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Liquid Chromatograph Mass Spectrometer LCMS-8045

Application News

A Fast, Simple and Sensitive Method for Estimation of Leuprolide in Human Plasma using Shimadzu LCMS-8045

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User Benefits

- Rapid, simple and sensitive method with LLOQ of 25 pg/mL
- Low plasma volume helps to reduce matrix effect and increase lifespan of column and LCMS
- Single step SPE method helps boost overall productivity, saving time and reducing errors.

1. Introduction

Leuprolide acetate is a synthetic nonapeptide analog of naturally occurring gonadotropin releasing hormone (GnRH or LH-RH). The analog possesses greater potency than the natural hormone. The 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) and the structure is provided in Fig.1 [1)].

Leuprolide also known as Leuprorelin acetate, is orally inactive and generally given subcutaneously or intramuscularly. Leuprorelin may be used in the treatment of hormone-responsive cancers such as prostate cancer and breast cancer. It may also be used for estrogen-dependent conditions such as endometriosis or uterine fibroids. It may be used for precocious puberty in both males and females, and to prevent premature ovulation in cycles of controlled ovarian stimulation for in vitro fertilization. It may be used to reduce the risk of premature ovarian failure in women receiving cyclophosphamide for chemotherapy [2)].

The peptide drug is released from the depot formulations at a functionally constant daily rate for 1, 3 or 4 months, depending on the polymer type [polylactic/glycolic acid (PLGA) for a 1-month depot and polylactic acid (PLA) for depot of >2 months], with doses ranging between 3.75 and 30mg.

Mean peak plasma leuprorelin concentrations of 13.1, 20.8 to 21.8, 47.4, 54.5 and 53 µg/L occur within 1 to 3 hours of depot subcutaneous administration of 3.75, 7.5, 11.25, 15 and 30 mg, respectively, compared with 32 to 35 µg/L at 36 to 60 min after a subcutaneous injection of 1mg of a non-depot formulation. Sustained drug release from the PLGA microspheres maintains plasma concentrations between 0.4 and 1.4 µg/L over 28 days after single 3.75, 7.5 or 15mg depot injections [3)]. It indicates that the method required for pharmacokinetic evaluations need to achieve a sensitivity limit of sub-picogram level as low as 400 pg/mL.

Such method should address many problems posed by peptides viz., poor ionization, non-specific adsorption, carry-over, and low extraction recovery.

We have therefore developed a method with high chromatographic resolution and ample sensitivity giving lowest limit of quantification (LLOQ) of 25 pg/mL for leuprolide in human plasma using LCMS-8045 (refer Fig.2). Method was developed keeping some key criteria in focus- namely simpler extraction procedure, highly optimized chromatography and enhanced sensitivity. These factors enable selective and high-throughput analysis of leuprolide for the pharmacokinetic investigation

2. Salient Features

- Quantitative method for estimation of leuprolide in human plasma was developed. Method was partially validated as per US major guidelines; results are presented in table 1.
- Effective throughput for quantitative assessment is increased by use of a quick simple extraction procedure.
- Heated ESI along with New UF-Qarray ion guide technology contributes by increasing ion production and enhancing transmission respectively. This ensures sensitive and selective quantification of leuprolide at 25 pg/mL.
- Low plasma volume helps to reduce matrix effect and increase lifespan of column and LCMS
- Customized gradient method enhances the chromatographic resolution of leuprolide with consistent and reproducible peak area and retention time.
- Method was partially validated as per US major guidelines for
  - ✓ Selectivity
  - ✓ Linearity
  - ✓ Inter-day and intra-day precision and accuracy (PA)
  - ✓ Recovery
  - ✓ Matrix effect

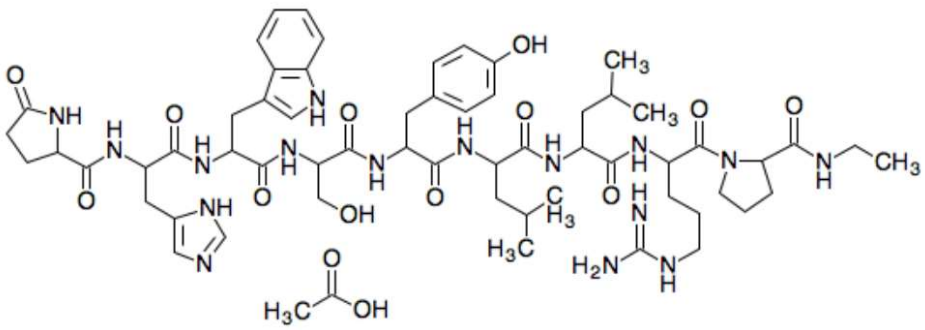


Fig.1 Structure of Leuprolide [1)]

Table 1 Partial Method Validation Summary

Calibration curve range	25.00 pg/mL to 30000 pg/mL	
Intraday precision and accuracy (For LLOQ QC)	Accuracy (%Nominal)	117.33
	Precision (%RSD)	15.50
Intraday precision and accuracy (For LQC, MQC and HQC)	Accuracy (%Nominal)	104.64 to 107.45
	Precision (%RSD)	1.97 to 6.08
Global precision and accuracy (For LLOQ QC)	Accuracy (%Nominal)	111.11
	Precision (% RSD)	16.13
Global precision and accuracy (For LQC, MQC and HQC)	Accuracy (%Nominal)	102.17 to 105.27
	Precision (% RSD)	3.00 to 6.37
Global % recovery	Recovery (%)	73.50
	Precision (% RSD)	5.08
Matrix effect	LQC	0.98
	HQC	0.87

Note: LLOQ QC- Lower Limit of Quantification Quality Control, LQC- Lower Quality Control, MQC- Middle Quality Control and HQC- Higher Quality Control

3. Experimental

3.1. Sample preparation and analytical conditions

- Fifty microliters of internal standard (50 ng/mL of Leuprolide-d10) was added to 200 µL of the pre-spiked calibration curve standards and quality control samples.
- To the blank and non-zero standard, 50 µL diluent was added to compensate the volume of internal standard.
- Samples were vortexed to mix and further precipitated with precipitating agent.
- Leuprolide was extracted by vortexing the samples for 5 minutes at 2000 rpm followed by centrifugation for 10 minutes at 5 °C.
- 200 µl of supernatant was transferred into autosampler vials and injected 25 µl on LCMS-8045 system

3.2. Instrument parameters on LCMS-8045

Refer to the Table 2 for analytical conditions and instrument parameters and Table 3 for MRM transition.

Table 2 Analytical conditions and instrument parameters

Parameter	HPLC
Column	Shim-pack™ GIST C18, 3um, 2.1 × 50 mm (P/N: 227-30008-03)
Mobile Phase	Pump A-0.1% formic acid in Water Pump B-0.1 % formic acid in Acetonitrile
Flow Rate	0.6 mL
Oven Temp	40 °C
Injection volume	25 µL
Parameter	MS
Interface	ESI
Interface Voltage and temp	3 kV and 400 ˚C
MS Mode	MRM, Positive
Heat Block Temp	500 ˚C
DL Temp	350 ˚C
CID Gas	230 kPa
Nebulizing Gas	3 L/min
Drying Gas	10 L/min

Table 3 MRM transition and parameters of Leuprolide on LCMS

Compound	MRM (m/z)	CE (V)
Leuprolide	605.5-249.1	-35.0
Leuprolide	605.6-159.1	-35.0
Leuprolide-D10	610.6-221.1	-40.0
Leuprolide-D10	610.6-249.1	-40.0
Leuprolide-D10	610.6-159.2	-40.0
Leuprolide-D10	610.6-110.1	-40.0

Fig.2 Nexera™ X2 with LCMS-8045 system

4. Result and Discussion

4.1. Method Development

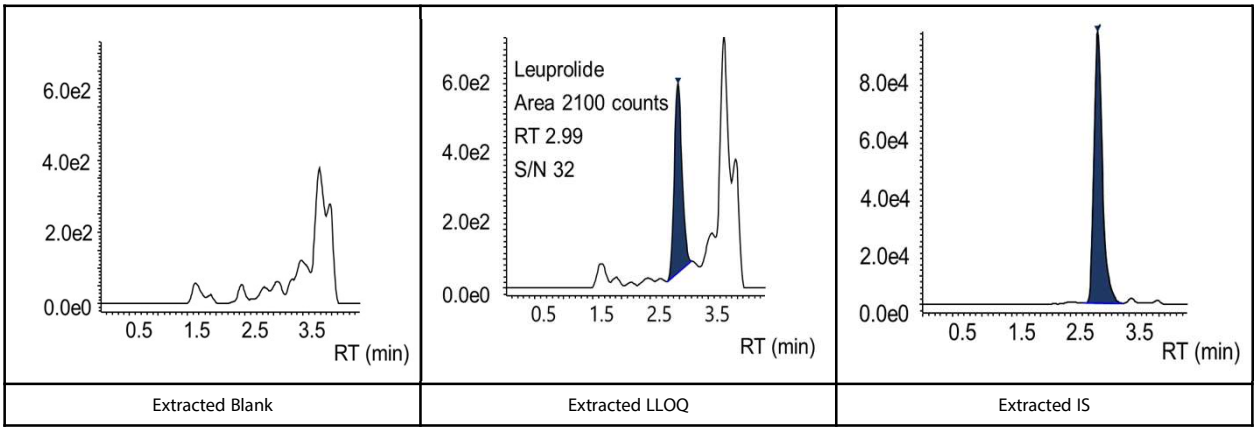
Optimization of the mass spectrometric condition

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. In the positive ESI interface, both leuprolide and its IS leuprolide D10 formed predominantly doubly charged protonated molecules $[M^+2H]2^+$ at *m/z* 605.5 and m/z 610.6 in Q1 while the [M+H]+ ions at m/z 1209.9 and m/z1168.6 were less than 5% relative abundance of $[M^+2H]2^+$.The corresponding product ions was selected as the precursor ion. Leuprolide and IS both have fragment ions at *m/z* 159.1 and *m/z* 249.1. The optimal collision energy for leuprolide and IS were both set at 35 eV and 40 eV respectively.

Optimization of the sample preparation and chromatographic condition

Different extraction techniques such like SPE, LLE and PPT were tried to extract leuprolide and leuprolide D10 from human plasma. However, protein precipitation extraction technique was found to be more economic and time saving without compromising on sensitivity and recovery of leuprolide and leuprolide-d10. The extraction process includes addition of two hundred microliter of precipitating reagent to 200ul of the plasma samples, followed by vortex and centrifugation. Two hundred microliter of the supernatant was aliquoted in the autosampler vials and 25 ul of samples was injected on LCMS-8045 system.

LC conditions were optimized to maximize sensitivity, speed, and peak shape. Mobile phase composition was optimized through flow injection analysis, utilizing varying percentages of organic solvents. Comparison between acetonitrile and methanol revealed that both solvents yielded similar sensitivity, yet acetonitrile proved more effective for chromatographic separation of leuprolide and Leuprolide-d10. Additionally, the addition of acidic modifiers, such as formic acid, to the mobile phase enhanced sensitivity by facilitating analyte ionization. Consequently, Shim-pack™ C18 column was employed for the analysis. The use of Shimadzu Shim-pack C18 column achieved excellent separation for leuprolide and leuprolide-d10, allowing for a total analysis run time of 4.0 minutes under the specified chromatographic conditions on Shim-pack C18 Column.



Extracted Blank	Extracted LLOQ	Extracted IS

Fig.3 Extracted Chromatograms of Leuprolide Blank, LLOQ level and IS

4.2. Partial Method Validation

Assay Selectivity

Selectivity of the method was evaluated by analyzing 6 different lots of blank human plasma and blank plasma spiked with leuprolide and Leuprolide-d10. No significant interference was observed at the retention time and MRM of analyte(s) and IS (refer to the Table 4 and Fig.3).

Table 4 Selectivity

Plasma lot no.	Leuprolide		
	Blank Plasma	LLOQ area	% Interference
V3071	159	1,362	11.67
V1889	267	1,416	18.86
V1789	208	1,912	10.88
V1166	0	1,371	0.00
V3074	374	2,051	18.24
V3077	168	1,267	13.26

Plasma lot no.	Leuprolide-d10		
	Blank Plasma	LLOQ area	% Interference
V3071	205	93,692	0.22
V1889	243	86,611	0.28
V1789	638	89,217	0.72
V1166	690	89,838	0.77
V3074	574	91,397	0.63
V3077	864	89,865	0.96

Linearity of calibration curve and lower limit of quantification

The linear regression of the peak-area ratios vs. concentrations were fitted over the concentration range of 25 pg/ml to 30,000 pg/ml for leuprolide in human plasma. A typical equation of the calibration curve on a validation run was as follows: y = 0.0618078) X + (0.000289935) (r^2 = 0.9971) where y represents the peak-area ratio of analyte to IS and x represents the plasma concentration of leuprolide (refer Fig.4). Good linearity was obtained in this concentration range. The lower limit of quantification was established as 25 pg/ml for leuprolide. The precision and accuracy values corresponding to LLOQ are shown in Table 1.

Intra-day precision and accuracy

Intraday precision and accuracy were conducted using 6 replicates of LLOQ-QC, LQC, MQC and HQC over one P&A batch. Quantitative data is summarized in Table 5.

Table 5 Intra-day precision and accuracy

Intra-day (n=6)			
Nominal Conc (pg/mL)	Observed Conc (pg/mL)	Accuracy (%)	Precision (% RSD)
LLOQ QC (25.00 pg/mL)	29.33	117.33	15.5
LQC (340.00 pg/mL)	365.33	107.45	6.08
MQC (18900.00 pg/mL)	19776.33	104.64	1.97
HQC (25050.00 pg/mL)	26892.67	107.36	2.27

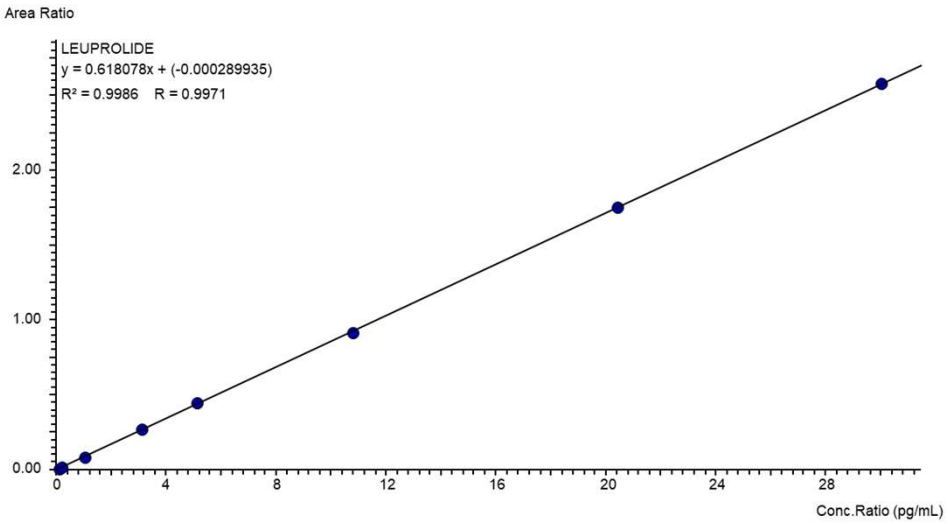


Fig.4 Calibration curve

Global precision and accuracy

Global precision and accuracy were evaluated in 3 batches. Excellent accuracy and repeatability were observed with % RSD less than 6.37 % and percent accuracy between 102.17 % to 105.27 % for LQC, MQC and HQC level. At LLOQ QC level, the %RSD was found to be 16.13 % and percent accuracy as 111.11 %. The results are presented in Table 6. These values were within the acceptable range, and the method was thus judged to be suitably accurate and precise.

Table 6 Global precision and accuracy

Intra-day (n=18)			
Nominal Conc (pg/mL)	Observed Conc (pg/mL)	Accuracy (%)	Precision (% RSD)
LLOQ QC (25.00 pg/mL)	27.78	16.13	111.11
LQC (340.00 pg/mL)	351.61	6.37	103.42
MQC (18900.00 pg/mL)	19310.22	4.24	102.17
HQC (25050.00 pg/mL)	26370.33	3.00	105.27

Carry-over effect

Carryover was evaluated by injecting extracted samples in the sequence of extracted blank, extracted highest calibrator, extracted blank and extracted lowest calibrator. No carryover was present/observed at the retention time and MRM transition of the analyte in the extracted blank sample following the highest standard calibrator.

Extraction recovery

Recovery of leuprolide extracted from plasma were 76.97 %, 73.98 % and 69.55 % at concentrations of LQC, MQC and HQC levels (n=6) as shown in Table 7. Refer to the Table 8 for Global recovery.

Table 7 Statistics of Recovery

	LQC		MQC		HQC	
	Ext-Sample	PE-Sample	Ext-Sample	PE-Sample	Ext-Sample	PE-Sample
Recovery	6,133	7,137	2,51,587	3,42,538	4,28,310	6,10,029
	5,946	7,365	2,39,034	3,45,354	4,18,507	6,19,069
	5,785	7,679	2,61,793	3,46,481	4,42,164	6,02,507
	5,723	7,679	2,64,605	3,47,864	4,17,093	6,18,910
	5,875	8,413	2,54,268	3,45,665	4,14,249	6,23,025
	6,588	8,564	2,56,364	3,37,096	4,44,670	6,14,441
Mean	6008.3	7806.2	254608.5	344166.3	427498.8	614663.5
SD	317.7	568.7	9016	3881.4	13230.8	7438.6
% CV	5.29	7.29	3.54	1.13	3.09	1.21
% Recovery	76.97		73.98		69.55	
Note: Read Ext-Sample as extracted sample and PE-Sample as post extracted sample						

Table 8 Global Recovery

QC level	Recovery
LQC (n=6)	76.97
MQC (n=6)	73.98
HQC (n=6)	69.55
Mean	**73.50**
SD	**3.73**
% RSD	**5.08**

Matrix effect

Matrix effect was studied for both leuprolide and Leuprolide-D10 internal standard using LQC and HQC samples. Mean matrix factor was found to be 0.98 and 0.87 at LQC and HQC respectively. Representative data of matrix effect is shown in Table 9. The results confirm the suitability of method for quantitative estimation of leuprolide in human plasma.

Table 9 Matrix factor

Leuprolide	Response ratio of Aqueous standard	Response ratio of Post extracted sample	Matrix factor
LQC	0.019	0.020	1.01
	0.020	0.018	0.90
	0.017	0.017	0.96
	0.019	0.018	0.95
	0.018	0.018	1.04
	0.020	0.019	0.93
Mean			**0.98**
SD			**0.05**
% RSD			**5.48**

Leuprolide	Response ratio of Aqueous standard	Response ratio of Post extracted sample	Matrix factor
HQC	1.294	1.167	0.9
	1.305	1.158	0.9
	1.364	1.156	0.8
	1.364	1.152	0.8
	1.335	1.149	0.9
	1.36	1.165	0.9
Mean			**0.87**
SD			**0.02**
% RSD			**2.66**

5. Conclusion

By optimizing the chromatographic conditions, a sensitive and rapid LC–MS/MS method for the quantification of leuprolide in human plasma was developed and partially validated. This method was sensitive enough to monitor low-dosage pharmacokinetic or depot formulation studies of leuprolide in human plasma. Compared with previously reported analytical methods, this method showed high throughput (4.0 min each sample) and greater sensitivity, with an LLOQ of 25 pg/mL. It could be applied to characterize the pharmacokinetics of leuprolide in healthy volunteers.

6. References

1) https://www.kegg.jp/entry/D00989 (accessed Jun 14, 2024).
2) https://en.wikipedia.org/wiki/Leuprorelin (accessed Jul 08, 2019).
3) Periti P, Mazzei T, Mini E. Clinical pharmacokinetics of depot leuprorelin. Clin Pharmacokinetic. 2002; 41(7):485-504.

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