

Pharmacokinetics and Bioavailability of the GnRH Analogs in the Form of Solution and Zn²⁺-Suspension After Single Subcutaneous Injection in Female Rats

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Abstract

Background and Objectives Although many synthetic gonadoliberin analogs have been developed, only a few of them, including buserelin, were introduced into clinical practice. Dalarelin, which differs from buserelin by just one aminoacid in the position 6 (D-Ala), is not widely used so far. Gonadotropin-releasing hormone (GnRH) analogs are used to treat many different illnesses and are available in different forms like solution for injection, nasal spray, microspheres, etc. Unfortunately, none of the above drug formulations can release the hormones for 24 h. We assumed that classical suspension could solve this problem. **Methods** Two sets of experiments were performed. In the first one, buserelin and dalarelin were injected into mature female rats in two forms: suspension, in which the analogs are bounded by Zn²⁺ ions and solution. The pharmacokinetic parameters and bioavailability of the analogs were calculated, based on their concentration in the plasma measured by high-performance liquid chromatography method (HPLC). In the second experiment, the hormones in two different forms were injected into superovulated immature female rats and then the concentration of Luteinizing hormone (LH),

Follicle-stimulating hormone (FSH) and 17β-estradiol in the serum was measured by radioimmunological method.

Results The Extent of Biological Availability (EBA), calculated on the base of AUC_{0–∞}, showed that in the form of solution buserelin and dalarelin display, respectively, only 13 and 8 % of biological availability of their suspension counterparts. Comparing both analogs, the EBA of dalarelin was half (53 %) that of buserelin delivered in the form of solution and 83 % when they were delivered in the form of suspension. The injection of buserelin or dalarelin, in the form of solution or suspension, into superovulated female rats increased LH, FSH and estradiol concentration in the serum. However, after injection of the analogs in the form of suspension, the high concentration of LH and FSH in the serum persisted longer.

Conclusion Performed studies indicate that GnRH analogs in the form of suspension have higher bioavailability than their solution counterparts. It influences the effects of their action, especially in relation to LH and FSH.

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Key Points

Pharmacokinetic parameters (absorption rate constant (k_a) and elimination rate constant (k_e)) of gonadotropin releasing hormone (GnRH) analogs differ depending on their formulation. The prolonged absorption and elimination is observed if analogs are delivered in the form of suspension.

Extent of Biological Availability (EBA) of both buserelin and dalarelin in the form of suspension is higher than in the form of solution.

Higher bioavailability of GnRH analogs in the form of suspension correlates with hormonal profiles of LH and FSH in the serum.

1 Introduction

Since the discovery of gonadoliberin, many synthetic analogs, both its agonists or antagonists, have been developed. However, so far only a few of them were introduced into clinical practice. Active analogs in the agonists group are most often made by modifications in the position 6 and 10. Usually, glycine in position 6 is replaced with more lipophilic D-aminoacid like D-Phe, D-Leu or D-Trp and glycylamide in position 10 is substituted by ethylamide. These substitutions result in more hydrophobic compounds having not only increased affinity to GnRH receptors, but also prolonged half-life due to increased resistance to proteolytic degradation. As such, synthetic analogs are characterized by higher activity than endogenous GnRH [1–3].

One of the agonists used in clinical practice is buserelin of m.w. 1239 D. Replacement of Gly in position 6 by D-Ser(tBu) and Gly-NH₂ in position 10 by ethylamide N-EtNH₂ increases its activity, in relation to natural GnRH, by 20 times [4]. Dalarelin, in contrary to buserelin, is not so broadly used. It also belongs to agonists group, have m.w. 1167 D and is different from buserelin by having in position 6 D-Ala. Its relative potency is roughly 14 times higher than natural gonadoliberin [4]. Studies performed by Colin and Jameson [5] on the cells from rats anterior pituitary proved that this peptide, like other GnRH analogs, binds to GnRH receptor and activates intracellular pathways.

GnRH analogs are commonly used in clinical practice. They are used in the diagnosis of hypothalamus–pituitary–gonadal axis, to induce and normalize activities of pituitary gland, in vitro fertilization (IVF) programs and long-term inhibition of pituitary–gonadal axis [4, 6–8]. Such a diversity of applications leads to the development of few formulations of drugs containing GnRH analogs. They are available as solution for injection, nasal spray, short release [9] or prolonged release [10] forms like implants, microspheres [11], micro- and nanoparticles [9, 11, 12]. In case of long-term depot (even few months), GnRH analogs are embedded in biodegradable polymer like poly-D,L-lactide (PLA) and poly D,L-lactide-co glycolide (PLGA) [13]. Microspheres, micro- and nanoparticles are delivered subcutaneously or intramuscularly in the form of suspension and are characterized by slow release or controlled release with zero-order kinetics. No formulation is available which would release analogs for 24 h. In our opinion, such a formula could be a classical suspension where analog is bound to inorganic ion, as in some insulin preparations. Such a drug formula could be very useful if only a few days of treatment with analogs is required. Presently, the only option in such a situation is to deliver

the hormone 2–3 times daily in the form of solution by injection or nasal spray. Kochman et al. [14] studied the influence of Cu²⁺, Ni²⁺ and Zn²⁺ ions forming the complexes with GnRH on the release of FSH and LH from pituitary (in vivo model) and compared affinity of these complexes to binding sites of GnRH receptors from anterior pituitary of female rats (in vitro model). The studies in vivo show that complex of GnRH with Cu²⁺ ions causes big surge of both FSH and LH from pituitary. Effectiveness of complexes containing Zn²⁺ ions was comparable to natural GnRH and the less effective was complexes with Ni²⁺ ions. Taking above into consideration, we decided to analyze the classical suspensions in which GnRH analogs form complexes with Zn²⁺ ions.

The aim of our study was to analyze and compare the basic pharmacokinetic parameters and bioavailability of buserelin and dalarelin delivered to female rats in 2 different forms: solution for injection and suspension containing hormone complexed with Zn²⁺ ions. We have also analyzed the biological effect of both forms by assessing the hormonal profiles of FSH, LH and 17β-estradiol in the serum of superovulated immature female rats.

2 Materials and Methods

2.1 Animals

The animal experiments were performed at the Center for Experimental Medicine, Medical University of Silesia in Katowice, Poland. The rats were housed at 22 °C with a regular 12/12 light–darkness cycle with access to standard Murigran chow and water ad libitum. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted. Research was approved by the Local Ethical Committee for Animal Experimentation at the Medical University of Silesia.

Experiment 1. To validate biological availability and to measure basic pharmacokinetic parameters, we used female rats (Wistar strain) weighting 200–210 g. Animals were divided into 4 groups (5 rats each) which received subcutaneous injection (6 mg/kg) of buserelin acetate or dalarelin acetate in the form of solution (group I and group III, respectively) or in the form of suspension (group II and group IV, respectively). At 15 and 30 min, then 1, 2, 3, 4, 5, 8, 12 and 24 h after analogs injection 250 μl of blood was drawn from tail vein into heparinized pipettes. The collected blood was kept on ice in the tubes containing heparin and, to inactivate proteases, 500 KIU/ml of Aprotinin (Traskolan, Jelfa S.A., Jelenia Góra, Poland). The tubes were then centrifuged for 20 min at 0 °C and 5000 rev/min. The obtained plasma was mixed with 10 %

trichloroacetic acid (1:1, v/v) and centrifuged again for 15 min at 15000 rev/min. The concentrations of buserelin and dalarelin were measured by HPLC method.

Experiment 2. To validate hormonal profile in the serum, we used immature female rats (Wistar strain) weighting 40–45 g. Animals were divided into 4 experimental groups and 1 control group (40 rats each). Experiment was performed according to Ovarian Ascorbic Acid Depletion (OAAD) method [15]. All the rats received subcutaneous injection of 50 IU PMSG (Serogonadotropin, Biowet S.A., Drwalew, Poland), and then after 60 h 25 IU of HCG (Biogonadył, Biomed Sp. z o.o., Lublin, Poland). 6 days later rats received subcutaneous injection (1 µg/kg) of buserelin acetate or dalarelin acetate in the form of solution (group I and group III, respectively) or suspension (group II and group IV, respectively). The control group received 0.9 % saline. The blood was drawn 0.5, 1, 2, 4, 6, 8, 12 and 18 h after analogs injection. The blood was allowed to clot for 2 h and then was centrifuged for 15 min at 3000 rev/min. The received serum was kept at -70°C until analyzed.

2.2 GnRH Analogs

The buserelin acetate (Buserelin acetate, Univesitas Opoliensis, Opole, Poland) and dalarelin acetate (Dalarelin acetate, BAPEX, L.T.D., Riga, Latvia) were used in the study. Chemical purity was 99.9 % for buserelin and 98.3 % for dalarelin as by HPLC method. Specimens in the form of solution or suspension were prepared in the Department of Applied Pharmacy Medical University of Silesia, Poland. The pH of the solutions was 7.3–7.5. In suspensions, the ratio of hormone—Zn was 1: 50, pH 7.5 and the concentration of albumin (BSA, Sigma-Aldrich Saint Louis, USA) was 0.25 mg/ml.

2.3 Measurement of GnRH Analogs Concentration by HPLC

Concentration of studied analogs was measured by modified HPLC method acc. to Kertscher et al. [16]. The modifications were: change in flow speed (from 1 ml/min to 0.8 ml/min) and change in excitation wave (from 280 nm to 274 nm) and emission wave (from 365 nm to 350 nm). Measurements were performed on Beckman HPLC apparatus with Shimadzu fluorescence detector and Nucleosil C18 (Sigma-Aldrich, Saint Louis, USA) column. The following buffers were used: buffer A—acetonitril (HPLC grade, Sigma-Aldrich, Saint Louis, USA)—water (5:95 v/v) and buffer B—acetonitril—water (35:65 v/v). Both buffers contained 0.05 % trifluoroacetic acid (HPLC grade, Sigma-Aldrich, Saint Louis, USA).

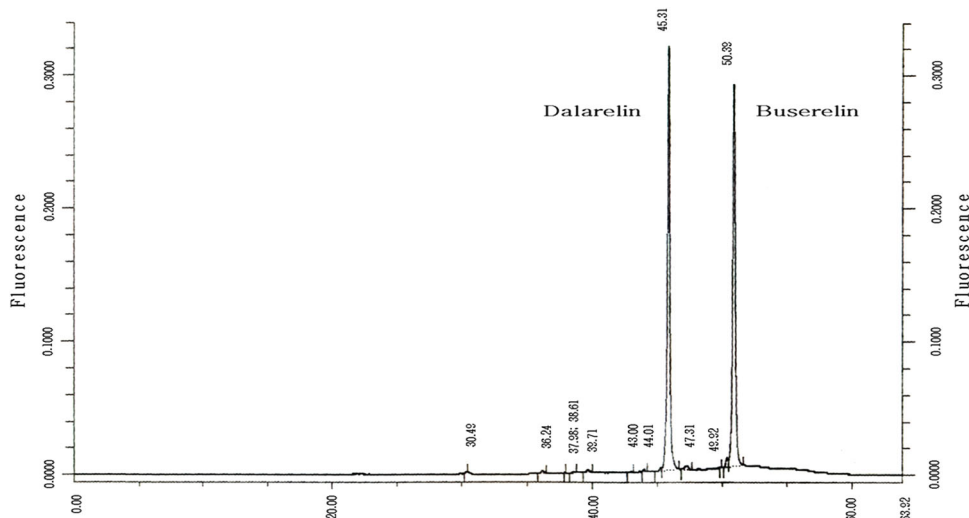
50 µl of supernatant was loaded on the column and then resolution was performed according to following scheme: isocratic flow—buffer A—linear gradient, 10 min; buffer B—0–17 %, 10 min; 17–100 %, 25 min; 100–10 min; buffer A—0–100 %, 5 min, 100 %, 2 min. Average retention time was: dalarelin—45.3 min; buserelin—50.4 min (Fig. 1).

Concentration of analogs was read according to standard curve. To make the standard curve, pure analogs were added to the plasma with heparin and aprotinin, deproteinized with 10 % TCA, centrifuged for 15 min at 15,000 rev/min and then 50 µl of supernatant was loaded on the column. The concentrations of hormones were: 0, 1.5, 3.0, 6 µg. The obtained standard curve represented concentration of hormones depending on peak area.

On the basis of obtained results, we established the biological availability and the following pharmacokinetic parameters: $\text{AUC}_{0-\infty}$ —area under plasma concentration—time curve (determined according to the trapezoidal

Fig. 1 HPLC chromatograph.

Mean retention time:
dalarelin—45.31 min;
buserelin—50.39 min



method) from time zero to the ∞ ; C_{\max} —the maximum plasma concentration; t_{\max} —the time to reach C_{\max} ; the extent of biological availability (EBA)—was calculated from the AUC according to the formula: $EBA = (AUC_{\text{solution}}/AUC_{\text{suspension}}) \times 100 \%$, $(AUC_{\text{dalarelin solution}}/AUC_{\text{buserelin solution}}) \times 100 \%$ and $(AUC_{\text{dalarelin suspension}}/AUC_{\text{buserelin suspension}}) \times 100 \%$; absorption rate constant (k_a) and elimination rate constant (k_e) by linear regression; half-life of absorption ($t_{1/2a}$) and half-life of elimination ($t_{1/2e}$) by $0.693/k_a$ or $0.693/k_e$; CL_{tot} —total body clearance by $\text{dose}/AUC_{0-\infty}$. The equation $C_t = Ae^{-k_e \times t} - Be^{-k_a \times t}$ describes time-dependent GnRH analogs concentration changes in the plasma.

2.4 Measurement of LH, FSH and 17 β -Estradiol Concentration

Concentration of FSH, LH and 17 β -estradiol in the serum was measured by radioimmunological method using diagnostic kits (Immunotech Beckman Coulter Co., USA) acc. to the manufacturer's instruction. The sensitivity of the method was the following: FSH-0.2 ng/ml, LH-0.02 ng/ml, 17 β -estradiol-2 pg/ml. Radioactivity of the samples was measured on γ scintillation counter-Clinigamma (LKB Pharmacia, Stockholm, Sweden) and then C_{\max} and $AUC_{0-\infty}$ for analyzed hormones as well as $AUC_{\text{LH}}/AUC_{\text{FSH}}$, $AUC_{\text{E}_2}/AUC_{\text{FSH}}$ and $AUC_{\text{E}_2}/AUC_{\text{LH}}$ ratios were calculated.

2.5 Statistical Analysis

Statistical analysis was performed using Statistica 10 (Systat software). Gaussian distribution and variance homogeneity were tested with Shapiro–Wilk and Levene's tests.

The results were then statistically analyzed using one-way ANOVA and HSD Tukey's tests.

3 Results

3.1 Bioavailability and Pharmacokinetics

The concentration and the half-life of studied GnRH analogs in the plasma differed depending on the formulation, solution or suspension, used for injection. In case of solution 5 h after injection, none of the analogs was present in the plasma. If injected in the form of suspension, both buserelin and dalarelin were detectable in the plasma even after 24 h (Fig. 2). Maximal concentrations of both analogs in the plasma appeared much faster (Table 1), but were 2 times lower if delivered in the form of solution comparing to their suspension forms (statistically significant $p \leq 0.001$). Absorption rate constants (k_a) for the analogs in the form of solution were higher (Table 1) which resulted in their higher concentration in the plasma 15 min after delivery (buserelin solution— $1.02 \pm 0.17 \mu\text{g/ml}$ vs. buserelin suspension— $0.68 \pm 0.13 \mu\text{g/ml}$ and dalarelin solution— $0.901 \pm 0.19 \mu\text{g/ml}$ vs. dalarelin suspension— $0.53 \pm 0.14 \mu\text{g/ml}$) (Fig. 2). Peptides delivered in the solution form were also faster eliminated. Half-time of elimination $t_{1/2e}$ for buserelin and dalarelin in the form of the solution was, respectively, 120 min and 100 min shorter if compared to their suspension forms. Values of the total body clearance (CL_{tot}) in case of solutions were 10 times higher than noted in groups of animals receiving suspensions (Table 1). Also, value of $AUC_{0-\infty}$ was much higher if analogs were in the suspension form. Biological availability of dalarelin was lower than buserelin, both in the form of solution and suspension, which was confirmed by its lower $AUC_{0-\infty}$ values (Table 1). The extent of biological availability (EBA), calculated on the base of $AUC_{0-\infty}$, showed that in the form of solution buserelin and dalarelin display, respectively, only 13 and 8 % of biological availability of their suspension counterparts. Comparing both analogs, the EBA of dalarelin was half

Fig. 2 Plasma concentration of buserelin and dalarelin after single s.c. injection at the same dose (6 mg/kg) in the form of solution and suspension. Each point represents the mean \pm SD

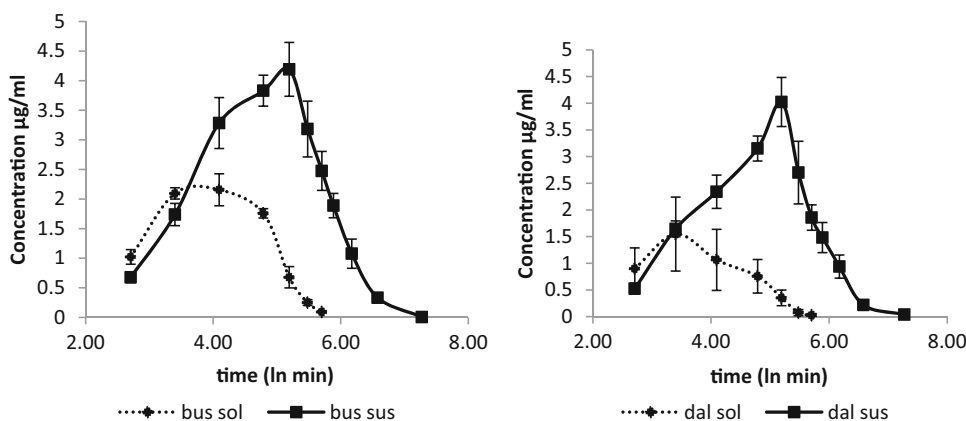


Table 1 Pharmacokinetic and bioavailability parameters of buserelin acetate and dalarelin acetate in female rats after single s.c. injection of the solution and suspension

Parameters	Buserelin solution (I)	Buserelin suspension (II)	Dalarelin solution (III)	Dalarelin suspension (IV)
A ($\mu\text{g/ml}$)	14.761	9.44	18.297	10.242
B ($\mu\text{g/ml}$)	4.764	8.605	2.641	9.703
k_e	0.0164	0.0043	0.0192	0.0052
k_a	0.1048	0.0166	0.1887	0.0133
$t_{1/2e}$ (min)	42.26	161.16	36.09	133.27
$t_{1/2a}$ (min)	6.61	41.75	3.67	52.11
CL_{tot} (ml/min)	30.34 ^{a,d} \pm 2.12	3.89 \pm 0.18	59.58 ^b \pm 8.41	4.75 \pm 0.54
t_{max} (min)	45	180	30	180
C_{max} ($\mu\text{g/ml}$)	2.16 ^a \pm 0.38	4.19 \pm 0.61	1.55 ^b \pm 0.44	4.02 \pm 0.65
AUC _(0–∞) ($\mu\text{g}\cdot\text{min/ml}$)	195.77 ^a \pm 16.64	1544.72 ^c \pm 96.92	100.78 ^b \pm 20.79	1272.66 \pm 186.78

^a Significantly ($p \leq 0.001$) different from group II

^b Significantly ($p \leq 0.001$) different from group IV

^c Significantly ($p \leq 0.01$) different from group IV

^d Significantly ($p \leq 0.001$) different from group III

(53 %) that of buserelin delivered in the form of solution and 83 % when they were delivered in the form of suspension.

3.2 Hormonal Profile

3.2.1 Concentration of LH

In the control group, LH concentration in the serum during the whole period of experiment was in the range of 0.02–0.21 ng/ml, while in the groups receiving GnRH analogs in the range of 0.021–23 ng/ml. In the latter groups, high concentration of LH, over 10 ng/ml, was noted for 4 h after injection of the analogs in the form of the solution and 6 h if they were in the form of suspension (Fig. 3a). Maximal LH concentration was noted, for both injection formulas, 2 h after injection—up to 23 ng/ml (Table 2). Calculated AUC_{0– ∞} was significantly lower ($p \leq 0.05$) if analogs were delivered in the form of solution (Fig. 4).

3.2.2 Concentration of FSH

Like in the case of LH, the FSH concentration in the serum of animals from control group was, during the whole period of experiment, lower than in the experimental groups and was in the range of 0.3–0.9 ng/ml (Fig. 3b). The highest concentration of FSH was noted 2 h after injection (for both analogs delivered as solution, but also dalarelin in form of suspension) and 4 h after injection in groups receiving buserelin in suspension form (Fig. 3b). Higher FSH concentration was noted in groups receiving dalarelin (in case of solutions the difference was statistically significant— $p \leq 0.05$) (Table 2). Accordingly, the AUC_{0– ∞} values were

significantly higher ($p \leq 0.001$) in dalarelin receiving groups, both for solution and suspension forms (Fig. 4)

3.2.3 Concentration of 17 β -Estradiol

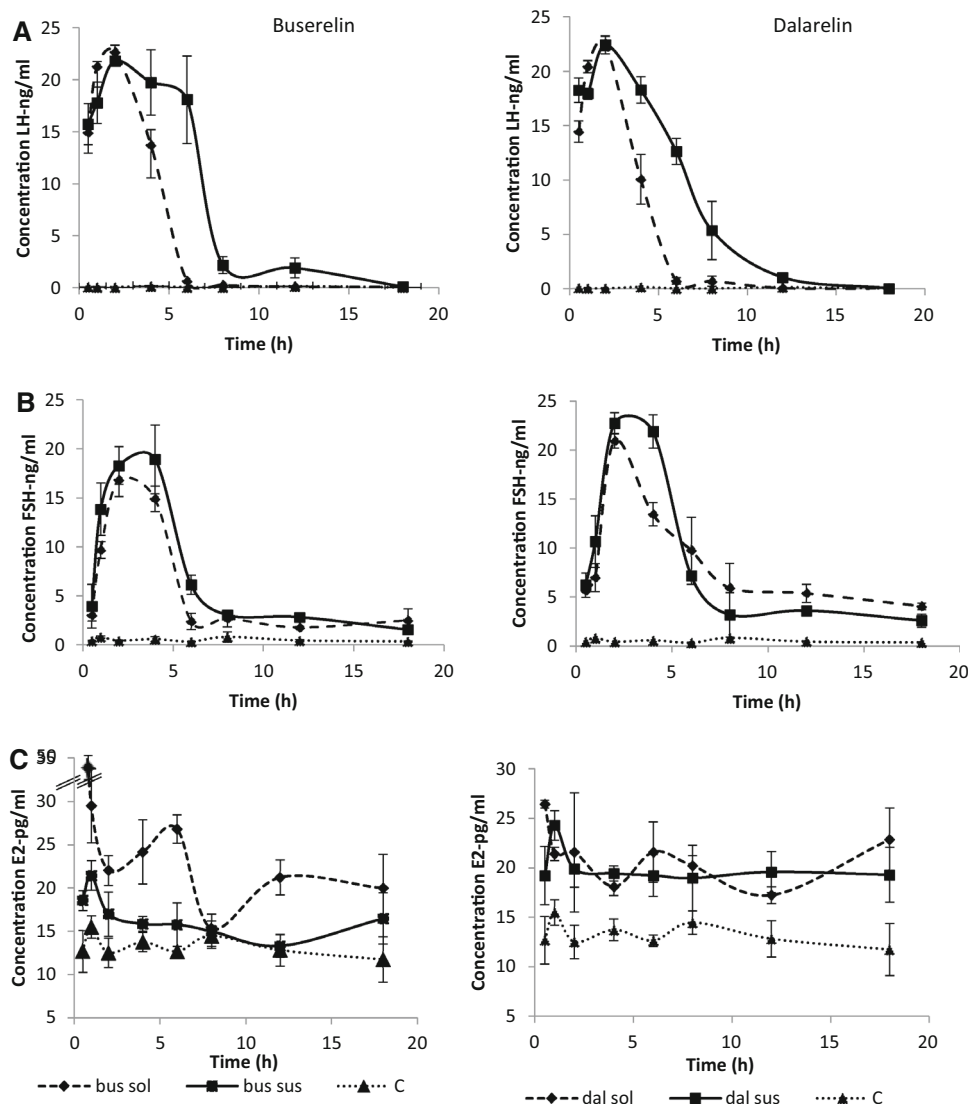
17 β -estradiol (E2) concentration (like LH and FSH) was significantly higher in experimental groups being in the range of 15–46 pg/ml (Fig. 3c). The estradiol profile in the serum differed depending on the type of analog and its form. In groups which received buserelin or dalarelin in form of solution, the maximal concentration of estradiol was noted 30 min after injection (Fig. 3c) and was, respectively, 45.7 pg/ml in buserelin, and 26.4 pg/ml in dalarelin receiving groups (Table 2). In groups which received GnRH analogs in suspension form, the highest estradiol concentration was noted after 1 h (Fig. 3c) and was 21.4 pg/ml (buserelin) and 24.2 pg/ml (dalarelin), respectively (Table 2). AUC_{0– ∞} was significantly higher ($p \leq 0.001$) if analogs were delivered in the form of solution (Fig. 5).

Besides the individual concentrations of particular hormones, very important are their relative ratios in the blood. Compared to control in all experimental groups, AUC_{E2}/AUC_{FSH} and AUC_{E2}/AUC_{LH} values were significantly lower ($p \leq 0.001$). Only AUC_{LH}/AUC_{FSH} ratio, excluding group in which animals received dalarelin in form of solution, was comparable to control (Table 2).

4 Discussion

The most known classical hormone containing suspensions are insulin suspensions in which hexamer structure of insulin is stabilized by the addition of protamine and Zn.

Fig. 3 Serum concentration of LH (a), FSH (b) and E2 (17 β -estradiol) (c) in the superovulated immature female rats after single s.c. injection of the buserelin acetate (bus) and dalarelin acetate (dal) in two different forms: solution (sol) and suspension (sus) at the same dose (1 μ g/kg) and 0.9 % NaCl (C control). Each point represents the mean \pm SD



Subcutaneous injection of such formulas leads to the pro-tamine breakdown and Zn dissipation which destabilizes hexamer structure of insulin and results in slow release of insulin in the form of dimers and monomers [17]. Zn is used not only in classical insulin suspensions, but also new, biodegradable, controlled release formulas containing insulin glargine [18]. In case of insulin, addition of Zn is justified as in β cells; hexamer structure of insulin is warranted by Zn presence [17]. However, besides insulin, suspensions of other hormones, like long acting form of ACTH, are also completed with Zn. It was shown that 30 min after intramuscular injection of ACTH-Zn, cortisol concentration in cows blood raises, reaching maximum after 4 h, and stays at high level for 10 h [19].

We have studied two GnRH agonists delivered into female rats in the form of solution or suspension and found significant differences both in their basic pharmacokinetic

parameters and biological availability. Despite that we injected relatively high dose of both analogs (6 mg/kg); their plasma levels throughout the whole period of experiment were relatively low. This could be explained by their fast distribution and elimination from the organism, especially if hormones are injected in solution form. Sandow et al. [10] in patients with prostate cancer used PLG implants (polylactide/glycolide; 75:25) containing 6.6 mg of buserelin with 2 months release profile. Concentration of buserelin in blood and urine was measured by specific HPLC/RIA method using anti buserelin antibody. The studies show that concentration of buserelin in the urine (per 1 g of creatinine) was definitely higher than measured in the blood during the whole period (2 months) of experiment. The mean coefficient value calculated as the ratio of buserelin concentration in the urine to its concentration in the blood was 20. The authors stated that

Table 2 LH/FSH, E2/FSH and E2/FSH AUC ratios and C_{\max} of LH, FSH and E2 (17β -estradiol) in the serum of the superovulated immature female rats after single s.c. injection of the buserelin acetate

	Buserelin solution (I)	Buserelin suspension (II)	Dalarelin solution (III)	Dalarelin suspension (IV)	Control (C)
AUC_{LH}/AUC_{FSH}	0.91 \pm 0.03	1.22 ^a \pm 0.21	0.55 ^{a,b} \pm 0.05	0.98 \pm 0.05	1.08 \pm 0.03
AUC_{E2}/AUC_{FSH}	5.54 ^c \pm 0.32	2.31 \pm 0.03	3.14 ^d \pm 0.18	2.55 \pm 0.03	26.54 ^e \pm 5.27
AUC_{E2}/AUC_{LH}	6.13 ^c \pm 0.53	1.93 ^a \pm 0.29	5.74 ^d \pm 0.17	2.60 \pm 0.15	215.6 ^e \pm 65.9
C_{\max}					
LH (ng/ml)	22.67 \pm 0.64	21.82 \pm 0.03	22.54 \pm 0.67	22.42 \pm 0.84	0.15 ^e \pm 0.06
FSH (ng/ml)	17.21 ^f \pm 1.14	20.23 \pm 1.41	20.93 \pm 0.72	22.74 \pm 0.42	0.82 ^e \pm 0.53
E2 (pg/ml)	45.70 ^e \pm 20.18	21.45 \pm 2.71	26.57 \pm 6.02	24.29 \pm 1.49	15.48 ^e \pm 1.29

^a Significantly ($p \leq 0.05$) different from group IV

^b Significantly ($p \leq 0.05$) different from groups II, C

^c Significantly ($p \leq 0.001$) different from groups II, III

^d Significantly ($p \leq 0.001$) different from group IV

^e Significantly ($p \leq 0.001$) different from group II

^f Significantly ($p \leq 0.001$) different from groups III, IV

^g Significantly ($p \leq 0.001$) different from all groups

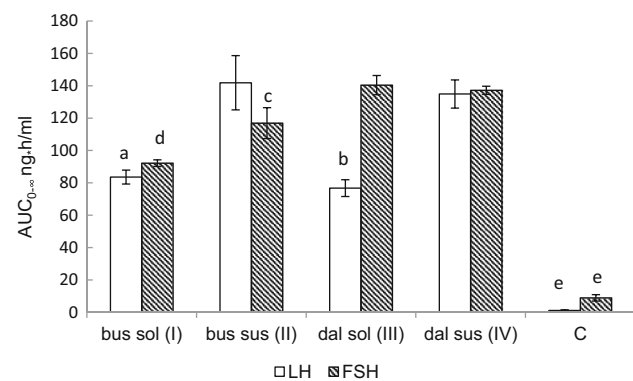


Fig. 4 $AUC_{0-\infty}$ for LH and FSH after single s.c. injection of dalarelin acetate (dal) and buserelin acetate (bus) in the form of solution (sol) or suspension (sus). **a** significantly ($p \leq 0.05$) different from group II, **b** significantly ($p \leq 0.05$) different from group IV, **c** significantly ($p \leq 0.01$) different from group III, IV, **d** significantly ($p \leq 0.001$) different from group II, III, IV, **e** significantly ($p \leq 0.001$) different from all groups

biological half-life of buserelin could be established only after injection of very high doses of this hormone.

In our study, after single subcutaneous injection of GnRH analogs in the solution form we observed their fast absorption from the delivery site and fast elimination from the plasma. When the analogs were delivered in suspension form, their absorption and elimination were slower and, consequently, their measured AUC values were higher. It can be explained by the fact that in the form of suspension hormones bind Zn and albumin what slows-down their absorption from delivery site but also slows-down their enzymatic degradation and elimination. However, it must

and dalarelin acetate at two different forms: solution and suspension at the same dose (1 μ g/kg) and 0.9 % NaCl (control)

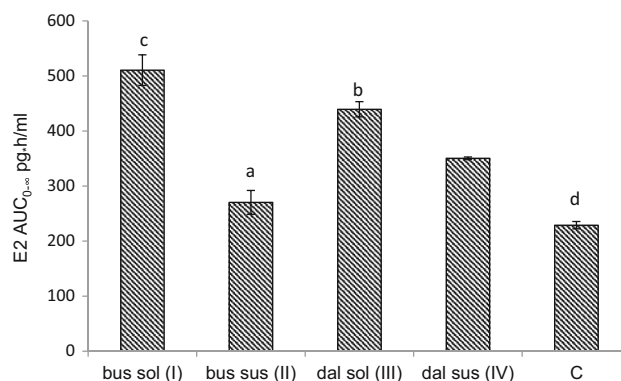


Fig. 5 $AUC_{0-\infty}$ for 17β -estradiol (E2) after single s.c. injection of dalarelin acetate (dal) and buserelin acetate (bus) in the form of solution (sol) or suspension (sus). **a** Significantly ($p \leq 0.01$) different from group IV, **b** Significantly ($p \leq 0.001$) different from groups II, IV, **c** significantly ($p \leq 0.001$) different from groups II, III, IV, **d** significantly ($p \leq 0.001$) different from all groups

be stated that mechanism of slower release of GnRH analogs from these types of suspensions is not clear. The studies indicate that only a small percentage of GnRH as well as its analogs bind albumin in the blood [20]. On the other hand, in the immunization procedures, using GnRH-albumin complexes is quite efficient [21]. Considering biological availability, hormones delivered in the form of solution were much less bioavailable. However, the differences in chemical structure of GnRH analogs also affect their availability. The calculated Extent of Biological Availability (EBA) showed that dalarelin is less bioavailable than buserelin both in the form of solution and suspension. It seems that substitution D-Ser(tBu) in buserelin

in place of glycine in GnRH is more effective than substitution of alanin (D-Ala) in dalarelin.

The known effect of GnRH agonists activity is the stimulation of LH and FSH release directly after their delivery (flare-up effect) which results in increased concentration of estrogens, mostly 17β -estradiol [22]. We observed this effect after injection of agonists both in the form of solution and suspension. Murase et al. [23] noted increased concentration of LH and FSH in the blood of ovariectomized female rats 2-5 h after delivery of leuprolide acetate in the form of depot. The similar effect was observed by Okada et al. [11] after subcutaneous injection of leuprorelin acetate in the form of depot injectable microspheres. Our own studies show that delivery of buserelin or dalarelin, both in the form of solution or suspension, into superovulated female rats increases LH, FSH and estradiol concentration in the serum. Maximal concentrations of LH were comparable, however, in the groups receiving the analogs in the form of suspension they lasted longer. In case of FSH the higher concentrations, both maximal and through the whole period of experiment, were observed after injection of dalarelin no matter of the formulation used. It indicates that FSH releasing profile depends mostly on the type of analog used. GnRH analogs are used in the treatment of many hormone-dependent illnesses. They are also used in IVF programs. There are many protocols to induce controlled hyperstimulation of the ovaries including the use of GnRH analogs [4, 24]. One of the complications connected with classical HCG hyperstimulation is ovary hyperstimulation syndrome (OHSS) [25]. The risk of OHSS is lower if GnRH analogs are used [26]. Studies indicate that women with OHSS syndrome have very high blood concentration of estrogen [27]. Ajonuma et al. [28] inducing OHSS in rats by injection of PMSG/HCG show that estrogen concentration in the blood of animals was 8 times higher than in control female rats in estrous and diestrous phases. In our study to induce superovulation, we employed the OAAD (Ovarian Ascorbic Acid Depletion) method described by Parlow [29]. We stated that in control female rats 6 days after HCG injection the serum levels of LH and FSH were low. 17β -estradiol concentrations were comparable to those noted in the blood of adult female rats in late estrous/early metestrous phase [30]. Probably, the difference in observed concentration of estrogens is due to the use of other model to induce superovulation, and mostly lower doses of PMSG and HCG, shorter PMSG delivery time and the use of younger female rats. However, relations between the hormones in the control group shown as E2/FSH and E2/LH ratios were respectively 10 times and 100 times higher comparing to groups receiving analogs. In general, in all groups receiving hormones the 17β -estradiol concentration was higher than in control group.

On the basis of performed experiments, we can state that estradiol concentration in the serum of female rats and its secretion profile depends, like in case of LH, mostly on the type of formula used for injection of both analogs. In the serum of animals which received analogs in form of solution, maximal concentration of estradiol is reached faster and then slightly fluctuates. In female rats that received analogs in the suspension form, concentration of estradiol after reaching its maximal level stays relatively constant. Estradiol concentrations in the serum of rats receiving buserelin and dalarelin in the form of solution are generally higher than in the serum of female rats receiving these analogs in form of suspension. From clinical point of view, extremely important are relations between the hormones. Calculated, on the basis of AUC, E2/FSH and E2/LH ratios were 2 times higher in groups receiving hormones in solution form. It suggests that in case of suspensions slower absorption and elimination of hormones lead to longer stimulation of pituitary gland which promotes release of gonadotropins. Obviously, the above results cannot be directly compared with those obtained in IVF programs mostly due to completely different hyperstimulation scheme. However, it is possible that the use of suspension instead of solution forms of analogs would lower the number of doses currently applied to patients. Our study indicates also that the hormonal profile depends on the type of analog used. On one hand, it seems obvious that the differences in chemical structure of agonists have influence on their biological activity. On the other hand, the data obtained from IVF programs [24] indicate that the type of analog used has no effect on number of newborns.

5 Conclusion

Performed studies indicate that GnRH analogs in the form of suspension have higher bioavailability than their solution counterparts. It influences the effects of their action, especially in relation to LH and FSH.

Compliance with Ethical Standards

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Conflict of Interest ASS, FR, BD, RD, AD, LF and RW declare no conflict of interest.

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