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# A VALIDATED ESI-LC-MS/MS METHOD FOR QUANTIFICATION OF METFORMIN FORM HUMAN URINE

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**Abstract:**

Metformin, marketed under the tradename Glucophage among others, is the first-line medication for the treatment of type 2 diabetes and PCOS (Polycystic Overian Syndrom).The current research work describes a selective and sensitive LC-MS/MS method using Electro spray ionization as an ion source in quantification of Metformin from pooled urine. The separation of Metformin and the internal standard, Metoprolol, was achieved on Inertsil $C_8$ column (150 mm X 4.6 mm i. d., 5 µ) using 1.50 mL/min isocratic mobile phase flow with splitter (with ratio of 1:8). The developed Bioanalytical method was validated using US FDA Bioanalytical guidelines over the concentration range of 3.228 ng/mL to 1478.072ng/mL ($r^2$> 0.99) for Metformin from urine.

The overall mean percent recovery of LQC, MQC and HQC is 98.62. The method met acceptance criteria for all the validation parameters and can be successfully applied to human pharmacokinetic and bioequivalence studies of Metformin. The bioanalytical method was highly sensitive and selective for estimation of Metformin in Urine samples containing the drug.

**Keywords:** Metformin, Metoprolol, LC-MS/MS, Bioanalytical Method validation, Urine

## 1. Introduction

Metformin is an anti-hyperglycemic agent which improves glucose tolerance in patients with type 2 diabetes, lowering both basal and postprandial plasma glucose. Its pharmacologic mechanisms of action are different from other classes of oral antihyperglycemic agents. Metformin decreases hepatic glucose production, decreases intestinal absorption of

glucose, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization. Unlike sulfonylureas, metformin does not produce hypoglycemia in either patients with type 2 diabetes or normal subjects and does not cause hyperinsulinemia. With metformin therapy, insulin secretion remains unchanged while fasting plasma insulin levels and day-long plasma insulin response may actually decrease.

## 2. Materials and Methods

**2.1 Urine samples:** The drug free Urine samples of six different healthy volunteers were procured from Apex Hospital, Miraroad, Thane - 401104, (Maharashtra) India.

**2.2 Chemicals and reagents:** The organic solvents and chemicals used for sample processing under study are of analytical grade and procured from Qualigens Fine Chemicals, Mumbai, India. Acetonitrile, methanol and distilled water were procured from Merck, Mumbai, India. The working standards Metformin hydrochloride (98.84% purity, as is basis) and Metoprolol succinate (98.79 % purity, as is basis) were procured from Simson Pharma, Mumbai, India.

The chemical structures of Metformin hydrochloride and Metoprolol succinate are shown in Fig.01 and Fig.02 respectively.

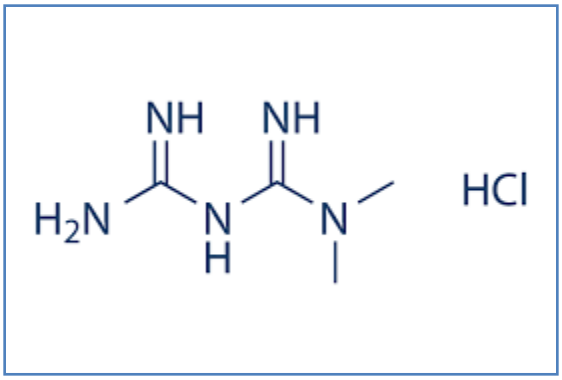



**Fig. 01 Structure of Metformin HCl**

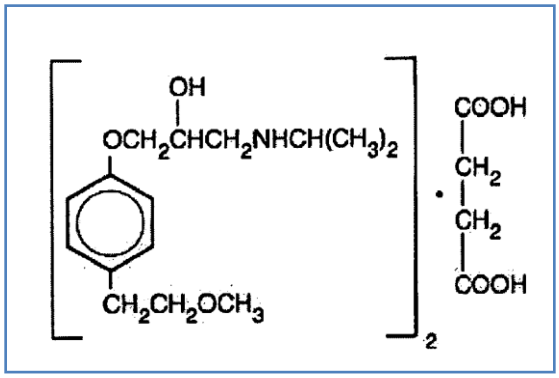



**Fig. 02 Structure of Metoprolol succinate**

**2.3 LC-MS/MS Instrumentation:** Front hand used consisted of Surveyor LC pump plus max equipped with Surveyor Autosampler Plus as an autosampler temperature controllable with column compartment. At the flow rate of 1.50 mL/min., with splittter ratio as 1:8, injection volume as 2 µL the column used was Inertsil $C_8$ (150 mm X 4.6 mm i. d., 5 µ) was set for chromatographic conditions.

Mass analysis was performed using Tripple - quadrupole Tandem Mass spectrometer, TSQ Quantum Discovery Max equipped with electrospray ionization source (ESI) (ThermoFisher Scientific). For data acquisition and analysis Analyst software LC Quan 2.5.6 was used with Selected Reaction Monitoring in positive mode. Sample solutions of 100 ng/mL

Metformin and 100 ng/mL of Metoprolol diluted in methanol were infused at 5.0 µL/min using syringe pump method. The MS parameters optimized were as follows: Spray voltage, Capillary temperature, Sheath Gas pressure, Auxillary Gas pressure, Tube Lens Offset, Collision Pressure, Collision Energy as 4000 Volts, 351ºC, 20 psi, 10 psi, 87 units, 1.5 mTorr and 26 Volts respectively.

**2.4 Other supportive instruments:** For weighing purpose Analytical semimicro balance of Denver (TB215DDE) was used. Micropipettes ranging as 10 – 100 µL (Biohit, 720050), 100- 1000 µL (Biohit, 720060) and 20-200 µL (Biohit, 720070) were used. For storage of prepared working solutions and the working standards the temperature of refrigerator (Croma, CRAR0125) was maintained at 2-8°C.For storage of urine samples deep freezer (Hally, HF-20°C) was set at -20°C ± 5°C for long term storage . The micro centrifuge (Heareusmultifugeplus 1-S, Thermo) was used at 4°C ± 2°C at 10,000 rpm.

**2.5 Preparation of calibrator and quality control (QC) samples:**Two sets of Metforminstock solutions of concentrations as 1002082.435 ng/mL and 1005165.766 ng/mL,for preparation of calibrator and quality control samples, respectively, were prepared by independent weighing in methanol. The diluent used was acetonitrile and HPLC grade water in the ratio of 65:35 *v/v*for further spiking solution preparation.The eight point calibration curve with concentrations as3.228, 5.380, 13.451, 26.901, 51.733, 129.332, 369.518, 1478.072 ng/mL (i.e. STD A to STD H) and four quality control samples at the concentration levels 1005.166, 517.661, 207.064 and 13.459 ng/mL (i.e. higher, middle, lower middle and low quality control samples) for MET were prepared in blank urine samples by using constant spiking volume method. Metoprolol stock solution of concentration 1014685.892 ng/mL was prepared in methanol. The stock was further diluted to obtain final concentration of spiking solution as10146.859 ng/mL.

**2.6 Sample Processing and Preparation:**The biological matrix (i.e. blank human urine samples) stored at -20°C ± 5°C was thawed and brought to room temperature for processing. The samples were brought to uniformity by vortex mixing of samples for 30 seconds. One portion of all the lots was pooled in equal quantity in a conical flask. The flask was capped and thenvortexed for 30 seconds for uniformity of the pooled matrix. 285µL of this pooled matrix was taken into the prelabelled polypropylene (PP) tube. 15 µL of respective drug spiking solution was spiked into the samples. The samples were vortexed for 30 seconds followed by the addition of 2.70 mL of HPLC grade water. The samples were vortexed for 30 seconds and an aliquot of the volume 250 µL was taken separately into the prelabbeled PP tube. 10 µL of

Metoprolol (10146.859 ng/mL) was spiked in all the tubes except in blank followed by vortexing for 30 seconds. 740 µL of the acetonitrile was added into each tube and all the tubes were vortexed for 30 seconds. The tubes were centrifuged at 10000 rpm in a micro centrifuge at 4°C ± 2°C for 5 minutes. 40 µL of the supernatant was further diluted with960 µL of diluent. These processed samples were then loaded into the autosampler vials for analysis purpose.

**3. Bioanalytical Method Validation**

The developed ESI-LC-MS/MS method for determination of Metformin from human urine thoroughly validated in accordance with the US FDA guideline for bioanalytical validation[20].

**3.1 System Suitability:** System suitability tests are integral part of liquid chromatographic Methods. The test was performed to verify that whether system was adequate for the intended analysis. Replicate injections of the standard solution of Metformin0.5 µg/mL in combination with standard solution of Metoprolol (n = 6), were compared to ascertain whether requirement for precision meet. The data generated by these injections was used to calculate % relative standard deviation (% RSD).

**3.2 Linearity (Calibration Curve):** Four calibration curves consistingof 8 point concentration levels of range 3.228 - 1478.072 in ng/mL of Metformin in human urine were prepared and analyzed on four consecutive days. The calibration curves were a plot of peak area ratios of drug (Metformin) and IS vs the concentration of Metformin constructed on the basis of weighted linear regression (1/ x2). Deviations of the back-calculated concentrations for the calibration standards were set at ±15 %, whereas for Lower Limit of Quantification (LLOQ) it was ± 20 %.

**3.3 Selectivity and Sensitivity:** Blank urine samples obtained from six different lots were processed and analyzed in order to investigate the potential interferences from endogenous substances. The chromatograms obtained for blank, zero standard (spiked with IS only), non-zero standard (spiked with analyte and IS) and an *in vivo* study sample were compared to ascertain the selectivity of the method.

The limit of detection (LOD) and limit of quantitation (LOQ) for the developed method was determined by injecting progressively low concentrations of the standard solution of Metformin. Limit of detection (LOD = 0.750 ng/mL) and limit of quantitation (LOQ = 3.228 ng/mL) were established at a signal to noise ratio of 3:1 and 10:1 respectively.

**3.4 Precision and Accuracy:** The precision and accuracy (P & A) of the system was determined by measuring repeatability of sample application and measurement of concentration for six replicates at three different concentrations

of 1005.166, 517.661, 207.064, 13.459 and 3.365 ng/mL. Intra and inter-day variation for the determination of Metformin were carried out. The intraday precision was carried out on the same day while inter-day precision (intermediate precision) was studied by comparing assays performed on three different days. The precision and accuracy of the system and method were expressed as percent Relative Standard Deviation (% R.S.D.) and % Nominal, respectively. The acceptance criteria for the intra-day and inter-day precision and accuracy are within ±15% for all levels.

**3.5 Dilution Integrity:** A dilution integrity experiment was performed with the aim of validating the method to be carried out for the samples with concentrations beyond the linear range during *in vivo* sample analysis. Six replicates at 2-fold and 10-fold dilutions were prepared by giving appropriate dilutions to the approximately two times highest level of the calibration curve with blank urine.

**3.6 Ruggedness:** Ruggedness was determined by change in column and analyst of assay procedure[10]. To authenticate the ruggedness of the proposed method, it was done on two precision and accuracy batches. The first batch was analyzed by different analyst while the second batch was analyzed on different column.

**3.7 Stability:**

Stock solution stability of Metformin was assessed by evaluation of short-term stability for 24 h and long-term stability for up to 30 days. The area responses for the stability solutions were compared with that of the freshly prepared solutions with an acceptable deviation of ± 10% [7]. The stability of MET in human urine was ascertained by analyzing in six replicates the quality control samples kept under following storage conditions: bench-top stability for 6hrs; process stability for 24 h at 8°C; long-term stability was evaluated by processing the samples kept at -20 ± 5°C for 30 days; freeze-thaw stability over five cycles; wet extracts were stored at 2-8°C for 24 h. Stability for dry extract was not applicable to the method hence, was not performed. These stability samples were compared with freshly extracted quality control samples. The percentage difference between the stability and freshly prepared samples was of ± 15 % and was considered acceptable[7].

**3.8 Recovery:** The recovery was evaluated by analyzing the quality control levels according to the methods described by Koo, *et.al*. The extraction recovery of Metformin and IS were determined by peak areas of analyte/IS spiked before processing the samples with peak areas for analyte/IS spiked after processing the samples.

**3.9 Matrix Effect:** By comparing the peak areas of the analyte standards, standards spiked before and after extraction into different lots of urine, and the peak area ratios of analytes to an IS, the recovery and ion suppression or enhancement associated with a given lots of urine were assessed[3].

## 4. Results and Discussions

**4.1 Mass spectrometry in detection and optimization:** The mass spectrometric parameters for Metformin and Metoprololwere optimized by direct syringe infusion method under both positive and negative mode. The molecules exhibited the higher sensitivity in positive ion modes of ESI in comparison with negative mode. MS parameters to enhance the sensitivity are shown in **Table 01**.

**Table 01: MS parameters of Metformin and Metoprolol**

| **Parameters** | Metformin | Metprolol |
|---|---|---|
| **m/z** | 131.100to 71.140 | 268.200 to 133.095 |
| **Polarity** | + ve | + ve |
| **Spray Voltage (volts)** | 4000 | 4000 |
| **Capillary Temperature (°C)** | 351 | 351 |
| **Sheath Gas Pressure** | 20 | 20 |
| **Auxillary Gas Pressure** | 10 | 10 |
| **Tube Lens Offset** | 87 | 46 |
| **Collision Pressure (mTorr)** | 1.5 | 1.5 |
| **Collision Energy (Volts)** | 26 | 22 |

**4.2 Optimization of chromatographic conditions:** The various mobile phases were tried by keeping methanol and acetonitrile as organic phase in combination with distilled water. The peaks obtained with acetonitrile and water as mobile phase in the ratio (10:90 *v/v*) were sharper as compared to the methanol and water as mobile phase. The addition of small concentration of ammonium acetate buffer helped in retaining the peak and reduced the tailing effect to some extent. Amongst the concentrations of ammonium acetate (2-10 mM) tried in the mobile phase, 5 mM was the best fit for mobile phase. Addition of formic acid or acetic acid made no changes in retention time or did not show any changes in peak shapes, so acidification of the mobile phase was rejected.

Rinsing solution was selected as water and acetonitrile in the ratio 35:65 *v/v* which gave no carry over effect for both the molecules. The molecule showed retention time more in $C_{18}$ as compared to $C_8$ column. Hence, Inertsil $C_8$ column was chosen for separation purpose. To lessen the retention time more and due to the higher response of the molecules even after dilution flow rate was set as 1.50 mL/min with the splitting ratio 1:8.

Under the optimized chromatographic conditions the retention times of Metformin and Metoprolol were 1.65 ± 0.2 minutes and 1.75 ± 0.2 minutes respectively.

**4.3 Optimization of sample processing and preparation procedure:**

There are numerous methods reported earlier to analyze the drug in plasma[12,18,22] and few are reported to analyze the drug from urine[22]. The reported methods for extraction of drug reported are time consuming as well as are by precipitating the plasma proteins. Injecting protein precipitated plasma samples into sensitive instrument like LC-MS/MS should be avoided as we are precipitating only plasma proteins but rest of the plasma is still getting injected. This may lead to high matrix effect, low sensitivity and may contaminate the curtain plate and quadrupole. Whereas, extraction of drug from urine as per earlier reported method, includes LLE using ethyl acetate followed by the dilution (1:5000) of samples before injections. Though the method is selective but, it is more time and solvent consuming as compared to current developed and validated method, as it involves the dilution of samples. Current method involves the dilution but due to the splitting of the inlet to LC-MS/MS less concentration is getting injected into the system. For first dilution HPLC grade water was used. Then the samples were aliquoted 250 µL. 10 µL of IS was spiked and 740 µL acetonitrile was added into it. This step was performed to precipitate any proteins (if present) in the urine samples. The samples were then centrifuged in microcentrifuge at 4º C for five minutes at 10000 rpm. Then 40 µL of this supernatant was diluted with 960 µL of diluent and the samples were injected after 30 vortexing followed by the loading in the autosampler vials.

**4.4 Selection of Internal standard:**

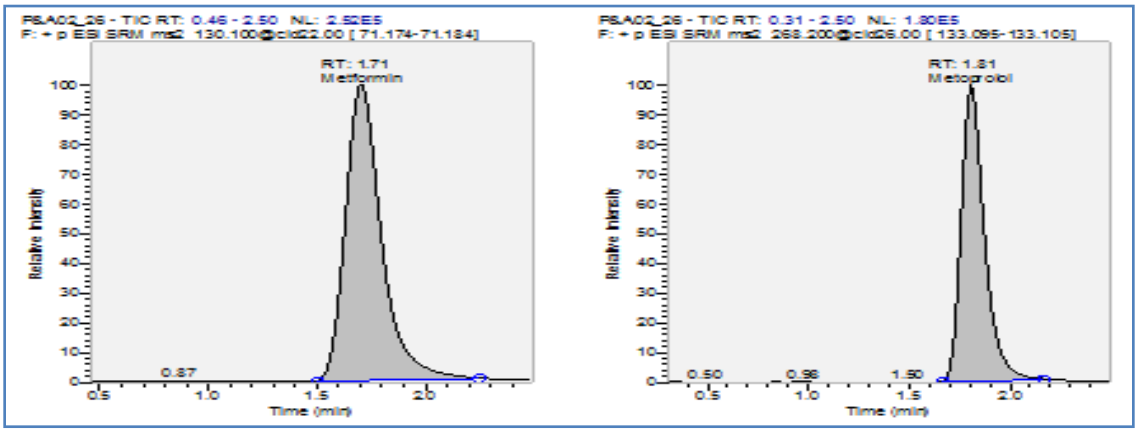
For quantitative bioanalysis, it is necessary to employ an appropriate IS that would mimic the analyte in the entire sample extraction, chromatographic elution and detection. In modern hyphenated techniques like HPLC-MS/MS, a stable isotope labelled IS that meets above criteria should be used ideally. Taking cost and difficulty in synthesizing stable isotope labeled compounds in consideration, several other compounds were screened to find suitable IS. Metoprolol was the best fit for the developed method for Metformin. hence, it was chosen as IS.

**4.5 System Suitability:** The system suitability was evaluated by calculating % R.S.D. for areas and retention times obtained for MET and MPL in experimental setup. On calculation % R.S.D. was found to be 1.79 and 0.81 for area ratio values and retention times respectively (**Table 02.**) and the representative chromatogram is given in **Fig. 03**.

**Table 02. System Suitability.**

| Sample code | Injection number | Area ratio | Retention time of MET |
|---|---|---|---|
| SYS 01 | 1 | 0.159 | 1.65 |
| | 2 | 0.160 | 1.65 |
| | 3 | 0.162 | 1.62 |
| | 4 | 0.161 | 1.63 |
| | 5 | 0.167 | 1.65 |
| | 6 | 0.164 | 1.65 |
| **Mean** | | 0.162 | 1.642 |
| **S.D.** | | 0.003 | 0.013 |
| **% R.S.D.** | | 1.790 | 0.810 |
| **Acceptance criteria** | | % R.S.D. ≤ 5 | % R.S.D. ≤ 2 |

**Fig. 02: Representative chromatogram of system suitability**




**4.6 Selectivity and Sensitivity:** Selectivity was carried out to evaluate the ability of the method to quantify the Analyte and the IS from other urine components after extraction. This was evaluated by injecting extracted blank urine samples from six different lots and comparing any interference with the response of the extracted LLOQ. The % interference was not seen for Metformin and was found to be not more than 0.15.

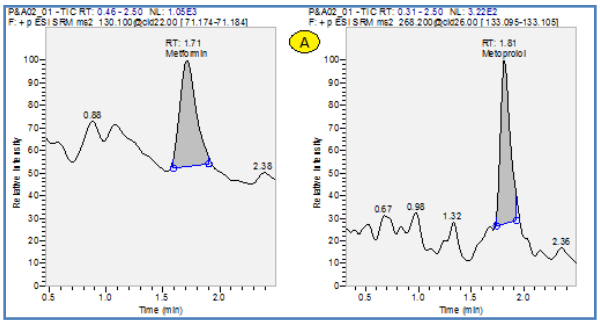
Current method was found to be sensitive with LOD and LOQ of 0.750 ng/mL and 3.228 ng/mL respectively. This indicates that proposed method is sensitive enough to determine lower concentrations of Metformin from samples effectively.

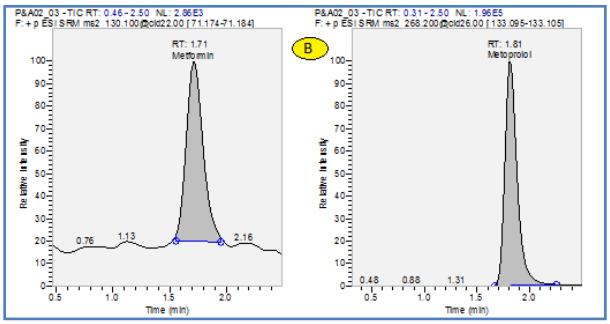
The signal to noise ratio for Metformin LLOQ met the acceptance criteria as per US FDA guidelines.

Representative chromatograms are given in **Fig.04.**

**Fig.04: Representative chromatograms (A &B)**

**A-Representative Blank urine chromatogram.** **B- Representative LLOQ chromatogram.**




**4.7 Matrix Effect:** The matrix factors of analyte and IS were evaluated in six blank/control human urine samples at low and high concentrations. The matrix factor for analyte and IS was 0.52 and 0.55 and 0.56 and 0.57, with a relative standard deviation of <15%. These results indicated that theion suppression or enhancement of the analyte and IS in the matrix was negligible.

**4.8 Linearity (Calibration curve):** The typical regression equation for eight-point calibration curve (3.228 to 1478.072 ng/mL) obtained by least squares regression for SWM was y = (0.0005 ± 0.0001) x + (-0.0032 ± 0.0018), r2 = (0.9988 ± 0.0026), where y represents ratio of analyte peak area to that of IS and x represents the urine analyte concentration. Further, the results presented in **Table 03.** Signify that the calibration curve has a good linear detector response over Metformin concentration range.

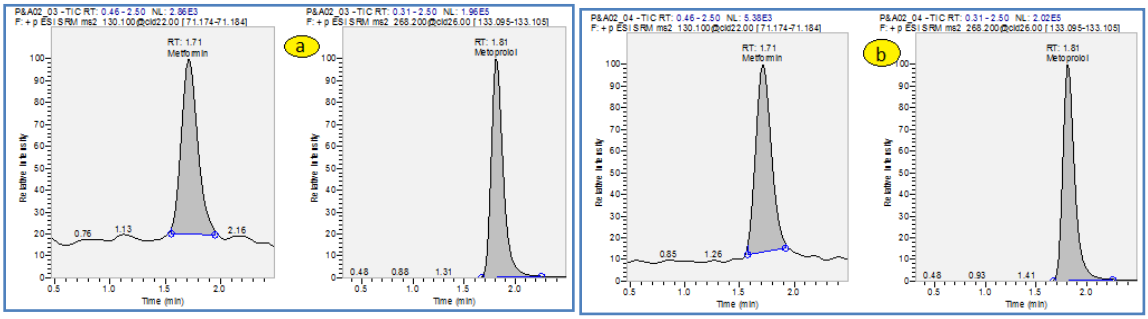
**Table 03. Summary of mean back-calculated concentrations for calibration standards of Metofrmin present in human urine (n=4) .**

| **Nominal concentrations (ng/mL)** | **3.228** | **5.380** | **13.451** | **26.901** | **51.733** | **129.332** | **369.518** | **1478.072** |
|---|---|---|---|---|---|---|---|---|
| **Mean calculated concentrations (ng/mL)** | 3.498 | 5.368 | 13.859 | 27.052 | 53.489 | 124.429 | 334.281 | 1448.330 |
| **S.D.** | 0.028 | 0.171 | 0.140 | 0.505 | 0.213 | 0.766 | 1.197 | 1.978 |
| **% R.S.D.** | 0.81 | 3.18 | 1.01 | 1.87 | 0.40 | 0.62 | 0.36 | 0.14 |
| **% Nominal** | 108.36 | 99.78 | 103.03 | 100.56 | 103.39 | 96.21 | 90.46 | 97.99 |
| **% Bias** | 8.36 | -0.22 | 3.03 | 0.56 | 3.39 | -3.79 | -9.54 | -2.01 |

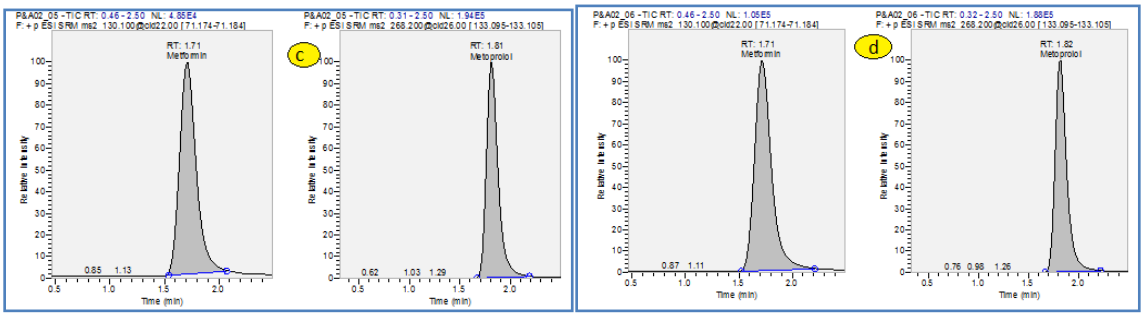
Representative chromatograms are given in **Fig.05.**

**Fig.05: Representative chromatograms of Linearity**

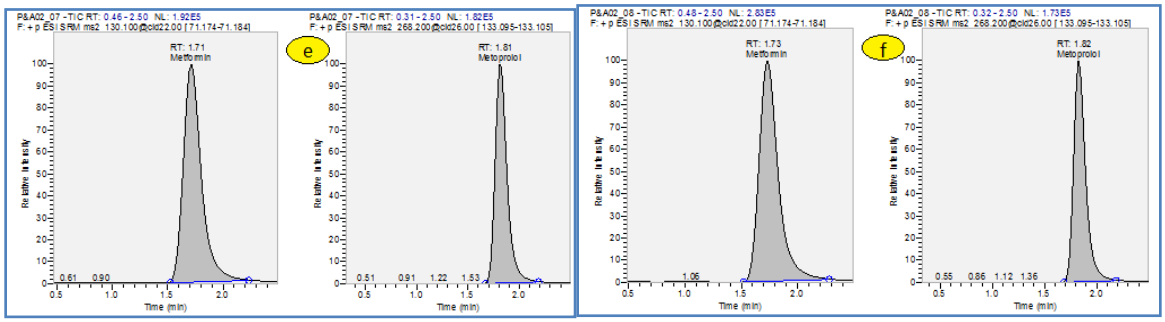
**a: STD A-3.228 ng/mL** **b: STD B- 5.380 ng/mL**



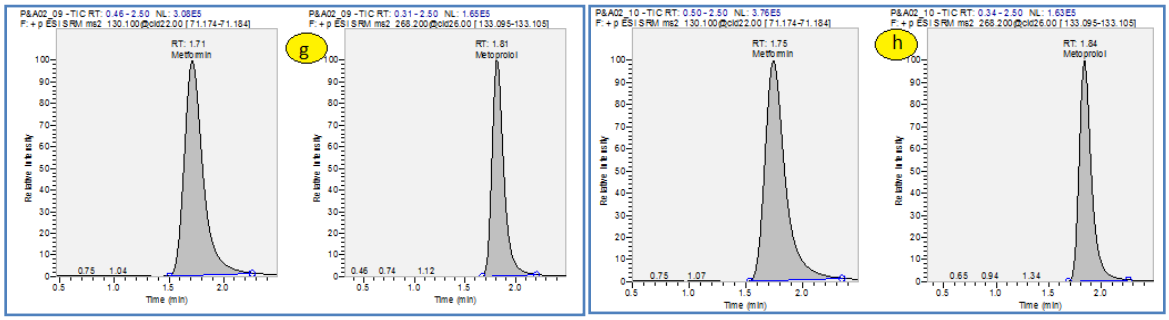
**c: STD C- 13.451 ng/mL** **d: STD D- 26.901 ng/mL**



**e: STD E- 51.733 ng/mL** **f: STD F- 129.322 ng/mL**



**g: STD G- 369.518 ng/mL** **h: STD H -1478.072ng/mL**



**4.9 Precision and Accuracy: Table 04.** Represents a summary of accuracy and precision results, which are within the acceptable range, indicative of the method being accurate and precise. The data obtained indicates current method has a satisfactory precision, accuracy and reproducibility.

**Table 04. Summary of inter-day precision and accuracy for quality control samples of Metformin spiked in human urine.**

| Nominal concentrations (ng/mL) | 13.459 | 207.064 | 517.661 | 1005.166 |
|---|---|---|---|---|
| **Inter-day assay (n = 6 replicates at each concentration, 3 days)** | | | | |
| **Mean ± S.D. (ng/mL)** | 13.060 ± 0.103 | 185.506 ± 11.282 | 531.026 ± 26.525 | 996.850 ± 95.426 |
| **Precision (% R.S.D.)** | 0.79 | 6.08 | 5.00 | 9.57 |
| **Accuracy (% Nominal)** | 97.04 | 89.59 | 102.58 | 99.17 |
| **Intra-day assay (n = 6 replicates at each concentration)** | | | | |
| **Mean ± S.D. (ng/mL)** | 13.090 ± 0.057 | 204.187 ± 18.871 | 504.678 ± 48.317 | 975.628 ± 78.210 |
| **Precision (% R.S.D.)** | 0.44 | 9.24 | 9.57 | 8.02 |
| **Accuracy (% Nominal)** | 97.26 | 98.61 | 97.49 | 97.06 |

**4.10 Dilution Integrity:** The % RSD and % Nominal obtained from 2-fold and 10-fold dilutions of spiked urine were 1.90, 92.81 and 1.24, 112.68 respectively. These results indicate the acceptability of both 2-fold and 10-fold dilutions prior to analysis of *in vivo* samples.

**4.11 Recovery:** The recovery (%) of Analyte and IS from Urine was determined by comparing the mean peak area of six extracted and six unextracted samples at three different concentrations (LQC, MQC and HQC). The percent recovery of Metforminwas 98.62 % and that was for Metoprolol as 96.12 %.

**4.12 Stability: Table 05.** Briefly summarizes the results of stability experiments performed for Metformin in urine. The data demonstrate that Metformin in blank human urine was stable for 6 hrs at room temperature and 24 hrs in the autosampler. Further, Metformin in blank human urine remained unaffected, even after the samples were subjected to five freeze-thaw cycles stored at -20 ± 5°C for up to 30 days. Data obtained by wet extract stability proved the stability of Metformin.

**Table 05. Summary of stability for quality control samples spiked in blank human urine (n=6).**

| Stability | Nominal concentrations (ng/mL) | Mean calculated concentration (ng/mL) | Presicision (% RSD) | Accuracy (% Nominal) | % Difference |
|---|---|---|---|---|---|
| **Fresh samples** | 13.459 | 13.920 ± 0.081 | 0.58 | 103.43 | - |
| | 1005.166 | 1055.630 ±19.606 | 1.86 | 105.02 | |
| **Autosamplerstability[a]** | 13.459 | 14.075 ±0.096 | 0.68 | 104.58 | 1.08 |
| | 1005.166 | 10199.539 ± 20.313 | 1.99 | 101.40 | -3.45 |
| **Bench top stability[b]** | 13.459 | 14.074 ±0.107 | 0.76 | 104.47 | 1.08 |
| | 1005.166 | 1039.539 ± 16.220 | 1.56 | 103.42 | -1.52 |
| **Freeze thaw stability[c]** | 13.459 | 14.117 ± 0.064 | 0.45 | 104.89 | 1.39 |

| | | | | | |
|---|---|---|---|---|---|
| | 1005.166 | 1046.088 ± 22.810 | 2.18 | 104.07 | -0.90 |
| **Long term stability[d]** | 13.459 | 14.089 ± 0.059 | 0.42 | 104.68 | 7.92 |
| | 1005.166 | 965.029 ± 22.417 | 2.32 | 96.01 | 5.97 |
| **Wet extract stability[e]** | 13.459 | 14.090 ± 0.055 | 0.39 | 104.69 | 1.22 |
| | 1005.166 | 1127.250 ± 17.860 | 1.58 | 112.15 | 6.78 |

[a] : After 24 hrs in autosampler at 10 ± 2ºC
[b] : After 6 hrs at room temperature
[c] : After five freeze/thaw cycles at -20° C
[d] : At -20° C for 30 days
[e]: Samples stored upto 24 hrs at 2-8 ºC

## 5. Conclusion:

To the author's knowledge, this is first ESI-LC-MS/MS method reported for the quantification of MET using sample three step dilution as sample pretreatment process from human urine validated as per US FDA guidelines. This method afforded satisfactory results in terms of sensitivity, selectivity, precision, accuracy, reproducibility and recovery. A low urine volume (285µL) for sample preparation step resulted in a detection limit of 0.750 ng/mL and quantification limit of 3.228 ng/mL of Metformin for the developed method. The method can be hence, successfully applicable to pharmacokinetic study for analysis of Metformin from the urine samples.

## 6. References


1. A. Dubey and I. C. Shukla, *Journal of the Indian Chemical Society, 81*(1), 84-86 (2004).
2. A.T. Barden, B. L. Piccoli, N.M. Volpato, M. Steppe, *Analytical Methods, 5*(20), 5701-5708 (2013).
3. B. K. Matuszewski, M. L. Constanzer, C. M. Chavez-eng, Analytical chemistry, 75, 3019-3030 (2003).
4. B. K. Matuszewski, Journal of Chromatography B, 830, 293–300 (2006).
5. B.M. Ayoub, *RSC Advances*, *5*(116), 95703-95709 (2015).
6. L. Adhikari, S. Jagadev, S. Sahoo, P. N. Murthy, U. S. Mishra, *International Journal of ChemTech Research, 4*(2), 625-630 (2012).
7. D. C. Reddy, A. T. Bapuji, V. Suryanarayanarao, V. Himabindu, D. R. Raju, S. Sayed, H. L. Ravikiran, E-Journal of Chemistry, 9(2): 899-911 (2012).
8. F.S. Bandarkar and I.S. Khattab, *Journal of Liquid Chromatography and Related Technologies, 33*(20), 1814-1830 (2010).

9. 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, Center for Veterinary Medicine (CV), May 2001.
10. J. Ahmad, S. Amin, S.R. Mir, *Journal of pharmaceuticals and biosciences*, ISSN: 2321-0125 (2014).
11. J. Brohon and M. Noel, *Journal of Chromatography B: Biomedical Sciences and Applications, 146*(1), 148-151 (1978).
12. K. Heining, and F. Bucheli, Journal of Pharmaceuticals and Biomedical analysis, 34, 1005-1011 (2004).
13. M. M. Annapurna, K.E.V. Nagoji, S.S. Rao, S.S. Petro, *Asian Journal of Chemistry, 14*(3-4), 1775-1776 (2002).
14. N.M. Bhatia, A.K. Mullani, I. Singhavi, M.S. Bhatia, *Journal of Chemistry, 20*(1), 236-240 (2008).
15. P.K. Chaturvedi and R. Sharma, *ActaChromatographica, 20*(3), 451-461 (2008).
16. S. Ashour and R. Kabbani, *Analytical Letters, 36*(2), 361-370, (2003).
17. S.D. Bhende, M. B. Varanasi, K. Abbulu, M. DivyaSwetha, V. Shravanthi, J. KarunaKumari and T. Shyamala, *Oriental Journal of Chemistry, 28*(1), 463-469 (2012).
18. S. L. Bonde, R. P. Bhadane, A. Gaikwad,, D. Katale, S. Gavali, A. S. Narendiran, *International Journal of Pharmacy and Pharmaceutical Sciences*, 0975-1491, 5(3), 463-470 (2013).
19. T. S. Koo, S. J. Kim, J. Lee, D. J. Ha, M. Baek, H. Moon, Biomedical Chromatography, 25: 1389 (2011).
20. US DHHS, FDA and CDER. Guidance for Industry: Bioanalytical Method Validation. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine, Washington DC, 2001. Available from: http://www/fda.gov/cder/guidance/index.htm
21. Y. Chen, L. Zhao, F. Lu, Y. Yu, Y. Chai, &Y. Wu, *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*(5), 595-603 (2009).
22. X. Zhang, X. Wang, D. I. Vernokovskaya, M. V. Fokina, T. Nanoviskaya, G.D.V. Hankins, M. S. Ahmed, Biomedical Chromatography, DOI 10.1002/bmc.3314 (2014).

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