VALIDATION OF LC-MS/MS METHOD FOR THE QUANTIFICATION OF NORETHINDRONE IN HUMAN PLASMA

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Summary

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) methods are described, one for the quantitative determination of Norethindrone (NOR) in human. The plasma method is based on solid-phase extraction. Detection takes place by ion-spray tandem mass spectrometry in the positive ion mode. Method validation results show that the method is sufficiently selective and capable of quantifying the analytes with good precision and accuracy in the concentration range of 0.1608 to 34.9782 ng/mL. The developed LC/MS-MS method was found to be selective, simple, sensitive, accurate and linear for the analysis of Norethindrone in human plasma.

Keywords: Norethindrone, Validation, LC/MS-MS

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Introduction

Norethindrone is chemically 17-Hydroxy-19-Nor-17α-pregn-4-en-20-yn-3-one. Norethindrone [NOR] and on pharmacodynamic variables that may be increased in the event of reduced contraceptive efficacy (concentrations of serum luteinizing hormone [LH], follicle-stimulating hormone [FSH], and progesterone.These methods have reported recovery to be 67-84.29%. The exact recovery can be obtained by comparing the response of processed spiked plasma with that of aqueous samples at the same concentration. The matrix effect can be evaluated by comparing spiked processed plasma blanks with aqueous samples at the same concentration. By knowing the recovery and matrix effect, the sensitivity of the method can be improved.

A specific and sufficiently sensitive method was required for the quantification of plasma NOR levels in clinical trials. The lowest limit of quantification was kept at 0.1608 ng/mL and the upper limit was 34.9782 ng/mL. Only limited methods are available in biological and analytical[1-7].In this study we reported a simple, robust, sensitive and reproducible method has been developed and validated to estimate NOR concentrations in human plasma. The validated method was applied to quantify plasma NOR concentrations in rat to determine the effect of Norethindrone on plasma NOR levels.

Materials and Methods

Chemicals and reagents

Norethindrone was obtained from Lupin Pharmacare, Pithampur, India and the internal standard (IS), Norethindrone – d_6 was from Varda Biotech (P) Ltd, Mumbai, India. The All solvents and other reagents were of analytical grade. Control human plasma (EDTA anticoagulant) for the preparation of quality control (QC) samples was obtained from a blood bank and stored at –70°C before use.

Column liquid chromatography

The column was an Acquity UPLC @ BEH column (100 mm x 2.1 mm, 1.7 µm particle size). The column was kept at 40ºC temperature. The mobile phase consisted of ammonium formate buffer (2 mM, and acetonitrile (40:60, v/v). The flow rate was 0.6 mL/min, and the total run time was 5.5 min.

Mass spectrometry

The liquid chromatograph (Waters Acquity; USA) was coupled to a mass spectrometer with a turbo electrospray ion source (4000 Qtrap) and was used in positive ionization mode with the following source settings. The turbo ion-spray interface was. Analyst software (version 1.4.2) was used for data registration.

Sample preparation

Samples were thawed in water. Withdraw the required number of quality control samples, calibration curve standards and clinical samples for validation and bio-study from ultra low temperature freezer/deep freezer and allow them to thaw in ice-water bath.Vortex the thawed samples to ensured complete mixing of contents. Pipette 50 µL of internal standard solution into all RIA vials except blank. Added 600 µL of sample into respectively labeled RIA vials and Added 50 µL of IS to all the samples and vortexed. Conditioned the strata cartridge with 1 mL of Methanol followed by

1 mL of water. Loaded the samples into cartridges and applied the gas.Washed the cartridges with 1 mL 0.05 ammonium acetate followed by 1 mL of 5% methanol in water twice. Dried the cartridges approximately for 3 minutes and elute with 300 µL of mobile phase. Loaded the processed samples into LC-MS/MS.

Standard curves

The calibration curve (CC) standards were prepared in water by adding known amounts of NOR. Lower limit of quantification (LLOQ) QC and low-quality control (LQC) samples were obtained by spiking NOR in methanol. Quality control samples Lower Quality Control (LQC), Middle-quality control (MQC) and high-quality control (HQC) samples were obtained by spiking in plasma with concentrations of 0.4682 11.1473 and 27.8682 ng/mL, respectively. The bulk-spiked CC and QC samples were stored at –70°C. All calibration curves consisted of one blank sample and eight calibration points in the concentration range of 0.1608–34.9782 ng/mL. The concentrations were corrected for potency and amount weighed. The resulting peak area ratios were plotted against the concentrations.

Validation

Specificity

A specificity exercise was performed for both water and plasma. Individual blank plasma samples, LLOQ QC samples, and water (blank) (n = 6) were prepared according to the sample preparation procedure described above and screened for interference.

Recovery

The recovery exercise was performed at all QC levels by comparing the response (area) of processed QC samples with those of directly injected QC samples. The dilutions were made in suitable solution to keep conditions the same.

Matrix effect

To study the matrix effect, blank plasma samples were processed and spiked later to obtain MQC and HQC concentrations. The response (area) was compared with directly injected samples at MQC and HQC levels.

Inter-assay and intra-assay imprecision and accuracy

Inter-assay and intra-assay imprecision and accuracy were evaluated by spiking known amounts of NOR and IS in plasma. Four different concentrations were used, and samples were prepared according to the procedure mentioned above. Intra-assay imprecision and accuracy were assessed within one batch, whereas inter-assay imprecision and accuracy were assessed on three separate occasions.

Stability

The stability of NOR was studied in human plasma and water at room temperature (bench top) for 16 h. and in an autoinjector for 35 h. The bulk-spiked plasma and water samples stored at –70°C underwent three freeze-thaw cycles. Stock solution stability studies were performed at room temperature for 18 h, long-term stability study was done in human plasma stored at –70°C. The stability of NOR was assessed in an autoinjector for 20 h. Stock solution stability studies were performed at room temperature for 4 h and in refrigeration for 18 hours. Stock solution stability studies were carried out at the MQC level, and working IS was prepared from fresh and

refrigerated stock solutions. The drug and IS response ratios of stored and fresh stocks were compared. In other stability studies, five replicates of LQC and HQC were analyzed.

Results

A high-performance liquid chromatographic mass spectrometric method for the estimation of NOR in human plasma has been developed and validated according to the principles of Good Laboratory Practices. The plasma was validated over a concentration range of 0.1608 to 34.9782 ng/mL. Sample cleanup was accomplished by solid-phase extraction using cartridges (Figure 1). The reconstituted samples were analyzed by LC-MS/MS using a Acquity column. The retention times of NOR and NOR-D6 were between 1.94 and 1.92 min, with a total run time of 5.5 min (Figure 2). The lower limit of quantitation was 0.1685 ng/mL for NOR. The linearity of the method was determined by a weighted least-squares regression analysis of an eight point standard curve. The calibration lines were shown to be linear from 0.1608 to 34.9782 ng/mL. Best-fit calibration lines of the ratio of NOR to IS peak area versus the concentration of calibration standards were determined by least-squares regression analysis with weighting factors of $1/x^2$. The r^2 values were consistently >0.99 during the course of validation (Figure 3).

Figure 1 Representative Chromatogram of a Blank Plasma Sample for Norethindrone

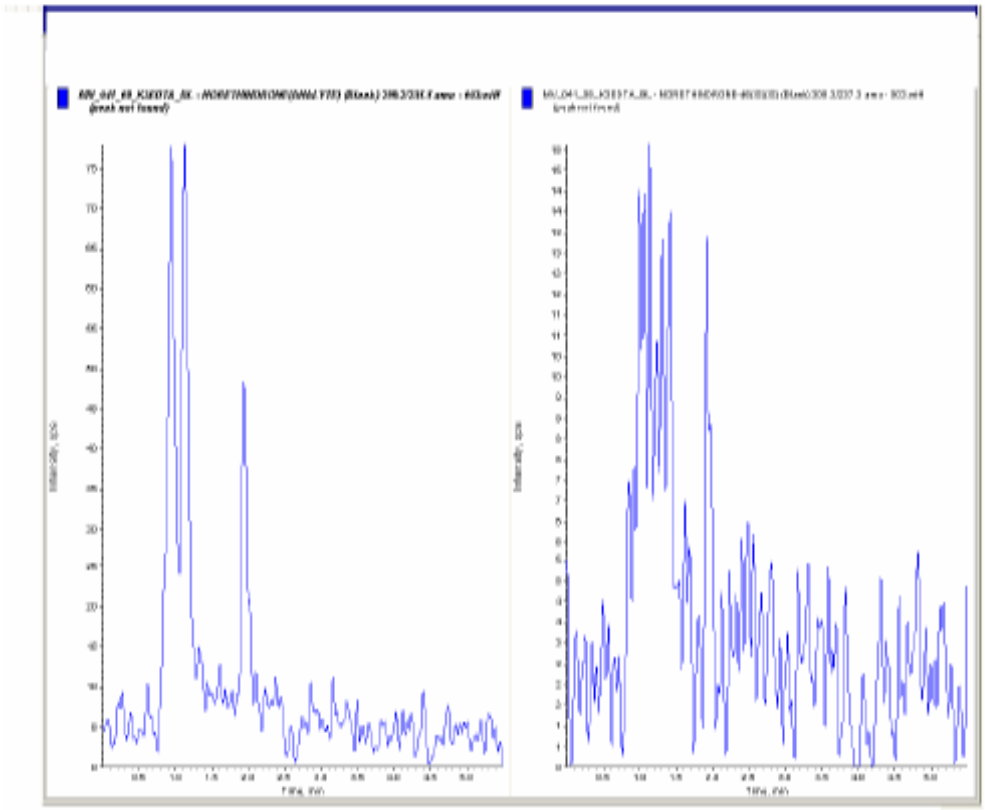


Figure 2 Representative Chromatogram of an Aqueous Standard Solution for Norethindrone

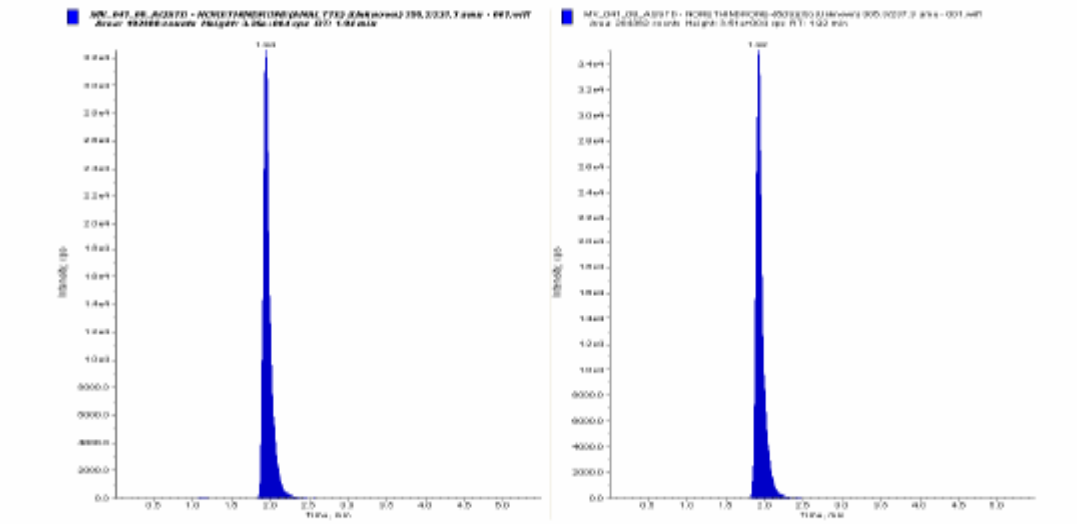
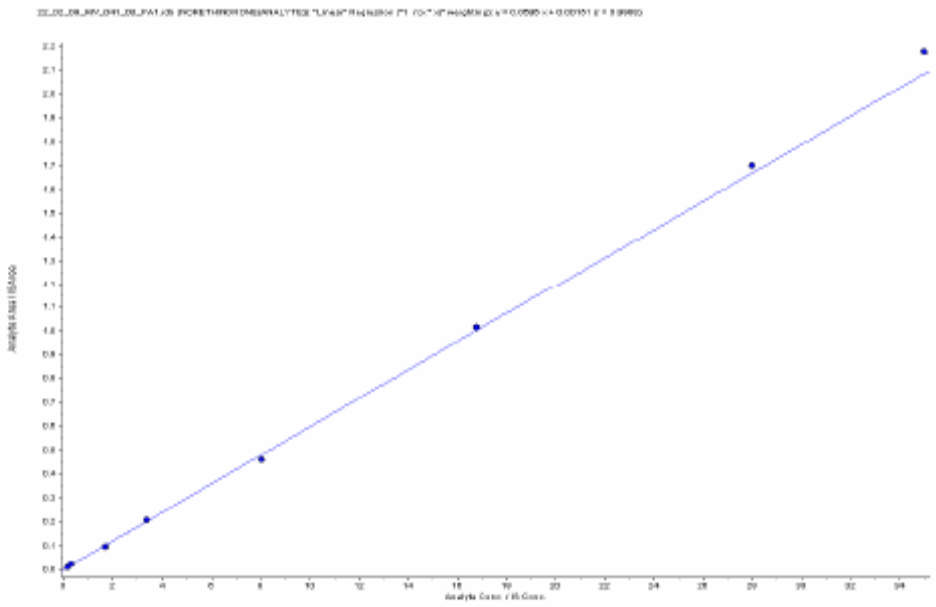


Figure 3 Representative Regression Analysis of a Calibration Curve for Norethindrone



The imprecision of the assay was measured by the percentage coefficient of variation over the concentration range of LLOQ QC, LQC, MQC, and HQC samples during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the QC samples to their respective nominal values, expressed as percentages (Table 1 and 2). The percentage matrix effect was 97.71% for analyte and 99.45% for IS.

Table 1 Intra-batch Precision and Accuracy of Norethindrone

P&A batch ID	LOQQC	LQC	MQC	HQC
Actual (ng/mL)	0.1685	0.4682	11.1473	27.8682
P&A - 01	0.1690	0.5410	11.3896	29.8600
	0.1831	0.5317	11.2837	29.4626
	0.1410	0.4343	11.5185	30.2071
	0.1840	0.4530	11.7377	29.3471
	0.2044	0.5069	11.8787	29.5877
	0.1626	0.5105	11.4291	29.6667
MEAN	0.17402	0.49623	11.53955	29.68853
SD	0.021684	0.043091	0.225775	0.308733
%CV	12.46	8.68	1.96	1.04
%NOMINAL	103.27	105.99	103.52	106.53
P&A - 02	0.1741	0.4819	11.6412	28.2651
	0.1997	0.4707	11.2827	29.2452
	0.1936	0.4781	11.2074	28.4509
	0.2096	0.4933	11.5094	28.9396
	0.1732	0.4426	11.3327	NA
	0.2234	0.4278	11.2612	NA
MEAN	0.19560	0.46573	11.37243	28.72520
SD	0.019767	0.025191	0.167472	0.448442
%CV	10.11	5.41	1.47	1.56
%NOMINAL	116.08	99.47	102.02	103.08
P&A - 04	0.1949	0.4854	11.0386	28.7622
	0.1588	0.4647	11.291	28.4668
	0.1814	0.506	11.3303	28.3643
	0.2121	0.4708	11.0163	27.9403
	0.2007	0.4617	10.9406	28.6174
	0.1496	0.4797	11.0909	27.9708
MEAN	0.18292	0.47805	11.11795	28.35363
SD	0.024517	0.016347	0.157387	0.336697
%CV	13.40	3.42	1.42	1.19
%NOMINAL	108.56	102.10	99.74	101.74
P&A - 05	0.1784	0.4724	10.6905	27.4923
	0.1444	0.5140	11.2112	27.4799
	0.1536	0.5141	10.9011	27.2954
	0.1929	0.4582	11.1956	27.4975
	0.1942	0.4580	16.1027	28.7337
	0.1948	0.4499	11.4944	28.6409
MEAN	0.17638	0.47777	11.93258	27.85662
SD	0.022251	0.029024	2.061657	0.648529
%CV	12.62	6.07	17.28	2.33
%NOMINAL	104.68	102.04	107.04	99.96
P&A -0 6	0.1835	0.4978	11.5169	28.6621
	0.1954	0.4876	11.3131	28.5946
	0.1967	0.4923	11.1494	28.6150
	0.1887	0.4801	11.0921	28.5943
	0.1666	0.4719	11.0347	28.3288
	0.1722	0.4979	11.4514	28.0298
MEAN	0.18385	0.48793	11.25960	28.47077
SD	0.012292	0.010337	0.198309	0.246013
%CV	6.69	2.12	1.76	0.86
%NOMINAL	109.11	104.21	101.01	102.16
P&A -07	0.1846	0.4389	11.2045	29.1945
	0.1543	0.4303	11.2891	29.2146
	0.1593	0.4644	11.0470	28.4336
	0.1693	0.4510	11.0569	28.7715
	0.1465	0.4407	11.4776	28.5286
	0.1613	0.4653	11.3140	27.9250
MEAN	0.16255	0.44843	11.23152	28.67797
SD	0.013188	0.014321	0.164890	0.492446
%CV	8.11	3.19	1.47	1.72
%NOMINAL	96.47	95.78	100.76	102.91

Table 2 Inter-day or Total Precision and Accuracy of Norethindrone

	LOQQC	LQC	MQC	HQC
Actual Concentration (ng/mL)	**0.1685**	**0.4682**	**11.1473**	**27.8682**
	0.1690	0.5410	11.3896	29.8600
	0.1831	0.5317	11.2837	29.4626
	0.1410	0.4343	11.5185	30.2071
	0.1840	0.4530	11.7377	29.3471
	0.2044	0.5069	11.8787	29.5877
	0.1626	0.5105	11.4291	29.6667
	0.1741	0.4819	11.6412	28.2651
	0.1997	0.4707	11.2827	29.2452
	0.1936	0.4781	11.2074	28.4509
	0.2096	0.4933	11.5094	28.9396
	0.1732	0.4426	11.3327	NA
	0.2234	0.4278	11.2612	NA
	0.1949	0.4854	11.0386	28.7622
	0.1588	0.4647	11.291	28.4668
	0.1814	0.506	11.3303	28.3643
	0.2121	0.4708	11.0163	27.9403
	0.2007	0.4617	10.9406	28.6174
	0.1496	0.4797	11.0909	27.9708
	0.1784	0.4724	10.6905	27.4923
	0.1444	0.5140	11.2112	27.4799
	0.1536	0.5141	10.9011	27.2954
	0.1929	0.4582	11.1956	27.4975
	0.1942	0.4580	16.1027	28.7337
	0.1948	0.4499	11.4944	28.6409
	0.1835	0.4978	11.5169	28.6621
	0.1954	0.4876	11.3131	28.5946
	0.1967	0.4923	11.1494	28.6150
	0.1887	0.4801	11.0921	28.5943
	0.1666	0.4719	11.0347	28.3288
	0.1722	0.4979	11.4514	28.0298
	0.1846	0.4389	11.2045	29.1945
	0.1543	0.4303	11.2891	29.2146
	0.1593	0.4644	11.0470	28.4336
	0.1693	0.4510	11.0569	28.7715
	0.1465	0.4407	11.4776	28.5286
	0.1613	0.4653	11.3140	27.9250
MEAN	**0.17922**	**0.47569**	**11.40894**	**28.62311**
SD	**0.020777**	**0.028339**	**0.839898**	**0.701412**
%CV	**11.59**	**5.96**	**7.36**	**2.45**
%NOMINAL	**106.36**	**101.60**	**102.35**	**102.71**

Discussion

In this study, a reverse-phase HPLC method with mass spectrometric detection using D7-NOR as an IS was developed. Various combinations of organic and aqueous phases were tried, and better chromatography with lower baseline was achieved using 2mM ammonium formate buffer and acetonitrile (20:80, v/v) as the mobile phase. Response was observed in the range of 0.6 mL/min flow rate. Better sample cleanup and reproducibility were obtained using strata cartridges. The method described is sensitive, selective, precise, and accurate for the determination of NOR in human plasma at very low concentrations. The method has been validated for a maximum batch size of samples.

The matrix effect was determined by comparing spiked processed blank plasma QC samples against the same aqueous concentrations for NOR and NOR-D6. A significant matrix effect was observed. The low recovery is attributable to the matrix effect. During method development, six different plasma samples were spiked to add 10 ng/mL to endogenous NOR. The response observed in spiked plasma was increased proportionately compared with that in blank plasma, and similar results were seen with QC samples. It can be concluded that the presence of some constant endogenous substance other than NOR or any reagent effect during sample processing

can contribute to the matrix effect. Hence, this method can be used to obtain accurate plasma NOR concentrations.

The method was successfully applied to estimate human plasma NOR levels after a single oral dose of Norethindrone (0.35 mg).

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