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UPLC simultaneous determination of empagliflozin, linagliptin and metformin

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The first UPLC method for simultaneous determination of empagliflozin, linagliptin and metformin hydrochloride in the different combinations of their pharmaceutical dosage forms was developed. Chromatographic separation was achieved on a Symmetry® Acclaim™ RSLC 120 C_{18} column (100 mm × 2.1 mm, 2.2 μm) applying isocratic elution based on potassium dihydrogen phosphate buffer pH (4)–methanol (50 : 50, v/v) as the mobile phase. The linearity, accuracy and precision were found to be acceptable over the concentration ranges of 1–32 μg mL^{-1}, 0.5–16 μg mL^{-1} and 1–100 μg mL^{-1} for empagliflozin, linagliptin and metformin hydrochloride, respectively. All the variables were studied to optimize the chromatographic conditions. The optimized method was validated and proved to be suitable for the quality control of the mentioned drugs in their different pharmaceutical dosage forms.

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1. Introduction

Empagliflozin (EGN), (2*S*,3*R*,4*R*,5*S*,6*R*)-2-[4-chloro-3-[[4-[(3*S*)-oxolan-3-yl]oxyphenyl]methyl] phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol (Fig. 1(a)), is an inhibitor of sodium glucose co-transporter-2 (SGLT-2), which accounts for about 90 percent of glucose reabsorption into the blood.[1] Linagliptin (LGP), 8-[(3*R*)-3-aminopiperidin-1-yl]-7-(but-2-yn-1-yl)-3-methyl-1-[(4-methylquinazolin-2-yl) methyl]-3,7-dihydro-1*H*-purine-2,6-dione (Fig. 1(b)), belongs to a dipeptidyl-peptidase-4 inhibitor class which stimulates glucose-dependent insulin release.[2] Metformin hydrochloride (MET), *N*,*N*-dimethylimidodicarbonimidic diamide (Fig. 1(c)), is a biguanide hypoglycemic drug that exerts its effect mainly by increasing peripheral utilization of glucose.[3]

To the best of the author's knowledge, no published methods are available for the determination of EGN either alone or as part of a combination in pharmaceutical dosage forms. A literature review showed that few methods have been described for the determination of LGP in pharmaceutical preparations including spectrophotometry and chromatographic methods.[4–9] In addition, numerous analytical methods have been reported for the determination of MET in pharmaceutical preparations including spectrophotometry and chromatographic methods.[10–25]

The aim of the new proposed UPLC method is to present the first method for simultaneous determination of EGN, LGP and MET, in bulk and in the different combinations of their pharmaceutical dosage forms. Furthermore, it is the first method for determination of empagliflozin either alone or as part of a combination. In addition, the established UPLC method has many advantages over the routine HPLC methods reported for LGP and MET as UPLC is more economic, consuming less organic solvent and less time. Finally, the newly developed UPLC method has major advantages in comparison to the reported methods for LGP and MET,[4–25] including simultaneous determination of EMP with LGP and MET, reduced retention times, enhanced resolution, better sensitivity

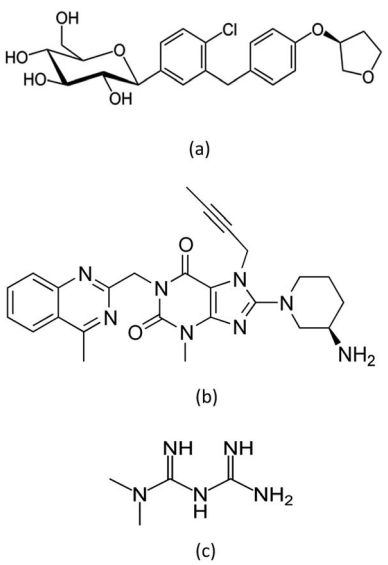

Fig. 1 Chemical structures of empagliflozin (a), linagliptin (b) and metformin (c).

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Table 1 System suitability tests for the proposed UPLC method[a]

Item	MET	LGP	EGN
N	1584	2614	2874
T	1.02	1.01	1.00
R	2.08	4.81	
%RSD for 6 injections (peak area)	0.32	0.21	0.19
%RSD for 6 injections (retention time)	0.18	0.15	0.33

[a] N: number of theoretical plates, T: tailing factor, R: resolution between two consecutive peaks, %RSD: percentage relative standard deviation.

and a simple mobile phase. Most of the advantages may be attributed to moving from HPLC to UPLC.

2. Experimental

2.1. Instrumentation

The liquid chromatography system consisted of a Thermo Fisher UPLC SYSTEM Model Ultimate 3000 Complete Ultra High Performance Liquid Chromatography (USA). For the UPLC system, a Symmetry® Acclaim™ RSLC 120 C_{18} column (100 mm × 2.1 mm, 2.2 μm) (USA) was used. The system was equipped with a diode array detector (DAD-3000RS, USA) and an auto-sampler (WPS-3000TRS, Thermo scientific, USA). An Elmasonic S 60 H (Germany) was used for the degassing of the mobile phases. A Jenway digital pH meter was used to adjust and determine the hydrogen ion concentration (pH) of the buffer solutions.

2.2. Reagents and reference samples

Pharmaceutical grade EGN and LGP certified to contain 99.70% and 99.90% of the drugs, respectively; Jardiance® tablets nominally containing 25 mg of EGN per tablet; Tradjenta® tablets nominally containing 5 mg of LGP per tablet; Jentadueto® tablets nominally containing 2.5 mg of LGP and 500 mg of metformin hydrochloride per tablet; Synjardy® tablets nominally containing 12.5 mg of EGN and 850 mg of MET per tablet; and Glyxambi® tablets nominally containing 5 mg of linagliptin and 10 mg of empagliflozin per tablet were supplied by Boehringer Ingelheim pharmaceutical company (Germany). Metformin hydrochloride, certified to contain 99.80% of the drug, was supplied by Chemical Industries Development (CID) Co., (Giza, Egypt). Glucophage® tablets nominally containing 500 mg metformin per tablet were supplied by Merck Serono (Egypt).

HPLC grade methanol was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Orthophosphoric acid (85%) was purchased from VWR Chemicals (Pool, England). HPLC grade potassium dihydrogen phosphate was purchased from Sigma Aldrich (Deisenhofen, Germany). Bi-distilled water was produced in-house (POLNA, DE 10, Poland). PTFE membrane filters, 47 mm, 0.20 μm (100/pk), (UK) were used. All other chemicals and reagents used were of analytical grade unless indicated otherwise.

2.3. Standard stock solutions

Standard stock solutions of EGN, LGP & MET (1 mg mL^{-1}) were prepared by dissolving 100 mg of the drug in methanol using a 100 mL volumetric flask and completing to volume with methanol. The mixture was sonicated for ten minutes and then the required concentrations were prepared by serial dilution using the mobile phase.

2.4. Working solutions

A working solution of EGN (40 μg mL^{-1}) was prepared by dilution of 4 mL of its stock solution in a 100 mL volumetric flask by completing to volume with the mobile phase. A working solution of LGP (20 μg mL^{-1}) was prepared by dilution of 2 mL of its

Table 2 Results obtained using the proposed UPLC method for simultaneous determination of EGN, LGP and MET

Item	MET	LGP	EGN
UPLC-UV detection	225 nm	225 nm	225 nm
Retention time	1.6	2.3	4.7
Linearity	1–100 μg mL^{-1}	0.5–16 μg mL^{-1}	1–32 μg mL^{-1}
Regression equation	AUP = $1.3213C_{\mu g\ mL^{-1}}$ + 0.128	AUP = $3.4148C_{\mu g\ mL^{-1}}$ + 0.0301	AUP = $4.7462C_{\mu g\ mL^{-1}}$ + 0.0481
Regression coefficient (r^2)	1	0.9998	0.9999
Standard deviation of slope	0.012	0.026	0.035
Standard deviation of intercept	0.03	0.08	0.05
Accuracy (mean ± SD)	100.65 ± 1.18	98.88 ± 1.06	99.81 ± 0.90
Dosage form (mean ± SD)	99.28 ± 0.77	99.62 ± 1.16	99.25 ± 1.23
Drug added (standard addition)	99.38 ± 0.86	98.64 ± 0.83	100.59 ± 1.33
LOD	0.21 μg mL^{-1}	0.12 μg mL^{-1}	0.26 μg mL^{-1}
LOQ	0.63 μg mL^{-1}	0.36 μg mL^{-1}	0.78 μg mL^{-1}
Intraday %RSD	0.16–0.27	0.22–0.34	0.19–0.28
Interday %RSD	0.12–0.30	0.10–0.26	0.14–0.31

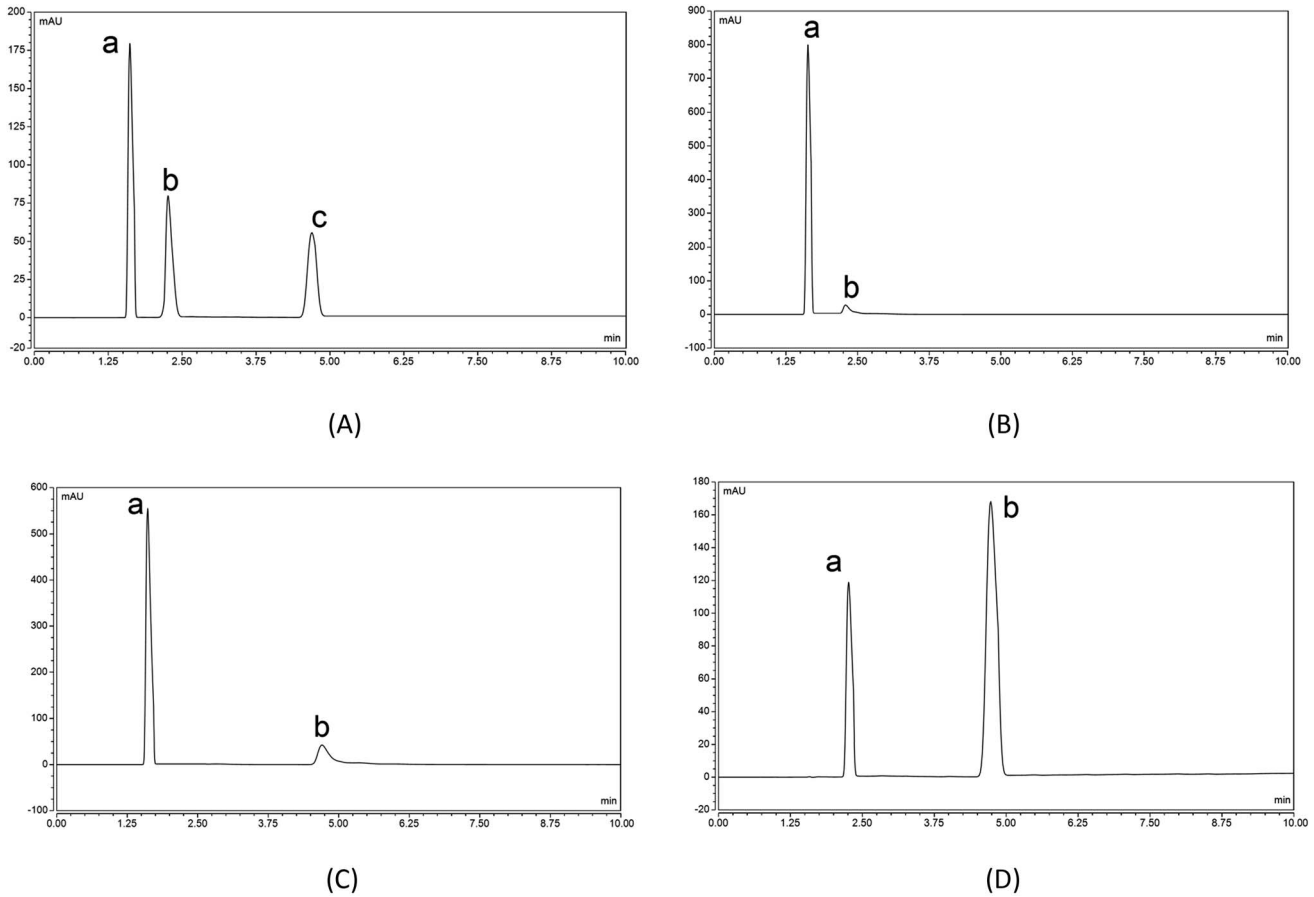

Fig. 2 (A) UPLC chromatogram of a laboratory prepared mixture of (a) metformin hydrochloride (20 μg mL^{-1}), (b) linagliptin (1.5 μg mL^{-1}) and (c) empagliflozin (1.5 μg mL^{-1}). (B) UPLC chromatogram of the Jentadueto® tablet extract in methanol containing (a) metformin hydrochloride (100 μg mL^{-1}) and (b) linagliptin (0.5 μg mL^{-1}). (C) UPLC chromatogram of the Synjardy® tablet extract in methanol containing (a) metformin hydrochloride (68 μg mL^{-1}) and (b) empagliflozin (1 μg mL^{-1}). (D) UPLC chromatogram of the Glyxambi® tablet extract in methanol containing (a) linagliptin (2.5 μg mL^{-1}) and (b) empagliflozin (5 μg mL^{-1}).

stock solution in a 100 mL volumetric flask by completing to volume with the mobile phase. While the working solution of MET (100 μg mL^{-1}) was prepared by dilution of 10 mL of its stock solution in a 100 mL volumetric flask by completing to volume with the mobile phase.

2.5. Chromatographic conditions

Chromatographic separation was achieved on a Symmetry® Acclaim™ RSLC 120 C_{18} column (100 mm × 2.1 mm, 2.2 μm) applying isocratic elution based on potassium dihydrogen phosphate buffer pH (4)–methanol (50 : 50, v/v) as the mobile phase. The ultraviolet detector was operated at 225 nm. The buffer solution was filtered through a 0.2 μm membrane filter and degassed for 30 min in an ultrasonic bath prior to use. The mobile phase was pumped through the column at a flow rate of 0.4 mL min^{-1}. The column temperature was adjusted to 50 °C and the injection volume was 10 μL.

2.6. Sample preparation

Twenty tablets of Jardiance®, Tradjenta®, Glucophage®, Jentadueto®, Synjardy® and Glyxambi® were separately weighed, powdered and mixed in a mortar. Accurately weighed amounts of the finely powdered Jardiance®, Tradjenta® and Glucophage® tablets, equivalent to 10 mg of EGN, LGP and MET, respectively, were separately made up to 100 mL with methanol and sonicated to dissolve them. Also accurately weighed amounts of the finely powdered Jentadueto® tablets equivalent to 0.5 mg of LGP and 100 mg of MET, Synjardy® tablets equivalent to 1 mg of EGN and 68 mg of MET, and Glyxambi® tablets equivalent to 2.5 mg of EGN and 5 mg of LGP were separately made up to 100 mL with methanol and sonicated to dissolve them. The solutions were filtered, followed by serial dilution to the required concentrations using the mobile phase for each experiment and inclusion of the necessary amount of drug for the standard addition technique.

2.7. Procedure

2.7.1. Linearity. Accurately measured aliquots of the stock solutions equivalent to 10–320 μg, 5–160 μg and 10–1000 μg for EGN, LGP and MET, respectively, were transferred separately into a series of 10 mL volumetric flasks, completed to volume with the mobile phase, transferred into the vials of the auto

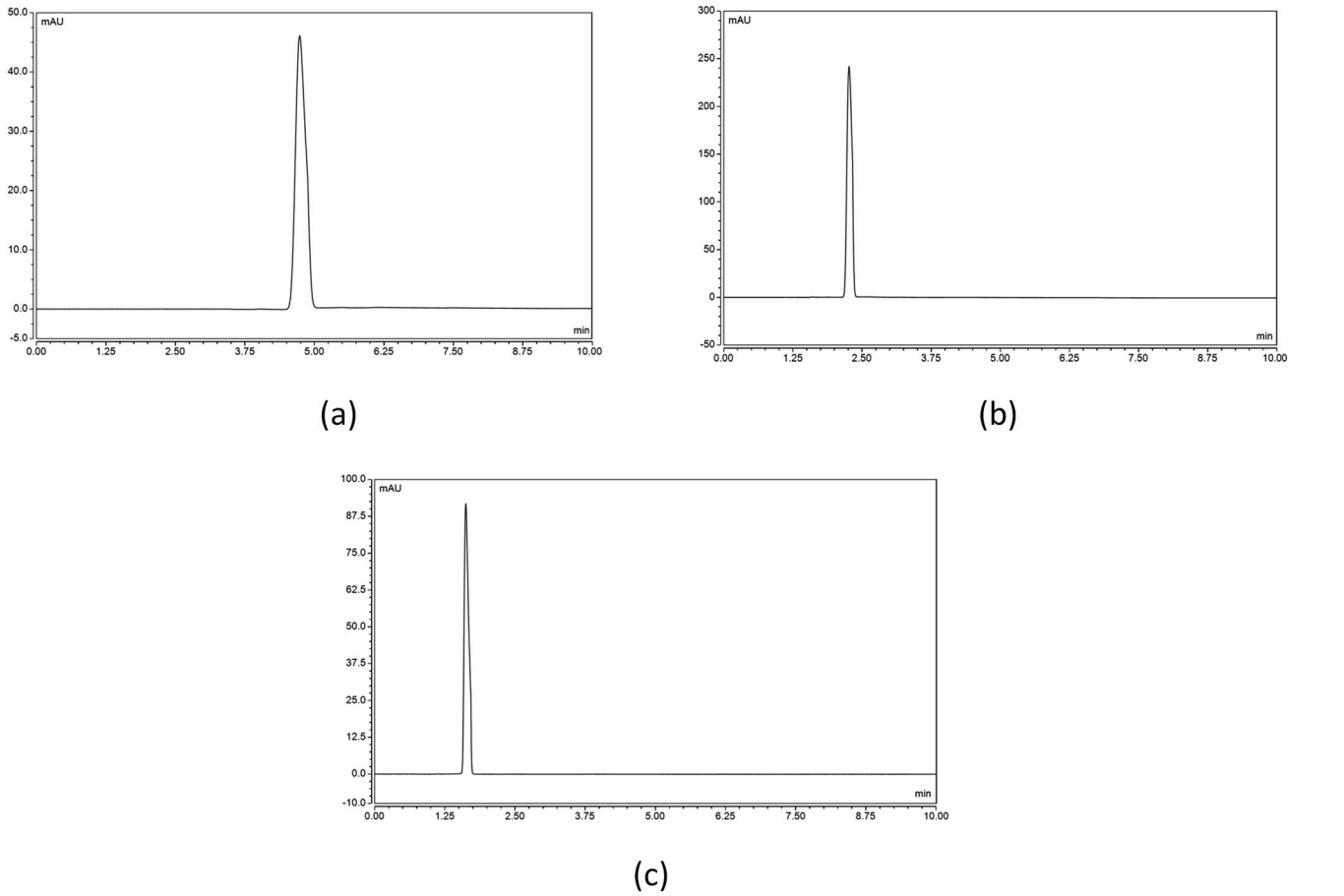

Fig. 3 (a) UPLC chromatogram of the Jardiance® tablet extract in methanol containing empagliflozin (1 μg mL^{-1}) after serial dilution. (b) UPLC chromatogram of the Tradjenta® tablet extract in methanol containing linagliptin (5 μg mL^{-1}) after serial dilution. (c) UPLC chromatogram of the Glucophage® tablet extract in methanol containing metformin hydrochloride (10 μg mL^{-1}) after serial dilution.

sampler and only ten micro liters was injected. A calibration curve was obtained by plotting the area under the peak (AUP) against concentration (C).

2.7.2. Assay of EGN, LGP and MET in bulk (accuracy), the lab prepared mixtures, and the Jardiance®, Tradjenta®, Glucophage®, Jentadueto®, Synjardy® and Glyxambi® tablets. The procedure mentioned under 2.7.1. was repeated using concentrations equivalent to 5, 10, 15, 20 and 25 μg mL^{-1} EGN, concentrations equivalent to 3, 6, 9, 12 and 15 μg mL^{-1} LGP and concentrations equivalent to 10, 30, 50, 70 and 90 μg mL^{-1} MET. Also different ratios of EGN, LGP and MET were prepared using the laboratory prepared mixtures. For the determination of EGN, LGP and MET in the Jardiance®, Tradjenta®, Glucophage®, Jentadueto®, Synjardy® and Glyxambi® tablets, the sample solutions prepared as detailed under 2.6. were serially diluted and then the procedure mentioned under 2.7.1. was repeated. The concentrations of the mentioned drugs were calculated using their specified calibration equations.

Table 3 Statistical comparison between the results of the proposed UPLC method and the reference method for LGP and MET

Statistical term	Reference method, LGP[5]	UPLC method, LGP	Reference method, MET[5]	UPLC method, MET
Mean	99.45	98.88	100.40	100.65
S.D.±	1.34	1.06	1.05	1.18
%RSD	1.35	1.07	1.05	1.18
n	5	5	5	5
V	1.8	1.12	1.1	1.39
t (2.306)[a]		0.75		0.35
F (6.39)[a]		1.61		1.26

[a] Figures in parentheses are the theoretical *t* and *F* values at $p = 0.05$.

2.7.3. Precision of the proposed method. Three different concentrations of EGN (16, 20 and 24 μg mL^{-1}), LGP (8, 10 and 12 μg mL^{-1}) and MET (40, 50 and 60 μg mL^{-1}) were analyzed three times, within the same day, using the procedure mentioned under 2.7.1. Also the mentioned concentrations were analyzed on three successive days using the same procedure.

2.7.4. Robustness of the chromatographic method. The flow rate of the mobile phase was changed from 0.4 mL min^{-1} to 0.38 mL min^{-1} and 0.42 mL min^{-1}. The proportion of the organic solvent in the mobile phase was changed by ±1%. Finally, the value of the pH of the phosphate buffer was varied from 4.0 to 3.9 and 4.1.

3. Results and discussion

3.1. Advantages of the newly proposed UPLC method

It is the first method for simultaneous determination of EGN, LGP and MET with possible application to six approved dosage forms including EGN (Jardiance®), LGP (Tradjenta®), MET (Glucophage®), LGP & MET (Jentadueto®), EGN & MET (Synjardy®), and EGN & LGP (Glyxambi®). Also, to the best of the author's knowledge, no published methods are available for sensitive determination of EGN either alone or as part of a combination in pharmaceutical dosage forms.

In addition, it is the first method that applies UPLC for LGP and MET rather than HPLC, with many associated advantages such as that UPLC operates at much higher pressures. This ultra pressure ensures the advantages of improved resolution and fewer consumables. One of the key advantages is the resolution, as defined by the peak shape. HPLC typically produces broad peaks that skilled operators can characterize very well, including peak heights and peak widths. Another important advantage is a faster run time. The significant reduction in solvent use is another important advantage of UPLC. Not only does the higher pressure system require less solvent, but shorter run times also require less solvent. Moving from HPLC to UPLC means higher resolution coupled with increased throughput analysis, decreased solvent use, and decreased cost.

3.2. Method development for the chromatographic method

During the optimization process, several columns were tested for the experiment, but the C_{18} column showed the best results with good peak intensity, sharp peaks with enhanced resolution, and high throughput elution. Using a cyano column reversed the elution with less resolution and less symmetric peaks. Various mobile phase compositions, containing different ratios of organic and aqueous phases were tried in an isocratic mode. Methanol was found to be the optimum for the elution process. Besides, the use of different buffers at different pH values was attempted along with the methanol. The pH value of the buffer was adjusted to be 4.00 to ensure that its value was below or above the pK_a values of the studied drugs by more than two, as the pK_a of EGN is 12.6 and the pK_a of MET is 12.4, while LGP has two pK_a values which are 1.9 and 8.6.

UV detection at 225 nm was selected according to the UV spectra of the studied drugs and the phosphate buffer showed the optimum conditioning at this wavelength rather than acetate, formate or other buffers. Adjusting the flow rate to 0.4 mL min^{-1} was crucial for the proposed method to enhance the resolution between the three peaks. Applying a flow rate of more than 0.4 increased the back pressure of the UPLC system to more than 400 psi which is not favorable. The marked increase in the column temperature to 50 °C allowed greater symmetry of the eluted peaks with less noise and less tailing, while an increase of the column temperature to over 50 °C was not applicable in order to avoid thermal degradation of the processed samples.

3.3. System suitability tests for the UPLC method

System suitability tests were used to verify that the detection sensitivity, resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The resolution factor shows the accuracy of the quantitative analysis, it is specified to ensure that closely eluting compounds are resolved from each other, the column efficiency is specified as a system suitability requirement and is a measure of peak sharpness, which is important for the detection of trace compounds, while the tailing factor is a measure of peak symmetry. Different parameters affecting the chromatographic separation were studied, including the column efficiency (number of theoretical plates), the tailing of the chromatographic peak, the peak resolution factor, and the %RSD of the peak area and retention time of six injections, as shown in Table 1.

3.4. Method validation

The method was validated according to ICH guidelines.[26]

3.4.1. Linearity. In this study, a linear relationship between the area under the peak (AUP) and the component concentration (C) was obtained using six chosen concentrations for each drug and the regression equations were then computed for the chromatographic method. The linearity of the calibration curves was validated by the high value of the correlation coefficient, acceptable values of the regression coefficients, the standard deviation of the slope and the standard deviation of the intercept, as shown in Table 2.

3.4.2. Accuracy. The accuracy of the results was calculated using the %recovery for 5 different concentrations of each drug. The results, including the mean of the recovery and the standard deviation, are shown in Table 2.

3.4.3. Precision.

3.4.3.1. Repeatability. Three concentrations of EGN (16, 20 and 24 μg mL^{-1}), three concentrations of LGP (8, 10 and 12 μg mL^{-1}) and three concentrations of MET (40, 50 and 60 μg mL^{-1}) were analyzed three times, within the same day, using the procedure mentioned under 2.7. The %RSD was calculated and found to be less than 2% using the three concentrations, as shown in Table 2.

3.4.3.2. Intermediate precision. The above mentioned concentrations were analyzed on three successive days using

the procedure mentioned under 2.7. The %RSD was calculated and the results are shown in Table 2.

3.4.4. Robustness of the chromatographic method. The robustness was investigated by deliberately changing the chromatographic conditions. The most important parameter to be studied was the resolution factor between the two peaks of MET and LGP and also between the two peaks of LGP and EGN. The flow rate of the mobile phase was changed from 0.4 mL min^{-1} to 0.38 mL min^{-1} and 0.42 mL min^{-1}, and the resolution factors obtained were (2.08, 4.81), (1.98, 4.67) and (2.19, 4.94), respectively. Additionally, the proportion of methanol was changed from 50% to 49% and 51%, and the resolution factors obtained were (2.08, 4.81), (2.13, 4.69) and (2.16, 4.86), respectively. Finally, the value of the pH of the phosphate buffer was varied from 4.0 to 3.9 and 4.1, and the resolution factors obtained were (2.08, 4.81), (1.90, 4.85) and (2.10, 4.97), respectively. There was no significant difference in the results obtained for all these variations, indicating a good robustness of the proposed LC method.

3.4.5. Specificity. Specificity is the ability of an analytical method to measure the analyte response in the presence of interference sources including other drugs or excipients. In the present work, the specificity was checked by analyzing 5 different concentrations of each drug in the presence of the excipients of different pharmaceutical dosage forms. The results including the mean of the recovery and the standard deviation are shown in Table 2.

3.4.6. Limit of detection and limit of quantification. The limit of detection (LOD), which represents the concentration of the analyte at a S/N ratio of 3.3, and the limit of quantification (LOQ) at which the S/N ratio is 10 were determined and the results are shown in Table 2. Low values of the LOD and LOQ indicate sensitivity of the applied method for determination of the mentioned drugs in tablets.

3.4.7. Pharmaceutical dosage forms & the standard addition technique. The proposed chromatographic method was successfully applied to different pharmaceutical dosage forms and to check the validity of the proposed method, a standard addition technique was applied by adding different known concentrations of the pure drug (2, 4 and 8 μg mL^{-1} of EGN, 1, 2 and 4 μg mL^{-1} of LGP and 10, 20 and 40 μg mL^{-1} of MET) to different known concentrations of each drug product and the procedures mentioned under 2.7.1. were adopted (Fig. 2 and 3). The concentrations were calculated using the corresponding regression equations in Table 2.

3.4.8. Statistical analysis. Statistical analysis of the results obtained using the proposed method and the well established HPLC reference method for the determination of LGP and MET[5] was carried out using "SPSS statistical package version 11". Significant differences between the reference method and the described method were investigated using a *t*-test and *F*-test at $P = 0.05$, as shown in Table 3. The tests ascertained that there was no significant difference among the methods. No statistical comparison was carried out for EGN as the proposed method is the first method for its determination either alone or as part of a combination.

4. Conclusion

The newly proposed method was proven to be simple, accurate, precise and reproducible for the simultaneous determination of EGN, LGP and MET in bulk and in the different combinations of their pharmaceutical dosage forms, in a reasonable run time with high throughput analysis. The method was validated, showing satisfactory data for all the parameters tested. The developed UPLC method can be conveniently used by quality control laboratories and has the advantages of a simple mobile phase, saving time and a decreased cost due to less solvent being used in UPLC.

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