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Development and validation of a liquid chromatography–tandem mass spectrometry method for the determination of goserelin in rabbit plasma

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ABSTRACT

A rapid and sensitive liquid chromatography–electrospray ionization tandem mass spectrometry method (LC–ESI-MS/MS) was developed and validated for the determination of goserelin in rabbit plasma. Various parameters affecting plasma sample preparation, LC separation, and MS/MS detection were investigated, and optimized conditions were identified. Acidified 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 mobile phase A. The separation was achieved on a Capcell-Pak C18 (2.0 mm × 150 mm, 5 μm, AQ type) column with a gradient elution of solvent A (0.05% acetic acid in deionized water/acetonitrile = 85/15; v/v) and solvent B (acetonitrile) at a flow rate of 250 μL/min. The LC–MS/MS system was equipped with an electrospray ion source operating in positive ion mode. Multiple-reaction monitoring (MRM) of the precursor–product ion transitions consisted of m/z 635.7 → m/z 607.5 for goserelin and m/z 424.0 → m/z 292.1 for cephapirin (internal standard). The proposed method was validated by assessing specificity, linearity, limit of quantification (LOQ), intra- and inter-day precision and accuracy, recovery, and stability. Linear calibration curves were obtained in the concentration range of 0.1–20 ng/mL (the correlation coefficients were above 0.99). The LOQ of the method was 0.1 ng/mL. Results obtained from the validation study of goserelin showed good accuracy and precision at concentrations of 0.1, 1, 5, 10, and 20 ng/mL. The validated method was successfully applied to a pharmacokinetic study of goserelin after a single subcutaneous injection of 3.6 mg of goserelin in healthy white rabbits.

1. Introduction

Goserelin (pGlu-His-Trp-Ser-Tyr-D-Ser(tBu)-Leu-Arg-Pro-(Aza)Gly-NH_2; pGlu: pyro-glutamic acid, His: histidine, Trp: tryptophan, Ser: serine, Tyr: tyrosine, D-Ser(tBu): D-serine *tertiary*-butyl ester, Leu: leucine, Arg: arginine, Pro: proline, and (Aza)Gly: aza-glycine; $C_{59}H_{84}N_{18}O_{14}$; monoisotopic mass = 1268.64; isotopic average mass = 1269.41; Fig. 1(A)) is a parenteral synthetic decapeptide analogue of luteinizing hormone-releasing hormone (LHRH) [1].

Synthetic LHRH analogues, such as goserelin, buserelin, leuprolide, and triptorelin, act as agonists that stimulate the pituitary gland. In an initial stage, LHRH analogues stimulate the pituitary gland to release luteinizing hormone (LH). However, with continuous administration, the number of unoccupied LHRH receptors decreases, the pituitary gland becomes desensitized, and, ultimately, LH secretion is reduced. As a result of the LH decrease, serum testosterone in males or estradiol in females is decreased to castration or post-menopausal levels, respectively [2].

Goserelin is used for the treatment of patients with hormone-sensitive prostate and breast cancers, as well as several benign gynecological disorders, including endometriosis, uterine fibroids, and endometrial thinning [3–6]. Subcutaneous injection of sustained release formulations containing 3.6 mg of goserelin maintains lower blood concentrations of testosterone or estradiol over a 4-week period [7]. Modification of the administration route for goserelin acetate from an injectable micro-implant to a microsphere (powder) injection system developed by Dongkook Pharmaceutical Co., Ltd. (Seoul, South Korea) [8] has alleviated injection pain.

Pharmacokinetic studies of goserelin have been performed using a radioimmunoassay (RIA) [7,9,10], which was shown to be

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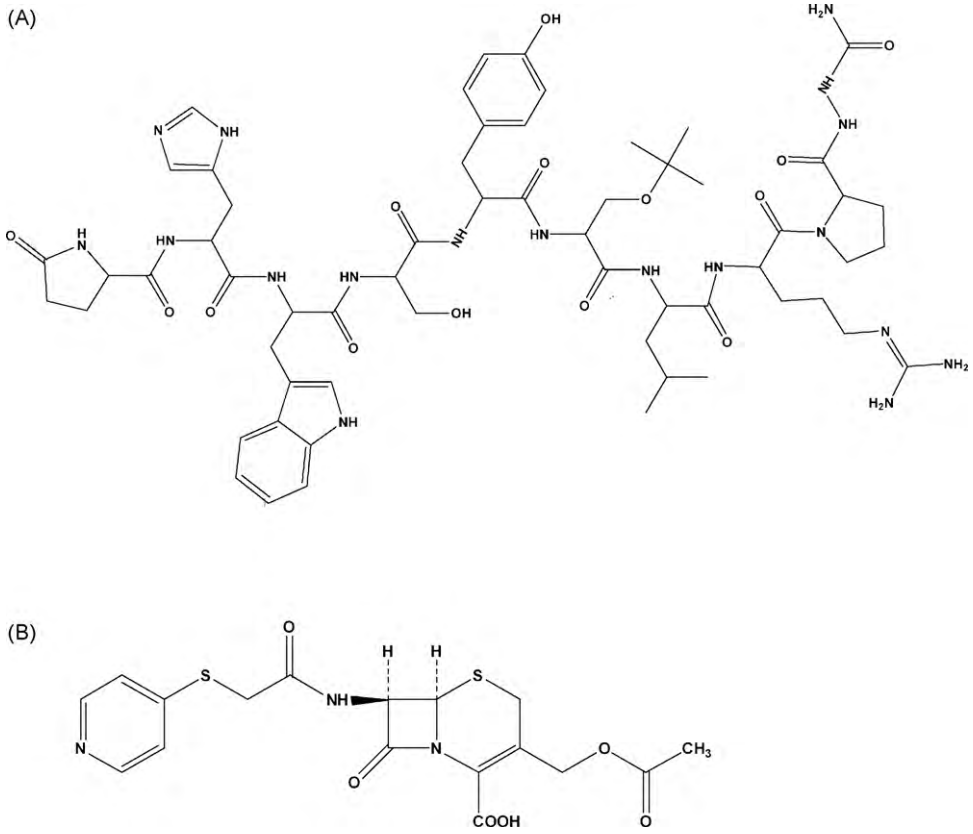

Fig. 1. Chemical structures of (A) goserelin and (B) cephapirin (internal standard).

selective and sensitive. The limit of detection (LOD) of this method was approximately 0.15 ng/mL. Nevertheless, it has limitations due to the cross-reactivity with structurally similar peptides, which interfere with the precise quantitation of goserelin in plasma. Although a number of analytical methods for the qualitative or quantitative determination of goserelin, including radioimmunoassay [7,9,10], liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) [11–14], capillary zone electrophoresis (CZE)-UV/MS [15], multiple-injection CZE (MICZE) [16], CE hydrogen deuterium exchange-MS (CE-H/D-MS) [17], fast atom bombardment-MS (FAB-MS) [18], and quadrupole time-of-flight MS (Q-TOF MS) [19], have been published, these reports have usually been concerned with the crude synthetic peptide mixtures, degradation products, and pharmaceutical formulations. Michalet et al. [19] used LC-Q-TOF tandem mass spectrometry for the quantitation of goserelin, and this is the only reported method, other than immunoassays, for goserelin analysis in biological fluids. A drawback of the LC-Q-TOF method is that an internal standard was not used. Moreover, detection sensitivity (the limit of quantification was 0.3 ng/mL) and a method validation procedure were not sufficient to monitor goserelin plasma concentration-time profiles in pharmacokinetic studies. Furthermore, another important drawback of this method was the long chromatography run time (>25 min), which is not ideal for pharmacokinetic studies. Therefore, there is still a need to develop and to validate a more accurate, faster, and more sensitive analytical method for quantifying goserelin in plasma.

In the present study, we have developed a rapid and sensitive liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) method for the determination of goserelin in rabbit plasma. To the best of our knowledge, this is the first report of a plasma concentration-time profile of goserelin in rabbits following a single subcutaneous injection. This method could be readily adapted to clinical pharmacokinetic studies by increasing the plasma sample size.

2. Materials and methods

2.1. Chemicals and reagents

Goserelin acetate was supplied by Dongkook Pharmaceutical Co., Ltd. (Seoul, South Korea). Cephapirin sodium (internal standard) was obtained from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile, water, methanol, acetic acid, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Hydrochloric acid was purchased from Duksan Pure Chemicals Co., Ltd. (Gyeonggi, South Korea), and rabbit plasma was supplied by Orient Bio Inc. (Gyeonggi, South Korea). Oasis® HLB SPE cartridges (1 cc, 30 mg) were obtained from Waters (Milford, MA, USA). Nylon membrane filters (0.22 μm) were purchased from Whatman (Maidstone, England). All other reagents were of analytical grade except those for HPLC.

2.2. LC–MS/MS instrumentation

The LC–MS/MS method was performed with a Waters Alliance 2795 HPLC system (Milford, MA, USA) and a Waters Quattro Micro API tandem mass spectrometer equipped with an ESI source, a pump, an autosampler, a degasser, an automatic thermostatic column oven, and a computer. MassLynx software (Waters, Ver. 4.1) was used to control the HPLC and mass spectrometer and to process the data.

2.3. Preparation of stock solutions and quality control (QC) samples

Stock solutions (1000 μg/mL) were prepared by dissolving 10 mg of goserelin acetate and the internal standard (cephapirin sodium) in 10 mL of 20% acetonitrile. A series of working standard solutions of goserelin in the concentration range of 1–200 ng/mL was prepared by diluting the stock solution with mobile phase A. Internal standard stock solution was diluted with 20% acetonitrile to a final concentration of 250 ng/mL. Standard plasma samples were prepared by spiking 50 μL of suitable working standard solution in 450 μL of blank rabbit plasma. Finally, standard plasma concentrations for goserelin were 0.1, 0.5, 1, 2, 5, 10, and 20 ng/mL. Quality control samples (0.1, 1, 5, 10, and 20 ng/mL) were prepared in the same manner. All of the solutions were stored at −70 °C until analysis.

2.4. Sample preparation

All frozen rabbit plasma samples were thawed at room temperature. A 500 μL volume of rabbit plasma sample was placed in a 2-mL Eppendorf tube, and 50 μL internal standard working solution (250 ng/mL cephapirin) and 50 μL 3 M hydrochloric acid were added, and then the mixture was vortexed for 30 s. For the solid-phase extraction (SPE), the SPE cartridge was pre-treated with 1 mL methanol, followed by 1 mL deionized water. The sample mixture was gently loaded onto the SPE cartridge and left for 30 s. The SPE cartridge was washed with 1 mL deionized water and then dried for 60 s before elution with 1 mL methanol. The eluate was collected in a clean microtube and was evaporated to dryness at 40 °C under a stream of nitrogen. The dry residue was reconstituted with 100 μL mobile phase A (0.05% acetic acid in deionized water/acetonitrile = 85/15, v/v) and centrifuged at 13,000 rpm for 10 min. The clear supernatant was transferred to an autosampler vial, and 20 μL of the supernatant was injected into the LC–MS/MS system.

2.5. LC–MS/MS operation conditions

The chromatographic separation was performed on a Capcell-Pak C18 AQ type (2.0 mm × 150 mm, particle size 5 μm, Shiseido, Kyoto, Japan) at a column temperature of 40 °C. The mobile phase consisted of solvent A (0.05% acetic acid in deionized water/acetonitrile = 85/15; v/v) and solvent B (acetonitrile), filtered through a 0.22 μm membrane filter. A flow rate of 250 μL/min was used for the sample analysis. The gradient elution was, as follows: 100% solvent A for 1.0 min, then the solvent B percentage was gradually increased to 30% over 2.0 min and maintained for 3.0 min, then reequilibrated to the initial conditions over 0.1 min and maintained for 10 min. The temperature of the autosampler was kept at 4 °C. Detection was performed with triple quadruple tandem mass spectrometry in the positive ion mode using multiple-reaction monitoring (MRM). Nitrogen was used as a nebulizer and drying gas, and argon was used as a collision gas. The desolvation temperature was set at 400 °C, and the source temperature was 100 °C. The desolvation gas flow was 800 L/h, and the cone gas flow was 50 L/h. Multiple-reaction monitoring of the precursor-product ion transitions were m/z 635.7 → m/z 607.5 for goserelin and m/z 424.0 → m/z 292.1 for cephapirin. Precursor-product ion mass spectra for each analyte and cephapirin (I.S.) are shown in Figs. 2 and 3. MS/MS parameter settings were, as follows: capillary voltage at 3.5 kV; cone voltage at 22 V; extractor at 4.0 V; RF lens at 0.3 V; collision cell entrance potential at −2.0 V; collision energy at 18 eV; collision cell exit potential at 1.0 V; multiplier at 650 V; and dwell time was set to 0.2 s.

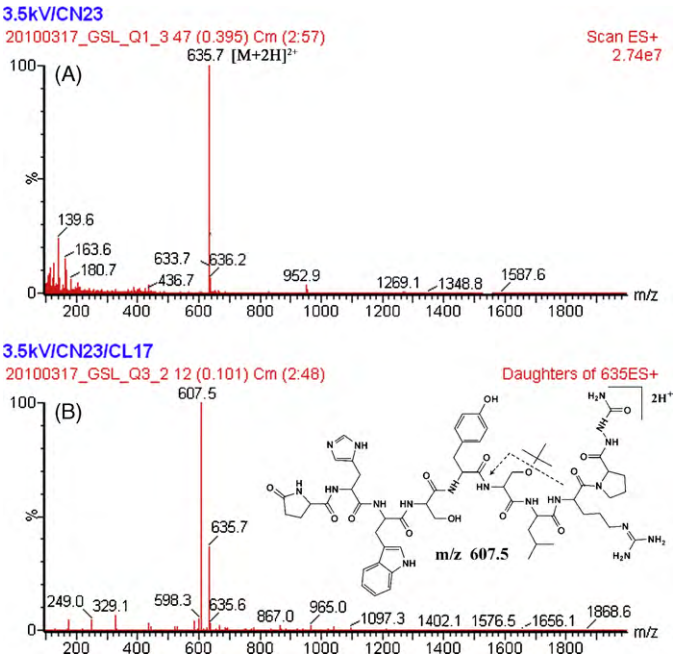

Fig. 2. Precursor ion (A) and product ion (B) scan spectra of goserelin produced by. LC–ESI-MS/MS.

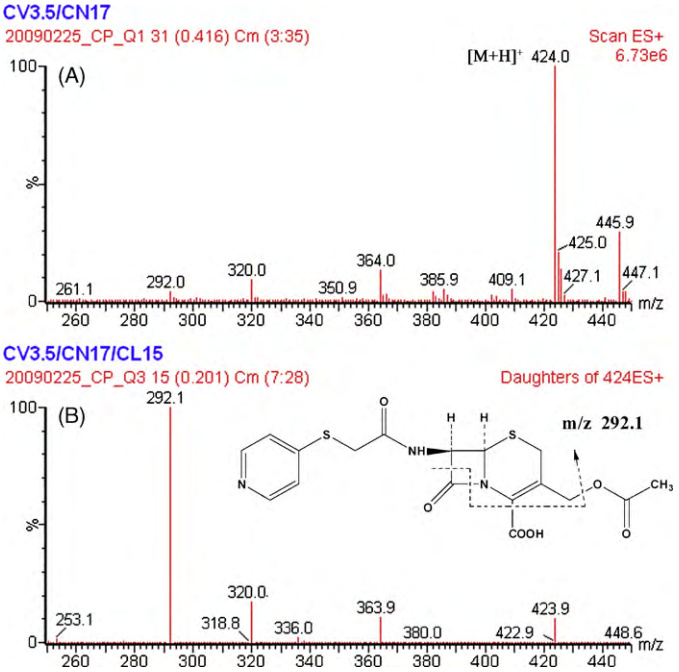

Fig. 3. Precursor ion (A) and product ion (B) scan spectra of I.S. (cephapirin) produced by LC–ESI-MS/MS.

2.6. Validation of the LC–MS/MS method

In this study, the developed method was validated in terms of specificity, linearity, limit of quantification (LOQ), precision, accuracy, recovery, and stability according to the guidelines of the Food and Drug Administration (FDA) for the validation of bioanalytical methods [20,21]. The specificity of the method was investigated by screening six different batches of blank rabbit plasma samples. Double blank samples (processed without internal standard) and blank samples (processed with internal standard only) were prepared and tested for confirmation that endogenous compo-

nents did not interfere with the analyte and the internal standard. Calibration curves were constructed by plotting the peak area ratio of goserelin to internal standard against the analyte concentration. The intra- and inter-day precision and accuracy were determined by analyzing five replicates of QC samples at five different concentrations (0.1, 1, 5, 10, and 20 ng/mL) within one day or over five consecutive days. Accuracy was expressed as the percentage of observed value to true value, and precision was expressed as the relative standard deviation (coefficient of variance, CV). The LOQ was determined as the concentration in which the signal-to-noise (S/N) ratio was greater than 10, with a precision of <20% and an accuracy between 80% and 120% for both intra- and inter-day assays. The recovery of an analyte in the assay is the detector response obtained from the amount of analyte added to and extracted from the biological matrix, as compared to the detector response obtained for the true concentration of the pure authentic standard. In this study, recovery was determined by analyzing QC samples with three different concentrations (0.5, 5, and 20 ng/mL) and by comparing the peak area ratios of goserelin and internal standard in the pre-extraction and the post-extraction spiked samples. Six replicates were measured at each concentration level to determine the extraction recovery. The stability of goserelin in rabbit plasma was also tested using various conditions for the plasma sample handling process and sample storage with QC samples at three concentrations (0.5, 5, and 20 ng/mL). The protocol for the stability assay included freeze–thaw stability, short-term and long-term temperature stability, and stock solution and autosampler stability. Freeze–thaw stability was assessed after three freeze and thaw cycles. Short-term temperature stability was tested using QC samples kept at ambient temperature for 6 h. Long-term temperature stability (−78 °C in plasma) was checked using QC samples kept for 35 days. Stock solution stability was evaluated after being kept at 25 °C for 24 h. Autosampler stability was determined from QC samples kept at the autosampler temperature (10 °C) for 12 h. All stabilities were calculated as the ratio of average concentration of QC samples and freshly prepared QC samples ($n = 3$).

2.7. Application of the method to a pharmacokinetic study in rabbits

New Zealand white rabbits (13 males, 11 females) were used in the pharmacokinetic experiments. Rabbits were housed in individual cages with free access to food and water in a room with automatically controlled illumination (a 12 h light–dark cycle). Conscious New Zealand white rabbits were used after division into two groups (G1 and G2). The G1 group (five males, three females) received a reference formulation 3.6 mg dose, and the G2 group (eight males, eight females) received a DKF301 formulation (test drug) 3.6 mg dose by subcutaneous injection. Before and at 0.25, 1, 2, 4, 7, 14, 21, 28, 33, and 35 days after injection, 8-mL venous blood samples were collected in heparinized tubes. Blood samples were centrifuged at 3,000 rpm for 10 min (4 °C) to separate the upper plasma and stored at −70 °C until analysis.

3. Results and discussion

3.1. Optimization of the sample preparation

Although mass spectrometry is a powerful technique for the selective determination of peptides in a complex biological matrix, bioanalytical sample preparation is still an essential step in the analysis process. Unless appropriate sample preparation steps are taken, a number of matrix components can cause contamination and ion suppression in the ionization source. Protein precipitation (PPT), liquid–liquid extraction (LLE), and SPE are the most common techniques used for the extraction of peptides from biological samples [22].

The first study for the determination of goserelin in rat plasma, reported by Michalet et al. [19], used the protein precipitation method. However, several matrix components co-eluting with goserelin had the same precursor–product ion transition and could not be distinguished from goserelin by triple stage quadrupole mass spectrometry. Therefore, a high-resolution Q-TOF instrument was used to perform all analyses. Initially, we also tested a protein precipitation method for goserelin quantification from rabbit plasma samples, but we observed poor recovery, peak distortion, and strong ion suppression from the remaining plasma matrix components. Hence, solid-phase extraction was chosen as an alternative sample preparation method. C18-reverse phase silica is the most commonly applied SPE sorbent for the separation of peptides in biological fluids [22]. Goserelin was obtained from the rabbit plasma matrix with good recovery and selectivity with an Oasis® HLB cartridge.

In particular, the loss of peptides due to adsorption to the surfaces of the experimental apparatus polymer and glass, including tubes, pipette tips, SPE cartridges, vials and parts of the LC–MS/MS system [23–27], is considerable during the sample preparation and LC–MS/MS analysis. Peptide adsorption to container surfaces depends on the specific amino acid side chains that make up the peptide. Goserelin is a decapeptide that contains histidine (His) and arginine (Arg), basic amino acids, and tryptophan (Trp), leucine (Leu), proline (Pro), and serine *tert*-butyl ester, nonpolar amino acids, in its structure. The presence of the two basic amino acids can induce electrostatic interactions with glass surface silanol groups. In addition, the nonpolar side chains of goserelin may produce hydrophobic interactions with the polymer surface of plastic containers.

To reduce the adsorptive loss of goserelin, 50 μL 3 M HCl was added [24,28] to 500 μL rabbit plasma before loading the samples onto SPE cartridges. As a result, the recovery could be improved to over 90%.

3.2. Optimization of the HPLC conditions

Column packing materials strongly influence the separation and sensitivity of an HPLC method. Many types of stationary phases have been used to separate peptides, including C18- and C8-reverse

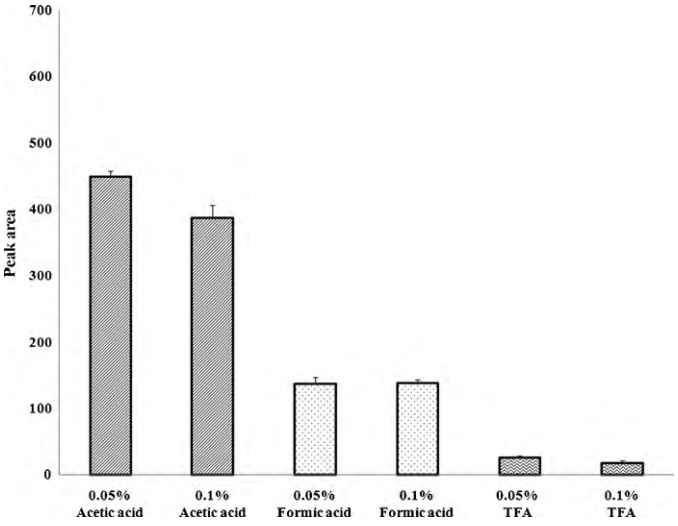

Fig. 4. Comparison of goserelin peak areas (10 ng/mL) using different mobile phases additives.

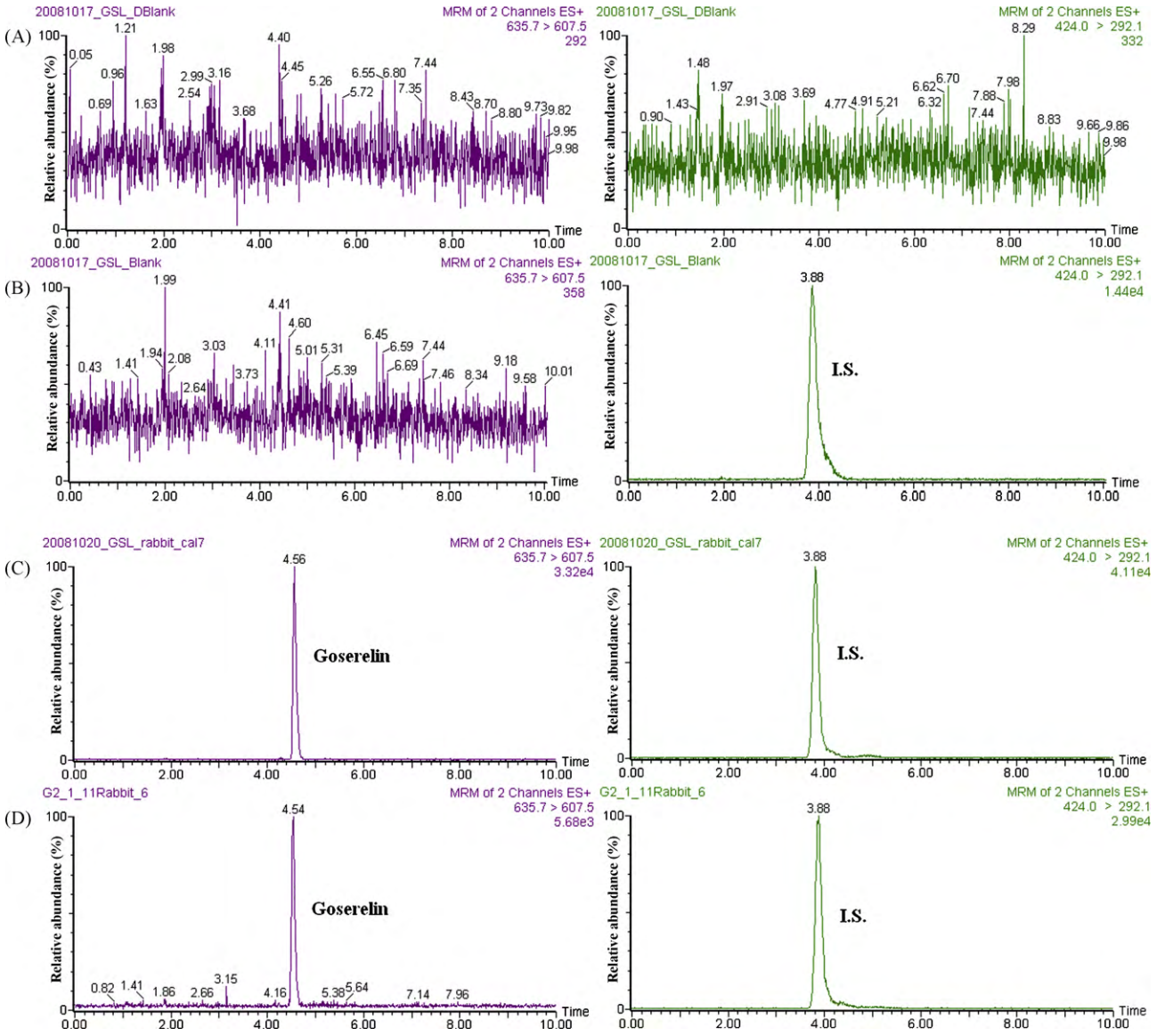

Fig. 5. Multiple-reaction monitoring chromatograms of (A) double blank rabbit plasma, (B) blank rabbit plasma, (C) goserelin (20 ng/mL) plasma standard with 50 μL of I.S. (cephapirin, 250 ng/mL) and (D) plasma sample 14 days after subcutaneous injection of 3.6 mg of goserelin to rabbit G2-11 (calculated concentration was 3.98 ng/mL).

phase, CN phase, phenyl phase, and hydrophilic-interaction liquid chromatography (HILIC) [29]. Generally, C18-reverse phase has been considered the gold standard in peptide analysis due to its simplicity and ruggedness. For the analysis of highly basic peptides using silica-based stationary phases, however, end-capped materials have been used to avoid peak tailing.

In the present study, several C18-reverse phase columns, including Capcell-Pak C18 AQ (2.0 mm × 150 mm, 5 μm), Atlantis dC18 (2.1 mm × 150 mm, 3 μm), YMC-pack Pro C18 R5 (2.0 mm × 100 mm, 3 μm), Thermo Hypersil GOLD (2.1 mm × 150 mm, 5 μm), Waters Xbridge C18 (2.1 mm × 100 mm, 3.5 μm) and Waters XTerra MS C18 (2.1 mm × 150 mm, 5 μm) were compared on the basis of peak separation and sharpness in order to evaluate column performance (data not shown). The Capcell-Pak C18 AQ column, which was designed and developed to retain highly polar compounds and enable usage in 100% water by varying the type and amount of silicone polymer coating and functional groups, exhibited the most satisfactory performance in chromatographic separation and sensitivity. Thus, this column was selected as the stationary phase for the determination of goserelin

Table 1
Precision and accuracy data for the LC–MS/MS analysis of goserelin in rabbit plasma.

Spiked quantity (ng/mL)	Intra-day			Inter-day		
	Measured quantity (ng/mL)	Accuracy (%)	RSD	Measured quantity (ng/mL)	Accuracy (%)	RSD
0.1	0.09	86.5	18.8	0.09	86.3	16.8
1	0.90	86.3	14.5	0.95	95.1	9.7
5	4.50	89.8	14.1	4.55	90.9	9.9
10	8.61	86.1	7.7	9.70	97.0	13.9
20	17.78	88.9	8.8	19.24	96.2	13.5

in rabbit plasma. A double peak for goserelin was observed on some of the other columns, and a similar result was reported in a leuprolide analysis [30]. However, further studies are needed to fully investigate this peak splitting phenomenon with specific columns and conditions.

The separation of peptides is strongly influenced by acidic additives in the mobile phase, trifluoroacetic acid (TFA), acetic acid, and formic acid, which act as ion-pairing agents and pH modifiers [31,32]. Since these acids increase the hydrophobicity of a peptide by forming ionic pairs and denature the peptide to a single molecular conformation, the interaction of a peptide with the hydrophobic stationary phase is improved, which results in better chromatographic separation and sharper, more symmetrical peaks. Nevertheless, these ion-pairing reagents suppress the ion formation of peptides in the ESI source, which has been attributed to the charge neutralizing effect of ion-pair formation.

Therefore, we evaluated the effects of TFA, acetic acid, and formic acid on the chromatographic separation and ion suppression of goserelin using two concentrations (0.05% and 0.1%) of each acid. In this study, the type and concentration of the acids in the mobile phase did not greatly affect chromatographic separation. However, TFA formed strong ion pairs with goserelin, which not being degraded in the ionization source could lead to suppression of goserelin ionization and reduced detection sensitivity [33,34]. Formic and acetic acids showed less signal suppression in the ESI source than TFA. Nevertheless, acetic acid gave better sensitivity than formic acid, and 0.05% acetic acid gave better sensitivity than 0.1% acetic acid (Fig. 4).

Post-column addition of highly concentrated weak acids and organic solvents (dioxane, 2-propanol, acetonitrile, and methanol) has been attempted to overcome the suppressive effect of ion-pairing reagents; however, contrary to theoretical expectation and Sanz-Nebot et al.'s reports [12,13], this did not improve the signal intensity.

As described in Section 2.5, the gradient elution profile was optimized in order to obtain shorter run times, to separate matrix components, and to maintain a minimal peak width. Accordingly, goserelin and the internal standard were fairly resolved with a gradient elution within 5 min (Fig. 5).

3.3. Optimization of the MS/MS parameters

Currently, several methods have been reported for the qualitative analysis of goserelin in a crude synthetic mixture using capillary zone electrophoresis (CZE)-MS [15] and LC–ESI-MS [11–14] and for its quantification in rat plasma using LC-Q-TOF [19]. However, due to long run times, low sensitivity, and insufficient method validation and sample preparation of these methods, a highly sensitive, selective, and rapid tandem mass spectrometry method (MS/MS) is necessary to quantify goserelin in biological fluids for pharmacokinetic studies.

Quantitative MS analysis of peptides is mostly performed by ESI instead of APCI because ESI is easier to use and more sensitive than APCI [32]. Goserelin has two basic amino acids, which are readily protonated; therefore, positive ion detection mode improves sensitivity to goserelin. The intensity of fragment ions of goserelin was dependent on the mass spectrometry ionization parameters and could be affected by the formation of charged states. Parameters, such as desolvation temperature, gas flow, capillary voltage, cone voltage [12], RF lens voltage, and collision energy, were optimized to obtain the highest signal response for the precursor and product ions of goserelin. In particular, RF lens voltage affected the goserelin signal intensity. The optimal RF lens voltage for goserelin and internal standard was found to be 0.3 V. Electrospray ionization of peptides in the positive ion mode produces multiply charged ions of the type $[M+nH]^{n+}$.

According to the review of van den Broek et al. [32], $[M+H]^+$ or $[M+2H]^{2+}$ were generally selected as the precursor ion of peptides with molecular weights below about 2000 Da, whereas $[M+3H]^{3+}$ or $[M+4H]^{4+}$ were measured for 2000–4000 Da peptides. For measuring goserelin, the double protonated molecule ($[M+2H]^{2+}$) at m/z 635.7 was selected as the precursor ion (most abundant ion) for MS1, and the product ion at m/z 607.5 was selected for MS2 (Fig. 2). This preference for the $[M+2H]^{2+}$ ion could be explained by the presence of the two basic amino acids, His and Arg, in the peptide. Cephapirin (internal standard) produced a protonated precursor ion ($[M+H]^+$) at m/z 424.0 and a major product ion at m/z 292.1 (Fig. 3). The $[M+H]^+$ ion at m/z 1269.1 was less than 1% of the relative abundance of $[M+2H]^{2+}$.

3.4. Validation of the developed analytical method: specificity, linearity, limit of quantification (LOQ), intra- and inter-day precision and accuracy, recovery, and stability

The proposed method was validated by assessment of its specificity, linearity, LOQ, intra- and inter-day precision as well as accuracy, recovery and stability. The specificity of the method was determined by comparing the chromatograms of blank rabbit plasma with standard-spiked rabbit plasma. Goserelin and cephapirin eluted at about 4.5 and 3.9 min, respectively, and were detected without apparent interference from endogenous components of the rabbit plasma (Fig. 5).

Good linearity was obtained at concentration ranges of 0.1–20 ng/mL for goserelin in rabbit plasma, as demonstrated by the high correlation coefficient (r^2) value above 0.99. Linear regression was performed using the weighting factor ($1/x$) because of the wide calibration range. The LOQ was 0.1 ng/mL.

Precision and accuracy were assessed from replicated experiments ($n=5$) at five different concentrations, 0.1, 1, 5, 10, and 20 ng/mL, of the QC samples. Table 1 shows the results for intra- and inter-day precision and accuracy. The intra-day precision ranged from 7.7% to 18.8%, and the inter-day precision ranged from 9.7% to 16.8%. The intra-day accuracy was 86.1–89.8%, and the inter-day accuracy was 86.3–97.0%. For the recovery, three concentration levels (0.5, 5 and 20 ng/mL) of goserelin were evaluated from replicated experiments ($n=6$). Extraction recovery by the established method was 90.8–101.2%. Total recoveries with all three sample concentrations were greater than about 90%. The results are shown in Table 2.

Chemical and biological degradation of peptides is caused by oxidation, reduction, and hydrolysis of amino acid residues and enzyme hydrolysis [18,35]. To overcome enzymatic degradation of gonadorelin, the C-terminal glycinamide moiety is replaced by an azaglycinamide moiety to give goserelin, which has an increased human plasma half-life [35]. Nevertheless, this azaglycinamide moiety is chemically unstable in neutral and alkaline solutions, and elimination of *tert*-butyl moiety of the *O*-*tert*-butyl-D-serine residue is favored under acidic conditions (Fig. 2) [18,35]. Therefore, the stability of goserelin was evaluated by analyzing QC samples (0.5, 5, and 20 ng/mL) under different storage and analytical processing conditions.

Table 2
Recovery of goserelin in rabbit plasma ($n=6$).

Spiked quantity (ng/mL)	Measured quantity (ng/mL)	Recovery (%)	
		Mean	RSD
0.5	0.45	90.8	0.9
5	5.06	101.2	5.5
20	19.16	95.8	2.4

Table 3
Results for determination of goserelin stability ($n = 3$).

Concentration (ng/mL)	Stability (%)				
	Freeze–thaw stability	Short-term temperature stability	Long-term stability	Stock solution stability	Stability of samples in autosampler
0.5	86.1	84.4	94.5	114.1	98.8
5	108.0	99.5	103.7	109.3	96.3
20	95.0	107.3	102.3	112.5	96.6

As shown in Table 3, goserelin was generally stable under the plasma storage and analytical process conditions used throughout the study.

3.5. Application of the method to a pharmacokinetic study in rabbits

Using the liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) method described in this paper, we were able to measure plasma concentrations of goserelin for a pharmacokinetic study in rabbits (13 males and 11 females) after subcutaneous injection (3.6 mg) of a reference formulation and DKF301 formulation. Blood samples were taken at specific times, and samples were analyzed by this LC–MS/MS method. Concentrations of goserelin in plasma samples were calculated using the peak area ratios (peak area of analyte/peak area of I.S.) and regression equations of the calibration curves. The mean goserelin plasma concentrations versus time profiles are shown in Fig. 6.

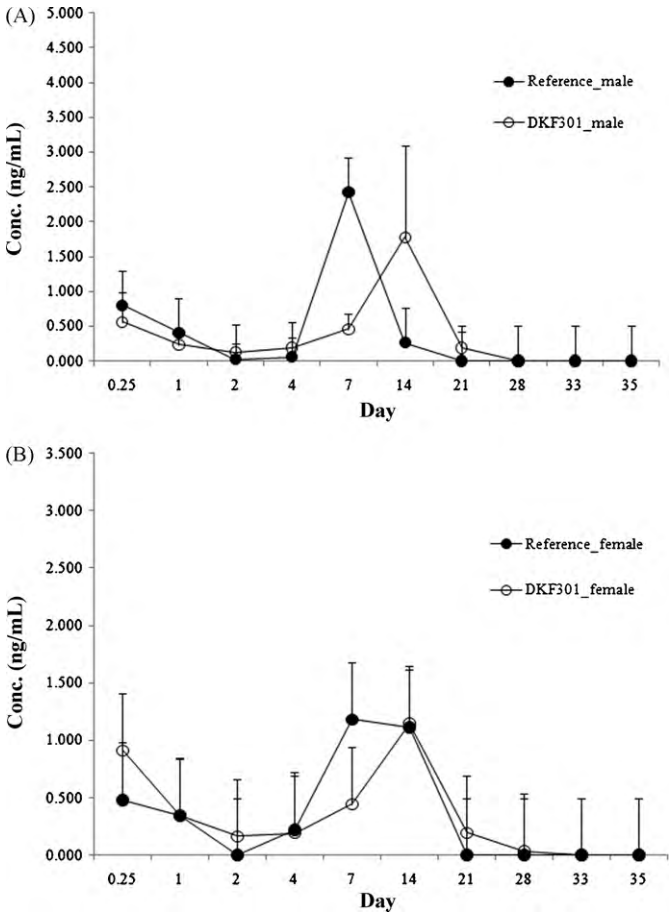

Fig. 6. Mean (±SD) plasma concentration-time curves of goserelin in (A) male and (B) female rabbits following subcutaneous injection of reference drug (●) and DKF301 drug (○) with a 3.6 mg goserelin dose.

4. Conclusions

A rapid and sensitive LC–ESI-MS/MS method for the determination of goserelin in rabbit plasma was developed and validated. Plasma samples were acidified using hydrochloric acid and were then loaded onto SPE cartridges, which were used to isolate and concentrate the analyte. As a result of this improved sample preparation, the adsorptive loss of goserelin and interference by endogenous components of plasma samples were reduced. Under the optimized HPLC and MS/MS conditions, goserelin and an internal standard were well separated, and sharp peaks were obtained with high sensitivity within 5 min. Finally, the established method was suitable for the determination of goserelin in rabbit plasma and was satisfactorily applied to a pharmacokinetic study.

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