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Rapid and sensitive liquid chromatography–tandem mass spectrometry method for the determination of leuprolide in human serum

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ABSTRACT

Leuprolide is a synthetic nonapeptide that has two basic amino acids, arginine and histidine, in its structure. By selection of an appropriate analytical column and optimizing the mobile phase composition, an improved analytical method has been developed and validated to determine leuprolide concentrations in human serum. After methanol-induced protein precipitation of serum samples and Oasis® HLB cartridge solid-phase extraction, leuprolide and an internal standard (alarelin) were analyzed on a C_{18} column interfaced with a triple quadrupole tandem mass spectrometer with positive electrospray ionization. The mobile phase consisted of acetonitrile–water–propionic acid (20:80:0.05). The analyte and internal standard were both detected in the selective reaction monitoring mode. The method exhibited a linear range of 0.018–45.2 ng/mL for leuprolide. The intra- and inter-assay precisions were 11.5% or less relative standard deviation (R.S.D.), and accuracy was within ±2.8% relative error (RE). The lower limit of quantification (LLOQ) was identifiable and reproducible at 0.018 ng/mL, with acceptable precision and accuracy. The validated LC–MS/MS method was tested to a clinical pharmacokinetic study of leuprolide after a single subcutaneous injection of 1 mg leuprolide acetate in healthy male Chinese volunteers.

1. Introduction

Peptides and proteins are becoming increasingly important in the pharmaceutical industry, especially as a novel class of therapeutic agents for cancer therapy [1]. Many peptide therapeutics are often synthetic analogs of endogenous peptides. The studies of pharmacologically active peptides are therefore significant for the development of new therapeutic methods for disease therapy.

Leuprolide (pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHC_2H_5), a synthetic nonapeptide, is a potent gonadotropin releasing hormone (GnRH) analog that has increased duration of effect and affinity for the pituitary receptor, and that can induce medical castration [2]. Leuprolide has been used in the treatment of sex hormone-related disorders, including advanced prostate cancer, endometriosis, and precocious puberty [2]. Due to the low bioavailability after oral administration or intraduodenal (ID) injection [3], leuprolide is presently administered by subcutaneous or intramuscular injection [4].

Single daily injections of leuprolide or its retard formula, administered every 4 or more weeks, have been introduced for prostate cancer, endometriosis, and other sex hormone-related disorders [5]. The recommended dose for Lupron® (leuprolide acetate injection) is 1 mg administered as a single daily subcutaneous injection [6]. In patients with advanced prostate cancer, leuprolide acetate 3 month depot (11.25 mg leuprolide acetate) is effective in suppressing the testosterone levels for at least 13 weeks [7]. At week 13, therapeutic effective levels of the mean values of leuprolide in serum were reported as 174 ± 77 pg/mL in 19 patients [7].

To date, the main method for studying the pharmacokinetics of leuprolide is by immunoassay [2,8–11]. More recently, Sofianos et al. [12] established a method using HPLC, coupled to a hybrid quadrupole linear ion trap, to determine leuprolide in mouse plasma. They obtained a lower limit of quantification (LLOQ) of 0.100 ng/mL using 100 μL mouse plasma, but separation required a relatively long chromatographic run time (12 min). Therefore, analytical methods of more sensitivity are still required for studying leuprolide and its pharmacokinetic profiles in human plasma or serum.

To our knowledge, determination of leuprolide in human serum or plasma by a LC–MS/MS method has not yet been reported. In the present study, we have optimized the chromatographic conditions and serum sample preparation to initiate a more rapid and sensitive LC–MS/MS method for determining leuprolide in human serum. The LLOQ of this method has been decreased to 0.018 ng/mL and the chromatographic run time was less than 5 min. The validated method was applied successfully to a pharmacokinetic study of leuprolide after a single subcutaneous injection of 1 mg leuprolide acetate.

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2. Experimental

2.1. Materials

Leuprolide acetate (chemical purity 99.3%, peptide content 90.2%) was kindly provided by H&J Beijing Clinical Research Ltd. (Beijing, China). Alarelin (internal standard, peptide content 86.7%) was obtained from Anhui Xinli Pharmaceutical Co., Ltd. (Hefei, China). HPLC-grade methanol, acetonitrile, formic acid, and propionic acid were purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetic acid was purchased from Tedia (Fairfield, OH, USA). Millipore Milli-Q gradient purified water (Molsheim, France) was used throughout the study. An Oasis® HLB cartridge (30 mg, 1 mL) was purchased from Waters Corp. (Milford, MA, USA). Drug-free human serum was supplied by the Fourth Military Medical University (Xijing Hospital, Xi'an, China).

2.2. Instrument

The Agilent 1100 liquid chromatography system used was equipped with a G1311A quaternary pump, a G1322A vacuum degasser, a G1316A thermostated column oven and a G1367A autosampler (Agilent, Waldbronn, Germany). Mass spectrometric detection was performed on a Thermo Finnigan TSQ Quantum Ultra triple quadrupole instrument (San Jose, CA, USA). The Agilent Chemstation and Finnigan Xcalibur software packages were used to control the LC–MS/MS system, as well as for data acquisition and processing.

2.3. Chromatographic conditions

Chromatographic separation was achieved on a Venusil ASB-C_{18} column (150 mm × 4.6 mm i.d., 5 μm, Agela, Newark, DE, USA) protected by a SecurityGuard C_{18} guard column (4 mm × 3.0 mm i.d.; Phenomenex, Torrance, CA, USA). A mixture of acetonitrile–water–propionic acid (20:80:0.05) was used as the mobile phase at a flow-rate of 0.50 mL/min. The column temperature was maintained at ambient temperature (25 °C). A post-column diverter valve was used to direct HPLC column eluate to a waste container for the first 2 min of the chromatographic run and then to the ionization source.

2.4. Mass spectrometric conditions

The mass spectrometer was operated in the positive ion mode using an electrospray ionization (ESI) source. The tuning parameters were optimized for leuprolide and IS by infusing a solution containing 226 ng/mL of both the analyte and IS, at a flow-rate of 20 μL/min into the mobile phase (0.50 mL/min), using a post-column "T" connection. Nitrogen was used as sheath gas (35 Arb), auxiliary gas (5 Arb), and ion sweep gas (2 Arb), to assist with nebulization and desolvation. The spray voltage was set at 4200 V and the capillary temperature was maintained at 320 °C. For collision-induced dissociation (CID), argon was used as the collision gas at a pressure of 1.2 mTorr. Quantification was performed using selected reaction monitoring (SRM) of the transitions m/z 605.5 → m/z (221.0 + 249.0) for leuprolide and m/z 584.5 → m/z (221.0 + 249.0) for alarelin (IS), with a dwell time of 200 ms per transition. The optimized collision energy of 35 eV was used for the analyte and IS. The mass spectrometer was operated at unit mass resolution (peak width at half-height set at 0.7 Da) for both Q1 and Q3.

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

A stock solution of leuprolide with a concentration of 376 μg/mL (calculated as peptide content) was prepared by dissolving an accurately weighed sample of leuprolide acetate in methanol. A series of standard working solutions with concentrations in the range of 0.090–226 ng/mL for leuprolide were obtained by further dilution of the stock solution with methanol–water (50:50, v/v). Calibration curves were prepared by spiking 40 μL of the appropriate standard solution into 200 μL of blank human serum. Quality control (QC) samples were similarly prepared at concentrations of 0.045, 1.80, and 40.6 ng/mL in human serum, by a separate weighing of the reference compound. A 50.0 ng/mL (calculated as peptide content) internal standard (IS) working solution was prepared by diluting a stock solution of alarelin with methanol–water (50:50, v/v). All of the solutions were stored at 4 °C and were brought to room temperature before use. QC samples were aliquoted into 1.5 mL polypropylene vials and stored at −20 °C until analysis.

During the study, no adsorption of leuprolide or alarelin was observed on the different tube materials.

2.6. Sample preparation

Frozen serum samples from the human study subjects were thawed to room temperature prior to preparation. After vortexing, a 40 μL aliquot of the IS solution (alarelin, 50.0 ng/mL), 40 μL of methanol–water (50:50, v/v), and 200 μL methanol were added to 200 μL of serum sample. The mixture was vigorously vortexed for 1 min and centrifuged at 11,300 × g for 5 min. The supernatant was mixed with 300 μL water and then transferred to Oasis® HLB 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 1 mL methanol–water solution (10:90, v/v), then leuprolide and IS were eluted with 2× 1 mL of methanol containing 1% formic acid. The eluate 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 (15:85:0.15, v/v/v). A 20 μL aliquot was injected onto the LC–MS/MS system for analysis.

2.7. Method validation

To ensure the selectivity, accuracy, reproducibility, and sensitivity, the method was validated described as follows.

The selectivity of the method was evaluated by analyzing six blank serum samples and six spiked serum samples at LLOQ level from six different sources. 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 [13]. The internal standard was present in the bioanalytical assay to compensate for the variability of extraction in LC-MS/MS analysis. A highly variable internal standard can be an indication of an uncontrolled process during sample analysis [14].

The linearity was evaluated by analyzing duplicate calibration curves for leuprolide in human serum on 3 separate days. In addition, a blank (no leuprolide or IS) and a zero serum sample (no leuprolide, but with IS) were run to eliminate the presence of interferences. The standard curves were calculated by a weighted ($1/x^2$) least squares linear regression 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 [13]. The lower limit of quantification (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 [13].

Precision and accuracy of the method were assessed by the determination of QC samples at three concentration levels (0.045, 1.80 and 40.6 ng/mL), in six replicates, on 3 validation days. The precision was expressed by relative standard deviation (R.S.D.) and calculated using the one-way analysis of variance (ANOVA) [15]. The assay accuracy was expressed as relative error (RE), i.e. (observed concentration − nominal concentration)/(nominal concentration) × 100%. The intra- and inter-day precisions were required to be below 15%, and the accuracy to be within ±15% [13].

To evaluate the precision and accuracy of the method in the sample dilution process, QC samples at 90.2 ng/mL were diluted 4-fold before being analyzed in six replicates on the first day of pharmacokinetic study.

The matrix effect was investigated by measuring the matrix factor, as defined by the ratio (analyte peak area in presence of serum matrix from six different sources)/(analyte peak area in absence of serum matrix), and was expressed as percent response relative to the neat solution. In our experiment, the matrix effect was determined at two concentration levels (0.045 and 40.6 ng/mL). The inter-subject variability of matrix effect at each concentration level should be less than 15% [16].

The recovery of leuprolide was estimated at three concentration levels (0.045, 1.80 and 40.6 ng/mL) by comparing the peak-area ratios of the analyte to IS. Samples that were spiked with the analyte prior to extraction were compared with samples to which the analyte was added post-extraction. The IS was added to the two sets of samples post-extraction. The extraction recovery of the IS was determined in a similar way, using the QC samples at medium concentration as a reference.

The stability of leuprolide in human serum was assessed by analyzing replicates ($n = 3$) of serum samples at the concentrations of 0.045 and 40.6 ng/mL, which were exposed to different conditions (time and temperature). The short-term stability was determined after the exposure of the spiked samples to room temperature for 2 h, or by exposure of the ready-to-inject samples (after extraction, in the mobile phase) to the autosampler rack (room temperature) for 24 h. The long-term stability was evaluated after storage of the standard spiked serum samples at −20 °C for 21 days. The freeze/thaw cycle stability was assessed after three complete freeze/thaw cycles (−20 to 25 °C) on consecutive days. The analytes are considered to be stable in serum when 85–115% of the initial concentrations were found.

2.8. Application to a pharmacokinetic study

The LC–MS/MS method described above was applied to determine the serum concentrations of leuprolide from a clinical pharmacokinetic study in which 10 healthy male Chinese volunteers were enrolled. The pharmacokinetic study was approved by the Medical Ethics Committee of Xi'an Xijing Hospital. Informed consent was obtained from all subjects after explaining the aims and risks of the study. A single subcutaneous dose of 1 mg leuprolide acetate, equivalent to leuprolide base at 0.95 mg, was injected into each subject. Venous blood samples were collected into tubes before and 0.17, 0.33, 0.5, 0.75, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10, 14 and 24 h after dosing. Samples were kept at 4 °C for 30 min and then centrifuged at 2000 × *g* (4 °C) for 10 min to separate the serum fractions. The collected serum samples were stored at −20 °C until analysis.

The pharmacokinetic parameters of leuprolide were calculated by non-compartmental assessment of data using the computer program WinNonlin (V5.0.1, Pharsight, Mountain View, CA, USA). The maximum serum concentrations (C_{max}) and their time of occurrence (T_{max}) were both obtained directly from the measured data. The area under the serum concentration–time curve from time zero to the time of the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal method. The terminal elimination rate constant (k_e) was estimated by log-linear regression of concentrations observed during the terminal phase of elimination. The corresponding elimination half-life ($t_{1/2}$) was then calculated as $0.693/k_e$.

3. Results and discussion

3.1. 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 favorable 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. In the positive ESI interface, both leuprolide and IS formed predominantly double protonated molecules $[M+2H]^{2+}$ at *m/z* 605.5 and *m/z* 584.5 in Q1 full-scan mass spectra, respectively (see Fig. 1), while the $[M+H]^+$ ions at *m/z* 1209.9 and *m/z* 1168.6 were less than 5% relative abundance of $[M+2H]^{2+}$. The corresponding product ion mass spectra are depicted in Fig. 2, where $[M+2H]^{2+}$ of each compound was selected as the precursor ion. Leuprolide and IS both have fragment ions at *m/z* 221.0 and *m/z* 249.0. In order to improve the sensitivity, the two major diagnostic fragment ions were both acquired in the SRM for leuprolide and IS. The optimal collision energy for leuprolide and IS were both set at 35 eV.

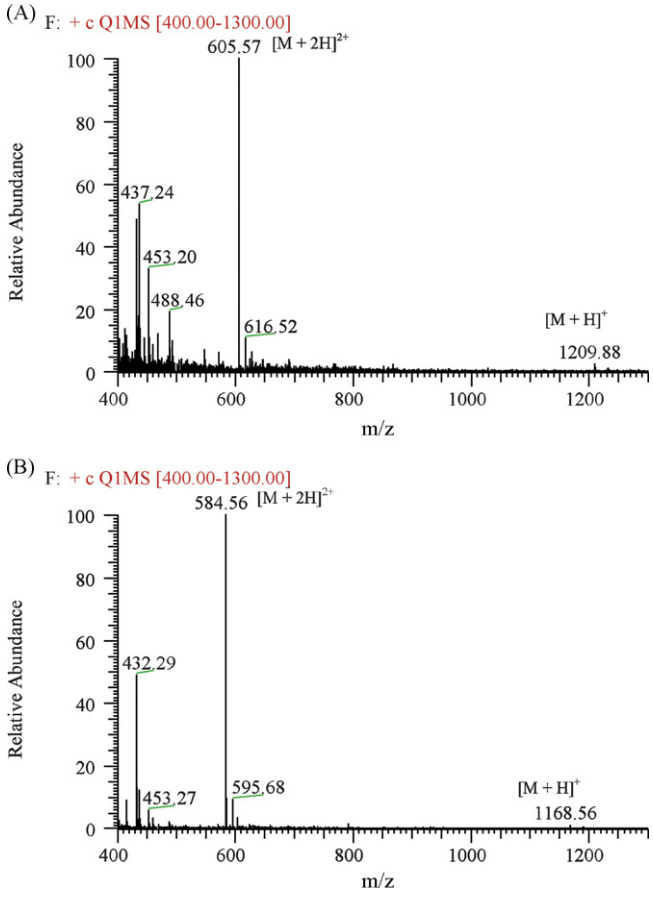

Fig. 1. Full-scan mass spectra of leuprolide (A) and alarelin (B).

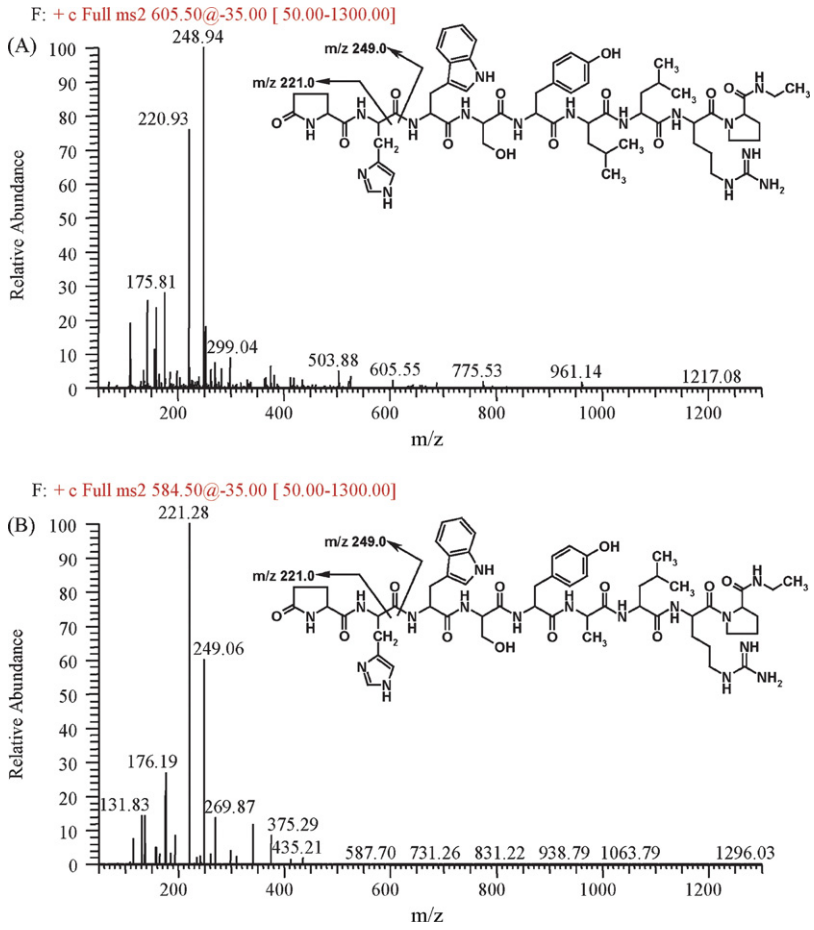

Fig. 2. Product ion mass spectra of $[M+2H]^{2+}$ ion of leuprolide (A) and alarelin (B).

3.2. Optimization of the sample preparation and chromatographic condition

Currently, the most commonly employed techniques for the extraction of peptides from biological matrices are solid-phase extraction (SPE), protein precipitation (PPT) and liquid–liquid extraction (LLE) [17]. PPT, a simple pre-treatment technique, was used by Sofianos et al. [12] to separate leuprolide from mouse plasma samples. During our initial method development, we also tested a PPT method to separate leuprolide from serum samples, but we encountered strong ion suppression from the endogenous substances in serum samples and a broad peak occurred under isocratic HPLC conditions. Although improvements in ion suppression might be improved by gradient chromatographic conditions, as described in the literature, this would have meant sacrificing our short analytical run time.

The use of solid-phase extraction (SPE) cartridges also allows enrichment of analytes and therefore better sensitivity for determination. SPE has been widely chosen for the separation of peptides from biological matrixes [18–20]. During our method development, we investigated a variety of SPE cartridges (Strata-E cartridge, Strata-ph cartridge, Orochem C_{18} cartridge, Strata-X cartridge and Oasis® HLB cartridge) for serum pre-treatment and optimized the different loading, washing and elution steps. The Oasis® HLB cartridge provided the highest and most stable recovery. When 1% formic acid in methanol was used as elution solvent, the recovery of leuprolide could be improved from the 50% seen with methanol alone to about 80%. Through optimization, we established 1% formic acid in methanol (2× 1 mL) to be the elution solvent that achieved the highest recovery (more than 80%) for both leuprolide and IS.

A waters symmetry C_4 column using gradient elution has been successfully used to separate leuprolide from mouse plasma [12]. However, a relatively long analytical run time (about 12 min) was needed to equilibrate the column and to avoid ion suppression. Leuprolide is a strongly hydrophilic and basic compound. In the study, all the tested columns were suitable for analysis of high-polarity compounds, including Diamonsil C_{18} (Dikma, China), Synergi 4 μ Hydro-RP (Phenomenex, USA), Capcell PAK AQ C_{18} (Shiseido, Japan), Atlantis HILIC Silica (Waters, USA) and Venusil ASB-C_{18} (Agela, USA). Strong ion suppression was observed on Diamonsil C_{18} column. As shown in Fig. 3, there was an obvious double peak on the Capcell PAK C_{18} AQ column and the peak shape on Phenomenex Synergi 4 μ Hydro-RP 80 A column was tailed. We also attempted a hydrophilic-interaction liquid chromatography (HILIC) column, but the retention time of leuprolide was over 10 min. After a series of trials, the Venusil ASB-C_{18} column was considered to be optimal and leuprolide and IS were well retained on the column (Fig. 3). However, the retention time, peak shape, and MS response were easily affected by the composition of the mobile phase.

Acetonitrile led to a higher mass spectrometric response and a lower background noise than did methanol, and was chosen as the organic phase. A small amount of acidic modifiers in the mobile phase was found to improve MS response of leuprolide and IS. The MS response of leuprolide using acetic acid as acidic modifier, was about 2 times higher than that obtained with formic acid, but the leuprolide peak was split with mobile phases con-

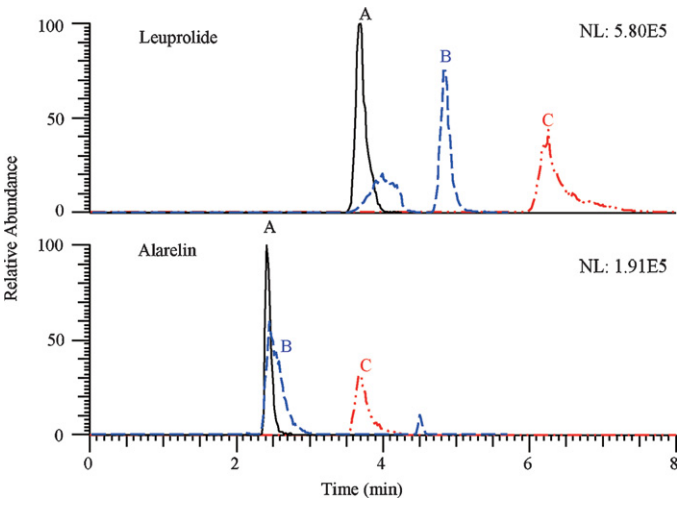

Fig. 3. Typical chromatograms of leuprolide in human serum acquired at different columns to evaluate the chromatographic performance of leuprolide and alarelin. Peak A: acetonitrile–water–propionic acid (20:80:0.05, v/v/v) on a Venusil ASB-C_{18} column (4.6 mm × 150 mm i.d., 5 μm, Agela, USA), $t_{R,leuprolide}$ = 3.71 min; Peak B: acetonitrile–water–propionic acid (20:80:0.05, v/v/v) on a Capcell PAK C_{18} AQ column (4.6 mm × 150 mm i.d., 5 μm, Shiseido, Japan), $t_{R,leuprolide}$ = 4.86 min; Peak C: acetonitrile–water–propionic acid (30:70:0.05, v/v/v) on a Phenomenex Synergi 4 μ Hydro-RP column 80 A (4.6 mm × 150 mm i.d., 4 μm, Phenomenex, USA), $t_{R,leuprolide}$ = 6.27 min.

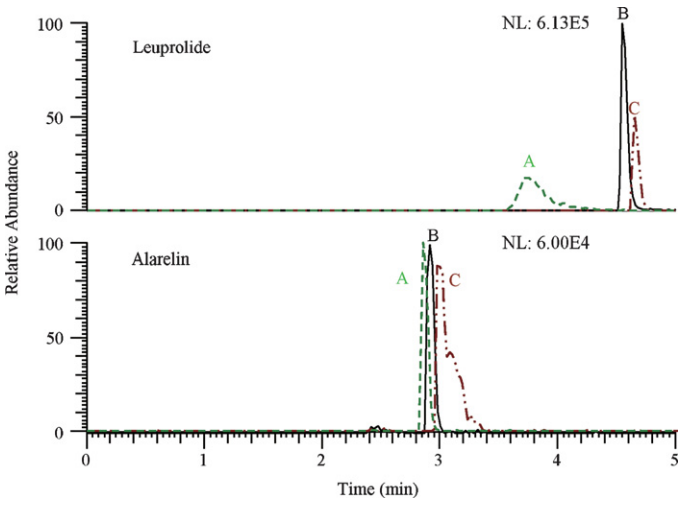

Fig. 4. Typical chromatograms of leuprolide and alarelin in human serum obtained on a Venusil ASB-C_{18} column when acetonitrile–water–propionic acid (20:80:0.05, v/v/v) was used as the mobile phase to evaluate the effect of the reconstitution solvent on the chromatographic peak shape and MS response; Peak A: acetonitrile–water–propionic acid (20:80:0.05, v/v/v), $t_{R,leuprolide}$ = 3.73 min; Peak B: acetonitrile–water–propionic acid (15:85:0.15, v/v/v), $t_{R,leuprolide}$ = 4.55 min. Peak C: acetonitrile–water–propionic acid–acetic acid (15:85:0.04:0.11, v/v/v/v), $t_{R,leuprolide}$ = 4.64 min.

taining 0.05% acetic acid. The peak-splitting issue for leuprolide could be resolved by introducing 5 mM ammonium acetate to the mobile phase, but then the IS peak became broadened. Propionic acid in place of acetic acid resulted in a slightly lower MS response, but a reasonable peak shape and retention time was achieved, and no peak-splitting was observed. We supposed that leuprolide and the acidic modifier might have a transition process between ion-pairing and dynamic ion-exchange mechanism. The retention of leuprolide in the ion-pair chromatography system tended to increase with longer carbon chain acidic modifiers. The mobile phase system of acetonitrile–water–propionic acid (20:80:0.05) may have provided a condition where only one mechanism contributed to chromatographic separations. However, other aspects of this peak-splitting phenomenon are the subject of further study.

As shown in Fig. 4, the MS response and peak shape of leuprolide was improved by increasing propionic acid content of the reconstitution solvent. A sharp chromatographic peak with suitable retention time (4.6 min) was obtained when a solution of acetonitrile–water–propionic acid (15:85:0.15) was used as the reconstitution solvent. The analytical run time of each sample was less than 5 min.

3.3. Method validation

3.3.1. Assay selectivity and matrix effect

As for the impact of internal standard variability, the variability with incurred samples of leuprolide was insignificant. Fig. 5 shows typical chromatograms of a blank human serum, human serum sample spiked with leuprolide at LLOQ and IS, and a serum sample from a volunteer 4.0 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 4.5 and 3.0 min, respectively.

The absolute matrix effects of leuprolide in six different lots of human serum at concentrations of 0.045 and 40.6 ng/mL were 141% and 146%, respectively. The relative matrix effect was 6.5% and 8.6%, respectively. The absolute and relative matrix effect for IS (10.0 ng/ml in serum) were 126% and 10.5%, respectively. Although there was an extent to the matrix enhancement for leuprolide in the present condition, it did not influence the accurate determination of leuprolide in human serum.

3.3.2. Linearity of calibration curve and lower limit of quantification

The linear regression of the peak-area ratios versus concentrations were fitted over the concentration range of 0.018–45.2 ng/mL for leuprolide in human serum. A typical equation of the calibration curve on a validation run was as follows:

$$y = 1.02 \times 10^{-3} + 0.402x \quad (r^2 = 0.9923)$$

where y represents the peak-area ratio of analyte to IS and x represents the serum concentration of leuprolide. Good linearity was obtained in this concentration range.

The lower limit of quantification was established as 0.018 ng/mL for leuprolide. The precision and accuracy values corresponding to LLOQ are shown in Table 1. Under the present LLOQ of 0.018 ng/mL, the serum concentration of leuprolide could be determined up to 24 h after a single subcutaneous administration of 1 mg leuprolide acetate to healthy male Chinese volunteers. This is sensitive enough to investigate the pharmacokinetic behavior of leuprolide in clinical studies.

3.3.3. Precision and accuracy

The method showed good precision and accuracy. Table 1 summarizes the intra- and inter-day precision and accuracy values for

Table 1
Precision and accuracy of the LC–MS/MS method to determine leuprolide in human serum (in 3 consecutive days, six replicates for each day).

Concentration (ng/mL)		R.S.D. (%)		RE (%)
Added	Found	Intra-day	Inter-day	
0.018	0.0177 ± 0.0020	11.5	6.6	−1.8
0.045	0.0444 ± 0.0036	7.9	10.4	−1.6
1.80	1.77 ± 0.14	8.5	5.8	−1.9
40.6	40.4 ± 1.85	4.3	6.1	−0.5
90.2	92.9 ± 9.02	9.8	–	2.8

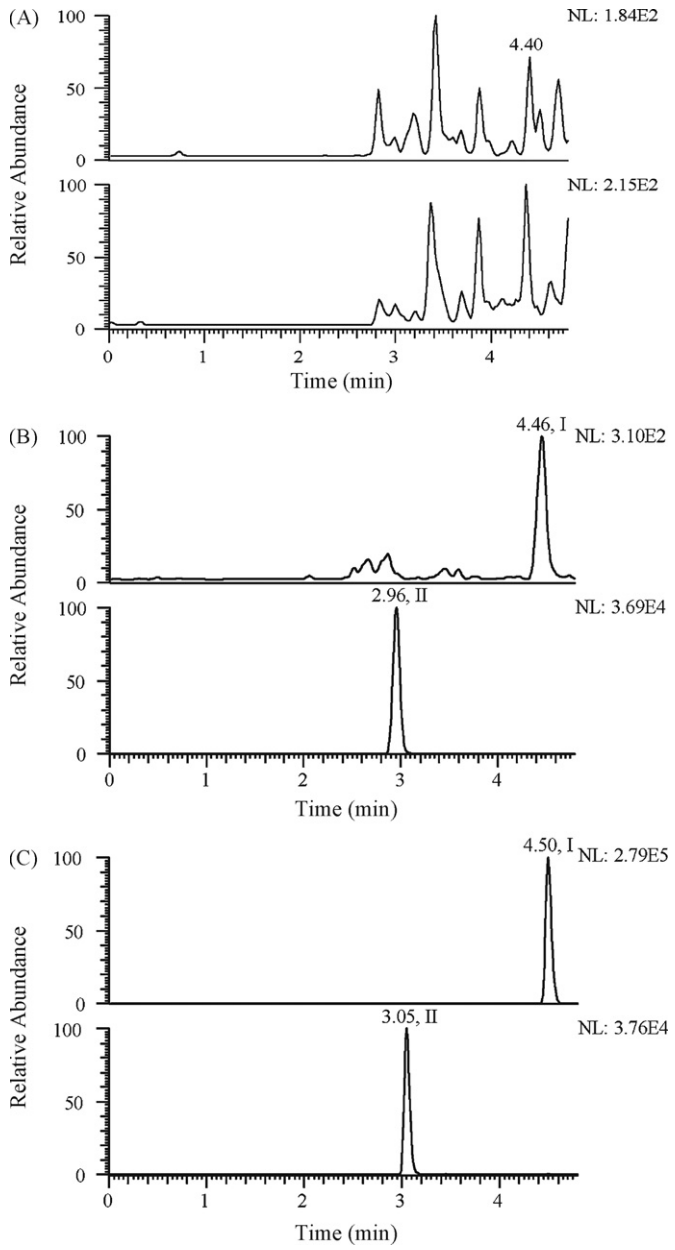

Fig. 5. Representative SRM chromatograms for leuprolide (I) and alarelin (IS, II) in human serum: (A) a blank serum sample; (B) a blank serum sample spiked with leuprolide at 0.018 ng/mL and alarelin (IS) at 10.0 ng/mL; (C) a volunteer serum sample of 14.9 ng/mL obtained at 4.0 h after a single subcutaneous injection of 1 mg leuprolide acetate (equivalent to 0.95 mg leuprolide base).

leuprolide from QCs. In this assay, the intra- and inter-assay precisions were measured to be below 11.5% and 10.4%, respectively, with relative errors from −1.9% to 2.8%. These values were within the acceptable range, and the method was thus judged to be suitably accurate and precise.

3.3.4. Extraction recovery

The recoveries of leuprolide extracted from serum were 81.6 ± 3.2%, 86.8 ± 7.2% and 83.6 ± 1.9% at concentrations of 0.045, 1.80, and 40.6 ng/mL, respectively (n = 6). Mean recovery for the IS was 81.0 ± 2.5% (n = 6).

3.3.5. Stability

The stability tests of the leuprolide were designed to cover anticipated conditions of handling of typical clinical samples. As seen in Table 2, the analyte was stable under the typical serum storage and processing conditions used throughout the current study.

Table 2
Summery of stability of leuprolide under various storage conditions (n = 3).

Storage conditions	Concetrations (ng/mL)		R.S.D. (%)	RE (%)
	Added	Found		
Three freeze–thaw cycles	0.045	0.0444 ± 0.0040	8.9	−1.6
	40.6	40.1 ± 1.9	4.8	−1.2
Freezing for 21 days (−20 °C)	0.045	0.0451 ± 0.0048	10.6	0.2
	40.6	39.0 ± 3.5	8.9	−3.9
Autosampler for 24 h (25 °C)	0.045	0.0408 ± 0.0015	3.7	−9.6
	40.6	41.0 ± 3.4	8.4	0.9
Short-term (2 h at 25 °C)	0.045	0.0464 ± 0.0062	13.4	2.9
	40.6	39.9 ± 1.4	3.6	−1.8

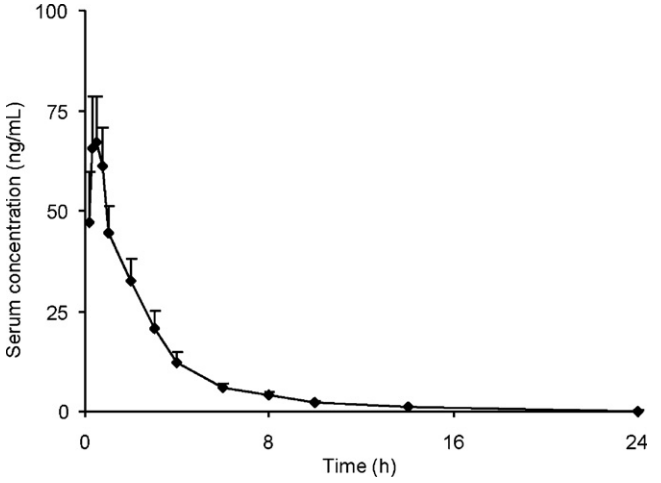

Fig. 6. Mean serum concentrations of leuprolide after a single subcutaneous injection of 1 mg leuprolide acetate (equivalent to 0.95 mg leuprolide base) to 10 healthy male Chinese volunteers (mean ± S.D.).

3.4. Application of the method to a pharmacokinetic study in healthy volunteers

The validated LC–MS/MS method was successfully applied to a pharmacokinetic study of leuprolide in healthy male Chinese subjects. Using this analytical method, we were able to measure post-injection serum concentration of leuprolide for up to 24 h in all subjects, after a single subcutaneous administration of 1 mg leuprolide acetate (equivalent to 0.95 mg leuprolide base). In order to quantify the higher leuprolide concentrations observed in human serum samples, the samples were diluted 2-fold before being analyzed. Fig. 6 shows the profile of the mean leuprolide serum concentration versus time and the main pharmacokinetic parameters for leuprolide are presented in Table 3. Compared with pharmacokinetic results reported previously [8], the values of AUC_{0-24} and

Table 3
Mean pharmacokinetics parameters for leuprolide after a single subcutaneous injection of 1 mg leuprolide acetate (equivalent to 0.95 mg leuprolide base) to healthy male Chinese volunteers (mean ± S.D., n = 10).

Parameters	Mean ± S.D.
C_{max} (ng/mL)	64.9 ± 8.36
T_{max} (h)	0.407 ± 0.178
AUC_{0-24} (ng h/mL)	164 ± 21.3
$AUC_{0-\infty}$ (ng h/mL)	164 ± 21.6
$t_{1/2}$ (h)	3.47 ± 0.732

C_{max} from the present study were about 2 times higher, while the $t_{1/2}$ did not differ significantly. The reported method [8] was determined by a modified radioimmunoassay procedure. The differences of pharmacokinetic parameters might result from the different assay methods or inter-ethnic differences.

According to the literature [7], the therapeutic effective levels of leuprolide acetate depot in serum were 174 ± 77 pg/mL. Therefore high detection sensitivity will be necessary for any pharmacokinetic study of leuprolide depot.

4. Conclusion

By optimizing the chromatographic conditions, a sensitive and rapid LC–MS/MS method for the quantification of leuprolide in human serum was developed and validated. This method was sensitive enough to monitor low-dosage pharmacokinetic or depot formulation studies of leuprolide in human serum. Compared with previously reported analytical methods, this method showed high throughput (4.8 min each sample) and greater sensitivity, with an LLOQ of 0.018 ng/mL. It was successfully applied to characterize the pharmacokinetics of leuprolide in healthy male Chinese volunteers.

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