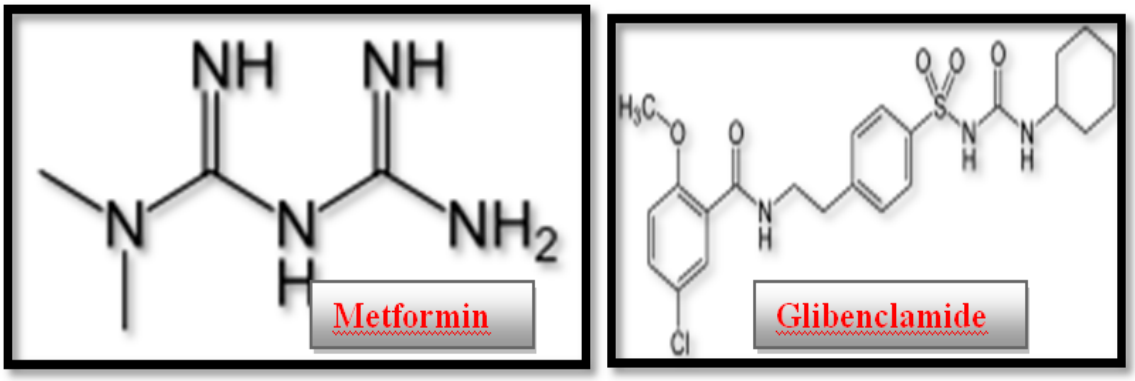
CHAPTER-4



LCMS-MS method for the simultaneous Analysis of Metformin and Glibenclamide in Human Plasma (In-vivo)

4.1 Introduction

4.1.1 Metformin

Metformin is a potent anti-diabetic drug of class biguanide class. It is considered as the first line treatment of diabetes mellitus particularly in overweight and obese people and people with normal kidney function [1, 2, and 3]. It has been extensively used in the treatment of non-alcoholic fatty liver disease (NAFLD) and premature puberty, three other diseases that feature insulin resistance.

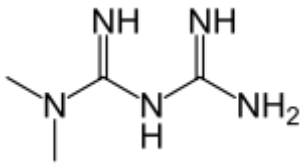


Figure 4.A: Structure of Metformin

It is used in the treatment of gestational diabetes and also being used in the treatment of polycystic ovary syndrome. It has been investigated for the other diseases where insulin resistance plays vital role. Metformin is the only anti diabetic drug that has been conclusively shown to prevent the cardiovascular complications of diabetes. It also reduces LDL Cholesterol and triglceride levels and not associated with gaining weight. Up to 2010, Metformin is one of only two oral anti diabetics in the World Health Organization Model List of Essential Medicines (the other being brokers bazaar, glibenclamide) [4].The literature reveals that Metformin is anti-diabetic is a class of biguanide, which also includes the withdrawn agents phenformin and buformin, originates from the French lilac or goat's rue (*Galega officinalis*), a plant used in folk medicine for several centuries [5].The drug was first described in the scientific literature in 1922, by Emil Werner and James Bell, as a product in the synthesis of *N*,*N*-dimethyl guanidine [6]. In 1929, Slotta and Tschesche discovered its sugar-lowering action in rabbits, noting it was the most potent of the biguanide analogs they studied [7]. Metformin found to reduce blood glucose levels in 1920's but later it was forgotten and next decades the focus of research was shifted to insulin and other anti-diabetic drugs. In 1940's it was the use of Metformin was rekindled and several studies were carried to state the action of the drug in reducing blood glucose levels.

In 1957, French physician Jean Sterne published the first clinical trial of metformin as a treatment for diabetes. It was introduced to the United Kingdom in 1958, Canada in 1972, and the United States in 1995. Metformin is now believed to be the most widely prescribed anti diabetic drug in the world; in the United States alone, more than 48 million prescriptions were filled in 2010 for its generic formulations [8, 9]. Metformin suppresses glucose production by the liver (using hepatic gluconeogenesis mechanism) and decreases hyperglycemia. When a type 2 diabetes patient is treated with Metformin that person will have gluconeogensis reduced by three times the average rate of gluconeogensis. The inhibition of the mitochondrial respiratory chain (complex I), activation of AMP-activated protein kinase (AMPK), inhibition of glucagon-induced elevation of cyclic adenosine monophosphate (cAMP) and consequent activation of protein kinase A (PKA), and an effect on gut microbiota have been proposed as potential mechanisms [10,11]. In addition it increases insulin sensitivity by suppressing glucose production, enhanced by peripheral glucose uptake (by inducing the phosphorylation of GLUT4 enhancer factor), decreases insulin-induced suppression of fatty acid oxidation [12] and decreases absorption of glucose from the gastrointestinal tract. Increased peripheral utilization of glucose may be due to improved insulin binding to insulin receptors [13].

The drug is available in different forms as tablet, capsule and liquid formulations with different brand names. Metformin is marketed with several brand names, including Glucophage XR, Carbophage SR, Riomet, Fortamet, Glumetza, Obimet, Gluformin, Dianben, Diabex, Diaformin, Siofor and Metfogamma. The liquid formulation of the drug is marketed as Riomet, which is available in 5ml formulation and equivalent to 500mg of tablet form of metformin. According to pharmacokinetic studies, Metformin has an oral bioavailability of 50–60%under fasting conditions, and is absorbed slowly [14,15]. After in taking of the drug in pure form within two to three hours Peak plasma concentrations were reached and four to eight hours with the extended-release formulations [14,16].

The most common side effects of the drug is gastrointestinal irritation, including diarrhea, cramps, nausea, vomiting and increased flatulence; metformin is more commonly associated with gastrointestinal side effects than most other antidiabetic drugs [17]. Among serious side effects lactic acidosis and vast majority of these cases seem to be related to comorbid conditions, such as impaired liver or kidney function [18].

Metformin has also been reported to decrease the blood levels of thyroid-stimulating hormone in people with hypothyroidism [19].

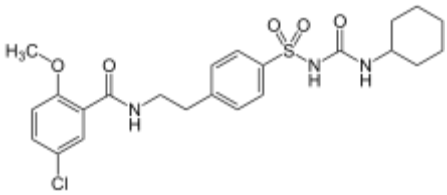


Figure 4.B: Structure of Glibenclamide

4.1.2 Glibenclamide

Glibenclamide acts as anti-diabetic drug belongs to the class of sulfonylureas commonly known as sulfa drugs. The drug was first developed in 1966 in a cooperative study between Boehringer Mannheim (now part of Roche) and Hoechst (now part of Sanofi-Aventis) [20]. The drug is marketed with different trade names Diabeta, Glynase and Micronase in the United States and Daonil, Semi-Daonil andEuglucon in the United Kingdom and Delmide in India. The drug is also available in the market in combination with Metformin under the brand name of Glucovance, Benimet and Glibomet. The drug is effectively used in the treatment of type 2 diabetes. Up to 2003 it was most famous sulfonylurea drug [21]. It is also helpful in improving the out coming results on animal stroke models by preventing brain swelling and enhancing neuro protection [22]. In 2011 survey it has been reported that Glibenclamide is one of only two oral anti diabetic drugs in World Health Organization Model List of Essential Medicines (the other being metformin) [23]. The mechanism of action of drug is illustrated by binding and activation of the sulfonylurea receptor 1 (SURI 1), which is considered as regulatory subunit of the ATP-sensitive potassium channels, in pancreatic beta cells (K_{ATP}) [24]. This leads to cell membrane depolarization opening voltage-dependent calcium channel due to inhibition.

The increase in intracellular calcium in the beta cell and subsequent stimulation of insulin release is resulted by the membrane depolarization of the cell membrane of pancreas.

Glibenclimide proved to be best drug which can show it efficacy in case of ischemic insult the blood brain barrier, it potentially reaches central nervous system. Glibenclamide more efficiently bind to ischemic hemisphere. Moreover, sulfonylurea receptor 1 under ischemic conditions, the regulatory subunit of the K_{ATP-} and the NC_{Ca-ATP}-channels, is expressed in neurons, astrocytes, oligodendrocytes, endothelial cells and by reactive microglia [25, 26].

The common side effects of the in take of drug are hypoglycemia and Cholestatic jaundice. However, the drug is restricted to patients suffering with G6PD deficiency as it may cause acute hemolysis [27]. Recent studies reveled that Glibenclamide is associated with significantly higher annual mortality when combined with metformin than other insulin-secreting medications, and has potential to lower some of the side effects [28].

4.2 Review of Literature

The literature study reveals that few analytical methods have been established for simultaneous determination the Metformin and Glibenclamide in pharmaceutical dosage forms. Reverse phase high performance liquid chromatography and spectrophotometer methods have been described in the literature. Some of the researchers estimated only Metformin or Glibenclamide in pharmaceutical dosage form, some other analysts performed simultaneous determination of Metformin, Glibenclamide and some other anti-diabetic drugs in combination were also estimated.

Seema M. Dhole et al [29] described simple, accurate, rapid, precise and sensitive UV spectrophotometric absorption correction method for the simultaneous determination of Pioglitazone HCl, Metformin HCl and Glibenclamide in combined tablet dosage form. Ethanol (95%) was used as solvent. The wavelengths selected for the analysis using absorption correction method were 237 nm, 268 nm and 300 nm for estimation of Metformin HCl, Pioglitazone HCl and Glibenclamide, respectively. Beer's law obeyed in the concentration range of 3-30 µg/ml, 10-100 µg/ml and 1-10 µg/ml for Pioglitazone HCl, Metformin HCl and Glibenclamide, respectively. The mean percentage drug content for Pioglitazone HCl, Metformin HCl and Glibenclamide were found to be 99.48%, 99.77% and 99.35%, respectively and the % RSD value was found to be less than 2 which show the precision of method. The developed method was validated statistically and by recovery studies. The high recovery and low coefficients of variation conforms the suitability of the method for simultaneous analysis of three drugs in combined tablets. Statistical analysis proves that the method was found to be suitable for the routine quality control analysis of Pioglitazone HCl, Metformin HCl and Glibenclamide in pure and pharmaceutical dosage forms.

Ketan P. Dadhania et al [30] developed a simple, rapid, accurate, precise, specific and economical spectrophotometric method for simultaneous estimation of Gliclazide (GLC) and Metformin hydrochloride (MET) in combined tablet dosage form. It employed formation and solving of simultaneous equation using two wavelengths 227.0 nm and 237.5 nm. This method obeys Beer's law in the employed concentration ranges of 5-25

µg/ml and 2.5-12.5 µg/ml for Gliclazide and Metformin hydrochloride, respectively. Results of analysis were validated statistically and by recovery studies.

Shweta Havele et al [31] proposed a simple and sensitive, HPTLC method has been developed for the quantitative estimation of metformin in its single component tablet formulation. Metformin was chromatographed on silica Gel 60 F254 TLC plate using ammonium sulfate (0.5%): 2-propanol: methanol in the ratio of 8.0:1.6:1.6 (v/v/v) as mobile phase. Metformin showed R_f value of 0.50±0.03 was scanned at 238 nm using Camag TLC Scanner 3. The linear regression data for the calibration plot showed a good relationship with r =0.999. The method was validated for precision and recovery. The limits of detection and quantification were 95 and 200 ng/spot respectively. The developed method was successfully used for the assay of metformin tablet formulations. The method is simple, sensitive and precise; it can be used for the routine quality control testing of marketed formulations.

Patil Sudarshan S et al [32] developed a Simple spectrophotometric method for simultaneous estimation of Glibenclamide and Metformin HCl in combined dosage form. The method employed simultaneous equation method for analysis using methanol as a solvent. The two wavelengths 229.5 nm and 237 nm were selected for estimation of Glibenclamide and Metformin HCl respectively. Linearity was observed in the concentration range of 3-15 µg/ml and 2-10 µg/ml for Glibenclamide and Metformin HCl respectively. The recovery studies ascertained the accuracy of the proposed method and the results were validated as per ICH guidelines. The method can be employed for estimation of pharmaceutical formulations with no interference from any other excipient's and diluents.

Wagh Vinod. T. et al [33] proposed a simple and accurate method of analysis to determine Glimepiride (GLM), Pioglitazone hydrochloride (PIO) and Metformin hydrochloride (MET) in combined dosage forms using second-derivative spectrophotometry and. GLM, PIO and MET in combined preparations (tablets) were quantified using the second-derivative responses at 233.4 nm for GLM, 265.4 nm for PIO and 252.6 nm for MET in spectra of their solutions in methanol. The calibration curves

were linear [correlation coefficient (r) = 0.9990 for GLM, 0.9990 for PIO and 0.9990 for MET] in the concentration range of 5-25 g/ml for GLM, 5- 25 g/ml for PIO and 2- 12 g/ml for MET. The method was validated and found to be accurate, precise, and specific. The method was successfully applied to the estimation of GLM, PIO and MET in combine tablet formulations.

Pradeep G Shelke et al [34] developed a selective and sensitive reverse phase high performance liquid chromatographic (RP-HPLC) method for the separation and quantification of Metformin HCl (MET) vildagliptin (VILD) in tablet dosage form. The determination was carried out using phenomex C18 column (4.6´150 mm) as a stationary phase and mobile phase comprised of phosphate buffer (pH6.0): methanol (65:35v/v). The pH of phosphate buffer is adjusted to 6.0 by using ortho phosphoric acid. The flow rate was maintained at 1.0ml/min and the eluent was monitored at 255nm.The retention time of MET and VILD were 1.503 min and 5.530 min respectively. The method was validated in terms of linearity, precision, accuracy, ruggedness, specificity and robustness. The method was linear over the range 50-150 µg/ml for both MET (r = 0.999) and VILD (0.998). For precision studies; RSD for MET and VILD were 0.24 and 0.14 respectively. The method was linear over the range 50-150 µg/ml for both MET (r = 0.999) and percentage recoveries for both drugs from their tablets were 100.16 and 99.98 respectively. Inter-day; intra-day RSD for both drugs were found be 0.27 and 0.26, 0.13 and 0.29 respectively.

G. Satya Sri et al [35] stated Metformin is a biguanide anti hyperglycemic agent used for treating non-insulin- dependent diabetes mellitus (NIDDM). It improves glycemic control by decreasing hepatic glucose production, decreasing glucose absorption and increasing insulin-mediated glucose uptake. Metformin is the only oral anti hyperglycemic agent that is not associated with weight gain. Alogliptin is a selective, orally bioavailable inhibitor of enzymatic activity of dipeptidyl peptidase-4. A new reversed-phase High Pressure Liquid Chromatographic (RPHPLC) method was developed for the determination of Metformin & Alogliptin (ALG) based on isocratic elution using a mobile phase consisting of potassium dihydrogen phosphate buffer [pH 4.0] and Acetonitrile [HPLC Grade] (70:30, *v/v*) at a flow rate of 1 ml min−1with UV detection at

235nm. The chromatographic separation was achieved on a XTerra column (250 mm × 4.6 mm, 5 µm). The run time was maintained for 8mins. The Inter day and intraday precision was found to be within the limits. The Accuracy values were within specified limits (98-102%) The calibration curve for Metformin was linear from (300-700 µg ml) and for Alogliptin from (7.5-17.5 µg /ml).The Limit of Detection for Metformin and Alogliptin was found to be 0.175 and 0.050 µg/ml respectively. The Limit of Quantification for Metformin and Alogliptin was found to be 0.57and 0.20 µg/ml respectively. The proposed method was adequate sensitive, reproducible, and specific for the determination of Metformin and Alogliptin bulk as well as in its tablet dosage forms. The validation of method was carried out utilizing ICH-guidelines. The described HPLC method was successfully employed for the analysis of pharmaceutical formulations containing combined dosage form. The present work was undertaken with the aim to develop and validate a rapid and consistent RP-HPLC in which the peaks will be appear with short period of time as per ICH Guidelines. The proposed method was simple, fast, accurate and precise method for the Quantification of drug in the dosage form, bulk drug as well as for routine analysis in Quality control. Overall the proposed method was found to be suitable and accurate for the Quantitative determination of the drug in Pharmaceutical dosage form.

F. S. Bandarkar et al [36] proposed a rapid, precise, sensitive, economical, and validated analytical method is reported for simultaneous separation and quantification of three anti-diabetic drugs, *viz.*, glibenclamide (GLB), gliclazide (GLC), and metformin hydrochloride (MHC) using ultra-fast liquid chromatography (UFLC). The separation of the three drugs was achieved using a XR-ODS C18 column (30°C) with a mobile phase comprised of acetonitrile-water-tri fluoro acetic acid-tri ethylamine (54:46:0.1:0.1v/v) in isocratic elution mode at a flow rate of 0.38 ml/min and detected at 230 nm. System suitability tests essential for the assurance of quality performance of the method were performed. The method was validated for accuracy, precision, reproducibility, robustness, detection (LOD), and quantification (LOQ) limits according to FDA and ICH guidelines. MHC (Rt = 0.98 min), GLC (Rt = 4.10 min), and GLB (Rt = 6.40 min) separated with good resolution in a single chromatographic run of 7.5 min. Linear relationship

($r^2 > 0.999$) was observed between the peak area and concentration for all the three compounds within the range of 5–50 µg/ml. Accuracy ranged from 98 to 103% and the coefficient of variation for precision was found to be less than 3%; in all cases. LOD and LOQ values were 10 ng/ml and 20 ng/ml, respectively, for GLC and GLB; whereas 25 ng/ml and 35 ng/ml, respectively, for MHC. The method was found to be robust with minor changes in injection volume and column temperature. Validation results indicated that the method shows satisfactory linearity, precision, accuracy, and ruggedness. The extremely low flow rate, short run time, and simple mobile phase composition makes the method cost effective, rapid, non-tedious, and can also be successfully employed for simultaneous analysis of the three anti-diabetic drugs from commercial products.

B. L. Kolte et al [37] developed a high-performance liquid chromatography (HPLC) method for the simultaneous determination of metformin and glibenclamide in a combined dosage form using a Zorbax XDB C_{18}, 15-cm column. The mobile phase was composed of the buffer and acetonitrile in the ratio 68:32, vol/vol; pH was adjusted to 7.5 with orthophosphoric acid. The buffer used in the mobile phase contains 10 mm disodium hydrogen phosphate and 10 mm sodium dodecyl sulphate (SDS) in double-distilled water. The mobile phase flow rate was 1 ml/min, column oven temperature was maintained at 40°C, and analytes were detected at a wavelength of 226 nm. The developed method was validated and shown to be linear. The correlation coefficients for metformin and glibenclamide were 1.0 and 0.9999, respectively. The relative standard deviations for six replicate measurements in two sets of each drug in the tablets were always less than 2%.

Asit Kumar De et al [38] developed a high performance reverse phase liquid chromatographic procedure is developed for simultaneous estimation of Metformin hydrochloride and Glibenclamide in combined tablet dosage form. The method was carried out on a Agilent Hypersil ODS (25cm x 4.6mm, i.d. 5µ) column with a mobile phase used consisting of acetonitrile**:** mono basic sodium phosphate Buffer (50:50) and the pH of buffer was adjusted to 2.5 using 2M Ortho phosphoric acid. The detection of the combined dosage form was carried out at 228 nm and a flow rate employed was 1 ml/min and column oven temperature at 300C. The retention times of Metformin HCl &

Glibenclamide were 2.709 & 9.216 minutes respectively. The developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantification as per ICH norms. The proposed method can be used for the estimation of these combined drugs.

Narendra Nyola et al [39] gave a analytical method for estimation of Saxagliptin and Metformin in active pharmaceutical ingredient. The method was validated in terms of linearity, accuracy, precision, specificity, limit of detection and limit of quantitation. The optimum conditions for the analysis of the drug were established. The maximum wavelength (λ max) of Saxagliptin and Metformin were found to be 274 nm and 231 nm respectively. The percentage recovery of Saxagliptin and Metformin were 100.1 and 99.98 respectively. Beer's laws were obeyed in the concentration range 50-90µg/ml for Saxagliptin and 2-10µg/ml for Metformin. The linear equation for Saxagliptin and Metformin were found to be y = 0.012x- 0.462, r^2 = 0.987 and y = 0.055x - 0.031, r^2 = 0.990 respectively. Validation was performed as per ICH guidelines.

Anandkimar R. Tengli et al [40] developed a Reverse phase high performance liquid chromatographic method for the simultaneous estimation of metformin, pioglitazone and glibenclamide in tablet dosage for using gliclazide as an internal standard. The separation was achieved at ambient temperature with low pressure gradient mode by using 5 µ size phenomenex luna CN (100R 250×4.60 (mm) column with mobile phase containing acetonitrile, water and buffer (0.5% potassium dihydrogen phosphate) pH 2.5 adjusted with ortho phosphoic acid in the ratio of 60:20:20. The flow rate was 1 ml min-1 and eluent was monitored at 230 nm by using UV detector. The selected chromatographic conditions effectively separated Metformin, pioglitazone and Glibenclamide with the retention time of 2.2, 2.8 and 5.8 min respectively. The linearity range for metformin, pioglitazone and glibenclamide is found in the range of 50-300 µg ml-1, 1.5-9.0 µg ml-1 and 0.5- 3.0 µg/ml respectively. The developed method was found to be accurate, simple, specific and reproducible. It can also be used for routine quality control analysis of these antidiabetic drugs in combinational dosage forms.

4.3 Materials and Methods

4.3.1 Instrumentation

An HPLC system (Shimadzu, Kyoto, Japan) consisting of an advance C18 column, a binary LC-20AD prominence pump, an auto-sampler (SIL-HTc) and a solvent degasser (DGU-20A3) was used for the study. Aliquots of the processed samples (20 ml) were injected into the column, which was kept at 30 1 C. The isocratic mobile phase was delivered into the electro-spray ionization chamber of the mass spectrometer. Quantitation was achieved with MS–MS detection in positive ion mode for both the analytes using a MDS Sciex API-4000 mass spectrometer equipped with a Turbo ion spray TM interface at 500 1 C. The ion spray voltage was set at 5500 V. The source parameters, viz. the nebulizer gas, curtain gas, auxiliary gas and collision gas were set at 45, 20, 45 and 10 psi, respectively. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM)

4.3.2 Chemicals and Standard Drugs

The working standard drug Metformin having a purity of 99.05% and Glibenclamide with 99.55% pure were kindly provided by Cipla Pharmaceuticals Ltd, Hyderabad; AP, India. All the chemicals used were of laboratory reagent grade and were purchased from Merck chemicals private limited, Mumbai; Maharashtra, India.

4.3.3 Preparation of Solutions:

4.3.3.1 pH 4.4 Acetate buffer (USP):

Weigh accurately dissolve 136 g of sodium acetate and 77 g of ammonium acetate in water and dilute to 1000.0ml with the same solvent; add 250.0 ml of glacial acetic acid and mix well to get a buffer solution of pH 5.1.

4.3.3.2 Preparation of Mobile Phase:

Measure accurately buffer solution and Methanol in the ratio of 40:60 (v/v) and sonicate the solution for ten minutes mix the contents. The content was mixed and degased using ultrasonic sonicater, and then it was filtered through 0.45µ nylon membrane filter paper using vacuum filtration set. The solution was stored at room temperature and used within 7 days from the date of preparation.

4.3.3.3 Preparation of Diluent:

An 80:20 (v/v) ratio of Methanol and Water was used as diluent in the analysis. For the preparation of diluent, 80ml of methanol was transferred into a 100ml reagent bottle and 20ml of Water was added, mixed and sonicated for 5 minutes. The solution was stored at room temperature and use within 7 days from the date of preparation.

4.3.3.4 Rinsing Solution:

50:50 ratios of methanol and Acetonitrile were used as rinsing solution. To this 50ml of methanol was mixed with 50ml of acetonitrile in a 100ml beaker. Mix the solution well and then it was filtered through membrane filter paper. The solution was used as rinsing solution to rinse useful things. The solution was stored at room temperature and used within 7 days from the date of preparation.

4.3.3.5 Preparation of Extraction Solution:

Diethyl ether and dichloromethane in the ratio of 60:40 (v/v) was used for the extraction of drugs from the biological matrix. 60 ml of Diethyl ether was added to 40ml of dichloromethane. Mix the solution well and then it was filtered and used for the extraction. The solution was stored at room temperature and used within 7 days from the date of preparation.

4.3.3.6 Preparation of Standard Stock Solution for Metformin:

A stock solution of mg/ml (1000mcg/ml) was prepared by accurately weighing 25 mg of the standard drugs Metformin and was dissolved in 25ml of methanol. The standard stock solution was prepared as per the potency of Metformin. A standard concentration of 990.5mcg/ml was obtained. The solution was filtered and was used as standard stock solution. The solution was preserved safely and was used when it required.

4.3.3.7 Preparation of Aqueous Calibration Curve Dilutions for Metformin:

From the standard stock solution of Metformin, pre-calculated dilutions were made accurately and a working standard stock solution concentration of 990.5ng/ml was prepared. From the working standard stock solution, aqueous calibration dilutions were prepared as per the table 4.1.

Table 4.1: Preparation of Aqueous Calibration Curve Dilutions for Metformin

S. No	Concentration (ng/ml)	Volume taken (ml)	Volume added (ml)	Final volume (ml)	Final concentration (ml)	Vial code (ml)
1	990.5	1.5	3.5	5	297.15	AQ-CC 1
2	990.5	1.0	4.0	5	198.1	AQ-CC 2
3	990.5	0.8	4.2	5	158.48	AQ-CC 3
4	990.5	0.6	4.4	5	118.86	AQ-CC 4
5	990.5	0.4	4.6	5	79.24	AQ-CC 5
6	990.5	0.2	4.8	5	39.62	AQ-CC 6

4.3.3.8 Preparation of Standard Stock Solution for Glibenclamide:

A stock solution of mg/ml (1000mcg/ml) was prepared by accurately weighing 4.20mg of the standard drugs Glibenclamide and was dissolved in 10ml of methanol. The standard stock solution was prepared as per the potency of Glibenclamide. A standard concentration of 1008.91mcg/ml was obtained. The solution was filtered and was used as standard stock solution. The solution was preserved safely and was used when it required.

4.3.3.9 Preparation of Aqueous Calibration Curve Dilutions for Glibenclamide:

From the standard stock solution of Glibenclamide, pre-calculated dilutions were made accurately and a working standard stock solution concentration of 995.50ng/ml was prepared. From the working standard stock solution, aqueous calibration dilutions were prepared as per the table 4.2.

Table 4.2: Preparation of Aqueous Calibration Curve Dilutions for Glibenclamide

S. No	Concentration (ng/ml)	Volume taken (ml)	Volume added (ml)	Final volume (ml)	Final concentration (ml)	Vial code (ml)
1	497.75	3.0	17.0	20	74.6625	AQ-CC 7
2	497.75	2.5	17.5	20	62.21875	AQ-CC 8
3	497.75	2.0	18.0	20	49.775	AQ-CC 9
4	497.75	1.5	18.5	20	37.33125	AQ-CC 10
5	497.75	1.0	19.0	20	24.8875	AQ-CC 11
6	497.75	0.5	19.5	20	12.44375	AQ-CC 12

4.3.3.10 Plasma Spiked Calibration Curve for Metformin:

The prepared aqueous dilutions were used to spike the screened blank human plasma matrix to prepare plasma calibration curve standards. The plasma spiked calibration curve was prepared with in the concentration range of 801.272ng/ml 40.064ng/ml. The preparation of solution was given in table 4.3.

Table 4.3: Preparation of Plasma Spiked Calibration Curve Dilutions for Metformin

S. No	Concentration (ng/ml)	Volume taken (ml)	Volume added (ml)	Final volume (ml)	Final concentration (ml)	Vial code (ml)
1	990.5	0.5	4.5	5.0	99.05	PS-CC 1
2	990.5	1.0	4.0	5.0	198.10	PS-CC 2
3	990.5	1.5	3.5	5.0	297.15	PS-CC 3
4	990.5	2.0	3.0	5.0	396.20	PS-CC 4
5	990.5	2.5	3.5	5.0	495.25	PS-CC 5
6	990.5	3.0	4.0	5.0	594.30	PS-CC 6

4.3.3.11 Plasma Spiked Calibration Curve for Glibenclamide

The prepared aqueous dilutions were used to spike the screened blank human plasma matrix to prepare plasma calibration curve standards. The plasma spiked calibration curve was prepared with in the concentration range of 908.019ng/ml-20.178ng/ml. The preparation of solution was given in table 4.4.

Table4.4: Preparation of Plasma Spiked Calibration Curve Dilutions for Glibenclamide

S. No	Concentration (ng/ml)	Volume taken (ml)	Volume added (ml)	Final volume (ml)	Final concentration (ml)	Vial code (ml)
1	995.50	0.25	9.75	10	24.887	PS-CC 1
2	995.50	0.50	9.5	10	49.775	PS-CC 2
3	995.50	0.75	9.25	10	74.6625	PS-CC 3
4	995.50	1.00	9.0	10	99.55	PS-CC 4
5	995.50	1.25	8.75	10	124.438	PS-CC 5
6	995.50	1.50	8.50	10	149.325	PS-CC 6

4.3.3.12 Extraction of Drugs from Plasma:

Prior to sample analysis, 100µL of each solution was extracted using 300µL of diethyl ether: dichloromethane (60:40% v/v) for protein precipitation. Further, each of the mixtures was vortex for a period of 5 min in a vortex mixer with subsequent centrifugation at 10000 rpm, for a period of 10 min at 4°C using a centrifuge. For each sample, an aliquot of a supernatant was isolated and subjected to dryness. The residue was reconstituted in 100µL of mobile phase and subsequently centrifuged at 10000 rpm for10 min at 4°C in a centrifuge. The supernatant was finally collected and directly injected for analysis. This procedure was followed for all samples of calibration curve plasma spiked dilutions and plasma spiked samples.

4.4 Method Development

The objective of this work was to develop and validate a simple, rapid and sensitive assay method for the quantification of Metformin and Glibenclamide, suitable to determine the drugs in plasma. To achieve the objective, different options was evaluated to optimize sample extraction, detection parameters and chromatography during method development. The standard solutions of both drugs were analyzed by LC-MS/MS system using direct injection probe with ESI and APCI interfaces.

In order to find the suitable mobile phase, which provides a good ionization and a maximum sensibility of the analytical method, a solvent screening was achieved. In all cases the organic solvent used was Methanol in proportion of 60% and to water phase were added salts or organic acids in order to modify the conductibility and the pH. It was compared the ratio signal/noise (S/N) for a standard solution containing both drugs with standard concentration in negative (MS2), positive (MS3) ionization. The suitable mobile phase proved to be a mixture of buffer solution and Methanol in the ratio of 40:60 (v/v) using ESI positive ionization. The pump delivered it at 0.8ml/min.

Both the analytes were extracted from human plasma by solid-phase extraction technique using Waters Oasis® HLB 1 cm3 (30 mg) extraction cartridge. The reconstituted samples were chromatographed on a BDS Hypersil C18 column (50 mm×4.6 mm, 5µm).

The full scan MS and M S/M S spectra of each analyte were obtained by direct infusion of the respective sample solution at a concentration of 594.30ng/ml of Metformin and 908.019ng/ml of Glibenclamide solution prepared in the mobile phase. The flow rates of sheath gas and auxiliary gas were optimized and set to 30 psi and 5 psi, respectively. The needle spray voltage was set to 4.5 k V. Helium was used as collision gas tuned for each analyte to obtain good signal intensity in MS2experiment. The drugs were analyzed using multiple react ions monitoring (MRM) mode.

Table 4.5 Optimized Chromatographic Conditions

S. No	Parameters	Conditions
1	API	Metformin and Glibenclamide,
2	Mobile phase	buffer solution and Methanol in the ratio of 40:60 (v/v)
3	column	BDS Hypersil C18 column (50 mm×4.6 mm, 5µm).
4	Flow rate	0.8ml/min
5	Run time	10 min
6	Retention time	Metformin : 2.91min Glibenclamide : 6.07min

Figure 4. C: Standard LC chromatogram of Metformin and Glibenclamide

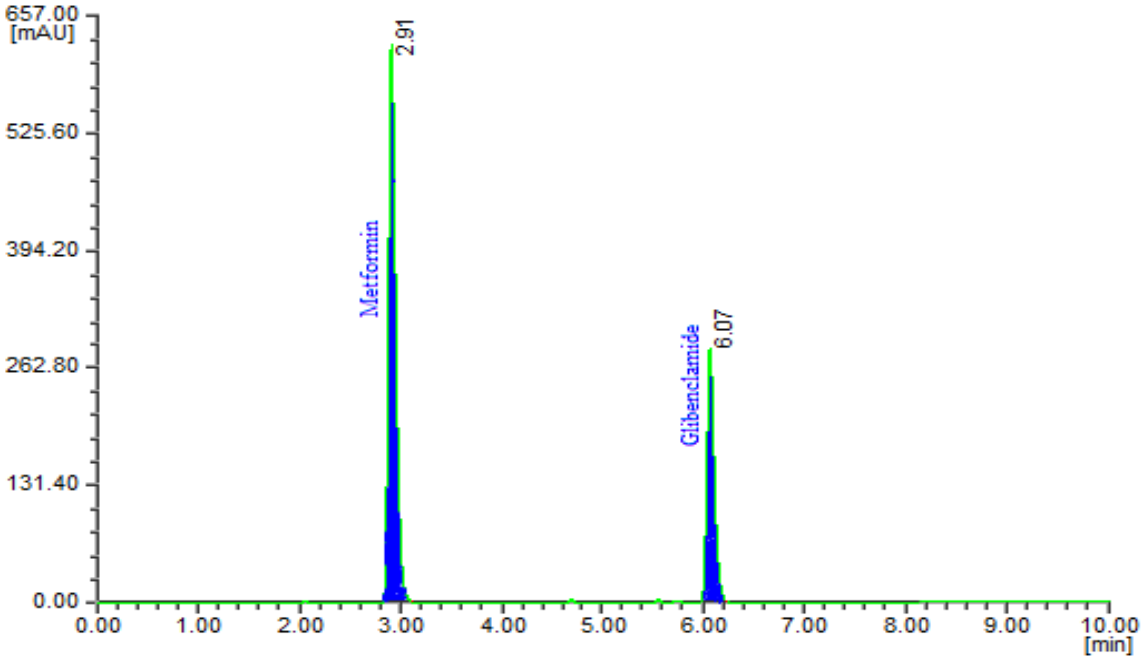


Figure 4.D: Blank chromatogram of Metformin and Glibenclamide

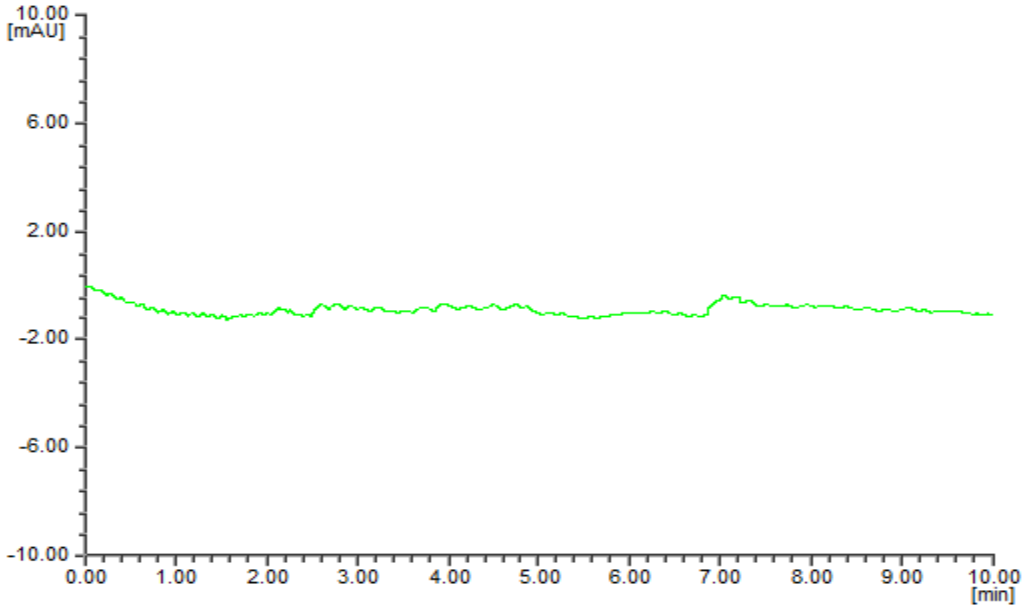


Figure4.E: Standard LC chromatogram of Metformin

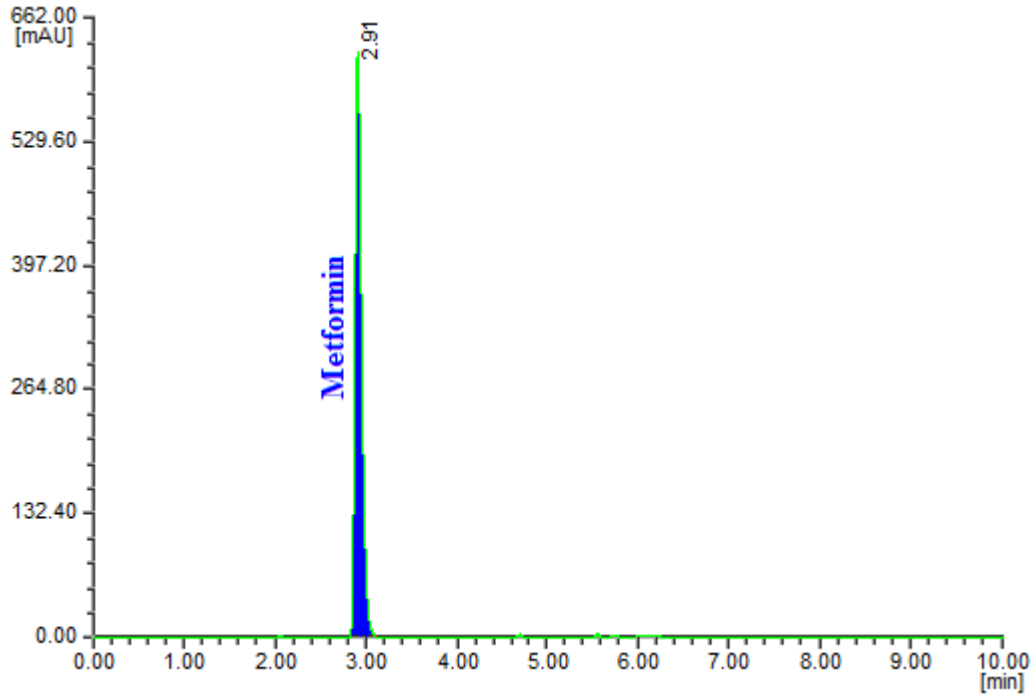


Figure 4.F: Standard LC chromatogram of Glibenclamide

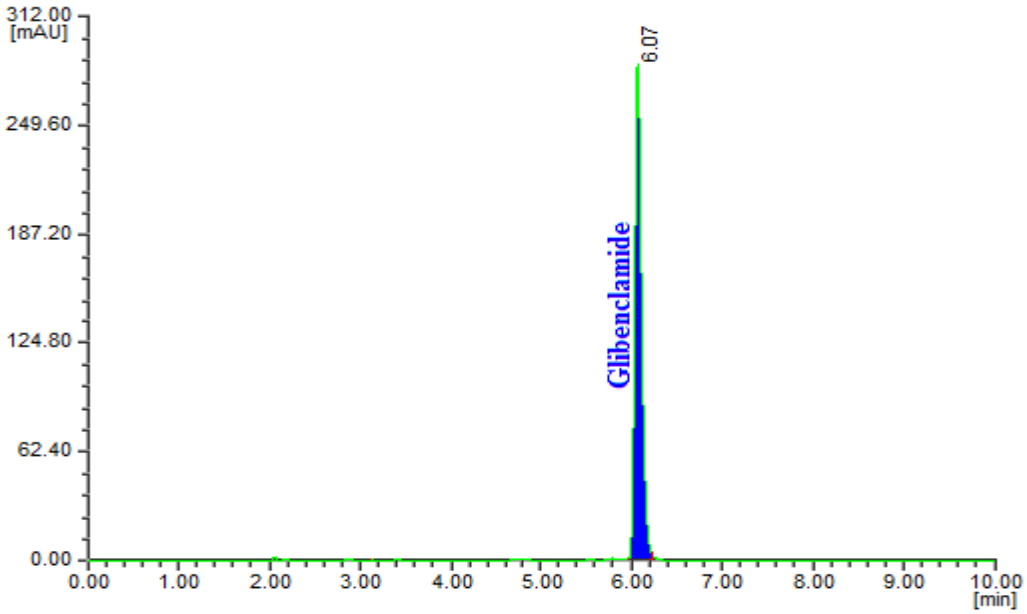


Figure 4.G: Mass spectrum of Metformin

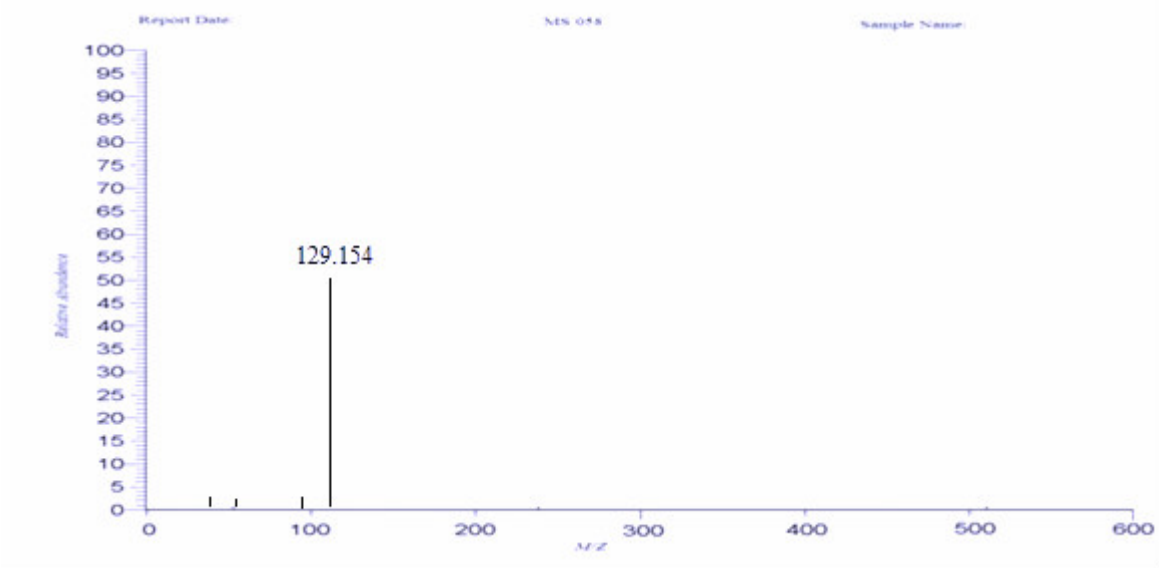
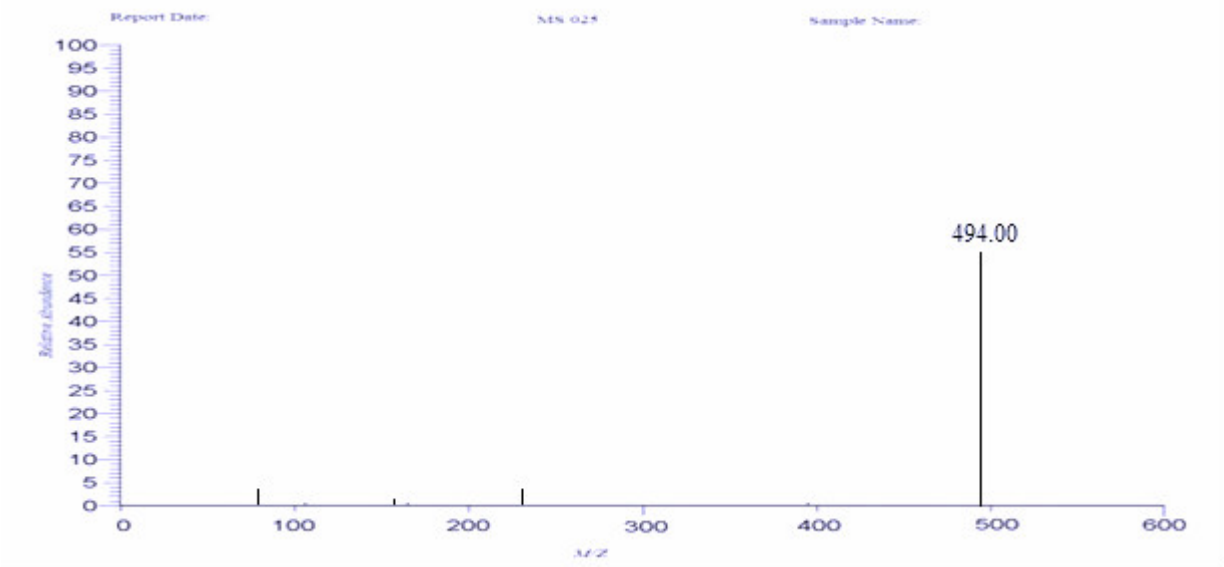


Figure4.H: Mass spectrum of Glibenclamide



4.5 Method Validation

A full validation according to the FDA guidelines (US DHHS, FDA, CDER, 2001) was performed for the assay in plasma. The following parameters were determined for the validation of the analytical method developed for quetiapine in human plasma: matrix effect, selectivity, linearity, precision, accuracy, LLOQ, recovery and stability. During pre-study validation, six validation runs were conducted on six separate days. Each validation run consisted of a set of the calibration standards at eight concentrations over the concentration range.

4.5.1 Selectivity:

The specificity of the method was evaluated by analyzing plasma samples from at least six different lots to investigate the potential interferences at the LC peak region for both the analytes. The responses of the interfering substances or background noises at the retention time of the Metformin and Glibenclamide acceptable if they are less than 20% of the response of the lowest standard curve point or LLOQ. There is no remarkable noise was observed at the retention time of Metformin and Glibenclamide and hence the proposed method was selective for the standard drugs Metformin and Glibenclamide only and hence the method selective.

4.5.2 Linearity

The six point calibration curve (99.05ng/ml to 594.3ng/ml for Metformin and 19.83ng/ml to 118.98ng/ml for Glibenclamide) was constructed by plotting the peak area of the analyte against the nominal concentration of calibration standards in plasma. Following the evaluation of different weighing factors, the results were fitted to linear regression analysis with the use of 1/X2 (X = concentration) weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.99 or better. The acceptance criteria for each back-calculated standard concentration were ± 15% deviation from the nominal value except at LLOQ, which was set at ± 20% (US DHHS, FDA, CDER, 2001). A good correlation of 0.998 and 0.999 with a regression equation of y = 3075.x + 16113 and y = 7535.x + 3644 was observed for Metformin and Glibenclamide respectively. The results were given in table.4.6, and linearity graphs were given in figure 4.I.

Table4.6: Plasma Spiked Calibration Curve for Metformin and Glibenclamide

S.NO	Metformin		Glibenclamide		Sample vial code
	Concentration	Area at the retention	Concentration	Area at the retention time	
1	99.05ng/ml	328471	19.83ng/ml	142597	PSCC 001
2	198.1ng/ml	649085	39.66ng/ml	296593	PSCC 002
3	297.15ng/ml	899174	59.49ng/ml	479864	PSCC 003
4	396.2ng/ml	1219369	79.32ng/ml	614692	PSCC 004
5	495.25ng/ml	1528690	99.15ng/ml	809568	PSCC 005
6	594.3ng/ml	1869058	118.98ng/ml	952143	PSCC 006
	Slope Intercept r^2	3075 16113 0.998	Slope Intercept r^2	7535 3644 0.999	

Figure4.I: Linearity Graph for Metformin

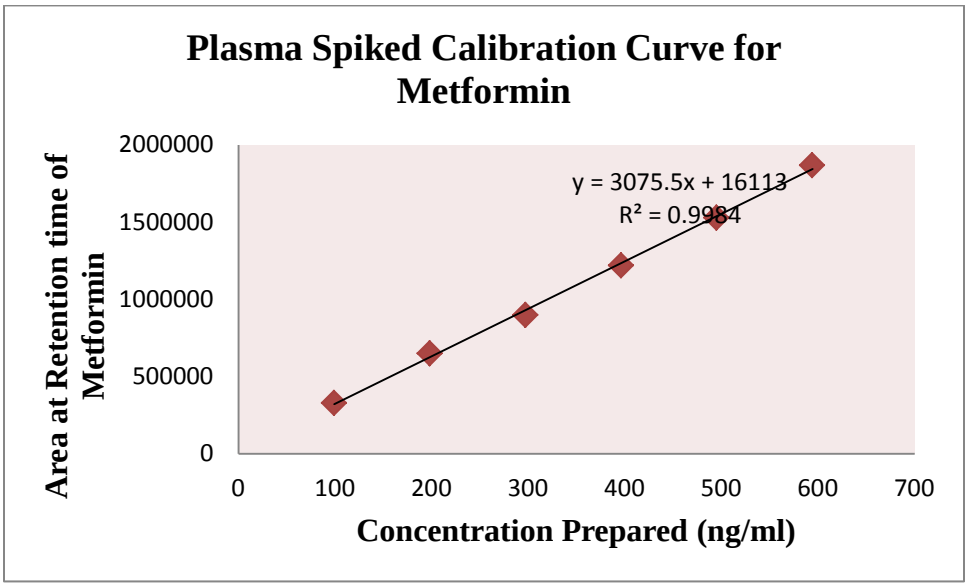
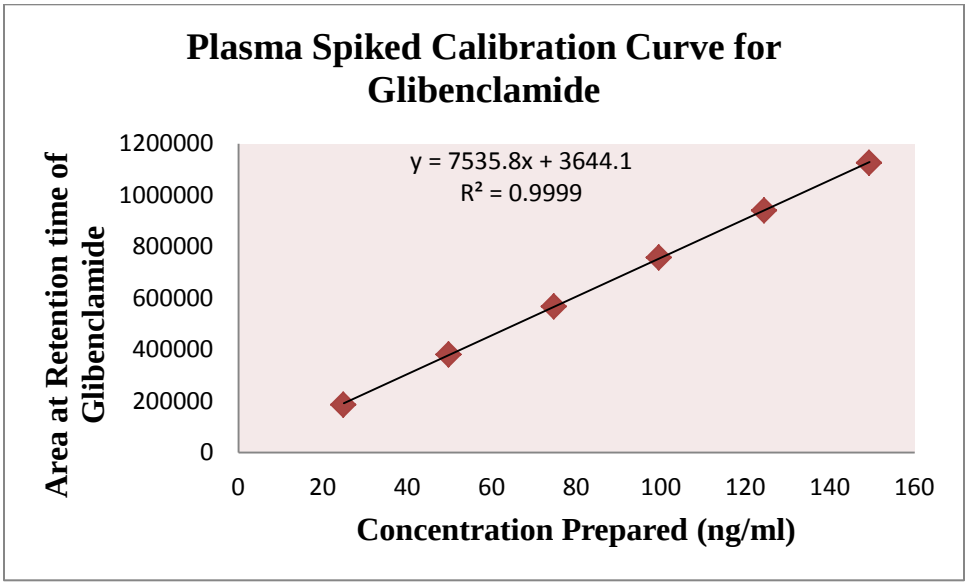


Figure4.J: Linearity Graph for Glibenclamide



4.5.3 Precision and Accuracy:

Inter- and intraday precision values using this method were estimated by assaying control plasma containing different concentrations of plasma spiked standards six times on the same day and on three separate days to obtain the relative standard deviation (RSD). Precision was carried out at HQC, MQC, LQC and LLOQC for both the drugs in calibration curve range. Detector response at the retention time of both the drugs in each level was determined and the %CV of the response was calculated. The acceptance criteria included accuracy within ±15% deviation (SD) from the nominal values, except LLOQ QC, where it should be ±20% and a precision of ≤15% relative standard deviation (RSD), except for LLOQ QC, where it should be ±20%. Whereas batch acceptance criteria included 67% for overall quality control samples and 50% at each level respectively. The results confirmed that the method was found to be precise and accurate. Results were given in table 4.7 and 4.8

Table 4.7: Precision at HQC and LQC levels

Precision at HQC					
S.NO	Sample ID	Metformin		Glibenclamide	
		Area obtained	Observed Concentration	Area obtained	Observed Concentration
P and A at HQC	PA001	1858732	591.0167	1120256	148.585
	PA002	1860893	591.7038	1101890	146.149
	PA003	1861896	592.0227	1121637	148.768
	PA004	1859969	591.41	1122029	148.82
	PA005	1869253	594.362	1129694	149.837
	PA006	1860269	591.5054	1102586	146.241
Nominal Conc.		801.272ng/ml		908.019ng/ml	
N		6		6	
Average		1861835	592.0034	1116349	148.067
SD		3781.024	1.202243	11419.82	1.51467
%CV		0.20308	0.20308	1.022962	1.02296
Accuracy (%)		99.961		99.949	
Precision at MQC					
S.NO	Sample ID	Metformin		Glibenclamide	
		Area obtained	Observed Concentration	Area obtained	Observed Concentration
P and A at MQC	PA007	891128	294.491	565812	74.3888
	PA008	894780	295.6979	566238	74.4448
	PA009	895014	295.7752	565906	74.4011
	PA010	896628	296.3086	566089	74.4252
	PA011	895217	295.8423	562147	73.9069
	PA012	897582	296.6239	563828	74.1279
Nominal Conc.		170.27ng/ml		100.891ng/ml	
N		6		6	
Average		895058.2	295.7898	565003.3	74.2825
SD		2208.388	0.729806	1656.012	0.21772
%CV		0.246731	0.246731	0.293098	0.2931
Accuracy (%)		98.5011		99.1535	

Table 4.8: Precision at LQC and LLOQC levels

Precision at LQC					
S.NO	Sample ID	Metformin		Glibenclamide	
		Area obtained	Observed Concentration	Area obtained	Observed Concentration
P and A at LQC	PA013	645870	197.1188	197.1188	49.4537
	PA014	656912	200.4888	200.4888	49.1498
	PA015	656475	200.3554	200.3554	49.2933
	PA016	647901	197.7386	197.7386	49.4001
	PA017	647014	197.4679	197.4679	49.3077
	PA018	646891	197.4304	197.4304	49.0164
Nominal Conc.		70.111ng/ml		50.445ng/ml	
N		6		6	
Average		650177.2	198.4333	377850.8	49.2702
SD		5090.34	1.553566	1242.963	0.16208
%CV		0.782916	0.782916	0.328956	0.32896
Accuracy (%)		99.9197		98.8681	
Precision at LQC					
S.NO	Sample ID	Metformin		Glibenclamide	
		Area obtained	Observed Concentration	Area obtained	Observed Concentration
P and A at LLOQC	PA019	321858	97.05586	186825	24.9479
	PA020	328589	99.08558	186979	24.9685
	PA021	329587	99.38653	185969	24.8336
	PA022	329968	99.50142	189085	25.2497
	PA023	327017	98.61155	186471	24.9006
	PA024	328014	98.91219	187958	25.0992
Nominal Conc.		40.064ng/ml		20.178ng/ml	
N		6		6	
Average		327505.5	98.75885	187214.5	24.9999
SD		2965.198	0.894151	1128.078	0.15064
%CV		0.905389	0.905389	0.602559	0.60256
Accuracy (%)		99.768		99.492	

4.5.4 Recovery

The accuracy of the optimized methods was determined by relative and absolute recovery experiments. The extraction recovery was determined by comparing the peak areas obtained from the extracted samples in plasma with those of direct injected standards, at the same concentrations. The mean recoveries were determined in triplicate. Accuracy was determined as the percentage of the nominal concentration. Recovery of the analytes from the extraction procedure was determined by comparing the peak areas of the analytes in spiked plasma samples (six each of HQC, MQC, and HQC samples) with those of the analytes in samples prepared by spiking the extracted drug-free plasma samples with the same amounts of the analytes at the step immediately prior to chromatography. The percentage recovery values for Metformin ranged from 89.7736 to 82.00784% and for Glibenclamide from 86.52834-83.8784%. The coefficient of variation (%) of these values was less than 4.00%. It is indicative that the developed methods are accurate and reliable. Table 4.9 and 4.10 showing the results of recovery obtained in the developed method for Metformin and Glibenclamide respectively.

Table 4.9: Recovery of Metformin

S. NO	Recovery at HQC level				Recovery at MQC level				Recovery at LQC level			
	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery
1	1795123	215840	494.258	83.16646	887214	985678	267.466	90.0105	635547	749614	167.956	84.783
2	1756842	214251	487.305	81.99653	885624	987265	266.558	89.7048	632641	748541	167.427	84.516
3	1785420	213544	496.892	83.60963	883351	983245	266.961	89.8404	630544	747124	167.189	84.396
4	1732584	215427	477.981	80.42755	884125	986458	266.324	89.6262	632518	749854	167.102	84.352
5	1763254	214253	489.088	82.29648	880250	980326	266.816	89.7916	631012	743145	168.209	84.911
6	1748526	213215	487.373	82.00784	886541	987530	266.762	89.7736	632340	742492	168.711	85.164
SD	23258.2	103124	6.583	1.107686	2542.26	2792.79	0.389	0.13078	1751.782	3234.171	0.63470	0.3204
Mean	1763625	214423	488.816	82.25075	8845175	9850837	266.814	89.7912	632433.7	746795	167.765	84.687
CV	1.31876	0.4883	1.347	1.346718	0.28742	0.28357	0.146	0.14565	0.277	0.433	0.378	0.3783
Standard Deviation					3.848							
Average recovery of three levels					85.576							

Table 4.10: Recovery of Glibenclamide

S. NO	Recovery at HQC level				Recovery at MQC level				Recovery at LQC level			
	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery
1	1235871	1432568	102.643	86.269	542368	642131	50.247	84.4638	172548	203568	33.6165	84.762
2	1258743	1424876	105.108	88.340	548723	645632	50.561	84.9901	174254	206584	33.453	84.350
3	1268547	1412543	106.851	89.8059	548691	645478	50.570	85.0054	170589	203568	33.235	83.799
4	1258643	1415364	105.805	88.927	547262	644591	50.507	84.9007	172842	203256	33.7255	85.036
5	1274584	1424857	106.432	89.453	548760	644215	50.675	85.1827	175237	205487	33.8216	85.279
6	1265348	1462351	102.951	86.528	548714	646254	50.511	84.9069	173250	206549	33.266	83.878
SD	13412.48	17975.64	1.782	1.498	2542.87	1465.16	0.1430	0.24046	1587.13	1557.287	0.2419	0.610
Mean	1260289	1428760	104.965	88.22	547419.	6447168	50.512	84.9082	173120	204835.3	33.519	84.517
CV	1.064238	1.258129	1.6978	1.698	0.46451	0.22722	0.283	0.2832	0.917	0.760	0.722	0.722
Standard Deviation					2.035							
Average recovery of three levels					85.882							

4.5. 5 Stability of the Drug in Solution

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions

4.5.5.1 Short Term Stability

To evaluate the short-term stability, six replicates plasma samples at a concentration of 594.3ng/ml of Metformin and 149.325ng/ml of Glibenclamide were left to stand at room temperature for 4 hours (which exceeds the expected duration that samples could be maintained at room temperature and then processed according to the plasma sample preparation as previously described. The samples were quantified with a set of calibration samples that had been processed immediately after preparation. The samples qualified the test if the deviation was within ±15%. %CV was found to be 0.52 and the stability range of 96.94926 to 98.21865 for Metformin and a %CV of 0.901 and the stability range of 96.756 to 98.5512 for Glibenclamide. High stability values were observed for the proposed method.

Table 4.11 Short Term Stability Results for Metformin and Glibenclamide

S. NO	Metformin			Glibenclamide		
	Fresh Stock	Room Temperature stock	% Stability	Fresh Stock	Room Temperature stock	% Stability
1	1936457	1895271	97.87313	1120457	1092481	97.5032
2	1937186	1879123	97.00271	1101257	1075842	97.6922
3	1916475	1867598	97.44964	1135786	1098941	96.756
4	1928919	1874654	97.18677	1125478	1085423	96.4411
5	1909568	1851312	96.94926	1105894	1089872	98.5512
6	1925614	1891312	98.21865	1121876	1079365	96.2107
SD	10985.0	16097.84	0.507915	12798.02	8557.539	0.88446
Mean	1925703	1876545	97.44669	1118458	1086987	97.1924
CV	0.57044	0.857845	0.521223	1.144256	0.787271	0.91001
% Stability	98.855			1876545		
% Change	1.145			0.857845		

4.5.5.2 Long Term Stability

Six replicates human plasma samples at 594.3ng/ml of Metformin and 149.325ng/ml of Glibenclamide were stored at -20^{o}C for 11 days. These samples were processed and quantified using a fresh set of calibration samples. The stability samples were bracketed by freshly prepared quality control samples, one each concentrations of Metformin and Glibenclamide by the developed method . These QC samples were prepared with an independent stock solution. The samples qualified the test if the deviation was within ±15%. High % CV of 1.543 for Metformin and 0.721 for Glibenclamide was observed for the developed method. % stability of more than 97% was observed for both the drugs. Hence in the method, the solutions were stable over a long period. Results were given in table 4.12

Table 4.12: Long Term Stability Results for Metformin and Glibenclamide

S. NO	Metformin			Glibenclamide		
	Fresh Stock	Room Temperature stock	% Stability	Fresh Stock	Room Temperature stock	% Stability
1	1860457	1795271	96.49624	1198540	1173547	97.9147
2	1847186	1786123	96.69427	1165847	1142340	97.9837
3	1816475	1797598	98.96079	1186254	1152314	97.1389
4	1803919	1768754	98.05063	1174510	1146851	97.6451
5	1820568	1785312	98.06346	1154573	1132540	98.0917
6	1815614	1767312	97.33963	1142572	1140725	99.8383
SD	21635.82	12860.04	0.935526	20521.8	14117.62	0.91656
Mean	1827370	1783395	97.60084	1170383	1148053	98.1021
CV	1.183987	0.721099	0.958522	1.753426	1.229701	0.9343
% Stability	98.457			1783		
% Change	1.543			0.721		

4.5.5.3 Freeze Thaw Stability

Six replicates human plasma samples at two quality control samples concentrations; HQC and LQC for both the drugs were subjected to three freeze-thaw cycles of -20^{o}C during 24 h. After the completion of third cycle, these samples were processed and analyzed comparing with fresh samples, and quantified with a standard set of calibration samples. The samples qualified the test if the deviation was within ±15%. Response at the retention time of the each drug was noted and the % stability was calculated. % stability was found to be 999.83 and 99.736 for

Metformin; 99.392 and 97.921 for Glibenclamide in HQC and MQC levels respectively. High stability was observed for the proposed method. Results were given in table 4.13.

Table 4.13: Freeze Thaw Stability Results for Metformin and Glibenclamide

S.NO	Metformin				Glibenclamide			
	At HQC		At LQC		At HQC		At LQC	
	Fresh	stability	Fresh	stability	Fresh	stability	Fresh	stability
1	594.225	594.104	198.258	197.898	149.683	149.014	49.145	48.858
2	594.395	593.014	198.114	197.108	148.254	147.698	49.958	48.475
3	594.581	593.547	197.758	197.558	149.574	148.557	49.582	48.225
4	594.663	593.697	198.996	198.254	149.687	148.674	49.558	48.696
5	594.085	592.205	198.858	198.201	148.996	148.041	49.969	48.998
6	594.157	593.495	197.758	197.582	149.874	148.636	49.705	48.471
N	6	6	6	6	6	6	6	6
SD	0.235137	0.659157	0.53275	0.437006	0.61247	0.478756	0.30585	0.28445
Mean	594.351	593.3437	198.29	197.7668	149.345	148.4367	49.6528	48.6205
% CV	0.039562	0.111092	0.26867	0.220971	0.4101	0.322532	0.61597	0.58504
Accuracy	100.0086	99.83908	100.096	99.83182	100.013	99.4051	99.7546	97.6806
Stability	99.830		99.736		99.392		97.921	

4.5.5.4 Bench-Top Stability

The stability of Metformin and Glibenclamide standard solutions at HQC and MQC were prepared using the standard procedure. The prepared solution was kept 6 hours on bench top (at room temperature, in the presence of ambient light). The solutions were analyzed using the standard optimized conditions and the values were compared with the freshly prepared solutions. Results were found to be stable up to 6 hours as per the acceptance criteria. The percent stability was found to be 99.653 at HQC and 100.159 at LQC for Metformin and 97.797 at HQC and 96.703 at LQC for Glibenclamide. Results were given in table 4.14.

Table4.14 : Bench-top Stability Results for Metformin and Glibenclamide

S. NO	Metformin				Glibenclamide			
	At HQC		At LQC		At HQC		At LQC	
	Fresh	stability	Fresh	stability	Fresh	stability	Fresh	stability
1	595.214	593.663	197.785	196.698	148.858	146.689	50.154	48.898
2	593.998	592.258	197.558	196.997	148.582	146.824	49.669	48.996
3	595.124	593.696	196.989	196.68	149.695	146.558	50.447	47.785
4	595.635	594.254	197.055	196.747	149.82	145.582	50.693	47.858
5	596.258	592.179	197.114	199.693	147.586	143.69	49.581	48.582
6	594.581	592.365	197.295	198.858	146.698	142.258	49.217	47.758
N	6	6	6	6	6	6	6	6
SD	0.789579	0.905055	0.31406	1.32012	1.21478	1.884317	0.56435	0.57878
Mean	595.135	593.0692	197.299	197.612	148.54	145.2668	49.9602	48.3128
% CV	0.132672	0.152605	0.15918	0.66803	0.81781	1.297142	1.1296	1.19798
Accuracy	**100.140**	**99.793**	99.5958	99.7537	**99.474**	**97.282**	100.372	97.0624
Stability	**99.653**		**100.159**		**97.797**		**96.703**	

4.5.5.5Auto-Sampler Stability:

Auto-sampler stability was studied following 24h storage period in the auto sampler tray with control concentrations. Auto sampler stability was studied at HQC and LQC level. % stability was found to be 99.801 and 96.971 for Metformin and 99.794 and 96.393 for Glibenclamide e at HQC and LQC levels. Stability results were found to be accepted. Results were given in table. 4.15

Table 4.15: Auto-Sampler Stability Results for Metformin and Glibenclamide

S. NO	Metformin				Glibenclamide			
	At HQC		At LQC		At HQC		At LQC	
	Fresh	stability	Fresh	stability	Fresh	stability	Fresh	stability
1	595.582	594.582	199.205	197.858	150.124	148.885	48.996	48.714
2	594.582	593.179	198.826	197.558	149.968	148.101	50.585	47.586
3	593.854	593.552	197.778	196.696	148.587	149.987	50.107	47.996
4	596.669	592.287	198.881	168.981	148.693	149.258	50.258	47.582
5	594.582	593.693	199.582	197.747	147.586	147.774	50.229	48.969
6	594.693	595.582	198.274	197.582	147.582	146.698	50.257	48.749
N	6	6	6	6	6	6	6	6
SD	0.98803	1.14314	0.64702	11.6453	1.10635	1.1707	0.55057	0.62151
Mean	594.994	593.813	198.758	192.737	148.757	148.451	50.072	48.266
% CV	0.16606	0.19251	0.32553	6.04205	0.74373	0.78862	1.09956	1.28767
Accuracy	100.117	99.918	100.332	97.2928	99.6194	99.4144	100.597	96.9684
Stability	99.801		96.971		99.794		96.393	

4.6 Discussion of the Results

A sensitive and specific high-performance liquid chromatography combined with electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS) method, operating in the positive ionization mode, for simultaneous quantifying of Metformin and Glibenclamide in human plasma was developed and validated. Both the analytes were extracted by simple one step liquid/liquid extraction with a mixture of diethyl ether: dichloromethane in the ratio of 60:40% v/v.Chromatographic separation was performed on BDS Hypersil C18 column (50 mm×4.6 mm, 5µm) using mobile phase mixture of buffer solution and Methanol in the ratio of 40:60 (v/v) using ESI positive ionization. The pump delivered it at 0.8ml/min. The retention time for metformin was found to be 2.91min and 6.07min for Glibenclamide. There is no additional detections were detections were observed in the chromatogram.

Blank human plasma containing heparin and ethylene diamine tetraacetic acid (EDTA) were extracted and analyzed to determine the extent to which endogenous human plasma components may contribute to chromatographic interference with the analyte or the internal standard. No significant interferences were observed in eight different lots of human heparin plasma (including one lipemic and one haemolysed plasma) samples and two lots of human EDTA plasma samples.

The specificity and selectivity of the method were investigated by comparing the chromatograms of six different batches of blank human plasma with the peak response of Metformin and Glibenclamide at LLOQ. The product ion chromatograms extracted from plasma are depicted in Figure 4.C. As shown, the chromatogram retention times for Metformin and Glibenclamide were about 2.91 and 6.07min, respectively, and there was no interference from endogenous substances observed at the retention time of the analytes. The total LC-MS/MS analysis time was 4.0 min per sample.

No significant matrix effect was observed in all the eight batches of human plasma for the analytes at LQC and HQC concentrations. The precision and accuracy for Metformin and

Glibenclamide at HQC concentration were found to be 99.961 and 99.949, at MQC level they were 98.501 and 99.1535, at LQC were 99.919 and 98.868 and LLOQC was found to be

99.768 and 99.492 respectively. Similarly, the precision and accuracy for amlodipine at LQC concentration were found to be 1.69% and 102.52%, and at HQC level they were 1.12% and 100.12%, respectively.

It was observed that the optimized methods were linear within a specific concentration range for Metformin and Glibenclamide. The calibration curves were plotted between response factor and concentration of the standard solutions against concentration of the analyte. The linearity ranges were found to be 99.05ng/ml to 594.3ng/ml for Metformin and 19.83ng/ml to 118.98ng/ml for Glibenclamide. The calibration curves were constructed on 11 different days over a period of four weeks to determine the variability of the slopes and intercepts. The results indicated no significant interday variability of slopes and intercepts over the optimized concentration range.

The stability studies of plasma samples spiked with selected drugs were subjected to three freeze-thaw cycles, short term stability at room temperature for 3 h and long term stability at – 70^{o}C over four weeks. In addition, stability of standard solutions was performed at room temperature for 6 h and freeze condition for four weeks. The mean concentrations of the stability samples were compared to the theoretical concentrations. The results indicate that selected drugs in plasma samples can be stored for a month without degradation in frozen state. The results of short term storage at room temperature stability and freeze thaw cycles indicate no degradation of the selected drugs in plasma as well as in sample solution and hence plasma samples could be handled without special precautions.

4.7 Conclusion

In this study it was concluded that sensitive, selective, accurate, precise and rapid electrospray LC-MS/MS method for the simultaneous determination of Metformin and Glibenclamide in human plasma is described. The method was successfully validated; stability

studied and was found to meet the entire requirement of current Thai FDA guidelines. It was shown that this method has high sensitivity and specificity, and is capable of support for pharmacokinetic assays, such as in bioequivalence studies.

4.8 References

1. Clinical Guidelines Task Force, International Diabetes Federation (2005). "Glucose control: oral therapy" PDF (100 KB). In: *Global Guideline for Type 2 Diabetes*. Brussels: International Diabetes Federation, 35–8.
2. National Collaborating Centre for Chronic Conditions. *Type 2 diabetes: national clinical guideline for management in primary and secondary care (update)* [pdf]. London: Royal College of Physicians; 2008.
3. American Diabetes Association. Standards of medical care in diabetes—2009. *Diabetes Care*. 2009; 32 Suppl 1:S13–61.
4. (March 2010) *WHO Model List of Essential Medicines* PDF (433 KB), 16th edition, World Health Organization,. 24.
5. Witters L. The blooming of the French lilac. *J Clin Invest*. 2001, 108(8):1105–7.
6. Werner E, Bell J. "The preparation of methylguanidine, and of ββ-dimethylguanidine by the interaction of dicyanodiamide, and methylammonium and dimethylammonium chlorides respectively", *J Chem Soc, Transactions*. 1921, 121: 1790–5.
7. See Chemical Abstracts, v.23, 42772 (1929) "K. H. Slotta, R. Tschesche. Uber Biguanide. II. Die Blutzuckersenkende Wirkung der Biguanides. *Berichte der Deutschen Chemischen Gesellschaft B: Abhandlungen"*. 1929, 62: 1398–1405.
8. Bailey CJ, Day C. Metformin: its botanical background. *Practical Diabetes International*. 2004, 21(3):115–7.
9. "The Use of Medicines in the United States: Review of 2010" PDF (1.79 MB). IMS Institute for Healthcare Informatics (April 2011).
10. Rena, G.; Pearson, ER. Sakamoto, K. (Sep 2013). "Molecular mechanism of action of metformin: old or new insights?" *Diabetologia* 56 (9): 1898–906.
11. Burcelin, R. (Jul 2013). "The antidiabetic gutsy role of metformin uncovered?" *Gut*.
12. Collier CA, Bruce CR, Smith AC, Lopaschuk G, Dyck DJ. Metformin counters the insulin-induced suppression of fatty acid oxidation and stimulation of triacylglycerol storage in rodent skeletal muscle. *Am J Physiol Endocrinol Metab*. 2006, 291(1):E182–E189.
13. Bailey CJ, Turner RC. Metformin. *N Engl J Med.* 1996, 334(9):574–9.

14. Bristol-Myers Squibb. U.S. Food and Drug Administration. Glucophage (metformin hydrochloride tablets) Label Information; August 27, 2008.
15. Jayasagar G, Krishna Kumar M, Chandrasekhar K, Madhusudan Rao C, Madhusudan Rao Y. "Effect of cephalexin on the pharmacokinetics of metformin in healthy human volunteers", *Drug Metabol Drug Interact*. 2002, 19(1):41–8.
16. Heller JB. "Metformin overdose in dogs and cats". *Veterinary Medicine*. 2007(April):231-233.
17. Bolen S, Feldman L, Vassy J, "Systematic review: comparative effectiveness and safety of oral medications for type 2 diabetes mellitus". *Ann Intern Med*. 2007, 147(6):386–99.
18. Khurana R, Malik IS. Metformin: safety in cardiac patients. *Heart*. 2010;96(2):99–102.
19. Vigersky RA, Filmore-Nassar A, Glass AR. "Thyrotropin suppression by metformin". *J Clin Endocrinol Metab*. 2006, 91(1):225–7.
20. Riddle MC (February 2003). "Editorial: sulfonylureas differ in effects on ischemic preconditioning--is it time to retire glyburide?" *J. Clin. Endocrinol. Metab,* 88 (2): 528–30.
21. Simard JM, Chen M, Tarasov KV, Bhatta S, Ivanova S, Melnitchenko L, Tsymbalyuk N, West GA, Gerzanich V, "Newly expressed SUR1-regulated NC(Ca-ATP) channel mediates cerebral edema after ischemic stroke", *Nat. Med.2006,* 12 (4): 43340
22. WHO Expert Committee "The selection and use of essential medicines". *World Health Organ Tech Rep Ser* ,2011, (965): i–xiv, 1–249.
23. Serrano-Martín X, Payares G, Mendoza-León A "Glibenclamide, a blocker of K+(ATP) channels, shows antileishmanial activity in experimental murine cutaneous leishmaniasis".*Antimicrob. Agents Chemother,2006,* 50 (12): 4214–6.
24. Ortega FJ, Gimeno-Bayon J, Espinosa-Parrilla JF, Carrasco JL, Batlle M, Pugliese M, Mahy N, Rodríguez MJ , "ATP-dependent potassium channel blockade strengthens microglial neuroprotection after hypoxia-ischemia in rats". *Exp. Neurol.2012,* 235 (1): 282–96.
25. Simard JM, Woo SK, Schwartzbauer GT, Gerzanich V "Sulfonylurea receptor 1 in central nervous system injury: a focused review". *J. Cereb. Blood Flow Metab.2012,* 32 (9): 1699–717.

26. Meloni G, Meloni T "Glyburide-induced acute haemolysis in a G6PD-deficient patient with NIDDM". *Br. J. Haematol.* 1996, 92 (1): 159–60.
27. Monami M, Luzzi C, Lamanna C, Chiasserini V, Addante F, Desideri CM, Masotti G, Marchionni N, Mannucci E "Three-year mortality in diabetic patients treated with different combinations of insulin secretagogues and metformin". *Diabetes Metab Res Rev,2006,* 22 (6): 477–82.
28. Seema M. Dhole, Pramod B. Khedekar, Nikhil D. Amnerkar, "UV Spectrophotometric Absorption Correction Method for the Simultaneous Estimation of Pioglitazone HCl, Metformin HCl and Glibenclamide in Multicomponent Formulation", *International Journal of Analytical and Bioanalytical Chemistry*, 2013; 3(1): 18-22.
29. Ketan P. Dadhania, Parthika A. Nadpara , Yadvendra K. Agrawal, "Development And Validation Of Spectrophotometric Method For Simultaneous Estimation Of Gliclazide And Metformin Hydrochloride In Bulk And Tablet Dosage Form By Simultaneous Equation Method", *International Journal of Pharmaceutical Sciences and Research*, 2(6): 1559-1563.
30. Shweta Havele and Sunil Dhaneshwar, Journal of Nanomedicine & Nanotechnology, 1(1).
31. Patil Sudarshan S., Bonde C. G., "Development and Validation of analytical method for Simultaneous Estimation of Glibenclamide and Metformin HCl in Bulk and Tablets using UV – visible spectroscopy", *International Journal of ChemTech Research*, 2009, 1(4):905-909.
32. Wagh Vinod T, "Simultaneous Estimation Of Glimepiride, Pioglitazone Hydrochloride And Metformin Hydrochloride By Derivative Spectrophotometry Method", Pharmatutor.
33. *Pradeep G Shelke, Anil P Dewani, Sanjay N Vasu, Alok S Tripathi, Ravindra L Bakal, Anil V Chandewar,"* A validated RP-HPLC method for simultaneous determination of Metformin HCl and Vildagliptin in pharmaceutical formulation", *International Journal of Advances in Pharmaceutical analysis*, 2013, 3(2) .
34. G. Satya Sri, S. Ashutosh Kumar, J. Saravanan, Manidipa Debnath, V. Greeshma, N. Sai Krishna, "A New RP-HPLC Method Development For Simultaneous Estimation Of Metformin And Alogliptin In Bulk As Well As In Pharmaceutical Formulation By Using

Pda Detector", *World Journal Of Pharmacy And Pharmaceutical Sciences*, 2(6): 6720-6743.

35. F. S. Bandarkar and I. S. Khattab, "Simultaneous Estimation Of Glibenclamide, Gliclazide, And Metformin Hydrochloride From Bulk And Commercial Products Using A Validated Ultra Fast Liquid Chromatography Technique", 33(20).
36. B. L. Kolte, B. B. Raut, A. A. Deo, M. A. Bagool, D.Shinde, "Metformin and Glibenclamide: HPLC Determination", *Encyclopedia of Chromatography*, Third Edition.
37. Asit Kumar De, Ayan Kumar Dey, Angshuman Biswas, "Simultaneous Estimation Of Metformin Hydrochloride And Glibenclamide By Rphplc Method From Combined Tablet Dosage", *International Journal of Science Inventions Today*, 2012, 1(2).
38. Narendra Nyola, Govinda Samy Jeyabalan, "Development and validation of uv-vis specctroscopy method for simultaneous estimation of saxagliptin hydrochloride and metformin hydrochloride in active pharmaceutical ingredient", *J Pharm Educ Res,2012, 3(2).*
39. Anandkimar R. Tengli, Gurupadayya BM, Neeraj Soni and Vishwanathan B, "Method Development and Validation of Metformine, Pioglitazone and Glibenclamide in Tablet Dosage Form by using RP-HPLC", *Biochem Anal Biochem*, 2 (2) .
40. International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of Analytical Procedure: Methodology (ICH – Q 2B), 1996, 1-8.
41. Madhusudhanareddy Induri, Bhagavan Raju Mantripragada, Rajendra Prasad Yejella, Pavankumar Reddy Kunda, Meechel Arugula, Rajkumar Boddu "Development and Validation of a spectrophotometric method for Quatification and Dissolution studies of Glimipride in tablets",*Tropical J Pharm Res*, 2011, 10(4): 475-81.