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Measurement of the novel decapeptide cetrorelix in human plasma and urine by liquid chromatography–electrospray ionization mass spectrometry

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Abstract

A sensitive LC–MS quantitation method of cetrorelix, a novel gonadotropin releasing hormone (GnRH) antagonist, was developed. Plasma and urine samples to which brominated cetrorelix was added as an internal standard (I.S.) were purified by solid-phase extraction with C_8 cartridges. The chromatographic separation was achieved on a C_{18} reversed-phase column using acetonitrile–water–trifluoroacetic acid (35:65:0.1, v/v/v) as mobile phase. The mass spectrometric analysis was performed by electrospray ionization mode with negative ion detection, and the adduct ions of cetrorelix and I.S. with trifluoroacetic acid were monitored in extremely high mass region of m/z 1543 and 1700, respectively. The lower limit of quantitation was 1.00 ng per 1 ml of plasma and 2.09 ng per 2 ml of urine, and the present method was applied to the analysis of pharmacokinetics of cetrorelix in human during phase 1 clinical trial.

Keywords: Cetrorelix

1. Introduction

Cetrorelix ([Ac-D-Nal[1]-(p-Cl)-D-Phe[2]-D-Pal[3]-Ser[4]-Thy[5]-D-Cit[6]-Leu[7]-Arg[8]-Pro[9]-D-Ala[10]-NH_2], Fig. 1) is the decapeptide synthesized by Bajusz et al. [1], which exhibits a potent antagonistic activity of gonadotropin releasing hormone (GnRH) [2]. Cetrorelix is now under clinical trial as a therapeutic agent for gynecological diseases such as hysteromyoma. During the course of our pharmacokinetic studies on cetrorelix, establishing a sensitive analytical method for cetrorelix in biological samples is an essential prerequisite.

Plasma level of cetrorelix has been investigated by radioimmunoassay (RIA) [3]. However, it is not clarified yet whether present RIA method can discriminate the unchanged molecule from other metabolized peptide fragments or not. Considering increasing requirements for analytical methods validation [4], we had planned to develop a physicochemical analytical method, which could provide better selectivity for unchanged cetrorelix.

Recently, Raffel et al. [5] have developed a high-performance liquid chromatographic (HPLC) method

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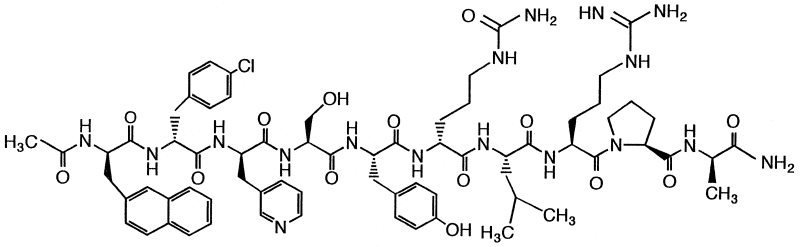

Fig. 1. Chemical structure of cetrorelix.

for the quantitation of cetrorelix in human plasma. Although the lower limit of quantitation of this HPLC method was as low as 2 ng/ml with 4 ml of plasma, the analytical method that utilizes less plasma is favorable for therapeutic monitoring. On the other hand, the assay range of RIA reported by Csernus et al. [3] was 0.032–12.8 ng/ml with 0.02 ml serum.

We then attempted to enhance the sensitivity in the determination of cetrorelix utilizing electrospray ionization (ESI)–MS. Up to now the LC–MS method for GnRH analogs and their degradation products with thermospray [6] and electrospray [7] ionizations were reported, but few attempts were made to apply ESI–MS to therapeutic monitoring of GnRH analogs. We report here a sensitive and specific LC–ESI–MS method combined with solid-phase extraction. Rapid assay of cetrorelix by the LC–MS method would be of particular interest for the therapeutic monitoring and pharmacokinetic studies of cetrorelix in humans.

2. Experimental

2.1. Materials

Cetrorelix acetate salt was supplied by ASTA Medica (Frankfurt, Germany). Sep-Pak Vac C_8 cartridge was purchased from Waters corporation (Milford, MA, USA). Hydrophilic PTFE filter, Ultrafree-MC/LCR was purchased from Nihon Millipore Ltd. (Tokyo, Japan). Other solvent and reagents were of analytical or HPLC grade.

2.2. Synthesis of the internal standard

Brominated cetrorelix was synthesized from cetrorelix acetate and bromine. Ten mg of cetrorelix acetate was dissolved in 1 ml of glacial acetic acid. To this solution 0.015 ml of bromine in acetic acid (10%, w/v) was added slowly while stirring at room temperature. After stirring for 1 h, the resulting mixture was evaporated to dryness under nitrogen stream to remove excess reagent. The residue was dissolved in glacial acetic acid and stored at 4°C.

2.3. LC–MS

The chromatographic system consisted of a Model PU-980 pump (Jasco, Tokyo, Japan) and a Model 7123 sample injector (Rheodyne, Rohnert Park, CA, USA) was employed. Chromatographic separation was achieved on a μ-Bondasphere C_{18} column (Nihon Waters K. K., Tokyo, Japan, 150 mm length, 2.1 mm I.D., 5 μm particle size, 300 Å pore size). The mobile phase was composed of acetonitrile–water–trifluoroacetic acid (35:65:0.1, v/v/v). The mobile phase was degassed before use and its flow-rate was 0.2 ml/min.

A Platform single stage quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ion source and a gas nebulizer probe was employed. Nebulizer and drying gas were nitrogen, and delivered at flow-rates of 30 and 300 l/h, respectively. The source temperature was maintained at 120°C. Capillary voltage was set at 3 kV.

2.4. Solutions

Cetrorelix stock solution was prepared by dissolving cetrorelix acetate salt into HPLC mobile phase, and the concentration was 1 mg/ml. Cetrorelix working solutions were prepared by diluting stock solution with HPLC mobile phase to appropriate concentrations to add to plasma or urine. Cetrorelix stock solution was stable for at least 9 months at −20°C. I.S. working solutions were prepared by

diluting synthesized I.S. (stored in acetic acid) with HPLC mobile phase to 0.02 mg/ml.

2.5. Extraction procedures

To 1 ml of human plasma spiked with cetrorelix acetate (in 0.02 ml working solution), 0.01 ml of I.S. working solution was added. The resulting plasma was vortex-mixed, diluted with methanol–water (10:90, v/v, 10 ml) and then passed through Sep-Pak Vac C_8 cartridge (125 Å pore size, 500 mg), previously activated with 20 ml of methanol and rinsed with 20 ml of water. The cartridge was washed with methanol–water (10:90, v/v, 10 ml and 50:50, v/v, 10 ml), and then I.S. and cetrorelix were eluted with methanol–water–trifluoroacetic acid (90:10:0.1, v/v/v, 10 ml). The eluate was evaporated to dryness under the reduced pressure. The residue was dissolved in mobile phase (0.1 ml) and vortex-mixed. The mixture was transferred to Ultrafree-MC/LCR (0.2 μm pore size, 0.2 cm^2) and filtered by centrifuge at 3500 *g* for 20 min at 4°C. A 0.04 ml filtrate was injected onto the column.

To 2 ml of human urine spiked with cetrorelix acetate (in 0.05 ml working solution), 0.2 ml of blank human plasma was added. After addition of 0.01 ml of I.S. working solution to the urine, the resulting mixture was diluted with methanol–water (10:70, v/v, 8 ml) and transferred onto Sep-Pak Vac C_8 cartridge. The cartridge was washed with methanol–water (10:90, v/v, 10 ml and 50:50, v/v, 10 ml), and I.S. and cetrorelix were eluted with methanol–water–trifluoroacetic acid (90:10:0.1, v/v/v, 10 ml). Then the collected fraction was dried, dissolved in mobile phase and filtered as mentioned in regard to plasma.

3. Results and Discussion

3.1. LC–MS

Fig. 2 shows the ESI mass spectra of cetrorelix and I.S. Intense and characteristic peaks at *m/z* 1429, 1543 and 1657 for cetrorelix and 1586, 1700 and 1813 for I.S. were observed. The ion *m/z* 1429 was considered as the quasi-molecular ion $[M-H]^-$ for cetrorelix, because the accurate monoisotopic mass calculated for $[M-H]^-$ was 1428.6620. The ion observed at *m/z* 1543 for cetrorelix was estimated to be $[M+CF_3COOH-H]^-$. By comparing mass spectra of I.S. and cetrorelix, I.S. was identified as cetrorelix dibromide, based on the suggested molecular weight difference of approximately 158Da. The ion *m/z* 1429 was considered as the quasi-molecular ion $[M-H]^-$ for I.S., because the accurate monoisotopic mass calculated for $[M-H]^-$ was 1585.4908. The ion observed at *m/z* 1700 for I.S. was considered to be the second isotopic ion of $[M+CF_3COOH-H]^-$.

As the sample cone voltage increased, these adduct ions were expected to converge to quasi-molecular ions by collision with atmospheric gas molecule, in low vacuum region of the mass spectrometer. Whereas even at 120 V of cone voltage, complete convergence was not observed (Fig. 3), suggesting strong interaction between decapeptide and trifluoroacetic acid molecules. Considering that an adduct ion of $[M+CF_3COOH-H]^-$ at *m/z* 1543 with cetrorelix was the most stable ion around 90 V of cone voltage, *m/z* 1543 and 1700 were chosen as monitoring ions at 90 V of cone voltage.

Fig. 4 shows the typical selected ion recordings of extracts obtained from human blank plasma and plasma spiked with 200 ng of I.S. and 5.00 ng of cetrorelix acetate. Fig. 5 shows the selected ion recordings of extracts obtained from human blank urine and urine spiked with 200 ng of I.S. and 10.5 ng of cetrorelix acetate. As is obvious from those recordings, no interference peaks for cetrorelix and I.S. were observed and the assay specificity was confirmed.

3.2. Calibration curve

Calibration standards were prepared in the levels of 0, 1.00, 2.00, 5.00, 10.0, 15.0 and 20.0 ng for plasma and 0, 2.09, 5.23, 10.5, 20.9, 52.3 and 104.7 ng for urine. The calibration curve was obtained by the least-squares analysis of cetrorelix acetate amounts added per tube versus peak area ratios of cetrorelix to I.S. The parameters of calibration curves obtained throughout the study (expressed as mean±SD) were as follows: A (slope); 0.0117004±0.0029546, B (intercept); 0.0069344±0.0102659 and r (correlation coefficient); 0.99815±0.00092 for plasma ($n=6$, $n=1$ per day), and A; 0.0097720±0.0012877, B;

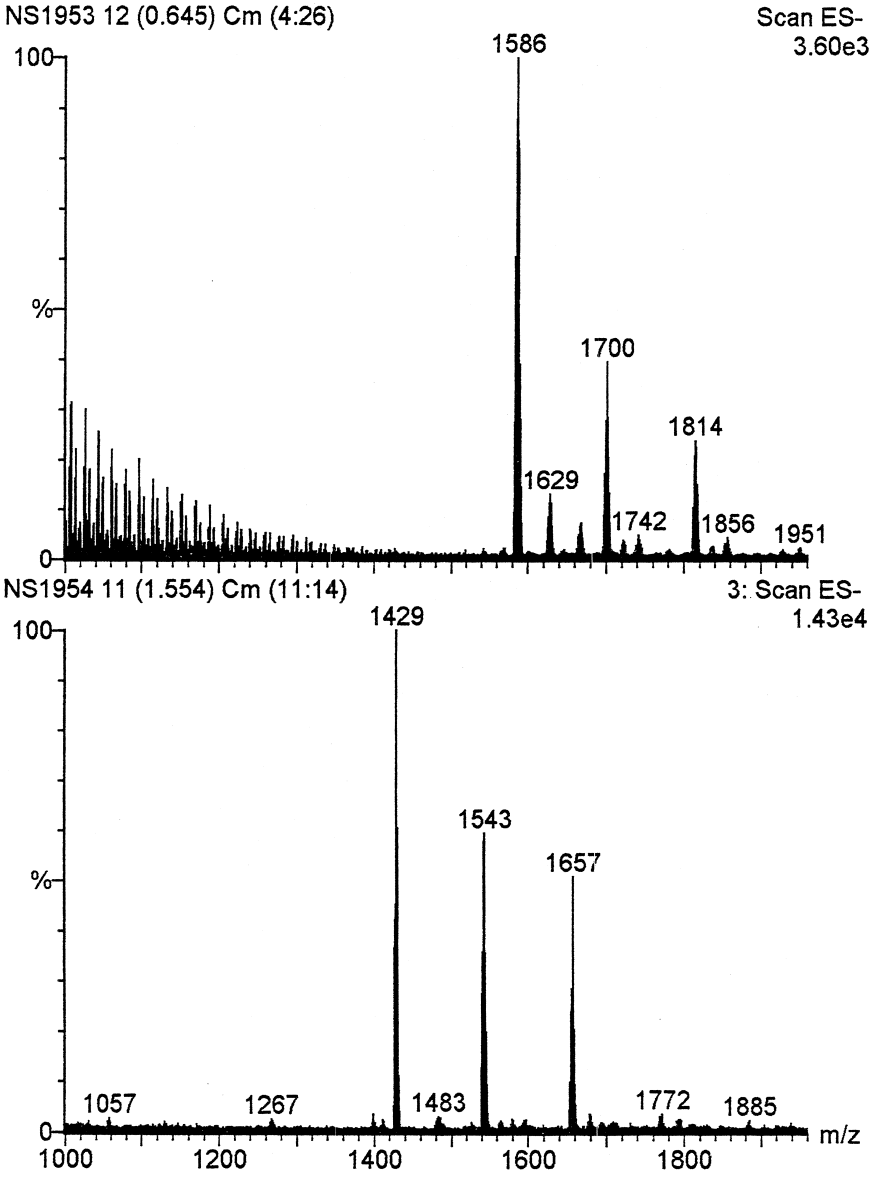

Fig. 2. Electrospray-negative mass spectra of I.S. (800 ng, upper) and cetrorelix (400 ng, lower). The solvent was acetonitrile–water–trifluoroacetic acid (35:65:0.1, v/v/v) and the sample cone voltage was 90V. Ions observed with I.S. at *m/z* 1586, 1700 and 1813 correspond to $[M\text{-}H]^-$, $[M+CF_3COOH\text{-}H]^-$ and $[M+2CF_3COOH\text{-}H]^-$, respectively. Ions observed with cetrorelix at *m/z* 1429, 1543 and 1657 correspond to $[M\text{-}H]^-$, $[M+CF_3COOH\text{-}H]^-$ and $[M+2CF_3COOH\text{-}H]^-$, respectively.

0.0097543±0.0035426 and r; 0.99887±0.00111 for urine ($n=6$, $n=1$ per day). The correlation coefficients for both plasma and urine indicate good linearity. The backextrapolated value of each calibration point was calculated to investigate on the reproducibility of the calibration curve (Tables 1 and 2). As the RSD did not exceed 15% except for the lowest standard level (15.1% at plasma 1.00 ng and

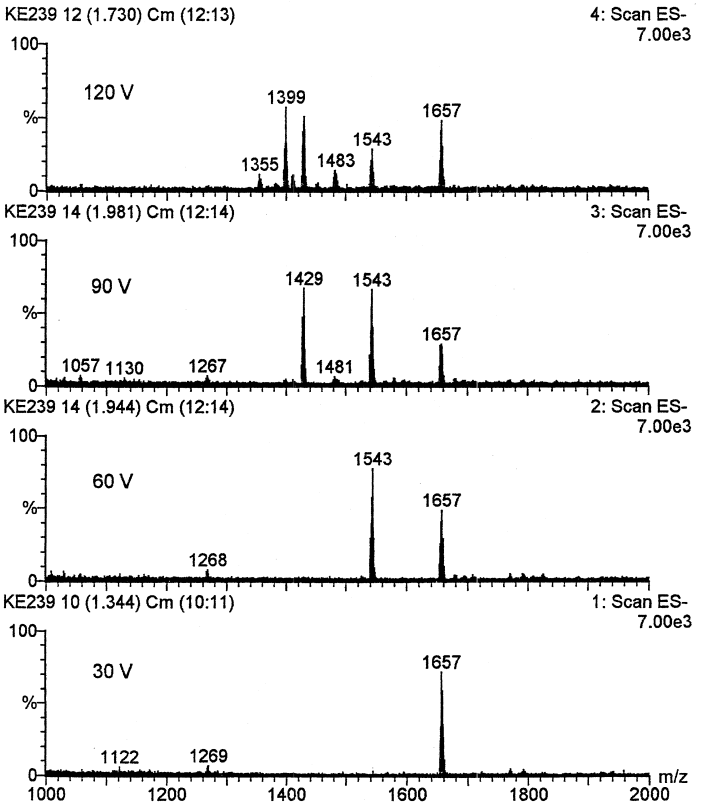

Fig. 3. Electrospray-negative mass spectra of cetrorelix (500 ng) with varied cone voltage (120, 90, 60 and 30 V, from top to bottom).

15.3% at urine 2.09 ng), the inter-day variation of the calibration curves were thought to be acceptable. Intra- and inter-day accuracy and precision are further discussed in Section 3.4.

3.3. Absolute recovery

The absolute recoveries of cetrorelix and I.S. in plasma and urine are shown in Tables 3 and 4, respectively. The recoveries of cetrorelix in both plasma and urine were constant over the range of the calibration curve. On the other hand, the recovery from 20 and 100 ng per 2 ml urine without addition of 0.2 ml plasma were 26.4±3.5% and 38.2±5.4% (n=5), respectively. The reason why the recovery from urine was improved by the addition of plasma has not been clarified, but considering that cetrorelix binds strongly with the Sep-Pak Vac C_8 cartridge when added without plasma (data not shown), the plasma ingredients might cover the undesired binding site of Sep-Pak Vac C_8, which could strongly interact with cetrorelix. The absolute recoveries of the I.S. in both plasma and urine were close to those of cetrorelix.

3.4. Accuracy and precision

The accuracy and precision of the present LC–MS method were examined by quantitating the amount of cetrorelix acetate added to human plasma and urine. Intra- and inter-day accuracy and precision of the quantitation in plasma are shown in Table 5. The intra-day RSD did not exceed 7.0%, and the bias of the mean did not exceed 14.0%. The inter-day RSD did not exceed 13.5%, and the bias of the mean did not exceed 4.0%. Intra- and inter-day accuracy and

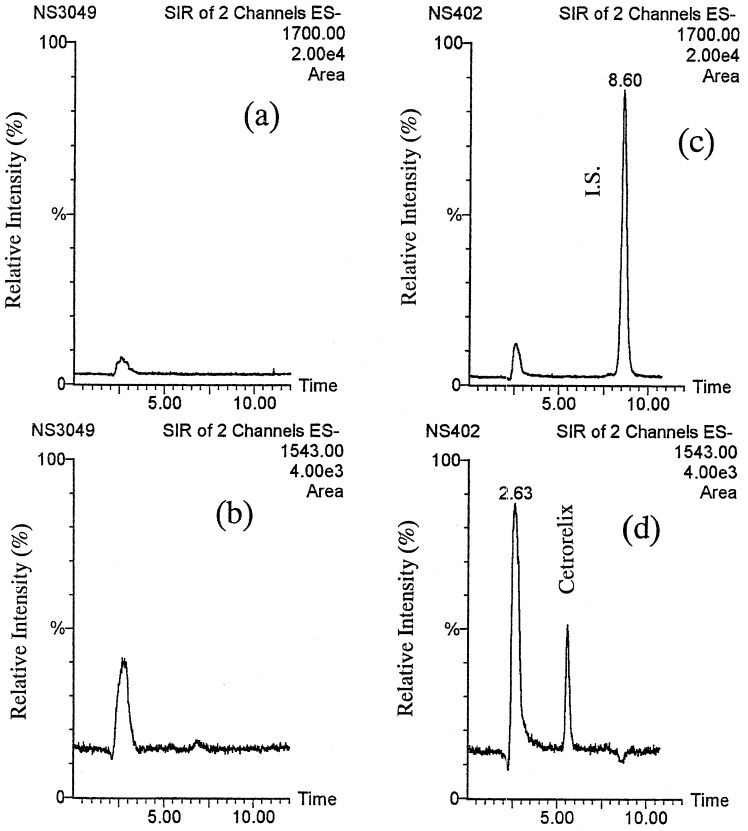

Fig. 4. Chromatogram of extracted human blank plasma ((a), (b)) and plasma spiked with 200 ng of the I.S. and 5.0 ng of cetrorelix ((c), (d)). (a) And (c) show SIR of m/z 1700, and (b) and (d) show SIR of m/z 1543, respectively.

precision of the quantitation in urine are shown in Table 6. The intra-day RSD did not exceed 14.6%, and the bias of the mean did not exceed 9.6%. The inter-day RSD did not exceed 13.8% except 17.1% at the lowest level (2.09 ng), and the bias of the mean did not exceed 13.4%. From these results, the accuracy and precision of the procedure were found to be satisfactory, because the RSD and the bias of the mean were within 15% except the lowest level and within 20% at the lowest level [4]. The lower limit of quantitation of cetrorelix was 1.00 ng per 1 ml of plasma, and 2.09 ng per 2 ml of urine. This LC–MS method proved to have two times higher sensitivity compared to the previous HPLC assay with fluorescence detection [5] with only 1 ml of plasma.

3.5. Application to the analysis of human plasma and urine

The method was applied to the analysis of the pharmacokinetics of cetrorelix in phase 1 clinical trials. Fig. 6 shows the change in plasma level of cetrorelix (expressed as free base) after a single subcutaneous administration of cetrorelix acetate to women volunteers at the dose of 3 mg (free base equivalent) per volunteer. The mean plasma level of cetrorelix reached the maximum of 25.8 ng/ml at 1 h after the administration and then decreased by bi-exponential manner with half-lives of 5.0 h (α-phase) and 25.7 h (β-phase). The plasma cetrorelix of all volunteers at 48 h after administration could be detected by the present method. The urinary excre-

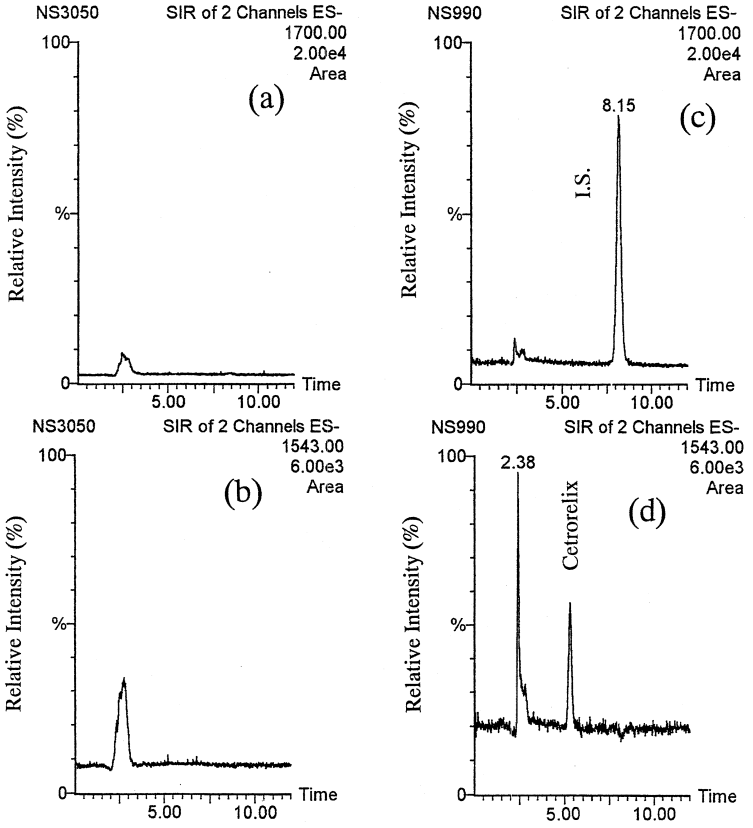

Fig. 5. Chromatogram of extracted human blank urine ((a), (b)) and urine spiked with 200 ng of the I.S. and 10.5 ng of cetrorelix ((c), (d)). (a) And (c) show SIR of m/z 1700, and (b) and (d) show SIR of m/z 1543, respectively.

Table 1
Reproducibility of backextrapolated values of calibration curves for human plasma ($n=6$)

Added (ng)	1.00	2.00	5.00	10.0	15.0	20.0
Found (ng)						
Mean	0.93	1.86	5.07	10.33	15.0	19.8
SD	0.14	0.26	0.50	0.70	0.5	0.5
RSD (%)	15.1	14.0	9.9	6.8	3.3	2.5

Table 2
Reproducibility of backextrapolated values of calibration curves for human urine ($n=6$)

Added (ng)	2.09	5.23	10.5	20.9	52.3	104.7
Found (ng)						
Mean	1.83	4.93	10.33	21.1	55.1	103.3
SD	0.28	0.37	0.93	1.3	2.6	1.1
RSD (%)	15.3	7.5	9.0	6.2	4.7	1.1

Table 3
Absolute recovery of cetrorelix and I.S. in plasma (1 ml)

Cetrorelix (n=4)		I.S. (n=12)	
Added (ng)	Absolute recovery (%) (mean±SD)	Added (ng)	Absolute recovery (%) (mean±SD)
1.00	68.1±11.1		
5.00	65.3±7.1	200	63.2±5.3
20.0	70.4±5.5		

Table 4
Absolute recovery of cetrorelix and I.S. in urine (2 ml)

Cetrorelix (n=4)		I.S. (n=16)	
Added (ng)	Absolute recovery (%) (mean±SD)	Added (ng)	Absolute recovery (%) (mean±SD)
2.09	63.4±6.4		
10.5	66.5±3.5	200	68.5±5.8
52.3	65.7±5.7		
104.7	67.9±7.6		

Table 5
Intra- and inter-day accuracy and precision for the quantitation of cetrorelix in human plasma

Added (ng)	Intra-day (n=4)			Inter-day (n=6; n=2 per day, 3 days)		
	Found (ng, mean±SD)	RSD (%)	Bias (%)	Found (ng, mean±SD)	RSD (%)	Bias (%)
1.00	1.14±0.08	7.0	14.0	0.96±0.13	13.5	−4.0
5.00	5.17±0.25	4.8	3.4	5.08±0.38	7.5	1.6
20.0	19.4±1.1	5.7	−3.0	19.5±1.3	6.7	−2.5

tion rate of cetrorelix in women volunteers was also investigated. The results were shown in Table 7. The mean cumulative excretion rate of cetrorelix up to 72 h after administration was 3.42% of dose, suggesting the extensive metabolism and/or biliary excretion of cetrorelix in human.

Table 6
Intra- and inter-day accuracy and precision for the quantitation of cetrorelix in human urine

Added (ng)	Intra-day (n=4)			Inter-day (n=6; n=2 per day, 3 days)		
	Found (ng, mean±SD)	RSD (%)	Bias (%)	Found (ng, mean±SD)	RSD (%)	Bias (%)
2.09	1.89±0.25	13.2	−9.6	1.81±0.31	17.1	−13.4
10.5	9.88±1.44	14.6	−5.9	10.07±1.39	13.8	−4.1
52.3	53.6±3.5	6.5	2.5	49.1±3.5	7.1	−6.1
104.7	101.3±12.5	12.3	−3.2	96.4±12.1	12.6	−7.9

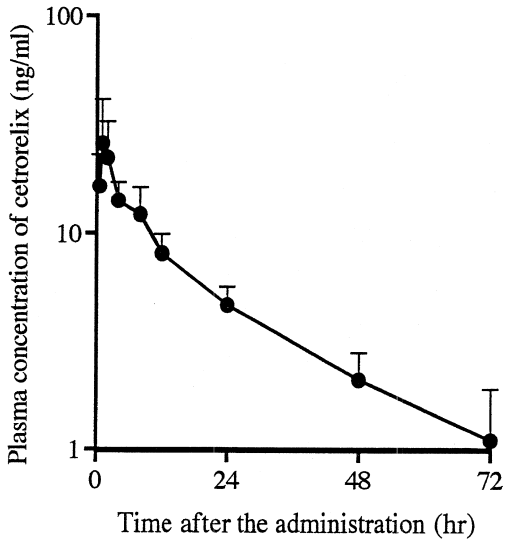

Fig. 6. Time course of the plasma concentration of cetrorelix in healthy women volunteers after single subcutaneous administration of cetrorelix (3 mg free base equivalent). Each point represents the mean±SD of four volunteers.

Table 7
Urinary excretion of cetrorelix following single subcutaneous administration of cetrorelix acetate (3 mg free base equivalent) to 4 female volunteers

Urinary excretion per dose (%, mean±SD)	
0–24 h	2.27±0.92
24–48 h	0.70±0.47
48–72 h	0.46±0.29
0–72 h	3.42±1.09

4. Conclusion

The present study demonstrated that cetrorelix in human plasma and urine can be accurately and precisely analyzed by a solid-phase extraction utilizing Sep-Pak Vac C_8 cartridges followed by reversed-phase liquid chromatography and mass spectrometric analysis. Selected ion recordings in high mass region of m/z 1543 enabled the detection of cetrorelix at concentrations of 1.00 ng per 1 ml in plasma and 2.09 ng per 2 ml in urine without any disturbances from endogenous substances co-existing in biological matrices. The present LC–MS method may be useful for the further investigation of pharmacokinetic study and therapeutic monitoring of cetrorelix.

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