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Short Communication

High-performance liquid chromatographic assay for the determination of the decapeptide cetrorelix, a novel luteinizing hormone-releasing hormone antagonist, in human plasma

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Abstract

This is the first paper which describes a HPLC method for the determination of the decapeptide cetrorelix, a potent luteinizing hormone-releasing hormone (LH-RH) antagonist, in human plasma by liquid–liquid extraction, concentration by back-extraction with diluted acid into a smaller volume, and fluorescence detection, using the decapeptide D-21740 as internal standard. The excitation (227 nm) and emission wavelengths (340 nm) for cetrorelix and the internal standard are identical. The extraction yield for both peptides is *ca.* 50% and the assay is linear over the concentration range 2–20 ng/ml in plasma. The mobile phase components are ammonium acetate buffer (0.05 mol/l, pH 4) as solvent A and methanol–acetonitrile (1:1, v/v) as solvent B. The elution condition for the peptides from the column (Lichrospher 60 RP-Select B 5 μm, 250 × 4 mm I.D.) is isocratic with a 49:51 mixture of solvent A–solvent B. The lower limit of quantitation for cetrorelix is 2 ng/ml human plasma.

1. Introduction

The decapeptide cetrorelix (SB-75) (Fig. 1) was synthesized by Bajusz *et al.* [1] and is now under development for oncological and gynaecological indications in our laboratories. Cetrorelix was characterized as a potent antagonist of LH-RH (luteinizing hormone-releasing hormone) [2]. LH-RH antagonists can be used for the treatment of hormone sensitive tumors like prostate [3–5] and mammary [6] carcinomas. Unlike older antagonists, cetrorelix is devoid of side effects such as histamine release and induction of edema [4]. Toxicological studies confirmed the safety of cetrorelix. Up to now blood levels in healthy human volunteers were determined by radioimmunoassay (RIA). However, it has not been established that RIA can discriminate between the parent compound and/or metabolites, because up to this time nothing is known about the metabolism of cetrorelix in humans. For this reason, as well as for special pharmacokinetic and metabolism studies, a sensitive HPLC method was developed.

This is the first paper that describes a HPLC assay for the quantitative determination of cet-

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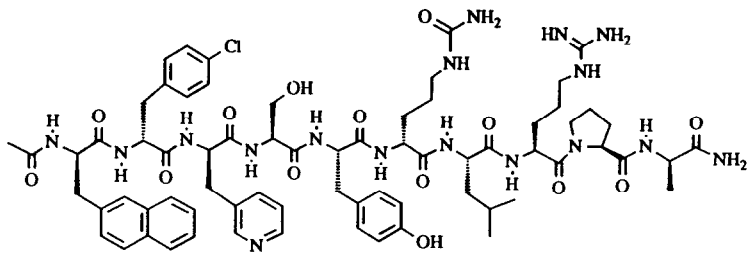

Fig. 1. Structural formula of cetrorelix.

rorelix in human plasma using an internal standard.

2. Experimental

2.1. Reagents and chemicals

Cetrorelix acetate salt (D-20761, [Ac - D - Nal^1 - (*p*-Cl) - D - Phe^2 - D - Pal^3-Ser^4 - Tyr^5 - D - Cit^6 - Leu^7 - Arg^8 - *Pro*^{9} - D - Ala^{10}-NH_2] acetate) and the internal standard (I.S.) D-21740 ([Ac - D - Nal^1 - (*p* - Cl) - D - Phe^2 - D - Pal^3 - Ser^4 - Tyr^5 - D - Cit^6 - Leu^7 - Arg^8 - *Ala*^{9} - D - Ala^{10} - NH_2] TFA) were synthesized by the Chemical Department of ASTA Medica (Frankfurt, Germany). Differences in amino acid composition are typed in italics.

All other chemicals and solvents of analytical grade (HPLC grade) were from E. Merck (Darmstadt, Germany).

2.2. Instrumentation and chromatographic conditions

The HPLC equipment consisted of a Hewlett-Packard 1090 M liquid chromatograph with a diode array detector, a Hewlett-Packard 1046 A fluorescence detector and the Hewlett-Packard HPLC Pascal workstation (Hewlett-Packard, Waldbronn, Germany). LiChrospher 60 RP-Select B columns (250 × 4 mm I.D., 5 μm particle size) for the analysis of basic components were supplied by E. Merck. The mobile phase components were ammonium acetate buffer (0.05 mol/l, pH 4) as solvent A and a mixture of methanol–acetonitrile (1:1, v/v) as solvent B. Elution was carried out isocratically with a mixture of 49% ammonium acetate buffer (0.05 mol/l, pH 4) and 51% methanol–acetonitrile (1:1, v/v) at a flow-rate of 0.4 ml/min. Cetrorelix and I.S. were detected by fluorescence: excitation wavelength, 227 nm; emission wavelength, 340 nm.

2.3. Plasma extraction procedure

To 4 ml of human plasma spiked with cetrorelix acetate calibration standards was added 200 μl of 1 *M* HCl. After addition of 100 ng internal standard (I.S.) to each tube the plasma was transferred to a centrifuge tube and extracted with 9 ml of a mixture of ethyl acetate–1-butanol (9:1, v/v) for 15 min on a Reax mixer (Heidolph, Kehlheim, Germany). After centrifugation at 1000 *g* for 3 min a 5-ml volume of the organic layer was transferred to a pointed centrifuge tube. Once again 5 ml of the extraction solution were added to the plasma sample and extracted for a further 15 min. After centrifugation (1000 *g*, 3 min) 5 ml of the organic layer were removed and combined with the first extract. The peptide material was reextracted into 150 μl 10 m*M* HCl. After centrifugation (1000 *g*, 3 min) the organic layer was aspirated with a thin glass capillary and 120 μl of the HCl-phase were injected onto the HPLC system.

2.4. Solutions

Both cetrorelix acetate salt and I.S. were soluble in diluted acetic acid (0.01 mol/l). Stock solutions, containing 1 mg cetrorelix acetate salt or I.S. per ml solvent, were diluted with the

acetic acid solution to a concentration of 1 μg/ml to obtain the so called working solutions.

2.5. Calibration

A calibration curve was prepared from 2 to 20 ng cetrorelix/ml human plasma by processing 4 ml of human plasma spiked with 100 μl working solution of I.S. and 100 μl of working solution of cetrorelix.

2.6. Clinical study

In a clinical phase I study plasma levels after intravenous and subcutaneous administration of cetrorelix should be determined. In a pilot study 0.35 mg cetrorelix were administered intravenously to four healthy volunteers. Five minutes after application blood was sampled from the volunteers and plasma levels were determined.

3. Results and discussion

3.1. Chromatography

The decapeptide cetrorelix shows a strong natural fluorescence. The excitation scan of cetrorelix shows a maximum at wavelength 227 nm, the emission scan has a broad maximum around 347 nm. The internal standard can be measured at the same wavelengths with nearly the same intensity (Fig. 2). Further, no interferences from the plasma were observed and thus both compounds were baseline separated under the HPLC conditions used (see Figs. 3 and 4). A chromatogram of a characteristic study plasma sample is illustrated in Fig. 5. This plasma sample is one of 4 samples obtained from healthy volunteers 5 min after intravenous administra-

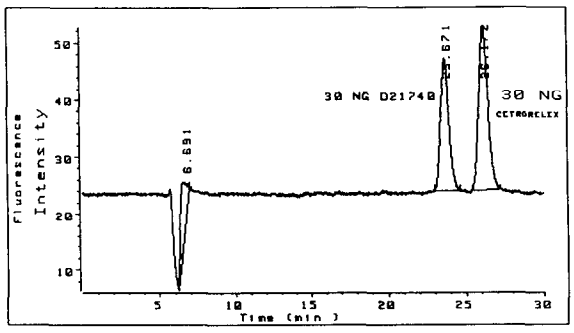

Fig. 2. HPLC run of equal amounts working solution of cetrorelix and D-21740 (I.S.).

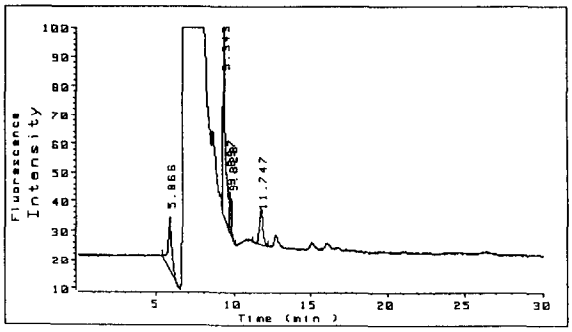

Fig. 3. Chromatogram of extracted blank plasma.

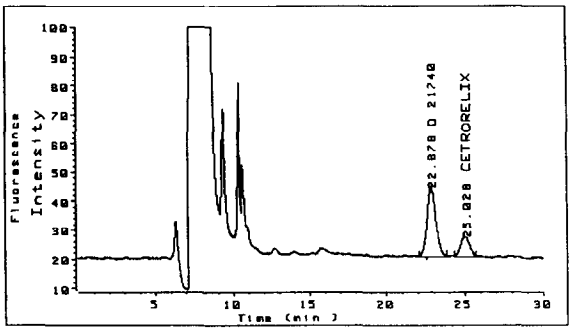

Fig. 4. Human plasma was spiked with 5 ng/ml cetrorelix acetate salt and 30 ng/ml I.S. as internal standard, extracted and analysed by HPLC.

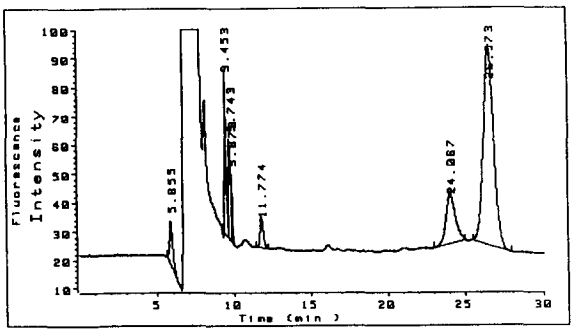

Fig. 5. Characteristic chromatogram of extracted plasma obtained from a healthy volunteer 5 min after intraveneous administration of 0.35 mg cetrorelix.

tion of 0.35 mg cetrorelix, and demonstrates the usefulness of the HPLC assay. For quantitative determination of cetrorelix the plasma extracts have been diluted (1:1 with 10 m*M* HCl) because the cetrorelix concentration in the samples exceeded the upper concentration of the calibration curve. The concentrations of cetrorelix in plasma samples of the volunteers vary between 20 and 30 ng/ml plasma.

3.2. Linearity and recovery

The assay is linear in the evaluated range of 2–20 ng/ml plasma. This concentration range was chosen because, after subcutaneous application (intended route in man) of the pharmacological dose, the expected blood levels in human are in that range, as indicated from data derived from healthy volunteers whose blood levels were determined by RIA (unpublished results).

The deviations of the calibrators between spiked and measured concentrations observed by the evaluation of a typical calibration curve by least-squares regression are shown in Table 1. The calculated curve follows the equation $y = 48x - 8.7$ with the correlation coefficient $r^2 = 0.98$. The deviation of the calibrations over the whole calibration range is less than 15%.

To minimize the absorption of cetrorelix to the glass walls it is advantageous to acidify (pH 3–4) the plasma samples. The recovery of both cetrorelix and I.S. is relatively low but reproducible (cetrorelix: 50.1 ± 4.9%; I.S. (D-21740): 46.1 ± 5.5%; $n = 10$). In conjunction with the chromatographic results this indicates that D-21740 is an internal standard well suited for cetrorelix analysis.

Table 1
Deviation of the calibrators between spiked and calculated concentrations

Concentration (ng/ml)		Deviation (%)
spiked	calculated	
2.00	1.89	−5.50
5.00	5.73	+14.60
10.00	9.98	−0.20
15.00	13.06	−12.93
20.00	20.34	+1.70

3.3. Precision and limit of quantitation

The coefficients of variation of the peak areas for serial determinations ($n = 10$) of 5 and 10 ng/ml plasma were found to be 8.26% and 3.92% respectively. The deviations (mean ± S.D., $n = 10$) between measured and originally spiked concentration were 6.7% ± 3.6% for 5 ng/ml and 3.3% ± 2.4% for 10 ng/ml. The limit of quantitation was found to be 2 ng cetrorelix acetate salt/ml human plasma. This is the lowest concentration for which the deviation from spiked to measured concentration, in each measurement, was less than 20%.

4. Conclusions

In summary, the described HPLC assay is suitable for the determination of cetrorelix in plasma samples from humans. Because of its high natural fluorescence the decapeptide cetrorelix is detectable at relatively low plasma concentrations. As a specific assay the HPLC assay can be used for studying the *in vitro* and *in vivo* metabolism of cetrorelix and for controlling the specificity of the RIA, because nothing is known about the metabolism of cetrorelix and about the cross-reaction of the RIA with its metabolites.

5. References

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