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ORIGINAL PAPER

Fluorescence Spectroscopic Determination of the Critical Aggregation Concentration of the GnRH Antagonists Cetrorelix, Teverelix and Ozarelix

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Abstract The fluorescence spectroscopic behavior of three 2-naphthylalanine containing Gonadotropin-releasing hormone (GnRH) antagonists Cetrorelix, Teverelix and Ozarelix has been investigated concerning their aggregation process in comparison to the non-aggregating peptide D-Phe^6-GnRH. The aim of the present investigation consisted in developing a method to determine the critical aggregation concentration for these decapeptides. This was achieved by monitoring the incorporation of the fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS) into aggregates and utilizing the modification of band shape and intensity of the intrinsic peptide fluorescence emission depending on the analyzed peptide concentrations. Finally an approach for the explanation of the observed band characteristics is presented analyzing the fluorescence of fragments Cetrorelix$_{1–4}$ and Cetrorelix$_{1–6}$.

Keywords Gonadotropin-releasing hormone antagonists · Cetrorelix · 1-anilino-8-naphthalene sulfonate · Critical aggregation concentration · Tyrosine fluorescence

Introduction

The Gonadotropin-releasing hormone (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, GnRH) [1] controls the secretion of luteinizing and follicle-stimulating hormone from the anterior pituitary gland. More than 3000 synthesized and analyzed GnRH analogues are described [2]. GnRH agonists have a high similarity in their primary structure to GnRH sharing at least eight amino acids with the native hormone. The use of D-amino acids or bulky residues at position 6 enhances the stability against proteolysis [3]. Initially designed to stimulate secretion of gonadotropins, continuous application results in a decrease of pituitary hormone secretion, which is based on a desensitization of the gonadotrophic cells and down-regulation of pituitary receptors. In contrast, developed GnRH antagonists induce an immediate reduction of gonadotropin levels by inhibition of their secretion from the anterior pituitary gland [4]. The decapeptide D-Phe^6-GnRH is an example for a GnRH agonist and Cetrorelix, Ozarelix and Teverelix are potent antagonists (Fig. 1). Due to their ability to suppress the production of gonadotropins, potential medical applications for antagonists concern the treatment of prostate and breast cancer, endometriosis, female infertility or controlled ovarian stimulation [2, 4–8]. Cetrorelix (Cetrotide) is already approved in endocrine therapy.

The antagonistic peptides show intrinsic fluorescent activity which is dominated by the side chain of 2-naphthylalanine as observed for poly-naphthylalanine species [9]. Because of their hydrophobic properties these three peptides tend to aggregate. A formation of aggregates can be monitored by incorporation of a fluorescent probe into the hydrophobic core of peptide units [10]. Furthermore, a change of intrinsic fluorescence can also be detected during this process.

In the present study, a method has been developed to determine the critical aggregation concentration (*cac*) for the described peptides. Therefore 1-anilino-8-naphthalene sulfonate (ANS) has been used as a probe for extrinsic fluorescence. Beyond that, the *cac* could be identified by analysis of the intrinsic fluorescence characteristics of these peptides.

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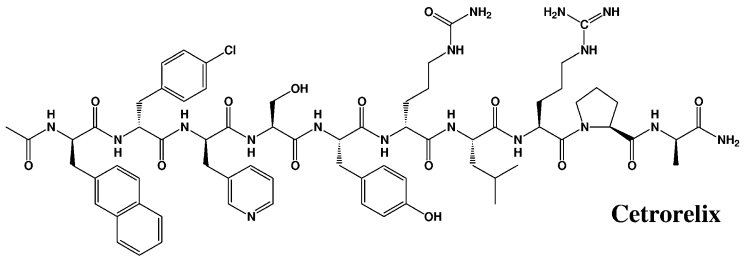

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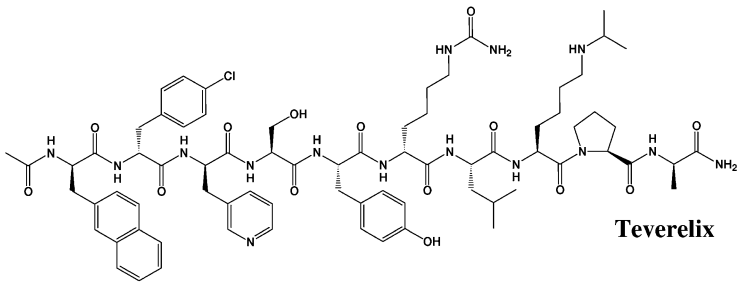
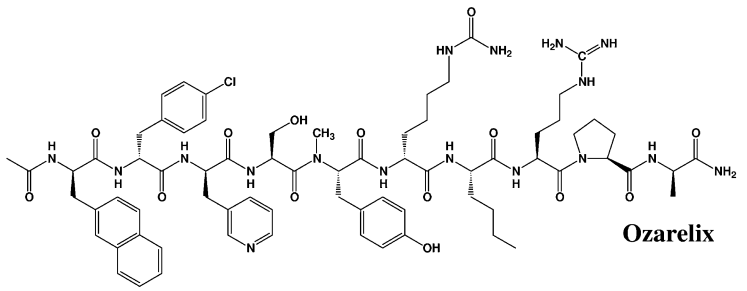
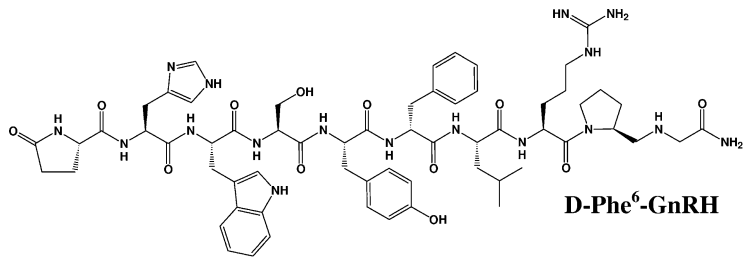


Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Hcit-Leu-Lys(iPr)-Pro-D-Ala-NH_2



Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-NMe-Tyr-D-Hcit-Nle-Arg-Pro-D-Ala-NH_2



pGlu-His-Trp-Ser-Tyr-D-Phe-Leu-Arg-Pro-Gly-NH_2

Fig. 1 Primary structures of analyzed GnRH analogues

Experimental

Cetrorelix, Cetrorelix$_{1-4}$, Cetrorelix$_{1-6}$, Teverelix, Ozarelix and the aromatic amino acids were purchased from AEterna Zentaris, D-Phe^6-GnRH from Veyx Pharma. Sample concentration and purity were verified by HPLC and UV measurement using a Specord S 100 B UV-spectrometer (Analytik Jena).

Peptide solutions for fluorescence measurement were prepared in 10 mM ammonium acetate pH 7.0 and incubated for 7 days at room temperature to ensure reproducible fluorescence intensities. The pH of the samples was measured using a Mettler-Toledo, MP220 pH meter (Inlab 410 electrode).

Ammonium 1-anilino-8-naphthalene sulfonate (ANS) was purchased from Biochemika, Fluka. Aliquots of probe solution containing 0.2 mM ANS were mixed with peptide solutions at the fluorescence cell to get a final ANS concentration of 5 μM. All samples were equilibrated for 15 min at 22 °C before measurement.

Fluorescence spectra were recorded with a Hitachi F-4500 Fluorescence Spectrophotometer equipped with a 150 W Xenon lamp and standard photomultiplier as detector using 10 mm × 10 mm / 5 mm × 5 mm sample cells from Hellma. Scan speed was 60 nm/min, excitation and emission slit were at 5 nm and photomultiplier voltage was 400 V for intrinsic and 700 V for probe fluorescence measurements. Excitation wavelength for intrinsic fluorescence measurement was 280 nm and emission was recorded between 300 and 450 nm. Excitation wavelength for extrinsic

fluorescence measurement was 346 nm and emission of ANS was recorded between 400 nm and 650 nm.

Results and discussion

Determination of the critical aggregation concentration using extrinsic fluorescence

ANS as a probe for the hydrophobicity of the micro environment in a solution is applicable for monitoring the interaction and association of peptides. Accumulation of aggregates in aqueous solution leads to the formation of a hydrophobic core. An incorporation of ANS into such cores is reflected by a change of the maximum of fluorescence emission and an increase of the intensity [10, 11]. For Cetrorelix, Teverelix and Ozarelix an abrupt shift of fluorescence maximum of ANS from 520 nm to 465 nm and an increase of intensity could be detected reaching a defined peptide concentration.

This specific concentration, where the change of the fluorescence behavior occurs, was defined as the critical aggregation concentration (*cac*) an analogy to describe critical micelle concentrations for surfactants [12, 13]. Plotting intensity values at 490 nm against the concentration demonstrates this behavior (Fig. 2).

In contrast, no significant increase of the fluorescence intensity could be detected for the more hydrophilic peptide D-Phe^6-GnRH (Fig. 3) and no shifting of the maximum emission of the probe to wavelengths below 520 nm was observed. Therefore, this decapeptide was used to define an ANS reference for the monomeric peptide state in solution.

In addition to the presented method the critical aggregation concentration can be obtained as the inflection point of a sigmoid curve by plotting the quotient of fluorescence intensities at 465 nm and 520 nm against the peptide concentrations (Fig. 4).

Both described analysis methods result in rounded critical aggregation concentrations of 40 µg/ml for Cetrorelix, 50 µg/ml for Teverelix and 170 µg/ml for Ozarelix. The dominant change in the primary structure of Ozarelix in comparison to Cetrorelix and Teverelix is the N-methylation of tyrosine at position 5. Haviv et al. discussed that tyrosine N-methylation at GnRH antagonists leads to an increase of solubility by distortion and restriction of the peptide backbone and a better interaction with the aqueous solvent [14]. The highly soluble and non-aggregating D-Phe^6-GnRH has no detectable *cac* at the analyzed concentrations up to 10 mg/ml.

Intrinsic fluorescence of the analyzed GnRH antagonists

The fluorescence emission spectra for solutions of monomeric Cetrorelix, Ozarelix and Teverelix at low concentrations show a typical emission pattern as observed for D-2-naphthylalanine (Fig. 5).

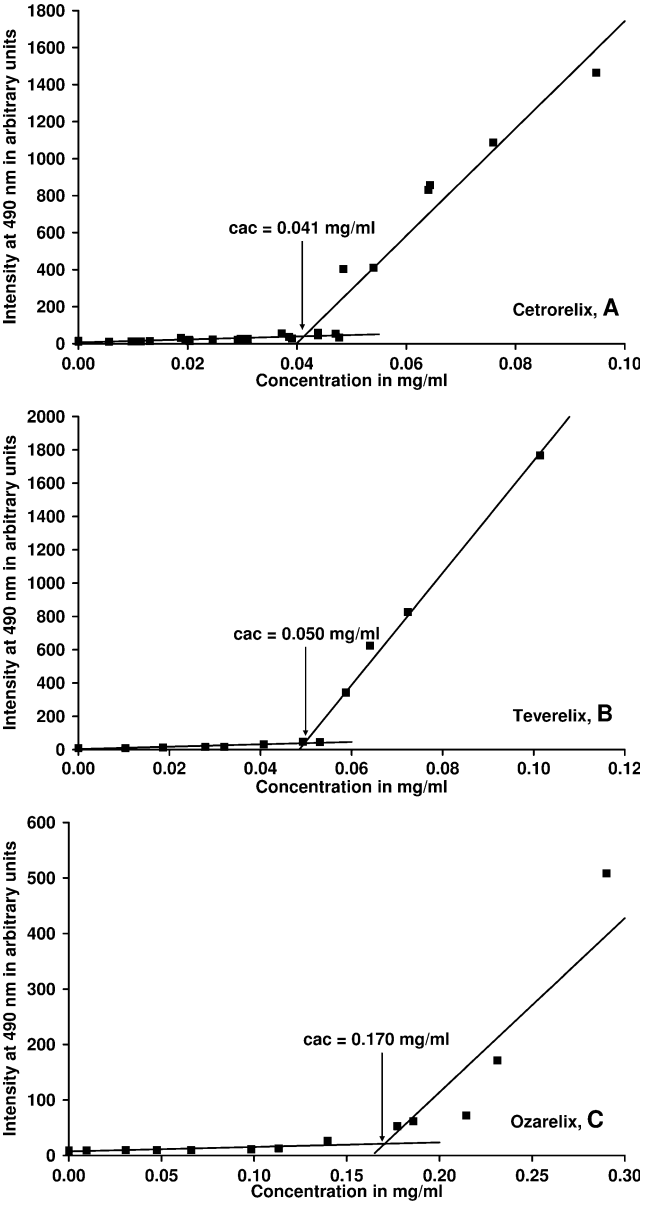


Fig. 2 Fluorescence of ANS at 490 nm against peptide concentration; Cetrorelix (**A**), Teverelix (**B**) and Ozarelix (**C**)

For all spectra there is a global maximum for the emission at 335 nm, a second maximum at 327 nm, and two shoulders between 350–360 nm and 365–380 nm can be detected. A weak minimum at 329 nm is observable. These characteristics are in good agreement with results extracted from published fluorescence spectra of poly(L-2-naphthylalanine) and N-acetyl-2-naphthylalanine [9]. The intrinsic fluorescence of the interesting aromatic amino acids was checked using the described conditions. D-p-Cl-phenylalanine and D-3-pyridylalanine show no fluorescence emission. A described tyrosine fluorescence emission near 305 nm [15] can

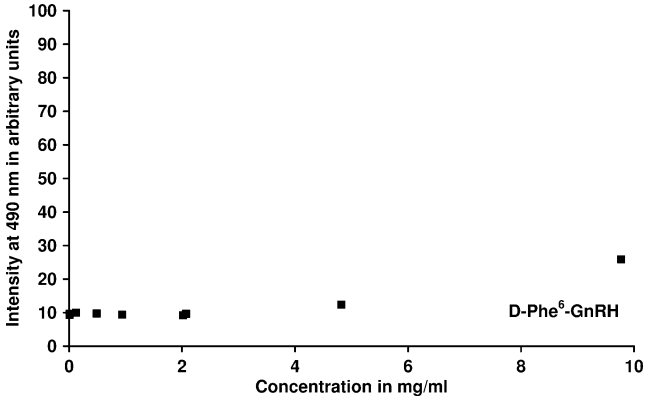


Fig. 3 Fluorescence of ANS at 490 nm against peptide concentration for D-Phe^6-GnRH

be detected for the pure amino acid, but no contribution to peptide fluorescence is observable at all.

Evaluation of the band shifting using intrinsic fluorescence

In addition to the examination of the ANS probe fluorescence the intrinsic fluorescence properties of samples with concentrations near *cac* was analyzed. A change of the fluorescence band structure was observed, which depends on the concentration of the analyzed peptide solutions. For Cetrorelix and Teverelix there is a change of the emission maximum from 335 nm to 332 nm, reaching the specified aggregation concentration (Figs. 6A and 7). At low sample concentrations, the emission of 2-naphthylalanine dominates the spectra (Fig. 6B).

Beginning band change can be detected near 0.040 mg/ml for Cetrorelix and 0.050 mg/ml for Teverelix. Ozarelix also shows a change of the band shape and the emission maximum shifts from 335 nm to 334 nm at the highest analyzed concentration. Because of the proximity of the new emission to the original fluorescence maximum for monomeric Ozarelix solutions, the modified band structure can only be detected clearly for high peptide concentrations above 0.300 mg/ml (Fig. 8).

To increase the sensitivity of the detection of a beginning band deformation for Ozarelix the characteristic extreme values (cf. Fig. 5) and inflection points were determined for the fluorescence spectra by calculation of the first and second derivatives and the detection of corresponding zero points (Fig. 9).

A beginning shift of the maximum near 335 nm (e) can be confirmed for concentrations above 0.15 mg/ml. The extreme values (a, c) disappear and a shifting of the corresponding inflection point (b) to higher wavelengths can be observed. Its position moves gradually from 328.3 nm to 329.1 nm starting at a peptide concentration of 0.15 mg/ml and ranging up to 0.40 mg/ml (Fig. 10).

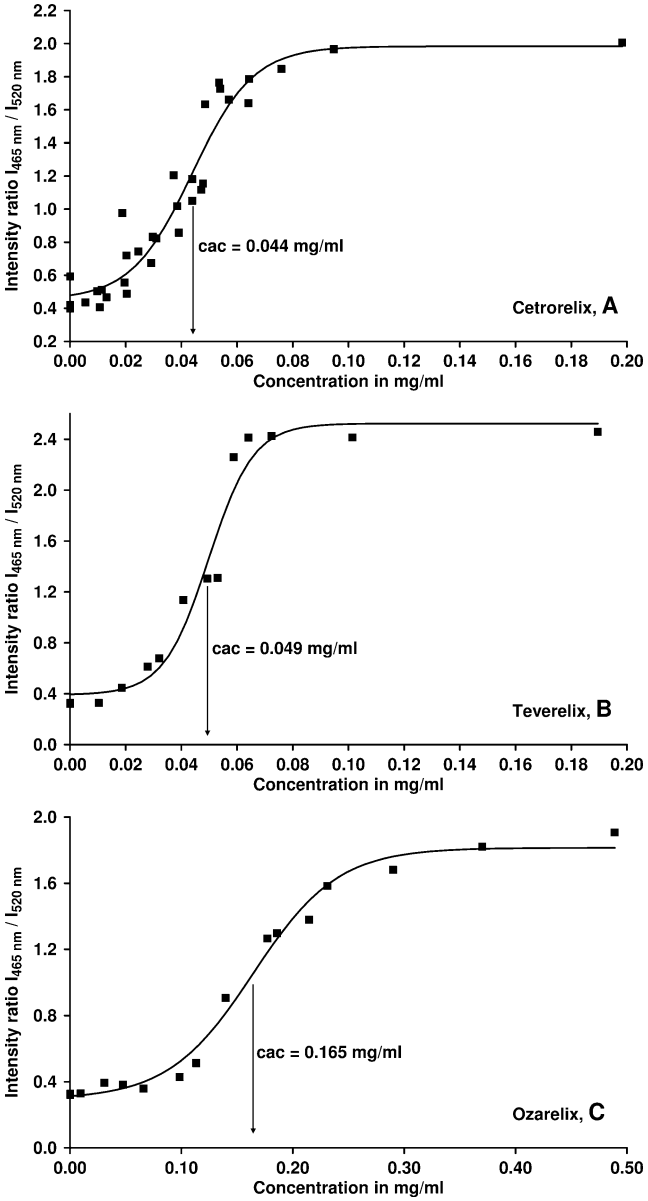


Fig. 4 Intensity ratio of ANS fluorescence of 465 nm and 520 nm against peptide concentration; Cetrorelix (**A**), Teverelix (**B**), Ozarelix (**C**)

Below a concentration of 0.15 mg/ml no significant shift of the inflection point is observable and above 0.40 mg/ml the specified inflection point no longer exists. This method allows a sensitive detection of a change in spectra characteristics starting near the detected *cac*, which was already determined by probe fluorescence measurements. The same procedure was applied to Cetrorelix and Teverelix spectra, but there is no additional information gained in comparison to the already described analysis.

Fluorescence spectra of the non-aggregating reference system D-Phe^6-GnRH do not show a band change and the fluorescence is dominated by the emission of tryptophan with a maximum at 348 nm (Fig. 11).

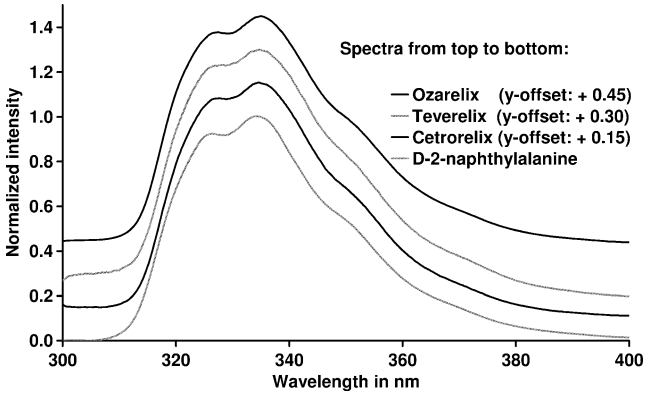


Fig. 5 Normalized fluorescence spectra for 2-naphthylalanine, monomeric Cetrorelix, Ozarelix and Teverelix

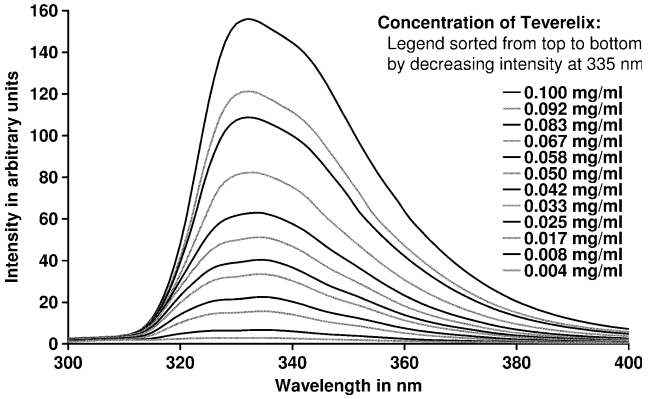


Fig. 7 Intrinsic fluorescence of Teverelix solutions

In the next step the change of fluorescence intensity versus peptide concentration was analyzed. Measured spectra of Cetrorelix show a nearly proportional slope of the fluorescence intensity at 335 nm with increasing peptide concentration until a critical concentration is reached. Beyond this peptide level a change of the slope is visible (Fig. 12A). Using bilinear regression a critical aggregation concentration of 40 µg/ml for Cetrorelix can be detected.

Teverelix shows a similar behavior as Cetrorelix and a critical aggregation concentration can be detected at 50 µg/ml (Fig. 12B). These results are in agreement with the obtained values of the extrinsic measurements.

No change of the slope can be detected for Ozarelix exploring intrinsic fluorescence intensities. The concentration dependent growth of intensity is not linear (Fig. 13). For that reason, no aggregation concentration can be determined for Ozarelix using an intrinsic intensity plot.

To prove that the small changes of the observed maximum for the analyzed peptides is not an artifact of self-absorption for solutions with high sample concentrations, fluorescence spectra were recorded using a configuration with an angle of 30° between excitation and emission beam and small path length. These results display identical band characteristics (not shown).

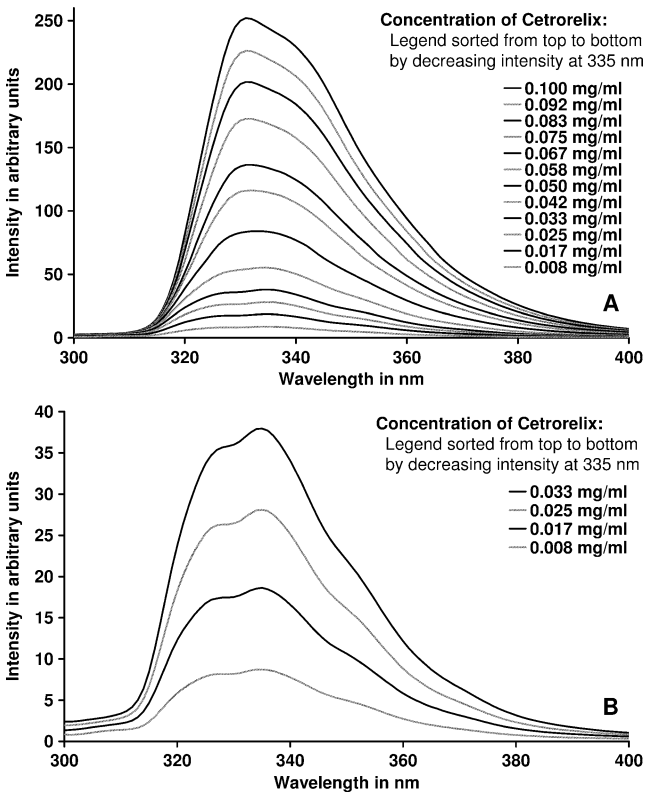


Fig. 6 Intrinsic fluorescence of Cetrorelix solutions (**A**), dominating 2-naphthylalanine fluorescence at low sample concentrations, zoomed (**B**)

Analysis of cetrorelix fragments

In addition to the measurement of the complete Cetrorelix peptide, the synthesized fragments Cetrorelix$_{1–4}$ (Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser) and Cetrorelix$_{1–6}$ (Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Cit) were analyzed to explore the nature of band change during aggregation process.

It was confirmed that no change in band shape is visible for the shortest fragment Cetrorelix$_{1–4}$, lacking tyrosine. In

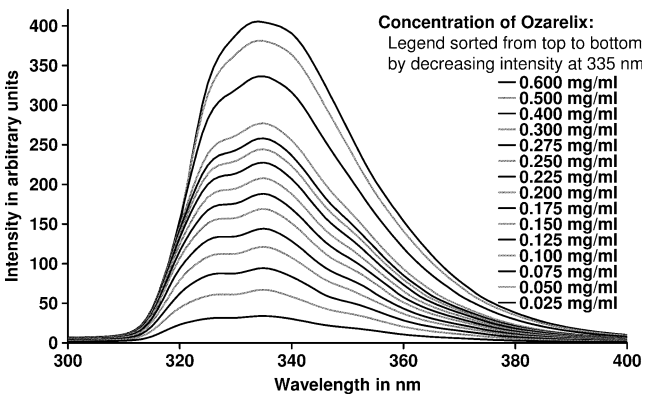


Fig. 8 Intrinsic fluorescence of Ozarelix solutions

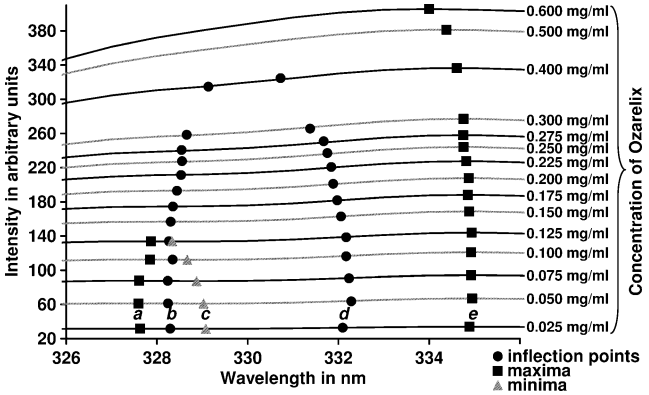


Fig. 9 Visualization of characteristic points of Ozarelix fluorescence spectra with increasing peptide concentration

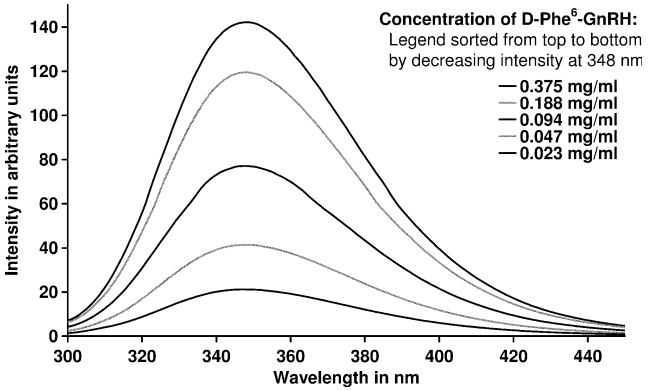


Fig. 11 Intrinsic fluorescence of D-Phe^6-GnRH solutions

fact, the dominating fluorescence of 2-naphthylalanine is observable for all analyzed peptide concentrations regardless of the aggregation state of the solution (Fig. 14). Aggregates are already visible macroscopically by formation of a gel at a concentration of 0.500 mg/ml.

For the hydrophobic tyrosine-containing fragment Cetrorelix$_{1–6}$, it was demonstrated that the aggregate typical band shape is already visible for the lowest analyzed concentration of 1.7 µg/ml (Fig. 15).

Investigating the possibility to obtain a monomer typical spectrum for this fragment, hydrochloric acid was added to samples, resulting in precipitation of the peptide. After centrifugation a monomeric type spectrum is visible and native tyrosine emission near 305 nm can be detected.

Monitoring the fluorescence of Cetrorelix fragments allows the conclusion, that the fluorescent amino acid tyrosine is an essential part for a change in the fluorescence behavior of aggregating species.

Searching for an explanation for the observed change of the band shape leads to an examination of potential fluorescent species of the analyzed peptides. The fluorescence of the monomeric peptides is dominated by 2-naphthylalanine. When aggregation begins a change of intrinsic fluorescence emission is observed by gradual modification of the band structure and finally a new band becomes visible between 330 nm and 334 nm.

It is assumed that the new band is caused by tyrosinate-like emission. Therefore, a short overview of known tyrosine and tyrosinate fluorescence and their characteristic wavelengths shall be given. Fluorescent properties of tyrosine depend on its environment. Comprehensive reviews regarding fluorescence of tyrosine as amino acid or incorporated in peptides and proteins are presented by Lakowicz [11, 16] and Creed [17]. The normal fluorescence emission of tyrosine (excitation at 275 nm) is observed near 305 nm [15, 18, 19] and can be

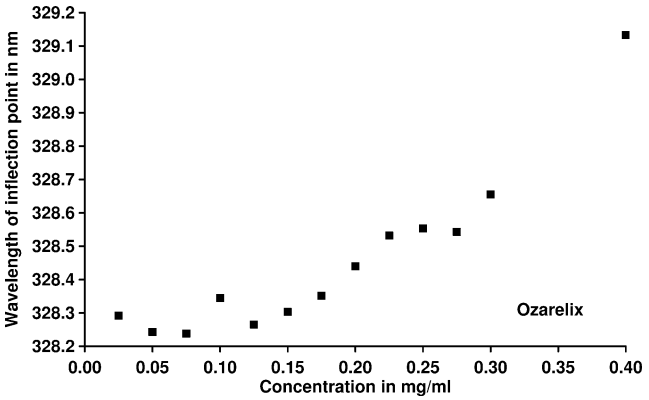


Fig. 10 Shift of the inflection point between 328 and 329 nm of Ozarelix spectra with increasing peptide concentration

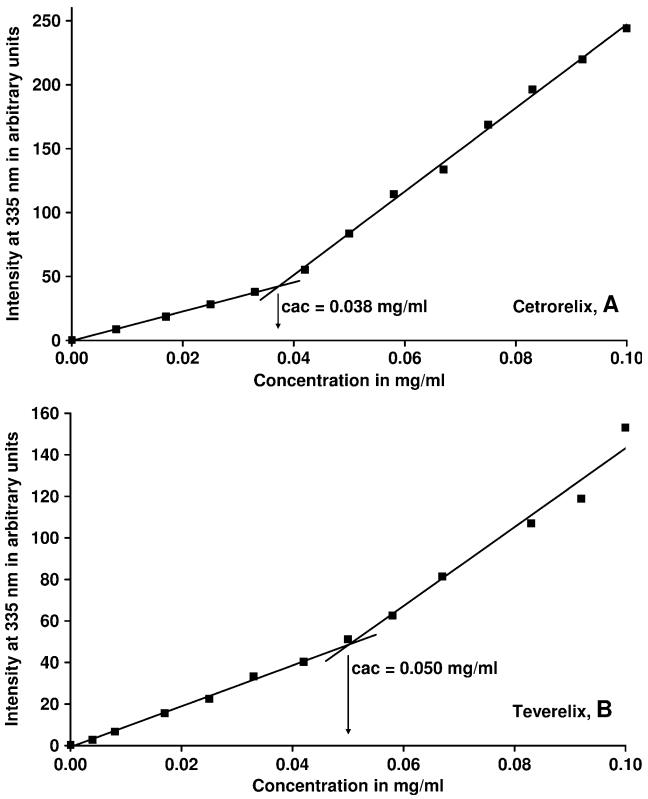


Fig. 12 Change of fluorescence intensity for Cetrorelix (**A**) and Teverelix (**B**) solutions at 335 nm

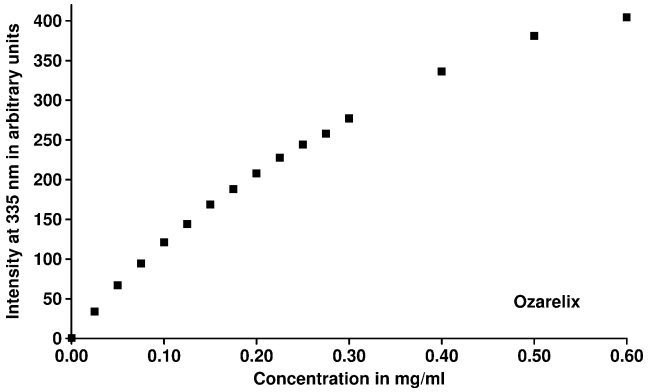


Fig. 13 Change of fluorescence intensity for Ozarelix solutions at 335 nm

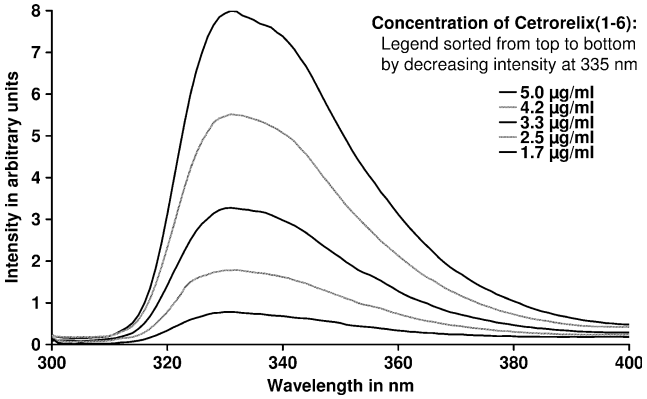


Fig. 15 Intrinsic fluorescence spectra of Cetrorelix$_{16}$

shifted towards 345 nm as tyrosinate fluorescence using strong alkaline conditions [20]. However, such a fluorescence could be monitored for proteins at pH 7 as well and it was suggested that it originated from a proton transfer of phenolic hydroxyl to a potential acceptor group in the protein [18]. Willis and Szabo examined tyrosine solutions with varying potential acceptor groups as phosphate, acetate, formate, imidazole and tris buffers and identified related fluorescent species with emission maxima reaching from 315 to 335 nm [15]. The shifted fluorescence was explained as formation of a ground state hydrogen-bonded complex that persisted in excited state. The anomalous fluorescence at 330 nm of the tryptophan-free protein adrenodoxin was analyzed and finally identified as tyrosine fluorescence [21–23], where the phenolic group of tyrosine might be involved in a hydrogen bond. The originally specified glutamate acceptor groups [24, 25] could not be confirmed by our analysis of the adrenodoxin 3D structure published by Pikuleva et al. [26]. Voicescu et al. used synthesized covalent bonded models for an analysis of tyrosine imidazole interactions. The observed species with emission maxima between 328 and 334 nm are attributed to the formation of hydrogen bonds [27].

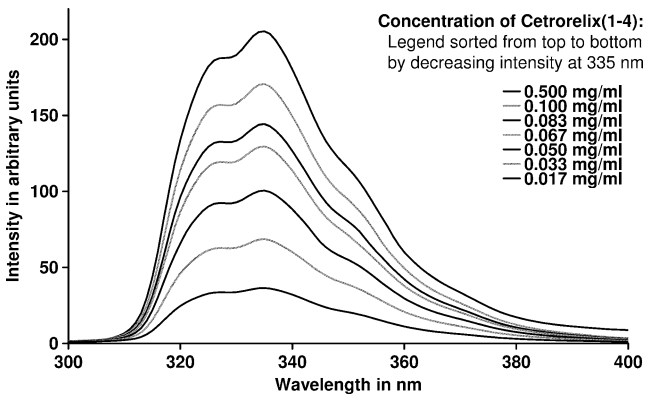


Fig. 14 Intrinsic fluorescence spectra of Cetrorelix$_{1-4}$

The above references show that tyrosinate-like emission is observed between 315 and 335 nm and at 345 nm for tyrosinate. Comparing discussed fluorescence experiments for tyrosine containing proteins and our results leads to assignment of the new emission band at 332 nm for Cetrorelix and Teverelix and at 334 nm for Ozarelix to a formation of a tyrosinate-like emission.

Additionally, the formation of intermolecular hydrogen bonds at the phenolic hydrogen of tyrosine at aggregates is proposed. In excited state, the pKa of the phenolic group of tyrosine decreases from 10.3 to 4.2 [28]. Thus, a proton transfer could take place to suitable acceptor molecules. The amino acid 3-pyridylalanine (pKa 4.9 for the aromatic nitrogen group [29]) is suggested as an acceptor for the analyzed decapeptides. However, there are more possibilities for the formation of hydrogen bonds within the analyzed molecules.

Conclusion

The critical aggregation concentration of three GnRH antagonists could be determined using extrinsic and newly established intrinsic fluorescence methods. ANS was utilized as a reference probe for the beginning formation of peptide aggregates. The modification of intrinsic fluorescence emission of these peptides was discovered and proved to be attached to the aggregation process. The resulting tendency to aggregate has the following order:

Cetrorelix ≈ Teverelix	> Ozarelix	>> D-Phe^6-GnRH
40 µg/ml	50 µg/ml	170 µg/ml	not detected

The D-Phe^6-GnRH reference peptide does not form any aggregates. All other peptides share a hydrophobic N-terminus with the amino acid sequence: Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3). Cetrorelix and Teverelix have nearly the same affinity to form aggregates. Ozarelix with a modified

peptide backbone tends to agglomerate in a different concentration range. The decreasing potential of aggregation about a factor of 3–4 could be demonstrated comparing backbone unmethylated and methylated species.

The important characteristics of tyrosine fluorescence are essential for the change of intrinsic fluorescence of the investigated peptides. The mechanism could be enlightened by the analysis of two fragments of Cetrorelix which differ in containing tyrosine or not. A tyrosinate-like fluorescence and an appropriate acceptor are discussed.

In summary, there is a new method established to describe the aggregation process of GnRH antagonists which utilizes an adjusted ANS assay as well as the intrinsic fluorescence of the peptides. The latter is advantageous because of its possibility to study the system as it is without a disturbing influence of additives.

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