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Liquid chromatographic–mass spectrometric studies on the enzymatic degradation of gonadotropin-releasing hormone

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) derivatives are used in cancer therapy, but relatively little is known about their metabolic fate in the organism. This paper describes the application of high-performance liquid chromatography combined with electrospray mass spectrometry to identify the degradation products resulting from the incubation of two GnRH analogues, D-Phe^6–GnRH and DSer(OtBu)^6–desGly^{10}–GnRH–ethylamide (buserelin) with rat kidney membranes. Reversed-phase columns were applied with gradient elution using a flow-rate of *ca.* 2 μl/min to the mass spectrometer. Post- and precolumn stream splitting were employed to adjust the flow-rates for columns of 2 and 0.32 mm I.D. The pattern of peptide degradation products obtained with this method indicates that a defined proteolytic membrane enzyme system is responsible for these catabolic processes.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) derivatives are used in cancer therapy, but relatively little is known about their metabolic fate in the organism [1–3]. To increase their stability against proteolytic degradation and their biological activity, these synthetic "superactive" analogues have D-amino acids substituted for glycine in position 6 of the GnRH peptide chain (Fig. 1). Some analogues are additionally modified at the C-terminus of the peptide chain (*e.g.*, buserelin). Although stabilized in this way, these peptide analogues are known to be extensively degraded in the organism and thus inactivated.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH$_2$ (1 2 3 4 5 6 7 8 9 10) — GnRH, natural sequence

pGlu-His-Trp-Ser-Tyr-D-Phe-Leu-Arg-Pro-GlyNH$_2$ (1 3 6 10) — D-Phe^6-GnRH (PG)

pGlu-His-Trp-Ser-Tyr-D-Ser(OtBu)-Leu-Arg-Pro-NHEt (1 3 6 9) — Buserelin (SG)

Fig. 1. GnRH and analogues studied.

Recently, the kidney has been identified as the organ with special relevance concerning the pharmacokinetic fate of GnRH analogues [4]. In an attempt to minimize *in vivo* studies, we were able to demonstrate a close correlation between experiments with GnRH analogues in rats and with rat kidney membranes. Therefore, degradation experiments in this study were carried out by incubation of GnRH analogues with rat kidney membranes. Two GnRH analogues, D-Phe^6–GnRH and D-Ser(OtBu)^6–desGly^{10}–GnRH–ethylamide (buserelin) (where tBu = *tert.*-butyl), were used as representative molecules of the two types of superactive analogues mentioned above (Fig. 1). The objectives of this study were (i) to obtain more information on the pattern of degradation products of these peptides, (ii) to investigate the feasibility of on-line LC–MS experiments for this problem and (iii) a comparison of HPLC columns of 2 and 0.32 I.D. using stream splitting.

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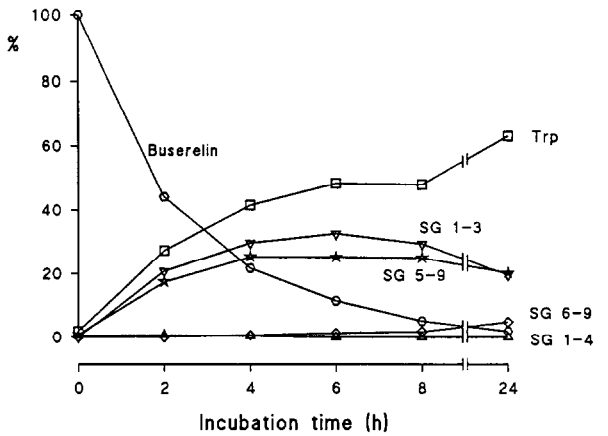

Fig. 2. Time course of the degradation of buserelin by rat kidney membranes and resulting degradation products measured by relative $[(Q/P)\cdot 100]$ chromatographic peak areas (UV detection at 215 nm; P = peak area of buserelin at zero time and Q = peak area at the corresponding incubation time).

LC–MS, especially with fast atom bombardment ionization, has already been applied for the study of peptide proteolysis [5,6]. We used electrospray ionization (ESI) mass spectrometry [7], which is a relatively new technique with great potential for this purpose. We report here the unambiguous identification of the degradation products of the two GnRH analogues produced by a selective enzyme system of the kidney membranes. The kinetics of the metabolites (Fig. 2) were also studied, but will be reported in detail in a separate publication.

EXPERIMENTAL

Materials

The peptides D-Phe^6–GnRH and buserelin and their partial sequences were obtained from Berlin-Chemie (Berlin, Germany) and Hoechst (Frankfurt, Germany), respectively. Trifluoroacetic acid (TFA) of HPLC gradient grade was obtained from Baker (Gross-Gerau, Germany). Acetonitrile (MeCN) of HPLC gradient grade and all other chemicals were purchased from Merck (Darmstadt, Germany).

Incubation and sample preparation

Membrane fractions of rat kidney were prepared as described previously [4] and incubated with 10–30 nmol/ml of peptide at 37°C in 20 m*M* phosphate or Tris buffer (pH 7.4) (100 m*M* NaCl, 0.1 mg/ml human serum albumin, *ca.* 20 mg kidney membrane per millilitre). Samples were taken at time intervals, the reaction stopped by heating and the samples were frozen at −20°C. In the off-line mode these samples were thawed, the peptides were adsorbed on C_{18} cartridges, eluted with MeCN–0.05% TFA (1:1) and the solvent evaporated by vacuum centrifugation. The residue was dissolved in the HPLC eluent, separated on a 4 mm I.D. column (see below) with fractionation, again evaporated and the residue dissolved in methanol–water (1:1) containing 1% acetic acid for mass spectrometric measurement. If TFA or pentafluoropropionic acid (PFP) was used (both 0.05%) instead of acetic acid in the off-line MS measurement, lower signal intensities were obtained. The signal ratio measured with the methanol–water system for acetic acid–PFP–TFA was 100:20:6 using buserelin (16.7 pmol/μl).

Chromatography

Peptides were separated in the off-line mode with 4 mm I.D. HPLC columns (Nucleosil 120 C_{18}, 5 μm, length 250 + precolumn 11 mm) using a Shimadzu LC-6A gradient system (RF-535 fluorescence detector, $\lambda_{ex} = 280$ nm, $\lambda_{em} = 365$ nm). Gradient elutions were run with (A) MeCN–water (5:95) and (B) MeCN–water (35:65), both containing 0.05% TFA, from 5% to 100% B within 25 min. For the isolation of the dipeptide pGlu-His ($t_R = 6$ min) the gradient was preceded by a 10-min isocratic elution period with MeCN–water (0.5:99.5). The systems used for the LC–MS experiments [Fig. 3, (A) columns of 2 mm I.D., (B) columns of 0.32 mm I.D.] consisted of an Applied Biosystems Model 140B dual-syringe pump and a Model 785A UV detector equipped with a micro flow cell (light path 0.1 mm, quartz capillary). The injectors were Rheodyne Model 7125 (20-μl loop) and Model 7520 (0.5-μl internal loop) and Valco Model C14W (60-nl internal loop). The split systems were made of Valco T (ZT1C) and quartz capillaries (75 and 50 μm I.D.) of appropriate length to achieve a stream splitting ratio of 100:1. With the precolumn split (Fig. 3B), a Lee (Frankfurt, Germany) visco jet micro mixer was applied. Separations were run with columns of

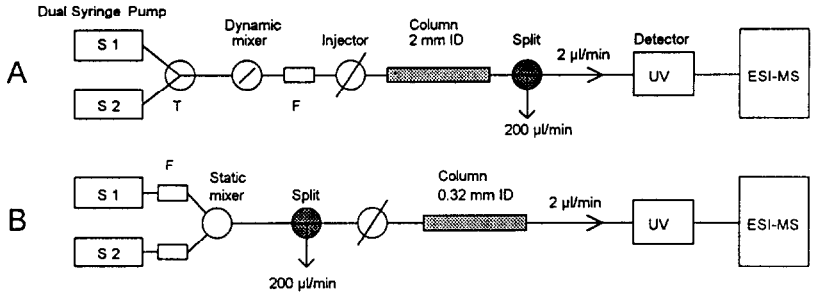

Fig. 3. HPLC set-up in the on-line mode for columns of 2 mm I.D. (variant A) and 0.32 mm I.D. (variant B) to deliver an eluent flow-rate of *ca.* 2 µl/min to the ESI mass spectrometer. Sample volumes were (A) 20 µl and (B) 60 nl or 0.5 µl.

Nucleosil 300 C_{18}, 5 µm (100 + 11 mm × 2 mm) (Macherey–Nagel, Düren, Germany) and Vydac 300A C_{18}, 5 µm (100 × 0.32 mm) (LC-Packings, Frankfurt, Germany).

Gradient elution in the on-line mode was performed with mobile phases of (A) MeCN–water (5:95) and (B) MeCN–water (1:1), both containing 0.05% TFA, from 5% to 60% B within 30–40 min. When large sample volumes (>60 nl) were applied to the capillary column, a 5-min equilibrium time with eluent A was inserted after sample injection before starting the gradient.

Mass spectrometry

The mass spectrometric equipment consisted of a TSQ 700 tandem quadrupole mass spectrometer (Finnigan MAT, Bremen, Germany) with an electrospray ion source (Analytica of Branford, Branford, CT, USA) operating in the positive-ion mode. Off-line ESI mass spectra were obtained by syringe infusion with a flow-rate of 1 µl/min of methanol–water (1:1) containing 1% acetic acid without sheath liquid, a drying gas temperature of 140°C and a high voltage of 2.8 kV. In the on-line mode the ESI ion source was operated at a sample flow-rate of about 2 µl/min, a 2-methoxyethanol sheath liquid flow-rate of 0.5 µl/min with a drying gas temperature of 210°C and a high voltage of 3.2 kV.

RESULTS AND DISCUSSION

Identification of degradation products

Incubation of the GnRH analogues PG and SG with rat kidney membranes results in a very similar pattern of HPLC peaks. As can be seen in Fig. 4, three major and two minor UV absorption peaks appear, two of them having no counterpart in the fluorescence trace (*i.e.*, no tryptophan). Using the off-line procedure, the peaks were fractionated and analysed by ESI-MS as described above. The results in Table I clearly demonstrate that both types of analogues, despite their structural differences at the C-terminus, are degraded in the same way, as depicted in Fig. 5 for buserelin. These results indicate that a selective proteolytic enzyme system is responsible for the metabolic events at the kidney membranes. By this method the structures of all degradation products (individual HPLC–UV peaks) were revealed. The weakness of this analytical procedure concerns the small hydrophilic molecules that elute together in or near the void volume of the RP chromatogram and are difficult to analyse by ESI-MS. Therefore, serine had to be identified by the dansylation procedure [8]. Obviously, the expected dipeptide pGlu-His appeared in the same fraction but could primarily not be detected by the LC–MS procedure. Therefore, the chromatographic conditions were changed to elution with a low MeCN content (see Experimental), resulting in the identification of pGlu–His.

On-line LC–MS studies

ESI-MS is an increasingly used method for detection in LC–MS experiments. As this method is ideally suited to the LC–MS of peptides, we tried to substitute an on-line LC–MS method for the laborious off-line procedure described above.

The feasibility of this approach was tested

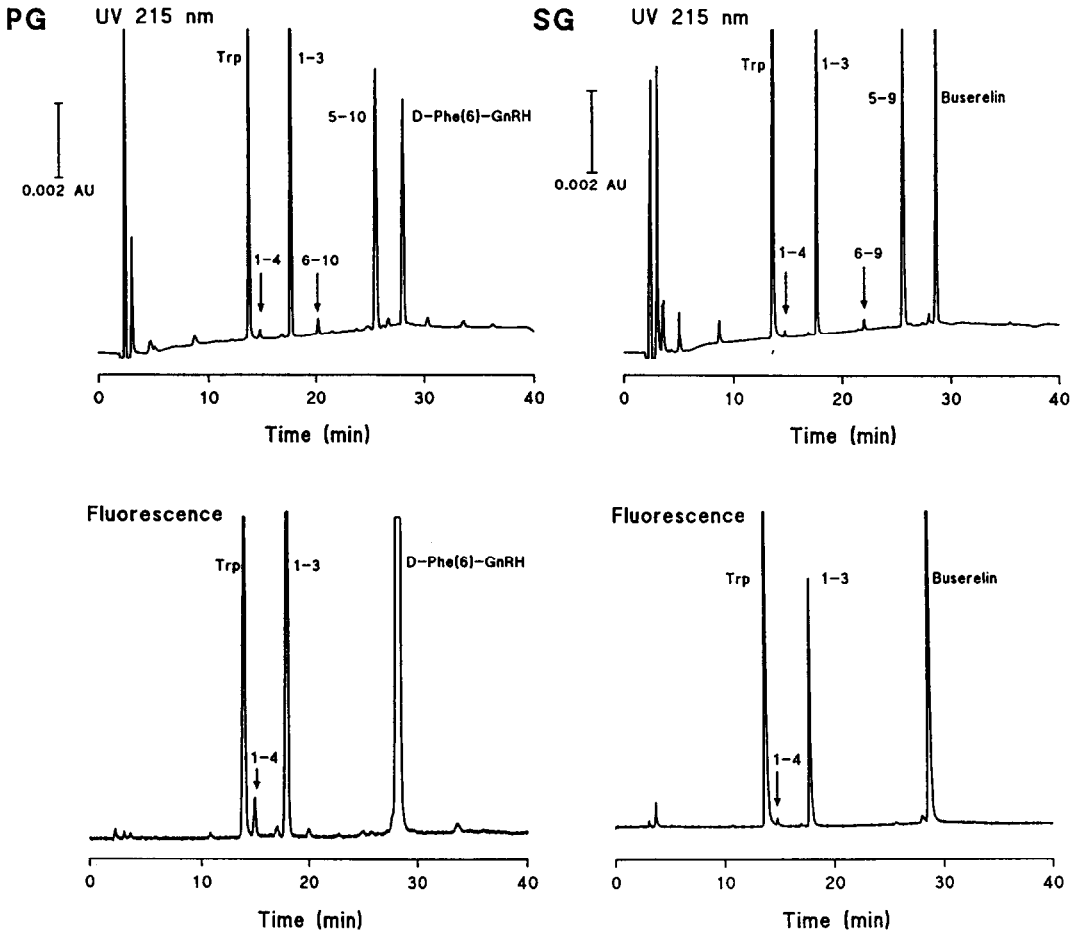

Fig. 4. HPLC separation of the degradation mixtures of D-Phe^6-GnRH (PG) and buserelin (SG), incubation time 3 h, with a Nucleosil 120 C_{18}, 5 μm column (250 + 11 mm × 4 mm) using UV and fluorescence detection. Gradient elution with acetonitrile–water–trifluoroacetic acid (see Experimental, off-line mode) was applied. Minor peaks with low retention times resulting from the kidney membrane matrix were not identified in the UV trace.

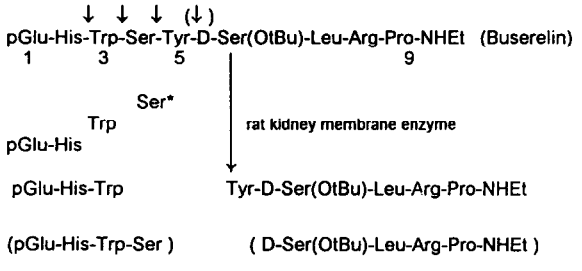

Fig. 5. Degradation pattern of buserelin produced by incubation with rat kidney membranes (minor products in parentheses). Primary and secondary splitting by the enzyme complex occurs around the serine residue. Serine was identified by the dansylation method [8].

using two known HPLC variants (Fig. 3A and B) using columns of 2 and 0.32 mm I.D., respectively. In set-up A, 2 mm were preferred to 4 mm I.D. columns to reduce sample and solvent consumption. Both variants were applied to the described peptide mixtures produced by the degradation of buserelin and to a test mixture of the same peptides prepared for quantitative comparison. Figs. 6 and 7 show two examples of these four sets of LC–MS experiments. As expected, the amount of peptide delivered at the same flow-rate to the MS system was the critical factor for the limit of detection.

In Fig. 6, a run with variant A is depicted with

TABLE I

STRUCTURAL ASSIGNMENT BY ESI-MS OF THE PEPTIDE DEGRADATION PRODUCTS OF D-Phe^6-GnRH AND BUSERELIN

Sequence assignment	Sequence No.	M_r (calc.)[a]	m/z[b]	
			MH^+	MH_2^{2+}
Trp	3	204.1	205.1	–
pGlu–His	1–2	266.1	267.0	–
pGlu–His–Trp	1–3	452.2	453.1	–
pGlu–His–Trp–Ser	1–4	539.2	540.2	–
Tyr–D-Phe–Leu–Arg–Pro–Gly–NH_2	PG 5–10	750.4	751.5	376.3
D-Phe–Leu–Arg–Pro–Gly–NH_2	PG 6–10	587.3	588.4	294.6
Tyr–D-Ser(OtBu)–Leu–Arg–Pro–NHEt	SG 5–9	717.4	718.4	359.7
D-Ser(OtBu)–Leu–Arg–Pro–NHEt	SG 6–9	554.3	555.5	–
D-Phe^6-GnRH	PG 1–10	1271.6	1272.8	636.9
Buserelin	SG 1–9	1238.6	1239.8	620.3

[a] Monoisotopic masses.
[b] m/z Values for the $[M + H]^+$ and $[M + 2H]^{2+}$ ions in the ESI mass spectra.

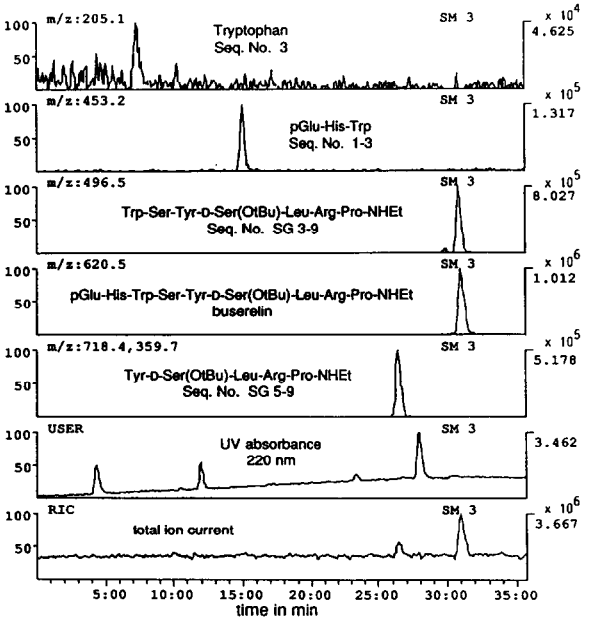

Fig. 6. LC–MS experiment with a test mixture (20 pmol/μl of each component) of buserelin SG 1–9 and the peptide sequences 3, 1–3, SG 5–9 (*cf.*, Table I) and SG 3–9 [Trp–Ser–Tyr–D-Ser(OtBu)–Leu–Arg–Pro–NHEt]. The separation was carried out with experimental set-up A (Fig. 3). The sample volume was 20 μl and the splitting ratio 250 to 2.1. The amount of each component introduced into the mass spectrometer was 3.4 pmol. The dead volume between the UV detector and the ESI ion source causes a delay of about 3 min of the mass chromatograms relative to the UV trace. The sequence SG 3–9 (not separated by the chromatographic system) is detected in the ion chromatogram of the $[M + 2H]^{2+}$ ion (m/z 496.5).

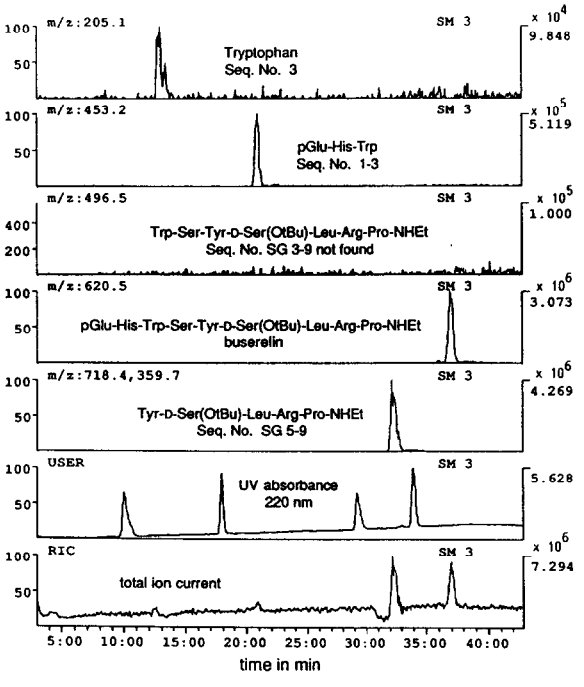

Fig. 7. LC–MS experiment with a degradation mixture of buserelin after incubation for 4 h with rat kidney membranes. Experimental set-up B (Fig. 3) was employed (sample volume 500 nl, eluent flow-rate to the ESI ion source 2.1 μl/min, delay time between mass chromatograms and UV trace *ca.* 3 min). The amount of each of the major components injected and delivered to the MS system was *ca.* 10 pmol. The sequence SG 3–9 was not detected in the respective ion chromatogram (m/z 496.5).

the peptide test mixture of known concentration (20 pmol/μl for each substance). Although 400 pmol of each peptide were injected, only 3.4 pmol per substance were delivered to the MS system (because of the postcolumn stream splitting), which is near the limit of detection. This mixture additionally contained the potential degradation product SG 3–9. We were not able to separate SG 3–9 from the starting peptide buserelin with our chromatographic system but both peptides were clearly detected by the MS system (Fig. 6, traces SG 1–9 and SG 3–9).

Fig. 7 shows a run with a degradation mixture using variant B. The amounts injected were *ca.* 10 pmol of each of the major components, which were also completely delivered to the MS system in this set-up. As can be seen from the ion current traces, peptides SG 1–3, SG 5–9, the starting material SG 1–9 and tryptophan were identified. However, the small amounts (<1 pmol) of SG 1–4 and SG 6–9, still detectable by the UV detector, are below the limit of mass spectrometric detection. The sequence SG 3–9 ($m/z = 496.5$) was not detected. Therefore, this peptide can be ruled out as a major degradation product.

CONCLUSIONS

Peptide metabolites resulting from the enzymatic degradation of the modified peptide structures of two GnRH analogues were unambiguously identified by combined HPLC and ESI-MS methods. A comparison of the off-line and on-line modes demonstrates that each of them has advantages in some respects.

The off-line mode is preferable if small amounts of unknown peptide have to be identified in the chromatogram. High enrichment factors can be attained and TFA can be replaced with acetic acid before the sample is introduced into the mass spectrometer, which results in higher sensitivity in this measurement.

The on-line mode is fast, reliable and less laborious than the off-line procedure. If only a very small amount of material is available (*e.g.*, cell fractions), the use of a 0.3 mm I.D. capillary column (variant B) is advantageous. Even with this small column, relatively high sample volumes can be used in the applied gradient system to increase the amount of peptide introduced into the mass spectrometer.

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REFERENCES

1 A.S. Dutta, *Drugs Future*, 13 (1988) 43.
2 J. Kiesel, J. Sandow, K. Bertges, G. Jarabek-Sandow, H. Trabant and B. Runnebaum, *J. Clin. Endocrinol. Metab.*, 68 (1989) 1167.
3 H. Ueno and S. Matsuo, *J. Chromatogr.*, 566 (1991) 57.
4 H. Berger, N. Heinrich, E. Albrecht, U. Kertscher, J. Oehlke, M. Bienert, H. Schäfer, I. Baeger and B. Mehlis, *Regul. Pep.*, 33 (1991) 229.
5 R.B. van Breemen and R.G. Davis, *Anal. Chem.*, 64 (1992) 2233.
6 W.E. Seifert, Jr., W.T. Moore and R.M. Caprioli, in D.M. Desiderio (Editor), *Mass Spectrometry of Peptides*, CRC Press, Boca Raton, FL, 1991, p. 201.
7 E.C. Huang, T. Wachs, J.J. Conboy and J.D. Henion, *Anal. Chem.*, 62 (1990) 713A.
8 Y. Tapuhu, D.G. Schmidt, W. Lindner and B.L. Karger, *Anal. Biochem.*, 115 (1981) 123.