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Determination of Leuprolide in Beagle Dogs' Serum by High Performance Liquid Chromatography-Mass Spectrometry and Study on Bioequivalence

D.M. XIONG, S.L. WEN, H. CHEN, X. BAO* and L.M. YE

West China School of Pharmacy, Sichuan University, Chengdu 610041, P.R. China

*Corresponding author: Fax: +86 28 85502305; Tel: +86 13980986791; E-mail: yeliminglaoshi@126.com

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An HPLC-MS/MS method was used to calculate the pharmacokinetic parameters and to evaluate the bioequivalence *versus* equal dose of test or reference leuprolide microspheres in male healthy beagle dogs. The MS-MS detection was made by monitoring 605.5→221.3 (*m/z*) for leuprolide and 584.4→221.3 (*m/z*) for the I.S. The dynamic range was from 0.103-103 ng mL^{-1}. The recovery rate was 78.75-90.77 % and matrix effect was -5.25-10.3 %. The intra- and inter-assay precisions were with relative standard deviations (RSDs) of ≦ 5.19 % (n = 6) and ≦ 6.48 % (n = 6), respectively. Evaluation of bioequivalence was made by analysis of variance, Double-sided *t*-test and (1-2α) confidence interval method. The results show the test agents, the $AUC_{0\to t}$ leuprolide 90 % confidence interval for the reference preparation of the corresponding parameters of 99.6-102.5 %, the $AUC_{0\to\infty}$ leuprolide 90 % confidence interval for the reference preparation of the corresponding parameters of 90.2-103.9 %, C_{max} 90 % confidence interval for the reference preparation of the corresponding parameters of 91.0-129.8 %.

Keywords: Leuprolide acetate, HPLC-MS/MS, Determination, Bioequivalence.

INTRODUCTION

Leuprolide acetate (LA) is a synthetic nonapeptide analog of naturally occurring gonadotropin-releasing hormone which possesses greater potency than the natural hormone. Its chemical name is 5-oxo-l-prolyl-l-histidyl-l-tryptophyl-l-seryl-l-tyrosyl-d-leucyl-l-leucyl-l-arginyl-N-ethyl-l-prolinamide acetate, the molecular is 1246.46, dissolving in water, ethanol and propylene glycol, pKa 6.9. Leuprolide acetate desensitizes and down regulates pituitary luteinizing hormone-releasing hormone (LHRH) receptors, thus reducing luteinizing hormone (LH) synthesis and release[1]. Long-term taking leads to the suppression of gonadotropin and gonadal steroid. Currently, leuprolide acetate has been used for the treatment of a large number of diseases related with the regulation of sexual hormones, like masculine and feminine infertility, uterine myomas and prostatic and mammalian tumors[2].

Leuprolide acetate has no oral bioavailability and relatively short half-life which reduces testosterone level only when administered on a continuous basis. Therefore developing a sustained-releasing formulation is becoming the research hotspot. Biodegradable poly (lactic-co-glycolic acid) (PLGA) microspheres have been widely used for long-term controlled delivery of various peptides and proteins[3-5]. Recently, sustained depot formulations of some of luteinizing hormone-releasing hormone (LHRH) and somatostatin analogues have become commercialized and several additional products are under clinical investigation.

We are aimed at developing a specific and sensitive analytical method for the determination of drugs in biofluides. A sensitive and specific high performance liquid chromatography-mass spectrometry (HPLC-MS/MS) method has been developed and validated to determine leuprolide concentrations in beagle dogs' serum and was used in the bioequivalence study of leuprolide microspheres. For its clinical research and application provides a reference.

EXPERIMENTAL

Leuprolide acetate (chemical purity 92.0 %, peptide content 87.2 %) was kindly provided by European Directorate for the Quality Control of Medicines (EDQM). Alarelin acetate (internal standard, peptide content 86.95%) was obtained from Shanghai taishi Biological Technology Co., Ltd. (TASH). HPLC-grade methanol and propionic acid were purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile from Fisher (USA), HPLC-grade formic acid from TEDIA (USA), Distilled water was purified "in-house" using a milli-Q20 system Millipore (MA, USA).An Oasis® CEC18 cartridge (200MG, 3ML, 50PKG) was purchased from Waters Corp.

(UCT, USA). Test formulation: Test leuprolide microspheres was kindly supplied by Chengdu, a flat Medical Science and Technology Development Co., Ltd, reference leuprolide microspheres was from Japan's Takeda Pharmaceutical Industries, Ltd. Specification 3.75 mg. Six healthy beagle dogs, ♂, weight of 9-11 Kg, were from Chengdu Dashuo Bio-Technology Co., Ltd. The concentration of leuprolide was detected using Agilent 1100 HPLC system (Agilent Technologies Inc., USA) consisting of MS/MS detector (API3000, AB, USA). Chromatographic separation was achieved on an Ultimate XB-C_{18} column (2.1 mm × 50 mm, 5 μm). The column was maintained at 25 °C and eluted with a linear gradient of 0-100 % B, where A = ultrapure water with 0.1 % formic acid and B = acetonitrile with 0.1 % formic acid. The duration of the gradient program for beagle dogs' serum sample was 0-1 min, 5-20 % B; 1-6 min, 20-100 % B; 6-7 min, keeping 100 % B; 7-10 min, 100-95 % B; 10-16 min, maintaining 95 % B, at a ow rate of 0.20 mL min^{-1}. For analysis, 10 μL aliquots of sample were loaded. The column elution was analyzed by MS/MS spectrometry. The mass spectrometer was operated in the positive ion mode using an electrospray ionization (ESI) source. The tuning parameters were optimized for leuprolide by infusing a solution containing 10.36 μg mL^{-1} of the analyte and IS was 8.41 μg mL^{-1}, respectively, at a ow-rate of 10 μL min^{-1} into the mass detection. A high electrospray ionization voltage of 4.5 kV was applied to the sprayer. The turbo gas temperature was 500 °C. The settings of nebulizer gas, curtain gas and collision gas flows were 9, 9 and 10, respectively. All the gas used in this experiment was high purity nitrogen. Collision energy was set at 40 eV for leuprolide acetate, 35 eV for IS, respectively. The detection was monitored by the most intensive precursor →fragment transitions at *m/z* 605.5→221.3 for leuprolide and at *m/z* 584.4→221.3 for alarelin (IS). The dwell time was 200 ms for both the analyte and the I.S. In this assay, the mass spectrometer was operated at low mass resolution (peak width at half-height set at 0.7Da) for Q1 and unit mass resolution for Q3. For each injection, the total data acquisition time was 16 min.

Sample preparation: Frozen serum samples from the beagle dogs were thawed to room temperature prior to preparation. After vortexing briefly, a 40 μL aliquot of the IS solution (alarelin, 104.5 ng mL^{-1}), 40 μL of methanol-water (50:50, v/v) were added to 200 μL of serum sample, vortexing 0.5 min and then added 200 μL methanol. The mixture was vigorously vortexed for 1min and stand for 5 min, then centrifuged at 12,000 × g for 10 min. The supernatant was mixed with 700 μL water and then transferred to Oasis→ CEC18 solid-phase extraction tubes that had been pre-treated sequentially with 2 × 1 mL of methanol and 2 × 1 mL of water. After loading the serum sample, the cartridge was washed with 2 × 1 mL of water, then leuprolide and IS were eluted with 2 × 1 mL of methanol containing 1 % formic acid. The elute was evaporated to dryness at 40 °C under a gentle stream of nitrogen and the residue was reconstituted by addition of 100 μL of acetonitrile-water-propionic acid (15:85:0.05, v/v/v). The supernatant was transferred to an autosampler vial for HPLC insert and the autosampler vials were placed in the autosampler for analysis.

Method validation: Validation of the optimized HPLC-MS/MS method was done with respect to following parameters.

The selectivity of the method was evaluated by analyzing six test beagle dogs' blank serum samples (serum samples prepared as 2.6. Sample preparation except 40 μL of methanol-water (50:50, v/v) instead of 40 μL aliquot of the IS solution and six spiked serum samples at LLOQ level from six different sources and a test beagle serum sample 2 h after i.h. of 3.75 mg leuprolide acetate (All the serum samples prepared as 2.6. Sample preparation). Peak areas of endogenous compounds coeluting with the analytes should be less than 20 % of the peak area of the LLOQ standard according to international guidelines[6] .

The linearity, precision and accuracy of the method were evaluated as the pharmacopoeia rules. The standard curves were calculated by a weighted (1/x) least squares method through the measurement of the peak-area ratio of the analyte to IS. The acceptance criterion for each back-calculated standard concentration was 15 % deviation from the nominal value[6]. The lower limit of quantization (LLOQ), taken as the lowest concentration on the calibration curve that could be measured with acceptable accuracy and precision, was determined in six replicates on 3 consecutive validation days. The precision should be equal or less than 20 % and accuracy between 80 and 120 % of nominal concentrations for both within and between-assay. The intra- and inter-day precisions were required to be below 15 %, accuracy were required to be between 85 and 115 % of nominal concentrations for both within and between-assay[6].

The matrix effect was investigated by measuring the matrix factor, as defined by the ratio [ME % = (analyte peak area in presence of serum matrix from six different sources-analyte peak area in absence of serum matrix)/(analyte peak area in absence of serum matrix)] and was expressed as per cent response relative to the neat solution. In our experiment, the matrix effect was determined at three concentration levels (0.2051, 5.128 and 82.05 ng mL^{-1}). The inter-subject variability of matrix effect at each concentration level should be less than 15 %[7].

The recovery of leuprolide was estimated at three concentration levels (0.2051, 5.128 and 82.05 ng mL^{-1}) by comparing the peak-area ratios of the analyte to IS. The extraction recovery of the IS was determined by using the QC samples at medium concentration as a reference.

The stability of leuprolide in beagle dogs' serum was done at three different concentration levels of 0.2051, 5.128 and 82.05 ng mL^{-1}, which were exposed to different conditions (time and temperature). The analytes are considered to be stable in serum when the rate of changing less than 15 %.

Application to the bioequivalence study of leuprolide microspheres: The LC-MS/MS method described above was applied to determine the 6 healthy male beagle dogs' serum concentrations of leuprolide from different time point after dosing tested and reference prepared leuprolide microspheres. Six beagle dogs were randomly divided into two groups: the test and the reference. They were fasted for 12 h before the test, without drinking within 2 h and without food within 4 h

after injecting. In the duration give them the same food at same time. Each of the test beagle dogs was injected a single subcutaneous dose of 3.75 mg test leuprolide acetate and each of the reference beagle dogs was injected reference leuprolide acetate at the same dose. 5 mL venous blood samples were collected into tubes before and 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 24, 48, 72, 168, 336, 504, 672, 840, 1008 and 1176 h after dosing. Samples were standing for 1 h at room temperature and then centrifuged at 3000 rpm for 15 min to separate the serum fractions. The collected serum samples were stored at "80 °C until analysis. The pharmacokinetic parameters of leuprolide were calculated using DAS2.0. AUC0→t, AUC0→∞ and Cmax analysis of variance, Double-sided t test and (1-2α) confidence interval method were used to evaluate bioequivalence.

RESULTS AND DISCUSSION

Optimization of the sample preparation and chromatographic condition: Although LLE is a traditional method of sample pre-treatment, leuprolide is a strongly hydrophilic and basic compound, the solubility of it in an organic solvent is less than in the aqueous phase. LLE was not considered. Peptide, a simple pretreatment technique, was used by Sofianos *et al.*[8] to separate leuprolide from mouse plasma samples. But at isocratic conditions there was a strong ion suppression from the endogenous substances in serum samples. The use of solid-phase extraction (SPE) cartridges has been widely chosen for the separation of peptides from biological matrixes[9-11]. Before the use of SPE, we use peptide first. During our method development, we investigated the precipitated protein's effect of acetonitrile and methanol, because of low extraction recovery of acetonitrile and big difference extraction recovery for leuprolide acetate and IS, last we selected methanol to precipitate protein.

During our method development, we investigated the influence of different percentage of methanol in elution solvent for extraction recovery and matrix effect. The content of methanol in the elution solvent varied from 20-100 % (v/v). A complex and interesting extraction recovery phenomenon was noticed for both the analyte and the I.S. We can find when 1 % formic acid in 20-40 % methanol-water(v/v) was used as elution solvent, the recovery of leuprolide and IS could be increased. When the percentage of methanol changed from 40 to 100 %, the response of leuprolide and IS hardly increased. (Fig. 1). Considering the elute easily evaporated to dryness and shut down the time of sample pretreatment, we established 1 % formic acid in methanol (2 × 1 mL) to be the elution solvent.

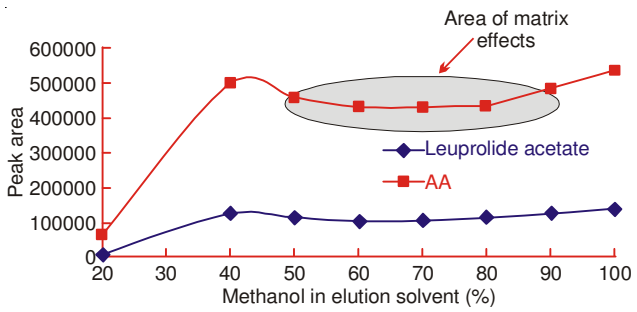


Fig. 1. Optimization of elution conditions for the SPE of leuprolide acetate from dog serum

In the study, all the tested columns were suitable for analysis of high-polarity compounds, including Tigerkin C_{18} (150 mm × 4.6 mm, 5 μm), CACELL PAK C_{18} (150 mm × 4.6 mm, 5 μm), Inertsil ODS-3 (100 mm × 2.1 mm, 5 μm). However, the retention time, peak shape and MS response were easily affected by the composition of the mobile phase. In order to improve the sensitivity and get good peak shape, we switched to choose phenomenex C_{18} (2.0 mm × 50 mm, 3 μm), phenomenex C_{18} (2.0 mm × 50 mm, 5 μm) and Ultimate XB-C_{18} (2.1 mm × 50 mm, 5 μm) to investigate. After comparation, we selected Ultimate XB-C_{18} (2.1 mm × 50 mm, 5 μm).

Optimization of the mass spectrometric condition: Leuprolide is an oligopeptide containing a histidine (His) and an arginine (Arg) in its structure. The presence of these two basic amino acids resulted in favourable sensitivity for leuprolide in the positive ESI ionization mode. Alarelin is the analogue of leuprolide, the tiny difference in their structure is that a leucine of leuprolide group is substituted by an alanine group[12]. In the positive ESI interface, leuprolide formed predominantly protonated molecules $[M + 2H]^{2+}$ at *m/z* 605.5 in Q1 full-scan mass spectra, (Fig. 2), while the $[M+H]^+$ ions at *m/z* 1210.0 less than 20 % relative abundance of $[M + 2H]^{2+}$; IS formed predominantly protonated molecules $[M + 2H]^{2+}$ at *m/z* 584.4, while the $[M + H]^+$ ions at *m/z* 1168.2 less than 20 % relative abundance of $[M + 2H]^{2+}$. The corresponding product ion mass spectra are depicted in Fig. 3, where $[M + 2H]^{2+}$ of each compound was selected as the precursor ion. Leuprolide and IS both have fragment ions at *m/z* 221.3 and *m/z* 249.2. Initially, in order to improve the sensitivity, the two major diagnostic fragment ions were both acquired in the MRM for leuprolide and IS, but subsequently, we found that when we use *m/z* 249.2, there were serious matrix effect. So *m/z* 221.3 was selected as the diagnostic fragment ion. The optimal collision energy for leuprolide and IS were set at 40 and 35 eV, respectively.

Assay selectivity and matrix effect: As for the impact of internal standard variability, the variability with incurred samples of leuprolide was insignificant. Fig. 4 shows typical chromatograms of a blank beagle dogs' serum, beagle dogs' serum sample spiked with leuprolide at LLOQ (0.103 ng mL^{-1}) and IS (104.5 ng mL^{-1}) and a serum sample from a test beagle dog 2 h after subcutaneous injection with leuprolide. No significant interference from endogenous substances was observed at the retention times of leuprolide and IS. Typical retention times for leuprolide and IS were 7.10 and 6.87 min, respectively. The relative matrix effects of leuprolide in six different lots of beagle dogs' serum at concentrations of 0.2051, 5.128 and 82.05 ng mL^{-1} were 10.3, 7.85 and -5.25 %, respectively. The relative matrix effect for IS (104.5 ng mL^{-1} in serum) was -7.24 %. Although there was an extent to the matrix enhancement for leuprolide in the present condition, it did not inuence the accurate determination of leuprolide in beagle dogs' serum.

Linearity of calibration curve and lower limit of quantization: The linear regression of the peak-area ratios *versus* concentrations were fitted over the concentration range of 0.103-103 ng mL^{-1} for leuprolide in beagle dogs' serum. Over the above curve range, the linear correlation coefficients were found to be better than or equal to 0.9982. This validated

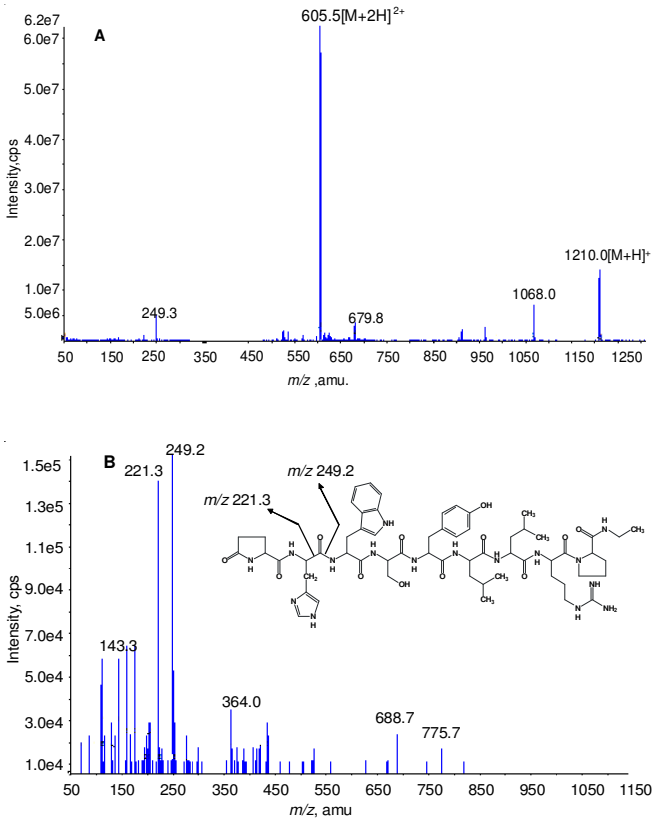


Fig. 2. MS/MS spectra of LA:Q1(A) and MS2(B)

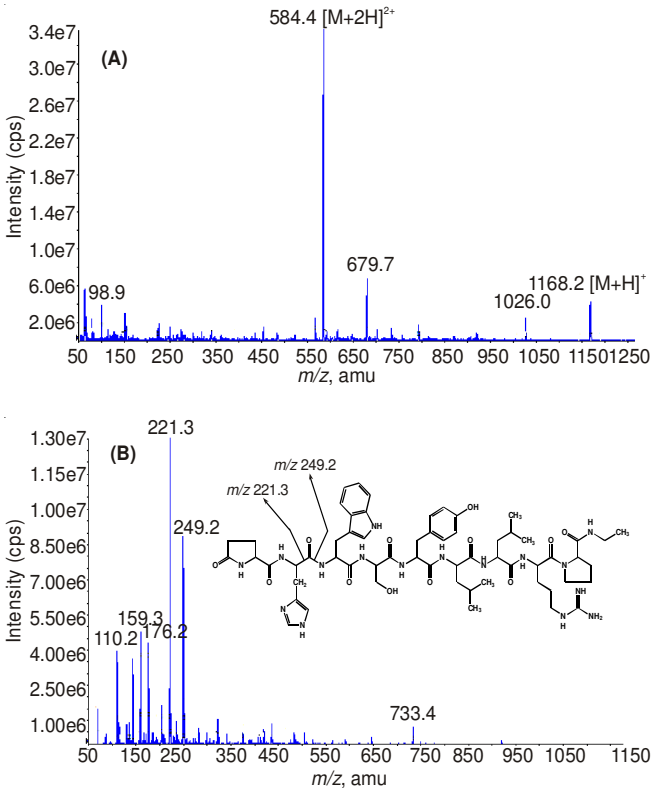


Fig. 3. MS/MS spectra of alarelin:Q1(A) and MS2(B)

assay had an LLOQ of 0.103 ng mL^{-1} in serum, which is sensitive enough to investigate the pharmacokinetic behaviour of leuprolide in this study.

Precision: The method showed good precision. Table-1 summarizes the intra- and inter-day precision values for leuprolide from quality controls. In this assay, the intra- and inter-assay precisions were measured to be ≦ 5.19 % (n = 6) and ≦ 6.48 % (n = 6), respectively. These values were within the acceptable range and the method was thus judged to be suitably accurate and precise.

TABLE-1
INTRA-DAY AND INTER-DAY PRECISION AND ACCURACY OF QUALITY CONTROL SAMPLES

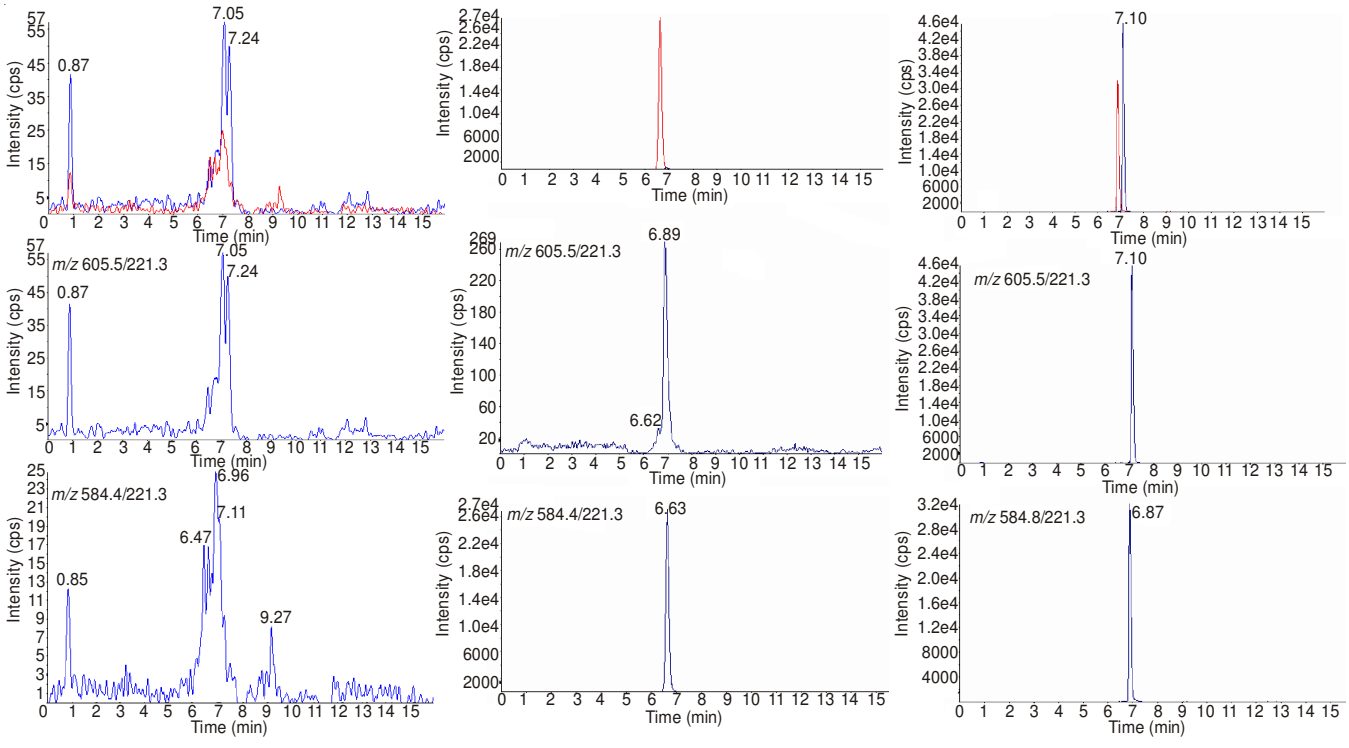
Leuprolide in serum (ng mL^{-1})				
	Low-0.2051	Medium-5.128	High-82.05	LLOQ
Intra-day (n = 6)				
Mean (ng mL^{-1})	0.200	5.20	84.5	0.113
SD	0.01	0.27	3.70	0.00
RSD (%)	4.56	5.19	4.37	3.35
Inter-day (n = 18)				
Mean (ng mL^{-1})	0.209	5.32	84.6	–
SD	0.01	0.30	5.48	–
RSD (%)	6.46	5.67	6.48	–

Extraction recovery and stability: The recoveries of leuprolide extracted from serum were 78.8 ± 5.73, 90.8 ± 3.6 and 86.0 ± 11.69 % at concentrations of 0.2051, 5.128 and 82.05 ng mL^{-1}, respectively (n = 6). Mean recovery for the IS was 87.2 ± 10.09 % (n = 6).

The stability experiments aimed at testing all possible conditions that the samples might experience during the sample shipping and handling such as freezing-thawing and a short storage at room temperature (bench-top) and during analysis such as extracted samples sitting in sample tray or refrigerator, *etc*. All stability results are summarized in Table-2. Three freeze-thaw cycles and 8 h room temperature storage for quality control samples had no substantial effect on the results. Keeping extracts at room temperature for approximately 12 h prior to injection did not affect the quantitative determination of leuprolide acetate in samples. The 3-month storage stability of quality control samples has also been tested and the data were included in Table-2. The results show a better than 10.22 % RSD (n = 3 for each level QC) and 85.2-113.9 % accuracy, indicating that the QC samples were stable for at least 3 months if storing frozen at *ca.* -20 ºC. As seen in Table-2, the analyte was stable under the typical serum storage and processing conditions used throughout the current study.

Pharmacokinetic results: Test preparation and reference preparation pharmacokinetic parameters of single dose t_{max} were 1.67 ± 0.577 h and 2 ± 0 h; C_{max} were 27.3 ± 4.854 and 25.1 ± 4.681 ng mL^{-1}, $t_{1/2}$ were 193.95 ± 51.709 and 235.09 ± 94.021 h; $AUC_{0\to t}$ were 778.293 ± 123.118 and 784.987 ± 199.441 μg h L^{-1}, $AUC_{0\to\infty}$ were 817.019 ± 133.862 and 846.375 ± 169.02 μg h L^{-1}. Fig 5 shows concentration-time curve of beagles after subcutaneous injection of 3.75 mg leuprolide acetate.

Evaluation of bioequivalence was made by analysis of variance, double-sided t test and (1-2α) confidence interval method. The results show the test agents, the $AUC_{0\to t}$ leuprolide 90 % confidence interval for the reference preparation of the corresponding parameters of 99.6-102.5 %, the $AUC_{0\to\infty}$



(A) (B) (C)

Fig. 4. Specific chromatograms of leuprolide acetate in serum with MS/MS detection: blank serum (A); a blank serum sample spiked with leuprolide acetate (0.103 ng mL^{-1}) and I.S. (104.5 ng mL^{-1}) (B) a test beagle serum sample 2 h after i.h. of 3.75 mg leuprolide acetate (C)

TABLE-2
STABILITY DATA

Leuprolide acetate in serum (ng mL^{-1})			
	0.2051	5.128	82.05
Bench-top ambient ~ 24 h (n = 6)			
Mean (ng mL^{-1})	0.220	4.96	83.7
SD	0.00	0.42	4.74
RSD (%)	1.20	8.43	5.66
Rate of changing (%)	4.60	-1.46	4.32
Extracts stored ambient ~12 h (n = 6)			
Mean (ng mL^{-1})	0.221	4.73	78.5
SD	0.02	0.18	2.69
RSD (%)	8.11	3.85	3.43
Rate of changing (%)	5.23	-6.02	-2.20
Reinjection ~ 3 times (n = 6)			
Mean (ng mL^{-1})	0.209	5.68	89.4
SD	0.01	0.08	2.26
RSD (%)	4.35	1.37	2.53
Rate of changing (%)	-1.88	-0.12	0.04
Three freeze–thaw cycles (n = 6)			
Mean (ng mL^{-1})	0.190	5.08	83.0
SD	0.00	0.34	9.21
RSD (%)	1.33	6.77	11.1
Rate of changing (%)	-9.8	0.86	3.36
Three-month QC samples storageat -20 ℃ (n = 3)			
Mean (ng mL^{-1})	0.224	4.62	82.9
SD	0.00	0.22	1.71
RSD (%)	1.43	4.70	2.06
Rate of changing (%)	6.66	-8.27	3.28

leuprolide 90 % confidence interval for the reference preparation of the corresponding parameters of 90.2-103.9 %, C_{max} 90 % confidence interval for the reference preparation of the corresponding parameters of 91.0-129.8 %. Calculated as leuprolide, the two preparations were bioequivalence *in vivo*

TABLE-3
RESULTS OF DOUBLE-SIDED T TEST AND (1-2α) CONFIDENCE INTERVAL ANALYSIS FOR THE PHARMACOKINETICS PARAMETERS OF LA

Parameters	t_1 (Test)	t_2 (Reference)	90 % confidence interval (%)	Standards (%)
$AUC_{0\to t}$	35.023	31.920	99.6-102.5	80-125
$AUC_{0\to\infty}$	5.723	7.666	90.2-103.9	80-125
C_{max}	4.4519	2.4224	91.0-129.8	75-133

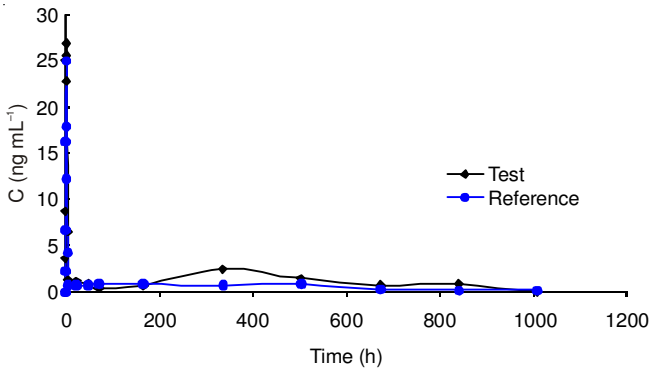


Fig. 5. Mean concentration-time curve of 3 beagle dogs after sc. of 3.75 mg leuprolide acetate

Beagle dogs, see Table-3. Rank sum test was used for statistical analysis using non-parametric test method according to the distribution characteristics of t_{max}. The value of T of test and reference preparations were 9 and 12, respectively, both were in critical range (6-15) of T 0.05 (n1 = n2 = 3), showing no difference of t_{max} between test and reference leuprolide.

Conclusion

The validated HPLC-MS/MS method is simple, sensitive and accurate. It was suitable for determination of drug concentration of leuprolide in beagle dog's serum. Determination of the sample used in this study has accepted satisfactory results. This method can be further used in the bioequivalence study of leuprolide microspheres.

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