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Stability indicating HPLC-Fluorescence detection method for the simultaneous determination of linagliptin and empagliflozin in their combined pharmaceutical preparation

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ABSTRACT

A sensitive, accurate, and precise liquid chromatographic method has been developed and validated for the determination of Linagliptin (LNG) and Empagliflozin (EMP) in their combined tablets. Chromatographic separation was carried out on ODS-3 Inertsil® C18 column (150×4.6 mm, 5 µm). The mobile phase A (consisting of 0.30% Triethyl amine buffer (TEA) at pH = 4.5, adjusted using ortho-phosphoric acid); the mobile phase B (consisting of acetonitrile) was pumped through the column whose temperature was maintained at 40 °C, with a flow rate 1.7 mL/min, using gradient elution from 0-3 min A:B (75:25, *v:v*), then from 3-6 min the ratio changed to be A:B (60:40, *v:v*). Fluorescence detection (FLD) was performed at 410 nm after excitation at 239 nm. Acceptable linearity, accuracy and precision values of the proposed method were found over the concentration ranges of 0.5-15 µg/mL for LNG and 1.0-30 µg/mL for EMP with correlation coefficients of 0.9997 and 0.9998 in the case of LNG and EMP, respectively. The recoveries and relative standard deviations percentages were found in the following ranges: 98.56-101.85 and 0.53-1.52% for LNG and 98.00-101.95 and 0.31-1.05% for EMP. The detection and quantification limits were 0.15 and 0.45 µg/mL for LNG and 0.22 and 0.67 µg/mL for EMP. The optimized method was validated and proved to be specific, robust, accurate and reliable for the determination of the drugs in pure form or in their combined pharmaceutical preparations. No significant difference was found regarding accuracy and precision upon statistical comparison between the obtained results of the proposed method and those of the reported method. Furthermore, the proposed method is proved to be a stability-indicating assay after exposure of the studied drugs to variable forced degradation parameters, such as acidic, alkaline and oxidative conditions, according to the recommendations of the International Conference on Harmonization guidelines. The simplicity and selectivity of the proposed method allows its use in quality control laboratories.

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1. Introduction

Linagliptin is chemically known as 8-[(3*R*)-3-amino piperidin-1-yl]-7-but-2-ynyl-3-methyl-1-[(4-methylquinazolin-2yl) methyl] purine-2,6-dione [1]. It is a dipeptidyl peptidase-4 (DPP-4) inhibitor used for treatment of Type-II diabetes. LNG acts by blocking the action of DPP-4 enzyme that destroys the glucagon-like peptide-1 hormone (GLP-1), which helps to increase insulin secretions and inhibits the release of glucagon resulting in decreasing the glucose level in the circulation. LNG binds tightly but not irreversibly to DPP-4 enzyme [2]. Empagliflozin is chemically known as (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 [1]. It is a sodium-glucose co-transporter-2 (SGLT-2) inhibitor. So that it can be used for the treatment of type II diabetes by blocking the reabsorption of glucose in the kidneys and promoting the excretion of excess glucose in urine [3]. The structure of the studied drugs is presented in Figure 1.

Literature surveys released several analytical designs that have been established for the determination of both LNG and EMP mixtures in dosage forms and biological fluids; these methods include chromatographic [4-17], spectrophotometric methods [18-21], spectroflourimetric method [22] and electrochemical method [23].

High-performance liquid chromatographic methods have many advantages in analytical chemistry, as they are mostly the preferred tools for separation and quantitative analysis of many component mixtures, Liquid chromatographic techniques can be used to analyze greater variety of samples than other techniques as they are quick and efficient, they are the methods

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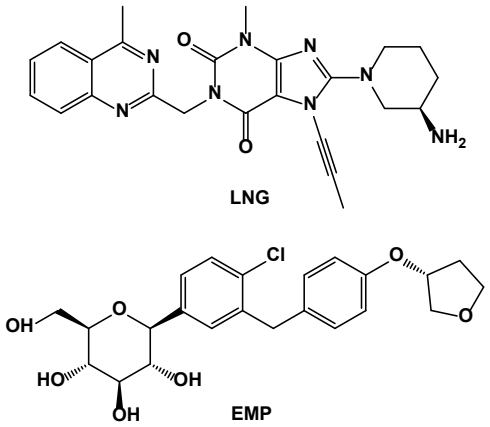

Figure 1. Display of chemical structures of LNG and EMP.

of choice for checking the purity of drugs and for resolving drug mixtures.

Since the studied drugs are recently approved, they are not yet official in pharmacopeia. Linagliptin was approved by the food and drug administration (FDA) on 2011 [24], while Empagliflozin was approved by the food and drug administration (FDA) on 2014 [25]. To our knowledge, there are no HPLC-FLD methods developed for the determination of LNG and EMP either in pure form or in combined tablets. In the present study, the proposed method was exploited as stability indicating assay after stress degradation studies of both drugs. Although other HPLC methods have been published for determination of LNG and EMP using LC-MS/MS with a lower limit of detection and quantification [26,27], yet mass detector is considered quite expensive and less notorious; it may not be used regularly in quality control laboratories.

It is important to mention that HPLC methods utilizing UV detectors are most widely used in analytical laboratories but in general the new approach easy spectral analysis using FLD detector is more preferred because of the UV-absorbing matrix will not interfere with monitoring the fluorescence signal if the matrix doesn't absorb at the excitation and emission wave lengths of the detectable drugs so it is more flexible, specific, and selective.

The aim of this work is to develop a new validated simple, rapid, accurate, precise, and robust HPLC-FLD method for simultaneous determination of LNG and EMP in laboratory-prepared mixtures (Lab prepared mix) and synthetic combined dosage forms (co-formulated tablets) based on the native fluorescence of the mentioned drugs. To fulfill this aim, the optimization of HPLC conditions and method validation are explained.

2. Experimental

2.1. Instrument

Agilent technologies 1200 series HPLC system (USA), equipped with Agilent 1200 series quaternary pump (G1311A), Agilent 1200 series Thermostat Column compartment (G1316B), Agilent 1200 series auto sampler (G1329A), S/No.DE64771876 and 1260 Agilent technology FLD (G1321B). The data was analyzed using Chemstation software. The mobile phase was filtered through Charles Austen Pumps Ltd. Filter; model-B100 SE (England, UK) using 0.45 µm milli-pore filters (Gelman, Germany). An ODS-3 Inertsil® C18 (150×4.6 mm, 5 µm, Japan) column was equilibrated and saturated for 30 min at a flow rate 1.7 mL/min before the injection of the samples. The fluorescence detector was set at excitation wavelength 239 nm and emission wavelength 410 nm. Cellulose acetate filter paper, dimension 47 mm, pore size 0.45 µm, Rankem, New Delhi, was used for filtration.

2.2. Materials

2.2.1. Drug substances

LNG (Batch No. Rm-01-018-163), manufactured by Lee pharma limited, Tenalanga, India and It was kindly provided by Rameda Pharmaceutical Company, Egypt. Its purity was certified to be 99.78%. EMP (Batch No. OT-EMP-S1/001/139), manufactured by Optrix laboratories private limited, Tenelanga, India. It was kindly provided by Hikma Pharma Company, Egypt. Its purity was certified to be 99.50%.

2.2.2. Dosage forms

Trajenta tablets (Batch No. 661442B, 5 mg LNG) and Jardiance tablets (Batch No. 607106, 10 mg EMP) were manufactured by Boehringer Ingelheim, Germany and they were purchased from local market. Synthetic mixtures were prepared by mixing the above-mentioned tablets to mimic the combined Glyxambi® tablets (5 mg LNG, 10 mg EMP) and (5 mg LNG, 25 mg EMP).

2.2.3. Chemicals and reagents

All reagents and solvents were of HPLC grade while chemicals were of analytical grade. HPLC grade methanol (Fisher Chemicals, UK), acetonitrile, glacial acetic acid, triethyl amine and formic acid were bought from (Sigma Aldrich, Germany). Hydrogen peroxide 30% (*w*:*v*) (Panreac, Spain), potassium dihydrogen phosphate, sodium hydroxide (ELNASR Pharmaceutical Chemicals Co., Cairo, Egypt), orthophosphoric acid (85%) (Scharlab-Scharlau, Spain), ammonium formate (SD fine chem. Limited, Mumbai, India), ammonium acetate (Oxford Laboratory Reagents, India), and hydrochloric acid (fine-chem. Industries, India) were used in the work.

2.3. Standard solutions

2.3.1. Standard stock solution

Accurately weighed 12.5 mg of LNG and 25.0 mg of EMP were transferred into a 25 mL volumetric flask, dissolved, and diluted to a volume with methanol to obtain final concentrations of 0.5 mg/mL for LNG and 1.0 mg/mL for EMP. The standard stock solution was stored at 4-8 °C in a refrigerator for not more than 2 weeks.

2.3.2. Standard working solution

Prepared by diluting 1.0 mL of the stock solution to 10 mL with diluent (distilled water) to obtain the final concentration of 50 µg/mL for LNG and 100 µg/mL for EMP, then serial dilutions were prepared in the range of (0.5-15 µg/mL) for LNG and (1.0-30 µg/mL) for EMP.

2.3.3. Laboratory prepared mixtures

Laboratory prepared mixtures equivalent to different concentration ratios (5 µg/mL LNG with 10 µg/mL EMP, 5 µg/mL LNG with 25 µg/mL EMP and 10 µg/mL LNG with 15 µg/mL EMP) were prepared and examined under the same chromatographic conditions.

2.4. Chromatographic conditions

Column: ODS-3 Inertsil® C18 column (150×4.6 mm, 5 µm); Mobile phase: consisting of a mixture of mobile phase A (0.30% Triethyl amine buffer (TEA) at pH = 4.5, adjusted with ortho-phosphoric acid) and mobile phase B (acetonitrile) with gradient elution from (0-3) min A: B (75:25, *v*:*v*) then till the end of elution (3-6) min the ratio of mobile phase was changed to be A: B (60:40, *v*: *v*). Filtration of the buffer solution was done using 0.20 µm membrane filter and then it was degassed for 10 min. in an ultrasonic bath prior to use. Flow rate was selected to be 1.7 mL/min with 10 µL as injection volume, column temperature was adjusted at 40 °C and fluorescence detection was performed at 410 nm for emission after excitation at 239 nm.

2.5. Procedures

2.5.1. Preparation of calibration graphs

Aliquots of standard working solution equivalent to 5-150 µg/mL for LNG and 10-300 µg/mL for EMP were accurately transferred into 10 mL volumetric flasks in series and then the final volume was completed with diluent (distilled water) from each solution volume of 10 µL was injected to the column and eluted under the previously mentioned chromatographic conditions. The calibration graphs were constructed by plotting area under the peak (AUP) against the corresponding concentration (C) of each drug and then the regression equations were computed.

2.5.2. Assay of laboratory prepared mixtures

Triplicate injections for each mixture of the laboratory-prepared mixtures were injected and eluted under the previously mentioned chromatographic conditions. The concentrations (C) of each drug were calculated using the regression equation of the standard calibration graph.

2.5.3. Application to combined pharmaceutical formulations

Five tablets of Trajenta® and five tablets of Jardiance® were finely powdered and weighed. Accurately weighed portions of the fine powder equivalent to 5 mg of LNG and 10 mg of EMP were then mixed well and transferred into a 100 mL volumetric flask containing 25 mL methanol. The flask was sonicated for 30 min and made up to the volume with the same solvent, mixed well, and filtered to separate out the insoluble excipients. The obtained solution was with a final concentration of 50 µg/mL for LNG and 100 µg/mL for EMP. The required concentrations were prepared by serial dilutions and the studied drugs were analyzed by the proposed method and their concentrations were calculated using the computed regression equations of the standard calibration graphs. Assay of Trajenta® and Jardiance® tablets applying standard addition technique was performed using different known concentrations of the pure drugs that were added to the studied drugs in dosage forms and then the computed regression equations of the standard calibration graphs were used to calculate the concentrations of standard added.

2.5.4. Forced degradation study of LNG and EMP

According to ICH guidelines Q1A (R2) [28], forced degradation studies of LNG and EMP were applied. The proposed degradation conditions (acidic hydrolysis with 1 N HCl, alkaline hydrolysis with 1 N NaOH, and oxidative hydrolysis with 30% H_2O_2) were applied to LNG and EMP stock solutions with a concentration of 1 mg/mL. Acidic degradation was carried out in conical flasks by mixing 1 mL using a pH meter. Then the contents of the flask were quantitatively transferred to 100 mL volumetric flasks and the final volume was completed with diluent (distilled water), this is to get a neutral final concentration of 10 µg/mL for both drugs in each flask before injection. The same procedure was applied for alkaline degradation using 1 N NaOH. Neutralization of the resulted solution at time intervals was carried out using 1 N HCl using a pH meter followed by dilution as previously mentioned. Oxidative degradation was carried out following the same procedure mentioned above using 1 mL of 30% H_2O_2. At applicable time the flasks were cooled and the final volume in 100 mL volumetric flasks was completed with distilled water. All resulted solutions were analyzed by the developed chromatographic method.

2.5.5. Selectivity and specificity

According to ICH guidelines [29], selectivity is defined as the ability to detect accurately the analyte of interest in the presence of other components without interference which was established by the proposed HPLC method. Selectivity of the method has been verified by the analysis results of different laboratory prepared mixtures with good recovery, and the assay results of different synthetic prepared mixtures for dosage forms without any interference from other components such as additives or excipients. According to ICH guidelines [29], specificity is defined as the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. This might include (degradation products, impurities, matrix, etc.). The specificity of the proposed method could be investigated through the standard addition method with good recoveries and %RSD values. It could be further explained by injecting a placebo solution prepared by dissolving the tablets matrix components in solvent which did not produce any response, blank solution, standard solution, and sample solution of tablets dosage form. The chromatograms of the studied drugs in tablets explain that the excipients did not interfere with the retention time of the chromatographic peaks of the studied drugs.

3. Results and discussion

The proposed HPLC-FLD is proved to be highly selective and sensitive for the simultaneous determination of LNG and EMP in bulk and in combined pharmaceutical dosage forms. This work also includes a stability indicating study for both drugs under different stress conditions.

3.1. Method development

The chromatographic conditions of the proposed method achieved efficient separation and good resolution between the two drugs in gradient elution mode, where good separation was

shown by the difference in the R_t values of LNG (R_t = 2.17 min), and EMP (R_t = 4.19 min). To earn best separation and resolution of the two drugs; different columns as well as different mobile phases were tried in different ratios and with different pH values. Optimization of the chromatographic parameters to ensure good separation was done as follows:

3.1.1. Wavelength selection

Several trials have been done using different excitation and emission wave lengths but the best detector sensitivity with symmetrical peaks was achieved at λ_{ex} = 239 nm and λ_{em} = 410 nm. At λ_{ex} = 210 nm, λ_{em} = 302 nm and λ_{ex} = 224 nm, λ_{em} = 302 nm, only EMP peaks could be detected while LNG peak did not appear. At λ_{ex} = 230 nm, λ_{em} = 355 nm no peaks appeared. At λ_{ex} = 239 nm, λ_{em} = 355 nm and λ_{ex} = 293 nm, λ_{em} = 355 nm only LNG peak could be detected while EMP peak did not appear. So, the best detector sensitivity was achieved at λ_{ex} = 239 nm and λ_{em} = 410 nm with minimum noise.

3.1.2. Mobile phase composition

Various mobile phases have been investigated to obtain the best selectivity, sensitivity and suitability in a short time of separation of both drugs with good resolution. The studied variables such as: pH, flow rate and temperature are shown in Table 1. First analysis of the studied drugs was tried using different combinations of either methanol or acetonitrile with distilled water, yet an acceptable separation could not be obtained, so another study was conducted using buffer. The proposed method was developed with a simple mobile phase which enables its further application on different detectors. The optimum separation was achieved using the a mobile phase consisting of mobile phase A (0.30% TEA buffer at pH = 4.5, adjusted with ortho-phosphoric acid) and mobile phase B (acetonitrile), with gradient elution A:B (75:25, *v*:*v*) from (0-3) min, then till the end of elution from 3-6 min, the ratio of mobile phase was changed to be A:B (60:40, *v*:*v*). This pH ensured the highest number of theoretical plates with good resolution and good peak symmetry. High pH values above pH = 4.5 caused overlapping of peaks. Selection of acetonitrile as the main organic modifier helped in peak sharpening to get well resolution, increasing the ratio of organic modifier from 25 to 40% resulted in a suitable retention time, good peak resolution and better separation. Decreasing the percentage of buffer caused deformation of LNG peak and the enhancement of EMP peak, so the gradient mode was found to be the best for the elution of the mobile phase.

It was found that using mobile phases with various combinations of either methanol or acetonitrile with water gave shorter retention time of both drugs but asymmetric peaks (i.e., more than 1.2) were obtained, while using different types of buffers such as; potassium dihydrogen orthophosphate, ammonium formate and ammonium acetate gave broad peak for EMP, the best buffer was found to be 0.3% TEA. Since using isocratic elution delayed the retention time of EMP with increasing tailing factor; so gradient elution was purposeful, also different pH values were studied to obtain the optimum value, when pH value either below or above 4.5 (for instance pH = 3.6 and 5.1) was used broadening of the peaks of both drugs was observed (Table 1).

3.1.3. Flow rate of the mobile phase

The flow rate of the mobile phase mainly affects the retention time of the drugs peaks, peaks shapes, and the resolution between peaks. Different flow rates were investigated over the range 1.0-2.0 mL/min (Table 1). The best flow rate was found to be 1.7 mL/min; which helped to reach a reasonable retention time, high number of theoretical plates, good peak shape, and better resolution between peaks. It is obvious that low flow rate consumes less solvents, increases the retention time of peaks, and gives tailed peaks, while high flow rate consumes more solvent, decreases the retention time of peaks, and gives bad baseline and bad peak shape; so, the optimum flow rate was found to be 1.7 mL/min as it offered good peak shape within a suitable retention time and high number of theoretical plates.

3.1.4. Stationary phase optimization

Different stationary phases were used to obtain best separation, resolution, good peak shape, sharpness, and higher number of theoretical plates. ODS-3 Inertsil® C18 column (150×4.6 mm, 5 µm), S/N. 1A5142903, made in Japan is the column of choice due to it has high efficiency with high number of theoretical plates and small values of height equivalent to theatrical plates (HEPT) that resulted in good separation and resolution between peaks, sharpness of peaks, adequate tailing factor, and excellent peak symmetry. Different columns were tested to choose the best one which achieves good separation and good peak shapes within a reasonable retention time. The C8 column (150 mm) and C18 column (100 mm) resolved drug peaks in a shorter retention time than the longer C18 column (250 mm), but all columns have low efficiency with low number of theoretical plates and high tailing factor value (more than 1.2) as shown in Table 1. The best performance of the proposed method for analysis of the studied drugs was obtained upon using Inertsil® C18 column (150 mm).

3.1.5. Column temperature

Temperature of the column affects the interactions of the analyte with the stationary phase and mobile phase, so it affects the retention time and peak shape. Room temperature resulted in tailed broad peaks while increasing the temperature to 40 °C gave more symmetric peaks. Increasing temperature decreases the retention time and increases the number of theoretical plates, but increasing the temperature up to 50 °C caused deformation of peaks shapes as shown in Table 1. So, column temperature of 40 °C was selected in order to give a reasonable retention time, good separation and resolution between peaks, high number of theoretical plates, and to obtain sharp peaks. After optimization of the previous variables, the best peak shape, lowest peak tailing, good separation and resolution between peaks, and good sensitivity was achieved in a reasonable run time as shown in Figure 2.

Briefly, stability studies of both drugs were carried out under multiple stress conditions of oxidative, acidic and alkaline degradation. It was found that the degradation products have no fluorescence so the proposed method could be effectively used in the analysis of LNG and EMP in the presence of their degradation products, hence it could be applied successfully as a stability-indicating assay for both drugs.

3.2. Method validation

Validation of the proposed method was assessed according to the ICH Q2 (R1) recommendation [29]. The method was validated for parameters such as: Linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, robustness, ruggedness, specificity, and system suitability. The obtained results confirmed the validity of the proposed method showing better resolution and sharp peaks.

3.2.1. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of the analyte in the sample.

Table 1. Optimization of the conditions of the proposed HPLC-FLD method for the simultaneous determination of LNG and EMP.

Parameters	Variation	LNG			EMP			
		Capacity factor (K')	No. of theoretical plates (N)	Tailing factor (tf)	Capacity factor (K')	No. of theoretical plate (N)	Tailing factor (tf)	Selectivity (α)
Mobile phase composition	Water: methanol (50:50, *v:v*)	1.26	445	1.53	3.75	1096	1.20	2.98
	Water: acetonitrile (50:50, *v:v*)	1.13	524	1.51	3.40	3033	1.12	3.01
	Ammonium formate buffer, pH = 4 adjusted with formic acid: acetonitrile (80:20, *v:v*)	2.55	1942	1.16	5.63	4016	0.84	2.21
	Ammonium acetate buffer pH = 4.5 adjusted with glacial acetic acid (A): acetonitrile (B) (A:B) (80:20, v:v) changed to (65:35, *v:v*)	2.30	1901	1.16	5.10	4207	0.86	2.22
	Potassium dihydrogen phosphate buffer pH = 4.5 adjusted with orthophosphoric acid (A): acetonitrile (B) (A:B) (80:20, *v:v*) changed to (65:35, *v:v*)	2.25	779	1.20	4.80	4148	1.40	2.13
	0.3% Tri ethyl amine buffer, pH = 4.5 adjusted with orthophosphoric acid (A): acetonitrile (B) (A:B) (75:25, *v:v*) changed to (60:40, *v:v*)	2.12	2522	1.08	4.64	9784	1.03	2.19
pH	3.6	2.40	1883	1.27	5.23	3332	0.90	2.18
	4.5	2.12	2522	1.08	4.64	9784	1.03	2.19
	5.1	1.90	1535	1.30	4.29	3225	0.81	2.26
Flow rate (mL/min)	1.0	2.90	707	1.35	5.44	2627	1.27	1.88
	1.7	2.12	2522	1.08	4.64	9784	1.03	2.19
	2.0	1.80	1018	1.29	4.13	3399	1.21	2.29
Stationary phase	C_8 column (150 mm)	1.70	554	1.30	3.93	1596	1.16	2.31
	C_{18} column (100 mm)	1.83	985	1.31	4.21	5053	1.24	2.30
	C_{18} column (150 mm)	2.12	2522	1.08	4.64	9784	1.03	2.19
	C_{18} column (250 mm)	3.10	789	1.40	5.75	2027	1.14	1.85
Temperature (°C)	25	2.46	853	1.40	5.37	1840	1.26	2.18
	30	2.24	856	1.13	4.88	2508	1.26	2.18
	40	2.12	2522	1.08	4.64	9784	1.03	2.19
	50	1.88	2940	1.20	4.27	11052	1.54	2.27

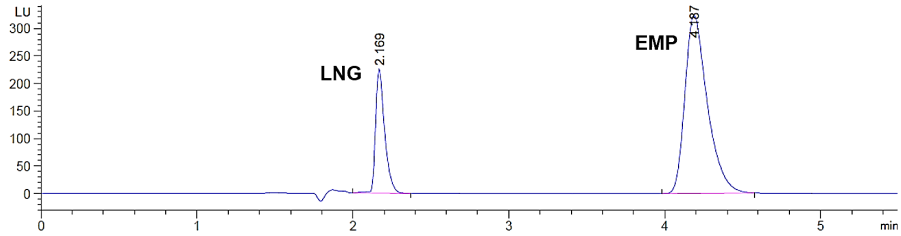

Figure 2. HPLC chromatogram of LNG (5 µg/mL) and EMP (10 µg/mL) at the optimum chromatographic conditions of the proposed HPLC-FLD method.

Good linear relationships were established between area under the peak (AUP) and concentrations of drugs in the range of 0.5-15 µg/mL for LNG and 1.0-30 µg/mL for EMP. The high values of correlation coefficient proved good linearity of the proposed method. The analytical data of each calibration curve are summarized and listed in Table 2.

The regression equations were computed and found to be:

$y = 406.52 \times x + 47.80$ $r = 0.9997$ for LNG (1)

$y = 442 \times x - 6.60$ $r = 0.9998$ for EMP (2)

where y is the area under the peak and x is the concentration of drug in µg/mL.

3.2.2. Limit of detection (LOD) and limit of quantification (LOQ)

The linearity assessment performed above was used for the determination of the LOD which was determined by establishing the minimum level at which the analyte can be reliably detected and LOQ which was determined by establishing the lowest concentration that can be measured according to ICH recommendations below which the calibration graph is nonlinear. LOD and LOQ values were predicted using the following formula and the precision was established at these predicted levels.

$$LOQ = 10\ \sigma / S \quad (3)$$

$$LOD = 3.3\ \sigma / S \quad (4)$$

where σ = Standard deviation of the *y*-intercept of the regression line and S = Slope of the calibration curve. The calculated LOD and LOQ were found to be 0.15 and 0.45 µg/mL for LNG and 0.22 and 0.67 µg/mL for EMP as shown in Table 2.

3.2.3. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy of the proposed method was proved by good recovery values and low values of percentage error as indicated in Table 2. It was further evaluated by statistical comparison between the results obtained by the proposed method with those obtained by the reported method for simultaneous determination of LNG and EMP [8]. The results obtained by applying student's t-test and variance ratio F-test [30] showed no significant difference between the performance of the two methods regarding accuracy and precision respectively (Table 3).

Table 2. Analytical performance data of the proposed HPLC-FLD method for simultaneous determination of LNG and EMP in laboratory prepared mixtures.

Parameters	LNG				EMP			
	Conc. taken (µg/mL)	AUP	Conc. found (µg/mL)	%Recovery	Conc. taken (µg/mL)	AUP	Conc. found (µg/mL)	%Recovery
	0.50	248.1	0.49	98.56	1.00	444.0	1.02	101.95
	1.50	652.0	1.49	99.10	3.00	1330	3.02	100.80
	2.50	1057	2.48	99.31	5.00	2190	4.97	99.40
	3.00	1251.3	2.96	98.70	6.00	2590	5.87	97.91
	5.00	2073	4.98	99.64	10.00	4486	10.16	101.64
	6.00	2504	6.04	100.71	12.00	5285	11.97	99.77
	7.50	3089	7.48	99.75	15.00	6560	14.86	99.04
	10.00	4188	10.19	101.85	20.00	8913	20.18	100.90
	15.00	6100	14.89	99.26	30.00	13222	29.93	99.80
Linearity range (µg/mL)	0.50-15.00				1.00-30.00			
Correlation coefficient (r)	0.9997				0.9998			
Intercept (a)	47.80				-6.60			
Slope (b)	406.52				442			
Confidence interval of intercept. at 95%confidence limit	2.83-92.71				76.04-62.84			
Confidence interval of slope. At 95% confidence limit	400.23-412.80				437.1-446.81			
S_a (standard error of intercept)	19.00				29.37			
S_b (standard error of slope)	2.66				2.05			
Residual sum of square (R.S.S)	8458.36				20197.51			
L.O.D (µg/mL)	0.15				0.22			
L.O.Q (µg/mL)	0.45				0.67			
Standard error (S.E.)	0.35				0.43			
Percentage error (% Er)	0.34				0.43			
Drugs in lab prepared mix (mean±SD)	99.65±1.04				100.13±1.30			
Intra-day precision (%RSD)	0.53-1.52				0.31-1.05			
Inter-day precision (%RSD)	1.42-1.77				0.41-1.71			

Table 3. Statistical comparison between the results of the proposed HPLC-FLD method and the reported HPLC-UV method performed on LNG and EMP in laboratory-prepared mixtures

Parameters	LNG				EMP			
	Conc. Taken (µg/mL)	Conc. Found (µg/mL)	%Recovery Proposed method [HPLC-FLD]	%Recovery Reported method [8] [HPLC-UV]	Conc. Taken (µg/mL)	Conc. Found (µg/mL)	%Recovery Proposed method [HPLC-FLD]	%Recovery Reported method [8] [b] [HPLC-UV]
	1.50	1.49	99.10	99.77	3.00	3.02	100.80	99.84
	2.50	2.48	99.31	101.93	5.00	4.97	99.40	98.54
	5.00	4.98	99.64	98.81	10.00	10.16	101.64	101.87
	7.50	7.48	99.75	99.90	15.00	14.86	99.04	100.33
	10.00	10.19	101.85	100.64	20.00	20.18	100.90	99.70
% Mean recovery	99.93			100.21	100.36			100.06
SD (standard deviation)	1.10			1.16	1.09			1.21
SE (standard error)	0.49			0.52	0.49			0.54
No. of experiments	5			5	5			5
Variance	1.22			1.35	1.20			1.46
Student's t-test	0.39 [2.31] [a]				0.41 [2.31] [a]			
F value	1.11 [6.39] [a]				1.22 [6.39] [a]			

[a] Values between parentheses are the tabulated F and t values respectively, at p = 0.05 [30].
[b] Reference HPLC-UV method for the simultaneous determination of LNG and EMP (8) using a C18 column (250×4.6 mm, 5 µm) at ambient temperature and a mobile phase consisting of 0.1% aqueous formic acid (pH = 3.6): methanol: Acetonitrile (40:20:40, v:v:v), the flow rate was 1 mL/min, and UV detection was performed at 226 nm.

3.2.4. Selectivity

Selectivity of the HPLC-FLD method was realized by the analysis of different laboratory prepared mixtures containing different concentrations of LNG and EMP within the linearity range including the same ratios as present in market pharmaceutical dosage form (5 µg/mL LNG and 10 µg/mL EMP), (5 µg/mL LNG and 25 µg/mL EMP) with the same conditions of the proposed HPLC-FLD method. The data presented in Table 4 manifest the high selectivity of the proposed method for analysis of the studied drugs in mixtures without interference from other components, with good resolution between peaks and good recoveries and %RSD values.

3.2.5. Specificity

The specificity of a method is the extent to which it can be used for analysis of a particular analyte without interference from other components. The ability of the proposed method to measure the analyte response in the presence of interferences including degradation products, additives, excipients and related substances is assessed by applying the standard addition technique. Furthermore, forced degradation studies were also performed to assess the specificity of the proposed method and the results obtained indicated that the proposed HPLC-FLD method is specific for determination of LNG and EMP.

The chromatograms of the samples were checked for the appearance of any extra peaks. No chromatographic interference from any of the excipients was found at the retention times of the examined compounds as well as the chromatogram of each compound in the sample solution of the combined dosage form was found identical to the chromatogram received by the standard solution of pure form at the wavelengths applied (Figure 3). These results demonstrate the absence of interference from other materials in the pharmaceutical formulations and therefore confirm the specificity of the proposed method.

Table 4. Simultaneous determination of LNG and EMP in laboratory prepared mixtures by the proposed HPLC-FLD method

Laboratory prepared mixtures	Authentic conc. (µg/mL)		Peak area		Conc. found (µg/mL)		% Recovery	
	LNG	EMP	LNG	EMP	LNG	EMP	LNG	EMP
1	5	10	2090.3	4510.0	5.02	10.22	100.40	102.20
			2039.0	4507.0	4.90	10.21	98.00	102.12
			2113.0	4390.0	5.08	9.95	101.60	99.50
%Mean recovery							100.00	101.27
S.D.							1.83	1.54
%RSD							1.83	1.52
2	5	25	2042.3	10804.0	4.91	24.46	98.20	97.84
			2076.5	10815.6	4.99	24.50	99.81	98.00
			2051.3	10856.4	4.93	24.60	98.60	98.40
%Mean recovery							98.87	98.10
S.D.							0.84	0.288
%RSD							0.85	0.294
3	10	15	4109.2	6420.7	9.99	14.54	99.91	97.00
			4078.2	6453.5	9.91	14.62	99.14	97.50
			4091.3	6551.6	9.95	14.84	99.50	98.93
%Mean recovery							99.52	97.81
S.D.							0.385	1.00
%RSD							0.387	1.02

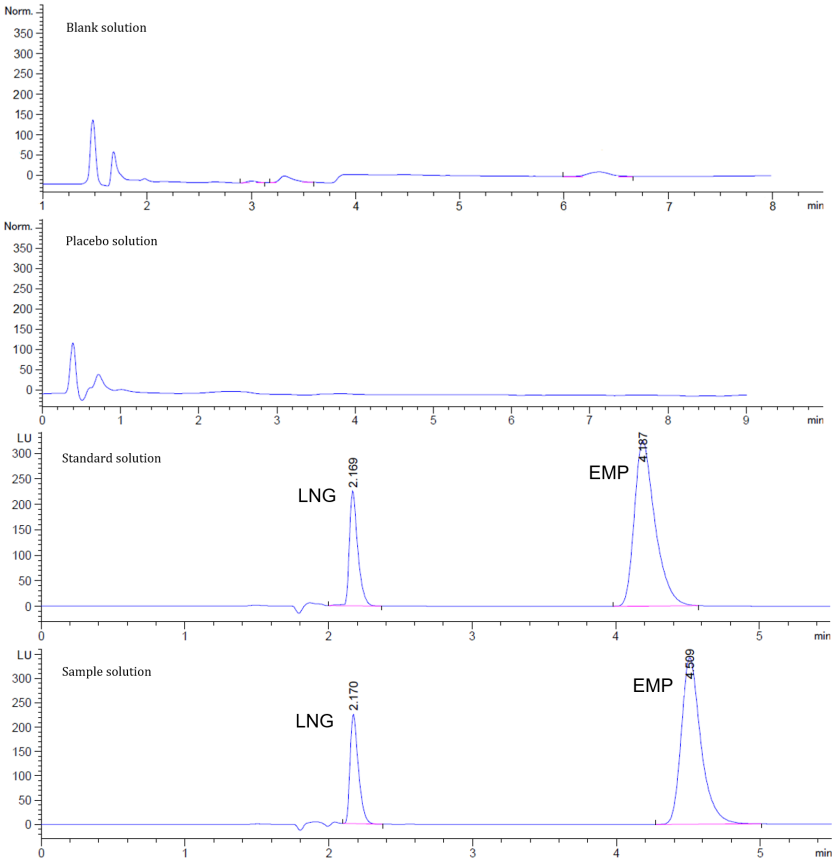

Figure 3. HPLC-FLD chromatograms of blank solution (diluent), placebo solution (excipients), standard solution (pure form) 5 µg/mL LNG and 10 µg/mL EMP and sample solution (dosage form) 5 µg/mL LNG and 10 µg/mL EMP.

3.2.6. Precision (repeatability and intermediate precision)

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple samplings of the same homogenous sample under the prescribed conditions. The precision of the proposed method was expressed as RSD was determined by the analysis of three different concentrations of pure drugs within linearity range. Repeatability (intra-day precision) was performed though replicate analysis of both drugs at different concentration levels and each concentration was analyzed three times within the same day.

Low values of %RSD and % Er < 2% indicate high accuracy & high precision of the proposed method (Table 5). The intermediate precision (inter-day precision) to confirm reproducibility was obtained through replicate analysis of different concentration levels of both drugs, and each concentration was analyzed on three successive days. The results presented in Table 5 prove the good reproducibility of the proposed method.

3.2.7. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberately variations in method parameters and provides an indication of its reliability during normal usage.

Table 5. Repeatability and intermediate precision data of the proposed HPLC-FLD method for determination of LNG and EMP in laboratory prepared mixtures.

Parameters	LNG						EMP					
	Intra-day (repeatability)		precision	Inter-day (intermediate precision)		precision	Intra-day (repeatability)		precision	Inter-day (intermediate precision)		precision
Conc. (µg/mL)	5.0	7.5	10.0	5.0	7.5	10.0	10.0	15.0	20.0	10.0	15.0	20.0
%Recovery	102.08	102.34	99.32	98.80	102.40	96.20	96.60	97.93	101.42	96.43	96.80	100.95
	100.20	101.53	101.32	99.92	102.49	98.51	97.53	97.33	99.32	97.00	98.02	101.80
	101.77	102.55	102.34	101.62	99.33	99.53	96.23	97.62	100.35	97.20	97.64	98.50
%Mean recovery	101.35	102.14	100.99	100.11	101.41	98.10	96.80	97.63	100.36	96.90	97.50	100.42
±SD	1.01	0.54	1.54	1.42	1.80	1.71	0.67	0.30	1.05	0.40	0.62	1.71
%RSD	0.99	0.53	1.52	1.42	1.77	1.74	0.69	0.31	1.05	0.41	0.64	1.71
%Er	0.57	0.30	0.88	0.82	1.03	1.01	0.40	0.17	0.60	0.24	0.37	0.98

Table 6. Robustness results of the proposed HPLC-FLD method for determination of LNG and EMP in laboratory prepared mixtures.

Parameters	Theoretical conc. (µg/mL)		% Recovery		Retention time (Rt)		Resolution (Rs)	
Flow rate 1.7±0.1 mL/min	**LNG**	**EMP**	**LNG**	**EMP**	**LNG**	**EMP**	**LNG**	**EMP**
1.6 mL/min	5	10	101.20	98.80	2.204	4.245	15.30	-
1.7 mL/min			100.08	102.00	2.140	4.184	15.24	-
1.8 mL/min			100.80	102.20	2.092	4.120	15.05	-
%Mean recovery			100.70	101.00				
S.D.			0.57	1.91				
%RSD			0.56	1.89				
Column temperature (40±5.0 °C)	**LNG**	**EMP**	**LNG**	**EMP**	**LNG**	**EMP**	**LNG**	**EMP**
35 °C	10	20	101.10	98.15	2.198	4.233	14.00	-
40 °C			101.10	100.65	2.172	4.215	14.27	-
45 °C			100.90	99.32	2.152	4.196	14.55	-
%Mean recovery			101.03	99.37				
S.D.			0.12	1.25				
%RSD			0.11	1.26				
pH of mobile phase (pH = 4.5±0.2)	**LNG**	**EMP**	**LNG**	**EMP**	**LNG**	**EMP**	**LNG**	**EMP**
pH = 4.3	10	20	97.60	97.50	2.145	4.187	14.13	-
pH = 4.5			98.20	99.95	2.140	4.184	14.67	-
pH = 4.7			97.00	98.55	2.147	4.202	14.07	-
%Mean recovery			97.60	98.67				
S.D.			0.60	1.23				
%RSD			0.61	1.25				

The robustness of the proposed method was studied by determination of the same sample by applying minor changes in the experimental parameters such as: PH of the mobile phase (4.5±0.2), flow rate (1.7±0.1) and temperature of column (40±5 °C). The effect of small variations in chromatographic conditions on the resolution between the two peaks of LNG and EMP revealed no significant difference as presented in Table 6.

The results shown in Table 6, confirm that the proposed method is robust and unaffected by small changes of experimental conditions. The efficiency of the separation and the integrated peak areas of LNG and EMP were not affected by those variables, indicating the reliability of the suggested method. Therefore, the HPLC-FLD method is robust to small changes in experimental chromatographic conditions.

3.2.8. Ruggedness

Ruggedness is evaluated through analysis of three different concentrations from both drugs mixtures three times by different analysts, in different day and on the same device. The results obtained are shown in Table 7 and are found to be within the acceptance limits that indicate the ruggedness of the proposed HPLC-FLD method for determination of LNG and EMP.

3.2.9. System suitability

According to ICH guide lines system suitability tests are essential part of many analytical methods; especially chromatographic methods, they are used to manifest that the resolution and reproducibility of the chromatographic system are adequate for the analysis performed. Ten microliters of the working standard solution with a concentration of 5 µg/mL for LNG and 10 µg/mL for EMP were injected and chromatographic conditions were applied. Parameters including capacity factor (Ќ'), tailing factor (T_f), resolution (R_s), column efficiency (number of theoretical plates; N), height equivalent to theoretical plate (HETP) and selectivity factor (α) were calculated according to the USP guidelines [31] and listed in Table 8. Where capacity factor (Ќ') is a means of measuring the retention of an analyte on the column, a high value indicates that the drug is highly retained and has spent a significant period of time interacting with the stationary phase. The retention or capacity factor (Ќ') is equal to the ratio of retention time of the analyte on the column to the retention time of a non-retained compound. The most important issue in HPLC method is to obtain the optimum resolution in a short time; a resolution value greater than 2 between two peaks of the studied drugs will ensure a very good baseline and a degree of separation at which the area under the peak will be accurately measured.

3.3. Method applications

3.3.1. Determination of LNG and EMP in combined dosage form

The proposed HPLC-FLD method is successfully applied for the determination of LNG and EMP in their combined dosage forms without interference from the inactive ingredients and the frequently encountered tablet excipients which proves the selectivity and specificity of the proposed method for determination of the studied drugs.

The assay (% recovery) of the marketed mixed dosage forms were found to be within limits as shown in Table 9. We have compared our results with those of a reported method [8] to confirm the suitability of the proposed method for the routine analysis of the studied drugs in their pharmaceutical formulations. Statistical analysis [30] of the results obtained using student's t-test and variance ratio F-test revealed no significant difference between the performances of the two methods regarding accuracy and precision, respectively (Table 10).

Table 7. Ruggedness results of the proposed HPLC-FLD method for determination of LNG and EMP in laboratory prepared mixtures.

Parameters	LNG						EMP					
	Analyst (1) Intra-day precision			Analyst (2) ruggedness			Analyst (1) Intra-day precision			Analyst (2) ruggedness		
Conc. (µg/mL)	5	7.5	10	5	7.5	10	10	15	20	10	15	20
%Recovery	102.08	102.34	99.32	100.36	102.12	98.45	96.60	97.93	101.42	102.30	98.00	98.15
	100.20	101.53	101.32	100.60	102.38	102.30	97.53	97.33	99.32	101.20	99.60	97.55
	101.77	102.55	102.34	99.80	100.87	101.10	96.23	97.62	100.35	99.00	98.70	100.66
%Mean recovery	101.35	102.14	100.99	100.25	101.80	100.62	96.80	97.63	100.36	100.83	98.80	98.80
±SD	1.01	0.54	1.54	0.41	0.81	1.97	0.67	0.30	1.05	1.68	0.80	1.65
%RSD	0.99	0.53	1.52	0.41	0.79	1.96	0.69	0.31	1.05	1.67	0.81	1.67
%Er	0.57	0.30	0.88	0.24	0.46	1.13	0.40	0.17	0.60	0.96	0.47	0.96

Table 8. Parameters of System Suitability of the developed HPLC-FLD method

Parameter	LNG (5 µg/mL) (R_t = 2.32 min)	EMP (10 µg/mL) (R_t = 4.56 min)	Reference values [31]
Capacity Factor ('K')	2.12	4.64	0-10
Tailing Factor (T_f)	1.08	1.03	0.9-1.2
Resolution (R_s)		9.93	>2
Selectivity (α)		2.19	>1
Column efficiency (N) (No. of theoretical plates)	2522	9784	>2000 High values indicate separation efficiency
Height equivalent to theoretical plate (HETP)	0.059	0.015	The smaller the value, the higher the column efficiency

Table 9. Accuracy of the proposed method for determination of LNG ad EMP in Trajenta and Jardiance tablets by applying the standard addition technique.

% Test (Tablet sample) Taken	% Standard added	Standard added (µg/mL)		Test + Standard (claimed total conc.) µg/mL)		Total conc. (Found) µg/mL)		% Recovery	
		LNG	EMP	LNG	EMP	LNG	EMP	LNG	EMP
80%	-	-	-	4.00	8.00	3.94	7.94	98.39	99.28
	40	2.00	4.00	5.94	11.94	6.04	12.12	101.75	101.46
	80	4.00	8.00	7.94	15.94	7.91	15.71	99.73	98.56
	120	6.00	12.00	9.94	19.94	10.12	20.27	101.88	101.63
Mean recovery								101.12	100.55
%RSD								1.19	1.72
Min.								99.73	98.56
Max.								101.88	101.63

Table 10. Application of the proposed HPLC-FLD method for simultaneous determination of LNG and EMP in mixed tablet dosage forms.

Parameters	LNG				EMP			
	Conc. Taken (µg/mL)	Conc. Found (µg/mL)	%Recovery Proposed method [HPLC-FLD]	%Recovery Reported method [8] [HPLC-UV]	Conc. Taken (µg/mL)	Conc. Found (µg/mL)	%Recovery Proposed method [HPLC-FLD]	%Recovery Reported method [8] [HPLC-UV]
	2.00	1.97	98.46	101.11	4.00	4.04	100.91	99.54
	3.50	3.57	102.07	97.77	7.00	7.09	101.28	98.71
	5.00	5.01	100.14	101.90	10.00	10.04	100.42	99.48
	6.50	6.62	101.89	99.22	13.00	12.82	98.62	100.80
	7.50	7.62	101.54	99.86	15.00	14.84	98.91	98.23
% Mean recovery	100.82			99.97	100.03			99.35
SD (Standard deviation)	1.52			1.62	1.20			0.98
SE (Standard error)	0.68			0.72	0.54			0.36
(N) No. of experiments	5			5	5			5
Variance	2.31			2.61	1.43			0.95
Student's t-test	0.85 [2.31] [a]				0.98 [2.31] [a]			
F value	1.13 [6.39] [a]				1.50 [6.39] [a]			

[a] Values between parentheses are the tabulated t and F values at p = 0.05 [30].

The accuracy of the proposed method was further assessed by applying the standard addition technique to tablets of LNG and EMP. The results presented in Table 9 demonstrate the ability of the proposed method to analyze both drugs without interference from the excipients that present in the combination dosage form.

Table 11 shows the results of comparison between the proposed HPLC-FLD method and other methods in the literature used for simultaneous determination of LNG and EMP. It is obvious that the proposed HPLC-FLD method is more sensitive than the other methods mentioned in the literature.

3.3.2. Stability indicating study

Forced degradation (stress degradation) is a degradation of the drug substances at conditions more than accelerated conditions and they were performed as per ICH guidelines [28]. When both drugs were subjected to liquid state forced degradation (acidic, alkaline and oxidative) the chromatograms of both drugs were monitored for the detection of any extra peaks appeared. No chromatographic interference from any degradation product was observed at the retention time of both drugs, no interference from any peaks of degradation products with both drugs was observed. This may be due to that the degradation products have no fluorescence hence; the proposed method can be used as a stability indicating assay for both drugs without any interferences.

Both drugs were susceptible to acidic, alkaline and oxidative degradation, and the drugs content were found to be degraded up to 86.00, 65.40% in acidic degradation, 54.46, 42.43% in alkaline degradation and 54.78% in oxidative degradation for LNG and EMP; respectively. The results are summarized in Table 12.

3.4. Stability of standard solutions

To monitor the stability of standard solutions, they were stored in a refrigerator (4-8 °C) for two weeks and then they were reanalyzed by the proposed HPLC-FLD method, no significant difference was found.

Table 11. Comparison of some analytical methods for simultaneous determination of LNG and EMP in bulk and in pharmaceutical formulations.

Method	LNG Linear range (µg/mL)	EMP Linear range (µg/mL)	Reference
The proposed HPLC-FLD	0.5-15	1.0-30	-
RP-HPLC/ UV	12.5-75	12.5-75	[5]
RP-HPLC/ UV	2.5-15	5.0-30	[6]
RP-HPLC/ PDA	12.5-75	25-150	[1]
RP-HPLC/ PDA	20-100	10-50	[7]
RP-HPLC/ PDA	50-150	50-150	[13]
UPLC/PDA	5.0-15	10-30	[15]
Spectrometry	2.0-6.0	5.0-15	[18]
Spectrometry	2.5-12.5	2.0-10	[19]
Spectrometry	5.0-40	2.5-30	[21]

Table 12. Summary of preliminary investigation of LNG and EMP forced degradation.

Parameters	**LNG**											
Stress degradation conditions	**Acid degradation**				**Alkaline degradation**				**Oxidative degradation**			
Time intervals (min)	30	60	120	180	30	60	120	180	30	60	120	180
% Remained	84.44	54.13	23.45	14.00	96.10	96.00	94.00	45.54	99.54	95.90	46.12	46.00
% Regraded	15.56	45.87	76.55	86.00	3.90	4.00	6.00	54.46	0.46	4.10	53.88	54.00
Degradation products	Not detected (no extra peaks appeared)											
Parameters	**EMP**											
Stress degradation conditions	**Acid degradation**				**Alkaline degradation**				**Oxidative degradation**			
Time intervals (min)	30	60	120	180	30	60	120	180	30	60	120	180
% Remained	93.50	87.23	65.60	34.60	93.40	81.26	66.85	57.57	77.70	55.20	33.00	22.00
% Degraded	6.50	12.77	34.40	65.40	6.60	18.74	33.15	42.43	22.30	44.80	67.00	78.00
Degradation products	Not detected (no extra peaks appeared)											

The % recoveries of stored solutions were calculated against fresh standard solutions and were found to be within the acceptable limit 100.53% in case of LNG and 99.58% in case of EMP which indicates good stability of standard drug solutions in methanol for more than two weeks in refrigerator.

4. Conclusion

A gradient RP-HPLC-FLD method is developed and validated for simultaneous determination of both LNG and EMP in drug substances and in drug products without interference from the frequently used additives. The proposed method is found to be sensitive, simple, specific, precise, linear, accurate, and robust; thus, this method can be implemented in quality control laboratories for routine analysis of both drugs. The proposed method is also found to be a stability indicating assay as it achieved good separation of both drugs from potential degradation products.

Disclosure statement

Conflict of interests: The authors declare that they have no conflict of interest.
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