Comparative Study of Different Derivative Spectrophotometric Techniques for Analysis and Separation of Metformin, Empagliflozin, and Glimepiride

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Abstract

Mean centering, double divisor, ratio spectra-zero crossing, and successive derivative were applied for estimation of metformin, empagliflozin, and glimepiride in the concentration range of 1.0-10, 2.5-30, and 1.0-10 μgmL^{-1}, respectively in their prepared laboratory mixtures and in pharmaceutical tablets, without prior chemical separation. In some cases, lifestyle changes aren't enough to keep type 2 diabetes under control, there are several medications that may help. Metformin, can lower your blood sugar levels. Glimepiride, make more insulin. Empagliflozin, prevent the kidneys from reabsorbing sugar into the blood and sending it out in urine.

The absorption spectra of these drugs were recorded in the range of 200-400nm, mean centering for metformin were measured at 232 and 244 nm, empagliflozin and glimepiride had amplitude values at 262 and 278nm, respectively. The derivative of double divisor was measured at 234, 278, and 288nm for metformin, empagliflozine and glimepiride, respectively. The ratio spectra-zero crossing were quantifying at the amplitude values of analytical signal at 234 and 274nm for metformin and empagliflozine, respectively, whereas glimepiride was determined at 242 and 286nm. The successive ratio of metformin, empagliflozin, and glimepiride were determined at 284, 242, and 266nm, respectively.

The methods were studies and optimized, upon the validation linearity, precision, accuracy LOD, LOQ and selectivity were proved to be effective for analysis of the mentioned drugs in pharmaceutical dosage form. Statistical comparison done between the proposed methods with the reported methods with respect to accuracy and precision no significant difference was found by student's t-test, F-test and one-way ANOVA.

Key words: Mean centering, Double Divisor, Ratio Derivative-Zero-Crossing, Successive derivative Ratio, Ternary Mixture.

1. Introduction:

In type 2 diabetes, however, hyperglycemia indicates insulin resistance coupled with abnormalities of insulin production and secretion and other endocrinopathies that collectively cause a highly heterogeneous and progressive disorder [1]. The effective management of type 2 diabetes mellitus is extremely important because of its increasing prevalence worldwide [2]. Treatment success can be limited by medication side effects, such as weight gain and hypoglycemia, and a recent joint statement from the American Diabetes Association and the European Association for the Study of Diabetes recommends that these side effects should play a major role in the selection of drug therapy for the management of T2DM [3].

Chemically, metformin hydrochloride (Fig. 1a) N, N-dimethylimido carbonimidic diamide hydrochloride. Metformin, a biguanide is traditionally prescribed as first line oral therapy for type 2 diabetes mellitus [4]. metformin is closely related to its capabilities in suppression of hepatic glucose production and intestinal glucose absorption, and promotion of β-cell functions and insulin sensitivity [5,6].

When metformin alone does not maintain glycemic control. Sulfonylurea drugs are a recommended second-line option [7]. Glimepiride (Fig. 1b) belongs to second generation sulfonylurea which is being used for the treatment of non-insulin dependent diabetes mellitus which preserves a more physiological adjustment of insulin secretion [8]. Glimepiride, (1-[[p-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)ethyl]phenyl]sulfonyl]-3-(trans-4methyl cyclohexyl) urea, is acts as an insulin secretagogue. Lowers blood sugar by stimulating the release of insulin by pancreatic beta cells and by inducing increased activity of intracellular insulin receptors [8,9].

Empagliflozin from the gliflozin class that was approved for the treatment of type 2 diabetes as a sodium glucose co-transporter 2 inhibitor enhancing urinary glucose excretion [10]. Empagliflozin (Fig. 1c), (2S,3R,4R,5S,6R)-2-[4-chloro-3-[[4-[(3S)-oxolan-3-yl]oxy phenyl] methyl] phenyl] -6- (hydroxymethyl) oxane-3,4,5-triol. The drug inhibits reabsorption of filtered glucose in the proximal tubules of the kidneys and lowers the renal threshold for glucose, thereby increasing urinary glucose excretion (UGE) and reducing blood glucose levels [11].

Combination therapy is common in type II diabetes, when glycemic control is not accomplished with ordinary monotherapy combination oral therapy becomes an accessible choice [12]. Combination of a second oral diabetes medication with metformin in the same tablet allows for simplified dosage regimens, particularly attractive among a population of patients that may have other comorbidities and require multiple medications [13].

Metformin, regarded as the main compound in mixed therapies [2], for example, metformin given in combination with sulfonylurea lower blood glucose concentration more than either drug alone and is less likely to cause hypoglycemia [14]. Adding empagliflozin and glimepiride as a second therapy to metformin [15]. Triple combination of glitazone, biguanide, and a sulfonylurea is useful for treating diabetes and improving glycemic control [16,17].

Literature review revealed that several analytical methods have been developed for estimation of metformin, empagliflozin, and glimepiride in a single dose or in combination dosage forms, some methods have been reported for determination of metformin in combination with some oral diabetic drugs in pharmaceutical formulations and biological fluids and by using different methods like spectrophotometry and derivative spectroscopy [18-21], and different chromatographic methods [22-29].

Several spectrophotometric determination methods were used for resolving mixtures of compounds with overlapping spectra. When these methods are compared with each other, the range of application of derivative spectrophotometry is more reliable with respect to utility and sensitivity than normal spectrophotometry [30]. Among the methods used to reduce or eliminate such interference or irrelevant absorption is mean centering spectrophotometry, double divisor ratio-derivative, ratio derivative – zero crossing and successive derivative ratio spectra methods. The suggested methods allowed the quantitative determination of ternary mixtures and quaternary mixture without prior separation

In this present work, four techniques of derivative spectrophotometry (mean centering ratio spectra (MCRS), double divisor ratio spectra(DDRS), ratio derivative-zero crossing

spectrophotometric (DRZC) and successive derivative ratio spectra(SDRS)) have been applied successfully for simultaneous determination of metformin, empagliflozine and glimepiride in their overlapped ternary mixture spectra.

Mean centering ratio spectra was developed by Afkhani and Bahrami [31] for analysis of binary and ternary mixtures, in this method signal-noise ratio was enhanced by eliminating derivative steps. Binary mixture of empagliflozin and metformin were determined in pharmaceutical combination by using mean centering ratio spectra [32]. Dinc was developed double divisor ratio spectra derivative spectrophotometric, this method based on the use of the coincident spectra of derivative of the ratio spectra obtained [33-34]. Derivative ratio spectra-zero crossing method was used for determination of binary and ternary mixtures, this method developed by Nevado, were based on the simultaneous use of the first derivative of the ratio spectra and measurement of zero-crossing wavelength [35]. Successive derivative ratio spectra [36]

1.1. Theoretical Background

1.1.1. Mean Centering Ratio Spectra (MCRS)

The three compound present in the mixture are; Metformin (MTF), Glimepiride (GLM) and Empagliflozine (EMG), where Beer's law is obeyed over the whole wavelength range used for all compounds. Then:

$$Am = \propto_{MTF} C_{MTF} + \propto_{GLM} C_{GLM} + \propto_{EMP} C_{EMP} \qquad (1.0)$$

where Am is the mixture absorbance vector, $\propto_{MTF}$, $\propto_{EMG}$ and $\propto_{GLM}$ are the absorptivity vectors, and C_{MTF}, C_{GLM}, and C_{EMG} are the concentrations of MTF, GLM, and EMG in the ternary mixture, respectively.

For the determination of MTF, if the equation (1.0) is divided by $\propto_{GLM}$ corresponds to the spectrum of standard solution of EMG, the first divisor, though we get first ratio spectrum

$$\frac{Am}{\propto_{GLM}} = \frac{\propto_{MTF} C_{MTF}}{\propto_{GLM}} + C_{GLM} + \frac{\propto_{EMG} C_{EMG}}{\propto_{GLM}} \qquad (1.1)$$

equation (1.2) was obtained by mean centering of equation (1.1)

$$mc\frac{Am}{\propto_{GLM}} = mc\frac{\propto_{MTF} C_{MTF}}{\propto_{GLM}} + mc\frac{\propto_{EMG} C_{EMG}}{\propto_{GLM}} \qquad (1.2)$$

we obtain the second ratio spectrum by dividing equation (1.2) by mc $\frac{\propto_{EMG}}{\propto_{GLM}}$, the second divisor,

$$\frac{mc\frac{Am}{\propto_{GLM}}}{\text{mc}\frac{\propto_{EMG}}{\propto_{GLM}}} = \frac{mc\frac{\propto_{MTF} C_{MTF}}{\propto_{GLM}}}{\text{mc}\frac{\propto_{EMG}}{\propto_{GLM}}} + C_{EMG} \qquad (1.3)$$

for the mean centering of equation (1.3), we get;

$$mc\frac{\frac{mc\frac{Am}{\propto_{GLM}}}{\text{mc}\frac{\propto_{EMG}}{\propto_{GLM}}}}{} = mc\frac{\frac{mc\frac{\propto_{MTF} C_{MTF}}{\propto_{GLM}}}{\text{mc}\frac{\propto_{EMG}}{\propto_{GLM}}}}{} \qquad (1.4)$$

Then:

$X=ZC_{MTF}$ (1.5)

where, $X= mc \frac{\frac{Am}{\propto_{GLM}}}{\text{mc}\frac{\propto_{EMG}}{\propto_{GLM}}}$ and $Z = mc \frac{\frac{\propto_{MTF} C_{MTF}}{\propto_{GLM}}}{\text{mc}\frac{\propto_{EMG}}{\propto_{GLM}}}$

The mean centered second ratio spectrum that obtained in equation (1.4) is dependent only on the concentration of $C_{MTF,}$ but independent on the concentration of C_{EMG} and C_{GLM} in the mixture. We can determine the concentration of (EMG and GLM) by using the same procedure.

1.1.2. Double Divisor Ratio Spectra (DDRS)

In this method, MTF was determined in the ternary mixture containing Metformin (MTF), Empagliflozin (EMG), and Glimepiride (GLM), the stored spectra of pure MTF and its mixture were divided by the spectra of binary mixture (GLM and EMG), the UV-VIS spectra of ternary mixture at wavelength λi can be given by:

$$Am = \propto_{MTF} C_{MTF} + \propto_{EMG} C_{EMG} + \propto_{GLM} C_{GLM} \text{ .....(1.0)}$$

where Am denotes the absorbance of the ternary mixture at λi, $\propto_{MTF}$, $\propto_{EMG}$, and $\propto_{GLM}$ denote the absorptivities of MTF, EMG and GLM, respectively.

A similar expression of the binary mixture (a double divisor) of two compounds in ternary mixture can be written:

$$Am = \propto_{EMG} C_{EMG} + \propto_{GLM} C_{GLM} \text{ ..... (1.6)}$$

If Eq. (1.0) is divided by Eq. (1.6) (double divisor) and the resulting expression for the ratio spectra can be given as:

$$\frac{d}{d\lambda}\frac{Am}{\propto_{EMG} C^0_{EMG} + \propto_{GLM} C^0_{GLM}} = \frac{d}{d\lambda}\frac{\propto_{MTF} C_{MTF} + \propto_{EMG} C_{EMG} + \propto_{GLM} C_{GLM}}{\propto_{EMG} C^0_{EMG} + \propto_{GLM} C^0_{GLM}} \text{ ... (1.7)}$$

The ratio of $(\frac{\propto_{EMG} C_{EMG} + \propto_{GLM} C_{GLM}}{\propto_{EMG} C^0_{EMG} + \propto_{GLM} C^0_{GLM}})$ is equal to constant (k) (or very close to 1) with respect to λi in a certain range of wavelength and if this above constant is replaced in Eq. (1.7), it can be obtained Eq. (1.8):

$$\frac{d}{d\lambda}\frac{Am}{\propto_{EMG} C^0_{EMG} + \propto_{GLM} C^0_{GLM}} = \frac{d}{d\lambda}\frac{\propto_{MTF} C_{MTF}}{\propto_{EMG} C^0_{EMG} + \propto_{GLM} C^0_{GLM}} + K \text{ ... ... (1.8)}$$

In the double divisor procedure, the standard concentrations of C^0_{GLM} and C^0_{EMG} in Eq. (1.8) are equal to each other ($C^0_{GLM} = C^0_{EMG}$) which can be expressed as:

$$\propto_{EMG} C^0_{EMG} + \propto_{GLM} C^0_{GLM} = C^0_{EMG}[\propto_{EMG} + \propto_{GLM}] \dots\dots\dots\dots (1.9)$$

When Eq. (1.9) is substituted to Eq. (2.9) the following equation can be obtained:

$$\frac{d}{d\lambda}\left[\frac{Am}{C^0_{EMG}[\propto_{EMG} + \propto_{GLM}]}\right] = \frac{d}{d\lambda}\left[\frac{\propto_{MTF} C_{MTF}}{C^0_{EMG}[\propto_{EMG} + \propto_{GLM}]}\right] + K \dots\dots\dots. (2.0)$$

The first derivation of Eq. (2.0) can be taken with respect to λi in the selected region of wavelength, in this case, the derivation of a constant (k) is zero and we can have obtained Eq. (2.1):

$$\frac{d}{d\lambda}\left[\frac{Am}{C^0_{EMG}[\propto_{EMG} + \propto_{GLM}]}\right] = \frac{d}{d\lambda}\left[\frac{\propto_{MTF} C_{MTF}}{C^0_{EMG}[\propto_{EMG} + \propto_{GLM}]}\right] + zero \dots\dots\dots. (2.1)$$

This derivative analytical signal of MTF is dependent exclusively on the concentration C_{MTF} and C^0_{MTF}; but is independent on the concentrations, C_{EMG} and C_{GLM} in ternary mixtures. In this method, the concentration C_{MTF} is proportional to the derivative signals in the coincided points corresponding to the maximum and minimum of wavelength for pure MTF and its ternary mixture with EMG and GLM. As explained above, the analogue procedures can be used for the estimation of C_{EMG} and C_{GLM} in ternary mixture. Eq. (2.1) is the expression of double divisor-ratio spectra derivative method for the resolution of ternary mixture [33].

1.1.2. Ratio Derivative-Zero Crossing

For the determination of MTF, Eq. (1.0) was divided by the corresponding equation for the spectrum of a standard solution of one of the components (EMG at a certain concentration C^0_{EMG}) and the first derivative of the result is obtained, the following equation can be written:

$$Am = \propto_{MTF} C_{MTF} + \propto_{EMG} C_{EMG} + \propto_{GLM} C_{GLM} \dots\dots\dots\dots (1.0)$$

$$\frac{d}{d\lambda}\left[\frac{A_m}{\alpha_{EMG} C^0_{EMG}}\right] = \frac{C_{MTF}}{C^0_{EMG}} \frac{d}{d\lambda}\left[\frac{\alpha_{MTF}}{\alpha_{EMG}}\right] + \frac{C_{GLM}}{C^0_{EMG}} \frac{d}{d\lambda}\left[\frac{\alpha_{GLM}}{\alpha_{EMG}}\right] \dots\dots\dots (2.2)$$

Eq. (2.2) indicates that the derivative ratio spectrum of the ternary mixture is independent of the value of C_{EMG}, and dependent of the value of C_{MTF}, C_{GLM} and C^0_{EMG} in the ternary mixtures. The

content of MTF and GLM can be resolved by the zero-crossing method by measuring at adequate wavelengths and by use of calibration graphs. Two calibration graphs are obtained by recording and storing the spectra of solution of pure MTF and pure GLM at different concentrations and the spectrum of a solution of pure EMG of concentration C^0_{EMG}. The amplitudes of the spectra of MTF and GLM are then divided, wavelength by wavelength, by the corresponding amplitudes for EMG. The "ratio spectra" thus obtained are then differentiated with respect the derivative values $\frac{C_{MTF}}{C^0_{EMG}}\frac{d}{d\lambda}\left[\frac{\alpha_{MTF}}{\alpha_{EMG}}\right]$ were plotted against C_{MTF} for a given wavelength corresponding to zero crossing of ratio spectrum of GLM (in this wavelength $\frac{C_{GLM}}{C^0_{EMG}}\frac{d}{d\lambda}\left[\frac{\alpha_{GLM}}{\alpha_{EMG}}\right] = 0$), to give a calibration graph.

The calibration graph of GLM was plotted by plotting derivative values $\frac{C_{GLM}}{C^0_{EMG}}\frac{d}{d\lambda}\left[\frac{\alpha_{GLM}}{\alpha_{EMG}}\right]$ against C_{GLM} at λ_i corresponding to zero crossing of the ratio spectrum of MTF (at this wavelength $\frac{C_{MTF}}{C^0_{EMG}}\frac{d}{d\lambda}\left[\frac{\alpha_{MTF}}{\alpha_{EMG}}\right] = 0$). Application of the method to the sample containing MTF, EMG, and GLM and use of the calibration graphs, will then give the values of C_{EMG} and C_{GLM} in the ternary mixtures. EMG can be determined by an analogous procedure. We use a spectrum of standard solution of MTF of concentration C^0_{MTF} we can determine EMG and GLM in the presence of MTF [35].

1.1.4. Successive Ratio Derivative Spectra

MTF was determined where Eq. (1.0) is divided by $\propto_{GLM}$ corresponding to the spectrum of a standard solution of GLM in ternary mixture, the first ratio spectrum is obtained (for possibility of dividing operation, the zero values of $\propto_{GLM}$ should not be used in the divisor)

$$Am = \propto_{MTF} C_{MTF} + \propto_{EMG} C_{EM} + \propto_{GLM} C_{GLM} \quad \text{.....(1.0)}$$

$$\left[\frac{A_m}{\alpha_{GLM}}\right] = \frac{\alpha_{MTF} C_{MTF}}{\alpha_{GLM}} + \frac{\alpha_{EMG} C_{EMG}}{\alpha_{GLM}} + C_{GLM} \quad \text{.....(2.3)}$$

If the first derivative is taken, since the derivative of a constant (C_{GLM}) is zero, Eq. (2.4) would be obtained

$$\frac{d}{d\lambda}\left[\frac{A_m}{\alpha_{GLM}}\right] = \frac{d}{d\lambda}\left[\frac{\alpha_{MTF} C_{MTF}}{\alpha_{GLM}}\right] + \frac{d}{d\lambda}\left[\frac{\alpha_{EMG} C_{EMG}}{\alpha_{GLM}}\right] \quad \text{.....(2.4)}$$

By dividing the previous equation by (d/dλ) ($\alpha_{EMG}/\alpha_{GLM}$), corresponding to the derivative of the ratio of the spectra of the standard solutions of EMG and GLM, the second ratio spectrum is obtained Eq. (2.5)

$$D = \frac{\frac{d}{d\lambda}\left[\frac{A_m}{\alpha_{GLM}}\right]}{\frac{d}{d\lambda}\left[\frac{\alpha_{MTF}}{\alpha_{GLM}}\right]} = \frac{\frac{d}{d\lambda}\left[\frac{\alpha_{MTF}C_{MTF}}{\alpha_{GLM}}\right]}{\frac{d}{d\lambda}\left[\frac{\alpha_{MTF}}{\alpha_{GLM}}\right]} + C_{EMG} \ldots\ldots\ldots\ldots \quad (2.5)$$

If the first derivative of the previous equation is taken since the derivative of a constant (C_{EMG}) is zero, the following equation would be obtained

$$\frac{dD}{d\lambda} = \left[\frac{\frac{d}{d\lambda}\left[\frac{A_m}{\alpha_{GLM}}\right]}{\frac{d}{d\lambda}\left[\frac{\alpha_{MTF}}{\alpha_{GLM}}\right]}\right] = \frac{\frac{d}{d\lambda}\left[\frac{\alpha_{MTF}C_{MTF}}{\alpha_{GLM}}\right]}{\frac{d}{d\lambda}\left[\frac{\alpha_{MTF}}{\alpha_{GLM}}\right]} \quad \ldots\ldots\ldots\ldots \quad (2.6)$$

Eq. (2.6) is the mathematical foundation of multicomponent analysis that permits the determination of concentration of each of the active compounds in the solution (MTF in this equation) without interference from the other compounds of the ternary system (EMG and GLM in these equations). As Equation shows there is a linear relation between the amount of dD/dλ and the concentration of MTF in the solution. A calibration curve could be constructed by plotting dD/dλ against concentration of MTF in the standard solutions of MTF or in the standard ternary mixtures. For more sensitivity the amount of dD/dλ corresponding to maximum or minimum wavelength should be measured. Calibration graphs for EMG and GLM could be also constructed as described for MTF [37].

2. Experimental

2.1. Instrumental and software

A double beam UV spectrophotometer (SHIMADZU, Japan) model UV-1800 PC with quartz cell with 1cm path length, was connected to a lap top having 3.0 GB RAM, and the software used SHIMADZU UV prop data system program (version 1.1).

The Data for MCR were processed using IBM SPSS statistics 25.

2.2. Materials and Reagents

2.2.1. Pure samples

Glimepiride CAS no. [93479-97-1] certified to be >98%, and empagliflozin CAS no. [864070-44-0] certified to be 99.05 were purchased from ApexBio Technology (Generon, UK). Metformin pure standard was manufactured and kindly supplied by Pioneer Co (Pioneer company for pharmaceutical Industries, Sulaimani, Iraq). Glucophage® tablets containing 500mg of metformin were supplied by Bristol-Myers Squibb, Jardiance® tablets contain 25mg of

empagliflozin were supplied by Boehringer Ingelheim, Amaryl® tablets contain 3mg of glimepiride supplied by Boehringer Ingelheim,

2.2.2. Standard solutions

Stock standard solutions of MTF, EMG and GLM were prepared by accurately weighing 100 mg of MTF, EMG, and GLM separately into three separate 100-mL volumetric flasks and then filling to the mark with methanol. Working standard solutions of MTF, EMG and GLM were prepared from their respective stock standard solutions by diluting 10ml in three 100-mL volumetric flasks with methanol. HPLC grade methanol was used as a solvent from Sigma-Aldrich Germany.

2.3. Procedures

2.3.1. Spectral characteristics

The zero order absorption spectra of MTF, EMG and GLM each 5.0 µg mL^{-1}, were recorded over the range of 200-400 nm using methanol as a blank. The overlapped spectra are shown Fig. 2.

2.3.2. Construction of calibration curve

Different aliquots from the working standard solution equivalent to 10-100, 25-300, and 10-100 µg of MTF, EMG and GLM were dispensed into three separate sets of 10ml volumetric flasks, then filled by methanol. The absorption spectra were recorded for each set in the wavelength ranges of (200-400) nm.

2.3.3. Mean centering of the ratio spectra spectrophotometric (MCRS)

For MTF determination, the stored spectrum of were divided by the standard spectrum of 2.0 µg mL^{-1} GLM the first divisor, then the first ratio spectra was mean centered, these vectors were divided by the second divisor $mc\frac{\propto_{EMG}}{\propto_{GLM}}$ corresponding to the mean centering of standard spectra in which the concentration of GLM and EMG are 2.0 and 2.5µg mL^{-1} , respectively then the second ratio spectra were mean centered.

Similarly, EMG and GLM were determined according to their corresponding method was operated for MTF determination using their related divisors specified in Table 1. The amplitudes value of mean centered second ratio spectra were measured at 234 and 248nm for MTF and the amplitude values at 276 and 262nm for EMG and GLM respectively. The calibration curve was plotted between the amplitude value of the mean centered second ratio spectra against the concentration of each component, and regression equations were determined.

2.3.4. Double divisor spectrophotometric (DDRS).

For the MTF determination, the stored absorption spectra were divided by a spectrum of a standard binary mixture of GLM and EMG (double divisor with equal concentration 3.0 µgmL^{-1} of each). The first derivative of the ratio spectra was obtained at the amplitude values was measured at 234nm by using $\Delta\lambda$=20 and a scaling factor of 10. The calibration curve was

constructed against the corresponding concentration of MTF and regression equation was calculated.

EMG and GLM were determined by applying the same procedure used for MTF determination, equal concentration of a divisor mixture was used of the other two drugs (3.0 μgmL^{-1} of each), the amplitude values of ratio spectra of EMG and GLM were measured at 278, and 288nm respectively, the calibration curves were constructed according to their corresponding concentrations, and the regression equations was calculated.

2.3.5. Derivative ratio spectra-zero crossing (DRZC)

For MTF determination, the stored absorption spectra of MTF and GLM and their ternary mixture with EMG were divided by the spectrum of the standard solution of 2.5 μgmL^{-1} EMG (the divisor), and the first derivative of ratio spectra were obtained with intervals of $\Delta\lambda = 20$ and a scaling factor of 10. The amplitude values were measured at 234nm for MTF (at zero-crossing point of GLM) and at 242nm for GLM (at zero-crossing point of MTF), respectively.

By using the same method, the stored absorption spectra of EMG and GLM and their ternary mixture with MTF were divided by the spectrum of the standard solution of 4.0μgmL^{-1} MTF (the divisor), and the first derivative of ratio spectra obtained with intervals of $\Delta\lambda = 20$ and a scaling factor of 10, the amplitude values were measured at 274nm for EMG (at zero-crossing point of GLM) and at 286nm for GLM (at zero-crossing point of EMG), respectively. According to these results, the calibration curves with respect to the corresponding concentrations of MTF, EMG, and GLM were plotted at the analytical signal, and the regression equation was calculated.

2.3.6. Successive derivative ratio spectra (SDRS)

The absorption spectra of MTF were divided by the spectrum of the standard solution of 2.5 μgmL^{-1} of EMG, the ratio spectra were obtained. The first derivative of the ratio spectra were gained with $\Delta\lambda$= 20 nm, and these vectors were divided by the first derivative of the ratio spectra [d/dλ] [GLM/EMG], concentration was 2.0 μgmL^{-1} of GLM, then the second ratio spectra were obtained. The first derivative of these spectra were obtained from which MTF was determined by measuring the peak amplitude at 284 nm.

For determination of EMG, the recorded spectra were divided by the spectrum of 2.0 μgmL^{-1} GLM, and the first derivative of the ratio spectra were divided by the first derivative ratio spectra [d/dλ] [MTF/GLM], MTF concentration was 2.0 μgmL^{-1}, then the second ratio spectra were obtained. The first derivative of these vectors were obtained with $\Delta\lambda$= 20nm.

Similarly, the recorded spectra of the different concentration of GLM were divided by the spectrum of 3.0μgmL^{-1} MTF, and the first derivative of the ratio spectra were divided by [d/dλ] [EMG/MTF], concentration of EMG was 2.5 μgmL^{-1}, then the second ratio spectra were obtained. The first derivative of these vectors were obtained with $\Delta\lambda$= 20nm.

The calibration curves were created by plotting the amplitude values at 284, 242, and 266nm for MTF, EMG, and GLM, respectively with respect to the corresponding concentration of each component.

2.3.7. Laboratory prepared mixtures

Different synthetic mixtures containing different ratios of MTF, EMG and GLM with respect to their calibration ranges were prepared, the zero order spectra of these mixture recorded in the range of 200-400nm, as mentioned at 2.3.2.

2.3.8. Pharmaceutical assay

Ten tablets of Jardiance, Glucophage, and Amaryl were separately weighed, powdered and mixed, weighed an accurately amount of the powdered equivalent to 10mg of each drugs were separately dissolved in 100 mL of methanol, the solution was filtered. Different concentrations were prepared by serial dilution from the previously prepared pharmaceutical solution. For each method calibration curves were plotted and the regression equation were determined to find the concentration of MTF, EMG, and GLM. To ensure the accuracy of the proposed methods a standard addition technique was carried out, by spiking excess amounts of standard MTF, EMG, and GLM, respectively to a pre-analyzed solution of 5.0, 2.5, and 3.0 μgmL^{-1} MTF, EMG, and GLM, respectively.

3. Results and discussions

The absorption spectra of MTF, EMG, and GLM in their ternary mixture were overlapped closely in the spectral range of (210-300) nm are shown in Fig 2. Were usual spectrophotometric method and classical derivative spectrophotometric method was tested from (first to fourth) unable to determine three compounds in the mixtures.

3.1. Optimization:

3.1.1. Mean centering of ratio spectrophotometric (MCRS)

To optimize the MCR method, the divisor concentration has a great effect on the selectivity of the method. Thus, different concentrations of MTF, EMG and GLM were tested; Table 3 shows that the selectivity of the method can be affected by the divisor concentrations. Reproducible and good results were obtained for determination of MTF, EMG and GLM. The optimum concentrations were 2 μgmL^{-1} of MTF, 2.5 μgmL^{-1} of EMG and 2 μgmL^{-1} of GLM.

3.1.2. Double divisor spectrophotometric (DDRS)

There are many factors must be tested to optimize this method for determination of ternary mixture. An important step is selection of the working wave length,

In the operation of this method; choosing the working wavelength is an important step. The absorption spectra of the pure compound and its ternary mixture were recorded and divided by the spectrum of the standard solution (double divisor), the first derivative of ratio spectra were obtained, these two spectra must coincide in the wavelength spectral region corresponding to maximum or minimum point of the wavelength. For the determination of the compound in the

ternary mixture and to be certain that co-formulated drugs do not interfere the coincident point obtained in the derivative ratio spectra must be selected as a working wavelength region.

Another factor that affects the selectivity of the method is the divisor concentration. For determination of MTF, EMG and GLM, different concentrations were tested because the concentration of the divisor is the basic step for selecting an optimum concentrations of the mixture to use as a divisor by using equal concentration of the divisors, as shown in the Table 3.

For the determination of MTF, the optimum concentration of the divisor was 3.0 μgmL^{-1} of EMG and GLM. The selected wavelength coincident point for the determination of MTF in the ternary mixture with those of pure MTF was at 234nm, showing no interference from co-formulated drugs. For the determination of EMG in the ternary mixture, optimum concentrations 3.0 μgmL^{-1} of MTF and GLM were founded. The selected wavelength coincident point for the determination of EMG in ternary mixture with those of pure EMG was at 278nm. Finally, the optimum concentrations 3.0 μgmL^{-1} of MTF and EMG were founded suitable for the determination of GLM, while the selected wavelength coincident point in the ternary mixture with its pure were founded at 288nm.

3.1.3. Ratio derivative-zero crossing(RDZC)

To achieve the better definite curve of the first derivative of ratio spectra, the essential instrument parameters conditions were optimized. The main factors that affect the ratio spectra was the concentration of the standard solution used as a divisor, which studied by testing different concentrations of the divisors Table 3. The optimum standard solution of 2.5μgmL^{-1} EMG for determining of MTF and GLM and 4.0μgmL^{-1} of MTF for EMG and GLM in their ternary mixture were founded suitable.

The influence of the wavelength interval $\Delta\lambda$ for obtaining the first derivative of the ratio spectra were tested, and the value of $\Delta\lambda$=20 was considered suitable to determine each component in the ternary mixture high recovery were obtained.

3.1.4. Successive derivative ratio spectra (SDRS)

The divisors concentration was effected on the selectivity of the method, different concentrations of MTF, EMG and GLM were tested, the results was attended that the concentration of the divisor has a powerful response on the methods selectivity. The optimum divisors concentrations were 3.0, 2.0, and 2.5μgmL^{-1} for MTF, EMG, and GLM, respectively.

3.2. Method Application

3.2.1. Mean centering of ratio spectra(MCRS)

The absorption spectra of the components in the quaternary mixture in the concentrations ranges of 1.0-10, 2.5-30, and 1.0-10μgmL^{-1} for MTF, EMG, and GLM, respectively, were recorded in the range of 210-330 nm. Upon using the divisors shown in Table 1, mean centered second ratio spectra amplitude value at 234 and 248nm for MTF, the amplitude values at 276 and 262nm for EMG and GLM respectively, as shown in (Fig. 3a, 3b, and 3c). The calibration curve

was plotted between amplitude values of the mean centered third spectra against the concentrations of each component; regression equations are shown in Table 2.

3.2.2. Double divisor ratio spectra (DDRS)

The absorption spectra were recorded in the range (210-330) nm for different concentrations of MTF in the range 1.0-10µgmL^{-1} were divided by the spectrum of standard mixture of 3.0µgmL^{-1} of each EMG and GLM (as a double divisor), then the first derivative of ratio spectra were obtained at the spectra amplitude value at 234nm, as shown in (Fig. 4a).

For determination of EMG, absorption spectra were recorded in the range (210-330) nm in the different concentration ranges 2.5-30 µgmL^{-1} then these vectors were divided by a spectrum of standard mixture consist of equal concentration 3.0µgmL^{-1} for each MTF and GLM, the first derivative of ratio spectra were obtained (Fig. 4b), the spectral amplitude value were measured at 278nm.

For determination of GLM, the stored spectra in the concentration ranges 1-10µgmL^{-1} were divided by the spectrum of standard mixture consist of equal concentration 3.0µgmL^{-1} for each MTF and EMG, the first derivative spectra were obtained at the amplitude value 288nm as shown in (Fig. 4c).

Calibration curves were constructed for each drugs, the concentration ranges and regressions were shown in Table 2.

3.2.3. Ratio derivative-zero crossing(RDZC)

In this method, the absorption spectra of the ternary mixture solution of MTF, EMG, and GLM were divided by the spectrum of the standard solution of 2.5 µgmL^{-1} EMG, and the ratio spectrum of MTF-GLM was obtained. The first derivative of the ratio spectra which was calculated with intervals of $\Delta\lambda$=20. The concentration of MTF and GLM in the ternary mixture were determined by measuring the signals at 234nm for MTF (at zero-crossing point of GLM), and at 242nm for GLM (at zero-crossing point of MTF), as shown in (Fig. 5a).

Similarly, the absorption spectra of the ternary mixture solution of MTF, EMG, and GLM were divided by the spectrum of the standard solution of 4.0 µgmL^{-1} MTF and the ratio spectrum of EMG-GLM was obtained. The first derivative of the ratio spectrum was calculated with interval $\Delta\lambda$=20 from the ratio spectrum. The concentration of EMG and GLM were determined in the ternary mixture by measuring the signals at 274nm for EMG (at zero-crossing point of GLM) and at 286nm for GLM (at zero-crossing point of EMG), respectively, as shown in (Fig. 5b).

Calibration curves were constructed for each drugs, the concentration ranges and regressions were shown in Table 2.

3.2.4. Successive derivative ratio spectra(SDRS)

For determination of MTF, the absorption spectra of different concentrations of MTF were divided by the absorption spectrum of 2.5 µgmL^{-1} EMG, and the ratio spectra were obtained. First derivative of the ratio spectra at $\Delta\lambda$=20, and a scaling factor of 10 were obtained,

then the second ratio spectra were obtained by dividing the first derivative of the ratio spectra MTF by the first derivative spectrum of [d/dλ] [2.0 μgmL^{-1} GLM / 2.5 μgmL^{-1} EMG]. The first derivative of the second ratio spectra at Δλ=20, and a scaling factor of 1.0 from which the concentration of MTF was determined by measuring the peak amplitude at 284nm as shown in (Fig. 6).

For determination of EMG, the recorded spectra of different concentrations of EMG were divided by the spectrum of 2.0 μgmL^{-1} GLM, and the first derivative of the ratio spectra at Δλ=20, and a scaling factor of 10 were obtained, then the second ratio were divided by the first derivative ratio spectra [d/dλ] [2.0 μgmL^{-1}MTF / 2 μgmL^{-1}GLM]. The first derivative of these vectors were obtained with Δλ= 20nm, and a scaling factor of 1.0 from which the concentration of MTF was determined by measuring the peak amplitude at 242nm as shown in (Fig. 7).

By the same method, the recorded spectra of the different concentrations of GLM were divided by the spectrum of 3.0 μgmL^{-1} MTF, and the first derivative of the ratio spectra were divided by [d/dλ] [2.5 μgmL^{-1}EMG / 3.0 μgmL^{-1}MTF], to obtain the second ratio spectra. The first derivative of these vectors were optimized at Δλ= 20nm, and a scaling factor of 1.0, from which the concentration of MTF was determined by measuring the peak amplitude at 266nm as shown in (Fig. 8)

The calibration curves were constructed by plotting the amplitude values at 284, 242, and 266nm for MTF, EMG, and GLM, respectively with respect to the corresponding concentration range of 1.0-10, 2.5-30, and 1.0-10μgmL^{-1} for MTF, EMG, and GLM, respectively, from which the regression equations were calculated as shown in Table 2.

3.3. Methods validation

Methods validation was performed according to ICH guideline [38]

3.3.1. Range and linearity

Linearity was studied for MTF, GLM, and EMG. A linear relationship between peak amplitude and component concentration was obtained, ranging among 1.0-10,1.0-10 and 2.5-30μgmL^{-1} for MTF, GLM, and EMG, respectively. The regression equations were also computed. Estimation of methods linearity by preparing different calibration curves, repeating each concentration three times. The linearity of the calibration curves was validated by the high value of correlation coefficients. The concentration ranges and other statically parameters for three methods were listed in Table 2. The analytical data of the calibration curves including standard deviations for the slope, intercept, and residual (Sb, Sa, and $S_{y/x}$) are summarized in Table 2.

3.3.2. Limit of Detection and Quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated as LOD = 3.3 (σ/S) and LOQ = 10 (σ/S), where 'σ' represents standard deviation of the response and 'S' is the slope of the calibration line, as shown in Table 2.

3.3.3. Accuracy

The accuracy of the proposed method under linearity was checked by determination of different concentration of pure samples by the standard addition technique, this performed by the addition of known amount of pure drugs to the known concentration of pharmaceutical solution, good accuracies were obtained from the recoveries which applied as shown in Table 3.

3.3.4. Precision

Evaluation of repeatability by analyzing of the drugs by using three different concentrations of MTF (3.0, 6.0, and 9.0µgmL^{-1}), EMG (7.5, 15, and 25µgmL^{-1}), and GLM (5.0, 8.0, and 10µgmL^{-1}) were replicated three times within the same day.

Intermediate precisions were estimated by using three concentrations of the drugs replicated on three different days. The % RSD was calculated and found to be less than 2% as shown in Table 3.

3.3.5. Selectivity

Selectivity of the methods was achieved by the study of different laboratory prepared mixture of MTF, EMG, and GLM for the four spectrophotometric methods within the linearity range. The good percentage recoveries with high selectivity of the proposed method were obtained as in Table 4. Table 5 show no interference from excipients.

3.3.6. Recovery

The proposed methods were successfully applied to different pharmaceutical dosage forms and to check the validity of the proposed method, the recovery of the drugs at different dosage (5.0, 3.0 and 2.5 µg mL^{-1} for MTF, GLM and EMG, respectively) were determined. The recovery for six replicated were founded in the range between %99.0-%100, and %RSD were founded to be less than 2. The standard addition technique was applied by adding different known concentrations of the pure drug (1.0, 3.0 and 5.0 µg mL^{-1} of MTF, 2.0, 4.0 and 6.0 µg mL^{-1} of GLM, and 5.0, 10, and 15µg mL^{-1} of EMG) to different known concentrations of each drug product, repeated three times. The recoveries ranging from 98% to 102% at different analyte concentrations, and %RSD<2. Results obtained shown in Table 5.

3.3.7. Statistical analysis

Table 6 shows the resulted obtained by proposed methods were statistically compared with the reported methods [39, 40, 41]. The calculated student's-t and F- testsat P=0.05, regarding both accuracy and precision; where the results are less than theoretical ones, no significant difference was found. One-way ANOVA was applied to compare the developed methods; Table 7 showed there was no significant difference between the methods and reported one for determination of MTF, EMG, and GLM. The data analysis was carried out by Minitab 17.

4. Conclusion

In the present work four different, accurate, precise, and selective derivative spectroscopic techniques were applied MCRS, DDRS, RDZC, and SDRS for determination of metformin,

empagliflozin, and glimepiride in the synthetic ternary mixture and the pharmaceutical tablets. The methods were able to resolve the overlapped spectra without prior separation of these components in the mixtures. The resulted were obtained by four proposed methods showing good agreement were statistically compared with the reported method.

Upon Validation Linearity, Precision, Accuracy, LOD, LOQ and selectivity were proved to be convenient and effective for the analysis of these drugs in Pharmaceutical Dosage Form. The measured signal showed to be linear over the concentration range 1.0-10,1.0-10 and 2.5-30 μgmL^{-1} for MTF, GLM, and EMG, respectively, with the correlation coefficient between 0.9990-0.9999. The RSD for all parameters were found to be within the prescribed limits. The resulted were obtained by four proposed methods showing satisfactory results were compared with the reported methods. Based on the results obtained and statistical analysis, it is concluded that the method is suitable for estimation of these drug in marketed tablet formulation without any interference of the excipients present in formulation.

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