# Development, validation, and utilization of a highly sensitive LC-MS/MS method for quantification of levonorgestrel released from a subdermal implant in human plasma

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**Abstract**

Levonorgestrel (LNG) is a synthetic progestin that is available in oral contraceptive tablets, a subdermal implant, and an intrauterine system for contraception. LNG pharmacokinetics is a pivotal determinant of contraceptive efficacy and can be useful in assessing drug-drug interactions influencing LNG exposure following different routes of LNG administration. A highly sensitive LC-MS/MS method was developed and validated to quantify levonorgestrel in human plasma. Liquid-liquid extraction was utilized with a sample volume of 500 μL to extract levonorgestrel from plasma. Chromatographic separation of LNG was achieved with a Fortis™ C18 (3 μm: 100mm x 2.1mm) reverse phase analytical column. The mobile phases consisted of de-ionized water plus 0.1% NH4OH (100:0.1%, v/v) (A), and methanol plus 0.1% NH4OH (100:0.1%, v/v) (B) flowing at 400 μL/min. Detection of LNG and internal standard (D-(-)-norgestrel-d7) was achieved using positive polarity mode monitoring at 313.2-245.2 amu and 320.1-251.2 amu, respectively. The assay was linear over the calibration range of 49.6 to 1500 pg/mL. This method was used to quantify plasma LNG released by subdermal implant in support of a drug interaction study among women with HIV receiving efavirenz- or nevirapine-based antiretroviral therapy.

**Keywords**

LC-MS/MS, liquid-liquid extraction, levonorgestrel, subdermal implant, plasma

**1. Introduction**

Levonorgestrel (LNG) is a synthetic progestin and is the biologically active levorotatory enantiomer of norgestrel. As a progestin, its contraceptive effect stems from inhibition of ovulation, as well as thickening cervical mucus and altering endometrial implantation [1]. LNG is used in various formulations of hormonal contraception, including combined oral contraceptives co-formulated with ethinyl estradiol, alone as emergency contraceptive tablets, LNG-releasing intrauterine systems, and subdermal implants [2]. After systemic distribution, LNG is metabolized by cytochrome P450 (CYP) 3A4 and further biotransformed by glucuronidation and sulfation, then eliminated primarily in the urine as metabolites (40% to 68%) [3]. Due to differences in administered dose, peak plasma LNG concentrations (Cmax) following non-oral administration via subdermal implants or intrauterine systems are lower as compared to oral administration. For example, the subdermal implant yields peak LNG concentrations that are substantially lower than peak concentrations following the oral tablet (Cmax 723 ± 344 pg/mL versus 19,100 ± 9700 pg/mL, respectively) [4, 5]. The subdermal implant is approved for use up to 5 years after insertion, at which time LNG concentrations have been reported to be 279 ± 123 pg/mL [1].

Detection of plasma LNG released by subdermal implantation requires an analytical assay that is highly sensitive and specific for LNG. To date, radioimmunoassays (RIA) have provided the most sensitive method for quantification of LNG released from subdermal implants [6], with an assay lower limit of quantification (LLOQ) of 50 pg/mL [7]. While highly sensitive, RIA lacks specificity, as the assays have cross reactivity with other steroids and metabolites, as well as with non-water soluble metabolites that remain following extraction [8, 9].

While other reports describe LC-MS/MS methods for quantifying LNG, most are validated to detect plasma LNG after oral administration, which merits LLOQs ranging from 100 to 265 pg/mL, and is inadequate for accurately detecting LNG from a subdermal implant [10-13].

Herein, we describe a highly sensitive, highly specific, non-automated, rapid assay using liquid-liquid plasma LNG extraction with LC-MS/MS detection, developed and validated for quantification of LNG from subdermal implant in women living with human immunodeficiency virus (HIV) enrolled in a clinical pharmacokinetic (PK) study [14].

**2. Materials and Methods**

**2.1 Chemicals**

LNG (MW: 312.45, 98% pure) was purchased from Sigma, UK. The internal standard D-(-)-norgestrel-d7 (MW: 319.49, 98% pure) was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Acetonitrile (LC/MS grade), methanol (HPLC grade), and tert-Butyl methyl ether (reagent grade >98% pure) were purchased from Sigma, UK. Ammonia in aqueous solution (25% AnalaR NORMAPUR®) was purchased from VWR International (Lutterworth, UK). HPLC grade de-ionized water was produced from an Elga Option S water purifier (Elga LabWater, High Wycombe, UK) and was further purified to 18.2 MΩ with a Purelab Ultra system (Elga LabWater, High Wycombe, UK). Blank human plasma (K2EDTA) was obtained from the National blood service (Liverpool, UK).

**2.2 Instruments**

An Accela HPLC autosampler and pump were interfaced with a Thermo Quantum Access triple quadrupole mass spectrometer operating in the ESI, positive polarity mode (Thermo Scientific, Hemel Hempstead, UK). Chromatographic separation of LNG was achieved with a Fortis™ C18 (3 μm: 100mm x 2.1mm) reverse phase analytical column (Fortis Technologies, Cheshire, UK) at an oven temperature of 40°C and interfaced with a 2 μm Quest pre-column (Thermo Scientific, Hemel Hempstead, UK). The mobile phases consisted of de-ionized water plus 0.1% NH4OH (100:0.1%, v/v) (Mobile Phase A), and methanol plus 0.1% NH4OH (100:0.1%, v/v) (Mobile Phase B) flowing at 400 μL/min. Needle wash solvent consisted of acetonitrile and de-ionized water plus formic acid (80:20:0.1%, v/v/v). All reagent solutions were stored at room temperature. TSQ Tune software was used for tuning and optimizing the analytes; data acquisition and processing was performed using LC Quan™ software (Version 2.7, Thermo Scientific, Hemel Hempstead, UK).

**2.3 Calibration, IS, and QC Solutions**

Two weighed 1 mg/mL stock solutions of LNG (calibration primary stock, and QC primary stock) were prepared independently by separately dissolving 2 mg aliquots of LNG, weighed on an AnD GR-202 balance (Scientific Laboratory Supplies, East Yorkshire, UK), in appropriate volumes of methanol. The primary LNG stocks (1 mg/mL) were used for preparation of working calibration standards and quality controls (QCs). From the calibration standard primary stock (1 mg/mL), an intermediate solution solution (1 μg/mL) was prepared by serial dilution with the appropriate volume of methanol. A calibration standard spiking solution was prepared from the 1 μg/mL intermediate solution by diluting in the appropriate volume methanol to a final concentration of 75 ng/mL. Working calibration standards were then prepared by spiking blank human plasma to yield 124, 750, and 1500 pg/mL. On the day of analysis, the working calibration standard solutions were serially diluted to yield final plasma concentrations of 49.6, 120, 250, 500, 750, 1010, 1280, and 1500 pg/mL of LNG. QCs were prepared from LNG QC primary stock. These consisted of High QC (1200 pg/mL; 80% of the highest calibration standard), Medium QC (420 pg/mL), Low QC (134 pg/mL; 3x the lowest calibration standard) and LLOQ (49.6 pg/mL; equivalent to the lowest calibration standard). They were prepared with blank human plasma spiked with appropriate volumes of the QC primary stock. All standards and QC were prepared using calibrated air-displacement pipets. All stock solutions were stored at 2 to 8°C in tightly sealed glass tubes. Working calibration standards and QC in plasma were stored at -40°C.

The primary stock of 1 mg/mL internal standard (IS) solution was prepared by dissolving the calculated quantity of D-(-)-norgestrel-d7 in methanol. A 10 μg/mL intermediate-stock was prepared by diluting the primary stock in the appropriate volume of methanol. The intermediate solution was stored at 4°C in a 10 mL tightly sealed glass tube for up to 6 months. The working IS solution (5 ng/mL) was prepared by dilution of the intermediate in the appropriate volume of methanol. Working IS was prepared fresh for each analytical run.

**2.4 Sample Preparation**

Calibrators, blanks, QCs and unknowns (500 µL) were added to labeled glass tubes and working IS solution (50 μL) was added to each extraction tube using a calibrated positive displacement pipet and vortexed. To normalize the volume of the blank tubes, 50 μL of methanol was added to each blank.Liquid-liquid extraction was performed by adding tert-Butyl methyl ether (4 mL) to all tubes using a repeater pipette, followed by prompt capping and tumbling for 30 minutes at room temperature. All tubes were ultra-centrifuged in a Heraeus Multifuge 3SR Plus (Scientific Laboratory Supplies, East Yorkshire, UK) at 2,000 revolutions per minute (rpm) for 5 minutes at room temperature. After centrifugation, all tubes were placed in a cryogenic bath until the aqueous layer was frozen (~45 seconds). The organic layer containing the analytes of interest was poured into appropriately labeled 12 mm x 75 mm glass tubes. All samples were evaporated to dryness under a stream of nitrogen at 40°C for 25 minutes. The residue was reconstituted with 100 μL reconstitution solvent (methanol:water:formic acid, 50:50:0.1 v/v/v) and transferred to appropriately labeled autosampler vials (Chromacol, Herts, UK). The samples were centrifuged for 10 minutes at 4000 rpm at room temperature. The vials were loaded onto the autosampler trays and 25 μL was injected into the LC-MS/MS system for analysis.

**2.5 LC-MS/MS Conditions**

A chromatographic step-gradient at a flow rate of 400 µl/min was used. Initial conditions consisted of 80% mobile phase A, increasing in organic content to 80% mobile phase B in 0.8 min, maintained over 3.7 min and equilibrated back to the initial conditions over a total run time of 6 minutes. The column temperature was 40ºC. The mass spectrometer used heated electron spray ionization with positive polarity. The electrospray voltage was 5000 V, and the capillary temperature and vaporizer temperature were set at 300°C and 350°C, respectively. The sheath and auxiliary gas pressures were set at 50 and 20 (arbitrary units). Argon, used as the collision gas, was delivered at a pressure of 200 bar. Selective reaction monitoring (SRM) scan was used for quantification by monitoring the precursor-product ion transitions **(Table 1).**

**2.6 Method Validation**

The assay was validated in accordance with the United States Food and Drug Administration’s (US FDA) guidance on Bioanalytical Method Validation [15].

**2.6.1 Accuracy and Precision**

Five separate batches consisting of the calibration curve and six LQC, MQC, and HQC were analyzed to examine inter and intra-assay accuracy and precision. Three separate batches consisting of six LLOQs were analyzed. Assay precision was measured over each QC concentration by the percent coefficient of variation [CV% = (standard deviation/mean)\*100]. Assay accuracy (% bias) was defined as the absolute value of the ratio of the calculated mean values of QC concentrations to the respective nominal values. Acceptance criteria for accuracy and precision were 15% for LQC, MQC, and HQC and 20% for LLOQ (49.6 pg/mL).

**2.6.2 Stability**

Stability of LNG in plasma was assessed with QC samples (n=6 per concentration) for all conditions tested. QC samples were analyzed on a freshly prepared calibration curve. Multiple conditions were used to determine stability of LNG in plasma before and after processing. These included stability of LNG in plasma during freeze-thaw cycles, bench-top stability, long-term stability, processed sample stability, and stability after heat inactivation. Reinjection reproducibility was also assessed. LNG instability was considered if detected concentrations deviated from the nominal value by at least 15%.

**2.6.3 Dilution Integrity**

Dilution integrity of LNG in plasma was also determined. Aliquots of blank plasma were spiked at a concentration of 2600 pg/mL. Dilutions of 1:2 and 1:4 were prepared with blank plasma (n=6 per dilution), resulting in concentrations within the assay calibration range. Each sample concentration was back calculated with the appropriate dilution factor against a fresh calibration curve.Accuracy was determined if the percent deviation from the nominal value was <15% the nominal concentration. At least 67% (n=8) of the total diluted samples needed to meet these acceptance criteria.

**2.6.4 Specificity and Selectivity**

Selectivity was assessed by screening six different lots of blank human plasma for LNG and IS. The peak area responses of interference (%) at the LNG or IS retention times were acceptable if <20% of the mean LLOQ (49.6 pg/mL) area response, and <5% of the mean IS area response, respectively (n=6).

**2.6.5 Carry-over**

Analyte carry-over (%) was determined by injecting the upper limit of quantification (ULOQ) (1500 pg/mL) calibration standard immediately followed by injection of a blank plasma sample. The peak area eluted in the blank sample was measured. Carry-over was considered to be negligible if the LNG peak area in the blank sample was <20% of the LLOQ (49.6 pg/mL) peak area and <5% of the IS peak area.

**2.6.6 Matrix Effects and Recovery**

Matrix effects were tested using a modification of the Matuszewski et al. method [16]. Six different plasma lots were spiked to LQC (134 pg/mL), MQC (420 pg/mL), and HQC (1200 pg/mL) concentrations. Three QC sets were prepared by spiking LNG and IS in blank plasma samples before extraction, after extraction, and in aqueous phase (non-extracted). To assess recovery, LNG peak areas from pre-extracted QCs were compared to post-extracted QC samples. To assess process efficiency, LNG peak areas from pre-extracted QCs were compared to non-extracted QCs. Matrix effects were assessed by comparing QCs spiked post-extraction to non-extracted QC samples.

**2.6.7 Clinical Application**

Plasma aliquots were analyzed from HIV-infected women using LNG-releasing subdermal implant (150 mg LNG), either alone, or in combination with oral efavirenz- or nevirapine-based antiretroviral therapy prior to implant placement and then at week 1, 4, 12, 24, 36, and 48 using the method described herein [14].

**3. Results**

The chemical structures of LNG and IS are shown in **Figure 1. Figures 2.1 and 2.2**depict a blank sample chromatogram and a typical chromatogram obtained when the LLOQ calibrator (49.6 pg/mL) was analyzed. **Figures 3.1 and 3.2** depict the chromatogram of LNG and the corresponding IS, respectively, from a participant plasma sample. Monitored m/z values for precursor and product ions are listed in **Table 1.** Calibration standards were analyzed by back calculating the concentration of LNG to IS ratio using a weighted 1/(concentration)2 linear regression. This value was compared to the nominal value of each calibration standard, and %CV and percent deviation from the nominal concentration were determined to assess assay precision and accuracy, respectively. Linearity was demonstrated over the analytical range (49.6 to 1500 pg/mL) by the coefficient of determination (R2), with all calibration standard curves achieving R2 of ≥ 0.991.

**3.1 Accuracy and Precision**

Inter- and intra-day accuracy and precision were assessed using day-to-day (n=30, LLOQ n=18) and within-batch (n=12, LLOQ n=6) LNG measurements from all QC concentrations and LLOQs. Five independent validation batches were run over four different days for all QC levels, and three additional batches including the LLOQs were run over three different days. Inter- and intra-day accuracy and precision were within ±15% for all QC levels and ±20% for LLOQs, as shown in **Table 2**.

**3.2 Stability**

Stability of LNG extracted from plasma samples was determined under multiple conditions and analyzed using a freshly prepared calibration curve **(Table 3).** LNG was stable during 3 freeze-thaw cycles, with the greatest deviation from the nominal concentration of -4.1% (LQC 134 pg/mL). Bench-top stability was demonstrated over 5 hours with the largest deviation from the nominal concentration of -13.4% (LQC 134 pg/mL). Processed plasma samples demonstrated stability for at least 24 hours at 2-8°C, with the greatest deviation from the nominal concentration of -10.1% (LQC 134 pg/mL). Stability of LNG following heat inactivation at 58°C for 40 minutes was demonstrated with the greatest deviation from the nominal concentration of -4.12% (LQC 134 pg/mL). The impact of heat inactivation was determined due to laboratory procedures for safe handling of plasma specimens acquired from participants with HIV infection. Long-term LNG stability was demonstrated over 39 days at -40°C, with the greatest deviation from nominal concentration of 7.2% (HQC 1200 pg/mL). Reinjection reproducibility was demonstrated over 72 hours at 10°C, with the greatest deviation from nominal concentration of 10.0% (HQC 1200 pg/mL). These stability data indicate that no significant LNG decomposition occurred under the conditions tested. With the exception of heat inactivation stability data, which was specifically assessed for purposes of handling bio-hazardous clinical specimens for analysis with this method, all stability data fell within similar ranges described previously [9, 10, 12, 13].

**3.3 Specificity and Selectivity**

After screening six different plasma lots, variations between area ratios of LNG and IS were less than 20% and 5%, respectively, for five of the six samples.

**3.4 Dilution Integrity**

Dilution integrity was demonstrated for both 1:2 and 1:4 plasma dilutions of LNG, with deviation from the nominal concentration and precision of -5.9% and 3.9%, respectively, for both dilution factors.

All six diluted samples at both 1:2 and 1:4 dilutions demonstrated individual accuracy within 15% of the nominal value.

**3.5 Carry-over**

LNG carry-over was determined to be negligible after injecting the ULOQ (1500 pg/mL) calibration standard immediately followed by injection of a blank plasma sample. The LNG peak area eluted in the blank sample was measured and determined to be <14.4% of the LLOQ (49.6 pg/mL) response and <5% of the IS response across all six batches.

**3.6 Matrix Effect and Recovery**

Plasma matrix effect testing found no significant variation in LNG peak area among six separate plasma lots, suggesting that endogenous plasma components did not affect analyte recovery **(Table 4).** The mean overall recovery for LNG and IS from plasma was 85.0% and 82.6%, respectively, with precision of 4.72% and 3.29%, respectively.

**3.7 Study Participant Plasma Analysis**

Plasma samples from 60 women living with HIV and using LNG-releasing subdermal implant (2-rods, 75 mg LNG/rod) alone or with efavirenz- or nevirapine-based antiretroviral therapy (ART) over one year were analyzed for LNG using this method and have been previously published [14]. The LNG concentrations detected ranged from 89.3 to 21,357.6 pg/mL (n=414 samples, median concentration 579.9 pg/mL).

**4. Discussion**

LNG is an exogenous progestin included in many formulations of contraception, resulting in highly variable concentrations that depend on the dose and route of administration. For example, LNG 1.5 mg administered orally as a single dose for emergency contraception results in peak concentrations twenty-six times higher than LNG-releasing subdermal implants used as long acting reversible contraception [4]. Clinically significant drug-drug interactions between progestin-releasing contraceptives and efavirenz have been demonstrated [14, 17-19]. Furthermore, LNG concentrations may be approximately 50% lower when combined with other CYP inducers, including anticonvulsants [20], and significant decreases are expected with agents like rifamycins [21]. In fact, the European and British guidelines have recently been updated to recommend a two-fold higher dose of LNG for emergency contraception (3 mg once) for women receiving CYP3A4 inducers [22, 23], highlighting the importance of understanding LNG pharmacokinetics in relationship to contraceptive effectiveness. In addition, LNG exposure may be increased by strong CYP inhibitors such as azole antifungals or HIV protease inhibitors [1], although the clinical significance of inhibition of LNG metabolism is unclear.

A simple, rapid, and highly sensitive analytical assay is necessary for determining plasma LNG concentrations in the context of drug-drug interactions. High sensitivity is particularly critical, as CYP3A enzyme inducers like efavirenz further decrease systemic LNG. While RIA provided an early method for sensitive LNG quantification, radioactive tracers complicate its use, and it is both labor intensive and lacks high throughput. To this end, LC-MS/MS has emerged as a preferable method for plasma LNG quantification. Comparison between LNG extraction techniques found that solid-phase extraction (SPE) produces more signal interferences and has decreased specificity compared to liquid-liquid extraction [11, 24]. Liquid-liquid extraction of plasma LNG has been described [25-27], however these methods are less sensitive, with LLOQs greater than or equal to 100 pg/mL. Furthermore, specialized semi-automated processes are required by one liquid-liquid extraction assay [25], which may potentially limit the application of this method in other laboratories. A method using an online SPE LC-MS/MS system following liquid-liquid extraction of LNG from plasma has been described, with a quantifiable linear range from 50 to 1500 pg/mL [24]. However online SPE LC-MS/MS requires additional costly equipment and maintenance that may not be available in all laboratory settings. Furthermore, the risk of carryover may complicate online SPE [28].

Steroidal drugs are poorly ionizable compounds and hence detection on a mass spectrometer poses analytical challenges. For example, LNG possesses only a single -OH functional group that can be ionized. Another challenge in measuring steroidal compounds is the potential for interference from endogenous components. With this in mind, of the mobile phases that were tested during early development, we opted for basic mobile phase conditions as this achieved increased separation of LNG from endogenous interferences (background interference) and improved the sensitivity of the method.

Using the method described herein, plasma extracts were prepared using a rapid and highly sensitive liquid-liquid extraction with good recovery. Similar to Liceo-Perez et al. [25], we developed a LC-MS/MS method using a liquid-liquid extraction technique, however we did not utilize semi-automated instruments during sample processing and the analyte extraction procedure, which permits our method to be utilized in laboratories without automated resources. Furthermore, we achieved similar sensitivity as Moser et al., with a lower limit of detection of 49.6 pg/mL [24]. Vieria et al. used a similar LC-MS/MS method to determine the concentration of the subdermal implant progestin, etonogestrel, using a highly sensitive assay that was linear over a range of 49.6 to 1500 pg/mL [17]. One limitation of our current method is the large plasma sample volume required for analysis (500 μL). Plasma sample volumes of 200 μL have been utilized [26, 27], but this in turn corresponded with substantially higher assay LLOQs (625 pg/mL and 5000 pg/mL). Moser et al. similarly used a plasma sample volume of 500 μL [24].

**5. Conclusion**

We have developed and validated a rapid and highly sensitive and specific LC-MS/MS method to determine plasma LNG concentrations. Given the high sensitivity of this assay, this approach is ideal for application in PK studies involving low systemic circulating LNG during placement of LNG-releasing subdermal implants, including detection of LNG in the context of drug-drug interactions.

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**Conflict of interest**

MS has received research grants from Janssen Pharmaceutica NV and ViiV.

LRC, KKS, ATP and LCW:None to declare.

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**Table 1: Mass transitions for LNG and D-(-)-norgestrel-d7**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Parameter** | **Parent ion** **(m/z)** | **Product ion** **(m/z)** | **Tube Lens (units)** | **Collision Energy (V)** |
| LNG | 313.2 | 245.2 | 98 | 23 |
| D-(-)-norgestrel-d7 | 320.1 | 251.2 | 92 | 23 |

Abbreviations: LNG, Levonorgestrel

**Table 2: Precision (%CV) and accuracy (%bias) data for LNG (inter- and intra-assay statistics)**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Interday** | **LLOQ (49.6 pg/mL)** | **LQC (134 pg/mL)** | **MQC (420 pg/mL)** | **HQC (1200 pg/mL)** |
| Mean (pg/mL) | 49.91 | 123.70 | 414.43 | 1263.42 |
| S.D. (pg/mL) | 2.84 | 9.42 | 16.44 | 65.13 |
| CV (%) | 5.68 | 7.62 | 3.97 | 5.15 |
| Bias (%) | -0.63 | -7.69 | -1.33 | 5.28 |
| n | 18 | 30 | 30 | 30 |
| **Intraday** | **LLOQ (49.6 pg/mL)** | **LQC (134 pg/mL)** | **MQC (420 pg/mL)** | **HQC (1200 pg/mL)** |
| Mean (pg/mL) | 50.57 | 124.45 | 421.17 | 1309.60 |
| S.D. (pg/mL) | 2.83 | 7.59 | 15.23 | 50.75 |
| CV (%) | 5.59 | 6.10 | 3.62 | 3.88 |
| Bias (%) | -1.95 | -7.12 | 0.28 | 9.13 |
| n | 6 | 12 | 12 | 12 |

Abbreviations: LNG, levonorgestrel; LLOQ, lower limit of quantification; LQC, low quality control; MQC, medium quality control; HQC, high quality control.

Each analytical run consisted of the [standard curve](http://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/standard-curve), blanks and 6 [quality control](http://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/quality-control) samples at 3 specified concentrations (LQC, MQC, HQC); assays were run on 5 separate occasions. The LLOQ samples were run on 3 separate occasions.

**Table 3: Short and long-term stability data of levonorgestrel under different conditions**

|  |  |  |
| --- | --- | --- |
| **Condition** | **QC** | **Accuracy (%bias)** |
| Freeze-thaw | LQC | -4.12 |  |
|   | MQC | -3.27 |  |
|   | HQC | 4 |  |
| Bench-top | LQC | -13.42 |  |
|   | MQC | -0.98 |  |
|   | HQC | 8.64 |  |
| Processed | LQC | -10.11 |  |
|   | MQC | -1.03 |  |
|   | HQC | 6.52 |  |
| Heat-Inactivation | LQC | -4.12 |  |
|   | MQC | -3.27 |  |
|   | HQC | 4 |  |
| Long-Term | LQC | -4.79 |  |
|   | MQC | -0.91 |  |
|   | HQC | 7.19 |  |
| Re-injection | LQC | 4.83 |  |
|  | MQC | 8.07 |  |
|  | HQC | 10.07 |  |

Abbreviations: LNG, levonorgestrel; LQC, low quality control; MQC, medium quality control; HQC, high quality control.

**Table 4: Recovery (%) and matrix effect (%) data for LNG**

|  |  |  |
| --- | --- | --- |
| **LQC (134 pg/mL)** | **%Recovery** | **%Matrix** |
| Mean | 88.944 | 97.856 |
| S.D. | 7.416 | 3.189 |
| C.V.(%) | 8.338 | 3.259 |
| n | 6 | 6 |
| **MQC (420 pg/mL)** | **%Recovery** | **%Matrix** |
| Mean | 89.954 | 92.392 |
| S.D. | 2.731 | 2.987 |
| C.V.(%) | 3.292 | 3.233 |
| n | 6 | 6 |
| **HQC (1200 pg/mL)** | **%Recovery** | **%Matrix** |
| Mean | 80.932 | 97.706 |
| S.D. | 4.94 | 0.48 |
| C.V.(%) | 6.104 | 0.491 |
| n | 6 | 6 |
| **Overall** | **%Recovery** | **%Matrix**  |
| Mean | 85.036 | 98.782 |
| S.D. | 4.01 | 1.736 |
| % CV | 4.715 | 1.757 |

Abbreviations: LNG, levonorgestrel; LQC, low quality control; MQC, medium quality control; HQC, high quality control.

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Figure 1. Chemical structures of LNG and D-(-)-norgestrel-d7 (IS)

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**Figure 2.1. Chromatogram of Blank Sample**



Figure 2.2. Chromatogram of LLOQ (49.6 pg/mL) LNG



**Figure 3.1 Chromatogram of LNG from Study Participant Sample**



Figure 3.2 Chromatogram of corresponding IS from Study Participant Sample