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Identification and determination of GnRH antagonist gelling at injection site

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Abstract

The purpose of this study was to first observe whether orntide, a GnRH antagonist, gels at the injection site and if so, to develop and validate an extraction method to quantitate the peptide amount as well as assess chemical stability in the gel. After subcutaneous injection of a large dose of orntide acetate solution, a white gel and local traumatized effect were observed at the injection site. Orntide remaining at the injection site was recovered by tissue excision, homogenization and tissue protein precipitation with perchloric acid and quantified by high performance liquid chromatography (HPLC) following separation on a C18 column. The standard curve was linear in the detection range and there was no interference from either blank tissue or excipients of the orntide formulation. The recovery from spiked tissue or that immediately following injection was in the range of 90–110%. MALDI-FT mass spectrometry (MS) of the peak fraction indicated that the orntide recovered from the injection site was in the intact form. The results showed that orntide solution, when injected at a large dose, formed a gel at the injection site. The gel delayed the release from the injection site and caused discernible tissue reaction.

Keywords: LHRH antagonist; Orntide; Gelling; Precipitation; Tissue

1. Introduction

A number of luteinizing hormone releasing hormone (LHRH) agonists and antagonists have been developed for clinical use in various disease states, primarily hormone-sensitive cancers (e.g. prostate cancer), endometriosis and precocious puberty (Nestor et al., 1982; Karten and Rivier, 1986; Filicori and Flamigni, 1988; Friedman, 1990). Two LHRH superagonists, leuprolide and nafarelin have been marketed successfully for some of these applications. However, LHRH antagonists have been reported to be more advantageous, since they block receptors from interacting with native LHRH and, therefore, do not produce

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the initial phase of testosterone elevation and disease flair up seen at the beginning of LHRH agonist therapy (Haviv et al., 1993; Kostanski et al., 2000a,b). For example, this transient stimulatory phase may persist for up to 1 week in rats and as long as 4 weeks in humans before chemical castration is achieved (Garnick et al., 1987; Kienle and Lubben, 1996).

Orntide (Fig. 1), a decapeptide analog of LHRH, is one of the most promising LHRH antagonists developed to date. It combines high antigonadotropic potency and low side effect of histamine release (Bowers et al., 1996).

Several papers have reported LHRH analogs to exhibit concentration dependent aggregation in aqueous media, forming liquid crystalline or gel systems upon standing (Powell et al., 1991, 1994; Cannon et al., 1995). For example, Powell et al. noted that the salts, specifically anions, affected the liquid crystal stability, as well as the temperature at which birefringent liquid crystals formed in aqueous formulations of deterelix. Leuprolide was also reported to induce gel formation with increasing peptide concentration, introduction of salts and gentle agitation (Tan et al., 1998). An antagonist of LHRH, A-75998, was observed by Cannon et al. to exhibit reverse or thermal gelation (Cannon et al., 1995). However, to date limited data are available regarding LHRH analogs on peptide gelling in vivo, which could be a concern in the safety and design of a dosage form. It seems prudent to address whether these peptides gel or precipitate upon injection and assess the content and stability of the peptide in the gel. Therefore, the purpose of this study was to investigate whether orntide gel formation or precipitation would occur following subcutaneous injection of a large solution dose in rats. If gelling was observed, the second objective was to develop a feasible and reliable extraction technique for high performance liquid chromatography (HPLC) and mass spectrometric (MS) analysis in order to quantify peptide amount and assess stability in the gel. In order to enhance the gelling, the injected amount of orntide acetate used was considerably higher than the pharmacologically active daily dose in the rat model.

2. Materials and methods

2.1. Materials

Orntide acetate was supplied by California Peptide Research Inc. All excipients in the orntide and placebo formulations were USP grade. HPLC

Orntide acetate ($C_{81}H_{107}N_{18}O_{14}Cl$)

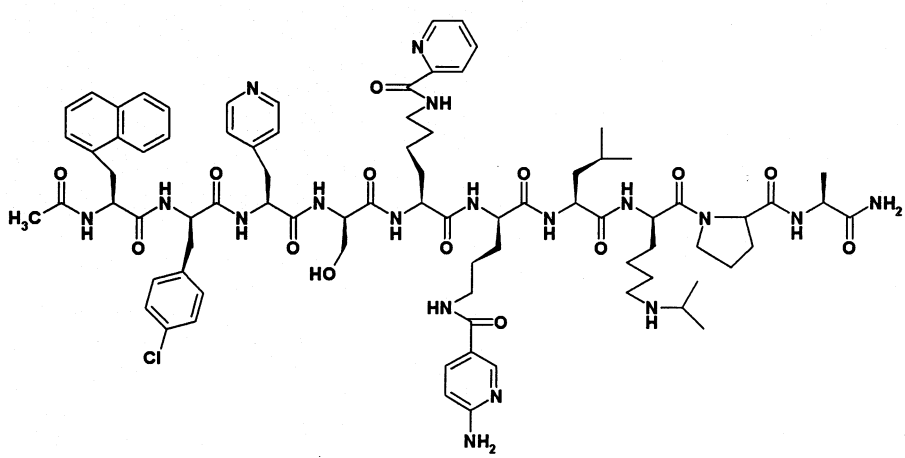

Fig. 1. Structure of orntide.

grade acetonitrile and perchloric acid were obtained from Fisher Scientific (Fair Lawn, NJ). Male Sprague–Dawley rats were purchased from Harlan (Indianapolis, IN).

2.2. Preparation of standard solutions

A stock solution of 1 mg/ml orntide was prepared in 0.1 M acetate buffer and kept at 4 °C. The standard solutions for constructing the standard curve were then prepared by dilution of the stock solution to 5–100 μg/ml with 1:2 (v/v) acetate buffer: 5% $HClO_4$. The volume ratio of acetate buffer and $HClO_4$ was same as in the final tissue extraction samples.

2.3. HPLC analysis

The chromatography was carried out using a Shimazu-6A instrument equipped with an autosampler and UV spectrophotometric detector interfaced with a Maxima peak integration software package. The analytical column used was a Bondclone 10 C18 (5-μm particle size, 150×3.9 mm), which was protected with a guard column (Phenomenex). Samples were analyzed using an isocratic mobile phase of acetonitrile and distilled water (31:69) with 0.1% TFA, using a flow rate of 1 ml/min, detection wavelength of 220 nm and an injection volume of 100 μl.

2.4. MALDI-FT MS

MALDI mass spectra were obtained with a Fourier transform ion cyclotron resonance mass spectrometer (IonSpec, Irvine, CA) using a 4.7 T superconducting magnet, and 2,5-dihydroxybenzoic acid as the matrix.

2.5. Preparation of orntide solution and placebo for animal Study

The orntide solution contained orntide acetate 0.25%, D-mannitol 3.5%, benzyl alcohol 0.9% in water, adjusted to pH 5 with glacial acetic acid. The placebo was formulated in the same way except sodium acetate 0.021% w/v replaced the orntide acetate.

2.6. Animal administration

2.6.1. Single dosing

Rats # 1 and 2 were injected with 5 ml of orntide solution subcutaneously on the right side of the abdomen. Similarly, 5 ml of placebo was injected on the left side of the abdomen of each rat. The rats were sacrificed after 4 h and the skin was separated to expose the subcutaneous space. Digital images of both injection sites were captured with a Largan digital camera. The tissue at the orntide injection sites was excised carefully and stored at −20 °C.

2.6.2. Multiple dosing

Rats # 3 and 4 were injected with 5 ml of orntide solution on the right side of the abdomen while rats # 5 and 6 were injected with 5 ml of placebo on the right side of abdomen. The doses were repeated on days 2, 3 and 4. Four hours after the fourth injection the rats were sacrificed and the injection sites were exposed as for the single dose and photographs taken. Tissue was removed from the injection sites as above.

2.7. Method validation for tissue collection and analysis

Prior to extraction of the treated tissue, four controls were run to develop and validate the analytical method including (a) blank tissue, (b) blank tissue mixed with placebo solution, (c) blank tissue spiked with known concentrations of orntide, and (d) zero time injection site tissue samples following known concentration of orntide administration to a fresh rat specimen. The controls and samples obtained from rats that received the orntide injections were homogenized in 10 ml 0.1 M acetate buffer (pH 4.0) followed by centrifugation at 4000 rpm for 15 min. The supernatant was transferred to a clean tube and stored at −70 °C until assay. Three milliliters of the above supernatant were mixed with 6 ml 5% $HClO_4$ to precipitate the tissue proteins. The tubes were then centrifuged at 4000 rpm for 30 min. The supernatant was diluted into the linear range and injected directly onto the column. The peak fraction was collected and analyzed by MALDI-FT MS.

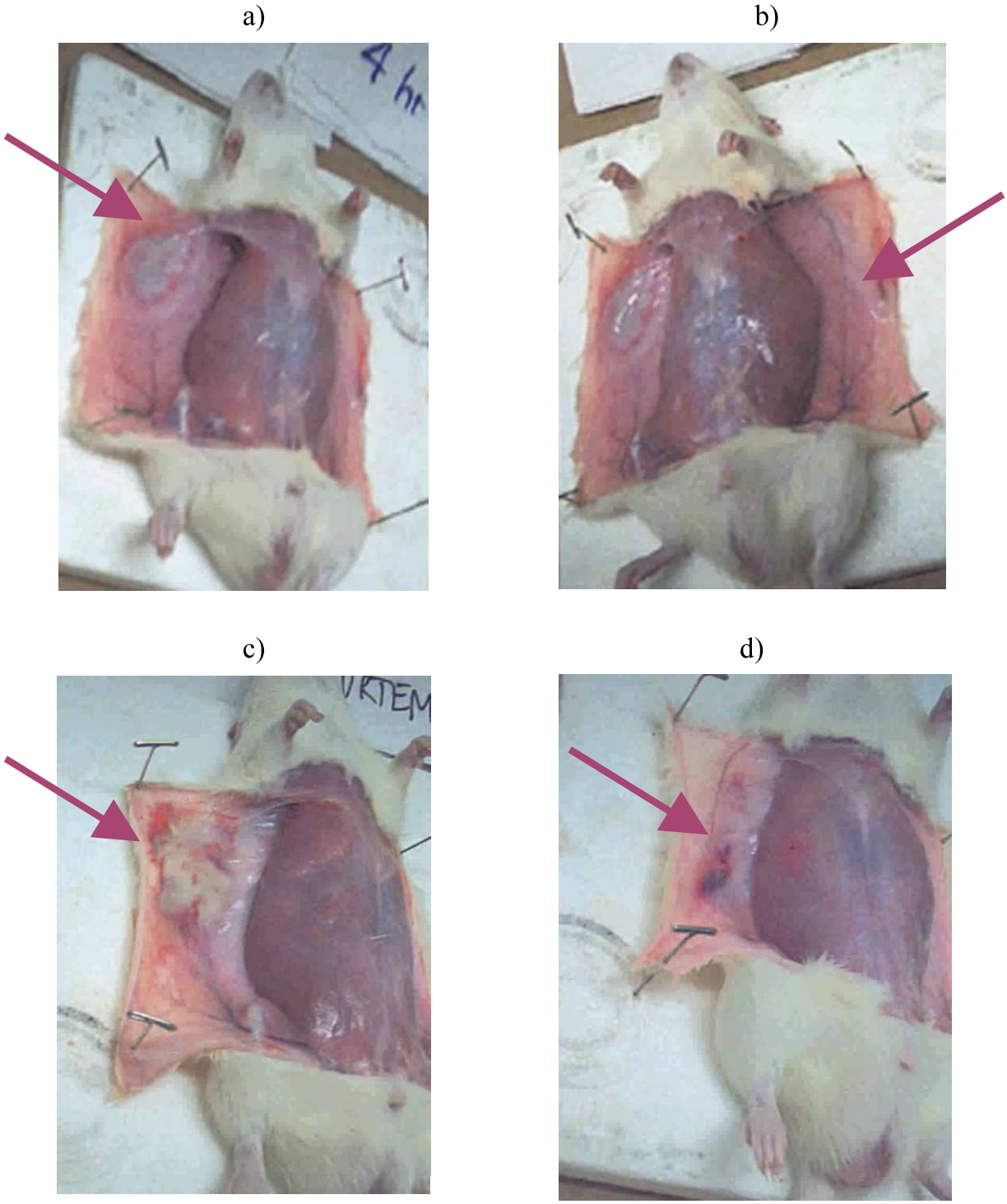

Fig. 2. Photographs of orntide gelling at subcutaneous injection site, (a) 4 h after single injection of orntide solution; (b) 4 h after single injection of placebo; (c) 4 h after the fourth injection of orntide solution; (d) 4 h after the fourth injection of placebo.

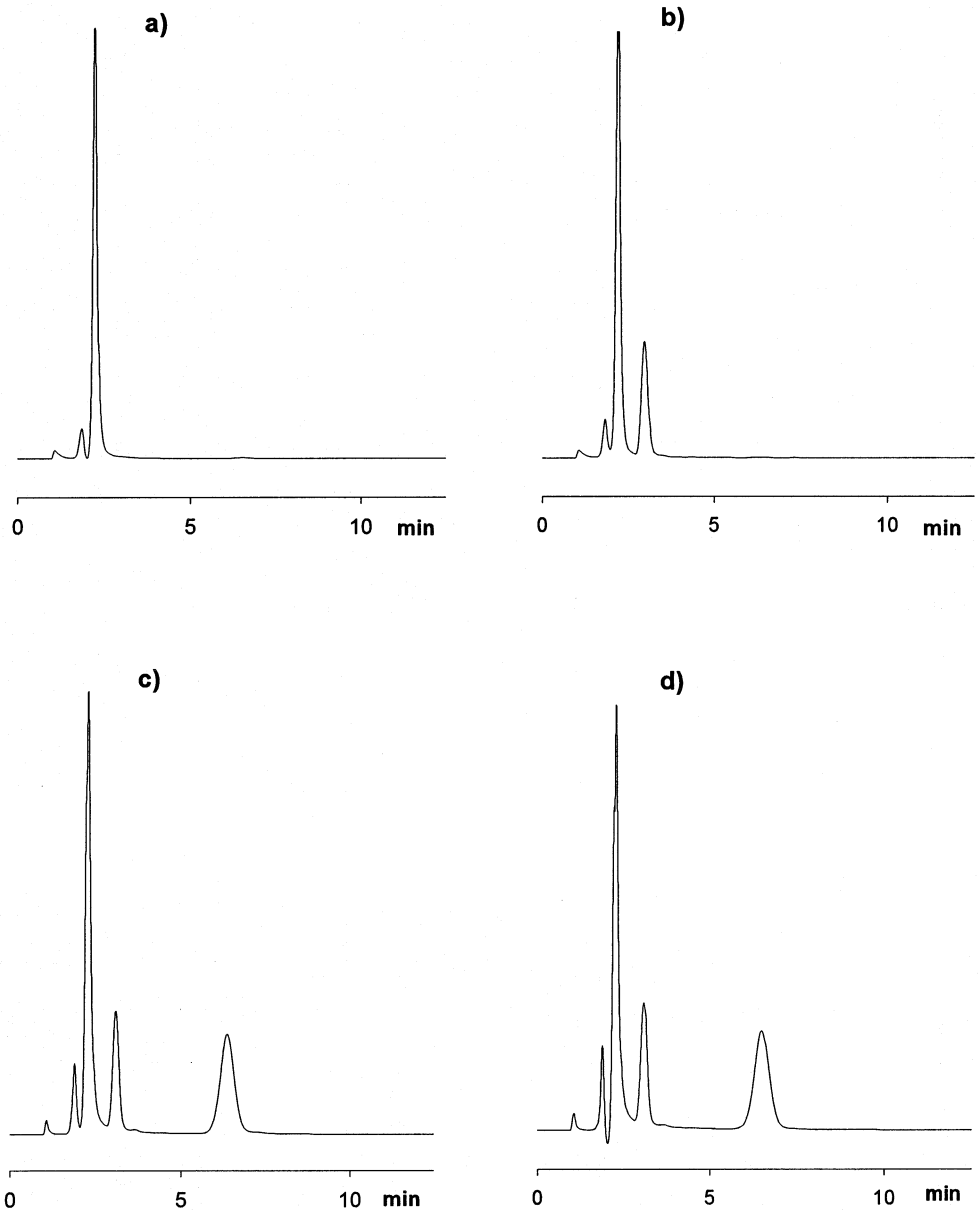

Fig. 3. Chromatograms of controls, (a) blank tissue; (b) blank tissue mixed with placebo; (c) blank tissue spiked with known concentration of orntide. (d) Zero time injection site tissue sample following known concentration of orntide administration to a new rat specimen.

3. Results and discussion

Immediately after single dosing of drug as well as placebo, there was noticeable swelling on both sides of the abdomen. However, within a few minutes the swelling at the placebo injection site subsided considerably while that at the orntide injection site was essentially unchanged. Within 2 h the swelling at the orntide injection site had subsided considerably to essentially that of the placebo. When the skin was separated 4 h after the administration, the exposed orntide injection

Table 1
Method validation for orntide extraction and analysis from tissue ($n = 2$)

Controls	Extraction	Interference	Recovery
A	Blank tissue	None	NA
B	Blank tissue mixed with placebo	None	NA
C	Blank tissue spiked with orntide	None	$105.2 \pm 2.6\%$
D	Zero time injection of orntide to a new rat	None	$99.9 \pm 2.1\%$

site looked very different from that of the placebo injection site. Fig. 2a shows a discernable gel-like white area at the orntide injection site, an occurrence which was absent at the placebo site (Fig. 2b). Similar behavior was observed from multiple dosing. As illustrated on Fig. 2c and d, the size and consistency of the white area was larger than that with a single injection, whereas the placebo treated animals did not display such behavior.

To identify whether the gel formed at the injection site contained orntide and to quantify the amount, tissue extraction and RP-HPLC assay methods were developed. In the method validation, over a concentration range of 5–100 μg/ml, linearity of standard was satisfactory and the correlation coefficient was > 0.999. In Fig. 3a and b, chromatograms of blank tissues (control a) and blank tissue with placebo (control b) showed no interfering peak from either endogenous constituents of tissue or placebo. The assay was validated using untreated tissue mixed with orntide formulation solution (control c). Orntide was found to elute at 6.4 min as a clear peak. The control d, i.e. zero time injection site tissue sample following known concentration of orntide administration to a new rat specimen, was included to quantify orntide recovery and ensure the completeness of collecting tissue containing the peptide. The means ($\pm$ S.D.) of recovery of controls c and d were 105.2 ± 2.6 and $99.9 \pm 2.1\%$ (Table 1). The satisfactory recovery rates confirm that the tissue extraction and analytical methods were suitable to measure orntide in the tissue.

Fig. 4 shows typical chromatograms of a sample obtained from a treated subject. The retention time for orntide was 6.4 min. Furthermore, there were no discernable degradation peaks observed. When the peak fraction was assayed with MALDI-FT MS, the sample had an identical same monoisotopic peak at MW of 1591.79 as the standard (Fig. 5), which demonstrated that orntide precipitated or gelled in the intact form. The orntide standard was made in 0.1 M acetate buffer, therefore, a much higher MNa^+ peak was observed compared with the sample from HPLC elution.

The amounts of orntide at the injection sites in four treated subjects are shown in Table 2. Four hours following single dose treatment of orntide solution, around 40% of orntide was recovered at the inject site. With respect to the multiple dosing, two rats showed relatively large intersubject variance of the precipitated orntide amount (32.6 and 15.2%). However, the amount from multiple dosing was higher than the single dose, which indicated an accumulation of peptide from four daily injections, i.e. the orntide gel or precipitate could not be eliminated within 24 h.

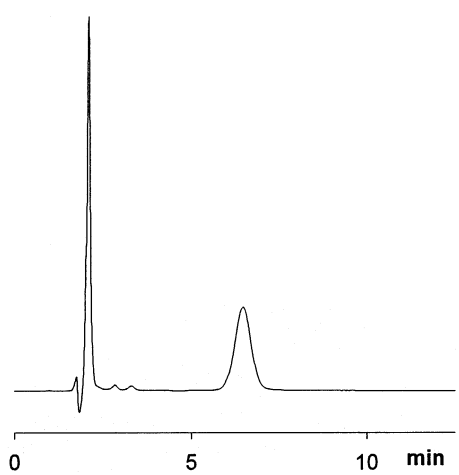

Fig. 4. Chromatograms of tissue gelling sample of rat # 1.

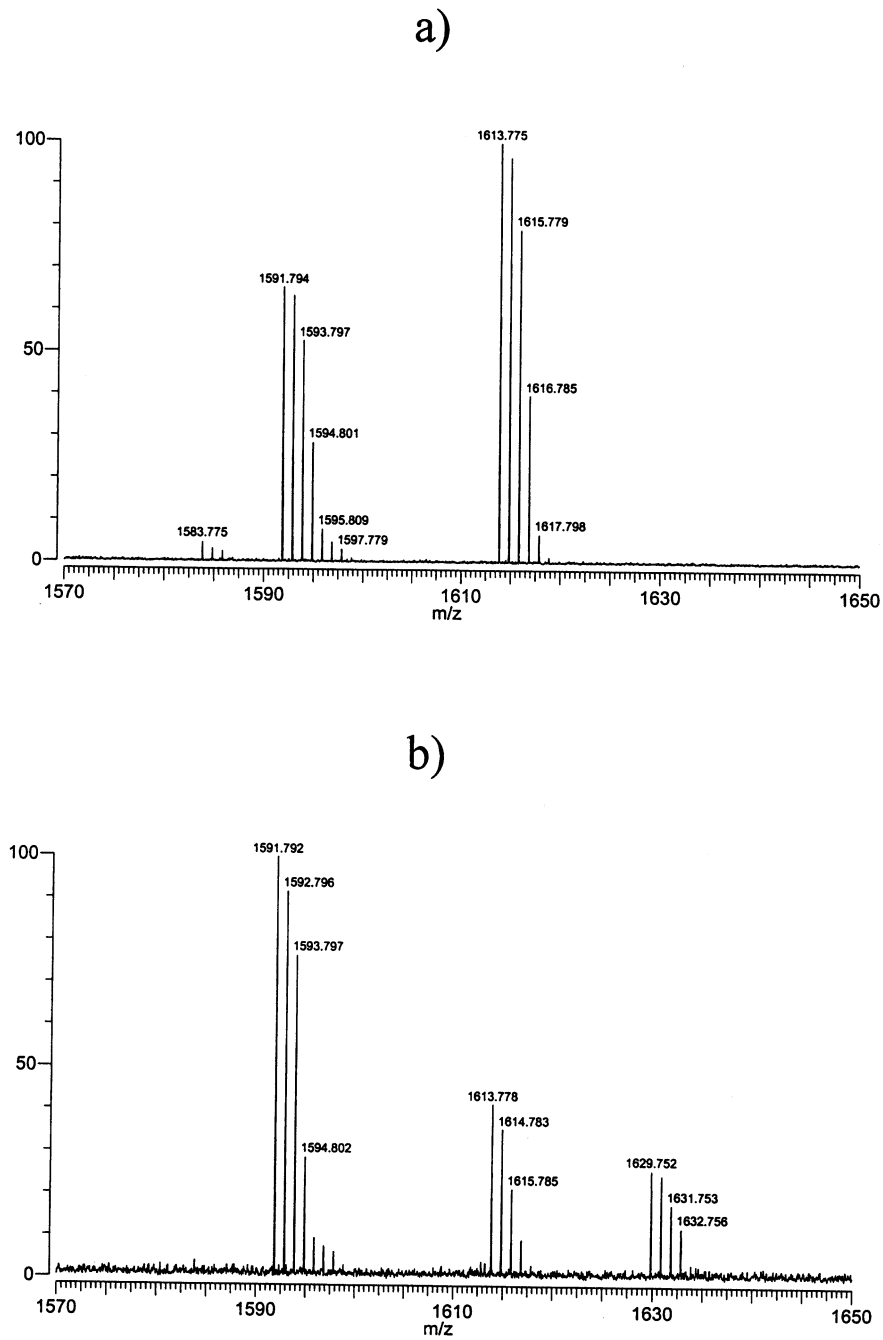

Fig. 5. MALDI-FT MS spectrum of (a) orntide standard in acetate buffer; (b) fraction of orntide peak from HPLC elution of rat #1 sample.

The detailed mechanism of gel formation in vivo is not clear yet. A possible reason of orntide gelling in the tissue could be a pH change post-injection and/or the water and other excipients in the solution formulation diffused rapidly after injection, leaving the orntide more concentrated to aggregate, precipitate or form the gel. Another reason could be the body fluid contains some salts, which induce association of orntide molecules. It was reported with increasing leuprolide concentration, the peptide may transition from a β-turn structure, through an unordered conformation to a β-sheet structure, which would fulfill the peptide hydrogen bonding requirements, and allow a more ordered, solvated packing structure (Tan et al., 1998). Like leuprolide, the ninth amino acid is also proline in orntide, therefore, the same conformational change could occur with orntide as well to form the gel. Additionally, orntide is highly branched with hydrophobic residues, the β-sheet structure would be stabilized by hydrophobic force among those residues at the interior of the β-sheet.

Peptide drugs are normally administered parenterally and require frequent administrations due to short biological half-lives (usually within 1 h). Peptide gelling in the tissue may provide a depot effect and impose a necessary dosing regimen adjustment. Compared with the half life of orntide itself in body fluid, a much longer time is needed for the orntide gel to be absorbed from the injection site, which may become a rate limiting step and induce drug accumulation when the patient is dosed frequently over extended periods of time.

The observed gelling of orntide was believed to be due to a high dose of free peptide. This phenomenon may not be severe or even occur in single therapeutic doses of 100 μg/kg daily to rats. However, treatment of prostate cancer requires extended suppression of testosterone to castration levels. For multiple injections of a solution form, it would be necessary to investigate whether the gel forms and tissue damage occurs in a dose dependent manner.

Currently, controlled release delivery systems ensure release of therapeutic peptides over extended periods of time, such as the microsphere preparations of leuprolide acetate (Lupron®, Takeda-Abbott Pharmaceutical) and trptorelin acetate (Decapeptyl®, Debiopharm-Ferring). A sustained dosage form of orntide acetate has also been undertaken as previously reported (Kostanski et al., 2000a,b). For example, a microsphere formulation enabled orntide to be delivered continuously in small doses over 30 days with a total dose of 2.2 mg/kg. Although the initial burst release from microspheres might be higher than the targeted 100 μg/kg daily dose, the subsequent daily amount would be lower than that required from multiple injections. This is due the sustained release kinetics and the lower amount required as a result of down regulation or desensitization of the LH receptors. Furthermore, since the desired daily dose of orntide is liberated gradually from the microspheres, a low local orntide concentration is expected and therefore, the amount released at the injection site is not likely to induce gelling or tissue reaction as seen with the high local concentration.

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Table 2
Orntide gelling amount at the injection site of four treated subjects

Subjects	Orntide solution treatment	Total orntide treated (μg)	Total orntide in tissue (μg)	Orntide precipitated in tissue (%)
1	Single injection	12 500	5602	44.8
2	Single injection	12 500	4643	37.1
3	Four injections	50 000	16 304	32.6
4	Four injections	50 000	7588	15.2

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