Original Article

Novel UV Spectroscopic Methods for Simultaneous Assessment of Empagliflozin, Linagliptin and Metformin in Ternary Mixture

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ABSTRACT

Background and Aim: Trijardy XR® consisting of empagliflozin, linagliptin, and metformin hydrochloride, a fixed-dose combination (tablets) that improves glycemic control in individuals with diabetes mellitus (type 2). The present work presents four spectrophotometric methods that are quick, effortless, accurate and reproducible for the concurrent assessment of the ternary mixture. **Materials and Methods:** The 1st approach works on the principle of solving established equations (simultaneous) by measuring absorbance at 224.6, 226 and 237.2 nm for empagliflozin, linagliptin and metformin hydrochloride, respectively. The second method namely ratio difference spectroscopy works by measuring the difference in amplitude at two different wavelengths in the ratio spectra. Whereas, the derivative ratio spectrum zero-crossing approach (third approach) relied on the utilization of the derivative ratio signals at zero-crossing locations. The fourth approach is the double divisor-ratio spectra derivative approach in which the first derivative of ratio spectrum was acquired and the concentrations of all 3 drugs in their combination were quantified. **Results and Discussion:** All the three drugs exhibited excellent linear correlation in the concentration series of 2-10 µg/ml for simultaneous equation method and 0.5-10 µg/ml for all the other methods with an exceptional correlation coefficient value. Furthermore, the projected approaches were validated as per ICH strategies and which displayed good precision, accuracy and sensitivity. **Conclusion:** The developed spectrophotometric approaches when compared to other analytical procedures are regarded to be more cost-effective because they do not require expensive solvents or sophisticated instruments. Therefore, the projected methods could be effectively employed for the concurrent assessment of empagliflozin, linagliptin and metformin hydrochloride in ternary mixture.

Keywords: Empagliflozin, Linagliptin, Metformin, Simultaneous Equation Method, Ratio Difference Spectroscopic method, Derivative Ratio Spectrum-Zero crossing approach, Double Divisor Ratio Spectra Derivative approach, Ternary mixture.

INTRODUCTION

Diabetes Mellitus Type 2 (DMT2), is a common endocrinological condition that is already huge and developing at an alarming rate around the world. Diabetic patients would number more than 500 million by 2030, and more than 700 million by 2045, according to projections. Several trials have been conducted to discover more effective anti-diabetic medications with better glucose control and fewer side effects. Sodium-glucose cotransporter-2 inhibitors (SGLT-2) have of late been licenced for the treatment of DMT2 either individually or in conjunction with existing diabetes medications.[1] Empagliflozin (EPZ) having the chemical term (2S,3R,4R,5S,6R)-2-[4-chloro-3-[[4-[(3S)-oxolan-3-yl]oxyphenyl] methyl]phenyl]-6 (hydroxymethyl)oxane-3,4,5-triol has been classified as sodium-glucose co-transporter-2 (SGLT2) inhibitor. SGLT2 is the transporter that is principal factor for reabsorption of glucose in the

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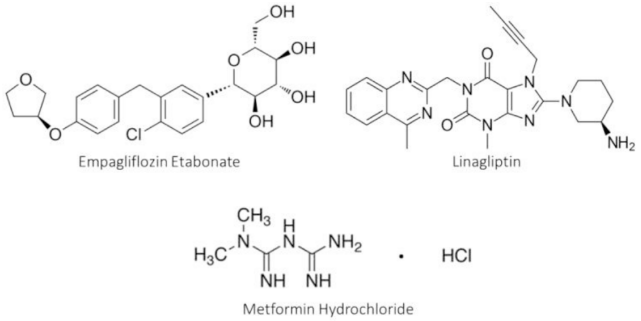

Figure 1: Chemical constructions of EPZ (Empagliflozin); LGT (Linagliptin) and MTH (Metformin hydrochloride).

kidney. It can be utilized to manage type 2 diabetes mellitus as an adjuvant to controlled eating habits and regular workout, frequently in conjunction with another drugs.[2-3] Linagliptin (LGT) chemically identified as (R)-8-(3-aminopiperidin-1-yl)-7-but-2-ynyl-3-methyl-1-(4-methylquinazolin-2-ylmethyl)-3,7 dihydro-purine-2,6-dione is a competitive, reversible DPP-4 inhibitor. GLP-1 and glucose reliant insulinotropic polypeptide (GIP) breakdown is delayed once this enzyme is inhibited. GLP-1 and GIP suppress liberation of glucagon from beta cells of pancreas thereby promoting release of insulin. Together, these processes inhibit glycogenolysis in the liver and enhance insulin liberation.[3-4] Metformin hydrochloride (MTH) a biguanide antidiabetic is chemically identified as 1,1-dimethyl biguanide hydrochloride. It is used to treat diabetes mellitus (type 2) and is the medicine primarily used for the patients who are overweight. Instead of promoting insulin secretion, they exert anti-diabetic effect in presence of some insulin. The delay in glucose absorption from the GI tract, increased sensitivity to insulin, release of glucose into cells and suppression of gluconeogenesis in the liver are all possible mechanisms of action.[5-8] Chemical constructions of all three analytes are given in Figure 1. Multiple medications are needed to effectively regulate blood sugar in diabetic patients. In 2019, the newer combination containing EPZ, LGT and MTH was accepted for phase II clinical trial. According to the USFDA research, the combination operates through 3 harmonious processes to assist regulate blood sugar in adults with type 2 diabetes who could have advantage taking EPZ and LGT along with MTH as part of their treatment schedule.

Trijardy XR® consisting of empagliflozin, linagliptin, and metformin hydrochloride formulated as tablets (extended release) is a fixed-dose combination that improves glycemic control in individuals with diabetes mellitus (type 2) if given in conjunction with controlled diet and regular workout.[9-11] Literature survey disclosed numerous analytical approaches for the assessment of EPZ, LGT and MTH independently and in combined dosage form by means of UV spectrophotometry,[12-13] HPLC,[14-20] HPTLC.[21-24] Moreover, few methods were described for the estimation of EPZ, LGT and MTH in combined formulation/synthetic mixture using HPLC,[25-29] and HPTLC.[2] Nevertheless, the simultaneous estimation of EPZ, LGT and MTH in the ternary mixture has not been reported so far by anyone by UV spectroscopic methods. Therefore, this paper is the first to detail the development and validation of four easy, reproducible, responsive, profitable and exact UV spectroscopic approaches for determining EPZ, LGT, and MTH in a ternary combination. The following are some of the advantages of the proposed methods: they provide a relatively easy standard and sample preparation approach, have a vast concentration range with high sensitivity, and have all been validated according to ICH recommendations. UV-spectroscopic approaches are considered as easy, quick, and cost-effective analytical procedures for assessing the quality of pharmaceuticals that are used on a regular basis. However, effect of multicomponent formulations and formulation additives are the major drawback of direct UV spectroscopic approaches. Therefore, in addition to simultaneous equation method, derivative and ratio derivative UV spectroscopic approaches were also developed to overcome these effects, and those were found to be appropriate for the concurrent estimation of EPZ, LGT and MTH lacking any interference.

MATERIALS AND METHODS

Chemicals and Reagents

Gift sample of EPZ, LGT and MTH reference standard employed for the entire investigation was collected from Dalton PharmaChem, Vadodara, Gujarat, India. All other solvents, chemicals or excipients (specificity study) of AR grade used in the study was purchased from Loba Chemie Pvt. Ltd., Mumbai, India.

Instruments

UV-1800, UV Probe, Shimadzu Corporation, Kyoto, Japan (Double beam UV visible spectrophotometer) having equivalent sample compartment (Quartz; 1cm) was employed for the entire investigation. Adventurer Pro AVG264C (Electronic balance), Ohaus Corporation, Pine Brook, NJ, USA was utilized for the measurement of weight.

Preparation of Standard Solution

The preparation of stock solution of all three drugs (EPZ, LGT and MTH) were carried out by taking weight

of 10 mg of standard analytes individually and shifting into a 100 ml standard flask separately. Concentration of standard analytes were lowered using methanol and capacity was filled up to 100 ml to attain the concentration of the analytes 100 µg/ml. Additionally, reduction in concentration was achieved by dilution employing methanol.

Preparation of Synthetic Mixture

The synthetic mixture of EPZ, LGT and MTH was formulated in the proportion of 25:5:1000 w/w/w. Conventional excipients such as Hydroxypropyl methylcellulose 0.34 gm, Magnesium stearate 0.015 gm, Talc 0.03 gm were measured appropriately and transferred to a mortar together with EPZ, LGT and MTH pure drugs and mixed thoroughly. (Above mentioned calculation is for laboratory prepared synthetic mixture which is equivalent to 1 tablet).[2,29-30]

Preparation of Mixed Standard Solution

A series of the mixed standard solution was made by transferring a suitable volume of EPZ, LGT and MTH standard solutions (0.05-1 ml) into a sequence of 10 ml standard flask separately and volume was filled with methanol to reach desired concentration (0.5-10 µg/ml).

Preparation of Sample Solution

Equivalent quantities of laboratory-prepared synthetic mixture (EPZ: 5 mg; LGT: 1 mg and MTH: 200 mg) were weighed and put into a 100 ml standard flask. In the 100 ml standard flask, pure EPZ (195 mg) and LGT (199 mg) were added. 50 ml methanol was poured to the standard flask and shaken for 10 min. Methanol was used to make a volume of up to 100 ml, which was then passed through Whatman filter paper No 1. 1 ml of the above-mentioned solution was shifted to a 100 ml standard flask and methanol was used to make the volume up to 100 ml. Afterwards, 2.5 ml of the subsequent solution was shifted to a 10 ml standard flask, and the capacity was filled with methanol to achieve the desired concentration (EPZ, LGT, and MTH: 5 µg/ml each).

Procedure /Method

Simultaneous Equation Method (SEM)

EPZ, LGT, and MTH in a laboratory prepared synthetic combination were estimated using the simultaneous equation approach. The UV spectrum of individual standard drugs under study was recorded in the UV region of 200-400 nm. The overlapping UV spectra were utilized for the selection of appropriate wavelength for the determination of proposed analytes in a laboratory-prepared synthetic mixture. The overlain zero-order spectra of EPZ, LGT and MTH displayed an absorption maximum at 224.6, 226, 237.2 nm for EPZ, LGT and MTH, respectively (Figure 2). Subsequently, the absorptivity of all three drugs were calculated and tabulated (Table 1). The quantity of drug present in the synthetic mixture was quantified using the formula.

$$Cx = \frac{(A1(ay2az3 - az2ay3) - ay1(A2az3 - az2A3) + az1(A2ay3 - ay2A3))}{ax1(ay2az3 - az2ay3) - ay1(ax2az3 - az2ax3) + az1(ax2ay3 - ay2ax3)}$$

$$Cy = \frac{(ax1(A2az3 - az2A3) - A1(ax2az3 - az2ax3) + az1(ax2A3 - A2ax3))}{ax1(ay2az3 - az2ay3) - ay1(ax2az3 - az2ax3) + az1(ax2ay3 - ay2ax3)}$$

$$Cz = \frac{(ax1(ay2A3 - A2ay3) - ay1(ax2A3 - A2ax3) + A1(ax2ay3 - ay2ax3))}{ax1(ay2az3 - az2ay3) - ay1(ax2az3 - az2ax3) + az1(ax2ay3 - ay2ax3)}$$

where, C_X, C_Y and C_Z are the amount of EPZ, LGT and MTH, sequentially in the synthetic mixture and the sample solutions.

A1, A2 and A3 are the absorbances of the sample at 224.6, 226, 237.2 nm. ax1, ax2 and ax3 are the absorptivity of EPZ at 224.6, 226, 237.2 nm, sequentially. ay1, ay2 and ay3 are the absorptivity of LGT at 224.6, 226, 237.2 nm, sequentially. az1, az2 and az3 are the absorptivity of MTH at 224.6, 226, 237.2 nm, respectively.[31-32]

Ratio Difference Spectroscopic Method (RDS)

The ratio spectra were generated by recording the UV absorption band of drug solutions made at various concentrations of EPZ and of the mixture of 3 drugs and dividing them by the total of the LGT and MTH (1 µg/ml of both the drugs in methanol) absorption spectra as the double divisor. Then the amplitude of the peak at 252.4 nm was deducted from the amplitude of the peak at 236.6 nm of the ratio spectrum. Further, a calibration curve was plotted using amplitude difference against concentration. To get the ratio spectra, absorption band of solutions generated at various concentrations of MTH and the ternary mixture were stored and divided by the total of the absorption band of EPZ and LGT (2 µg/ml separately in methanol) solutions as the double divisor. Then the peak amplitude at 245.6 nm was deducted from the peak amplitude at 240.4 nm of the ratio spectrum. Further, a calibration curve was plotted using amplitude difference against concentration. The absorption spectra of solutions generated with various concentrations of LGT and the ternary mixture were saved and divided by using EPZ and MTH (4 µg/ml separately in methanol) as a double divisor and ratio spectra were saved. Then the peak amplitude at 223 nm was deducted from the peak amplitude at 233 nm of the ratio spectrum. Further, a calibration curve was plotted using amplitude difference against concentration.[33-39]

Table 1: Average absorptivity values of EPZ, LGT and MTH at various wavelengths for SEM method.

EPZ			LGT			MTH		
224.6 nm	226 nm	237.2 nm	224.6 nm	226 nm	237.2 nm	224.6 nm	226 nm	237.2 nm
385.31*	385.85*	136.95*	694.8*	709.03*	214.71*	439.26*	482.92*	760.26*

*(*n* = 6) Average of six determinations.

Derivative Ratio Spectrum-Zero crossing Method (DRZC)

Firstly, the UV absorption bands of EPZ, LGT and MTH and their combination (3 drugs) at different concentrations (linearity range) were recorded. Later, the ratio spectra were plotted by dividing the absorption band of EPZ, MTH, and their ternary combination with LGT using a standard spectrum of LGT. Subsequently, first order ratio spectra were produced by converting the ratio spectra into their first derivative. The first derivative ratio signals at 284.4 nm (zero-crossing spot for MTH) and 254.4 nm (zero-crossing spot for EPZ) in the first derivative of ratio spectra were proportional to the concentrations of EPZ and MTH in the ternary mixture, respectively. The peak amplitudes of first derivative ratio spectra were noted down against ascending concentrations of pure EPZ and pure MTH, with pure LGT as a divisor, to produce calibration graphs. The above-mentioned calibration graphs can be used to determine the contents of EPZ and MTH. Similarly, the saved spectra of LGT, MTH, and their ternary mixture with EPZ were divided by a reference spectrum of EPZ to get the ratio spectra. Further, the first derivatives of the ratio spectra were recorded. In the first derivative of the ratio spectra, LGT and MTH were proportional to derivative ratio signals at 277.6 nm (zero-crossing spot for MTH) and 236.4 nm (zero-crossing spot for LGT), correspondingly. The calibration graphs for LGT and MTH were created by computing the first derivative ratio values in relation to ascending concentrations of standard LGT and standard MTH, and utilizing standard EPZ as a divisor. LGT and MTH can be determined using this method. By using both of the above-mentioned spectrophotometric procedures, the amount of MTH in the ternary mixture can be found.[40-42]

Double Divisor Ratio Spectra Derivative Method (DDRS)

The ratio spectra were generated by recording the absorption band of drug solutions made at various concentrations of EPZ and of the mixture (3 drugs) and dividing them by the total of the absorption band of LGT and MTH (1 µg/ml separately in methanol) as the double divisor. Subsequently, the first derivatives of the ratio spectra were constructed. The quantity of EPZ was then estimated by taking the amplitude at 231.4 nm equivalent to maxima or minima in the spectral region (nm) chosen. To get the ratio spectra, absorption spectra of solutions generated at various concentrations of MTH and the ternary mixture were saved and divided by the total of the absorption spectra of EPZ and LGT (2 µg/ml separately in methanol) solutions as the double divisor and subsequently, their first derivative spectra were plotted. The amount of MTH was calculated by taking the first derivative of ratio spectra and detecting signals at 252 nm, which correspond to maxima or minima. The absorption spectra of solutions generated with various concentrations of LGT and the ternary mixture were saved and divided by using EPZ and MTH (4 µg/ml separately in methanol) as a double divisor and ratio spectra were saved. Subsequently, the 1st derivative of ratio spectra was recorded and their signals at 310 nm corresponding to the maxima or minima were measured for the estimation of LGT.[43-47]

Analysis of Sample Solution

Sample solution was prepared and diluted as discussed in the previous section. For SEM, amount of analyte was calculated by solving simultaneous equation using standard absorptivity values (Table 1) and absorbances of sample solutions at their respective wavelengths. Whereas, for the other three methods (RDS, DRZC, DDRS), peak amplitude was measured and the quantification of analytes was done using the regression equations.

Validation of Spectroscopic Methods

The proposed approaches were validated using the guidelines of the "International Conference on Harmonization".[31,40,42-44,46-49]

Specificity

A specificity investigation was conducted to determine whether the tablet excipients employed in the formulation interfered with the drug substance. All of the tablet excipients (in accordance with the marketed formulation) were combined proportionately and diluted with methanol before being filtered with the help

of Whatman filter paper no 41. Further, all placebo and standard solutions were compared by scanning in the UV zone in order to examine the interference between excipients and analytes.

Linearity and Range

Linear correlation of all the four approaches and their range were assessed by evaluating all the standard solutions individually, consisting of EPZ, LGT and MTH in methanol and absorbances were recorded at 224.6, 226 and 237.2 nm in SEM method; whereas, amplitude difference (EPZ: 236.6-252.4 nm; LGT; 223-233 nm; MTH; 245.6-240.4 nm) was measured in RDS method. However, the amplitude was measured in DRZC (EPZ: 284.4; LGT: 277.6; MTH: 254.4 nm) and DDRS (EPZ: 231.4; LGT: 310; MTH: 252 nm) approach. Calibration plots of absorbances of standard analyte solutions against concentration in SEM approach; amplitude difference of reference analyte solutions against concentration in RDS approach; peak amplitude versus concentration in DRZC and DDRS method were constructed. Regression assessment was done applying least square approach to get the correlation coefficient, slope and intercept values. Each response was the average of six determination.

Precision

Repeatability, intra-day and inter-day precision were conducted to assess the method precision. The repeatability of proposed approaches was assessed by analysing sample solutions (EPZ, LGT, and MTH: 2 and 8 µg/ml) six times and calculating the percent RSD by measuring the responses of all the drugs at various wavelengths depending on the method. Within the linearity range, intra-day precision was assessed by testing sample solutions in triplicate at two dissimilar concentrations (EPZ, LGT, and MTH: 2 and 8 µg/ml) on the same day for three times. Whereas, Inter-day precision was checked on three different days taking two dissimilar concentrations (EPZ, LGT and MTH: 2 and 8 µg/ml) of sample solutions inside the concentration range and calculating the percentage RSD.

Accuracy

In order to perform recovery studies and thereby confirm the suitability and reliability of the projected approaches, standard addition procedure was utilized. To a pre-analyzed sample solution (EPZ, LGT and MTH: 2, 4 and 6 µg/ml), having an equivalent amount, standard EPZ, LGT and MTH of known concentration at 50, 100 and 150% level was added and reanalyzed by projected approaches and thereafter % recoveries were computed. The accuracy study outcomes was analysed on the basis of percentage of reference EPZ, LGT and MTH recuperated from the pharmaceutical preparation by making use of the below mentioned formula: -

$$\% \text{ Recovery} = \frac{\begin{matrix}(\text{Quantity of analytes after adding of standard analytes} - \\ \text{Quantity of analytes before adding of standard analytes})\end{matrix}}{(\text{Quantity of standard analytes added})} \times 100$$

LOD and LOQ

The assessment of sensitivity of the projected methods was performed by measuring LOD and LOQ. The below mentioned formula as given in ICH guidelines was utilized for the measurement of LOD and LOQ of the drugs under study

$$\text{LOD} = 3.3 \times \frac{\sigma}{S}$$

$$\text{LOQ} = 10 \times \frac{\sigma}{S}$$

Where σ = The standard deviation of the response, S = The slope of the calibration curve

Stability of the Solution

The solution stability was assessed by retaining the drug solutions at ambient temperature and in refrigerated conditions (6°C) and observing changes in absorbance and shape of the spectra in comparison with fresh solutions and analysing them at recurrent intervals.

RESULTS AND DISCUSSION

The developed spectrophotometric approaches are thought to be appropriate for use in quality control units where cost and time are critical. UV spectroscopic approaches are broadly utilized for regular investigation of pharmaceutical preparation owing to their easy, rapid, cheap and reproducible results. It was found that these spectrophotometric methods are superior and advantageous over many other analytical techniques. However, analysing all analytes without previous separation in the case of multi component formulations which have overlapping UV spectra is tough. The proposed work formulates a simple and cost-effective approach to the simultaneous analysis of EPZ, LGT and MTH in ternary mixtures having overlapping spectra.

Simultaneous Equation Method (SEM)

A simultaneous equation approach was developed and validated for the assessment of EPZ, LGT and MTH in a laboratory formulated synthetic mixture with satisfactory sensitivity and selectivity. The zero-order UV spectra showed highest absorbance at 224.6, 226 and 237.2 nm for EPZ, LGT and MTH, respectively

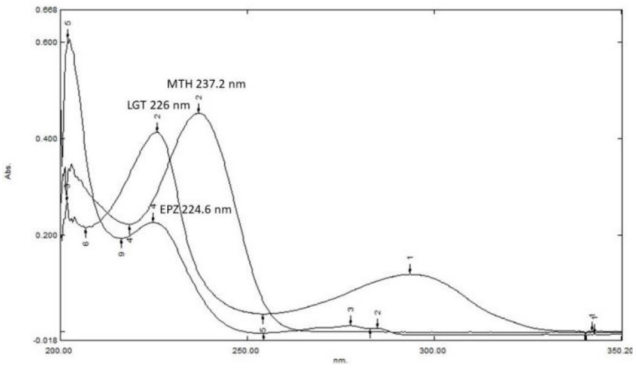

Figure 2: Overlain UV spectra of EPZ; LGT and MTH (6 µg/ml).

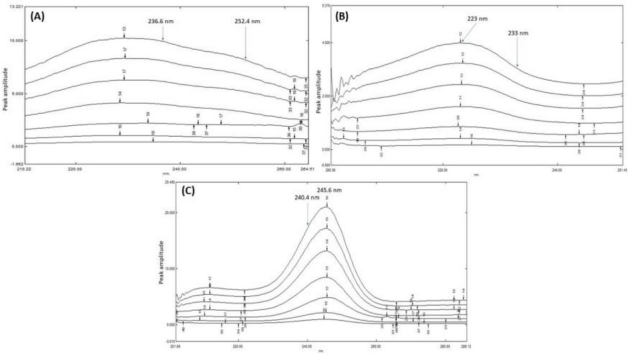

Figure 3: (A) Overlain ratio spectra of EPZ taking 0.5 µg/ml (LGT + MTH) as divisor; (B) Overlain ratio spectra of LGT taking 4 µg/ml (EPZ + MTH) as divisor; (C) Overlain ratio spectra of MTH taking 2 µg/ml (LGT + EPZ) as divisor.

(Figure 2). The zero-order UV spectra of EPZ, LGT and MTH showed overlapping of spectra as shown in (Figure 2) which facilitates simultaneous estimation of EPZ, LGT and MTH in the ternary mixture. The amount of drugs present in the mixture was calculated using a simultaneous equation. Absorptivity values and method validation parameters are shown in Table 1 and 2, respectively.

Ratio Difference Spectroscopic method (RDS)

Derivatization of UV spectra results in increase of specificity and selectivity of drugs in combined formulation by improving the resolution of spectra is a well-established fact. Derivatization also allows us to compute one analyte coexisting with other analyte and eradicates the excipient effects. The basis of the ratio spectroscopic method is to divide the mixture spectrum using one of the spectrums of analyte to get the ratio spectrum, which is devoid of divisor analyte and excipient interferences. Additionally, using an optimized spectrum as a divisor lowers noise and investigational mistakes. A further benefit of the ratio spectra approach is that measurements are taken in relation to the peaks, making them more precise, sensitive and specific. Therefore, ratio spectroscopic methods were developed which gives better results compared to other spectroscopic techniques. In order to select a double divisor of suitable concentration, different concentrations of EPZ, LGT, and MTH were tried. Finally, LGT and MTH (1 µg/ml separately in methanol), EPZ and LGT (2 µg/ml separately in methanol), EPZ and MTH (4 µg/ml separately in methanol) was selected for the estimation of EPZ, LGT and MTH in their ternary mixture for this method. Ratio difference spectroscopic method was performed by generating ratio spectra by recording the absorption band of solutions made at various concentrations of EPZ and of the ternary mixture and dividing them by the total of the absorption spectra of solutions of LGT and MTH (1 µg/ml separately in methanol) as the double divisor, as shown in Figure 3(A). Then the peak amplitude at 252.4 nm was deducted from the peak amplitude at 236.6 nm of the ratio spectrum. Subsequently, correlation coefficients and regression equations were calculated from linearity graphs plotted taking amplitude difference values and the corresponding amount of EPZ. To get the ratio spectra, absorption band of solutions produced at various concentrations of MTH and the ternary mixture were saved and divided by the total of the absorption band of EPZ and LGT (2 µg/ml separately in methanol) solutions as the double divisor, as shown in Figure 3(C). Then the peak amplitude at 245.6 nm was deducted from the peak amplitude at 240.4 nm of the ratio spectrum. Subsequently, correlation coefficients and regression equations were calculated from linearity graphs plotted taking amplitude difference values and the respective amount of MTH. The UV spectra of solutions generated with various concentrations of LGT and the ternary mixture were saved and divided by using EPZ and MTH (4 µg/ml separately in methanol) as a double divisor and ratio spectra were saved, as shown in Figure 3(B). Then the peak amplitude at 223 nm was deducted from the peak amplitude at 233 nm of the ratio spectrum. Subsequently, correlation coefficients and regression equations were calculated from linearity graphs plotted taking amplitude difference values and the equivalent quantity of LGT.

Optimization of Divisor and Scaling Factor for First Derivative of Ratio Spectra

To get the best definite curve of the 1st derivative of ratio spectra, optimization of different settings of instrumental parameters were carried out. Optimization of the divisor and scaling factor were the most important among them. In order to select a divisor of suitable concentration, dissimilar concentrations of

EPZ, LGT, and MTH were tried. Finally, 2 µg/ml of LGT was selected for the quantification of MTH and EPZ. Similarly, 2 µg/ml of EPZ was selected for the assessment of LGT and MTH in their ternary mixture for DRZC method. Furthermore, the scaling factor was fixed /optimized as 4 as it was found most suitable for the first derivative of the ratio spectra. Various wavelengths (2, 4, 8, 10 nm) were attempted for the first derivative spectra in order to find the best one. The results indicated that an optimum wavelength of 8 nm was suitable and this wavelength was chosen and employed with a scaling factor of 4.

Derivative Ratio Spectrum-zero crossing Method (DRZC)

An analytical approach namely the Derivative ratio spectrum zero-crossing approach was developed and validated. In this method, solutions of various concentrations of EPZ and MTH in linearity range was scanned and saved between 200–400 nm. Subsequently, the recorded spectra were divided by using the spectrum of the reference solution of 2 µg/ml LGT. The ratio spectra thus achieved were converted to their first derivative spectra. The concentrations of EPZ and MTH in the ternary mixture were estimated by tracing the derivative ratio analytical signals in the 1st derivative spectra of the ratio spectra at 284.4 nm for EPZ and 254.4 nm for MTH, as shown in Figure 4. In the same manner, the absorption spectra of LGT and MTH were divided by the spectrum of a reference solution of 2 µg/ml EPZ, yielding ratio spectra in the 200–300 nm range. Ratio spectra obtained by this procedure was then transformed into their first derivative with Δλ=8 and scaling factor 4. The concentrations of LGT and MTH in the ternary mixture were estimated by tracing the signals in the first derivative spectra of the ratio spectra at 236.4 for MTH and 277.6 nm for LGT, as shown in Figure 5.

Double Divisor Ratio Spectra Derivative Method (DDRS)

Firstly, different concentrations of ternary mixtures of EPZ, MTH and LGT were made and scanning was performed between 200 to 400 nm. Subsequently, these spectra were divided by 1 µg/ml LGT and 1 µg/ml MTH taken as a double divisor to get their respective ratio spectra. Further, the first derivative of ratio spectra was attained using Δλ= 8 and scaling factor as 4, as shown in Figure 6 (A) and (B). The amplitude of the first derivative ratio spectra at 231.4 nm was measured and the amount of EPZ was determined from the calibration graph plotted using these amplitude values

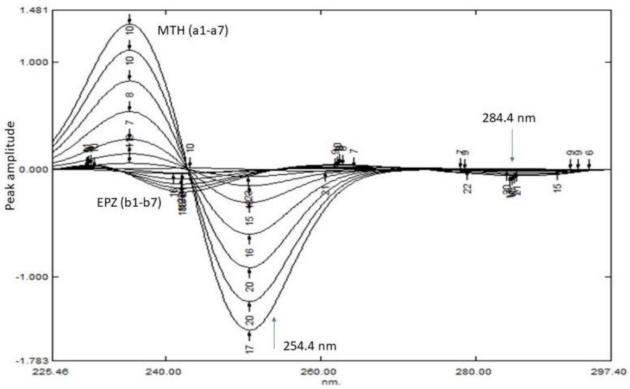

Figure 4: First derivative ratio spectra of MTH (a1) 0.5, (a2) 1, (a3), 2 (a4) 4, (a5) 6, (a6) 8, (a7)10 and of EPZ b1(0.5), b2(1), b3(2), b4(4), b5(6), b6(8), b7(10) (2 µg/ml LGT as divisor (Δλ =8 nm).

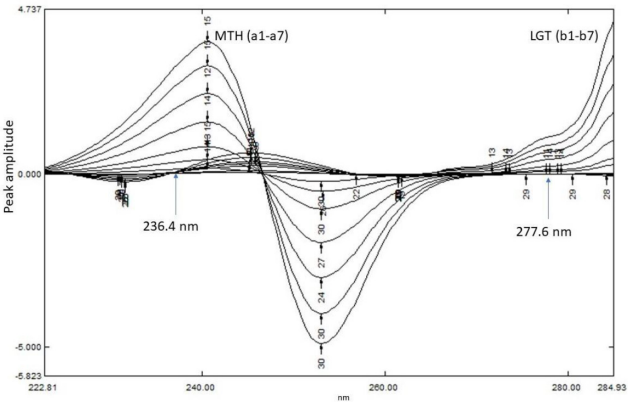

Figure 5: First derivative ratio spectra of MTH (a1) 0.5 (a2) 1 (a3) 2 (a4) 4 (a5) 6 (a6) 8 (a7) 10 and of LGT b1(0.5), b2(1), b3(2), b4(4), b5(6), b6(8), b7 (2 µg/ml EPZ as divisor (Δλ =8 nm).

against concentration at 231.4 nm. Similarly, a second set of ratio spectra were acquired by dividing the ternary mixtures of EPZ, MTH and LGT with 4 µg/ml of EPZ and 4 µg/ml MTH taken as the double divisor. The first derivative of ratio spectra was then acquired utilizing Δλ as 8 and scaling factor as 4, as shown in Figure 7 (A) and (B). The amplitude of the first derivative of ratio spectra at 310 nm were measured and the amount (concentration) of LGT was determined from the calibration graph plotted using these amplitude values against concentration at 310 nm. The final set of ratio spectra were acquired by dividing the ternary mixtures of EPZ, MTH and LGT with 2 µg/ml of EPZ and 2 µg/ml LGT taken as the double divisor. The first derivative of ratio spectra was then acquired utilizing Δλ as 8 and scaling factor as 4, as shown in Figure 8 (A) and (B). The amplitude of the first derivative of ratio spectra at 252 nm were measured and the amount of MTH was

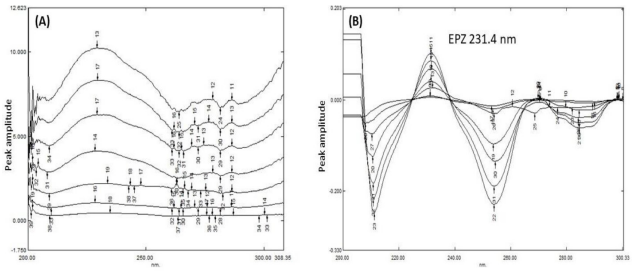

Figure 6: (A) Ratio spectra and (B) 1st derivative ratio spectra of EPZ using 1 µg/ml MTH+ 1 µg/ml of LGT as double divisor at Δλ=8.

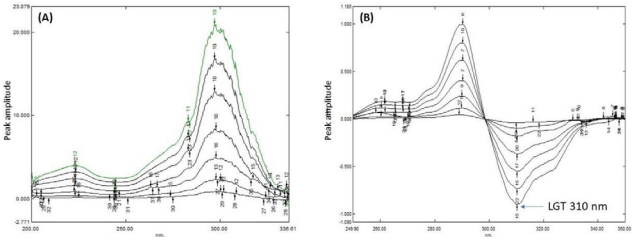

Figure 7: (A) Ratio spectra and (B) 1st derivative ratio spectra of LGT using 4 µg/ml MTH+ 4 µg/ml of EPZ as double divisor at Δλ=8.

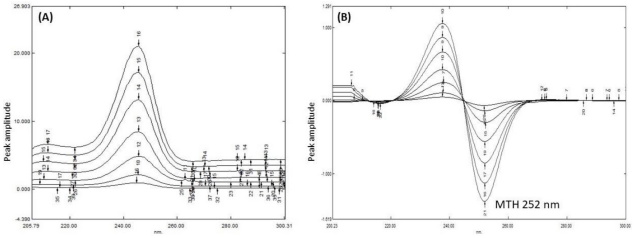

Figure 8: (A) Ratio spectra and (B) 1st derivative ratio spectra of MTH using 2 µg/ml LGT+ 2 µg/ml of EPZ as double divisor at Δλ=8.

determined from the calibration graph plotted using these amplitude values against concentration at 252 nm.

Method Validation

Validity of all the proposed procedures were assessed as per "International Conference on Harmonization" strategies. Outcome of various validation parameters are discussed in the following section.

Specificity

Overlain spectra of placebo (mixture of common excipients as used in marketed formulations, as indicated in the previous section) and drug solutions indicate that excipients and standard pharmaceuticals did not interact.

Linearity and Range

Linear correlation and range were assessed by measuring absorbance at specified wavelengths for SEM method; whereas, amplitude difference was measured in RDS method. However, peak amplitude was measured in DRZC and DDRS method. Linear correlation was observed for all three drugs in the concentration variation of 2-10 µg/ml for SEM method and 0.5-10 µg/ml for RDS, DRZC and DDRS method. The value of correlation coefficient advocates the linearity of all the developed method (Table 2). Each response was the average of six determination.

Precision

Results of precision studies (repeatability, intra and inter-day) stated in % RSD ensure ICH recommendation limits (<2), which confirms the excellent repeatability, less intra and inter-day changeability of all the developed method (Table 2).

Accuracy

Accuracy of the projected procedures were calculated on the basis of retrieval of analytes by standard addition approach. The outcome of recovery studies was in the range of 97-103 % for each drug displaying the correctness of all the developed procedures (Table 3).

LOD and LOQ

The values of LOD and LOQ for all the four methods was proved to be very less which demonstrates the extent of sensitivity of the projected procedures (Table 2).

Stability of the Solution

Solution stability was carried out at ambient temperature and refrigerated condition (6°C) and it was found to be stable up to 2 days at ambient temperature and 10 days at refrigerated condition.

Determination of EPZ, LGT and MTH in ternary mixture

The projected approaches were effectively employed for the assessment of EPZ, LGT and MTH. Six replicate determinations have been carried out to achieve statistically proven data set and which was between 97 and 102 % for all three analytes. Hence, the established methods can be utilized for the concurrent assessment of EPZ, LGT and MTH in ternary mixture (Table 4).

CONCLUSION

Four different methods namely SEM, RDS, DRZC and DDRS spectroscopic approaches were proposed for simultaneous assessment of EPZ, LGT and MTH in the ternary mixture. The methods developed were validated in accordance with ICH recommendations. The proposed methods were accurate, easy, responsive, reproducible,

Table 2: Data of method validation parameters and linear regression equation for the projected approaches.

Parameters/Drugs	SEM			RDS			DRZC			DDRS		
	EPZ	LGT	MTH	EPZ	LGT	MTH	EPZ	LGT	MTH	EPZ	LGT	MTH
Wavelengths (nm)	224.6	226	237.2	236.6-252.4	223-233	245.6-240.4	284.4	277.6	254.4	231.4	310	252
Linearity range (µg/ml)	2-10			0.5-10								
Correlation coefficient	0.9985	0.9995	0.9976	0.9989	0.9995	0.9981	0.9933	0.9992	0.9995	0.9932	0.9995	0.9994
Regression equation	y = 0.0377x + 0.0025	y = 0.0675x + 0.0143	y = 0.0756x + 0.002	y = 0.1498x + 0.0045	y = 0.1013x - 0.0017	y = 0.3449x + 0.0328	y = 0.0059x - 0.0008	y = 0.1044x - 0.0001	y = 0.1221x + 0.0026	y = 0.0105x + 0.0015	y = 0.091x + 0.0045	y = 0.1397x + 0.0042
LOD (µg/ml)	0.3080	0.2626	0.0626	0.0243	0.0179	0.0306	0.07955	0.0469	0.0510	0.0930	0.0423	0.0354
LOQ (µg/ml)	0.9333	0.7958	0.1898	0.0736	0.0542	0.0926	0.2411	0.1421	0.1546	0.2818	0.1283	0.1073
Specificity	Specific (No interference)											
Precision (% RSD) Repeatability of measurement (n=6)* Intra-day (n=3)* Inter-day (n=3)*	 1.5578 1.5162 1.5382	 1.2832 1.3081 1.1558	 1.2097 1.2574 1.0735	 0.9312 0.9603 0.7143	 1.0970 1.2335 1.2595	 0.8975 1.0062 0.8421	 1.4990 1.7799 1.3761	 1.1270 1.4703 1.2990	 1.0142 1.1421 0.8466	 1.4536 1.6343 1.5408	 1.2734 1.5609 1.3528	 0.9495 0.6807 0.9804

*n = number of determinations, % RSD (Percentage relative standard deviation).

Table 3: Recovery data of the projected methods.

Drugs	Level (%)	Recovery (%) *				RSD (%)			
		SEM	RDS	DRZC	DDRS	SEM	RDS	DRZC	DDRS
EPZ	50	98.57 ± 0.61	100.14±1.64	98.43 ± 0.47	98.43±0.91	0.62	1.63	0.48	0.92
	100	98.75 ± 0.67	99.78±1.54	99.40±0.76	98.6±0.43	0.68	1.55	0.76	0.44
	150	98.46 ± 0.79	100.64±1.63	97.43±0.99	99.24±1.9	0.80	1.62	1.01	1.92
LGT	50	98.27 ± 0.34	98.35±1.15	100.17±1.70	99.08±0.78	0.35	1.17	1.70	0.79
	100	98.91 ±0.91	99.80±1.16	98.80±0.72	99.01±0.68	0.92	1.17	0.73	0.69
	150	101.58 ± 1.85	100.82±1.19	99.17±1.23	101.16±1.34	1.82	1.18	1.25	1.32
MTH	50	100.27 ± 0.93	98.29±0.45	100.67±0.70	97.67±0.41	0.93	0.46	0.70	0.42
	100	100.84 ± 1.47	99.2±0.78	101.43±0.85	98.68±1.06	1.46	0.79	0.84	1.08
	150	98.67 ± 0.18	100.21±1.37	99.74±0.96	100.7±1.18	0.19	1.36	0.97	1.17

*Mean ± SD (n = 3), SD (Standard deviation), % RSD (Percentage relative standard deviation).

Table 4: Results of formulation analysis utilizing different approaches.

Drugs	Labelled Amount (mg/tab)	Amount Found (mg/tab)				Amount Found (%) *				RSD (%)			
		SEM	RDS	DRZC	DDRS	SEM	RDS	DRZC	DDRS	SEM	RDS	DRZC	DDRS
EPZ	25	24.68	24.63	24.71	24.92	98.72 ± 1.24	98.52± 1.02	98.85± 1.17	99.69± 1.59	1.26	1.03	1.8	1.60
LGT	5	4.92	4.95	4.90	4.94	98.4 ± 1.49	99.00± 1.86	98.00± 0.95	98.83± 0.95	1.51	1.88	0.97	0.96
MTH	1000	997.27	99.53	993.30	1000.89	99.73 ± 1.56	99.52± 1.64	99.33± 1.72	100.09± 1.81	1.57	1.64	1.73	1.81

*Mean ± SD (n = 6), SD (Standard deviation), % RSD (Percentage relative standard deviation).

and profitable. Furthermore, all of the established UV-spectrophotometric approaches necessitate less sample preparation steps and offer an extended range of concentration with good sensitivity. The developed spectrophotometric approaches are thought to be appropriate for use in quality control units where cost and time are critical. Moreover, when compared to other analytical procedures, these approaches are regarded to be more cost-effective because they do not require expensive solvents or sophisticated instruments. As a result, all of the established methods may be utilized for scheduled quality control analysis of EPZ, LGT, and MTH in synthetic mixtures and mixed tablet dosage forms successfully.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

EPZ: Empagliflozin; **LGT:** Linagliptin; **MTH:** Metformin hydrochloride; **SGLT$_2$:** Sodium-glucose co-transporter-2; **DPP-4:** Dipeptidyl peptidase-4; **USFDA:** United State Food and Drug Administration; **ICH:** International Conference on Harmonization; **SEM:** Simultaneous Equation Method; **RDS:** Ratio Difference Spectroscopic Method; **DRZC:** Derivative Ratio Spectrum-Zero Crossing Method; **DDRS:** Double Divisor Ratio Spectra Derivative Method.

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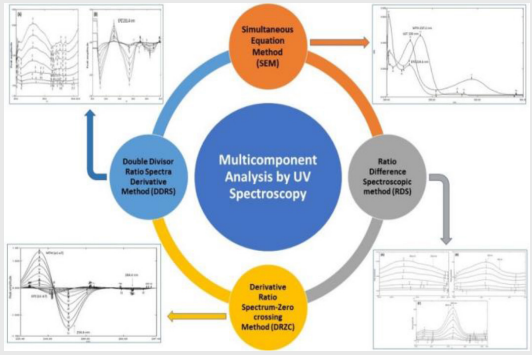
PICTORIAL ABSTRACT



SUMMARY

Trijardy XR®, containing empagliflozin, linagliptin, and metformin hydrochloride, improves glycemic management in diabetics (type 2). This paper proposes four quick, easy, accurate, and reproducible spectrophotometric procedures for assessing ternary mixtures simultaneously. The first approach solves established equations (simultaneously) by measuring empagliflozin, linagliptin, and metformin hydrochloride absorbance at 224.6, 226 and 237.2 nm, respectively. The second method, ratio difference spectroscopy, measures the amplitude difference at two different wavelengths. Third approach uses derivative ratio signals at zero-crossing sites. The fourth approach is the double divisor-ratio spectra derivative approach, which quantifies the concentrations of all 3 medicines in combination. All three medications showed excellent linear correlation in concentration series of 2-10 μg/ml for simultaneous equation approach and 0.5-10 μg/ml for all other methods. The proposed methodologies were validated according to ICH strategies and showed good precision, accuracy, and sensitivity. Spectrophotometric approaches are cost-effective since they don't require expensive solvents or complicated apparatus. The proposed approaches may effectively assess empagliflozin, linagliptin, and metformin hydrochloride in ternary mixture.

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