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Note

Isotachophoretic determination of anions and cations in peptides*

P. S. L. JANSSEN* and J. W. VAN NISPEN
Organon Scientific Development Group, P.O. Box 20, 5340 BH Oss (The Netherlands)
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In the synthesis and purification of peptides, many different organic and inorganic acids may be used. Basic compounds are also widely used in peptide chemistry and in chromatographic purification procedures. In addition, peptides are stored and biologically and pharmacologically tested preferably as their acetates or chlorides.

We have previously reported that analytical capillary isotachophoresis (ITP) is a rapid qualitative and quantitative method for the determination of anions or cations present in peptide preparations[1,2]. As an extension of these earlier studies, we have adapted a system for the determination of chloride, bromide and iodide ions. Moreover, an improved system for the determination of ammonium ions, frequently used in the purification of peptides, was developed.

EXPERIMENTAL

Materials

Electrolyte systems. Four discontinuous electrolyte systems were used for the determination of anionic compounds (with the exception of halides), halides, cationic compounds (with the exception of ammonium) and ammonium ions. These systems are summarized in Table I.

All chemicals were of the highest commercially available quality. Ultra-pure water (Milli-Q, electrical resistance at 25°C, 18 MΩ · cm) was used in the preparation of the electrolyte solutions. To increase the zone-separating performance, the separation capillary was occasionally treated overnight with a 4 g/l aqueous solution of hydroxypropylmethylcellulose (HPMC).

Reference and standard compounds. A reference mixture of eight anions was prepared, containing formic acid, sodium citrate, methanesulphonic acid, trifluoroacetic acid, monochloroacetic acid, sodium acetate, *p*-toluenesulphonic acid and 1-hydroxybenzotriazole, the concentration of each compound being 0.5–0.75 mmol/l. The standard for the determination of acetic acid was sodium acetate at a concentration of 0.5 mmol/l. A reference mixture of three halides was prepared, containing potassium chloride, potassium bromide and potassium iodide, the concentration of

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TABLE I

ELECTROLYTE SYSTEMS

Species	*Leading electrolyte**			*Terminating electrolyte**	
	Leading ion	*Counter ion*	*pH***	*Terminating ion*	*Addition of*
Anions	Cl^-	Histidine	5.75	MES	Tris to pH 5.75
Halides	NO_3^-	Cadmium	5.60	Tartaric acid	NaOH to pH 5.60
Cations	K^+	Acetate	4.50	β-Alanine	HOAc to pH 4.50
Ammonium	H^+	Chloride	2	Na^+	Citrate, pH 8

* Concentrations 0.01 mol/l, except 0.001 mol/l for morpholinoethanesulphonic acid (MES) and 0.004 mol/l for cadmium nitrate.

** Accuracy of the pH values ±0.05.

each compound being 0.5–0.75 mmol/l. A reference mixture of eleven cations was prepared, containing hydrazine, sodium citrate, trimethylamine, tetramethylammonium chloride, pyridine, piperidine, 4-dimethylaminopyridine, N-ethylmorpholine, N,N-diisopropylethylamine, triethylamine and dicyclohexylamine, the concentration of each compound being 0.05–0.5 mmol/l. The standard for the determination of ammonium was ammonium chloride at a concentration of 1 mmol/l. The standard for the determination of tetramethylammonium was tetramethylammonium chloride at a concentration of 2.5 mmol/l.

All reference and standard compounds used were the purest commercially available and, if necessary, were further purified by recrystallization[1]. The solutions of the standards were prepared by weighing the compounds accurately to 0.1 mg (Mettler B6 analytical balance) and dissolving them in the appropriate volume of ultra-pure water (Milli-Q).

Peptides. All peptides were synthesized by our Peptide Chemistry Group. Ultra-pure water (Milli-Q) was used in the preparation of the peptide solutions.

Methods

Apparatus. All ITP experiments were performed with a home-made apparatus constructed according to Everaerts *et al.*[3]. The polytetrafluoroethylene separation capillary was 250 mm long and 0.2 mm I.D. In general, resistance detection was 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 registered using a Model BD 9-725 dual-channel recorder (Kipp & Zonen, Delft, The Netherlands).

Procedure. In general, 0.1–1.0 mg of the peptide preparation to be analysed was weighed in duplicate (accurate to 1 μg; Cahn electrobalance or Mettler ME30 balance) and dissolved in 1.00 ml of water. The lower electrode compartment and the separation capillary were filled with leading electrolyte (LE) and the upper electrode compartment was filled with terminating electrolyte (TE). A volume of 1–4 μl of the peptide solution was carefully injected at the separation boundary of the LE and TE. During the separation process a constant current was applied (in general 50 μA, but 30μA for the separation of the halides; voltage gradient 5–12 kV). The re-

sistance of the separated ion zones was recorded at a chart speed of 200 mm/min (the zone height gives the nature of the ion and the zone length allows quantification). For an easy and more accurate measurement of the zone lengths, the differential signal of the linear resistance trace was also registered on the same recording.

The nature and the content of each anionic or cationic compound present in the peptide preparation under investigation was determined by comparison with the isotachophoretic pattern of the appropriate standard compound(s).

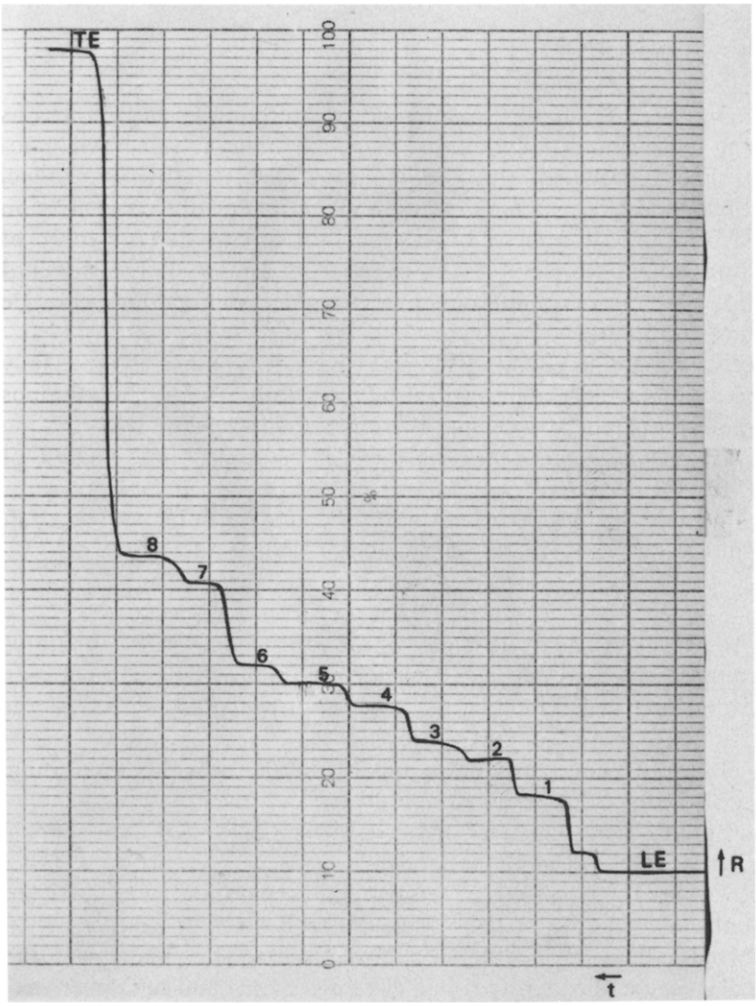

Fig. 1. Isotachopherogram of a reference mixture of eight anions. 1 = Formate; 2 = citrate; 3 = methanesulphonate; 4 = trifluoroacetate; 5 = monochloroacetate; 6 = acetate; 7 = *p*-toluenesulphonate; 8 = 1-hydroxybenzotriazole (2–3 nmol of each compound). *R* = increasing resistance; *t* = increasing time. The small sulphate zone between LE and 1 originates from an impurity in the HPMC.

RESULTS AND DISCUSSION

Anions (in general)

The isotachopherogram of the anion reference mixture, showing the component characteristic zone heights of eight anionic compounds frequently occurring in peptide chemistry, is given in Fig. 1.

Acetic acid

Special attention has been given to the determination of acetic acid in peptides. In addition to its use in the synthesis and purification of peptides, the final step in the preparation of a peptide is often conversion into the acetate form to obtain improved stability and a suitable form for biological and pharmacological testing.

We have compared the known titration[4] and gas chromatographic (GC)[5] methods for the determination of acetic acid in peptides with our ITP procedure, using the N-terminal 24-peptide of the adrenocorticotropic hormone, ACTH-(1–24), as a model. Nine batches of the 24-peptide were analysed in triplicate. The agreement between the overall mean values found for the weight percentage of acetic acid, by three methods was good *viz.*, titration 10.0%, GC 9.9% and ITP 9.7%.

The reproducibility of the determination of acetic acid by ITP was determined using 84 different peptide preparations analysed over a period of 1 year and varying in acetic acid content (0.21–17.10%). These 84 preparations were arranged according to their acetic acid content (weight-%) in four groups of 21 peptides and the coefficient of variation (CV) of each group was determined (see Table II).

We found that the limit of detection of acetic acid using the ITP method, applied routinely, *i.e.*, without further precautions, is *ca.* 5 ng.

A typical example of a determination of acetic acid in a peptide preparation by ITP is given in Fig. 2.

Halides

Chloride, the leading ion in the system used for the determination of the eight anions, can also be determined in this system by measuring the elongation of the leading ion zone for the sample under investigation. However, from an analytical point of view this is not an attractive procedure as the results are less accurate. Chloride can also be determined in a non-aqueous medium[6]; however, for our purposes this approach is not suitable because of the possibility of peptide insolubility and of damage to the Perspex parts of the apparatus.

Therefore, we adapted the system of Boček *et al.*[7], in which the chloride ion

TABLE II

REPRODUCIBILITY OF THE DETERMINATION OF ACETIC ACID USING 84 PEPTIDE PREPARATIONS (4 GROUPS OF 21)

HOAc (wt.-%)	*C.V. (%)*
0.21– 1.35	4.9
1.35– 2.40	2.6
2.40– 5.10	1.5
5.10–17.10	1.4

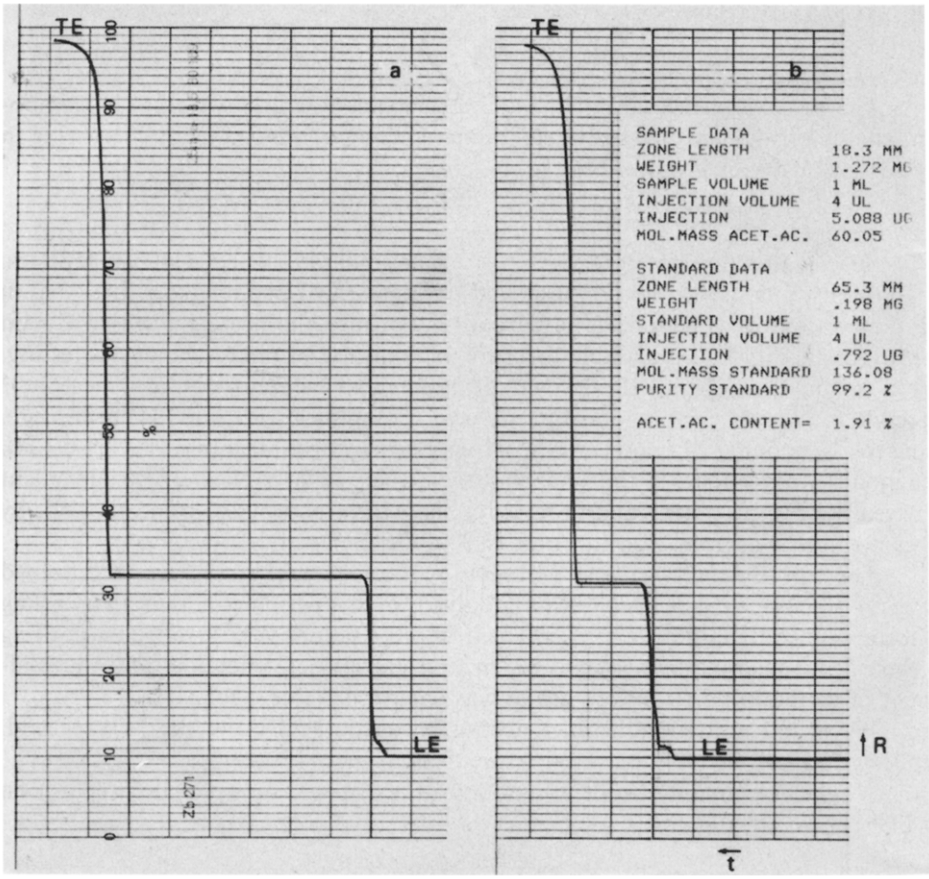

Fig. 2. Isotachophoretic determination of acetic acid in β-endorphin-(8–17). (a) Acetate standard (5.8 nmol); (b) peptide preparation. The computer print out with the relevant data for the content calculation is also shown.

is determined by means of complex formation with cadmium as the counter ion. In addition, bromide and iodide, which are less frequently applied in modern peptide chemistry, can be determined simultaneously in this system.

Fig. 3 shows the separation pattern of a reference mixture of chloride, bromide and iodide. As a typical example, the ITP patterns of a chloride determination in a commercial batch of D-lysine · HCl is also given. The chloride content of 19.3% obtained by ITP agrees well with the value of 18.9% that was found by argentimetric titration.

Cations (in general)

Fig. 4 shows the ITP separation pattern of a reference mixture of eleven basic compounds frequently applied in peptide synthesis and purification.

Cations used in preparative HPLC

In addition to partition and ion-exchange chromatography, preparative high-

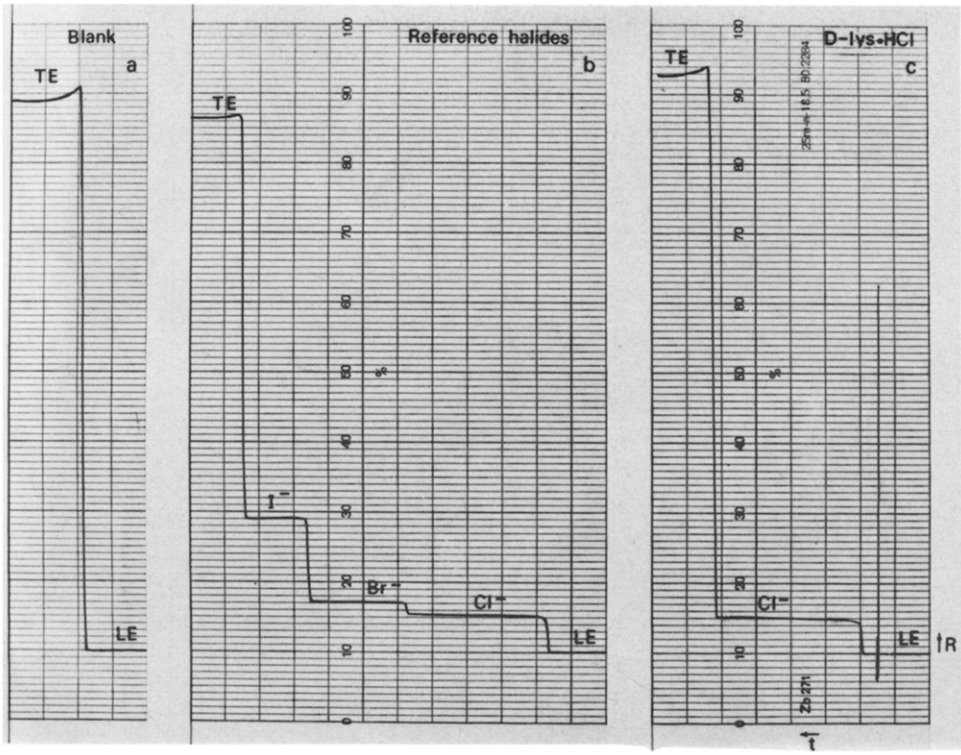

Fig. 3. ITP of halides. (a) Blank; (b) reference mixture of chloride, bromide and iodide (2–3 nmol of each compound); (c) chloride in D-lysine · HCl.

performance liquid chromatography (HPLC) is a rapidly growing technique in the purification of peptides. In general, the reversed-phase mode (RP-HPLC) is applied, consisting of a silica matrix with an apolar *n*-alkyl bonded stationary phase in combination with a more polar mobile phase, typically water–methanol or water–acetonitrile mixtures. The addition of buffers, such as ammonium acetate, triethylammonium phosphate or formate or tetramethylammonium phosphate, to the mobile phase considerably improves the HPLC separation pattern[8–12].

ITP proved to be a suitable technique for checking the possible presence of residual cations after preparative HPLC.

The non-volatile alkylammonium phosphate buffers can be removed by ion-exchange chromatography. As an alternative, we have removed tetramethylammonium phosphate buffer from the peptide solution by ultrafiltration (Amicon UM05 filter; cut-off range 500 daltons). Fig. 5 shows that this filtration procedure is effective in removing the tetramethylammonium ions.

Ammonium acetate, often used in preparative HPLC, is widely considered to be volatile, which would mean it could easily be removed by direct lyophilization of the column eluate containing the pooled fractions.

The ammonium ion, having too high an effective mobility to allow its determination in the system applied for the other cations, can be determined in a system

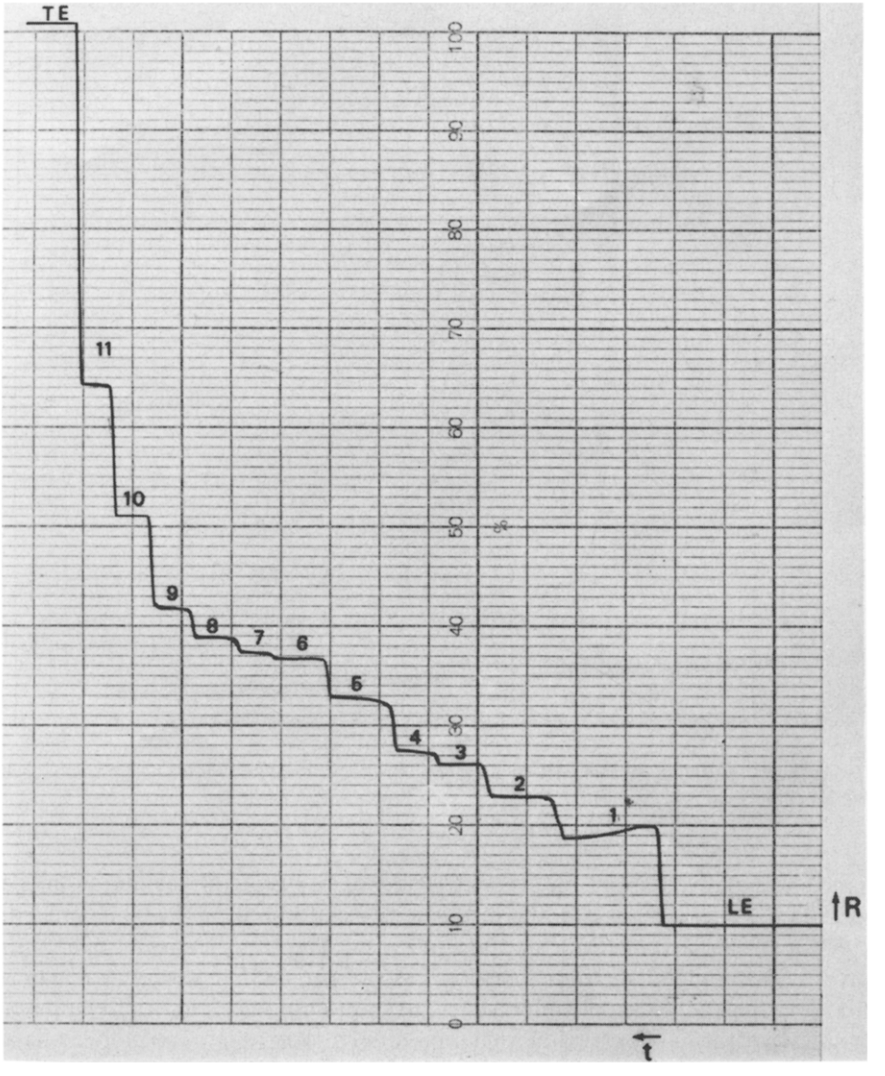

Fig. 4. Isotachopherogram of a reference mixture of eleven cations. 1 = Hydrazine; 2 = sodium; 3 = trimethylamine; 4 = tetramethylammonium; 5 = pyridine; 6 = piperidine; 7 = 4-dimethylaminopyridine; 8 = N-ethylmorpholine; 9 = N,N-diisopropylethylamine; 10 = triethylamine; 11 = dicyclohexylamine (0.2–2 nmol of each compound).

with H^+ as the leading ion and $Tris^+$ (2-amino-2-hydroxymethylpropane-1,3-diol) as the terminating ion, although sometimes disturbances are encountered[3]. For the same reasons as mentioned for the determination of the halides (possible low solubility of peptides and damage to the Perspex parts of the apparatus), we cannot use the published[6] non-aqueous systems. By replacing the terminating $Tris^+$ ion for Na^+, the ammonium system suited our purposes. Less disturbances were experienced in this system. Owing to the relatively narrow mobility interval (leading ion H^+ and

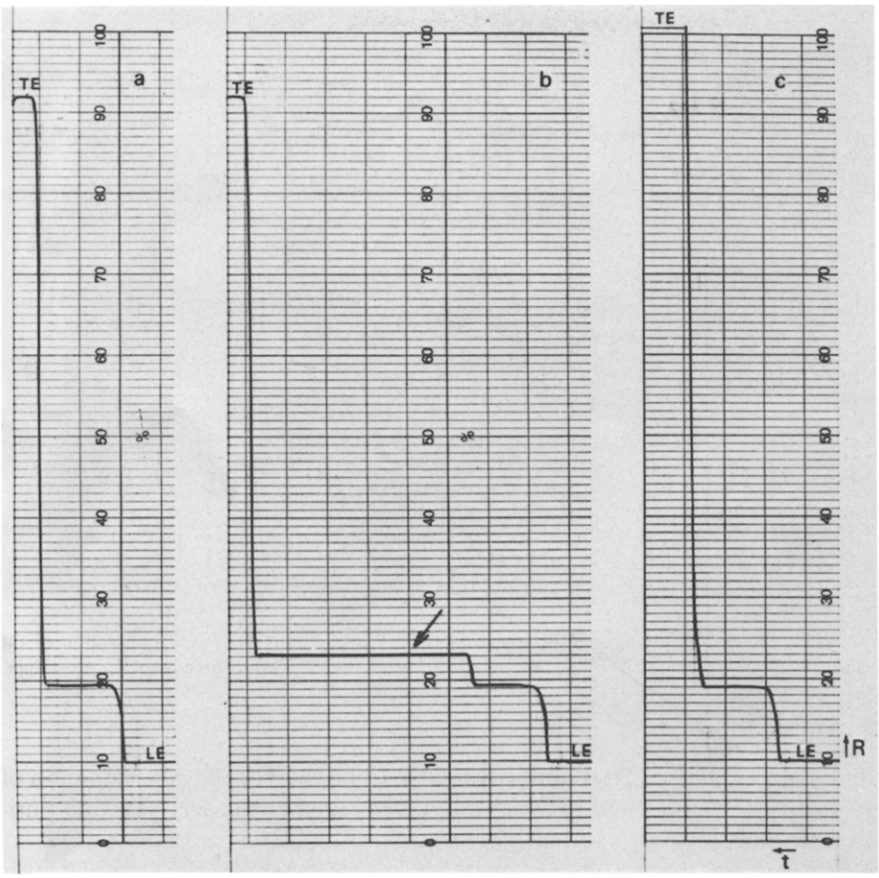

Fig. 5. ITP guidance of removal of tetramethylammonium phosphate by ultrafiltration. (a) Blank (Na^+ zone); (b) standard tetramethylammonium, indicated by arrow (10 nmol); (c) solution of AVP-(1–8) in 0.05 mol/l tetramethylammonium phosphate (1 mg/ml) after ultrafiltration.

terminating ion Na^+), this system has the additional advantage of allowing easy interpretation (only the limited number of cations with a mobility between H^+ and Na^+ are recorded on the ITP pattern).

This improved system was applied in the determination of ammonium ions and we found that the degree of salt removal by lyophilization depends strongly on the nature of the peptide. With the basic peptide [8-L-arginine-des-9-glycinamide] vasopressin, AVP-(1–8)*, we found that two lyophilization steps are necessary to remove the ammonium level to less than 1% of the original amount. For a solution of the neutral peptide β-endorphin-(6–17)*, three successive lyophilizations were necessary to attain this 1% level. Finally, for the acidic peptide H-Glu-Gly-OH*, even after lyophilizing three times a considerable residual percentage of ammonium (up to 9%) was found (Fig. 6).

* Initial solution 1 mg/ml in 0.05 mol/l ammonium acetate.

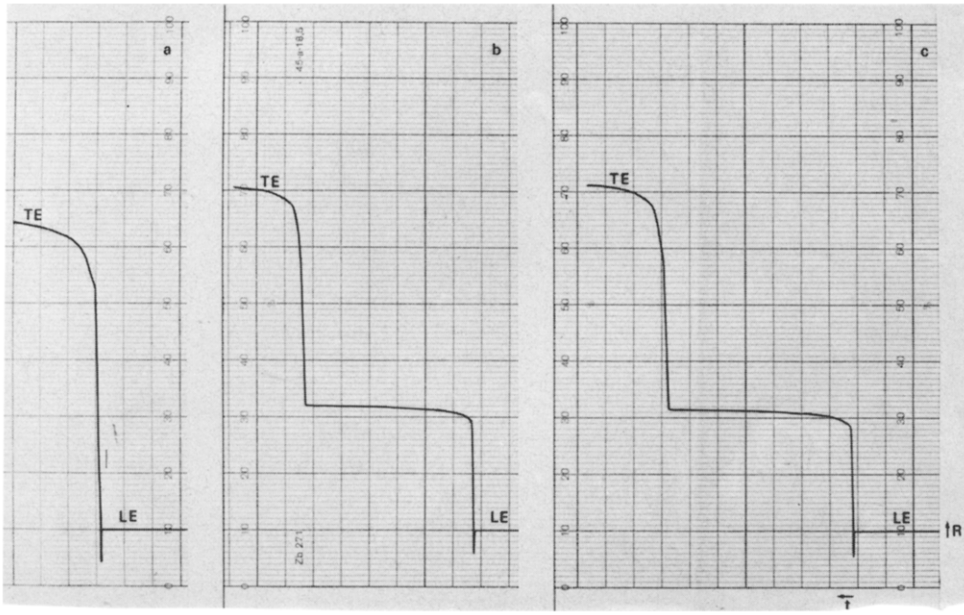

Fig. 6. Determination of ammonium. (a) Blank; (b) standard ammonium (4 nmol); (c) solution of the acidic peptide H-Glu-Gly-OH in 0.05 mol/l ammonium acetate (1 mg/ml) after three lyophilization steps.

CONCLUSION

Analytical capillary ITP is a most valuable technique for the determination of the large variety of anionic and cationic compounds used in the synthesis and purification of peptides.

Advantages of this direct, non-destructive ITP determination method over other techniques are simultaneous determination of various anionic or cationic compounds in a single run; high sensitivity (ng range); easy performance of the analysis; short time of analysis (10–15 min); only small amounts of peptide material are needed (0.1–1 mg).

In addition to other analysis techniques used in peptide chemistry, such as amino acid analysis, thin-layer chromatography and HPLC, we now routinely use analytical capillary ITP to check the purity of our peptide preparations.

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