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COMMUNICATION

Storage Conditions for Serum Deslorelin

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ABSTRACT

The stability of a luteinizing hormone-releasing hormone (LHRH) analog in rat serum was studied under reproduced experimental analysis conditions. Serum samples of deslorelin [D-Trp^{6}, Des-Gly, NH$_2^{10}$] LHRH ethylamide were exposed to multiple freeze-thaw cycles to determine the maximum number of cycles the serum sample can be exposed to without producing any quantitative changes in radioimmunoassay (RIA) measurements of deslorelin. A significant cycle effect was observed after completion of the sixth cycle. Serum samples were also stored at standardized −50°C conditions for variable periods of time to determine the effects of acute and chronic storage time on deslorelin stability. Matched-pair t*-test analysis showed no significant changes in deslorelin values as measured by RIA for a 3-week, 4-month, or 2-year storage period. The conditions in which the rat serum samples were stored prior to analytical analysis were sufficient to prevent detectable degradation of the deslorelin peptide.*

INTRODUCTION

In peptide therapeutics, the issue of stability can be divided into two main categories: chemical and physical. Chemical stability is related intrinsically to the structural components of the peptide such, as the amino acid (aa) sequence and the bonds that form between them. Chemical activity in the functional groups of aa side chains results in changes in primary and secondary structure. Physical stability refers to the structural conformation of the peptide in its environment. Physical interactions typically are defined as those in which no chemical changes are induced into the overall structure of the molecule (1). Both forms of stability directly affect the pharmacological properties of the peptide and its potential for therapeutic application.

Studies on the in vivo degradation of peptides can result in a greater understanding of the relationship between structure and stability. Identification of degradation pathways yields insight into the aa bonds that are the most susceptible to proteolysis or instability. Modifications can then be made at these positions, directly affecting the chemical stability and biological activity of the peptide.

The native luteinizing hormone-releasing hormone (LHRH) molecule consists of the 10 aa linear sequence: Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly. In vivo and in vitro studies have shown that the primary site for endopeptidase degradation on the LHRH molecule is the Gly aa at position 6 (2). Elimination of Gly-aa bonds by substitution of Gly at positions 6 and 10 with a D-aa leads to stabilization toward membrane enzymes and results in an increase in the amount of peptide presented to the blood (3). This substitution of an L-aa residue for a D-aa residue produces a conformational change in the peptide backbone that is thought to alter the analogue-enzyme complex, resulting in diminished cleavage activity of the peptidase (4,5). For LHRH agonists, those produced by substituting Gly^6 with a D-aa display total resistance to both endopeptidase 24.11 and 24.15[2].

Exopeptidases (typically located in blood) are known to cleave at either the Nα- or C-terminus of a nonmodified peptide. Typical Nα terminus modifications include acetylation and desamination, while amides and alcohols are in standard use to modify the C-terminus. The LHRH analog [D-trp^6, Des-Gly, NH_2^{10}] ethylamide (deslorelin) is structurally different from the native LHRH molecule due to a D-aa substitution at position 6, as well as a C-terminus modification. The chemical stability of modified LHRH results in a highly potent analog with increased biological activity.

The chemical modifications made to peptides to produce in vivo stability do not necessarily have effects on the stability issues encountered in solution or during storage. Although peptides consisting of 10 or fewer aa (such as deslorelin) tend to be fairly resistant to extreme conditions compared to larger peptides and proteins, the individual limits must be determined when evaluating a peptide for therapeutic application. Proper storage conditions result in protection of the peptide structure from proteolytic enzymes and denaturing mechanisms.

For preclinical in vivo studies, the issue of serum stability of the peptide needs to be explored to ensure accurate quantitative and qualitative determinations of the biological. Acute and chronic storage, as well as repeated freezing and thawing, are typical conditions to which serum samples are exposed during analytical analysis. Optimal conditions for the serum sample must be determined to minimize potential degradation of the peptide during these processes.

The objective of this study was to determine the effects of storage time and freeze-thaw cycles on the stability of deslorelin in rat serum. Conditions similar to those to which the serum samples were subjected during radioimmunoassay (RIA) analysis were evaluated.

MATERIALS AND METHODS

Freeze-Thaw Study

Serum samples from the deslorelin implant study that had been stored at −50°C were used in the study. To minimize the potential of individual serum variability effects on the outcome of the study, three pooled samples were created. Each pool consisted of 30 μl of serum from rats 21–35 taken on days 1, 8, 34, 71, and 98 for a total of 750 μl/pooled sample. The pools were then gently agitated, aliquoted into 9 individual microfuge tubes, labeled, and stored at −50°C for the duration of the study.

One sample from each of pools I, II, and III was taken through a series of freeze-thaw cycles that ranged from 1 to 6 cycles. A freeze-thaw cycle is defined here as a 24/1 increment. Following storage at −50°C for a minimum of 24 hr, samples are then placed at room temperature (20°C) for 1 hr. The thaw period of 1 hr was chosen because it represents the maximum amount of time a sample is exposed to room temperature conditions during initial pipetting in the deslorelin assay. After completion of the predetermined number of cycles, all samples were stored at −50°C to await further analysis.

Stability Study

Serum samples from a previous deslorelin implant study were used for this study. Serum samples from 10 rats were evaluated by RIA after a defined period of storage time in standardized conditions at −50°C. Serum deslorelin values for the baseline analysis were obtained from the original assay data.

Serum samples collected on day 55 of the deslorelin study split were evaluated following 3 weeks of storage. The serum samples taken on day 48 of the deslorelin study were evaluated after a 4-month period of storage. Serum samples taken on day 21 of the deslorelin study were evaluated after a 2-year storage period. All samples remained in their original vials for the duration of the study, were stored in the same cryovial boxes, and were placed in a specified area of the −50°C freezer to standardize the storage conditions.

Serum samples for both the freeze-thaw and the stability studies were evaluated by the deslorelin RIA protocol developed in our laboratories. The homologous double-antibody RIA utilizes anti-[D-Trp^6, Des-Gly-NH_2^{10}] LHRH-EA (Dr. LaBrie, Quebec, Canada) as primary antisera and deslorelin (Bachem, Los Angeles, CA) as labeled antigen and standard. The separation

phase of the assay utilizes 4% polyethylene glycol as the precipitant.

For the freeze-thaw study, analysis of variance (ANOVA) was conducted to determine significant ($p < .05$) differences in serum deslorelin values among cycles. Duncan's new multiple range test was used to compare the differences among cycle means. For the stability study, inferences regarding mean differences between the two dependent groups were determined using a matched-pair *t* test. Here, significance was determined at $p < .05$.

RESULTS AND DISCUSSION

Freeze-Thaw Study

In this study, the effects of multiple freeze-thaw cycles on serum deslorelin values were determined. A significant cycle effect was observed. Mean serum deslorelin values were significantly higher after the sixth freeze-thaw cycle compared to the initial four cycles. The results of Duncan's new multiple range test (Table 1) indicates that differences in serum deslorelin values for the sixth cycle were not significantly different from those of the fifth cycle, but they were significantly different from those of cycles 1 to 4. As seen in Fig. 1, serum deslorelin values show minimal increases during the first four cycles, with increases in deslorelin values appearing after the fifth cycle, and variable increases (as seen by the large SEM) after completion of the sixth freeze-thaw cycle. Since the effect of multiple freeze-thaw cycles was not seen until the sixth cycle, the implications are that the structure of deslorelin remained unchanged until the sixth cycle or changes in the structure of deslorelin occurred during each cycle, but did not have an effect on antibody affinity until the sixth cycle. High-performance liquid chromatography (HPLC) data are not available for the same serum samples, hence both explanations are plausible. Similar stability results have been reported when plasma samples of deslorelin were stored at −20°C and passed through one freeze-thaw cycle with no detectable differences in RIA values (6). Likewise, LHRH analog in a sterile solution of normal saline and 10% mannitol was subjected to seven freeze-thaw cycles with no apparent effect on either its HPLC profile or its biological activity (7).

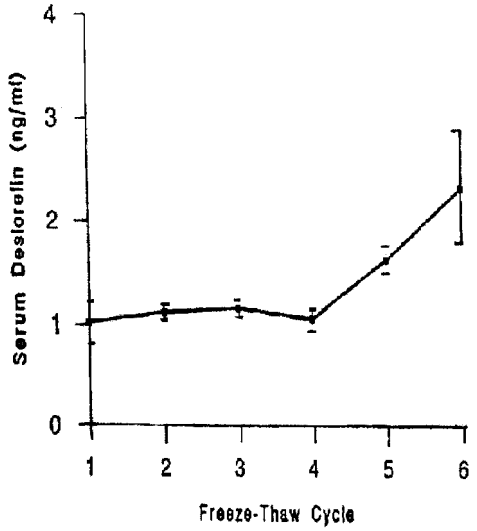

Figure 1. Mean serum deslorelin values (ng/ml + SEM) versus freeze-thaw cycles.

Table 1

Duncan's New Multiple Range Test Results

Number of means	2	3	4	5	6
Critical Range	23.30	24.39	25.05	25.48	25.78

Mean	*N*	Cycle	Duncan Grouping
58.57	3	6	A
40.37	3	5	A, B
28.90	3	3	B
27.77	3	2	B
26.37	3	4	B
25.47	3	1	B

Alpha = 0.05; *df* = 12 *f*; MSE = 171.4994.

Table 2

Matched-Pair t*-Test Stability Study: Results*

Time	*N*	Mean	SD	*T*	Prob > T
3 weeks	10	0.37	3/67	−/32	−/76
4 months	8	16.64	28.16	1.67	0.14
2 years	10	−1.6	4.54	−0.95	0.37

Stability Study

In this study, the storage conditions remained fixed, while the effect of length of time the serum samples were stored at −50°C was evaluated. The results of study A indicate that there is no significant difference ($\alpha > 0.05$) in the serum deslorelin values after an acute storage period of 3 weeks compared to the values obtained in the original assay. A lengthier storage period of 4 months also resulted in no significant changes ($\alpha > 0.05$) in serum deslorelin values. Interestingly, when the storage period was increased substantially to 2 years, there were still no significant differences ($\alpha > 0.05$) in serum deslorelin values (Table 2). These results are consistent with previous studies, which have shown that LHRH can be stored in normal saline or 1% glycine for at least a year without any changes in its biological activity (8).

CONCLUSION

Insight into handling conditions that maintain peptide stability during analytical analysis was achieved. Multiple freeze-thaw cycles do not appear, as determined by RIA, to have an effect on the deslorelin peptide. Results also indicate that, when stored at −50°C, unextracted serum deslorelin samples remain stable for up to 2 years. Further analysis by HPLC and bioassays would determine whether any subtle conformation changes occur over time.

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