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Isocratic RP-UHPLC method development and validation of stability-indicating for simultaneous determination of teneligliptin and metformin in fixed-dose combination

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A B S T R A C T

The pharmaceutical combination of Teneligliptin Hydrobromide hydrate (TEN) and Metformin Hydrochloride (MET) drugs is used in the treatment of type 2 diabetes mellitus. A new analytical method: QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) has been developed for the quantification of Teneligliptin (TEN) and Metformin (MET) in bulk and tablet dosage forms. The analysis was performed on Agilent symmetry analytical column Eclipse plus C18 (150 mm × 4.6 mm, 5 µm) ultra- performance liquid chromatography-Diode Array Detectors (UHPLC-DAD), while the detection was performed on 233 nm using Diode Array Detectors. Buffer and acetonitrile (65:35 v/v) were the mobile phase, run at a flow rate of 0.7 mL min^{-1} for isocratic elution. The buffer used in the mobile phase contained 50 mM potassium di-hydrogen phosphate, pH adjusted to 3.5±0.02 with orthophosphoric acid. The mean values of recovery were found to be 100.50% and 99.81%. The proposed method could be ideal for quantitative evaluation in pharmaceutical preparations of these drugs and also for their quality control in bulk manufacturing. Stress test covers: acid, base, peroxide, thermal and photolytic degradation; were conducted to show the specificity of the method and degradation.

1. Introduction

India is one of the region's six IDF (International Diabetes Federation) SEA (South-East Asia) countries. 425 million public worldwide have diabetes and 82 million in the SEA region, which may increase to 151 million by 2045. In 2017, India had more than 72,946,400 cases of diabetes. It has been likely that there are around 451 million people with diabetes globally in 2017, aged between 18-99 years. With such a high number of diabetic patients by 2045, there is a high chance that this figure may touch 693 million. In today's world, it is estimated that nearly 50% of the living population remains undiagnosed for being diabetic. Furthermore, it has been expected that around 374 million people impaired with glucose sufferance (IGT) were affected by some form of hyperglycemia, and approximately 21.3 million live births to women may be affected by several kinds of hyperglycemia, for the period of pregnancy. In 2017, around 5 million deaths worldwide were attributed to diabetes.

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The global healthcare expenses on diabetes patients in 2017 are expected at USD 850 billion[1]. In modern society, diabetes and its related concern are a major emergent issue. Many oral anti-diabetics with diverse mechanisms of action (MOA) have been developed to reduce glucose levels and delay the possibility of severe complication in type 2 diabetes patients[2]. Treatment with a conventional oral anti-diabetic drug is not effective in managing diabetes levels. Therefore, people with type 2 diabetes are suggested a combination therapy approaches including contributory drugs. Ideally, combination therapies have several advantages such as: being tolerable, easy to test, contraindications of relatively small quantities, and reduced risk of hypoglycemia and weight gain. In combination treatment such as the combination of biguanide and metformin, an insulin sensitizer and dipeptidyl peptidase-4 (DPP-4) inhibitors that function as an insulin secretagogue, may be successful in the short and long term. Combination treatment in this case makes the impaired β cell to function more securely, thus making the treatment profitable due to cost-effective diagnosis with a lower tablet load than their monotherapy [3,4].

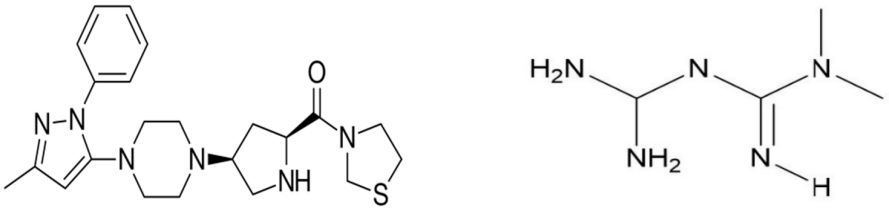
Teneligliptin (TEN) **(Fig. 1(a))** is (3-[(2S, 4S)-4-[4-(3-methyl-1-phenyl-1H-pyrazol-5-yl) piperazin-1-yl] pyrrolidin-2-ylcarbonyl] thiazolidine) is a new extremely powerful, long-lasting and specifically active oral DPP-4 inhibitor for use in type 2 diabetes treatment.[5] The HPTLC and UV methods for estimating TEN in bulk and tablet dosage forms have been developed and validated[6] by RP-HPLC and UPLC MS / MS degradation product degradation[7]. There are only a few LC-MS/MS analytical methods reported for TEN alone [8,9].

Metformin (MET) **(Fig. 1(b))** is N, N - dimethyl biguanide, which is an antihyperglycemic agent in the class of biguanide[10]. The MET was determined by spectrophotometric methods with mixtures in bulk and various pharmaceutical formulations[11,12] and by HPLC[13]. MET was estimated using LC-MS techniques in human plasma mixtures[14–19]. TEN co-administered with MET resulted in an important reduction in HbA1c in type 2 diabetes mellitus (T2DM) patients without the risk of hypoglycemia is improved[20]. TEN and MET were estimated using different spectrophotometric methods with mixtures in bulk and various pharmaceutical formulations.[21,22] and by HPLC methods[22–25]. TEN and MET were studied in human plasma using hydrophilic interaction by LC-MS/MS[26]. UHPLC-QTOF-MS methods for quantification of TEN and MET were studied in rat plasma[27] and by UHPLC-MS/MS[28].

Furthermore, the creation of the present contribution was also inspired by a more beneficial UHPLC method, which is an HPLC derivative, which demonstrates a significant increase in sensitivity, resolution, and speed of analysis due to the usage of column particle size smaller than 2 μm. It can operate at higher pressure with the mobile phase operating at faster linear speeds relative to HPLC with a significant decrease in analytical time, sample volume and solvent consumption, along with superior chromatographic separation [29,30].

The purpose of this work was thus focused on investigating a rapid and effective separation process using an updated QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) method which can simultaneously estimate TEN and MET in bulk drugs. For this, the pretreatment method for higher recoveries was optimized using different solvents, columns and pH. To evaluate authentic methods, the established method was successfully used.

The UHPLC-DAD methods were therefore developed and validated for the simultaneous quantitation of TEN and MET in bulk and tablets. The UHPLC methods developed have been validated under the ICH guidelines[31]. The methods proposed are ideal for evaluating the quality control and determining the purity of bulk and tablets that contain both the drugs.



(a) (b)

Fig. 1. Chemical structures of (a) Teneligliptin, (b) Metformin

2. Results and Discussion

TEN and MET were developed and validated with DAD detection in bulk product and pharmaceutical formulations as per ICH guidelines for validation of the analytical method, Q2 (R1).

2.1 Method Development

A much more advantageous UPLC method, which is an HPLC derivative but shows a dramatic improvement in speed, resolution, and analytical sensitivity, has encouraged and inspired the development of the present contribution. Furthermore, no method is available in the literature to simultaneously analyze the TEN and MET contents in a drug. Herein, different chromatographic conditions have been examined and designed for TEN and MET determination, such as mobile phases with varying compositions of pH and stationary phases with different packaging content, etc. The UV emissions showed a maximum of TEN and MET absorption at 233 nm.

Attempts were made using three types of UHPLC columns (Zorbax Eclipse Plus C18 (50×4.6 mm, 1.8 µm), Inertsil ODS-2 (150×4.6 mm, 5 µm) and Zorbax Eclipse Plus C18 (150×4.6 mm, 5 µm) columns with different compositions and ratios for the mobile phases. Broad characteristic peaks were obtained in all the following columns using various ratios of methanol/acetonitrile and water (20:80, 40:60, 50:50, 60:40, 80:20). No peak change in form was observed while column temperature was raised to 45°C. The theoretical plates with the methanol or acetonitrile combination solution with water as the mobile phases were below 2000, showing insufficient separation capacity for column chromatography. For the above two forms of mixing solutions, the peak symmetry and peak shape were both imperfect, which could be due to low mobile phase polarity. So, some phosphate buffer of specific concentration (20, 30, 40, 50 and 60 mM) was used to improve the mobile phase polarity, resulting in a narrow peak. However, the overall form and peak symmetry remained unsatisfactory. Therefore, acetonitrile was used instead of methanol. Finally, the phosphate buffer (50 mM) and acetonitrile (65:35 %, v/v) mixture solution was found to be the correct mobile phase, if peak shape and peak symmetry was to be improved. Buffer pH was still crucial in evaluating separation and process optimization, except for mixture solution composition. The effect of buffer pH on the retention period has to do with the solute's ionizing process. A series of mixing solutions with different pH values (2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) was used to investigate the retention time and resolution in which the other chromatographic parameters were maintained or remained unchanged for TEN and MET.

To conclude, phosphate buffer (50 mM potassium dihydrogen orthophosphate) and acetonitrile in the ratio of 65:35 v/v (pH 3.5 ± 0.02, modified with orthophosphoric acid) was chosen as the mobile phase and was found to be optimum with more theoretical plates (almost 4920), higher peak symmetry (0.99 and 0.98) and low retention time (2.81 and 1.72, less than 5 min). A strongly symmetrical and sharp characteristic peak of TEN and MET with a flow rate of 0.7 ml/min further obtained was based on the optimum mobile phase with Zorbax Eclipse Plus C18 (150×4.6 mm 5µm). A typical UPLC chromatogram obtained during the simultaneous TEN and MET determination is given in (**Fig. 2**).

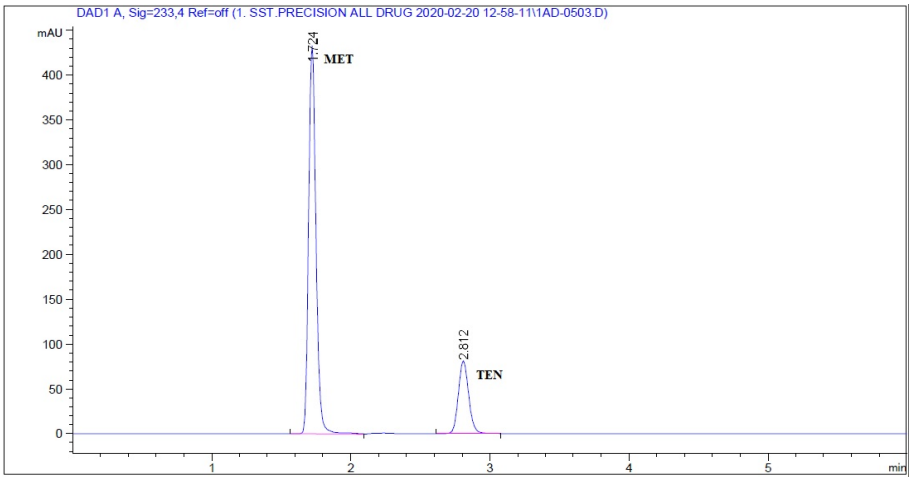


Fig. 2. UPLC chromatogram of TEN and MET. Final chromatographic conditions: Zorbax Eclipse Plus C-18 (150 mm × 4.6 mm i.d., particle size 5 µm); mobile phase phosphate buffer (50 mM Potassium dihydrogen phosphate, pH adjusted to 3.5 ± 0.02 with ortho phosphoric acid) and acetonitrile (65:35 v/v); flow rate of 0.7 $mLmin^{-1}$; and UV detection at 233 nm.

2.2 Validation of the Proposed Methods

Before actual usage, an automated system has to be checked. The system suitability testing was performed as per ICH guidelines for validation analytical system Q2 (R1). Within the following sections, the validation experiments carried out are prescribed.

2.2.1 Specificity

The specificity studies proved that there was no interference, as no other peak appeared during MET and TEN retention time (1.71 and 2.81 min). In comparison, interaction experiments showed that the analytes did not interconnect with each other and were far below the RSD acceptability level of 2.0%.

2.2.2 Range and Linearity

For linearity studies, nine separate concentrations (20, 30, 40, 50, 60, 70, 80, 90 and 100 μgmL^{-1}) of the TEN and MET mixture was prepared. Calibration curves with respective TEN and MET residual plots are shown in **(Fig. 3)**.

Table 2. Linearity Data of TEN and MET

STD. Concentration Range (µg/ml)	Peak Area (mAs)		Found Concentration (%)	
	TEN	MET	TEN	MET
20	191	677	20.12	19.62
30	280	1007	29.60	30.62
40	376	1336	39.88	41.13
50	465	1596	49.47	49.44
60	570	1985	60.67	61.87
70	662	2289	70.50	71.58
80	746	2579	79.50	80.85
90	848	2946	90.49	92.56
100	938	3172	100.06	99.81

In their respective calibration curves, a linear association was found between peak area and concentration. For TEN and MET, the linear regression equations were found to be $y = 9.378x + 1.364$, and $y = 31.63x + 56.05$, respectively. The regression coefficient (R^2) for TEN and MET were found to

be 0.999 and 0.998, respectively. The results revealed that within the selected concentration range, there was an excellent correlation between peak area and drug concentration. The findings verified the linearity of assay method and its reproducibility. The regression features of the suggested UHPLC system are carried out in **Tables 2 and 3**.

Table 3. Linearity parameters for the TEN and MET

Linearity Parameter	TEN	MET
Range (µg mL^{-1})	20-100	20-100
Slope	9.37	31.63
Intercept	1.36	56.05
Regression coefficient (R^2)	0.999	0.998
Standard error of Intercept	3.69	27.56
Standard deviation of intercept	11.08	82.67
Confidence limit of the slope	9.378±0.71	31.63±1.21
Confidence limit of the intercept	1.364±3.67	56.05±8.64

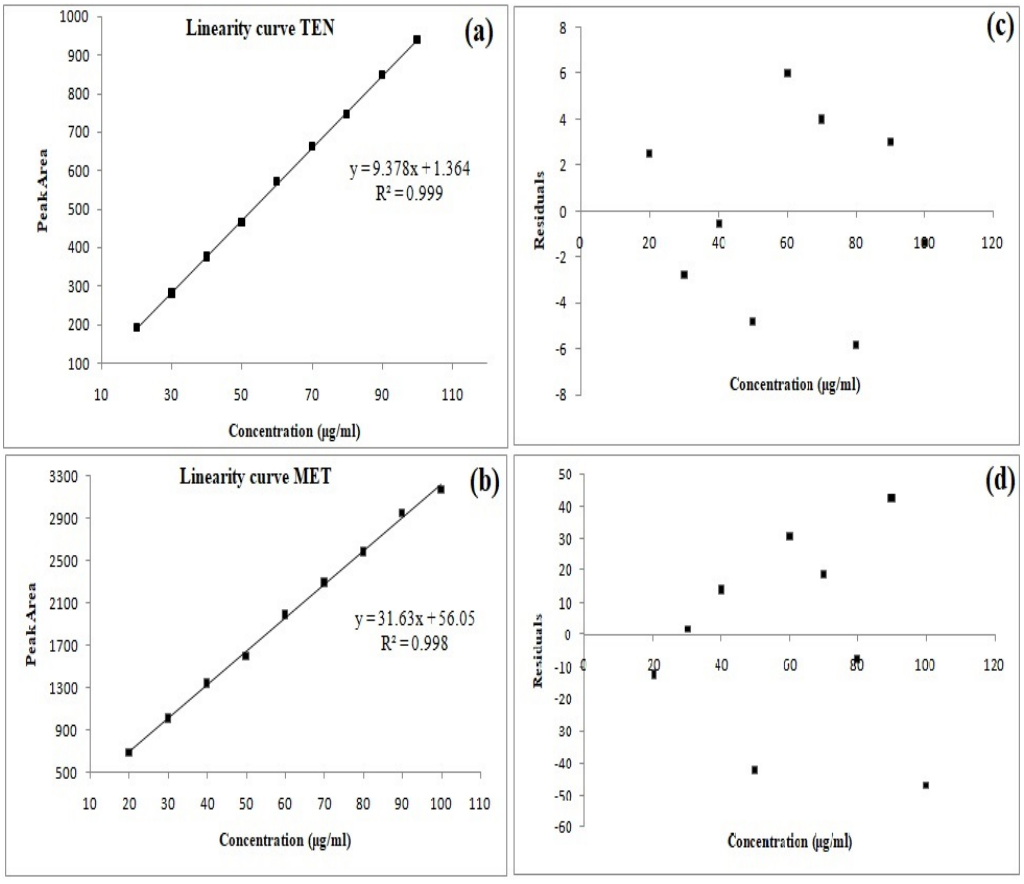


Fig. 3. Linearity plots for TEN (a) and MET (b) with corresponding residual plots for the TEN (c) and MET (d)

2.2.3 Accuracy

The recovery tests conducted by introducing recognized concentrations of drugs in placebo at three levels: 50%, 100% and 150% of the commercially developed product. For each stage of recovery, three samples were prepared. The approaches were then evaluated by calculating the percentage recoveries from the calibration curve. Results from recovery studies reported in **Table 4** revealed that for TEN and MET; the overall recovery, RSD % and RE % were in the range of (100 ± 1) %, < 2% and < 2.0%, respectively.

Table 4. Percent accuracy results of TEN and MET

Drug	% simulated dosage nominal	% Mean (n=3)	RSD(%)	RE%
TEN	50	100.39 ± 0.87	0.87	0.39
MET	50	99.81 ± 0.41	0.41	-0.19
TEN	100	99.61 ± 0.51	0.52	-0.39
MET	100	99.97 ± 0.34	0.34	-0.04
TEN	150	100.26 ± 0.32	0.32	0.26
MET	150	100.29 ± 0.23	0.23	0.29

2.2.4 Precision

The intra-day precision of the evolved LC method was determined by sampling three concentrations and three replicates of the same batch of tablets each. The inter-day precision was also calculated by three consecutive days of assaying the tablets in triplicate per day. The low %RSD infers that the approach is precise below the 2% acceptance limit. **Table 5** provides the intra- and inter-day variation or precision details. The findings thus illustrated that the system developed was of high precision. The tests obtained from intra-day and inter-day were statistically analyzed using the F-test and the student's t technique. The measured value of F-test and student's t test showed that the intra-day and inter-day results were not substantially different in terms of precision.

Table 5. Statistical treatment of the precision data and Intermediate precision (Assay)

Analysis Date	Intra-day		Inter-day		f test		t test	
Assay	**TEN**	**MET**	**TEN**	**MET**	**TEN**	**MET**	**TEN**	**MET**
% Assay Mean	100.42	99.86	100.65	99.79	1.47	1.53	0.72	0.42
% RSD	0.56	0.48	0.68	0.60	(1.93)[a]	(1.93)[a]	(2.00)[a]	(2.00)[a]

[a]The critical value for t-value and f-ratio at P=0.05

2.2.5 Limit of Detection and Limit of Quantitation

To see the sensitivity of the method, the LOD and LOQ for TEN and MET was estimated. The LOD and LOQ were calculated by injecting a sequence of dilute solutions with established concentrations at a signal to noise ratio of 3:1 and 10:1, respectively and are reported in **Table 6.**

Table 6. The results of LOD and LOQ

Drug	LOD (µg/mL)	LOQ (µg/mL)
TEN	1.29	3.93
MET	2.87	8.71

2.2.6 Robustness and Ruggedness

Robustness is the capacity of the method to remain unaltered by deliberate changes in parameters.

Table 7. Robustness and Ruggedness Results of TEN and MET

Parameter	conditions	% RSD (n=3)		tR (min)		N		AS	
		TEN	**MET**	**TEN**	**MET**	**TEN**	**MET**	**TEN**	**MET**
Change in λ_{max} 233±2 nm	231	0.15	0.25	2.83	1.72	7010	4975	0.99	0.96
	235	0.33	0.36	2.83	1.72	7017	4965	0.99	0.95
Change in flow rate 0.7±2 mL/min	0.5	0.34	0.74	3.96	2.41	8808	6633	0.91	0.98
	0.9	0.23	0.7	2.21	1.34	5647	3726	0.93	1.02
Change in Temp. 30±5 °C	25	0.09	0.22	2.77	1.72	6976	5058	0.89	0.95
	35	0.16	0.5	2.86	1.72	7044	4952	0.99	0.96
Change in pH 3.5±2	3.49	0.25	0.5	2.80	1.71	7719	5138	0.92	0.96
	3.52	0.23	0.33	3.81	1.76	7805	5180	0.93	1.03
Ruggedness									
Different analyst	Analyst 1	0.49	0.12	2.81	1.72	6945	5003	0.99	0.96
	Analyst 2	0.21	0.23	2.82	1.73	6978	4984	0.99	0.96

(tR: retention time; N: number of theoretical plates; AS: Symmetric factor)

The experimental conditions were changed deliberately, and the chromatographic resolution of TEN and MET were assessed. The minor changes in different parameters like wavelength, flow rate, temperature and pH are shown in **Table 7.** The method's ruggedness is defined by changing the analyst and carrying out the analysis with the system established. The calculated % RSD is reported in **Table 7.**

2.2.7 Study of Tablets Formulations

The method developed was successfully applied in evaluating TEN and MET in the preparation of the marketed tablets. The recovered sums expressed as a percentage of the demand for the mark. Analysis was conducted on the marketed tablet (Teniza-M 500, Torrent Pharma) using the optimized handheld process and UHPLC conditions **(Fig. 4).** The average percentage of tablet drug content obtained from the proposed method for TEN and MET was 100.54% and 99.82%, respectively. The results of this analysis are mentioned in **Table 8.**

Table 8. Analysis of Marketed Tablets

Tablet (Teniza M-500) Replicate number	tR (min)		A^a (mAs)		AS		N		% Assay	
	TEN	MET	TEN	MET	TEN	MET	TEN	MET	TEN	MET
1	2.813	1.725	415	1589	0.99	0.97	6959	4999	99.57	99.76
2	2.814	1.724	419	1592	0.99	0.96	6940	5015	100.77	100.09
3	2.813	1.724	417	1589	0.93	0.96	6937	4995	100.91	99.72
4	2.863	1.726	417	1586	0.99	0.96	7036	4953	100.48	99.53
5	2.863	1.726	418	1593	0.99	0.96	6974	4978	100.59	100.16
6	2.867	1.727	417	1587	0.94	0.97	6925	5021	100.88	99.67
Mean ± SD	2.839 ±0.03	1.725 ±0.00	417 ±1.44	1589 ±2.73	0.99 ±0.01	0.96 ±0.01	6962 ±40.27	4994 ±25.03	100.54± 0.50	99.82 ±0.25
%RSD	0.99	0.07	0.34	0.17	0.52	0.54	0.58	0.50	0.50	0.25

(tR: retention time; A^a Area; AS: Symmetric factor; N: number of theoretical plates)

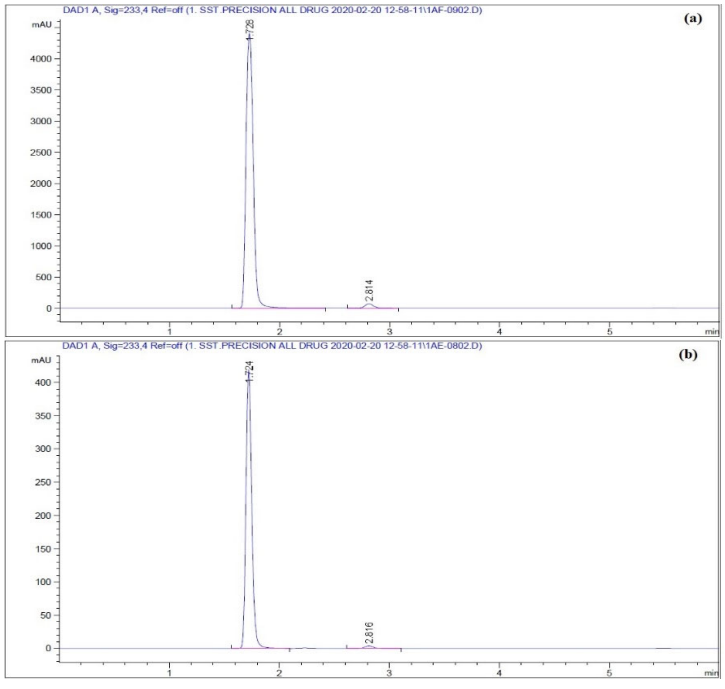


Fig. 4. UHPLC chromatogram of (a) 50 µgmL^{-1} TEN and (b) 50 µgmL^{-1} MET from Teniza-M 500 tablet sample solution

2.2.8 Forced degradation study

In forced degradation study, UHPLC and different conditions were responsible for separating TEN and MET from tablet samples. Significant drug degradation peaks were observed under neutral and straightforward (H_2O_2) conditions. **Table 12** shows the stability tests. During the study time, the samples were neutralized, except for samples treated with thermal, ultraviolet, peroxide and diluted with diluent (water and acetonitrile in 70:30 v/v ratio). The samples were filtered using 0.45 µm Millipore membrane filters. TEN and MET were found to be stable under acid, thermal, and photolysis conditions. Pure product chromatograms and their stress conditions are seen in **(Fig. 5(b), (c), (d), (e) and (f)). Tables 9 and 10** report the recorded peak retention period, TEN and MET percentage of degradation under different stress conditions.

Table 9. Outcomes of degradation study of TEN

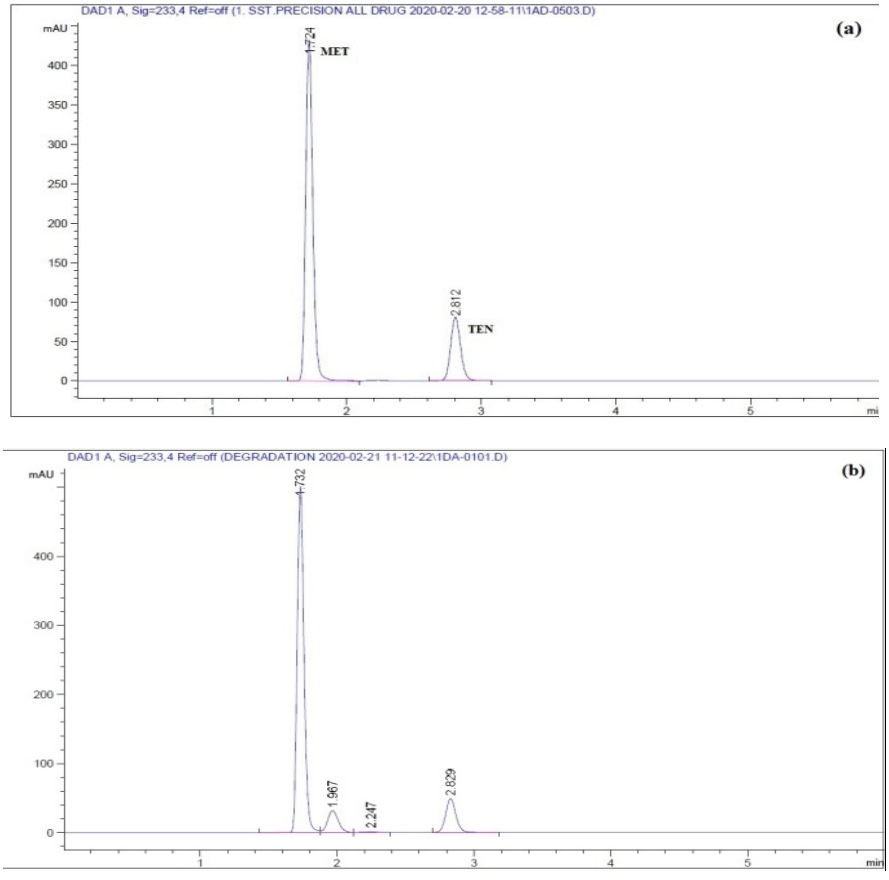
Sr. No.	Condition	tR (min)	Recovery ± SD	%RSD	% Drug degraded
1	Acid hydrolysis	2.83	97.50±0.40	0.41	2.10
2	Base hydrolysis	2.97	76.24±0.31	0.41	23.04
3	Oxidative degradation	2.82	71.27±0.36	0.50	27.59
4	Thermal degradation	2.83	96.83±0.16	0.17	2.67
5	Photo degradation	2.83	98.88±0.20	0.21	0.99

tR: retention time

Table 10. Outcomes of degradation study of MET

Sr. No.	Condition	tR (min)	Recovery ± SD	%RSD	% Drug degraded
1	Acid hydrolysis	1.73	98.33±0.23	0.23	1.38
2	Base hydrolysis	2.00	75.36±0.46	0.62	23.42
3	Oxidative degradation	1.73	89.51±0.34	0.38	9.41
4	Thermal degradation	1.72	98.50±0.24	0.25	0.82
5	Photo degradation	1.72	98.86±0.27	0.27	0.73

tR: retention time



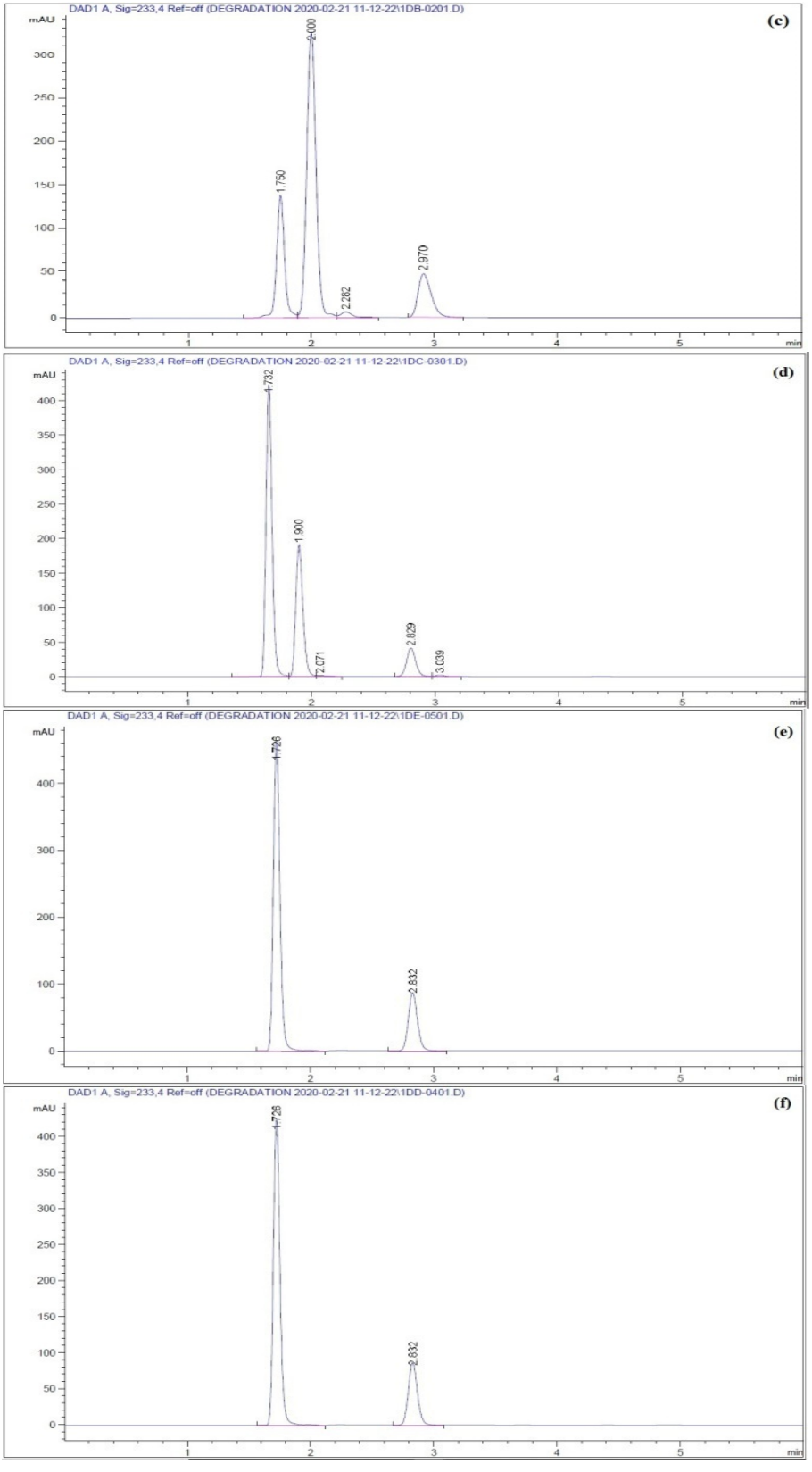


Fig. 5. (a) A UHPLC chromatogram comprising TEN and MET as standard sample. UHPLC chromatogram of TEN and MET found from degradation studies, (b) Acid hydrolysis (1 N HCl); (c) Base hydrolysis (1 N NaOH); (d) Oxidative degradation (3% H_2O_2); (e) Thermal degradation (80°C); (f) Photo degradation (25°C with UV radiation at 320-400 nm)

2.2.9 System Suitability

Six replicates of standard mixed solution were injected for system suitability parameters. All critical parameters on all days met warm requirements. Parameters which were measured and their outcomes are summarized in **Table 11.**

Table 11. Outcomes of system suitability data for TEN and MET

Parameters	TEN	MET
Peak area (A) (mAs)	422.12 ± 0.95	1593.98±2.93
Relative standard deviation (RSD)	0.22%	0.18%
Retention time (tR)	2.81	1.72
Theoretical plates (N)	6949	4934
Symmetry factor (AS)	0.99	0.98
Resolution	9.33	-
Retention factor K′	2.95	1.42

3. Conclusion

We have developed and validated a simple isocratic reversed-phase UHPLC method for the simultaneous estimation of TEN and MET as per ICH guidelines. Validation studies have proven that the UHPLC system is linear as well as accurate, precise and specific in the proposed working range. The substantial percentage in tablet forms on recovery shows that the placebo does not interfere with resolution. Also, the RSD% was less than 2, which indicated a high degree of method precision. Moreover, the proposed method was credited stable in terms of the flow rate and mobile phase composition. Additionally, this simple isocratic easy extraction and elution method has provided a quick and cost-effective drug analysis. The projected method can be used in combined dosage form for routine analysis of TEN and MET and also in quality control in bulk industrial. The technique developed is also reliable and qualified during the stability studies to validate and notice any anticipated changes in the asses of the drug product. Peak purity was tested for TEN and MET peaks, indicating they are pure from all other placebo or impurities or derivative materials. Thus, during stability studies, the analytical method is reliable and qualified to validate because any expected changes in the assay of the drug product was noticed.

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4. Experimental

4.1 Materials and Chemicals

Teneligliptin (TEN) with purity (> 99.00%) and Metformin (MET) with purity (> 99.10%) was obtained from Clearsynth Labs Ltd. (Mumbai, India). Generic product tablet (Teneza-M 500) dosage forms teneligliptin (20 mg) and metformin hydrochloride (500 mg) was procured from Torrent Pharmaceuticals Ltd. Throughout the study, potassium di-hydrogen ortho-phosphate and ortho phosphoric acid used were of AR Grade, while acetonitrile and water used were of HPLC Grade used. Other chemicals used were of analytical or HPLC grade purchased from S. D. Fine-chem Ltd (Ahmadabad, India).

4.2 Instrumentation

A chromatographic system consisting of Agilent 1290 series (US-CA); a device installed with Agilent quaternary pump G4204A, Agilent DAD G4212A (Diode array detector) 10 mm Max-Light cartridge flow cell, Agilent thermostated column compartment TCC G1316C and Agilent G4226A

autosampler fitted with Agilent G1330B thermostat was used. For separation and quantification, Agilent Zorbax Eclipse Plus C18 (150×4.6 mm, 5 μm) was used. The pH of the solutions was measured with pH meter–EUTECH Instruments (Singapore).

4.3 Chromatographic Conditions

The separation was performed at 30°C temperature on Agilent columns Zorbax Eclipse Plus C18 (150×4.6 mm, 5 μm) isocratic reversed process. The instrument was controlled by station Open LAB CDS Chem (version A.01.05), which is installed with data collection and acquisition equipment. The analysis was conducted for methods with a detection wavelength of 233 nm, using a 0.7 mL min^{-1} flow rate with the injection volumes of 5 μL.

4.4 Solution Preparation

4.4.1 Mobile Phase and Dilution Medium

Potassium phosphate buffer (pH 3.5) was prepared in 1000 mL of HPLC water by dissolving 50 mM of Potassium di-hydrogen ortho-phosphate. The pH of the solution was then adjusted to 3.5 ± 0.5 with phosphoric acid. The mobile phase was prepared by combining potassium phosphate buffer pH 3.5 with a ratio of acetonitrile (65:35 v/v) and filtering via a nylon membrane filter of 0.45 microns. A harmonized mixture of water and acetonitrile ratio (70:30) was prepared to be used as the dilution medium (diluent).

4.4.2 Reference and Sample Stock Solutions

A 100 mL sample stock solution contained 75 mg Teneligliptin hydrobromide hydrate (equivalent to 50.90 mg TEN) and 50 mg MET, dissolved in diluent through 10 minutes sonication. The solution was further diluted to a concentration of 50 μg mL^{-1} TEN and MET in the solution.

4.4.3 Teneza-M 500 tablets Preparation

Twenty Teneza-M 500 tablets, each of which contained 20 mg TEN and 500 mg MET, were accurately weighed and crushed into a homogenized fine powder using a mortar pestle. Accurate weight of this powder equal to the one tablet content was measured, moved into a 100 mL volumetric flask, and solubilized in diluent by sonication (for 40 minutes). Once dissolved, the volumetric flask was filled up-to the mark by the diluent. To remove any un-dissolved drug in the prepared solution, the 100 mL solution was centrifuged at 3500 RPM for 15 minutes. 5 mL of the filtrate was then diluted to 20 mL with diluent and this solution was further diluted by diluting 2 mL of it to 50 mL. The solution was then filtered using a hydrophilic PVDF 0.22 μm syringe filter, the concentration of TEN and MET in which was 50 μg mL^{-1}.

4.5 Method Validation

Following the ICH guidelines Q2 (R1), the optimized chromatographic conditions were validated by evaluating the specificity, linearity, range, accuracy, precision, detection limit (LOD), quantification limit (LOQ), system suitability and robustness parameters. The linearity and range of the defined method were determined using nine separate standard mix concentrations (20, 30, 40, 50, 60, 70, 80, 90 and 100 μg mL^{-1}). The study was performed in triplicate and the peak area values were measured for the corresponding concentrations. The method's accuracy was measured by conducting the sample assay (spiked placebos), prepared at three concentration levels of 50%, 100%, and 150% of average concentration, three replicates each. The percentage recovery and percent of RSD was determined for each of the replica samples. A sampling of three levels and three replicates of the same batch of tablets was used to identify the intraday precision of this method. The detection limit (LOD) and quantification

limit (LOQ) of this method was calculated using the standard deviation response (σ) and the slope approach, as specified in ICH guidelines. The LOD was determined using the formula: (3.3×σ/slope), and the LOQ was determined using the formula: (10×σ/slope). Robustness of the system under a variety of conditions including wavelength, flow rate, temperature, and pH of the mobile phase investigated. The ruggedness was measured by an analyst and an elapsed assay time[32].

4.6 Forced Degradation Study

To analyze the stability-indicating properties and the specificity of the process intentional, forced degradation experiments were performed degradation by exposing the formulations to 5 different stress conditions, which are listed in **Table 12**. The stressed samples were regularly examined and the existence of related peaks and overall purity were checked for the active ingredients[33].

Table 12. Forced degradation stress and conditions

Sr. No.	Stress	Conditions
1	Acid hydrolysis	50 µg mL^{-1} in 1 N HCl at 60°C for 2hrs
2	Base hydrolysis	50 µg mL^{-1} in 1 N NaOH at 60°C for 2hrs
3	Oxidative degradation	50 µg mL^{-1} in 3 % H_2O_2 at 60°C for 2hrs
4	Thermal degradation	50 µg mL^{-1} in 60°C for 48hrs
5	Photo degradation	50 µg mL^{-1} in 25°C for 48hrs with UV radiation at 320-400 nm

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