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Development and validation of a HPLC method for routine quantification of the decapeptide Cetrorelix in liposome dispersions

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Abstract

The development and validation of an HPLC method for the quantification of the decapeptide Cetrorelix (acetyl-D-2-naphthylalanyl-D-4-chlorphenylalanyl-D-3-pyridylalanyl-seryl-tyrosyl-D-citrullyl-leucyl-arginyl-prolyl-D-alaninamide), a potent antagonist of the luteinising hormone-releasing hormone in liposome dispersions is described. An isocratic reversed phase method with UV-detection appeared most appropriate. Several detergents were tried to disrupt liposomes. Furthermore, detergents turned out to be useful, because they minimised unwanted loss of Cetrorelix due to adsorption to the vial surfaces. Triton X-100 was found most effective, while sodium cholate led to quantification problems. In the presence of 2.5% Triton X-100 calibration curves with a high degree of linearity were achieved in the desired range of 0.2–10 μg/ml. The limits of detection and quantification of Cetrorelix were calculated from the peak-to-noise ratio to be 11 and 37 ng/ml, respectively. The repeatability of the method in presence of phospholipid and Triton was good with relative standard deviations (R.S.D.) ranging from 0.8% (at 0.05 μg/ml) to 1.5% (at 0.2 μg/ml). The presence of liposomes at phospholipid contents of up to 0.25 mg/ml did not significantly affect the slope or linearity of the calibration curve, nor the peak-to-noise ratio.

Keywords: HPLC; Peptide; Cetrorelix; Detergent; Validation

1. Introduction

Specific challenges occur in the analysis of peptides due to their special properties, e.g. surface adsorption, aggregation and denaturation. The peptide under investigation here is Cetrorelix (acetyl-D-2-naphthylalanyl-D-4-chlorphenylalanyl-D-3-pyridylalanyl-seryl-tyrosyl-D-citrullyl-leucyl-arginyl-prolyl-D-alaninamide), a potent antagonist of the luteinising hormone-releasing hormone, which was first synthesised by Bajusz [1] and is currently used in infertility treatment. It is however also thought to be suitable for the treatment of hormone dependant tumours or benign prostate hypertrophy (BPH). Treatment of the latter would clearly benefit from the availability of a sustained release formulation. Our approach for a sustained release formulation is based on semisolid phospholipid formulation of vesicular morphology,

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so called vesicular phospholipid gels [2], which shall serve as a depot for Cetrorelix upon implantation, releasing Cetrorelix in a sustained manner. This gives rise to the need to perform long-term in vitro release studies, where the presence of varying amounts of phospholipid in the final fractions can be expected. Therefore, the method has to be suitable for the quantification of Cetrorelix in the presence of lipids and of detergents, which are needed to disrupt the liposomes. The development of a suitable method is described in this paper.

Several methods of analysis for Cetrorelix are reported in the literature for various purposes, e.g. a radioimmunoassay [3], and three liquid chromatographic methods for the determination of Cetrorelix in human plasma samples. The latter methods use either fluorescence detection with excitation wavelength at $\lambda = 227$ nm and emission wavelength at $\lambda = 340$ nm [4] or mass spectrometry [5] for the detection of Cetrorelix, respectively. One of the methods [6] used combinations of UV-detection at 226 nm and detection of radioactivity or LC–MS. As we expected to analyse rather large numbers of samples from in vitro release studies, our aim was to establish an analysis method (a) that possesses short analysis time, (b) avoids an eluent gradient, (c) is suitable for quantification of Cetrorelix in the presence of liposomes (phospholipid), and (d) has a highly linear and reproducible response in the expected concentration range (0.2–10 μg/ml Cetrorelix).

2. Materials and methods

2.1. Materials

Cetrorelix acetate was a gift from Zentaris GmbH (Frankfurt, Germany). Acetic acid 30% (w/w) was diluted from Suprapur® acetic acid (Merck, Darmstadt, Germany); acetonitrile was HPLC grade (Merck, Darmstadt, Germany). Hexane was purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) and Sodium cholate were purchased from Sigma-Aldrich (Steinheim, Germany). Triton X-100 was purchased from Sigma-Aldrich (Steinheim, Germany) or Merck (Darmstadt, Germany; Triton X-100 for molecular biology). Polysorbate 20 was obtained from ICI Surfactants (Eversberg, Belgium). E80 (egg phospholipid with at least 80% phosphatidylcholine) and E80-3 (saturated egg phospholipid with at least 80% phosphatidylcholine) were a gift from Lipoid (Ludwigshafen, Germany). Cholesterol was obtained from Croda Chemicals (Goole, UK) and re-crystallised from methanol. Waters total recovery vials (Waters, Milford, MA, USA) were of glass type1, class A with 950 μl maximal injectable volume.

2.2. Sample preparation

For investigating the influence of lipids, the lipid was dispersed in distilled water and diluted to concentrations of up to 0.25 mg/ml. This was assumed to be around the maximum concentration, which can be expected to be released in one fraction during our release test. The liposomes in the peptide containing dispersions were disrupted by adding various detergents. Alternatively, samples were extracted with organic solvents by mixing the aqueous Cetrorelix solution with equivalent volumes of organic solvents. The mixtures were vortexed, centrifuged and the lower aqueous phase was injected into the HPLC.

2.3. HPLC method

The HPLC system consisted of a Waters 2690 Separation Module with a 996 photo diode array (PDA) detector, alternatively with a combination of a 474 SFD fluorescence detector and a 2487 dual wavelength UV detector (all: Waters, Milford, MA, USA). Millennium 32 software (Waters, Milford, MA, USA) was used. The column system consisted of a Symmetry® C18 analytical column (3.9 mm × 150 mm, 5 μm) combined with a Symmetry® Sentry™ guard column (3.9 mm × 20 mm, 5 μm) (both: Waters, Milford, MA, USA). The temperature of the column heater was set to 25 °C, while the temperature of the sample chamber was set to 10 °C. The sample volume was 50 μl. A short cleaning cycle with 95% acetonitrile was performed after each analysis. All experiments using UV absorbance were performed at 227 nm, the maximum absorption wavelength of Cetrorelix. The use of the more specific secondary absorption maximum at 265 nm led to a much smaller AUC and was therefore abandoned. Fluorescence detection was carried out with excitation at $\lambda = 227$ nm and emission at $\lambda = 340$ nm.

3. Results and discussion

A starting point was taken in the HPLC method reported by Raffel et al. [4] who were using fluorescence detection and a gradient method using ammonium acetate buffer (0.05 mol/l, pH 4) and methanol-acetonitrile (1:1, v/v) as mobile phase, leading to a retention time of Cetrorelix of around 25 min. The limit of quantification for this method was reported to be 2 ng/ml. The long retention time and the use of an eluent gradient made this method inappropriate for our purposes. For the same reasons, a method used by Schwahn et al. [6] was considered inappropriate, as the run time for a single determination was above 3 h. Niwa et al. [5] used acetonitrile–water–TFA (35:65:0.1, v/v/v) for the LC–MS determination of Cetrorelix and found a quantification limit for Cetrorelix of 1 ng/ml. We prioritised UV- or fluorescence- over MS-detection with respect to the expected better precision and repeatability of quantification for the samples under investigation. We decided to apply a classical reversed phase HPLC-approach, using a Symmetry® C18 analytical column (3.9 mm × 150 mm, 5 μm) together with a Symmetry® Sentry™ guard column and an isocratic eluent system consisting of a mixture of acetonitrile and 0.05% aqueous TFA as mobile phase. The percentage of trifluoroacetic acid was reduced in comparison to the method reported by Niwa in order to minimise eventual disturbances due to the high UV absorbance of trifluoroacetic acid at the desired wavelength. The pH of the aqueous phase was measured to be 2.25, a value assumed to be sufficiently acidic to ensure a stable charge of Cetrorelix (pK_a of the pyridine moiety: 5.49).

The influence of the mobile phase was the first aspect to be investigated during the development of the new HPLC method. In order to determine the influence of the acetonitrile/water mixing ratio on retention time, we varied the content of acetonitrile between 33 and 25% (v/v), which corresponds to percentages of 0.05% aqueous TFA between 67 and 75% (v/v). The following retention times were found for Cetrorelix, which was dissolved in 0.03% acetic acid and injected at concentrations of 10 μg/ml (Table 1).

These results indicate that small changes in the percentage of acetonitrile had a strong influence on the retention time. Quite short retention times could be achieved. Since our aim was to analyse a considerable number of samples from our release studies and the detection of eventual impurities or degradation products was of minor relevance in this context, we chose isocratic elution with 30% acetonitrile for further experiments resulting in retention times of 3–4 min for Cetrorelix.

Table 1
Influence of the amount of acetonitrile in the mobile phase on the retention time of Cetrorelix

Acetonitrile (%)	0.05% TFA (%)	Retention time (min)
33	67	2.0
31	69	2.9
30	70	3.6
29	71	4.7
27	73	8.9
26	74	12.8
25	75	19.8

The next subject under investigation was the influence of different sample preparation procedures. In order to disrupt liposomes, which are expected to be present during routine analysis, various tensides were tried as well as extraction of the analyte by organic solvents. Upon addition of Triton X-100 E80-liposome dispersions became clear, i.e. the liposomes were transferred into mixed micelles at concentrations of 2.5% and above. In contrast, E80-3/cholesterol liposomes (5.5:4.5 mol/mol) needed to be warmed to 60 °C for 8–10 min upon addition of Triton to disrupt the liposomes (data not shown).

When analysing Triton-containing Cetrorelix solutions by HPLC an additional benefit was seen: Triton led to an increase of the area under the curve (AUC) of the Cetrorelix peak, i.e. the HPLC-response was enhanced over aqueous Cetrorelix solutions (Table 2).

Table 2
Response curves of Cetrorelix established with five concentrations in the range 0.2–4 μg/ml in two different concentrations of Triton X-100

Concentration Triton X-100 (%)	Linearity (r-value)	Slope	Intercept
0	0.97258	24,191	+1010
2.5	0.99808	149,642	−536
5	0.99977	155,491	−612

Each value measured in triplicate.

Table 3
AUC of various concentrations of Cetrorelix in water and Triton X-100

Type of vial	Solvent	0.2 μg/ml	1 μg/ml	4 μg/ml	Slope
Polypropylene	2.5% Triton	37,871	196,977	758,096	187,209
Glass	2.5% Triton	30,505	154,948	616,273	154,114
Polypropylene	Water	10,144	60,254	484,303	123,642
Glass	Water	5,901	38,164	168,806	42,588

Comparison of glass and polypropylene vials. Detection at $\lambda = 227$ nm. Mean AUC, $n = 3$ each. Slope of linear fit given ($0.9949 \leq r \leq 1.0000$).

This was found to be due to a reduced adsorption of Cetrorelix to the surfaces of the vials.

The adsorptive loss of Cetrorelix varied not only in dependence on the presence of Triton but also with the type of vials used. Although Triton enhanced the quality of the analysis by reducing this adsorption, a further increase in performance was found when exchanging glass vials and equipment with polypropylene vials and equipment (Table 3). It can be seen that irrespective of the medium (water or Triton solution), higher responses were achieved when polypropylene equipment was used. The adsorption phenomenon has been investigated and described in more detail elsewhere [7].

Triton unfortunately, generated multiple peaks at retention times from 4 min on (wave-like chromatogram; Fig. 1), irrespective of origin and batch, with all qualities of Triton X-100. Triton X-100 of Sigma origin usually made lower disturbances than the one of Merck origin and was therefore chosen for further experiments. Two other detergents, sodium cholate or polysorbate 20 were tried as detergents at final concentrations of 20 and 8.1 mM (corresponding to 1% (m/m) polysorbate), respectively. With sodium cholate a double peak appeared quite regularly at the retention times of the Cetrorelix peak (Fig. 2). The UV spectra of both peaks appeared identical with a maximum at $\lambda = 227$ nm (data not shown), which indicates that both peaks contain Cetrorelix. Injection of sodium cholate solution produced no peak at this retention time. It was assumed that part of the Cetrorelix elutes at a slightly different retention time in presence of sodium cholate. Whether there possibly occurs aggregation or degradation of Cetrorelix could not be clarified. With Polysorbate 20 lower AUCs were found for Cetrorelix as compared to the other two tensides, leading to the assumption that polysorbate 20 is less efficient in the prevention of the adsorption of Cetrorelix to surfaces (data not shown). Furthermore,

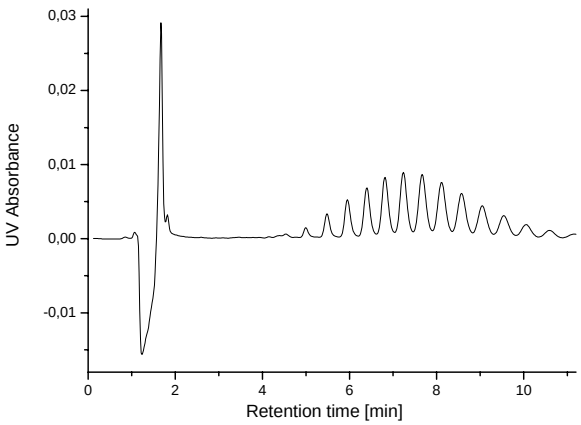

Fig. 1. Chromatogram of 2.5% Triton X-100 (Sigma, Lot 11K0216). UV absorbance at 227 nm.

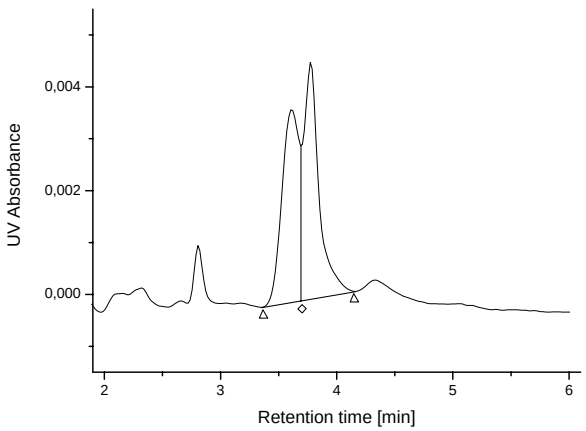

Fig. 2. Chromatogram of a release cell fraction, containing unknown amounts of phospholipid, Cetrorelix and 20 mM Sodium cholate. UV absorbance at 227 nm measured with PDA detector.

polysorbate 20 could not be used in combination with a fluorescence detector due to its own fluorescence.

Sample preparation by solvent extraction using several organic solvents and mixtures, namely chloroform, chloroform-methanol (2:1, v/v), hexane–2-propanol–water (40:50:8, v/v/v) led to high losses of substance. Only pure hexane resulted in a good correlation between the detected AUCs of extracted and untreated Cetrorelix solutions. Extraction with hexane is considered suitable for the determination of Cetrorelix in presence of phospholipids with unsaturated fatty acids, but the use of large quantities of organic solvents is not desirable. It was therefore concluded that Triton X-100 is the best choice as long as the peaks caused by Triton X-100 are clearly separated from the Cetrorelix peak and that solvent extraction by hexane is second choice.

Furthermore, the influence of various detectors, including a PDA detector, a fluorescence detector and a dual-wavelength UV detector was investigated in samples containing 2.5% Triton X-100. Fluorescence detection was carried out with excitation at $\lambda = 227$ nm and emission at $\lambda = 340$ nm. As the presence of Triton X-100 leads to a less stable base line compared to a baseline upon injection of water based samples, the detection and quantification limit of Cetrorelix were somewhat higher than of other methods reported in literature. The peak-to-noise ratio was measured at 4 concentrations between 10 and 100 ng/ml, plotted against the concentration and a polynomial fit was fitted to the data. From this fit the corresponding concentrations of a peak-to-noise of 3:1 for the limit of detection and of a peak-to-noise ratio of 10:1 for the limit of quantification were extracted. The difference between the 996 PDA and the 2487 dual wavelength UV detector using a wavelength of 227 nm for quantification was only marginal. In the presence of 2.5% Triton X-100 the PDA detector showed a limit of detection of 17 ng/ml while the limit of quantification was found to be 56 ng/ml. The UV detector showed under same conditions a limit of detection of 11 ng/ml and a limit of quantification of 37 ng/ml. However, the PDA detector has a clear advantage over a dual wave-length UV detector, namely ensuring a higher specificity of the method, as the UV absorbance spectrum of the peaks can be shown. Fluorescence detection with a 474 SFD was not used for routine analysis, as no superiority was found. A noisy baseline (Fig. 3)

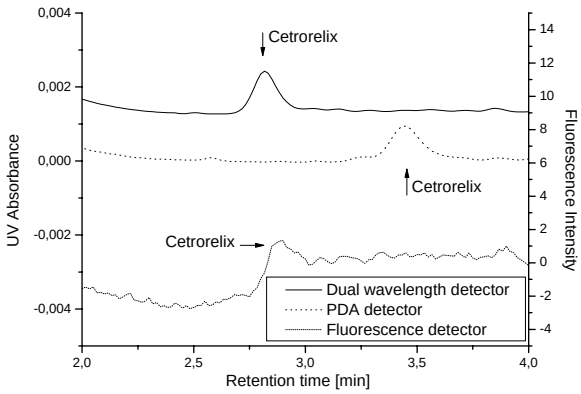

Fig. 3. Comparison of peak-to-noise ratio of various detectors; 50 ng/ml Cetrorelix. Dual wavelength detector and PDA: absorbance at 227 nm; fluorescence detector: excitation at 227 nm; and emission at 340 nm. Curve with dual wavelength detector shifted by +0.001. Longer retention time with PDA detector due to different HPLC.

led to a poorer peak-to-noise ratio yielding in a limit of detection of 37 ng/ml and a limit of quantification of 124 ng/ml. Considering the limits achieved with each detector and the specificity of the method, the PDA detector was decided to be first choice.

Investigating the specificity of the method, no signs of interference with Triton or phospholipid were detected. The peak purity was ensured by comparing the UV spectrum of the Cetrorelix peak of numerous chromatograms in the up- and down-slopes. No difference between up- and down-slopes were observed, suggesting that the UV spectrum is consistent with that of reference Cetrorelix.

For investigation of the precision (repeatability and intermediate precision), Cetrorelix samples (4 μg/ml) in both 2.5 and 5% Triton solutions were analysed on four days in six replicates each. The precisions as well as the day-to-day variance (intermediate precision) are given as relative standard deviations (R.S.D.) of the series. Both samples in Triton solution were within the set limits (< 2%, Table 4). Robustness: the influence of the composition of the mobile phase was discussed earlier (Table 1). No influence of the solvent on the retention time was discovered. The relative standard deviation of the retention time on the same day (24 measurements) was up to 3.9% (up to 0.12 min). The day-to-day variation of the average retention time was up to 8.7%. The variation between two columns of the

Table 4
Precision: repeatability given as relative standard deviation (in %) of AUC of 4 μg/ml Cetrorelix solution

Solvent	Repeatability				Intermediate precision
	Day 1	Day 2	Day 3	Day 7	
2.5% Triton	0.26	0.17	0.16	0.82	1.21
5% Triton	0.88	0.19	0.27	0.18	0.63

All values measured six times. Intermediate precision given as relative standard deviation of all 24 measurements in one solvent.

same type was up to 5.4%, herewith being lower than the normal day-to-day variation.

The stability of the solution was required to be 98% after a specified time of 7 days, as this is the maximal standing time after release experiments. Comparing the AUCs from Day 1 and Day 7 of Cetrorelix in 2.5 and 5% Triton solution yielded in recoveries of 100.5 and 99.0%, respectively, fulfilling the requirements set.

In order to study the influence of liposomes, calibration curves in presence of E80 and E80-3 as phospholipids were established. In a first experiment the influence of phospholipid on the base line was investigated by mixing a 0.5 mg/ml E80 and a 0.5 mg/ml E80-3/Cholestrol (5.5:4.5) dispersion, respectively, with the same volume of Triton X-100. It was found that the presence of phospholipid did not increase the noise of the base line. The lipid dispersion was mixed with a Cetrorelix stock solution containing 10 μg/ml Cetrorelix and 5% Triton X-100 and diluted to the final concentration with 5% Triton X-100, which led to a clear solution in case of E80. The solutions under investigation contained five different concentrations in the range 0.2–4.0 μg/ml Cetrorelix, 0.25 mg/ml E80 or E80-3/cholesterol (5.5:4.5) mixture and 2.5% Triton X-100. Preliminary experiments showed no difference in AUC of Cetrorelix in the presence of 0.05–0.25 mg/ml E80. The linearity of the hexane extraction method and its recovery of Cetrorelix were investigated in presence of E80-3/cholesterol (5.5:4.5). The recovery was between 88.5 and 100.8% (mean 95.3 ± 4.0%). The precision (% R.S.D.) in presence of phospholipid was found to be between 0.21 and 1.91%. The linearity is given in Fig. 4; all *r*-values were higher than 0.999. With the Triton method, all calibration curves showed *r*-values higher than 0.999 (Fig. 5). The *y*-intercept, which is required

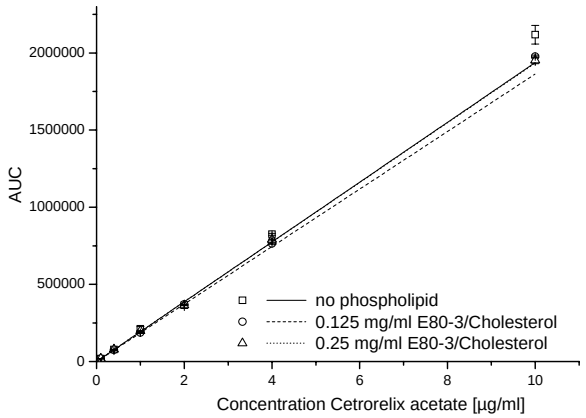

Fig. 4. Response curve of Cetrorelix after extraction with hexane in presence of various concentrations of E80-3/Chol (5.5:4.5). Mean ± standard deviation, $n = 3$ each, $r > 0.999$.

to be below 2%, was between 0.25 and 0.96% of the maximum absorption. With the given linearity and precision, accuracy of the method was inferred. The recovery of Cetrorelix in presence of phospholipid was found to be between 96 and 107%. The precision (given as relative standard deviation) was between 0.10 and 1.79%. The slightly higher fluctuation of the recovery here is due to the fact that a separate solution was prepared for every concentration. It can be seen that phospholipids of 0.25 mg/ml had no influence on the linearity or the AUC of analysis of Cetrorelix when 2.5% Triton X-100 was present. It

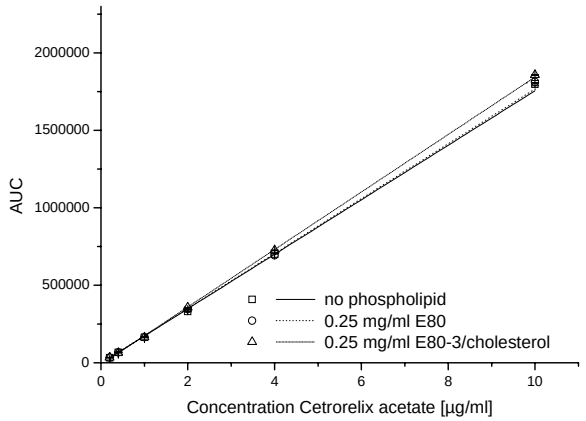

Fig. 5. Response curves of Cetrorelix in 2.5% Triton X-100 in absence and presence of phospholipid. Mean ± standard deviation, $n = 3$ each, $r > 0.999$.

was therefore concluded that this HPLC method is suitable for the routine analysis of Cetrorelix in the presence of phospholipid.

4. Conclusion

The development of a HPLC method for a peptide is often far from trivial. A fast method for the determination of the decapeptide Cetrorelix is described in this paper. The method is using acetonitrile and 0.05% aqueous trifluoroacetic acid (30:70, v/v) as mobile phase with a flow rate of 1 ml/min. The solid phase was consisting of a C18 analytical reversed phase column with guard column. UV detection proved to be around three times more sensitive than fluorescence detection reaching a limit of detection at 11 ng/ml and a limit of quantification at 37 ng/ml. 2.5% Triton X-100 was sufficient to ensure reliable analysis of Cetrorelix in the presence of up to 0.25 mg/ml egg phosphatidylcholine. It was furthermore found that polypropylene equipment is superior to glass equipment.

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