

Developing and validating bio analytical method biology essay

[Science](#), [Biology](#)



LC-MS/MS has become the technique of choice for pharmaceutical industry for quantitative bio analysis of drugs and active metabolites in bio fluids such as plasma, serum to support clinical development and bio equivalence of new chemical entity and generics drug respectively. LC-MS/MS has become technique of choice when compared with HPLC, GC due to selectivity and specificity which in turn results in shorter run time of the assays 16. Shorter run times results in high throughput of samples which is the need of hour to support the faster development of molecules in clinical steps. The introduction of automated sample preparation techniques of solid phase extraction, liquid-liquid extraction and protein precipitation has brought about high-throughput approach to bio-analysis 17. Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites (analytes) are critical for the successful conduct of preclinical and/or biopharmaceutics and clinical pharmacology studies. Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use. The fundamental parameters for this validation include (1) accuracy, (2) precision, (3) selectivity, (4) sensitivity, (5) reproducibility, and (6) stability 18.

2. 1 BIO-ANALYTICAL METHODS METHOD DEVELOPMENT , VALIDATION AND ITS APPLICATIONS

LC-MS based screening approaches are used to evaluate the potential of drug-drug interaction either in vitro or in vivo. It is very important to

understand their concentration in order to adjust the dosage. Hence rugged and robust method will require monitoring the levels in circulatory concentration. Attempt has been made to develop and validate method for below drugs. Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method. 18

2. 2 IN-VITRO METABOLISM

The strategy of these experiments is to obtain the early indication of inhibition or induction of metabolism of compounds by cytochrome P450(CYP isozymes). Specific CYP isozymes, typically those are commonly responsible for the metabolism of drug are studied because inhibitor of CYP mediated metabolism is often the mechanism of drug –drug interaction. These studies provide information on whether a drug may inhibit the bio-transformation of another when two are co-administered. Knowledge of the specific CYP isoform responsible for metabolism of drug is critical for the prediction of the drug –drug interaction and generally based individual variation on drug metabolism. CYP isoform selective substrate have been identified and are being commonly used for probing the role of specific CYP isoforms in drug metabolism. Evaluation of enzyme activity is traditionally performed by using a single CYP isoform at a time, which is labor-intensive, time consuming and cost effective. Attempt has been made for the development for simultaneous

determination of multiple probe substrates for CYP isoform in a single LC-MS/MS run.

2.3 AIM OF THE WORK

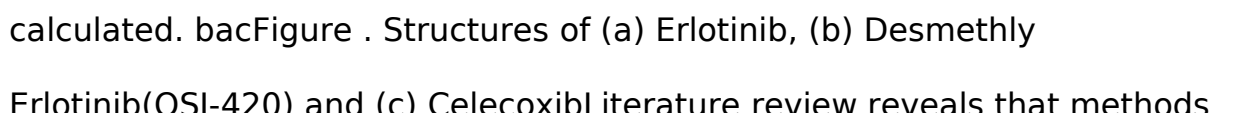
Present research work aims at developing and validating bio analytical method using LC-MS/MS for the selected classes of the drugs from anti-cancer to anti-diabetic & its application to drug-drug interaction/ pharmacokinetics study and simultaneous determination of multi-probe substrate for in-vitro metabolism study.

2.4 OBJECTIVES

Simultaneous determination of celecoxib, erlotinib and its metabolite desmethyl erlotinib (osi-420) in rat plasma by liquid chromatography/tandem mass spectrometry with positive/negative ion-switching electrospray ionization: application for a pharmacokinetic study
Liquid chromatography-tandem mass spectrometry for the simultaneous quantitation of glipizide, cilostazol and its active metabolite 3, 4-dehydro-cilostazol in rat plasma: application for a pharmacokinetic study
Simultaneous determination of methotrexate, dasatinib and its active metabolite N- deshydroxyethyl dasatinib in rat plasma by LC-MS/MS: Method validation and application to pharmacokinetic study
Simultaneous quantitation of IC87114, roflumilast and its active metabolite roflumilast n-oxide in plasma by LC-MS/MS: application for a pharmacokinetic study
Simultaneous determination of multi-probe substrate for in-vitro metabolism study

3. Simultaneous determination of celecoxib, erlotinib and its metabolite desmethyl erlotinib (osi-420) in rat plasma by liquid chromatography/tandem mass spectrometry with positive/negative ion-switching electrospray ionisation

3. 1. Introduction

The epidermal growth factor receptor (EGFR) is recognized as an important molecular target in cancer therapy. Erlotinib (ERT) is an orally active and potent inhibitor of the EGFR tyrosine kinase (TKI) used in lung cancer and several other cancers 19-20. Celecoxib(CCB) is a selective cyclooxygenase-2 (COX-2) inhibitor²¹. COX-2 is an inducible enzyme that is over expressed in pancreatic cancer. Through the conversion of arachidonic acid to prostaglandin, the COX-2 enzyme modulates angiogenesis and metastasis. Current ongoing clinical trial utilizes erlotinib and celecoxib in non small cell lung cancer/ head and neck cancer 22-23. A fast, sensitive and specific LC/MS/MS method for the simultaneous determination of ERT and its active metabolite OSI-420 and CCB in rat plasma is described. After administration of ERT and CCB, blood samples were periodically collected from male wistar rats. The pharmacokinetics parameters of ERT, OSI-420 and CCB were calculated. . Literature review reveals that methods have been reported for analysis of CCB, ERT and OSI-420, HPLC and LC-MS/MS method for determination of CCB, ERT, and OSI-420 in combination with other drugs reported 24-35. To our knowledge, no prior reports have described a LC-MS/MS-based method for simultaneous determination of ERT, OSI-420 & CCB from plasma. Hence, we developed a

reverse phase HPLC method for simultaneous estimation of ERT, OSI-420 & CCB on C18 column using tandem mass spectroscopy detection and validated the method before applying in preclinical experiments. The current study describes a rapid, specific and simple protein precipitation method using LC-MS/MS for the simultaneous determination of CCB and ERT along with its major active metabolite OSI-420 in rat plasma suitable for pharmacokinetic and drug-drug interaction studies. The method was validated using authentic pure standards. This method was successfully applied to the pharmacokinetic study of CCB with ERT in rats after oral administration of CCB and ERT.

3. 2. Materials and Methods

3. 2. 1 Chemical and Reagents

Pure reference standards of celecoxib (CCB), Erlotinib (ERT) and Sitagliptin (SIT) were obtained from Sigma-Aldrich (Germany). Desmethyl erlotinib (OSI-420) was obtained from Selleckchem (USA). Efavirenz (EFV) was obtained from SBC (USA). Acetonitrile (HPLC grade), methanol (HPLC grade) and ammonium acetate (GR-grade) were procured from E Merck (India) Ltd., India. Formic acid was obtained from Sigma-Aldrich (Germany). Ultra pure water of 18 MΩ/cm was obtained from Millipore: Milli-Q purification system (USA).

3. 2. 2 Stock solution, calibration standards and quality control samples

Standard stock solutions of CCB, ERT, OSI-420, SIT and EFV were prepared in methanol with a final concentration of 1 mg/ml. These solutions were stored

at 2-8°C until use. SIT and EFV stock solution were diluted to achieve a final concentration of 1.2 µg/mL and 1.15 µg/mL respectively with the methanol. Analytical standards for CCB, ERT and OSI-420 were prepared in acetonitrile: water (70:30, v/v) over a concentration range of 1.6 ng/mL to 1144.4 ng/mL, 1.8 ng/mL to 1289.2 ng/mL, and 1.5 ng/mL to 1177.2 ng/mL respectively by serial dilution, and same concentration range for calibration curve were prepared in blank normal rat plasma. Quality control (QC) samples at four different concentration levels 1.6, 3.9, 384.5, 915.6 ng/mL for CCB, 1.8, 4.4, 433.2, 1031.4 ng/mL for ERT and 1.5, 3.8, 375.4, 893.8 ng/mL for OSI-420 as LLOQC (lower limit of quantitation QC), low medium and high level of QC respectively) were prepared in three sets independent of the calibration standards. During analysis, low, medium and high QC samples were placed after every sixth position of unknown samples.

3.2.3 Sample preparation

An aliquot of 50 µL of plasma was transferred to a 1 mL eppendorf micro centrifuge tube, 150 µL of internal standard solution (contains final concentration of 1.2 µg/ml and 1.15 µg/ml, SIT and EFV in methanol) were added and the sample was vortex-mixed for 5 min. After centrifuging at 10000 rpm for 5 min at 4°C, 0.1 mL of the supernatant was collected in to HPLC and 10 µL of the same was injected onto the LC-MS/MS system.

3.2.4. Chromatographic condition

A Shimadzu SIL – 20 AC HT (Shimadzu Corporation, JAPAN) consisting of flow control valve, vacuum degasser operated in isocratic mode to deliver the mobile phase at flow rate of 0.8 ml/min. The chromatographic system

consisted of reverse phase C18 column (50mm×4.6mm i. d., 3μ) (YMC®-PACK, JAPAN) and mobile phase consists of 80% v/v solvent A: methanol and 20% v/v solvent B: ammonium acetate buffer, 2mM (pH ~4.0 adjusted with 0.1% formic acid). The samples (10 μL) were injected on to the LC-MS/MS system through an auto injector. The auto sampler temperature was kept at 10°C and the column oven was maintained at 40°C.

3. 2. 5. Mass spectrometric condition

Mass spectrometric detection was performed on Thermo Scientific - Finnigan TSQ Quantum Ultra tandem mass spectrometer equipped with a Heated Electron Spray Ionization (HESI) source (San Jose, CA, USA), Selective Reaction Monitoring (SRM) mode was used for data acquisition with Xcalibur 1.2 software (Thermo-Scientific, San Jose, CA, USA). Peak integration and calibration were carried out by using LC Quan 2.5.2 software (Thermo-Scientific). MS and MS/MS condition for pure standards of CCB, ERT, OSI-420, SIT and EFV were optimized by continuous infusion at 5μl/min using inbuilt syringe pump. The transitions monitored were m/z -380.1 > -316.3, 394.5 > 278.1, 380.3 > 278.1, and 408.1 > 127.0, -313.8 > -243.9 for components CCB, ERT, OSI-420, SIT and EFV, respectively. All analyses were carried out in positive and negative ion HESI, for positive polarity, spray voltage set at 3.5 kV, heated capillary temperature was set 150°C. Nitrogen sheath gas and auxiliary gas were set at 40, 30 KPa. For negative polarity, spray voltage set at 3.0 KV, heated capillary temperature was set 300°C. Nitrogen sheath gas and auxiliary gas were set at 30, 40 KPa and capillary offset was set at -35. The argon gas collision induced dissociation was used

with a pressure of 1.5 m Torr and the energy selected to be 2100 eV. Total run time for an LC-MS/MS analysis was 5.0 min.

3.2.6. Assay Validation

Specificity was assessed by analysis of six different samples of blank matrix with and without spiking with CCB, ERT, OSI-420 and IS. Calibration curves were constructed from working standard solutions of CCB, ERT and OSI-420 at concentration range 1.5–1150 ng/mL by plotting peak area ratio (y) of analyte(s) to the internal standard, versus analyte concentration (x).

Linearity was assessed by weighted ($1/x^2$) linear regression of calibration curves generated in triplicate on three consecutive days using analyte internal standard peak area ratios. Quality control samples (around 1.5, 4, 400 and 900 ng/mL) were prepared to evaluate the accuracy, precision, recovery, stability, and matrix effect of the assay. Accuracy (expressed as percent nominal, SD) and between and within-day precision (expressed as percent co-efficient of variation- %CV) were assessed by assay of six replicate QC samples on three different days. The limit of quantification (LOQ) was defined as the lowest concentration in the calibration curve that can be determined with accuracy and precision of no more than 80-120%, $\pm 20\%$ respectively. The limit of detection (LOD) was defined as a signal to noise ratio of 3: 1. The extraction recovery for the analytes and IS were determined by assaying two sets of samples: plasma extracts spiked with analytes and IS after extraction (set 1), and plasma spiked with analytes and IS before extraction (set 2). CCB, ERT and OSI-420 of each batch were prepared at levels of 4, 400 and 900 ng/mL. The percent extraction

recoveries of CCB, ERT, OSI-420 and IS were calculated as the percent ratio of set 2 peak area to set 1 peak area. Matrix effect was evaluated to verify whether potential ion suppression or enhancement due to the co-elution matrix components existed in the analysis. The peak areas of analytes and the internal standard from the spike-after protein precipitation samples were compared to those of the standard solutions in the mobile phase at the same concentrations. This experiment was carried out with blank plasma samples from six different rats at low and high QC concentrations of CCB, ERT and OSI-420. Potential sample carry-over was tested by analyzing the upper limit of quantitation (ULOQ, 1150 ng/mL) calibrator of CCB, ERT and OSI-420 of respective samples followed by blank samples. Stability experiments were performed to evaluate the analyte stability in stocks solutions and in plasma samples under different conditions. Stock solution stability was performed by comparing area response of stability sample of analyte with the area response of sample prepared from fresh stock solutions. To meet the acceptance criteria, percent change should be within $\pm 10\%$ when compare to fresh stock solution. Bench-top stability, long-term stability, freeze-thaw cycles stability and auto-sampler stability were performed at 4 ng/mL and 900 ng/mL of QC levels using six replicates at each level. To meet the acceptance criteria, the percent nominal should be within $\pm 15\%$ of their respective nominal concentrations. The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which should be encountered during real samples analysis. A set of plasma samples was prepared containing CCB, ERT & OSI-420 at a concentration of 4500, 5100 & 4500

ng/mL respectively, and placed in a -70°C freezer overnight prior to analysis. After thawing, certain aliquot was diluted either or 4 & 8 times with wistar rat plasma and analyzed respectively. The results of this experiment indicated that the dilution integrity of all the plasma samples was found to be less than 15% of their respective nominal concentrations.

3.3 Application of method in pharmacokinetic study

Healthy male Wistar rats weighing 200 ± 30 g were obtained from Mahaveera Enterprises, Hyderabad and housed at Incozen Therapeutics Pvt Ltd, Hyderabad in appropriate cages. They were maintained in standard laboratory conditions with regular 12 h day-night cycle in well-ventilated room with an average temperature of 24–27°C and relative humidity of 40–60%. Standard pelleted laboratory chow diet (Provimi Animal Nutrition India Private Limited, Bengaluru, India) and water was allowed ad libitum to rats. All applicable national and international ethical guidelines for maintenance and experimental studies with Wistar rats were followed. The method was successfully applied to generate the plasma concentration versus time profile of test drugs (CCB and ERT) as well as to detect its active metabolite (OSI-420) in plasma following simultaneous oral administration at 20mg/kg dose of ERT and 10 mg/kg dose of CCB in six male wistar rats. Oral formulations were prepared in suspension form by triturating accurately weighed amount of powdered compound in methyl cellulose solution (0.5%, w/v water) in gravimetric dilution pattern. Oral doses of ERT and CCB (20, 10mg/kg,) were administered using an oral gavage at 5 ml/kg volume in rats after overnight fast (12 hr) and fasting restriction was continued till 4 hr post

dose. The blood samples (0.15 ml) were collected from retro orbital sinus at predose, 10, 15, 30 min and 1, 2, 4, 6, 8, 12 and 24 hrs post dose in to K2-EDTA(di potassium ethylene diamine tetra acetic acid) tubes and were kept on ice bath till further processing. These samples were separated for plasma by centrifugation at 4°C for 10 min at 3000 rpm and then stored at -70°C till further analysis. These samples were simultaneously estimated for the levels of CCB, ERT and its active metabolite OSI-420. Pharmacokinetic parameters, including the area under the concentration–time curve (AUC), maximum plasma concentration (C_{max}) and time to reach the maximum concentration (T_{max}), were estimated by means of a non-compartmental analysis using Phoenix WinNonlin (Pharsight Inc., USA, version 6.1). Statistical parameters like mean, standard deviation and C. V were calculated by using MS-Excel 2007 (Microsoft®). Incurred sample reanalysis (ISR) was performed to reconfirm the initial values and to demonstrate that the assay was reproducible. In the study, ISR was performed on 18 plasma samples from six different rats at T_{max} and the second time point covering the phase of elimination.

3. 4 Results and discussion

3. 4. 1 Mass spectrometry

In order to find most sensitive ionization mode for the components studied, ESI positive ion mode and ESI negative ion mode were tested with various combination of mobile phase, i. e. acetonitrile and water/ammonium acetate buffer (2 mM)/formic acid (0.1%) in positive and negative ionization mode. It was observed that the signal intensity for [M + H]⁺ ions in ESI positive ion

mode were 2–10-fold higher for ERT, OSI and SIT in analyses using acetonitrile: ammonium acetate buffer (2 mM), versus experiments run with ESI negative ion mode. The protonated molecular ion of $[M + H]^+$, m/z 446.4, 370.2, 368.3 and 491.4 amu were obtained for ERT, OSI-420 and SIT & $[M - H]^-$, m/z -380.1 and -313 amu were obtained for CCT and EFV, respectively. No significant solvent adduct ions or fragment ions were observed in the full scan spectra of all the compounds. Thus, it was decided to utilize positive ion mode & negative ion switching mode for detection and quantitation, which on fragmentation gave prominent and stable product ions. The optimized tube-lens potentials for the protonated $[M+H]^+$ of component ERT, OSI-420 and SIT were found to be 95, 67, and 122 eV respectively, negatively charged $[M-H]^-$ of component CCT, and EFV were found to be 110 and 76 eV respectively. Product ions selected for final mass transition are given in Fig. 2.

3. 4. 2. Liquid chromatography

Acetonitrile rather than methanol was chosen as the organic modifier because of analytes better peak shape. Moderately high acidic ammonium acetate buffer 2 mM, pH~4, was required to achieve acceptable peak width and shapes. A reverse phase C18 column (50mm×4.6mm i. d., 3 μ) (YMC-PACK®, JAPAN) with methanol: ammonium acetate buffer at flow rate of 0.8 mL/min in isocratic mode was applied in final LC method. With the total analysis time of 5.0min, all components were eluted between in 1.2–4.2min. Representative chromatograms of blank and LOQ level are shown in Fig. 3.

3. 4. 3. Optimization of LC–MS/MS condition

Final SRM transitions were selected on the basis of signal to noise ratio (S/N) with on-column injection analysis. Nitrogen sheath gas, auxiliary gas, argon gas collision induced dissociation, ion spray voltage and capillary temperature were set to 40, 30 KPa, 1. 5m Torr, 3500 eV and 300°C, respectively. The transitions selected were m/z -380. 1 > - 316. 3, 394. 5 > 278. 1, 380. 3 > 278. 1, and 408. 1 > 127. 0, -313. 8>-243. 9 for components CCB, ERT, OSI-420, SIT and EFV, respectively.

3. 5 Method validation

Accuracy, precision, selectivity, sensitivity, linearity and stability were measured and used as the parameter to assess the assay performance. LC–MS/MS analysis of the blank plasma samples showed no interference with the quantification of components CCB, ERT, OSI-420 and internal standards. The specificity of the method was established with pooled and individual plasma samples from six different sources. The retention times of all the analytes and the IS showed less variability with a percent co-efficient of variance (% C. V) well within acceptable limits of 5%.

3. 5. 1 Limit of detection (LOD) and quantification (LOQ)

Two criteria were used to define LOQ, i. e., (1) the analytical response at LOQ must be five times the baseline noise and (2) the analytical response at LOQ can be detected with sufficient accuracy (80-120%) and precision (20%).

LOD is defined as the lowest concentration of the analyte at which the signal is larger than three times the baseline noise. The measured LOQ and LOD values were 20 and 5 arbitrary units for all three analytes. LOQ was set at 1.

5 ng/mL. These results well met the requirements of quantifications of all analytes in plasma.

3. 5. 2 Linearity

The peak area ratios of analytes to IS in rat plasma were linear over the concentration range 1. 6-1144. 4 ng/mL for CCB and 1. 8-1289. 2 ng/mL for ERT and 1. 5- 1117. 2 ng/mL for component OSI-420. The calibration model was selected based on the analysis of the data by linear regression with and without intercepts ($y = mx + c$ and $y = mx$) and weighting factors ($1/x$, $1/x^2$ and $1/\log x$). The best fit for the calibration curve could be achieved by a linear equation of $y = mx + c$ and a $1/x^2$ weighting factor for all components. Mean slope for CCB, ERT and OSI420 were found to be 0. 00026, 0. 07124 and 0. 00889 respectively. Mean intercept CCB, ERT and OSI420 were found to be -0. 00208, 0. 01945 and -0. 00522. The correlation coefficients (R^2) for all components were above 0. 99 over the concentration range used.

3. 5. 3. Precision and accuracy

The within day precision (expressed by coefficient of variation of replicate analyses) was estimated on the four quality control levels and the within batch precision on the nine calibration standard levels. Table 1 shows the results obtained for the within batch and between batch precision for CCB, ERT and OSI-420. The precision for all these analytes under investigation were not exceeded 15% at any of the concentrations studied and well met the requirements of validation.

CCBERT

OSI-420EFV

SIT

Figure . Fragmentation pattern and product ion spectra of CCB, ERT, OSI-420, EFV and SIT.

AB

CD

E

Figure . Representative chromatograms for (A) CCB, (B) ERT, (C) OSI-420, (D) SIT and (E)EFV in the extracted blank plasma and extracted LOQ rat

plasmaTable . Summary of precision and accuracy from QC samples in wistar rat plasma

Drug**Spiked concentration****(ng/mL)****Within batch (n= 6)****Between batch (n= 3)****Measured concentration (ng/ml)****(mean ± SD)****% Accuracy****%****Measured concentration (ng/ml)****(mean ± SD)****%****Accuracy****% C. V****% C. V**

CCB1. 61. 64±0. 125102. 27. 61. 62±0. 139101. 08. 63. 93. 91±0. 263100.

26. 73. 95 ± 0. 271101. 36. 9384. 5405. 07±21. 351105. 35. 3399. 93 ± 20.

331104. 05. 1915. 5916. 14±56. 283100. 16. 1921. 38 ± 61. 516100. 66.

7ERT1. 81. 81± 0. 179100. 39. 91. 85±0. 151102. 98. 24. 44. 29± 0. 28597.

46. 74. 45±0. 394101. 18. 9433. 2403. 37± 31. 34793. 17. 8432. 34±41.

37999. 89. 61031. 41058. 52± 44. 444102. 64. 21010. 71±54. 71298. 05.

41. 51. 48±0. 14996. 210. 01. 5±0. 13897. 59. 2OSI-4203. 83. 84±0.

308100. 08. 04. 02±0. 231104. 75. 7375. 4387. 43±17. 18103. 24. 4389.

71±17. 606103. 84. 5893. 8930. 88±26. 121104. 12. 8917. 36±41. 916102.
64. 6

3. 5. 4. Recovery

The recovery of CCB, ERT and OSI-420 from plasma was estimated at their respective low, medium and high QC levels. Plasma samples (in six replicates) containing all analytes at QC concentration level was also spiked with respective internal standards. The absolute recoveries ranged from 73.7 to 78.7, 88.7 to 90.3% and 92.2 to 94.4% for CCB, ERT and OSI-420 respectively. The results are indicated in Table 2. Table : Extraction recovery in rat plasma (n= 6)

Drug

Concentration (ng/ml)

Recovery (%)

% C. V

CCB3. 973. 79. 3384. 572. 79. 4915. 578. 78. 5ERT4. 488. 77. 4433. 290. 36.
51031. 486. 36. 4OSI-4203. 893. 33. 4375. 494. 42. 3893. 892. 23. 3

3. 5. 6. Stability

QC samples were subjected to short term and long term storage condition (–70°C), freeze-thaw stability, auto-sampler stability and bench top stability studies. All stability studies were carried out at two concentration levels (low and high QC) in six replicates. The bench top stability was studied for low and high QC samples kept at room temperature (25°C) for 6 hours. Freeze-thaw stability of low and high QC samples was evaluated after 3 freeze thaw

cycles. The autosampler stability was studied for low and high QC samples stored at autosampler at 10°C for 24 hour. The freezer storage stability of the drug in plasma was determined by comparing the low and high QC samples stored for 30 days at -70°C. The results indicated that each analyte had an acceptable stability under those conditions, as shown in Table 3.

3. 5. 7 Application to pharmacokinetic study

The method described above was successfully applied to a PK drug-drug interaction study in which plasma concentration of pure markers was determined for up to 24 h after simultaneous oral administration at 20 mg/kg dose of ERT and 10 mg/kg dose of CCB in male wistar rats. The plasma concentration time profiles of CCB, ERT & its active metabolite OSI-420 are shown in Fig. 4, and could be traceable up to 24 h, 12h and 12h respectively. The pharmacokinetic parameters of CCB, ERT and OSI-420 are presented in Table 4. During ISR it was observed that all of the samples were within $\pm 20\%$ of initial concentration value, further demonstrating that this method is capable of producing reproducible results over time. Table . Stability of analytes in rat plasma (n= 6)

Drug**Nominal****Sample condition****concentration****Bench top stability a****Autosampler stability****Freeze-thaw stability c****30 days****storage stability d**

(ng/ml)% Accuracy% CV% Accuracy% CV% Accuracy% CV% Accuracy%

CVERT4. 490. 27. 41015. 5106. 34. 5100. 67. 51031. 498. 66. 4100. 56.

7100. 69. 2112. 75. 50SI-4203. 8101. 79. 4107. 95. 4112. 84. 4948. 4893.

8103. 64. 3103. 29. 7109. 76. 593. 26. 6CCB3. 996. 87. 299. 12. 397. 54.

41054. 4915. 599. 26. 698. 63. 51004. 695. 17. 6a Exposed at ambient

temperature (25°C) for 6h, b Kept at autosampler temperature (10°C) for

24h, c After three freeze-thaw cycles, d Stored at -70°C Table .

Pharmacokinetic parameters (Mean \pm S. D.) after single dose oral

administration of CCB and ERT simultaneously in wistar rats

Parameters**Units****CCB****ERT****OSI-420****(metabolite)**

C max $\mu\text{g/ml}$ 1. 91 \pm 0. 22. 72 \pm 0. 230. 48 \pm 0. 07 AUC 0-24 $\mu\text{g. h/ml}$ 13. 72 \pm 0. 67 27. 2 \pm 3. 125. 57 \pm 0. 54 AUC 0-inf $\mu\text{g. h/ml}$ 14. 94 \pm 0. 92 31. 78 \pm 6. 447. 48 \pm 1. 28 t 1/2 h 6. 19 \pm 0. 97. 94 \pm 2. 15 11. 96 \pm 2. 01 T max h 1. 08 \pm 0. 492. 17 \pm 0. 98 2 \pm 1. 1 K elh -10. 11 \pm 0. 020. 09 \pm 0. 020. 06 \pm 0. 01

Figure . Mean plasma concentration vs. time after single dose oral administration of CCB and ERT in six wistar rats.

3. 6 Conclusion

An LC-MS/MS bioanalytical method for simultaneous determination of three analytes, CCB, ERT and OSI-420 was developed and validated in rat plasma. The method was good enough to detect low concentration of 1. 5 ng/mL for all these analytes in 50 μL rat plasma and further can be improved by increasing the plasma volume. Analytes recovery from spiked control samples were > 73%, using convenient and fast protein precipitation method. Intra- and inter-day accuracy and precision of the validated method were within the acceptable limits of <15% and 85 to 115% respectively at low and <10% at other concentrations. The method was successfully applied to generate stability profile as well as PK evaluation of simultaneous administration of CCB, ERT in rat following oral administration.

4. Liquid chromatography–tandem mass spectrometry for the simultaneous quantitation of glipizide, cilostazol and its active metabolite 3, 4-dehydro-cilostazol in rat plasma: application for a pharmacokinetic study

4. 1. Introduction

Therapeutic drug management of antidiabetic agents (ADA) and antiplatelet drugs is an aid in the clinical management of diabetic patients with peripheral arterial diseases (PAD). Monitoring these drugs is desirable for adjusting doses, to provide safe, effective, avoiding side effects and assessing patient compliance. Among ADAs, Glipizide (CAS 29094-61-9), a second generation sulphonyl urea derivate, stimulates the release of insulin from beta cells of the pancreatic islet tissues. 36. It increases pancreatic insulin secretion by competitively binding to sulphonyl urea receptors (SUR), thereby inhibiting KATP channels and thus reducing blood glucose levels in diabetic patients 37-38. The insulinotropic response to a meal occurs within 30 minutes after an oral dose of glipizide (GLZ) in diabetic patients, but elevated insulin levels do not persist beyond the time of the meal challenge. Extra pancreatic effects may play a part in the mechanism of action of sulphonyl ureas³⁹⁻⁴⁰. It is metabolized predominantly by CYP2C9 to its hydroxy metabolites and defective CYP2C9*3 allele making the exposure of glipizide drastically lower than other subjects⁴¹⁻⁴². Cilostazol (CAS 73963-72-1) is the most often used drug for anti-platelet therapy in diabetics along with other ADAs. Cilostazol (CLZ), a selective PDE-III enzyme inhibitor, showed antithrombotic, vasodilator, antimitogenic and cardiotoxic properties used for the treatment of intermittent claudication in PAD⁴³⁻⁴⁴. CYP3A4 and

CYP2C19 are the major enzymes involved in metabolism of CLZ that were found during the in vitro metabolism studies using human liver microsomes⁴⁵. The major active in vivo metabolite found in rat and human after administration of CLZ was 3, 4-dehydro cilostazol (DCLZ) and the minor was 4'-trans-hydroxy-cilostazol along with the other inactive metabolites ⁴⁶.

(b)(c)Figure . Structures of (a) Glipizide, (b) Cilostazol and (c) 3, 4 dehydro cilostazol

The possibilities of drug-drug interactions are more common in diabetics since they are commonly prescribed with anti-platelet agents along with ADAs. The prescriptions of using GLZ and CLZ in combination are expected in treatment of diabetics and there is no such information of drug-drug interaction effect available on these two drugs. During literature survey, we found several publications quoting the methods for the determination of either individual or simultaneous estimation of multiple analytes in biological fluids by reverse phase chromatography ⁴⁶⁻⁵⁸. Individual methods for the estimation of these analytes published so far have shown long analysis time, sensitivity issues and complex extraction procedures. These may impact on analysis time, column life and also significant consumption of reagents. To our knowledge, no prior reports have described a LC-MS/MS-based method for simultaneous determination of GLZ, CLZ & DCLZ from plasma. Hence, we developed a reverse phase HPLC method for simultaneous estimation of GLZ, CLZ and DCLZ on C18 column using tandem mass spectroscopy detection and validated the method before applying in preclinical experiments. The current study describes a rapid, specific and simple liquid-liquid extraction method using LC-MS/MS for the simultaneous determination of GLZ and CLZ along with its major active metabolite DCLZ in rat plasma

suitable for pharmacokinetic and drug-drug interaction studies. The method was validated using authentic pure standards. This method was successfully applied to the pharmacokinetic and drug-drug interaction study of GLZ with CLZ in rats after oral administration of GLZ and CLZ.

4. 2 Materials and Methods

4. 2. 1 Chemical and Reagents

Pure reference standards of cilostazol (CLZ), glipizide (GLZ) and glimepiride (CAS 93479-97-1) used as internal standard were obtained from Sigma-Aldrich (Germany). 3, 4-dehydro-cilostazol (DCLZ) was synthesized at Incozen Therapeutics Pvt Ltd. Hyderabad. Acetonitrile (HPLC grade), methyl t-butyl ether (TBME), methanol (HPLC grade) and Ammonium acetate (GR-grade) were procured from E Merck (India) Ltd., India. Formic acid was obtained from Sigma Aldrich, Germany. Ultra pure water of 18 MΩ/cm was obtained from Milli-Q purification system (Millipore, USA).

4. 2. 3 Stock solution, calibration standards and quality control samples

Standard stock solutions of GLZ, CLZ, DCLZ and IS were prepared in methanol with a final concentration of 1 mg/ml. These solutions were stored at 2-8°C until use. The IS stock solution was diluted to achieve a final concentration of 2.5 µg/ml with the diluent (acetonitrile: water, 70: 30 v/v) solution. Analytical standards for GLZ, CLZ and DCLZ were prepared in acetonitrile: water (70: 30, v/v) over a concentration range of 25.4 ng/mL to 2080.0 ng/mL, 24.3 ng/mL to 1980.0 ng/mL and 22.9 ng/mL to 1870.0 ng/mL, respectively by serial dilution, and same concentration range for

calibration curve were prepared in blank normal rat plasma. Quality control (QC) samples at four different concentration levels (25.4, 51.5, 823.2, 1646.4 ng/mL for GLZ, 24.3, 50.9, 814.2, 1628.5 ng/mL for CLZ and 22.9, 52.5, 840.0, 1680.0 ng/mL for DCLZ as LLOQC (lower limit of quantitation QC), low medium and high level of QC respectively) were prepared in three sets independent of the calibration standards. During analysis, low, medium and high QC samples were placed after every seventh position of unknown samples.

4.2.3. Sample preparation

An aliquot of 50 μ L of plasma was transferred to a 2 mL eppendorf micro centrifuge tube, 20 μ L of IS (final concentration of 90.4 ng/mL) and 50 μ L of 0.1 N HCl, 1.5 mL of TBME were added and the sample was vortex-mixed for 5 min. After centrifuging at 10000 rpm for 5 min at 4°C, the supernatant was collected and dried under nitrogen stream. (Nitrogen Evaporator, Caliper Instruments, USA). The residue was reconstituted in 100 μ L of mobile phase and 5 μ L of the same was injected onto the LC-MS/MS system.

4.2.4. Chromatographic condition

A Shimadzu SIL – 20 AC HT (Shimadzu Corporation, Japan) consisting of flow control valve, vacuum degasser operated in isocratic mode to deliver the mobile phase at flow rate of 0.4 ml/min. The chromatographic system consisted of reverse phase C18 column (50mm \times 4.6mm i. d., 5 μ) (Hypurity®, Thermo Electron, USA) and mobile phase consists of 90% v/v solvent A: acetonitrile and 10% v/v solvent B: ammonium acetate buffer, 2 mM (pH ~3.2 adjusted with 0.1% formic acid). The samples (5 μ L) were

injected on to the LC-MS/MS system through an auto injector. The auto sampler temperature was kept at 10°C and the column oven was maintained at 40°C.

4. 2. 5. Mass spectrometric condition

Mass spectrometric detection was performed on Thermo Scientific - Finnigan TSQ Quantum Ultra tandem mass spectrometer equipped with a Heated Electron Spray Ionization (HESI) source (San Jose, CA, USA), a Selected Reaction Monitoring (SRM) mode was used for data acquisition with Xcalibur 1. 2 software(Thermo- Scientific, San Jose, CA, USA). Peak integration and calibration were carried out by using LC Quan 2. 5. 2 software (Thermo-Scientific). MS and MS/MS condition for pure standards of GLZ, CLZ, DCLZ and IS were optimized by continuous infusion at 5µl/min using inbuilt syringe pump. The transitions monitored were m/z 446. 4 > 321. 1, 370. 2 > 288. 3, 368. 3 > 286. 2, and 491. 4 > 352. 2 for components GLZ, CLZ, DCLZ and IS, respectively. All analyses were carried out in positive ion HESI with spray voltage set at 2. 5kV. The heated capillary temperature was set 300°C. Nitrogen sheath gas and auxiliary gas were set at 30, 40 kPa. The argon gas collision induced dissociation was used with a pressure of 1. 5 m Torr and the energy selected to be 2100 eV. Total run time for an LC-MS/MS analysis was 3. 5 min.

4. 2. 6. Assay Validation

Specificity was assessed by analysis of six different samples of blank matrix with and without spiking with GLZ, CLZ, DCLZ and IS. Calibration curves were constructed from working standard solutions of GLZ, CLZ and DCLZ at

concentration range 25–2000 ng/mL by plotting peak area ratio (y) of analyte(s) to the internal standard, versus analyte concentration (x). Linearity was assessed by weighted ($1/x^2$) linear regression of calibration curves generated in triplicate on three consecutive days using analyte internal standard peak area ratios. Quality control samples (around 25, 50, 800 and 1600 ng/mL) were prepared to evaluate the accuracy, precision, recovery, stability, and matrix effect of the assay. Accuracy (expressed as percent nominal, SD) and intra- and inter-day precision (expressed as percent coefficient of variation- CV) were assessed by assay of six replicate QC samples on three different days. The precision of IS was calculated from the mean and standard deviation of the internal standard peak areas in QC samples. The lower limit of quantification (LLOQ) was defined as the lowest concentration in the calibration curve that can be determined with accuracy and precision of no more than 80-120%, $\pm 20\%$ respectively. The limit of detection (LOD) was defined as a signal to noise ratio of 3: 1. The extraction recovery for the analytes and IS were determined by assaying two sets of samples: plasma extracts spiked with analytes and IS after extraction (set 1), and plasma spiked with analytes and IS before extraction (set 2). GLZ, CLZ and DCLZ of each batch were prepared at levels of 50, 800 and 1600 ng/mL. The percent extraction recoveries of GLZ, CLZ, DCLZ and IS were calculated as the percent ratio of set 2 peak area to set 1 peak area. Matrix effect was evaluated to verify whether potential ion suppression or enhancement due to the co-elution matrix components existed in the analysis. The peak areas of analytes and the IS from the spike-after LLE samples were compared to those of the standard solutions in the mobile phase at the same

concentrations. This experiment was carried out with blank plasma samples from six different rats at low and high QC concentrations of GLZ, CLZ and DCLZ. Potential sample carry-over was tested by analyzing the upper limit of quantitation (ULOQ, 2000ng/mL) calibrator of GLZ, CLZ and DCLZ of respective samples followed by blank samples. Stability experiments were performed to evaluate the analyte stability in stocks solutions and in plasma samples under different conditions. Stock solution stability was performed by comparing area response of stability sample of analyte with the area response of sample prepared from fresh stock solutions. To meet the acceptance criteria, percent change should be within $\pm 10\%$ when compare to fresh stock solution. Bench-top stability, long-term stability, freeze-thaw cycles stability and auto-sampler stability were performed at 50 ng/mL and 1600ng/mL of QC levels using six replicates at each level. To meet the acceptance criteria, the percent nominal should be within $\pm 15\%$ of their respective nominal concentrations. The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which should be encountered during real samples analysis. A set of plasma samples was prepared containing GLZ, CLZ & DCLZ at a concentration of 6240, 5940 & 5610 ng/mL respectively, and placed in a -70°C freezer overnight prior to analysis. After thawing, certain aliquot was diluted either or 4 & 8 times with wistar rat plasma and analyzed respectively. The results of this experiment indicated that the dilution integrity of all the plasma samples was found to be less than 15% of their respective nominal concentrations.

4. 2. 7. Application of method in pharmacokinetic study

Healthy male Wistar rats weighing 200 ± 30 g were obtained from Mahaveera Enterprises, Hyderabad and housed at Incozen Therapeutics Pvt Ltd, Hyderabad in appropriate cages. They were maintained in standard laboratory conditions with regular 12 h day-night cycle in well-ventilated room with an average temperature of 24–27°C and relative humidity of 40–60%. Standard pelleted laboratory chow diet (Provomi Animal Nutrition India Private Limited, Bengaluru, India) and water was allowed ad libitum to rats. All ethical guidelines for maintenance and experimental studies with Wistar rats were followed. The method was successfully applied to generate the plasma concentration versus time profile of test drugs (GLZ and CLZ) as well as to detect its active metabolite (DCLZ) in plasma following simultaneous oral administration at 1mg/kg dose of GLZ and 10 mg/kg dose of CLZ in six male wistar rats. Oral formulations were prepared in suspension form by triturating accurately weighed amount of powdered compound in methyl cellulose solution (0.5%, w/v water) in gravimetric dilution pattern. Oral doses of GLZ and CLZ (1, 10mg/kg,) were administered using an oral gavage at 5 ml/kg volume in rats after overnight fast (12 hr) and fasting restriction was continued till 4 hr post dose. The blood samples (0.15 ml) were collected from retro orbital sinus at predose, 10, 15, 30 min and 1, 2, 3, 4, 6, 8, 12 and 24 hrs post dose in to K2-EDTA(di potassium ethylene diamine tetra acetic acid) tubes and were kept on ice bath till further processing. These samples were separated for plasma by centrifugation at 4°C for 10 min at 3000 rpm and then stored at -70°C till further analysis. These samples were simultaneously estimated for the levels of GLZ, CLZ and its

active metabolite DCLZ. Pharmacokinetic parameters, including the area under the concentration–time curve (AUC), maximum plasma concentration (C_{max}) and time to reach the maximum concentration (T_{max}), were estimated by means of a non-compartmental analysis using Phoenix WinNonlin (Pharsight Inc., USA, version 6. 1). Statistical parameters like mean, standard deviation and C. V were calculated by using MS-Excel 2007 (Microsoft®). Incurred sample reanalysis (ISR) was performed to reconfirm the initial values and to demonstrate that the assay was reproducible. In the study, ISR was performed on 18 plasma samples from six different rats at T_{max} and the second time point covering the phase of elimination.

4. 3. Results and discussion

4. 3. 1 Mass spectrometry

In order to find most sensitive ionization mode for the components studied, ESI positive ion mode and ESI negative ion mode were tested with various combination of mobile phase, i. e. acetonitrile and water/ammonium acetate buffer (2 mM)/formic acid (0. 1%) in positive and negative ionization mode. It was observed that the signal intensity for [M + H]⁺ ions in ESI positive ion mode were 2–10-fold higher for all components in analyses using acetonitrile: ammonium acetate buffer (2 mM), versus experiments run with ESI negative ion mode. The protonated molecular ion of [M + H]⁺, m/z 446. 4, 370. 2, 368. 3 and 491. 4 amu were obtained for GLZ, CLZ, DCLZ and IS, respectively. No significant solvent adduct ions or fragment ions were observed in the full scan spectra of all the compounds. Thus, it was decided to utilize positive ion mode for detection and quantitation, which on

fragmentation gave prominent and stable product ions. The optimized tube-lens potentials for the protonated $[M+H]^+$ of component GLZ, CLZ, DCLZ and I. S., were found to be 140, 122, 139 and 130 eV respectively.

4. 3. 2. Liquid chromatography

Acetonitrile rather than methanol was chosen as the organic modifier because of its better peak shape. Moderately high acidic ammonium acetate buffer 2 mM, pH~3.2, was required to achieve acceptable peak width and shapes. A reverse phase C18 column (50mm×4.6mm i. d., 5 μ) (Hypurity®, Thermo Electron, USA) with acetonitrile: ammonium acetate buffer in gradient mode was applied in final LC method. Within the total analysis time of 3.5 min, all components were eluted in 2.0–2.3 min. A column with lesser particle size (Inertsil ODS-3, 100mm×4.6mm i. d., particle size 3 μ , GL sciences, Japan) was tested in the development stage with the solvent flow rate decreased to 0.3 mL/min. Retention was delayed for component GLZ. Additionally, the analysis time expanded to 5.0 min.

4. 3. 3. Optimization of LC–MS/MS condition

Final SRM transitions were selected on the basis of signal to noise ratio (S/N) with on-column injection analysis. Nitrogen sheath gas, auxiliary gas, argon gas collision induced dissociation, ion spray voltage and temperature were set to 30, 40 KPa, 1.5m Torr, 2100 eV and 400°C, respectively. The transitions selected were m/z 446.4 > 321.1, 370.2 > 288.3, 368.3 > 286.2, and 491.4 > 352.2 for components GLZ, CLZ, DCLZ and IS respectively. The fragment ions selected for final SRM method are given in Fig. 1. The selection of glimipiride, a sulphonyl urea derivative as IS was based on its

structural similarity with GLZ. Hence, glimipiride was expected to behave closely in terms of ionization giving better results for linearity and quantitation. (A) GLZ (B) CLZ (C) DCLZ (D) IS

Figure . Chemical structures and parent/product ion spectra of $[M + H]^+$ of (A) GLZ (B) CLZ (C) DCLZ and (D) IS

4.3.4. Sample extraction

The next step was to develop simple and efficient sample clean up devoid of matrix effect and interference from endogenous plasma components for estimation of the analytes in rat plasma. Hence, precipitation method was tried initially with acetonitrile (1.5 mL) which showed ion enhancement for all the analytes. Further liquid-liquid extraction (LLE) using ether and different combinations of hexane and ethyl acetate (85-15%, v/v), n-hexane and isopropyl alcohol (2-5%, v/v) was tried but none of these was found suitable to give good and consistent recovery for all analytes. Finally, LLE using TBME was tried and found suitable to give optimal recovery for all analytes.

4.4. Method validation

Accuracy, precision, selectivity, sensitivity, linearity and stability were measured and used as the parameter to assess the assay performance. LC-MS/MS analysis of the blank plasma samples showed no interference with the quantification of components GLZ, CLZ, DCLZ and the IS. The specificity of the method was established with pooled and individual plasma samples from six different sources. Representative chromatograms of extracted blank rat plasma and blank plasma fortified with analytical standards and IS,

demonstrating the specificity and selectivity of the method are shown in Fig. 2. The retention times of all the analytes and the IS showed less variability with a relative standard deviation (R. S. D.) well within acceptable limits of 5%. (A) GLZ (B) CLZ (C) DCLZ (D) IS

Figure . Representative Chromatograms for (A) GLZ, (B) CLZ, (C) DCLZ and (D) IS in the extracted drug-free rat blank plasma and extracted LOQ rat plasma.

4. 4. 1. Limit of detection (LOD) and quantification (LOQ)

Two criteria were used to define LOQ, i. e., (1) the analytical response at LOQ must be five times the baseline noise and (2) the analytical response at LOQ can be detected with sufficient accuracy (80-120%) and precision (20%).

LOD is defined as the lowest concentration of the analyte at which the signal is larger than three times the baseline noise. The measured LOQ and LOD values were 20 and 5 arbitrary units for all three analytes. The limit of quantification (LOQ) was set at 25 ng/mL. These results well met the requirements of quantifications of all analytes in plasma.

4. 4. 2 Linearity

The peak area ratios of analytes to IS in rat plasma were linear over the concentration range 25. 4–2080. 0 ng/mL for GLZ and 24. 3–1980. 0 ng/mL for CLZ and 22. 9- 1870. 0 ng/mL arbitrary unit for component DCLZ. The calibration model was selected based on the analysis of the data by linear regression with and without intercepts ($y = mx + c$ and $y = mx$) and weighting factors ($1/x$, $1/x^2$ and $1/\log x$). The best fit for the calibration curve could be achieved by a linear equation of $y = mx + c$ and a $1/x^2$ weighting

factor for all components. The correlation coefficients (R) for all components were above 0.993 over the concentration range used.

4.4.3. Precision and accuracy

The intra-day precision (expressed by coefficient of variation of replicate analyses) was estimated on the four quality control levels and the inter-day precision on the nine calibration standard levels. Table 1 shows the results obtained for the intra-assay (variation intra-day) and inter-assay (variation inter-day) precision for GLZ, CLZ and DCLZ. The precision for all these analytes under investigation were not exceeded 15% at any of the concentrations studied and well met the requirements of validation.

4.4.4. Recovery

The recovery of GLZ, CLZ and DCLZ from plasma was estimated at their respective low, medium and high QC levels. Plasma samples (in six replicates) containing all analytes at QC concentration level was also spiked with IS at the working concentration of 90.4 ng/mL. The absolute recoveries ranged from 87.3 to 91.8%, 80.1 to 80.6% and 76.0 to 78.0% for GLZ, CLZ and DCLZ respectively. The results are indicated in Table 2. Table .
Summary of precision and accuracy from QC samples in wistar rat plasma

Drug**Spiked concentration****(ng/mL)****Intra-day (n= 6)****Inter-day (n= 3)**Measured concentration (ng/ml) (mean \pm SD)% Nominal**%**C. VMeasured concentration (ng/ml) (mean \pm SD)% Nominal**%**

C. VGLZ25. 424. 7 \pm 1. 097. 112. 625. 3 \pm 2. 699. 610. 551. 553. 4 \pm 3.
 7103. 67. 052. 4 \pm 4. 4101. 78. 5823. 2817 \pm 37. 899. 34. 6773. 3 \pm 14.
 793. 91. 91646. 41630. 3 \pm 45. 999. 02. 81646. 6 \pm 51. 2100. 03. 1CLZ24.
 323. 5 \pm 2. 596. 610. 522. 8 \pm 3. 593. 814. 550. 952. 7 \pm 3. 5103. 66. 753. 7
 \pm 2. 5105. 54. 7814. 2826. 4 \pm 28. 1101. 53. 4817. 6 \pm 49. 2100. 46. 01628.
 51636. 6 \pm 62. 2100. 53. 81649. 5 \pm 59101. 33. 6DCLZ22. 924. 1 \pm 1. 5105.
 36. 324. 3 \pm 2. 6106. 310. 952. 554. 4 \pm 5. 2103. 69. 653. 6 \pm 3102. 25.
 7840. 0848. 5 \pm 17101. 02. 0863. 7 \pm 46. 2102. 85. 316801636. 8 \pm 5797.
 43. 51712. 7 \pm 32. 7101. 91. 9

Table . Extraction recovery of the analytes in rat plasma (n= 6)

Drug**Concentration (ng/ml)****Recovery (%)****% C. V**

GLZ51. 587. 34. 4823. 291. 72. 41646. 491. 84. 0CLZ50. 980. 11. 0814. 280.
21. 01628. 580. 60. 7DCLZ52. 576. 08. 1840. 078. 07. 1168076. 25. 0

4. 4. 5. Stability

QC samples were subjected to short term and long term storage condition (-70°C), freeze-thaw stability, auto-sampler stability and dry residue stability studies. All stability studies were carried out at two concentration levels (low and high QC) in six replicates. The bench top stability was studied for low and high QC samples kept at room temperature (25°C) for 6 hours. Freeze-thaw stability of low and high QC samples was evaluated after 3 freeze thaw cycles. The autosampler stability was studied for low and high QC samples stored at autosampler at 10°C for 24 hour. The freezer storage stability of the drug in plasma was determined by comparing the low and high QC samples stored for 30 days at -70°C . The results indicated that each analyte had an acceptable stability under those conditions, as shown in Table 3. Table . Stability in rat plasma (n= 6)

Drug**Nominal****concentration****(ng/ml)****Sample condition**

Bench top stability aAutosampler stabilityFreeze-thaw stability c30 days

storage stability d% NominalRSD% NominalRSD% NominalRSD%

NominalRSDGLZ51. 597. 68. 399. 79. 1104. 97. 399. 38. 41646. 496. 66.

299. 25. 399. 36. 4111. 25. 9CLZ50. 9105. 39. 2106. 54. 3111. 37. 092. 89.

11628. 5100. 74. 4101. 83. 9108. 34. 992. 03. 6DCLZ52. 598. 65. 897. 88.

196. 25. 8103. 68. 81680. 0102. 45. 297. 34. 798. 73. 193. 82. 9a Exposed at

ambient temperature (25°C) for 6h; b Kept at autosampler temperature

(10°C) for 24h ; c After three freeze-thaw cycles; d Stored at -70°C

4. 4. 6. Comparison of methods

Previous methods describing the determination of cilostazol (CLZ) and its metabolites involved liquid-liquid partitioning followed by Solid phase extraction (SPE) using HPLC Other methods using HPLC-UV were involved with various gradient and isocratic conditions comprised of multiple steps of liquid-liquid extraction (LLE) or SPE procedures for estimating CLZ and its metabolites 49, 51, 57. Most of the available methods for determination of GLZ were involved using UV and MS/MS detector 48, 52-55, 58. However, the reported method involves complex step extraction procedures and larger run times. Another method using MS detector is being published recently and it described the multiple analyte monitoring of anti-hyperglycemic agent, but

involved gradient method 58. The aim of present investigation is to develop and validate a simple LC-MS/MS method using isocratic mode with sufficient accuracy and precision for simultaneous estimation of GLZ & CLZ along with its active metabolite DCLZ and its subsequent use in pharmacokinetic studies in rats. The present method involves simple LLE procedure with good sensitivity and an isocratic reverse-phase HPLC analysis for all analytes of interest. This method is specific for GLZ & CLZ and its major active metabolite DCLZ with no interference and with good linearity, accuracy and precision. The LLOQ can be achieved to very low concentrations by increasing the plasma volume. The chromatographic conditions of this method were optimized for a short 3.5 min run time on LC-MS/MS.

4.5. Application to pharmacokinetic study

The method described above was successfully applied to a PK drug-drug interaction study in which plasma concentration of pure markers was determined for up to 24 h after simultaneous oral administration at 1 mg/kg dose of GLZ and 10 mg/kg dose of CLZ in male wistar rats. The plasma concentration time profiles of GLZ, CLZ & its active metabolite DCLZ are shown in Fig. 3, and could be traceable up to 24 h, 12h and 12h respectively. The pharmacokinetic parameters of GLZ, CLZ and DCLZ are presented in Table 4. During ISR it was observed that all of the samples were within $\pm 20\%$ of initial concentration value, further demonstrating that this method is capable of producing reproducible results over time. Table . Pharmacokinetic parameters (Mean \pm S. D.) after single dose oral administration of GLZ and CLZ simultaneously in wistar rats

Parameters

Units

GLZ

CLZ

DCLZ (metabolite)

C_{max} $\mu\text{g/ml}$ 5.97 \pm 1.68 1.90 \pm 0.51 1.39 \pm 0.30 AUC 0-24 $\mu\text{g. h/ml}$ 40.00 \pm 5.04 5.35 \pm 1.11 4.69 \pm 0.65 AUC 0-inf $\mu\text{g. h/ml}$ 42.83 \pm 7.16 5.84 \pm 1.16 5.37 \pm 0.99 *T_{max} h 2.00 \pm 0.41 0.25 \pm 0.43 2.00 \pm 0.41 # t_{1/2} h 6.67 \pm 1.78 3.02 \pm 1.47 3.94 \pm 1.95 K_{el} h⁻¹ 10.104 \pm 0.02 60.230 \pm 0.06 60.176 \pm 0.065

Figure . Mean plasma concentration vs. time after single dose oral administration of GLZ and CLZ in six wistar rats.

4. 6. Conclusion

An LC-MS/MS bioanalytical method for simultaneous determination of three analytes, GLZ, CLZ and DCLZ was developed and validated in rat plasma. The method was good enough to detect low concentration of 25 ng/mL for all these analytes in 50 μL rat plasma and further can be improved by increasing the plasma volume. Glimipiride, a sulphonyl urea derivative with similar structural nucleus to GLZ was used as IS in assay method to account the variations due to matrix effect, extraction variability and instrument performance. Analytes recovery from spiked control samples were > 76%, using convenient and fast LLE method. Intra- and inter-day accuracy and precision of the validated method were within the acceptable limits of <15% and 85 to 115% respectively across the concentrations. All these features confirm that this method is simple, quick, specific, precise and accurate. The

method was successfully applied to generate stability profile as well as PK evaluation of simultaneous administration of GLZ, CLZ in rat following oral administration.