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[Ser^7]neurotensin: isolation from guinea pig intestine

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Using three antisera to neurotensin of defined regional specificity, a novel neurotensin has been identified in extracts of guinea pig brain and small intestine. The primary structure of the peptide was established as: pGlu Leu Tyr Glu Asn Lys Ser Arg Arg Pro Tyr Ile Leu. Guinea pig neurotensin differs from bovine neurotensin by substitution of a prolyl residue by a seryl residue at position 7. Synthetic [Ser^7]neurotensin showed identical chromatographic and immunochemical properties to guinea pig neurotensin. This difference in primary structure may account for some of the anomalous pharmacological effects of bovine neurotensin on guinea pig tissues.

Neurotensin *(Guinea pig)* *Gastrointestinal peptide* *Primary structure* *HPLC* *Radioimmunoassay*

1. INTRODUCTION

The tridecapeptide, neurotensin, was originally isolated from bovine hypothalamus [1] and shown to possess limited homology to the amphibian peptide, xenopsin [2]. A peptide identical to hypothalamic neurotensin was subsequently isolated from bovine intestine [3] and immunocytochemical and radioimmunoassay studies have confirmed a dual brain/gut distribution in other mammals [4,5]. Using antisera of defined regional specificity in radioimmunoassays, evidence was obtained that the primary structure of neurotensin is probably conserved in the human, dog, rat and pig [6,7]. In the guinea pig, many otherwise highly conserved regulatory peptides, e.g. insulin [8], glucagon [9], vasoactive intestinal peptide [10], cholecystokinin-octapeptide [11] and gastrin [12] possess appreciably different primary structures. Here, we report the presence of a novel neurotensin, [Ser^7]neurotensin in extracts of guinea pig brain and small intestine and characterization of the peptide from the gut.

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2. MATERIALS AND METHODS

2.1. *Isolation procedures*

The entire small intestine (607 g) and brain (90 g) from 40 fed, anaesthetized guinea pigs were separately homogenized at 4°C with 8 ml/g ethanol/0.7 M HCl (3:1, v/v) using a Waring blender. The homogenates were stirred overnight at 4°C, centrifuged (20000 × *g* for 1 h) and ethanol removed from the supernatants under reduced pressure. An aliquot of each solution was removed for gel-permeation chromatography and radioimmunoassay. After further centrifugation (20000 × *g* for 1 h), trifluoroacetic acid was added to the supernatants to a final concentration of 0.1% (v/v) and the solutions pumped at a flow rate of 10 ml/h through 8 Sep-pak C18 cartridges (Waters Associates) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0:28.9:0.1) and the effluent lyophilized.

2.2. *Gel-permeation chromatography*

Aliquots (5 ml) of extracts of brain and small intestine were chromatographed on a column (100 × 2.5 cm) of Sephadex G-50 (fine) equilibrated with

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1 M acetic acid. The column was eluted at a flow rate of 25 ml/h and fractions (5 ml) collected. Aliquots (500 μl) of each fraction were lyophilized, reconstituted in 50 mM sodium phosphate buffer (pH 7.4) containing 140 mM NaCl and 0.2% (w/v) bovine serum albumin and subjected to region-specific radioimmunoassay. The column was calibrated with blue dextran (V_0), synthetic bovine neurotensin (SNT) and potassium chromate (V_t).

2.3. *High-performance liquid chromatography*

The intestinal extracts, after Sep-pak concentration, were redissolved in 0.1% (v/v) trifluoroacetic acid (1 ml) and injected onto a column (25 × 1 cm) of Ultrasphere-ODS (Beckman Instruments) equipped with a Spherisorb-ODS guard column. The column was eluted at a flow rate of 2.5 ml/min with a linear gradient (total volume 150 ml) formed from water/trifluoroacetic acid (99.9:0.1) and acetonitrile/water/trifluoroacetic acid (42.0:57.9:0.1). Fractions (1 min) were collected and the UV absorbance measured at 214 nm. Neurotensin-like immunoreactivity (NT-LI) in the fractions was measured at a dilution of 1:100 using the C-terminally directed antiserum NT 6.

The fraction containing maximum NT-LI was lyophilized, redissolved in 0.1% (v/v) trifluoroacetic acid (1 ml) and injected onto a Supelcosil LC-3DP column (250 × 4.6 mm) (Supelco). The column was eluted at a flow rate of 1.5 ml/min with a linear gradient (7.5 ml) formed from water/trifluoroacetic acid (99.9:0.1) and acetonitrile/water/trifluoroacetic acid (21.0:78.9:0.1) followed by a linear gradient (90 ml) to acetonitrile/water/trifluoroacetic acid (35.0:64.9:0.1). Peaks of UV-absorbing material were collected manually and assayed as previously. The peak of NT-LI was chromatographed on a Supelcosil LC-18-DB column (250 × 4.6 mm) eluted at a flow rate of 1.5 ml/min with a linear gradient (15 ml) formed from water/trifluoroacetic acid (99.9:0.1) and acetonitrile/water/trifluoroacetic acid (16.5:83.4:0.1) followed by a linear gradient (45 ml) to acetonitrile/water/trifluoroacetic acid (28.0:71.9:0.1).

2.4. *Structural analysis*

Amino acid composition was determined in duplicate using approx. 1 nmol peptide as described [13]. Guinea pig neurotensin (approx. 1 nmol) was incubated with L-pyroglutamyl-peptide hydrolase (EC 3.4.11.8) (Boehringer, Mannheim) according to [14]. The primary structure of guinea pig des[pGlu^1]neurotensin was determined by automated Edman degradation using an Applied Biosystems model 470A gas-phase sequencer [15]. The detection limit for PTH-amino acids was 0.5 pmol.

2.5. *Radioimmunoassay methods*

NT-LI was determined using three antisera to neurotensin of defined regional specificity according to [16,17]. Antiserum NT6 is directed towards the C-terminal region of neurotensin and shows full cross-reactivity with neurotensin (8–13) but no detectable cross-reactivity with neurotensin (1–8) and neurotensin (1–11). Antiserum GNT-21 is directed towards a site in the N-terminal to central region of the molecule and shows full cross-reactivity towards neurotensin (1–8) and neurotensin (1–11) but approx. 3% with neurotensin (1–6). Antiserum NT3 is directed towards a conformation existing in intact neurotensin and shows 1% cross-reactivity with neurotensin (2–13) and 18% cross-reactivity with neurotensin (8–13). Antisera of similar specificity have been described by others [6].

2.6. *Synthesis of guinea pig neurotensin*

[Ser^7]neurotensin was synthesized by the solid-phase method of [18] using the Cambridge Research Biochemicals Pepsynthesiser and purified by reverse-phase HPLC under the conditions described previously.

3. RESULTS

3.1. *NT-LI in guinea pig tissues*

The concentrations of neurotensin measured with the three regionally specific antisera in extracts of guinea pig brain and gut are shown in table 1. The total content of neurotensin in the intestinal extract, determined with C-terminally directed antiserum NT6, was 11 nmol. The NT-LI in both brain and gut extracts was eluted from a Sephadex G-50 gel permeation column as a single peak with the same elution volume as bovine neurotensin (fig.1). The ratios of immunoreac-

Table 1

Concentration of neurotensin-like immunoreactivity (pmol/g wet wt tissue) in extracts of guinea pig brain and small intestine measured with regionally specific antisera

Antiserum	Brain	Small intestine
NT 6	9.60	18.10
GNT 21	0.23	1.57
NT 3	3.90	3.48

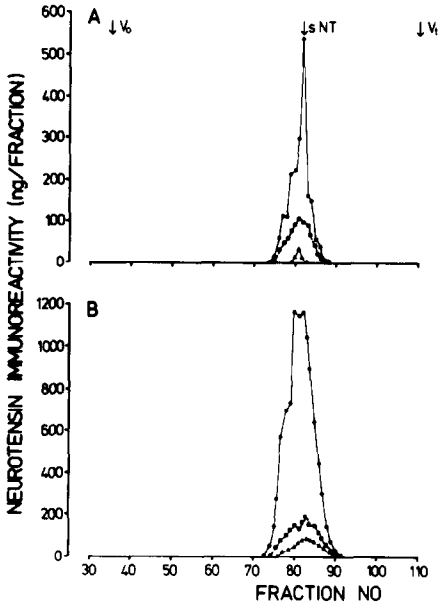

Fig.1. Elution profile on Sephadex G-50 of the neurotensin-like immunoreactivity in extracts of guinea pig. (A) Whole brain, (B) small intestine. Fractions were assayed with regionally specific antisera to neurotensin: NT 6 (●—●), NT 3 (■—■) and GNT 21 (▲—▲). Elution conditions and antisera specificities are described in the text.

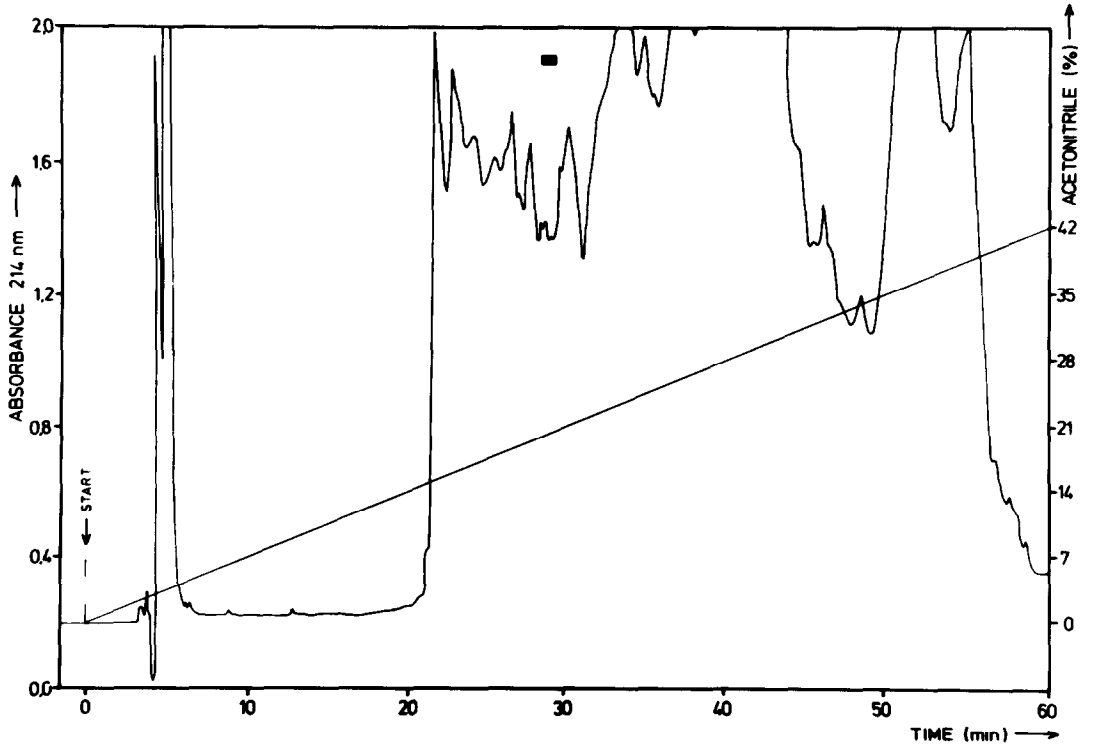

Fig.2. Reverse-phase HPLC on a semi-preparative Ultrasphere-ODS column of extracts of guinea pig small intestine, after concentration on Sep-Pak C18 cartridges. The bar indicates the fraction with maximum neurotensin-like immunoreactivity determined with antiserum NT 6. (——) Concentration of acetonitrile in the elution solvent.

tivities measured with the three regionally specific antisera were the same in the chromatographic peaks as in the crude extracts.

3.2. *Purification of the peptide*

Using the semi-preparative reverse-phase HPLC column (fig.2), the NT-LI in the Sep-pak concentrated extract of guinea pig gut was eluted as a single major peak with retention time between 29 and 30 min (synthetic bovine neurotensin 29.5 min). After rechromatography of this peak on a diphenylmethylsilylsilica column (fig.3), NT-LI was associated with a single, sharp peak of UV-absorbing material. This component was purified to homogeneity by chromatography on an analytical octadecyldimethylsilylsilica column (fig.4). Guinea pig neurotensin was eluted as a symmetrical peak with retention time 37.2 min compared with a retention time of 38.8 min for bovine neurotensin. The absorbance at 280 nm indicated the presence of tyrosine and/or tryptophan residues in the peptide. The yield of guinea pig neurotensin was 2.8 nmol representing 26% of that in the original extract.

3.3. *Structural analysis*

Amino acid analysis of guinea pig neurotensin (table 2) indicated that the peptide differed from

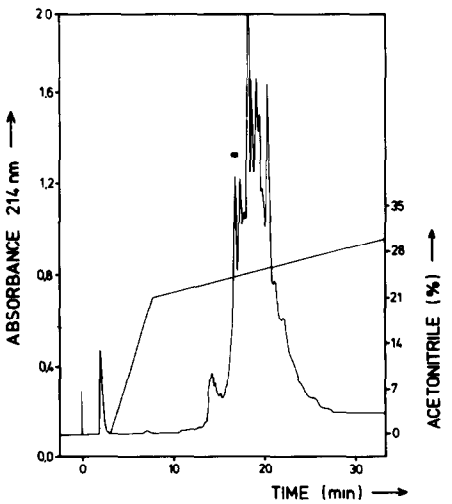

Fig.3. Reverse-phase HPLC of partially purified guinea pig neurotensin on a Supelcosil LC-3DP column. The peak with neurotensin-like immunoreactivity is indicated by the bar. (——) Concentration of acetonitrile in the elution solvent.

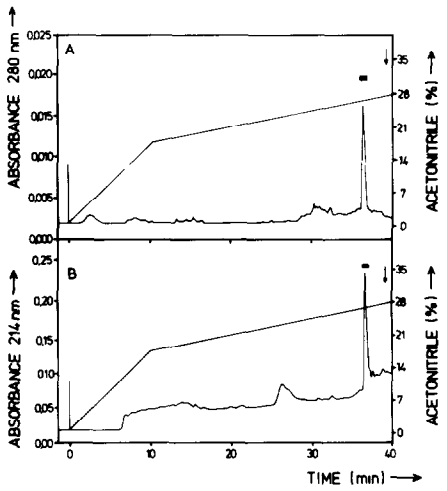

Fig.4. Purification to homogeneity of guinea pig neurotensin (■) on a Supelcosil LC-18-DB column. Absorbance was measured at (A) 280 nm and (B) 214 nm. (——) Concentration of acetonitrile in the elution solvent. The arrow indicates the retention time of synthetic bovine neurotensin.

Table 2

Amino acid compositions of guinea pig neurotensin and synthetic [Ser^7]neurotensin

Residue	Relative amount	
	Guinea pig neurotensin	[Ser^7]neurotensin
Asx	1.12	1.00
Ser	1.33	0.89
Glx	2.33	2.39
Pro	1.14	0.71
Ile	0.87	0.93
Leu	1.97	1.99
Tyr	1.99	1.97
Lys	0.95	1.02
Arg	1.83	2.10

Data represent the means of two determinations

bovine neurotensin by loss of a prolyl residue and gain of a seryl residue. Treatment with pyroglutamyl aminopeptide resulted in a significant decrease in the retention time on HPLC of the product, indicating the presence of a pyroglutamyl residue at the N-terminus of the peptide. Unambiguous assignation of residues (1–12) of guinea

Table 3

Automated Edman degradation of guinea pig des[pyroglutamyl[1]]neurotensin

Cycle no.	PTH-amino acid	Yield (pmol)
1	Leu	647
2	Tyr	703
3	Glu	167
4	Asn	708
5	Lys	499
6	Ser	167
7	Arg	183
8	Arg	266
9	Pro	374
10	Tyr	287
11	Ile	161
12	Leu	107

The repetitive yield was 89.4% calculated from Tyr^2 and Tyr^{10}

pig des[pGlu[1]]neurotensin was possible by automated Edman degradation (table 3) and the sequence analysis indicated that the prolyl residue at position 7 of the bovine neurotensin sequence had been substituted by a seryl residue. The structure of guinea pig neurotensin was confirmed by chemical synthesis. Synthetic [Ser^7]neurotensin (amino acid composition shown in table 2) was eluted from a reverse-phase HPLC column with the same retention time as guinea pig neurotensin under the elution conditions shown in fig.4. The reactivities of the synthetic and natural peptides towards the regionally specific antisera were similar (antiserum NT6, natural 100%, synthetic 100%; antiserum GNT-21, natural 6.6%, synthetic 4.8%; antiserum NT3, natural 15.6%, synthetic 13.0%).

4. DISCUSSION

The isolation of [Ser^7]neurotensin from guinea pig gut has provided the first evidence that the primary structure of neurotensin is not fully conserved in mammals. The substitution of a prolyl residue in bovine neurotensin for a seryl residue in the guinea pig requires only a single base change in the corresponding DNA sequence. In chicken neurotensin, the C-terminal hexapeptide sequence is conserved but three substitutions occur in the N-terminal region [19]. The substitution of Pro^7 for Ala^7 in chicken neurotensin also requires only a single base change in the codon. The immunochemical properties of guinea pig neurotensin are consistent with its proposed structure. Strong reactivity towards antiserum NT 6 is to be expected from the conservation of the C-terminal region of the molecule. Markedly reduced reactivity towards antiserum GNT-21 suggests that the prolyl residue at position 7 is important in the binding of neurotensin to this antiserum. As antiserum NT 3 requires a determinant that exists only in the intact neurotensin molecule for full expression of binding [16], the reduced reactivity of guinea pig neurotensin [comparable to that of the (8–13) fragment] suggests that the peptide adopts an appreciably different conformation to that of bovine neurotensin.

Neurotensin exerts a powerful pharmacological effect upon gastrointestinal motility in mammals. The predominant effect in vivo is the inhibition of peristaltic activity. Studies in vitro have shown that bovine neurotensin exerts an anomalous effect upon the guinea pig gastrointestinal tract. Thus, neurotensin induces relaxation of the rat duodenum [1] and ileum [20] but causes contraction of the guinea pig ileum [1,20] and taenia coli [20]. Although the guinea pig ileum has been used extensively for structure-activity studies of neurotensin and its analogues, the present study supports the conclusion [21] that this species is inappropriate for the study of neurotensin pharmacology. The availability of synthetic [Ser^7]-neurotensin will permit an investigation of the biological effects of this peptide in the guinea pig.

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