Short communication

Quantitation of slow release triptorelin in beagle dog plasma by liquid chromatography–tandem mass spectrometry

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A B S T R A C T

A sensitive method based on liquid chromatography–tandem mass spectrometry has been developed for the determination of triptorelin levels in beagle dog plasma. Plasma samples were applied to Oasis® HLB solid-phase extraction (SPE) cartridges. Extracted samples were evaporated under a stream of nitrogen and then reconstituted with 100 μl methanol:water:formic acid (60:40:0.08, v/v/v). The separation was achieved on a Venusil MP-C18 column (2.1 mm × 50 mm, 3 μm, Agela) with a gradient elution. Detection utilized a QTrap5500 system operated in the positive ion mode with multiple reaction monitoring of the analyte at m/z 656.5 → 249.1 and of the L.S. at m/z 510.8 → 120.1. The proposed method was validated by assessing the specificity, linearity, precision and accuracy, recovery, matrix effects, and stability. Linear calibration curves were obtained in the concentration range of 0.01–10 ng/ml (the correlation coefficients were above 0.995). The lower limit of quantification (LLOQ) of the method was 0.01 ng/ml. The method was successfully applied to a pharmacokinetic study of a slow release triptorelin formulation in beagle dogs following a single intramuscular injection.

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1. Introduction

Peptides and proteins are becoming increasingly common pharmacotherapies for the treatment of many human diseases. For example, peptides are emerging as a novel class of therapeutic agents in the pharmaceutical industry, especially in cancer chemotherapy. Peptide therapeutic agents are often synthetic analogs of endogenous peptides, such as hormones or neurotransmitter peptides.

Recent findings have suggested that the regression of various hormone-dependent disorders and of prostate tumors can be achieved after the administration of luteinizing hormone–releasing hormone (LHRH) agonists [1–4]. Natural LHRH is rapidly degraded in the blood (elimination t1/2, 3–4 min) by enzymatic cleavage at position 6, position 10, or both; however, the replacement of the amino acids at these positions allowed the synthesis of analogs with enhanced resistance to enzymatic degradation, resulting in a 10–200-fold increased potency compared with natural LHRH [5,6]. Among these analogs, triptorelin ([D-Trp6]-LHRH) has been widely used, and its biological and clinical effects are well documented [7].

Triptorelin, 5-oxo-Pro-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH₂, is a synthetic peptide used in hormonal therapy for the treatment of prostate cancer. This peptide inhibits the production of hormones by the pituitary gland, leading to a reduction in the level of the male hormone testosterone. Prostate cancer is often sensitive to the levels of these hormones in the body, and reducing the levels of these hormones may result in slowing of the growth of the cancer [8].

Although expected effects are obtained with daily injections of triptorelin, depot formulations are needed to increase the flexibility of administration; this increased flexibility will increase the convenience for patients and possibly improve patient compliance. A microsphere-based, slow release formulation of triptorelin acetate 3.75 mg has been developed. This formulation is injected intramuscularly, and the drug is released over 1 month.

With this type of formulation, the assessment of the release of and the plasma concentration of triptorelin is of particular importance to assure the desired biological effects.

Pharmacokinetic studies of triptorelin have been performed using a radioimmunoassay (RIA) [9], which was shown to be sensitive. The limit of detection (LOD) of this method was approximately 0.01 ng/ml using 500 μl of plasma. Nevertheless, this method has limitations due to the high cost and the cross-reactivity with structurally similar peptides, which interfere with the precise quantitation of the triptorelin level in plasma.

In the present study, we developed a highly specific and sensitive liquid chromatography–electrospray ionization tandem mass
spectrometry (LC–ESI-MS/MS) method for the determination of triptorelin levels in beagle dog plasma, and we applied this method to the evaluation of the slow release profile of triptorelin acetate biodegradable microspheres after the intramuscular injection of 0.3 mg/kg per month (peptide base). To the best of our knowledge, this is the first report of the use of the LC–MS/MS method for the determination of the plasma concentration of triptorelin.

2. Experimental

2.1. Reagents and chemicals

Triptorelin acetate (peptide base content 86%) was supplied by the National Institute for the Control of Pharmaceutical and biological products (Beijing, PR China). Octreotide acetate (purity > 99%) (Fig. 1) for use as internal standard (I.S.) was purchased from Shanghai TASH Biotechnology (Shanghai, PR China). HPLC grade methanol was purchased from Merck Scientific (New Jersey, USA). HPLC grade formic acid and acetic acid were supplied by Fluka (St. Louis, MO, USA). Millipore Milli-Q gradient purified water (Molsheim, France) was used throughout the study. Oasis\textsuperscript{®} HLB cartridges (30 mg, 1 cm\textsuperscript{3}) was purchased from Waters (Milford, MA, USA). Centrifuge tubes were supplied by Eppendorf (Hamburg, Germany).

2.2. Preparation of calibration standards and quality control (QC) samples

A stock solution of triptorelin acetate (containing triptorelin 1 mg/ml) in methanol–water–formic acid (80:20:0.04, v/v/v) was diluted with methanol–water–formic acid (60:40:0.08, v/v/v) to give a series of standard solutions with triptorelin concentrations in the range 0.01–10 ng/ml. A series of calibration standards was then prepared by spiking blank plasma samples (100 μl) with 100 μl of aliquots of standard solutions to give triptorelin concentrations in plasma of 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 ng/ml. Low, medium and high QC samples were prepared by spiking blank plasma samples with QC solutions (0.03, 0.3 and 8 ng/ml) prepared independently in the same way.

2.3. Sample preparation

All frozen dog plasma samples were thawed at room temperature prior to preparation. After vortexing, 100 μl of IS solution (Octreotide, 5 ng/ml), 100 μl of methanol–water–formic acid (60:40:0.08, v/v/v), and 500 μl of methanol were added to 100 μl of plasma. The mixture was vigorously vortexed for 1 min and centrifuged at 15,000 \( \times \) g for 10 min. The supernatant was...
mixed with 600 µl of water and then transferred to an Oasis HLB SPE column, which had been previously activated by successive applications of 1 ml of methanol and 1 ml of Milli-Q water. The column was then washed with 1 ml of methanol–water (60:40, v/v). The peptide adsorbed on the two monomers (hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene) surface was eluted with 1 ml of methanol (containing 1% formic acid). The eluate was placed in the glass tubes and evaporated under a gentle stream of nitrogen at 40 °C. The residue was reconstituted in 100 µl of methanol–water–formic acid (60:40:0.8, v/v/v) solution. A 10 µl aliquot was transferred into the LC–MS/MS system.

2.4. LC–MS/MS

The HPLC system (Agilent 1290 series) consisted of a binary pump, an autosampler, a column oven maintained at 40 °C and a Venusil MP-C18 (2.1 mm × 50 mm, 3 µm, Agela, Tianjin, PR China). The mobile phase consisted of solvent A (0.02% acetic acid in MilliQ water) and solvent B (methanol) at a flow rate of 0.6 ml/min. The gradient involved: 10% B for 1.2 min; a linear increase to 60% B over 1.1 min; 60% B for 0.4 min, an increase to 98% B in 0.01 min; 98% B for 1.3 min (to wash the column); a decrease to 10% B in 0.01 min; and 10% B for 1.5 min (to equilibrate the column).

Detection was performed on a Qtrap 5500 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) with an ESI source operated in the positive ion mode. Multiple reaction monitoring (MRM) involved triptorelin at m/z 656.5 → 249.1 transition and octreotide at m/z 510.8 → 120.1 transition. Optimized MS conditions were as follows: curtain gas, gas 1 and gas 2 (all nitrogen) 35, 55 and 55 units respectively; ion spray voltage 5500 V; source temperature 575 °C; de-clustering potentials 50 V for triptorelin and 55 V for octreotide; collision energies 42 eV (m/z 656.5 → 249.1) and 90 eV (m/z 656.5 → 110.1) for triptorelin and 60 eV for octreotide.

2.5. Assay validation

Assay validation was performed according to FDA guidelines [10]. The specificity was determined by analyzing blank plasma samples from six different subjects. The linearity was assessed by weighted (1/x^2) least-squares linear regression of the analyte:I.S. peak area ratios for the calibration curves. The precision (represented by the relative standard deviation, RSD) and the accuracy (represented by the relative error, RE) were evaluated based on assay of the LLOQ QC samples in three consecutive days, six replicates for each day. The matrix effects were evaluated by comparing the peak areas of the analyte in post-extraction spiked blank plasma samples with the peak areas of the standard solutions [11]. To evaluate the precision and accuracy of the method with respect to the sample dilution process, QC samples of 40 ng/ml were diluted five-fold before being analyzed in six replicates on the day of method validation. The recovery was determined by comparing the peak areas of the QC samples with the peak areas of the post-extraction spiked blank plasma. The stability of the stock solution at 4 °C for two months was evaluated. The long-term and short-term stability were studied by assaying samples after storage for one month at −35 °C and after storage at room temperature for 2 h, respectively. The stability during three successive freeze–thaw cycles and in processed samples stored at room temperature (25 °C) for 6 h was also evaluated.

2.6. Application of the method to a pharmacokinetic study in beagle dog

The method was applied to determine the plasma concentrations of triptorelin in healthy beagle dogs (males, N = 5) after the intramuscular injection of triptorelin acetate biodegradable microspheres (0.3 mg/kg peptide base). Blood samples (0.5 ml at each time point) were collected from the antebrachium vein prior to dosing and at 0.25, 1, 6, and 24 h, and 2, 3, 4, 6, 9, 11, 14, 16, 19, 23, 26, and 30 d after dosing. The plasma concentration–time profiles for triptorelin in each subject were analyzed by noncompartmental analysis using DAS 2.1.

3. Results and discussion

3.1. Method development

The full-scan product ion spectra of [M+H]^2+ ions of triptorelin and octreotide are shown in Fig. 2. Even though both the m/z 656.5 → 249.1 and m/z 656.5 → 110.1 transitions had the same signal in the extracted ion chromatogram when the collision gas (CAD) was set to 9 units, a better peak shape and a better signal-to-noise ratio were obtained for the m/z 656.5 → 249.1 transition than the m/z 656.5 → 110.1 transition due to the former having a higher selectivity. Thus, the m/z 656.5 → 249.1 transition was selected as the basis for quantification. The m/z 510.8 → 120.1 transition was used to monitor octreotide.

The chromatographic conditions were optimized based on the signal-to-noise and the peak shapes of the analyte and I.S. The Venusil MP-C18 column was chosen because it gave the best resolution and peak shapes. Mobile phase A solutions containing 0.02%, 0.05%, or 1.0% formic acid; 0.02%, 0.05%, or 1.0% acetic acid; or 0.01% or 0.02% trifluoroacetic acid were evaluated. Either methanol or acetonitrile was used as mobile phase B and 0.02% acetic acid aqueous solution–methanol was selected because it provided the highest sensitivity. Using a flow rate of 0.6 ml/min, the retention times for the analyte and I.S. were 2.33 and 2.26 min, respectively.

Solid-phase extraction (SPE) was chosen as the sample preparation method. During the development of our method, we
investigated a variety of SPE cartridges (Oasis MAX cartridge, Oasis MCX cartridge, Oasis HLB cartridge) for plasma pre-treatment, and we optimized the different loading, washing and elution steps. The Oasis HLB cartridge provided a higher and more stable recovery than other SPE cartridges that we used. Compared with methanol, 1% formic acid in methanol could improve the recovery of triptorelin from the 40% to approximately 70% and was therefore was used as the elution solvent.

3.2. Method validation

The assay was found to be free from interference because there were no endogenous peaks in the plasma at the retention times of triptorelin and octreotide. Representative chromatograms of blank plasma, plasma spiked with triptorelin at the LLOQ (0.01 ng/ml) and a study sample taken 6 d after intramuscular injection of triptorelin acetate biodegradable microspheres are shown in Fig. 3. The assay was linear in the range of 0.01–10 ng/ml ($y=0.561x+0.000899$, $r=0.9978$), with intra- and inter-day precisions of $<13.8$% and $<14.8$, respectively, and an accuracy in the range of $-1.05$ to $1.52$% (Table 1). The matrix effects were shown to be minimal based on the ratios of peak areas for triptorelin in the low, medium and high post-extraction spiked blank plasma samples to the peak areas in the corresponding standard solutions; these ratios were $104.7 \pm 9.3\%$, $97.0 \pm 2.8\%$ and $104.9 \pm 4.5\%$, respectively. The precision and accuracy of the dilution process were found to be acceptable, with an RSD $<3.96\%$, an RE $<-1.5\%$. The recoveries of triptorelin from the low, medium and high QC samples were also satisfactory, with concentrations of $70.6 \pm 5.2\%$, $72.4 \pm 1.2\%$ and $64.0 \pm 2.0\%$ of the nominal values, respectively. In terms of stability (Table 2), triptorelin was shown to be stable under all of the conditions examined.

3.3. Pharmacokinetic study

The mean concentration–time profile of triptorelin after the intramuscular injection of triptorelin acetate biodegradable microspheres is shown in Fig. 4. The mean peak in the plasma

Fig. 3. Representative MRM chromatograms of triptorelin and octreotide in beagle dog plasma. (A) Blank plasma; (B) blank plasma spiked with 0.01 ng/ml triptorelin (I) and 5 ng/ml octreotide (II); (C) plasma sample from a beagle dog volunteer 6 d of triptorelin after intramuscular injection triptorelin acetate biodegradable microspheres 0.3 mg/kg (as peptide base).
Table 1
Precision and accuracy for the determination of triptorelin in beagle dog plasma (in three consecutive days, six replicates for each day).

<table>
<thead>
<tr>
<th>Spiked conc. (ng/ml)</th>
<th>Calculated conc. (mean ± SD, ng/ml)</th>
<th>Intra-day RSD (%)</th>
<th>Inter-day RSD (%)</th>
<th>Accuracy RE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0100</td>
<td>0.0101 ± 0.0014</td>
<td>13.8</td>
<td>14.8</td>
<td>1.2</td>
</tr>
<tr>
<td>0.030</td>
<td>0.0301 ± 0.0023</td>
<td>7.09</td>
<td>10.03</td>
<td>0.39</td>
</tr>
<tr>
<td>0.300</td>
<td>0.305 ± 0.019</td>
<td>6.5</td>
<td>1.01</td>
<td>1.52</td>
</tr>
<tr>
<td>8.00</td>
<td>7.92 ± 0.44</td>
<td>3.64</td>
<td>2.98</td>
<td>−1.05</td>
</tr>
</tbody>
</table>

Table 2
Stability of triptorelin under various conditions (data are means ± SD of 3 replicates).

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Initial conc. (ng/ml)</th>
<th>Final conc. (ng/ml)</th>
<th>RE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beagle dog plasma at room temperature for 2 h</td>
<td>0.03</td>
<td>0.032 ± 0.001</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>0.29 ± 0.02</td>
<td>−3.3</td>
</tr>
<tr>
<td></td>
<td>8.00</td>
<td>8.01 ± 0.27</td>
<td>0.1</td>
</tr>
<tr>
<td>Processed samples at room temperature for 6 h</td>
<td>0.03</td>
<td>0.032 ± 0.001</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>0.29 ± 0.01</td>
<td>−2.6</td>
</tr>
<tr>
<td></td>
<td>8.00</td>
<td>7.80 ± 0.42</td>
<td>−2.5</td>
</tr>
<tr>
<td>Beagle dog plasma after three freeze/thaw cycles</td>
<td>0.03</td>
<td>0.031 ± 0.001</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>0.30 ± 0.01</td>
<td>−0.9</td>
</tr>
<tr>
<td></td>
<td>8.00</td>
<td>8.04 ± 0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Beagle dog plasma for 1 month at −35 °C</td>
<td>0.03</td>
<td>0.028 ± 0.001</td>
<td>−6.8</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>0.29 ± 0.02</td>
<td>−4.1</td>
</tr>
<tr>
<td></td>
<td>8.00</td>
<td>7.81 ± 0.36</td>
<td>−2.4</td>
</tr>
<tr>
<td>The stability of the stock solution for 2 months</td>
<td>0.03</td>
<td>0.032 ± 0.002</td>
<td>6.7</td>
</tr>
<tr>
<td>at 4 °C</td>
<td>0.30</td>
<td>0.31 ± 0.02</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>8.00</td>
<td>8.47 ± 0.35</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Fig. 4. Plasma concentration–time profile of intramuscular injection triptorelin acetate biodegradable microspheres 0.3 mg/kg (as peptide base) to healthy beagle dog. Data are mean ± SD for 5 dogs (5 males).

concentration (Cmax) was 33.9 ± 9.5 ng/ml at a time (Tmax) of 1 h after the injection was given. The mean area under the plasma concentration–time curve (AUC0–1) was 20.89 ± 7.44 ng d/ml and the mean elimination half-life (t1/2) was 4.34 ± 1.47 d. From the first day to the third day following administration, the triptorelin plasma concentration continuously decreased from 33.9 ng/ml to 0.487 ng/ml, and then reached a plateau at concentrations of 0.091–0.268 ng/ml.

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