DEVELOPMENT AND VALIDATION OF RAPID STABILITY-INDICATING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE DETERMINATION OF LINAGLIPTIN AND EMPAGLIFLOZIN IN PURE AND DOSAGE FORMS

RAGAA EL SHEIKH¹, WAFAA S HASSAN², EMANH YOUSSEF³, ABDULRAHMAN Y HAMDI³, NAIF AHMED BADAHDAH¹, MUNEER ESA ALZUHRI², AYMAN ABOU ELFETOUH GOUDA¹*¹

¹Department of Chemistry, Faculty of Science, Zagazig University, Zagazig, Egypt. ²Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt. ³Poison control and Medical Forensic Chemistry Center, Makkah, Saudi Arabia. ⁴Department of Faculty of Public Health and Health Informatics, Umm Al-Qura University, Makkah, Saudi Arabia.

Email: aymangouda77@gmail.com

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ABSTRACT

Objective: A new, simple, rapid, sensitive, and accurate stability-indicating high-performance liquid chromatography (HPLC) method was developed and validated for the quantitative determination of linagliptin (LNG) and empagliflozin (EMP) in pure and tablet dosage forms.

Methods: An isocratic HPLC method, using a C₁₈ reversed-phase column (150 mm x 4.6 mm i.d., particle size 5 μm) with an isocratic binary mobile phase consisting of phosphate buffer and acetonitrile (55:45, v/v), was investigated to separate the drug from its stress degradation products. The flow rate was 1.0 mL/min at ambient temperature and photodiode array detector is used at 226 nm for detection. The developed method was validated for system suitability, linearity, accuracy, precision, limits of detection and quantitation, specificity, stability, and robustness.

Results: The retention time of LNG and EMP was found to be 3.27±0.002 and 7.96±0.0006 min, respectively. The calibration curve was found to be linear with the equation y=158926.39X+11.139, with a correlation coefficient of R²=0.9991 for LNG and y=22688.45X+4.259, with a correlation coefficient of R²=0.9994 for EMP over a concentration range of 2.5–7.5 µg/mL and 5.0–15 µg/mL for LNG and EMP, respectively. The limits of detection were 0.29 and 0.48 µg/mL for LNG and EMP, respectively. The limits of quantification were 0.89 and 1.5 µg/mL for LNG and EMP, respectively. The recovery values of this method are 101.11% and 101.48% for LNG and EMP, respectively, and the reproducibility is within 0.070 and 0.277 for LNG and EMP, respectively.

Conclusion: The proposed method is a rapid stability-indicating HPLC method that can be applied for the determination of LNG and EMP in pure and tablet dosage forms.

Keywords: Linagliptin, Empagliflozin, Rapid stability-indicating high-performance liquid chromatography method, Method validation, Dosage forms.

INTRODUCTION

Linagliptin (LNG) is a more potent dipeptidyl peptidase (DPP)-4 inhibitor than other drugs that belong to the same class for the treatment of Type II diabetes. LNG is a competitively reversible DPP-4 enzyme inhibitor that slows the breakdown of insulinotropic hormone glucagon-like peptide-1 for better glycemic control in diabetes patients. Empagliflozin (EMP) is a sodium glucose cotransporter-2 inhibitor indicated as an adjunct to diet and exercise to improve glycemic control in adult patients with Type 2 diabetes. The combination of LNG and EMP is served as a nadurivant to diet and exercise to improve glycemia control in adults with Type-2 diabetes who know to have the cardiovascular disease [1-3]. LNG chemically β-[[3R]-3-amino-2-phenyl-4H-pyran-2-one]-3-methyl-1-[4-(methyl quinazolin-2-yl) methyl]-3,7-dihydro-1H-purine-2,6-dione EMP, chemically designated as 2S, 3R, 4R, 5, 6S)-2-[4-chloro-3-[[4-[[3S]-oxolan-3-yloxa y]phenyl][methyl]phenyl]-6-(hydroxyethyl)oxane-3,4,5-triyl (Fig. 1). Literature review revealed that few methods were described for the determination of LNG and EMP alone or in combination with other drugs from pharmaceutical dosage forms and in human plasma including spectrophotometry [4-7], ultra-performance liquid chromatography (LC) [8], LC-mass spectroscopy [9], and high-performance LC (HPLC) [10-25] techniques.

The aim of the present work is to develop and validate simple, fast, and reliable stability-indicating reverse-phase HPLC method with ultraviolet (UV) detection for the simultaneous determination of LNG and EMP in pure and pharmaceutical dosage forms. The proposed method can overcome the problems in all previously reported HPLC methods such as long time of analysis and expensive detectors, as shown in Table 1.

METHODS

Instrumentation

HPLC apparatus (Agilent 1200, Agilent, USA) equipped with UV detector DAD system. The pH measurements were made on a Hanna pH meter equipped with a combined glass-calomel electrode (Portugal) (HI: 9321).

Chemicals and reagents

Analytical HPLC grade solvents were used in all experiments, including acetonitrile and methanol (LAB-SCAN, Analytical Sciences, Gliwice, UL, Sowinskiego, Poland). Potassium dihydrogen orthophosphate, sodium hydroxide (NaOH), hydrochloric acid (HCl), and hydrogen peroxide (H₂O₂, 30%, v/v) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Double distilled water was used.

LNG (99.40%) and EMP (99.70%) were kindly supplied by Zeta Pharma, Egypt. The pharmaceutical dosage forms used were Glyxambi® tablets (Eli Lilly and Company; Boehringer Ingelheim Pharmaceuticals, Inc., USA).
Sheikh et al.

Asian J Pharm Clin Res, Vol 13, Issue 4, 2020, 172-177

Table 1: Chromatographic methods reported for the determination of LNG and EMP in dosage forms

| Chromatographic conditions | LOD (μg/mL) | Concentration range (μg/mL) | References |
|----------------------------|-------------|-----------------------------|------------|
| Mobile phase               | LNG         | EMP                         |            |
| Phosphoric acid:acetonitrile (45:50, v/v) | 0.43         | 12.5–75                      | [19]       |
| 0.1% perchloric acid:acetonitrile (60:40, v/v) | 0.3         | 12.5–75                      | [20]       |
| 0.01% phosphoric acid (pH 4.5):acetonitrile (68:32, v/v) | 0.03         | 0.01–10                      | [12]       |
| Methanol:phosphate buffer (KH₂PO₄, K₂HPO₄) (pH 3.0) (70:30, v/v) | 2.17         | 20–1000                     | [21]       |
| Methanol:acetonitrile 0.1% ortho phosphoric acid (30:60:10, v/v) | 0.06         | 2.5–15                      | [22]       |
| Potassium dihydrogen phosphate buffer pH (3.4):methanol (70:30, v/v) | 0.76         | 50–150                      | [23]       |
| Phosphate buffer 0.01 M:acetonitrile (65:35, v/v) | 0.48         | 2.5–7.5                     | Proposed work |

5.0 mg LNG and 10 mg EMP per tablet and Empaalpha plus tablets (Zeta Pharma, Egypt, 5.0 mg LNG and 10 mg EMP per tablet).

Chromatographic conditions

The chromatographic separation was performed using ODS-3 Inertsil C18 (150 mm×4.6 mm), 5.0 μm particle size column; the column temperature was maintained at 25±2°C. The autosampler utilized methanol as a rinse solution, the total run time was 6.0 min. The elution quaternary pump ran an isocratic flow using mobile phase consisting of a mixture of phosphate buffer and acetonitrile (65:35, v/v) at a flow rate of 1.0 mL/min. The eluate was monitored at 226 nm using UV diode array detector. The retention time of the drug was found to be LNG and EMP which were found to be 3.276±0.002 and 6.966±0.0006 min, respectively. The injection volume was 20 μL. Mobile phase was used as diluent during the standard and test samples preparation.

Preparation of standard solutions

A stock solution of LNG or EMP (10 μg/mL) was prepared by dissolving 10 mg of LNG and EMP in mobile phase in 100 mL volumetric flask, then shaken and sonicated for 10 min till completely dissolved and then complete the volume to 100 mL with mobile phase. The working standard solutions were prepared by diluting aliquots of stock solution with mobile phase to obtain final concentrations ranging from 2.5–7.5 μg/mL to 5.0–15 μg/mL for LNG and EMP, respectively. Working solutions of the drugs were stable for 1 week.

Construction of calibration curves

Aliquots of standard solution, ranging from 2.5–7.5 μg/mL to 5.0–15 μg/mL for LNG and EMP, respectively, were prepared in a series of 10 mL volumetric flasks, 20 μL were injected into the instrument. Detection was performed at the wavelength of 226 nm. The calibration graph was constructed by plotting the peak areas obtained at the wavelength of 226 nm versus the corresponding injected concentrations.

Assay for tablets dosage forms

Twenty tablets of Glyxambi and Empacaalpha plus were weighed, finely powdered, and an accurately weighed amount of the powdered tablets equivalent to 5.0 mg LNG and 10 mg EMP which were transferred to 100 mL measuring flask and dissolved in 50 mL of mobile phase, sonicated for 10 min, and the solution was filtered through a 0.45 μm membrane filter and then the final solution was completed to volume with mobile phase. The proposed procedure was then completed as mentioned above.

RESULTS

DISCUSSION

Method optimization

The conditions affecting the chromatographic performance of LNG and EMP were carefully studied to recognize the most suitable chromatographic system. Hence, the optimum chromatographic performances were achieved when using isocratic mobile phase composed of phosphate buffer and acetonitrile (65:35,v/v) with a flow rate of 1.0 mL/min, injection volume 20 μL, column temperature 25°C, and detection wavelength 226 nm. The results of the three runs indicate high system suitability (Table 2). The retention time (tᵣ) values LNG and EMP were found to be 3.276±0.002 and 6.966±0.0006 min, respectively (Fig. 2).

Method validation

The developed method was validated for system suitability, linearity, sensitivity, precision, accuracy, robustness selectivity, and
Sheikh et al.

Asian J Pharm Clin Res, Vol 13, Issue 4, 2020, 172-177

specificity and is applied for forced degradation studies as per the ICH guidelines [26].

**Linearity**
The linearity of LNG and EMP was established by eight-point calibration curve, concentration ranging from 2.5 to 7.5 µg/mL and 5.0 to 15 µg/mL for LNG and EMP, respectively. The graph of the peak area against concentration proved linear graph with regression equations; y=158,926.39x+11.139 and y=22,688.45x+4.259, with a correlation coefficients ($R^2$=0.9991 and 0.9994) for LNG and EMP, respectively.

**Sensitivity**
The limit of detection is defined as the injected quantity giving $S/N$ of 3 (in terms of peak area) and were found to be 0.29 and 0.48 µg/mL for LNG and EMP, respectively. The limit of quantification is defined as the injected quantity giving $S/N$ of 10 (in terms of peak area) and was found to be 0.89 and 1.50 µg/mL for LNG and EMP, respectively (Table 2).

**Precision**
The intraday repeatability (precision) of the developed method was assessed by analyzing six replicate injections of the standard solution at three different concentrations on the same day. The same was done for interday precision test except that the injection of the samples was every day for 5 days. The precision of the method was determined by calculating relative standard deviation (RSD %). The results in Table 3 show that the method is reproducible and there were high intra- and inter-day precisions (RSD ≤0.435%).

**Accuracy**
Accuracy of the method was established by performing recovery studies according to the ICH guidelines. Spiked samples were prepared by spiking pre-analyzed sample solutions with standard drug at three different concentration levels (50%, 100%, and 150% level). Mean percentage recovery values at three different concentrations of the two drugs were calculated. The % mean recovery was ranged from 100.13 to 101.20% and 100.40 to 101.70% for LNG and EMP, respectively (Table 3).
Robustness
The robustness of the present method was evaluated in terms of temperature, flow rate, column to column, wavelength of detection, and injection volume (Table 4). The slight variations in the examined factors had no significant effect on the shape of the peak. The results of coefficient of variation % indicate that the method is more sensitive to changes in the wavelength and the flow rate greater than to changes in the other factors. Compared with retention times (t_R-values), peak areas were more affected with the slight changes in the chromatographic conditions.

Selectivity and specificity of the method
The resulted peak after tablet analysis is found to be homogeneous and there are no coeluting peaks indicating specificity of the method. Comparison between the chromatogram of the raw LNG and EMP and that of extracted LNG and EMP from tablets indicate that the excipients in the formulation did not interfere with the determination of LNG and EMP.

Accuracy and application
Analysis of LNG and EMP in Empacoza plus and Glyxambi tablets by the proposed method showed high accuracy with a mean recovery range of 100.71±0.541% and 100.81±0.589% and 101.48±0.254% and 101.64±0.289% for LNG and EMP, respectively (Table 5). The results were compared with a reported method [20]. The values of t and f indicate that there is no significant difference between both methods.

Stability tests
The results (Fig. 3) of stress degradation indicate that LNG and EMP are strongly affected with reflux with HCl or NaOH. Reflux with H_2O and

Table 3: Intra- and interday precision and accuracy of LNG and EMP (n=5)

| Injected amount (μg/mL) | Intraday                      | Interday                      |
|------------------------|-------------------------------|-------------------------------|
|                        | Observed amount±SD | RSD %* | Accuracy (recovery %)** | Observed amount±SD | RSD %* | Accuracy (recovery %)** |
| LNG                    |                    |       |                      |                    |       |                      |
| 2.5                    | 2.53±0.003         | 0.119 | 101.20               | 2.52±0.004         | 0.159 | 100.80               |
| 5                      | 5.05±0.003         | 0.059 | 101.00               | 5.01±0.002         | 0.040 | 100.20               |
| 7.5                    | 7.58±0.033         | 0.435 | 101.07               | 7.51±0.013         | 0.173 | 100.13               |
| EMP                    |                    |       |                      |                    |       |                      |
| 5                      | 5.06±0.007         | 0.138 | 101.20               | 5.02±0.011         | 0.219 | 100.40               |
| 10                     | 10.17±0.003        | 0.029 | 101.70               | 10.08±0.015        | 0.149 | 100.80               |
| 15                     | 15.23±0.016        | 0.105 | 101.56               | 15.11±0.01        | 0.066 | 100.73               |

*RSD (%)=SD×100/mean. **Accuracy (recovery %)=(Observed amount/Injected amount)×100. SD: Standard deviation, RSD: Relative standard deviation, LNG: Linagliptin, EMP: Empagliflozin

Fig. 3: Separation of (a) linagliptin and (b) empagliflozin from degradants after stress conditions
Table 4: Robustness of the proposed method

| Changes factors | Temp. (°C) | Flow rate (ml/min) | Column to column | Wavelength of detection (nm) | Injected volume (μL) |
|-----------------|-----------|--------------------|------------------|-----------------------------|----------------------|
| LNG             |           |                    |                  |                             |                      |
| Changes         | 23, 25, and 27 | 0.95, 1.0, and 1.05 | ODS-3V, ODS      | 224, 226, and 228           | 19.9, 20, and 20.1   |
| Tested parameter | Peak area | ts                  | Peak area        | ts                          |                      |
| CV (%)          | 0.125     | 0.51                | 0.131            | 0.98                        |                      |
| EMP             |           |                    |                  |                             |                      |
| Changes         | 23, 25, and 27 | 0.95, 1.0, and 1.05 | ODS-3V, ODS      | 224, 226, and 228           | 19.9, 20, and 20.1   |
| Tested parameter | Peak area | ts                  | Peak area        | ts                          |                      |
| CV (%)          | 0.502     | 0.47                | 0.228            | 0.67                        |                      |

LNG: Linagliptin, EMP: Empagliflozin, CV: Coefficient of variation

Table 5: Statistical analysis of results obtained by the proposed method applied on tablets compared with a reported method

| Tested parameter | Mean recovery | ±SD | ±RSD% | Variance | t-value | F-valuea | F-valueb |
|------------------|---------------|-----|-------|----------|---------|----------|----------|
| LNG              | Empacoza plus tablets | 100.71 | 0.541 | 0.537 | 0.292 | 0.150 | 1.014 |
|                  | Glyxambi tablets    | 100.81 | 0.589 | 0.584 | 0.346 | 0.212 | 0.856 |
|                  | Average of five determinations (n=5). | 100.52 | ±0.545 | ±0.542 | ±0.297 | ±0.212 | ±0.856 |
| EMP              | Empacoza plus tablets | 101.48 | 0.254 | 0.251 | 0.065 | 0.114 | 0.547 |
|                  | Glyxambi tablets    | 101.64 | 0.214 | 0.210 | 0.046 | 0.096 | 1.146 |
|                  | Average of five determinations (n=5). | 101.29 | ±0.289 | ±0.286 | ±0.084 | ±0.129 | ±1.845 |

REFERENCES

1. Kawamori R, Inagaki N, Araki E, Watada H, Hayashi N, Horie Y, et al. Linagliptin monotherapy provides superior glycaemic control versus placebo or voglibose with comparable safety in Japanese patients with Type 2 diabetes: A randomized, placebo and active comparator-controlled, double-blind study. Diabetes Obes Metab 2012;14:348-57.
2. Jyothirmai N, Kumar MA, Nagaraju B. Novel UV and visible spectrophotometric methods for the analysis of empagliflozin in A 2 diabetic drug in bulk and pharmaceutical formulations. J AJ 2016;3:177-87.
3. Raedler LA. Glyxambi (Empagliflozin/linagliptin): A dual-acting oral medication approved for the treatment of patients with Type 2 diabetes. Am Health Drug Benefits 2015;8:171-5.
4. Banik S, Karnakar P, Miah MA. Development and validation of a UV-spectrophotometric method for determination of vilagliflozin and linagliptin in bulk and pharmaceutical dosage forms. Bangladesh Pharm J 2015;18:163-8.
5. Sangeetha RK, Subashri T. Analysis of linagliptin in bulk and pharmaceutical dosage forms. J Afr Pharm Sci 2016;10:9.
6. Padmaja P, Veerabadram G. Development and validation of an analytical method for simultaneous estimation of empagliflozin and linagliptin in bulk drugs and combined dosage forms using UV-visible spectroscopy. Pharm Lett 2015;7:306-12.
7. Bassam MA. Development and validation of simple spectrophotometric and chemometric methods for simultaneous determination of empagliflozin and metformin: Applied to the recently approved pharmaceutical formulation. Spectrochim Acta Part A 2016;168:118-22.
8. Ayoub BM. UPLC simultaneous determination of empagliflozin, linagliptin and metformin. RSC Adv 2015;11:95703-9.
9. Maha FA, Omar AA, Miriam FA, Mariam MT. Pharmaceutical analysis of linagliptin and empagliflozin using LC-MS/MS. Pharma Chem 2016;8:186-9.
10. Madhusudhan P, Radhakrishna MR, Devanna N. RP-HPLC method

CONFLICTS OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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AUTHORS’ CONTRIBUTIONS

Prof. Dr. Ragaa El Sheikh has generated the research idea and interpreted the data and helped to draft the manuscript. Prof. Dr. Wafa El Sayed Hassan has suggested the research idea and participated in the design of the study. Miss. Eman Helmy Youssef was prepared the solutions, carried out the experiments, interpreted the data, and helped to draft the manuscript. Dr. Abdulrahman Y. Hamdi has suggested the research idea and participated in the design of the study. Mr. Naif Ahmed Badahdah has participated in the design of the study and carried out the experiments. Prof. Dr. Ayman A. Gouda helped in checking the plagiarism, interpreting the data, reviewed the manuscript, and submitted the manuscript for publication.
development and validation for simultaneous determination of linagliptin and empagliflozin in tablet dosage form. Int Adv Res J Sci Eng Technol 2015;2:95-9.
11. Kavitha KY, Geetha G, Hariprasad R, Kaviarasu M. Development and validation of stability indicating RP-HPLC method for the simultaneous estimation of linagliptin and metformin in the pure and pharmaceutical dosage form. J Chem Pharm Res 2013;5:230-5.
12. Donepudi S, Achanta S, validated HPLC-UV method for simultaneous estimation of linagliptin and empagliflozin in human plasma. Int J Appl Pharm 2018;10:56-61.
13. Padmaja N, Veerabhadram G. Development and validation of a novel stability-indicating RP-HPLC method for the determination of empagliflozin in bulk and pharmaceutical dosage form. Int J Pharm Sci Res 2016;7:4523-30.
14. Godasu SK, Sreenivas SA. A new validated RP-HPLC method for the determination of metformin HCl and empagliflozin in bulk and pharmaceutical dosage and forms. Int J Pharm Sci Res 2017;8:2223-32.
15. Patil SD, Amrutkar SV, Chatpalliwar VA, Upasani CD. Development and validation of RP-HPLC method for empagliflozin and metformin HCL. J Innov Pharm Biol Sci 2017;4:185-9.
16. Sujatha K, Seshagirirao JV. A new RP-HPLC method for the estimation of linagliptin in tablet dosage forms. Indo Am J Pharm Res 2013;3:8376-81.
17. Badugu LR. A validated RP-HPLC method for the determination of linagliptin. Am J PharmaTech Res 2012;2:462-70.
18. Afzal SJ, Asif M, Khan PM. Validation of stability indicating high performance liquid chromatographic method for simultaneous determination of assay of linagliptin and metformin drugs in the pharmaceuticals tablet formulations using bupropion as a common internal standard. J Innov Pharm Biol Sci 2018;5:21-8.
19. Madhusudhan P, Reddy R, Deanna N. RPHPLC method development and validation for simultaneous determination of linagliptin and empagliflozin in tablet dosage form. Int Adv Res J Sci Eng Technol 2015;2:95-9.
20. Naazneen S, Sridevi A. Development and validation of stability indicating RP-HPLC method for the simultaneous estimation of empagliflozin and linagliptin in tablet formulation. Pharm Lett 2016;8:57-65.
21. Jayalaxmi, Rajesh T, Kumar GV. A validated RP-HPLC method for the simultaneous estimation of empagliflozin and linagliptin in its bulk and pharmaceutical dosage forms. Int J Chem Pharm Sci 2016;4:634-40.
22. Jyothirmai N, Begum KMD, Supriya P. Novel stability indicating RP-HPLC method for the simultaneous estimation of empagliflozin and linagliptin in bulk and pharmaceutical formulations. J Atoms Mol 2016;6:977-86.
23. Bakshi A, Mounika A, Bhutada S, Raju MB. Simultaneous estimation of empagliflozin and linagliptin by RP-HPLC method. World J Pharm Sci 2018;7:1062-71.
24. Shyamala, Nirmala K, Mounika J, Nandini B. Validated stability-indicating RP-HPLC method for determination of empagliflozin. Pharm Lett 2016;8:457-64.
25. El-Bagary RI, Elkady EF, Ayoub BM. Liquid chromatographic determination of linagliptin in bulk, in plasma and in its pharmaceutical preparation. Int J Biomed Sci 2012;8:209-14.
26. ICH Guidelines for Validation of Analytical Procedures: Text and Methodology Q2 (R1). Geneva: ICH; 2005. p. 1-14.