RESEARCH ARTICLE

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B. Syama Sundhar, Int.J.A.PS.BMS,2016,Vol.5(1) 027-043

A simple and rapid ESI-LC-MS/MS method for simultaneous estimation of Metformin and Glibenclamide in plasma

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Abstract:

Metformin and Glibenclamide are the combined medication having complimentary mechanisms to improve glycemic control in type 2 diabetes. A quick and high-through LC-MS/MS method has been developed and validated for simultaneous estimation of Metformin and Glibenclamide in human plasma. Liquid-liquid extraction was used for sample preparation and analysis, followed by liquid chromatography tandem spectrometric analysis and an electrospray-ionization interface. Compounds were analyzed on a BDS Hypersil C18 column (250×4.6mm×5µm) column with the mobile phase of pH 4.4 Acetate buffer solution, Methanol in the ratio of 40:60 (v/v) in isocratic condition at a flow rate of 0.8m L/min for 10min. a retention time of 2.91min and 6.07min were observed for Metformin and Glibenclamide respectively. The method was validated as per ICH guidelines as Linearity, precision, accuracy, recovery and different stability studies. All the results obtained were found to be within the acceptance limit. Hence the developed LC/MS/MS method was successfully applied for the determination of Metformin and Glibenclamide in human plasma.

Key Words: LCMS/MS Method, Metformin and Glibenclamide, Plasma, Matrix effect

Drug Introduction:

Metformin

Metformin is a potent anti-diabetic drug belongs to biguanide class used as first line treatment of diabetes mellitus particularly in overweight and obese people and people with normal kidney function [1-3]. 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. It is used in the treatment of gestational diabetes, polycystic ovary syndrome. Metformin is the only anti diabetic drug that has been conclusively shown to prevent the cardiovascular complications of diabetes. It also reduces LDL Cholesrol and triglceride levels and not associated with gaining weight. The mechanism of action of drug by suppresses glucose production by the liver (using hepatic gluconeogenesis mechanism) and decreases hyperglycemia [4-7]. Metformin is available in different forms as tablet, capsule and liquid formulations with different brand names.

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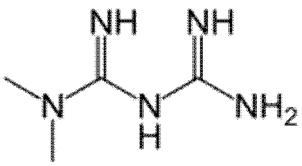


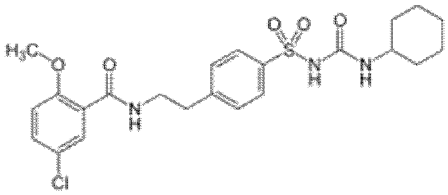


Figure A: Structure of Metformin **Figure B: Structure of Glibenclamide**

Glibenclamide

Glibenclamide acts as anti-diabetic drug belongs to the class of sulfonylureas commonly known as sulfa drugs developed in 1966 [8]. It is also helpful in improving the out coming results on animal stroke models by preventing brain swelling and enhancing neuro protection [9]. The mechanism of action of drug is 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}) [10]. 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. The drug is marketed with different trade names Diabeta, Glynase and Micronase in the United States and Daonil, Semi-Daonil and Euglucon in the United Kingdom and Delmide in India. The drug is restricted to patients suffering with G6PD deficiency as it may cause acute haemolysis [11]. 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 [12]. The Glibenclamide and metformin drugs are available in the market in combination form under the brand name of Glucovance, Benimet and Glibomet.

The literature study reveals that few analytical methods spectrophotometry [13-17], RP-HPLC [18-25] have been established for simultaneous determination the Metformin and Glibenclamide in pharmaceutical dosage forms. The present work is aimed to develop a new LC-MS/MS method for simultaneous estimation of Metformin and Glibenclamide in plasma

Instrumentation:

An HPLC system (Shimadzu, Kyoto, Japan) consisting of anadvance 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 (20mL) 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. Quantization was achieved with MS–MS detection in positive ion mode for both the analytes using a MDSSciex API-4000 mass spectrometer equipped with a Turbo-ion spray TMinterface 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)

Materials and Method:

Chemicals and reagents:

All chemicals and solvents were of analytical grade. HPLC grade Acetonitrile, Methanol and water were purchased from Merck (Mumbai, India). Ammonium acetate, glacial acetic acid, diethyl ether and dichloromethane were obtained in their highest grade from SD fine chemicals limited (Mumbai, India). 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

Preparation of stock and standard solutions:

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. Similarly mg/ml (1000mcg/ml) concentration of *Glibenclamide* was prepared by accurately weighing 10.20mg 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 stock solutions were preserved safely and were used when it required.

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.

Chromatographic conditions:

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.

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.

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.

Method Validation:

To demonstrate the feasibility of the newly developed method, validation was performed in relation to specificity, linearity, LOQ, LOD, accuracy, precision, robustness, and solution stability. These parameters were validated in agreement with the ICH guidelines.

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. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences, but these data were not used to construct the calibration curve.

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 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.

Stock solution stability was determined by comparing the peak areas of freshly prepared solutions (LQC and HQC) with stability samples. Main stock solutions of Metformin and Glibenclamide were freshly prepared and aliquots of stocks were kept at room temperature for 8hours (stability sample). Areas of stability samples and freshly prepared samples were compared to determine mean % nominal concentration during stability period. The mean % change calculated. The areas of stability samples and freshly prepared samples were compared to determine mean % nominal concentration during stability period.

For the Long term stock solutions (LQC and HQC), the working solution of Metformin and Glibenclamide was prepared and stored in the refrigerator at 2-10°C for 11 days. Working solutions of Metformin and Glibenclamide was compared against fresh stock solution prepared. The mean % concentration calculated. The mean % concentration was calculated by comparing freshly prepared and stability samples.

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 prepared solution HQC and LQC solutions were kept 6 hours on bench top (at room temperature, in the presence of ambient light) and in auto sampler. 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 and 12H for bench top and auto-sampler sterilities as per the acceptance criteria.

Results and Discussion:

Sample preparation is an important part in the pharmaceutical analysis, because matrix effects in trace analysis were enlarged, causing loss of sensitivity, abnormal recovery, and analyte instability. Different diluents were evaluated with respect to chromatographic efficiency. Solubility of both Metformin and Glibenclamide were good in methanol. Good response and proper peak shapes were obtained for both drugs when Methanol and Water at a ratio of 8:2 was used as the diluent. Good recoveries (more than 90%) were also observed for both Metformin and Glibenclamide when this solution was used as a diluent. Therefore, Methanol and water in the ratio of 8:2 (v/v) was employed as the diluent throughout the analysis.

The present method was developed by testing different stationary phases to achieve good separation of the peaks. It is important to achieve proper separation among the two components, because of similar chemical. In order to obtain a short analysis time, various analytical columns like Kromasil C18 150 mm × 4.6 mm, 3.5 µm, Hypersil BDS C8 150 mm × 4.6 mm, 3.5 µm and Aquasil-C18 (250×4.6mm×5µm) columns were evaluated. On BDS Hypersil C18 column (250×4.6mm×5µm) column, the separation and responses for both the compounds were found good. On this column, the analytes were well retained and separated from each other. This separation is achieved due to polar group technology that 'shields' the silica residual silanol surface from highly basic analytes; this reduced silanol activity for the symmetry column significantly improved the peak shape and resolution. The possibility of using electrospray ionization (ESI) source under positive ion detection mode was evaluated during the early stage of method development. The signal intensity in positive mode was much higher than that in negative mode. Further, the method development was carried out with ESI source operated in positive polarity mode. The ion source parameters were optimized to get proper response. The representative mass spectra of Metformin and Glibenclamide are shown in Figure 2 and 3 respectively and LC chromatogram of Metformin and Glibenclamide were given in figure 4-7.

The linear calibration curve of Metformin and Glibenclamide were given in figure 8 and 9 respectively. Accurate calibration curve was obtained for both the drugs in the study. Table 1 gives the results of the calibration curve for both the drugs. The precision of the method was evaluated at two levels, viz. repeatability and intermediate precision. 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. The results of the recovery conforms that

the % recovery was found to be 85.576 for Metformin and 85.882 for Glibenclamide in three levels. The results of the recovery were given in table 2 and 3 for Metformin and Glibenclamide respectively.

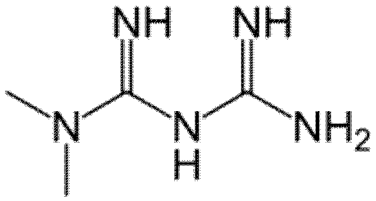
Solution at 594.3ng/ml of Metformin and 149.325ng/ml of Glibenclamide was used for stability study. Solution Stability studies like short term, long term studies were studied. % stability was found to be within the range of more than 98% for both the drugs in short term and long term stability studies. Stability of the drug in biological matrix was studies by bench top, freeze thaw and auto injector stabilities were studied. Accurate range of results was obtained for all the stability studies. Results were represented in table 4-7.

Conclusion:

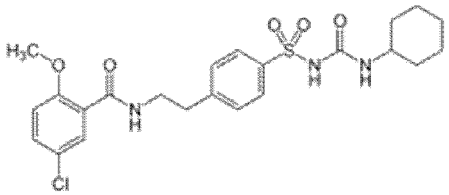
In conclusion, we developed and verified an LCMS-MS method for simultaneously quantifying the Metformin and Glibenclamide in plasma. The sample preparation is cheap and easy for only protein precipitation followed by a liquid–liquid extraction. The LC-MS running time is short to 10 minutes. The method 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. The method presented here could be very useful for the simultaneous estimation of Metformin and Glibenclamide in plasma and other biological samples.

List of figures and Tables:

Figure 1: structure of Metformin and Glibenclamide



Metformin



Glibenclamide

Figure 2: Mass spectrum of Metformin

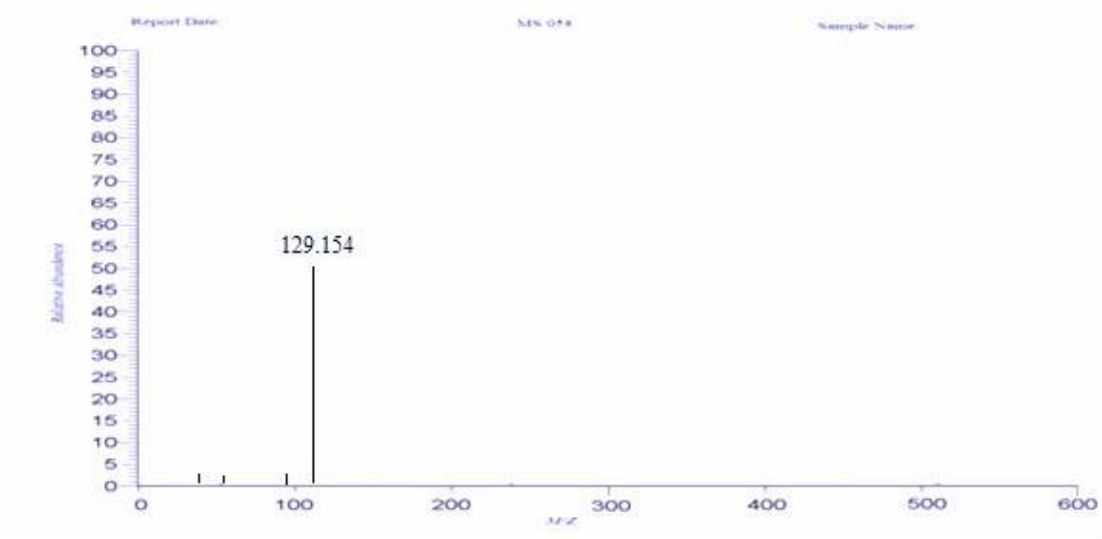


Figure 3: Mass spectrum of Glibenclamide

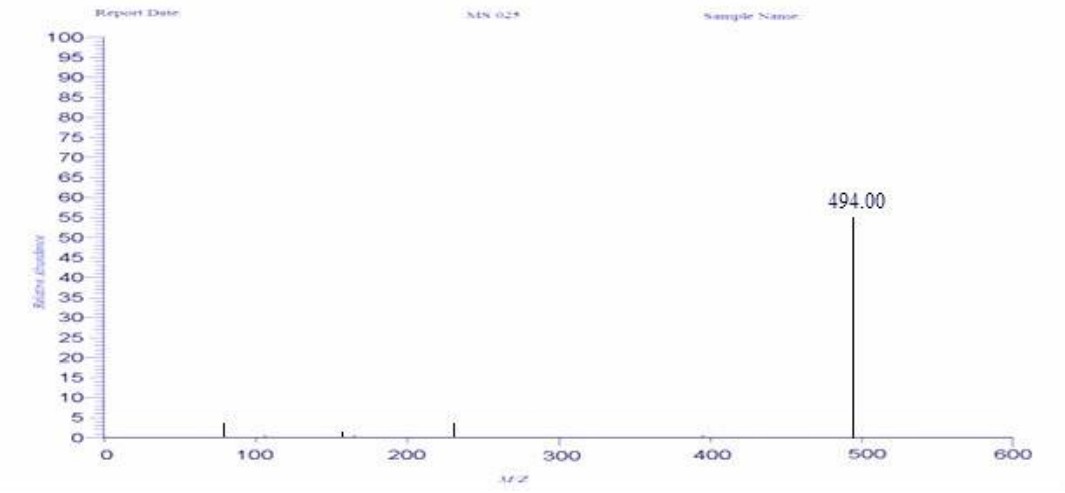


Figure 4: Standard LC chromatogram of Metformin and Glibenclamide

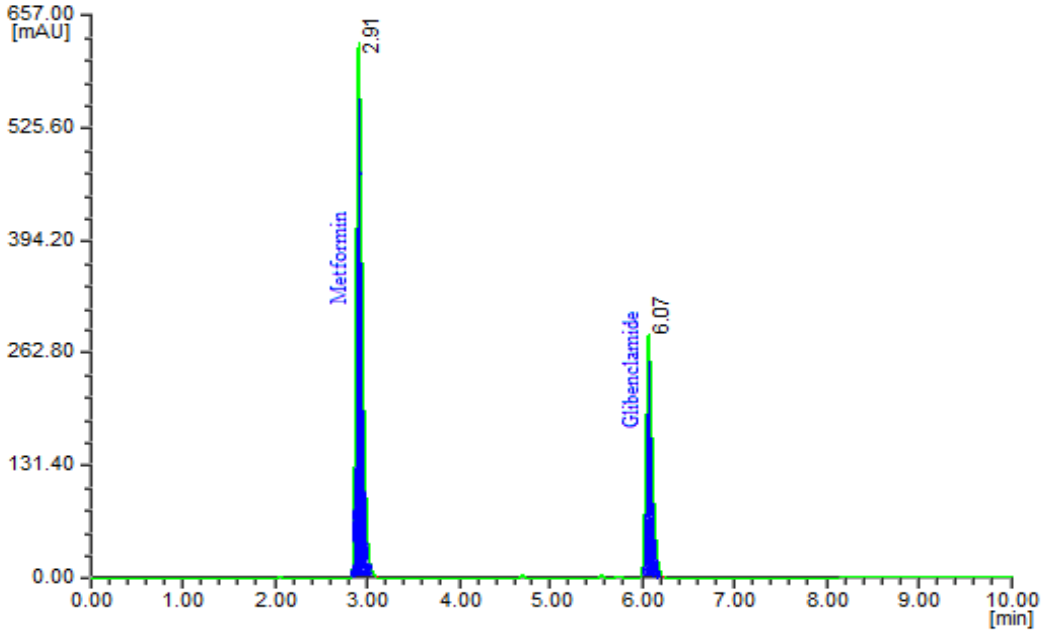


Figure 5: Blank chromatogram of Metformin and Glibenclamide

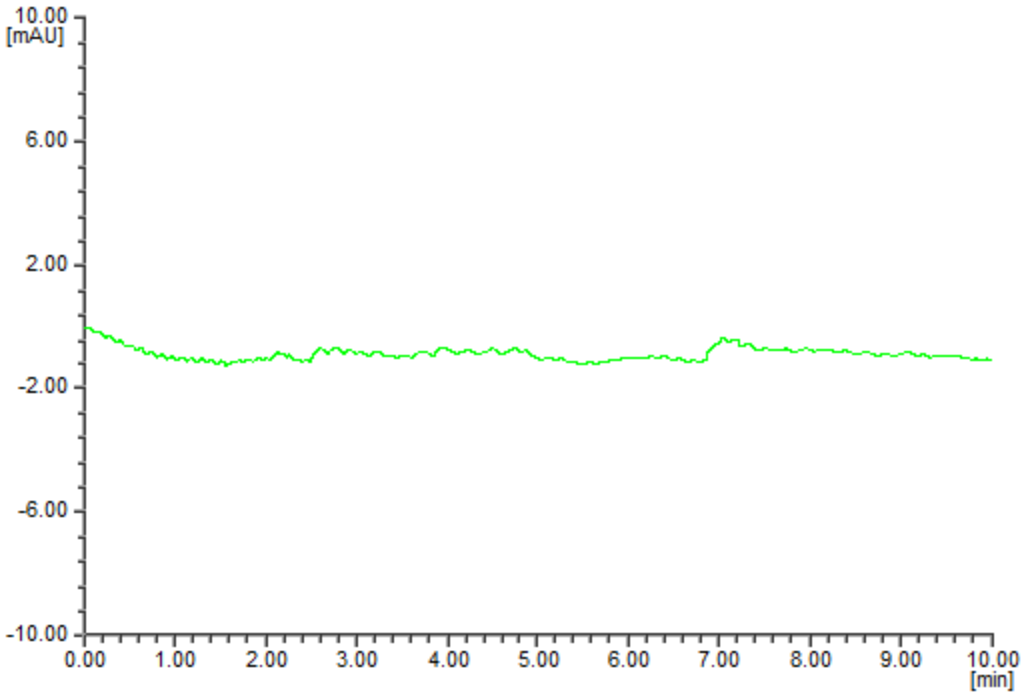


Figure 6: Standard LC chromatogram of Metformin

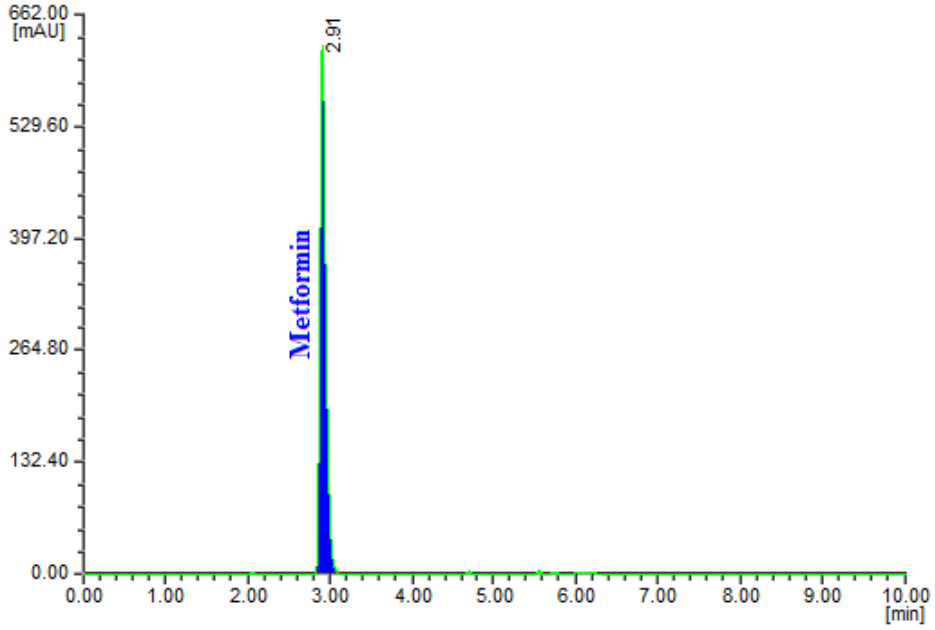


Figure 7: Standard LC chromatogram of Glibenclamide

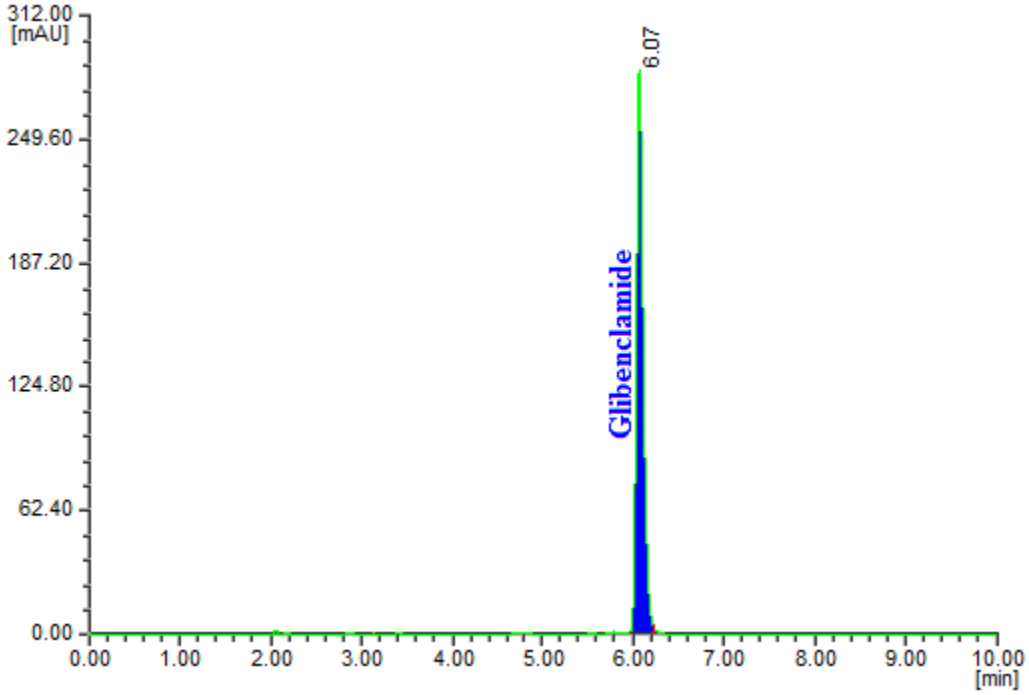


Figure 8: Linearity graph for Metformin

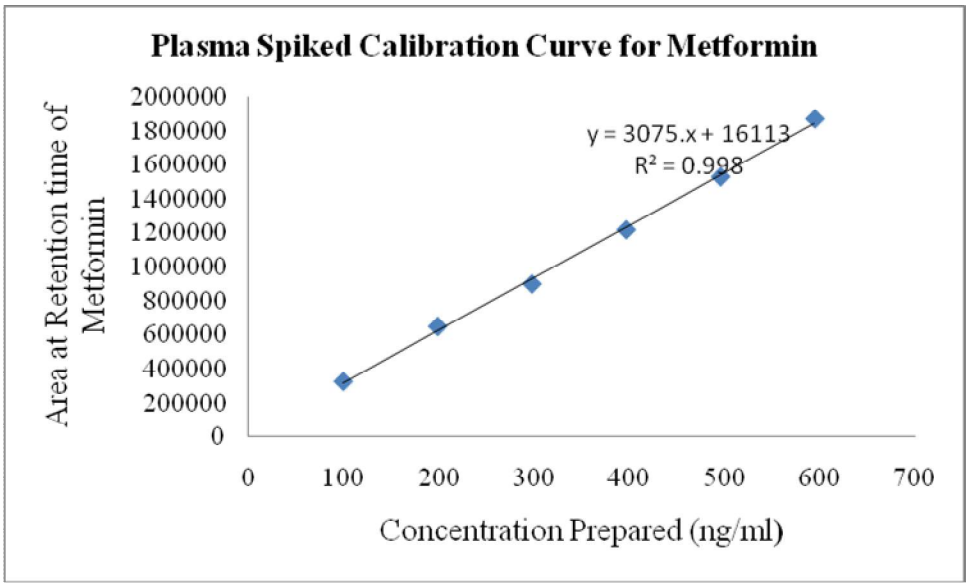


Figure 9: Linearity graph for Glibenclamide

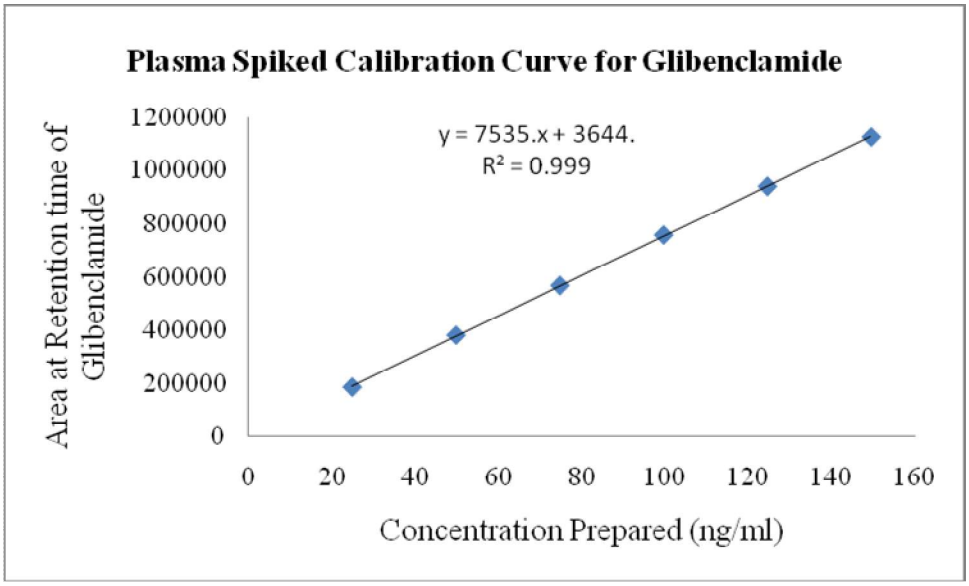


Table 1: Plasma spiked calibration curve

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	

Table:2 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:3 Recovery of Glibenclamide

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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**							

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Table 4: 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		

Table 5: 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

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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**		

Table 6: 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**	

Table 7: 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**	

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