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Original Article

SIMULTANEOUS DETERMINATION OF METFORMIN, LINAGLIPTIN IN JENTADUETO AND METFORMIN, SAXAGLIPTIN IN KOMBIGLYZE BY LC-MS METHOD

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ABSTRACT

Objective: The objective of the present investigation was to develop a novel, simple and economic method for the determination of metformin (MET), linagliptin (LIN) and saxagliptin (SAX) in jentadueto and kombiglyze sample by employing the liquid chromatography and mass spectrometric method for estimation in bulk and pharmaceutical dosage form in presence of degradation products.

Methods: The chromatographic separation was achieved by using the mobile phase composition of methanol and ammonium acetate buffer pH 4.5 (85:15 % v/v) on the Hypurity advance C-18 column at a flow rate of 0.5 ml/min. Ion signals m/z "130.10/70.10, 473.10/420.40 and 316.30/180.20" for metformin, linagliptin and saxagliptin respectively measured in positive ion mode. The detailed validation of the method was performed as per ICH guidelines.

Results: The results of all validation parameters found within acceptance limits. The linearity of the drugs was found to be in the concentration range of 50–5000 ng/ml for all the drugs. Accuracy of the drugs was found to be from 94-102% and precision was found 4.67% RSD for all three drugs. The validated method was employed for the determination of drugs in the formulation and also determined the drugs in the presence of degradation products under stress conditions.

Conclusion: The method was developed and validated as per guidelines. Hence, this method can be used for the simultaneous determination of metformin, linagliptin and metformin, saxagliptin in bulk and combined dosage forms.

Keywords: Linagliptin, LC-MS, Method development, Metformin, Saxagliptin, Validation

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INTRODUCTION

Biguanide class drug metformin hydrochloride (N,N-dimethyl imido carbonamide diamide), an oral anti-diabetic drug is used as a first-line choice for the treatment of type 2 diabetes, particularly in overweight or obese people and those with normal kidney function. Metformin reduces hyperglycemia, primarily through its suppressive action on hepatic gluconeogenesis and also by increasing glucose transport across the cell membrane in skeletal muscle [1-3].

Linagliptin, 8-[(3R)-3-aminopiperidin-1-yl]-7-(but-2-yn-1-yl)-3-methyl-1-[(4-ethylquinazolin-2-yl) methyl]-3,7-dihydro-1H-purine-2,6-dione] is a novel dipeptidyl peptidase-4 inhibitor representing a new therapeutic approach by stimulating glucose-dependent insulin release and reduction of glucagon levels. It acts through inhibiting the inactivation of incretins particularly glucagon-like peptide-1 and gastric inhibitory polypeptide [4, 5].

Saxagliptin is a potent, selective, reversible dipeptidyl peptidase 4 (DPP4) inhibitor specifically designed for extended inhibition of the DPP4 enzyme. Saxagliptin is recently approved for treatment of type-II diabetes mellitus [6]. DPP-4 inhibitors represent a new therapeutic approach to the treatment of type-II diabetes that functions to stimulate glucose-dependent insulin release and reduce glucagon levels. This is done through inhibition of incretins inactivation, particularly glucagon-like peptide-1 and gastric inhibitory polypeptide, thereby improving glycemic control [7].

A few analytical methods were reported for the determination of MET, LIN and SAX in individual/combined dosage forms [8-23]. The present investigation was to develop a novel, simple and economic method for the determination of MET, LIN and SAX in jentadueto and Kombiglyze sample by employing the liquid chromatography and mass spectrometric method for estimation in bulk and pharmaceutical dosage form. Further, it is applied for studying of stress degradation of the drugs.

MATERIALS AND METHODS

Instrumentation

The instruments employed in the present study are as follows; HPLC-Agilent 1200 Series, AB Sciex API-3200 with Analyst Software 1.4.2, Agilent technologies, Santa Clara, USA, centrifuge apparatus-Multifuge, Thermofisher scientific, Mumbai, India, sonicator-Sharp Analytical, Hyderabad, India, rotary shaker-Vibramax, Germany, analytical balance-Metler Toledo, Mumbai, India, Pipettes-Thermo Electron, UK.

Chemicals

Metformin hydrochloride (MET, 99.35%), linagliptin (LIN, 98.26%) and saxagliptin (SAX, 99.59%) are from Clearsynth Company, Mumbai, India. ammonium acetate, formic acid and methanol are purchased from Merck Ltd, Mumbai, India.

Stock and working solution preparation

Preparation of MET standard stock solution: 6.240 mg of MET has weighed accurately and transferred to 5 ml volumetric flask, then added methanol to dissolve and made up to mark with methanol to get the 1 mg/ml solution of MET.

Preparation of LIN *standard stock solution*: 5.124 mg of LIN was weighed accurately and transferred to 5 ml volumetric flask, then added methanol to dissolve and made up to mark with methanol. The resultant solution was 1 mg/ml. a solution of LIN.

Preparation of SAX *standard stock solution*: 5.214 mg of SAX was weighed accurately and transferred to 5 ml volumetric flask, then

added methanol to dissolve and made up to mark with methanol. The resultant solution was 1 mg/ml solution of SAX.

Preparation of Mobile phase: Weighed and transferred 0.7708 g of ammonium acetate dissolved it in 1000 ml of water and adjusted the pH of the solution to 4.5 with formic acid. 150 ml of above solution was diluted with 850 ml of methanol, mixed and sonicated. This mixture was used as mobile phase.

Sample preparations

Sample preparation for metformin in jentadueto: Ten tablets were weighed and the coats were removed by carefully rubbing with a clean tissue wetted with methanol, 5 ml of methanol was added to an accurately weighed amount of the finely powdered jentadueto tablets equivalent to 500 mg of metformin and 2.5 mg of linagliptin, sonicated for 25 min and then made up to 10 ml with methanol. The resulting solutions were found to be 250 µg/ml of linagliptin and 50,000µg/ml of metformin.

To 0.050 ml of sample, 950.00 ml of the mobile phase was added and vortexed. Then the sample was transferred to autosampler vial, loaded the vials into autosampler and injected 10 µl of sample into LC/MS/MS system. The same procedure was followed for the preparation of metformin samples of Kombiglyze sample.

Sample preparation for linagliptin in jentadueto: Ten tablets of jentadueto was weighed and then finely powdered. An accurately weighed portion of the powder equivalent to 2000 mg of metformin and 10 mg of linagliptin was transferred to 100 ml volumetric flask and 50 ml methanol was added and sonicated for 25 min. Then the volume was made up to 100 ml with methanol and was filtered. 0.05 ml of jentadueto sample was diluted with 150.0 ml of mobile phase and vortexed. Then the sample was transferred to autosampler vial, loaded the vials into autosampler and injected 10 µl of sample into LC/MS/MS system.

Sample preparation for saxagliptin in kombiglyze: 0.050 ml of kombigylze sample was diluted with 150.0 ml of mobile phase and vortexed. Then the sample was transferred to autosampler vial, loaded the vials into autosampler and injected 10 µl of sample into LC/MS/MS system.

Instrument parameters and characterization of production using mass spectrometry

One micromole of MET, LIN and SAX solutions were separately infused into the mass spectrometer at a flow rate of 10 µl/min, to characterize the productions of each compound. The precursor ions $[M^+H]^+$ and the pattern of fragmentation were monitored using the positive ion mode. The major peaks observed in the MS/MS scan were used to quantify MET, LIN and SAX. The chromatographic conditions and multiple reaction monitoring (MRM) settings of the instrument were presented in [table 1].

Table 1: Chromatographic and mass conditions of the instrument (LC-MS)

Chromatographic conditions		**MRM conditions****	
In source	Turbo ion spray	Curtain gas (CUR)	25.0 PSI
Column	Hypurity advance C-18	Collision gas (CAD)	5.0 PSI
Mobile phase	Methanol: ammonium acetate buffer pH 4.5 (85:15 %v/v)	Temperature (TEM)	550.0 °C
Flow rate	0.5 ml/min	Ion spray voltage	5500V
Volume of injection	10 µl	Gas-1 and Gas-2	40.0 PSI
Run time	3 min	Entrance potential	10.0 V
Detection ions		Collision cell exit potential	MET-2.0 V LIN-5.0 V SAX-2.0 V
Metformin	130.10* amu (parent) 70.10* amu (product)	Declustering potential (DP)	MET-25.0 V LIN-55.0 V SAX-40.0 V
Linagliptin	473.10* amu (parent) 420.40* amu (product)	Collision energy	MET-35.0 V
Saxagliptin	316.30* amu (parent) 180.20* amu (product)		LIN-33.0 V SAX-33.0 V

Note: *parameters may change by 0.5units. **these parameters may vary from one instrument to another instrument due to their mass calibration parameters.

Method validation

The validation parameters like specificity, linearity, sensitivity, and accuracy, precision were done according to the ICH guidelines [24] and also referred few publications on method development and validation [25, 26]. Selectivity is studied by comparing the chromatograms obtained from placebo sample with the chromatogram obtained from tablets. Calibration standards are prepared by spiking required volume of working standard of MET, LIN and SAX solution in different 10 ml volumetric flasks and volume made up with methanol to yield concentration range of 50-5000 ng/ml of drugs. The resultant peak areas of drugs were measured. The linearity of the method was determined by plotting the peak area (y) of drugs (MET, LIN and SAX) against normal concentration (x) of drugs, respectively.

The lower limit of quantification (LLOQ) for drugs was defined as the lowest concentration giving a signal-to-noise ratio of at least 10-fold, acceptable accuracy (80–120%), and precision (within 20%); this was verified by the analysis of 10 replicates. Intra-and inter-day accuracy and precision of this method were determined at three different concentration levels on three different days, and on each day, three replicates were analyzed. The accuracy and precision were expressed as percentage accuracy and relative standard deviation (R. SD, %) respectively, and calculated using following equation (1) and (2).

$$\text{Accuracy (\%)} = \frac{\text{Mean observed concentration}}{\text{Nominal concentration}} \times 100 \quad \text{Eq.(1)}$$

$$\text{\% RSD} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100 \quad \text{Eq.(2)}$$

RESULTS

Method development

The drugs are in polar nature, so the reverse-phase chromatography was adopted using hydrophobic Hypurity advance, C-18 column (5µ, 100 × 4.6 mm) at a 0.5 ml/min flow rate than evaluated various compositions of mobile phases to obtain better resolution and symmetric peak shapes of analyte as well as suitable retention time. Buffers like ammonium acetate, ammonium formate and acetic acid in various strengths were tried along with methanol as organic solvent. These buffers gave sharp peak shape, but poor response. Finally, the mobile phase consisting of 10 mmol ammonium acetate buffer: methanol (15:85) gave a sharp peak shape and good response.

The mass spectrometric detection of drugs (MET, LIN and SAX) were investigated by ESI positive ion mode. In the positive ion mode, MET gave protonated molecular ions, $[M^+H]^{+,}$ as the major species. The fragmentation patterns of the protonated molecular ions were evaluated by increasing the collision energy. The greatest intensities

were observed at 130.10/71.10 amu for MET, 473.40/420.40 amu for LIN and at 136.30/180.20 amu for SAX, respectively. The mass parameters were optimized by observing the maximal response of the productions. The transition of 130.10→71.10 amu, 473.40→ 420.40 amu and 136.30 → 180.20 amu were used for detection of MET, LIN and SAX respectively. After optimization of the above conditions, the method was validated according to the ICH guidelines. The chromatograms of MET, LIN and SAX were shown in [fig. 1. a, 1. b, 1. c].

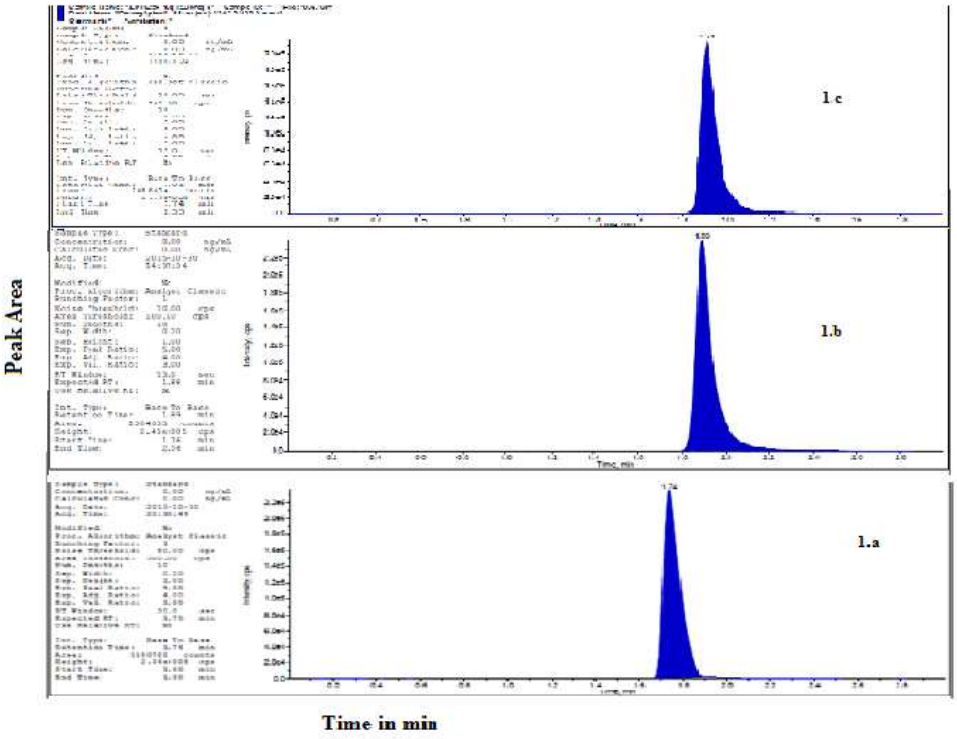

Fig. 1: Standard chromatogram of MET (1a), LIN (1b) and SAX (1c)

Method validation

Selectivity: The selectivity of the present method is established by checking the blank sample and observed the chromatogram. There is no interference found in retention times of MET, SAX and LIN in the blanks concludes the selectivity of the method.

Linearity: The linearity of this method was evaluated by linear regression analysis, using the least square method. The drug concentrations were linear in a range of 50-5000 ng/ml. Calibration standards were prepared by spiking required volume of working standard (10000 ng/ml) solution along with internal standard into different 10 ml volumetric flasks and volume made up with methanol to yield concentrations 50, 100, 300, 1000, 2000 and 5000 ng/ml of drugs. The resultant peak area of the drug was measured. A calibration curve was plotted between the peak area of the drug against the concentration of the drug.

The [fig. 2] represents the linearity graph, regression coefficient (r^2) including the slope and y-intercept.

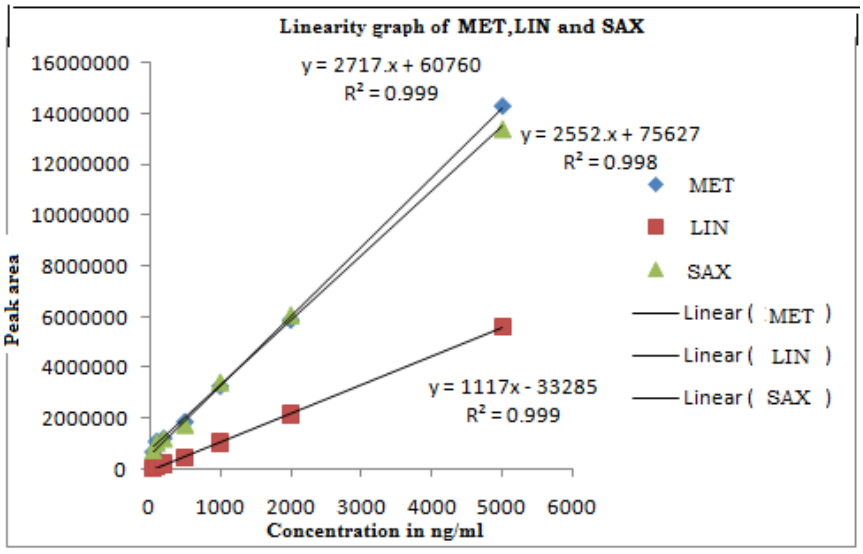

Fig. 2: Linearity graph of MET, LIN and SAX, n=6

Sensitivity: The lower limit of quantitation (LLOQ) was found to be 50 ng/ml. The percent accuracy of LLOQ was 109.68%, and precision denoted by % RSD was 4.73%.

Intra-day and Inter-day Precision and Accuracy: The intra-and inter-day: precision and accuracy of this assay were determined by analyzing replicates of QC samples at three concentrations on three different days. The coefficients of variation for the intra-and inter-day precisions were<4.67%. The intra-and inter-day accuracies were 94.50-102.50%. The low levels of coefficients of variation, i.e.: 2.51%-4.67% [table 2] indicate the method is accurate and precise. All these criteria were acceptable and follow ICH.

Table 2: Intra-day and inter-day accuracy and precision of MET, LIN and SAX

	Standard	MET		LIN		SAX	
		Avg. conc.	Accuracy	Avg. conc.	Accuracy mean±SD, % RSD	Avg. conc.	Accuracy mean±SD, % RSD
Intra-day	150.35	142.09	94.5±2.38, 2.51	143.63	95.5±0.70, 0.73	142.1	94.5±2.38, 2.51
(n=6)	1500	1501.55	102.42±3.64,3.56	1500.97	100.06±0.08, 0.08	1501.5	102.4±3.64, 3.56
	4000	4106.55	102.42±3.64,3.56	4000.93	100±0.03, 0.03	4106.49	102.4±3.64,3.56
Inter-day	150.35	142.77	96.96±3.98, 4.10	143.96	95.53±1.26, 4.10	142.69	96.96±3.98, 4.10
(n=18)	4009.15	1507.29	102.50±4.78,4.67	1501.87	99.82±0.48,0.48	1507.3	102.50±4.78,4.67
	4000	4109.29	102.50±4.78,4.67	4000.39	99.91±0.15,0.15	4109.4	102.50±4.78,4.66

Note: Actual concentrations of drugs mentioned in ng/ml. Values are expressed in mean±SD

Ruggedness: Ruggedness was studied along with precision and accuracy of batches where the effect of column change and analyst change were observed. The observed value for column variation and results obtained for precision and accuracy were within the acceptance criteria (i.e. there were no changes in the retention time, recovery and precision of the drug) according to US-FDA and ICH.

Analysis of formulation of jentadueto and kombiglyze

The samples were studied for various stress conditions like acid, base, hydrogen peroxide and photolytic conditions and the drug quantities were measured after standard and sample jentadueto and kombiglyze samples individually injected. Metformin standard solution and mixture of linagliptin and saxagliptin standard solution separately injected in six replicates.

Along with the samples (jentadueto and kombiglyze) the standard solution samples were interspersed in a run and all the results are tabulated in [table 3] and [table 4]. The chromatograms of drugs, both the sample for jentadueto and kombiglyze were shown in [fig. 3 (a) and 3 (b), and 4a and 4b] respectively.

Table 3: Degradation data of drugs in jentadueto sample

Sample	MET (100ng/ml)*	LIN (1000 ng/ml)*
Standard	1199057±31741	1256839±65739
0.1N HCl	3167698±96408	26158±1166
1N HCl	2931780±100795	11637±1365
0.1N NaOH	2324546±96459	25797±2432
1N NaOH	1149570±39839	11944±754
H_2O_2	3585314±168909	43640±1024
Photolytic	3871644±149582	0.00±0.0

*Values expressed in mean±SD, n=3

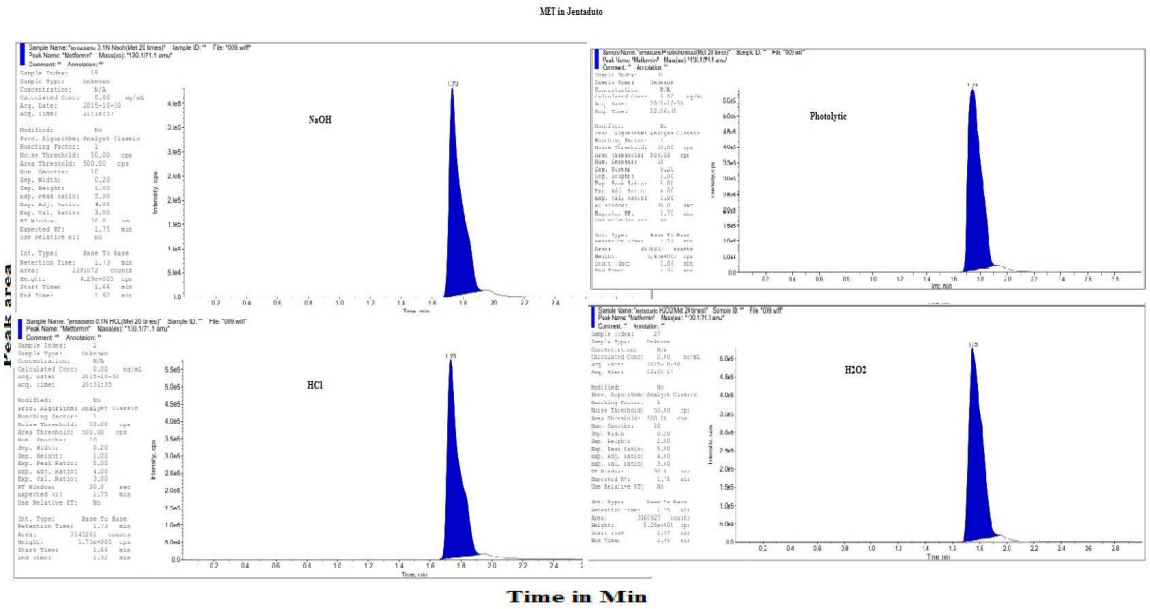

Fig. 3(a)

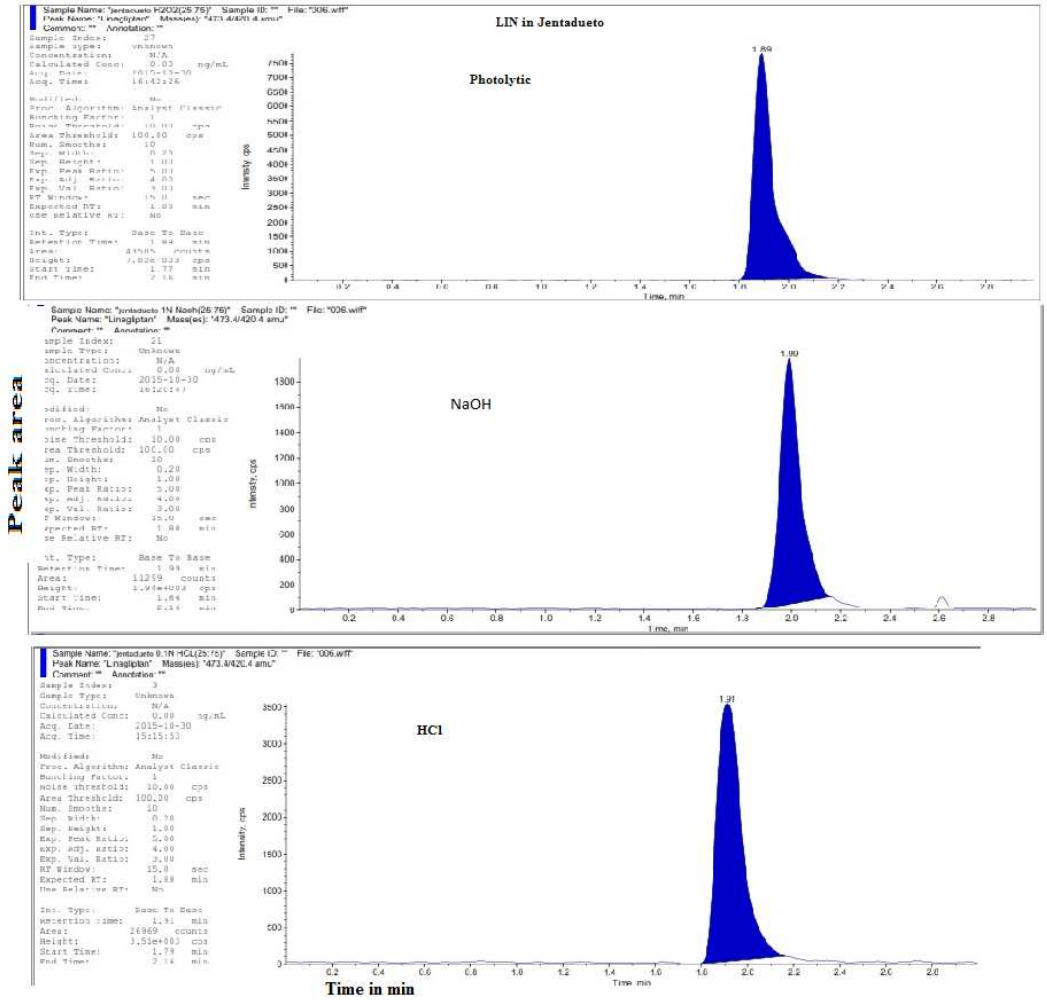

Fig. 3(b)

Fig. 3a: Chromatograms of MET in jentadueto sample and fig. 3. b. Chromatograms of LIN in jentadueto sample under stress conditions

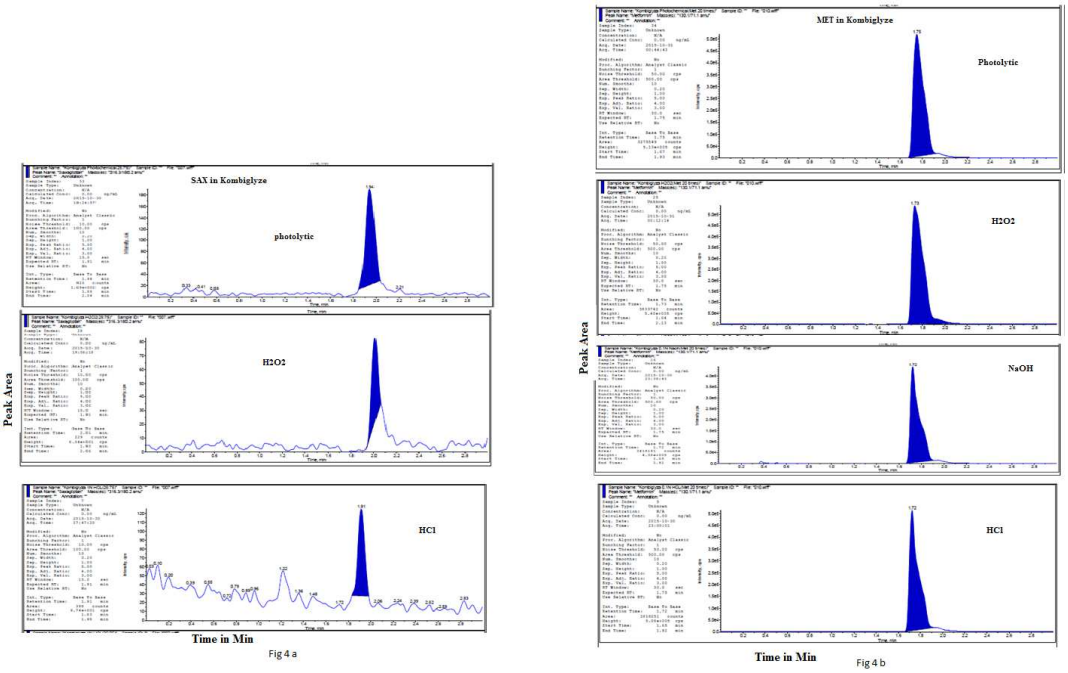

Fig. 4: Chromatograms of MET (4a) and SAX (4b) in kombiglyze sample under stress conditions

Table 4: Degradation data of drugs in kombiglyze sample

Sample	MET (100ng/ml)*	SAX *(200 ng/ml)*
Standard	1199057±31741	1087995±23445
0.1N HCl	2844320±80666	582.40±105
1N HCl	1872539±106140	175.80±174
0.1N NaOH	2200686±154669	0.00±0.00
1N NaOH	1054260±29420	0.00±0.00
H_2O_2	3589928±194918	45.80±102.41
Photolytic	3430214±223323	905.60±73.21

*Values expressed in mean±SD, n=3

DISCUSSION

The chromatographic method was optimized by changing various parameters, such as pH of the mobile phase, organic modifier and buffer used in the mobile phase. Under the presently prescribed conditions, the recoveries were found to be 94.5%-102.50% for MET, 95.5%-100.06% for LIN and 94.5%-102.5% for SAX. This indicates that commonly used excipients in jentadueto and kombiglyze samples were not interfering in the proposed method. Hence, this method is very useful for determination of MET, LIN and SAX in pharmaceutical dosage forms. It was also found that the differences of less than 5.0 % for three intra-and inter-day data reflect the precision of the method. The observation of % C. V less than 5.0 for both intra-and inter-day measurements also indicates a high degree of precision. In this study, the developed chromatographic method the linearity range of 50-5000ng/ml for three drugs will cover all the concentrations of drugs in a sample of jentadueto and kombiglyze. The LOQ of the method was 50ng/ml for all three drugs, it is a more sensitive method compared to reported method Asiya et al.2013 [28]. Owing to the high sensitivity the method was used to detect the drugs in different stress degradation conditions i.e. acidic, alkaline, and photolytic.

The stress studies were conducted for LIN and MET combination by HPLC method. In acid and alkali hydrolysis of both MET and LIN were degraded by increasing the strength of acid and alkali and similarly degradation was also observed with peroxide. Further studies in photolytic degradation, MET was quantifiable significantly whereas LIN was found to be quantifiable but very low. LIN was completely degraded in the photolytic condition and detectability in either the drug or its degradants was not possible and hence no quantification of linagliptin by LCMS was observed. SAX was completely degraded in the alkaline and peroxide solutions but partially degraded in acidic and photolytic conditions. The saxagliptin (SAX) or its degradants were not detectable. Hence no quantification of saxagliptin was attempted.

In the present study, Jentadueto was subjected to stress conditions like acid, base, peroxide, and photolytic degradation. This lead to there was no interference of degradants with the drug peaks. The degradation studies of jentadueto reveal that the developed method was stability indicating hence, this method can easily and conveniently adopted for routine quality control analysis of metformin and linagliptin in pure and its pharmaceutical dosage forms like jentadueto, This study was in agreement with Kavitha *et al.*,. 2013 (27) HPLC method.

Further, in LC-MS analysis Stress-induced studies performed under different conditions employed like acid, base, peroxide, and photolytic. In each degradation study for both metformin and linagliptin it was observed that metformin was found to be stable in acidic media as compared alkaline media the degradation was more in alkali whereas, there are no changes in metformin content in peroxide and photolytic degradation. In case of linagliptin degradation was increased with acidity, similarly with alkali. In case of peroxide-induced degradation of linagliptin was found to be significant, but in case of photolytic degradation there was not a single fragment eluted in LC-MS method.

Saxagliptin was completely degraded in the alkaline and peroxide solutions but partially degraded in acidic and photolytic conditions. The saxagliptin or its degradants were not detectable. Hence, no quantification of saxagliptin was attempted. It was found that the degradation of saxagliptin was found to be very highly significant ($P<0.001$) as mentioned from the [table 4].

Linagliptin was completely degraded in the photolytic condition and detectability in either the drug or its degradants was not possible and hence no quantification of linagliptin by LCMS was attempted. The stress studies were conducted for linagliptin and metformin combination by HPLC method. In acid and alkali hydrolysis of both metformin and linagliptin were degraded by increasing the strength of acid and alkali and similarly degradation was also observed with peroxide. Further studies in photolytic degradation, metformin were quantifiable significantly, whereas linagliptin was found to be quantifiable but very low. The degradation was highly significant ($P<0.001$) as mentioned in [table 3]. It may also be due to the concentration of linagliptin employed for the study was very low in case of the LCMS.

CONCLUSION

The present developed method indicates more sensitive than earlier methods reported in the literature. Jentadueto sample stress degradation studies indicate linagliptin was found to be sensitive against acidic, alkali, peroxide media but in photolytic studies totally degraded and not a single fragment detectable in LC-MS. In the case of kombiglyze sample, stress studies indicate saxagliptin was found to be highly sensitive against alkali media not a single fragment of saxagliptin was detectable. Further, saxagliptin was found to be quantifiable in other forced degradation studies like acidic, photolytic and peroxide. The method is highly useful for for quantification of metformin, linagliptin and saxagliptin in their combinations like Jentadueto and Kombiglyze. The method is novel and specific and can be applied as an alternative to HPLC as the analytical method is supported by forced degradation studies and the comparative data indicates the method is robust and specific

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Authors' contribution: P. B. N. Prasad has carried the experimental work, Dr. K. Satynarayana has gave the idea of extending the HPLC studies to LC-MS and Dr. G. Krishnamohan has done the comparative study and interpretation.

AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Declared none

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