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# An ultra high performance liquid chromatography - tandem mass spectrometry method for quantification of linagliptin in human plasma

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**Abstract**

A simple, rapid, sensitive, reliable and selective ultra high performance liquid chromatography (UHPLC)-tandem mass spectrometry (MS/MS) method was developed, for quantification of linagliptin (LGN) in human plasma. LGN and its deuterated internal standard (IS), LGN-d4 was extracted from low plasma sample volume (300 µL) by simple liquid-liquid extraction protocol. Efficient estimation of analyte and IS at mean retention time (RT) of 1.75 and 1.74 min respectively, with a rapid 3.5 min run time per sample was chromatographically established on Gemini C18 (100 mm × 4.6 mm, 3µ) column under simple isocratic elution conditions, using a mixture of 10 mM ammonium formate: methanol [20:80 (v/v)] delivered at a flow rate of 0.5 mL/min. Following separation of compounds, protonated precursor → product ion transitions were monitored, for LGN (m/z: 473.3 → 420.1) and IS (m/z: 477.5 → 424.3) on a triple quadrupole mass spectrometer, operating in multiple reaction monitoring (MRM) mode. Most recent regulatory guidelines were adopted during method validation. Method demonstrated very good analyte and IS recovery (not less than 71.0%), precision (≤ 8.6 %CV), accuracy (range: 86.7% to 95.6%) and linearity (r>0.99) across clinically relevant LGN plasma concentration range: 50.3 to 12115.5 pg/mL. Validated method was successfully applied to pharmacokinetic study samples for measuring linagliptin plasma levels.

**Key words:** DPP-4, linagliptin, human plasma, ultra high performance liquid chromatography, tandem mass spectrometry, anti-diabetic drug

## 1.0 Introduction:

An astonishing figure of 415 million global population are diabetes with estimated health expenditure touched USD 673 billion in 2015, and dramatic disease elevation to 642 million people could be possible by 2040, if the same trend continues. Up to 91% of effected adults accounts for type 2 diabetes in high income countries.[1] DPP-4 (Dipeptidyl peptidase-4) inhibitors, being relatively new class of blood glucose lowering drugs, acts by stimulating the insulin secretion through the degradation of glucagon like peptide-1 (GLP-1), an endogenous substance[2] and they are well tolerated as their adverse effects profile matches with placebo.[3] Linagliptin (LGN) is an orally active and competitive DPP-4 enzyme inhibitor having xanthine based chemical structure[4,5] used to treat type 2 diabetes, who failed to achieve glycemic control with metformin alone.[6] It is approved in US, Europe, Japan and other countries[7,8] and administered as once daily 5 mg dose in adults either alone or in combination with metformin as twice a dose daily.[9] LGN exhibits similar pharmacokinetic (PK) profile in healthy subjects and in type 2 diabetes. Peak plasma levels (Tmax) achieved about, 1.5 hours post dose of single 5 mg tablet (in healthy subjects) with maximum plasma concentration (Cmax) of 8.9 nmol/L and resulting area under curve (AUC) of 139 nmol*h/L. It's absolute bioavailability~30% and exhibits concentration dependent plasma protein binding.[10]

Though extensive literature available on LGN human PK/clinical studies, performed either alone[7,11-16] or in combination[17-21] with various other drugs, till date to the best of our knowledge scientific reporting of the optimized bioanalytical method, for estimating LGN with full validation, addressing essential method details required for reproducing the results, is lacking. These studies reported with respective LC-MS/MS method provide only incomplete method details (refer section: 3.4), for linagliptin determination in human plasma sample[16-18,20] or in both human plasma and urine sample[12-15,19,21] with 13C3 labelled IS.[12-16,18-21] One of this method[15] along with LGN, simultaneously measured main inactive

metabolite of LGN (CD 1790) in single analytical run. For measurement of LGN, these methods vary in terms of sensitivity (range: 0.049 to 0.25 ng/mL, 1 ng/mL of LGN = 2.116 nM[19,20]), upper limit of calibration curve (range: 49.14 to 250 ng/mL), volume of plasma sample (range: 50 µL to 150 µL), retention time and run time (50 min and 70 min respectively with gradient elution), where all these methods adopted expensive Solid Phase Extraction (SPE) technique for sample clean up. Further it is important to note that none of these methods neither comprehensively captured method validation experiments with results nor followed latest regulatory guidelines for method validation.[22-24]

It is essential to establish reliable bioanalytical method with complete validation for PK and Bioequivalence [BE] study application, intended for regulatory submission. Therefore in view of non-availability of sufficient literature for LGN estimation in biological samples, we aimed to develop, simple, sensitive, selective, high throughput and reliable bioanalytical method with full quality validation conducted as per the most recent version of regulatory guidelines.[22-24]

## 2.0 Experimental

### 2.1 Materials and reagents

Both LGN working standard (purity, 98.26%) and LGN-d4, IS (purity, 98.39%) **(Fig. 1)**, were procured from Clearsynth Labs Ltd. (Mumbai, India). Methanol, water, ethyl acetate [HPLC grade], ammonium formate (extra pure grade), ammonia solution and formic acid [EMPARTA grade] was procured from Merck specialities Pvt. Ltd. (Mumbai, India). K2EDTA (Dipotassium ethylene diamine tetra-acetic acid) healthy human control plasma (including the haemolyzed and lipemic nature) was obtained from Deccan's Pathological Labs, Hyderabad.

### 2.2 Preparation of mobile phase buffer and stock solutions

About 0.31 g of ammonium formate (mobile phase buffer) was weighed into reagent bottle containing 500 mL of HPLC water and content was mixed before filtration with 0.22 µ filter.

The primary stock solution (PSS) of LGN and LGN-d4 was prepared in methanol (0.5 mg/mL and 0.4 mg/mL respectively) and stored in refrigerator at 2-8°C. As and when required, freshly prepared ISS (intermediate stock solution) from PSS, using diluent [mixture of methanol-water (50:50, v/v)] at 50000 ng/mL for LGN and at 4000 ng/mL for IS. CC/QC spiking solutions were prepared in appropriate concentration range from analyte ISS using same diluent.

### 2.3 Preparation of calibration curve standards (CC's) and quality control samples (QC's).

Prepared 8 CC standards **[**50.3 (LLOQ-lower limit of quantitation), 114.5, 318.0, 1325.3, 4016.2, 6693.8, 10298.1 and 12115.5 pg/mL (ULOQ-upper limit of quantitation)] and 4 level QC samples [50.6 (LLOQ QC), 142.8 ( low QC or LQC), 4760.3 (middle QC or MQC) and 9520.6 (high QC or HQC)] by spiking screened blank K2 EDTA human plasma with respective CC/QC spiking solutions at 2% v/v. LGN-d4 working solution was prepared freshly ( from PSS) as and when required at 40 ng/mL using same diluent as mentioned above. Replicate CC's, QC's, standard blanks (blank plasma with out spiking analyte and IS) and zero samples ( blank plasma spiking with 50 µL LGN-d4 working solution during sample processing ) in 0.4 mL quantity were aliquoted into pre-labelled polypropylene vials and stored in deep freezer at (-70 ±15°C and -20 ± 5°C) for conducting various validation parameters.

### 2.4 Instrumentation and LC-MS/MS conditions

Analysis was performed on UHPLC system [(Shimadzu, Kyoto, Japan) consisting of binary pump - LC Nexera X2 LC 30 AD, degasser - DGU 20 A 5R , an auto sampler - Nexera X2 SIL 30AC$_{MP}$ and column oven - Prominence CTO 20AC], having interface with tandem mass spectrometer of API 4000 triple quadrupole (AB SCIEX, Singapore), internally equipped with an electrospray ionization (Turbo v$^{TM}$) source. Nitrogen generator (Peak scientific, INFINITY 1031) was used to provide highly pure nitrogen for mass spectrometer. Chromatographic profile for analyte and IS [ RT at 1.72 (± 0.30) and 1.71 (± 0.30) min respectively with run time of 3.5 min] was accomplished with isocratic mobile phase [a mixture of 10 mM ammonium formate: methanol [20:80 (v/v)], degassed for 5 min ultrasonically using ultrasonic bath (HWASHIN TECH) and delivered at a flow rate of 0.5 mL/min, on to Gemini 3µ, C18, 100 mm × 4.6 mm (phenomenex) column, maintained at 35 ± 1°C, while injecting sample volume of 20 µL. Only 30% of the eluent from column exit was split in to the mass ionization source to facilitate MRM (multiple reaction monitoring) quantification (in positive ion mode) at common optimized source and compound specific parameters, for both LGN and LGN- d4 with resulting precursor to product ion transitions observed at m/z: 473.3/420.1 and 477.5/424.3 respectively **(Fig.2).** Ion source temperature and spray voltage was maintained at 500ºC and 5500 V respectively and Quadrupoles Q1 and Q3 were set on unit resolution with a dwell time of 200 ms for each mass transition. GS1(nebulizer gas), GS2 (turbo gas), curtain and CAD (collision associated dissociation) gas source parameters were set at 40, 45, 40 and 6 psi, respectively. Compound dependent parameters, viz. DP (declustering potential), CE (collision energy), EP (entrance potential) CXP (collision cell exit potential), were settled at 90, 31, 10 and 13 V respectively. Instrument management and data acquisition was performed using Analyst software version 1.6.2 (AB Sciex, Singapore).

### 2.5 Protocol for sample extraction procedure

A simple LLE (liquid- liquid extraction) method was followed for isolating both analyte and IS from plasma sample. Each 0.3 mL aliquot from CC standard, QC sample, standard blank, zero sample and unkown subject sample was spiked with 50 µL of LGN- d4 IS working solution (except for standard blank sample) followed by vortex mixing and addition of 0.4 mL extraction buffer [10% v/v ammonia solution in water]. The resulting sample content was first vortex mixed followed by the addition of 2.5 mL of extraction solvent (ethyl acetate), then further mixing on a vibramax reciprocating shaker for 20 min at 2000 rpm before subjecting to centrifugation for 10 min ( 3500 rpm at 5ºC ) on Eppenddorf 5810 R (Eppenddorf, Germany) centrifuge machine. Two (2.0) mL of supernatant organic layer obtained post centrifugation was evaporated to dryness under nitrogen (Rapid 50, Crescent scientific India) gas at 50°C. The dried residue was reconstituted with 400 µL of the mobile phase (reconstitution solution) and reconstituted sample, after vortex mixing was loaded in to autosampler vials of UHPLC system, for injecting 20 µL onto column.

### 2.6 Method validation

Method validation was performed as per the most recent version of US-FDA, EMA and ANVISA guidelines.[22-24] Precision (P) of the method was expressed in terms of % CV (coefficient of variation), calculated as the ratio of standard deviation/mean. Accuracy (A) was defined as: percent difference of mean observed value from nominal value, expressed as % nominal or calculated concentration/nominal concentration*100. Eight point calibration standards and four level QC samples at appropriate level (refer, section 2.3) in replicates were used for establishing majority of validation experiments as described below.

**2.6.1 System suitability:** A mixture of aqueous sample containing LGN at MQC level and IS at working concentration level, was injected in six replicates to check the

precision in peak area ratio ( ≤ 5.0% CV) and retention time ( ≤ 3.0% CV). It was performed on each day during method validation.

**2.6.2 Autosampler carryover:** Assessed along with aqueous and extracted LLOQ samples, by subsequently injecting reconstitution solution and extracted standard blank after aqueous and extracted ULOQ standard.

**2.6.3 Selectivity:** Established with 10 different matrix lots of K2 EDTA plasma (6 normal and 2 each of hemolytic and lipemic). Acceptable criteria used to assess method selectivity was: Interfering peak response at RT of analyte and IS in each lot standard blank sample (without analyte and IS) should be ≤ 20.0% and ≤ 5.0% respectively from LLOQ response of corresponding lot.

**2.6.4 Specificity:** Evaluated (using the same interference criteria build for selectivity) by injecting replicates of 1. Standard blank, 2. Concomitant blank [blank with concomitant medication at concentration equivalent to Cmax for nicotine, caffeine, cetrizine, domperidone, ranitidine, acetaminophen, diclofenac, ibuprofen, and metformin and 3. Zero sample, by comparing the interference at RT of analyte and IS in 1 and 2 and RT of analyte in 3 with mean LLOQ sample response.

**2.6.5 Sensitivity:** Established by processing and analyzing six LLOQ samples, which were part of P&A batch, where analyte (LGN) peak response should be quantifiable, reproducible and discrete with defined precision (≤ 20.0%) and accuracy (± 20%), while the average signal to noise ratio (S/N) should be ≥ 5.

***2.6.6 Analyte* recovery:** *Determined in six replicates at LQC, MQC and HQC level by comparing mean peak area of analyte in extracted samples (analyte spiked before sample extraction) against the mean peak area of analyte in post extracted samples (analyte spiked after sample extraction). Precision at each QC level should be within 15.0% and global recovery (average mean recovery of 3 QC's) should not be more than 115.0%.*

**2.6.7 IS recovery:** Evaluated at MQC level of analyte ( n = 6) by comparing the mean peak area of IS in the extracted samples with that of the post extracted samples where % recovery should not be more than 115.0% (≤ 15.0% CV).

**2.6.8 Matrix effect by evaluating matrix factor (MF):** 10 lots of screened plasma including two lots each of hemolytic and lipemic were evaluated to determine the MF for analyte and IS (calculated by: individual peak area in presence of matrix ions/ mean peak area in absence of matrix ions) and IS normalized MF (calculated by MF of analyte/ MF of IS). Post extracted samples for each lot at concentration equivalent to HQC and LQC were injected along with 6 replicate injections of aqueous sample having same concentration. % CV for IS normalized MF at each QC level should be ≤ 15.0%.

**2.6.9 Dilution integrity:** Conducted by using six dilution integrity quality control samples of *analyte* having concentration (19834.7 pg/mL) equivalent to 1.6 times of ULOQ, were diluted 5 fold with previously screened plasma and analyzed along with three P&A batches on with - in and between - run basis.

**2.6.10 Linearity, precision and accuracy (P&A):** 3 P&A batches were analyzed on two different days to determine linearity, P&A of the method, where each batch was organized in following sequence: Aqueous standard (MQC level), reconstitution solution or mobile phase, standard blank, zero sample, 8 CC standards and 6 replicates of QC's at HQC, MQC, LQC and LLOQ QC level. Residual square means for each CC standard were calculated in presence of weighing factors i.e. 'none', 1/x & $1/x^2$ to select appropriate factor which give minimum mean residual. Batch should be accepted, when each CC standard back calculated concentration would lie within ± 15.0% of its nominal concentrations except for LLOQ (within ± 20.0%). At least 75% of total CC standards including the ULOQ and LLOQ should comply with this criteria with resultant coefficient of correlation (r) of curve should be ≥ 0.98.

Within - run and between - run P&A was established where precision should lie within 20.0 % CV for LLOQ QC and within 15% CV incase of other QC's. Accuracy incase of LLOQ QC should be with in ± 20.0 % ( from their nominal concentrations) and for other QC's, it should be with in ± 15.0 % . At least 67% of overall QC samples and 50% at each QC level should meet this criterion.

**2.6.11 Auto sampler re-injection reproducibility:** Evaluated by re-injecting accepted P&A batch after storing samples in auto sampler temperature (10±1°C). This batch should meet acceptance criteria as described above for P&A.

**2.6.12 Ruggedness of the method:** Was performed by different analyst (who was not involved in previous analysis) on two separate batches covering HQC, MQC and LQC levels, where one batch was analysed using different column of same make and other batch with different equipment of same configuration.

**2.6.13 Stability studies:** Conducted to assess the stability of analyte and IS in stock solutions and stability of analyte in plasma sample.

**Stock solutions stability:** Comprising of short term stock solution stability (STSSS) and long term stock solution stability (LTSSS) for analyte (LLOQ and ULOQ level) and IS (working concentration level), by comparing the mean peak area of the stability stock with comparison stock, determined by injecting six replicates.

In case of STSSS, primary stock kept at 25±5°C was assigned as stability stock vs corresponding level of stock retrieved from refrigerator condition (2-8°C) was assigned as comparison stock. For LTSSS, respective stock prepared from fresh weighing was designated as comparison stock vs corresponding stock stored at 2-8°C in the refrigerator and retrieved for analysis was designated as stability stock.

Stock solution was considered stable if the mean peak area of respective stability sample was with in ± 10% (90-110%) from the mean peak area of the corresponding comparison sample, while meeting the precision criteria of ≤ 15% for both stability and comparison samples.

**Stability studies in *plasma* sample:** Bench top stability (BTS), Freeze thaw stability (FTS), Dry extract stability (DES), Stability of extract (SE), Long term stability (LTS) , Stability of analyte in K2EDTA blood (SAB) was conducted at LQC and HQC level in 6 replicates, where respective stability samples mean back calculated concentrations (calculated using freshly spiked CC's) were compared to that of nominal concentrations (excepting SAB) with common P&A acceptance criteria of ± 15.0%.

BTS samples were kept on bench at ambient temperature (25±5°C) before processing for analysis. FTS was determined after freezing the samples in deep freezer at -70±15°C and -20±5°C for a minimum period of 12 hours and then completely thawing at ambient temperature in a water bath. DES was conducted (since sample processing involve evaporation step), after storing dried extract samples in refrigerator (2-8°C). SE was performed **(**after immediate reconstitution with mobile phase), at ambient (25±5°C) and at refrigerator (2-8°C) temperature. LTS was evaluated for samples stored in deep freezer at -20±5°C and -70±15°C.

SAB was studied separately at ambient (25±5°C) and ice water bath (10±3°C) condition comparing mean peak area ratio (analyte/IS) of these stability samples with freshly prepared comparison samples.

**2.6.14 Batch Size experiment:** 30 QC samples each from LLOQC, LQC, MQC and HQC were *analyzed* against the prepared CC standards under separate P&A batch. These QC samples were earlier prepared and stored at -70±15°C in deepfreezer until analysis. This batch was considered acceptable, when it meets the same acceptance criteria set for P&A experiment.

### 2.7 Application of the method to biological samples

Successfully validated method was subjected to measure plasma linagliptin concentrations of 12 healthy adult male Indian subjects whose samples previously collected for PK study (conducted as per ICH-GCP norms) after administration of single 5 mg tablet under fasting conditions.

## 3.0 Results and discussion

### 3.1 Method development

To fulfill the objective of developing the simple, sensitive, selective, rapid, and reliable bioanalytical method of LGN in human plasma using LC-MS/MS method, thorough review of the collective literature on LGN, from physicochemical,[8,10,25] pharmacokinetic aspects[8,10] and reported bioanalytical methods[7,11-21] contributed a vital role, for development process which include selection of appropriate chromatographic - mass spectrometric conditions and extraction protocol before optimization.

#### 3.1.1 Optimization of mass spectrometric conditions

Development began, tuning the triple quadrupole mass spectrometer by separately injecting 100 ng/ mL solution (prepared in methanol: water 80:20 v/v and injected at a flow rate of 10.0 µL /min using infusion pump) of LGN and LGN-d4 in to ionization source, to determine their respective Q1 mass of the parent ion and Q3 mass of the product ion **( Fig.2 )** and to generate the optimal source and compound specific parameters as explained in section *2.4*. For the analyte and IS, mass parameters were tested initially in both positive and negative ionization modes. MRM method in positive ion mode was implemented in optimized method since it gave a consistent mass response where observed precursor to product ion mass transition of LGN matches with earlier literature.[15]

### 3.1.2 Selection of the IS

In support of literature [23,26,27] it was decided to use an isotopically labeled and stable IS (LGN-d4) in order to minimize the variations in analyte quantification, expecting similar RT and extraction recovery as that of LGN. Though previous methods adopted $^{13}C_3$ LGN, [12-20] not preferred the same because its non easy availability from authorized commercial sources and custom synthesizing the same would be time consuming and very expensive.

### 3.1.3 Optimization of the UHPLC conditions

Because of the UHPLC platform involved in the method and to achieve faster analysis of analyte and IS (with reproducible chromatography accomplishing well defined base line separated symmetric peaks and consistent RT's), short length C18 columns in combination with isocratic mobile phase having different proportions of volatile buffers and minimal possible consumption of the organic solvents[28,29] suitable for mass analysis were tested. C18 columns studied were : Beta 150*4.6 mm, 5 µ, Gemini 100*4.6 mm, 3µ, Gemini 50*4.6 mm, 3µ (Phenomenex), Zorbax SB 75*4.6 mm, 3.5 µ (in combination with following eluting systems: MEOH(methanol) : 2 mM amm.(ammonium) acetate pH - 4.0 (85:15 and 80:20 % v/v, with corresponding flow rates (FR) of 0.8 and 0.4 mL/min), MEOH:0.1% formic acid (80:20%v/v), FR : 0.5mL/min, MEOH:10 mM amm.formate pH-4.00 (80:20 %v/v), FR : 0.6 mL/min, acetonitrile : 5mM amm.acetate pH-4.01(85:15% v/v), FR :0.8 mL/min. Best chromatographic and mass optimization **( Fig. 3)** achieved (while maintaining the consistent column back pressure between 1400 to 1600 psi until 12 hours continuous analysis) with Gemini 4.6*100 mm, 3µ (Phenomenex) and MEOH:10mM ammonium formate (80:20 %v/v), delivered at a flow rate of 0.5 mL/min with sample injection volume of 20 µL. Only C18 columns are tested based on prior knowledge[13,15] and also the logP of the linagliptin molecule.[25]

### 3.1.4 Optimization of the extraction protocol

Various sample cleaning techniques[30] were studied prior to implementation of the best suited one for establishing reliable, simple and cost effective method for isolating the analyte from interfering substances present in plasma sample. PP method was not adopted as it would not be technically ideal option based on the fact that LGN at therapeutic drug concentrations, exhibit concentration-dependent protein binding in human plasma[10] and none of the previously reported methods or literature also not implemented this method.[7,11-20]

Though SPE technique was adopted in some of earlier reports [12,13,15-20] not given priority as this method in comparison with LLE is time consuming and requires expensive materials.[31] Therefore, in the present work simple LLE method as explained under section 2.5, was implemented, having the merits of minimizing the experimental cost, introducing clean sample for spectral analysis in general[32] and short sample processing time along with very good and consistent overall recoveries of the analyte and IS (not less than 70.9%) while confirming for the absence of any significant matrix effects. Various extraction solvents investigated for recovery experiments include, ethyl acetate : N-hexane in different volume ratio's, 100% TBME in presence of different buffer conditions, acid-0.1N HCl, base-25 mM $K_2HPO4$. 100% ethyl acetate when used in presence of base - 5% ammonia solution in water gave comparatively low recovery, where as 20% ammonia solution gave inconsistent recovery at LQC level.

## 3.2 Assay performance and validation

**3.2.1 System suitability:** Analytical instrument proved its performance for testing the proposed method with consistent and reproducible chromatography for analyte and IS retention times and their peak area ratio's with reported precision not more than 2.0% throughout the validation.

**3.2.2 Autosampler carryover:** Carry over was not observed at retention time of the analyte and IS for both aqueous and extracted samples which confirms the proposed method was devoid of potential carry over impacts.

**3.2.3 Selectivity:** All 10 different sources of blank plasma tested for selectivity were shown insignificant endogenous matrix components interference, at analyte and IS retention times. Refer **Fig. 3A**, representative chromatogram demonstrating the selectivity of method.

**3.2.4 Specificity:** Some of the commonly used concomitant and potential OTC drugs most likely to be administered in clinical situation of LGN were evaluated. Performance of the method was proved in presence of these drugs, due to the absence of significant response at retention time of *analyte* and IS in standard blanks, concomitant blanks and zero sample (only absence of significant analyte response).

**3.2.5 Sensitivity:** Sensitivity of the method for analyte was confirmed at LLOQ concentration of 50.3 pg/mL with reported precision, accuracy and average S/N of 6.3% , 102.4% and 9.03 respectively. Refer **Fig. 3C** for typical chromatogram depicting peak shape and S/N level at LLOQ level.

**3.2.6 Recovery of analyte and IS:** Overall recovery reported for analyte was 71.0 %. Refer **table no.1** for results, which met predefined acceptance criteria. Almost consistent results for IS (like analyte) was reported with % mean recovery not less than 78.2 and % CV not more than 6.3%. Observed consistency in recovery could be due to implementation of the optimized LLE procedure while adopting the deuterated internal standard.

**3.2.7 Matrix effect by evaluating matrix factor (MF):** Proposed method preclude the possible influence of the endogenous matrix components on the ionization process of analyte and IS as there was insignificant change in peak area response in extracted sample in reference to aqueous sample. Refer **table no.2,** for results.

**3.2.8 Dilution integrity:** Within - run and between- run precision of 1/5 dilution of the dilution integrity quality control sample was not more than 3.5%. Within - run and between- run accuracy was 89.2% and 93.3% respectively.

**3.2.9 Linearity, precision and accuracy (P&A) :** Linearity of the method with proportional peak area ratio (analyte/IS) Vs corresponding nominal concentration was proved over concentration range of 50.3 to 12115.5 pg/mL, using linear regression model with $1/x^2$ weighing produced best result (r = 0.9994). For CC standards observed P & A were 0.9 to 3.2 % CV and - 5.3 to 2.8 % nominal respectively. Refer **table no.3**, for summary of results on P & A of the method.

**3.2.10 Auto sampler re-injection reproducibility**: Established for 46 hours 51 min, where observed precision over reinjected samples was not more than 8.0% and their accuracy range was - 4.0 to 3.4%.

**3.2.11 Ruggedness of the method:** Both the batches analyzed for ruggedness were demonstrated linear calibration curve with goodness of fit greater than 0.99 while precision across 4 QC's tested was not more than 12.3% and accuracy ranging from 94.4% to 111.4%.

**3.2.12 Stability studies:** Designed to cover expected conditions of exposure to analyte (LGN) and IS during clinical study conduct. From the results as described below, it was evident that various conditions studied for stability had negligible impact on performance of the method for quantification of LGN and IS, due to in significant drug loss.

**Stability of analyte and IS in stock solutions:** For analyte and IS, short term stock solution stability was established for 07 hours 40 min and 06 hours 16 min respectively. Their long term stock solution stability was recorded for the duration of 19 days, 21 hours and 2 days, 23 hours respectively. Mean peak area of stability samples was in the range of 91.3 to 103.8% in relation to comparison samples. Observed precision range among stability and comparison samples was 1.0 to 9.1 %.

**Stability of analyte in plasma sample:** Designed studies were met predefined acceptance criteria. Refer **table no.4** for summary of results.

**Stability of analyte in K2 EDTA blood**: LQC and HQC level of stability samples kept at ambient and refrigerator condition for a period of 02 hours 08 minutes were not deviated by more than 4.1% from freshly prepared samples.

**3.2.13 Batch size experiment:** Total 120 QC samples analyzed across 4 levels against the CC standards in a separate batch. P & A for these QC samples ranging from 3.0 to 10.9% and 88.5 to 93.0% respectively. CC for this batch was found to be linear with goodness of fit more than 0.99.

### 3.3 Application:

Fully validated method was subsequently applied to PK study samples with successful incurred samples reanalysis in a separate batch where % variability was within 20% for more than 90% samples. A representative linear plasma concentration vs time profile for one of the subject was shown in **Fig.4.**

### 3.4 Comparison of proposed method with previous published reports

Refer **table no.5**, to highlight the merits of our proposed method (PM) in comparison with existing literature. Common unfavorable attributes of these published methods[12-21] which were not designed as per the latest regulatory requirements, were accounted with an un reporting of detailed procedures for (1) optimization of LC and MS/MS conditions (2) sample cleanup by SPE (3) conducting various validation parameters to prove the reliability of the method along with their results. Further all these methods employed (1) $^{13}C_3$ LGN as internal standard which is very difficult to obtain from authorized commercial sources and custom synthesizing the same may be very expensive and time consuming (2) expensive *and* time consuming SPE protocol for sample clean up. (3) Very wide calibration range most importantly to note with respect to upper limit (50 to 250 ng/mL), which is abnormally very

high to reflect the low concentrations of LGN from clinical studies, especially when conducted on approved oral doses of 2.5 mg (multi drug combination) and 5 mg tablet resepctively[8,10]. As per the regulatory requirements and in scientific perspective in order to appropriately characterize the PK profile, it is mandatory to choose appropriate calibration range based on the expected concentrations of analyte of interest in clinical study programs[22-24]. *Several drawbacks were found even in latest LC-MS/MS method reported by Shafi SS M et al[33] which include: in appropriated CC and QC range (10 ng/ mL to 5000 ng/ mL and 10 ng/ mL to 4000 ng/ mL receptively) used in method development and validation, insensitive characteristic of the method with validated LLOQ (10 ng/mL ) which was almost 2.4 times higher than the reported Cmax [8.9 nmol/L or 4.2 ng/ mL (1ng/mL of LGN = 2.116 nM)] of LGN at maximum oral dose of 5 mg, usage of large plasma sample volume (450 µL), incomplete method validation experiments in line with latest regulatory guidelines, usage of normal IS instead of stable labeled IS etc. However there were some close difference between their method and our proposed method with respect to extraction protocol, usage of C18 column and sample run time.* Our proposed method except comparatively use high plasma volume than the previous reports had superior results in all other aspects while fulfilling our aim of establishing the simple, rapid, reliable, sensitive and selective method for estimating LGN in human plasma. Further, LOQ of our method at pg/mL (50.3 pg/mL) could help to address expected lower plasma levels even from low dose linagliptin (dose lower than 5 mg) studies.

## 4.0 Conclusions:

To the best of our knowledge, first time a sensitive, rapid, rugged, selective and simple bioanalytical method was developed using UHPLC-MS/MS in human plasma with successful full validation as per the combined requirements of most recent regulatory

guidelines. Validated method was further tested to analyse PK study samples with successful incurred sample reanalysis.

All potential features as described in section 3.4, collectively makes this method attractive for high throughput analytical demands from large cohort clinical, PK and BE studies of linagliptin intended especially for regulatory submission and further expected to advance new clinical study programs on linagliptin.

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**Conflicts of interest**

Authors disclose no conflicts.

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**Figure Captions**

**Figure 1**. Chemical structure of **(A)** linagliptin, molecular weight: 472.54 g/mol and **(B)** linagliptin-d4, molecular weight: 476.57 g/mol.

**Figure 2.** Product ion mass spectra of protonated **(A)** linagliptin (m/z: 473.3 → 420.1, scan range: 100 - 500 Da) and **(B)** linagliptin-d4 (m/z: 477.5 → 424.3, scan range: 50 - 600 Da) in ESI mode.

**Figure 3.** UHPLC-MS/MS/MRM ion extracted plasma chromatograms for **(A)** standard blank (with out spiking analyte and IS) **(B)** zero sample (spiked with only IS - 50 µL of 40 ng/mL solution) **(C)** LLOQ ( 50.3 pg/mL) and **(D)** post dose subject sample (spiked with IS - 50 µL of 40 ng/mL solution) at 2.5 hours. Here in each figure left panel represents linagliptin and right panel shown with linagliptin-d4.

**Figure 4**. A representative plasma concentration vs time profile of one of the subject following administration of single 5 mg tablet of linagliptin

*Note: In this manuscript image with heading " First time reporting of quality Linagliptin assay in human plasma using UHPLC-ESI-MS/MS'' reflects table of contents entry for graphical abstract.*

RSC Author Templates - ChemDraw (CDX) - Single Column Artwork

All text and images must be placed within the frame.

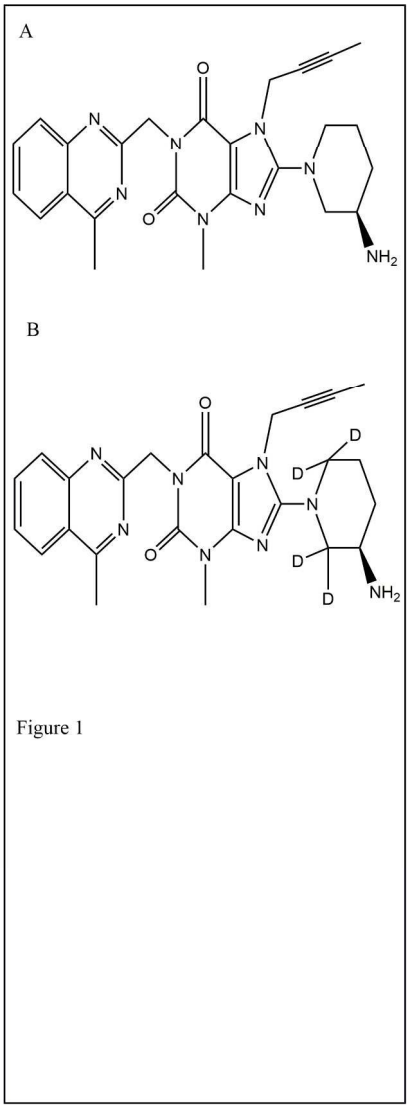


Figure 1

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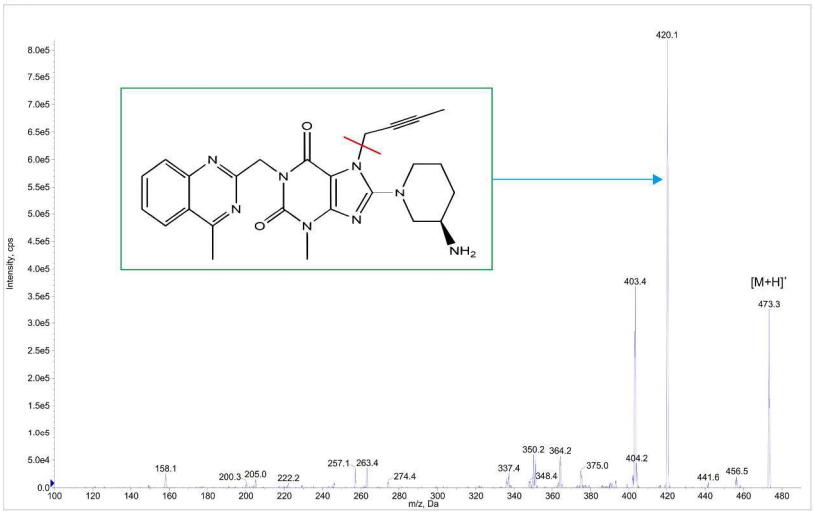
(A)

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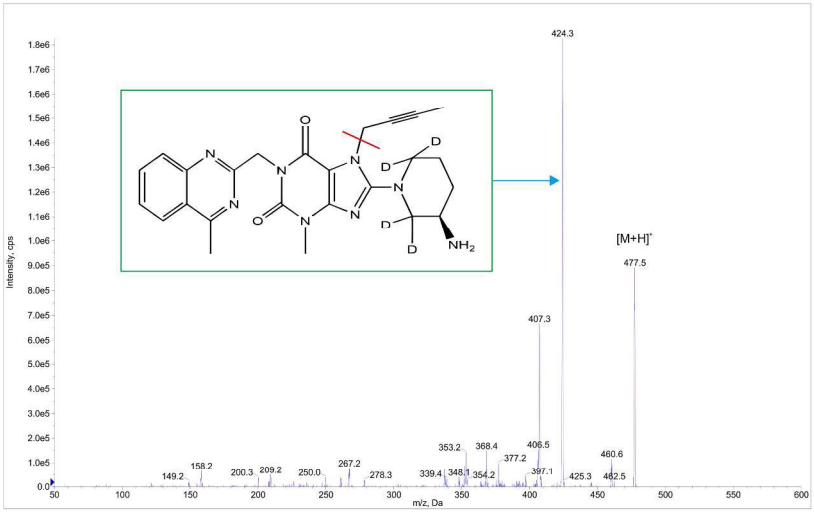


Figure 2

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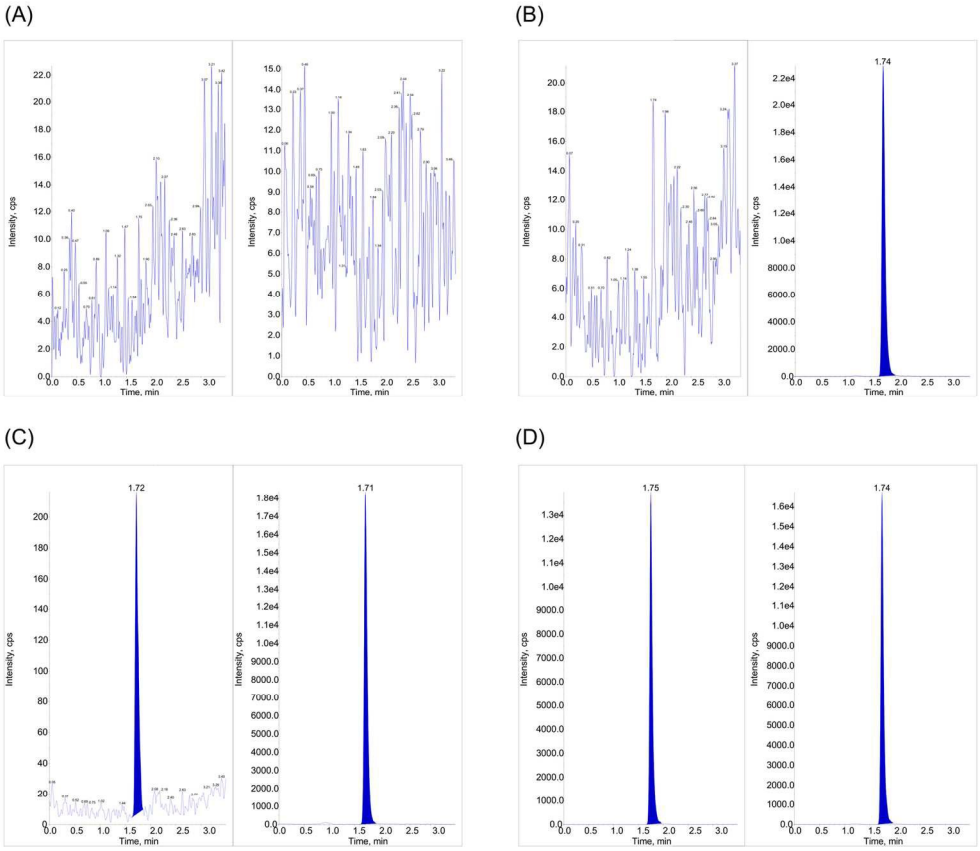


Figure 3

153x138mm (300 x 300 DPI)

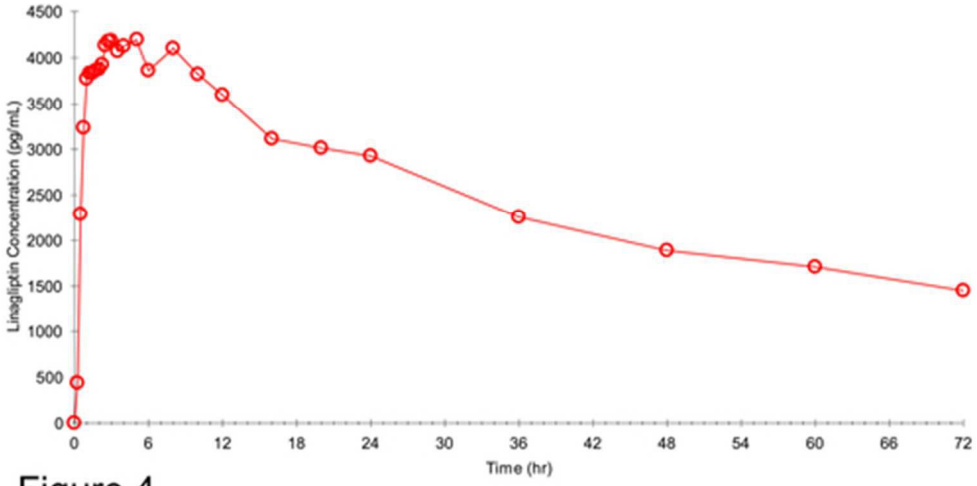


Figure 4

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**Table no.1**: Recovery of linagliptin in human plasma

| QC sample | post extracted sample peak area | | extracted sample peak area | | mean recovery [a] | global recovery [b] |
|---|---|---|---|---|---|---|
| ( n=6) | (mean ± SD ) | % CV | (mean ± SD ) | % CV | % | % |
| LQC | 2496.7 ± 70.3 | 2.8 | 1635.0 ± 120.9 | 7.4 | 65.5 | |
| MQC | 85163.5 ± 2310.9 | 2.7 | 59773.8 ± 3373.0 | 5.6 | 70.2 | 71.0 |
| HQC | 161749 ± 6952.2 | 4.3 | 124999.8 ± 9805.7 | 7.8 | 77.3 | |

Abbreviations: LQC: Low Quality Control, MQC: Middle Quality Control, HQC: High Quality Control, SD: Standard Deviation, % CV: Percentage Coefficient of Variation, n=6: total number of observations

[a] $\%\ \text{mean recovery} = \dfrac{\text{Mean extracted sample peak area}}{\text{Mean post extracted sample peak area}} \times 100$

[b] Average mean recovery of 3 QC's.

**Table no.2:** Matrix effect assessment by evaluating the matrix factor at LQC and HQC level of linagliptin in 10 different plasma matrices

| Plasma lot no. | MF | | IS normalized MF at HQC level (9520.6 pg/ mL ) | MF | | IS normalized MF at LQC level (142.8 pg/ mL) |
|---|---|---|---|---|---|---|
| | HQC | IS | | LQC | IS | |
| 1 | 1.19 | 1.23 | 0.97 | 1.14 | 1.13 | 1.01 |
| 2 | 1.16 | 1.19 | 0.97 | 1.17 | 1.11 | 1.05 |
| 3 | 1.06 | 1.07 | 0.99 | 0.95 | 0.98 | 0.97 |
| 4 | 1.07 | 1.06 | 1.01 | 1.11 | 1.03 | 1.08 |
| 5 | 1.04 | 1.03 | 1.01 | 1.00 | 0.96 | 1.04 |
| 6 | 1.04 | 1.00 | 1.04 | 0.99 | 1.00 | 1.00 |
| 7 (L) | 1.01 | 1.03 | 0.98 | 1.05 | 1.00 | 1.06 |
| 8(L) | 1.04 | 1.05 | 0.99 | 0.98 | 1.03 | 0.95 |
| 9 (H) | 0.98 | 0.99 | 0.99 | 1.01 | 1.02 | 1.00 |
| 10 (H) | 1.05 | 1.05 | 1.00 | 0.98 | 0.98 | 1.00 |
| Mean | 1.06 | 1.07 | 0.99 | 1.04 | 1.02 | 1.01 |
| SD | | | 0.022 | | | 0.042 |
| % CV | | | 2.2 | | | 4.1 |

Abbreviations: LQC: Low Quality Control, HQC: High Quality Control, % CV: Percentage Coefficient of Variation, SD: Standard Deviation, MF: Matrix Factor, IS: Internal Standard, L: Lipemic plasma, H: Hemolytic plasma.

$$\text{IS normalized MF} = \frac{\text{MF of analyte}}{\text{MF of IS}}$$

**Table no. 3**: Within - run and between - run precision and accuracy of method established with QC samples in plasma.

| Nominal Conc. of QC's (pg/mL ) | Within - run (n=6) | | | Between - run (n=18) | | |
|---|---|---|---|---|---|---|
| | Conc. found mean[a] ± SD (pg/mL) | Precision (%CV) | Accuracy (%) | Conc. found mean[b] ± SD (pg/mL) | Precision (%CV) | Accuracy (%) |
| LLOQ QC (50.6) | 47.7 ± 4.1 | 8.6 | 94.3 | 47.1±3.2 | 7.0 | 93.1 |
| LQC (142.8) | 123.8 ± 7.2 | 5.8 | 86.7 | 130.1±7.8 | 6.0 | 91.2 |
| MQC (4760.3) | 4276.05 ± 48.5 | 1.1 | 89.8 | 4440.8±149.9 | 3.4 | 93.3 |
| HQC (9520.6) | 8695.9 ± 95.3 | 1.1 | 91.3 | 9100.3±325.8 | 3.6 | 95.6 |

[a] Mean of six replicate sample observations from one analytical validation run

[b] Mean of eighteen replicate sample observations from three separate analytical validation runs

n = total number of observations

Abbreviations: LLOQ QC**:** Lower Limit of Quantitation Quality Control, LQC: Low Quality Control, MQC: Middle Quality Control, HQC: High Quality Control, % CV: Percentage Coefficient of Variation, SD: Standard Deviation.

**Table no. 4**: Stability of linagliptin in human plasma under different conditions

| Condition of storage & stability duration | QC sample | Nominal concentration (pg/mL) | Calculated concentration ( mean ± SD ) | Precision (%CV) | Accuracy (%) | % change [a] |
|---|---|---|---|---|---|---|
| BTS (25 ± 5°C, 7.15 h) | LQC | 142.8 | 141.0 ± 6.6 | 4.7 | 98.7 | -1.26 |
| | HQC | 9520.6 | 9631.3 ± 458.9 | 4.8 | 101.2 | 1.16 |
| FTS (after 5 cycle, -70 ± 15°C) | LQC | 142.8 | 148.3 ± 7.2 | 4.9 | 103.8 | 3.85 |
| | HQC | 9520.6 | 9539.8 ± 249.3 | 2.6 | 100.2 | 0.20 |
| FTS (after 5 cycle, -20 ± 5°C) | LQC | 142.8 | 144.9 ± 6.0 | 4.1 | 101.5 | 1.47 |
| | HQC | 9520.6 | 9421.4 ± 128.4 | 1.4 | 99.0 | -1.04 |
| DES (2-8°C, 26.39 h) | LQC | 142.8 | 136.0 ± 6.1 | 4.5 | 95.2 | -4.76 |
| | HQC | 9520.6 | 9686.9 ± 65.3 | 0.7 | 101.7 | 1.74 |
| SE (25 ± 5°C, 4.14 h) | LQC | 142.8 | 125.8 ± 3.1 | 2.5 | 88.1 | -11.90 |
| | HQC | 9520.6 | 9073.8 ± 199.6 | 2.2 | 95.3 | -4.69 |
| SE ( 2-8°C, 26.39 h) | LQC | 142.8 | 132.6 ± 5.9 | 4.4 | 92.9 | -7.14 |
| | HQC | 9520.6 | 9122.5 ± 223.1 | 2.4 | 95.8 | -4.18 |
| LTS (-70 ± 15°C, 118 days) | LQC | 142.8 | 140.2 ± 6.1 | 4.4 | 98.2 | -1.82 |
| | HQC | 9520.6 | 9247.2 ± 157.9 | 1.7 | 97.1 | -2.87 |
| LTS (-20 ± 5°C, 118 days) | LQC | 142.8 | 153.0 ± 8.1 | 5.3 | 107.7 | 7.14 |
| | HQC | 9520.6 | 10276.6 ± 213.9 | 2.1 | 108.5 | 7.94 |

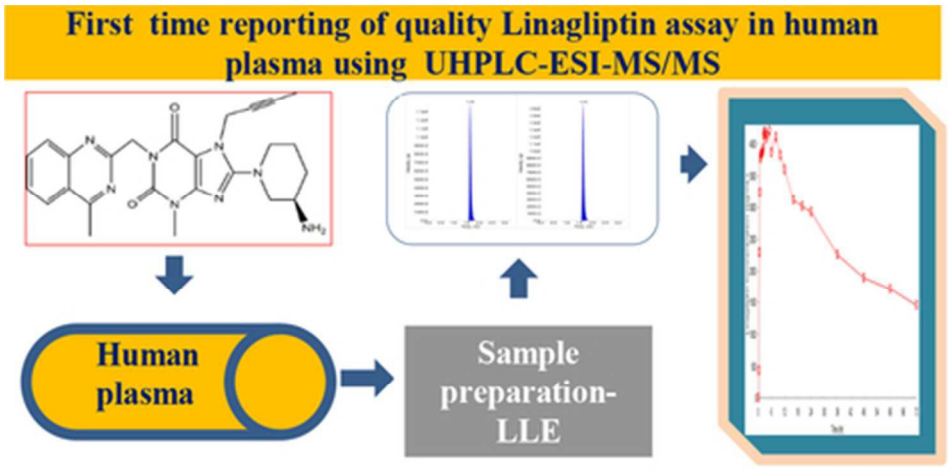
Abbreviations: BTS: Bench Top Stability, FTS: Freez -Thaw Stability, DES: Dry Extract Stability, SE: Stability of Extract, LTS: Long Term Stability, LQC: Low Quality Control, HQC: High Quality Control, SD: Standard Deviation, % CV: Percentage Coefficient of Variation.

$$^{a}\%\ \text{Change} = \frac{\text{Mean calculated concentration} - \text{Nominal concentration}}{\text{Nominal concentration}} \times 100$$

**Table no.5:** Comparison of proposed method (PM) with previous literature

| S.N | Method | IS | Biological matrix[a] | EM | SV | Cal. Range[b] | RTPS | RT | Reporting full method details (Yes/No) | Reporting of MV exp. with results (Yes/No) | Followed latest reg. guid.[c] | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | ( µL) | (ng/mL) | (min) | (min) | | | | |
| 1 | LC-MS/MS | $^{13}C_3$ LGN | Human K2 EDTA plasma | SPE[e] | 50 | 0.25 to 250 | - | - | No | No | No | 12,13 |
| 2 | LC-MS/MS | $^{13}C_3$ LGN | Human plasma | - | - | - | - | - | No | No | No | 14 |
| 3 | LC-MS/MS | $^{13}C_3$ LGN & $^{13}C_3$ CD 1750[d] | Human plasma | SPE[e] | 150 | 0.049 to 49.14 | 70 | 50 | No | No | No | 15 |
| 4 | LC-MS/MS | $^{13}C_3$ LGN | Human EDTA plasma | SPE[e] | 150 | 0.05 to 50 | - | - | No | No | No | 16-20 |
| 5 | LC-MS/MS | - | Human EDTA plasma | - | - | - | - | - | - | - | - | 21 |
| *6* | *LC-MS/MS* | *Telmisartan* | *Human plasma* | *LLE* | *450* | *10.0 to 5000* | *3.0* | *1.42* | *No* | *PR* | *No* | *33* |
| 7 | UHPLC-MS/MS | LGN- d4 | Human K2 EDTA plasma | LLE | 300 | 0.05 to 12.1 | 3.5 | 1.75 | Yes | Yes | Yes | PM |

Abbreviations: S.N: Serial number, IS: Internal standard, EM: Extraction method, SV: Sample volume, Cal. Range: Validated calibration curve range, RTPS: Run time per sample, RT: Retention time of LGN (linagliptin), MV: Method validation, exp: Experiment, reg.guid.: Regulatory guideline, Ref: Reference, SPE: Solid phase extraction, LLE: Liquid-liquid extraction, [a]Most of these published methods analysed LGN in both human plasma and urine sample using different level of calibration range and calibration range in this table pertains only to LGN in human plasma. [b] Calibration range given in published reports in nM/L converted to ng/mL (1ng/mL of LGN = 2.116 nM[19,20]), [c] US FDA (2013), EMEA(2011) and ANVISA (2012), [d]Inactive main metabolite (CD 1750) of LGN also estimated, [e]SPE procedure was not reported in detail**,** – not reported, *PR: Partial reporting*

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