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Development, Optimization and Validation of a Stability-Indicating RP-HPLC Method for Simultaneous Estimation of Metformin Hydrochloride and Linagliptin in Bulk Drug Using Design of Experiments

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ABSTRACT: A systematic reverse-phase high-performance liquid chromatographic (RP-HPLC) method was developed for simultaneous estimation of Metformin Hydrochloride and Linagliptin in bulk drug. Preliminary chromatographic trials were followed by a three-factor Design of Experiments (DoE) study in which flow rate, column temperature and mobile-phase pH were investigated as independent variables. Peak area, retention time, theoretical plate count and tailing factor for both analytes were evaluated as responses. The DoE comprised 15 observations and the response models were assessed by analysis of variance (ANOVA) and regression analysis. Column temperature significantly affected Linagliptin retention time ($p=0.0044$) and theoretical plate count ($p=0.0177$). Metformin Hydrochloride retention time was significantly affected by flow rate ($p=0.0056$), column temperature ($p=0.0052$) and mobile-phase pH ($p=0.0048$). The Metformin Hydrochloride retention-time model showed $R^2=0.7671$, adjusted $R^2=0.7035$ and predicted $R^2=0.5002$, while the corresponding Linagliptin retention-time model showed $R^2=0.5886$, adjusted $R^2=0.4764$ and predicted $R^2=0.1982$. Numerical optimization predicted a solution at 1.000 mL/min flow rate, 40.0°C column temperature and pH 4.5, with desirability 0.664. The predicted retention times were 5.154 min for Metformin Hydrochloride and 6.751 min for Linagliptin. The available validation dataset demonstrated excellent reported linearity ($r=1.000$ for both analytes), mean recoveries of 99.7–100.2% for Metformin Hydrochloride and 99.7–99.9% for Linagliptin, and method precision of 0.2% and 0.1% RSD, respectively. Forced-degradation experiments under acidic, alkaline and oxidative conditions produced additional chromatographic responses while retaining identifiable principal analyte peaks. Importantly, the DoE optimum is a model prediction and should be experimentally confirmed at 40°C/pH 4.5 before those conditions are claimed as the final validated method.

INTRODUCTION

Metformin Hydrochloride is an oral antidiabetic drug belonging to the biguanide class and is widely used for management of type 2 diabetes mellitus. It acts principally by reducing hepatic gluconeogenesis and hepatic glucose output and is included in major pharmacopoeias. The chemical structure of Metformin Hydrochloride is presented in Figure 1.

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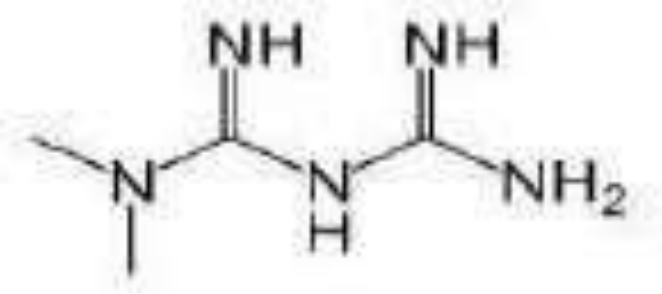


Figure 1. Chemical structure of Metformin Hydrochloride.

Linagliptin is an orally active dipeptidyl peptidase-4 (DPP-4) inhibitor used in the management of type 2 diabetes mellitus. It increases the activity and persistence of incretin hormones and thereby contributes to improved glucose regulation. The chemical structure of Linagliptin is presented in Figure 2.

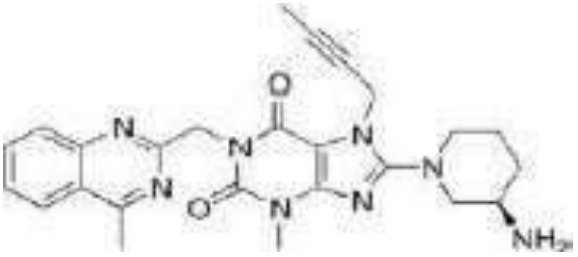


Figure 2. Chemical structure of Linagliptin.

RP-HPLC is widely applied to simultaneous analysis of multicomponent pharmaceutical systems because it provides selective separation and quantitative measurement. Earlier literature has reported analytical procedures for Metformin and Linagliptin individually and in combination with other antidiabetic drugs. However, conventional one-factor-at-a-time development does not efficiently characterize simultaneous effects of several chromatographic variables. DoE permits multiple factors to be investigated in a structured experimental framework and can identify the variables that most strongly influence critical analytical responses.

The objective of the present study was to develop and validate a stability-indicating RP-HPLC method for simultaneous estimation of Metformin Hydrochloride and Linagliptin and to integrate the available DoE dataset into method development and optimization.

2. MATERIALS AND METHODS

2.1 Materials

Metformin Hydrochloride and Linagliptin reference standards were used for method development and validation. HPLC-grade solvents, analytical-grade reagents and purified water were used throughout the study.

2.2 Instrumentation

Chromatographic analysis was performed using an HPLC system equipped with a solvent-delivery system, autosampler/injector, column compartment and UV/PDA detector.

2.3 UV Spectral Studies

Standard solutions of the two analytes were scanned in the UV-visible region. Metformin Hydrochloride showed a maximum absorption around 233 nm, whereas Linagliptin showed a prominent absorption response around 295 nm. The spectral observations were used in selecting the chromatographic detection condition.

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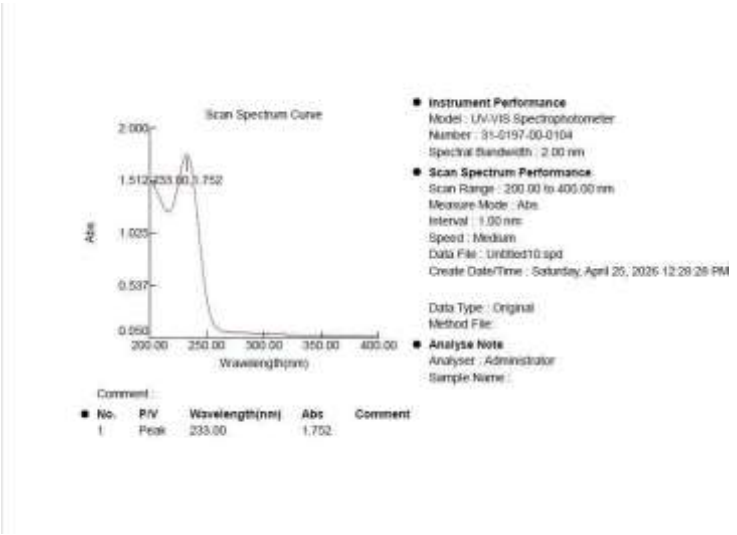


Figure 3. UV spectrum of Metformin Hydrochloride.

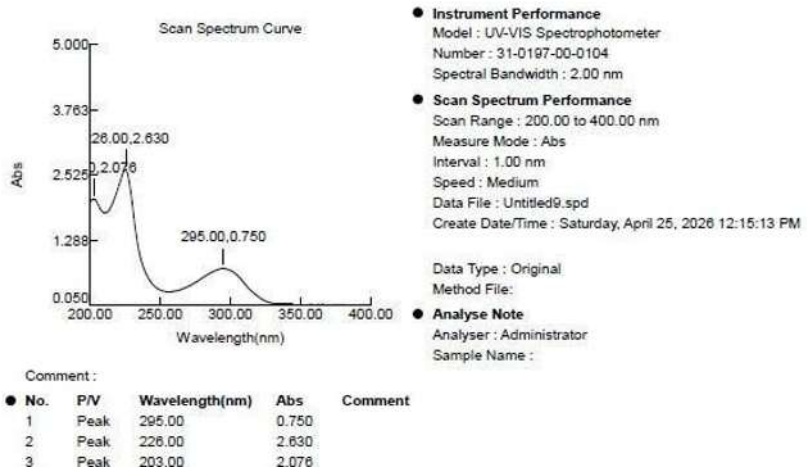


Figure 4. UV spectrum of Linagliptin.

2.4 FTIR Characterization

FTIR characterization was performed to support the identity of the reference APIs. The recorded spectra showed characteristic absorption bands corresponding to the functional groups of the investigated compounds.

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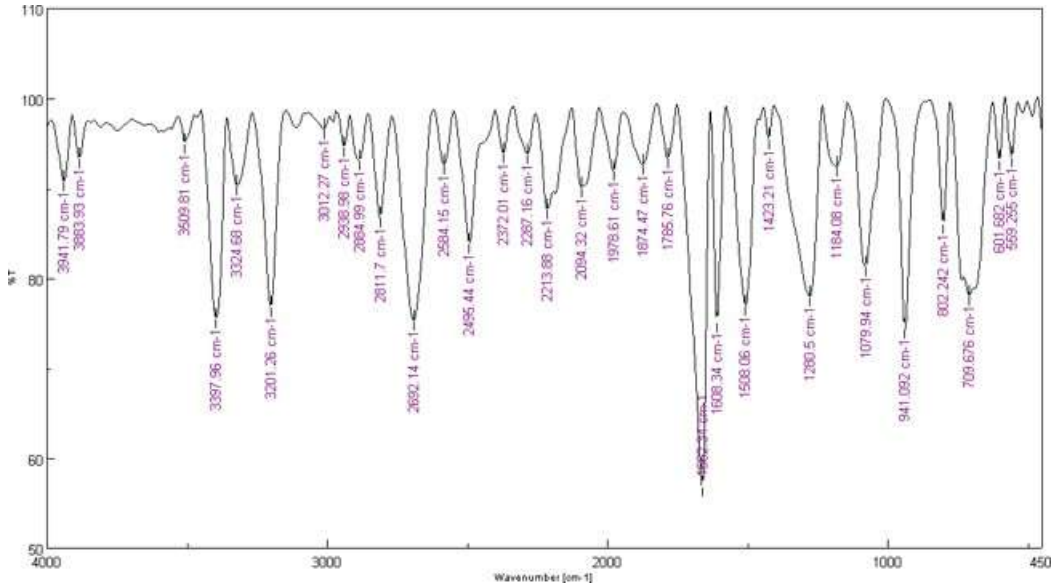


Figure 5. FTIR spectrum of Metformin Hydrochloride.

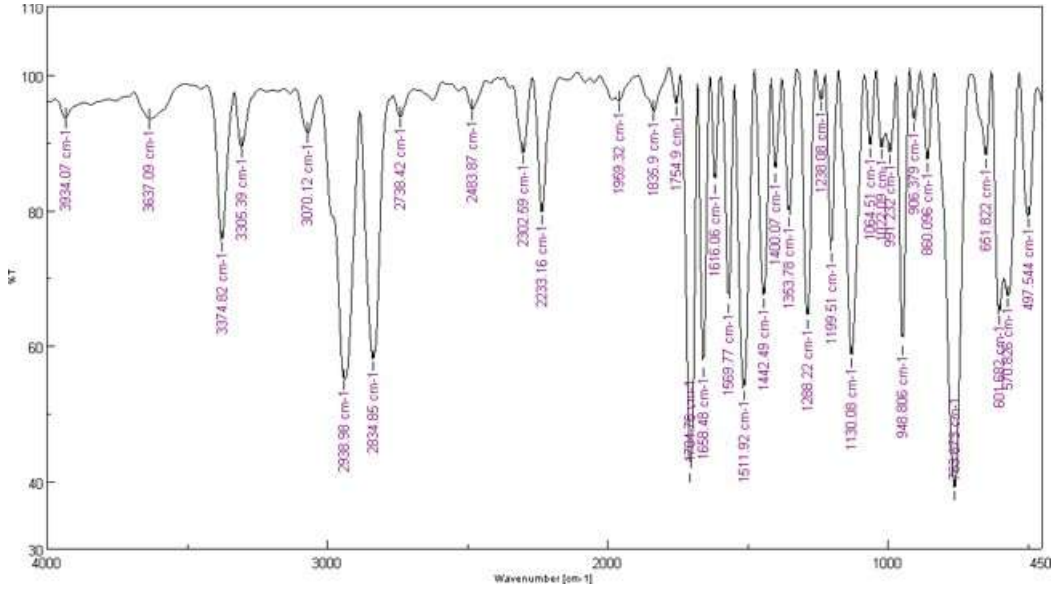


Figure 6. FTIR spectrum of Linagliptin.

2.5 Preliminary Chromatographic Method Development

Preliminary chromatographic trials were performed using a reversed-phase C18 column. The source document reports a tentative chromatographic procedure consisting of a C18 column (250 × 4.6 mm, 5 µm), phosphate buffer:acetonitrile (70:30, v/v), flow rate 1.0 mL/min, detection at 233 nm, injection volume 20 µL, column temperature 30°C and a 10-min run time. Under these conditions, retention times were approximately 5.10 min for Metformin Hydrochloride and 6.78 min for Linagliptin.

Parameter	Tentative condition
Column	C18, 250 × 4.6 mm, 5 µm
Mobile phase	Phosphate buffer : Acetonitrile (70:30, v/v)

Flow rate	1.0 mL/min
Detection wavelength	233 nm
Injection volume	20 µL
Column temperature	30°C
Run time	10 min
Metformin HCl retention time	5.10 min
Linagliptin retention time	6.78 min

These 30°C conditions are retained here as the experimentally documented preliminary/validation condition. They must not be conflated with the later DoE-predicted optimum at 40°C.

2.6 Preparation of Standard Solutions

Accurately weighed quantities of Metformin Hydrochloride and Linagliptin were transferred separately into volumetric flasks, dissolved using the selected diluent and diluted to the required working concentrations. Approximately 100.1 mg of Metformin Hydrochloride and 100.0 mg of Linagliptin were used in preparation of the respective standard solutions.

2.7 Design of Experiments

A three-factor DoE was used to investigate the effects of flow rate, column temperature and mobile-phase pH on chromatographic performance. The available DoE output reports 15 observations, corresponding to 14 total degrees of freedom in the ANOVA models. Eight chromatographic responses were considered.

Factor/Response	**Description / range**
A	Flow rate: 0.9–1.1 mL/min; target 1.0 mL/min
B	Column temperature: 35–45°C; target 40°C
C	Mobile-phase pH: 4.3–4.7; target 4.5
Response 1	Linagliptin peak area
Response 2	Linagliptin retention time
Response 3	Linagliptin theoretical plates
Response 4	Linagliptin tailing factor
Response 5	Metformin Hydrochloride peak area
Response 6	Metformin Hydrochloride retention time
Response 7	Metformin Hydrochloride theoretical plates
Response 8	Metformin Hydrochloride tailing factor

2.8 DoE Response Optimization

Numerical optimization simultaneously considered the three chromatographic factors and the analytical responses. The DoE software targeted flow rate 1.0 mL/min, column temperature 40°C and mobile-phase pH 4.5. Linagliptin retention time was targeted at 6.7 min and Metformin Hydrochloride retention time at 5.1 min; theoretical plate counts were maximized and tailing factor was constrained at approximately 1.1.

Optimization criterion	Goal	Lower limit	Upper limit
Flow rate	Target = 1.0	0.9	1.1
Column temperature	Target = 40	35	45
Mobile-phase pH	Target = 4.5	4.3	4.7
Linagliptin area	In range	3.0221×10^6	3.02991×10^6
Linagliptin RT	Target = 6.7 min	6.13	7.58
Linagliptin plates	Maximize	9901	12709
Linagliptin tailing	Fixed/none	1.1	1.1
Metformin area	Minimize	2.05112×10^6	2.14998×10^6
Metformin RT	Target = 5.1 min	4.62	5.69
Metformin plates	Maximize	9708	11095
Metformin tailing	Fixed/none	1.1	1.1

3. RESULTS AND DISCUSSION

3.1 Chromatographic Separation

The experimentally documented chromatogram showed distinct Metformin Hydrochloride and Linagliptin peaks at approximately 5.10 and 6.78 min, respectively. These data correspond to the 30°C chromatographic condition reported in the source manuscript.

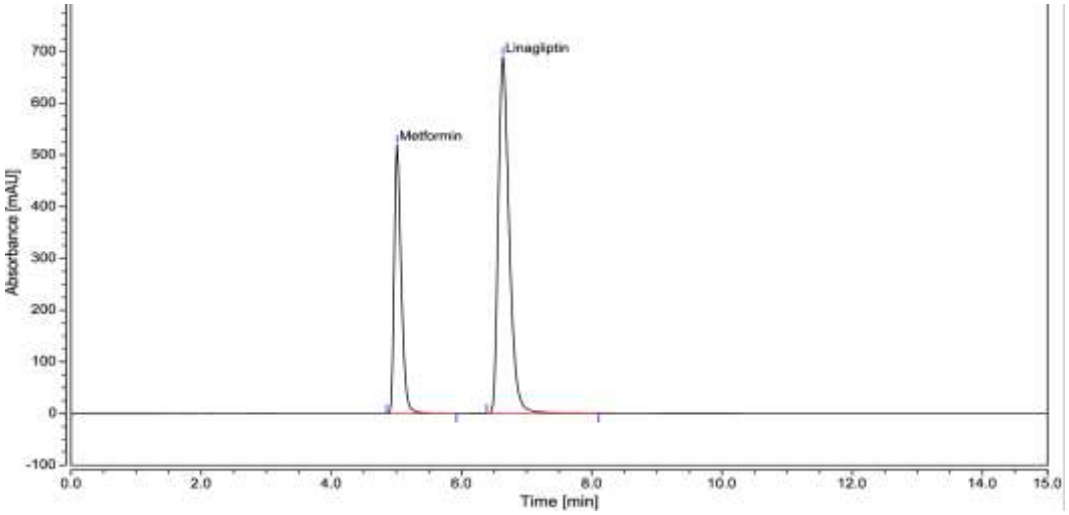


Figure 7. Untreated chromatogram showing Metformin Hydrochloride and Linagliptin.

3.2 DoE Statistical Analysis

The DoE output was evaluated using linear models where factor-dependent variation was supported and mean models where the response remained essentially constant. The statistical interpretation below is based directly on the supplied DoE output.

3.2.1 Linagliptin Peak Area

The linear model for Linagliptin peak area was not statistically significant ($F=2.94$, $p=0.0803$). Column temperature was the only individual factor reaching statistical significance ($p=0.0463$). Flow rate and pH were not significant ($p=0.2191$ and 0.1764, respectively).

Source	F-value	p-value	Interpretation
Model	2.94	0.0803	Not significant
Flow rate (A)	1.70	0.2191	Not significant
Column temperature (B)	5.04	0.0463	Significant
Mobile-phase pH (C)	2.09	0.1764	Not significant

Statistic	Value
R^2	0.4452
Adjusted R^2	0.2939
Predicted R^2	-0.1318
CV (%)	0.0709
Adequate precision	6.1156

Linagliptin Area = 3.027×10^6 + 885.00A + 1525.00B − 981.20C

3.2.2 Linagliptin Retention Time

The Linagliptin retention-time model was statistically significant (F=5.25, p=0.0172). Column temperature was the significant individual factor (p=0.0044), whereas flow rate and pH were not significant at the 5% level. The negative coefficient for temperature indicates a decrease in predicted retention time as temperature increased within the investigated range.

Source	F-value	p-value	Interpretation
Model	5.25	0.0172	Significant
Flow rate (A)	0.0006	0.9802	Not significant
Column temperature (B)	12.71	0.0044	Significant
Mobile-phase pH (C)	3.03	0.1095	Not significant

Statistic	Value
R^2	0.5886
Adjusted R^2	0.4764
Predicted R^2	0.1982
CV (%)	7.40
Adequate precision	6.5289

Linagliptin RT = 6.75 + 0.0040A − 0.5630B − 0.2750C

3.2.3 Linagliptin Theoretical Plate Count

The overall linear model for Linagliptin theoretical plates was not significant (F=3.21, p=0.0659). Nevertheless, column temperature showed a significant individual effect (p=0.0177).

Source	F-value	p-value	Interpretation
Model	3.21	0.0659	Not significant
Flow rate (A)	0.0134	0.9100	Not significant
Column temperature (B)	7.76	0.0177	Significant
Mobile-phase pH (C)	1.84	0.2017	Not significant

Statistic	Value
R^2	0.4665
Adjusted R^2	0.3210
Predicted R^2	0.1106
CV (%)	8.11
Adequate precision	5.2164

Linagliptin Plates = 10886.13 + 32.30A − 777.60B − 379.00C

3.2.4 Linagliptin Tailing Factor

Linagliptin tailing factor remained essentially constant at 1.10 throughout the DoE dataset. A mean model was therefore selected, with the fitted response equal to 1.10.

Linagliptin Tailing Factor = 1.10

3.2.5 Metformin Hydrochloride Peak Area

The DoE output selected a mean model for Metformin Hydrochloride peak area. The factor-dependent model was not significant, and the estimated mean response was approximately 2.123×10^6.

Statistic	Value
R^2	0.0000
Adjusted R^2	0.0000
Predicted R^2	-0.1480
CV (%)	1.95
Mean response	2.123×10^6

Metformin Area = 2.123×10^6

3.2.6 Metformin Hydrochloride Retention Time

Metformin Hydrochloride retention time showed the strongest DoE model. The overall model was highly significant (F=12.07, p=0.0008). Flow rate, column temperature and mobile-phase pH were all statistically significant individual factors, with p-values of 0.0056, 0.0052 and 0.0048, respectively.

Source	F-value	p-value	Interpretation
Model	12.07	0.0008	Significant
Flow rate (A)	11.75	0.0056	Significant
Column temperature (B)	12.07	0.0052	Significant
Mobile-phase pH (C)	12.40	0.0048	Significant

Statistic	Value
R^2	0.7671
Adjusted R^2	0.7035
Predicted R^2	0.5002
CV (%)	5.28
Adequate precision	12.7661

Metformin RT = 5.15 − 0.2950A − 0.2990B − 0.3030C

3.2.7 Metformin Hydrochloride Theoretical Plate Count

The model for Metformin Hydrochloride theoretical plates was not statistically significant (F=1.05, p=0.4093). None of the three individual factors reached statistical significance.

Source	F-value	p-value	Interpretation
Model	1.05	0.4093	Not significant
Flow rate (A)	0.3955	0.5423	Not significant
Column temperature (B)	1.36	0.2681	Not significant
Mobile-phase pH (C)	1.39	0.2630	Not significant

Statistic	Value
R^2	0.2225
Adjusted R^2	0.0105
Predicted R^2	-0.4637
CV (%)	4.62
Adequate precision	3.6439

Metformin Plates = 10340.13 + 95.10A + 176.40B + 178.40C

3.2.8 Metformin Hydrochloride Tailing Factor

Metformin Hydrochloride tailing factor remained essentially constant at 1.10 across the DoE dataset. A mean model was therefore selected.

Metformin Tailing Factor = 1.10

3.3 Overall DoE Interpretation

The DoE results indicate that retention time was more responsive to the investigated chromatographic variables than peak symmetry. Linagliptin retention time and plate count were particularly sensitive to column temperature. In contrast, all three investigated factors significantly affected Metformin Hydrochloride retention time. Peak tailing remained stable at approximately 1.10 for both analytes, indicating that peak symmetry was maintained across the investigated design space.

Response	Model p-value	R^2	Adjusted R^2	Key observation
Linagliptin area	0.0803	0.4452	0.2939	Model not significant; temperature individually significant
Linagliptin RT	0.0172	0.5886	0.4764	Temperature significant
Linagliptin plates	0.0659	0.4665	0.3210	Temperature significant individually
Linagliptin tailing	Mean	0.0667	0.0667	Constant at 1.10
Metformin area	Mean	0.0000	0.0000	Mean model; CV 1.95%
Metformin RT	0.0008	0.7671	0.7035	All three factors significant
Metformin plates	0.4093	0.2225	0.0105	No factor significant
Metformin tailing	Mean	0.0667	0.0667	Constant at 1.10

3.4 Numerical Optimization

Numerical optimization of the DoE models generated two closely related solutions. The first solution was selected by the DoE software as the optimum. It used a flow rate of 1.000 mL/min, column temperature 40.000°C and mobile-phase pH 4.500, with an overall desirability of 0.664.

Solution	Flow rate (mL/min)	Column temp. (°C)	pH	Linagliptin RT (min)	Metformin RT (min)	Desirability	Status
1	1.000	40.000	4.500	6.751	5.154	0.664	Selected
2	1.000	40.038	4.500	6.747	5.152	0.664	Alternative

Predicted response at selected solution	Predicted value
Linagliptin area	3,027,212.867
Linagliptin retention time	6.751 min
Linagliptin theoretical plates	10,886.133
Linagliptin tailing factor	1.100
Metformin area	2,122,545.000

Metformin retention time	5.154 min
Metformin theoretical plates	10,340.133
Metformin tailing factor	1.100

These values are model-predicted responses and not experimental confirmation. Because the validation dataset in the manuscript was obtained at the documented 30°C condition, the 40°C DoE optimum should be confirmed experimentally before being designated as the final validated chromatographic condition.

3.5 System Suitability

System suitability was evaluated using five replicate injections. Metformin Hydrochloride showed a mean peak area of 2,152,008 with SD 2645.2 and %RSD 0.1%; retention time was 5.10 min, theoretical plates were approximately 10,542–11,873 and tailing factor was 1.10–1.11. Linagliptin showed a mean peak area of 3,025,985 with SD 325.3 and reported

%RSD approximately 0.0%; retention time was 6.78 min, theoretical plates were approximately 12,552–13,098 and tailing factor was 1.10.

Parameter	Metformin HCl	Linagliptin
Mean peak area	2,152,008	3,025,985
SD	2645.2	325.3
%RSD	0.1	0.0
Retention time (min)	5.10	6.78
Theoretical plates	10,542–11,873	12,552–13,098
Tailing factor	1.10–1.11	1.10

Table: System Suitability and Method Precision of Metformin and Linagliptin

Analyte	Injection	Area	Retention Time	Tailing Factor	Theoretical Plates
Metformin HCl					
Metformin HCl	1	2,151,122	5.10	1.11	11,091
Metformin HCl	2	2,151,231	5.10	1.10	11,109
Metformin HCl	3	2,150,924	5.10	1.11	10,542
Metformin HCl	4	2,156,672	5.10	1.11	10,924
Metformin HCl	5	2,150,092	5.10	1.10	11,873
Metformin HCl	**Average**	**2,152,008 ± 2,645.2**	**5.10 ± 0.00**	**1.11 ± 0.01**	**11108 ± 485**
Metformin HCl	Method precision	100 ± 0.2	—	—	Assay: 100.0 ± 0.2 %
Linagliptin					
Linagliptin	1	3,026,529	6.78	1.10	12,762
Linagliptin	2	3,025,667	6.78	1.10	13,098
Linagliptin	3	3,025,876	6.78	1.10	12,552
Linagliptin	4	3,025,987	6.78	1.10	12,860
Linagliptin	5	3,025,865	6.78	1.10	12,652
Linagliptin	**Average**	**3,025,985 ± 325.3**	**6.78 ± 0.00**	**1.10 ± 0.00**	**12785 ± 210**
Linagliptin	Method precision	100 ± 0.1	—	—	Assay: 99.9 ± 0.1 %

Figure 8. System suitability and method precision data.

3.6 Linearity

Linearity was assessed over five concentration levels. For Metformin Hydrochloride, concentrations of 50.1, 80.1, 100.1,

120.1 and 150.2 µg/mL produced mean peak areas of 1,075,506; 1,722,654; 2,153,648; 2,582,865; and 3,235,885, respectively. The reported regression equation was y = 21571.63x − 5640.07 with r=1.000.

For Linagliptin, concentrations of 50, 80, 100, 120 and 150 µg/mL produced mean peak areas of 1,512,255; 2,428,732; 3,025,668; 3,633,975; and 4,536,547, respectively. The reported regression equation was y = 30227.49x + 4686.09 with r=1.000.

Analyte	Concentration (µg/mL)	Mean peak area
Metformin HCl	50.1	1,075,506
Metformin HCl	80.1	1,722,654
Metformin HCl	100.1	2,153,648
Metformin HCl	120.1	2,582,865
Metformin HCl	150.2	3,235,885
Linagliptin	50	1,512,255
Linagliptin	80	2,428,732
Linagliptin	100	3,025,668
Linagliptin	120	3,633,975
Linagliptin	150	4,536,547

Analyte	Range (µg/mL)	Regression equation	r
Metformin HCl	50.1–150.2	y = 21571.63x − 5640.07	1.000
Linagliptin	50–150	y = 30227.49x + 4686.09	1.000

Table 8: Linearity of Linagliptin

Linearity Level (%)	mL of stock solution	Final Volume (mL)	Final Concentration (ppm)	Area 1	Area 2	Area 3	Average	SD	%RSD
50	2.50	50	50.0	1512332	1512093	1512339	1512255	140.1	0.0
80	4.00	50	80.0	2420099	2428553	2437543	2428732	8723.4	0.4
100	5.00	50	100.0	3025584	3025632	3025789	3025668	107.2	0.0
120	6.00	50	120.0	3631176	3630927	3639823	3633975	5065.8	0.1
150	7.50	50	150.0	4530924	4539974	4538743	4536547	4908.4	0.1

Correlation coefficient	1.0000
Slope	30227.4931
Intercept	4686.0897
Y-Intercept	0.129

Figure 9. Linearity data for Linagliptin.

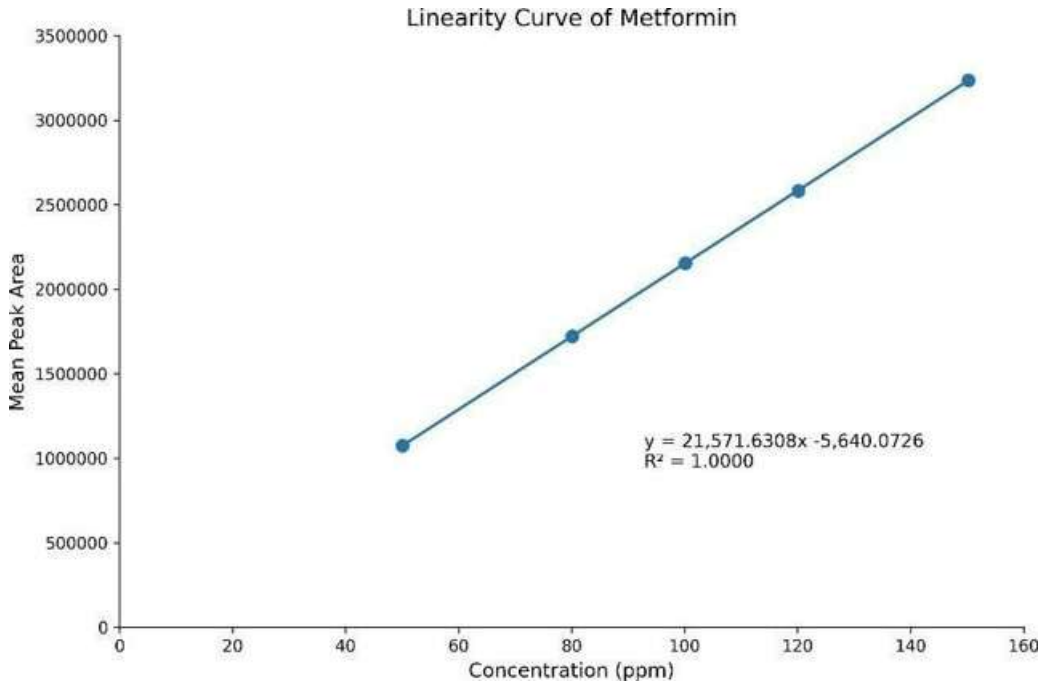


Figure 10. Calibration curve of Metformin Hydrochloride.

Table 7: Linearity of Metformin

Linearity Level (%)	mL of stock solution	Final Volume (mL)	Final Concentration (ppm)	Area 1	Area 2	Area 3	Average	SD	%RSD
50	2.50	50	50.1	1076654	1074532	1075332	1075506	1071.6	0.1
80	4.00	50	80.1	1722554	1720953	1724454	1722654	1752.6	0.1
100	5.00	50	100.1	2150922	2156889	2151132	2153648	4540.3	0.2
120	6.00	50	120.1	2584432	2584210	2579953	2582865	2524.3	0.1
150	7.50	50	150.2	3234456	3242231	3230967	3235885	5766.3	0.2

Correlation coefficient	1.0000
Slope	21571.6308
Intercept	-5640.0726
Y-Intercept	-0.2184

Figure 11. Linearity data for Metformin Hydrochloride.

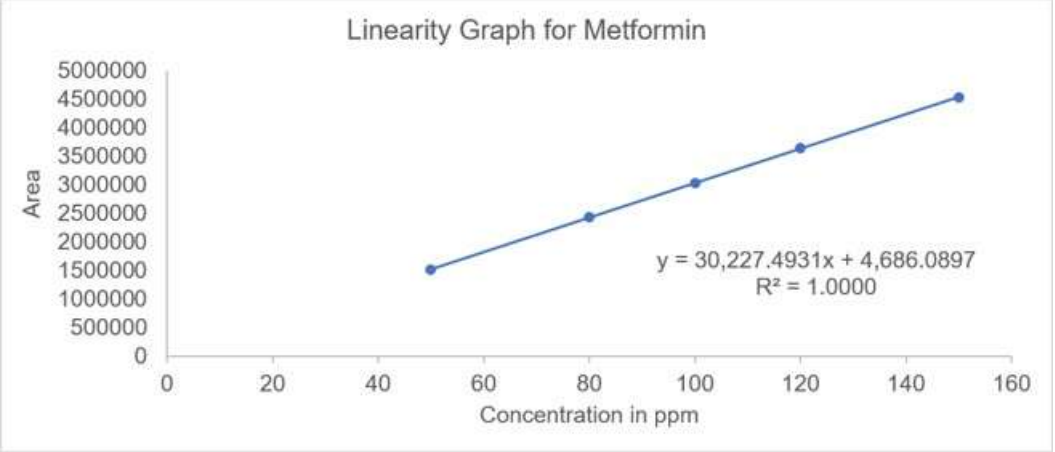


Figure 12. Calibration curve of Linagliptin.

3.7 Precision

Method precision was evaluated using six independent sample preparations. Metformin Hydrochloride showed assay values

from 99.8 to 100.3%, with mean assay approximately 100.0% and %RSD 0.2%. Linagliptin showed assay values from 99.8 to 100.0%, with mean assay approximately 99.9% and %RSD 0.1%.

Analyte	Mean assay (%)	%RSD
Metformin Hydrochloride	100.0	0.2
Linagliptin	99.9	0.1

3.8 Accuracy

Accuracy was evaluated by standard addition at 50%, 100% and 150% levels. Metformin Hydrochloride gave mean recoveries of 99.7%, 100.1% and 100.2%, with %RSD values of 0.1%, 0.1% and 0.3%, respectively. Linagliptin gave mean recoveries of 99.7%, 99.9% and 99.8%, with %RSD values of 0.1%, 0.1% and 0.1%.

Analyte	Level	Mean recovery (%)	%RSD
Metformin HCl	50%	99.7	0.1
Metformin HCl	100%	100.1	0.1
Metformin HCl	150%	100.2	0.3
Linagliptin	50%	99.7	0.1
Linagliptin	100%	99.9	0.1
Linagliptin	150%	99.8	0.1

Table: Accuracy of Metformin HCl and Linagliptin

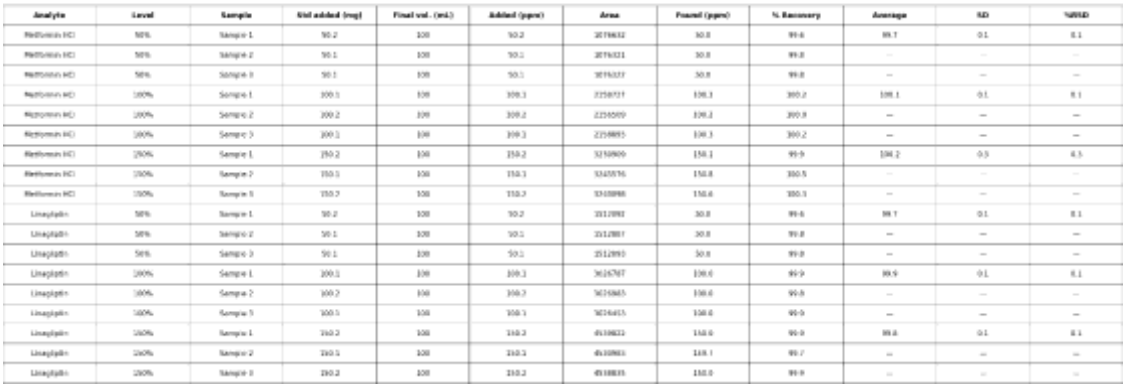


Figure 13. Accuracy data for Metformin Hydrochloride and Linagliptin.

3.9 Robustness

Robustness was assessed by deliberate changes in flow rate, detection wavelength and pH. The maximum reported %RSD was 0.35% for Metformin Hydrochloride and 0.21% for Linagliptin, indicating low variability under the investigated variations.

Parameter varied	Metformin HCl (%RSD)	Linagliptin (%RSD)
Flow rate – low	0.28	0.00
Flow rate – high	0.00	0.00
Wavelength – low	0.28	0.14
Wavelength – high	0.14	0.14
pH – low	0.35	0.00
pH – high	0.00	0.21

Robustness Study (Mean ± SD)

		METFORMIN HCl				LINAGLIPTIN			
Parameter	Condition	Area	RT (min)	Tailing Factor	Theoretical Plates	Area	RT (min)	Tailing Factor	Theoretical Plates
Flow rate	Low	2173453 ± 2899	5.68 ± 0.00	1.10 ± 0.00	10362 ± 396	3056432 ± 1389.6	7.53 ± 0.00	1.10 ± 0.00	12209 ± 512
Flow rate	High	2154592 ± 2519	4.64 ± 0.00	1.10 ± 0.00	10451 ± 348	3055075 ± 2519.4	6.16 ± 0.00	1.10 ± 0.00	12330 ± 348
Wavelength	Low	2154993 ± 3707	5.10 ± 0.00	1.10 ± 0.00	10957 ± 458	3026465 ± 2466	6.78 ± 0.00	1.10 ± 0.00	12222 ± 438
Wavelength	High	2151354 ± 1971	5.10 ± 0.00	1.10 ± 0.00	10629 ± 217	3037670 ± 1385	6.78 ± 0.00	1.10 ± 0.00	12032 ± 419
pH	Low	2144786 ± 3851	5.68 ± 0.00	1.10 ± 0.00	9851 ± 102	3025640 ± 372	7.53 ± 0.00	1.10 ± 0.00	11182 ± 780
pH	High	2147229 ± 3761	4.64 ± 0.00	1.10 ± 0.00	10448 ± 361	3077203 ± 3776	6.16 ± 0.00	1.10 ± 0.00	10338 ± 377

Figure 14. Robustness study results.

3.10 Forced Degradation Studies

Forced degradation studies were performed under acidic, alkaline and oxidative conditions to assess the stability-indicating capability of the method. The untreated chromatogram showed principal peaks at approximately 5.1 min for Metformin Hydrochloride and 6.7–6.8 min for Linagliptin. Acidic, alkaline and oxidative treatments produced additional chromatographic responses while the principal analyte peaks remained identifiable. The oxidative condition involved hydrogen peroxide; acid and base treatments were performed under the stress conditions documented in the source study.

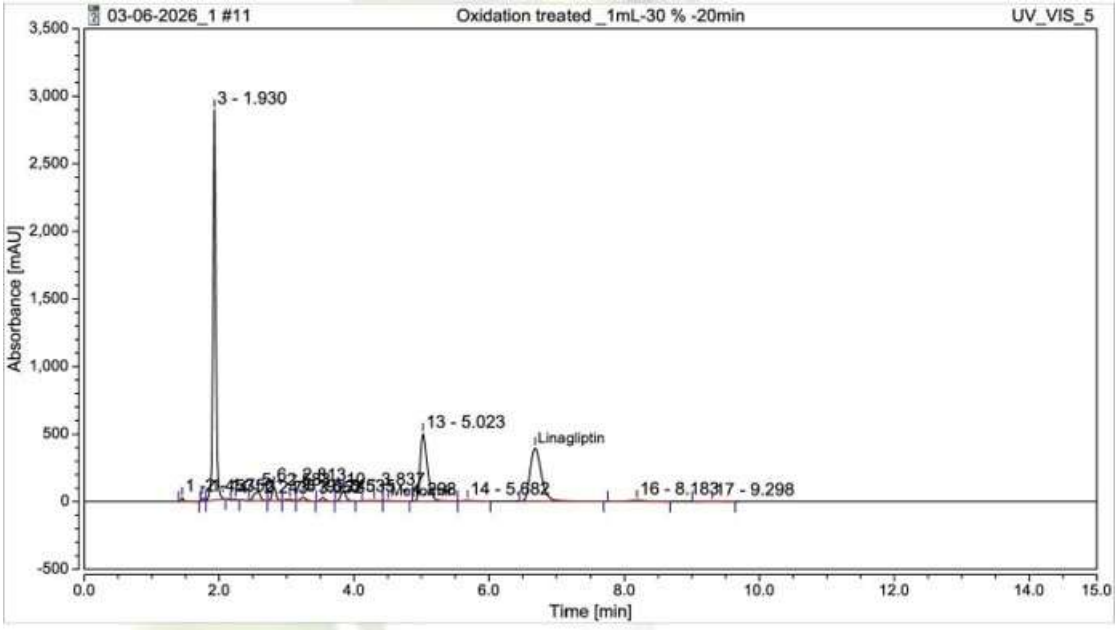


Figure 15. Oxidative-stress chromatogram.

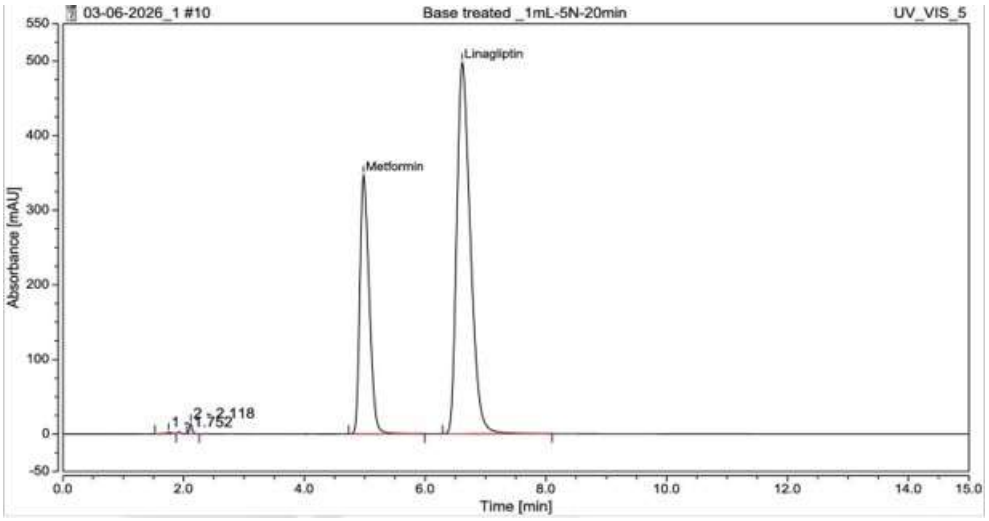


Figure 16. Base-treated chromatogram.

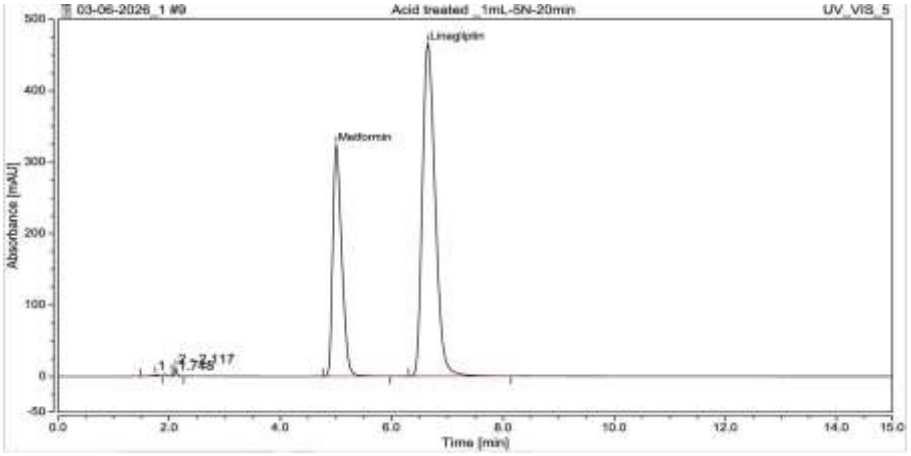


Figure 17. Acid-treated chromatogram.

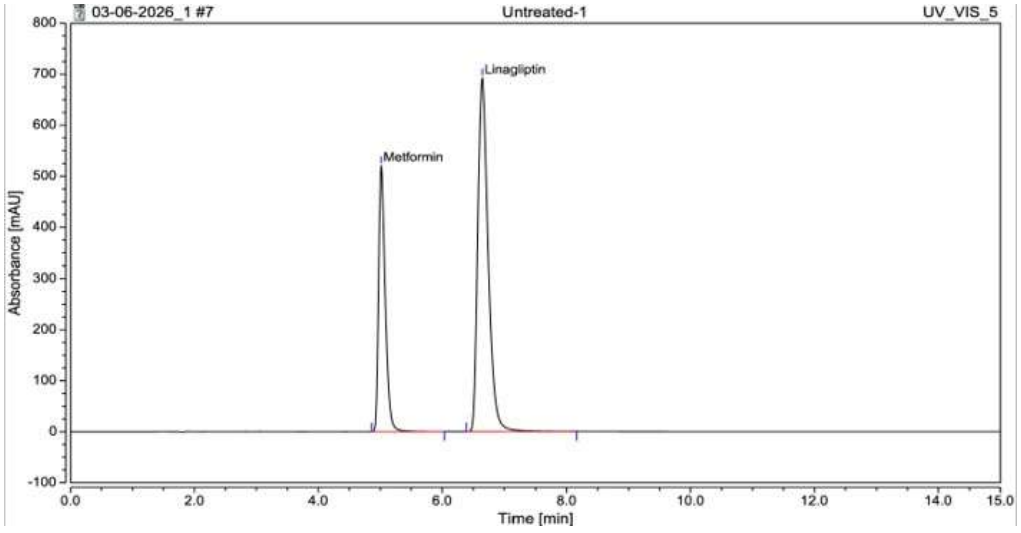


Figure 18. Untreated chromatogram.

The chromatograms support use of the procedure for monitoring degradation-related chromatographic changes. Because numerical peak-area degradation data and complete mass-balance calculations are not provided in the available dataset, percentage degradation and mass balance are not reported here.

4. INTEGRATED DISCUSSION

The combined preliminary development, DoE and validation results provide complementary information on the analytical procedure. The experimentally documented 30°C method produced clear separation and demonstrated strong linearity, accuracy, precision and conventional robustness. The DoE added a multivariate assessment of three chromatographic variables and showed that retention time, particularly for Metformin Hydrochloride, was sensitive to changes in flow rate, temperature and pH.

The Metformin Hydrochloride retention-time model was the strongest of the investigated response models, with R^2=0.7671, adjusted R^2=0.7035 and predicted R^2=0.5002. The Linagliptin retention-time model was also significant, although its predictive strength was lower. Column temperature was the most influential individual factor for Linagliptin retention time and plate count. In contrast, the tailing factor for both analytes remained essentially unchanged at 1.10.

The desirability-based optimization predicted 1.0 mL/min, 40°C and pH 4.5 as the selected operating point. However, this prediction differs from the experimentally documented 30°C validation condition. The manuscript therefore distinguishes clearly between experimentally observed validation results and model-predicted optimization results. A confirmation injection at the DoE-predicted optimum, followed by confirmation of system suitability and relevant validation characteristics, is required before the 40°C condition can be claimed as the final validated method.

5. CONCLUSION

A systematic RP-HPLC procedure for simultaneous estimation of Metformin Hydrochloride and Linagliptin was developed and evaluated using conventional analytical validation together with DoE-based statistical optimization. The available validation data showed excellent reported linearity, satisfactory accuracy, good precision and low variability during conventional robustness testing. Forced-degradation experiments generated additional chromatographic responses while retaining identifiable principal analyte peaks.

The DoE investigation demonstrated significant effects of column temperature on Linagliptin retention time and theoretical plate count and significant effects of flow rate, column temperature and mobile-phase pH on Metformin Hydrochloride retention time. Numerical optimization predicted a desirable condition of 1.0 mL/min, 40°C and pH 4.5 with desirability 0.664. This condition is a model prediction and must be experimentally confirmed. The final validated method should therefore be designated only after the DoE optimum has been confirmed experimentally.

6. DATA INTEGRITY AND REPORTING NOTE

The present rewrite intentionally does not invent a 15-run raw DoE response table because the supplied DoE presentation provides the statistical models, ANOVA outputs, optimization constraints and numerical solutions, but the complete run-by-run response matrix is not available as extractable data in the supplied materials. Only values explicitly supported by the supplied manuscript and DoE output have been incorporated..

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