HA filters (0.45 μm, Millipore) were used for the filtration of the aqueous mobile phases and Type FM filters (0.5 μm) for those containing acetonitrile.

ITP

All experiments were performed with a home-made apparatus constructed according to Everaerts *et al.*[12]. The polytetrafluoroethylene separation capillary was 320 mm long × 0.2 mm I.D. Resistance detection and UV detection (254 nm) were applied. A constant electric driving current was obtained from a Wallis (Worthing, U.K.) VCS 303/1 power supply (0–30 kV; 0–100 μA). Isotachopherograms were recorded using a Model BD 9-725 dual-channel recorder (Kipp & Zonen, Delft, The Netherlands).

Details of the isotachophoretic procedure were described previously[11]. For the electrolyte systems applied, see Table II.

Peptide characteristics

Human, porcine and bovine insulin were obtained from Diosynth (Oss, The Netherlands). All other peptide preparations were synthesized by the peptide chemistry group of Organon (Oss, The Netherlands); several of them had purposely

TABLE I

CHROMATOGRAPHIC CONDITIONS

All separations were performed at a flow-rate of 1.0 ml/min and a temperature of 45°C.

System	*Mobile phase*	*Elution profile*			
		Time (min)	*A (%)*	*B (%)*	*C (%)*
1	(A) 0.5 *M* NaH_2PO_4 + 0.05 *M* SOS[a] and H_3PO_4 to pH 2.1	0	30	30	40
	(B) Water	3	30	30	40
	(C) Acetonitrile–water (60:40)	10	30	25	45
		20	30	25	40
2	(A) 0.5 *M* Na_2SO_4 + 0.3 *M* NaH_2PO_4 and $HClO_4$ to pH 2.5	0	20	30	50
	(B) Water	3	20	30	50
	(C) Acetonitrile–water (60:40)	30	20	22.5	57.5
3	(A) 0.5 *M* NaH_2PO_4 and H_3PO_4 to pH 2.1	0	20	70	10
	(B) Water	3	20	70	10
	(C) Acetonitrile–water (60:40)	40	20	40	40

[a] Sodium octanesulfonate.

TABLE II

ELECTROLYTE SYSTEMS

System	*Analytes*	*Leading electrolyte*[a]			*Terminating electrolyte*[a]
		Leading ion	*Counter ion*	*pH*	
1	Neutral and basic peptides	Na^+	Acetate	4.50	β-Alanine–CH_3COOH to pH 5.00
2[b]	Acidic peptides/anions	Cl	Histidine	5.60	MES[c]–Tris to pH 5.60
3	Halides	NO_3^-	Cadmium	5.60	Tartaric acid–NaOH to pH 5.60
4	Cations	K^+	Acetate	4.80	β-Alanine–CH_3COOH to pH 4.80
5	Ammonium	H^+	Chloride	2.00	Sodium citrate–HCl to pH 7.50

[a] Concentrations 0.01 mol/l, except for cadmium nitrate (0.004 mol/l).
[b] Additive to the leading electrolyte: 0.2% hydroxypropylmethylcellulose.
[c] Morpholinoethanesulphonic acid.

not been purified before use. For the primary structures of the peptides, see Table III.

Insulin. Insulin is the well known polypeptide hormone concerned with the regulation of the carbohydrate metabolism. It contains 51 amino acid residues arranged in two covalently linked chains (A and B). Minor differences in primary structure are found between the bovine, porcine and human insulins. At room temperature and especially in acid solution insulin undergoes deamidation, principally at the A-21 asparagine residue, which is converted into the corresponding aspartic acid residue. For a reliable and rapid discrimination between the three insulins a clear mutual separation is necessary between six closely related polypeptides, *viz.*, bovine, porcine and human insulin and their respective monodesamido (MDA) derivatives.

ACTH. The adrenocorticotropic hormone ACTH contains 39 amino acid residues and has as its principal activity the stimulation of the adrenal cortex to produce and release steroid hormones. The N-terminal part ACTH-(1–24) possesses full biological activity. Fragments of ACTH-(1–24) and their analogues have been the subject of many biological and pharmacological investigations.

Org 2766. Structure–activity studies of peptides derived from ACTH with regard to avoidance behaviour in rats resulted in the development of the modified ACTH fragment Org 2766. During the synthesis of this peptide in solution the histidine residue can racemize, *i.e.*, L-histidine may be partially converted into D-histidine. A rapid and accurate determination of possible racemization is essential in this aspect. For further quality control, racemization studies of the other amino acid residues are also important.

β-Endorphin. β-Endorphin, the C-terminal 31-peptide of β-lipotropin, was originally isolated from pituitaries of several species. It was shown to have a high affinity for opiate-binding sites in the brain and to induce many behavioural effects.

In order to support metabolism studies of the candidate antipsychotic drug β-endorphin-(6–17) (Org 5878), we aimed at the separation of the parent compound and its possible metabolites. Especially the separation of β-endorphin-(6–17) from the -(7–17), -(8–17) and -(9–17) fragments was essential in this respect.

Vasopressin. The pituitary nonapeptide 8-arginine vasopressin (AVP) is long known for its influence on blood pressure and diuresis. Later it was found that AVP is also involved in behavioural processes. It has been shown that the complete

TABLE III

PEPTIDE SEQUENCES

Peptide	*Sequence*

Species	A-8	A-10	B-30
Bovine	Ala	Val	Ala
Porcine	Thr	Ile	Ala
Human	Thr	Ile	Thr

Insulin (porcine)

H-Gly-Ile-Val-Glu-Gln-Cys(10)-Cys-Thr-Ser-Ile(10)-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys(20)-Asn-OH (A-chain)

H-Phe-Val-Asn-Gln-His-Leu-Cys(10)-Gly-Ser-His(10)-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys(20)-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala(30)-OH (B-chain)

ACTH-(1–24) H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly(10)-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val(20)-Lys-Val-Tyr-Pro-OH

β-Endorphin (human) H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser(10)-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn(20)-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly(30)-Glu-OH

AVP H-Cys(5)-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

CCK-8S H-Asp-Tyr(SO_3H)-Met-Gly-Trp(5)-Met-Asp-Phe-NH_2

Org 2766 H-Met(O_2)-Glu-His-Phe-D-Lys(5)-Phe-OH

nonapeptide structure is essential for full endocrine activity while the central activity of the 9-desglycinamide fragment [AVP-(1–8) or DGAVP] remains nearly intact.

CCK. Cholecystokinin (CCK) was originally isolated from the gastrointestinal tract and found to be involved in digestive functions and feeding behaviour. Later, high concentrations, especially of a sulphated octapeptide sequence (CCK-8S), were demonstrated to be present in several brain regions.

RESULTS AND DISCUSSION

HPLC

Over a 10-year period we have performed many critical RP-HPLC peptide separations[13–15]. Some recent characteristic examples are given here.

β-Endorphin-(6–17) family. The application of ion-pair HPLC proved to be necessary in the development of an adequate mobile phase system for the separation of β-endorphin-(6–17) and fourteen fragment peptides in a 100-min run[14]. If the chromatographic parameters pH, type and concentration of the ion-pairing agent, buffer concentration and temperature are chosen well, β-endorphin-(6–17) and its N-terminally shortened fragments -(7–17), -(8–17) and -(9–17) are baseline separated in a 20-min gradient run (see Fig. 1).

Insulin. At an earlier stage we reported on the separation of bovine, porcine and human insulin and their respective MDA derivatives in a 60-min run[13]. By application of a mobile phase containing sodium sulphate and sodium dihydrogenphosphate acidified to pH 2.5 with perchloric acid, a clear separation between the six components is obtained within 30 min, as can be seen in Fig. 2. By comparison with a reference insulin preparation, three-fold information is obtained in one run: identification of species of origin, purity determination and biological potency determination. With regards to the last aspect, insulin formulations are still tested for their potency in a bioassay[16,17]. However, applying adequate reference insulin preparations of known

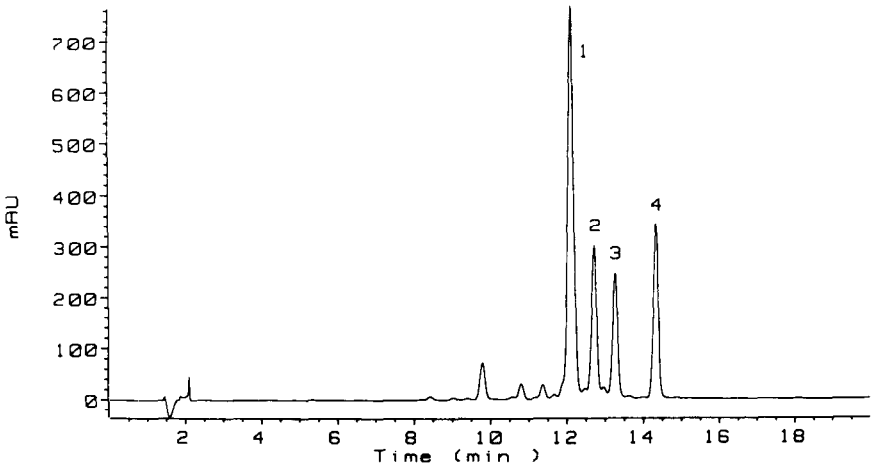

Fig. 1. HPLC separation of β-endorphin-(6–17) and its N-terminally shortened fragments. For the chromatographic conditions, see system 1, Table I. 1 = β-Endorphin-(6–17); 2 = -(7–17); 3 = -(8–17); 4 = -(9–17). mAU = milli absorbance units.

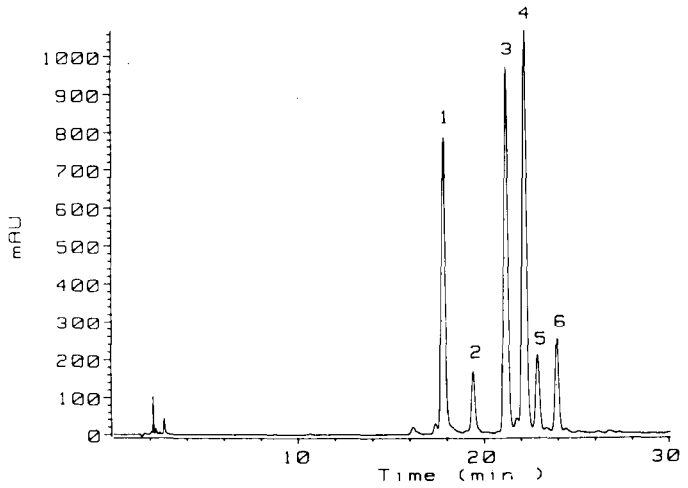

Fig. 2. HPLC separation of insulins. For the chromatographic conditions, see system 2, Table I. 1 = Bovine insulin; 2 = bovine MDA insulin; 3 = human insulin; 4 = porcine insulin; 5 = human MDA insulin; 6 = porcine MDA insulin.

potency in combination with a well validated assay procedure, HPLC seems an attractive alternative to the time-consuming, expensive and less reproducible bio-assays.

ACTH fragment analogue (Org 2766). In continuation of an earlier study in which we reported on the HPLC separation of Org 2766 and its D-histidine analogue[13], we developed a procedure in which Org 2766 and its six single D- or L-amino acid substituted diastereoisomeric peptides are separated in one run (see Fig. 3).

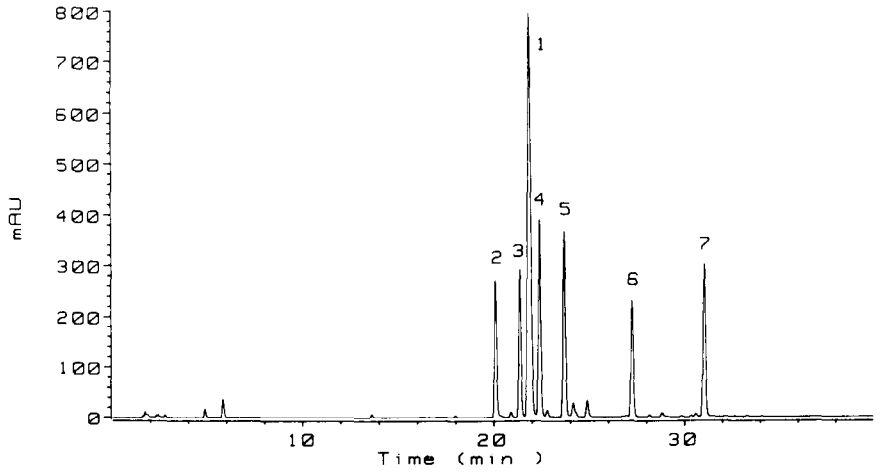

Fig. 3. HPLC separation of Org 2766 and its six diastereoisomers. For the chromatographic conditions, see system 3, Table I. 1 = Org 2766; 2 = D-Glu analogue; 3 = D-His analogue; 4 = D-Met(O_2) analogue; 5 = C-terminal D-Phe analogue; 6 = 4-D-Phe analogue; 7 = L-Lys analogue.

ITP

The application of ITP in peptide analysis is two-fold, *viz.*, purity determination of the native peptides and determination of ionic compounds of non-peptidic nature possibly present in the preparations.

ITP of the peptides. As an indication of the high resolving power of ITP in peptide analysis, we present here some characteristic examples of the separation of closely related components.

Fig. 4 shows the separation of β_h-endorphin from the (2–31) sequence in which only the N-terminal tyrosine residue is lacking. Although this separation is critical, it is reproducible and the considerable difference in UV response between the two peptides is an extra help in the interpretation of the isotachopherogram. Fig. 5 shows the separation of five closely related basic ACTH fragments. We found a good correspondence between the effective mobilities and the calculated isoelectric point (p*I*) values[18] of these peptides. The same holds for ACTH-(1–24) and its -(1–10) and -(11–24) fragments and for AVP and the desglycinamide fragment DGAVP (see Figs. 6 and 7, respectively). The isotachopherogram of the sulphated and non-sulphated forms of an analogue of the octapeptide CCK-8 is shown in Fig. 8.

Anions and cations in peptides. During the synthesis and purification of peptides, a wide variety of ionic compounds are applied. The ITP determination of these non-peptidic substances in peptide preparations proves to be an important additional purity check.

For reference purposes a mixture of ten anions, which are frequently applied

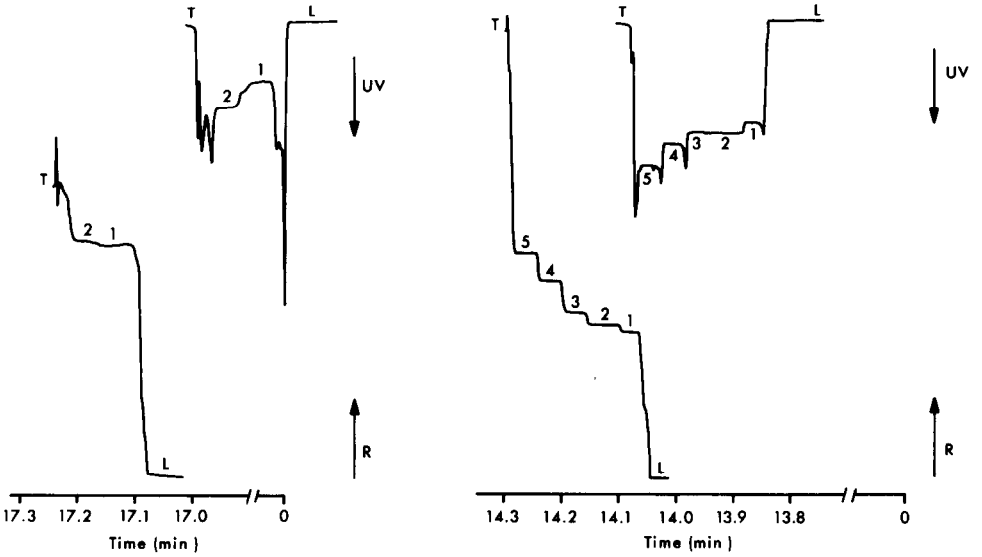

Fig. 4. ITP separation of β-endorphin from β-endorphin-(2–31). Electrolyte system 1, Table II. 1 = β-endophin-(2–31); 2 = -(1–31). *R* = Resistance.

Fig. 5. ITP separation of closely related ACTH fragments. Electrolyte system 1, Table II. 1 = ACTH-(1–18), p*I* 11.5; 2 = -(1–17), p*I* 10.9; 3 = -(1–16)NH_2, p*I* 10.9; 4 = -(1–16), p*I* 10.4; 5 = -(1–13)NH_2, p*I* 10.3.

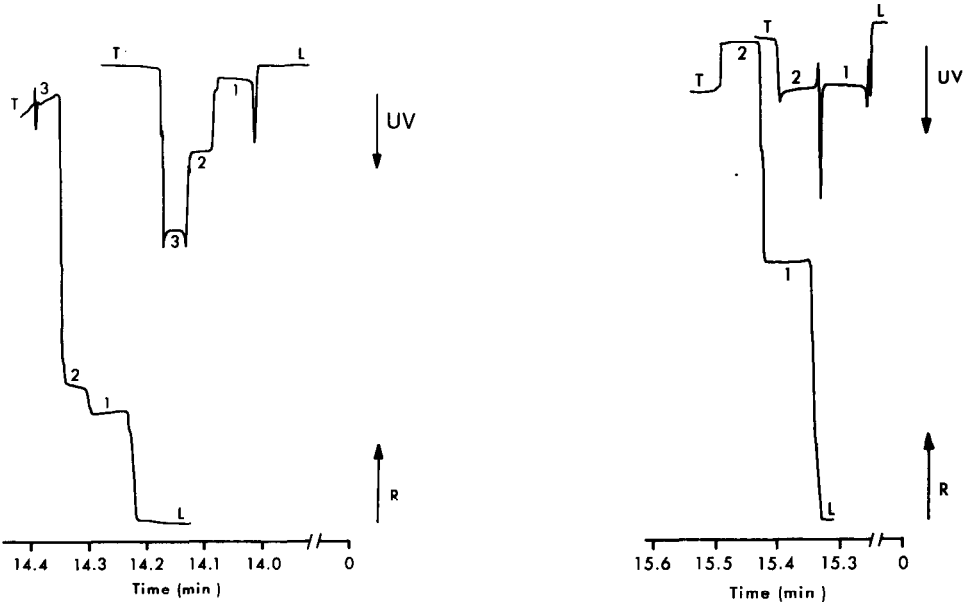

Fig. 6. ITP separation of ACTH-(1–24) and fragments. Electrolyte system 1, Table II. 1 = ACTH-(11–24), p*I* 11.7; 2 = -(1–24), p*I* 11.0; 3 = -(1–10), p*I* 7.6.

Fig. 7. ITP separation of AVP and DGAVP. Electrolyte system 1, Table II. 1 = AVP, p*I* 9.3; 2 = DGAVP, p*I* 8.3.

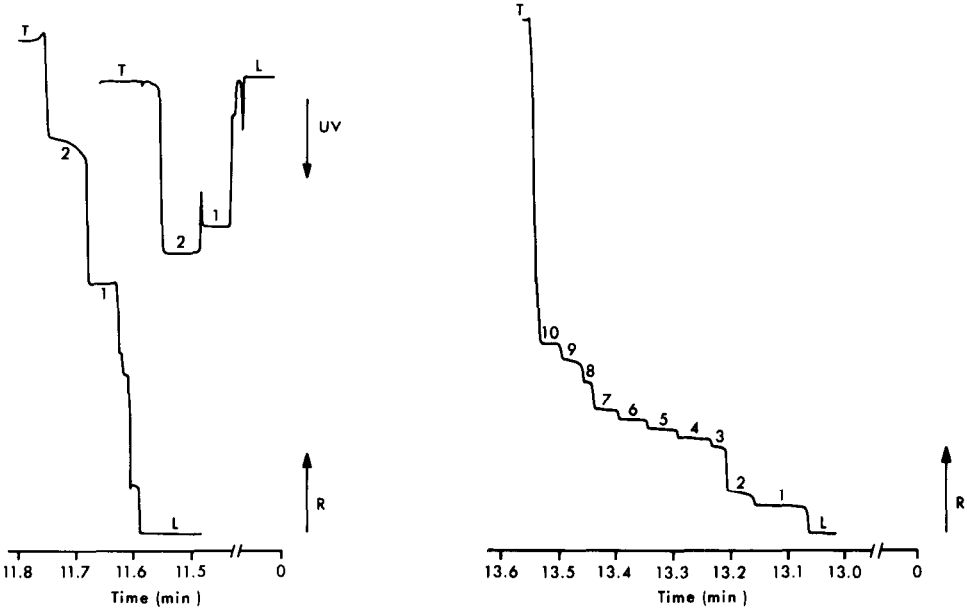

Fig. 8. ITP separation of a sulphated and a non-sulphated analogue of CCK-8. Electrolyte system 2, Table II. 1 = Sulphated analogue; 2 = non-sulphated analogue.

Fig. 9. ITP separation of the anion reference mixture. Electrolyte system 2, Table II. 1 = Sulphate; 2 = formate; 3 = methanesulphonate; 4 = trifluoromethanesulphonate; 5 = trifluoroacetate; 6 = monochloroacetate; 7 = acetate; 8 = phosphate; 9 = *p*-toluenesulphonate; 10 = 1-hydroxybenzotriazole.

during peptide synthesis and isolation, is used. The separation of this mixture is shown in Fig. 9.

As most of our peptide preparations are converted into their acetate salts, which have good stability and are a suitable form for biological testing, we paid special attention to the determination of acetic acid in peptide preparations[11].

An illustration of the additional information that ITP can give is the simultaneous determination of acetic acid and trifluoroacetic acid (TFA). TFA is frequently applied as a deprotecting agent in peptide synthesis and is also used as a volatile modifier in preparative HPLC[19]. One should ensure that in the final product the TFA has been removed completely, *e.g.*, by repeated lyophilization and/or by treatment of the preparation with an anion-exchange resin in which TFA is exchanged for acetic acid. A characteristic example of the combined use of HPLC and ITP is shown in Fig. 10. Preparative HPLC purification of a peptide was performed with TFA as modifier in the mobile phase. The bulk of TFA was removed by evaporation and finally an anion-exchange treatment (Dowex 2-X8 in the acetate form) was performed to convert the TFA salt into the acetate form. However, an ITP check on this product indicated that a considerable amount of residual TFA was present (ratio of acetate to TFA = 1:0.12). Three successive ion-exchange treatments were necessary to remove the TFA completely.

The same phenomenon as for TFA was also found for phosphate, which is also widely used as a mobile phase additive to improve chromatographic performance[20]. As with TFA, three successive ion-exchange treatments were required to remove the phosphate completely.

For the determination of halides we applied system 3 in Table II, which was adapted from Boček *et al.*[21] as reported previously[11].

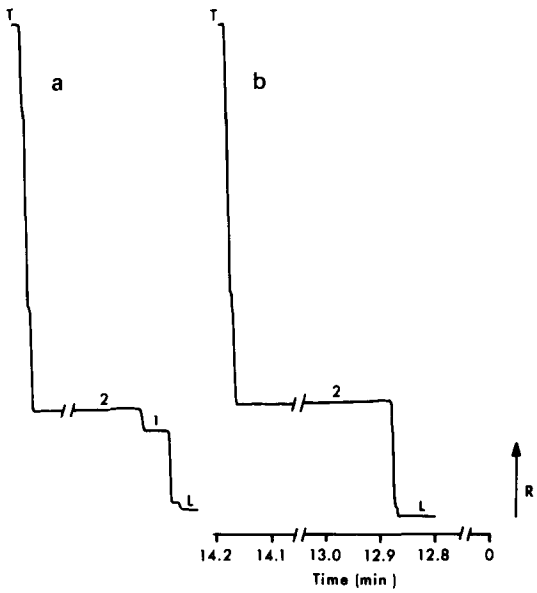

Fig. 10. Check by ITP on TFA removal after preparative HPLC; (a) after one ion-exchange treatment; (b) after three ion-exchange treatments. Electrolyte system 2, Table II. 1 = Trifluoroacetate; 2 = acetate.

Like anionic compounds, cations are also frequently used in peptide synthesis and purification. As with the anions, we used a cation reference mixture, the separation of which is given in Fig. 11.

Alkylamine buffers such as triethylamine (TEA) or tetramethylammonium (TMA) phosphate are often added to the mobile phase to reduce the non-specific interaction of the compounds with the unreacted silanol groups of the material[22,23]. In preparative HPLC these buffer components have to be removed in the final preparation. ITP proved to be a suitable technique for checking the desalting procedure applied.

Non-volatile buffers such as TMA phosphate can be removed by (repeated) ion-exchange procedures. As an alternative desalting procedure for the micrograms to milligrams range, Böhlen *et al.*[24] proposed the use of an octadecylsilica HPLC column. The salt-containing peptide preparation is loaded on a C_{18} column and elution of the salt ions is performed with an aqueous mobile phase, whereas the peptide in question is retained on the hydrophobic support. Elution of the peptide is achieved with an organic solvent-containing mobile phase (methanol, *n*-propanol). The effectiveness of the desalting procedure is monitored by on-line conductivity measurement.

We applied this procedure in the desalting of several peptides of different nature, in which we monitored not only the UV but also the conductivity signal. As expected, not only the salt fractions but also the peptide itself, in a sense being an amphoteric electrolyte, gave an increase in conductivity. Hence the possible co-elution of peptide and residual salt could not be deduced from the HPLC conductivity signal. A careful check of the HPLC-desalted fractions with the sensitive ITP technique indicated that up to 4% of residual TMA salt was present, although the HPLC conductivity

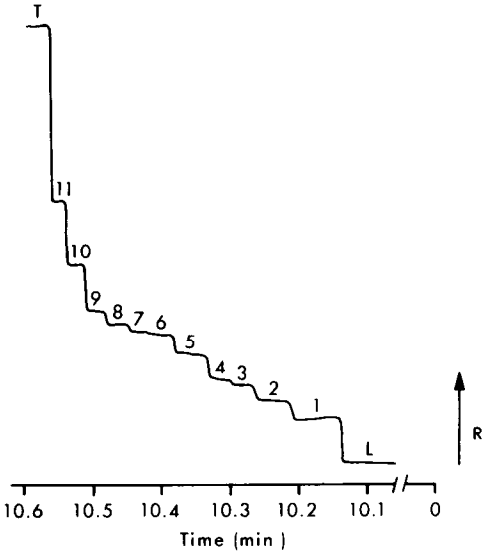

Fig. 11. ITP separation of the cation reference mixture. Electrolyte system 4, Table II. 1 = Hydrazine; 2 = sodium; 3 = trimethylamine; 4 = tetramethylammonium; 5 = pyridine; 6 = piperidine; 7 = 4-dimethylaminopyridine; 8 = N-ethylmorpholine; 9 = N,N-diisopropylethylamine; 10 = triethylamine; 11 = dicyclohexylamine.

monitoring has led us to believe that we had removed all of the salt present[15]. A more efficient desalting procedure proved to be ultrafiltration. Applying a filter with a cut-off range of 500 daltons (Amicon UM 05), complete removal of TMA from a DGAVP preparation was obtained[11].

In the application of the check by ITP on the effective salt removal in peptide preparations, care should be taken to avoid misinterpretation due to the co-migration of the salt ion and the peptide under investigation. This is illustrated by the basic peptide ACTH-(1–24), which was purified by preparative HPLC using a mobile phase containing the volatile salt TEA formate. To control the effectiveness of the TEA removal by lyophilization, we performed ITP in the normally applied cation system at pH 4.8 (see Table II, system 4). However, owing to co-migration of the peptide and TEA, only one zone was monitored in an ACTH-(1–24)–TEA reference mixture. Lowering the pH of the system to 4.5 had no effect. However, increasing the pH to 5.1 resulted in the clear separation of the two components (see Fig. 12).

Ammonium-containing buffers have also been proposed as a separation-improving medium in preparative HPLC[25]. Ammonium acetate is generally considered to be volatile, so in theory it can be removed fairly easily by lyophilization of the pooled HPLC fractions. Two control this procedure by ITP, the "general" cation system cannot be applied owing to the high effective mobility of the ammonium ion. For this reason we developed an electrolyte system in which ammonium can be determined adequately[11].

Application of this system to three ammonium acetate-containing peptides of basic, neutral and acidic nature indicated that the degree of salt removal depends strongly on the nature of the peptide. With the basic peptide two lyophilization steps were necessary to remove the salt to a level of less than 1%; the neutral peptide required an additional step to attain this 1% level and finally the acidic peptide contained up to 9% ammonium even after three lyophilization steps (see Table IV).

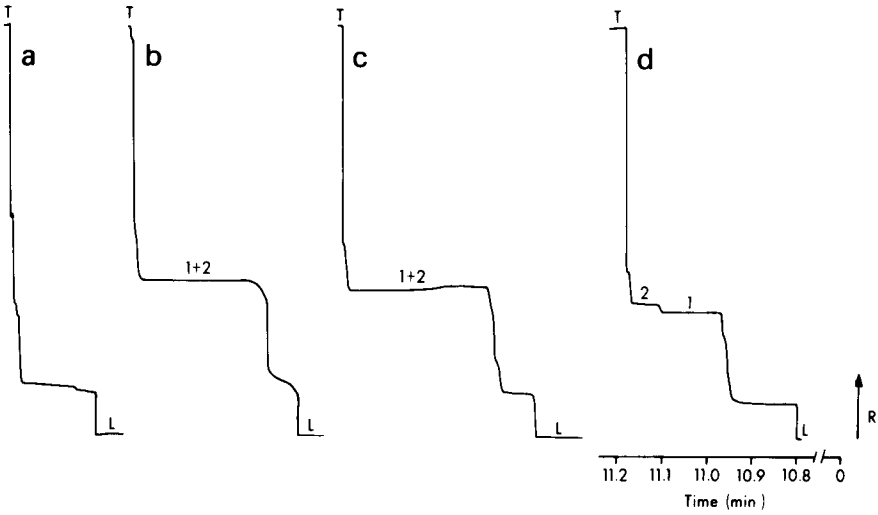

Fig. 12. Influence of pH on the separation of ACTH-(1–24) and TEA. Electrolyte system 4, Table II. (a) Blank; pH set at (b) 4.5, (c) 4.8, (d) 5.1. 1 = TEA; 2 = ACTH-(1–24).

TABLE IV

RESIDUAL AMMONIUM IN PEPTIDES AFTER ONE, TWO AND THREE LYOPHILIZATION STEPS

Peptide nature	*Ammonium (wt.-%)*		
	Step 1	*Step 2*	*Step 3*
Basic	14	0.9	
Neutral	42	21	1
Acidic	49	28	9

CONCLUSION

Owing to its versatility in performance and its high separation power, HPLC is an established technique in peptide chemistry. In the analytical mode HPLC can give information on both chemical and optical purity. Moreover, HPLC can be used to determine the species of origin of a preparation (*e.g.*, insulin) and in the determination of the biological potency of a peptide.

Characteristic general features of carrier-free capillary ITP are a high resolution capacity, high sensitivity (nanogram range), simultaneous determination of various anions or cations, easy performance, short analysis time, small amount of sample preparation needed (0.1–1 mg), low running costs and the absence of undesirable analyte–carrier interactions.

The ITP technique complements the HPLC method regarding the analysis of peptides. For a check on the purity of the peptide itself ITP is a valuable alternative. In this respect it is important to bear in mind that the ITP approach to purity determination is based on a different physico-chemical parameter, *viz.*, electrophoretic mobility, than that used in RP-HPLC analysis, in which the peptide hydrophobicity is the main separation mechanism. Moreover, ITP may give a general indication of the p*I* value of a peptide under investigation.

Complementary to this information, ITP is a reliable and sensitive technique for determining quantitatively non-peptidic ionic compounds in preparations (intermediates and end-products). A wide variety of anions and cations used in peptide synthesis and the purification of a peptide are easily monitored. The use of ITP as a check on the effective removal of unwanted mobile phase components introduced by preparative HPLC employed for peptide purification is especially illustrative.

In our opinion, the ITP technique is a most valuable addition to the current methods of analysis in the peptide field.

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