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RESEARCH PAPER

An analytical strategy to characterize the pharmacokinetics and pharmacodynamics of triptorelin in rats based on simultaneous LC–MS/MS analysis of triptorelin and endogenous testosterone in rat plasma

Jiangbin Han · Shu Zhang · Wanhui Liu · Guangyi Leng · Kaoxiang Sun · Youxin Li · Xin Di

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Abstract Triptorelin, a gonadotropin-releasing hormone agonist, has been used in the treatment of hormone-responsive prostate cancer by inducing testosterone suppression. Research on the relationship between the time courses of triptorelin and testosterone is very important, but accurate quantification of triptorelin and testosterone simultaneously in biological specimens is a challenging analytical problem. In the present study, a rapid, sensitive, and selective method for simultaneous determination of triptorelin and testosterone in rat plasma by solid-phase extraction and liquid chromatography–tandem mass spectrometry was developed using a ZORBAX RRHD Eclipse Plus C8 column (2.1×50 mm, 1.8 μm) with a 0.05 % propionic acid/methanol gradient. In view of the polarity difference between the two analytes, two internal standards, i.e., leuprolide and testosterone-$^{13}C_3$, were used for individual quantitation of triptorelin and testosterone. Endogenous testosterone was determined by reference to a calibration curve prepared using testosterone-D_3 as a surrogate analyte. The method exhibits excellent linearity over three orders of magnitude for each analyte. The lower limit of quantification was 0.01 ng/mL for triptorelin and 0.05 ng/mL for testosterone, with consumption of 100 μL of plasma. The method was successfully applied to characterize the pharmacokinetics and pharmacodynamics of slow-release 28-day form triptorelin acetate biodegradable microspheres in rats after intramuscular injections of three consecutive doses of 0.6 mg/kg per 28 days. The results revealed that the pharmacokinetic profile of triptorelin produced an initial flare-up in testosterone levels, rapid castration within 5 days after injection, and long-term castration until the next dose.

Keywords Triptorelin · Testosterone · LC–MS/MS · Pharmacokinetics/pharmacodynamics · Plasma

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Introduction

Prostate cancer is the most commonly diagnosed cancer and has become the second leading cause of cancer-related deaths among men in Western countries. The American Cancer Society estimates that 238,590 new cases of prostate cancer may occur in the United States and that 29,720 American men may die of this disease in 2013 [1]. Based on the fact that prostate cancer cell growth is dependent on the presence of androgens, hormone therapy aimed at blocking androgen secretion/activity to achieve castration (testosterone levels ≤0.5 ng/mL) has played an important role in the treatment of prostate cancer [2].

Triptorelin (Pyr-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2), a synthetic analogue of naturally occurring gonadotropin-releasing hormone (GnRH), is one of the most common hormone therapies used for prostate cancer. As a GnRH agonist, it initially stimulates and then desensitizes pituitary GnRH receptors, resulting in decreased gonadotropin release, decreased testosterone production, decreased prostate growth, and a beneficial effect in prostate cancer [3]. Research on the relationship between the time courses of triptorelin and

testosterone levels will be helpful to better understand drug efficacy and safety. Knowledge of pharmacokinetic (PK) and pharmacodynamic (PD) properties of triptorelin will also provide useful information for the optimization of dosing regimens and drug delivery systems.

Some analytical methods have previously been developed to separately determine triptorelin and testosterone in biological specimens. For example, investigators have employed radioimmunoassay [4, 5] or LC–MS/MS [6] for the determination of triptorelin in human and dog plasma. The analysis of endogenous testosterone in human serum, urine, and saliva has been accomplished using immunoassay [7], gas chromatography–mass spectrometry (GC–MS) [8], or liquid chromatography–tandem mass spectrometry (LC–MS/MS) [7, 9–12]. To date, simultaneous determination of triptorelin and testosterone in biological specimens has not been reported.

It is well known that high-throughput bioanalytical methods are essential to support PK/PD study. LC–MS/MS with its inherent advantages of high speed, sensitivity, and selectivity is well suited for rapid simultaneous determination of triptorelin and testosterone in biological samples. However, the difference in terms of the intrinsic polarity between the two compounds makes it difficult to optimize the LC–MS/MS conditions to achieve adequate retention, good peak shape, and sufficient MS response for both the analytes. The evident dissimilar hydrophobic character also poses a challenge when selecting suitable sample preparation methods in order to extract the two analytes efficiently from biological fluids. In addition, quantitative determination of endogenous testosterone in biological specimens by LC–MS/MS presents a number of complications due to the lack of testosterone-free biological matrix during method validation.

In the present work, a rapid and sensitive method based on solid-phase extraction (SPE) followed by gradient LC–MS/MS analysis using a ZORBAX RRHD Eclipse Plus C8 column was developed for simultaneous determination of triptorelin and testosterone in rat plasma. Quantification of endogenous testosterone was performed using the surrogate-analyte approach that has not been used so far in the published non-derivatization LC–MS/MS methods for the analysis of testosterone in biological specimens. The method was fully validated in terms of selectivity, linearity, precision, accuracy, extraction recovery, and stability, and then applied to the evaluation of the PK/PD study of slow-release 28-day form triptorelin acetate biodegradable microspheres after intramuscular injections of three consecutive doses of 0.6 mg/kg (peptide base).

To the best of our knowledge, this is the first demonstration of simultaneous determination of triptorelin and endogenous testosterone in biological specimens by LC–MS/MS and also the first using analytical strategy for the characterization of the PK and PD of triptorelin based on LC–MS/MS.

Experimental

Materials

Triptorelin acetate (peptide base content 86 %) was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P.R. China). Leuprolide acetate (purity>99 %) was purchased from Shanghai TASH Biotechnology (Shanghai, P.R. China). They were both stored in brown Schering bottles at 4 °C.

Testosterone solution (1.00 mg/mL in 1,2-dimethoxyethane), testosterone-D_3 solution (100 μg/mL in 1,2-dimethoxyethane), and testosterone-$^{13}C_3$ solution (100 μg/mL in 1,2-dimethoxyethane) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemical purity of testosterone, testosterone-D_3, and testosterone-$^{13}C_3$ were 99.6 %, 99.5 %, and 98.7 %, respectively. The isotopic purity of testosterone-D_3 and testosterone-$^{13}C_3$ were 99.8 atom% D (determined by LC–MS/MS) and 99 atom% ^{13}C (provided by the manufacturer).

Methanol (HPLC grade) was purchased from Merck Scientific (New Jersey, USA). Formic acid (HPLC grade) and propionic acid (HPLC grade) were supplied by Fluka (St. Louis, MO, USA). High-purity water (18.2 MΩ) was obtained by passing house-distilled water through a Milli-Q system (Millipore, Molsheim, France). Strata™-X SPE cartridges (30 mg, 1 cm^3) were purchased from Phenomenex (Madrid Ave, Torrance, CA, USA). Centrifuge tubes were supplied by Eppendorf (Hamburg, Germany).

Preparation of calibration standards and quality control samples

A stock solution of triptorelin was prepared at 1 mg/mL in methanol–water–formic acid (60:40:0.08 v/v/v) and then serially diluted to prepare triptorelin working solutions at 0.02, 0.06, 0.2, 0.6, 2.0, 6.0, 20.0, and 60.0 ng/mL. A 100 μg/mL testosterone-D_3 solution was serially diluted with methanol–water–formic acid (60:40:0.08 v/v/v) to prepare testosterone-D_3 working solutions at 0.1, 0.2, 0.4, 1.0, 2.0, 6.0, 20.0, and 60.0 ng/mL. Individual working solutions of triptorelin and testosterone-D_3 were mixed in equal volumes to make eight mixed working solutions for preparation of calibration standards. Another set of working solutions for preparation of quality control (QC) samples was made from separately prepared stock solutions. A mixed internal standard (IS) working solution containing 10.0 ng/mL of leuprolide and 1.0 ng/mL of testosterone-$^{13}C_3$ was prepared in the same way as described above. All the solutions were stored at 4 °C until analysis and brought to room temperature before use.

Calibration standards were freshly prepared by spiking each mixed working solution into blank rat plasma to yield final plasma concentrations ranging from 0.01 to 30.0 ng/mL

for triptorelin and 0.05–30.0 ng/mL for testosterone-D_3. QC samples were prepared by the same procedure as above at plasma concentrations of 0.02, 1.0, and 24.0 ng/mL for triptorelin and 0.1, 1.0, and 24.0 ng/mL for testosterone-D_3.

Sample preparation

All frozen rat plasma samples were thawed at room temperature prior to preparation. After vortex mixing, an aliquot of 100 μL of plasma sample was pipetted into a 1.5-mL Eppendorf tube. Then 100 μL of IS working solution, 100 μL of methanol–water–formic acid (60:40:0.08 v/v/v), and 500 μL of methanol were added. The mixtures were briefly vortex-mixed and centrifuged at 13,500×*g* for 5 min at 4 °C. The supernatants were mixed with 500 μL of water and then submitted to a Strata™-X SPE cartridge as follows: (a) the cartridge was preconditioned with 1 mL of methanol followed by 1 mL of water, (b) the sample was loaded on the cartridge and aspirated in a dropwise flow under a light vacuum for 2 min, (c) the cartridge was washed with 1 mL of water followed by 1 mL of methanol–water (60:40, v/v), and (d) the analytes were eluted by rinsing the cartridge with 1 mL of methanol containing 0.01 % formic acid. The eluates were transferred to another tube and evaporated to dryness under a gentle stream of nitrogen in TurboVap LV evaporator at 50 °C. The residue was reconstituted in 100 μL of methanol–water–formic acid (60:40:0.08, v/v/v). An aliquot of 10 μL of the resulting solution was injected into the LC–MS/MS system.

LC–MS/MS conditions

LC–MS/MS analyses were performed using an Agilent 1290 series HPLC system (Agilent Technology, Waldbronn, Germany) coupled to a QTRAP 5500 mass spectrometer equipped with a Turbo V™ source (Applied Biosystems/MDS SCIEX, Toronto, Canada). Chromatographic separation was conducted on a ZORBAX RRHD Eclipse Plus C8 (2.1× 50 mm, 1.8 μm) column with a gradient mobile phase consisting of 0.05 % propionic acid (A) and methanol (B). The elution program was performed as follows: 0–1.2 min, 20 % B; 1.2–2.0 min, 20–60 % B; 2.0–2.6 min, 60 % B; 2.6–4.0 min, 60–80 % B; 4.0–4.2 min, 80 % B; 4.2–4.5 min, 80–98 % B; 4.5–4.7 min, 98 % B; 4.7–5.0 min; 98–20 % B; 5.0–6.0 min, 20 % B. The column temperature was maintained at 40 °C and the flow rate was set at 0.4 mL/min. The eluates from the analytical column was directed to waste for the first 1.8 min via a divert valve and then introduced directly into the ESI source which was operated in positive ionization mode. Nitrogen was used as nebulization and desolvation gas. Curtain gas, gas 1, and gas 2 flows were 40, 50, and 55 arbitrary units, respectively. The ionspray voltage was 5,500 V and source temperature was kept at 600 °C. The multiple reaction monitoring (MRM) transitions monitored for the analytes and IS are listed in Table 1. Data acquisition and processing were performed using Analyst 1.5.2 software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

Method validation

Method validation was performed according to the European Medicines Agency's guideline on bioanalytical methods validation [13]. The following characteristics of the method were assessed during method validation: selectivity, lower limit of quantification (LLOQ), linearity, carryover, precision, accuracy, matrix effects, extraction recovery, and stability. Selectivity was evaluated by the analysis of blank rat plasma from six different sources. LLOQ was defined as the lowest concentration that could be quantified with acceptable precision and accuracy. Calibration curves were plotted by the peak-area ratio (y) of the analyte to IS versus the nominal concentration (x) and then fitted using linear regression ($y = ax+b$) weighted by a factor of $1/x^2$. Carryover was evaluated by injecting an extracted blank sample after the injection of an extracted upper limit of quantification (ULOQ) sample.

Precision and accuracy were evaluated from six replicates of LLOQ and QC samples at four concentrations (LLOQ, low, medium, and high QC) on three different days (n =18). Intra- and inter-day precisions were calculated in terms of the relative standard deviation (% RSD) using one-way analysis of variance. Accuracy was expressed as a percentage of the nominal concentration and was calculated according to the formula [(mean measured concentration/nominal concentration)×100].

Matrix effect was investigated by six replicate analyses of QC samples prepared from six lots of rat plasma. The matrix factor (MF) of each analyte or IS was calculated in each lot of matrix by calculating the ratio of the peak area from post-extraction spiked samples to the peak area from pure solution. The IS normalized MF was calculated by dividing the MF of the analyte by the MF of the IS. Extraction recovery was determined by comparing the peak areas of the analytes and

Table 1 MRM transitions, declustering potentials (DP), and collision energies (CE)

Compound	MRM transitions	DP (V)	CE (V)
Triptorelin	656.5≥249.1	50	42
	656.5>110.1	50	90
Leuprolide (IS)	605.5>249.1	25	38
Testosterone	289.2≥97.0	87	26
	289.2>109.1	87	30
Testosterone-D_3	292.2≥97.2	87	26
	292.2>109.1	87	30
Testosterone-$^{13}C_3$ (IS)	292.2>100.2	87	26

internal standards obtained from QC samples with those from post-extraction spiked samples.

The stability of the analytes in rat blood was investigated at room temperature and in ice with or without aprotinin. The stability of analytes in rat plasma was investigated by analyzing three replicates of low, medium, and high QC samples under a variety of storage and processing conditions: (a) bench-top storage (2 h at room temperature), (b) freezer storage (3 months at −70 °C), (c) three freeze–thaw cycles, and (d) post-preparation stability (24 h at room temperature). In order to mimic the actual situation in study samples, stability experiments were also performed with unspiked samples.

Application to PK/PD study

The method was applied to determine the plasma concentrations of triptorelin and testosterone in eight male Sprague Dawley rats after intramuscular injections of Diphereline SR 28-day form biodegradable microspheres (Beaufour Ibsen Pharma, France). The rats were given 0.6 mg/kg (peptide base) in three cycles of 28 days each. Blood samples (0.5 mL each) were taken from the oculi chorioideae vein prior to dosing and at 0.25, 1, and 6 h after injection on day 1 and on days 2, 3, 4, 7, 14, 21, and 28 of each cycle. The whole blood was collected in chilled polypropylene tubes containing heparin and aprotinin [1 TIU (trypsin inhibitory unit) per milliliter of blood] and immediately centrifuged at 10,000×*g* for 5 min at 4 °C. The plasma obtained were stored at −70 °C until analysis. Pharmacokinetic parameters of triptorelin were calculated by non-compartmental analysis using Phoenix WinNonlin 6.3 (Pharsight, Mountain View, CA, USA).

Results and discussion

Optimization of LC–MS/MS conditions

Testosterone responded well in positive ion mode using either ESI [14–16] or APCI source [7]. Triptorelin gave strong response only in positive ESI mode [6]. Therefore, positive ESI mode was used to monitor triptorelin and testosterone in the present study. The compound-dependent parameters such as declustering potential and collision energy, etc. were optimized by infusing a standard solution of each analyte using a syringe pump. The full-scan product ion spectra of the $[M+2H]^{2+}$ ions of triptorelin and leuprolide, and those of the $[M+H]^{+}$ ions of testosterone, testosterone-D_3, and testosterone-$^{13}C_3$ are shown in Fig. 1. Two MRM transitions were monitored for each analyte, the first transition for quantitation and the second for confirmation (see Table 1).

In optimizing chromatographic conditions, a significant challenge encountered was selecting a suitable column to achieve adequate retention and good peak shape for the two analytes exhibiting great difference in the polarity. During the early phase of method development, three C18 columns, i.e., ACQUITY UPLC BEH130 (2.1×50 mm, 1.7 μm; Waters), ZORBAX RRHD Eclipse Plus (2.1×50 mm, 1.8 μm; Agilent), and SYNERGI Hydro-RP (2.0×50 mm, 4 μm; Phenomenex) columns were tested. Unfortunately, the late eluting testosterone suffered from serious peak tailing problem on these columns. The use of fast gradient elution may lead to some slight improvement in peak shape, but adequate retention of the first eluting triptorelin was not achieved. Next, ZORBAX RRHD Eclipse Plus C8 (2.1×50 mm, 1.8 μm; Agilent) and Eclipse XDB-CN (2.1×100 mm, 3.5 μm; Agilent) columns were tried. The first column proved to be a suitable stationary phase resulting in the best possible peak shape and retention for both triptorelin and testosterone. Using ZORBAX RRHD Eclipse Plus C8 column, different mobile phases consisting of methanol/water or acetonitrile/water mixture with the addition a small amount of formic acid or propionic acid were evaluated. The optimal result, selected as a compromise between the peak symmetry and MS detection sensitivity, was achieved when using methanol/0.05 % propionic acid gradient. Under the optimal chromatographic condition, the retention times of triptorelin and testosterone were 2.60 and 3.74 min, respectively.

Development of sample pretreatment

The simplest way to extract testosterone from the matrix is liquid–liquid extraction using organic solvents such as *tert*-butyl methyl ether or diethyl ether [17, 18]. For the extraction of triptorelin from the matrix, SPE appears to be a more suitable approach [4, 6]. In the present study, we evaluated the efficiency for SPE extraction of triptorelin and testosterone using Bond Elut plexa, Strata™-X and Oasis HLB cartridges. It was found that Strata™-X cartridge, which is capable of exhibiting hydrophobic, p–p, dipolar, and hydrogen bonding retention mechanisms, provided higher and more stable recoveries for the two analytes than other cartridges. Using Strata™-X cartridge, different loading, washing, and elution steps were further investigated and the optimal SPE procedures were obtained as described in the Experimental section.

Method validation

Selectivity

Representative chromatograms of blank plasma, blank plasma spiked with analytes at the LLOQ and internal standards, and real plasma sample taken 14 days after intramuscular injection

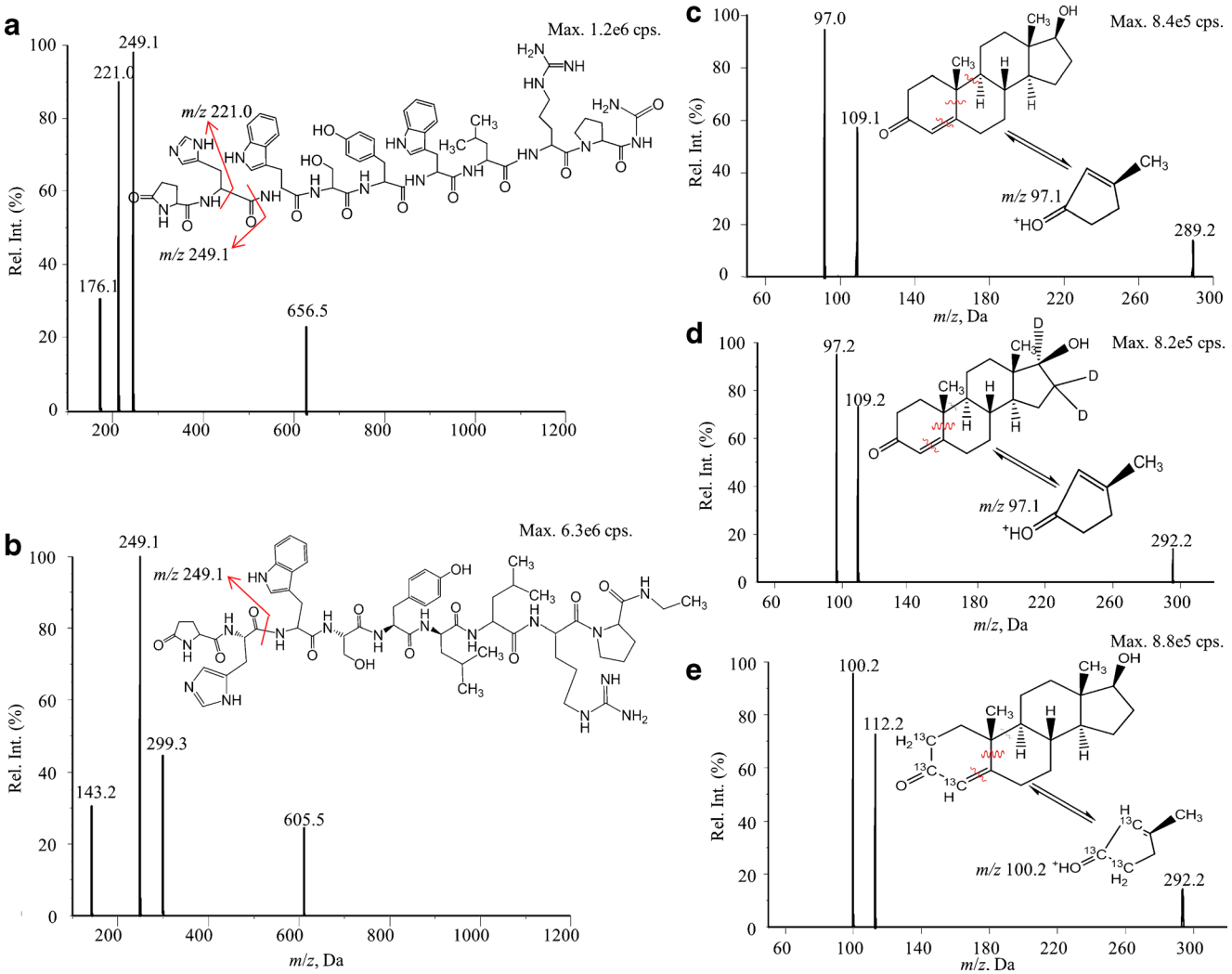

Fig. 1 Full-scan product ion mass spectra of $[M+2H]^{2+}$ for **a** triptorelin and **b** leuprolide, $[M+H]^{+}$ for **c** testosterone, **d** testosterone-D_3, and **e** testosterone-$^{13}C_3$

of Diphereline are shown in Fig. 2. No endogenous interfering peaks were found at the retention times of either the analytes or the internal standards. The crosstalk between the MS/MS channels used for monitoring triptorelin and leuprolide and the crosstalk among the channels of testosterone, testosterone-D_3, and testosterone-$^{13}C_3$ were examined by injecting their individual solutions at high concentrations. No cross-talk effect was found.

LLOQ and linearity

The LLOQ was 0.01 ng/mL for triptorelin and 0.05 ng/mL for testosterone, with acceptable precision (RSD≤20 %) and accuracy (80–120 % of the nominal value). The calibration curves for the two analytes showed good linearity up to 30 ng/mL. All correlation coefficients (r^2) were greater than 0.999 and the back-calculated concentrations of the calibration standards were within ±15 % of their nominal values.

Carryover

Neither testosterone-D_3 nor testosterone-$^{13}C_3$ peaks was observed in an extracted blank sample after the injection of an extracted ULOQ sample. However, a significant carryover effect was found for triptorelin and leuprolide, both of which were easily adsorbed on the outer surface of autosampler needle. When a flush port needle wash program was used, the carry over responses in blank were 16.0 % and 0.267 % of the response of LLOQ sample for triptorelin and leuprolide, respectively. Since the responses in blank were less than 20 % of the response detected in a LLOQ sample, the carryover effect was considered negligible.

Precision and accuracy

The precision and accuracy at LLOQ, low, medium, and high QC levels are summarized in Table 2. The intra- and inter-day precisions ranged from 1.9 to 10.3 %, and the accuracy was

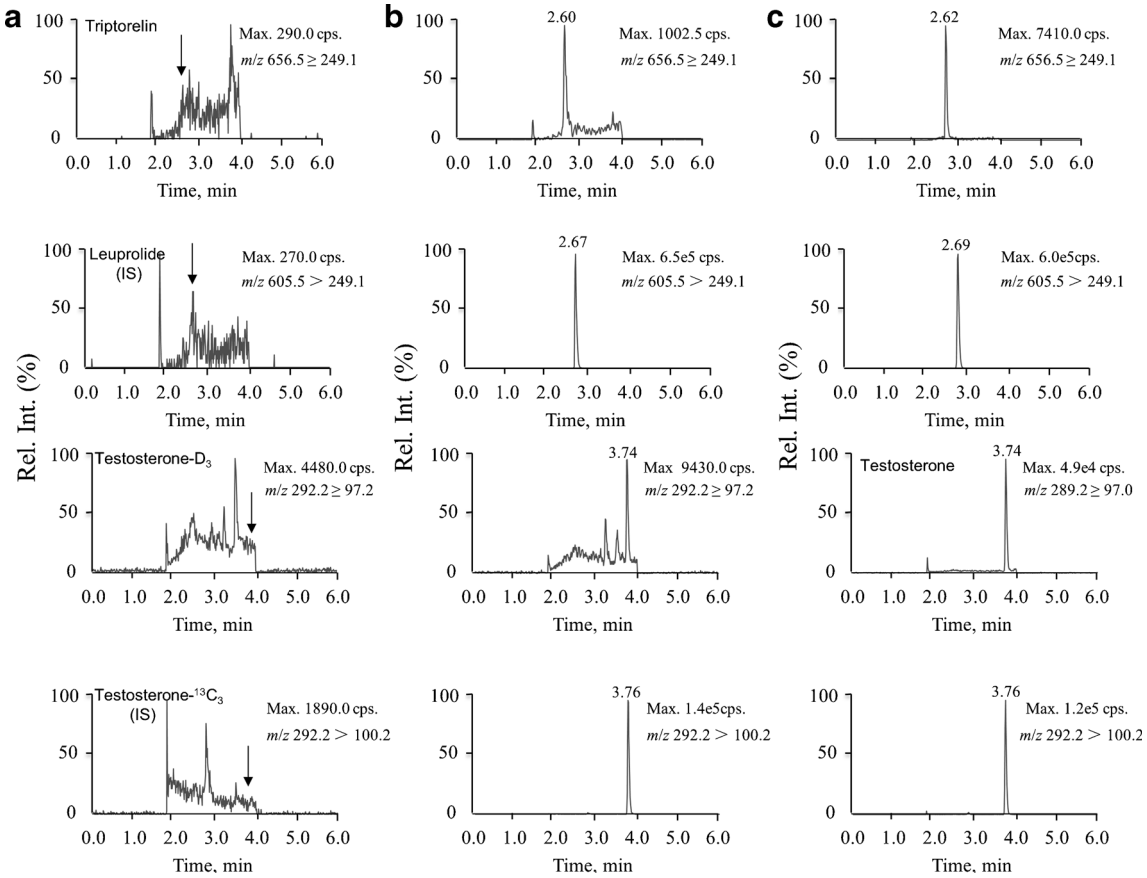

Fig. 2 Representative MRM chromatograms of analytes in rat plasma. **a** Blank plasma; **b** blank plasma spiked with analytes (LLOQ) and their corresponding internal standards; **c** real plasma sample taken 14 days after intramuscular injection of Diphereline

between 91.7 and 108.2 %. Assessment of precision and accuracy was also performed by analyzing six replicates of unspiked and spiked samples at concentrations of 1.0 and 20.0 ng/mL of testosterone against a calibration curve of testosterone-D_3. The results are listed in Table 3. At all levels the accuracy was within 85–115 % with RSD less than 15 %, indicating that the method is reliable and reproducible for the determination of triptorelin and testosterone in rat plasma.

Table 2 Precision and accuracy for the determination of triptorelin and testosterone-D_3 in rat plasma

Concentration	Triptorelin			Testosterone-D_3		
	Intra-day RSD (%)	Inter-day RSD (%)	Accuracy (%)	Intra-day RSD (%)	Inter-day RSD (%)	Accuracy (%)
LLOQ	6.5	10.3	108.2	4.9	8.2	97.9
	4.6		99.7	2.7		91.7
	10.2		102.6	10.2		96.2
Low	5.8	7.2	105.1	2.5	8.5	100.9
	6.9		99.3	5.4		95.4
	9.0		103.7	3.5		101.8
Medium	3.6	4.6	98.0	2.9	2.6	101.3
	4.8		97.3	2.1		102.2
	6.0		100.8	3.0		100.1
High	2.7	4.9	99.5	2.1	4.8	96.9
	1.9		101.3	2.4		100.1
	6.0		103.5	2.0		96.7

Table 3 Precision and accuracy for the determination of testosterone in rat plasma

Plasma sample	Concentration (ng/mL)		Precision (%)	Accuracy (%)
	Added	Found		
Unspiked	–	4.6	2.1	–
Spiked	1.0	5.6	7.7	104.8
	20.0	24.9	1.5	101.6

Extraction recovery and matrix effect

The extraction recoveries of triptorelin and testosterone-D_3 from the low, medium, and high QC samples ranged from 73.2 to 88.3 % with a maximum RSD of 5.4 %. The extraction recoveries of internal standards were 65.9±3.2 % for leuprolide and 88.6±3.8 % for testosterone-$^{13}C_3$. The results revealed that the SPE approach employed in the present work gave reproducible recoveries for the two analytes and internal standards. The IS normalized MF for both analytes ranged from 95.1 to 102.4 % with RSD less than 15 %, indicating that the internal standards can correct for matrix effects. The extraction recovery and matrix effect results are summarized in Table 4.

Stability

The stability results of triptorelin and testosterone in rat blood at room temperature and in ice with or without adding aprotinin are summarized in Table 5. Triptorelin was found to be unstable in blood at room temperature due to fast enzymatic degradation. By adding aprotinin (1 TIU/mL) into the blood collection tube in ice, triptorelin was found to be stable in blood for 1 h. Testosterone was stable in blood for more than 2 h under the condition mentioned above. Triptorelin and testosterone-D_3 were stable in rat plasma for at least 2 h at room temperature, for at least 3 months at −70 °C, and after three freeze–thaw cycles. They were also stable in the reconstituted solutions for at least 24 h at room temperature.

Table 4 Recovery and matrix effect for the determination of triptorelin and testosterone-D_3 in rat plasma

	Compound	Low	Medium	High
Recovery (%)	Triptorelin	73.2±4.0	75.7±3.8	73.5±2.9
	Testosterone-D_3	86.5±4.1	88.3±3.0	87.2±2.8
Matrix effect (%)	Triptorelin	102.1±5.2	99.5±2.0	102.4±3.1
	Testosterone-D_3	101.9±4.2	95.1±2.7	97.2±1.3

Table 5 Stability of triptorelin and testosterone in rat blood

Conditions	Time point (h)	% Deviation from zero time		
		Triptorelin		Testosterone
		0.2 ng/mL	20 ng/mL	–
Room temperature	0.50	−27.1	−29.7	−1.7
	1.00	−42.5	−40.6	−3.3
	2.00	−54.0	−53.3	2.6
Without aprotinin in ice	0.50	−13.7	−10.5	5.4
	1.00	−18.1	−16.2	−7.0
	2.00	−27.2	−21.5	8.4
With aprotinin in ice	0.50	−2.1	−1.5	9.2
	1.00	−6.9	−5.2	2.0
	2.00	−19.2	−18.9	8.8

Method comparison

The developed method was compared with the previously published LC–MS/MS methods for the determination of triptorelin and testosterone in plasma or serum separately. The present method demonstrated the same LLOQ of triptorelin (0.01 ng/mL) as the published method [6], but offered wider linear concentration range (0.01–30.0 ng/mL vs. 0.01−10.0 ng/mL). Due to the lack of testosterone-free matrix, LC–MS/MS analysis of testosterone in plasma or serum is a challenging task. In most of previously published LC–MS/MS methods for testosterone analysis [7, 10], a solvent for dissolving testosterone is frequently used as surrogate matrix, which may not simulate the authentic matrix for accurate LLOQ determination. Recently, Star-Weinstock et al. reported a novel derivatization method for the analysis of testosterone in human serum by LC–MS/MS using the surrogate–analyte method [12]. Despite its high sensitivity, this derivatization method is not suitable for the determination of triptorelin without ketone functionality. We believe that our method with its attractive combination of short run time of 6.0 min and accurate determination of 0.05 ng/mL of testosterone is well suited for simultaneous determination of triptorelin and endogenous testosterone in rat plasma for PK/PD study of triptorelin.

PK/PD study

The validated method was applied to characterize the PK and PD properties of triptorelin in rats after intramuscular injections of Diphereline SR 28-day form (acetate formulation). The main pharmacokinetic parameters for triptorelin are summarized in Table 6. The values for C_{max} on days 0 to 28, 28 to 56, and 56 to 84 were 19.9±4.5, 18.5±4.2, and 25.2±1.3 ng/mL, respectively. C_{max} at days 0 to 28 was achieved at 0.50±

Table 6 Pharmacokinetic parameters of triptorelin after intramuscular injections of three consecutive doses of Diphereline and their effects on testosterone

Analytes	Parameters	Days (0–28)	Days (28–56)	Days (56–84)
Triptorelin	C_{max} (ng/mL)	19.9±4.5	18.5±4.2	25.2±1.3
	T_{max} (h)	0.50±0.00	0.50±0.00	0.60±0.22
	AUC_{0-t} (ng h/mL)	421.8±147.5	505.6 ±106.4	519.2±65.1
Testosterone	C_{max} (ng/mL)	22.8±3.5	0.83 ±0.58	1.27±0.98
	T_{max} (h)	1.00±0.00	6.00±0.00	6.00±0.00

0.00 h and reached at 0.50±0.00 and 0.60±0.22 h after the injections on days 28 and 56, respectively. The values for AUC_{0-t} were 421.8±147.5, 505.6±106.4, and 519.2±65.1 ng h/mL for month 1 (days 0–28), month 2 (days 28–56), and month 3 (days 56–84), respectively.

The relationship between the time courses of triptorelin and testosterone is shown in Fig. 3. The results showed that triptorelin plasma levels increased rapidly and reached a peak within 0.50–1.00 h after injection, then decreased to 1.25±0.44 ng/mL at 6.00 h and a secondary flare-up occurred on day 1 or day 2, after which the triptorelin concentration decreased progressively during the first 4 days and then remained at a plateau until day 28. After a single injection, the mean plasma testosterone level increased initially (flare-up), and the C_{max} was 22.8±3.5 ng/mL at 1.00 h. This initial increase in testosterone levels was followed by a rapid and sustained decrease in testosterone levels due to pituitary desensitization and the castration level was reached within 5 days and maintained below this level until day 28. There was a transient escape of testosterone above castration levels on day 28, day 56 after the second and the third injection; the C_{max} were 0.83±0.58 ng/mL and 1.27±0.98 ng/mL, respectively. The surge in testosterone levels after the first injection was several folds higher than the small spike after the second and the third injection. When the treatment was interrupted, the level of testosterone went back to normal again. The results indicated that the PK/PD relationship of triptorelin was non-linear and time-dependent.

Conclusion

A rapid, sensitive, and selective LC–MS/MS method for simultaneous determination of triptorelin and endogenous testosterone in rat plasma has been developed and validated for the first time. Special emphasis was focused on the optimization of LC–MS/MS conditions and sample preparation. The use of testosterone-D_3 as a surrogate analyte made it possible to accurately quantify the endogenous testosterone. The LLOQ was 0.01 ng/mL for triptorelin and 0.05 ng/mL for testosterone. The developed method has been successfully applied to the PK/PD characterization of slow release 28-day form triptorelin acetate biodegradable microspheres in rats. The analytical methodology described in the present work

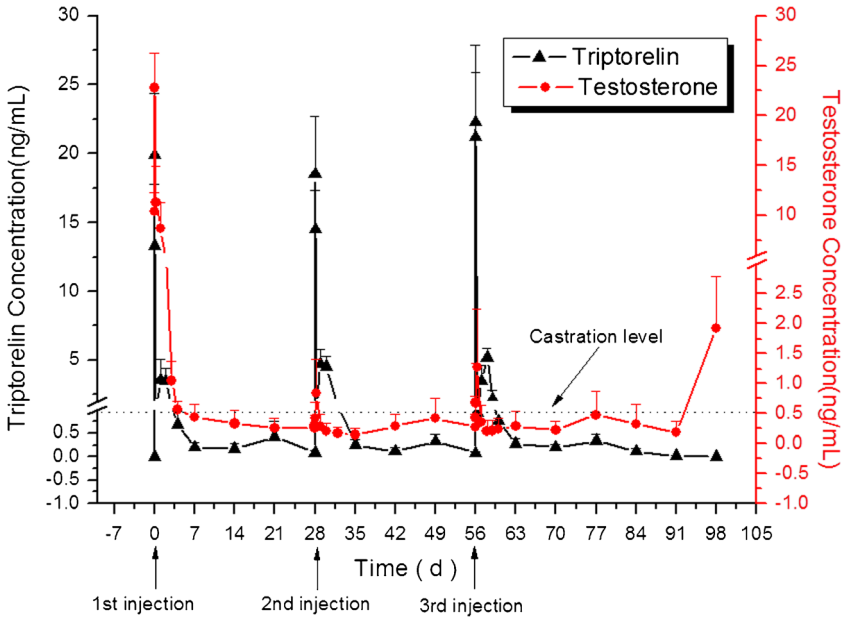

Fig. 3 Mean plasma concentration–time profiles for triptorelin and testosterone after intramuscular injections of three consecutive doses of 0.6 mg/kg of Diphereline (peptide base) per 28 days to rats

should be useful in the future work aiming to establish a model relating the PK/PD characteristics of triptorelin between animals and humans, and will be helpful in developing optimal delivery system of triptorelin.

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