Simultaneous quantification of empagliflozin, linagliptin and metformin hydrochloride in bulk and synthetic mixture by RP–LC method

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Abstract

Background: Extensive literature review revealed that no RP–LC method has been developed for simultaneous estimation of EMPA, LINA and MET in combined dosage form. This is a newer combination approved by USFDA on 4th June 2019 and it is launch in the United State Market on 27th January 2020.

Result: A simple, sensitive, specific, precise and accurate reverse phase—high performance liquid chromatography (RP- HPLC) method has been developed for simultaneous estimation of Empagliflozin, Linagliptin and Metformin HCl in bulk and synthetic mixture. Phenomenex C18 column (250 mm × 4.6 mm, 5 µm) was used as stationary phase for chromatographic separation through isocratic elution using Acetonitrile: Methanol: Water in a ratio (27: 20: 53, v/v/v) pH 4 adjusted with 1% Ortho-phosphoric acid as mobile phase at flow rate 1 ml/min. PDA detector was used for simultaneous analysis of all three drugs at common wavelength 223 nm and the each injection volume was 20 µl. The linearity range for Empagliflozin, Linagliptin and Metformin HCl was found to be 0.5–5 µg/ml, 0.25–2.5 µg/ml, and 50–500 µg/ml, respectively. The retention time for Empagliflozin, Linagliptin and Metformin HCl was found to be 14.5 min, 3.4 min and 2.01 min, respectively. The percentage (%) recovery was found to be 99.98–100.81% for Empagliflozin, 99.33–100.57% for Linagliptin and 100.65–101.35% for Metformin HCl respectively.

Conclusion: As per the international Conference on Harmonisation (ICH) Q2 (R1) guideline, proposed RP–LC method validation has been carried out. The proposed RP–LC method was repeatable and selective as per statistical analysis and it can be use for simultaneous estimation of Empagliflozin, Linagliptin and Metformin HCl in bulk and synthetic mixture. The proposed method might be applied for simultaneous estimation of all three drugs in pharmaceutical formulation.

Keywords: Empagliflozin (EMPA), Linagliptin (LINA), Metformin HCl (MET), Validation, RP–HPLC

Background

Empagliflozin (EMPA) is used as a sodium glucose co-transporter-2 (SGLT-2) inhibitor to improve glycemic control in adult patients with type 2 diabetes. SGLT-2 co-transporters reabsorb glucose from the glomerular filtrate in kidney and the glucuretic action resulting from inhibition of SGLT-2 which reduces renal absorption and lowers down the renal threshold for glucose, therefore
increases glucose excretion which reduces hyperglycemia and also helps in blood pressure reduction [1, 2]. Chemically EMPA is 1-chloro-4-(glucopyranos-1-yl)-2-(4-(tetrahydrofuran-3-yloxy)benzyl)benzene and having empirical formula is C₂₃H₂₇ClO₇ with molecular weight 450.91 g/mole (Fig. 1A).

Linagliptin (LINA) is having competitive, reversible DPP-4 inhibitory action which is responsible for DPP-4 breakdown reduction of GLP-1 and glucose-dependant insulinotropic polypeptide (GIP). From beta cells of the pancreas, GLP-1 and GIP stimulate the release of insulin during inhibiting release of glucagon from pancreatic beta cells. These effects together reduce the breakdown of glycogen in the liver and increase insulin release in response to glucose [2–4]. Chemically LINA is (R)-8-(3-aminopiperidin-1-yl)-7-but-2-ynyl-3-methyl-1-(4-methylquinazolin-2-ylmethyl)-3,7-dihydro-purine-2,6-dione and having empirical formula is C₂₅H₂₈N₈O₂ with molecular weight 472.5422 g/mole (Fig. 1B).

N-dimethylmethanimidamide hydrochloride is Metformin Hydrochloride (MET). Metformin is belongs to antihyperglycemic agent of the biguanide class which is used for the management of type II diabetes. MET is having empirical formula C₄H₁₁N₅.HCl with molecular weight 165.625 g/mole (Fig. 1C).

Metformin HCl is drug of choice for type II diabetes patient which lowers blood glucose concentrations in type II diabetes without causing hypoglycemia. It is also known as an insulin sensitizer which leads to decrease in insulin resistance which leads to significant reduction of plasma fasting insulin level [5].

On 4th June 2019, USFDA is approved a newer combination and is launch in the United State Market on 27th January 2020. Combination works by three complementary mechanisms which help in managing blood glucose in adult with type 2 diabetes [6].

A far-reaching literature survey carried out for quantitative analysis of EMPA, LINA and MET revealed that the attempts have been made to develop liquid chromatographic methods for the estimation of EMPA, LINA and MET alone and combination with other drugs [7–11, 11–14] which represent that no RP–LC method has been reported in literature review for simultaneous estimation of EMPA, LINA and MET in combined dosage form. LC methods are preferred method of analysis due to their accuracy, precision and sensitivity of instrument. RP–LC method is more advantageous over HTPLC which is require a small sample size which can be modified on depending on level of quantitation needed and gives reliable results. Therefore, attempt has been made for the simultaneous estimation of EMPA, LINA and MET in synthetic mixture by RP–LC method.

**Methods**

**Instruments**

HPLC system consisted of binary pump (Model Waters 515 HPLC pump), rhodyne loop injector and PDA detector (Waters 2998). Empower- version 2 software is used for data collection and analysis. Phenomenex C₁₈ column (250 mm × 4.6 mm, 5 µm) is used for separation through isocratic elution using Acetonitrile: Methanol: Water in a ratio (27: 20: 53, v/v/v) pH 4 adjusted with 1% Ortho-phosphoric acid as mobile phase at flow rate 1 ml/min. The photo diode array (PDA) detector is used for detection monitoring at common wavelength 223 nm and the each injection volume was 20 µl. The calibrated Sartorius CP124S (Sartorius Corporation, United State) instrument is used for weighing of all the active ingredients.
pharmaceutical ingredients, excipients and chemicals which is having weighing sensitivity 0.01 mg.

Chemicals and reagents
Analytically pure active pharmaceutical ingredients EMPA (99.23%), LINA (98.92%), and MET (99.12%) was procured as a gratis samples from Torrent Pharmaceutical Pvt. Ltd., Ahmedabad, Gujarat, India, Zydus Cadila Healthcare Ltd., Ahmedabad, Gujarat, India and Sun Pharmaceutical Pvt. Ltd., Vadodara, Gujarat, India respectively. Methanol (HPLC grade) and acetonitrile (HPLC grade) were obtained from SRL Pvt. Ltd. Mumbai, India, HPLC grade water—Milli Q integral Water purification system, Merck KGaA, Darmstadt, Germany, and Ortho-phosphoric acid (AR grade) obtained from SRL Pvt. Ltd. Mumbai, India.

Chromatographic system
Preparation of mobile phase
By taking premix Acetonitrile: Methanol: Water (27:20:53, v/v/v) pH 4.0 adjusted with 0.6 ml of 1% O—phosphoric acid in 200 ml reservoir was used as a mobile phase. For degassing the mobile phase prior to use, the reservoir was sonicated for 20 min and mixtures of solvent were used as mobile phase.

Preparation of standard stock solution
Precisely weigh 100 mg of MET, 2.5 mg of LINA and 5 mg of EMPA and transfer into the three different 10 ml volumetric flask containing 2.0 ml of methanol and the volume was made upto the mark with methanol which gives the stock solution having concentrations 10000 µg/ml of MET, 250 µg/ml of LINA and 500 µg/ml of EMPA, respectively. Pipette out the 2.5 ml of MET, 0.5 ml of LINA and 0.5 ml of EMPA from the above stock solution, transfer into volumetric flask of 50 ml and make up the volume with methanol to acquire the concentration of working standard stock solution 500 µg/ml of MET, 2.5 µg/ml of LINA and 5 µg/ml of EMPA.

Calibration curve
From above working standard stock solution, pipette out appropriate volume of aliquots of MET (500 µg/ml), LINA (2.5 µg/ml) and EMPA (5 µg/ml) are transferred to different 10 ml volumetric flask and volume was adjusted up to the mark with the mobile phase to give a final concentration range of 50, 100, 200, 300, 400 and 500 µg/ml for MET, 0.25, 0.5, 1, 1.5, 2 and 2.5 µg/ml for LINA and 0.5, 1, 2, 3, 4 and 5 µg/ml for EMPA. By using the proposed chromatographic conditions, each solution was analyzed and the chromatogram was recorded. Calibration curves were constructed by plotting peak area v/s concentration and regression equations were computed.

Validation
According to the International Conference on Harmonization (ICH) guidelines Q2 (R1) guidelines [15], validation of the proposed developed RP–LC method was carried out.

Linearity
Linearity was studied by preparing standard solution of 7 different concentrations of 50, 100, 200, 300, 400 and 500 µg/ml for MET, 0.25, 0.5, 1, 1.5, 2 and 2.5 µg/ml for LINA and 0.5, 1, 2, 3, 4 and 5 µg/ml for EMPA. The chromatogram of each concentration was recorded 5 times from freshly prepared seven different concentrations for MET, LINA and EMPA. The terms of slope, intercept and correlation coefficient of MET, LINA and EMPA was used for the assessment of linearity. The calibration curves were developed by plotting concentration v/s peak area (n=5).

Precision
Precision study was designed in expressions of intra-day and inter-day precisions. Intraday and Interday precision was carried out by analyzing sample solution at three levels covering low, medium and high concentration of the calibration curve three times on the same day for intraday precision study and over a period of three different days (n=3) for interday precision study and over a period of three different days (n=3) for interday precision study and over a period of three different days (n=3) for interday precision study and over a period of three different days (n=3). The chromatogram was recorded and peak areas obtained were used to calculate mean and % RSD values. The repeatability studies were carried out by estimating the response at 200 µg/ml for MET, 1.0 µg/ml for LINA and 2.0 µg/ml for EMPA 6 times and % RSD of area are reported.

Accuracy
The accuracy study was performed by calculating recovery of MET, LINA and EMPA with the help of standard addition method. Known amount of MET, LINA and EMPA at 80, 100, and 120% levels were spiked to pre quantified sample solution and the amount of MET, LINA and EMPA were estimated by putting the value of peak area to the regression equation of calibration curve.

Limit of detection and limit of quantification
The limit of detection (LOD) is defined as the lowest concentration of an analyte that can detect. The limit of quantification (LOQ) is the lowest amount of analyte that can be quantitatively determined with appropriate precision and accuracy. As per ICH guideline, LOD and LOQ were calculated using the following equation:

\[
\text{LOD} = 3.3 \times \frac{\sigma}{S}
\]

\[
\text{LOQ} = 10 \times \frac{\sigma}{S}
\]
where, $\sigma$ is the standard deviation of $y$-intercepts of regression lines and $S$ is the slope of the calibration curve.

**Robustness**

The robustness study has been carried out by a deliberate change in the proposed RP–LC chromatographic condition parameters like detection wavelength, flow-rate of mobile phase and the mobile phase solvent make on the results were examined for the concentration of 200 $\mu$g/ml for MET, 1 $\mu$g/ml for LINA and 2 $\mu$g/ml for EMPA. The average and %RSD of peak retention time were calculated.

**Specificity**

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically these might be including impurities, degradants, preservatives, and excipients for checking the interference which are used in synthetic mixture. The developed method was found to be specific.

**System suitability**

The adequate performance of liquid chromatographic system can be assessed by System suitability parameters. A system suitability test requires ensuring that a given operating system may be normally applicable because of normal variation in equipment, supplies and techniques. System suitability also require for the verification of the resolution and reproducibility of the chromatographic system which are adequate for the analysis to be done.

**Analysis of synthetic mixture [16]**

The synthetic mixture of MET, LINA and EMPA was prepared in ratio of 500 mg: 2.5 mg: 5 mg respectively. Common excipients like Hydroxypropyl methylcellulose 0.34 g [HPMC], Polyethylene glycol 0.24 g [PEG], Magnesium stearate 0.15 g, Talc 0.18 g were weighed accurately and transfer into motor pestle along with 0.1 g of EMPA, 0.05 g of LINA, and 10 g of MET which is equivalent to 20 tablets. Synthetic mixture powder (0.554 g) was accurately weighed and transferred into 100 ml volumetric flask containing 20 ml of methanol. It was sonicated for
15 min and solution was filtered using Whatman filter paper No.42. Filtrate was collected in another 100 ml volumetric flask and the residue was washed with few amount of methanol, the filtrate and residue was combined and methanol was added upto the mark. From the above solution, 0.4 ml of aliquot was pipette out into the 10 ml volumetric flask and methanol was added up to the mark to obtain absolute concentration of 200 µg/ml for MET, 1.0 µg/ml for LINA and 2.0 µg/ml for EMPA respectively. The chromatogram was recorded at 223 nm and the quantification was carried out by keeping these values in the regression equation of calibration curve.

### Results

**Selection of analytical wavelength**

The solution of EMPA, LINA and MET having the concentration of 10 µg/ml was scanned in the range of 200-400 nm, all three drug shows considerable absorbance at common wavelength 223 nm. Therefore 223 nm was a choice of an analytical wavelength for the analysis of all three drugs. The overlay spectra for selection of analytical wavelength are shown in Fig. 2.

**Optimization of mobile phase**

Several combinations of solvents were tried for optimization of mobile phase like Methanol: Water (80:20, v/v), Acetonitrile: Water (60:40, v/v), Acetonitrile: Methanol (50:50, v/v) and Acetonitrile: Methanol: Water (20:25:50, v/v/v) pH 4.0 adjusted with 1% Ortho-phosphoric acid (OPA). The mobile phase containing Acetonitrile (ACN): Methanol: Water (27:20:53, v/v/v) pH 4 adjusted with 0.6 ml of 1% Ortho-phosphoric acid (OPA) and showed satisfactory results at a flow rate of 1 ml/min. The retention time was found to be 2.01 min for MET, 3.2 min LINA and 14.5 min for EMPA. Where, total run time of analysis was 18 min (Fig. 3).

### Table 1 Regression Analysis of calibration curve

| Parameter                        | MET  | LINA | EMPA |
|----------------------------------|------|------|------|
| Range (µg/ml)                    | 50–500 | 0.25–2.5 | 0.5–5 |
| Regression coefficient (R²)      | 0.9981 | 0.9967 | 0.9968 |
| Slope of regression equation     | 50,738.2 | 91,942 | 43,503 |
| Standard deviation of slope      | 686.03 | 1928.75 | 244.10 |
| Intercept of regression equation | 755,315.8 | 50,312 | 3715.22 |
| Standard deviation of intercept  | 86,870.96 | 1146.97 | 475.10 |

![Chromatogram of MET (50 µg/ml), LINA (0.25 µg/ml) and EMPA (0.5 µg/ml) using Acetonitrile: Methanol: Water (27:20:53, v/v/v) pH 4.0 adjusted with 1% OPA](image-url)

**Fig. 3** Chromatogram of MET (50 µg/ml), LINA (0.25 µg/ml) and EMPA (0.5 µg/ml) using Acetonitrile: Methanol: Water (27:20:53, v/v/v) pH 4.0 adjusted with 1% OPA
The linearity study was performed by considering the clinical dose ratio of all the three drugs. The method was found to be linear in the range 50–500 µg/ml for MET, 0.25–2.5 µg/ml for LINA and 0.5–5 µg/ml for EMPA with the correlation coefficient 0.9981, 0.9967 and 0.9968 respectively. The data of regression analysis of calibration curve was shown in Table 1 and overlay spectra of MET (50–500 µg/ml), LINA (0.25–2.5 µg/ml) and EMPA (0.5–5 µg/ml) at 223 nm shown in (Fig. 4). The correlation coefficient values were found to be more than 0.9950 which indicate that the method is linear in relationship with respect to recorded area and concentrations.

Table 2 Summary of Validation Parameters

| Parameter                              | MET     | LINA    | EMPA    |
|----------------------------------------|---------|---------|---------|
| Range (µg/ml)                          | 50–500  | 0.25–2.5| 0.5–1   |
| Limit of detection (LOD)               | 5.65    | 0.04    | 0.03    |
| Limit of quantification (LOQ)          | 17.12   | 0.12    | 0.10    |
| Retention time                         | 2.01    | 3.2     | 14.5    |
| Accuracy (%)                           | 100.65–101.35 | 99.33–100.57 | 99.98–100.81 |
| Instrumental precision (%RSD)          |         |         |         |
| Intraday (n = 3)                        | 0.21–1.12 | 0.20–1.27 | 0.29–1.69 |
| Interday (n = 3)                        | 1.10–1.91 | 0.76–1.53 | 0.70–1.78 |

Table 3 Accuracy studies of proposed method

| Amount of drug taken from samples (µg/ml) | Amount of standard drug spiked (µg/ml) | Average Amount of recovered Standard (µg/ml) (n = 3) | % Recovery ± SD (n = 3) |
|------------------------------------------|----------------------------------------|-----------------------------------------------------|------------------------|
| MET                                      | LINA                                   | EMPA                                                | MET                    | LINA                                   | EMPA                                                | MET                    | LINA                                   | EMPA                                                |
| 200                                      | 1                                      | 2                                                   | 161                    | 0.81                                  | 1.58                                                | 100.62±0.95            | 101.25±0.62                                   | 98.75±0.80                                            |
| 200                                      | 1                                      | 2                                                   | 200                    | 1                                      | 2                                                  | 101.50±0.72            | 98.00±1.1                                     | 101.00±0.56                                            |
| 200                                      | 1                                      | 2                                                   | 240                    | 1.2                                    | 2.4                                                | 98.75±0.84             | 98.33±0.92                                     | 99.17±1.12                                            |
The intraday and interday variation in the method responses were studied using precision study. The intraday and interday precision was performed and the %RSD of MET, LINA and EMPA was found to be 0.21–1.12, 0.20–1.27 and 0.29–1.69 for intraday and % RSD of MET, LINA and EMPA was found to be 1.10–1.91, 0.76–1.53, and 0.71–1.78 respectively. Instrumental precision was carried out by performing injection repeatability test and the %RSD of MET, LINA and EMPA was found to be 1.12%, 0.97% and 0.62%. The data of intraday, interday and repeatability was shown in Table 2. The variability in the responses was less than 2% for intra-day, inter-day and repeatability study, which indicate that the method is precise.

### Accuracy

The percentage (%) recoveries of MET, LINA and EMPA was determined by a known amount of standard was spiked into pre-analyzed sample solutions. The recoveries were found to be 100.65–101.35% for MET, 99.33–100.57% for LINA and 99.89–100.81% for EMPA respectively. The accuracy data was shown in Table 3. The percentage (%) recoveries for all three drugs were found to be in the range of 95 to 102%, which indicate the method is accurate.

### Limit of detection and Limit of quantification

The LOD and LOQ were carried out by signal to noise ratio. The lowest amount of drug (LOD) for MET, LINA and EMPA was found to be 5.65 µg/ml, 0.04 µg/ml, 0.03 µg/ml and the lowest amount of quantification (LOQ) was 17.12 µg/ml, 0.12 µg/ml and 0.10 µg/ml respectively.

### Specificity

The specificity study was performed to check the interference of excipients used in the preparation of synthetic mixture and there is no any interference observed by excipients at the time of elution of all three different drugs at the respective retention time. The developed method was found to be specific.
Robustness
After an introducing small and deliberate changes in chromatographic condition parameters like change in detection wavelength, flow rate, and make of mobile phase solvent, the % RSD of retention time of all three drugs was found to be less than 2% which confirming that the proposed method is robust. The data of robustness studies is shown in Table 4.

System suitability
The results of system suitability test are reported in Table 5.

Analysis of synthetic mixture
The proposed RP–LC method is applied for the simultaneous estimation of MET, LINA and EMPA from the prepared synthetic mixture. The % amount of drug for MET, LINA and EMPA was found to be 100.92–101.39%, 98.55–100.76% and 99.33–101.22%, respectively. The overlay Chromatogram of standard and prepared synthetic mixture of MET (200 µg/ml) LINA (1 µg/ml) and EMPA (2 µg/ml) are shown in (Fig. 5).

Discussion
The proposed RP–LC method is used for simultaneous estimation of EMPA, LINA and MET in bulk and synthetic mixture. Optimization of chromatographic conditions was achieved and with the help of Phenomenex C18 column (250 mm × 4.6 mm, 5 μm) through isocratic elution by Acetonitrile: Methanol: Water (27: 20: 53, v/v/v) pH 4.0 adjusted with 1% Ortho—phosphoric acid as mobile phase at flow rate 1 ml/min, the separation of all three drugs were achieved. The linearity for MET, LINA and EMPA was found to be 50–500 µg/ml, 0.25–2.5 µg/ml and 0.5–5 µg/ml, respectively. Table 4 is represent accuracy data and the % recoveries for MET, LINA and EMPA were found to be 100.65–101.35%, 99.33–100.57% and 99.89–100.81%, respectively. The specificity study was performed to check the interference of excipients used in the preparation of synthetic mixture and there is no any interference observed by excipients at the time of elution of all three different drugs which represent the method is specific. Table 5 represent the data of Robustness of the proposed developed RP–LC method is a measure of its ability to remain unaffected by small but deliberate change of the chromatographic method parameters. It was estimated by small changes in the chromatographic conditions like change in detection wavelength (± 2 nm), change in flow rate (± 0.1 ml) and change in mobile phase solvent make which represents
the minor changes does not affect on the symmetry of peak and retention time (R_t) of MET, LINA and EMPA which confirming the reliability and robustness of the method. The percentage (%) amount of all three drugs were found to be more than 98% in the assay of synthetic mixture which specify that the method provides accurate and precise results. As compared to reported TLC method, the proposed RP–LC method is more sensitive, accurate, robust and precise [17].

Conclusions
The proposed study describes that RP–LC method was developed and validated for the simultaneous estimation of EMPA, LINA and MET in bulk and synthetic mixture as per ICH Q2(R1) guidelines and found to be sensitive, accurate and precise. The proposed RP–LC method was repeatable and selective as per statistical analysis and the proposed method might be applied for simultaneous estimation of all three drugs in pharmaceutical formulation. As compared to reported TLC method, the proposed RP–LC method is more sensitive, accurate, robust and precise therefore R–LC method provides more accurate results and requires less quantity of sample. Due to complete automation, HPLC is preferred over TLC method as a pharmacopoeia method of analysis. The results published HPTLC manuscript was found to be effective for qualitative analysis, however proposed RP–LC method was found to be more accurate for quantitative analysis.

Abbreviations
HPLC: High Performance liquid chromatography; RP–HPLC: Reverse Phase High Performance liquid Chromatography; EMPA: Empagliflozin, LINA: Linagliptin, MET: Metformin HCI, OPA: Ortho phosphoric acid, LOQ: Limit of Quantitation, LOD: Limit of Detection, %RSD: Relative standard deviation, Rt: Retention time.

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All authors associated with this research work declared that there is no conflict of interest for publication of work. All authors have read and approved the manuscript. The contribution of each authors are mentioned below: IMP: He is post graduate student and above work has been carried out by him as dissertation work. UKC: He is a mentor of IMP and under his noble guidance proposed method has been developed and validated as per ICH guideline. He is also giving training for ease of operation sophisticated reverse phase liquid chromatography instrument and involved in interpretation of data. HDJ: She is also involved in interpretation of data. DAS: Through his good relationship with pharmaceutical industry we have received all active pharmaceutical ingredients and he is having sound technical knowledge Waters HPLC software system. All authors read and approved the final manuscript.

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No competing interests to declare.

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