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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF GLIPIZIDE AND MOMORDICININ IN RAT PLASMA

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ABSTRACT: Momordicinin (MMR) is a major triterpenoid compound from *Momordica charantia* Linn fruit having antidiabetic potential, whereas glipizide (GPZ) is a well-known molecule in treatment of diabetes mellitus. The primary objective of the study is to develop and validate a simultaneous RP-HPLC method for upcoming pharmacokinetic studies to analyze herb-drug interactions. The study uses the protein precipitation method for plasma sample preparation. Chromatographic separation of GPZ and MMR was well achieved using hypersil gold C18 column (250 × 4.6, 5µm) and mobile phase constituting acetonitrile: phosphate buffer 10 mM with pH 3.5 (40:60, v/v). Quantification was done at wavelength 223 nm. The method was validated as per USFDA guidelines. The developed method showed good linearity over the range of 7-17 µg/ml, and the lower limit of quantifications was found to be 1.89 µg/ml and 1.99 µg/ml for MMR and GPZ, respectively. The extraction and chromatographic separation of both the drugs was found to be robust and reproducible. The %RSD for validation parameters was within the specified limits as per USFDA guidelines. The developed and validated bioanalytical RP-HPLC method for quantitative measurement of MMR and GPZ simultaneously was found to be accurate, rapid, simple, and reproducible, which will be applicable for future pharmacokinetic studies.

INTRODUCTION: Diabetes is a chronic metabolic disease that occurs either when the pancreas does not produce sufficient insulin or when the body cannot effectively use the insulin it produces. Insulin is a hormone that maintains blood sugar in the body. The common effect of uncontrolled diabetes is Hyperglycemia, or raised blood sugar, over time, leads to serious damage to many of the body's systems, especially the nerves and blood vessels [1-2].

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Oral antidiabetic agents exert their effects by various mechanisms (1) stimulation of beta cells in the pancreas to produce more insulin (sulfonylureas and meglitinides), (2) increasing the sensitivity of muscles and other tissues to insulin (thiazolidinediones), (3) decreasing gluconeogenesis by the liver (biguanides) and (4) delaying the absorption of carbohydrates from the gastrointestinal tract (alpha-glucosidase inhibitors) [3-4].

The limitations of currently available oral anti-diabetic agents either in terms of efficacy/safety coupled with the emergence of the disease into a global epidemy have encouraged a concerted effort to discover drugs that can manage type 2 diabetes more efficiently [5-6]. Also, with the increasing incidence of diabetes mellitus in rural populations throughout the world and due to adverse effects of

synthetic medicine, there is a clear need for the development of indigenous, inexpensive botanical sources for anti-diabetic crude or purified drugs [7-8]. Glipizide (GPZ), chemically known as N-[2 [4[[[(Cyclo-hexylamino) carbonyl] amino] sulfonyl] phenyl] ethyl]-5- methylpyrazine-2-carboxamide is an oral anti-hyper glycaemic agent [9]. Its molecular weight is 455.5 g $mol^{-1,}$ corresponding to the formula $C_{21}H_{27}N_5O_4S$. GPZ is a white or almost white crystalline powder with a melting point of 200-203 ºC, practically insoluble in water, freely soluble in di-methylformamide, very slightly soluble in methylene chloride and in acetone, and practically insoluble in ethanol (96%). It dissolves in dilute solutions of alkali hydroxides. GPZ belongs to the sulphonyl urea class of antidiabetics and is indicated for type II diabetes mellitus [10]. It mainly acts by stimulation of insulin release from β-cells of the pancreas by blocking the ATP- sensitive K+ channels, resulting in depolarization and Ca^{2+} reduction in hepatic glucose production [11-12]. Momordicinin (MMR) (13β,28-epoxy-urs-11-en-3-one) is chemical compound, a triterpene with formula $C_{30}H_{46}O_2$, found in the fresh fruit of Momordicacharantia linn (MC). The compound is soluble in ethyl acetate and chloroform but not in petrol. It crystallizes as irregular plates that melt at 146−147 °C. It was isolated in 1997 by S. Begum [13].

It is believed that diabetic patients may consume herbal products as a home remedy along with prescribed medication. Hence it is important to establish a quantitative method for determination of both antidiabetic drugs GPZ and MMR. No Bioanalytical methods have been published till date for the simultaneous determination of GPZ and MMR. This article focuses on the development of sensitive, specific, and validated bioanalytical methods for GPZ and MMR in order to assess their pharmacokinetic profile in future studies.

MATERIALS AND METHODS:

Chemicals and Reagents: GPZ was obtained as a gift sample from Supra Chemicals Limited, Mumbai, Momordicinin was isolated with 98% purity (by HPLC) [14]. Acetonitrile, potassium dihydrogen orthophosphate (HPLC grade) were purchased from Merck Limited. High purity deionized water was obtained from Millipore, Mili-Q (Bedford, MA, USA) water purification system.

Animals: Male wistar rats weighing 190±10 g were purchased from the National Institute of Biosciences. All rats were placed in cages having an adequate supply of food and water and maintained under controlled room temperature (25 ± 2 ºC) and humidity (60-70%) with day/night cycle (12/12h). The experimental protocol was duly approved by IAEC (Institutional Animal Ethical Committee) of CPCSEA (Committee for Control and Supervision of Experimentation on Animals) through approval number 1703/PO/Re/S/01/ CPCSEA.

Chromatographic Condition: The chromato-graphic separation was performed using Jasco HPLC (AS-4050, PU-2080, UV-2075) and C18 Thermo Hypersil Goldcolumn (250 × 4.6 mm id, 5 µm) along with supelcoguard column (4.6mm × 10 mm, 5 µm). Data acquisition and processing as done using ChromNav software. The mobile phasesodium dihydrogen phosphate buffer, 10 mM (pH 3.5): ACN (60:40 v/v) was eluted with 1ml/min flow rate using isocratic elution. The detection was done at 223 nm. The mobile phase was filtered through a membrane filter (0.45 mm, Millipore) and degassed prior to use.

Calibration Standards and Quality Control Sample Preparation: A stock solution was prepared by dissolving an accurately weighed quantity of GPZ and MMR in methanol to yield a final concentration of 1 mg /ml. The solution was sonicated for 5 min to ensure complete dissolution and allowed to equilibrate to room temperature, after which it was suitably diluted with methanol. From the stock solution, working standards having concentrations 7, 9, 11, 13, 15, and 17 µg/ml of GPZ and MMR were prepared by suitable dilution with methanol. The quality control (QC) samples were similarly prepared at concentrations of 7, 11, and 15 µL/mL for low, medium, and high concentration QC samples, respectively. All solutions were kept refrigerated (-80 ºC) and brought to room temperature before use.

Extraction Procedure of Momordicinin from *Momordica charantia* Linn: MC fruits were procured from the local farmer of Pune. The whole fruit was chopped, sun-dried, and grinded to get fine powder of MC. The dried powder was kept for defatting for 48 h using n-hexane to eliminate

sugars and fats. The marc was then dried to evaporate n-hexane.MC powder was then extracted with 95% alkalinized ethanol using the ultrasound-assisted method for 45 min followed by maceration for 72 h at room temperature. The supernatant was evaporated to dryness using a rotary evaporator to get sticky mass which was coded as EMC (Ethanolic extract of MC).

EMC was further suspended in water to get rid of water-soluble contents. The water-insoluble portion was dissolved in ethanol: water (1:1) and partitioned with ethyl acetate. The ethyl acetate fraction was evaporated to dryness and further injected in HPLC for separation of pure MMR.

Plasma Sample Preparation: The preparation of plasma samples was done by using protein precipitation method. Different solvents like ACN, methanol, ethyl acetate, ACN: methanol (1:1). To the 200 µl drug-free plasma 200 µl of drug solution was spiked. The mixture was vortexed and 400 µl of extracting solvent was added to it followed by vertexing for 2-3 min. The plasma and drug mix were centrifuged for 15 min at 15000 rpm. The supernatant was transferred to another clean tarson microtube and diluted with 400 µl of the mobile phase. The 20 µl of the sample was injected to HPLC for analysis.

Method Validation: The method was validated as per USFDA guidelines involving selectivity, linearity, precision, accuracy, repeatability, robustness, and stability.

Selectivity: The selectivity of the method was determined to check the ability of the method to quantify the analyte in the presence of other components. The selectivity was performed to ensure that the developed method was able to separate GPZ and MRN from the plasma matrix. Six blank plasma samples were injected and compared with plasma samples spiked with GPZ and MRN for any interference.

Linearity and Sensitivity: The calibration curves were constructed with six concentrations ranging from 7 to 17 µg/ml for both the drugs. The linearity was performed by injecting each sample thrice and evaluated by linear regression analysis. The calibration curve was plotted as the concentration of drug vs response at each level.

Limits of Detection (LOD) and quantitation (LOQ): Limits of detection (LOD) and quantitation (LOQ) were estimated from the signal-to-noise ratio. The detection limit was defined as the lowest concentration level resulting in a peak area of three times the baseline noise, and the equation is LOD = 3.3 × (SD)/S. LOQ is the lowest concentration that provides signal to noise ratio more than 10 and the equation is LOQ = 10 × SD/S, where 'SD' is the standard deviation and 'S' is the slope of the line.

Accuracy: The accuracy of an analytical method is defined as the similarity of the results obtained by the analytical method to the true value.16Accuracy was evaluated by multiple analyses of QC samples (7, 11, 15 µg/ml). The known amount of concentration was spiked in blank plasma, and recovery studies were carried out to determine the accuracy of the method. The mean value should be within 15% for all QC samples except LLOQ where the deviation should be within 20%.

Precision: The intra-day and inter-day precision were determined by analyzing three QC samples in triplicate on the same day at different time intervals and in individual days. The mean peak areas and %RSD were calculated. At each level %, RSD should not exceed 15% except for LLOQ.

Extraction Recovery: Extraction recovery is an important parameter in bioanalytical method validation in order to evaluate the capability of solvent to extract maximum drug concentration from plasma matrix. The study compares three QC samples spiked before extraction in plasma with the plasma samples spiked with drug post-extraction.

Robustness: Robustness was performed by deliberately changing the chromatographic condition. The study demonstrates the effect of chromatographic changes on retention time and peak area of the analyte. The deliberate variation was done in the flow rate and wavelength, one factor at a time, and %RSD was recorded for each QC sample.

Stability: The benchtop stability of the stock solution and QC samples spiked in plasma was determined under laboratory conditions. The samples were evaluated after 48 h. In the case of freeze-thaw analysis, samples were stored in frozen condition for 12 h followed by room temperature

for 12 h and again in frozen condition for 12 h. Three cycles were performed, and %RSDs of mean peak areas were calculated to report any deviation in drug sample. Long-term stability was performed after storing the samples at -80 °C for 3 months.

RESULTS AND DISCUSSION:

Method Development: Development of appropriate chromatographic conditions to achieve good separation of analytes includes two main parts sample preparation and optimization of mobile phase. Sample preparation plays an important role in bioanalytical method development to extract maximum drug concentration from plasma. It was found that due to the difference in solubility profiles of GPZ and MMR, the combination of ACN and methanol in 1:1 ratio was suitable to extract both the drugs from the plasma matrix using the protein precipitation method. The highest responses for both the drugs were recorded by UV spectrophotometer, and the results revealed a suitable detection wavelength that is 223 nm.

The various mobile phase composition was executed using ACN, methanol, and buffers like phosphate buffer and OPA. It was observed that peaks obtained from ACN: water and methanol: water proportions were lacking in resolution. Hence after taking full consideration of response and noise, sodium dihydrogen phosphate buffer with pH 3.5: CAN was employed, which showed good resolution of both the drugs with Rt of 4.60 min and 9.02 min respectively **Fig. 1**.

A good separation of both drugs was achieved within 15 min without interference of matrix.

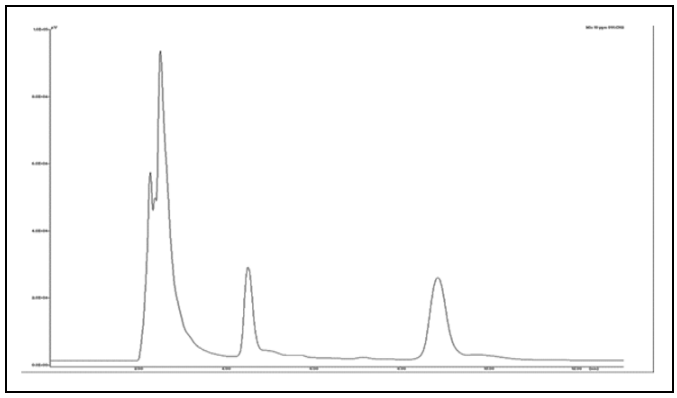

FIG. 1: HPLC CHROMATOGRAM OF MMR AND GPZ IN PLASMA

Method Validation: The RP-HPLC method developed in the present study was well-validated according to, 'Bioanalytical method validation Guidance for Industry' of the USFDA. The selectivity of the method was confirmed by comparing chromatograms from six independent blank samples with spiked samples. There was no interference reported at a retention time of GPZ and MMR.

The standard calibration curve was found linear over a concentration range of 7-17 µL/mL for MMR and GPZ. The correlation coefficients for GPZ and MMR were r = 0.997 and r = 0.998 with linear regression equations, y = 52464x + 359298 and y =32392x + 31170 respectively **Fig. 2**. The regression equation states the linearity degree.

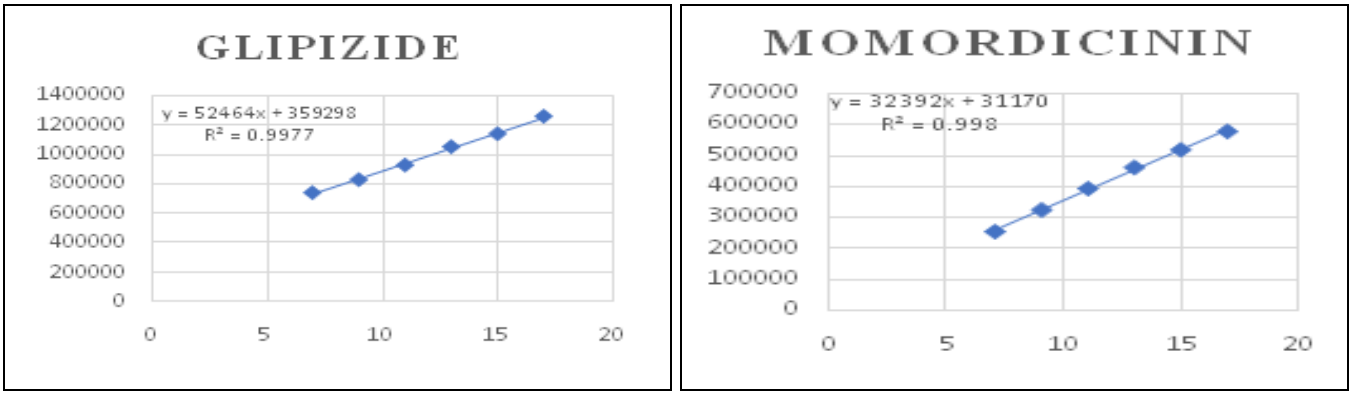

FIG. 2: LINEARITY OF GLIPIZIDE AND MOMORDICININ

The slope was reported from the regression equation, and standard deviation was calculated by statistical method. The values were used to calculate LOD and LLOQ of MMR and GPZ, which were found to be 0.60 µg/ml and 1.89 µg/ml for MMR and 0.65 µg/ml and 1.99 µg/ml for GPZ. The intra-day and inter-day precisions were tested by repeated analysis and were expressed as relative standard deviations (RSD). The intra-day RSD values for MMR and GPZ were found in the range

of 0.13% to 0.35% and0.14% to0.58% and for inter-day RSD values were 0.17% to0.56% to 0.05% to0.16% respectively. The deviations were reported within 15% as shown in **Table 2,** concluding that the developed method is precise. The accuracy was reported across the specified range of the analytical method, which confirmed the closeness of mean test concentration to the actual concentration. The extraction recovery using ACN as an extracting solvent was found to be in the range of 95.12% to 97.5% which were accepted as per USFDA guidelines. The maximum recovery resulted in the accurate quantification of GPZ and MMR from rat plasma. The results obtained from robustness were within the acceptable limit of 20% at LLOQ and 15% at higher concentrations. The % RSD was found to be within limit **Table 3**. The stability was conducted using three QC samples (7, 11, 15 µg/ml) to evaluate the influence of solvent or plasma matrix on active moiety. The result of stability studies is given in **Table 4**. There were no remarkable changes seen in the QC samples after three months, confirming the stability of the analyte in different storage conditions.

TABLE 1: INTRADAY AND INTER-DAY PRECISION AND ACCURACY DATA FOR MMR

Concentration (µg/ml)	Intra day			Inter day		
	Measured concentration	Accuracy	% RSD	Measured concentration	Accuracy	% RSD
7	6.64	94.89	0.13	6.75	96.46	0.17
11	10.96	99.66	0.17	11.01	100.13	0.22
15	14.90	99.33	0.35	15.04	100.32	0.56

TABLE 2: INTRADAY AND INTER-DAY PRECISION AND ACCURACY DATA FOR GPZ

Concentration (µg/ml)	Intra day			Inter day		
	Measured Concentration	Accuracy	% RSD	Measured Concentration	Accuracy	% RSD
7	7.08	101.18	0.58	7.02	100.41	0.05
11	10.31	93.78	0.14	10.74	97.713	0.08
15	14.69	97.98	0.26	14.85	99.01	0.16

TABLE 3: ROBUSTNESS OF GPZ AND MMR

Condition	Concentration (µg/ml)	Mean area		%RSD	
		MMR	GPZ	MMR	GPZ
Change wavelength					
λ221	7	287621.2	701834	0.19	0.88
	11	395197	895222	0.57	0.11
	15	587814.6	1076928	1.61	0.24
λ225	7	229529	764269	0.68	0.98
	11	339611.25	1021057	1.20	0.16
	15	449220	1262115	0.19	0.17
Change in flow rate					
0.8 ml/min flow	7	289200.75	767149	1.08	0.65
	11	439788	1014608	0.88	0.27
	15	574710.4	1252590	0.45	0.21
1.2 ml/min flow	7	230807	676628	1.54	0.45
	11	340409	859698	0.66	0.34
	15	454979	1067057	0.21	0.18

TABLE 4: RESULTS FOR STABILITY STUDY OF MMR AND GPZ

Matrix	Stability conditions	Theoretical Concentration (µg/mL)	Found concentration (µg/mL)		% Recovery		%RSD	
			MMR	GPZ	MMR	GPZ	MMR	GPZ
Plasma	Short term (at RT for 24 h)	7	6.76	7.11	96.62	101.65	1.14	0.20
		11	11.04	10.74	100.43	97.72	0.94	0.46
		15	14.90	14.71	99.38	98.07	0.74	0.28
	Freeze-thaw (in freezer at -30°C to 25°C, 4 cycles)	7	6.67	6.23	95.31	89.07	1.35	0.10
		11	10.82	9.90	98.41	90.04	0.18	0.32
		15	14.76	14.14	98.40	94.28	0.42	0.93

CONCLUSION: The simple, rapid, and sensitive RP-HPLC method was developed and validated for the identification of GPZ and MRN in rat plasma simultaneously. This method serves as an efficient tool for samples in bioanalysis with a simple sample preparation method. This is the first study for estimation of conventional antidiabetic drug glipizide and herb momordicinin at the same time in plasma. The study builds a foundation for future pharmacokinetic studies and evaluation of herb-drug interactions.

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CONFLICTS OF INTEREST: Nil

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