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Development and validation of a new analytical HPLC method for simultaneous determination of the antidiabetic drugs, metformin and gliclazide

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ABSTRACT

An efficient and simple HPLC method has been developed and validated for the simultaneous determination of gliclazide and metformin hydrochloride in bulk and was applied on marketed metformin and gliclazide products. The mobile phase used for the chromatographic runs consisted of 20 mM ammonium formate buffer (pH 3.5) and acetonitrile (45:55, v/v). The separation was achieved on an Alltima CN (250 mm × 4.6 mm x5 μ) column using isocratic mode. Drug peaks were well separated and were detected by a UV detector at 227 nm. The method was linear at the concentration range 1.25–150 μ g/ml for gliclazide and 2.5–150 μ g/ml for metformin respectively. The method has been validated according to ICH guidelines with respect to system suitability, specificity, precision, accuracy and robustness. Metformin limit of detection (LOD) and limit of quantification (LOQ) were 0.8 μ g/ml and 2.45 μ g/ml respectively while LOD and LOQ for gliclazide were 0.97 μ g/ml and 2.95 μ g/ml respectively.

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1. Introduction

Metformin is an effective biguanide antidiabetic agent that has been used to control blood glucose level of type II diabetic patients for decades and has been considered the first line treatment according to international guidelines [1,2]. Mitochondrial inhibition and activation of AMPK are key molecular effects of metformin to inhibit hepatic gluconeogenesis [1,2]. Metformin on the other hand can directly and indirectly improve skeletal muscle sensitivity towards insulin [3].

Gliclazide is a second-generation sulfonylurea which binds to a specific sulfonylurea receptor on the pancreatic β -cells to enhance insulin secretions [3,4]. In addition to its pancreatic effects, Gliclazide can play a significant role in the treatment of diabetic vascular disease through its antioxidant properties [5].

There are several HPLC methods either in pharmaceutical products or biological samples reported in the literature for determination of metformin alone [6,7], gliclazide alone [8,9], metformin with other agents [10–13], gliclazide with other therapeutics [14,15] or metformin and gliclazide together

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[16,17]. Some of these methods used gradient elution to separate the tested analytes [10] [18]. Literature also reveals the use of ion pairing technique [18] [19–21] and micellar liquid chromatography [22] to develop a successful HPLC method for the determination of gliclazide and/or metformin. The reported LODs of metformin in some literature were quite high 12 µg/ml [21], 22.93 µg/ml [23] and the reported sensitivity of other methods in terms of LODs for gliclazide were 1.19 µg/ml [15] and 3.9 µg/ml [24].

The present study is aiming to develop and validate a simple, sensitive, rapid, economic and isocratic HPLC method for the determination of both metformin and gliclazide on the same chromatographic run without the need for derivatization or precolumn treatment.

2. Experimental

2.1. Materials, reagents and pharmaceutical products

Gliclazide >98%, metformin hydrochloride 97% and analytical reagent grade ammonium formate were purchased from Sigma–Aldrich (St. Louis, USA). Metformin® (metformin 500 mg tablets, Sandoz) (Sydney, Australia) and Glyade® (gliclazide 80 mg tablets, Alphapharm) (Brisbane, Australia) were obtained from local pharmacy. Analytical reagent grade formic acid was obtained from Ajax Fine Chem Pty Ltd (Melbourne, Australia). HPLC grade acetonitrile was procured from Fisher Chemical, Thermo Fisher scientific (Melbourne, Australia). A Millipore Milli-Q water ultra-pure water system (Millipore, Australia) was used to obtain distilled water.

2.2. Instrumentation

The HPLC system used for the method development and validation consisted of Shimadzu, Japan equipped with a LC-20AT pump with inline degasser, SPD-20A_{SR} UV detector and SIL-20AC_{HT} Autosampler. Data acquisition, recording and chromatographic integration was performed by Labsolutions version 5.82. Analysis and separation has been done on an Alltima CN column 250 mm × 4.6 mm × 5µ at 227 nm in an air-conditioned lab (temperature maintained at 25 °C throughout all chromatographic runs). The mobile phase consisted of ammonium formate buffer (20 mM), pH 3.5 and acetonitrile at ratio (45:55, v/v), the flow rate was set at 1 ml/min in an isocratic mode and the injection volume was set at 20 µl for all samples.

2.3. Preparation of the buffer solution

A 20 mM buffer solution was prepared by dissolving 1.26 g ammonium formate in 1000 mL Milli-Q water and the final pH adjusted to 3.5 using formic acid. The buffer solution was then filtered through (0.45 Nylon NY membrane filter) and degassed in a sonicator for 10 min.

2.4. Preparation of standard stock solutions (A and B)

25 mg of metformin was accurately weighed and transferred into 100 ml volumetric flask and 20 mL of the mobile phase

mixture was added to metformin and sonicated for 10 min, the final volume was made up to 100 mL using the mobile phase mixture (Flask A). In a separate volumetric flask, the same procedure was followed to dissolve 25 mg gliclazide (Flask B).

2.5. Preparation of working solution (mixture)

An aliquot of 2 mls from flask A and 2 mls from flask B were transferred into 10 ml volumetric flask and the final volume was made up with the mobile phase to give a working solution of metformin (50 µg/ml) and gliclazide (50 µg/ml).

2.6. Preparation of pharmaceutical samples

20 tablets of Metformin® Sandoz were weighed and crushed. 583.35 mg powder equivalent to one Metformin® tablet (500 mg metformin) was placed in a 500 ml volumetric flask (Flask C), 20 tablets of Glyade® Alphapharm were weighed and crushed the same way and 157.3 mg powder equivalent to one Glyade® tablet (80 mg gliclazide) was transferred into a 200 ml volumetric flask (Flask D). 10 mls of distilled water were added to (Flask C) and (Flask D) and sonicated for 10 min and the final volume was made up to the mark of both Flask C and D with mobile phase mixture followed by 5 min shaking. Flask C and D were filtered and separately 10 mls of the filtrate from each flask were transferred into two separate 20 ml volumetric flasks (Flask C and Flask D) and final volume was made to the mark with the mobile phase mixture. An aliquot of 2 mls from Flask C and 5 mls from Flask D were transferred into two separate 20 ml volumetric flasks and mobile phase was added to the mark to produce a final concentration of 50 µg/ml metformin flask and 50 µg/ml gliclazide flask respectively.

2.7. Method development and optimisation

Due to the significant difference in the physical and chemical properties of metformin and gliclazide, several mobile phases and columns were initially trialed in order to have both eluents on the same chromatogram. The suitability of the column and the mobile phase used in the optimized method have been decided based upon the basis of the selectivity, sensitivity as well as acceptable chromatographic parameters of the produced peaks in terms of peak sharpness, peak symmetry, tailing factor and resolution between the two peaks. We used the mobile phase as a solvent for all samples to ensure minimum noise and to eliminate any unwanted solvent peaks.

2.7.1. Columns applied in our initial trials

- Apollo C18 (150 mm × 4.6 mm × 5µ).
- Phenomenex Luna C18 (150 mm × 4.6 mm × 5µ).
- Phenomenex Luna C18 (100 mm × 2 mm × 5µ).
- Phenomenex Jupiter C18 (250 mm × 4.6 mm × 5µ).
- Alltima HP CN (150 mm × 4.6 mm × 3µ).
- Alltima CN (250 mm × 4.6 mm × 5µ) for the optimised method

2.7.2. Examples of buffers trialed either with methanol or acetonitrile, based on previous literature

- Phosphate buffer, different pH values 2.5, 3, 3.5, 5.3,6 and 7.3.
- Acetate buffer pH 3.
- Formic acid 0.05%.
- Ammonium formate for the optimised method.

2.7.3. Selection of UV wavelength

Gliclazide has a λ_{\max} at 228 nm and metformin has λ_{\max} at 234 nm in a water and methanol mixture (60:40) [25]. An acceptable response was obtained upon detection of both drugs at 227 nm either individually or in combination.

2.8. Method validation

The optimized method for simultaneous determination of metformin and gliclazide has been validated as per International Conference of Harmonisation (ICH) guidelines Q2 (R1) [26] for evaluating system suitability, specificity, precision, accuracy, linearity, limit of detection (LOD), limit of quantitation (LOQ) and robustness.

2.8.1. System suitability

System suitability parameters with respect to tailing factor, repeatability, number of theoretical plates and resolution between metformin and gliclazide peaks were assessed by injecting a blank mobile phase followed by six replicates of metformin (50 $\mu\text{g/ml}$)/gliclazide (50 $\mu\text{g/ml}$) mixture.

2.8.2. Precision, repeatability (intra-day precision) and intermediate precision (inter-day precision)

System and method precision were assessed by injecting 6 independent combined samples of metformin and gliclazide (50 $\mu\text{g/ml}$ each) on the same day under same operating conditions.

Intermediate or inter-day precision was assessed by comparing the results of 6 independent determinations on 3 different days.

2.8.3. Specificity/selectivity

The effect of excipients commonly used in our tableting laboratory was checked, where (placebo matrix) was composed of anhydrous lactose NF, Letco medical (Decatur, USA), microcrystalline cellulose NF (Avicel), Letco medical (Decatur, USA), polyvinylpyrrolidone (PVP), Merck Pty Ltd (Melbourne, Australia), Potato starch, Fisher Scientific (Loughborough, UK), and magnesium stearate, BDH laboratories (Poole, UK). The specificity of the proposed HPLC method for the determination of metformin and gliclazide has been established by injecting the mobile phase, placebo matrix extracted solution and the pharmaceutical products (Metformin® Sandoz and Glyade® Alphapharm) into the HPLC system.

2.8.4. Linearity and range

Flask A, the standard stock solution of metformin is diluted in the concentration range of (2.5–150 $\mu\text{g/ml}$). Triplicates of such concentration range were prepared and plotted on a metformin calibration curve.

Flask B, the standard stock solution of gliclazide is diluted in the concentration range of (1.25–150 $\mu\text{g/ml}$). Triplicates of

Table 1 – System suitability and precision results (acceptance limit RSD % < 2).

	Retention time		Tailing factor		Number of theoretical plates		Resolution
	gliclazide	metformin	gliclazide	metformin	gliclazide	metformin	
1	4.117	6.965	1.226	1.166	6827	11,354	12.365
2	4.108	6.965	1.224	1.165	6857	11,399	12.437
3	4.103	6.963	1.23	1.163	6854	11,467	12.482
4	4.098	6.964	1.223	1.159	6888	11,475	12.525
5	4.093	6.964	1.224	1.156	6881	11,506	12.563
6	4.091	6.965	1.23	1.157	6904	11,525	12.591
Mean	4.101	6.964	1.23	1.161	6868.5	11,454.3	12.49
Standard deviation	0.009	0.0008	0.003	0.004	27.79	65.39	0.084
RSD%	0.239%	0.012%	0.255%	0.365%	0.405%	0.571%	0.671%

Precision results as peak area of different determinations on 3 different days, (metformin 50 $\mu\text{g/ml}$, gliclazide 50 $\mu\text{g/ml}$) (n = 6), acceptance limit RSD% <2)

	Day 1		Day 2		Day 3	
	gliclazide	metformin	gliclazide	metformin	gliclazide	metformin
1	2,657,308	4,169,612	2,584,188	4,165,847	2,632,224	4,614,954
2	2,646,317	4,134,736	2,588,337	4,214,888	2,611,676	4,624,234
3	2,641,722	4,133,151	2,587,760	4,159,595	2,625,581	4,650,749
4	2,625,888	4,128,304	2,615,001	4,176,160	2,627,764	4,621,892
5	2,636,926	4,128,241	2,597,393	4,184,036	2,634,300	4,593,732
6	2,631,707	4,110,705	2,596,967	4,207,524	2,648,357	4,600,653
Mean	2,639,978	4,134,124.8	2,594,941	4,184,675	2,629,983.667	4,617,702.333
Standard deviation	11,133.78	19,378.60	11,165.36	22,321.80	12005.91735	20,125.14913
RSD%	0.422%	0.469%	0.430%	0.533%	0.457%	0.436%

such concentration range were prepared and plotted on a gliclazide calibration curve. Slope, intercept and correlation coefficient of the calibration curves (peak area versus concentration) were determined to ensure linearity of the analytical method.

2.8.5. Accuracy study and recovery

Accuracy of the proposed method was confirmed by placebo spiking method, which was carried out by spiking a matrix of (lactose, Avicel, polyvinylpyrrolidone (pvp), starch and magnesium stearate) with metformin and gliclazide separately at 3 different levels 80%, 100% and 120%. Triplicate determinations of these 3 levels have been recorded to obtain the mean and % RSD.

2.8.6. Method sensitivity, LOD and LOQ

LOD and LOQ for metformin and gliclazide were calculated from the linear regression equation based on standard deviation of the intercept and the slope using the formula.

$$\text{LOD} = 3.3 \text{ Q/S and LOQ} = 10 \text{ Q/S}$$

where Q: the standard deviation of the intercept, S: slope of the calibration curve.

2.8.7. Robustness

Deliberate minute variations in the chromatographic conditions such as flow rate, mobile phase composition and pH of the buffer component have been made. These variations were also evaluated for resolution between metformin and gliclazide peaks, number of theoretical plates and tailing factor.

3. Working solution stability

The stability of the gliclazide and metformin mixed solution (50 µg/ml each) was assessed after 24 h in autosampler, after 24 h at room temperature, 25 °C (light protected to minimize possible light degradation) and after a week in the fridge (2–8 °C).

4. Results and discussion

4.1. Method development and optimisation

A volatile, mass compatible ammonium formate buffer was selected in our study due to the following pKa values of both analytes – gliclazide and metformin. Ammonium formate has a pKa of 3.74. The low pH 3.5 selected for the separation falls within the buffering pH range (2.74–4.74) of ammonium formate and as close as possible to its pKa. At this low pH of 3.5, the ionization of the sulphonamide moiety on the gliclazide molecule (weak acid, pKa 5.8) [18] is suppressed. Metformin is a small polar molecule (pKa 2.8 and 11.5, log p Octanol: Water, -2.6) [18].

In all tried C18 columns, metformin was too polar to be retained with gliclazide on the same column with isocratic mode using different mobile phases mentioned earlier. Early trials of reducing the organic proportion in the mobile phase failed to retain metformin on any of the C18 columns used while a significant delay of a wide asymmetric peak of gliclazide has been noticed. The same findings have been reported by others [27] where metformin tends to be eluted

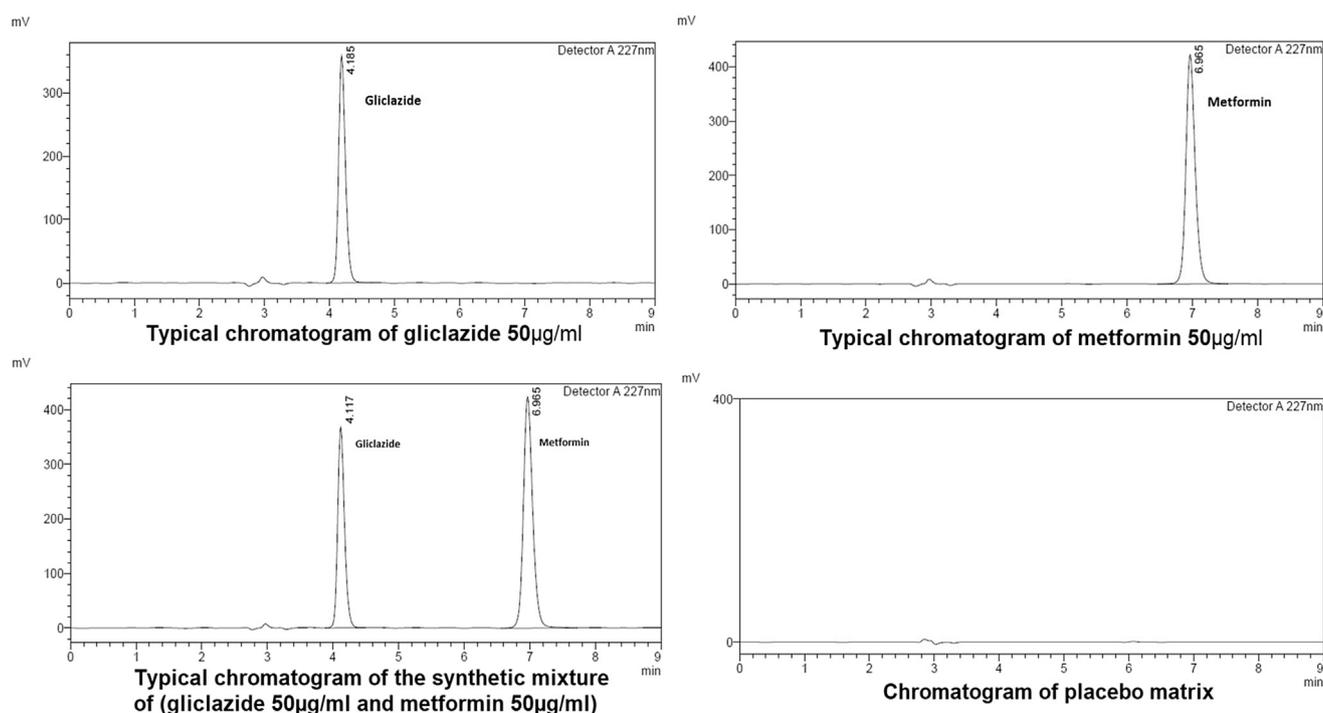


Fig. 1 – Chromatograms of placebo matrix, metformin, gliclazide and typical chromatogram of gliclazide and metformin mixture.

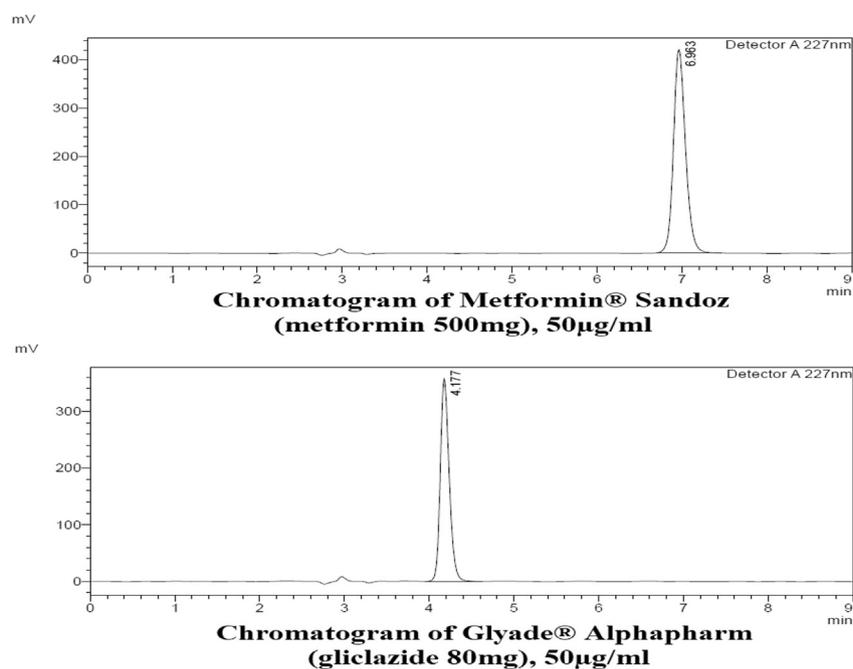


Fig. 2 – Chromatograms of Metformin® Sandoz (metformin 500 mg tablets) and Glyade® Alphapharm (gliclazide 80 mg tablets) both made to an injectable final dilution of 50 µg/ml.

rapidly from conventional C18 columns along with the dead volume of the column. Unretained metformin peak has been explained in another research where the polar metformin was not able to interact with lipophilic chains of the C18 stationary phase [21] and the difficulty in retaining metformin on conventional C18 columns or even with more polar phenyl columns has been reported in another investigation [28]. The authors of this research also commented on the use of other techniques such as cation exchange columns as a way of solving the unretained metformin problem. Additionally, the

method is tedious, and the time consumed is a further limiting factor [28].

Ion pairing [20,21,27,28] and micellar liquid chromatography [22] are alternative techniques to conventional reversed phase chromatography that have been used to retain polar analytes such as metformin either alone or among other therapeutic agents. The time consumed in the preparation stage, mobile phase complexity and in some cases long chromatographic runs [20] are the major disadvantages of these techniques.

Table 2 – Recovery results for gliclazide and metformin (acceptance limit recovery % = 98–102%).

Gliclazide recovery results				
Sample name	Theoretical (claimed) concentration in µg/ml	The concentration found in µg/ml	Recovery %	Statistical data
S1 80%	42	41.76	99.42	Mean = 99.85 Standard deviation = 0.51 RSD% = 0.507%
S2 80%		42.17	100.41	
S3 80%		41.88	99.73	
S1 100%	54.6	54.89	100.54	Mean = 100.4 Standard deviation = 0.4 RSD% = 0.401%
S2 100%		54.57	99.95	
S3 100%		54.99	100.72	
S1 120%	63	62.99	99.99	Mean = 99.7 Standard deviation = 0.46 RSD% = 0.461%
S2 120%		62.48	99.17	
S3 120%		62.96	99.94	
Metformin recovery results				
S1 80%	43	42.65	99.20	Mean = 98.98 Standard deviation = 0.41 RSD% = 0.412%
S2 80%		42.36	98.51	
S3 80%		42.67	99.23	
S1 100%	50.2	50.96	101.52	Mean = 101.19 Standard deviation = 0.52 RSD% = 0.512%
S2 100%		50.93	101.45	
S3 100%		50.50	100.59	
S1 120%	60.2	60.66	100.77	Mean = 100.55 Standard deviation = 0.54 RSD% = 0.538%
S2 120%		60.77	100.94	
S3 120%		60.16	99.93	

The use of cyano columns, a more polar stationary phase than C18 columns, was also tested during the development phase of the proposed analytical method to investigate not only the interaction on a different stationary phase but also the chromatographic separation as well as the resolution between metformin and gliclazide peaks. The elution pattern on CN columns tried was opposite to what was noticed on all attempted C18 columns, where retention time of metformin was delayed on the chromatogram while gliclazide was eluted first using 20 mM ammonium formate buffer (pH 3.5) and acetonitrile. The observed retention times for both gliclazide and metformin were too close to be separated on Alltima HP CN (150 mm × 4.6 mm × 3 μ) in the same chromatographic run, however the separation of either metformin alone or gliclazide alone on such a column could be optimized, so the decision was made to use a longer column yet the same stationary phase (cyano) in our study to have both eluents on the same chromatographic run.

In the present study, the use of Alltima CN (250 mm × 4.6 mm × 5 μ) showed good retention of both metformin and gliclazide and the method was optimized via trying different mobile phase ratios. Eventually, a 20 mM ammonium formate buffer (pH 3.5) and acetonitrile at ratio of (45:55, v/v) gave the best chromatographic results and metformin was retained at 6.9 min while gliclazide was retained at 4.1 min on an isocratic mode without the need for ion pairing, micellar chromatography or even gradient elution with good sensitivity compared to others [12,23,24].

4.2. System suitability

The obtained results of 6 replicate injections showed that the parameters tested were within the acceptable range. Gliclazide and metformin were repeatedly retained and well separated at 4.1 min and 6.9 min expressing excellent resolution between both peaks with RSD% of the recorded retention times <0.3 to

Table 3 – Robustness results for both metformin and gliclazide (acceptance limit RSD% < 2).

Condition	Retention time		Tailing factor		Theoretical plates		Resolution
	glic	metf	glic	metf	glic	met	
Flow rate 0.9 ml/min	4.579	7.666	1.220	1.152	7436	12,148	12.583
Buffer (pH 3.5): Acetonitrile	4.582	7.663	1.213	1.154	7404	12,096	12.534
45:55, v/v	4.580	7.661	1.215	1.155	7435	12,085	12.544
mean	4.58	7.66	1.216	1.154	7425	12,109.67	12.55
Standard deviation	0.002	0.003	0.004	0.002	18.19	33.65	0.026
RSD%	0.033%	0.033%	0.297%	0.132%	0.245%	0.278%	0.206%
Flow rate 1 ml/min	4.151	6.911	1.228	1.160	6744	11,284	11.934
Buffer (pH 3.5): Acetonitrile	4.151	6.910	1.230	1.156	6743	11,264	11.927
45:55, v/v	4.151	6.909	1.229	1.157	6763	11,238	11.925
mean	4.151	6.91	1.229	1.158	6750	11,262	11.93
Standard deviation	0	0.001	0.001	0.002	11.269	23.065	0.0047
RSD%	0.000%	0.014%	0.081%	0.180%	0.167%	0.205%	0.040%
Flow rate 1.1 ml/min	3.761	6.283	1.245	1.169	6135	10,491	11.532
Buffer (pH 3.5): Acetonitrile	3.759	6.283	1.249	1.169	6146	10,514	11.554
45:55, v/v	3.758	6.285	1.248	1.167	6129	10,512	11.560
mean	3.759	6.284	1.247	1.168	6136.7	10,505.67	11.55
Standard deviation	0.002	0.001	0.002	0.001	8.621	12.741	0.015
RSD%	0.041%	0.018%	0.167%	0.099%	0.140%	0.121%	0.128%
Flow rate 1 ml/min	4.335	6.577	1.217	1.165	6882	10,838	9.712
Buffer (pH 3.5): Acetonitrile	4.335	6.577	1.215	1.164	6874	10,827	9.706
47.5:52.5, v/v	4.337	6.577	1.215	1.164	6893	10,821	9.702
mean	4.336	6.577	1.216	1.164	6883	10,828.67	9.707
Standard deviation	0.001	1.09	0.001	0.0006	9.54	8.62	0.005
RSD%	0.027%	0.000%	0.095%	0.050%	0.139%	0.080%	0.052%
Flow rate 1 ml/min	3.945	7.302	1.253	1.152	6478	11,729	14.418
Buffer (pH 3.5): Acetonitrile	3.949	7.297	1.254	1.152	6520	11,724	14.393
42.5:57.5, v/v	3.951	7.3	1.252	1.154	6525	11,721	14.394
mean	3.948	7.3	1.253	1.153	6507.67	11,724.67	14.4
Standard deviation	0.003	0.003	0.001	0.001	25.81	4.041	0.014
RSD%	0.077%	0.034%	0.080%	0.100%	0.397%	0.034%	0.098%
Flow rate 1 ml/min	4.088	6.964	1.231	1.156	6881	11,544	12.602
Buffer (pH 3.4): Acetonitrile	4.084	6.962	1.232	1.157	6885	11,560	12.626
45:55, v/v	4.083	6.96	1.221	1.158	6911	11,588	12.648
mean	4.085	6.962	1.228	1.157	6892.3	11,564	12.625
Standard deviation	0.003	0.002	0.006	0.001	16.289	22.271	0.023
RSD%	0.065%	0.029%	0.495%	0.086%	0.236%	0.193%	0.182%
Flow rate 1 ml/min	4.186	6.96	1.215	1.174	6834	11,147	11.898
Buffer (pH 3.6): Acetonitrile	4.189	6.959	1.215	1.173	6850	11,151	11.886
45:55, v/v	4.186	6.964	1.215	1.175	6875	11,128	11.92
mean	4.187	6.961	1.215	1.174	6853	11,142	11.901
Standard deviation	0.002	0.003	0	0.001	20.663	12.288	0.017
RSD%	0.041%	0.038%	0.000%	0.085%	0.302%	0.110%	0.145%

indicate good repeatability of replicate injections on the integral HPLC system used, the tailing factor for both gliclazide and metformin peaks never exceeded 1.25 in all peaks indicating good peak symmetry (acceptance limit is <2) and the number of theoretical plates were always >2000 in all chromatographic runs to ensure good column efficacy throughout the developed separation process. Results are presented in Table 1.

4.3. Precision

The peak areas obtained following injecting 6 independent combined gliclazide and metformin samples were repeatable and precise over 3 consecutive days. The results for both intra-day and inter-day determinations ensure the high precision and repeatability of the designed method where, all data were expressed in RSD% and never exceeded 0.54% (acceptance limit RSD% <2). Results for intra and inter-day precision are given in Table 1.

4.4. Specificity

The analytical method was able to detect and assess metformin and gliclazide in the presence of placebo matrix of common tablet excipients. The representative chromatogram of placebo, metformin standard, gliclazide standard and typical chromatogram of metformin and gliclazide mixture are shown in Fig. 1. The specificity of the method has been confirmed where the optimized conditions were applied to detect gliclazide and metformin (from manufacturer's excipients) in Glyade® Alphapharm tablets and Metformin® Sandoz tablets respectively, representative chromatograms of gliclazide peak in Glyade® Alphapharm tablets and metformin peak in Metformin® Sandoz tablets are shown in Fig. 2.

4.5. Linearity

The analytical calibration curve constructed for both gliclazide and metformin were linear in the specified ranges, indicated by the closeness of the correlation coefficient R^2 to 1 ($R^2 = 0.9999$). The linear regression equation for gliclazide is ($Y = 45392x + 27194$, $R^2 = 0.9999$) and the linear regression equation for metformin is ($Y = 99511x + 39966$, $R^2 = 0.9999$).

4.6. Recovery

Accuracy of the proposed analytical method was evaluated by determining the added analytes in the placebo matrix in triplicates at 3 different levels (80%, 100% and 120%) and expressed in terms of % recovery of metformin and gliclazide from the spiked matrix. The closeness of the values of found analytes compared to the claimed theoretical concentrations at different levels proved the trueness/accuracy of the proposed method where, metformin and gliclazide $>99\%$ recovered from the spiked excipients. Results for gliclazide and metformin recoveries are shown in Table 2.

4.7. LOD and LOQ

The calculated LOD and LOQ were 0.97 $\mu\text{g/ml}$, 2.95 $\mu\text{g/ml}$ for gliclazide and 0.8 $\mu\text{g/ml}$, 2.45 $\mu\text{g/ml}$ for metformin. The method

sensitivity has been checked practically where experimental LODs were 0.8 $\mu\text{g/ml}$ for both gliclazide and metformin and the experimental LOQs for both agents were 2.4 $\mu\text{g/ml}$.

4.8. Robustness

No significant changes detected upon applying small variations to the chromatographic conditions ensuring that the method is robust to small deliberate changes applied in terms of the flow rate, pH of the buffer used or different mobile phase ratios. In all cases gliclazide and metformin peaks were symmetric (tailing factor <2) and were well separated (resolution >2) and the RSD% of gliclazide and metformin retention times were <0.1 ensuring the robustness of the proposed analytical method to small changes. Results for robustness are presented in Table 3.

5. Solution stability

Gliclazide/metformin sample solution was stable for 24 h in both autosampler and 25 °C (room temperature) and after 1 week in fridge when maintained at 2–8 °C. The stability results have been assessed for the percentage difference from zero-time injections, where no decrease in the peak areas of either gliclazide or metformin have been detected in the mentioned conditions.

6. Conclusion

The presented validated method is rapid, economic, simple, accurate, sensitive, robust, specific and linear. It can be used for routine analysis of metformin and gliclazide either alone or in combination products.

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REFERENCES

- [1] Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, et al. Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. *Diabetes* 2002;2074:51.
- [2] Rena G, Pearson E, Sakamoto K. Molecular mechanism of action of metformin: old or new insights? *Diabetologia* 2013;56:1898–906.
- [3] DeFronzo R. Pharmacologic therapy for type 2 diabetes mellitus. *Ann Intern Med* 1999;281–303.
- [4] Palmer KJ, Brogden RN. Gliclazide. *Drugs* 1993;46:92–125.
- [5] Lee KY, Kim J-R, Choi HC. Gliclazide, a KATP channel blocker, inhibits vascular smooth muscle cell proliferation through the CaMKK β -AMPK pathway. *Vasc Pharmacol* 2018;102:21–8.

- [6] Chhetri HP, Thapa P, Van Schepdael A. Simple HPLC-UV method for the quantification of metformin in human plasma with one step protein precipitation. *Saudi Pharmaceut J* 2014;22:483–7.
- [7] Kar M, Choudhury M. HPLC method for estimation of metformin hydrochloride in formulated microspheres and tablet dosage form. *Indian J Pharmaceut Sci* 2009;71:318–20.
- [8] Talar R, Arshosaz J, Mostafavi A, Nokhodchi A. Development and validation of a novel rp-hplc method for pharmacokinetic studies of gliclazide in rat. *FARMACIA* 2011;59:388–95.
- [9] Ghai D, Ganesh GL. HPLC method for determination of gliclazide in human serum. *Asian J Chem* 2009;21:4258–64.
- [10] Neelima K, Prasad YR. Analytical method development and validation of metformin, voglibose, glimepiride in bulk and combined tablet dosage form by gradient rp-hplc. *Pharmaceut Meth* 2014;5:27–33.
- [11] Lakshmi K, Rajesh T, Sharma S. Simultaneous determination of metformin and pioglitazone by reversed phase HPLC in pharmaceutical dosage forms. *Int J Pharm Pharmaceut Sci* 2009;1:162–6.
- [12] Shirode A, Maduskar P, Deodhar M, Kadam V. Rp-HPLC and HPTLC methods for simultaneous estimation of metformin hydrochloride and vildagliptin from bulk and marketed formulation: development and validation. *Br J Pharmaceut Res* 2014;4:2370–86.
- [13] Soni LK, Narsinghani T, Jain M. Development and validation of RP-HPLC method for simultaneous estimation of metformin hydrochloride and repaglinide in tablet dosage form. *J Liq Chromatogr Relat Technol* 2012;35:385–92.
- [14] Al Mahmud MA, Bhadra S, Haque A, Al Mamun ME, Haider SS. Development and validation of HPLC method for simultaneous determination of Gliclazide and Enalapril maleate in tablet dosage form. *J Pharmaceut Sci* 2015;13:51–6.
- [15] Rathinavel G, Uma Nath U, Valarmathy J, Samueljoshua L, Thanuja CS, Ganesh M, et al. RP-HPLC method for the simultaneous estimation of rosiglitazone and gliclazide in tablets. *J Chem* 2009;6:1188–92.
- [16] Havele S, Dhaneshwar S. Development and validation of a HPLC method for the determination of metformin hydrochloride, gliclazide and pioglitazone hydrochloride in multicomponent formulation. *Webmedcentral* 2010;1, WMC001078.
- [17] Mansoor NM, Jain A. Simultaneous estimation of metformin hydrochloride, pioglitazone hydrochloride and gliclazide by validated rp-hplc method in solid dosage form. *Int J Pharm Pharmaceut Sci* 2012;4:72–6.
- [18] Ranetti M-C, Ionescu M, Hinescu L, Ionica E, Anuta V, Ranetti AE, et al. Validation of a HPLC method for the simultaneous analysis of metformin and gliclazide in human plasma. *FARMACIA* 2009;57:728–35.
- [19] Rao BU, Nikalje AP. Determination of gliclazide in a tablet dosage form in the presence of metformin hydrochloride by ion pair reversed phase liquid chromatographic technique. *Afr J Pharm Pharmacol* 2011;5:1331–7.
- [20] 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 glimiperide in plasma. *J Chromatogr B* 2005;817:277–86.
- [21] Eva T, Leonard D, Gëzim B. Ion-pair HPLC method for the quantification of metformin in human urine. *J Appl Bioanal* 2016;2:16–24.
- [22] El-Wasseef DR. Simultaneous determination of metformin, nateglinide and gliclazide in pharmaceutical preparations using micellar liquid chromatography. *Int J Biomed Sci* 2012;8:144–51.
- [23] Psrcnpv D, Rao AL, Dinda SC. Simultaneous determination of metformin and vildagliptin in solid dosage form by stability indicating RP-HPLC method. *Int Res J Pharm* 2013;4:122–8.
- [24] Gumieniczek A, Berecka A, Pietras R, Ślebioda M. Stress degradation study of two oral antidiabetics, gliclazide and glipizide, and chemical analysis by LC and LC/MS methods. *Open Chem* 2014;12:80–9.
- [25] Dhabale P, Seervi C. Simultaneous UV spectrophotometric method for estimation of gliclazide and metformine hydrochloride in tablet dosage form. *Int J Chem Tech Res* 2010;2:813–7.
- [26] Guideline IHT. Validation of analytical procedures: text and methodology Q2 (R1). In: International conference on harmonization, Geneva, Switzerland, vol. 4; 2005. p. 1–13.
- [27] Vasudevan M, Ravi J, Ravisankar S, Suresh B. ION-pair liquid chromatography technique for the estimation of metformin in its multicomponent dosage forms. *J Pharmaceut Biomed Anal* 2001;25:77–84.
- [28] AbuRuz S, Millership J, McElnay J. Determination of metformin in plasma using a new ion pair solid phase extraction technique and ion pair liquid chromatography. *J Chromatogr B* 2003;798:203–9.