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QUANTITATIVE BIOANALYSIS OF LEVONORGESTREL IN HUMAN PLASMA USING LC-MS-MS

Bhargavi P*[1], Lakshmi K.S[1], Lakshmi S[1], Ravi Kumar K[2]

1. Department of Pharmaceutical Analysis, SRM College of Pharmacy, SRM University, Kattankulathur-603 203, Tamil Nadu, India.
2. Vimta Labs Pvt. Ltd., Cherlapally, Hyderabad-500 762, Andhra Pradesh, India.

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For Correspondence:

Bhargavi P

SRM College of Pharmacy,
SRM University,
Kattankulathur-603 203,
Tamil Nadu, India

E-mail:
bhargavi.pasagada@gmail.com

ABSTRACT

A rapid, sensitive and reproducible liquid chromatography-tandem mass spectrometry method for the determination of Levonorgestrel in human plasma was developed and fully validated. With a structural analogue Levonorgestrel-d_6 as the internal standard, Levonorgestrel was extracted from human plasma with 2% Ammonia in Methanol solution. The organic layer was evaporated to dryness and the residue was reconstituted in mobile phase of 0.1% Formic acid, Acetonitrile and Methanol. An aliquot of 20 μL was chromatographically analyzed on a Vertisep BDS C_{18} column with the mobile phase. Selected reaction monitoring was specific for mass detection employing positive turbospray ionization. The calibration standards were linear over the concentration range 0.5-100.0 ng mL^{-1}. The lower limit of quantitation of this method was 0.5 ng mL^{-1}. The results indicate that the method was efficient with a simple preparation procedure and had high selectivity, sensitivity, acceptable accuracy and precision at low cost. The validated LC-MS/MS method was successfully used for a bioequivalence study of Levonorgestrel tablets in healthy female volunteers.

INTRODUCTION

Levonorgestrel is a synthetic progestogen. It is a hormonally active levorotatory enantiomer of the racemic mixture norgestrel. It is used as an active ingredient in some hormonal contraceptives. It is widely used in emergency contraception for women who had unprotected sexual intercourse thus preventing unwanted pregnancy [1, 2]. As an emergency contraceptive, the use of Levonorgestrel has been increasing. Furthermore, due to the strong side effects, the detection of Levonorgestrel in plasma is important to provide information for pharmacokinetic or bioavailability studies. The knowledge of the pharmacokinetics of Levonorgestrel when used in different forms and the selection of the dose currently recommended is based on limited data [3-6].

Since the year 1979 several methods have been reported for the determination of Levonorgestrel such as thin-layer chromatography [7, 8], radioimmunoassay [9], spectrophotometry [10, 11], voltammetry [12] or HPTLC but the methods were not sensitive enough for pharmacokinetic analysis of low doses of Levonorgestrel. Although the GC-MS methods were selective and sensitive enough to support pharmacokinetic studies when low doses of the drugs were administered but complex sample preparation with multiple steps of derivatization prior to analysis was usually very time consuming and run times exceeded 20 min per sample, thus making them less suitable for the high-throughput analysis. In the recent years LC–MS/MS has become the method of choice for the analysis of the drugs in plasma or serum due of its high sensitivity and selectivity [13 and references therein]. F Vallée *et al* [14] employed LC-MS/MS to quantify Levonorgestrel in human plasma by liquid-liquid extraction with hexanes. Qinggang Wang *et al* [15] determined Levonorgestrel in human serum by an ether extraction procedure followed by high-performance liquid chromatography electrospray tandem mass spectrometric method using Norethindrone as an internal standard. David Matějíček *et al* [16] developed a high performance liquid chromatographic/ion-trap mass spectrometric method for simultaneous determination of estrogens in contraceptives and river water samples with LLOQ of 14 ng mL^{-1}. Liu Fei *et al* [17] determined Levonorgestrel in human plasma with Norethindrone as the internal standard and extracted from plasma using Ethyl acetate by liquid chromatography-tandem mass spectrometry method. All these methods employed LLE which required a large volume of organic solvents, limiting the sample preparations in tube format and thus difficult to automate. Consequentially they posed results with less sensitivity and occurrence of interfering peaks at low concentration levels. H. B. Theron *et al* [18] used atmospheric pressure photospray ionization source to lower the background noise, but the sample preparation procedure needed a freezing bath at −25 °C to achieve layer separation which made the operation complicated. Meanwhile, a large plasma volume (1 mL) was required to obtain a low LLOQ by high condensation.

The current work adopts a highly sensitive LC-MS/MS technique for the quantitative bioanalysis of Levonorgestrel in human plasma over a concentration range of 0.5-100.0 ng mL^{-1} with Levonorgestrel-d_6 as the IS using a solid–phase extraction (SPE) procedure. The LLOQ was 0.502 ng mL^{-1} using 500µL plasma sample. The proposed method was validated and applied successfully to the analyses of samples obtained from a pharmacokinetic study.

MATERIALS AND METHODS

Chemicals and Reagents

Levonorgestrel was supplied by Lupin Pharma Care (India). Levonorgestrel-d_6 (IS) was supplied by Syncom (India). Acetonitrile and Methanol (both Lichrosolv) were obtained from Merck (India). Formic acid (SQ-grade), Potassium dihydrogen orthophosphate (ExcelR) and Liquor Ammonia (ExcelR) were obtained from Qualigens (India). HPLC grade water obtained from Merck was used throughout the study. Blank plasma was supplied from a private blood bank located in Hyderabad (India).

Preparation of calibration curve standards and quality control samples

Pure reference standard of Levonorgestrel and Levonorgestrel-d_6 were separately dissolved in 60% Methanol to obtain their respective stock solutions which were spiked into a pool of blank K_2EDTA human plasma. By serial dilution of stock solutions with blank K_2EDTA human plasma, a calibration standard range between 0.5 and 100.0 ng mL^{-1} was obtained. Similarly, quality control standards (QC) were prepared but from different stock solutions at 0.5, 1.5, 8.0, 40.0 and 80 ng mL^{-1} concentrations. Sufficient calibration standards and quality controls were prepared to validate the method and assay all the study samples. Aliquots of the standards and quality controls were stored in polypropylene tubes together with the study samples at −20°C until processed.

Extraction procedure

0.500mL of 50mM Potassium dihydrogen orthophosphate was added to 0.500mL of Blank, calibration curve standards and quality control samples. 50µL of Levonorgestrel-d_6 (approximately 1.500 µg mL^{-1}) was added to all the above samples (except the plasma blank). 50µL of 60% Methanol in water solution was added to plasma blank. The samples were vortexed to ensure complete mixing of contents.

OASIS MAX 30mg/1cc cartridges, (new cartridge for each sample) was taken onto a positive pressure processor manifold and the procedure as below was followed –

i. *Conditioning:* 1.000mL each of Methanol and water was added separately. (Taken care not to dry cartridge).
ii. *Loading:* The sample was applied and was allowed to dry for approximately 2.00 minutes under positive pressure.
iii. *Washing:* The cartridges were washed with 1.000mL of 0.1% Formic acid in water solution followed by 1.000mL of 5% Methanol in water solution and allowed to dry under positive pressure for approximately 5.00 minutes.
iv. *Elution:* The drug was eluted into 1.000mL of 2% Ammonia in Methanol solution and allowed to dry under positive pressure for approximately 2.00 minutes. This organic layer was evaporated under a stream of Nitrogen gas at 45ºC. It was reconstituted with 0.250mL of Mobile phase. The sample was loaded into auto sampler vials. 20µL of sample was injected onto the LC-MS/MS system.

Liquid Chromatography

Chromatography was performed on Agilent 1200 series. 20μL of each of the extracts were injected into the HPLC Vertisep BDS C_{18} (4.6×100mm) column with 0.1% Formic acid, Acetonitrile and Methanol (10:10:80 v/v/v) as the mobile phase. The flow rate was 0.9 mL/min and the total run time was 6.5 minutes.

Mass Spectrometry

The Applied Biosystems API 4000, a triple-quadrupole tandem mass spectrometer equipped with turbospray ionization source was operated at unit resolution in the multiple reaction monitoring (MRM) mode for the quantification of Levonorgestrel.

After direct infusion of 1 ng mL^{-1} Levonorgestrel and IS standard solutions, high abundance of $[M+H]^+$ was found in positive mode owing to the oxygen atoms in the structures as proton acceptors. A collision gas pressure of 6 PSI in collision cell induces to fragment the protonated molecular ions of Levonorgestrel and IS. The transitions of $[M+H]^+$ precursor molecular ions were *m/z* 313.40 → 109.20 for Levonorgestrel and *m/z* 319.40 → 115.20 for IS with optimal collision energies of 26 V and 60 V respectively. MS detection was chosen because of its high sensitivity and selectivity. MS conditions were optimized to achieve high MS response. The most intensive ions at *m/z* 245.1 for Levonorgestrel and at *m/z* 233.1 for IS were obtained. For instrument control, data acquisition and processing, the instrument was interfaced with a computer running Applied Biosystems Analyst version 1.4.2 software, Canada.

The mass spectra of Levonorgestrel and its resulting product ion are presented in **Fig. 1**

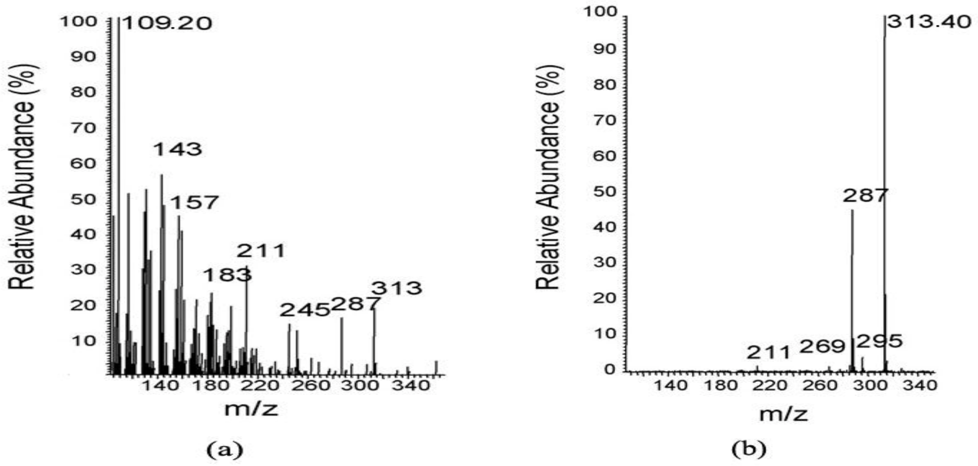

Fig. 1 The mass spectra of Levonorgestrel (**a**) and its resulting product ion (**b**)

Validation

The validation was carried out according to the recommendations and definitions provided by the “Guidance for Industry—Bioanalytical Method Validation” (FDA, USA, 2001) [19]. The method was validated for different parameters such as linearity, accuracy, precision, ruggedness, selectivity, carryover effect, sensitivity, stability, recovery, matrix effect and dilution integrity.

RESULTS AND DISCUSSION

Validation Parameters

The method was validated over a concentration range of 0.500 and 100.000 ng mL^{-1} using $1/x^2$ weighting method for the best linear fit of concentration Vs response relationship. Correlation coefficient (r) generated was 0.9996, which confirmed the assay was linear over the above concentration range and this is shown in the **Fig. 2**

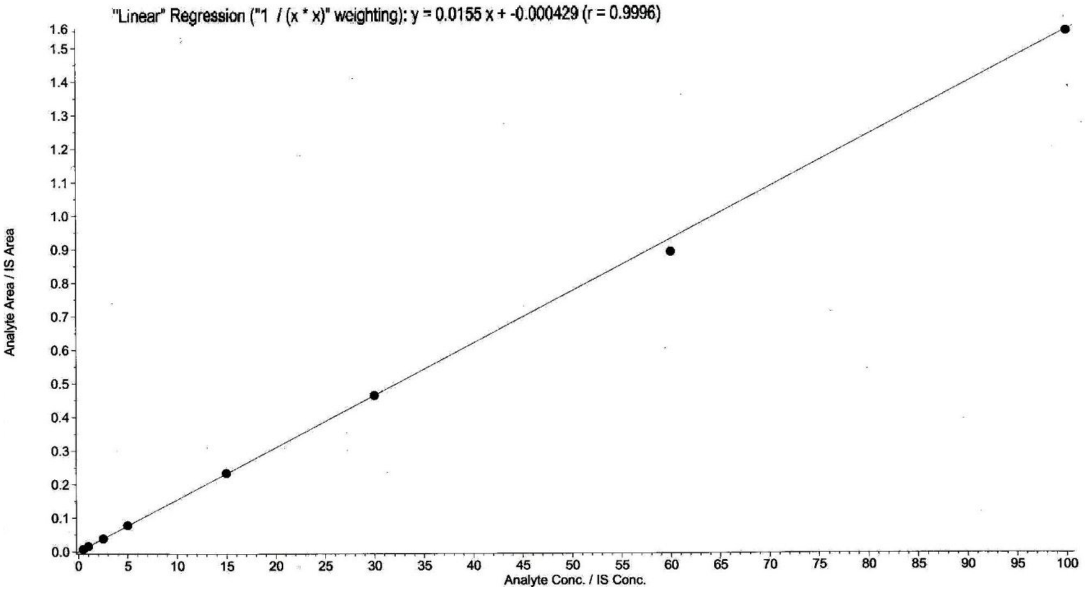

Fig. 2 A representative calibration curve for Regression analysis

Precision and Accuracy were evaluated in intra and inter precision and accuracy batches by determining three concentrations of quality control samples (LQC, MQC, HQC) in six replicates under one calibration curve. The results of precision and accuracy were summarized in **Table 1** which proved that the proposed method was acceptable. Ruggedness was performed for the changes in column and analyst which was studied during precision and accuracy batches.

Table 1: The precision and accuracy results of the validation of Levonorgestrel

1.	**PRECISION** (%CV < 15%)	LQC	MQC	HQC
	Inter-batch precision	3.46%	4.02%	2.19%
	Intra-batch precision	3.23% -3.77%	0.58% -5.17%	0.52% -1.49%
2.	**ACCURACY** (% Accuracy within 85%-115%)	LQC	MQC	HQC
	Inter-batch accuracy	100.58%	98.36%	94.44%
	Intra-batch accuracy	99.36%-101.75%	95.07%-101.62%	92.45%-96.72%

The selectivity of the method was established by checking the blank K_2EDTA human plasma obtained from 8 different blood donors (without spiking with Levonorgestrel-d_6) of which one from Haemolytic K_2EDTA plasma and other from Lipemic K_2EDTA plasma (which may contain any interfering compounds that elute along with Levonorgestrel and IS) with 6 samples of LLOQ concentration spiked from K_2EDTA human plasma of any one donor. The response of interfering peaks at the retention time of the Levonorgestrel and ISTD were less than 20% and 5% of the mean response obtained at LLOQ for Levonorgestrel and IS respectively. This can be observed in the **Fig. 3**

Sensitivity was determined by lower limit of quantitation quality control (LLOQ) sample that was measured with acceptable accuracy and precision. The LLOQ was established at 0.502 ng mL^{-1} (as depicted in Fig.3d), at which the calculated accuracy and precision was 99.73% and 8.17% respectively.

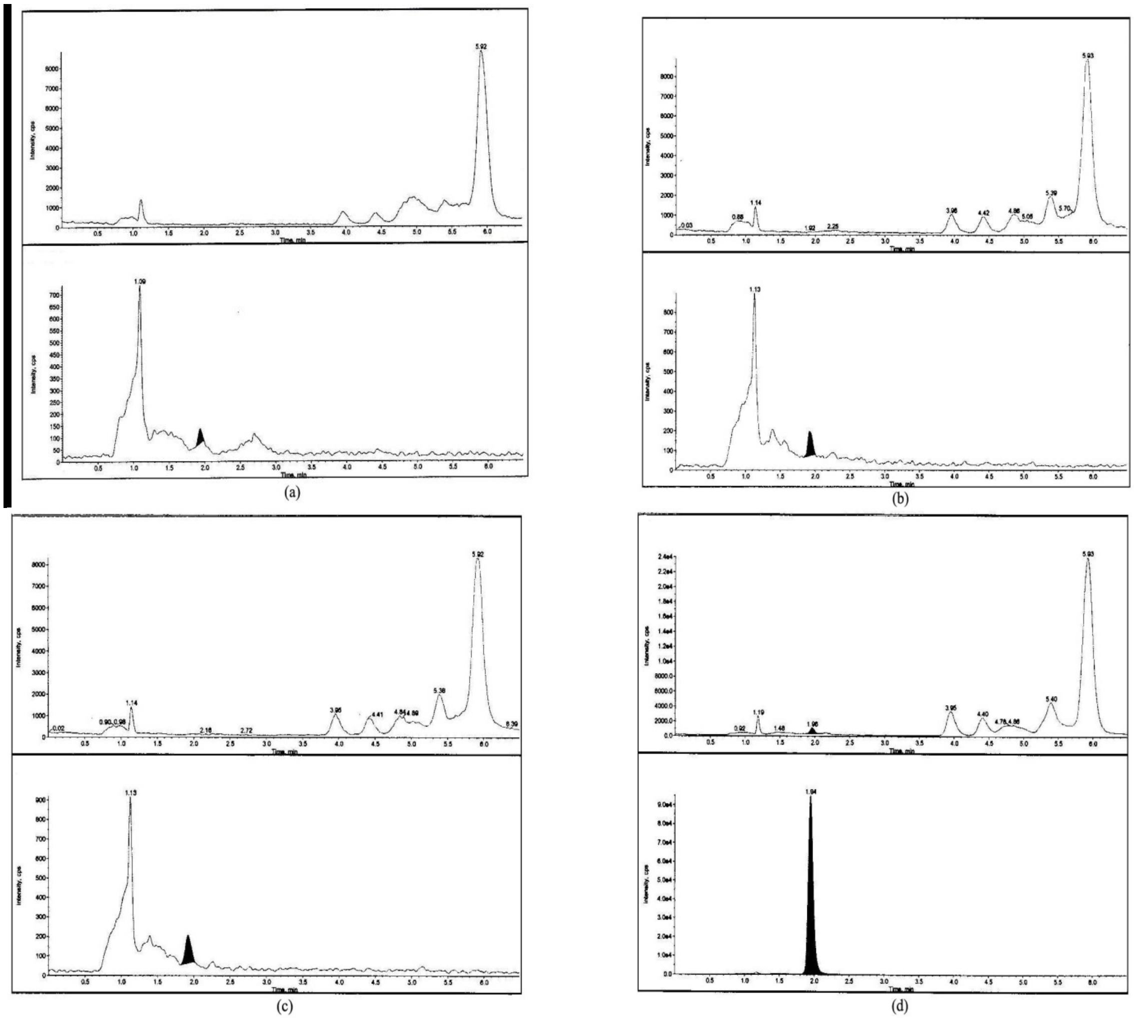

Fig. 3 Representative chromatograms for Levonorgestrel and IS in human plasma: **a** Blank Plasma; **b** Blank Haemolytic Plasma; **c** Blank Lipemic Plasma; **d** LLOQ

Two blank samples from biological matrix (plasma) for seven injections and two samples at ULOQ concentration for six injections were processed and analyzed alternately to determine no carryover in the blank sample due to ULOQ.

The stability of Levonorgestrel was estimated by subjecting the QC samples (LQC and HQC) to the effect of three freeze and thaw cycles, up to 11.00 hours delay on the Bench-top, up to 33.00 hrs of In-injector stability, up to 24.00 hrs of Wet extract stability and up to 24.00 hrs Dry extract stability. The **Table 2** summarizes the results of stability studies which proved that the drug was found to be stable under various stability conditions.

Table 2: The stability studies results of Levonorgestrel

Sl. No	STABILITY	% Stability (85% - 115%)	
		LQC	HQC
1.	Freeze & Thaw	105.08%	97.94%
2.	In-injector (after 33hr)	106.58%	97.83%
3.	Wet Extract (after 24hr)	102.70%	98.17%
4.	Dry Extract (after 24hr)	104.14%	97.57%
5.	Bench top (after 11hr)	101.33%	97.89%

The extraction efficiency of Levonorgestrel was determined by performing the recovery experiment in six replicates for Levonorgestrel along with IS and comparing the analytical results for extracted samples at three QC concentrations (LQC, MQC and HQC) with the extracted samples that represent 100% recovery. The average % recovery of Levonorgestrel and IS (Levonorgestrel-d_6) was found to be 73.93% and 74.22% respectively.

The suppression or enhancement of ionization of analytes by the presence of matrix components in the biological samples was estimated by matrix effect which was determined as IS-normalized factor at two QC concentrations (LQC and HQC) in 6 replicates each for analyte along with IS. The IS-normalized factor as calculated by the CV% was below 15% as per the acceptance criteria.

Dilution integrity was tested by diluting 6 replicates of 1.6 times the ULOQ concentration (160.512 ng mL^{-1}) with matrix blank (depending on the sample volume used for the assay) in 1:5 ratio. The % accuracy and CV% for 1:5 dilutions were found to be 98.94 % and 0.70% respectively.

Pharmacokinetic Study

The validated method was applied to a pharmacokinetic study which was conducted with 72 healthy female volunteers who had received a single oral dose of 1.5mg Levonorgestrel tablet. The drug was administered under fasting condition. Blood samples (4ml) were taken into heparinized evacuated glass tubes by venepuncture prior to dose and at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.33, 2.67, 3.00, 4.00, 5.00, 6.00, 8.00, 10.00, 12.00, 18.00, 24.00, 36.00, 48.00, 72.00, 96.00, 120.00h thereafter. Plasma samples were separated by centrifugation at 4,000g for 10min and stored at -20°C

The profile of the mean plasma concentration versus time is presented in the figure 1. The maximum plasma concentration (C_{max}) was 20.305 $\pm$ 8.840 ng mL^{-1}; area under curve ($AUC_{0\rightarrow120}$) was 385.492 $\pm$ 64.688 ng mL^{-1}; the time to maximum plasma concentration (T_{max}) was 2.72 $\pm$ 1.00 h; and the half-life ($t_{1/2}$) was 32.153 $\pm$ 4.494 h.

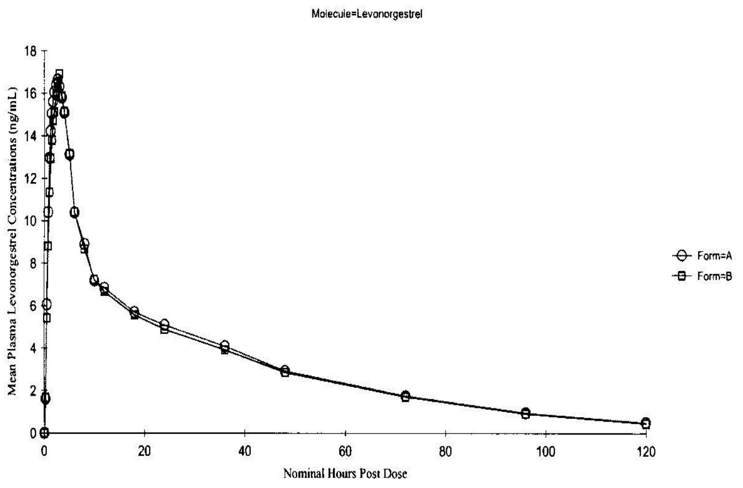

Figure 4: Mean Plasma Concentrations Vs Time Plot – Linear Scale

CONCLUSION

Levonorgestrel was determined in human plasma over a concentration range of 0.5-100.0 ng mL^{-1} based on the C_{max} of the drug which was ideal for the pharmacokinetic study. Following a single oral dose in a set of 72 healthy female volunteers, Levonorgestrel in human plasma was quantified for the first time with a structural analogue Levonorgestrel-d_6 as an IS by solid phase extraction technique using 2% Ammonia in Methanol solution. This work could eliminate the significant interfering peaks that co-elute with Levonorgestrel and IS at low concentration levels as reported by earlier researchers thus making the method more sensitive, specific and reliable.

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