Journal of Chromatography, 351 (1986) 283–293
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 176

APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ISOTACHOPHORESIS TO THE PURIFICATION AND CHARACTERIZATION OF PRODUCTS OF PEPTIDE SYNTHESIS

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(Received September 12th, 1985)

SUMMARY

The application of both high-performance liquid chromatography (HPLC) and capillary isotachophoresis (CITP) to the characterization of the products of peptide synthesis is demonstrated. CITP can give valuable information for the improvement of preparative HPLC, and the nature of by-products can be deduced. The two methods complement each other.

INTRODUCTION

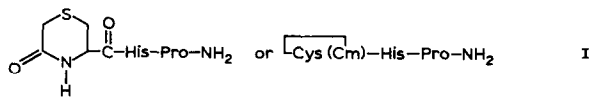
The use of chromatographic and electrophoretic methods in monitoring the purity of synthetic peptides is well established. Whereas reversed-phase high-performance liquid chromatography (RP-HPLC) is the most effective technique in this field (for a recent review see ref. 1), electrophoretic methods appear well suited to peptide separation because of the ionic character of these substances. However, the rather poor separations of peptides of comparable net charge and molecular masses and the insufficient reproducibility and low accuracy in quantitations are drawbacks of zone electrophoresis and even isoelectric focusing.

The advantages of capillary isotachophoresis (CITP)[2–15] are based on the step-like gradient of the electric field strength formed according to the mobility of ions in the discontinuous electrolyte system, without broadening by diffusion, and the detection within the capillary in the steady state of the consecutive zones of charged compounds[16–19]. CITP gives a measure of the amount of substance, the zone length being independent of the method of detection, *e.g.*, UV absorption, conductivity.

We present here an example in which the full power of both HPLC and CITP is demonstrated. A preparation of the thyrotropin-releasing hormone analogue I

0021-9673/86/$03.50

(TRH analogue I), synthesized in our laboratory[20,21], was selected which was expected to contain several impurities: D-lactam of S-carboxymethylcysteine; diketopiperazine cyclo(His–Pro); sulphoxides; diastereoisomers (racemization); partial sequences.



The analogue I is characterized by the substitution in TRH (II) of the pyroglutamyl residue by the lactam of S-carboxymethyl-L-cysteine. It is expected therefore that the cationic forms of both substances, having the same net charge (at imidazole of His), will behave identically in electrophoresis. The high separation efficiency of CITP in the case of TRH has already been demonstrated[4]. TRH and its analogues have been studied by HPLC, *e.g.*, refs. 22–25, and conditions for its determination in tissues were reviewed[26]. TRH (II) is thus an excellent standard for our purpose.

EXPERIMENTAL

Materials

Redistilled water was used in the preparation of the electrolyte solutions and of the solutions of samples for CITP. All chemicals were of the highest quality commercially available: 1 *M* potassium hydroxide solution (Testal Masslösung, VEB Feinchemie Sebnitz, G.D.R.); 100% acetic acid (Merck, Darmstadt, F.R.G.); pivalic acid (Fluka, Buchs, Switzerland); hydroxypropylmethylcellulose, HPMC (Methocel 90 HG 15 000 cps; Dow Chemical, Midland, MI, U.S.A.); β-alanine (Serva, Heidelberg, F.R.G.).

Peptide material

Thyrotropin-releasing hormone, TRH (Protirelin) was obtained from VEB Berlin-Chemie (Berlin, G.D.R.). The crude lyophilized preparation of the TRH analogue I was obtained after partition chromatography on Sephadex G-25 fine[27] of the reaction mixture from the last step in the synthesis[20]. Analogue I has also been synthesized independently[28].

Methods

CITP experiments were performed with the Tachophor 2127 (LKB Producter AB, Bromma, Sweden) equipped with a conductivity detector [conductivity detection signal (*C–D*) and rate of change of conductivity with time (dC/dt)], UV detector (filter 254 nm), polytetrafluoroethylene (PTFE) capillaries (230 and 430 × 0.5 mm I.D.) and a current-stabilized high voltage power supply. Isotachopherograms were registered at chart speeds of 5 and 6 cm/min with a two-channel recorder (LKB) or a three-channel recorder (W + W electronic, Basle, Switzerland). Injections were made with Hamilton syringes. For conditions see Table I.

HPLC experiments were performed with a SP-8700 liquid chromatograph

TABLE I

ELECTROLYTE SYSTEMS AND CONDITIONS FOR CITP

Leading electrolyte	KOH (6 m*M*) including 0.4% HPMC, with acetic acid to pH 5.0 (I, III) or pivalic acid to pH 5.1 (II)
Terminating electrolyte	β-Alanine (10 m*M*), no additive (I), with acetic acid to pH 5.0 (III) or pivalic acid to pH 4.9 (II)
Capillary	Assay, 230 × 0.5 mm I.D.; purity, 430 × 0.5 mm I.D. Temperature: 10°C (I, III); 12°C (II)
Detection	Conductivity including differential signal, d*C*/d*t*; UV absorbance at 254 nm
Current	Assay: 135 and 85 μA respectively to 9 kV; for detection, 35 μA (max. 5 kV). Purity: 140 and 100 μA respectively to 15 kV; for detection 40 μA (max. 9 kV for systems I and III, 12 kV for system II)
Injected volumes	Assay, 1 μl; purity, 2–12 μl)

(Spectra Physics, Santa Clara, CA, U.S.A.), equipped with a SP-8400 continuously variable-wavelength UV detector and a SP-4100 integrator. Analytical chromatography was performed on 150 × 4 mm columns filled with Separon SI-C18, preparative HPLC on 250 × 12.7 mm columns filled with the same adsorbent (Laboratorni Přistroje, Prague, Czechoslovakia). As mobile phases, mixtures of methanol or dioxane with aqueous buffer solutions were used. Buffer solutions: 0.05–0.1% trifluoroacetic acid (TFA) pH 2.0 or 2.5; 0.1 *M* ammonium acetate pH 7.0 or 8.1; 0.05 *M* phosphate buffer pH 3.8.

RESULTS AND DISCUSSION

CITP of the crude peptide preparation

Fig. 1 shows the isotachopherogram of the crude synthetic product (8 μl, 29.12 μg). Upon doubling the sample volume from 2 to 4 μl or 4 to 8 μl, cationic impurities were easily recognized from the resulting elongated zones. Two impurities, found even with 1 μl samples (3.64 μg), are the main by-products (see Fig. 2a). One compound (pre-zone 4) with a relative step height (related to the main product) in the *C-D* signal of 58.7% (no UV absorption at 254 nm) comprises 7.3% of the sum of all zone lengths, and a second compound (post-zone 8) with a relative step height of 117.8% (same UV absorption as the main product) comprises 7.4% of the zone lengths.

As a measure of the purity of compound I the isotachophoretic purity degree (ITP-PD) is used (see Table II). It is defined as the percentage of the zone length of the cation I^+ of the sum of all zone lengths for cations with mobilities between those of K^+ and β-Ala^+, corrected for impurities in the electrolyte system.

For quantitative analysis the d*C*/d*t* signal was used. The measurement of the distances between the zone boundaries (peak maxima) is more exact using this signal than with UV detection.

As expected, the analogue I shows the same relative step height in the *C–D* signal as does TRH (II) (relative to β-Ala^+) (see Table II). If both compounds were mixed before injection only one step height resulted. In contrast to the TRH zone, the zone of the analogue I shows a small UV absorption at 254 nm (2.3% of the overall absorbance) which is caused by its sulphide group. In this way, compounds I and II can be distinguished qualitatively.

Fig. 1. Isotachopherogram of crude I. Sample volume injected: 8 μl (29.12 μg). Chart speed: 5 cm/min. Capillary: 430 mm. Electrolyte system I. Zones: 1 = K^+; 2 = cationic impurities from the electrolyte system; 3–6 = pre-zones; 7 = analogue I^+; 8, 9 = post-zones; 10 = β-Ala^+. This isotachopherogram is representative only for qualitative use.

From calibration curves of the zone lengths at different TRH concentrations (see Fig. 2b), the concentration of compound I can be calculated from the zone length on the basis of the M_r ratio (I:II = 394.45:362.39). The results of these experiments are presented in Table II. The pre-zones showed no UV absorption; post-zone 9 (Fig. 1) showed a rather high UV absorption. The test solution contained 1.82 mg synthetic product per 0.5 ml. From the responses, the content of analogue I in the sample is calculated to be 66.4%.

The anionic analysis (For conditions see ref. 4) showed 1.9% acetic acid. The difference between the ITP-PD of 81.9% and the content of I in the sample (66.4%) is an indication of the presence of other compounds, *e.g.*, inorganic salts, electrophoretically immobile substances like water, organic solvents, etc.

Analytical HPLC of the crude peptide preparation

The analogue I was analyzed under various conditions. The elution pattern in

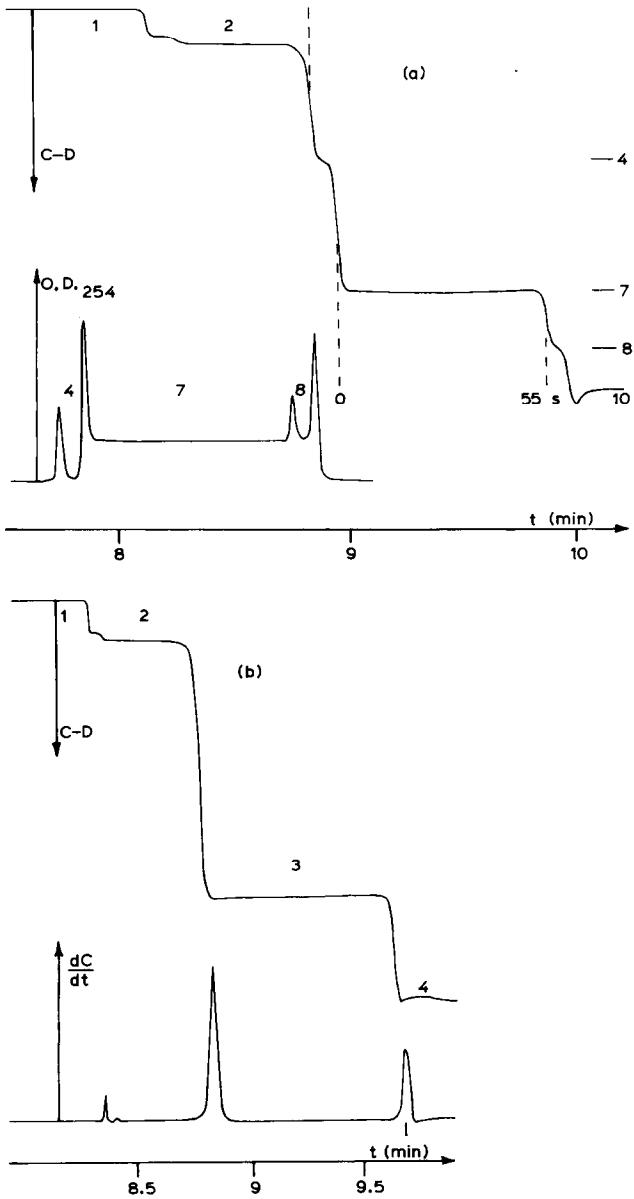


Fig. 2. (a) Isotachopherogram of crude I. Sample volume: 1 μl (3.64 μg). Chart speed: 6 cm/min. Capillary: 230 mm. Electrolyte system I. Zones: 1 = K^+; 2 = cationic impurities (see Fig. 1); 4 = pre-zone; 7 = analog I^+; 8 = post-zone; 10 = β-Ala^+ (numbering as in Fig. 1). (b) Isotachopherogram of TRH. Sample volume: 1 μl (2.3 μg). Calibration for assay of analogue I. Details as in (a). Zones: 1 = K^+; 2 = cationic impurities (see Fig. 1); 3 = TRH^+; 4 = β-Ala^+.

TABLE II

ISOTACHOPHORETIC PURITY-DEGREE (ITP-PD) AND ASSAY FOR THE CRUDE TRH ANALOGUE

$S.D._{rel.}$ for confidence level $p = 95\%$.

Sample	*ITP-PD (%)*	*Zones*		*Step height rel. to β-Ala*			*Response*		
		Pre-	*Post-*	*%*	*n*	*$S.D._{rel.}$ (%)*	*mm/μg*	*n*	*$S.D._{rel.}$ (%)*
TRH	97.8	2	1	74.0	16	±0.88	25.10*	6	±1.11
TRH analogue	81.9	4 (10.2%)	2 (7.9%)	74.8	5	±1.25	15.32	5	±0.31

* Relative to the pure water-free substance. Electrolyte system I. By making use of electrolyte system II (with pivalic acid) the range of mobilities between that of K^+ and of β-Ala^+ is more extended; however, no further zones were detected.

Fig. 3 shows the use of a methanol gradient with 0.05% TFA as the aqueous component of the mixture. The main peak was divided into small fractions, I–IV. The analysis of two fractions is shown in Fig. 4. In this case, 0.1 *M* ammonium acetate pH 8.1 was used as the aqueous component of the mobile phase. From these results it is clear that the main impurity seen in Fig. 5 (eluted earlier than the main peak

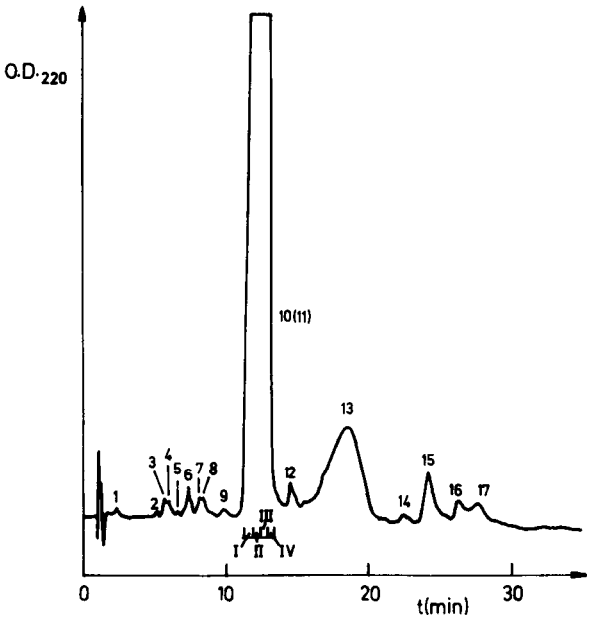


Fig. 3. HPLC analysis of a crude preparation of analogue I in a methanol gradient. Conditions: gradient 0–15.8% methanol plus 0.05% TFA in 20 min, flow-rate: 1 ml/min; column (150 × 4 mm), Separon SI-C18. Compound numbers as in Table III.

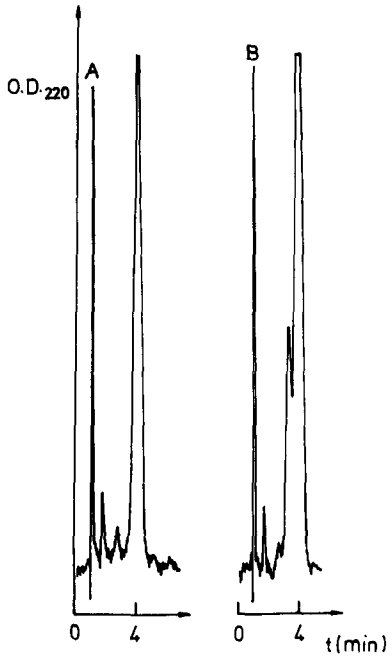


Fig. 4. HPLC analysis of two fractions obtained from gradient elution. Conditions: mobile phase, 16% methanol in 0.1 *M* ammonium acetate, pH 8.1; other details as in Fig. 3. (A) Analysis of fraction I (Fig. 3); (B) analysis of fraction IV.

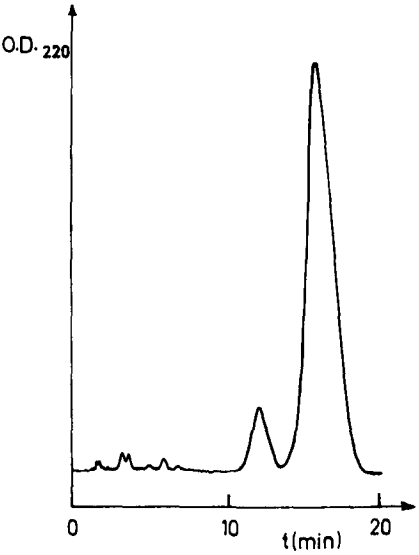


Fig. 5. HPLC analysis of the crude peptide preparation in basic buffer. Conditions: mobile phase, 1.25% dioxane in 0.1 *M* ammonium acetate, pH 8.1; other details as in Fig. 3.

under neutral or basic conditions) is under acidic conditions (Fig. 3) hidden in the main peak and it can be quantitated only by separation using neutral or basic buffers. Table III shows the composition of the mixture of the crude peptide material as evaluated from the UV absorption at 220 nm. In some cases we isolated the impurities in quantities sufficient for amino acid analysis, the results of which are also given. The "fingerprint" of the impurities is dependent on organic modifier used (not shown), but the elution order of the main components is not changed (Use of 1.25% dioxane in the mobile phase leads to the same k' values as does 7% methanol).

TABLE III

COMPOSITION OF THE CRUDE TRH ANALOGUE MIXTURE (SEE FIG. 3)

Compound	*Rel. amount according to O.D.$_{220}$ (%)*	*Amino acid composition*	*Probable structure*
1	0.06	Cys(Cm)	Cys(Cm)-lactam
2	0.04	Cys(Cm),His	
3	0.10	His,Pro	
4	0.07	His,Pro	
5	0.03	Cys(Cm),His,Pro	D-Amino acid containing tripeptide
6	0.31	His,Pro	Diketopiperazine
7	0.18	Cys(Cm),His,Pro	Sulphoxides of
8	0.19	Cys(Cm),His,Pro	the tripeptide
9	0.08		
10	82.90	Cys(Cm),His,Pro	L-Cys(Cm)-His-Pro-NH_2
11	8.90*	Cys(Cm),His,Pro	D-L-Cys(Cm)-His-Pro-NH_2
12	0.51		
13	5.49		
14	0.08		
15	0.72		
16	0.17		
17	0.17		

* Isolated under basic conditions.

The relative position of the main components is strongly dependent on the pH of the buffer (see Table IV), which is a little surprising because according to the amino acid analysis and the CITP results (see Table V, fractions 33–36) we are dealing with diastereoisomeric peptides. Similar, but not as pronounced, behaviour was found for a series of diastereoisomeric peptides containing glycine and leucine[29]. The separation of [D-His^2]-TRH from TRH was demonstrated earlier[23,25].

In order to determine whether the given peak contains sulphur-containing amino acids [Cys(Cm)] in the sulphide state, we employed the oxidation by sodium periodate as previously described[30] in the case of oxytocin analogues. Preparative purification of compound I was performed on the larger column. The same adsorbent as in the analytical experiments was used, with 4% methanol in 0.1 *M* ammonium acetate pH 8.1 as the mobile phase. This mobile phase is directly lyophilizable if the ammonium acetate is prepared from distilled acetic acid and distilled ammonia. The result is given in Fig. 6. The separated compounds, pure according to HPLC, were analyzed by CITP (see Table V).

TABLE IV

DEPENDENCE OF k' OF THE MAIN PRODUCT AND MAIN IMPURITY ON THE pH OF THE MOBILE PHASE

Conditions: Separon SI-C18 (150 × 4 mm), flow-rate, 1.5 ml/min, mobile phase 7.5% methanol + 0.1% TFA (pH adjusted by addition of 1 *M* NaOH).

	Mobile phase pH					
	2.3	*3.5*	*4.5*	*5.4*	*6.6*	*8.1*
k' of the main product (compound 10, Table III)	5.45	6.91	7.97	14.0	19.1	18.9
k' of the main impurity (compound 11, Table III)	5.83	7.12	8.16	14.0	15.1	14.6

TABLE V

ISOTACHOPHORETIC PURITY DEGREE OF FRACTIONS FROM PREPARATIVE HPLC SEPARATION (SEE FIG. 6)

Electrolyte system III.

Sample fractions	*ITP-PD (%)*	*Pre-zones*		*Post-zones*	
		Extent (%)	*UV absorption**	*Extent (%)*	*UV absorption**
33–36	93.3	6.2	1 > 2 ⩾	0.5	1 ⩾ 2 >
44–55	98.3	1.0	1 ⩾ 2 >	0.7	1 ⩾ 2 ⩽

* UV absorption of the pre- and post-zones estimated on the basis of the absorption of the zone for the pure TRH analogue, the numbers 1 and 2 indicate the consecutive zones of the impurities in chronological order.

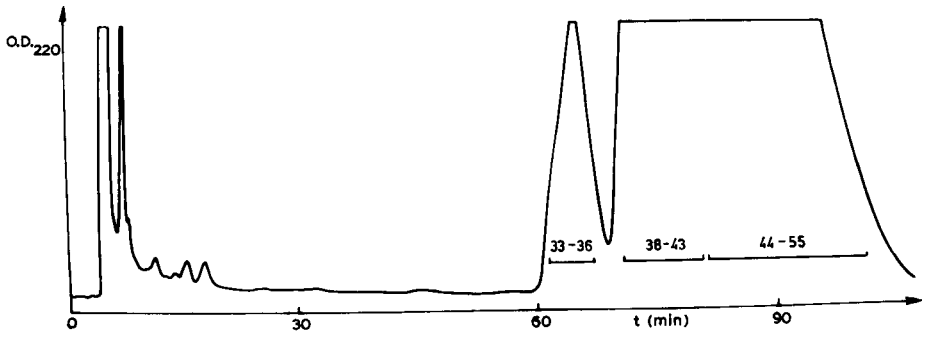


Fig. 6. Preparative HPLC of the crude peptide preparation. Conditions: mobile phase, 4% methanol in 0.1 *M* ammonium acetate, pH 8.1; flow-rate 7 ml/min; column (250 × 12.7 mm), Separon SI-C18.

CITP from preparative HPLC fractions

The isotachopherogram of the main fraction from preparative HPLC (see Fig. 6) is shown in Fig. 7. The ITP-PD is high. The detection of impurities in a "pure" HPLC fraction is a measure of the high separation efficiency of CITP and evidence of its suitability for monitoring the effectiveness of preparative HPLC for the purification of peptides. Because the separation principles of the two methods are different, the purity of I obtained in these experiments is largely validated.

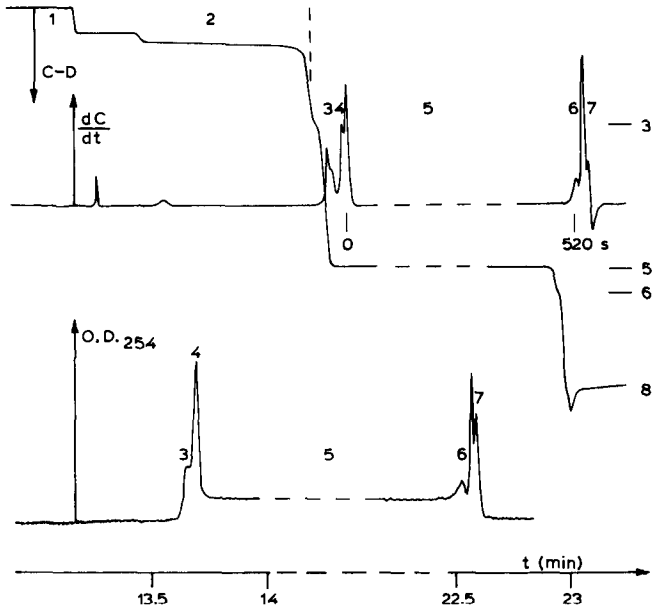


Fig. 7. Isotachopherogram of fractions 44–55 from preparative HPLC. Purity of the water-soluble component: a 10-μl volume was injected containing 22% I (5.06 mg dissolved in 0.5 ml). Chart speed: 6 cm/min. Capillary: 430 mm. Electrolyte system III. Zones: 1 = K^+; 2 = cationic impurities (see Fig. 1); 3,4 = pre-zones (zone 3 included blank value); 5 = analogue I^+; 6, 7 = post-zones; 8 = β-Ala^+. +, indicates only a cation, not the net charge.

The main impurity separated by preparative HPLC (fractions 33–36) behaves identically to analogue I in CITP. It has the same amino acid composition and has therefore to be regarded as a diastereoisomer of I. The results are summarized in Table V. The lyophilized fractions 33–36 and 44–55 from preparative HPLC contained water-insoluble material (adsorbent dissolved by the basic buffer). This fact had no influence on the reliability of the results.

A sample of the diketopiperazine cyclo(His–Pro) showed a relative (to TRH^+) step height of 71% in CITP. It is therefore not identical to one of the main impurities of the crude peptide preparation. This conclusion was verified by analytical HPLC. The diketopiperazine is eluted with approximately the same k' as that of peak 6 in Fig. 3, which is in accordance with previous findings[22].

CONCLUSIONS

Analytical CITP is a valuable method for the detection of impurities in the products of peptide synthesis and in monitoring the purity of material purified by HPLC. The results of CITP can be used to improve preparative HPLC by modifying the HPLC conditions till all impurities detected in CITP are found also in HPLC. The agreement between the results of CITP and analytical HPLC in the characterization of pure peptides is evident.

CITP and HPLC complement each other. While diastereoisomers can be separated by HPLC (without giving information as to their structure), their nature becomes evident from their behaviour in CITP.

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