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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-A sensitive and rapid ultra-performance liquid chromatography-tandem mass spectrometric (UPLC-MS/MS) method has been developed for the determination of Levonorgestrel in human plasma using Levonorgestrel-d6 as the internal standard (IS). After Solid phase extraction from 100μL human plasma, the analyte and IS were analyzed on Waters Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7μm) column using Acetonitrile-2 mM Ammonium Formate, pH 3.0 (90:10, v/v) as the mobile phase. The protonated analyte was quantified by multiple reaction monitoring in the positive ionization mode by triple quadrupole mass spectrometer. The calibration plots were linear over the concentration range 0.025-25.0 ng/mL. Intra-batch and inter-batch precision (% CV) and accuracy (%) for five quality control samples ranged within 0.96-2.84 % and 98.9-101.0 % respectively. The overall mean extraction recovery of Levonorgestrel from plasma ranged from 93.2 to 95.0 %. Matrix effect was assessed by post-column analyte infusion and the extraction recovery was> 93.0 % across four quality control levels for the analyte and IS. Stability was evaluated under different conditions like Bench top, Auto sampler, Dry Extract and Wet Extract (in cooling chamber), Freeze-thaw and Long term stability. The method was applied to support a bioequivalence study of 1.5 mg Levonorgestrel tablet formulation in 34 healthy subjects. Assay reproducibility was demonstrated by reanalysis of 122 incurred samples.

Key Words: Levonorgestrel; UPLC-MS/MS; sensitive; high throughput; bioequivalence study; incurred sample reanalysis

I. INTRODUCTION

In the present scenario of the drug delivery and development system, various conjugated forms and the natural or synthesized drug moieties have played a pivotal role in their own mode of actions. Depending upon the usage they have their equivalence and prevalence in the environment and also in live systematics as well. Levonorgestrel is a imitation progestin extensively used as a progestin-only emergency contraceptive (EC) for women who had insecure sexual intercourse [1]. It has only recently been found to be useful in pregnancy deterrence [2]. Although the use of levonorgestrel for EC is increasing, 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]. Furthermore, due to the strong side effects, the emergence of levonorgestrelin plasma is important to provide information for pharmacokinetic or bioavailability studies.

Several reports for the determination of levonorgestrel, including the use of radioimmunoassay [7], spectrophotometry [8, 9], voltammetry [10] or thin-layer chromatography [11, 12] have been studied, but the methods were not sensitive enough for pharmacokinetic analysis of low doses of levonorgestrel. Wang et al. [13, 14] described a liquid chromatography– tandem mass spectrometry (LC–MS–MS) method for the determination of levonorgestrel in human serum, with a lower limit of quantification (LLOQ) of 5 ng mL-1. Matejiceket al. [15-17] employed. This study reveals and focussed on the existence role and quantitative determination of Levonorgestrel drug using UPLC-MS/MS which have a sensitivity range of detection 0.0250-25.0 ng/mL as the most suitable and would be perhaps a conscientious method. This drug is generally used for the antiepileptic medications and provokes the hormonal influences for the sex hormones associated. The determination of this drug within the blood or a subsequent blood plasma is essential as this could be a direct evidence of the exposure of the drug. Presently this drug is in a vogue and applied in a larger scale to meet with the medicinal applications. This it needs to be analysed and a confirmation method along with its validation even at minute quantities using Ultra performance liquid chromatography (UPLC) is an ideal tool for rapid separation of complex mixtures in both isocratic and gradient modes. Improved separation efficiency and a decrease in the analysis time can be realized by reducing the particle size of the stationary phase. The advantage of UPLC over conventional HPLC is the ability to increase the speed without sacrificing efficiency [18].The present work emphasis a sensitive, selective and rapid UPLC-MS/MS method has been developed and fully validated for reliable measurement of Levonorgestrelin human plasma. The method requires only 100 µL human plasma for sample processing and demonstrates superior chromatographic performance in a run time of 1.5 min. It was applied to support a bioequivalence study in 34 healthy subjects.

II. EXPERIMENTAL

A. Chemicals and Material

Reference standard of Levonorgestrel (LEV, 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 format and LC-MS grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Solid phase extraction cartridges, Phenomenex StrataTM-X (1.5 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.

B. Liquid Chromatographic and Mass Spectrometric Conditions

The chromatographic analysis of Levonorgestrel and IS was carried out on Waters Acquity UPLC system (MA, USA) with UPLC BEH C18 (50× 2.1mm, 1.7µm) analytical column, maintained at 25°C.The mobile phase consisted of Acetonitrile-2 mM Ammonium Formate, pH 3.0 (90:10, v/v), pH 3.0, adjusted with formic acid and was delivered at a flow rate of 0.250 mL/min. The sample manager temperature was maintained at 5°C and the pressure of the system was 6200 psi. Quantization was done using multiple reaction monitoring (MRM) for protonated precursor → product ion transitions on Quattro Premier XETM mass spectrometer from Waters – Micro Mass Technologies (MA, USA), in the positive electro spray ionization mode. The mass parameters optimized for Levonorgestrel and IS are summarized in Table 1. MassLynx software version 4.1 was used to control all parameters of UPLC and MS.

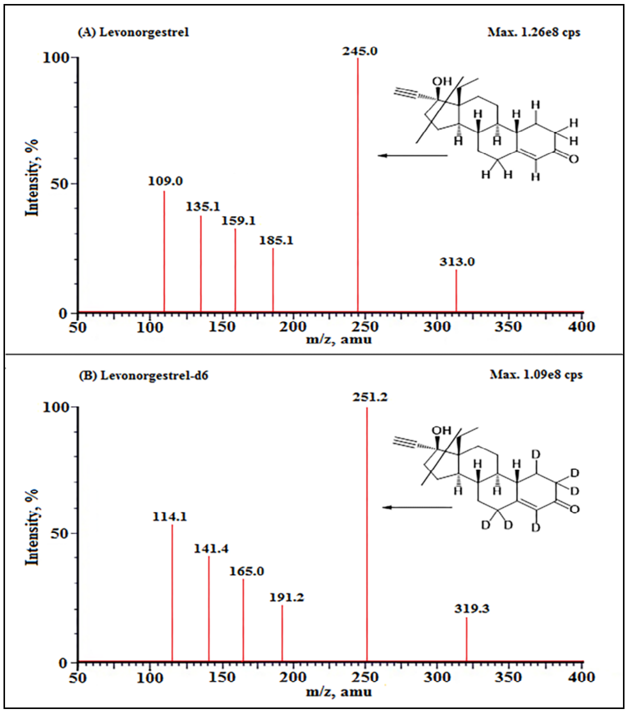

Fig. 1 . Product ion mass spectra of (a) Levonorgestrel(m/z 313.0 → 245.0, scan range 50-400 amu) (b) Levonorgestrel-d6 (m/z 319.3 → 251.2, scan range 50-400 amu) in positive ionization mode.

C. Standard Stock, Calibrators And Quality Control Samples

The standard stock solution of LEV (200 µg/mL) was prepared by dissolving requisite amount in methanol. Intermediate solutions (5.00 µg/mL and 0.250 µg/mL) for spiking were prepared in methanol:water (50:50, v/v). Calibration standards and quality control (QC) samples were prepared by spiking (2% of total plasma volume) blank plasma with stock/intermediate solutions. Stock solution (100 µg/mL) of the internal standard was prepared by dissolving 1.0 mg of Levonorgestrel-d6 in 10.0 mL of methanol. Its working solution (0.05 µg/mL) was 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) 20.0ng/mL (HQC, high quality control), (b) 10.0/0.750ng/mL (MQC-1/2, medium quality control), (c) 0.0750ng/mL (LQC, low quality control) and (d) 0.0250ng/mL (LLOQ QC, lower limit of quantification quality control).

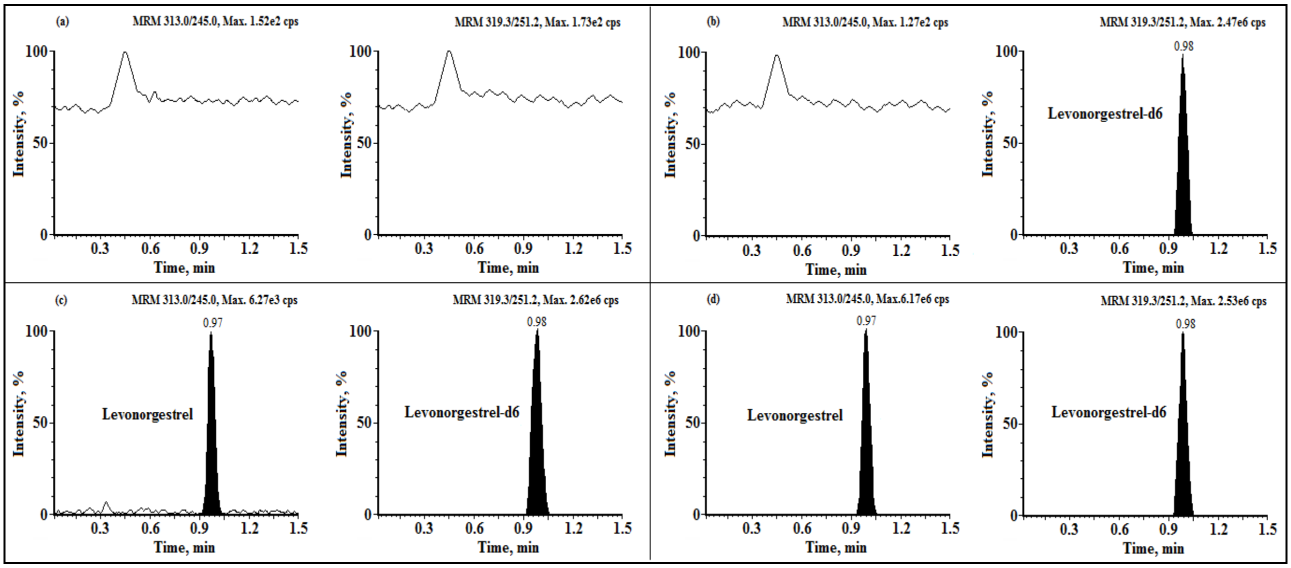

Fig. 2.Representative MRM ion-chromatograms of (a) double blank plasma (without analyte and IS),
(b) blank plasma with working solution of Levornorgestrel-d6 (m/z 319.3 → 251.2),IS
(c)Levonorgestrel at LLOQ (m/z 313.0 → 245.0) and IS,
(d) Levonorgestrel in subject sample at Cmax and IS afteroral administration of Pozatouni tablet (containing 1.5 mg of Levonorgestrel – Libs FarmaceuticaLtds., Brazil).

D. Protocol for Sample Preparation

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature for 30 min. To an aliquot of 100µL of spiked plasma sample, 25 µL of internal standardwas added, 0.100 mL of 2% Ortho Phosphoric Acid 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 (1.5 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 was 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 200µL of Mobile Phase vortexed for 15s and 10 µL was used for injection in the chromatographic system.

E. Procedures for Method Validation

The method validation was performed as per the USFDA guidelines [19].System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of LEV (10.0 ng/mL) and IS at the start of each batch during method validation. System performance was studied by injectingone extracted blank (without analyte and IS) and one extracted LLOQ sample with IS at the beginning of each analytical batch and before re-injecting any sample during method validation. The carryover effect of the auto-sampler was evaluated by sequentially injecting extracted blank plasma → ULOQ sample → extracted blank plasma → LLOQ sample → extracted blank plasma at the start and end of each batch.

Selectivity of the method was assessed for potential matrix interferences in ten batches (6 normal lots of Na-heparin plasma, 2 haemolysed, and 2 lipemic) of blank human plasma by extraction and inspection of the resulting chromatograms for interfering peaks. The selectivity of the method towards commonly used medications by human volunteers was also ascertained. This included paracetamol, chlorpheniramine maleate, diclofenac, caffeine, acetylsalicylic acid and ibuprofen. Their stock solutions (100 µg/ mL) were prepared by dissolving requisite amount in methanol. Further, 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.

Five calibration lines containing ten non-zero concentrations were used to determine linearity. A quadratic, 1/x2, least-squares regression algorithm was used to plot the peak area ratio (analyte/IS) from multiple reaction monitoring versus concentration. The linear equations were then used to calculate the predicted concentrations in all samples within the analytical runs. The correlation coefficient for each calibration curve must be ≥ 0.99 for LEV. 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. Reinjection reproducibility for extracted samples was also checked by reinjection of an entire analytical run after storage at 5 °C.

Intra-day accuracy and precision were evaluated by replicate analysis of plasma samples on the same day. The analytical run consisted of a calibration curve and six replicates of LLOQ, LQC, MQC-1/2 and HQC samples. The inter-day accuracy and precision were assessed by analysis of five precision and accuracy batches on three consecutive validation days. The precision (% CV) at each concentration level from the nominal concentration should not be greater than 15 %. Similarly, the mean accuracy should be within 85-115 %, except for the LLOQ where it can within 80-120 % of the nominal concentration.

Ion suppression/enhancement effects on the MRM LC-MS/MS sensitivity were evaluated by post column analyte infusion experiment. Briefly, a standard solution containing LEV (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 LEV 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 one 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 LEV 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.

F. Bioequivalence Study Design And Incurred Sample Reanalysis

The purpose of the study was to determine the bioequivalence between of Pozatouni tablet (containing 1.5 mg of Levonorgestrel – Libs FarmaceuticaLtds., Brazil) and Postinor Uno® Tablet tablet (containing 1.5 mg of Levonorgestrel - Manufactured by Gedeon Richer Ltd. – Budapest – Hungry and distributed by Ache Laboratories Farmaceutics S.A) in 34 healthy adult Indian subjects under fasting conditions. The design was an open label, balanced, randomized two-treatment, two-period, two-sequence, crossover study. The primary target variables of the study were Cmax, AUC0-240, and AUC0-inf, which were analyzed using the confidence interval approach. The secondary end points of the study included AUC0-240/AUC0-inf,Tmax, Kel and t1/2. The study was conducted as per International Conference on Harmonization, E6 Good Clinical Practice guidelines. The subjects were orally administered a single dose of Deflanil and Calcort formulations after recommended wash out period of 7 days with 240 mL of water. Blood samples were collected at 0.00 (pre-dose), 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 after oral administration. Plasma was separated by centrifugation and kept frozen at -70°C until analysis. During study, subjects had a standard diet while water intake was unmonitored. The pharmacokinetic parameters of LEVwere estimated by non-compartmental model using WinNonlin software version 5.2.1 (Pharsight Corporation, Sunnyvale, CA, USA). The statistical analysis for pharmacokinetic parameters of LEVincluded descriptive statistics, analysis of variance and two one-sided tests for bioequivalence was done using SAS® software version 9.2 (SAS Institute Inc., Cary, NC, USA). To determine whether the two formulations were pharmacokinetically equivalent, Cmax, AUC0-240 and AUC0-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 %.

An incurred sample re-analysis (ISR) for assay reproducibility was also done by reanalysis of 122 samples, by selecting samples near the Cmax 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. [21]

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

III. RESULTS AND DISCUSSION

A. Method Development

Mass spectrometric conditions were suitably optimized to obtain maximum sensitivity for Levonorgestrel and Levonorgestrel-d6 (IS). We have now developed a more sensitive, rapid and a rugged method based on UPLC–MS/MS. Three aspects were considered to modify in our previously developed method, (a) instrumentation-UPLC (b) extraction procedure-SPE and (c) the internal standard (IS)-a deuterated analog.The mass spectrometer between m/z 50 and 400 range. The present study was conducted using electrospray ionization (ESI) in the positive ionization mode.The Q1 mass full scan spectra contained protonated precursor $[M+H]^+$ ions at *m/z*313.0for LEV and *m/z* 319.3 for IS. The most stable and abundant product ions in Q3 mass spectra were observed at *m/z* 245.0 and 251.2 for LEV and IS respectively. The ion at *m/z* 245.0 for LEV can be ascribed to the fragment having the hydroxyl group and alkaene group as seen in Fig. 1.The fragmentation pathway for LEV and IS was largely dependent on collision energy. At low (14eV) collision energy both product ions at *m/z*245.0, and 251.2 were observed, which corresponded to elimination of hydroxyl and alkaene group respectively from the protonated precursor ions. Other mass parameters were suitably optimized to obtain a consistent and sufficient response for the analyte and IS. A dwell time of 50 ms gave a minimum of 27 data points across the peaks for quantitative reproducibility and sensitivity. In the present work, SPE was carried out on Phenomenex Strata^{TM}-X (1.5 mg,1cc),which required minimal steps for sample cleanup and ensured quantitative and precise recovery at all QC levels for the analyte and IS. Washing of cartridge with2 mL of 5% methanol was adequate for complete removal of interfering compounds. 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. Various combinations of organic diluents (methanol/acetonitrile) together with acidic buffers (ammonium formate/formic acid, ammonium acetate/acetic acid) with different ionic strengths (2-8 mM) in the pH range of 3.5-5.5 were tested. 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. Levonorgestrel-d6, a chemically and physically same properties having very similar structure was used as an internal standard. It adequately compensated for any variability during sample extraction and MS ionization. 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 I). The % CV value calculated for relative matrix effect in different plasma sources was 0.32, which is within the acceptance criteria of ≤3.0 %.

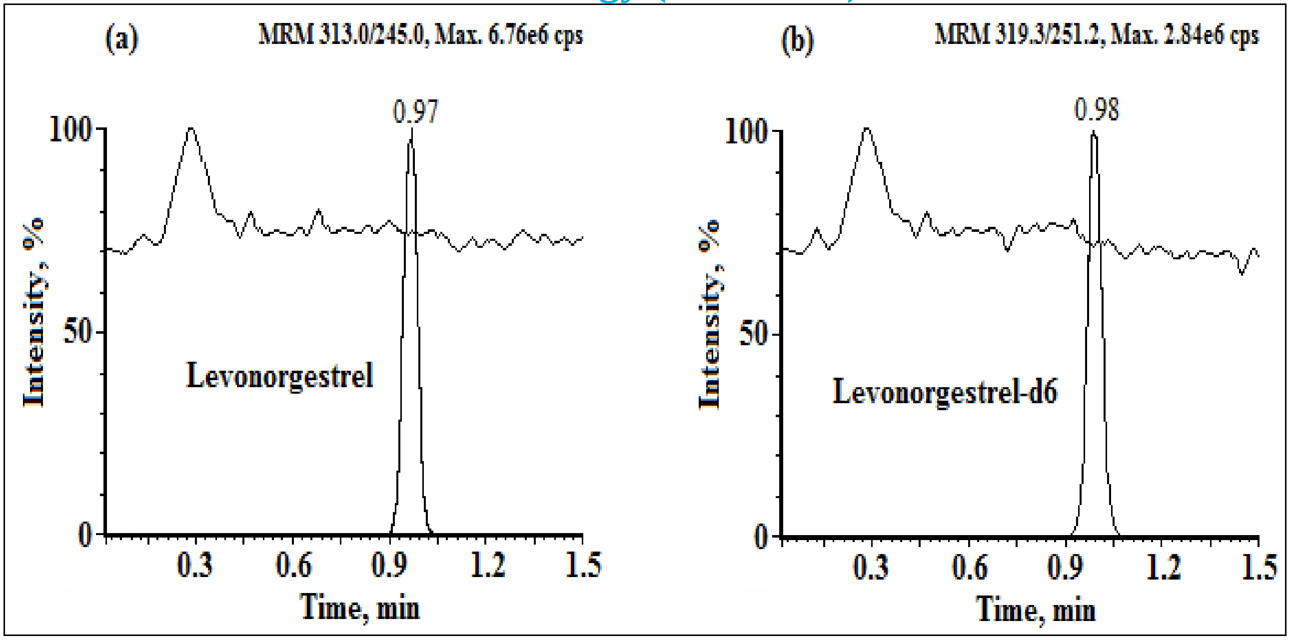

Fig. 3. Injection of extracted blank human plasma during post column infusion of (a) Levonorgestrelat HQC level and (b) Levonorgestrel-d6 (IS).

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

B. Assay Results

The precision (% CV) values for system suitability test varied from 0.35 to 0.84 % for the retention time and 0.76 to 1.29 % 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 (r2 ≥ 0.9997) in the studied concentration range of 0.0250-20.0ng/mL for Levonorgestrel. The mean linear equation for calibration curve concentrations was y=(0.0979 ± 0.0004) x + (0.000101 ± 0.000083). 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. The intra-batch precision (% CV) and accuracy varied from 1.25-2.84 while the accuracy was within 98.9-100.3 %. For the inter-batch experiments, the precision (% CV) ranged from 0.96-2.72 and the accuracy was between 100.4-101.0 %.

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.

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: mediumquality 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).

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-	20.00	20.04 ± 0.1921	0.22

8°C, 74 h	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

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.

C. *Application of the Method in Healthy Subjects and Incurred Sample Results*

Thus, a preliminary study was done with 34 healthy Indian males using Pozatouni tablet (containing 1.5 mg of Levonorgestrel – Libs Farmaceutica Ltds., Brazil). (Fig. 3) shows the plasma concentration vs. time profile of Levonorgestrelin healthy volunteers under fasting condition. The Cmax, AUC0-tand AUC0-infvalues obtained were higher compared to a previous study with similar formulations and identical dose strength. This difference could be ascribed to ethnicity, gender, food and other factors. However, Tmax and t1/2values for Levonorgestrel were nearly identical with their study. (Table 4) summarizes the mean pharmacokinetic parameters after oral administration of 1.5 mg Levonorgestrel; (Pozatoand Postinor Uno®) tablet formulations. No significant difference was found between the two formulations in any parameter. The ratios of mean log-transformed parameters (Cmax, AUC0-t, and AUC0-inf) and their 90 % CIs were all within the defined bioequivalence range of 80-125 %. Both formulations were well tolerated in healthy volunteers and there was no adverse event during the course of the study. This confirms the bioequivalence of the two formulations in terms of 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 $_{0\text{-}240\ h}$ (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; AUC0-120 h: area under the plasma concentration-time curve from 0 hour to 120 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.

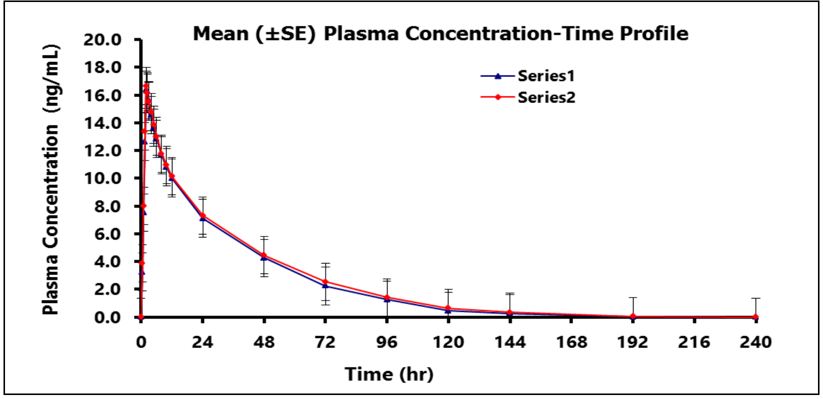

Fig. 4. Mean plasma concentration-time profile of 1.5 mg of Levonorgestrel (Pozatouni tablet- Libs Farmaceutica Ltds., Brazil) after oral administration (1.5 mg) of test and reference formulation (Postinor Uno® Tablet tablet (containing 1.5 mg of Levonorgestrel - Manufactured by Gedeon Richer Ltd. –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

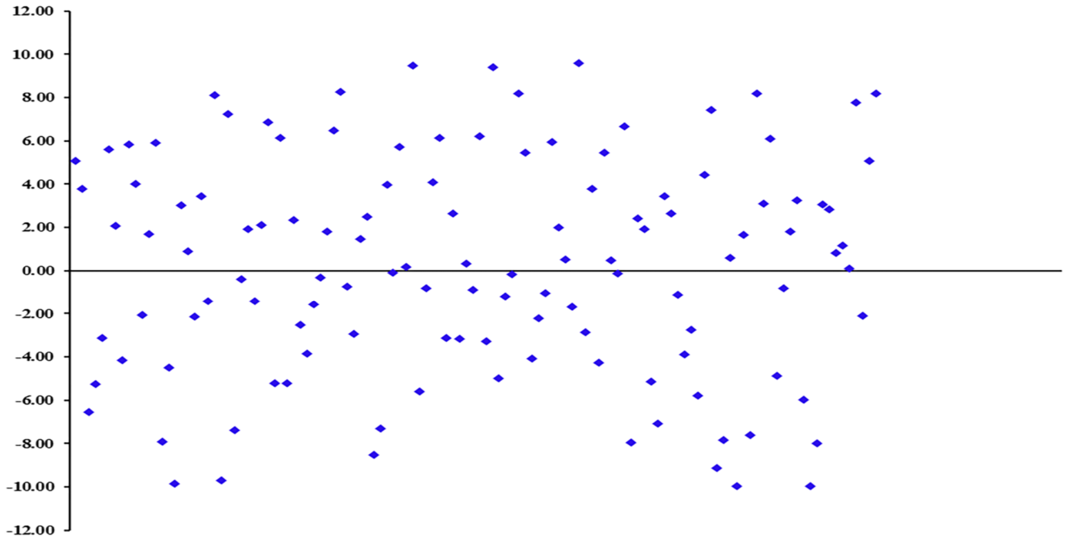

Fig 5. Graphical representation of % change in concentration during reanalysis of 122 incured samples for Levonorgestrel.

IV. CONCLUSION

A highly sensitive, selective and rapid UPLC-MS/MS method was developed for reliable measurement of Levonorgestrelin human plasma. The current method is superior to all other methods with respect to sensitivity, selectivity, ruggedness and faster rate of analysis. The method can be readily used in a clinical setting where large number of samples is to be analyzed. The absence of matrix effect is effectively shown by post-column infusion and by the precision (%CV) values for the calculated slopes of calibration curves from different plasma sources. Additionally, incurred sample reanalysis of 122 samples proves the reproducibility of the validated method.

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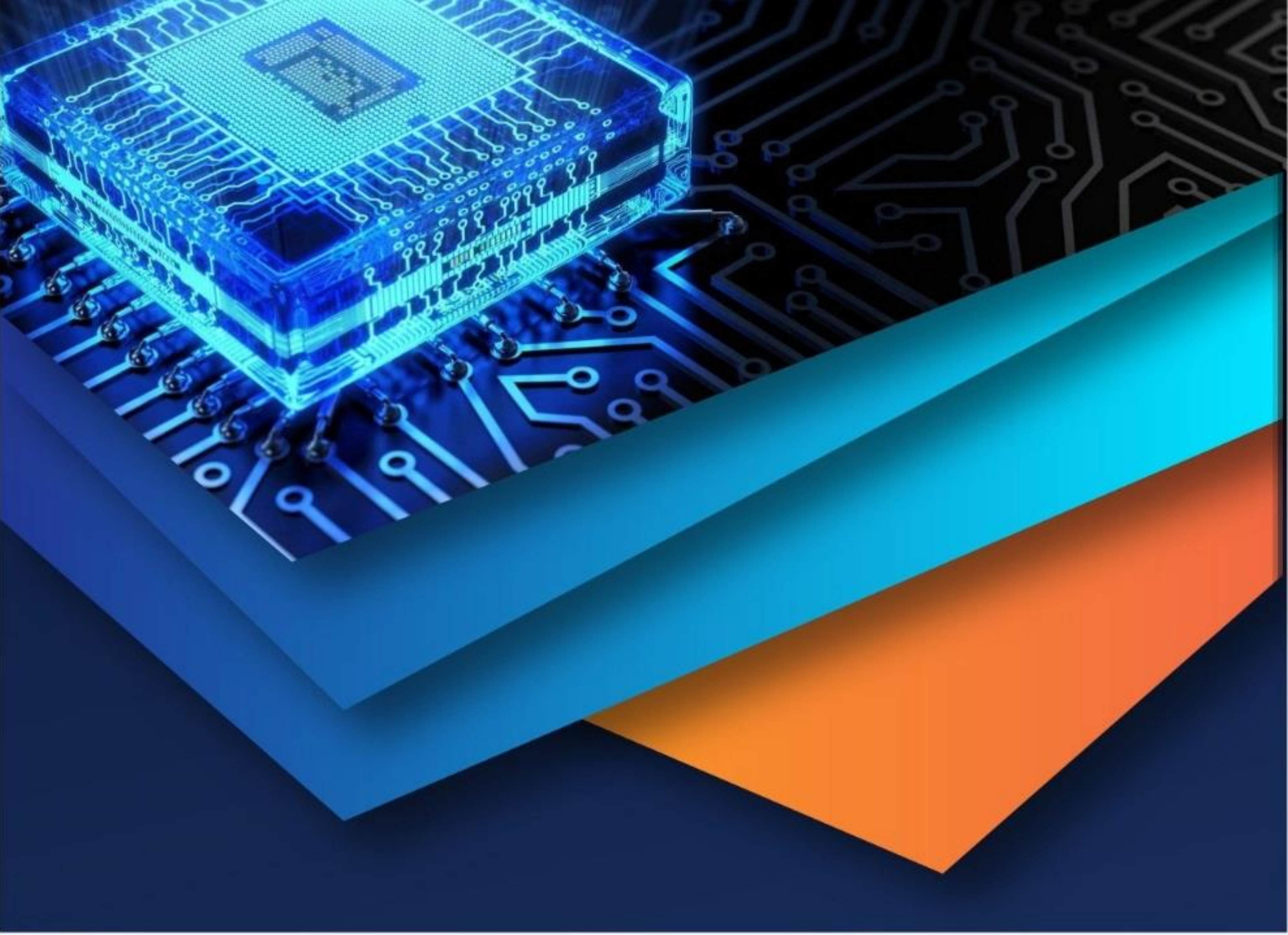

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