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Analysis of Levonorgestrel in Human Plasma by UPLC-MS/MS: Application to a Bioequivalence Study in Healthy Volunteers

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ABSTRACT:

The metabolic and the pharmacokinetics studies in the pharmaceutical industry at different development stages of drugs have gained a tremendous momentum by using a liquid chromatography interfaced with a tandem mass spectrometry. However, due to the advancement in the drugs analytical techniques like ultra-performance liquid chromatography linked with tandem mass spectrometry (UPLC-MS/MS) have emerged immensely in comparison to the conventional techniques due to advantages in speed, sensitivity, selectivity and resolution with a small particle size. Levonorgestrel (LNG) was determined in human plasma by UPLC-MS/MS while the determination of the internal standard LNG-d6 was done using the SPE mode of extraction using a Phenomenex Strata-X (30 mg, 1cc) cartridge. Waters Acquity UPLC BEH C18 column with the internal diameter of 1.7 µm and mobile phase composition as acetonitrile-2 mM ammonium formate, pH 3.0 (90:10, v/v) was used for the analysis which was then detected with the tandem mass spectrometer equipped with electrospray ionization (ESI) in positive ion mode with the multiple reaction monitoring (MRM). Calibration curve was linear over the range 0.025-25.0 ng/mL with the correlation coefficient value as ≥ 0.99. Precision and accuracy value of intra and inter-batch fall within the range of 0.96-2.84 % and 98.9-101.0 % respectively. Various parameters like sensitivity, selectivity, stability, ruggedness, carryover, dilution integrity, ion suppression/ enhancement was further validated to perk the developed method. The overall recoveries for LNG were within 93.2 to 95.0 %. Stability assessment was done for varied conditions like bench top, freeze and thaw, wet extract, dry extract and long-term stability. The propound method offers greater sensitivity, lower sample consumption, rapid and can be relevantly used for the pharmacokinetic study of 34 human volunteers with the reproducibility value as 122.

Keyword: *Levonorgestrel, sensitive, high throughput, UPLC-MS/MS, incurred sample reanalysis.*

INTRODUCTION

Contraceptives have been used both by men and women from past many years to control fertility by different artificial means, however all the types of means are not suitable. [1] So with the need to curb the growing population, varied forms of contraceptives are developed by constant research in this area. A synthetic second-generation progestin hormone levonorgestrel (17-α) -(+/-)-13 ethyl- 17 hydroxy-18,19-dinorpregn-4-en-20-yn-3-one also known as l-norgestrel or D-norgestrel is used as a progestin only emergency contraceptive to prevent pregnancy. [2][3] Levonorgestrel also acts as an active ingredient in many COC's pills, intrauterine systems, emergency contraceptives pills, progestogen only pills, and in HRT. [4] It does not undergo first pass metabolism, about 97-99% protein binding while LNG has majority of binding with the sex hormone binding globulin (SHGB), with a half-life of 17-20 h and peak plasma concentration reaching in less than 2 h. [5]

Various methods of analysis have been reported for the determination of levonorgestrel including Radio-immuno assay) [6], Enzyme Immuno Assay (EIA) [7], but these methods generally faced problem regarding sensitivity, time consuming, expensive and radioactive labelling other methods have also been reported including the analysis of Levonorgestrel in rat plasma [8], water samples [9] using HPLC, HPTLC [10] [11], simultaneous determination of ethinylestradiol and levonorgestrel by derivative spectrometry [12], spectrometric determination [13], voltametric analysis [14]. Desorption chemical ionization technique using membrane inlet mass spectrometry was proposed by Luaritsen et al [15]. Theron et al. used LC-MS operating in APPI mode to determine LNG in human plasma and serum [16]. Kumar et al. developed an ESI-LC-MS/MS to quantify LNG in human plasma by chemical derivatization [5].

However, to overcome all the problems in regard to sensitivity, throughput, time, sample preparation and hazards accompanying the use of radio labelling a selective, rapid, sensitive, robust UPLC-MS/MS method have been developed for the determination of LNG in human plasma. To the best of our knowledge this method has not been used so far. The advantages of the developed method are high sensitivity, robust, simple sample preparation, less time consuming, and the method also has been fully validated and successfully applied to the bioequivalence study of 34 healthy subjects.

2. Experimental

2.1 Chemicals and materials

Reference standard of Levonorgestrel (LNG, 99.93 %), and Levonorgestrel-d6 (IS, 99.89%) were obtained from Clearsynth Labs (P) Ltd. (Mumbai, India). HPLC grade methanol and acetonitrile were obtained from Mallinckrodt Baker, S.A.de C.V. (Estado de Mexico, Mexico). Bio ultra-grade ammonium formate and LC-MS grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Solid phase extraction cartridges, Phenomenex StrataTM-X (30 mg, 1 cc) were obtained from Phenomenex India (Hyderabad, India). Water was purified using Milli-Q water purification system from

Millipore (Bangalore, India). Blank human plasma was obtained from Supratech Micropath (Ahmedabad, India) and was stored at –20 °C until use.

2.2 Liquid chromatographic and mass spectrometric conditions

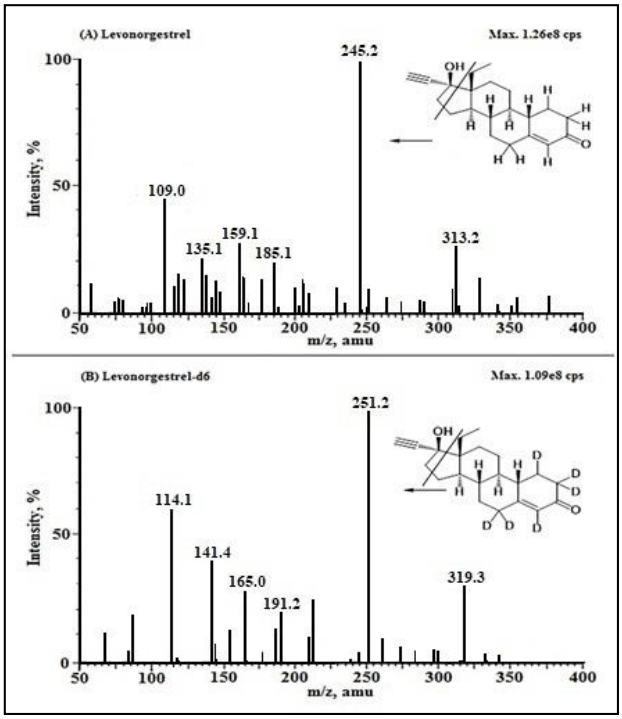
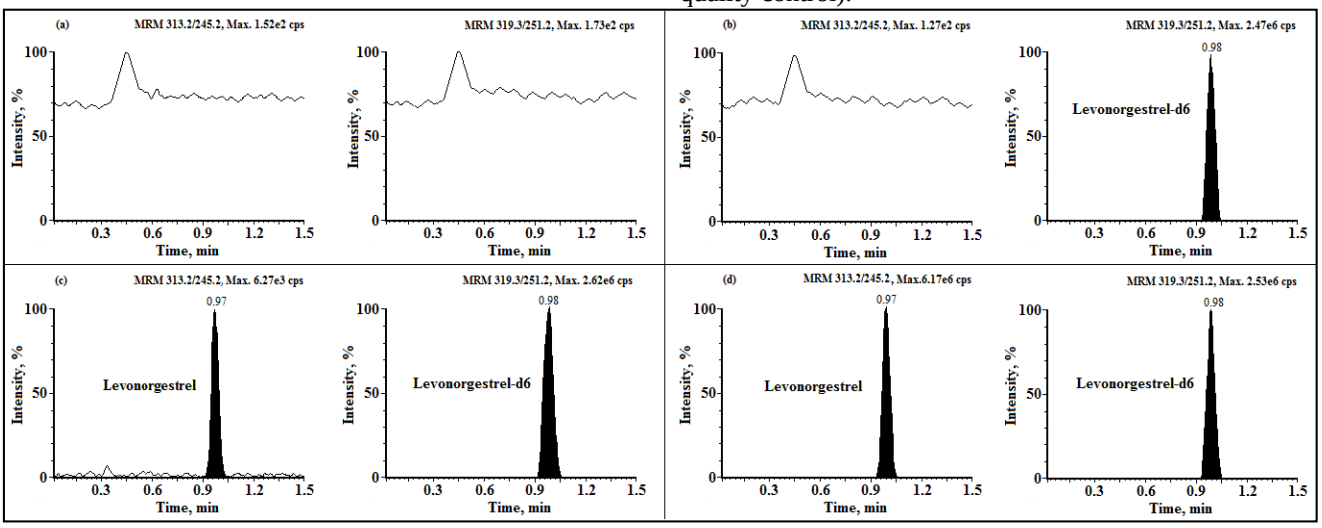


Fig. 1. Product ion mass spectra of (a) Levonorgestrel (m/z 313.2 → 245.2, scan range 50-400 amu), (b) Levonorgestrel-d6 (m/z 319.3 → 251.2, scan range 50-400 amu) in positive ionization mode.

Waters Acquity UPLC BEH C18 column (Milford, MA, USA) maintained at a temperature of 25 °C was used for the solvent and sample delivery which was then interfaced to a Waters Quattro Premier XE (Milford, MA, USA) triple quadrupole operating in positive ion electro-spray ionization mode with multiple reaction monitoring (MRM) for the detection of the compounds. Acetonitrile-2 mM Ammonium Formate (90:10, v/v) consisted of the mobile phase wherein pH 3.0 was adjusted by using formic acid this was then further delivered at a flow rate of 0.250 mL/min at 5°C. The system easily combats the pressure of 6200 psi due to the particle size of 1.7 µm. For quantitation and data acquisition Mass Lynx software of 4.1 version was used. Nitrogen of high purity was used a nebulizing gas. The other source dependent parameters studied were cone gas flow: 80 L/h; desolvation gas flow: 600 L/h; capillary voltage: 1.6 kV, source temperature: 120°C; desolvation temperature: 400°C; extractor voltage: 4.0V. The ion transitions for LNG and LNG-d6 were 313.0/245.0 and 319.3/251.2 with 50 ms dwell time.

2.3 Calibration standards and quality control samples

A standard stock solution of 200 µg/mL of LNG was prepared by dissolving the requisite amount in methanol. For spiking 5.00 µg/mL and 0.250 µg/mL were prepared in methanol: water (50:50, v/v) and were used as intermediate solutions. Calibration standards and quality control (QC) samples were prepared by spiking (2% of total plasma volume) blank plasma with stock/intermediate solutions. 1.0 mg of LNG-d6 dissolved in 10.0 mL of methanol resulted in (100 µg/mL) stock solution of intermediate solutions. Working solutions of (0.05 µg/mL) were prepared by appropriate dilution of the stock solution in methanol: water (50:50, v/v).). The QC samples were prepared from separately weighted amount of analyte. The stock solutions were stored at 5 °C, while calibration standards and quality control samples were stored at –70 °C until use. Calibration standards (CSs) were made at 0.0250, 0.0500, 0.100, 0.250, 0.500, 1.50, 3.00, 6.00, 12.5, 25.0 ng/mL concentrations. Five quality control samples were prepared (a) 20.0 ng/mL (HQC, high quality control), (b) 10.0/0.750 ng/mL (MQC-1/2, medium quality control), (c) 0.0750 ng/mL (LQC, low quality control) and (d) 0.0250 ng/mL (LLOQ QC, lower limit of quantification quality control).



2.4 Protocol for sample preparation

The first step of sample preparation is thawing all the frozen plasma samples, calibration standards and quality control samples followed by equilibrating at room temperature for 30 mins. 25 µL of internal standard LNG-d6 is added to an aliquot of 100 µL of spiked plasma samples. To which 0.100 mL of 2% ortho phosphoric acid is added and vortexed for 15s. Thereafter, the samples were centrifuged at 13148 × g for 5 min at 10 °C. Plasma samples were then applied to Phenomenex Strata-X (30 mg, 1cc) cartridges, which were pre-conditioned with 1 mL methanol followed by 1 mL of water. The samples were washed twice with 1 mL, 5 % methanol in water. Drying of cartridges was done for 1 min by applying nitrogen (1.72 x 105 Pa) at 2.4 L/min flow rate. The analyte and IS were eluted with 1 mL of 0.01 % (v/v) ammonia in acetonitrile into pre-labeled vials, evaporate to dryness all samples at 50°C under nitrogen gas and reconstitute with 200 µL of mobile phase vortexed for 15 s and 10 µL was used for injection in the chromatographic system.

2.5 Procedures for method validation

Various factors like selectivity, specificity, linearity, system suitability, system performance, precision, accuracy, recovery, carry over and matrix interference were effectively studied and

validated for the developed method. The validation of the developed method was done on the basis on USFDA guidelines [17]. LNG (10.0 ng/mL) and IS were injected six times consecutively at the start of each batch for carrying out system suitability whereas one extracted blank plasma (without analyte and IS) and one extracted LLOQ sample with IS was injected at the commence of each analytical run and before re-injection of sample to study system performance.

Carryover effect of the auto-sampler was analysed at the start and end of each batch by injecting extracted blank plasma, ULOQ sample, extracted blank plasma, LLOQ sample, extracted blank plasma. No interfering endogenous substance peaks were observed at the retention time of drugs in the ten batches (6 normal lots of Na-heparin plasma, 2 haemolysed, and 2 lipemic) of blank plasma samples indicated selectivity of the method. Selectivity was also assessed for the commonly used medications by human volunteers that included paracetamol, diclofenac, ibuprofen, caffeine, and acetylsalicylic acid. Stock solutions of (100 µg/ mL) were prepared by requisite amount in methanol, while the working solutions (1.0 µg/ mL) were prepared in the mobile phase and 10 µL was injected to check for any possible interference at the retention time of analyte and IS.

Calibration curve was established over ten non-zero concentrations and are satisfactorily described by quadratic, $1/x^2$unweighted least square linear regression to plot the peak area ratio (analyte/IS) versus concentration. Correlation coefficient value for each curve must be ≥ 0.99 for LNG. The lowest standard on the calibration line was accepted as the LLOQ, if the analyte response was at least ten times more than that of extracted blank plasma. The entire analytical run after storage at 5 °C was again reinjected to study the reinjection reproducibility for the extracted samples.

Intra-day and Inter-day accuracy and precision were analysed. Assays were repeated for six times in the same day to obtain the repeatability of the LLOQ, LQC, MQC-1/2 and HQC samples (Intra-day precision) while the assays were repeated for five batches on three consecutive days. Precision % CV should not be greater than 15%. The mean accuracy should fall within the limits of 85-115% while for the LLOQ it should be between 80-120% of the nominal concentration.

Post column infusion experiment was carried out to study the Ion suppression/enhancement effects. Briefly, a standard solution containing LNG (at HQC level) was infused post column into the mobile phase at 10 µL/min employing infusion pump. Aliquots of 10 µL of extracted control blank plasma sample were then injected into the column by the auto-sampler and chromatograms were acquired for the analyte and IS.

Extraction recovery of LNG and IS from human plasma was evaluated in six replicates by comparing the mean peak area responses of pre-extraction fortified samples to those of post-extraction fortified samples representing 93-97 % recovery. Absolute matrix effect was assessed by comparing the mean area response of post-extraction fortified samples with mean area of solutions prepared in mobile phase solutions (neat standards) at HQC, MQC-1/2 and LQC levels. Relative matrix effect was assessed from the precision (% CV) values of the slopes of the calibrations curves prepared from eight different plasma lots/sources, which included one haemolysed and lipemic plasma. To prove the absence of matrix effect, % CV should be less than 3-4 % for method applicability to support clinical studies. Stability tests were conducted for stock solutions of LEV and IS for short term and long-term stability at 25 °C and 5 °C respectively. The acceptance criterion was ±10.0 % deviation from the nominal value.

All stability results for spiked plasma samples were evaluated by measuring the area ratio response (analyte/IS) of stability samples against freshly prepared comparison standards at LQC and HQC levels. Auto sampler, Bench top, Dry extract and Wet extract in cooling chamber at 5 °C, Freeze-thaw (-20 °C and -70 °C) and Long term (-20 °C and -70 °C) stability of the analyte in plasma was studied at both these levels using six replicates. The samples were considered stable if the deviation from the mean calculated concentration of freshly prepared quality control samples was within ±15.0 %.

Method ruggedness was verified using two precision and accuracy batches. The first batch was analyzed on two different columns of the same make but different batch number, while the second batch was analyzed by two different analysts who were not part of method validation. The ability to dilute samples which could be above the upper limit of the calibration range was validated by analyzing six replicate samples containing 50.0 ng/mL of LNG after five-/ten-fold dilution respectively. The precision and accuracy for dilution reliability was determined by comparing the samples against freshly prepared calibration curve standards.

2.6 Bioequivalence study design and incurred sample reanalysis

A validated method was designed to carry out the bioequivalence study of the LNG tablet formulations. An open labelled, balanced, randomized, two –treatment, two-sequence, and two-period crossover design was used to execute the study. A total of 34 healthy adult female volunteers after complete physical, medical and biochemical examinations were chosen to participate in the entire study. They were priorly informed about all the aims and risks involved in the study. The study was performed based on International Conference on Harmonization, E6 Good Clinical Practice guidelines. The entire dosing periods were separated by a 7-day washout period with 240 mL of water. Bioequivalence study was carried out between Pozato uni tablet (by Libs Farmaceutica Ltds., Brazil comprising of 1.5 mg LNG) and Postinor Uno® tablet (by Gedeon Richer Ltd. – Budapest – Hungry and distributed by Ache Laboratories Farmaceutics S.A comprising of 1.5 mg LNG). A regular standardized meal was provided to the subjects with an unmonitored water intake. After the administration of the drug, the blood samples were collected in the heparinized tubes immediately before 0.00 (pre-dose) and after the dosing period at 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 4.00, 5.00, 6.00, 8.00, 10.0, 12.0, 24.0, 48.0, 72.0, 96.0, 120.0, 144.0, 192.0, and 240.0 h. The blood samples were further centrifuged at 1800g to collect plasma after which the samples are labelled and stored at -70°C till next analysis. A non-compartmental model using WinNonlin software version 5.2.1 (Pharsight Corporation, Sunnyvale, CA, USA) was used for the estimation of the pharmacokinetic parameters while SAS® software version 9.2 (SAS Institute Inc., Cary, NC, USA) was used to study the statistical analysis of the pharmacokinetic parameters of LNG including the descriptive statistics, analysis of variance and two on-sided tests for bioequivalence. C_{max}, $AUC_{0-t\,(240)}$ $AUC_{0\rightarrow\infty}$ were considered as primary variables, for the study. The secondary end points of the study included $AUC_{0\text{-}240}$/$AUC_{0\text{-}inf}$, T_{max}, K_{el} and $t_{1/2}$). To determine whether the two formulations were pharmacokinetically equivalent, $C_{max,}$ $AUC_{0\text{-}240}$ and $AUC_{0\text{-}inf}$ and their ratios (test/reference) using log transformed data were assessed. The drug formulations were considered pharmacokinetically equivalent if the difference between the compared parameters was statistically non-significant ($P \geq 0.05$) and the 90% confidence intervals (CI) for these parameters were within 80 to 125 %.

To assess the reproducibility of the assay an incurred sample reanalysis was carried out by re-analysing 122 samples, by selecting samples near the C_{max} and the elimination phase in the pharmacokinetic profile of the drug. The results were compared with initial pharmacokinetic study using the same procedure. As per the acceptance criterion at least two-thirds of the original and repeat results should be within 20 % of each other.

$$\%\text{Change} = \frac{\text{Repeat value} - \text{Initial value}}{\text{Mean of repeat and initial values}} \times 100$$

3.0 Results and discussion

3.1 Method development

The validated method used an electrospray ionization source (ESI) which provided a high signal intensity, satisfactory data and efficient quantitation for the plasma samples. Levonorgestrel formed predominantly protonated molecules $[M+H]^+$ of m/z 313.2 for LNG and 319.3 for I.S in full-scan spectra. LNG was determined in MRM mode. The product ion Q3 most abundant ion spectra was 245.2 for LNG 251.2 for I.S. The MS behavior of the analyte and the internal standard remained unchanged at the value of 3.0 kv for capillary voltage and 4 V for extractor volts which indicated that both these values do not influence the MS behavior. Hence m/z of 313.2-245.2 was selected for maximum sensitivity.

In the present study, a solid phase extraction (SPE) procedure carried out on Phenomenex Strata™-X (30 mg, 1 cc), which required minimal steps for sample cleanup and ensured quantitative and precise recovery at all QC levels for the analyte and IS was used. Washing of cartridges with 5% methanol was adequate for complete removal of interfering compounds. During the method development, it was observed that LNG was not able to detect with the protein precipitation.

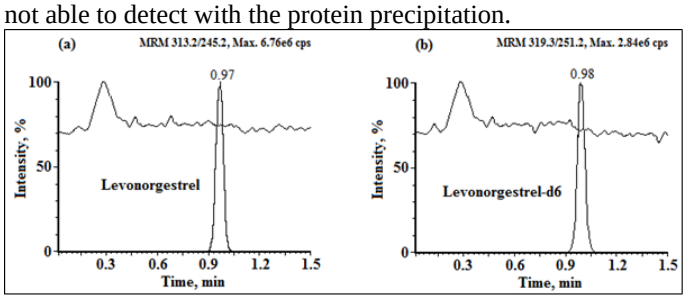


Fig. 3. Injection of extracted blank human plasma during post column infusion of (a) Levonorgestrel at HQC level and (b) Levonorgestrel-d6 (IS).

Chromatographic conditions were suitably optimized under isocratic conditions to get adequate response, acceptable peak shape and a short analysis time on Waters Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7 µm) column. Different ionic strengths (2-8mM) of acidic buffers (ammonium formate/formic acid, ammonium acetate/acetic acid) in combination with organic diluents (methanol/acetonitrile) in the pH range of 3.5-5.5 were investigated to identify the mobile phase producing the best peak shape, sensitivity and efficiency. The best mobile phase system which afforded adequate retention and peak shape was Acetonitrile-2 mM Ammonium Formate, pH 3.0 (90:10, *v/v*), adjusted with formic acid. The analyte and IS were eluted within 1.5 min with retention times of 0.97 and 0.98 min respectively. Representative MRM ion chromatograms in (**Fig. 2a-d)** verify the selectivity of the method to differentiate and quantify the analyte from endogenous components in the plasma matrix. The post column infusion experiment showed no interfering signal at the retention time of the analyte and IS (**Fig. 3**). Further, none of the commonly used medications by human volunteers interfered at the retention of Levonorgestrel. Absolute matrix effect values varied from 93.2 to 95.0 % indicating a minor ion enhancement (**Table 1**). The % CV value calculated for relative matrix effect in different plasma sources was 0.32, which is within the acceptance criteria of ≤3.0 %.

Table I: Relative matrix effect in eight different lots of human plasma for Levonorgestrel

Plasma lot	Levonorgestrel
Lot-1	0.098118
Lot-2	0.097642
Lot-3	0.097511
Lot-4	0.097981
Lot-5	0.097563
Lot-6 (heparinized)	0.098149
Lot-7 (haemolysed)	0.097535
Lot-8 (lipemic)	0.098267
Mean	0.097846
±SD	0.000314
%CV	0.32

SD: Standard deviation; CV: Coefficient of variation

3.2 Assay results

The precision (% CV) values for system suitability test varied from 0.10 to 0.24 % for the retention time and 0.56 to 0.85 % for the area response of Levonorgestrel and IS. The signal to noise ratio for system performance was ≥ 30. The auto-sample carryover experiment showed minimal carryover of analyte, ≤ 0.06 % of LLOQ area in the extracted blank sample after injection of ULOQ sample. The calibration curves showed good linearity (r^2 ≥ 0.9997) in the studied concentration range of 0.0250-25.0 ng/mL for Levonorgestrel. The mean linear equation for calibration curve concentrations was y = (0.09785 ± 0.00043) x + (0.000101 ± 0.000030). The accuracy (%) and precision (% CV) values for CSs ranged from 98.5 to 102.1 % and 0.91 to 2.92 % respectively. The lower limit of quantitation (0.0250 ng/mL) was measured at a signal-to-noise ratio (S/N) ≥ 30.

Table II: Extraction recovery and matrix factor for Levonorgestrel

QC level	Area response (n = 6)			Extraction recovery, % (B/A)	Matrix factor		
	A	B	C		Analyte (A/C)	IS	IS-normalized
Levonorgestrel							
LQC	1380	1305	1385	94.6 (96.4)[a]	0.997	1.071	0.930
MQC-2	13887	13025	14221	93.8 (95.9)[a]	0.976	1.084	0.900
MQC-1	185952	173251	187254	93.2 (97.0)[a]	0.993	1.076	0.922
HQC	379974	361164	382297	95.0 (96.8)[a]	0.993	1.066	0.931

[a] : values for internal standard, levonorgestrel-d6; A: mean area response of six replicates prepared by spiking in extracted blank plasma; B: mean area response of six replicates prepared by spiking before extraction; C: mean area response of six replicates

prepared by spiking in mobile phase (neat samples) n: number of replicates; IS: Internal standard; LQC: low quality control; MQC: medium quality control; HQC: high quality control.

The short-term and long-term stock solution stability of analyte and IS remained unchanged up to 29 h and for 28 days respectively with a % change ≤ 1.2. The detailed results of analyte stability in plasma are shown in (**Table 3)**.

Table III: Precision and accuracy data for Levonorgestrel

Nominal concentration (ng/mL)	Intra-batch			Inter-batch		
	Mean concentration found (ng/mL)[a]	% CV	% Accuracy	Mean concentration found (ng/mL)[b]	% CV	% Accuracy
HQC (20.0)	20.1	1.25	100.3	20.1	0.96	100.4
MQC-2 (10.0)	9.97	2.41	99.7	10.1	1.82	100.9
MQC-1 (0.750)	0.742	1.26	98.9	0.757	1.17	101.0
LQC (0.0750)	0.0748	2.41	99.8	0.0757	1.66	100.9
LLOQ QC (0.0250)	0.0248	2.84	99.3	0.0252	2.72	100.9

HQC: high quality control; MQC: medium quality control; LQC: low quality control; LLOQ QC: lower limit of quantitation quality control CV: Coefficient of variation; n: Number of replicates; The short-term and long-term stock solution stability of analyte and IS remained unchanged up to 29 h and for 28 days respectively with a % change ≤ 1.2. The detailed results of analyte stability in plasma are shown in (Table IV).

The precision (% CV) and accuracy values on different columns and analysts for method ruggedness ranged from 1.4 to 2.7 % and 97.9 to 102.0 % respectively across five QC levels. The precision values for dilution reliability with 1/5th and 1/10th dilution were 1.0 and 1.6 %, while the corresponding accuracy results were 97.9 and 101.2 % respectively.

Table IV: Stability of Levonorgestrel in plasma under various conditions (n = 6)

Storage conditions	Levonorgestrel		
	Nominal concentration (ng/mL)	Mean stability sample (pg/mL) ± SD	Change (%)
Bench top stability at 25 °C, 20 h	20.00	19.99 ± 0.2240	-0.03
	0.0750	0.0746 ± 0.0014	-0.59
Freeze & thaw stability at -20 °C	20.00	20.08 ± 0.2351	0.40
	0.0750	0.0756 ± 0.0016	0.85
Freeze & thaw stability at -70 °C	20.00	20.07 ± 0.3076	0.37
	0.0750	0.0745 ± 0.0017	-0.67
Autosampler stability at 4°C, 92 h	20.00	20.09 ± 0.1846	0.47
	0.0750	0.0755 ± 0.0014	0.64
Dry extract stability at 2-8°C, 74 h	20.00	20.04 ± 0.1921	0.22
	0.0750	0.0753 ± 0.0016	0.35
Wet extract stability at 2-8°C, 88 h	20.00	19.94 ± 0.1987	-0.29
	0.0750	0.0744 ± 0.0016	-0.80
Long term stability at -20 °C, 176 days	20.00	20.10 ± 0.1863	0.50
	0.0750	0.0759 ± 0.0015	1.15
Long term stability at -70 °C, 176 days	20.00	19.96 ± 0.2701	-0.19
	0.0750	0.0751 ± 0.0019	0.19

3.3 Application of the method in healthy subjects and incurred sample results

The developed validated method was further used to study the pharmacokinetic studies of LNG in 34 healthy female volunteers after its oral administration. **(Fig. 3)** shows the plasma concentration vs. time profile of Levonorgestrel in healthy volunteers under fasting condition. Pozato uni tablet (containing 1.5 mg of Levonorgestrel – Libs Farmaceutica Ltds., Brazil) was used for the study the values C_{max}, AUC_{0-t} and AUC_{0-inf} values obtained were higher compared to a previous study with similar formulations and identical dose strength which could be possible due to difference in gender, food and ethnicity while the T_{max} and $t_{1/2}$ were nearly identical for the test and reference (Pozato and Postinor Uno®) tablet formulations. The ratios of mean log-transformed parameters (C_{max}, AUC_{0-t}, and AUC_{0-inf}) and their 90 % CIs were all within the defined bioequivalence range of 80-125 %. Both the formulations showed no adverse effect with good tolerability profile in the volunteers. Thus, it concludes the bioequivalence in context to rate and extent of absorption. About 1428 samples were analyzed with the proposed method during a period of 6 days with acceptable precision and accuracy. Further, the reproducibility of the method was confirmed by reanalysis of 122 incurred samples with % change within ±10 % of the initial analysis results (Fig. 5).

Table V: Mean pharmacokinetic parameters and comparison of treatment ratios and 90% CIs of natural log (Ln)-transformed parameters following oral administration of 1.5 mg

levonorgestrel tablet formulation in 34 healthy Indian subjects under fasting

Parameter	Test (Mean ±SD)	Reference (Mean ±SD)	Ratio (test/reference), %	90% CI (Lower– Upper)	Power	Intra subject variation, % CV
Levonorgestrel						
C_{max} (ng/mL)	16.354 ± 3.612	16.180 ± 3.782	101.1	97.5-103.3	0.9998	3.56
$AUC_{0\text{-}240h(h.\ ng/mL)}$	349.56±145.64	356.562 ± 156.26	98.0	95.1-101.7	0.9996	5.41
$AUC_{0\text{-}inf\ (h.\ ng/mL)}$	430.16±150.23	445.816 ± 152.59	96.5	94.2-99.5	0.9999	4.46
T_{max} (h)	1.96 ± 0.72	1.91 ± 0.65	----	----	----	----
$t_{1/2}$ (h)	22.90 ± 3.96	23.64 ± 5.61	----	----	----	----
Kel (1/h)	0.030 ± 0.004	0.029 ± 0.005	----	----	----	----

CI: confidence interval; CV: coefficient of variation; Cmax: maximum plasma concentration; AUC0-240 h: area under the plasma concentration-time curve from 0 hour to 240 h; AUC0-inf: area under the plasma concentration-time curve from zero hour to infinity; Tmax: time point of maximum plasma concentration; t1/2: half-life of drug elimination during the terminal phase; Kel: elimination rate constant; SD: standard deviation.

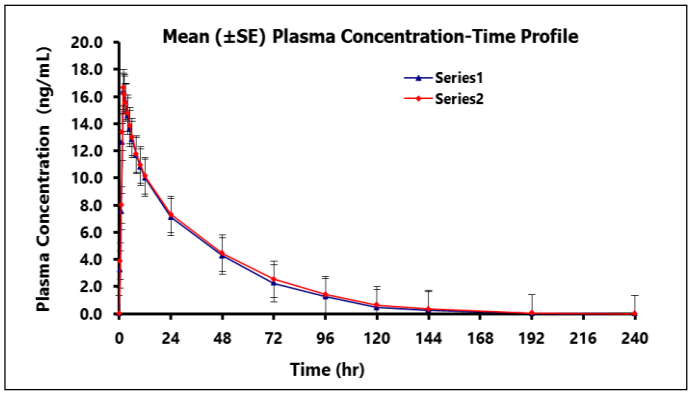


Fig. 4. Mean plasma concentration-time profile of 1.5 mg of Levonorgestrel (Pozatouni tablet- Libs Farmaceutica Ltds., Brazil) after oral administration (1.5 mg) of test and reference formulation (Postinor Uno® Tablet (containing 1.5 mg of Levonorgestrel - Manufactured by Gedeon Richer Ltd. – Budapest – Hungry and distributed by Ache Laboratories Farmaceutics S.A) to 34 healthy volunteers.

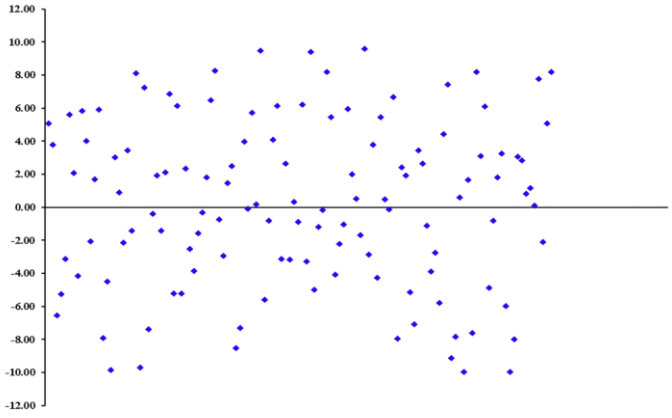


Fig 5. Graphical representation of % change in concentration during reanalysis of 122 incurred samples for Levonorgestrel.

4.0 Conclusion

A newly developed UPLC-MS/MS for the determination of LNG and its I.S in human plasma has been reported. The developed method comprised of an efficient, easy and faster sample pre-treatment technique encompassing solid phase extraction procedure. UPLC-MS/MS technique working in ESI mode generated good mass spectral response for LNG and its I.S. The validation was carried out on the basis of USFDA guidelines signified that the developed method offers good sensitivity, selectivity, response, reproducibility and extraction efficiency with minimal or no matrix interferences with a total run time of 1.5 mins. The method due to its superiority can be successfully applied to a clinical setting wherein large numbers of samples are to be analysed at a faster rate. A bioequivalence study was carried out on 34 healthy female volunteers; incurred sample reanalysis of 122 samples was also performed to check the reproducibility of the validated method.

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