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Note

Separation of diastereomers and analogues of neurotensin by anion-exchange high-performance liquid chromatography

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Neurotensin (NT) (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) is a recently discovered peptide hormone[1], which is distributed heterogeneously throughout the central nervous system[2,3], as well as in the digestive track of various mammals[2,4–6]. This peptide exhibits a large spectrum of biological activities[1,7–20].

A large number of fragments, diastereomers and analogues of NT have recently been synthesized by St-Pierre *et al.*[17] and tested for biological activity (hypertension, hypothermia, hyperglycemia, gut contraction, etc.). Structure–activity studies of these compounds showed that substitution of one amino acid by various D-amino acids or various other residues leads to important variations of biological activities of NT[14,17,20–22].

Racemization during the synthesis of peptides is an important problem because optical homogeneity of the peptides synthesized is usually required to study their physical, chemical and biological properties. On the other hand, analogues of peptides can have different biological activities as mentioned above in the case of NT. It is, therefore, desirable to separate diastereomers and analogues of NT, in particular, and of other biologically active peptides, in general, from each other and/or from their impurities by a chromatographic system. High-performance liquid chromatography (HPLC) is an excellent technique for separation of peptides. Reverse-phase (RP) HPLC has usually been employed to separate diastereomers and analogues of peptides[23–36].

We recently reported a method for separation of peptides by anion-exchange HPLC using mixtures of triethylammonium acetate and acetonitrile as the eluent[37–39]. In the present work, we demonstrate the application of this method to separation of some diastereomers and analogues of NT. This method has been developed to study the racemization of peptides by ionizing radiation.

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EXPERIMENTAL*

Apparatus

A Hewlett-Packard (Avondale, PA, U.S.A.) Model 1084B liquid chromatograph equipped with a microprocessor, an automatic injector and a variable-wavelength detector was used. Separations were carried out on a 30 × 0.4 cm MicroPak AX-10 (Varian, Walnut Creek, CA, U.S.A.), a difunctional weak anion-exchange bonded phase prepared on LiChrosorb Si-60 silica (10 μm)[40]. Prior to use, the column was equilibrated with at least 100 ml of 0.01 *M* triethylammonium acetate (TEAA) buffer (pH 6.0) to insure that column pH was also 6.0. Then after column equilibration with the starting solution, elution was begun with a gradient terminating at 100% buffer. Specific conditions are given in Figs. 1 and 2.

Materials

The synthesis of neurotensins is described elsewhere[17]. Triethylamine was purchased from Eastman Kodak (Rochester, NY, U.S.A.) and purified by distillation. TEAA buffer was prepared by titrating a 0.01 *M* acetic acid solution with triethylamine to pH 6.0 and filtered before use. Glass-distilled water and acetonitrile were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Amino acid analysis and recoveries

The eluted neurotensins were dried *in vacuo* and hydrolyzed with constant boiling hydrochloric acid (6 *N*) at 110°C in evacuated and sealed tubes. The dried hydrolyzates were analyzed on a Durrum D-500 amino acid analyzer. Recoveries were determined by comparing the amino acid analysis of these samples to similarly hydrolyzed samples which were injected without the column being present in the system.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of NT (peak 6) from its four diastereomers (D-Tyr[11])-NT, (D-Pro[10])-NT, (D-Arg[9])-NT and (D-Glu[1])-NT (peaks 2, 3, 5 and 7, respectively) by gradient elution at 50°C. This chromatogram also includes an analogue of NT, (Phe[11])-NT (peak 4), and its diastereomer (D-Phe[11])-NT (peak 1). Fig. 2 demonstrates the separation of NT and its five analogues by gradient elution at 40°C. Peak identification and amino acid analysis of all these peptides are given in Table I. The percent recovery of each peptide from the column along with the amount applied for that determination is also shown in Table I. Excellent recoveries for all peptides tested were obtained.

As Fig. 1 clearly shows, the chromatographic system used here was capable of separating NT from some of its available diastereomers. Except for (D-Arg[9])-NT (peak 5) all peptides in this particular mixture were completely separated from NT (peak 6). The resolution of (D-Arg[9])-NT from NT, however, appears to be sufficient

* Certain commercial equipment, instruments, or materials are identified in this paper in order to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the material or equipment identified are necessarily the best available for the purpose.

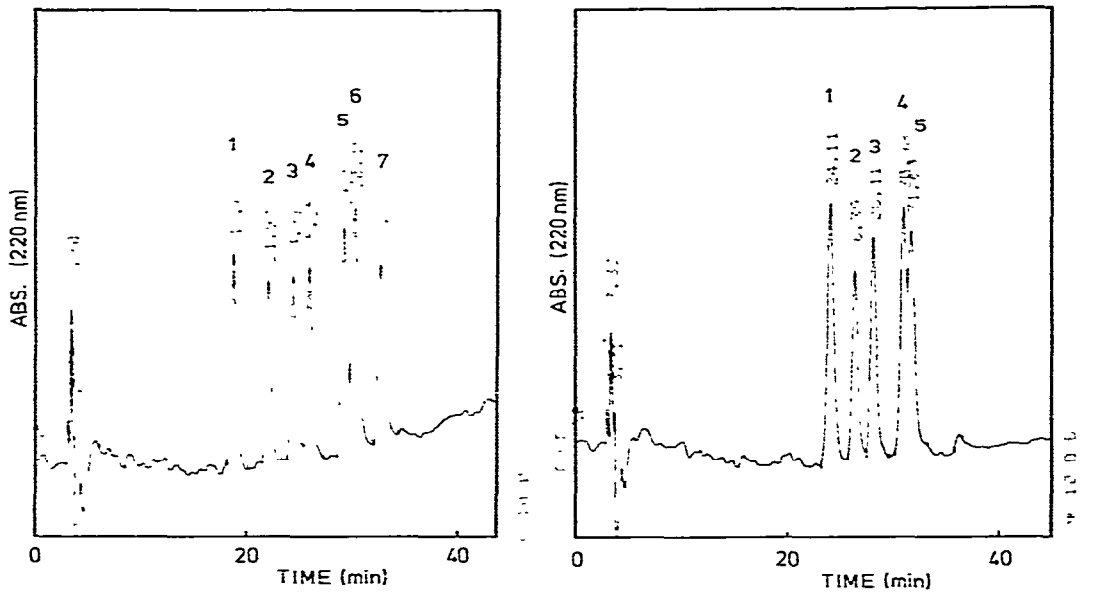

Fig. 1. Separation of some diastereomers of neurotensin. Column, MicroPak AX-10 (10 μm), 30 × 0.4 cm. Temperature, 50°C. Eluent: A, acetonitrile; B, 0.01 *M* triethylammonium acetate (pH 6.0), gradient program: linear starting from 23% B with a rate of 0.3% B/min. Flow-rate 1 ml/min. Peak identification is given in Table I. Amount of injection per peptide, *ca.* 1 nmol. a.u.f.s., 0.1 at 220 nm.

Fig. 2. Separation of some analogues of neurotensin. Column details as in Fig. 1 except temperature: 40°C.

TABLE I

PEAK IDENTIFICATION IN FIGS. 1 AND 2, AMINO ACID ANALYSES AND RECOVERIES

The numbers given for the various amino acids represent the numbers of these amino acids which were found in the corresponding peptide (experimentally, by amino acid analysis).

Peak	*Neurotensin*	*Glu*	*Leu*	*Tyr*	*Asp*	*Lys*	*Pro*	*Arg*	*Ile*	*Trp*	*Phe*	*Amount injected (nmol)*	*Amount recovered (nmol)*	*Recovery (%)*
Fig. 1														
1	(D-Phe^{11})-NT	2.04	2.00	0.96	1.03	1.01	1.94	2.05	0.96		0.98	6.2	5.6	90
2	(D-Tyr^{11})-NT	2.06	2.00	1.97	0.99	0.99	1.91	2.05	0.97			8.5	7.8	92
3	(D-Pro^{10})-NT	2.04	2.00	1.95	0.98	1.05	2.06	1.94	0.94			7.5	7.1	95
4	(Phe^{11})-NT	2.08	2.00	1.03	0.92	1.04	1.86	2.00	0.95		0.95	15.0	13.5	90
5	(D-Arg^{9})-NT	2.04	2.00	1.97	1.02	0.97	1.97	2.00	1.01			7.8	7.3	93
6	NT	2.09	2.00	1.98	0.94	1.02	1.89	2.11	0.99			5.5	5.0	90
7	(D-Glu^{1})-NT	2.06	2.00	1.90	0.93	0.98	2.02	2.04	1.03			8.8	8.1	92
Fig. 2.														
1	(Phe^{11})-NT	As above												
	(Trp^{11})-NT	2.07	2.00	0.88	0.99	1.05	1.88	2.10	1.03	0.88		9.5	9.1	96
2	(Leu^{11})-NT	1.99	3.00	0.91	0.94	1.03	2.09	2.02	1.02			7.0	6.9	98
3	NT	As above												
4	(Lys^{8})-NT	2.10	2.00	1.98	0.93	1.95	1.88	0.98	1.04			10.4	9.7	93
5	(Lys^{9})-NT	2.05	2.00	1.95	0.94	2.01	1.95	1.02	0.98			12.5	12.3	98

for a racemization test. Also remarkable is the excellent resolution of (Phe^{11})-NT (peak 4) from (D-Phe^{11})-NT (peak 1).

Five analogues of NT tested here were completely separated from NT (Fig. 2). (Phe^{11})-NT and (Trp^{11})-NT, however, could not be resolved from each other (peak 1). (Lys^{8})-NT (peak 4) and (Lys^{9})-NT (peak 5) were not completely separated.

A significant effect of temperature on retention of the neurotensins examined was observed. Retention times of these peptides at three different temperatures are given in Table II.

TABLE II

DEPENDENCE OF RETENTION TIMES ON TEMPERATURE*

Neurotensin	*Retention time (min) at*		
	30°C	*40°C*	*50°C*
Fig. 1			
(D-Phe^{11})-NT	16.1	17.5	18.7
(D-Tyr^{11})-NT	19.2	20.7	22.0
(D-Pro^{10})-NT	21.8	23.1	24.3
(Phe^{11})-NT	23.3	24.3	25.9
(D-Arg^{9})-NT	26.5	27.4	29.2
NT	27.6	28.2	30.2
(D-Glu^{4})-NT	29.9	31.2	32.6
Fig. 2.			
(Phe^{11})-NT	23.4	24.1	25.9
(Trp^{11})-NT	23.4	24.1	25.9
(Leu^{11})-NT	25.8	26.4	27.6
NT	27.6	28.1	30.0
(Lys^{8})-NT	30.3	31.0	32.8
(Lys^{9})-NT	30.9	31.7	33.4

* Other column details as in Fig. 1.

An increase in column temperature from 30 to 50°C increases the retention times of all peptides examined. However, the retention time of each peptide is differently affected by a change in temperature (Table II). Consequently, temperature can be varied to improve the resolution. For example, the mixtures in Figs. 1 and 2 were best separated at 50°C and 40°C, respectively.

CONCLUSIONS

The results obtained clearly show that the anion-exchange HPLC system applied here is capable of separating diastereomers and analogues of neurotensin. The effect of column temperature on retention can be exploited to improve the separation of a given mixture of peptides. The buffer used is volatile and thus facilitates a convenient and efficient isolation of separated peptides for further use and study. Moreover, the column used provides high recovery of chromatographed peptides.

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