SHIMADZU

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Highly sensitive quantitative analysis of Leuprolide from rat plasma using LC-MS/MS

Ashutosh Shelar, Shailendra Rane, Shailesh Damale, Purshottam Sutar, Deepti Bhandarkar, Anant Lohar, Navin Devadiga, Bhaumik Trivedi, Ajit Datar, Pratap Rasam and Jitendra Kelkar

Shimadzu Analytical (India) Pvt. Ltd., 1 A/B Rushabh Chambers, Makwana Road, Marol, Andheri (E), Mumbai-400059, Maharashtra, India.

1. Overview

Bioanalysis of xenobiotics is one of the important stage of drug discovery and development. In this study, physiologic fluids such as blood, serum, plasma, urine and tissues are analyzed to determine the absorption and disposition of a drug candidate administered to a test animal[1]. Pharmacokinetic data provides information that can guide future animal and clinical studies for the selection of the dose levels and frequency of administration. Hence, there is always a need of sensitive bioanalytical methods that can provide a good insight about pharmacokinetic behavior of given xenobiotic.

2. Introduction

Leuprolide (Figure 1) is a synthetic nonapeptide, analogue of gonadotropin releasing hormone. It is used to treat advanced prostate cancer, uterine fibroids and endometriosis. The drug is administered orally or by intramuscular injection. Due to the low bioavailability after oral administration, the plasma drug concentrations are very low. In addition, other critical challenges in pre-clinical studies are low sample volume and complex matrix.

This demands for a highly sensitive bioanalytical method to determine the analyte at lower concentrations. Over past many years LC-MS/MS has been considered as a gold standard for bioanalysis of small molecules owing to its sensitivity, selectivity and repeatability.

In this work, LC-MS/MS technology has been used to develop a highly sensitive bioanalytical method for analysis of Leuprolide from rat plasma.

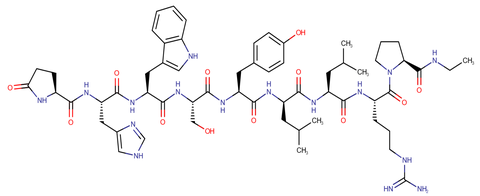

Figure 1. Structure of Leuprolide

3. Materials and methods

3-1. Sample preparation

• Preparation of spiked calibration standards and quality control (QC) samples

Leuprolide calibration standards at concentration levels from 10 pg/mL to 300000 pg/mL and quality control samples at concentration levels of LQC (45 pg/mL), MQC_1 (165 pg/mL), MQC_2 (1500 pg/mL), HQC_1 (24000 pg/mL) and HQC_2 (150000 pg/mL) were prepared in rat plasma.

• Sample extraction

Spiked calibration standards and quality control samples in rat plasma were taken in 1.5 mL microcentrifuge tubes, to which internal standard solution (goserelin) was added except in blank. Contents were vortexed and mild precipitant was added. Further the samples were centrifuged at 4500 rpm for 5 mins.

The samples were extracted by solid phase extraction technique as follows:
1.Conditioning (1 mL methanol followed by 1 mL water)
2.Loading (entire plasma sample)
3.Washing (1 mL water followed by 1 mL 20 % acetonitrile)
4.Elution (1 mL methanol)
SPE eluent was evaporated at 50 ^{0}C for 25 minutes in low pressure nitrogen evaporator. The residue was reconstituted in 100 µL reconstitution solution, vortexed and filled in HPLC vials for injection.

3-2. LC-MS/MS analysis

Figure 2. Nexera X2 with LCMS-8060

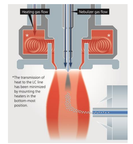

Figure 3. Heated ESI probe

LCMS-8060 triple quadrupole mass spectrometer by Shimadzu (Figure 2), sets a new benchmark in triple quadrupole technology with an unsurpassed sensitivity (UFsensitivity), ultra fast scanning speed of 30,000 u/sec (UFscanning) and polarity switching speed of 5 msec (UFswitching). This system ensures highest quality of data, with very high degree of reliability.

In order to improve ionization efficiency, the newly developed heated ESI probe (Figure 3) combines high-temperature gas with the nebulizer spray, assisting in the desolvation of large droplets and enhancing ionization. This development allows high-sensitive analysis of a wide range of target compounds with considerable reduction in background.

The details of analytical conditions are given in Table 1.

Table 1. Instrument parameters for analysis of Leuprolide

UHPLC condition (Nexera X2)	
Column	Shim-pack C18 (100 mm L × 2.1 mm I.D., 2.7 µm)
Mobile phase	A: Buffer
	B: Acetonitrile
Flow rate	0.5 mL/min
Elution mode	Flow and concentration gradient mode
Column temperature	40 ^{0}C
MS parameters (LCMS-8060)	
MS interface	Electro Spray Ionization (ESI)
Nitrogen gas flow	Nebulizing gas- 3 L/min; Drying gas- 10 L/min
Zero air flow	Heating gas- 15 L/min
MS temperatures	Desolvation line- 200 ^{0}C; Heating block- 400 ^{0}C; Interface- 300 ^{0}C
MRM transitions	Leuprolide (605 > 249) & Goserelin (635 > 221)

4. Results

4-1. Specificity and selectivity

LLOQ of 10 pg/mL was achieved for Leuprolide in rat plasma. Overlay of MRM chromatograms of blank and LLOQ spiked standards are shown in Figure 4. No interfering peaks were seen in blank plasma at the retention time of Leuprolide which confirms the absence of any interference.

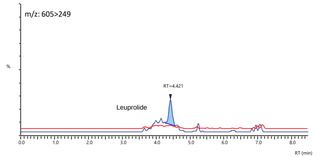

Figure 4. Overlay of MRM chromatograms of blank and 10 pg/mL spiked standard

4-2. Linearity

Linearity test was carried out using internal standard calibration method and correlation coefficient of 0.9952 was obtained for Leuprolide as shown in Figure 5.

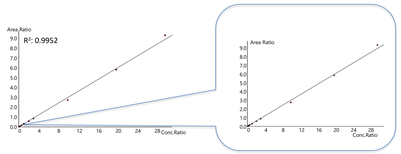

Figure 5. Calibration curve for Leuprolide spiked standards

4-3. Recovery

The recovery was more than 70 % for both Leuprolide and Goserelin as shown in Table 2 proving the efficiency of sample extraction method.

Table 2. Results of accuracy for Leuprolide spiked calibration standards

Batch	LQC	MQC_1	MQC_2	HQC_1
	Recovery (%)			
Analyte (Leuprolide)	86.79	80.28	66.08	76.71
	Average recovery			
	77.46			
Internal standard (Goserelin)	**72.86**			

4-4. Matrix effect

The interference in the response because of the presence of unintended analytes in the rat plasma matrix was evaluated. Table 3 shows the results for matrix effect experiment.

Table 3. Results of accuracy for Leuprolide spiked calibration standards

Analyte (Leuprolide)- Matrix factor			
LQC	**MQC_1**	**MQC_2**	**HQC_1**
0.90	0.80	0.66	0.78
Internal standard (Goserelin)- Matrix factor			
0.73			
Analyte matrix factor based on IS normalization			
1.23	1.10	0.90	1.07
Average matrix factor for given rat plasma			
1.07			

4-5. Precision and accuracy

Developed method was assessed for precision and accuracy batch. Results are summarized in Tables 4A and 4B, which are found to be within acceptable criteria for calibration and quality control samples.

Table 4A. Results of accuracy for Leuprolide spiked calibration standards

Name of compound	Standard concentration (pg/mL)	Calculated concentration from calibration graph (pg/mL)	% Accuracy
Leuprolide	10	9.60	95.90
	20	21.50	107.48
	60	59.10	98.50
	100	108.74	108.74
	200	201.50	100.75
	500	466.20	93.24
	1000	910.90	91.09
	2000	1842.20	92.11
	3000	2794.60	93.15
	10000	8643.90	86.44
	20000	18646.70	93.23
	30000	29985.40	99.95
	100000	107906.30	107.91
	200000	228991.60	114.49
	300000	339998.40	113.33

Table 4B. Results of accuracy and repeatability for quality control samples

Name of compound	Standard concentration (pg/mL)	Calculated average concentration from calibration graph (pg/mL) (n=6)	Average % accuracy (n=6)	Average % RSD for area counts (n=6)
Leuprolide	45 (LQC)	43.17	95.93	13.41
	165 (MQC_1)	176.47	106.95	10.23
	1500 (MQC_2)	1628.22	108.55	4.17
	24000 (HQC_1)	25756.82	107.32	5.96
	150000 (HQC_2)	158479.90	105.65	4.68

5. Conclusion

Leuprolide was analyzed from rat plasma over a concertation range from 10 pg/mL to 300000 pg/mL. Heated ESI probe and high efficiency ion transmission of LCMS-8060 has helped in achieving high sensitive analysis even with low plasma volume. Also, good linear dynamic range offered by LCMS-8060 has made analysis possible over 5 orders concentration range.

6. References

[1] Pradeep K Vuppala, Journal of B oJ ioequivalence & Bioavailability, Volume 5, Issue 4, (2013).
[2] EMA Guideline on Bioanalytical Method Validation, Rev.1 Corr. 2, EMEA/CHMP/EWP/192217/2009.
[3] US FDA Guidance for Industry, Bioanalytical Method Validation, Rev. 1, Draft September 2013.
[4] Japan MHLW, Guideline on Bioanalytical Method Validation in Pharmaceutical Development (25 July 2013).