SIMPLE AND ROBUST METHOD FOR THE QUANTIFICATION OF LEVONORGESTREL IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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Summary

A sensitive and specific liquid chromatography electrospray ionization mass spectrometry (LC–ESI–MS) method is developed and validated for the quantification of Levonorgestrel in human plasma. After the addition of the internal standard (IS) and plasma samples were extracted with n-hexane: ethyl acetate (20:80%v/v). The organic layer is evaporated under a stream of nitrogen at 40 °C. The residue is reconstituted with 400 µl mobile phase. The compounds were separated on a thermo hypersil gold (50 mm × 4.6 mm. 5µ) column using 87:13% v/v of methanol and 0.1% formic acid in water as mobile phase. Detection is performed on a triple quadrupole mass spectrometer by selected reaction monitoring (SRM) mode. The method proved to be sensitive and specific by testing six different plasma lots. Linearity was established for the range of concentrations 0.100–200.00 ng/ml with a coefficient of determination (r2) of ≥0.9989 with good accuracy and precision. The lower limit of quantification is identifiable and reproducible at 0.100 ng/ml. The proposed method enables the clear-cut identification and quantification of Levonorgestrel for pharmacokinetic, bioavailability or bioequivalence studies.
Keywords: Levonorgestrel; Human plasma; triple quadrupole

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Introduction

Levonorgestrel is a synthetic progestogen used as a progestin-only emergency contraceptive; it is administered either alone or along with an estrogen(1). Levonorgestrel acts by transforming proliferative endometrium into secretory endometrium and inhibiting secretion of pituitary gonadotropins, preventing follicular maturation and ovulation.

Levonorgestrel as a emergency contraceptive categorized by US FDA as Plan B, a backup method to birth control. It is in the form of two Levonorgestrel pills (0.75 mg in each pill) that are taken by mouth after unprotected sex. This can reduce a woman's risk of pregnancy when taken as directed after unprotected sex. Plan B contains Levonorgestrel, a synthetic hormone used in birth control pills for over 35 years. Severe side effects are relatively rare in women who are healthy and do not smoke while they are taking Levonorgestrel emergency contraceptives. On an average, more women have problems due to complications from getting pregnant than have problems with oral contraceptives. However, the potential for severe side effects may exist.

Clinical studies have shown Levonorgestrel emergency contraceptive is effective and well tolerated. Parenthood Association of Utah (PPAU) has reported a steady increase in the usage of Levonorgestrel contraceptive(2). Monitoring of plasma levels of this drug can be indicative to changes in formulation by the determination of effective dose and limiting the clinical side effects. In order to determine the drug in plasma a sensitive, accurate and reliable analytical method is needed. Various methods have been reported for the determination of Levonorgestrel on bio matrices including radio immunoassay (3**)** reaching low detection levels in pg/mL. These methods were found to be sensitive but are expensive, consumes more time for sample preparation and hazardous due to radio labeling. High Performance Thin Layer Chromatography (4), and gas chromatography with negative ionization mass spectrometric detection(1) have also been reported. However these methods are found to be time consuming due to complicated sample preparation procedures. A rapid HPLC–ESI–MS/MS method was also developed for the determination of Levonorgestrel in human plasma, but the method requires 0.500mL of plasma sample and the LLOQ is only 0.250ng/ml(5).

In this paper, we describe a simple, selective and highly sensitive method using High Performance Liquid Chromatography coupled with electrosprsy ionization (ESI) triple quadrupole mass spectrometry (MS/MS) for the determination of Levonorgestrel in human plasma.

Materials and Methods

Reagents:

Levonorgestrel ($C_{21}H_{28}O_2$. MW 312.45) was procured from USP, Internal standard; dexamethasone was obtained from Sigma Aldrich, USA. HPLC grade solvents- Methanol, n-hexane, and ethyl acetate were of Labscan products (Lab scan ltd, Thailand). Milli Q water (Millipore, USA) was produced in-house. Formic acid and ethyl acetate were from SD fine chemicals (SD fine chemicals, Mumbai). Human plasma with K_2-EDTA as the anticoagulant was obtained from Navjeevan Blood Bank, Hyderabad, India.

Liquid Chromatographic conditions:

A Surveyor liquid chromatograph from Thermo Finnigan Inc, USA, consisting of an auto sampler, and low pressure quaternary gradient pump was used for the chromatographic determination of Levonorgestrel and internal standard. The optimal response was obtained by the mobile phase composition of 87:13% v/v of methanol and 0.1% formic acid in water. Hypersil gold (Thermo, 50 mm × 4.6 mm, 5µ) column was used. The column oven temperature was optimized to 40^0C. The flow rate was 0.400mL/min and all the column effluent was delivered to the mass spectrometer interface.

Mass spectrometric conditions:

A TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo Fennigan Inc, USA) with an Electro Spray Ionization (ESI) interface operated in the positive ionization mode was used for the Selective Reaction Monitoring (SRM) in the HPLC–MS/MS analysis. Levonorgestrel and dexamethasone tuning solution was prepared by dissolving the standards in methanol.100 ng/mL solutions were used for tuning the analyte and the internal standard. The optimized instrument conditions were as follows: Spray voltage: 4500V; capillary temperature: 350 °C; Sheath gas pressure: 40 orb; Auxiliary gas pressure: 20 orb; collision energy: 44

V; for Levonorgestrel. The precursor to product ion transitions used for the selective reaction monitoring was for Levonorgestrel, *m*/*z* 313.10 → 108.89; and dexamethasone *m*/*z* 393.4 → 373.4, with scan width of 0.200 sec for analyte and internal standards respectively. The mass spectrometer was operated at unit mass resolution (half-height peak width set at 0.70 Da).

Preparation of stock solutions:
The stock solutions of Levonorgestrel and dexamethasone (IS) were prepared after applying correction factor to compensate the percentage purity.The main stock solutions of Levonorgestrel was prepared by dissolving 10.0 mg in 10 mL of methanol producing a concentration of 1.0 mg/ml and stored at 4 °C.The internal standard stock solution was prepared by dissolving 10.0 mg of dexamethasone in 10 mL methanol producing a concentration of 1.0 mg/mL and was stored at 4°C. This solution was further diluted with methanol: water (50:50%v/v) to prepare the internal standard working solution containing 500 ng/mL dexamethasone.Working solutions of dexamethasone were prepared daily during method validation with methanol: water (50:50%v/v) by appropriate dilution of the stock solution.

Calibration curves:
Intermediate stock of 20µg/mL was prepared from the main stock by using the diluent (water: methanol (50:50% v/v). For the purpose of validation, two different weights of the analyte were taken for the preparation of the stock solutions for calibration standards and quality control samples. The standard and QC spiking stock solutions were prepared from the intermediate stock solution (20µg/mL) at the desired concentrations. Nine calibration standards spiking stock solutions were prepared at 2.00, 4.00, 20.00, 200.00, 800.00, 1600.00, 2400.00, 3200.00 and 4000.00ng/mL concentrations. Final plasma concentration was 0.10, 0.20, 1.00, 10.00, 40.00, 80.00, 120.00,160.00 and 200.00ng/mL for the calibration standards. Dilution integrity samples were prepared at 2 × ULOQ (Upper Limit of Quantification) concentration.

Preparation of quality control samples
Quality control samples were prepared at four different concentration levels, Quality controls spiking stock solutions were

prepared at the concentration of 2.00, 6.00, 2000.00 and 3600.00ng/mL LLOQ (Lower limit of quantification), LQC (Low Quality Control), MQC (Medium Quality Control) and HQC (High Quality Control) respectively. QC samples at 0.10, 0.300, 100.00 and 180.00ng/mL for LLOQC, LQC, MQC and HQC respectively.
All calibration standards and QC samples required for validation were bulk spiked and aliquoted into polypropylene vials and stored in deep freezer at −70±5°C for further use.

Extraction procedure
QC and calibration curve plasma samples were extracted employing a liquid–liquid extraction technique. To each glass tube containing 0.300 mL plasma, 15µL of internal standard working solution was added and vortexed to mix. 2.00 ml n-hexane: ethyl acetate mixture (20:80, v/v) was added and then vortexed for 5 min. The samples were then centrifuged for 5 min at 3500rpm. The organic layer was separated and evaporated to dryness under a stream of nitrogen at 40 °C. The residue was reconstituted with 400µl mobile phase and vortexed for 30 sec. 25µL of this solution was injected into LC-MS system.

Data analysis
A weighted ($1/X^2$) linear regression was performed to determine the concentration of the analyte. All regressions and figures presented in this validation report were generated by LC Quan software version 2.5.6. Acceptance criteria were established to be >0.980 for the calibration curve coefficient of determination (r^2). The accuracy and precision were determined at LLOQ, low, medium and high QC samples. The inter-day and intra-day accuracy should be within ±15% of the nominal concentration and precision should be <15% except for LLOQ, where it should not exceed by more than 20%.

Method validation
The method validation assays were carried out according to the currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guidelines(6).The following parameters were considered.The specificity of the method was tested by screening six different lots of K2 EDTA, one lipemic and one hemolysed human blank plasma. Each blank sample was tested for interference using the proposed extraction procedure by injecting

extracted blank plasma followed by three replicates of LLOQ processed and extracted from the same batch of plasma.The matrix effect on the ionization of analyte was evaluated by comparing the peak area of analyte in post spiked sample with the aqueous equivalent samples. Matrix effect is evaluated for the six different lots of plasma by injecting three replicates of LQC and HQC from each lot.

Linearity was tested for the range of concentrations 0.10–200.0 ng/mL. For the determination of linearity, standard calibration curves of nine calibration standards (non-zero standards) were used. In addition, blank plasma samples were also analyzed to confirm absence of interferences but they were not used to construct the calibration function. Not more than 20% deviation at LLOQ and not more than 15% deviation for standards above the LLOQ were set as acceptable criteria. Six replicate analyses of LLOQ, LQC, MQC and HQC were done. The intra-day precision and accuracy of the assay was measured by analyzing six replicates of Levonorgestrel at each QC level (LLOQC, LQC, MQC and HQC). The inter-day precision and accuracy was determined over five days by analyzing 24 QC samples in each batch. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Accuracy should be should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%.The extraction efficiency of the method for Levonorgestrel was determined by comparing the area of the extracted samples with the post spiked samples at three different concentrations (low, medium and high) of the analyte. The recovery of IS was also determined. For sensitivity determination, the lowest standard concentration in the calibration curve was considered as the lower limit of quantification (LLOQ), and was to meet the following criteria: LLOQ response should be 5 times that of noise and be identifiable, discrete and reproducible within the precision deviation of 20%. Samples at the concentration 0.100 ng/ml were investigated as the lower limit of quantification and the reproducibility and precision were also determined.

Bench top stability: Stored plasma aliquots (six aliquots each from low and high concentrations) were thawed and kept at room

temperature for a period of time expected to be maintained at room temperature during the routine sample preparation (around 8 h).

Post-preparative (in injector) stability: The stability QC samples (low and high QC level) were kept in the auto sampler (5°C) and analyzed after 38hours with the freshly prepared calibration standards.

Freeze and thaw stability: LQC and HQC plasma samples were processed and analyzed after three freeze (−70 °C) and thaw (room temperature) cycles.

Long-term stability of Levonorgestrel in human plasma at −70 °C was studied for a period of 68 days by employing QC samples at two different levels.

The spiking stock stability of Levonorgestrel and internal standard working solutions were evaluated comparing the area of stability samples with that of the freshly prepared samples after 9 hours at room temperature. Stability of working solutions was expressed as mean percentage change.

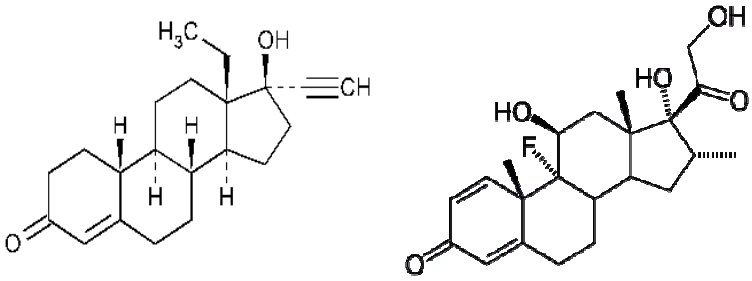
A calibration curve range was selected based on the reported Cmax of the analyte.

Dilution integrity: In some cases during determination of concentration of study samples the calculated concentration may exceed the upper limit of quantitation or insufficient volume of plasma sample was available to analyze. This case needs a test for sample dilution with blank matrix to determine the drug concentration in the sample, as a reason experiment was performed in ¼ and ½ dilutions of the QC samples.Reinjection reproducibility should be evaluated to determine if an analytical run could bereanalyzed in the case of instrument failure.

Results and Discussion

Selection of internal standard

The selection of the internal standard depends on the suitability with the chromatographic conditions of the analyte. In this method dexamethasone was used as an internal standard. Figure1 shows the structure of analyte and Internal Standard (IS)



Levonorgestrel (A) (Dexamethasone B)
Figure 1. Chemical structure of Levonorgestrel (A) and Dexamethasone (B)

Sample extraction technique

Protein precipitation technique was utilized during the method development but was found to be unsatisfactory. Extraction efficiency was increased with liquid–liquid extraction. Various solvents like ethyl acetate, dichloromethane, n-hexane and combination of n-hexane: ethyl acetate, dichloromethane: ethyl acetate. n-hexane: ethyl acetate were used and analyzed with reference to sample clean up and extraction efficiency. Combination of solvents n-hexane: ethyl acetate (20:80%v/v) exhibited good sample clean up and extraction efficiency of Levonorgestrel and internal standard and hence this was used in the present study.

Separation and specificity

Positive ion electrospray mass scan spectra of Levonorgestrel and IS are shown in Figure2 and Figure3, respectively. The major ions observed were $[M + H]^+$, $m/z = 313.4$ for Levonorgestrel and $m/z = 393.18$ for dexamethasone respectively. The ions of $[M + H]^+$, $m/z = 91.24$ for Levonorgestrel and $[M + H]^+$, $m/z = 373.16$ for dexamethasone were selected for the SRM(+) due to highly stable fragments and intensity.

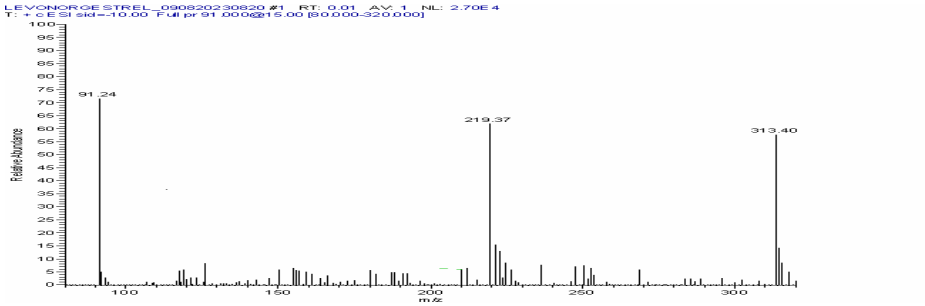


Figure 2. Positive ion electrospray mass scan spectrum of Levonorgestrel.

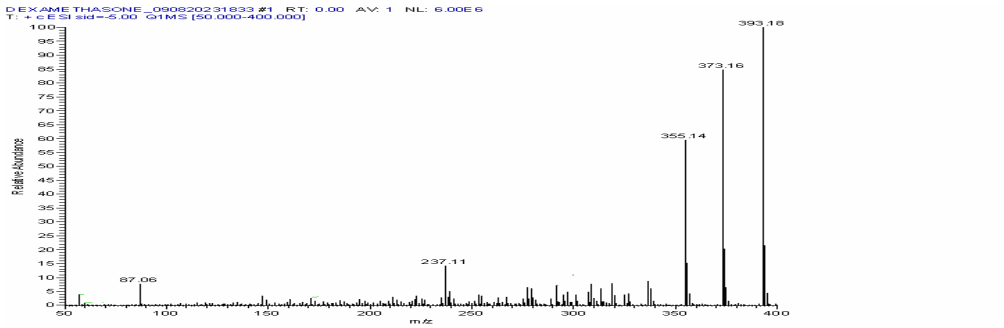


Figure 3. Positive ion electrospray mass scan spectrum of dexamethasone

The total HPLC–MS analysis time was 1.60 min per sample. No interferences of the analytes are observed because of the high selectivity of the SRM model. No ion suppression effects are observed under the developed sample preparation and chromatographic conditions. Figure3 shows an HPLC chromatogram for a blank plasma sample indicating no endogenous peaks at the retention positions of Levonorgestrel or internal standard (dexamethasone).

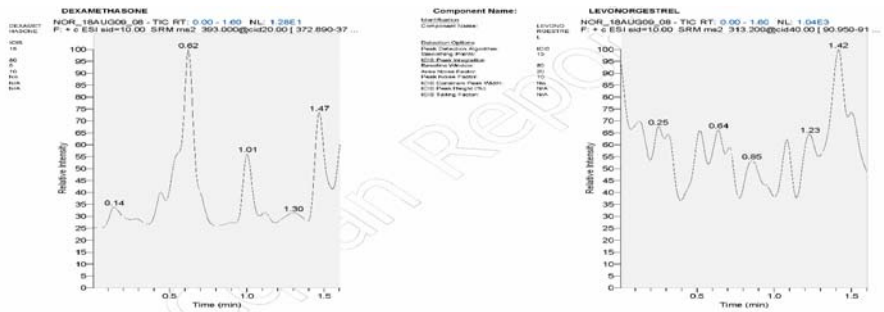


Figure 3. The SRM (+) chromatogram for a blank plasma sample

Selectivity

The lower limit of quantitation of Levonorgestrel was 0.100 ng/ml (LLOQ). Figure-4 shows the chromatogram of an extracted sample that contained 0.100 ng/ml (LLOQ) of Levonorgestrel.

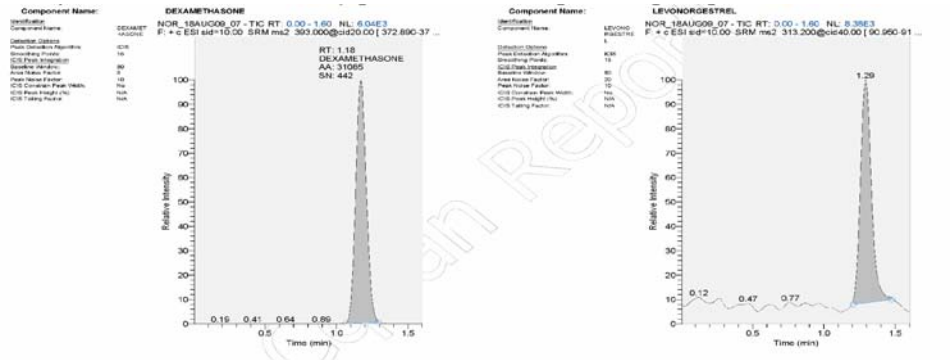


Figure 4. The SRM (+) chromatogram of LLOQ (0.100 ng/ml)

The SRM (+) chromatograms extracted from supplemented plasma are shown in Figure 5. As shown, the retention times of Levonorgestrel and the IS are 1.29 and 1.17 min, respectively.

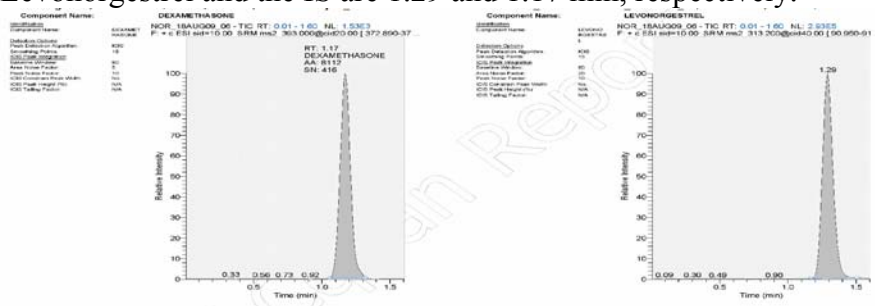


Figure 5. The SRM (+) chromatograms extracted from plasma. The retention times of Levonorgestrel and the IS were 1.29 min (A) and 1.17 min (B), respectively.

The purpose of this work was to develop a specific and sensitive procedure for the determination of Levonorgestrel in human plasma. HPLC–ESI–MS has several advantages for the analysis of Levonorgestrel. The combination of HPLC (under the isocratic conditions described) with ESI–MS leads to short run time and yields both high selectivity and sensitivity. ESI is a "gentle" ionization technique that produces high mass-to-charge $[M + H]^+$ precursor ions with minimal fragmentation of the analyte.

Carryover
LLOQ samples were injected followed by ULOQ samples and there was no significant carryover was observed

Matrix effect
Matrix effect was calculated by comparing the area three samples of of LQC and HQC post spiked to extracted blank plasma with aqueous equivalent LQC and HQC samples and No matrix effect was observed.

Linearity
The method exhibited a good linear response for the range of concentrations from 0.100 to 200.0 ng/ml with a coefficient of determination of 0.9989. Results of five representative calibration curves for Levonorgestrel HPLC–MS/MS determination are given in Table-I.

Linearity	Concentration(ng/mL)								
	Calibration standards								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	0.100	0.200	1.000	10.000	40.000	80.000	120.000	160.000	200.000
1	0.102	0.201	1.002	10.010	42.000	87.000	112.000	162.000	202.000
2	0.099	0.200	1.000	10.100	39.000	79.000	123.000	166.000	198.000
3	0.101	0.203	1.004	9.998	40.000	83.000	120.000	169.000	188.000
4	0.101	0.208	0.998	9.980	41.000	85.000	125.000	167.000	192.000
5	0.109	0.190	0.999	9.950	44.000	82.000	118.000	154.000	196.000
Mean	0.102	0.200	1.001	10.008	41.200	83.200	119.600	163.600	195.200
Mean % Nominal conc.	102.00	100.00	100.10	100.08	103.00	104.00	99.67	102.25	97.60
SD	0.004	0.007	0.002	0.056	1.924	3.033	5.030	5.941	5.404
%CV	3.92	3.50	0.20	0.56	4.67	3.65	4.21	3.63	2.77

Table I. Results of five representative calibration curves for Levonorgestrel HP LC–MS/MS determination.

Precision and accuracy
Data for intra- and inter-day precision of the method for Levonorgestrel as determined from the QC sample runs at the concentrations of 0.100, 0.300,100.000 and 180.000ng/ml are presented in Table II and Table III.

	Precision and Accuracy				
	Batch-1	Batch-2	Batch-3	Batch-4	Batch-5
LLOQC (0.100ng/mL)	0.101	0.103	0.102	0.106	0.108
	0.095	0.106	0.100	0.102	0.102
	0.096	0.101	0.095	0.092	0.100
	0.102	0.095	0.090	0.095	0.098
	0.103	0.092	0.102	0.090	0.096
Mean Calc. Conc.	0.099	0.099	0.098	0.097	0.101
SD	0.004	0.006	0.005	0.007	0.005
%CV	4.04	6.06	5.10	7.22	4.95
Mean % nominal Conc.	99.00	99.00	98.00	97.00	101.00
LQC (0. 300ng/mL)	0.303	0.290	0.289	0.308	0.306
	0.309	0.306	0.296	0.315	0.303
	0.292	0.305	0.292	0.296	0.300
	0.296	0.303	0.296	0.296	0.289
	0.308	0.301	0.306	0.291	0.292
Mean Calc. Conc.	0.302	0.301	0.296	0.301	0.298
SD	0.007	0.006	0.006	0.010	0.007
%CV	2.32	1.99	2.03	3.32	2.35
Mean % nominal Conc.	302.00	100.33	98.67	100.33	99.33
MQC (100. 00ng/mL)	101.200	106.235	106.230	106.236	102.365
	101.23	104.056	109.250	109.560	106.320
	106.00	98.956	99.850	102.360	108.620
	105.320	99.654	93.200	98.690	106.980
	103.250	90.562	102.300	96.023	101.234
Mean Calc. Conc.	103.400	99.893	102.166	102.574	105.104
SD	2.237	6.032	6.174	5.483	3.156
%CV	2.16	6.04	6.04	5.35	3.00
Mean % nominal Conc.	103.40	99.89	102.17	102.57	105.10
HQC (180.00ng/mL)	170.230	182.320	185.203	183.100	189.563
	189.100	179.650	176.235	180.230	192.456
	186.000	177.360	169.230	179.560	196.000
	192.230	175.000	168.023	168.530	180.235
	179.020	196.231	186.025	193.210	179.356
Mean Calc. Conc.	183.316	182.112	176.943	180.926	187.522
SD	8.801	8.346	8.519	8.827	7.419
%CV	4.80	4.58	4.81	4.88	3.96
Mean % nominal Conc.	101.84	101.17	98.30	100.51	104.18

Table-II: The intra-day precision and accuracy (n=5)

Precision and Accuracy	Mean Calculated concentration			
	LLOQC (0.100ng/mL)	LQC (0.300ng/mL)	MQC (100.00ng/mL)	HQC (180.00mL)
Batch-1	0.099	0.302	103.400	183.316
Batch-2	0.099	0.301	99.893	182.112
Batch-3	0.098	0.306	102.166	176.943
Batch-4	0.097	0.291	102.574	180.926
Batch-5	0.101	0.292	105.104	187.522
Mean Calc. Conc.	0.099	0.298	102.627	182.164
SD	0.001	0.007	1.899	3.835
%CV	1.01	2.35	1.85	2.11
Mean % nominal Conc.	99.00	99.33	102.63	101.20

Table-III. The interday precision and accuracy (n=5)

Recovery

The extraction recovery determined for Levonorgestrel was shown to be consistent, precise and reproducible. The relevant data below in Table-IV. The extraction recovery of IS is more than 85%.

QC LEVELS	Mean % Recovery	
	Levonorgestrel	**Dexamethasone**
LQC	82.53	82.52
MQC	84.77	
HQC	90.03	
Mean	85.78	
SD	3.850	
%CV	4.49	

Table IV: Recovery of Levonorgestrel and dexamethasone from plasma

Stability

Stability data of the short-term, freeze and thaw, in-injector stability and long-term stability test of Levonorgestrel and data is summarized in Table V. Table VI Summarizes Stock solution Stability.

Stability	LQC(0.300ng/mL) Mean % change	HQC(180ng/mL) Mean % change
Bench top stability (8 hrs @ room temp.)	2.18	-4.20
Freeze and thaw stability(After 3 cycles)	-3.26	-4.50
In injector stability(for 38hours @5^0C)	0.32	-4.03
Long-term stability(for 68 days @−70°C)	-2.05	-6.08

Table V: Data showing stability of Levonorgestrel in human plasma at different QC levels (n = 6)

Stock solution stability (9.0hours@ room temperature)	**Mean % change**		
	Main Stock	-1.05	2.19
	Spiking stock	-3.05	-1.89

Table VI: Data showing stock solution stability of Levonorgestrel QC levels (n = 6)

Short-term stability indicated reliable stability behavior under the experimental conditions of the regular runs. The results of freeze and thaw stability indicated that the analyte was stable in human plasma for three cycles of freeze and thaw, when stored at −70°C and thawed to room temperature. The in-injector stability of QC samples showed that Levonorgestrel was stable when kept at 5 °C in the auto sampler for 38 hours. The long-term stability test indicated that storage of Levonorgestrel in plasma at −70°C was stable for 68 days. The stability of analyte main stock, spiking stock and IS working solutions was tested at room temperature. Based on the results obtained, these solutions are found to be stable 9 hours.

Ruggedness

Method ruggedness was confirmed by analyzing four different precision and accuracy batches with two different analysts, different columns of same brand and different make of solvents. All the batches were found to be within the limits as specified in FDA guidelines. Results are presented in Table.VII. (n=4)

	Ruggedness			
	Analyst-1	Analyst-2	with respect to Column	with respect to solvent
LLOQC(0.100ng/mL)	0.109	0.101	0.106	0.103
	0.089	0.106	0.103	0.095
	0.092	0.098	0.109	0.098
	0.099	0.096	0.100	0.096
	0.106	0.106	0.090	0.095
Mean Calc. Conc.	0.099	0.101	0.102	0.097
SD	0.009	0.005	0.007	0.003
%CV	9.09	4.95	6.86	3.09
Mean % nominal Conc.	99.00	101.00	102.00	97.00

	Ruggedness			
	Analyst-1	Analyst-2	Column	Solvent
LQC(0.300ng/mL)	0.305	0.289	0.288	0.288
	0.310	0.294	0.309	0.284
	0.306	0.296	0.310	0.272
	0.303	0.305	0.313	0.302
	0.298	0.303	0.301	0.321
Mean Calc. Conc.	0.304	0.297	0.304	0.293
SD	0.004	0.007	0.010	0.019
%CV	1.32	2.36	3.29	6.48
Mean % nominal Conc.	101.33	99.00	101.33	97.67

	Analyst-1	Analyst-2	Column ruggedness	Solvent Ruggedness
MQC(100.00ng/mL)	1.080	1.090	1.002	0.970
	1.052	1.056	1.056	0.945
	1.036	1.045	1.089	0.990
	1.089	1.023	1.045	0.998
	1.002	0.985	1.008	0.984
Mean Calc. Conc.	1.052	1.040	1.040	0.977
SD	0.035	0.039	0.036	0.021
%CV	3.33	3.75	3.46	2.15
Mean % nominal Conc.	105.20	104.00	104.00	97.70

	Analyst-1	Analyst-2	Column ruggedness	Solvent Ruggedness
HQC(180.00ng/mL)	198.256	168.542	185.203	168.630
	165.200	185.560	176.235	169.470
	168.000	174.250	169.230	180.235
	194.256	198.000	168.023	174.020
	182.745	182.015	186.025	170.056
Mean Calc. Conc.	181.691	181.673	176.943	172.482
SD	14.940	11.284	8.519	4.803
%CV	8.22	6.21	4.81	2.78
Mean % nominal Conc.	100.94	100.93	98.30	95.82

Table: VII. Showing the results of ruggedness

Conclusion

A rapid and a highly sensitive method for the determination of Levonorgestrel in plasma was developed using high performance

liquid chromatographic separation with tandem mass spectrometric determination. The method is found to be highly sensitive and rugged than the reported methods. Simple liquid–liquid extraction procedure and short run time can reduce the time that is important for the analysis of large number of samples. The developed method is suitable for the analysis of Levonorgestrel in human plasma and hence, could be applied for pharmacokinetic, bioavailability or bioequivalence studies.

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References

1. Kook k, Gabelnick H, G.Duncan. Pharmacokinetics of Levonorgestrel 0.75 mg tablets. Contraception 2002;66(1):73-76.
2. Turok DK, Simonsen SE, Marshall N. Trends in Levonorgestrel Emergency Contraception Use, Births, and Abortions: The Utah Experience. J Med. 2009; 11(1):30.
3. Watson TG , Stewart BJ. A sensitive direct radioimmunoassay for assessing D-norgestrel levels in human plasma. Ann Clin Biochem. 1998; 25 (Pt 3) :280-7.
4 Amin M. Hassenbach M. Direct quantitative thin-layer chromatographic determination of Levonorgestrel and ethinyloestradiol in oral contraceptives by diffuse reflection and fluorescence methods. Analyst 1979; 104(1238):407-41.
5. Zhao LZ, Zhong GP, Bi HC, Ding L, et. al. Determination of Levonorgestrel in human plasma by liquid chromatography-tandem mass spectrometry method: application to a bioequivalence study of two formulations in healthy volunteers. Biomed Chromatography. Biomed Chromatogr.2008 May; 22(5):519-26.
6 Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), 2001 May.