A NOVEL VALIDATED STABILITY INDICATING METHOD FOR QUANTIFICATION OF EMPAGLIFLOZIN IN BULK AND MARKETED FORMULATION BY HPTLC APPLYING EXPERIMENTAL DESIGN APPROACH

Manojkumar K. Munde\textsuperscript{a,b},*, Nilesh S. Kulkarni\textsuperscript{b}, Ashim K. Sen\textsuperscript{a} and Dhanya B. Sen\textsuperscript{a}

(Received 12 June 2021) (Accepted 12 April 2023)

ABSTRACT

For the purpose of analyzing empagliflozin, a stability indicating high performance thin layer chromatographic method was developed. This method was optimized using design of experiment. In order to optimize the process, independent variables such as the proportion of isopropyl alcohol in the mobile phase, the duration of time that the chamber was saturated and the distance of mobile phase travelled were considered. On an aluminum plate that had previously been coated with silica gel, development was carried out with the assistance of twin trough glass chambers in ascending lines. The findings from these studies led to the selection of a mobile phase that had a composition of ammonium acetate (2 %), triethylamine and isopropyl alcohol in the ratio of 4:1:5 (V/V/V), and this mobile phase was utilized in the process of method development using central composite design approach. The saturation time was established at 10 minutes, and the ultraviolet detection was performed at a wavelength of 237 nm. The value 0.82 was discovered to be the retention factor (R\textsubscript{f}) for empagliflozin. The method was linear, precise and accurate over the entire concentration range examined (100-600 ng band\textsuperscript{-1}), along with correlation coefficient value of 0.992. The proposed method is quick and selective, and a straightforward method of sample preparation and analysis for empagliflozin in its bulk and commercially available dosage forms. The stability of the drug was tested under a variety of different stress conditions in accordance with ICH guidelines, and the results obtained from the force degradations indicate that the developed method is appropriate for stability studies.

Keywords: Empagliflozin, method development, validation, DoE, HPTLC, Forced degradation study

INTRODUCTION

Empagliflozin (EN) is a drug that is used to treat type 2 diabetes and is an inhibitor of the sodium glucose cotransporter-2 (SGLT-2). SGLT-2 inhibitors, also known as gliflozins, are recently developed anti-hyperglycemic medications. EN reduces blood sugar levels by preventing the kidneys from reabsorbing glucose. EN (Fig. 1) is 1-chloro-4-(glucopyranos-1-yl)-2-(4-{(tetrahydrofuran-3-yloxy} benzyl) benzene, according to its chemical structure\textsuperscript{1,2}. The review of literature for EN with its analytical method should include the following procedures for pharmaceutical dosage form, either alone or in combination with metformin hydrochloride/linagliptin. Thorough review of the literature revealed numerous high performance liquid chromatography (HPLC)\textsuperscript{3–17}, high performance thin layer chromatography (HPTLC)\textsuperscript{18–19} and spectrophotometry\textsuperscript{20–23} methods for the analysis of EN. A high performance thin layer chromatography (HPTLC) method has been developed for estimating EN in formulations using the central composite design (CCD) approach. The method that has been suggested will prove useful for the quantification of EN in bulk as well as for marketed dosage form. Using a CCD strategy, the proposed work aimed to develop a high performance thin layer chromatography (HPTLC) analytical method that could indicate stability.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{empagliflozin.png}
\caption{Chemical structure of empagliflozin (EN)}
\end{figure}

https://doi.org/10.53879/id.60.06.13038
MATERIALS AND METHODS

Drug and reagents

EN pure drug of analytical grade was a gift from Lupin Ltd. Pune, Maharashtra, India. Jardiance® (Boehringer Ingelheim, India), which was purchased from a local pharmacy with marking on the packaging containing 10 mg of EN per tablet, was the oral dosage form used in this study. From Loba Chemie Pvt. Ltd., Mumbai, India, AR grade methanol, ammonium acetate, isopropyl alcohol (IPA) and triethylamine were purchased.

Equipment and optimized experimental conditions

Solutions (standard drug) of varying concentrations were spotted in the form of bands with width of 6 mm on a silica gel aluminium Plate 60 F\textsubscript{254} using a sample applicator CAMAG Linomat V. A twin trough glass chamber was used to develop linear ascending development. Ammonium acetate (2%): triethylamine: isopropyl alcohol in the proportion 4:1:5 V/V/V was used as the mobile phase. 10 minutes at room temperature (25±2°C) served as the optimal chamber saturation period prior to chromatographic development. The chromatographic run was 8 cm long, and the mobile phase was covered in 15 min. Subsequent to the development, an air dryer was used to dry the HPTLC plates. Densitometric scanning was carried out by utilizing a CAMAG TLC scanner III in conjunction with the winCATS programmer. In order to achieve the highest possible level of sensitivity with the HPTLC method that uses ultraviolet detection, it is essential to make use of the appropriate wavelength. The densitometric measurements were recorded in UV (ultraviolet)-visible region of 200-700 nm. The overlaid spectrum of the developed plate was recorded on a Camag TLC scanner III. These measurements were made in the same region. EN was able to absorb light at 237 nm (Fig. 2). The data was treated statistically making use of a standard linear regression technique.

Standard stock solution preparation and construction of calibration curve

10 mg of EN was dissolved in 70 mL of methanol and the mixture was sonicated for 15 minutes; a standard stock solution was made by adding enough methanol so that the total volume was 100 mL to get a concentration of 100 µg mL\textsuperscript{-1}. Different volumes ranging from 1-6 µL were applied on the precoated TLC plate from the stock solution to get concentration of the drug in the 100–600 ng band\textsuperscript{-1}. 5 replicate measurement were carried out and an average peak area was considered for plotting calibration curve.

Analysis of tablet formulation

After carefully weighing 20 tablets, an average was obtained, which was subsequently utilized in the process of calculating the amount of EN contained in the tablet dosage form. These tablets were first crushed and ground into a powder with a glass mortar. After being sonicated for 15 minutes with methanol, the powder containing 10 mg equivalent of EN was filtered through Whatman filter paper No. 1 that had been previously moistened with methanol. This procedure was carried out in a volumetric flask with a capacity of 10 mL, methanol was used to bring the volume up to 10 mL, and a concentration of 1000 µg mL\textsuperscript{-1} was achieved as a result. 0.5 µL (500 ng band\textsuperscript{-1}) of filtered solution that was obtained from the stock solution was applied on the HPTLC plate and followed by development and scanning. There were three separate runs of the analysis performed.

Central composite design for method development

Using the Central Composite Design (CCD) method, the amount of isopropyl alcohol that is contained within the solvent system or mobile phase, the amount of time that the chamber is saturated, and the distance that the mobile phase travelled were all optimized, and their effects on the drug’s retardation factor (R\textsubscript{f}) (main, interaction, and quadratic) were assessed. CCD is an experimental design that can be used with response surface methodology to construct a second order polynomial (quadratic response surfaces) model for the response variable without conducting a full three-level factorial experiment. For the purpose of optimizing the DoE method’s parameters, important parameters and ranges were identified. Twenty
experiments with twelve centered points were designed using the isopropyl alcohol proportion in the mobile phase (A), the saturation time of chamber (B), the travelled distance (C), and the Rf of EN (responses) as shown in Table I. All three factors A, B, and C had nominal values of 5 mL, 10 min, and 8 cm, respectively. As a result, the amount of isopropyl alcohol that was used (A) was maintained at a level that ranged from 3 mL to 7 mL, the lower and higher ranges of the chamber saturation time (B) were set at 7 minutes and 23 minutes, respectively, and the value for C was kept at a distance that ranged from 5.3 cm to 8.6 cm.

Table I: Central composite design arrangement and responses

<table>
<thead>
<tr>
<th>Run</th>
<th>Type</th>
<th>Factor</th>
<th>Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Axial</td>
<td>4.5</td>
<td>6.591</td>
</tr>
<tr>
<td>2</td>
<td>Center</td>
<td>4.5</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>Axial</td>
<td>7.022</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Center</td>
<td>4.5</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>Axial</td>
<td>1.977</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Factorial</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>Axial</td>
<td>4.5</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>Center</td>
<td>4.5</td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>Factorial</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>Factorial</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>Factorial</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>Factorial</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>13</td>
<td>Factorial</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>14</td>
<td>Center</td>
<td>4.5</td>
<td>15</td>
</tr>
<tr>
<td>15</td>
<td>Factorial</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>16</td>
<td>Center</td>
<td>4.5</td>
<td>15</td>
</tr>
<tr>
<td>17</td>
<td>Factorial</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>18</td>
<td>Center</td>
<td>4.5</td>
<td>15</td>
</tr>
<tr>
<td>19</td>
<td>Axial</td>
<td>4.5</td>
<td>23.409</td>
</tr>
<tr>
<td>20</td>
<td>Axial</td>
<td>4.5</td>
<td>15</td>
</tr>
</tbody>
</table>

A= Isopropyl alcohol (mL), B=Chamber saturation time (min), C=Distance travel (cm), EN= Empagliflozin

METHOD VALIDATION

All parameters were evaluated as part of the validation process to ensure that it met ICH standards.

Linearity

Standard stock solution of EN containing 1000 µg mL⁻¹ was applied on the TLC plate in varied volume ranging from 1-6 µL to get concentration of the drug 100–600 ng band⁻¹. For the drug EN, a graph of peak area versus concentration in ng band⁻¹ was plotted.

Precision

Precision is the degree to which test results can be reproduced or repeated, and it is used to measure the reproducibility of analytical methods. When evaluating the precision of the method during both intra-day and inter-day precision studies, a total of six replicates and the concentration of 500 ng band⁻¹ was used.

Accuracy study

The accuracy of the method was tested three times at 80%, 100% and 120 % concentration levels. Recovery of EN was carried out by making use of the standard addition method, which involves the addition of known amounts of active pharmaceutical ingredient (API) of varying concentrations to the commercially available drug product and the resulting solutions were reanalyzed by the proposed method and % recoveries were calculated. The outcome of accuracy studies were assessed based on the % of standard EN recovered from the formulation by applying the following formula:

% Recovery = [(Total amount of analyte found after adding reference analytes – Amount of analytes found before adding reference analytes) / (Amount of reference analytes added)] x 100

LOD

The use of standard formulae was put to use in order to determine the detection limit of the method that was developed. The slope (S) and standard deviation (α) of the calibration curve that included the y-intercept were used in the calculation for the value of the LOD for EN. The formula used to calculate the limit of detection was LOD=3.3α/S.

Robustness

A study on the robustness of the method was carried out by evaluating intentional variations in the method parameters. These variations included shifts in the amount of time required for saturation, variation in mobile phase and volume of mobile phase. After calculating the relative standard deviations, the Rf value and peak area of EN were analyzed with regard to the changes in each parameter (RSD). This made it possible to assess how the various parameters interacted with one another. By computing the relative standard deviation (RSD) of each parameter, it was possible to evaluate the robustness of the proposed method.
Specificity

Analysis of the peak purity study for EN was done in order to fulfill the requirements of the specificity study. Both the sample and standard bands were recorded at three distinct levels, which were respectively known as the peak start, peak apex, and peak end in that order. These levels were in ascending order from lowest to highest. During the course of this investigation, a standard stock solution that contained EN at a concentration of 1000 µg mL\(^{-1}\) was prepared and used.

FORCED DEGRADATION STUDIES

Hydrolysis and oxidation

In a 10 mL volumetric flask, 5 mL of EN stock solution (1000 µg mL\(^{-1}\)) and 3 mL of HCl (0.1 N) were added. The solution was refluxed for 30 minutes at 40 °C. After reflux, methanol was used to make up the volume to 10 mL. The study was conducted in a dark setting to counteract any potential effects of light-induced degradation. With the aid of an applicator and nitrogen gas, a 6 mm band of the resulting 5 µL EN (500 ng band\(^{-1}\)) was applied on the 10.0 x 10.0 cm TLC plate and the chromatogram was allowed to run.

For base degradation and oxidation study, same solutions were made as acid degradation study. Solution of HCl (0.1N, 3 mL) was replaced with the NaOH (0.1N, 3 mL) and H₂O₂ (3 %, 3 mL), respectively, for base degradation and oxidation.

Heat degradation

EN was subjected to dry heat degradation for 24 h at 40 °C in an oven. Materials under dry heat degradation study were weighed and diluted using methanol in 10 mL volumetric flask. Further dilution was carried out to bring down the concentration to 500 µg mL\(^{-1}\). From the resulting solution, 5 µL (500 ng band\(^{-1}\)) was applied on the 10.0 x 10.0 cm TLC plate with the help of CAMAG Linomat V sample applicator.

Photo-degradation (Photolysis)

EN was exposed to UV light at a wavelength of 254 nm for 24 h in a photo stability chamber during the photo-degradation study. Further sampling was done, weighed, and diluted using methanol in 10 mL volumetric flask. Subsequently, dilution was carried out to bring down the concentration to 500 µg mL\(^{-1}\). From the resulting solution, 5 µL (500 ng band\(^{-1}\)) was applied on the 10.0 x 10.0 cm TLC plate with the help of CAMAG Linomat V sample applicator.

RESULTS AND DISCUSSION

HPTLC method optimization

The main goal of the proposed work was to select, a solvent system or mobile phase that can accurately measure EN. To accomplish this, the solvent system composition was varied by altering the polarity of system. Spots for standard API were applied to a TLC plate and scanned for EN in order to determine the analytical wavelength and quantify it. The wavelength of 237 nm was chosen for additional studies. The peak purity of EN in the commercially available tablet was determined by comparing the overlaid spectral band at the peak start, peak apex, and peak end with the standard EN (pure drug) (Fig. 3). The proposed mobile phase purity was confirmed (Fig. 4), which showed no interference at the 237 nm peak (blank). Numerous studies were conducted to optimize the mobile phase. Toluene with methanol, chloroform with methanol, methanol, hexane and glacial acetic acid were some of the different mobile phase combinations that were tested as solvent systems. The solvent system, which consisted of 2% ammonium acetate: triethylamine:
isopropyl alcohol in a ratio of 4:1:5 (V/V/V), provided a well-defined peak for EN at $R_f$ value of 0.820 (Fig. 5).

![Fig. 5: Typical densitogram of EN standard](image)

**Analysis of marketed formulation**

Jardiance® tablet was employed for the analysis of commercially available formulations. The chromatogram of the tablet displayed a peak for EN at $R_f$ value of 0.82 (Fig. 6), indicating that there is no excipient interference with the tablet formulation. Peak areas of the sample and the standard were compared to determine the content of EN (Table II).

**Preparation of calibration curve**

Linearity refers to the capacity of an analytical method to generate results that are directly proportional to the concentration of the analyte within a given range. Fig. 7 reveals a three-dimensional overlaid HPTLC densitogram for EN with calibration bands at 237 nm. The solution concentrations prepared in the range of 100-600 ng band$^{-1}$ of EN demonstrated a good correlation coefficient ($r^2 = 0.992$) (Fig. 8).

![Fig. 6: Typical densitogram of EN Pharmaceutical dosage form](image)

![Fig. 7: Three-dimensional densitogram for the linearity of EN](image)

**Table II: Assay results of the pharmaceutical dosage form**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Drug</th>
<th>Labeled amount (mg tablet$^{-1}$)</th>
<th>Amount found (mg tablet$^{-1}$)</th>
<th>% Amount found</th>
<th>Mean ± SD*</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>EN</td>
<td>10</td>
<td>9.942</td>
<td>99.42%</td>
<td>9.80% ± 1.028</td>
<td>1.030</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td></td>
<td>9.878</td>
<td>98.78%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td></td>
<td>10.12</td>
<td>101.21%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Average of three estimations
SD= Standard deviation
RSD= Relative standard deviation
Chromatographic conditions optimization using DoE

It was decided to use the CCD method because of its adaptability, and this was done in order to improve the separation. A three factorial central composite experimental design was used with the help of 20 experimental runs and 12 centre points. Based on the EN $R_f$ value, the quadratic model was chosen as the best fit for the model selection process. The models are significant if the p-value is less than 0.005. ANOVA data was validated using a quadratic model with DoE software, and the results are shown in Table III. As the ratio obtained is higher than 4 as the standard value,

<table>
<thead>
<tr>
<th>Response ($R_f$)</th>
<th>Type of model</th>
<th>Polynomial equation model for Y</th>
<th>Standard deviation</th>
<th>Model $p$-value</th>
<th>% CV</th>
<th>Adequate precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>EN</td>
<td>Quadratic</td>
<td>$EN = +0.8384 -0.0056 A -0.0051 B +0.0681 C -0.0725 AB -0.0025 AC +0.0350 BC + 0.0406 A^2 -0.0125 B^2 -0.0602 C^2$</td>
<td>0.0638</td>
<td>0.0068</td>
<td>7.82</td>
<td>9.549</td>
</tr>
</tbody>
</table>

$EN= Empagliflozin$

Table III: Predicted response model and statistical parameters obtained from the ANOVA for CCD

Table IV: Comparison of Experimental result and predicted values obtained from DoE

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Drug</th>
<th>Factors</th>
<th>Experimental Result ($R_f$)</th>
<th>Predicted Response ($R_f$)</th>
<th>Predicted error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EN</td>
<td>05 10 08</td>
<td>0.820</td>
<td>0.838</td>
<td>0.018</td>
</tr>
</tbody>
</table>

$A= Isopropyl alcohol (mL), B=Chamber saturation time (min), C=Distance travel (cm), EN= Empagliflozin$

The response to noise ratio exhibited by the precision measurements is satisfactory. For EN, the ratio value obtained was higher than 4. Ideally, the $R^2$ should be less than 10%, and the predicted $R^2$ value should be reasonably close to the adjusted $R^2$ value. It demonstrates the highest level of correlation between the data from experiments and the models that were fitted to those data. The last equation in Table III provides the relation between actual components and factors. The perturbation plots for the predicted model are displayed in Fig. 9. For the inspected procedures, saturation time of chamber showed significant effect on $R_f$ of EN. Fig. 10 shows a variation in the $R_f$ value of EN depending on the chamber’s saturation time and isopropyl alcohol concentration while maintaining a constant distance travelled. For EN, there was an inverse relationship between the $R_f$ value and the distance travelled. According to the CCD, the amount of isopropyl alcohol present (A) as well as the time spent in the chamber for saturation (B) have a significant impact on the responses in relation to the distance (C). Table IV illustrates a comparison of experimental results with predicted values derived from DoE, demonstrating the closeness of the $R_f$ value.
METHOD VALIDATION

Linearity

Peak area responses vs concentrations were found to be linear in the concentration range of 100-600 ng band\(^{-1}\) (\(R^2 = 0.992\)). In this case, the regression equation was \(y = 4.3211x - 13.534\). All concentrations tested yielded a linear response (Table V).

Precision (Intra-day and inter-day)

Acceptable repeatability, intraday variation, and interday variation were assessed for EN at a concentration of 500 ng band\(^{-1}\). The relative standard deviation (RSD) values were less than 2% (Table VI).

Accuracy

The percentage recovery studies are shown in Table VII and are satisfactory. For EN, the recovery ranged from 98.22% to 101.51%.

Specificity

The peak purity study for EN and dosage form revealed that it was specific at a selected wavelength of 237 nm, proving that the standard EN is in its purest form. This allowed for the confirmation of the drug’s peak purity. The method was very specific, even when a number of different excipients were present.

**Table V:** Summary of linear regression and validation data

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Parameter*</th>
<th>Empagliflozin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Linearity range</td>
<td>125-750 (ng band(^{-1}))</td>
</tr>
<tr>
<td>2.</td>
<td>Linear regression equation</td>
<td>(y = 4.3211x - 13.534)</td>
</tr>
<tr>
<td>3.</td>
<td>Slope</td>
<td>4.3211</td>
</tr>
<tr>
<td>4.</td>
<td>Intercept</td>
<td>13.534</td>
</tr>
<tr>
<td>5.</td>
<td>Correlation coefficient ((r^2))</td>
<td>0.9925</td>
</tr>
<tr>
<td>6.</td>
<td>Limit of detection ((\text{ng band}\ ^{-1}))</td>
<td>12.10</td>
</tr>
</tbody>
</table>

*Denotes an average of five estimations

**LOD**

According to ICH guidelines Q2 (R1), for the developed method, LOD was established to be 12.10 ng band\(^{-1}\), demonstrating the sensitivity of the suggested approach.

**Specificity**

The peak purity study for EN and dosage form revealed that it was specific at a selected wavelength of 237 nm, proving that the standard EN is in its purest form. This allowed for the confirmation of the drug’s peak purity. The method was very specific, even when a number of different excipients were present.

**Table VI:** Data of precision study

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Drug</th>
<th>Repeatability*</th>
<th>Interday precision*</th>
<th>Intraday precision*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>% RSD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>1.</td>
<td>Empagliflozin</td>
<td>2243.67 ±27.566</td>
<td>1.228</td>
<td>2548.44 ±26.441</td>
</tr>
</tbody>
</table>

*Average of six estimations, †Average of three estimations, ‡Mean peak area for empagliflozin

**Table VII:** Recovery study of the method (using the standard addition method)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Drug</th>
<th>Recovery level (%)</th>
<th>Initial amount</th>
<th>Amount added</th>
<th>% Recovery*</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Empagliflozin</td>
<td>80</td>
<td>10 mg</td>
<td>8 mg</td>
<td>98.22</td>
<td>1.228</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>10 mg</td>
<td>10 mg</td>
<td>101.51</td>
<td>0.920</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>10 mg</td>
<td>12 mg</td>
<td>101.17</td>
<td>1.185</td>
</tr>
</tbody>
</table>

*Average of three estimations at each level of recovery
Robustness study

The reliability of the method is supported by the results (Table VIII). These results showed that the peak area’s relative standard deviation was less than 2% (Table VIII).

Table VIII: Robustness study for the developed method

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Parameter</th>
<th>Mean* ± SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Change in saturation time (±2 min)</td>
<td>2259.33 ±28.5715</td>
<td>1.266</td>
</tr>
<tr>
<td>2.</td>
<td>Change in mobile phase (±1 mL)</td>
<td>2135.67 ±29.73</td>
<td>1.392</td>
</tr>
<tr>
<td>3.</td>
<td>Total mobile phase change (±1 mL)</td>
<td>2156.33 ±24.41</td>
<td>1.132</td>
</tr>
</tbody>
</table>

* Mean peak area of three estimations

Stability studies

In order to summarize the results of the various forced degradation studies conducted on EN, the mobile phase was composed of ammonium acetate (2%), triethylamine and isopropyl alcohol 4:1:5 (V/V/V) (Table IX).

Table IX: Stability studies for the developed method

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Degradation condition</th>
<th>Number of degradation products (Rf values)</th>
<th>Area of degradation products (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Acid</td>
<td>3 (0.50, 0.59, 0.67)</td>
<td>9.13, 7.84, 9.73</td>
</tr>
<tr>
<td>2.</td>
<td>Base</td>
<td>3 (0.32, 0.50, 0.67)</td>
<td>8.25, 7.19, 9.37</td>
</tr>
<tr>
<td>3.</td>
<td>Oxidative</td>
<td>3 (0.50, 0.58, 0.67)</td>
<td>2.07, 8.89, 4.24</td>
</tr>
<tr>
<td>4.</td>
<td>Heat</td>
<td>3 (0.54, 0.60, 0.78)</td>
<td>3.41, 2.31, 1.35</td>
</tr>
<tr>
<td>5.</td>
<td>Photo</td>
<td>2 (0.63, 0.73)</td>
<td>4.06, 4.37</td>
</tr>
</tbody>
</table>

Fig. 11: Typical densitogram of EN and degradation products in the acid degradation study
1. Degradation product – *Rf* 0.50 (9.13 %)
2. Degradation product – *Rf* 0.59 (7.84 %)
3. Degradation product – *Rf* 0.67 (9.73 %)
4. Standard empagliflozin- *Rf* (0.82)

Fig. 12: Typical densitogram of EN and degradation products in the base degradation study
1. Degradation product – *Rf* 0.32 (8.25 %)
2. Degradation product – *Rf* 0.50 (7.19 %)
3. Degradation product – *Rf* 0.67 (9.37 %)
4. Standard empagliflozin- *Rf* (0.82)

Fig. 13: Typical densitogram of EN and degradation products in the oxidative degradation study
1. Degradation product – *Rf* 0.50 (2.07 %)
2. Degradation product – *Rf* 0.59 (8.89 %)
3. Degradation product – *Rf* 0.67 (4.24 %)
4. Standard empagliflozin- *Rf* (0.82)
Acid induced degradation

The additional peaks were observed at R_f values of 0.50, 0.58, and 0.67 (approximately 2.07, 8.89 and 4.24% degradation), respectively (Fig. 11).

Base induced degradation

The additional peaks were observed at R_f values of 0.32, 0.50, and 0.67 (approximately 8.25, 7.19 and 9.37% degradation), respectively (Fig. 12).

Oxidative induced degradation

The additional peaks were observed at R_f values of 0.50, 0.59, and 0.67 (approximately 9.13%, 7.84% and 9.73% degradation), respectively (Fig. 13).

Heat degradation

The additional peaks were observed at R_f values of 0.54, 0.60, and 0.78 (approximately 3.41, 2.31 and 1.35% degradation, respectively, Fig. 14).

Photolytic degradation

The additional peaks were observed at R_f = 0.63 and R_f = 0.73 (approximately 4.06 and 4.37 % degradation), respectively, in a photo-degradation study (Fig. 15).

CONCLUSION

A stability indicating HPTLC method for EN was successfully developed and validated using the DoE methodology. Ammonium acetate 2%, triethylamine and isopropyl alcohol in the ratio of 4:1:5 (V/V/V) was used as the mobile phase. Prior to chromatographic development, the optimal chamber saturation period was 10 minutes at room temperature (25 ±2°C). Using the winCATS software and a CAMAG TLC scanner III, densitometric scanning was done. UV detector operating at 237 nm was used to detect the object. The developed and validated method was verified as per specifications of ICH Q2 (R1). Finding crucial details on the sensitivity of the R_f value for EN at various chromatographic variables is made easier with the help of the Central Composite Design (CCD) and response surface methodologies. Application of a DoE tool allowed for simultaneous optimization of the parameters as isopropyl alcohol content, saturation time of chamber and distance travelled. The R^2 value of 0.9925 over the concentration range of 100-600 ng\(^{-1}\) was found to be exact and accurate. The proposed methodology is novel, fast, cost effective specific and selective for method development and validation of EN by HPTLC using DoE. The forced degradation studies for EN including hydrolysis, oxidation, thermolysis, and photo-degradation were performed on the stability indicating parameters created in accordance with the ICH guidelines. The developed method can effectively be used for routine analysis and during stability studies as per regulatory requirements as it has ability to analyze the EN alone and has capability to identify the separate degradation products based on R_f value. The proposed method can be useful for the quantification of EN is bulk and in marketed dosage forms.
REFERENCES


