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Il Farmaco 58 (2003) 1131–1136
IL FARMACO
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Preparation and properties of selected Zn(II)–peptide complexes in suspension

Barbara Dolińska *, Florian Ryszka

Department of Applied Pharmacy and Drug Technology, Silesian Medical Academy, 41-205 Sosnowiec, ul. Kasztanowa 3, Poland

Received 24 April 2002; accepted 7 September 2002

Abstract

The preparation and properties of low soluble, suspended Zn(II) complexes containing the selected peptides: tyroliberin (TRH), gonadorelin (GnRH), dalarelin and corticothropin (ACTH) were studied. The amount of Zn(II) bound by 1 μM of the selected peptide (n) was defined, as well as affinity of Zn(II) to the peptide (Ka) and the durability of the created complex Zn(II)–peptide (Kd). ACTH associated the highest amount of Zn(II), and GnRH the lowest one: 1 μM of ACTH complexed 0.81 μM ± 0.03 Zn(II), the same quantity of GnRH—0.52 μM ± 0.07 and TRH and dalarelin associated 0.75 ± 0.03 and 0.79 ± 0.02 μM of Zn(II), respectively. The closest affinity was stated between Zn(II) and GnRH ($Ka = 157.692 \pm 21.300\ \mu M^{-1}$), the smallest—towards ACTH ($Ka = 1.136 \pm 0.042\ \mu M^{-1}$). The lower amount of Zn(II) associated by the studied peptide, the higher was its affinity versus this metal ($r = -0.942$). The analysis of the kinetics of the Zn(II)–peptide linkage revealed that the most stable complexes with this metal were formed by GnRH ($Kd = 0.006 \pm 0.001\ \mu M^{-1}$) and by dalarelin ($Kd = 0.020 \pm 0.001\ \mu M^{-1}$). Zn(II) with GnRH complexes are about 147 times more durable than ACTH ($Kd = 0.880 \pm 0.033\ \mu M^{-1}$) ones. It was established that the Zn(II)–peptide complexes were more stable in the case of lower molecular weight of the peptide ($r = 0.963$), and the inferior number of the amino acid residues accessible in the peptide ($r = 0.967$).

Keywords: Peptides; GnRH; Dalarelin; TRH; ACTH; Zn(II); Zn(II)–peptide complex; Suspension; Complex properties

1. Introduction

Sparingly soluble complexes of insulin and corticotrophin of Zn(II) with prolonged action in the form of suspension to be applied paranaterally have been used in therapy for 50 years. However, the mechanism of formation of such complexes is not clear until now. The process of formation of protein–peptide substances complexes with metals is known to be dependent on their properties, concentration and the kind of complexing factor as well as acidity of the solution [1–3]. The durability of the Zn(II)–amino acid complex is connected with its isoelectric point and its molecular weight [1].

It was stated that in order to obtain suspensions with the amino acids programmed, stated in advance parameters, that is, the suitable maintenance dose, prolonged release time and its suitable character the knowledge of the binding points Zn(II) in the amino acid, its affinity to the metal and the durability of the obtained complex seems essential [1,4].

The purpose of this research was to define the influence of the selected properties of the peptides upon formation and physicochemical properties of the sparingly soluble Zn(II)–peptide complexes obtained in the form of suspensions to be applied parenterally [5].

2. Materials and methods

2.1. Peptides

Gonadothropin-releasing hormone (GnRH), tyrothrophin-releasing hormone (TRH) were obtained from 'Sigma' Chemical Corp., St. Louis, USA; dalarelin (des-Gly10, [D-Ala^6]-LHRH ethylamide), the synthetic

* Corresponding author.
E-mail address: zekfar@infomed.slam.katowice.pl (B. Dolińska).

0014-827X/03/$ - see front matter © 2003 Published by Éditions scientifiques et médicales Elsevier SAS.
doi:10.1016/S0014-827X(02)00020-4

superactive GnRH analogue, was obtained from 'Biolar' Latvia, adrenocorticothropic hormone (ACTH) from 'Biochefa' Pharmaceutical Research Plant, Poland. The purity of the peptides, defined by HPLC method, was 95–98% (The Producer's Certificate of Quality).

The peptides selected for investigation differed by the molecular weight (mw), contained tyrosine and histidin rests, and possessed exactly defined biological functions.

2.2. Preparation of Zn(II)–peptide complexes in suspension

GnRH (20 μM) or TRH or dalarelin or 1.11 μM ACTH was added into an aqueous solution containing a predetermined amount of Zn(II) acetate. The molar ratio Zn(II)–peptide varied from 0.5:1 to 100:1. The Zn(II) content in solution varied from 10 to 2000 μM for the three above mentioned peptides and in the case of ACTH from 0.55 to 111 μM. The molar ratios Zn(II)–peptide were chosen after preliminary experiments had been carried out. The obtained mixtures were neutralised to pH 7.1–7.5 with 0.1 M aqueous NaOH solution and adjusted to 10 cm^3 with the distilled water. In this moment, the process of complexing takes place [1]. The whole substance was stirred by the means of a magnetic mixer during 20 min. After 1 h the whole substance was whirled at 5000 rotations per minute. The sediment was totally selected from the supernatant. The precipitate was discarded and the supernatant was further investigated. The Zn(II)–peptide complexes were prepared in the following conditions: pH 7.1–7.5; temperature: +20 °C; time of the static formulation of suspension: 1 h [2].

2.3. Quantitative determination of Zn(II) associated with peptide

The excess of the free, non-associated Zn(II) was separated by the gel filtration chromatography on a Sephadex G-25 column in 0.1 M acetic acid aqueous solution. Zn(II) acetate formed a fraction eluted at Rf = 0.29. Rf – the relation of the free volume of the Sephadex – filled column to the elute containing the examined substance. After the separation Zn(II) was determined by a spectrophotometric method. The absorbency of the supernatant samples taken before and after the complexation was measured. The absorbency of the solution containing free Zn(II) was measured at the wavelength $\lambda = 538$ nm after a derivatisation with dithizone. The measured absorbency values ranged from 0.2 to 0.6. A calibration curve was plotted for the amounts of Zn(II) associated to the peptides as a difference between the total amount used for the complex formation and the excess of the free, non-bonded Zn(II). The amounts of the associated Zn(II) were expressed in μM/μM peptide. Absorbency measurements were carried out in the 1 cm thick cells by means of the UV–Vis 'CE-3021' spectrophotometer (Cecil, UK).

2.4. Determination of complexation parameters for the selected peptides

The analysis of the complexation of the peptide with Zn(II) was carried out by a method introduced by Scatchard [6].

The amounts of Zn(II) associated to proteins were determined 1 h after the preparation of the complexes. The plots: $[B]/[F]$ versus $[B]$, where $[B]$ was quantity of the associated Zn(II) and $[F]$ is the quantity of the free, i.e. non-bonded Zn(II) were constructed. The following parameters characterising the peptide complexation were derived graphically from the geometry of the plotted curves:

- the amount of Zn(II) associated to 1 μM of the peptide (n);
- the affinity of the peptide versus the ligand (Ka);
- the dissociation constant for the Zn(II)–peptide complex (Kd).

Two asymptotes (straight lines) were constructed on diagrams. The first passed through the points situated in the proximity of the X-axis and the second through the points located in the proximity of the Y-axis. The points of intersection of the first asymptotes with the X-axis revealed the amount of Zn(II) associated by 1 μM of the peptide (n). The affinity constants (Ka) were found at points of intersection of second asymptotes with the Y-axis. The reciprocal value to (Ka) is the dissociation constant (Kd). The statistical significance of correlation was determined by the linear regression procedure and the obtained correlation coefficients, R^2.

2.5. Statistical calculations

The results were obtained as the means of five experiments. Statistical significance of the Zn(II)–peptide association was defined as a quotient of the amount of Zn(II) associated to the studied peptide versus the Zn(II) amount associated to the reference GnRH.

Analysis of variance was used to assess the significance of how the molecular weight (mw) of the studied peptides and the number of amino acid residues (r) could influence the amount of Zn(II) associated by 1 μM of the peptide (n), the peptide affinity (Ka) and the stability of the formed complex (Kd).

Linear correlation coefficients (R^2) were calculated for the studied regressions by means of the EXCEL and STATISTICA software, for the level of significance $P < 0.05$.

Table 1
Parameters of binding Zn(II) in the Zn(II)–peptide complexes

PEPTID	Properties of peptides		Properties of Zn(II)–peptid complex		
	mw	*r*	n (μM)	Ka (μM^{-1})	Kd (μM^{-1})
Tyroliberin	362.3	3	0.75±0.03	14.000±0.561	0.070
Gonadorelin	1182.3	10	0.52±0.07	157.692±21.300	0.006
Dalarelin	1167.3	9	0.79±0.02	50.633±1.280	0.020
Corticotrophin	4500	39	0.81±0.03	1.136±0.042	0.880

Table 2
Correlation coefficients (*r*)

	mw	*r*	n	Ka
n	0.378	0.367		
Ka	−0.376	−0.375	−0.942	
Kd	0.963	0.967	0.491	−0.559

3. Results

The studied peptides formed with Zn(II), in the above described conditions, colourless, low soluble, solid complexes in suspension. The peptides, with an exception of ACTH, formed complexes in the ligand concentration range from 1 to 200 μM. The Zn(II)–ACTH complexes were obtained in the Zn(II) concentration range from 0.055 to 11.10 μM. The Scatchard's diagrams presenting the amounts of Zn(II) associated to 1 μM peptide are depicted in Fig. 1. The calculated coefficients of regression of the fit, R^2, are presented too.

The quantity of Zn(II) associated to 1 μM peptide increased linearly with an increase of the added Zn(II). A decrease of the percent content of the associated Zn(II) was observed. The amounts of Zn(II) complexed to the selected peptides are statistically significant ($P < 0.05$).

The level of significance of Zn(II) association with the studied peptides was defined by the reference GnRH. An increase of the Zn(II) ion concentration involved a 4.5 times increase of the quantity of the bonded Zn(II) to ACTH, a 2.1 times increase of the Zn(II) quantity for TRH and a 1.3 times increase for GnRH. In Fig. 2 are shown the Scatchard's curves for the studied Zn(II)–peptide complexes.

The presented experimental Scatchard's curves form hyperbolas which indicate that the selected peptides could associate Zn(II) by means of two types of binding sites (n_1 and n_2). The determined number of the binding sites for Zn(II) could characterise the ability of the complex formation Zn(II)–peptide in the specified conditions. In Table 1 the parameters of Zn(II) binding to the studied peptides, and in Table 2 the observed correlation data are presented.

The highest amount of Zn(II) is associated to ACTH and the lowest one to GnRH. ACTH (1 μM) combines with 0.81±0.03 μM^{-1} Zn(II), while GnRH associates with 0.53±0.07 μM^{-1} Zn(II).

The molecular weight of peptide considerably influenced the amount of associated Zn(II).

The highest affinity to Zn(II) possessed GnRH (Ka = 157.692±21.300 μM^{-1}), and the lowest ACTH (Ka = 1.136±0.042 μM^{-1}). The affinity constant of GnRH versus Zn(II) is ca. three times higher than the affinity constant for dalarelin and ACTH. The obtained results suggests that the more Zn(II) is complexed by the peptide, the lower is its affinity versus Zn(II) ($r = -0.942$). The affinity of peptides is considerably affected by the molecular weight. The analysis of the complexation kinetics with Zn(II) proved that the most stable complexes formed were GnRH (Kd = 0.006±0.001 μM^{-1}) and dalarelin (Kd = 0.020±0.001 μM^{-1}). Relatively weak complexes were obtained with ACTH (Kd = 0.880±0.033 μM^{-1}). The GnRH complexes were ca. 147 times more stable than that the ACTH complexes.

4. Discussion

The knowledge of the Zn(II) binding site number in the studied peptides, the affinity versus the metal and the stability of formed complexes are necessary in order to prepare suspensions possessing determined parameters i.e. a convenient supporting dose, increased release time and appropriate formulation. So far, suggestions have been made that, e.g. suspensions containing ACTH associated with Zn(II) are formed as a result of an adsorption of this peptide on the surface of a precipitated sediment of $Zn(OH)_2$ [7]. Our results indicated that this peptide formed a low soluble complex with Zn(II) in form of solid particles. However, the ACTH affinity to Zn(II) is the lowest. Interesting relationships were found when comparing the proprieties of the Zn(II)–GnRH complexes with the proprieties of the dalarelin, which is the GnRH super active analogue. The difference between the latter and GnRH consisted in the different number of amino acid residues and in the fact that the latter had D-alanine instead of

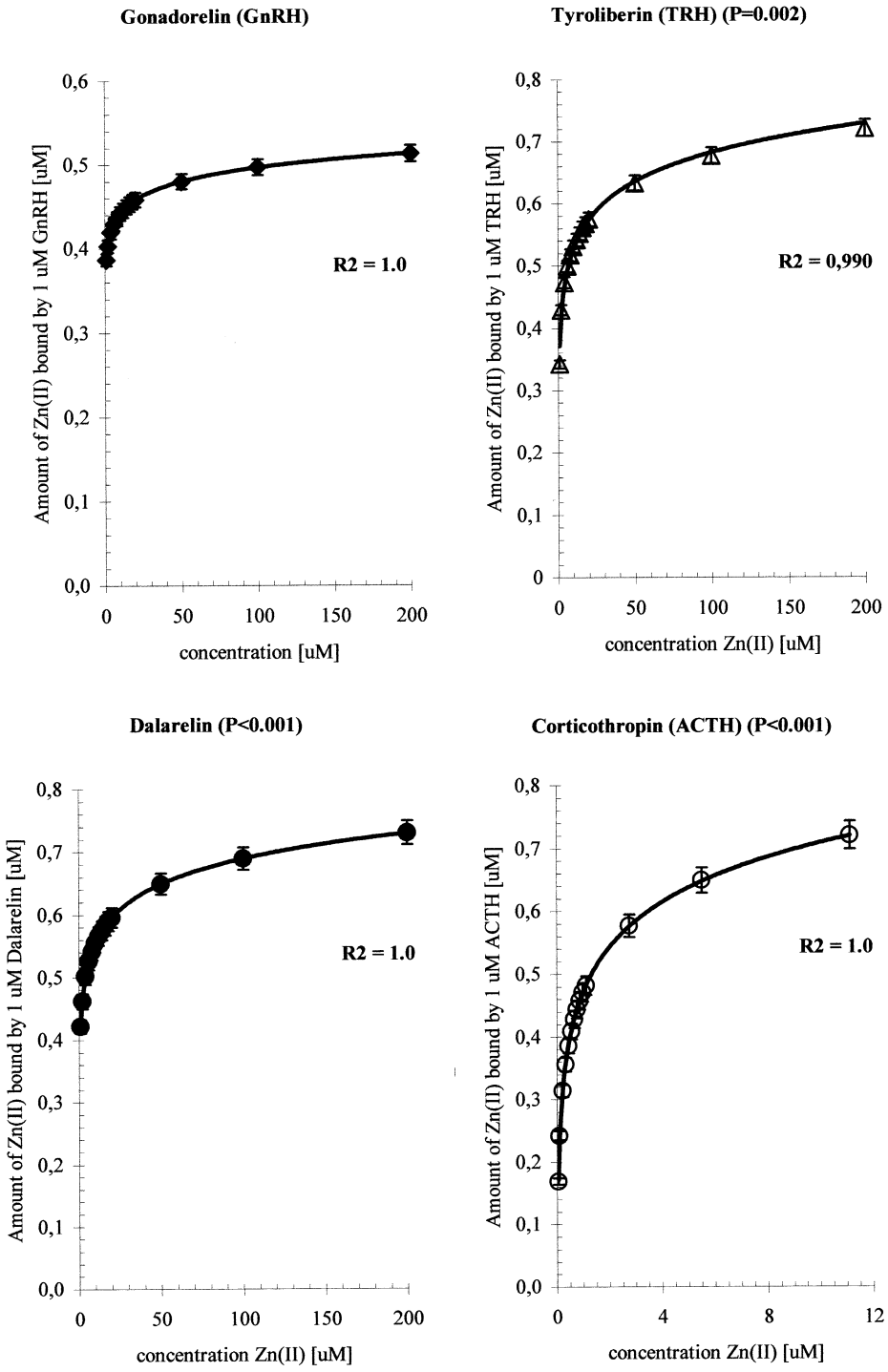

Fig. 1. Association ZN(II) with 1 μm peptides vs. the zinc concentration.

glycine. Alanine is the smallest linear aliphatic amino acid, which can be found in the domains where the peptide chains are considerably folded [3]. Alanine is an example of amino acid with non-coordinating side-chain. It creates very weak complex with Zn(II) [1]. This is probably the reason why the dalareline complex is less durable than Zn(II)–GnRH complex.

The complex formed by dalarelin is less stable than the Zn(II)–GnRH one. The stability of the Zn(II)–GnRH is ca. three times higher than the one of the complex formed by dalarelin with Zn(II). It was stated that the durability of Zn(II)–peptide complex depends on molecular weight of the peptide and the amount of its amino acids residues. The created Zn(II)–peptide com-

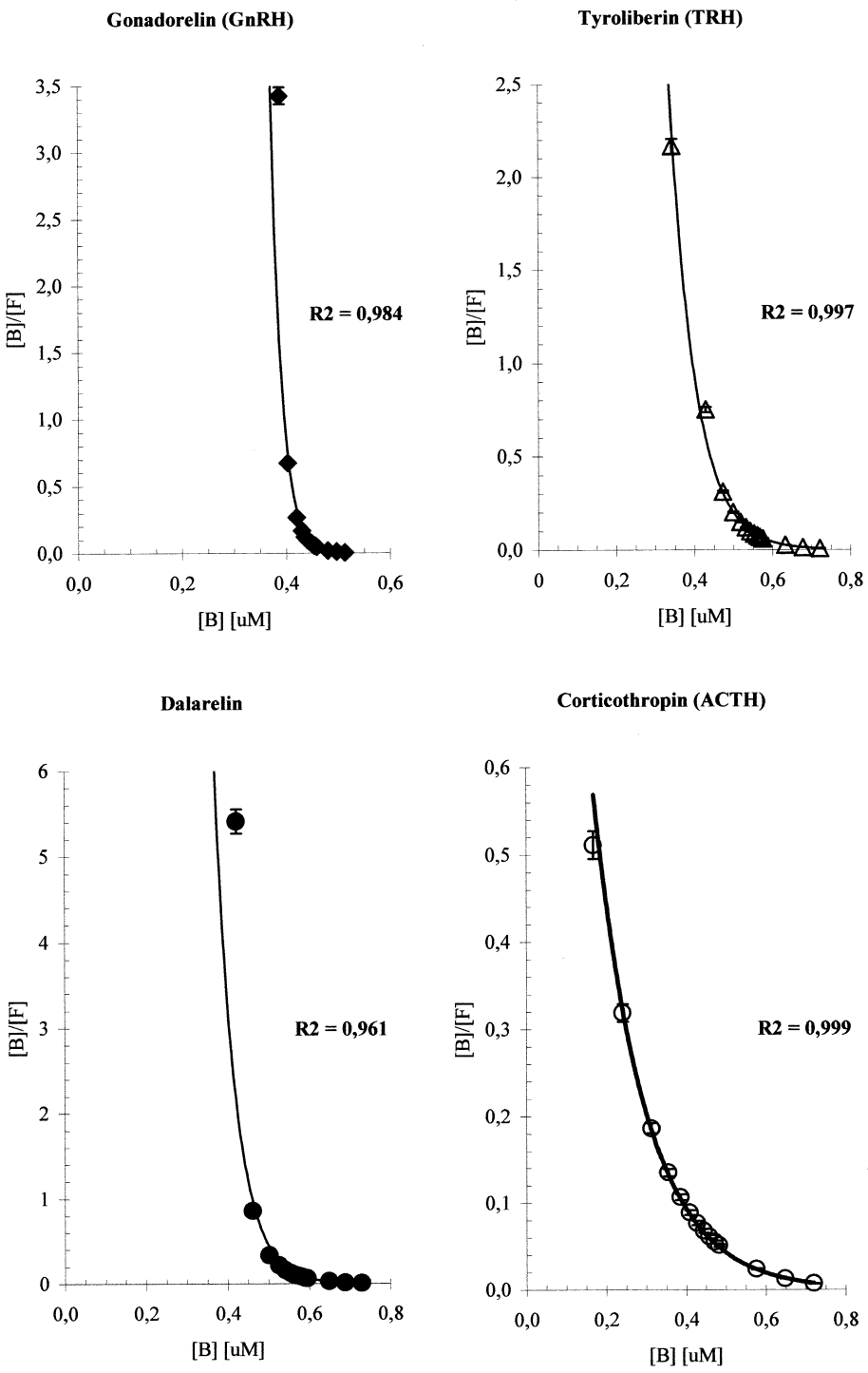

Fig. 2. Scatchard's curves of complexation of zinc with the studied peptides.

plex is the more durable the less is the peptide molecular weight and the amount of its amino acid residues. In the earlier research, we have stated that the durability of Zn(II)–amino acid is connected with the molecular weight and isoelectric point of the amino acid [1]. The most durable complexes are created by tryptophane and tyrosine.

Probably it blocks activity of proteolytic enzymes, which promotes a destruction of the hormone. So far, the following solid complexes with Zn(II) were obtained:

the first formed by GnRH and the second by buserelin, a GnRG analogue [9,10]. Authors [4] reported that a preparation of the GnRH–Zn(II) complex under other conditions was carried out. The NMR spectroscopy proved that the coordination between GnRH and Zn(II) took place at the nitrogen atom of the imidazole ring and at the oxygen atom of the carboxyl group in the peptide bond His–Trp. The imidazole rest of histidine played an important role in the protein–peptide complex formation with bivalent metals, which associated Zn(II). Interactions of Zn(II) with GnRH molecule caused a modification of the biological activity [4,8]. Investigations of the configuration of the GnRH molecule were carried out. It was found that all peptide bonds in GnRH had the trans conformation. The NMR spectra excluded the presence of strong hydrogen bonds and interactions between aromatic rings in this peptide [11]. The peptide chain in the GnRH molecule is flexible. A number of 300 low energy conformations could be theoretically possible, but taking into account a relative stiffness of the tetra peptide fragment of the medium part of GnRH (i.e. Tyr^5–Gly^6–Leu^7–Arg^8), the authors assumed that only eight low energy conformers were really present. The amino acids could coordinate through the amino group ($-NH_2$) or the carboxylic group ($-COO^-$) in relation to the pH values. In the presence of polar chains R, additional coordination possibilities could be found too.

Our results reported in this work suggest further investigations of the Zn(II) role in the modification of the character and the time of release of drug preparations with active protein and peptides components.

5. Conclusions

On the basis of the research conducted, it was stated that the molecular mass weight exercises the strong influence upon binding and affinity of Zn(II)–peptides.

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