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Original Article

Optimization and Validation for Simultaneous Estimation of Metformin and Furosemide by Using RP-UFLC

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ABSTRACT

Background: Metformin is a Biguanide derivative used to treat the type-II diabetes, Furosemide is a loop diuretic used in the treatment of hypertension associated with edema. Aim: Our aim is to create an analytical method for the simultaneous measurement of Metformin and Furosemide by using RP-UFLC techniques. **Materials and Methods:** In the present study, liquid chromatography with UV detector operating at 240 nm and a C-18 column was used. A gradient elution was performed using a mobile phase containing methanol and water containing 0.1% orthophosphoric acid in a ratio of 50:50 V/V and injected at a rate of 1 mL min^{-1}. **Results:** The developed method was validated according to the ICH guidelines. Linear response was detected over a concentration ranging from 400-2400 $ngmL^{-1}$ for both drugs with R^2=0.9982 for Metformin and R^2=0.9989 for Furosemide. **Conclusion:** The accuracy and precision of the method were within acceptable limit indicating its reliability for routine analysis.

Keywords: Metformin, Furosemide, RP-UFLC, Method development, Method validation.

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INTRODUCTION

Type-2 diabetes mellitus is caused by either decreased insulin production from β-pancreatic cells or insulin resistance, where cells do not response to generate insulin. The pancreatic β cells must secrete a considerable amount of insulin. However, functional defects in secretion develops over time resulting in type-2 diabetes mellitus (Magdy *et al*., 2023). Diabetes develops as a result of risk factors such as obesity, a secondary lifestyle and genetic vulnerability (AlThikrallah *et al*., 2023).

Metformin (MET) (1,1-dimethylbiguanide) as Shown in Figure 1(a), an oral antidiabetic medication of the biguanide family, (Al-Rimawi F, 2009; Satheesh Kumar *et al*., 2014) used to manage the type-2 diabetes mellitus (Sha'at M *et al*., 2022). It stimulates the activity of the 5-Adenosine Monophosphate activated Protein Kinase enzyme (AMPK). AMPK activation enhances insulin sensitivity in body cells, increases glucose consumption and uptake peripherally, decreases glucose absorption from the gastrointestinal tract (Abou-Omar *et al*., 2021; Gedawy *et al*., 2019) with an absolute bioavailability of 50-60% reported for a single 500 mg dose. After intake, plasma protein binding is minor and it is eliminated as an unchanged form in the urine (Zarghi *et al*., 2023).

Loop diuretics are a type of medicine that is commonly used in clinical practice to manage excessive fluid loads and maintain fluid balance. The pharmacologic effect of loop diuretics is that prevents the Na+, K+ and 2Cl- cotransporter, which transfuses from the tubular lumen to tubular cells. They block Na+ and Cl- reabsorption in the ascending limb of the loop of Henle, resulting in increased secretion of water, K+, Na+ and Cl- (Chen *et al*., 2021).

Furosemide (FU) chemically known as 4-chloro-N-furfuryl-5-sulfamoyl-anthranilic acid (Figure 1(b)) is a strong loop diuretic that is widely used to treat edema caused by chronic failure, hypertension or liver cirrhosis (Farthing *et al*., 1992; Chen *et al*., 2021). Fu is rapidly but incompletely absorbed after oral treatment and is strongly bound to plasma proteins (>90%) with up to 50% of the medication is eliminated in the urine, primarily as an unchanged drug (Al-Hashimi *et al*., 2022).

Several studies have been conducted to detect Metformin in either its single form (Sha'at M *et al*., 2022; Zarghi *et al*., 2023) or in combination with other antidiabetic medicines such as Telmisartan (Borse *et al*., 2022), Pioglitazone (Mohamed AM *et al*., 2015), Empagliflozin (Abou-Omar *et al*., 2021), Vildagliptin (Satheeshkumar *et al*., 2014) and Gliclazide (Gedawy *et al*., 2019). Similarly, other approaches were used to detect Furosemide in either alone or in combination with other medications such as Spironolactone (Sora *et al*., 2010), Canrenone (Naguib *et al*., 2018) and Carbamazepine (Al-Hashimi *et al*., 2022).

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Metformin is used to treat type-diabetes and Furosemide is also used to treat the hypertension associated with edema state. To the best of our knowledge, no analytical methods have been reported for simultaneously determining Metformin and Furosemide using RP-UFLC method. Therefore, our purpose is to develop and validated a simple and sensitive RP-UFLC analytical method for the simultaneous quantification of Metformin and Furosemide and the developed method was validated in compliance with ICH guidelines.

MATERIALS AND METHODS

Chemicals and solvents

Metformin (>98.0%) and Furosemide (>99.0%) were provided by Yarrow Chem Products (Mumbai). Methanol (HPLC grade) were supplied by Thermo Fisher Scientific India Pvt. Ltd., (Mumbai) whereas orthophosphoric acid (85w%, HPLC grade) was supplied by LOBA Chemie Pvt. Ltd., and Millipore water (HPLC grade) was provided by in house (B.G Nagar). All the chemicals and solvents were of analytical grade.

Chromatographic condition

For HPLC analysis, ultra-fast liquid chromatography with UV detector, Shimadzu (Japan) was used. The separation was achieved by using C-18 column (5 µm, 4.6x250 mm); mobile phase was consisting of methanol and water containing 0.1% orthophosphoric acid in a 50:50 V/V ratio. The mobile phase was pumped at a flow rate of 1 $mLmin^{-1}$ and wavelength was kept at 240 nm. The detection was carried out at 25°C; overall run time was 10 min with an injection volume was set to 20 µL. The mobile phase was prepared daily and degassed by ultra-sonicator before use. Peak integration and quantification were performed by using Lab-solution software.

Preparation of stock and working standard solution solutions

To prepare stock and working standard solution, a standard solution of Metformin and Furosemide was prepared by weighing 0.01 g of each drug, diluting with mobile phase and sonicated for 5 min. The volume was then adjusted to achieve a concentration of 1000 μgmL^{-1} of each drug. To prepare working solutions, the standard stock solution was serially diluted with mobile phase to achieve a final concentration of 1 μgmL^{-1} of both drugs.

Method validation

After optimization of chromatographic condition, the optimized method should be validated according to ICH guidelines that include linearity, system suitability, LOD and LOQ, accuracy, precision and robustness.

System suitability

System suitability parameters such as retention time, number of theoretical plates, peak area and tailing factor were evaluated for Metformin and Furosemide by injecting black followed by six replicates of a 2 μgmL^{-1} mixture containing two drugs.

Linearity

Linearity parameter was performed by serial dilution of stock solution by using mobile phase to achieve a concentration ranging from 400-2400 $ngmL^{-1}$ for both drugs. Slope, standard deviation of Y-intercept and correlation coefficient of the calibration curve were calculated to ensure that the analytical procedure was linear.

Accuracy study

Accuracy study was determined by spiking the marketed drugs with standard solution to get 50%, 100% and 150% concentrations. These mixtures were analysed by the proposed method in triplicate. % recovery, mean, standard deviation and % RSD of spiked drugs were calculated.

Precision

Both repeatability and reproducibility were assessed for Metformin and Furosemide. Repeatability was tested by injecting three different concentrations of Metformin and Furosemide in triplicate on the same day under same operational condition.

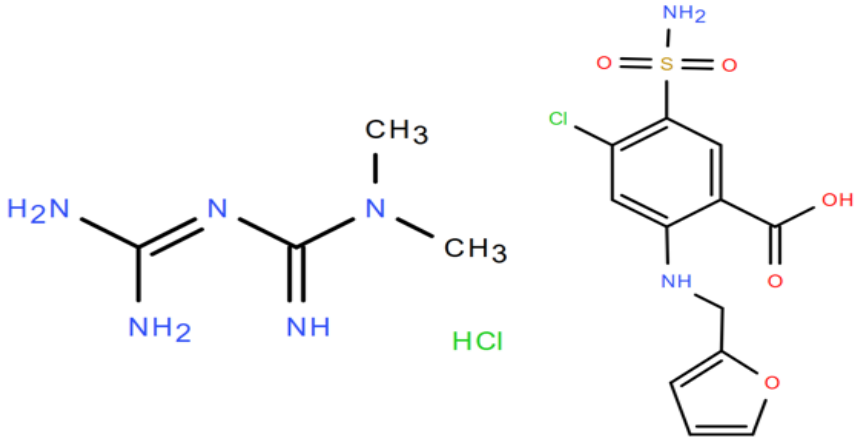


Figure 1: Structure of (a) Metformin and (b) Furosemide.

Inter-day precision was determined by comparing the results from three consecutive days.

Limit of detection and limit of quantification

The linear regression equation was used to calculate the LOD and LOQ for Metformin and Furosemide based on the standard deviation of the Y-intercept and slope.

$$LOD=(3.3*SD\ Y\text{-}inter)/slope$$

$$LOQ=(10*SD\ Y\text{-}inter)/slope$$

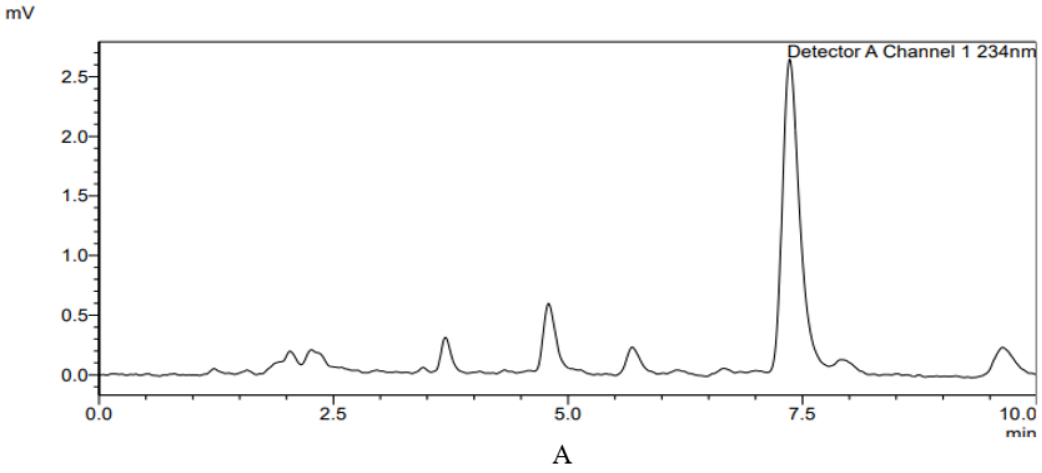
Robustness

The parameter has been studied by examining the influence of small but deliberate modification in chromatographic parameters such as mobile phase ratio (altered by ±2%), flow rate (altered by ±0.1 $mLmin^{-1}$), wavelength (modified by ±2 nm) and column oven temperature (changed by ±2°C). These chromatographic modifications were tested for resolution between Metformin and Furosemide peak areas, theoretical plate count and tailing factor.

RESULTS

Method development and optimization

Various mobile phase composition such as acetonitrile: water, methanol: water with 0.1% orthophosphoric acid in different ratios like 50:50, 55:45, 60:40 and 70:30 V/V were used for sensitive and selective determination of Metformin and Furosemide by RP-UFLC method for simultaneous estimation of Metformin and Furosemide. The optimized chromatographic condition was obtain using a C-18 column (5 µm, 4.6x250 mm) and a mobile phase was composed of methanol and water with 0.1% orthophosphoric acid in a 50:50 V/V ratio. The compounds were detected by using a UV detector with a wavelength of 240 nm. The flow rate was kept at 1 $mLmin^{-1}$ with an injection volume of 20 µL and temperature was maintained at 25°C. The entire run time was 10 min and the retention time for Metformin was 1.93 min and for Furosemide was 6.05 min as indicated in Figure 2.



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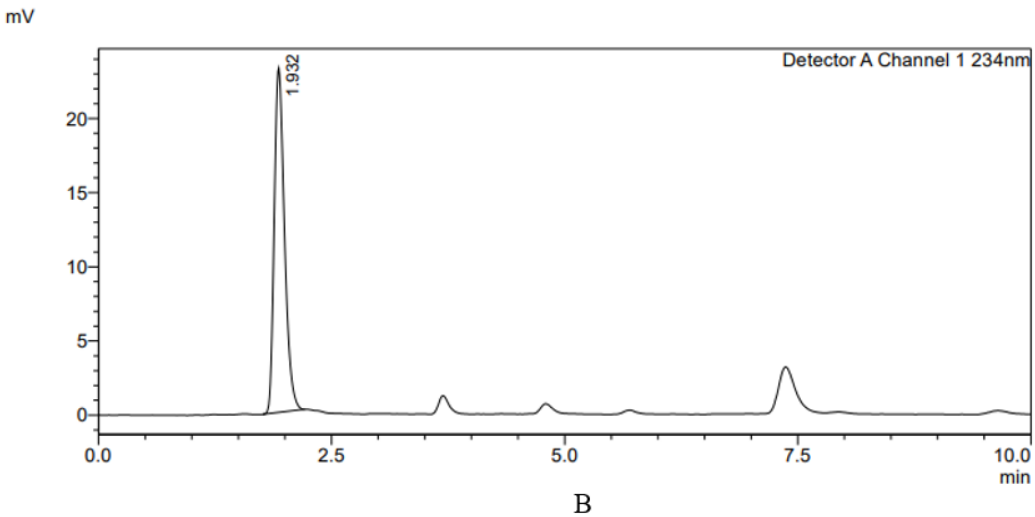

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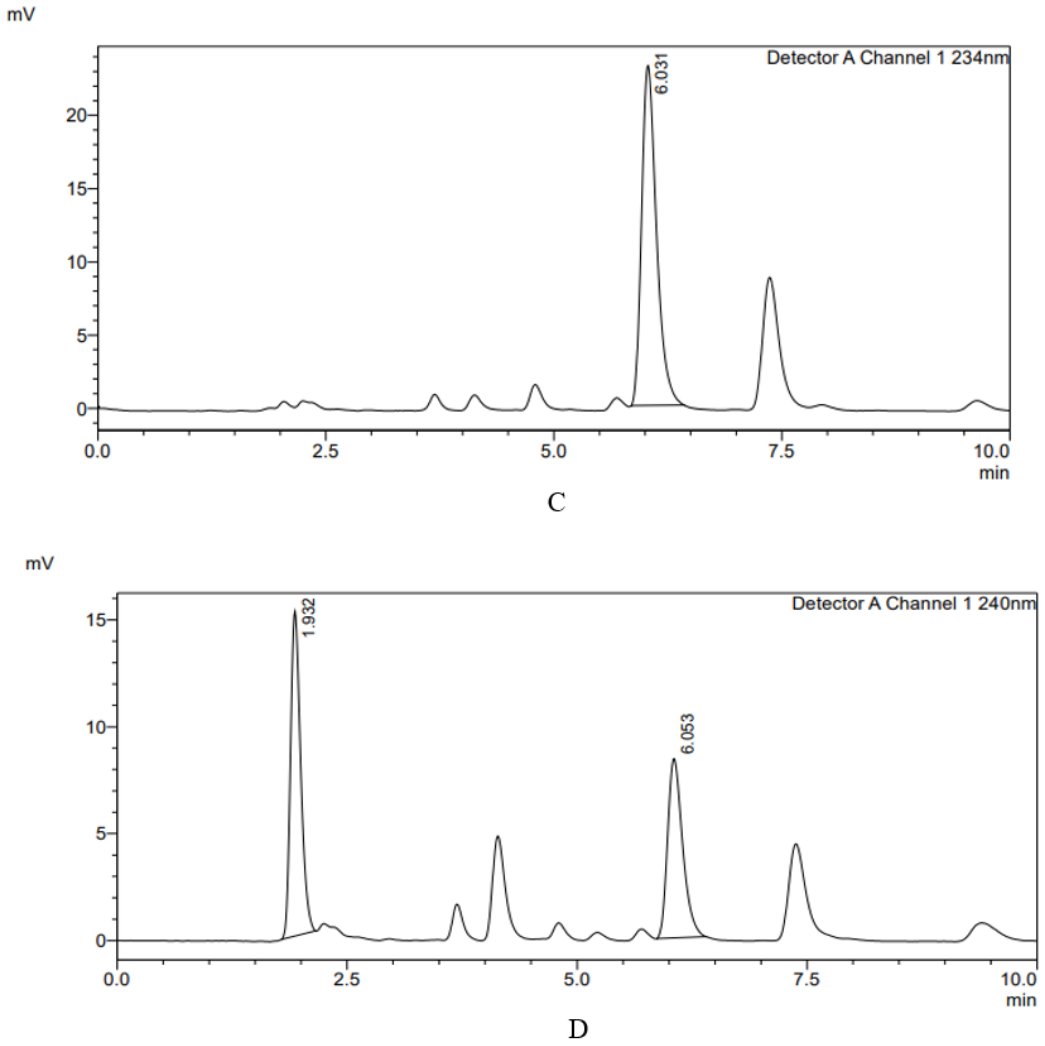

Figure 2: (A)-Blank chromatogram, (B)-Chromatogram containing only Metformin (1 µg/mL) (C)-Chromatogram containing only Furosemide (1 μgmL^{-1}), (D)-Chromatogram containing Metformin (1 μgmL^{-1}) and Furosemide (1 μgmL^{-1}) with a retention time of 1.93 min and 6.05 min respectively.

Validation studies

System suitability

Table 1: System suitability data of Metformin and Furosemide.

	Retention time.		Theoretical plate count		Peak Area.		Tailing Factor.	
	MET	FU	MET	FU	MET	FU	MET	FU
1	1.946	6.049	1345	6284	106784	110672	1.339	1.389
2	1.943	6.053	1362	6329	106973	107609	1.339	1.386
3	1.936	6.158	1350	6306	106405	108121	1.320	1.37
4	1.936	6.135	1366	6332	105635	107384	1.330	1.376
5	1.943	6.112	1357	6379	106773	108322	1.329	1.378
6	1.946	6.082	1339	6390	106365	108499	1.339	1.392
Mean	1.9416	6.0981	1353.16	6336.66	106489.16	108434.5	1.332	1.381
Standard deviation	0.0045	0.0443	10.342	41.064	480.3508	1175.28	0.0077	0.0084
% RSD	0.236	0.727	0.734	0.648	0.4510	1.083	0.582	0.614

Linearity

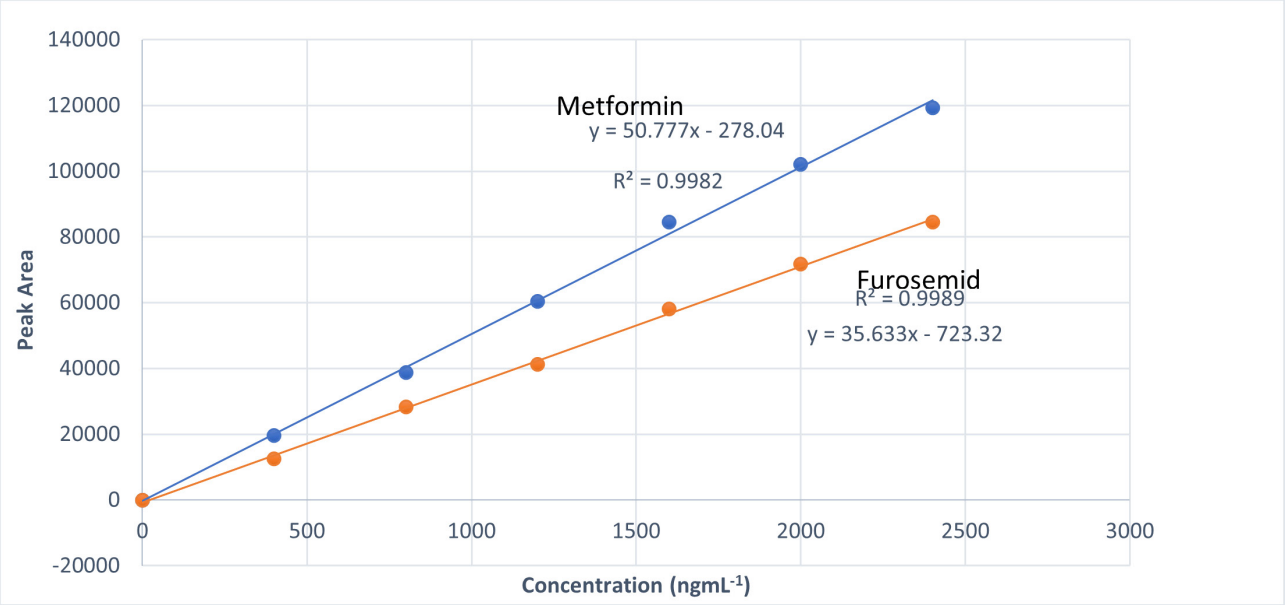

Figure 3: Linearity curve of Metformin and Furosemide.

Accuracy

Table 2: Accuracy studies of Metformin and Furosemide.

Drug	% of drug	Amt of std drug added ($ngmL^{-1}$)	Amt of marketed drug added ($ngmL^{-1}$)	Total amt of drug added ($ngmL^{-1}$)	Total amt of drug found ($ngmL^{-1}$)	% Recovery	Mean	SD	% RSD
MET	50%	400	200	600	597.12	99.52	98.68	1.25	1.27
					583.45	97.24			
					595.74	99.29			
	100%	400	400	800	800.15	100.01	100.06	0.10	0.10
					799.85	99.98			
					801.48	100.18			
	150%	400	600	1000	1002.42	100.24	100.23	0.15	0.15
					1000.60	100.06			
					1003.72	100.37			
FU	50%	400	200	600	615.53	102.58	103.53	0.81	0.78
					626.18	104.03			
					623.84	103.97			
	100%	400	400	800	821.18	102.64	102.08	0.63	0.61
					811.19	101.39			
					817.59	102.19			
	150%	400	600	1000	1046.72	104.67	104.86	0.18	0.17
					1050.43	105.04			
					1048.64	104.86			

Precision

Table 3: Repeatability and reproducibility data of Metformin and Furosemide.

Analyte	Concentration ($ngmL^{-1}$)	Repeatability	Reproducibility		
		Conc. found ($ngmL^{-1}$) (Avg); % RSD	Conc. found ($ngmL^{-1}$) (Avg); % RSD		
			Day-1	Day-2	Day-3
MET	400	399.79; 0.528	399.79; 0.528	397.95; 0.098	398.11; 0.038
	600	593.83; 0.458	593.83; 0.458	596.22; 1.044	593.61; 0.845
	800	795.57; 0.392	795.57; 0.392	792.66; 0.144	793.85; 0.140
FU	400	417.44; 1.656	417.44; 1.656	398.24; 0.535	397.15; 0.261
	600	625.85; 0.793	625.85; 0.793	600.01; 0.832	601.98; 0.343
	800	807.65; 0.207	807.65; 0.207	799.85; 0.156	800.37; 0.066

Robustness

Table 4: Robustness data of Metformin and Furosemide.

Condition	Variation	Retention time		Tailing factor		Theoretical plates	
		MET	FU	MET	FU	MET	FU
Flow rate (1±0.1 $mLmin^{-1}$).	0.9 $mLmin^{-1}$	1.99	6.10	1.360	1.413	1391	6220
	1.1 $mLmin^{-1}$	1.91	5.91	1.321	1.411	1382	6484
ACN ratio in Mobile phase ratio (50±2%).	48	1.94	6.14	1.362	1.382	1393	6133
	52	1.93	5.97	1.299	1.406	1390	6243
Wavelength (240±2 nm).	238 nm	1.94	6.03	1.342	1.432	1386	6174
	242 nm	1.94	6.03	1.335	1.398	1383	6278
Column oven temperature (25±2°C).	23°C	1.94	6.08	1.328	1.394	1381	6260
	27°C	1.94	6.04	1.338	1.414	1386	6274
Mean		1.941	6.037	1.335	1.406	1386.5	6258.25
Standard deviation		0.022	0.072	0.020	0.015	4.440	104.256
% RSD		1.149	1.206	1.536	1.074	0.320	1.665

DISCUSSION

System suitability

Six replicates of the Metformin (1 μgmL^{-1}) and Furosemide (1 μgmL^{-1}) mixture were injected to determine the system suitability parameters for peak area, retention time, number of theoretical plates and tailing factor. In order to ensure good efficacy, theoretical plate count for Metformin and Furosemide in all chromatographic run was >1000 for Metformin and >6000 for Furosemide. The tailing factors for the Metformin and Furosemide peak never exceeded 1.339 and 1.392 respectively in all peak indicating good symmetrical peak (acceptance limit <2) and presented in Table 1.

Linearity

Metformin and Furosemide were shown to have linearity between 400-2400$ngmL^{-1}$. The results showed that the correlation coefficient for Metformin and Furosemide were found to be 0.9982 and 0.9989 respectively. From the Figure 3, it was determined that the regression line equations for Metformin and Furosemide y=50.777x-278.04 and y=35.633x-723.32 respectively.

Limit of Detection and Quantification

LOD and LOQ were determined from the calibration curve of Metformin and Furosemide by using the formula.

LOD and LOQ of Metformin

The SD of Y-intercept and slope for Metformin were found to be 1381.2234 and 50.7770 respectively.

$$LOD=(3.3*1381.2234)/50.7770=89.765\ ngmL^{-1}.$$

$$LOQ=(10*1381.2234)/50.7770=272.017\ ngmL^{-1}.$$

LOD and LOQ of Furosemide

The SD of Y-intercept and slope for Furosemide were found to be 767.4846 and 35.633 respectively.

$$LOD=(3.3*767.4846)/35.633=71.077\ ngmL^{-1}.$$

$$LOQ=(10*767.4846)/35.633=215.385\ ngmL^{-1}.$$

Accuracy

The accuracy of the analytical method was assessed by creating a placebo of the drug formulation in accordance with the formulation procedure. To get concentrations of 600, 800 and 1000 $ngmL^{-1}$ (50%, 100% and 150%), a known quantity of standard solution of Metformin and Furosemide was added to the necessary amount of placebo. The results were presented in terms of the % recovery of Metformin and Furosemide from the spiked matrix. The suggested method's validity was demonstrated by how well the recorded analyte values matched the stated theoretical concentrations at various levels. From the Table 2, it was found that the % recovery for Metformin and Furosemide was 98.68-100.23% and 101.39-105.04% respectively.

Precision

The levels of the Metformin and Furosemide at three concentration levels (400, 600 and 800 $ngmL^{-1}$) in triplicates were assessed on the same day (repeatability) and in between the days (intermediate precision). According to the Table 3, results shows that the suggested method has good precision and repeatability for both intra and inter-day determination. All data are expressed as % RSD and were never exceeds the acceptable levels, which is <2.

Robustness

The developed method robustness was examined by checking the effect of small changes in the parameters of the experiment on the chromatographic response. After examining, % RSD reading was not significantly affected by the mobile phase (50±2%), flow rate (1±0.1 $mLmin^{-1}$), temperature (25±2°C) and wavelength (240±2 nm). The robustness of Metformin and Furosemide are shown in Table 4.

CONCLUSION

A robust and effective RP-UFLC method was successfully developed for the simultaneous determination of Metformin and Furosemide in raw material and pharmaceutical dosage form. The optimized chromatographic conditions provide excellent separation of drugs with a retention time of 1.90 min for Metformin and 6.01min for Furosemide. This developed method shows a good linearity for Metformin (R^2=0.9982) and for Furosemide (R^2=0.9989) with a good detection and quantification limits (LOD=89.765 $ngmL^{-1}$, LOQ=272.017 $ngmL^{-1}$) for Metformin and (LOD=71.077 $ngmL^{-1}$, LOQ=215.385 $ngmL^{-1}$) for Furosemide with a good accuracy and precision (% RSD is <2%). All the results are within the limit as per ICH guidelines. Therefore, the optimized method could be helpful for routine analysis of Metformin and Furosemide in raw materials as well as in pharmaceutical dosage form.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

ABBREVIATIONS

RP-UFLC: Reverse Phase-Ultra Fast Liquid Chromatography; **MET:** Metformin; **AMPK:** Adenosine monophosphate activated protein kinase; **FU:** Furosemide; **mL:** Millilitre; **°C:** Degree Celsius; **Min:** Minute; **nm:** Nanometre; **g:** Gram; **µg:** Microgram; **µm:** Micrometre; **mm:** Millimetre; **v/v:** Volume/volume; **ICH:** International Council for Harmonization; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **Conc:** Concentration; **ng:** Nanogram; **RSD:** Relative Standard Deviation; **SD:** Standard Deviation; **Avg:** Average.

AUTHOR CONTRIBUTIONS

Rakshitha A helps in methodology, validation, formal analysis and writing original draft. Rashmi N G helps in conceptualization, supervision and writing-review and editing.ETHICS APPROVAL AND CONSENT OF PARTICIPATEOur aim of study is to develop analytical method and validation. So, there is no point of taking ethical clearance, because we are not applying this method for animal study or human study.

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