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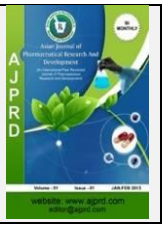

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Research Article

Development and Validation of RP-HPLC Method for Simultaneous Estimation of Metformin Hydrochloride, Linagliptin and Dapagliflozin in Synthetic Mixture

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ABSTRACT

A simple, accurate, precise, and reproducible reverse phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Metformin (MET), Linagliptin (LINA), and Dapagliflozin (DAPA) in a synthetic mixture. Chromatographic separation was achieved using a Shimadzu LC-2030 HPLC system equipped with a C18 column (250 mm × 4.6 mm, 5 µm particle size). The optimized mobile phase consisted of Methanol:10 mM phosphate buffer (pH adjusted to 4 using orthophosphoric acid) in the ratio of 65:35 (% v/v), delivered at a flow rate of 1.0 mL/min, and detection was carried out at 232 nm.

The retention times were found to be 2.337 min for Metformin, 3.324 min for Linagliptin, and 12.874 min for Dapagliflozin. The method demonstrated good linearity in the concentration range of 2.5-15 µg/mL for all three drugs, with correlation coefficients of 0.9969 for Metformin, 0.9944 for Linagliptin, and 0.9938 for Dapagliflozin. Recovery studies showed percentage recoveries of 102.89%, 99.20%, and 101.50% for Metformin, Linagliptin, and Dapagliflozin, respectively.

The developed method was validated according to International Council for Harmonisation guidelines for specificity, linearity, accuracy, precision, robustness, limit of detection (LOD), and limit of quantification (LOQ). The results indicated that the proposed method is simple, sensitive, accurate, and suitable for routine quality control analysis for the simultaneous estimation of Metformin, Linagliptin, and Dapagliflozin in pharmaceutical dosage forms.

Keywords: RP-HPLC, Metformin hydrochloride, Linagliptin, Dapagliflozin, Method validation, Synthetic mixture

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INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by elevated blood glucose levels due to defects in insulin secretion, insulin action, or both. Type 2 diabetes mellitus accounts for approximately 90-95% of all diabetes cases worldwide and requires long-term pharmacological management.

Metformin is a biguanide antidiabetic agent widely used as first-line therapy in type 2 diabetes mellitus due to its ability to decrease hepatic glucose production and improve insulin sensitivity.

Linagliptin is a dipeptidyl peptidase-4 inhibitor that increases incretin hormone activity and improves glycemic control.

Dapagliflozin is a sodium-glucose cotransporter-2 inhibitor that lowers blood glucose by promoting urinary glucose excretion.

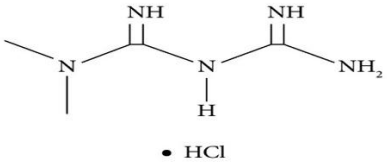
Combination therapy involving these three drugs offers enhanced glycemic control through multiple mechanisms of action. Literature review revealed several analytical methods for individual drugs and dual combinations; however, no RP-HPLC method has been reported for simultaneous estimation

of Metformin, Linagliptin, and Dapagliflozin in a single synthetic mixture.

Therefore, the present study aimed to develop and validate a simple, precise, and economical RP-HPLC method for simultaneous estimation of these three drugs.

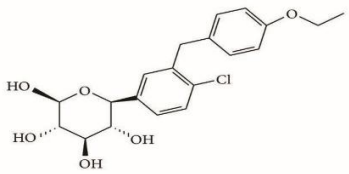
Drug Profile of Metformin Hydrochloride

Table 1: Drug Profile of Metformin hydrochloride

NAME OF DRUG	METFORMIN HYDROCHLORIDE
IUPAC Name	1,1-Dimethyl biguanide hydrochloride
Chemical Structure	
Molecular Formula	$C_4H_{11}N_5$.HCl
Molecular Weight	165.62 g/mol
CAS Id	1115-70-4
Category	Anti-hyper glycaemic agent
Description	White, hygroscopic crystalline powder
Solubility	Freely soluble: water, Methanol slightly soluble in alcohol Insoluble: Ether, chloroform, acetone, methylene chloride.
Pka	12.4
Log P	-2.6
Melting Point	232°C
Dose	500 mg twice daily
Pharmacopoeial Status	Official in IP,BP,EP,USP
Storgae	Store at 20-25ºC; protect from light and moisture
FDA Approval	October 22, 1998

Drug Profile of Dapagliflozin

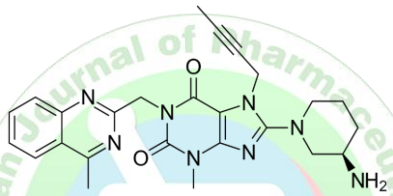
Table 2: Drug Profile of Dapagliflozin

NAME OF DRUG	DAPAGLIFLOZIN
IUPAC Name	(1S)-1,5-anhydro-1-(4-chloro-3-(4-ethoxybenzyl)phenyl]-D-glucitol.
Chemical Structure	
Molecular Formula	$C_{21}H_{25}ClO_6$
Molecular Weight	408.873 g/mol
CAS Id	461432-26-8
Category	Sodium-glucose cotransporter 2 inhibitors (SGLT2)

Description	Glassy, off-white amorphous solid
Solubility	Freely soluble: organic solvents such as Methanol, DMSO, and dimethyl formamide, also water-soluble, and hydrolytically stable in the aquatic environment.
Pka	12.6
Log P	2.7
Melting Point	65-70°C
Dose	Initial dose: 5 mg orally once a day and Maximum dose: 10 mg/day
Pharmacopoeial Status	Official in IP,BP,EP,USP
Storgae	Store in a slightly closed container, protected from light at a temperature below 25°C.
FDA Approval	2013

Drug Profile of Linagliptin

Table 3: Drug Profile of Linagliptin

NAME OF DRUG	**LINAGLIPTIN**
IUPAC Name	8-[(3R)-3-Aminopiperidin-1-yl]-7-(but-2-yn-1-yl)-3-methyl-1-[(4-methylquinazolin-2-yl) methyl]-3,7-dihydro-1H-purine-2,6-dione
Chemical Structure	
Molecular Formula	C25H26N8O2
Molecular Weight	472.54 g/mol
CAS Id	668270-12-0
Category	DPP-4 inhibitors
Description	White or yellowish, slightly hygroscopic solid substance
Solubility	Soluble in methanol ,Sparingly soluble in ethanol , Very slightly soluble in iso -propanol and acetone
Pka	pKa1 = 8.6; pKa2 = 1.9.
Log P	1.7
Melting Point	202 -204 ºC
Dose	5mg a day
Pharmacopoeial Status	Official in IP, USP, EP, BP
Storgae	In a closed container at room temperature, away from heat, moisture, and direct light.
FDA Approval	May 2, 2011

Materials and Methods

Materials

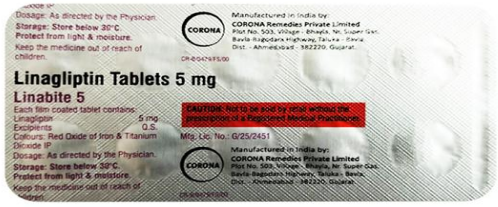
Standard Metformin hydrochloride and Dapagliflozin were obtained as gift samples from Exemed Pharmaceuticals, Vapi, Gujarat, India.

Standard Linagliptin was obtained from Bakul Pharma Pvt. Ltd., Ankleshwar, India.

Dapabite M 10/500 which contains a combination formulation of Dapagliflozin (10 mg) and metformin (500 mg) and Linabite 5 containing Linagliptin (5 mg) was purchased from local Pharmacy store, Gandhinagar, Gujarat.

Marketed formulations:

- Dapabite M 10/500
- Linabite 5



Linabite 5

Dapabite M 10/500

Instruments

Table 4: List of instruments used in Research work

Sr. No.	Instrument	Manufacturer	Model no.	Country
1.	Digital weighing balance	Sartorius	CP 124S	Germany
2.	Hot air oven	Electroquip	NA	India
3.	Ph meter	Electroquip	Ph Cal	India
4.	Vacuum filter assembly	J.B. Sawant Engg. Pvt. Ltd.	JEBIVAK	India
5.	High Performance Liquid Chromatography (HPLC)	Shimadzu Co.	LC 2030	Japan
6.	UV-visible spectrophotometer	Shimadzu Co.	1800	Japan
7.	Ultra sonicator bath	Frontline Electronic Pvt. Ltd.	NA	India

Chemicals and Reagents

Table 5: List of Chemical and Reagents used in Research work

Sr. no.	Name	Grade	Manufacturer
1.	Methanol	HPLC, AR	Rankem chemicals Ltd. Ahmedabad
2.	Acetonitrile	HPLC	Rankem chemicals Ltd. Ahmedabad
3.	Milli-Q water	HPLC	Rankem chemicals Ltd. Ahmedabad
4.	Potassium dihydrogen phosphate	HPLC	Rankem chemicals Ltd. Ahmedabad
5.	O-Phosphoric acid	AR	Finar chemicals Ltd. Ahmedabad

LIST OF APPARATUS

Table 6: List of apparatus used in Research work

Sr. no.	Components	Volume	Specification
1.	Volumetric Flasks	10ml,25ml,50ml,100ml	Borosilicate Type-1
2.	Pipette	1ml,2ml,5ml,10ml	Borosilicate Type-1
3.	Beaker	50ml, 100ml, 260ml, 500ml	Borosilicate Type-1
4.	Whatman filter paper	125 mm pore size	NA
5.	Membrane filter	0.45 µm	Merck Millipore

Methods

UV Spectroscopic Analysis

Standard stock solutions (1000 µg/mL) of Metformin, Linagliptin, and Dapagliflozin were prepared using methanol. Working solutions of 50 µg/mL were further diluted to obtain 10 µg/mL solutions, which were scanned using UV spectroscopy. Overlay spectra were recorded and 232 nm was selected as the optimized wavelength for analysis.

Preparation of Mobile Phase

Preparation of 10 mM Phosphate Buffer

Accurately weighed 0.36 g of potassium dihydrogen phosphate was transferred into a 250 mL volumetric flask and dissolved in HPLC-grade water. The pH was adjusted to 4.0 using 1% orthophosphoric acid. The solution was filtered using a vacuum filtration assembly.

Mobile Phase Composition

The optimized mobile phase consisted of:

Methanol : 10 mM phosphate buffer (65:35 % v/v)

The mobile phase was filtered and degassed before use.

Preparation of Standard Stock Solution

Accurately weighed 10 mg each of Metformin, Linagliptin, and Dapagliflozin were transferred separately into 10 mL volumetric flasks and diluted with methanol to obtain stock solutions of **1000 µg/mL**.

Preparation of Working Standard Solution

From each stock solution, 5 mL was transferred into a 100 mL volumetric flask and diluted with methanol to obtain working standard solutions of **50 µg/mL**.

Preparation of Calibration Curve

Aliquots of 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 2.5 mL, and 3.0 mL were withdrawn from working standard solutions and diluted to 10 mL with diluent to obtain concentrations ranging from **2.5-15 µg/mL**. These solutions were injected into the HPLC system and calibration curves were plotted by taking concentration versus peak area.

Optimized Chromatographic Conditions

Chromatographic separation of Metformin, Linagliptin, and Dapagliflozin was performed under optimized chromatographic conditions shown in Table 1.

Table 7: Optimized Chromatographic Conditions

Parameter	Condition
Instrument	Shimadzu Corporation LC-2030 HPLC
Column	Shimadzu Prominence C18 (250 mm × 4.6 mm, 5 µm)
Mobile Phase	Methanol:10 mM phosphate buffer
Mobile Phase Ratio	65:35 (% v/v)
pH	4.0
Flow Rate	1.0 mL/min
Detection Wavelength	232 nm
Injection Volume	20 µL
Run Mode	Low-pressure gradient mode
Column Temperature	Ambient temperature
Run Time	15 min

Under these optimized conditions, retention times were observed at **2.337 min** for Metformin, **3.324 min** for Linagliptin, and **12.874 min** for Dapagliflozin.

Results and Discussion

Method Development and Optimization

Selection of Column

Selection of an appropriate stationary phase is an important step in chromatographic method development to achieve adequate resolution and peak symmetry. Based on literature review and preliminary experimental trials, a reversed-phase C18 column was selected because of its wide applicability, high efficiency, and ability to separate compounds with varying polarities.

The Shimadzu Prominence C18 column (250 mm × 4.6 mm, 5 µm particle size) provided satisfactory separation of Metformin, Linagliptin, and Dapagliflozin with acceptable peak shape and resolution.

Selection of Detection Wavelength

Standard solutions of all three drugs (10 µg/mL) were scanned using UV spectrophotometry to determine the optimum wavelength for simultaneous detection. Overlay spectra indicated that all three drugs showed appreciable absorbance at **232 nm**, which was selected as the detection wavelength for RP-HPLC analysis.

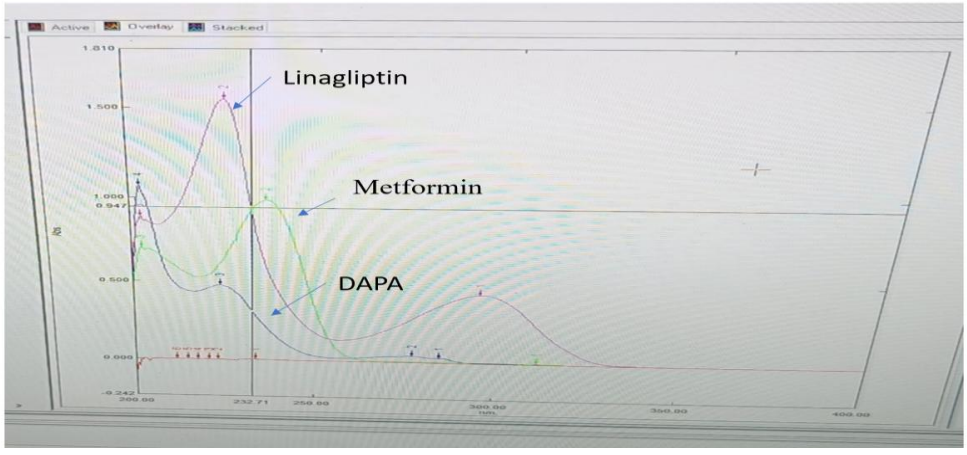


Figure 1: Overlay UV spectra of Metformin, Linagliptin and Dapagliflozin at 232 nm

Optimization of Mobile Phase Composition

Several mobile phase combinations were investigated to achieve proper peak separation, acceptable retention time, good peak symmetry, and better resolution. Different combinations of methanol, acetonitrile, water, phosphate buffer, orthophosphoric acid, and formic acid were evaluated.

Table 8: Mobile Phase Optimization Trials

Trial	Mobile Phase Composition	Observation
1	Methanol:Water (50:50-90:10)	No peak separation / broad peaks
2	ACN:Water (50:50-90:10)	Only MET and LINA eluted
3	Methanol:ACN	Improper peak shape
4	Methanol:Water (OPA pH 3.5)	No peak separation
5	Methanol:Water (Formic acid pH 3)	No peak separation
6	Methanol:ACN:Water	Peak separation with tailing
7	ACN:Phosphate buffer	Poor separation/tailing
8	Methanol:Phosphate buffer	Optimized separation achieved

Among all trials, **Methanol:10 mM phosphate buffer (65:35 % v/v, pH 4)** provided well-resolved peaks with acceptable retention time, theoretical plates, and peak symmetry.

Optimized Chromatographic Conditions

The optimized chromatographic conditions are summarized in Table 3.

Table 9: Optimized Chromatographic Conditions

Parameter	Condition
Column	Shimadzu Prominence C18 (250 mm × 4.6 mm, 5 µm)
Mobile Phase	Phosphate Buffer (10 mM): Methanol
Mobile Phase Ratio	35:65 % v/v
pH	4 ± 0.02
Flow Rate	1 mL/min
Detection Wavelength	232 nm
Injection Volume	10 µL
Column Oven Temperature	30°C
Run Time	15 min

Under optimized conditions, retention times were observed as:

- Metformin: **2.337 min**
- Linagliptin: **3.324 min**
- Dapagliflozin: **12.874 min**

The chromatogram showed well-resolved peaks with good symmetry and acceptable resolution.

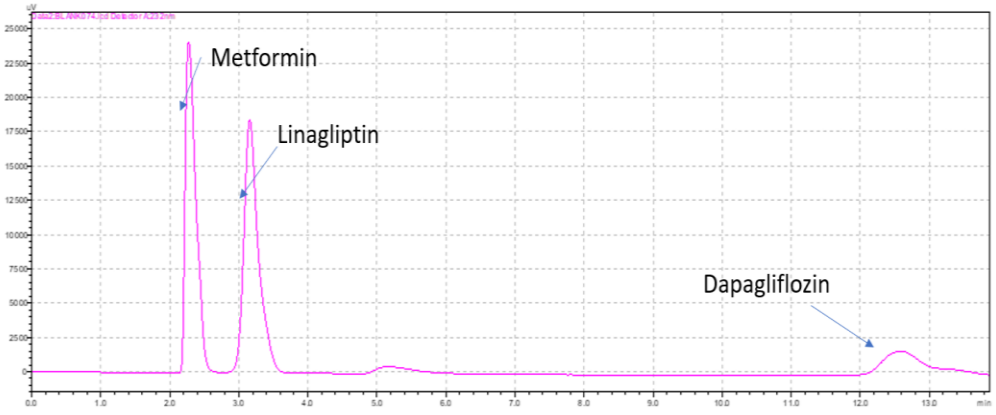


Figure 2: Standard chromatogram of Metformin, Linagliptin and Dapagliflozin

Method Validation

The developed RP-HPLC method was validated according to International Council for Harmonisation guidelines.

System Suitability

System suitability parameters confirmed the adequacy of chromatographic performance.

Table 10: System Suitability Results

Parameter	Metformin	Linagliptin	Dapagliflozin
Peak Area	277516	264758	84248
Retention Time	2.337 min	3.324 min	12.874 min
Asymmetry ± SD	1.537 ± 0.03	1.522 ± 0.02	1.477 ± 0.02
Resolution ± SD	—	3.097 ± 0.03	7.949 ± 0.02
Theoretical Plates ± SD	2523 ± 26.83	2874 ± 35.94	4298 ± 40.67

The %RSD was found to be less than 2%, indicating suitability of the chromatographic system.

Specificity

Specificity was evaluated by comparing chromatograms of blank, standard, and test solutions. No interfering peaks were observed at the retention times of the analytes, confirming specificity of the developed method.

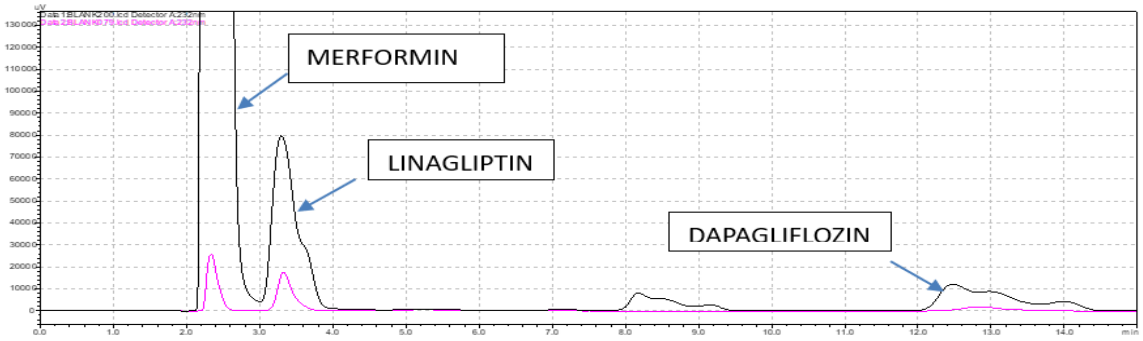


Figure 3: Overlaid chromatogram of standard and test solution

Linearity

Linearity was evaluated over a concentration range of **2.5-15 µg/mL** for all three drugs.

Table 11: Linearity Results

Drug	Linearity Range (µg/mL)	Correlation Coefficient
Metformin	2.5-15	0.9969
Linagliptin	2.5-15	0.9944
Dapagliflozin	2.5-15	0.9938

The method showed excellent linear response for all analytes.

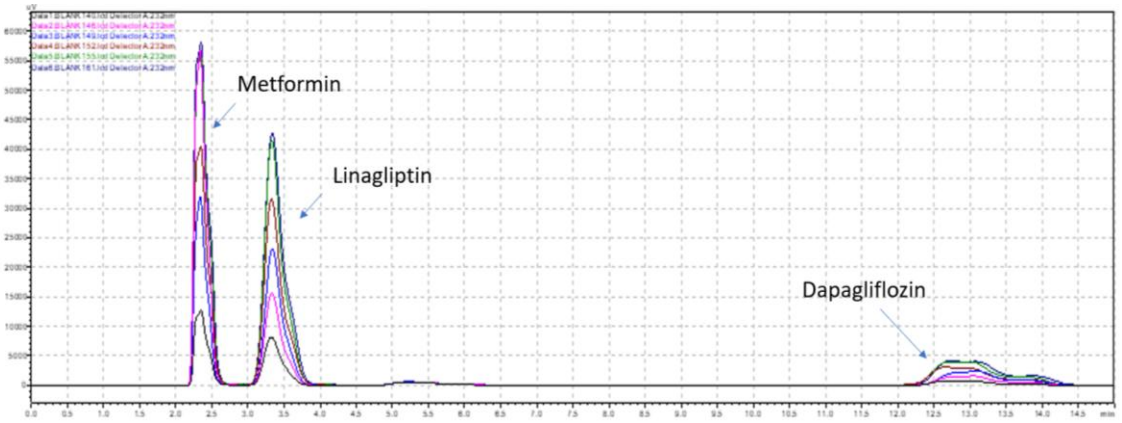


Figure 4: Overlaid RP-HPLC chromatogram of Metformin, Linagliptin & Dapagliflozin

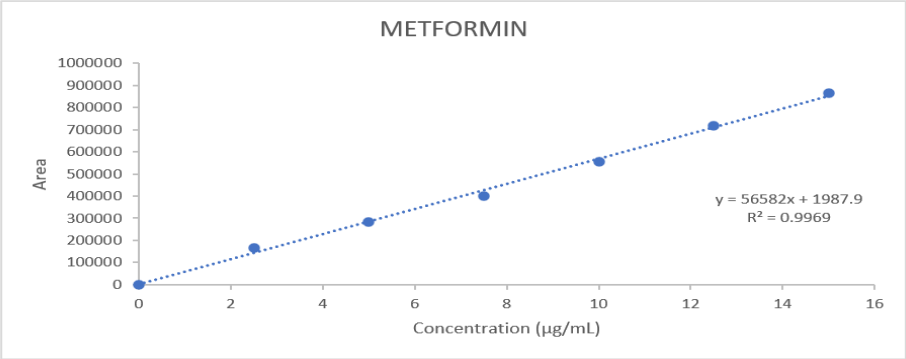


Figure 5: Calibration curve of Metformin

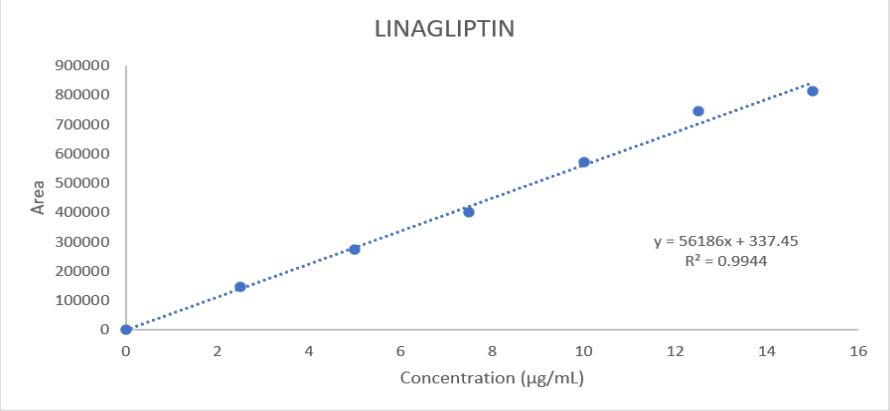


Figure 6: Calibration curve of Linagliptin

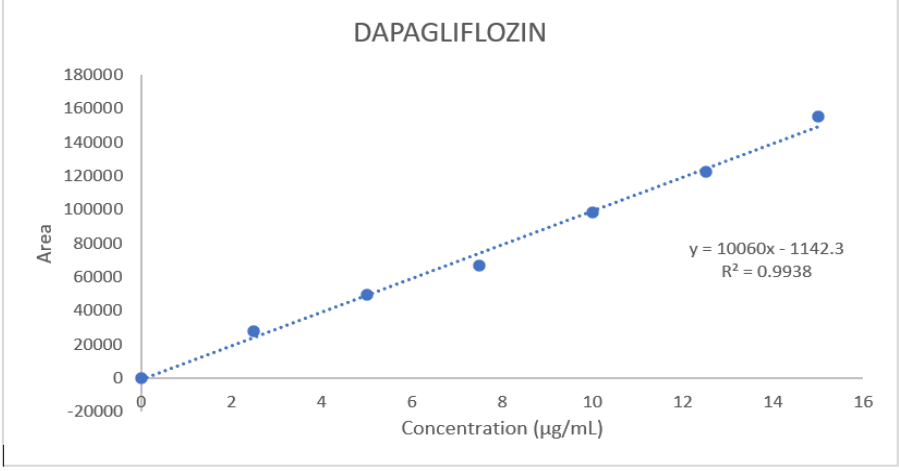


Figure 7: Calibration curve of Dapagliflozin

Sensitivity (LOD and LOQ)

Table 12: LOD and LOQ Results

Drug	LOD (µg/mL)	LOQ (µg/mL)
Dapagliflozin	0.27	0.82
Linagliptin	0.10	0.30
Metformin	0.061	0.18

Low LOD and LOQ values indicated high sensitivity of the developed method.

Accuracy

Recovery studies were performed at 50%, 100%, and 150% levels.

Mean Recovery Results

- Metformin: **97.46-103.70%**
- Linagliptin: **97.80-101.28%**
- Dapagliflozin: **102.13-104.36%**

The recovery values confirmed the accuracy of the developed method.

Precision

Repeatability, intraday precision, and interday precision studies were performed.

Repeatability (%RSD)

- Metformin: **1.19%**
- Linagliptin: **0.20%**
- Dapagliflozin: **0.39%**

Intraday Precision (%RSD)

All values were below **2%**

Interday Precision (%RSD)

All values were below **2%**

These results demonstrated excellent precision and reproducibility.

Robustness

Robustness was evaluated by making deliberate variations in:

- Flow rate
- Mobile phase composition
- Detection wavelength

The %RSD values remained below 2%, indicating robustness of the developed method.

Table 13: Variation in Robustness Parameter

Parameter	Variation	
Flow rate (1mL/min)	0.98 mL/min	1.02 mL/min
Mobile phase Composition (65:35%v/v)	63.7:36.3 %v/v	66.3:33.7 %v/v
λ_{max} (232nm)	230 nm	234 nm

Table 14: Results of Robustness

HPLC conditions	Drug	Mean area ± SD (mV) (n=3)	% RSD
λ_{max} (230nm)	Metformin	357209±449.5268	0.12
	Linagliptin	492329±200.0075	0.04
	Dapagliflozin	87695±153.8798	0.17
λ_{max} (234nm)	Metformin	373171.33±925.0672	0.24
	Linagliptin	318727.66±1005.372	0.31
	Dapagliflozin	62867±87.74395	0.13
Mobile phase composition (63.7:36.3%v/v)	Metformin	386095.66±136.6614	0.03
	Linagliptin	411814±323.0743	0.07
	Dapagliflozin	24260.66±84.50641	0.34
Mobile phase composition (66.3:33.7%v/v)	Metformin	386852±808.4844	0.20
	Linagliptin	410132.66±66.53069	0.016
	Dapagliflozin	21329.33±55.80621	0.26
Flow rate (0.98 mL/min)	Metformin	371736.66±466.3757	0.12
	Linagliptin	421719.33±736.8394	0.17
	Dapagliflozin	57404.33±45.8293	0.07
Flow rate (1.02 mL/min)	Metformin	352005±507.4377	0.14
	Linagliptin	403035.33±476.6344	0.11
	Dapagliflozin	54093±21.93171	0.04

Assay of Synthetic Mixture

The developed method was successfully applied for assay of synthetic mixture.

Table 15: Assay Results

Drug	Concentration Taken (µg/mL)	Concentration Found (µg/mL)	% Label Claim
Metformin	500	499.99	99.99
Dapagliflozin	10	10.17	101.77
Linagliptin	5	4.82	96.48

The assay results were within acceptable limits (95-105%), indicating applicability of the method for routine quality control analysis.

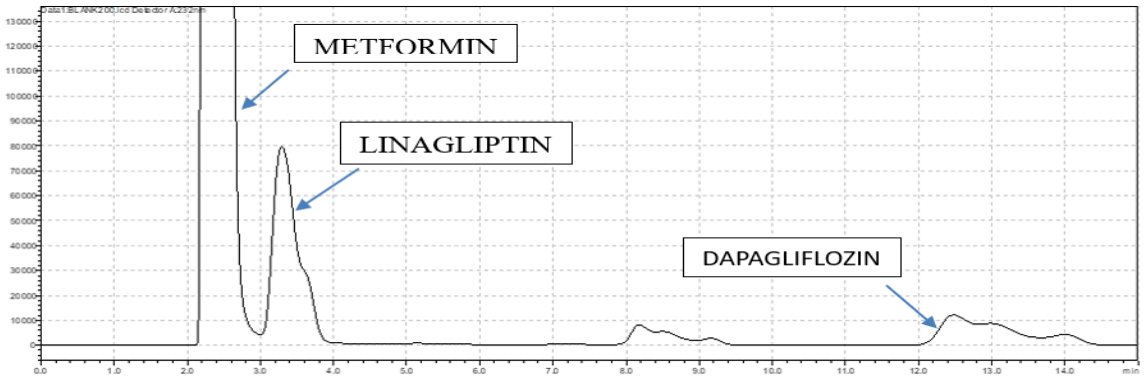


Figure 8: HPLC chromatogram of test sample

Table 16: Summary of validation parameters

System suitability parameters			
Parameters	**Metformin**	**Linagliptin**	**Dapagliflozin**
Retention time (min)	2.33 min	3.32 min	12.87 min
Theoretical plates	2523 ± 26.83	2874 ± 35.94	4298 ± 40.67
Asymmetry	1.537 ± 0.03	1.522 ± 0.02	1.477 ± 0.02
Resolution	-	3.097	7.949
Area	277516	264758	84248
Validation Parameters			
Specificity	specific	specific	specific
Linearity(n=3)	2.5-15µg/ml	2.5-15µg/ml	2.5-15µg/ml
R^2 value	0.9969	0.9944	0.9938
Repeatability(%RSD)	0.39	0.20	1.19
Intraday precision(%RSD)	0.19	0.16	0.37
Interday precision(%RSD)	0.15	0.35	0.59
Accuracy(%Recovery)	102.89%	99.20%	101.50%
Robustness	Robust	Robust	Robust
LOD(µg/mL)	0.27	0.10	0.016
LOQ(µg/mL)	0.82	0.30	0.18
%Assay	99.99%	96.48%	101.77%

CONCLUSION

A simple, accurate, precise, and reproducible RP-HPLC method was successfully developed and validated for the simultaneous estimation of Metformin, Linagliptin, and Dapagliflozin in synthetic mixture. The developed method utilized a Shimadzu Prominence C18 column (250 mm × 4.6 mm, 5 µm particle size) with a mobile phase consisting of Methanol:10 mM phosphate buffer (65:35 % v/v), where the pH of phosphate buffer was adjusted to 4.0 using orthophosphoric acid. Chromatographic separation was achieved using low-pressure gradient mode at a flow rate of 1 mL/min with UV detection at 232 nm.

The retention times were found to be 2.33 min for Metformin, 3.32 min for Linagliptin, and 12.87 min for Dapagliflozin with satisfactory peak symmetry, resolution, and theoretical plate count. The developed method showed excellent linearity in the concentration range of 2.5–15 µg/mL with correlation coefficients of 0.9969, 0.9944, and 0.9938 for Metformin, Linagliptin, and Dapagliflozin, respectively.

The method was validated according to International Council for Harmonisation guidelines for specificity, linearity, accuracy, precision, robustness, limit of detection (LOD), and limit of quantification (LOQ). Recovery studies demonstrated acceptable accuracy, while precision studies showed %RSD values below 2%, confirming reproducibility of the method. The developed method was found to be robust against minor variations in chromatographic parameters.

The assay results of commercially available formulations showed percentage assay values of 99.99% for Metformin, 96.48% for Linagliptin, and 101.77% for Dapagliflozin, indicating no interference from formulation excipients.

Therefore, the proposed RP-HPLC method can be successfully applied for routine quality control analysis and simultaneous estimation of Metformin, Linagliptin, and Dapagliflozin in pharmaceutical formulations.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

Author Contributions

Shaikh Munazza Iqbalahmed: Conceptualization, experimental investigation, method development, validation studies, data collection, statistical analysis, and manuscript drafting.

Amar M. Raval: Research supervision, methodology guidance, data interpretation, manuscript review, editing, and final approval of the manuscript.

Ethical Approval

This research work involved analytical method development and validation studies only and did not involve human participants, animal studies, or clinical samples. Therefore, ethical approval was not required for this study.

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