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 saxagliptin in bulk and combined dosage forms.

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

INTRODUCTION

Biguanide class drug metformin hydrochloride (N,N-dimethyl imido carbamidine 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-Meter 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 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 50.000 µg/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 vortexted. 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 kombiglyze sample was diluted with 1500 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>
<thead>
<tr>
<th>Chromatographic conditions</th>
<th>MRM conditions**</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In source</strong></td>
<td>Curtin gas (CUR)</td>
</tr>
<tr>
<td><strong>Column</strong></td>
<td>Collision gas (CAD)</td>
</tr>
<tr>
<td><strong>Mobile phase</strong></td>
<td>Temperature (TEM)</td>
</tr>
<tr>
<td><strong>Flow rate</strong></td>
<td>Ion spray voltage</td>
</tr>
<tr>
<td><strong>Volume of injection</strong></td>
<td>Gas-1 and Gas-2</td>
</tr>
<tr>
<td><strong>Run time</strong></td>
<td>Entrance potential</td>
</tr>
<tr>
<td><strong>Detection ions</strong></td>
<td>Collision cell exit potential</td>
</tr>
<tr>
<td>Metformin</td>
<td>Decustering potential (DP)</td>
</tr>
<tr>
<td>130.10* amu (parent) 70.10* amu (product)</td>
<td>MET-2.0 V</td>
</tr>
<tr>
<td>Linagliptin</td>
<td>MET-25.0 V</td>
</tr>
<tr>
<td>473.10* amu (parent) 420.40* amu (product)</td>
<td>MET-35.0 V</td>
</tr>
<tr>
<td>Saxagliptin</td>
<td>MET-33.0 V</td>
</tr>
<tr>
<td>316.30* amu (parent) 180.20* amu (product)</td>
<td>SAX-3.0 V</td>
</tr>
</tbody>
</table>

Note: *parameters may change by 0.5 units. **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).

\[
\text{Accuracy} (\%) = \frac{\text{Mean observed concentration} - \text{Nominal concentration}}{\text{Nominal concentration}} \times 100 \quad \text{Eq}(1)
\]

\[
\text{R. SD} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100 \quad \text{Eq}(2)
\]

**RESULTS**

Method development

The drugs are in polar nature, so the reverse-phase chromatography was adopted using hydrophobic Hypurity advance, C-18 column (5 µ, 100 x 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].

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](image)
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

<table>
<thead>
<tr>
<th>Standard</th>
<th>MET (Avg. conc. ±SD, % RSD)</th>
<th>LIN (Avg. conc. ±SD, % RSD)</th>
<th>SAX (Avg. conc. ±SD, % RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day (n=6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.35</td>
<td>142.09</td>
<td>94.5±2.38, 2.51</td>
<td>143.63</td>
</tr>
<tr>
<td>1500</td>
<td>1501.55</td>
<td>102.4±3.64, 3.56</td>
<td>1500.97</td>
</tr>
<tr>
<td>4000</td>
<td>4106.55</td>
<td>102.4±3.64, 3.56</td>
<td>4000.93</td>
</tr>
<tr>
<td>Inter-day (n=18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.35</td>
<td>142.77</td>
<td>96.9±3.98, 4.10</td>
<td>143.96</td>
</tr>
<tr>
<td>4009.15</td>
<td>1507.29</td>
<td>102.5±4.78, 4.67</td>
<td>1501.87</td>
</tr>
<tr>
<td>4000</td>
<td>4109.29</td>
<td>102.5±4.78, 4.67</td>
<td>4000.39</td>
</tr>
</tbody>
</table>

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 [4(a) and 4(b)] respectively.

Table 3: Degradation data of drugs in jentadueto sample

<table>
<thead>
<tr>
<th>Sample</th>
<th>MET (100ng/ml)</th>
<th>LIN (1000ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>1199505±31741</td>
<td>1256839±31439</td>
</tr>
<tr>
<td>0.1N HCl</td>
<td>3167698±96408</td>
<td>26158±1166</td>
</tr>
<tr>
<td>1N HCl</td>
<td>2931780±100795</td>
<td>11637±1365</td>
</tr>
<tr>
<td>0.1N NaOH</td>
<td>1149570±39839</td>
<td>11944±3934</td>
</tr>
<tr>
<td>1N NaOH</td>
<td>1149570±39839</td>
<td>11944±3934</td>
</tr>
<tr>
<td>H2O2</td>
<td>3585314±168909</td>
<td>43640e1024</td>
</tr>
<tr>
<td>Photolytic</td>
<td>3871644±149582</td>
<td>0.00±0.0</td>
</tr>
</tbody>
</table>

*Values expressed in mean±SD, n=3
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
conveniently adopted for routine quality control analysis of was stability indicating hence, this method can easily and degradation studies of Jentadueto reveal that the developed method In the present study, Jentadueto was subjected to stress conditions saxagliptin (SAX) or its degradants were not detectable. Hence no partially degraded in acidic and photolytic conditions. The completely degraded in the alkaline and peroxide solutions but quantification of linagliptin by LCMS was observed. SAX was either the drug or its degradants was not possible and hence no 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, alkalai, 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 alkalai 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 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 comparative data indicates the method is robust and specific

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Declared none

REFERENCES


