RESEARCH ARTICLE

Comparative Studies on Simultaneous Estimation of Metformin, Empagliflozin, Remogliflozin, Glimepride and Gliclazide by using RP-HPLC and Diol Technique

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ABSTRACT

Retaining extremely polar metformin and its combination with other non-polar antidiabetic drugs can be difficult and challenging by using the most common reverse-phase high-performance liquid chromatography (RP-HPLC). The retention time of metformin is comparatively short when separated, utilizing C18-based RPC. Peak fronting and tailing effects are commonly observed with metformin (MET), its combination with highly polar antidiabetic medicines, and early elution with void volume. However, normal phase chromatography may occasionally be rendered ineffective due to the low solubility of polar analytes in organic solvents. Therefore, a different and complementary strategy is required for the separation of such a highly polar drug. A more advantageous platform is mixed-mode chromatography. The use of this newer technique of separations has been increased because of its advantages over C18-based RPC for its efficiency, selectivity, sensitivity, specificity and reproducibility towards simultaneous quantification of selected drugs. In this study, we compared two established chromatographic methods for simultaneous quantification of metformin and other moderately polar antidiabetic medications, including empagliflozin, remogliflozin, glimepride, and gliclazide, both in their pure form and in combination tablet dosage. An isocratic elution based on solvent a 15 mM ammonium acetate-Methanol: acetonitrile (10:90 v/v) was used as the mobile phase in first method, which is reversed-phase HPLC with a Zodiac C18 column and chromatographic separation on a 150 × 4.6 mm acclaimed mix mode HILIC-1 column with a particle size of 5 µm. In accordance with ICH standards, both approaches underwent exhaustive validation for linearity, accuracy, precision, selectivity, and resilience. Metformin, empagliflozin, ramogliflozin, glimepride, and gliclazide have not yet been reported to be separated using any method.

Keywords: Mixed mode chromatography, Acclaim mixed mode HILIC-I Column.

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INTRODUCTION

Reverse-phase liquid chromatography (C18) is widely utilized to separate pharmaceutical formulation components. It was necessary to develop an alternative technique for the separation of mixtures containing extremely hydrophilic, hydrophobic, ionizable, and neutral drug moieties because metformin is too polar to retain on an ODS column because polar pharmaceutical amines having negative charges cannot retain in ion-pairing mode and their hydrophilic properties reduce their binding capacities to ODS.[1,2] Mixed-mode chromatography may be a more effective platform when using traditional reverse-phase liquid chromatography (RPLC) to retain highly polar medicinal components. Because of its improved speed, resolution power, specificity, reproducibility of results, and tolerance capacity for high sample concentrations, this more recent method of separations is being used more frequently.[3,4] The packing material utilized in the Acclaim Mixed-Mode HILIC-1 column is silica-based and has the ability to perform both RP and HILIC thanks to its alkyl long chain and hydrophilic polar terminal (Figure 1).[5,6] Separation of many different types of molecules, including polar and non-polar ones, shows promising results when using this packing medium. For individuals who are overweight and have type II diabetes mellitus, a condition defined by insulin secretion and sensitivity abnormalities, the medicine of choice is metformin HCL, which is taken orally pages.[7–9] The kidneys reabsorb glucose when given the powerful selective SGLT2 inhibitor empagliflozin.[10-12] The sodium-glucose transporter two inhibitor remogliflozin lowers blood sugar levels by facilitating the excretion of extra carbohydrates in urine.[13-15] Those who suffer from type

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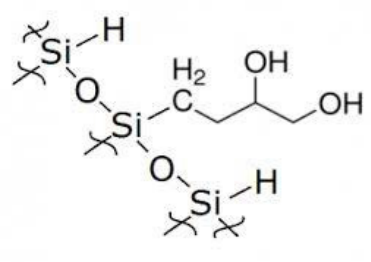

Figure 1: Diol stationary phase

2 diabetes mellitus are typically prescribed glimepiride, a sulfonylurea that is used as an oral antidiabetic medication. With its antidiabetic and antioxidative properties, gliclazide is a sulfonylurea drug that blocks sulfonylurea 1 (SUR1) receptors. Receptor inhibition causes K+ ion channels to close, which causes cell membrane depolarization, changes Ca^{2+} homeostasis, and stimulates insulin secretion. By reducing oxidative stress, gliclazide prevents mitochondrial damage and apoptosis caused by H_2O_2 *in-vitro*[16-18] (Figure 2).

MATERIALS AND METHODS

Materials

Drugs and chemicals

Yarrow Pharma Ltd. provided gift samples of reference standards, which include metformin, empagliflozin, remogliflozin, glimeperide, and gliclazide. We bought ammonium acetate from Merck Ltd. in Mumbai, India. We bought deionized water and HPLC-grade acetonitrile from Merck (Mumbai, India). Everyone else's chemicals and reagents were of HPLC quality.

Instrumentation

The whole investigation was carried out utilizing the Shimadzu SCL-10A VP HPLC system. The package includes an LC-10AT VP binary pump, an SPD-10A VP UV detector, and a P/N 77251 manual Rheodyne 20 µL loop capacity injector. The LC-Solution software was employed to interpret the HPLC results. An illustrious Ultrachrom Innovatives Pvt. Ltd. Mix-Mode HILIC-1 column, measuring 5 µm and having an internal diameter of 150 x 4.6 mm, was used throughout the study. The ultrasonicator Labman ® was acquired from UltraChrom Ltd. in India, and the digital weighing balance was acquired from Mettler Toledo. The Mettler-Toledo digital pH meter was bought in Mumbai, India. & 50 µ microsyringes from Hamilton, USA. Also, nylon membrane filters with 0.20 and 0.45 µ dimensions

Methods

Standard stock solutions

Metformin, empagliflozin, remogliflozin, glimeperide, and gliclazide were all made individually as standard stock solutions containing 1-mg/mL by dissolving in 10 mL of acetonitrile-methanol water in ratio (3:5:2 v/v) in 20 mL volumetric flask. Additionally, in order to execute validation experiments such as repeatability, precision, and robustness

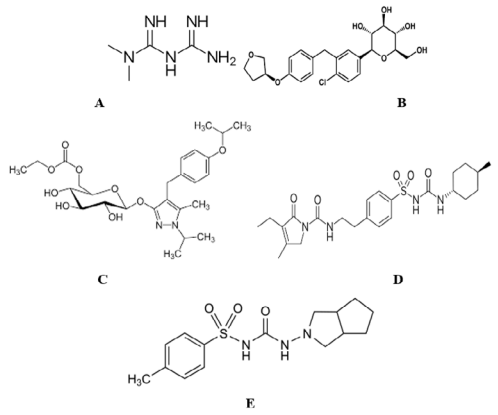

Figure 2: Chemical structures: A- Metformin; B- Empagliflozin; C- Remogliflozin; D- Glimepride; E- Gliclazide.

studies, freshly manufactured standards were combined to obtain the concentration of metformin (20 ppm), empagliflozin, remogliflozin, glimeperide and gliclazide (100 ppm), respectively. Before the HPLC analysis, the standard stock solution was ultrasonically sonicated for ten to twenty minutes and then filtered using 0.20 µ nylon filters.

Sample preparation

A total of 5 identical gubtulio-Met tablets, every containing 500 mg of metformin and 12.5 mg of empagliflozin manufactured by Lupin Laboratories, were individually weighed in order to calculate their average weight and it was ground into fine powder in a mortar pestle. A 10 mL mixture of acetonitrile, methanol, and water (4:4:2) was spiked with a precisely measured amount of the finely ground substance equivalent to 10 mg. Following an ultrasonication, a 0.45 µ nylon filter was used to filter it. Additionally, successive dilutions were performed to get the final concentration of 200 ppm of metformin and 5 ppm of empagliflozin. Following sonication, the solution was examined using the chromatographic conditions specified in the experimental section. By measuring and grinding each tablet into a fine powder and then placing them in the pestle and mortar, we were able to determine the average weight of five identical Reniva-M tablets, which encompass 500 and 100 mg of metformin remogliflozin correspondingly. Add 10 mL each of acetonitrile, methanol, and water to 10 mL of the finely powdered substance; stir until dissolved. It was then ultrasonically filtered using a 0.45 µ nylon filter. Both remogliflozin and metformin were serially diluted until a final concentration of 20 and 100 ppm, respectively, was reached. By weighing each Glimestar-M tablet independently, we were able to calculate their average weight. Metformin 500 mg and glimepiride 1-mg were the active ingredients in each tablet. Following mixing, a mortar and pestle were used to crush the ingredients into a fine powder. The concentration of the finely ground material was 10 mg, which was accurately measured in 10 mL of a 3:5:2 mixture of acetonitrile, methanol, and water.[19]

After ultrasonication, it was filtered using a 0.45 µ nylon filter. Further, by carrying out successive dilutions, the target

concentrations of 0.5 ppm glimeperide and 250 ppm metformin were reached. Subsequently, the solution underwent sonication and analysis. By measuring each tablet separately, we were able to determine the average weight of five Diamecron-XR pills, which contain 500 mg of metformin and 60 mg of gliclazide. After being mixed, they were pounded into a fine powder using a mortar and pestle. Ten milliliters of a mixture of acetonitrile, methanol, and water at a ratio of 3:5:2 contained a precisely measured amount of the finely ground substance equal to 10 mg. A 0.45 µ nylon filter was utilized for filtering it after ultrasonication. Furthermore, several dilutions were made to get ultimate concentrations of 12 ppm gliclazide and 100 ppm metformin. The sonication and examination of the solution followed the steps outlined in the experimental section.[20]

Validation Study

- *Sample preparation for linearity/calibration studies*

To make a similar mixture of each medication, standard stock solutions were mixed to yield the following concentrations: 50 to 3.5 µg/mL for metformin, empagliflozin, remogliflozin, glimeperide, and gliclazide; and 10 to 0.65 µg/mL for metformin. Samples were then ultrasonically purged, and chromatographic conditions designated in the experimental section were employed for analysis. Regression coefficient (R^2), LoD, and LoQ were resolute by plotting peak area *vs* known concentration on the calibration curve.

- *Precision studies of the proposed method*

Following homologous mixture was examined a total of three times in a single day (intraday precision) and three times in a subsequent day (intermediate precision) using chromatographic conditions: 20 ppm metformin, 100 ppm empagliflozin, 100 ppm remogliflozin, 100 ppm glimeperide, and 100 ppm gliclazide. The numbers were then averaged and their respective standard deviations and percentages were used to determine RSD.[21]

RESULT AND DISCUSSION

Selection of Analytical Wavelength

Before the HPLC analysis, the selected medications were first subjected to ultraviolet (UV) analysis using a wavelength range of 210 to 350 nm. There is a statistically significant peak at 230 nm for both remogliflozin and metformin as well as both empagliflozin and glimeptride. Crucially, gliclazide demonstrates considerable absorption at 230 nm, but its maximum UV absorbance (λ_{max}) limit is 290 nm. In order to identify all of the medicines at once, a wavelength of 230 nm was employed (Figure 3).

Chromatographic Conditions Octadesylane (C18) Column

Chromatographic separation was completed on a Zodiac C18 using 5, 5 µ, 150 x 4.6 mm columns. The isocratic elution was

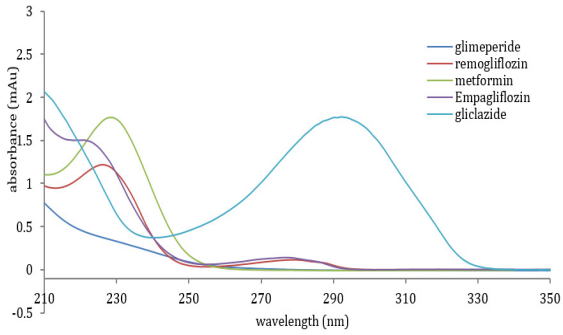

Figure 3: Overlay UV spectra of all selected antidiabetic drugs

Table 1: Results of separations by using octadesylane (C18) column

Peak#	*Retention time*	*Area*	*Area%*	*T. plate#*	*Resolution*	*k'*	*Tailing F*	*Separation*
Metformin	2.219	11907787	21.6242	1823.602	--	0	1.691	0
Remogliflozin	19.756	13544022	24.5955	96792.19	0.635	7.902	--	1.011
Empagliflozin	18.413	9746174	17.6988	98522.95	73.191	7.297	1.26	0
Gliclazide	22.431	10506880	19.0802	99564.16	4.32	9.108	0.995	1.061
Glimeperide	21.271	8380684	15.2191	113378.9	5.98	8.585	1.3	1.086

Table 2: Results of separations by using the diol column

Peak#	*Retention time*	*Area*	*Area%*	*T. Plate#*	*Resolution*	*k'*	*Tailing F.*	*Separation*
1	2.225	20507	0.2734	5388.319	--	0	1.256	0
2	2.46	122501	1.6331	4415.097	1.739	0.105	1.142	0
Metformin	3.350	454518	6.0594	2748.049	4.408	0.505	1.543	4.798
Empagliflozin	5.77	902242	12.0283	2622.471	6.851	1.592	1.057	3.152
Remogliflozin	7.33	958349	12.7763	3512.615	3.319	2.297	1.005	1.443
Glimeperide	8.90	336701	4.4887	4613.817	3.095	3.006	0.971	1.309
Gliclazide	13.32	4706187	62.7407	15612.84	9.275	4.99	0.973	1.66

performed using a solvent A mixture of 15 mM ammonium acetate-B and 10:90 v/v MeOH and ACN. It was at 230 nm when UV detector really started working. Before its use, the buffer solution underwent filtration over a 0.2 µm nylon membrane and was subsequently degassed in an ultrasonic bath for 10 to 20 minutes. A steady flow rate of 1-mL min^{-1} was maintained to move the mobile phase down the column. The injection volume was 20 µL, and the column temperature was adjusted to 28ºC.

Chromatographic Conditions for Diol Column

Using a gradient elution technique, an Acclaimed Mix Mode HILIC-1 column was used for chromatographic separation. For the first 0 to 5 minutes, the solvent mixture consisted of 15 mM ammonium acetate-acetonitrile (30:70, v/v). Then, for the next 5 to 23 minutes, the mobile phase was 15 mM ammonium acetate-acetonitrile (10:90 v/v). At 230 nm, UV detector was turned on. Before its use, the buffer solution underwent filtration over a 0.2 µm nylon membrane and was subsequently degassed in an ultrasonic bath for 10 to 20 minutes. A steady flow rate of 1-mL min^{-1} was maintained to move the mobile phase down the column. The injection volume was 20 µL, and the column temperature was adjusted to 28ºC.

Simultaneous Estimation by C18 Based RPC

Figure 4 shows that all of the antidiabetic medications that were considered had good separation, with the exception of metformin, but that the C18 column required more time for their elution. Hence, ICH guidelines do not support the simultaneous measurement of metformin and other antidiabetic medications, as metformin eluted with the void volume. As indicated, the k' values of early eluting substances should always be more than 0.5. Consequently, ODS separation has been disregarded in favor of alternate methods, such as the use of mix-mode chromatography, which allows for the simultaneous quantification of some substances (MMC) (Table 1).

Simultaneous Estimation by Using Diol Column

The five analytes that were chosen for separation followed all ICH requirements and were successfully separated using a diol column (Figure 5, and Table 2).

Method Validation Studies

System suitability tests

The proposed simultaneous estimation of all five selected drugs was tested to determine the basic separation factors of system suitability studies, including – theoretical plate

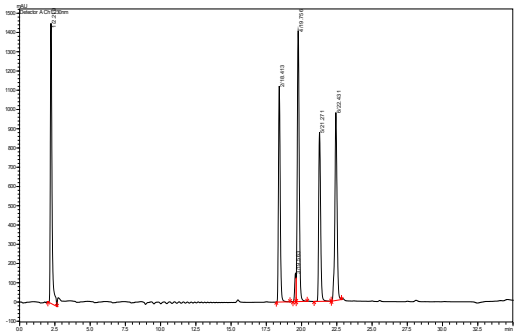

Figure 4: Run of simultaneous quantification of metformin, empagliflozin, remogliflozin, glimepride, gliclazide by using C18 column

Table 3: System suitability parameters for MET EMP, REMO, GLM, GLC

System suitability parameters	*Metformin (MTF)*	*Empagliflozin (EMP)*	*Remogliflozin (REM)*	*Glimeperide (GLM)*	*Gliclazide (GLC)*	*Acceptable range*
Theoretical plates (*N*)	2748	2622	3512	4613	15612	≥ 2000
Resolution (*R*)	---	6.85	2.29	---	9.27	≥ 1.5
Capacity factor (*K'*)	0.58	1.59	1.95	3.006	4.99	≥ 0.5
Asymmetry/Tailing factor (*T*)	1.53	1.05	1.005	0.97	0.97	≤ 1.5
Retention time (t_R) (Min)	3.35	5.77	7.33	8.90	13.32	---
Wavelength of detection (nm)	230	230	230	230	230	≥ 190
Repeatability (%RSD)	1.24	1.36	0.71	1.60	1.30	< 2.0%
Inter-day precision (%RSD)	0.74 - 0.90	0.47 - 0.97	0.12 - 0.72	0.47 - 0.97	0.43 - 0.95	< 2.0%
Linearity range (µg.ml^{-1})	3.9 – 62.5	3.9 – 62.5	3.9 – 62.5	3.9 – 62.5	3.9 – 62.5	NA
Regression equation	y = 36166x + 2417.5	y = 9113.2x + 305.38	y = 87524x + 15015	y = 3269.3x + 183.13	y = 47146x + 23693	NA
SE of intercept (S_e)	3232.4095	3113.822334	8895.354744	190.47012	32940.86289	NA
SD of intercept (S_a)	7227.887373	7627.275868	21789.0802	466.5546052	80688.30577	NA
Correlation coefficient (R^2)	1	1	1	1	0.999	NA
LoQ[a] (µg.mL^{-1})	2.00	3.42	1.01	0.58	6.99	NA
LoD[a] (µg.mL^{-1})	0.60	1.03	0.30	0.17	2.10	NA

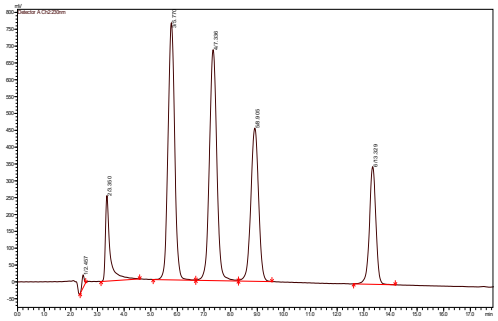

Figure 5: Method development of quantification of metformin, empagliflozin, remogliflozin, glimepride, gliclazide

Table 4: Results obtained from regression analysis

Drug	*MET*	*EMPA*	*REM*	*GLM*	*GLC*
Range (μg/mL)	0.625-10	3.12-50	3.12-50	3.12-50	3.12-50
Correlation coefficient	1	1	1	1	0.99
Std. error of intercept	3232.40	3113.82	8895.35	190.47	32940.86
Std. dev. of intercept	7227.88	7627.27	21789.08	466.55	80688.30

Table 5: Interday (intermediate) precision of metformin

Drug	*Concentration (ppm)*	*Area*	*Mean ± SD*	*%RSD*
	20	454518		
Metformin	20	448364	3314.286047	0.74
	20	449308		
	100	908242		
Empagliflozin	100	909343	4261.048111	0.47
	100	916111		
	100	950332		
Remogliflozin	100	952545	1113.602712	0.12
	100	951221		
	100	908240		
Glimepride	100	909342	4260.048110	0.46
	100	916110		
	100	4790298		
Gliclazide	100	4751020	32384.31186	0.68
	100	4726059		

Table 6: Repeatability statistics

	MET at 230 nm (20 ppm)	*EMPA at 230 nm (100 ppm)*	*Remo (100 ppm)*	*Glim (100 ppm)*	*GLZ (100 ppm)*
	Peak Area	*Peak Area*	*Peak Area*	*Peak Area*	*Peak Area*
	454518	902242	958349	336701	4706187
	439084	880207	945041	330207	4875041
	443869	871320	941334	331320	4801334
	440808	874947	941736	334947	4821736
	444048	874161	940204	344161	4807204
	441243	870623	946044	340623	4876044
Mean	443928.3333	878916.6667	945451.3333	336326.5	4814591
Std. dev.	5524.019213	11921.4513	6710.58977	5373.93516	62391.45417
RSD (%)	1.24	1.36	0.71	1.60	1.30

(N), capacity/retention factor (k'), resolution (R), separation factor (α), tailing factor (*T*) and relative standard deviation n(RSD). All separation parameters were in accordance with ICH guidelines (Table 3).

Linearity (Calibration) studies of selected drugs

As a measure of the HPLC-DAD method's linearity and calibration, the findings should be directly proportional to the known analyte concentration within the specified range, as measured against the peak area (mAu). As previously mentioned, the regression coefficients (R2) for MET, REM, EMPA, and GLM were all exactly 1, and for GLC, the value was 0.999, indicating that the corresponding areas were very proportionate over the known concentrations of GLC (Table 4).

Precision

We investigated and evaluated the corresponding mixture of MET, REM, GLP, and GLZ for three days in a row, using three replicates with similar dosages (interday/intermediate precision). Also, %RSD was calculated and found to be below 2% for all analytes that were included in the simultaneous HPLC-UV analysis. In addition, a correlation between the peak area and the chosen concentration could be observed because each sample had a percentage RSD of ≥ 2%. Results ensure that the devised method is very accurate and reproducible for intra- and inter-day measurements. With its reasonable degree of accuracy and minimal outliers, the proposed method is suitable for frequent analysis (Table 5).

Table 7: %recovery of all the drugs in marketed formulations

Metformin			*Empagliflozin*			*Ramogliflozin*			*Glimepiride*			*Gliclazide*		
Conc in µg/mL														
160	200	240	4	5	6	16	20	24	0.4	0.5	0.6	9.6	12	14.4
%drug recovery														
103.50	98.86	96.09	107.74	102.98	98.07	99.33	104.8	101.25	105.88	102.79	104.62	102.32	101.98	101.24

Repeatability

To determine how repeatable the procedure was, it was injected six times with a standard solution containing 20 µg/mL of MET and 100 µg/mL of EMPA, REMO GLM, and GLP, respectively (Table 6).

Drug accuracy studies

In order to compare the two methods of analysis, we ran analyses on the two analytes at three different concentrations (80, 100, and 150%) and determined the percentage of recovery for each (Table 7).

Limit of detection and limit of quantitation

Tab shows the results of calculating the limit of detection (LoD) and limit of quantitation (LoQ) using response SD and slope of the regression equation. Based on what was noted, LoD for MET, EMPA, REMO, GLM, and GlC were 0.60, 1.03, 0.30, 0.17, and 2.10 µg/mL, correspondingly, although LoQ were 2.00, 3.42, 1.02, 0.58, and 6.99 µg/mL. Therefore, the suggested method is applicable to routine HPLC analysis of pharmaceutical medications, whether for individual or simultaneous analysis of selected drugs.

CONCLUSION

While considering the reverse phase technique for the simultaneous study of metformin with other selected drugs, metformin is eluted at or close to the value that is not per ICH guidelines. When operating in reverse phase mode, a mixed-mode column must be considered. A reasonable amount of metformin was kept. Metformin and its combination with empagliflozin, remogliflozin, glimepride, and gliclazide from commercial formulation may be quantified simultaneously using the devised approach, which has a capacity factor of around 0.5.

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