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Development, validation and method stability study of a LC-MS method to quantify Leuprolide (Hormone analog) in Human plasma

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Abstract: A rapid, highly sensitive and simple high-performance liquid chromatographic-tandem mass spectrometric (LC-MS) assay is developed and validated for the quantification of leuprolide: a Gonadotropin Releasing Hormone analog (GnRH) in human plasma. Moreover, various parameters of the method stability are determined. After the addition of stable isotope (internal standard), the leuprolide was extracted from human plasma by a C₁₈ solid phase micro extraction (MEPS) cartridge and directly injected into LC-MS/MS system. Chromatographic separation was achieved using a Waters Atlantis HILIC, C₁₈, 150 × 2.1 mm, 5 μ column. Mobile phase was a mixture of acetate buffer (pH 3) and acetonitrile (25/75). Drug detection was performed by MS using electrospray ionization in positive mode. Multiple reaction monitoring (MRM) with a tandem mass spectrometer was used to detect the analytes. Precursor to product ion transitions of: m/z 605.5 → m/z 110.2 and m/z 609.1 → m/z 249.1 were used to quantify leuprolide and leuprolide-¹³C₆-¹⁵N, respectively. Sample analysis time was 3 min for each injection. The assay exhibited a linear dynamic range of 0.0500-40 ng/ml for each analyte with a lower limit of quantification (LLOQ) of: 0.0500 ng/ml. Furthermore, a complete analytic validation was carried out, including tests on: The specificity, precision, accuracy, matrix effect and stability under different storage conditions. Importantly, the obtained results established: an acceptable precision and accuracy for concentration over standard curve range. Nevertheless, it is to emphasize the simplicity, rapidity and also the high precision and accuracy of this novel LC-MS method, offering useful information about solution stability. Finally, this work is a good alternative to quantify Leuprolide concentration in human blood, especially on human clinic trials step.

Keywords: Leuprolide; LC-MS; Quantification; Validation; Solid phase micro extraction; GnRH analog; Human plasma.

1. Introduction

In recent years, many peptide drugs have been developed, which provide many advantageously characteristics such as less immunogenicity, easier synthesis and higher cure potency of Hormone-dependent cancers. Hormone-dependent cancers such as prostate and breast cancer are nowadays treated among others with peptide drugs Gonadotropin Releasing Hormone (GnRH), which is also known as Luteinizing Hormone Releasing Hormone (LHRH) [1, 2]. Pharmacological mechanism of GnRH is mainly due to suppressing the production of gonadotropin. This latter is responsible of androgens synthesis (testosterone) in the testes or estrogens synthesis (estradiol) in the ovaries. These hormones are well known to stimulate several cancers growth in men and women [1-3]. This inhibition leads to biochemical castration with an effective control of androgen-dependent (prostate) or estrogen-dependent (breast) cancers growth. Leuprolide acetate is a synthetic non-peptide of naturally occurring gonadotropin-releasing hormone (GnRH), which was discovered by Schally et al [1], 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 salt (Figure 1).

Leuprolide is used in sex hormone-related disorders treatment, including advanced prostate cancer, endometriosis, and precocious puberty [2]. Due to the low bioavailability after oral administration or intraduodenal injection (ID) [3], leuprolide should be administered subcutaneously or intramuscularly [4].

To date, main methods for studying leuprolide pharmacokinetics are: immunoassay (This method suffer from inherent cross-reactivity between drug and their metabolites and also has a limited sensitivity) [4-9], electrochemical DNA biosensor [10], HPLC, coupled to a hybrid quadrupole linear ion trap, to determine leuprolide in mouse plasma [11], fluorescence spectroscopy [12], Zhan *et al.*[5] validate a method using HPLC coupled to a triple quadrupole tandem mass spectrometer with positive electrospray ionization to determine leuprolide in human plasma. They obtained a separation required a relatively long chromatographic run time (5 min) with no robustness studies. Therefore, analytical methods of more rapid and sensitivity are still required for studying leuprolide and its pharmacokinetic profiles in human plasma or serum. In addition, solutions stability studies are indicated for a complete analytical validation.

The LC-MS technique is able to overcome these problems by offering more selective and sensitive detection [5, 6]. Therefore, there is a need to a complete existing analytical validation of leuprolide method quantification particularly in human plasma or serum.

In this study, the purpose was to develop and validate a rapid, sensitive, selective, specific, and short LC run-time LC-MS/MS method and to determine the analytical solution stability (robustness). Suitable for leuprolide determination in human plasma, which can be applied in a pharmacokinetic and bioavailability studies and serving also for evaluation in different conditions.

Figure 1.

2. Materials and methods

2.1. Materials

Leuprolide acetate and Leuprolide-¹³C₆-¹⁵N (B.G. Bio-Technology Co, Ltd, China); methanol, acetonitrile, formic acid, acetic acid, ammonium acetate and ammonium carbonate, which are HPLC grade (Sigma, St. Louis, MO, USA). Human plasma (Merck, France).

2.2. Instrument

Perkin Elmer liquid chromatography system used was equipped with a quaternary pump, a vacuum degasser, a thermostated column oven and a series 200, Perkin Elmer autosampler. Mass spectrometric detection was performed on a PE Sciex API 4000, Applied Biosystems. Analyst™ 1.4.2 software package was used to control LC-MS system, as well as for data acquisition and processing.

2.3. Chromatographic conditions

- Chromatographic separation was achieved on a Waters Atlantis HILIC (150 x 2.1 mm, 5 μm) HPLC column, protected by Waters security guard column Oasis HLB. A mixture of pH 3 acetate buffer (25 ml of ammonium acetate solution (0.2 M) and 320 mL of an acetic acid solution (0.522 M) were diluted up to 1 l with bidistilled water) /acetonitrile containing 1% of formic acid (25/75, V/V) was used as mobile phase at isocratic flow-rate of 1.0 ml/min. Column temperature was maintained at ambient temperature (25 °C).

Instrument	PE Sciex API 4000
Ionization source	Turbo V ion source, positive mode
Scan type	Multiple Reaction Monitoring (MRM)
Selected reaction monitoring	Leuprolide: m/z 605.5 → m/z 110.2 Leuprolide- ¹³ C ₆ - ¹⁵ N: m/z 609.1 → m/z 249.1
Dwell time per transition	100 ms
Ionspray voltage	5500 V
Curtain gas (Nitrogen)	10
Ion source (Nitrogen) gas 1	40
Ion source (Nitrogen) gas 2	40
Collision gas (Nitrogen)	9
Temperature	750°C
Acquisition time	3 min

2.4. Mass spectrometric conditions

The mass spectrometer (PE Sciex API 4000) was operated in the positive ion mode using a turbo V ion source. Nitrogen (40) was used as sheath, auxiliary and ion sweep gas to assist with nebulization and desolvation. Spray voltage was set at 5500 V and capillary temperature was maintained at 750 °C.

The tuning parameters were optimized for leuprolide and leuprolide-¹³C₆-¹⁵N (IS) by infusing a solution of both analyte and IS, at a flow-rate of 20 ml/min into the mobile phase (1 ml/min). The spray voltage was set at 5500V and the capillary temperature was maintained at 750 °C. For collision induced dissociation (CID), Nitrogen was used as the collision gas. Quantification of doubly protonated and doubly charged ion for Leuprolide and the ISTD [M+2H]²⁺ was performed using Multiple Reaction Monitoring (MRM) of the transitions m/z 605.5 → m/z 110.2 for leuprolide and m/z 609.1 → m/z 249.1 for leuprolide-¹³C₆-¹⁵N (IS), with a dwell time of 100 ms per transition.

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

Leuprolide stock solution as well as calibration curve was prepared as follows: Leuprolide acetate was dissolved by hand shaking in bi-distilled water, obtaining stock concentration solution of 0.125 µg/µl. Leuprolide working solution of 40 ng/ml concentration was freshly prepared by dilution of stock solution in bidistilled water. Moreover, internal standard stock solution (ISSS) was prepared by dissolving Leuprolide-¹³C₆-¹⁵N by hand shaking in bidistilled water, obtaining stock solution concentration of 1 µg/ml. Internal standard working solution of 20 ng/ml concentration was also freshly prepared by dilution of the stock solution (ISSS) in bidistilled water. Finally, calibration standards solutions containing acidified plasma (acidified with 1 % of formic acid) were prepared freshly by adding a leuprolide working solution volume (0.05, 0.1, 2.5, 5, 10, 25 and 40 ng/ml).

Samples were prepared at four different concentrations (0.0500, 0.100, 25.000 and 40.000 ng/ml). Each sample was shaken gently for one minute, and subsequently divided in aliquots. Aliquots were stored at -80 ± 10 °C in polypropylene tubes in order to be protected.

2.6. Extraction procedure

A micro extraction by packed sorbent (MEPS) was used to extract leuprolide from the plasma. Conditioning C₁₈ (MEPS) cartridge was carried out by dichloromethane and followed by pure acetonitrile, acetonitrile: water (50:50) and then acetonitrile: water (80:20). 100 µl of plasma were withdrawn using a 250 µl syringe fitted with a C₁₈ cartridge (SGE Analytical, Melbourne, Australia), and afterward, aqueous portion was discarded through the column. Leuprolide is adsorbed into the solid phase. Column was 3 times washed with 250 µl of water, and then, leuprolide (50 µl) was eluted by acetonitrile containing 1% formic acid. Fifteen (15) µl of the extracted samples were injected for LC-MS analysis.

2.7. Validation method

2.7.1. Specificity

Method specificity was determined by distinguishing interferences of endogenous compounds using screening analysis of drug free plasma as well as plasma spiked with leuprolide.

2.7.2. Linearity and LLOQ

Relationship between instrument response and leuprolide known concentrations was assessed by analysing plasma calibration standards on eight different runs.

Standard curves included eight points. Plasma concentration of leuprolide, which is lowest standard of calibration curve (LLOQ) was equal to 0.05 ng/ml and the highest standard (ULOQ) was equal to 40.0 ng/ml. The 1/x² weighed linear regression and not forced through the origin was used as calibration model. Calibration curves were established by the ratio of leuprolide peak area to internal standard peak area versus the plasma concentrations.

2.7.3. Selectivity

Acidified blank plasma, acidified blank plasma spiked with internal standard (IS) at current concentration and acidified blank plasma spiked with leuprolide at concentration of 0.050 ng/ml (LLOQ) were extracted and analysed for each human plasma.

2.7.4. Precision and accuracy

Repeatability (intra-run precision and accuracy) of analytical method was evaluated by analysing QC samples prepared at four concentrations (0.05, 0.10, 25.0 and 40.0 ng/ml). Six quality control samples were analysed at each concentration. Inter-run precision and accuracy were tested by analysing samples on eight different runs. Same concentration levels (0.05, 0.10, 25.0 and 40.0 ng/ml) as mentioned above in repeatability test were used. Two samples per concentration level were assayed in each run.

2.7.5. Extraction recovery

Leuprolide extraction recovery was carried out by comparing ratio peak area of extracted leuprolide and leuprolide-¹³C₆-¹⁵N as external standard to ratio peak area of leuprolide as pure product spiked with extracted blank matrix and peak area of leuprolide-¹³C₆-¹⁵N as external standard. Extraction recovery of leuprolide was then evaluated at two different concentrations (0.100 and 40.0 ng/mL). Six measurements were performed for each concentration level.

2.7.6. Matrix effect

Matrix effect was tested on six (6) different human plasma using one concentration level (25.0 ng/ml). Quality control samples were prepared for each batch of human plasma. Six QC samples were analysed per plasma.

2.7.7. Stability study

Stock solutions

Instrument response measured for stored solutions was compared to freshly prepared solutions. Leuprolide and leuprolide-¹³C₆-¹⁵N stabilities were calculated using as internal standard leuprolide-¹³C₆-¹⁵N and leuprolide, respectively.

Stability was evaluated using the following formula:

$$\frac{\text{ratio obtained for stored solution}}{\text{ratio obtained for freshly prepared solution}} \times 100$$

Leuprolide in solution

Leuprolide stability was evaluated in (0.125 mg/ml) solution, protected from light after **90 days** of storage at -20 ± 5 °C.

Leuprolide-¹³C₆-¹⁵N in solution

Leuprolide-¹³C₆-¹⁵N stability was evaluated in (0.100 mg/ml) solution, protected from light after **90 days** of storage at -20 ± 5 °C.

Extracted samples in mobile phase

This test was mainly carried out in order to determine if an analytical run could be reanalyzed by failure of the instrument. A QC samples at 0.05, 0.10, 25.0 and 40.0 ng/ml were prepared and injected after storage in mobile phase for 70 h at 5 ± 4 °C. All samples are protected against light.

Leuprolide in acidified plasma

Long-term stability of leuprolide in acidified plasma was determined by storing aliquots of each pool, which were prepared at 0.100 and 40.0 ng/ml, and subsequently stored at -80 ± 10 °C in polypropylene tubes. At each time of storage (0, 1, 2 and 3 months) QC samples were measured using a freshly prepared calibration curve.

3. Results and discussion

3.1. Mass spectrometry

HPLC–MS/MS is a powerful analytical technique, in particular for compounds determination in biological samples due to its simplicity, selectivity and specificity.

In this study, most abundant ion was found in the positive ESI interface using MRM. As a result, a good resolution and high intensity were obtained for compound quantification and no cross-talk effect was observed. Full mass scan spectra of leuprolide and the IS are shown in Figure 2.

Figure 2.

3.2. Validation results

A thoroughly and completely validation method was carried out according to the ICH Guidance (International conference on harmonization) [13].

3.2.1. Specificity

Method specificity was determined by distinguishing interferences of endogenous compounds using screening analysis of drug free plasma and plasma spiked with leuprolide. No significant chromatographic interferences were observed with leuprolide and IS at their retention times in drug-free plasma (Figure 3).

Figure 3.

3.2.2. Linearity and LLOQ determination

Calibration curve for leuprolide in plasma showed a good linearity over concentration range of 0.05 ng/ml to 40 ng/ml for leuprolide with a correlation coefficient of $R^2 = 0.999$. Typical linear regression equation of calibration curve was: $y = 26526 x + 7321$ (Figure 4).

Where ‘y’ is the peak-area ratio of the analyte to the IS and ‘x’ (ng/ml) is the plasma concentration of analyte. LLOQ of leuprolide was 0.05 ng/ml.

According to general ICH acceptance criteria [13], results showed that the linearity of the instrument response was occurred over used concentrations.

Figure 4.

3.2.3. Selectivity

Results of selectivity test are showed in table 1. In three investigated blank human plasma, no peak- interference has been observed at retention time of leuprolide. Same outcome are also obtained for the IS.

Table 1.

3.2.4. Precision and accuracy

Repeatability (intra-run precision and accuracy) of analytical method was evaluated by analysing samples prepared at four concentrations (0.050, 0.100, 25.0 and 40.0 ng/ml). Six quality control samples were analysed at each concentration. Results are presented in table 2.

Table 2.

It has to be pointed out that results were in the acceptance criteria as the mean measured concentration was within 85-115 % of the theoretical value and CV lower than 8 % at each concentration.

The inter-run precision and accuracy were tested by analysing samples on eight different runs. Same concentration levels (0.050, 0.100, 25.0 and 40.0 ng/ml) as those used in the repeatability test were analysed. Two samples per concentration level were assayed in each run. Results are presented in table 3.

Table 3.

Again, Results were in the acceptance criteria as the mean measured concentration was within 85-115% of the theoretical value and the CV lower than 15% at each concentration level.

3.2.5. Extraction recovery

The extraction recovery results are illustrated in table 4. As it can be seen, extraction recovery of leuprolide was evaluated at the concentrations of 0.100 and 40.0ng/ml.

The extraction recovery was equal to 64.28% at 0.100 ng/ml and 57.57% at 40.0 ng/ml.

Table 4.

3.2.6. Matrix effect

One quality control (QC) pools per plasma were prepared and only three samples were assayed. Interestingly, results demonstrated that there is no matrix effect this is probably due to the use of appropriate intern standard. These results (Table 5) show the mean measured concentrations of QC samples for each tested human plasma, which were within ± 4 % of the theoretical value. Moreover, CV calculated on measured concentrations was lower than 4 %. Importantly, the change of the matrix did not affect the precision and accuracy of the assay method.

Table 5.

3.3. Stability studies

3.3.1. Stability of leuprolide and leuprolide-¹³C₆-¹⁵N in solution

Stabilities of leuprolide and leuprolide-¹³C₆-¹⁵N were studied with a solution of 0.125 mg/ml stored during 90 days at $-20 \pm 5^\circ\text{C}$ and protected from light.

These results, which are in the acceptance criteria, are presented below in Table 6 and 7. Resulting data shows that difference between stored and freshly prepared solutions was lower than 5% and also the CV calculated from the determined ratios was lower than 5%.

Table 6.

Table 7.

3.3.2. Stability of extracted samples in mobile phase

The aim of this test was to determine if an analytical run could be re-analyzed in the case of instrument failure. A QC samples at 0.05, 0.10, 25.0 and 40.0 ng/ml were prepared and injected after storage in the mobile phase for 70 h at 5 ± 4 °C protected from light.

Results showed that accuracies calculated for 6 QC samples were in the acceptance criteria (85-115 % of the theoretical values). Note that a whole run could be injected after 70 h of storage at 5 ± 4 °C in mobile phase.

3.3.3. Long term stability of leuprolide in acidified plasma

Long-term stability of leuprolide in acidified plasma was determined by storing aliquots of each pool at -80 ± 10 °C in polypropylene tubes. The analysed concentrations of leuprolide are prepared at 0.100 and 40.0 ng/ml.

After 0, 1, 2 and 3 months, QC samples were analysed by means of a freshly prepared calibration curve. Each run performed for this test (Table 8) was validated by assaying freshly prepared QC samples.

Mean concentrations of stored QC pools were determined after a predetermined storage time (0, 1, 2 and 3 months), which were in all cases between 85-115% of theoretical values and with a CV lower than 15 % (Table 8). Leuprolide remained stable for 3 months upon storage in acidified plasma at -80 ± 10 °C (Table 8).

Table 8.

3.3. Summary and discussion of achievements of development and validation of the proposed method

First step of our work was to choose operating conditions of analysis method, so after several tests, it appeared to us that to have a good separation by LC-MS it was necessary to use:

- Mobile composition phase containing a mixture of pH 3 acetate buffer/acetonitrile containing 1% of formic acid (25/75, V/V)
- Quantification of Leuprolide was performed using selected reaction monitoring (SRM) of the transitions m/z (m/z 605.5 \rightarrow m/z 110.2) for leuprolide and (m/z 609.1 \rightarrow m/z 249.1) for Leuprolide- $^{13}\text{C}_6$ - ^{15}N . The dwell time was 100 ms per transition.
- Chromatographic separation was achieved on a Waters Atlantis HILIC (150 x 2.1 mm, 5 μm) HPLC column, protected by security guard. Column temperature was maintained at ambient temperature (25 °C).

Consequently, we followed recommendations of ICH [13] in terms of analytical validation. This allowed us to demonstrate unequivocally that method developed was specific, that it could quantify Leuprolide with good precision and repeatability at a lower limit of quantification (**0.05 ng/ml**), compared to less precision and selectivity and higher limit of quantification (around **3 ng/ml**) of classic immunoassay of leuprolode [14].

Additionally, we have chosen to study impact of conservation of leuprolide in mobile phase and also in human plasma. It was shown that Leuprolide in mobile phase can be stored for at least 90 days, similarly for samples in plasma maintained at -80 ° C. This result, is in accordance with preliminary work of M. RAHIMI et al [15], that established that leuprolide is stable in phosphate buffer at least 35 days.

4. Conclusions

From a practical point of view, this new validated analytical method offers an interesting potential in quality control of leuprolide. Importantly, rapid, a sensitive, specific, selective, accurate and cost-effective HPLC–MS/MS method for quantification of leuprolide in human plasma has been identified. In addition, different stabilities were evaluated, indicating possibility among others of being able to freeze samples of human plasma containing leuprolide. It has to be pointed out that these samples remained stable during 3 months.

Conflict of Interest: None

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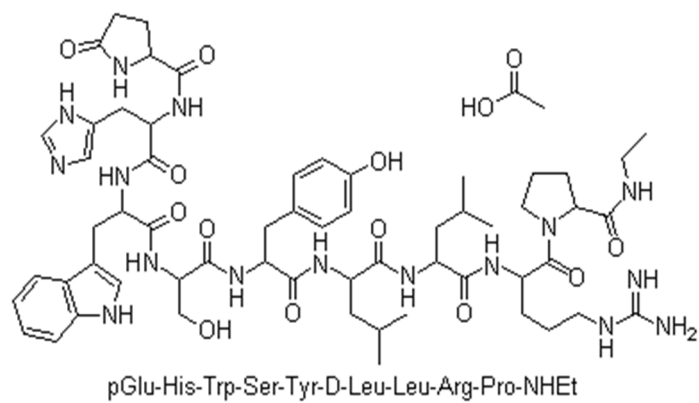


Figure 1: Chemical structure of leuprolide acetate.

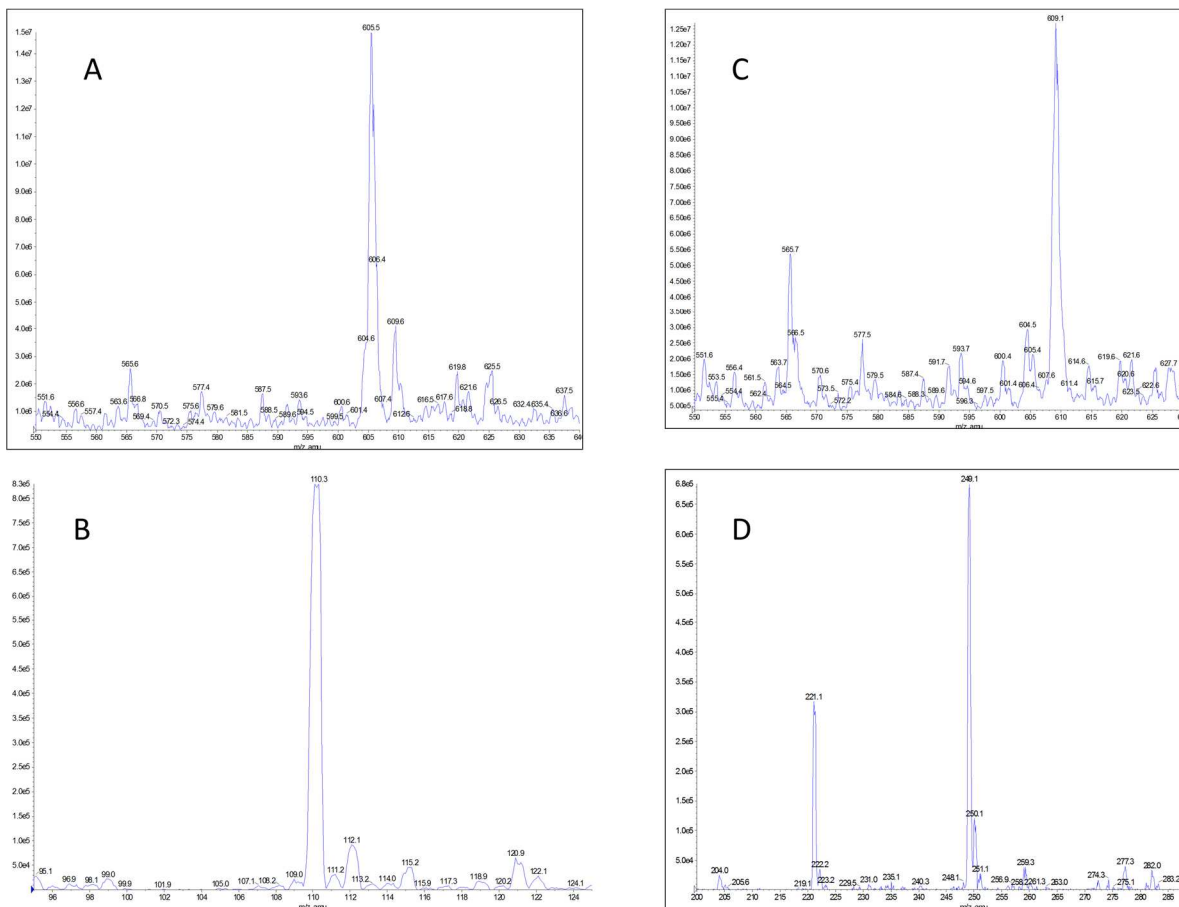


Figure 2: (A) Full-scan mass spectra of leuprolide at 0.100 ng/μl in H₂O/ACN (15/85), (B) full-scan mass spectra of leuprolide-¹³C₆-¹⁵N at 0.100 ng/μl in H₂O/ACN (15/85), (C) full-scan mass spectra of leuprolide at 0.100 ng/μl in H₂O/ACN (15/85), (D) full-scan mass spectra of leuprolide-¹³C₆-¹⁵N at 0.100 ng/μl in H₂O/ACN (15/85).

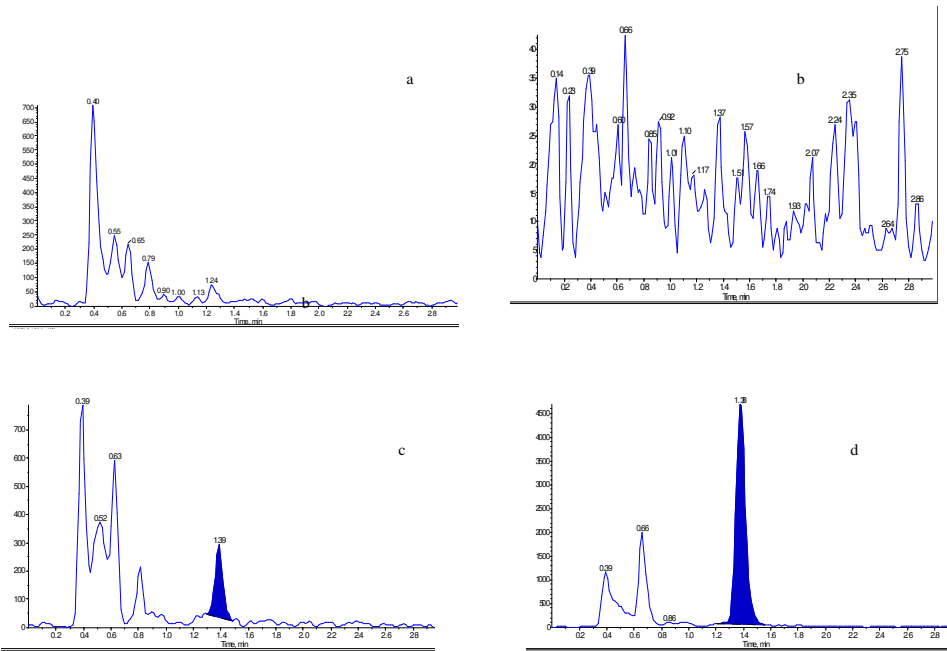


Figure 3: Representative SRM chromatograms for : (a) Acidified blank plasma, (b) mobile phase, (c) acidified blank plasma spiked with 0.0500 ng/ml of leuprolide and (d) acidified blank plasma spiked with 0.0500 ng/ml of IS.

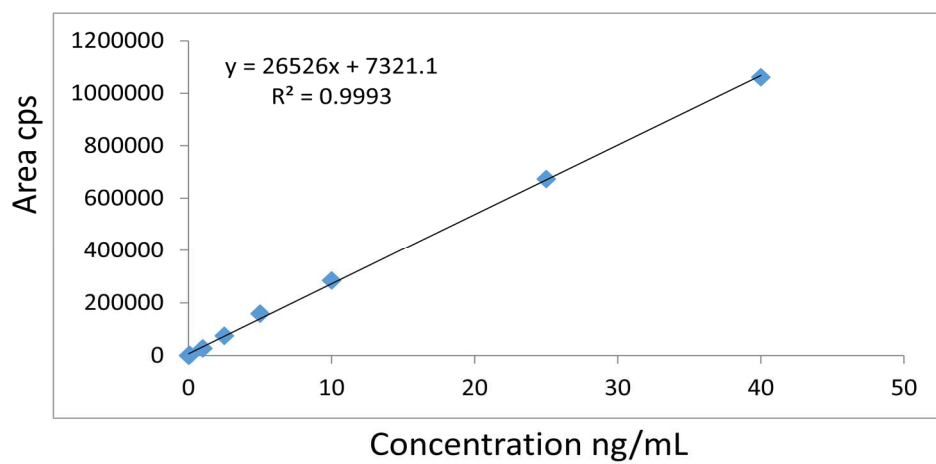


Figure 4: Calibration curve of leuprolide in human plasma.

Table 1: Selectivity of assay method towards endogenous compounds.

Selectivity for leuprolide (Results expressed as area).

Sample Plasma	Analyte peak area in acidified blank plasma (A)	Analyte peak area in acidified plasma spiked at the LLOQ (B)	Analyte peak area in acidified plasma spiked with IS (C)	A/B x 100	C/B x 100
1	302	1341	309	22.52	23.04
2	299	2008	299	14.89	14.8
3	303	2019	198	15	9.806

Selectivity for internal standard (IS) (Results expressed as area).

Sample Plasma	IS peak area in acidified blank plasma (A)	IS peak area in acidified plasma spiked with IS (B)	IS peak area in acidified plasma spiked at the LLOQ (C)	A/B x 100	C/B x 100
1	No peak	30140	No peak	ND	ND
2	No peak	29923	No peak	ND	ND
3	No peak	30994	No peak	ND	ND

ND: not determined.

Table 2: Intra-run precision and accuracy (ng/ml).

Theoretical concentration	0.0500	0.100	25.0	40.0
Assay 1	0.043	0.101	25.800	39.300
Assay 2	0.047	0.086	21.400	34.500
Assay 3	0.050	0.087	23.900	37.900
Assay 4	0.044	0.086	22.900	35.500
Assay 5	0.043	0.091	23.100	39.000
Assay 6	0.050	0.098	25.700	37.900
n	6	6	6	6
CV%	7.09	6.86	7.19	5.17
Accuracy%	93.23	91.86	95.2	93.37

Table 3: Inter-run precision and accuracy (ng/ml).

Samples	0.0500	0.100	25.0	40.0
1	0.042	0.101	25.800	39.8
	0.047	0.096	23.400	34.1
2	0.048	0.084	25.300	37.3
	0.048	0.099	24.900	36.5
3	0.044	0.099	23.900	38.1
	0.049	0.092	25.100	39.1
4	0.048	0.090	23.900	35.1
	0.049	0.094	25.300	36.9
5	0.049	0.100	24.000	39.4
	0.040	0.098	23.800	32.9
6	0.050	0.082	25.200	39
	0.041	0.080	23.200	31.8
N	12	12	12	12
CV%	8.03	7.99	3.56	7.27
Accuracy%	93.12	93.27	97.93	91.67

Table 4: Extraction recovery of leuprolide.

Concentration level	0,100ng/mL			40,0 ng/mL		
	Ratio 1	Ratio 2	Individual recovery %	Ratio 1	Ratio 2	Individual recovery %
Assay 1	0.079	0.054	67.764	20.800	11.100	57.913
Assay 2	0.071	0.049	61.630	18.900	12.600	65.739
Assay 3	0.060	0.039	49.885	21.100	10.200	53.217
Assay 4	0.075	0.049	62.262	18.100	11.300	58.956
Assay 5	0.103	0.059	74.765	16.900	10.900	56.869
Assay 6	0.090	0.055	69.389	19.200	10.100	52.695
n	6	6	6	6	6	6
mean	0.079	0.051	64.280	19.166	11.033	57.565
SD			8.560			4.732
CV%			13.32			8.22

Ratio 1: peakarea of non-extracted analyte/peak area of IS; Ratio 2: peak area of extracted analyte/peak area of IS; Individual recovery % = (ratio 2 / mean of ratio 1) x 100
The extraction recovery was equal to 64.28% at 0.100ng/ml and 57.57% at 40.0ng/ml.

Table 5:- Matrix effect.

	Matrix 1	Matrix 2	Matrix 3
Theoretical concentration (ng/mL)	25.0	25.0	25.0
Assay 1	25.1	24.9	24.9
Assay 2	25.9	24.7	25.1
Assay 3	24.9	24.9	23.3
Assay 4	25.0	25.9	25.9
Assay 5	23.9	24.9	24.7
Assay 6	26.8	25.9	25.8
N	6	6	6
Mean	25.27	25.20	24.95
Sd	0.99	0.55	0.94
CV%	3.90	2.17	3.77
Accuracy%	101	101	100

Table 6. Stability of leuprolide evaluated in solution at 0.125 mg/mL after 90 days of storage at -20 ± 5 °C protected from light.

	mean area ratio analyte/IS (stored)	mean area ratio analyte/IS (fresh)
Assay 1	3.84	3.71
Assay 2	3.79	3.96
Assay 3	3.86	3.91
Assay 4	3.88	3.99
Assay 5	3.91	3.94
n	5	5
mean	3.856	3.902
CV%	1.16	2.85
Coef sim (%)	98.82	

Table 7: Stability of leuprolide-¹³C₆-¹⁵N evaluated in solution at 0.125 mg/ml after 90 days of storage at $-20 \pm 5^{\circ}\text{C}$ protected from light.

	mean area ratio IS/analyte (storred)	mean area ratio IS/analyte (fresh)
Assay 1	0.298	0.272
Assay 2	0.303	0.291
Assay 3	0.287	0.299
Assay 4	0.301	0.298
Assay 5	0.311	0.307
n	5	5
mean	0.300	0.293
CV%	2.90	4.51
Coef sim (%)	102.25	

Table 8: Long-term stability of leuprolide in acidified plasma at $-80 \pm 10^{\circ}\text{C}$ (ng/ml).

Tested time	T = 0		1 month		2 months		3 months	
C th	0.10	40.00	0.10	40.00	0.10	40.00	0.10	40.00
Assay 1	0.11	38.10	0.10	37.20	0.10	37.50	0.10	39.50
Assay 2	0.10	37.30	0.10	35.80	0.10	36.60	0.09	37.50
Assay 3	0.11	38.80	0.09	38.40	0.09	38.00	0.10	37.60
Assay 4	0.10	37.10	0.10	38.00	0.10	39.10	0.10	38.70
Assay 5	0.10	38.50	0.10	39.30	0.10	37.00	0.10	38.20
mean	0.10	37.96	0.10	37.74	0.10	37.64	0.10	38.30
SD	0.01	0.74	0.00	1.32	0.00	0.97	0.00	0.83
CV%	4.87	1.95	2.36	3.50	4.64	2.58	3.86	2.16
Accuracy%	103.02	94.90	97.96	94.35	98.94	94.10	97.74	95.75