UNDER PEER REVIEW

Development and validation of stability indicating HPLC method for simultaneous determination of antidiabetic drugs metformin hydrochloride and glyburide in tablets

ABSTRACT

Aim: To develop and validate a stability indicating HPLC method for simultaneous estimation of metformin hydrochloride (MET) and glyburide (GLY) in their combined drug product.
Study Design: All variables were studied to optimize the chromatographic conditions.
Place and Duration of Study: Department of Chemistry, Faculty of Science, Aleppo University, Aleppo, Syria during six months.
Methodology: Bromocriptin mesylate (BCM) was used as internal standard. The determination was performed on a Hichrom 5 C8 column (250 × 4.6 mm i.d., 5 μm particle size); The mobile phase, consisting of a mixture of 0.1 M ammonium acetate (pH 5.0) and methanol (23:77, v/v), was delivered at a flow rate of 0.7 mL min^{-1}, and UV detection was at 230 nm.
Results: The retention times were about 4.2, 6.8 and 10.6 min for MET, GLY and BCM, respectively. Linearity ranges were 0.1-300.0 and 0.89-311.0 μg mL^{-1} with limit of detection values of 0.0267 and 0.089 μg mL^{-1} for MET and GLY, respectively. Correlation coefficients (r^2) of the regression equations were greater than 0.999 in all cases. Multicomponent dosage form was exposed to thermal, photolytic, acid, alkali and oxidative stress. The method distinctly separated the drugs and degradation products even in actual samples.
Conclusion: According to the validation results, the proposed HPLC method is simple, specific, precise, accurate and robust. Therefore it is appropriate for routine simultaneous quantitative analysis of MET and GLY in raw material and tablet formulation.

Keywords: Metformin hydrochloride; glyburide; liquid chromatography; pharmaceutical dosage forms.

1. INTRODUCTION

Diabetes mellitus is difficult to control with a single oral hypoglycaemic agent and the rate of mono therapy failure is high. Hence combination therapy with complementary classes of drugs that act on different aspects of glycemic control would be expected to be an effective strategy for the control of diabetes. Metformin hydrochloride is a biguanide hypoglycaemic agent used in the treatment of non-insulin-dependent diabetes mellitus. It is effective in glycemic control and decreases the intestinal absorption of glucose. It is chemically formulated of *N,N*-dimethylimidodicarbonimidic diamide hydrochloride [1]. Glyburide, 1-[[4-[2-

UNDER PEER REVIEW

[(5-Chloro-2-methoxybenzoyl)amino]ethyl] phenyl]sulphonyl] -3-cyclohexylurea, also known as glibenclamide, is a second generation sulfonylurea that is one of the most widely used oral hypoglycemic drugs for noninsulin-dependent diabetic patients and is effective even at very low dosages [1]. These two drugs are formulated as a tablet in a combined formulation. The British Pharmacopeia (BP) stated the nonaqueous titration method for the assay of metformin hydrochloride and glyburide [2]. Few methods for the determination of metformin hydrochloride and glyburide in combined dosage forms including HPLC and spectrophotometry [3,4] and HPTLC [5] have been reported. Metformin hydrochloride has been determined either alone or with other active ingredients in pharmaceutical formulations using spectrophotometry [6-8], high-performance liquid chromatography [9-16], capillary electrophoresis [17,18], HPTLC [19,20] and conductometry [21]. Several methods have been described for the determination of metformin hydrochloride and glyburide either alone or with other active ingredients in biological samples using high performance liquid chromatography [9-11,21-35], capillary electrophoresis [36,37] and voltammetry [38]. HPLC methods are the most convenient techniques because of their inherent simplicity, high sensitivity and wide availability in quality control laboratories. Unfortunately, the HPLC methods that have been reported for the determination of metformin hydrochloride and glyburide in their pharmaceutical formulations were associated with some major disadvantages, such as lack of selectivity, tedious extraction procedures and time-consumption.

The aim of this study was to develop and validate a new rapid, sensitive, economic and environmentally friendly analytical method based on reversed phase-high performance liquid chromatography with a simple composition and low cost of mobile phase, which was used for the first time in this work, for determination of metformin hydrochloride and glyburide present in raw material and tablet formulations, by the addition of internal standard, to improve the analytical performance and thus control undetermined changes in active pharmaceutical ingredient concentration and instrument response fluctuations, and also to reduce the problem of the many-fold dilution required in the classical batch procedures. Moreover, the lower solvent consumption leads to an environmentally friendly chromatographic procedure. The method serves as an alternative to the methods described in pharmacopoeias.

2. EXPERIMENTAL

2.1 Materials

The pure metformin hydrochloride (MET) and glyburide (GLY) powders were obtained from Cadila Health Care Limited, India, and pure bromocriptin mesylate (BCM) powder from Vasudha Pharma Chem Ltd. The structures of these compounds are shown in Fig. 1. HPLC grade methanol and water were purchased from Merck (Germany). Analytical reagent grade ammonium acetate from Merck (Germany) was used to prepare the mobile phase. Tablets were purchased from Syrian market, containing metformin hydrochloride 500 mg and glyburide 5 mg per tablet.

2.2 HPLC system

The chromatographic system consisted of Hitachi (Japan) Model L-2000 equipped with a binary pump (model L-2130, flow rate range of 0.000-9.999 mL min^{-1}), degasser and a column oven (model L-2350, temperature range of 1-85 ^{o}C). All samples were injected (10 μL) using a Hitachi L-2200 autosampler (injection volume range of 0.1-100 μL). Elutions of

UNDER PEER REVIEW

all analytes were monitored at 230 nm by using a Hitachi L-2455 absorbance detector (190-900 nm) containing a quartz flow cell (10mm path and 13 μL volume). Each chromatogram was analyzed and integrated automatically using the Ezchrom Elite Hitachi Software.

2.3 Chromatographic conditions

Separation was achieved on a reversed phase Hichrom 5 C8 column (250 × 4.6 mm, 5 μm particle size, Hichrom Limited, England). The mobile phase was consisted of methanol-0.1 M ammonium acetate adjusted to pH 5.0 (77/23, v/v) and was pumped at a flow rate of 0.7 mL min^{-1}. The mobile phase was filtered through a nylon 0.45 μm membrane filter and degassed by ultrasonic agitation before use. The injection volume was 10 μL. The system was operated at ambient temperature.

2.4 Standard solutions

Standard stock solutions of metformin hydrochloride and glyburide (1.0 mg mL^{-1} of both) were prepared by direct weighing of standard substance with subsequent dissolution in methanol. From these stock solutions the working standard solutions were prepared by further diluting of stock solution using methanol. Standard solution of bromocriptin mesylate (1.0 mg mL^{-1}) was prepared by dissolving appropriate amount of the compound in methanol. These solutions were stored in the dark at 2-8 °C and found to be stable for one month at least.

2.5 Assay procedure for dosage forms

Twenty tablets containing MET and GLY were weighed and finely powdered. Portions of the powder (each equivalent to the weight of five tablets) were accurately weighed into 50 mL volumetric flasks and 30 mL methanol was added. The volumetric flasks were sonicated for 15 min to effect complete dissolution of the MET and GLY, the solutions were then made up to volume with methanol. The sample solutions were filtered through 0.45 μm nylon filter. The aliquot portions of the filtrate were further diluted to get final concentration of 200μg mL^{-1} of MET and 2 μg mL^{-1} of GLY in the presence of 200 μg mL^{-1} of internal standard. Finally, 10 μL of each diluted sample was injected into the column and chromatogram was recorded for the same. Peak area ratios of MET and GLY to that of BCM were then measured for the determination. MET and GLY concentrations in the samples were then calculated using peak data and standard curves.

2.6 Forced degradation studies

To evaluate the stability indicating properties of the developed HPLC method, forced degradation studies were carried out in accordance to the International Conference on Harmonization (ICH) guidelines.

2.6.1 Acid-induced degradation study

3.75 mL methanolic stock solutions of MET and GLY from previously mentioned stock solutions of standard were transferred separately into 25 mL volumetric flask containing 5 mL each of 0.1 M hydrochloric acid. The mixtures were refluxed at 75 ^{o}C for 60 min and the volume was made up with methanol. The forced degradation was performed in the dark to exclude the possible degradation effect of light. The resulting solutions (150 μg mL^{-1} for MET and GLY) were run under optimized chromatographic conditions.

UNDER PEER REVIEW

2.6.2 Base-induced degradation study

3.75 mL methanolic stock solutions of MET and GLY from previously mentioned stock solutions of standard were transferred separately into 25 mL volumetric flask containing 5 mL each of 0.1 M NaOH. The mixtures were refluxed at 75 ^{o}C for 60 min and the volume was made up with methanol. The forced degradation was performed in the dark to exclude the possible degradation effect of light. The resulting solutions (150 μg mL^{-1} for MET and GLY) were run under optimized chromatographic conditions.

2.6.3 Hydrogen peroxide-induced (oxidation) degradation study

3.75 mL methanolic stock solutions of MET and GLY from previously mentioned stock solutions of standard were transferred separately into 25 mL volumetric flask containing 5 mL each of 3% hydrogen peroxide. The mixtures were refluxed at 75 ^{o}C for 60 min and the volume was made up with methanol. The forced degradation was performed in the dark to exclude the possible degradation effect of light. The resulting solutions (150 μg mL^{-1} for MET and GLY) were run under optimized chromatographic conditions.

2.6.4 Thermal (dry heat) degradation study

For dry heat degradation study, the standard powder drugs were placed in an oven at 60 ^{o}C for 24 h. Appropriate dilutions were prepared in methanol and then analyzed under the optimized chromatographic conditions.

2.6.5 Photo-degradation study

For the photo-degradation study, the standard powder drugs were exposed to UV light in a photo-stability chamber for 24 h. Appropriate dilutions were prepared in methanol and then analysed under the optimized chromatographic conditions.

2.7 Method validation

2.7.1 Linearity

A series of working standard drug solutions equivalent to 0.1-300.0 µg/mL for MET and 0.89-311.0 µg mL^{-1} for GLY were prepared by diluting the stock standard solution with the methanol. In each sample 2 mL of BCM was added (200 μg mL^{-1} in the final volume). To construct the calibration curve five replicates (10 µL) of each standard solution were injected immediately after preparation into the column and the peak area of the chromatograms were measured. Then, the mean peak area ratio of MET and GLY to that of the internal standard was plotted against the corresponding concentration to obtain the calibration graph.

2.7.2 Sensitivity

The sensitivity of the method was determined with respect to LOD and LOQ. The minimum level at which the investigated compounds can be reliably detected (limit of detection, LOD) and quantified (limit of quantitation, LOQ) were determined experimentally. LOD were expressed as the concentration of compound that generated a response to three times of the signal to-noise (S/N) ratio, and LOQ was 10 times of the S/N ratio [39]. The LOD and LOQ parameters were determined from regression equations of MET and GLY; $LOD_{(k=3)}=k\times S_a/b$,

UNDER PEER REVIEW

$LOQ_{(k=10)}=k\times S_a/b$ (where b is the slope of the calibration curve and S_a is the standard deviation of the intercept).

2.7.3 Precision and accuracy

Intra and inter-day precision of the methods were determined by performing replicate (n = 5) analyses of standards and samples. This procedure was replicated on different days (n = 5). Recovery studies were performed in view to justify accuracy of the proposed method. Precision was calculated from percentage relative standard deviation (RSD %) for repeated measurements, whereas accuracy was expressed as % of recovery.

2.7.4 Specificity

The specificity of the method was established through study of resolution factor of drug peaks from nearest resolving peak and also among all other peaks. Peak purity of both MET and GLY was assessed to evaluate the specificity of the method. Specificity was also studied by performing the forced degradation study using acid and alkali hydrolysis, chemical oxidation, dry heat and photo degradation studies.

2.7.5 Robustness

Robustness of HPLC method was determined by deliberately varying certain parameters like flow rate, percentage of organic solvent in mobile phase and pH of mobile phase. For all changes in conditions the samples were analysed in triplicate. When the effect altering one set of conditions was tested, the other conditions were held constant at optimum values.

2.7.6 System suitability

The system suitability test was performed to confirm that the LC system to be used was suitable for intended application. A standard solution containing 150 $\mu g\ mL^{-1}$ of MET and 150 $\mu g\ mL^{-1}$ of GLY in the presence of 200 $\mu g\ mL^{-1}$ of internal standard were injected seven times. The parameters peak area, retention time, resolution, capacity factor, theoretical plates, tailing factor (peak symmetry) and % RSD were determined.

3. RESULTS AND DISCUSSION

3.1 HPLC method development and optimization

During the analysis of drugs like MET and GLY, one of the well known problem in pharmaceutical industry is peak tailing. Since these compounds strongly interact with polar ends of HPLC column packing materials, causing severe peak asymmetry and low separation efficiencies. High purity silica backbone and advances in bonding technology have alleviated the tailing problem of polar compounds in HPLC to a significant extent. During the optimization of the method, different columns (Nucleodur C_8, 250 mm × 4.6 mm, 5 μm; Nucleodur C_{18} 250 mm × 4.6 mm, 5 μm; Hichrom 5 C_8 250 mm × 4.6 mm, 5 μm; ODS Hypersil C_{18} 250 mm × 4.6 mm, 5 μm) and two organic solvents (acetonitrile and methanol) were tested. The chromatographic conditions were also optimized by using different buffers like phosphate, acetate and citrate for mobile phase preparation. After a series of screening experiments, it was concluded that ammonium acetate gave better peak shapes than their buffers counterparts. With acetonitrile as solvent both the peaks shows less theoretical plates and more retention time compared to methanol. The chromatographic separation was

UNDER PEER REVIEW

achieved on a Hichrom 5 C_8, 250 mm × 4.6 mm, 5 μm column at 25 °C, by using a mixture of methanol– ammonium acetate (0.1 M, pH 5.0) (77:23, v/v) as mobile phase, and the peak shape of MET, GLY and BCM was found symmetrical (Fig. 1). The retention times of MTF, GLY and BCM were about 4.2, 6.8 and 10.6 min, respectively.

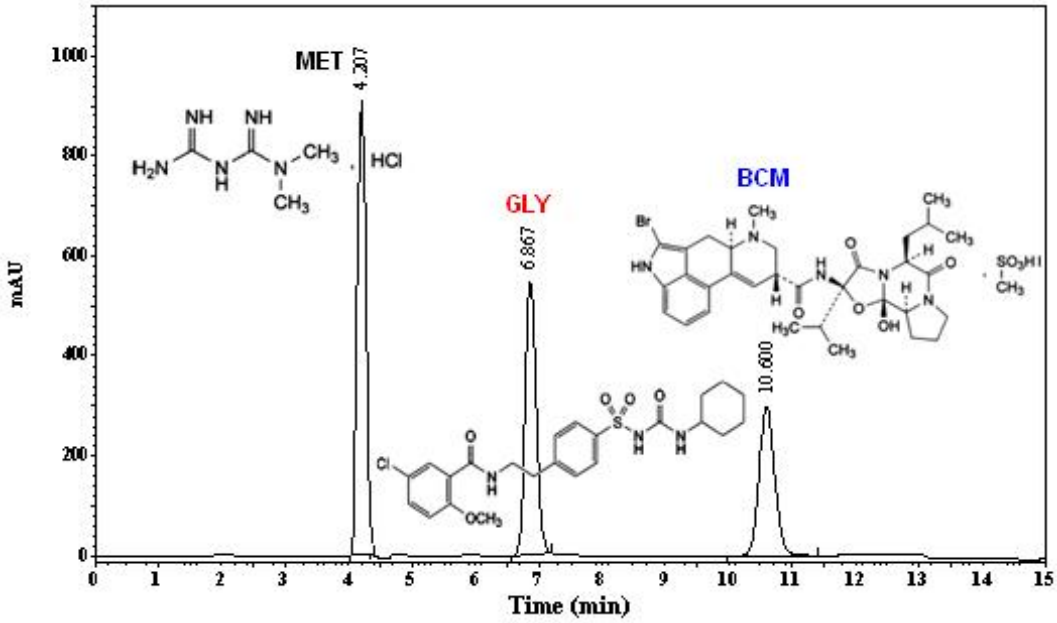

Fig. 1. A typical chromatogram of a mixture of MET (150 μg mL^{-1}), GLY (150 μg mL^{-1}) and BCM (200 μg mL^{-1}) at retention times 4.207, 6.867 and 10.60 min, respectively. Chromatographic conditions: RP-HPLC on C_8 column; mobile phase: ammonium acetate (0.1 M-pH 5.0) /methanol (23/77, *v/v*); flow rate 0.7 mL min^{-1} and detection at 230 nm.

The effect of composition of the mobile phase and flow rate on the retention time of MET, GLY and BCM was investigated. Results of the effect of methanol in the mobile phase are presented in Fig. 2. An increase in the percentage of methanol decreases the retention of compounds, MTF, GLY and the internal standard, BCM. Increasing methanol percentage to more than 85% MET peak is eluted with the solvent front, while at methanol percentage lower than 70% the elution of BCM peak is seriously delayed. The optimum methanol concentration was found to be 77%. The effect of pH in the chromatographic elution of the compounds was also investigated by changes the pH values of the aqueous component of the mobile phase from 3.5 to 6.0. For all experimental pH values, the drugs are eluted in order of MET, GLY and BCM. A value of pH 5.0 was chosen for the optimum separation of the compounds, as at this concentration the analyte peaks were well defined and resolved. The optimum wavelength for detection was at 230 nm, at which the best detector responses for all substances were obtained.

UNDER PEER REVIEW

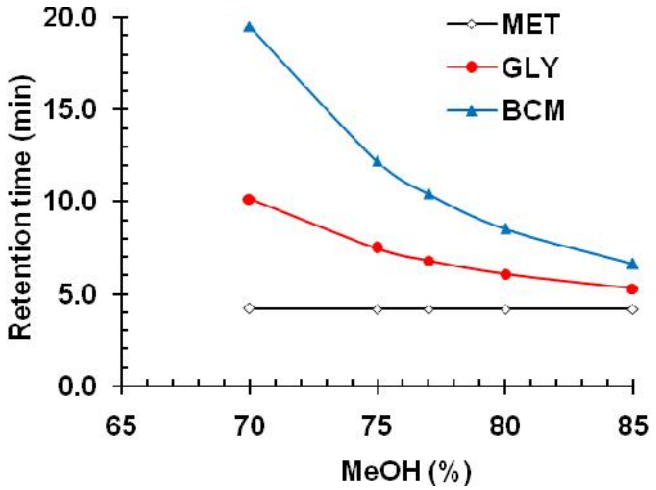

Fig. 2. plot of the retention time *vs*. methanol percentage in the mobile phase of MET, GLY and BCM.

3.2 Accelerated degradation

Forced degradations are performed to provide indications of the stability-indicating properties of an analytical method, particularly when there is no information available about the potential degradation products.

The results from the stress testing studies indicated that the method was highly specific for MET and GLY. The force degradation studies in acid, base, thermal and UV degradation conditions show insignificant degradation for MET and GLY (Fig. 3a–d). The drugs were found to be unstable in peroxide medium. The degradation products of MET were incompletely distinguishable from the parent compound. Peroxide stress leads to 170.11 and 84.24% recovery for MET and GLY, respectively, with two merge degradation peaks of MET and unknown at 4.227 min, whereas a prominent peak of GLY was stable at 6.753 min (Fig. 3e). Table 1 outlines the results of degradation study of MET and GLY at each stress condition.

UNDER PEER REVIEW

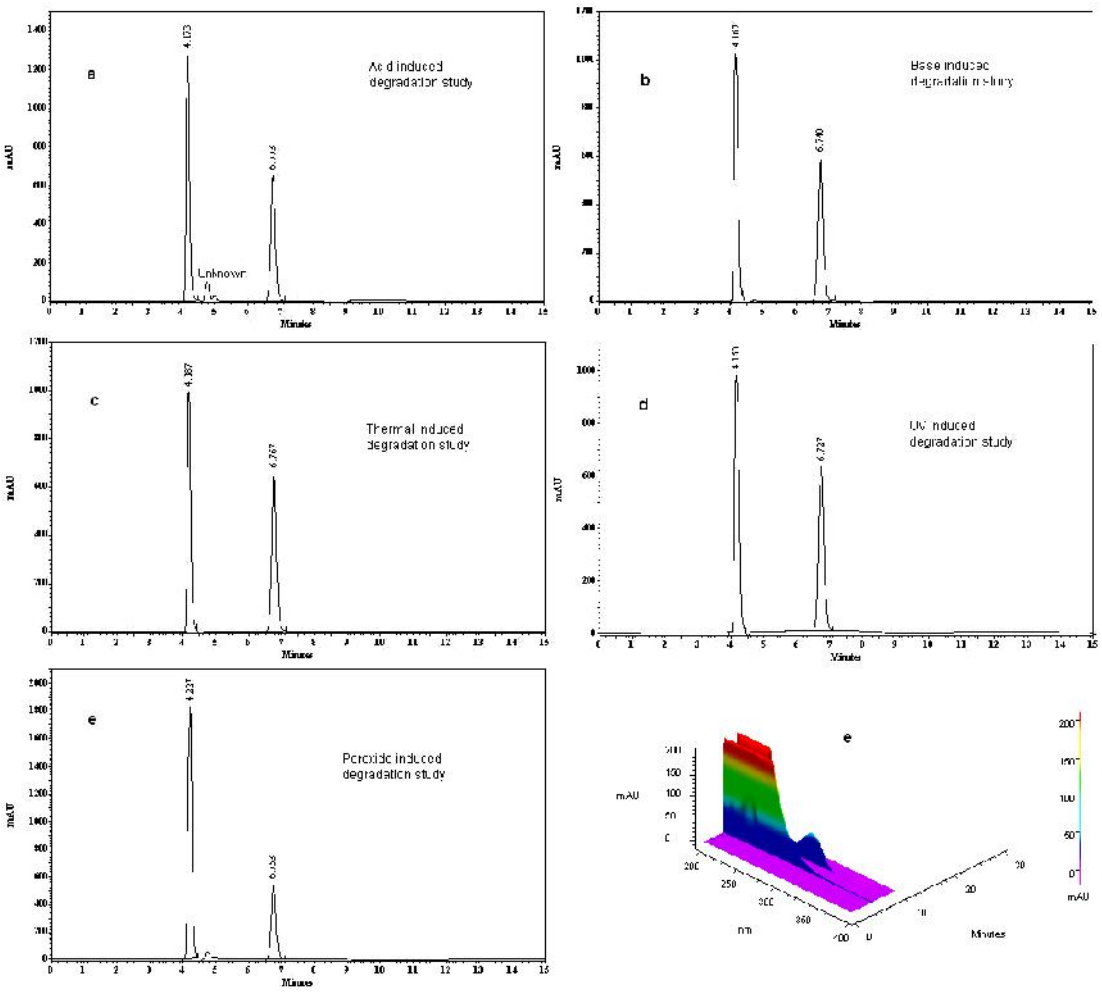

Fig. 3. HPLC chromatograms obtained for MET and GLY from degradation study.

Table 1. Degradation of MET and GLY at different stress conditions.

Mode of degradation	Retention time (min)				Area %	
	MET	MET	GLY	GLY	MET	GLY
Acid	4.207	4.173	6.867	6.773	99.06	99.39
Base		4.187		6.767	98.47	99.74
Oxidative		4.227		6.753	170.11	84.24
Dry heat		4.167		6.740	93.14	96.80
UV light		4.160		6.727	99.56	98.09

3.3 Method validation

The proposed method was validated with the aspect of system suitability, specificity, linearity, LOD and LOQ, accuracy, precision and robustness.

<u>3.3.1 System Suitability</u>

UNDER PEER REVIEW

The system suitability requirements for MET and GLY in the presence of internal standard was a % RSD for peak area less than 0.89, a peak tailing factor less than 1.23 and a R_s greater than 9.0 between adjacent peaks for all analytes. This method met these requirements. The results are shown in Table 2.

Table 2. System suitability parameters.

Parameters	Metformin	Glyburide	Bromocriptin
Theoretical plates (*N*)	4609	7501	9504
Resolution factor [a] (R_s)	--	9.38	9.77
Tailing factor (*T*)	1.23	1.12	1.06
Capacity factor (*k*)	3.17	5.80	9.43
%RSD for seven injections	0.42	0.11	0.89

[a] The resolution factor is calculated between each peak and its nearest preceding neighbor.

3.3.2 Specificity

The specificity of the method was established through study of resolution factor of drug peaks from nearest resolving peak and also among all other peaks. The specificity of the chromatographic method was determined to ensure separation of MET, GLY and the internal standard as illustrated in Fig. 1 where complete separation of MET and GLY was noticed. The HPLC chromatogram recorded for the analytes in tablet (Fig. 4) showed almost no peaks within a retention time range of 15 min. The figures show that MET and GLY are clearly separated and the peak of analytes were pure and excipients in the formulation did not interfere the analytes. Thus, the HPLC method presented in this study is selective for MET and GLY.

3.3.3 Linearity and limits of detection and quantification

For quantitative applications linear calibration graphs were obtained with correlation coefficients of the regression equations greater than 0.999 in all cases and results are summarized in Table 3. Calibration plots were linear for 0.10-300.0 µg/mL and 0.89-311.0 µg/mL for MET and GLY, respectively. Limits of detection (LOD) were 0.0267 $\mu g\ mL^{-1}$ for MET and 0.089 $\mu g\ mL^{-1}$ for GLY, limits of quantification (LOQ) was found to be 0.0892 $\mu g\ mL^{-1}$ for MET and 0.298 $\mu g\ mL^{-1}$ for GLY showed good sensitivity of the proposed method.

Table 3. Calibration data for the estimation of MET and GLY by HPLC.

Parameters	Metformin	Glyburide
Concentration range ($\mu g\ mL^{-1}$)	0.1 – 300.0	0.89 – 311.0
Regression equation	$A_{MTF} = 0.2355C_{MTF} + 0.289$	$A_{GLY} = 0.176C_{GLY} + 0.098$
Correlation coefficient (*n=5*)	0.9994	0.9988
Standard deviation of slope	0.0004	0.0089
Standard deviation of intercept	0.0264	0.0399
Regression equation*	$R_{MTF/BCM}=0.011C_{MTF}+0.014$	$R_{GLY/BCM}=0.008C_{GLY}+0.005$
Correlation coefficient (*n=5*)	0.9996	0.9989
Standard deviation of slope	0.011	0.0013
Standard deviation of intercept	0.0022	0.0083
LOQ ($\mu g\ mL^{-1}$)	0.0892	0.298
LOD ($\mu g\ mL^{-1}$)	0.0267	0.089

*Regression equation for ratio of peak area of drug to that of I.S. *vs*. concentration of drug in $\mu g\ mL^{-1}$.

3.3.4 Accuracy and precision

UNDER PEER REVIEW

The precision and accuracy of the method were determined by analysis of seven samples for drugs mixture. Intra-day assay variation was evaluated by injecting these samples in replicates of five in the same day. Inter-day assay variation was evaluated by injecting these samples in replicates of five on 5 different days from 1 to 30 after preparation. The relative standard deviation and recovery of different amounts tested were determined. Generally acceptable repeatability of the results within one day and day-today was observed. The accuracy of the method is indicated by the excellent recovery and the precision is supported by the low relative standard deviation, as recorded in Table 4.

Table 4. Accuracy and precision of within and between run analysis for the determination of metformin hydrochloride and glyburide by HPLC.

Drug	Nominal concentration ($\mu g\ mL^{-1}$)	*Intra-day (n=5)*			*Inter-day (n=5)*		
		Mean ± SD ($\mu g\ mL^{-1}$)	RSD (%)	Recovery (%)	Mean ± SD ($\mu g\ mL^{-1}$)	RSD (%)	Recovery (%)
Metformin	0.10	0.10±0.002	2.05	100.01	0.10±0.002	2.42	100.03
	2.50	2.50±0.04	1.68	100.26	2.49±0.04	1.53	99.60
	10.00	10.11±0.14	1.40	101.10	10.02±0.11	1.08	100.20
	50.00	51.00±0.28	0.56	102.00	51.12±0.48	0.93	102.24
	100.00	102.63±0.48	0.47	102.63	102.61±0.51	0.50	102.61
	200.00	201.37±0.84	0.42	100.68	200.81±0.62	0.31	100.40
	300.00	302.94±0.18	0.06	100.98	302.15±0.81	0.27	100.72
Glyburide	0.89	0.89±0.01	1.19	100.05	0.90±0.008	0.93	101.12
	2.50	2.52±0.02	0.84	100.80	2.51±0.02	0.65	100.40
	10.00	10.00±0.06	0.62	100.02	9.98±0.05	0.52	99.80
	50.00	50.37±0.30	0.60	100.74	50.52±0.25	0.49	101.04
	100.00	101.57±0.32	0.32	101.57	102.06±0.35	0.34	102.06
	200.00	204.93±0.22	0.11	102.46	204.05±0.39	0.19	102.02
	311.00	312.72±0.28	0.09	100.55	313.47±0.34	0.11	100.79

3.3.5 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness of the method was investigated under a variety of conditions including changes of pH of the mobile phase, flow rate, percentage of methanol in the mobile phase and column oven temperature. The standard solution is injected in five replicates and sample solution of 100% concentration is prepared and injected in triplicate for every condition and % RSD. of assay was calculated for each condition. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters has proven that the method is robust (Table 5).

3.4 Application of the assay

The performance of the proposed methods was assessed by comparison with the official method [4]. Mean values were obtained with a Student's t- and F-tests at 95% confidence limits for four degrees of freedom. The proposed procedures were applied to determine MET and GLY in their pharmaceutical formulations (Fig. 4). The results in Table 6 indicate the high accuracy and precision. As can be seen from Table 6, the proposed methods have the advantages of being virtually free from interferences by excipients such as glucose, lactose

UNDER PEER REVIEW

and starch or from common degradation products. The results obtained were compared statistically by the Student's t-test (for accuracy) and the variance ratio F-test (for precision) with those obtained by the official method for the samples of the same batch (Table 6). The values of t- and F-tests obtained at 95% confidence level did not exceed the theoretical tabulated value indicating no significant difference between the methods compared.

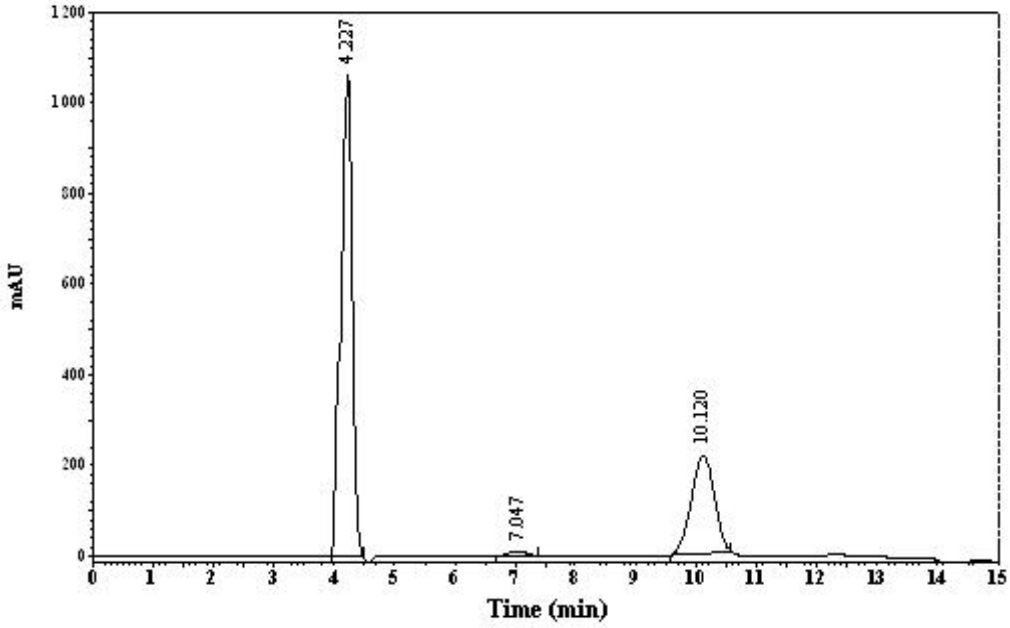

Fig. 4. A typical chromatogram of a mixture of MET (200 μg mL^{-1}), GLY (2 μg mL^{-1}) and the internal standard, BCM (200 μg mL^{-1}) in the mobile phase, prepared from Glicoral tablets. Chromatographic conditions: C_8 column; mobile phase: 0.1 M ammonium acetate and methanol (23:77, *v/v*); flow rate 0.7 mL min^{-1} and detection at 230 nm.

Table 5. Results of robustness study.

Factor	Level	Mean % assay (*n* =3)		% RSD of results	
		Metformin	Glyburide	Metformin	Glyburide
pH of mobile phase	4.9	100.19	100.14	0.82	1.45
	5.1	100.39	101.20	0.16	1.63
Flow rate (mL min^{-1})	0.6	100.85	99.59	0.14	0.12
	0.8	100.95	101.96	0.25	0.02
% of methanol	75	99.88	100.62	0.75	0.26
	80	99.08	99.70	0.05	0.14

4. CONCLUSION

Conclusively, the HPLC-method described in this paper is specific, sensitive, rapid and easy to perform. The proposed RP-HPLC method enables simultaneous determination of mtformin hydrochloride and glyburide using bromocriptin mesylate as internal standard with good separation and resolution of the chromatographic peaks. The sample recoveries from all formulations were in good agreement with their respective label claims, which suggested non-interference of formulations excipients in the estimation. Moreover, the present method is fast with respect to analysis time as compared to sophisticated chromatographic techniques. The method provided excellent specificity and linearity with a limit of

UNDER PEER REVIEW

quantification of 0.0892 and 0.298 μg mL^{-1} and limit of detection of 0.0267 and 0.089 μg mL^{-1} for MET and GLY, respectively. The major advantage of this method is the wide range of linearity.

Table 6. Determination of MTF and GLY in pharmaceutical formulations by the proposed and official methods.

Sample	% Recovery[a] ± SD			
	Metformin hydrochloride		Glyburide	
	Proposed method	Official method	Proposed method	Official method
Pure	100.68±0.86	100.45±0.85	101.17±0.04	100.61±0.05
t-value[b]	1.39	1.05	2.00	2.07
F-value[b]	1.02		1.56	
GLICORAL (500mg MET and 5 mg GLY/tablet)				
Mean±SD[a]	100.58±0.63	100.36±0.49	102.84±1.24	100.54±1.66
t-value[b]	1.87	1.46	1.94	1.79
F-value[b]	1.65		1.79	
GLICLAMET (500mg MET and 5 mg GLY/tablet)				
Mean±SD[a]	100.29±0.72	99.61±0.54	101.70±2.13	102.41±1.81
t-value[b]	1.61	2.02	1.08	1.21
F-value[b]	1.78		1.38	
GLYFORM (500mg MET and 5 mg GLY/tablet)				
Mean±SD[a]	99.71±0.17	100.73±0.11	103.30±1.36	103.02±1.41
t-value[b]	1.29	1.49	1.28	1.72
F-value[b]	2.38		1.07	

[a] Five independent analyses.
[b] Theoretical values for *t* and *F* at five degree of freedom and 95% confidence limit are *t* =2.776 and *F*=6.26.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Sweetman SC. Martindale-The Complete Drug Reference, The Pharmaceutical Press, London, 2007; 35th ed.
2. British Pharmacopœia, HMSO Publication, London. 2013.
3. Salem H. Determination of metformin hydrochloride and glyburide in an antihyperglycemic binary mixture using high-performance liquid chromatographic-UV and spectrometric methods. J. AOAC Int. 2010; 93(1): 133-40.
4. Contreras VU, Meneses-Nava MA, Ornelas-Soto N, Barbosa-García O, López-de-Alba PL, Maldonado JL, Ramos-Ortiz G, Acevedo-Aguilar FJ, López-Martínez L. Fast and environmentally friendly quantitative analysis of active agents in anti-diabetic tablets by an alternative laser-induced breakdown spectroscopy (LIBS) method and comparison to a validated reversed-phase high-performance liquid chromatography (RP-HPLC) method. Appl. Spectrosc. 2012; 66(11): 1294-1301.
5. Ghassempour A, Ahmadi M, Ebrahimi SN, Aboul-Enein HY. Simultaneous determination of metformin and glyburide in tablets by HPTLC. Chromatographia. 2006; 64(1):101-104.
6. Ashour S, Kabbani R. Direct spectrophotometric determination of metformin hydrochloride in pure form and drug formulations. Anal. Lett. 2003; 36(2): 361-370.

UNDER PEER REVIEW

7. Tubino M, Bianchessi LF, Vila MMDC. Quantitative spot-test analysis of metformin in pharmaceutical preparations using ultraviolet-visible diffuse reflectance spectroscopy. Anal. Sci. 2010; 26(1): 121-124.
8. Hegazy MA, El-Ghobashy MR, Yehia AM, Mostafa AA. Simultaneous determination of metformin hydrochloride and pioglitazone hydrochloride in binary mixture and in their ternary mixture with pioglitazone acid degradate using spectrophotometric and chemometric methods. Drug Test. Anal. 2009; 1(7): 339-349.
9. Sultana N, Arayne MS, Shafi N, Siddiqui FA, Hussain A. Development and validation of new assay method for the simultaneous analysis of diltiazem, metformin, pioglitazone and rosiglitazone by RP-HPLC and its applications in pharmaceuticals and human serum. Chromatogr. Sci. 2011; 49(10): 774-779.
10. Arayne MS, Sultana N, Zuberi MH, Siddiqui FA. Simultaneous determination of metformin, cimetidine, famotidine, and ranitidine in human serum and dosage formulations using HPLC with UV detection. J. Chromatogr. Sci. 2010; 48(9): 721-725.
11. El-Gindy A, Nassar MW, El-Abasawy NM, Attia KA, Al-Shabrawi M. Optimization and validation of an RP-HPLC method for direct determination of metformin hydrochloride in human urine and in a dosage form. J. AOAC Int. 2010; 93(6): 1821-1828.
12. Zhanga M, Moore GA, Lever M, Gardinera SJ, Kirkpatrick CMJ, Begg EJ. Development and validation of the liquid chromatographic method for simultaneous estimation of metformin, pioglitazone, and glimepiride in pharmaceutical dosage forms. J. Chromatogr. B. 2001; 766: 175–179.
13. Jain D, Jain S, Jain D, Amin M. Simultaneous estimation of metformin hydrochloride, pioglitazone hydrochloride, and glimepiride by RP-HPLC in tablet formulation. J. Chromatogr. Sci. 2008; 46: 501-504.
14. Lakshmi KS, Rajesh T, Sharma S. Simultaneous determination of metformin and pioglitazone by reversed phase HPLC in pharmaceutical dosage forms, Int. J. Pharm. Pharm. Sci. 2009; 1(2): 162-166.
15. Yardımcı C, Özaltın N. Simultaneous determination of rosiglitazone and metformin in pharmaceutical preparations by LC. Chromatographia. 2007; 66(7): 589-593.
16. Ali MS, Rafiuddin S, Ghori M, Khatri AR. Simultaneous determination of metformin hydrochloride, cyanoguanidine and melamine in tablets by mixed-mode HILIC. Chromatographia. 2008; 67(7): 517-525.
17. Hamdan II, Bani Jaber AK, Abushoffa AM. Development and validation of a stability indicating capillary electrophoresis method for the determination of metformin hydrochloride in tablets. J. Pharm. Biomed. Anal. 2010; 53(5): 1254-1257.
18. Yardımcı C, Özaltın N. Method development and validation for the simultaneous determination of rosiglitazone and metformin in pharmaceutical preparations by capillary zone electrophoresis. Anal. Chim. Acta. 2005; 549: 88–95.
19. Thomas AB, Patil SD, Nanda RK, Kothapalli LP, Bhosle SS, Deshpande AD. Stability-indicating HPTLC method for simultaneous determination of nateglinide and metformin hydrochloride in pharmaceutical dosage form. Saudi Pharm. J. 2011; 19: 221–231.
20. Modi DK, Patel BH. Simultaneous determination of metformin hydrochloride and glipizide in tablet formulation by HPTLC. J. Liq. Chromatogr. Relat. Techno. 2012; 35(1): 28-39.
21. Aboudan M, Ashour S, Aboudan H. Conductometric titration of metformin in pure form and in pharmaceutical preparations using sodium tetraphenylborate and cetylpyridiniumbromide. Asian J. Chem. 2001; 13(1): 1-7.
22. Zhang M, Moore GA, Lever M, Gardiner SJ, Kirkpatrick CMJ, Begg EJ. Rapid and simple high-performance liquid chromatographic assay for the determination of metformin in human plasma and breast milk. J. Chromatogr. B. 2002; 766(1): 175-179.
23. Zarghi A, Foroutan SM, Shafaati A, Khoddam A. Rapid determination of metformin in human plasma using ion-pair HPLC. J. Pharm. Biomed. Anal. 2003; 31: 197-200.

UNDER PEER REVIEW

24. Koseki N, Kawashita H, Niina M, Nagae Y, Masuda N. Development and validation for high selective quantitative determination of metformin in human plasma by cation exchanging with normal-phase LC/MS/MS. J. Pharm. Biomed. 2005; 36: 1063–1072.
25. Chen X, Gu Q, Qiu F, Zhong D. Rapid determination of metformin in human plasma by liquid chromatography−tandem mass spectrometry method. J. Chromatogr. B. 2004; 802: 377–381.
26. Niopas I, Daftsios AC. A validated high-performance liquid chromatographic method for the determination of glibenclamide in human plasma and its application to pharmacokinetic studies. J. Pharm. Biomed. 2002; 28: 653–657.
27. Albu F, Georgit C, David V, Medvedovici A. Determination of glibenclamide in human plasma by liquid chromatography and atmospheric pressure chemical ionization/MS-MS detection. J. Chromatogr. B. 2007; 846: 222–229.
28. Hsieh S, Selinger K. High-throughput bioanalytical method using automated sample preparation and liquid chromatography–atmospheric pressure ion spray mass spectrometry for quantitative determination of glybenclamide in human serum. J. Chromatogr. B. 2002; 772: 347–356.
29. Mistri HN, Jangid AG, Shrivastav PS. Liquid chromatography tandem mass spectrometry method for simultaneous determination of antidiabetic drugs metformin and glyburide in human plasma. J. Pharm. Biomed. 2007; 45: 97–106.
30. AbuRuz S, Millership J, McElnay J. The development and validation of liquid chromatography method for the simultaneous determination of metformin and glipizide, gliclazide, glibenclamide or glimperide in plasma. J. Chromatogr. B. 2005; 817: 277–286.
31. Wang M, Miksa IR. Multi-component plasma quantitation of anti-hyperglycemic pharmaceutical compounds using liquid chromatography–tandem mass spectrometry. J. Chromatogr. B. 2007; 856: 318–327.
32. Li N, Deng Y, Qin F, Yu J, Li F. Simultaneous quantification of metformin and glipizide in human plasma by high-performance liquid chromatography-tandem mass spectrometry and its application to a pharmacokinetic study. Biomed. Chromatogr. 2013; 27(2): 191-196.
33. Chen L, Zhou Z, Shen M, Ma A. Simultaneous determination and pharmacokinetic study of metformin and rosiglitazone in human plasma by HPLC-ESI-MS. J Chromatogr. Sci. 2010; 49(2): 94-100.
34. Zhang L, Tian Y, Zhang Z, Chen Y. Simultaneous determination of metformin and rosiglitazone in human plasma by liquid chromatography/tandem mass spectrometry with electrospray ionization: Application to a pharmacokinetic study. J. Chromatogr. B. 2007; 854: 91–98.
35. Polagani SR, Pilli NR, Gajula R, Gandu V. Simultaneous determination of atorvastatin, metformin and glimepiride in human plasma by LC–MS/MS and its application to a human pharmacokinetic study. J. Pharm. Anal. 2013; 3(1): 9–19.
36. Lai EPC, Feng SY. Solid phase extraction—non-aqueous capillary electrophoresis for determination of metformin, phenformin and glyburide in human plasma. J. Chromatogr. B. 2006; 843: 94–99.
37. Song JZ, Chen HF, Tian SJ, Sun ZP. Determination of metformin in plasma by capillary electrophoresis using field-amplified sample stacking technique. J. Chromatogr. B. 1998; 708: 277–283.
38. Skrzypek S, Mirčeski V, Ciesielski W, Sokołowski A, Zakrzewski, R. Direct determination of metformin in urine by adsorptive catalytic square-wave voltammetry. J. Pharm. Biomed. 2007; 45(2): 275-281.
39. Long GL, Winefordner JD. Limit of Detection. A Closer Look at the IUPAC Definition. Anal. Chem. 1983; 55(7): 712A–721A.