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Different mathematical processing of absorption, ratio and derivative spectra for quantification of mixtures containing minor component: An application to the analysis of the recently co-formulated antidiabetic drugs; canagliflozin and metformin

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ABSTRACT

In the presented work several spectrophotometric methods were performed for the quantification of canagliflozin (CGZ) and metformin hydrochloride (MTF) simultaneously in their binary mixture. Two of these methods; response correlation (RC) and advanced balance point-spectrum subtraction (ABP-SS) were developed and introduced for the first time in this work, where the latter method (ABP-SS) was performed on both the zero order and the first derivative spectra of the drugs. Besides, two recently established methods; advanced amplitude modulation (AAM) and advanced absorbance subtraction (AAS) were also accomplished. All the proposed methods were validated in accordance to the ICH guidelines, where all methods were proved to be accurate and precise. Additionally, the linearity range, limit of detection and limit of quantification were determined and the selectivity was examined through the analysis of laboratory prepared mixtures and the combined dosage form of the drugs. The proposed methods were capable of determining the two drugs in the ratio present in the pharmaceutical formulation CGZ:MTF (1:17) without the requirement of any preliminary separation, further dilution or standard spiking. The results obtained by the proposed methods were in compliance with the reported chromatographic method when compared statistically, proving the absence of any significant difference in accuracy and precision between the proposed and reported methods.

1. Introduction

Canagliflozin (CGZ); (2*S*, 3*R*, 4*R*, 5*S*, 6*R*)-2-{3-[5-(4-fluoro-phenyl)-thiophen-2-ylmethyl]-4-methyl-phenyl}-6-hydroxymethyltetrahydropyran-3,4,5-triol (Fig. 1) is a glucose lowering agent [1]. CFZ is a member of the gliflozin class or sodium-glucose co-transporter (SGLT2) inhibitors, which are indicated for the treatment of type 2 diabetes mellitus [2]. SGLT2 inhibitors block the reabsorption of glucose causing it to be excreted in the urine [3], they are possibly prescribed as monotherapy or in combination with any of the existing classes of glucose-lowering agents such as metformin [4]. Metformin hydrochloride (MTF); 1,1–Dimethyl biguanide monohydrochloride (Fig. 1) is a biguanide anti-diabetic [5]. It is administered orally for the treatment of type 2 diabetes mellitus where it is the drug of choice for patients suffering from overweight [6]. Biguanides possibly exert their action through delaying the absorption of glucose from the gastrointestinal tract, increasing the insulin sensitivity and glucose uptake into cells, and inhibiting liver glucose production. The main action of MTF lies in increasing the glucose transport across the cell membrane in skeletal muscles [6].

Canagliflozin and metformin hydrochloride are recently co-formulated in Vokanamet® tablets (50 mg CGZ and 850 mg MTF); this formulation is specifically recommended when MTF alone, or in combination with other diabetes medicines, including insulin, do not provide satisfactory control of diabetes.

MTF is official in both the United States Pharmacopoeia (USP) [5] and the British Pharmacopoeia (BP) [7], while as CGZ is a newly released drug in the market, thus, it is not yet official in any pharmacopoeia.

Literature survey has revealed that MTF and CGZ were determined as single components in pharmaceutical formulations as well as in biological fluids by several methods including spectrophotometry, where CGZ as a single component was directly determined at its λ_{max} in the zero order spectrum [1], for MTF; it was determined as a single component either through its reaction with ninhydrin in alkaline medium [8]

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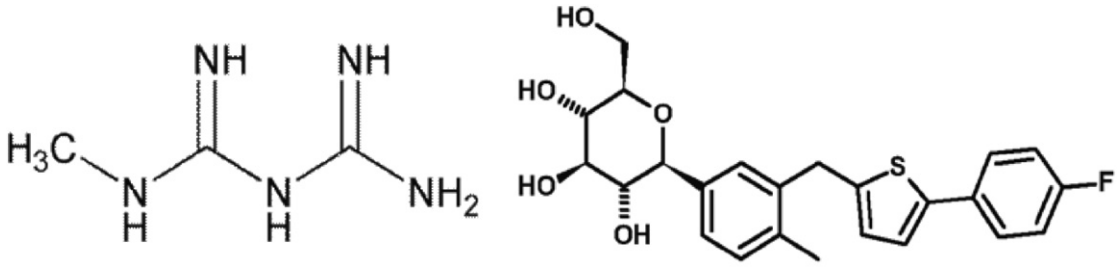

Metformin (MTF) **Canagliflozin (CGZ)**

Fig. 1. The chemical structure of metformin and canagliflozin.

or through the formation of a charge transfer complex with iodine in acetonitrile medium [9]. High performance thin layer chromatography (HPTLC) [10,11], HPLC-UV [12–15], HPLC-fluorescence detection [16], HPLC-MS/MS [17–20] and capillary electrophoresis [21,22] were also utilized. However, the simultaneous determination of MTF and CGZ was performed with the utilization of HPLC-UV [23–25] only. No spectrophotometric methods were reported for the analysis of the proposed drugs in their combination.

In the last few years the mathematical spectrophotometric methods of analysis as well as the chemometric techniques have powerfully emerged and to a pronounced extent have replaced the other chromatographic methods. This could be attributed to their simplicity, less time and solvent consumption, lack of prior extraction or separation steps and of course lower cost. Thus, nowadays the utilization of the spectrophotometric and chemometric methods became vital for the analytical studies which are performed in the quality control laboratories or for the routine analysis of different pharmaceutical products either in the research or industry laboratories. More precisely the spectrophotometric methods are considered advantageous than the chemometric techniques as they do not require purchasing a specific software or a trained person to operate the software.

The quantitative spectrophotometric resolution of the mixtures of two or more compounds having overlapped spectra is an interesting issue for analytical chemists [26–32]. For resolving complex mixtures, the analytical chemist needs new analytical methods or approaches to obtain accurate, precise and reliable results. Therefore, the analytical chemists have focused mainly on the use of a new mathematical technique or the combined use of the mentioned approaches together with traditional analytical techniques to analyze binary mixtures or ternary mixtures after its conversion to binary one by using successive resolution techniques includes successive ratio subtraction and successive derivative subtraction [33]. The diversity of the spectrophotometric methods is considered the most important feature of spectrophotometric analysis, where it gives the analyst a chance to choose the most suitable method for analysis.

The lack of simple mathematical spectrophotometric methods for the simultaneous determination of CGZ and MTF has encouraged the development of four UV-spectrophotometric methods with minimal manipulation steps. Accordingly, the aim of this work is to develop two novel methods namely; response correlation (RC) and advanced balance point-spectrum subtraction (ABP-SS). Moreover, a comparative study between these two methods and another two recently established methods; advanced amplitude modulation (AAM) and advanced absorbance subtraction (AAS) was conducted regarding their ability to resolve overlapped spectra of the binary mixture of CGZ and MTF without any preliminary separation. The proposed methods have showed significant advantages over the reported HPLC-UV methods [23–25] of being rapid, easily applied without any requirements for optimization of conditions such as pH, temperature or flow rate and thus highly economical and time saving. Additionally, these proposed spectrophotometric methods have eliminated the utilization of any harmful chemicals to both health and environment as the main utilized solvent is methanol which is considered less hazardous than the buffers (acetate or phosphate) and solvents (acetonitrile or a mixture of acetonitrile and methanol) that were used in the reported chromatographic methods [23–25]. Thus, our proposed methods could be considered as ecofriendly methods of analysis. Finally, all the developed methods were validated according to the ICH guidelines [34] to decide their appropriateness for the intended use.

1.1. Theory

1.1.1. Response Correlation Method (RC)

This novel method could be applied for a mixture composed of components X and Y whose zero order absorption spectra show partial or complete overlap and intersect in an isoabsorptive point (λ_{iso}) at which both components show the same absorptivities $a_X = a_Y$. The absorbance at this wavelength A_{iso} represent ($A_X + A_Y$) which is retained in the ratio spectra as P_{iso} ($P_X + P_Y$) using pure X as a divisor (X′) (either normalized spectrum of X or a concentration of X). In the application of this method on the binary mixtures two wavelengths (λ_{iso} and λ_2) were chosen one of them is the isoabsorptive point; where component X has equal absorbance at these wavelengths. The absorbance difference ΔA ($A_{iso} - A_2$) between the two chosen wavelengths on the mixture spectra is corresponding to the concentration of component Y only; while the difference for component X equals to zero.

This approach starts with computation of two regression equations using zero order and ratio spectra for different concentrations of pure Y representing the relationship between ($A_{iso} - A_2$) versus A_{iso} Eq. (1) and A_{iso} versus its corresponding P_{iso} Eq. (2).

$$\Delta A = \text{Slope } A_{Y(iso)} + \text{intercept} \quad (1)$$

$$A_{Y(iso)} = \text{Slope } P_{Y(iso)} + \text{intercept} \quad (2)$$

For a mixture of the two component (X) and (Y), recording its zero order absorption spectrum the $A_{Y(iso)}$ corresponding to component Y in the mixture can be calculated using Eq. (1) using the absorbance difference at the selected wavelengths. Then, substituting in Eq. (2) by the obtained $A_{Y(iso)}$ to get the corresponding $P_{Y(iso)}$ of component Y in the mixture.

The amplitude $P_{X(iso)}$ of constant ($\frac{X}{X'}$) in the mixture is calculated via subtracting the recorded amplitude ($P_{M(iso)}$) of the ratio spectrum of the mixture at λ_{iso} ($P_{X(iso)} + P_{Y(iso)}$) and that corresponding to Y in the mixture which is calculated by substitution in Eq. (2) at the same wavelength (λ_{iso}).

$$P_{X(iso)} = P_{M(iso)} - P_{Y(iso)} \quad (3)$$

The zero order absorption spectrum of X for each mixture was obtained by multiplying the calculated amplitude corresponding to X in the mixture $P_{X(iso)}$ by the divisor (X′) (normalized spectrum or concentration of X). While, zero order absorption spectrum of component Y

can be obtained by subtracting the zero order spectrum of X from the absorption spectrum of the mixture.

Thus, the concentrations of components X and Y in the mixture are calculated using the corresponding regression equation obtained by plotting the absorbance values of the zero order spectra of pure X or Y at their λ_{max} against their corresponding concentration.

The normalized spectrum is obtained by dividing the whole spectrum of the analyte by its corresponding concentration to obtain a new spectrum representing the absorptivity of the analyte of interest versus all the measured wavelengths.

This method has the advantage of getting the zero-order absorption spectrum of both drugs in the mixture which act as spectral profile of the drug subsequently the drugs were analyzed by using the absorbance value at their λ_{max} which offered maximum accuracy and precision with minimum number of manipulating steps. In addition, this method could be applied for analysis of binary mixtures where their spectra showing no extension or the spectrum of one of the components is extended and its spectrum shows low absorbance (due to its poor absorptivity or its low concentration) at the extended part which hinders the analysis of this mixture by the methods utilizing the constant at the extended region using either zero order absorption spectrum or derivative spectrum due to inaccurate determination of the constant value. Thus, this method is a suitable method for mixtures containing minor component whose spectrum shows slight or no extension.

1.1.2. Advanced Balance Point-spectrum Subtraction Method (ABP-SS)

This method is a modification of the derivative compensation method [35–37]. It is utilized when the absorption spectra of two components X and Y are severely overlapped or when the determination of a minor component in the presence of a major component is challenging. Consequently, the spectrum of the mixture of the two analytes is assumed to possess a gross curve which does not have the characteristics of either of the pure compounds.

This method can be applied on the zero order absorption spectrum D^0 or derivative in any order (n) (D^n). The method starts with the calculation of response ratio (RR) value of pure Y which is the absorbance ratio (AR) at two absorbance maxima in case of D^0 or the amplitude ratio (PR) at its maxima and minima (wavelength $D^n\lambda_1$ / wavelength $D^n\lambda_2$) in case of derivative spectra, where component X shows overlap at λ_1 and has no or slight contribution at λ_2.

For the analysis of mixture containing X and Y; the method monitors the change in the response ratio of the mixture (the absorbance ratio in case of D^0 or the amplitude ratio in case of D^n) upon subtracting different concentrations of the pure minor component (X) (C_X) above and below that expected to be in the mixture solution via spectrum subtraction using the data set manipulation step in spectrophotometer software. The mixture spectrum is minuend and spectrum of X is subtrahend i.e. [mixture (m) − minor component (r)] where ($m = D_X + D_Y$) and ($r = D_X$) to get the difference spectrum ($D_m - D_X$) for each concentration of X. Thus, the balance point characteristics of the mixture gradually approach pure drug (Y) as C_X increases and finally coincide with the response ratio of the mixture of pure drug (Y). At this balance point C_X in the mixture is equal to the subtracted C_X.

Simply, this balance point can be specified using regression equation representing linear relationship of the response ratio at the two chosen wavelengths ($D_m - D_X$) λ_1 / ($D_m - D_X$) λ_2 of each difference spectrum of the mixture versus the corresponding concentration of pure X (C_X).

$$(D_m - D_X)_1 / (D_m - D_X)_2 = \text{Slope } C_X + \text{intercept} \quad (4)$$

The concentration of the minor component X in the mixture can be calculated by substitution in regression equation (Eq. (4)) with the calculated (RR) value of pure Y (D_{Y1}) / (D_{Y2}) at the two chosen wavelengths. Specification of the balance point via regression equation (Eq. (4)) cancels tedious manipulation steps until the mixture spectrum and that of pure Y coincide over each other.

This method has advantage over the well-established compensation method [35–37] that it cancel the instrumental error which may be occurred upon the use of different concentrations of pure X in the reference cell instead of the blank.

2. Experimental

2.1. Apparatus and Software

Spectrophotometric measurements were carried on a JASCO V-530 double beam UV-VIS spectrophotometer. The spectral acquisition and elaboration of the obtained data was done using a Spectra Manager Program (JASCO) software. The light absorption in the ultraviolet region (200–400 nm) was measured using Quartz cuvettes of 1-cm pathlength.

2.2. Chemicals

Pure samples: Canagliflozin was purchased from Beijing Huikang Boyuan Chemical Technology Co. Ltd. (Beijing, China), its purity was found to be 100.23% ± 1.06 when checked by a reported method [23]. Metformin hydrochloride was kindly supplied by Chemical Industries Development (CID) Co. (Giza, Egypt) and its purity was found to be 100.02% ± 1.13 when checked by a reported method [23].

Market sample: Vokanamet® tablets (B.N EGZT300) manufactured by Janssen-Cilag International NV (Beerse, Belgium), labeled to contain 850 mg metformin hydrochloride and 50 mg canagliflozin. Vokanamet® tablets were purchased from online Canadian pharmacy.

Solvents: Methanol (HPLC grade, ≥99.9%) was obtained from sigma-Aldrich (Steinheim, Germany).

2.3. Standard Solutions

The stock solutions of CGZ and MTF were prepared each one separately by dissolving the appropriate weight of each analyte in methanol using 100-mL volumetric flasks to obtain stock solutions with the concentration of 1 mg/mL. The stock solutions were stable for one week when stored in a refrigerator at 4 °C.

The working solutions were freshly prepared on the day of analysis through dilution of the stock solutions with methanol to achieve a concentration of 0.1 mg/mL for each of CGZ and MTF.

2.4. Procedures

2.4.1. Spectral Characteristics

The zero-order absorption spectra (D^0) of the two analytes were recorded in the range of 200–400 nm using methanol in the blank cell.

2.4.2. Construction of Calibration Graphs

2.4.2.1. Response Correlation Method (RC). The absorption spectra of MTF solutions (1–20 μg/mL) were scanned and the corresponding absorbance at 231 nm, 254 nm and 236 nm was measured. A calibration graph was constructed representing the linear relationship between the difference in MTF absorbance at 231 nm and 254 nm ($A_{231\ nm} - A_{254\ nm}$) against the absorbance at 254 nm, consecutively the regression equation was calculated. Then the scanned absorption spectra of MTF (1–20 μg/mL) were divided by the normalized divisor of CGZ and the amplitude of the different obtained ratio spectra of MTF at 254 nm (P_{iso}) was recorded. A calibration graph representing the linear relation between A_{iso} of different concentrations of pure MTF versus its corresponding P_{iso} was plotted and the regression equation was computed.

Another calibration graph was constructed representing the linear relationship between the different concentrations of pure MTF and its corresponding absorbance at 236 nm (λ_{max}).

Lastly, the absorption spectra of CGZ solutions (1–30 μg/mL) were scanned and the corresponding absorbance at 290 nm was measured. A calibration graph was constructed representing the linear relationship between the different concentrations of pure CGZ and its corresponding absorbance at 290 nm (λ_{max}).

2.4.2.2. Advanced Balance Point-spectrum Subtraction Method (ABP-SS). Utilizing the second derivative: The second derivative spectra (D^2) of MTF (1–20 μg/mL) were obtained. A calibration graph was constructed representing the linear relationship between the different concentrations of MTF and its corresponding D^2 amplitude at 252 nm. Additionally, the D^2 amplitude ratios of different concentrations of MTF at 237 nm and 252 nm ($D^2_{237\ nm}$ / $D^2_{252\ nm}$) were determined and the mean of these amplitudes (PR) was calculated and was found to be −2.185.

Utilizing the zero order (D^0): The scanned spectra of MTF (1–20 μg/mL) were used to construct a calibration graph representing the linear relationship between the different concentrations of MTF and its corresponding absorbance at 239 nm. Additionally, the absorbance ratios (AR) of different concentrations of MTF at 254 nm and 239 nm ($A_{254\ nm}$ / $A_{239\ nm}$) were determined and the mean of these absorbance ratios (AR) was calculated and was found to be 0.1381.

2.4.2.3. Advanced Amplitude Modulation Method (AAM). The scanned absorption spectra of MTF (1–20 μg/mL) were divided by the normalized divisor of CGZ and the amplitude of the different obtained ratio spectra of MTF at 254 nm and 225 nm were recorded. Two calibration graphs were constructed; the first one representing the linear relationship between the different concentrations of MTF and its corresponding amplitudes at 254 nm, where a unified regression equation was calculated. The second calibration graph representing the linear relationship between the difference in the amplitudes of different concentrations of MTF at 225 nm and 254 nm ($P_{225\ nm} - P_{254\ nm}$) against the corresponding amplitude at 254 nm, consecutively the regression equation was computed.

2.4.2.4. Advanced Absorbance Subtraction Method (AAS). The absorption spectra of MTF solutions (1–20 μg/mL) were scanned and the corresponding absorbance at 231 nm and 254 nm was measured. Two calibration graphs were constructed; the first one representing the linear relationship between different concentrations of MTF and its corresponding absorbance at 254 nm (A_{iso}), where the unified regression equation was computed. The second calibration graph representing the linear relationship between the difference in MTF absorbance at 231 nm and 254 nm ($A_{231\ nm} - A_{254\ nm}$) against the absorbance at 254 nm, consecutively the regression equation was calculated.

2.4.3. Application to Laboratory Prepared Mixtures

Different aliquots of CGZ and MTF were transferred from their working solutions into a series of 10-mL volumetric flasks to prepare six mixtures containing different ratios of the analytes. The volumes were completed with methanol. The different mixtures were scanned in the range 200–400 nm and the corresponding spectra were stored in the computer. The different manipulation steps of each method were carried out and the concentrations of CGZ and MTF were determined by substitution in the corresponding regression equations.

Regarding the (ABP-SS) method in the second derivative: For determining the concentration of CGZ in the mixtures; spectrum subtraction was carried out between the D^2 spectra of each mixture and D^2 spectra representing different concentrations of CGZ above and below that expected to be in the mixture ($D^2_{mix} - D^2_{CGZ(Conc\ 1)}$), ($D^2_{mix} - D^2_{CGZ(Conc\ 2)}$), ($D^2_{mix} - D^2_{CGZ(Conc\ 3)}$), … and so on, consequently the D^2 amplitudes at 237 nm and 252 nm (obtained after subtraction) were recorded. Then a calibration graph was constructed for every mixture representing the relation between the different concentrations of CGZ which are above and below that present in the mixture against the $(D^2_{mix} - D^2_{CGZ})_{\lambda 237\ nm}$ / $(D^2_{mix} - D^2_{CGZ})_{\lambda 252\ nm}$ and the regression equation was calculated. By substitution in this equation with the calculated amplitudes ratio value (PR) which was equal to −2.185 will give the concentration of CGZ in the mixture. However, for the (ABP-SS) method in the zero order, the same previous steps were followed but using the zero order spectra instead of the D^2 spectra and the constructed calibration graph was representing the relation between the different concentrations of CGZ which are above and below that present in the mixture against the $(A_{mix} - A_{CGZ})_{\lambda 254\ nm}$ / $(A_{mix} - A_{CGZ})_{\lambda 239\ nm}$. Then the concentration of CGZ in the mixture was calculated from the corresponding regression equation by substitution with the calculated absorbance ratios (AR) value which was equal to 0.1381.

2.4.4. Application to Pharmaceutical Preparation

Ten Vokanamet® tablets were accurately weighed, finely powdered and mixed homogeneously. An accurate weight equivalent to one tablet was transferred into a 100-mL beaker followed by the addition of 50 mL methanol. The solution was ultrasonicated for 30 min, cooled and filtered into a 100-mL volumetric flask then the volume was completed with the same solvent to obtain Stock 1. One milliliter aliquot of Stock 1 was transferred to 100-mL volumetric flask and the volume was completed with methanol to obtain Stock 2. Finally, 2.3 mL aliquot of Stock 2 was transferred into a 10-mL volumetric flask to obtain a working solution with claimed concentration of 1.15 μg/mL CGZ and 19.55 μg/mL MTF in the ratio 1:17, respectively. All the proposed methods were applied for the analysis of the pharmaceutical preparations solutions using the procedures mentioned under analysis of laboratory prepared mixtures. The concentration of each analyte was calculated using the corresponding regression equation for each method.

3. Results and Discussion

Zero-order absorption spectra (D^0) of CGZ and MTF (Fig. 2a) were overlapped which suggested the necessity for the development of different spectrophotometric methods for the resolution of such a combination. Our work was devoted to the analysis of Vokanamet®

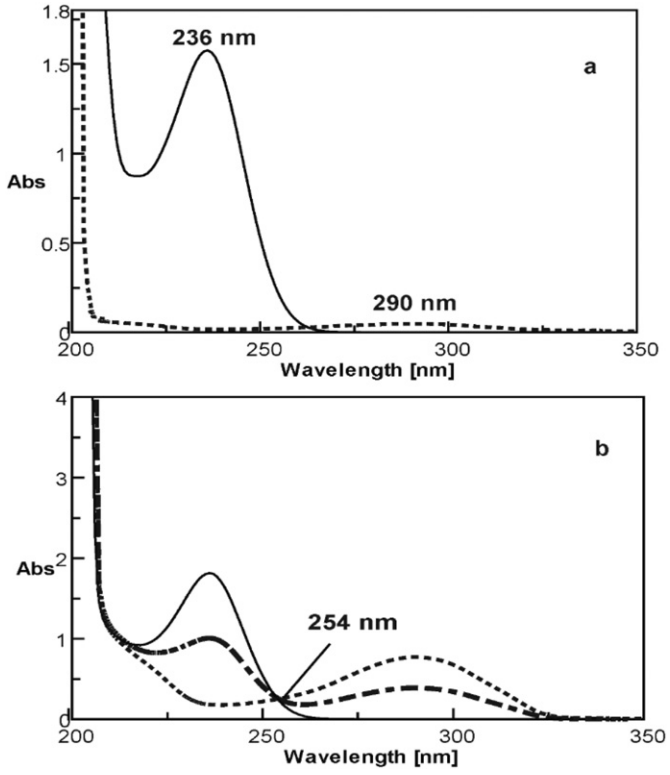

Fig. 2. Zero order spectra of: a - 17 μg/mL MTF (—) and 1 μg/mL CGZ (…..) and b - 20 μg/mL of MTF (—), 20 μg/mL of CGZ (…..) and the binary mixture of 10 μg/mL of each (–·–·).

tablets where MTF is co-formulated with CGZ in the ratio of 17:1, respectively. Unfortunately, MTF which is considered to be the major analyte in the pharmaceutical formulation is also of higher absorptivity, which encounters a difficulty for the determination of CGZ at its λ_{max}, at which CGZ shows low absorbance specifically when present in low concentrations. Additionally, CGZ although being the minor analyte however, it shows slight absorbance at 236 nm which is the λ_{max} of MTF. So novel, easy applicable and accurate spectrophotometric methods were developed based on measurements carried at two chosen wavelengths either in zero order, second derivative or ratio spectra, for the simultaneous analysis of CGZ and MTF in their bulk powders, in laboratory prepared mixtures as well as in pharmaceutical formulation. Furthermore, comparison of the novel methods with recently established ones and conductance of a statistical comparison between all these methods regarding their capability for the determination of the cited analytes were performed.

3.1. Response Correlation Method (RC)

This method was based on two main criteria: First the selection of two wavelengths λ_{iso} and λ_2 at which one component showed significant difference in absorbance at these two wavelengths while the other component showed equal absorbance. Second the presence of the isosbestic point in the zero order absorption spectrum would be retained at the same point (wavelength) in the ratio spectrum which is obtained after division by one component as a divisor.

The absorption spectra of CGZ and MTF demonstrated the presence of an isosbestic point (Fig. 2b) in which the mixture of the two analytes would act as a single component and would give the same absorbance value as the pure analytes. In this method two wavelengths were selected; 254 nm (λ_{iso}) and 231 nm (λ_2) at which MTF showed difference in absorbance at these two wavelengths, this difference in absorbance is directly proportional to the concentration of MTF while CGZ had equal absorbance (i.e. the absorbance difference is integrally equal to zero). A calibration graph was constructed for pure MTF (1–20 μg/mL) demonstrating the relationship between ($A_{231\ nm} - A_{254\ nm}$) and $A_{254\ nm}$ which showed a linear response. The calibration graph possessed the following equation:

$$(A_{231} - A_{254}) = 5.3619\ A_{254} - 0.0697\ (r^2 = 0.9998) \quad (5)$$

The absorbance of MTF at 254 nm (A_{iso} or A_{254} for MTF) in the mixture could be determined by substituting with the difference in absorbance between 231 nm and 254 nm of the mixture ($A_{231\ nm} - A_{254\ nm}$ for the mixture) in the previous equation.

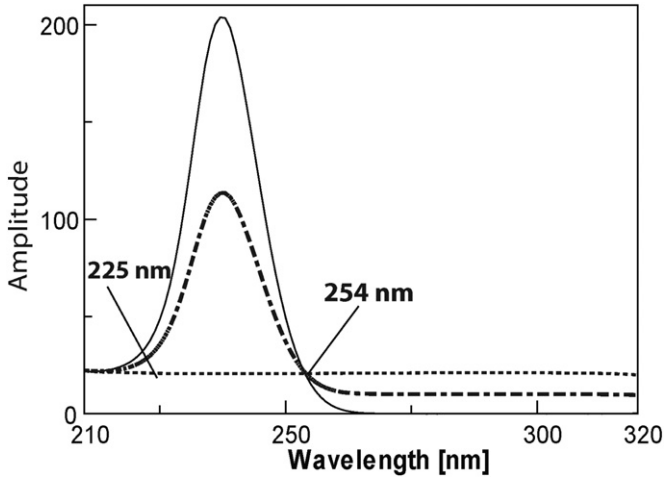

Fig. 3. Ratio spectra of 20 μg/mL of MTF (—), 20 μg/mL of CGZ (.....) and the binary mixture of 10 μg/mL of each (–·–) using the normalized spectrum of CGZ as a divisor.

Then the scanned absorption spectra of MTF (1–20 μg/mL) were divided by the normalized divisor of CGZ, where the P_{iso} is retained at 254 nm the same wavelength as A_{iso} (Fig. 3). Then the amplitudes of the obtained ratio spectra of MTF at 254 nm (P_{iso}) were recorded. A calibration graph representing the linear relation between A_{iso} of different concentrations of pure MTF versus its corresponding P_{iso} was plotted and the regression equation was found to be:

$$A_{254} = 0.0112\ P_{254} + 0.0033\ (r^2 = 0.9996) \quad (6)$$

Subsequently, the scanned absorption spectra of the different laboratory prepared mixtures were divided by CGZ normalized divisor. The amplitude of MTF only at 254 nm (P_{254} MTF) in the ratio spectra of the mixtures could be calculated by substituting the previously obtained A_{254} from Eq. (5) in Eq. (6). However, the amplitude of CGZ at 254 nm (P_{254} CGZ) in the ratio spectra of the mixture is calculated by subtracting the calculated (postulated) P_{254} MTF (using Eq. (6)) from the recorded (measured) P_{254} of the mixture (P_{254} CGZ = P_{254} mixture − P_{254} MTF)

The zero order absorption spectrum of CGZ could be obtained by multiplying the obtained P_{254} CGZ of each mixture by CGZ normalized divisor (Fig. 4). Then, the zero order absorption spectrum of MTF could be resolved (Fig. 4) by utilizing spectrum subtraction method, where the obtained absorption spectra of CGZ were subtracted from the corresponding absorption spectra of the mixtures. The concentrations of CGZ and MTF were calculated by substitution in the corresponding regression equation representing the linear relationship between the concentrations of pure CGZ and the absorbance at λ_{max} of each proposed drug (290 nm for CGZ and 236 nm for MTF).

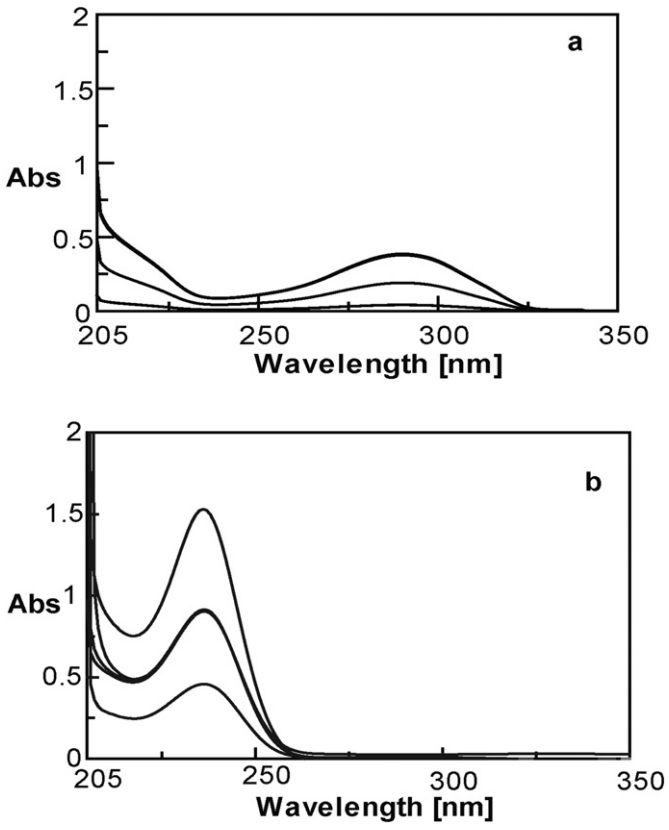

Fig. 4. a - The zero order spectra of CGZ in the different laboratory prepared mixtures obtained by multiplying the normalized spectrum of CGZ by the different Piso values of CGZ in the mixtures and b - the zero order spectra of MTF obtained after subtraction of the previously obtained zero order spectra of CGZ from the corresponding laboratory prepared mixtures.

This method was applicable for resolution of partial and completely overlapped spectra and offered several advantages: The results were not affected by the choice of divisor due to the utilization of the normalized divisor. Additionally, the presence of the isosbestic point allowed for the direct measurement of the minor component in the mixture due to the leveling effect exerted by the major component. Furthermore, both components were recovered in their zero order absorption spectra which act as fingerprints for the drug where the proposed drugs were measured at their λ_{max} with maximum accuracy and precision thus, diminishing the noise error.

3.2. Advanced Balance Point-spectrum Subtraction Method (ABP-SS)

3.2.1. Utilizing the second derivative spectra D^2

The D^2 spectra of CGZ and MTF are demonstrated in Fig. 5. Two wavelengths were chosen for MTF; 237 nm and 252 nm where CGZ showed overlap in the first one and had no contribution in the latter. The D^2 amplitude ratios of different concentrations of pure MTF (1–20 μg/mL) at the two selected wavelengths D^2_{237}/D^2_{252} were determined and the mean value of these amplitude ratios (PR) was calculated to be −2.185. Then spectrum subtraction was carried out (utilizing the spectrophotometer's software), where the D^2 spectra of different concentrations of CGZ were subtracted from the D^2 spectra of the laboratory prepared mixtures. As an example in the mixture with the concentration 1 μg/mL CGZ and 17 μg/mL MTF (the concentration present in the pharmaceutical dosage form); the D^2 spectra of 0.25, 0.5, 0.75, 1, 2.5 and 5 μg/mL CGZ were successively subtracted from the D^2 spectrum of the mixture and the amplitudes at 237 nm and 252 nm were recorded. A regression equation was constructed representing the linear relationship between the different utilized concentrations of CGZ against the corresponding D^2_{237}/D^2_{252}. The regression equation was computed and was found to be

$$P = -0.0226C - 2.1626 \ (r = 0.9996)$$

where P is the amplitude ratio of the mixture (D^2_{237}/D^2_{252}) and C is the concentration of pure CGZ.

The concentration of CGZ in the mixture was calculated by substituting in the regression equation with the mean amplitude ratio of pure CGZ previously determined ($D^2_{237}/D^2_{252} = -2.185$). The concentration of MTF in the mixture could be determined from the calibration graph representing the linear relationship between the different concentrations of MTF (1–20 μg/mL) and its corresponding D^2 amplitude at 252 nm as CGZ had no contribution at this wavelength.

3.2.2. Utilizing the zero order absorption spectrum (D^0)

Two wavelengths were chosen for MTF; 239 nm and 254 nm. The absorbance ratios of different concentrations of pure MTF (1–20 μg/mL) at the two selected wavelengths A_{254}/A_{239} were determined and the mean value of these absorbance ratios (AR) was calculated to be 0.1381. Then spectrum subtraction was carried out (utilizing the spectrophotometer's software), where the D^0 spectra of different concentrations of CGZ were subtracted from the D^0 spectra of the laboratory prepared mixtures. As an example in the mixture with the concentration 1 μg/mL CGZ and 17 μg/mL MTF (the concentration present in the pharmaceutical dosage form); the D^0 spectra of 0.25, 0.5, 0.75, 1, 2.5 and 5 μg/mL CGZ were successively subtracted from the D^0 spectrum of the mixture and the absorbance at 239 nm and 254 nm were recorded. A calibration equation was constructed representing the linear relationship between the different utilized concentrations of CGZ against the corresponding absorbance ratio A_{254}/A_{239}. The regression equation was computed and was found to be

$$A = -0.0088C + 0.147 \ (r = 0.9992)$$

where A is the absorbance ratio of the mixture (A_{254}/A_{239}) and C is the concentration of pure CGZ.

The concentration of CGZ in the mixture was calculated by substituting in the regression equation with the mean absorbance ratio (AR) of pure CGZ previously determined ($A_{254}/A_{239} = 0.1381$). The concentration of MTF in the mixture could be determined after spectrum subtraction of the absorption spectrum of 1 μg/mL CGZ from that of the mixture then the absorbance of MTF at 239 nm was recorded and its concentration was calculated by substitution in the calibration graph representing the linear relationship between the different concentrations of MTF (1–20 μg/mL) and its corresponding absorbance at 239 nm.

The main advantages of the ABP-SS method are: No need of using of the drug as a divisor. It is simpler, accurate and precise than compensated method [35–37] since it cancelled any instrumental error resulting from using minor drug as a blank instead of solvent. ABP-SS method utilizing zero order absorption spectrum has advantage over that of derivative as it eliminates derivative step so signal to noise ratio is enhanced, while its drawback that it gives unsatisfactory results in case of mixtures of severely overlapping spectra in contrast to ABP-SS method utilizing derivative which shows more accurate results.

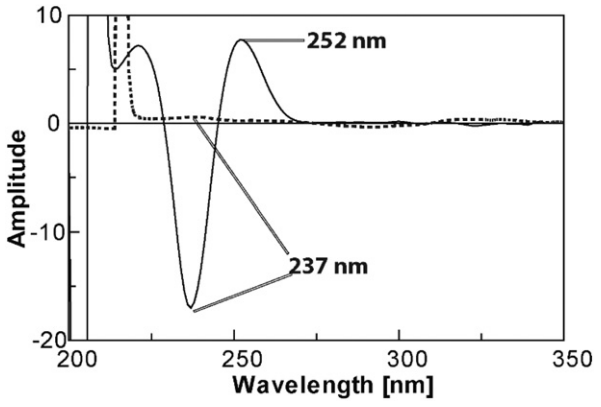

Fig. 5. Second order derivative spectra of 17 μg/mL MTF (—) and 1 μg/mL CGZ (.....) using a scale factor of 1000.

3.3. Advanced Amplitude Modulation Method (AAM)

This method was recently introduced by Lotfy et al. [28] and could be applied when the spectra of two components are severely overlapped and possess an isosbestic point (λ_{iso}) in the zero order spectrum and is thus retained in the ratio spectrum. Examining the zero order spectra of CGZ and MTF revealed the presence of isosbestic point at 254 nm (Fig. 2b), additionally, the ratio spectrum of MTF (using the normalized spectrum of CGZ as a divisor) showed the same isosbestic point at 254 nm (Fig. 3). To apply the AAM method, the absorption spectra of the laboratory prepared mixtures were divided by the normalized divisor of CGZ, where the ratio spectra were obtained. Two wavelengths were chosen λ_{iso} at 254 nm and λ_2 at 225 nm and the amplitude of the ratio spectra at these two wavelengths was recorded. A calibration graph was constructed representing the linear relationship between the differences of the ratio amplitudes ($P_{225} - P_{254}$) of different concentrations of pure MTF (1–20 μg/mL) against the corresponding ratio amplitude at 254 nm and the regression equation was computed and was found to be as follows:

$$(P_{225} - P_{254}) = 1.2211\ P_{254} - 1.0397 \ (r^2 = 0.9997)$$

By substituting with $P_{225} - P_{254}$ for the mixture, thus the postulated amplitude of MTF at 254 nm (λ_{iso}) in the mixture could be obtained. Subsequently, when subtracting the obtained postulated amplitude of MTF at λ_{iso} from the practically recorded amplitude of the mixture, then the postulated amplitude of CGZ at 254 nm could be obtained. Since these postulated amplitudes of MTF and CGZ at 254 nm were

modulated so they directly corresponded to their recorded concentrations in the mixture. However, to eliminate any error, the actual concentration of MTF and CGZ could be calculated using the unified regression equation representing the linear relationship between the amplitude of the ratio spectra of pure MTF or CGZ at 254 nm (λ_{iso}) against the corresponding concentration which was found to be as follows:

$$C_{recorded} = 0.9651\ C_{actual} + 0.2213\ (r^2 = 0.9998)$$

where $C_{recorded}$ represented the recorded amplitude corresponding to the concentrations of pure MTF or CGZ at $\lambda_{iso} = 254$ nm which was obtained from the ratio spectrum using normalized spectrum of CGZ as a divisor and C_{actual} represented their corresponding concentration.

The AAM method has the advantage of measuring the concentrations of the mixture components directly through the utilization of the ratio spectrum, additionally, using a unified regression equation for the determination of the two components' concentration.

3.4. Advanced Absorbance Subtraction Method (AAS)

This method was recently introduced by Lotfy et al. [28]. In this method two wavelengths were selected; 254 nm (λ_{iso}) and 231 nm at which MTF showed difference in absorbance at these two wavelengths, this difference in absorbance is directly proportional to the concentration of MTF while CGZ had equal absorbance (i.e. the absorbance difference is integrally equal to zero). A calibration graph was constructed for pure MTF (1–20 μg/mL) demonstrating the relationship between ($A_{231\ nm} - A_{254\ nm}$) and $A_{254\ nm}$ which showed a linear response. The calibration graph possessed the following equation:

$$(A_{231} - A_{254}) = 5.3619\ A_{254} - 0.0697\ (r^2 = 0.9998)$$

The postulated absorbance ($A_{postulated}$) of MTF at 254 nm (λ_{iso}) in the mixture could be determined by substituting with the difference in absorbance between 231 nm and 254 nm of the mixture ($A_{231\ nm} - A_{254\ nm}$ for the mixture) in the previous equation. Subsequently, the postulated absorbance of CGZ in the mixture at 254 nm was obtained by subtracting the postulated absorbance of MTF at 254 nm from the recorded absorbance of the mixture at 254 nm.

Where $A_{recorded} = A_{MTF} + A_{CGZ}$

In order to calculate the concentration of MTF and CGZ in the mixture; a unified regression equation was constructed which represented the linear relationship between the absorbance of the zero order spectra of either pure MTF or CGZ at 254 nm (λ_{iso}) versus the corresponding concentration.

$$A_{MTF\ or\ CGZ} = 0.0120\ C_{MTF\ or\ CGZ} + 0.0058\ (r^2 = 0.9999)$$

where $A_{MTF\ or\ CGZ}$ is the absorbance of pure MTF or CGZ at 254 nm (λ_{iso}) and $C_{MTF\ or\ CGZ}$ is the corresponding concentration of MTF or CGZ.

The absorbance subtraction method has the advantage that the concentration of the two components in the mixture can be determined using a unified regression equation at the λ_{iso}. Thus the AAS method is considered more advantageous than the traditional isoabsorptive point method [30,32] which is capable of determining the total concentration of the two components, however, one of the components is calculated by using another conventional spectrophotometric method as a complementary method.

4. Methods Validation

It is the process of documenting or proving that the selected method provides analytical data for the intended use. As per the ICH guidelines [34], the objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose as follows:

4.1. Linearity and Range

A linear relationship should be evaluated across the range of the analytical procedure. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration then, the regression line is calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte. The linear range that obeys Beer's law is dependent on the compound analyzed and the detector used. The working sample concentration and samples tested should be in the linear range. The linearity of all the proposed methods was assessed by constructing the different calibration graphs on three different days, where each concentration was repeated for three times. The calibration graphs were constructed within the concentration ranges of CGZ and MTF which were present in the pharmaceutical formulation and which achieved adherence to Beer's law. The linearity of the calibration graphs was judged by the high values of the correlation coefficients. The analytical data of the calibration graphs are summarized in Table 1. The linearity range was 1–20 μg/mL for each of CGZ and MTF by all the proposed methods except for the linearity range of CGZ by the RC method which was 1–30 μg/mL.

4.2. Accuracy

The accuracy of any analytical method could be defined as the nearness of the test results attained by the method to the true value. It is the degree of the exactness of the established analytical method. It might be determined by applying the method to samples or mixtures of excipients to which known amount of analyte was added both above and below the normal levels expected in the samples. Accuracy is then calculated from the test results as the percentage of the analyte recovered by the assay. The accuracy of the proposed spectrophotometric method was determined at three concentration levels of CGZ and MTF by analyzing three replicate samples of each concentration. The obtained percentage recoveries and standard deviations have indicated good accuracy of all the proposed methods as shown in Table 1.

Table 1
Assay parameters and validation sheet obtained by applying the proposed spectrophotometric methods.

Parameter	CGZ	CGZ or MTF		MTF		
	RC	AAM	AAS	RC	ABP-SS D^2	ABP-SS D^0
Wavelength (nm)	D^0 at 290	P at 254	D^0 at 254	D^0 at 236	P at 252	D^0 at 239
Calibration range[a] (μg/mL)	1–30	1–20	1–20	1–20	1–20	1–20
Slope	0.0383	0.9651	0.0120	0.0908	0.4532	0.0863
Intercept	0.0044	0.2213	0.0058	0.0030	0.0588	0.0109
Correlation coefficient	0.9999	0.9998	0.9990	0.9998	0.9999	0.9997
Mean[a]	99.82	101.09	99.09	99.62	99.98	100.55
RSD%	1.30	1.40	1.04	1.28	1.15	0.94
LOD (μg/mL)	0.32	0.31	0.29	0.28	0.31	0.32
LOQ (μg/mL)	0.98	0.95	0.89	0.86	0.95	0.97
Repeatability[a,b] RSD%	1.39	1.45	1.21	1.25	1.10	1.11
Inter-day precision[a,b] RSD%	1.56	1.77	1.44	1.67	1.29	1.46

The mean and RSD% correspond to the mean and relative standard deviation of the percentage recovery.

[a] Average of three experiments.

[b] Relative standard deviation of three concentrations of each drug (5, 10, 15 μg/mL), each concentration repeated for three times.

Table 2
Analysis of laboratory prepared mixtures and dosage form and the application of the standard addition technique by the proposed spectrophotometric methods.

Conc in μg/mL CGZ: MTF	AAM		AAS		RC		ABP-SS D^2		ABP-SS D^0	
	CGZ	MTF	CGZ	MTF	CGZ	MTF	CGZ	MTF	CGZ	MTF
1:10	100.81	100.46	98.74	98.60	98.85	97.69	100.27	99.98	100.65	100.13
1:17[b]	101.19	104.69	100.84	103.27	97.56	99.57	99.12	100.14	101.14	97.89
5:5	98.82	100.58	101.77	100.72	97.45	100.22	101.60	102.18	97.38	102.18
5:10	102.13	102.64	98.24	102.07	99.64	100.00	99.08	102.87	101.18	99.84
10:5	99.20	101.95	98.18	101.32	97.37	99.78	99.87	101.53	99.19	102.69
1:20	98.65	102.97	100.84	102.95	98.37	99.87	102.09	101.99	102.86	101.89
Mean[a] ± SD	100.13 ± 1.44	102.22 ± 1.59	99.77 ± 1.56	101.49 ± 1.71	98.21 ± 0.91	99.52 ± 0.92	100.34 ± 1.26	101.45 ± 1.16	100.40 ± 1.89	100.77 ± 1.82
Dosage form										
Mean[a] ± SD	97.80 ± 1.48	100.44 ± 1.49	97.00 ± 1.58	103.13 ± 1.89	96.80 ± 1.30	98.80 ± 1.53	98.00 ± 1.00	102.64 ± 1.09	101.81 ± 0.84	100.98 ± 0.71
Standard addition	96.87 ± 1.21	99.61 ± 0.79	98.87 ± 0.81	102.06 ± 0.42	97.07 ± 1.01	97.44 ± 1.39	98.73 ± 0.64	101.89 ± 1.17	100.87 ± 1.03	99.61 ± 0.79

The mean and SD correspond to the mean and standard deviation of the percentage recovery.
[a] Average of three experiments.
[b] Ratio present in dosage form.

4.3. Precision

The precision of any analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of homogenous samples. It is usually expressed as the relative standard deviation. Precision is the measurement of the degree of reproducibility or repeatability of the developed method under ordinary operational conditions. The repeatability implies the analysis of replicates of the samples by the same analyst using the same equipment and method over a short period of time while the intermediate precision demands precision study over different days (three successive days). The precision of the proposed spectrophotometric method was determined at three concentration levels, of CGZ and MTF by analyzing three replicate samples of each concentration. The relative standard deviations (RSD) for the results did not exceed 2% (Table 1), proving the high precision of the method. This good level of precision was suitable for quality control analysis of CGZ and MTF in their pharmaceutical formulation.

4.4. Selectivity

The selectivity of a method denotes the extent to which it can estimate particular analyte(s) under certain conditions in a complex mixture comprising other components of similar behavior without any interference from these components [38]. If the response is distinguished from all other responses, the method is said to be selective. The selectivity of the proposed methods was established through the analysis of laboratory prepared mixtures containing CGZ and MTF in different ratios within the linearity range with good percentage recoveries and low standard deviations. In addition satisfactory results of the analysis of the combined dosage form indicates the good selectivity of the methods for the analysis of the cited drugs in presence of the excipient as shown in Table 2.

4.5. Limits of Detection (LOD) and Limit of Quantification (LOQ)

The detection limit of an analytical procedure is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated as an exact value. The limit of quantification is the lowest amount of the analyte in the sample that can be quantitatively determined with definite precision under the specified experimental circumstances. LOQ is principally used for the determination of low levels of active constituent in a product.

$$\mathrm{LOD} = 3.3\frac{\mathrm{S.D}}{\mathrm{Slope}}$$

$$\mathrm{LOQ} = 10\frac{\mathrm{S.D}}{\mathrm{Slope}}$$

where, S.D is standard deviation of y-intercepts of regression lines and slope is the slope of the calibration graph. Both the LOD and the LOQ were calculated and the results are abridged in Table 1.

Table 3
Statistical comparison between the results obtained by the proposed spectrophotometric methods and reported methods [23] for the determination of CGZ and MTF in pure powder form.

Items	CGZ				MTF					
	AAM	AAS	RC	Reported method[b]	AAM	AAS	RC	ABP-SS D^2	ABP-SS D^0	Reported method[b]
Mean[a]	101.09	99.09	99.82	100.23	101.09	99.09	99.62	99.98	100.55	100.02
RSD%	1.40	1.04	1.30	1.06	1.40	1.04	1.28	1.15	0.94	1.13
SEM	0.63	0.46	0.58	0.48	0.63	0.46	0.57	0.51	0.42	0.51
Variance	2.0113	1.0710	1.6802	1.1287	2.0113	1.0710	1.6317	1.3196	0.8863	2.2651
n	5	5	5	5	5	5	5	5	5	5
Student's *t*-test[c]	1.090	1.724	0.544		1.320	1.364	0.529	0.064	0.808	
F value[d]	1.782	1.054	1.488		1.568	1.198	1.272	1.029	1.447	

SEM is standard error of the mean.
[a] Average of three experiments.
[b] The reported method is an HPLC method using C_{18} column (250 × 4.6 mm, 5 μm), mobile phase consisting of phosphate buffer: acetonitrile (65: 35 v/v) at flow rate of 1.0 mL/min and the measurement was carried out at 248 nm.
[c] The corresponding tabulated value of *t*-test equals to 2.306 at $p = 0.05$.
[d] The corresponding tabulated value of F equals to 6.3882 at $p = 0.05$.

Table 4
One way ANOVA testing for the different proposed and reported methods used for the determination of CGZ and MTF.

	Source of variation	DF	Sum of squares	Mean square	F value
CGZ	Between experiment	3	10.508	3.503	2.378 (3.239)
	Within experiment	16	23.565	1.473	
MTF	Between experiment	5	5.317	1.063	0.744 (2.621)
	Within experiment	24	34.305	1.429	

The values between parentheses are the theoretical F values.
The population means are not significantly different.

5. Application of the Proposed Methods

The proposed methods were applied for the analysis of CGZ and MTF in Vokanamet® tablets using the procedure described under Section 2.4.4. Application to pharmaceutical preparation. Good recoveries and standard deviations for both drugs were obtained, where recovery% ranged from 96.80% to 101.81% for CGZ and from 98.80% to 103.13% for MTF, while the SD has not exceeded the value of 2 for the analysis of both drugs utilizing any method of the proposed methods. The previous results proves the high accuracy and precision of the proposed methods for the determination of the drugs in their combined dosage form and further proves the tablet excipients have not encountered any interference. Additionally, the validity of the proposed methods was evaluated by applying the standard addition technique. The obtained results are abridged in Table 2.

6. Solution Stability Study

The solution stability of the stock solutions was studied at different time intervals. It was concluded that the stock solution prepared in methanol were stable for one week when stored in a refrigerator at 4 °C, as during this time the results were not decreased below the minimum percentage and this is in agreement with the literature [10,39] which have indicated that both drugs were stable. We have specified one week storage in a refrigerator at 4 °C to avoid any error resulting from solvent loss due to the volatility of methanol which could occur even when stored in the refrigerator and this leads to inaccurate results.

7. Statistical Analysis

The results obtained by the proposed methods were statistically [40] compared to those obtained by the reported method [23] as demonstrated in Table 3. The reported method is an HPLC method using C_{18} column (250 × 4.6 mm, 5 μm), mobile phase consisting of phosphate buffer: acetonitrile (65: 35 v/v) at flow rate of 1.0 mL/min and the measurement was carried out at 248 nm. No significant difference regarding the accuracy and precision of the proposed and reported methods was observed as indicated from the lower values of the calculated *t* and F tests than that of the theoretical ones. Moreover, one-way ANOVA was applied for the comparison of the developed methods, where from the data obtained it was obvious that there was no significant difference between the developed methods as demonstrated in Table 4.

8. Conclusion

This work presented the application of novel, simple, eco-friendly, cheap spectrophotometric methods for the investigation of the recently released binary combination of canagliflozin and metformin. The methods depended on measuring the response at two wavelengths using zero order absorbance, second derivative or ratio spectra. The proposed methods were successfully applied for the simultaneous determination of the cited analytes in pharmaceutical formulation without the need for prior chemical treatment or sophisticated apparatus or purchasing a specific software and they have not demonstrated any interference by common tablet excipients and additives. The advantages and the required conditions for each of the developed methods were discussed and they were superior to the reported HPLC methods which require prior steps such as extraction and other tedious analytical process during method development for optimization then analysis besides high cost and time consumption. Furthermore, while working with these methods one does not need to use toxic organic solvents. In other words, they belong to green analytical chemistry or environmentally-friendly analytical methods. The proposed methods could be efficiently utilized for the routine analysis of the studied analytes in quality control laboratories with acceptable accuracy and precision.

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