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Evaluation of remogliflozin, vildagliptin, and metformin in tablet dosage form using modern and cost-effective UV spectroscopic methods

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ABSTRACT

The fixed-dose combination (tablets) Remo® MV 500, consists of remogliflozin (RGF), vildagliptin (VGT), and metformin hydrochloride (MTH), enhances blood sugar regulation in people having diabetes mellitus (type 2). The proposed study introduces four newer spectrophotometric approaches for the concurrent assessment of the fixed dosage form that are fast, simple, accurate, and reproducible. The first strategy involves progressively measuring absorbance at 228, 210, and 236 nm for RGF, VGT, and MTH to decipher pre-existing equations (simultaneous equation). The second technique, called ratio difference spectroscopy, analyses the amplitude variation between spectra taken at two different wavelengths. In contrast, the third strategy was based on zero-crossings in the spectrum and the derivative ratio signals. The fourth procedure is all about collecting the first derivative of the ratio spectrum and thereby assessing using a double divisor-ratio spectral derivative methodology. Concentrations between 2.5 and 15 μg/ml, all four medicines showed a remarkable degree of linear correlation across all four approaches. Further, the projected approaches were verified as having adequate precision, accuracy, and sensitivity in accordance with International Conference on Harmonization protocols. Because they do not demand costly solvents or complicated instruments, the spectrophotometric approaches that have been proposed are generally considered to be more cost-effective than alternative analytical procedures. As a result, the proposed approaches could be used successfully intended for the concomitant assessment of RGF, VGT, and MTH in marketed formulation.

INTRODUCTION

Metabolic disorders like diabetes affected 463 million individuals globally till 2019 and this figure is predicted to climb to 578 million by 2030. This figure is again expected to climb to 700 million by 2045. Those living in high-income countries are more likely to be affected, as are those living in metropolitan areas. One out of every two diabetics does not know they have the disease. According to current predictions, there will be 374 million people with impaired glucose tolerance in 2019, 454 million by 2030, and 548 million in the year 2045 (Saeedi *et al.*, 2019). Anti-diabetic medications having enhanced glycemic control and lesser harmful effects have been the subject of numerous clinical investigations. Recent licensing of sodium-glucose cotransporter-2 inhibitors (SGLT-2) for use in the therapy of diabetes mellitus type 2 (DMT2) either in single or in the combined form alongside existing diabetic medications has increased their visibility in the medical community (Kalra, 2014). Remogliflozin etabonate (RGF), pro-drug of RGF with the chemical term [5-methyl-4-(4-(1-methylethoxy)benzyl)-1-(1-methylethyl)-1H-pyrazol-3-yl 6-O-(ethoxycarbonyl)-b-D-glucopyranoside] is classed as SGLT2 inhibitor. The kidneys' ability to reabsorb glucose is mostly dependent on a transporter called SGLT2. Often used in conjunction with other drugs, it can be helpful in controlling DMT2 when used additionally with controlled food

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intake and workout (Dharmalingam *et al.*, 2020; Tammisetty *et al.*, 2021). Vildagliptin (VGT) also recognized as (S)-1-[*N*-(3-hydroxy-1-adamantyl) glycyl] pyrrolidine-2-carbonitrile, an example of a competitive, reversible dipeptidyl peptidase-4 inhibitor. By blocking this enzyme, the collapse of glucagon-like peptide-1 (GLP-1) and glucose reliant insulinotropic polypeptide (GIP) is postponed. The discharge of insulin is stimulated by GLP-1 and GIP, which prevent glucagon from being secreted by pancreatic beta cells. All of these actions work together to reduce glycogenolysis in the liver and boost insulin production (Boovizhikannan and Palanirajan, 2013; Keating, 2014). A biguanide antidiabetic, metformin hydrochloride (MTH) is also known by its chemical name, 1,1-dimethyl biguanide hydrochloride. This medication is prescribed most often to individuals who are overweight and suffering from DMT2. They do not stimulate insulin secretion but rather have an anti-diabetic effect when some insulin is already present. Possible methods of action include postponing glucose absorption in the gastrointestinal tract, boosting insulin sensitivity, allowing glucose to enter cells, and inhibiting gluconeogenesis in the liver (Indian Pharmacopoeia, 2018; Martindale, 2009; Sen *et al.*, 2016; The Merck Index, 2001). The structures of the three analytes are displayed in Figure 1.

Patients with diabetes typically require a combination of drugs to control their blood sugar levels. The newer combination of RGF, VGT, and MTH was launched in October 2021 by Glenmark Pharmaceuticals Ltd., India; it works through three synergistic mechanisms to help control glycemic levels in people with diabetes (type 2) who may benefit from consuming RGF and VGT together with MTH. Remo® MV 500 is a fixed-dose combination of RGF, VGT, and MTH as tablets that lower glycemia in individuals with type 2 diabetes when used in conjunction with a healthy diet and frequent exercise (Glenmark Pharmaceuticals Ltd, 2021; Hussey *et al.*, 2013; Khaladkar *et al.*, 2022). From ultraviolet (UV) spectrophotometry (Attimarad *et al.*, 2021a, 2021b, 2021c; Banik *et al.*, 2015; Itigimatha *et al.*, 2022; Kumari and Khansili, 2020; Patel and Desai, 2022; Sen *et al.*, 2016, 2022) to HPLC (Attimarad *et al.*, 2022a, 2022b, 2022c; Boovizhikannan and Palanirajan, 2013; Itigimatha *et al.*, 2022; Patel and Desai, 2022; Shah *et al.*, 2020; Shakoor *et al.*, 2020; Tammisetty *et al.*, 2021), and HPTLC (Bendale *et al.*, 2018; El-Kimary *et al.*, 2016; Modi *et al.*, 2013; Patil *et al.*, 2020; Shah *et al.*, 2021; Thomas *et al.*, 2011) the literature review uncovered a wide variety of systematic approaches for the evaluation of RGF, VGT, and MTH alone or in conjunction with different analytes. However, only two research articles reported (Attimarad *et al.*, 2022c; Patel and Desai, 2022) simultaneous estimation of all three drugs under study. But reported methods show some shortcoming in

various aspects like range, sensitivity, specificity, etc. Therefore, the proposed methods justify their existence and have been compared with the reported methods (Table 6). The proposed procedures have been validated according to International Conference on Harmonization (ICH) criteria cover a broad concentration series with increased sensitivity, and are relatively simple in terms of standard and sample preparation. Analytical methods based on UV spectroscopy are considered simple, rapid, and inexpensive; they are ideal for determining the quality of commonly prescribed medications. Direct UV spectroscopic methods have the advantage of being simple to use and interpret, but they suffer from the fundamental limitation of being susceptible to the influence of multicomponent formulations and formulation additives (Attimarad *et al.*, 2012). Derivative and ratio derivative UV spectroscopic techniques were therefore devised in addition to the simultaneous equation method (SEM) to counteract these effects, and they were validated as suitable for the simultaneous estimation of RGF, VGT, and MTH without hindrances.

MATERIALS AND METHODS

Raw materials and reagents

Throughout the study, researchers relied on the RGF reference standard (99.6% *w/w*), procured from Glenmark Pharmaceuticals Ltd., Sinnar, Nasik, India. Dalton PharmaChem in Vadodara, Gujarat, India, was the source for the VGT (99.25% *w/w*) and MTH (99.42% *w/w*). Loba Chemie Pvt. Ltd., Mumbai, India supplied all diluents, raw materials, and excipients (specificity evaluation) made use in the research.

Instruments

The entire study used a Double beam UV visible spectrophotometer (UV-1800 with UV Probe), Shimadzu Corporation, Kyoto, Japan with a Quartz (1 cm) sample container that was essentially the same as the one used in the original instrument. For the purpose of determining precise masses, we used an electronic balance (Adventurer Pro AVG264C), Ohaus Corporation, Pine Brook, NJ.

Preparation of standard solution

Each drug's stock solution (RGF, VGT, and MTH) was made by adding 10 mg of reference analytes by weight to a 100 ml standard flask. Reference analytes were reduced to a concentration of 100 µg/ml using methanol by filling the container up to its capacity. The concentration was further lowered by diluting the solution with methanol.

Preparation of combined standard solution

A sequence of a combination of reference solutions (RGF + VGT + MTH, RGF + VGT, RGF + MTH, VGT + MTH) was made by shifting the right quantity of RGF, VGT, and MTH reference solutions (0.25–1.5 ml) into 10 ml standard flasks one at a time and filling them with methanol until they reached the required concentration (2.5–15 µg/ml).

Sample solution preparation

Powdered tablets comprising 10 mg of RGF, 5 mg of VGT, and 50 mg of MTH were measured out and placed in a 50
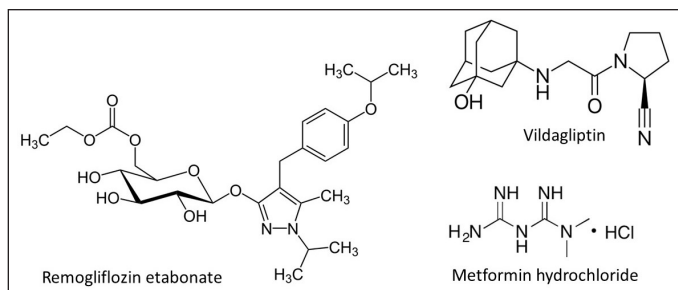

Figure 1. Chemical constructions of RGF; VGT and MTH.

ml standard bottle. The measured amount of pure RGF (40 mg) and VGT (45 mg) was put into the 50 ml standard flask. The standard bottle was occupied with 30 ml of methanol and agitated for 10 minutes. To reach 50 ml, methanol was poured, and filtration of the mixture was performed using Whatman filter paper 41. One milliliter of the aforementioned solution was transferred to a 10-ml bottle, and the remaining 9 ml were filled with methanol. Further, 1 ml of the subsequent solution was transferred to a volumetric bottle (10 ml) and the remaining space was occupied with the same solvent to produce optimum dilution (10 µg/ml of RGF, VGT, and MTH).

Procedure/method

Simultaneous equation method

The mentioned methodology was used to estimate RGF, VGT, and MTH in the formulation of tablets. The UV region of 200–400 nm was used to record the UV spectra of each standard analyte under investigation. The approximation of the suggested analytes in the tablet required the selection of an acceptable wavelength, which was done using the overlapping UV spectra. RGF, VGT, and MTH's overlaid zero-order spectra showed a maximum absorbance at 228, 210, and 236 nm, respectively (Fig. 2). Absorptivity of each medication was then computed and summarized (Table 1). Using the formula, the amount of medication in the formulation was calculated.

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

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

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

The amounts of RGF, VGT, and MTH that are progressively present in the sample solutions are represented in the equation above by C_x, C_y, and C_z.

A1, A2, and A3 are the sample's absorbances at 228, 210, and 236 nm, respectively. RGF's absorptivity at 228, 210, and 236 nm is represented by ax1, ax2, and ax3, respectively. The absorptivity of VGT at 228, 210, and 236 nm is represented by ay1, ay2, and ay3. Absorptivity of MTH at 228, 210, and 236 nm is represented by az1, az2, and az3, respectively (Beckett and Stenlake, 2005; Kalyani and Rao, 2018) as presented in Table 1.

Ratio difference spectroscopic approach (RDS)

The ratio spectra were acquired by taking the total of the VGT and MTH absorption spectra (2.5 µg/ml of both as a double divisor) and dividing it by the UV absorption band of several concentrations of RGF and the combined three analytes. The ratio spectrum's amplitude at 251.6 nm was then deducted from the spectrum's amplitude at 254.6 nm. Additionally, a standard curve was created using the relationship between amplitude difference and concentration. RGF and MTH (1 µg/ml) were used as a double divisor to divide the UV spectra of analyte solutions produced with varied dilutions of VGT and the triple drug combination, and ratio spectra were also recorded. Later, the amplitude at 260.6 nm was subtracted from the ratio spectrum's peak amplitude at 250 nm. Also, a calibration graph was formed by plotting the variation in amplitude versus the concentration. To get the ratio spectra, the absorption bands of different amounts of MTH and the ternary mixture were recorded and then divided by the whole absorption band of RGF and VGT solutions (2.5 µg/ml each) as a divisor. Afterward, the ratio spectrum's peak amplitude at 236 nm was subtracted from the peak amplitude at 240 nm. Also, a linear graph was built by taking the variation in amplitude against the concentration (Abdelrahman and Abdelwahab, 2014; Darwish *et al*., 2016; Eissa and Abou Al Alamein, 2018; Erk, 2001; Lotfy *et al*., 2013; Millership *et al*., 2005; Zaazaa *et al*., 2015).

Derivative ratio spectrum-zero crossing method (DRZC)

First, the response of RGF, VGT, and MTH, as well as the three drugs together in the UV region were saved at dissimilar concentrations (linearity series). Afterward, the ratio spectra were made by a division of the absorption band of RGF, MTH, and a combined mixture of 3 drugs with VGT by a reference spectrum of 2.5 µg/ml of VGT. After that, the ratio spectra were turned into the first derivative, which made the first-order ratio spectra. The amplitudes were found to be proportional to the ternary mixture concentration of RGF and MTH at 223.2 nm (zero-crossing spot for MTH) and 242.6 nm (zero-crossing spot for RGF) in the first derivative of ratio spectra. To make calibration graphs, the amplitudes of the first derivative ratio spectra were taken versus increasing concentrations
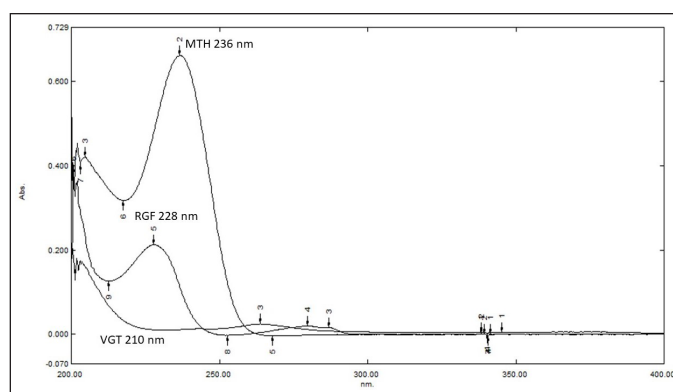
Figure 2. Overlain UV spectra of RGF, VGT and MTH (12.5 µg/ml).

Table 1. Average absorptivity values (RGF, VGT and MTH) at various wavelengths using the SEM method.

Drugs	RGF			VGT			MTH		
Wavelengths (nm)	228	210	236	228	210	236	228	210	236
Absorptivity[a]	217.66 (ax1)	98.63 (ax2)	116.61 (ax3)	17.78 (ay1)	69.81 (ay2)	8.37 (ay3)	390.26 (az1)	270.64 (az2)	542.89 (az3)

[a](*n* = 6) Average of six determinations.

of standard RGF and MTH, with standard VGT used as a divisor. The linear graph thus obtained can be used to find out the amount of RGF and MTH. In the same way, the ratio spectra were made by dividing the saved spectra of VGT, MTH, and their ternary mixture with RGF by reference spectra of 2.5 µg/ml RGF. Also, the first derivatives of the ratio spectra were documented. Signals at 350.4 nm (the zero-crossing point for MTH) in the first derivative of the ratio spectra represent VGT. The calibration graphs for VGT were made by figuring out the first derivative ratio spectra with regard to increasing concentrations of reference VGT using reference RGF as a divisor. This method can be used for the estimation of VGT (Alvi *et al.*, 2013; Dinç and Onur, 1998;).

Double divisor ratio spectra derivative method (DDRS)

The ratio spectra were made by taking the spectra of analyte solutions with different strengths of RGF and the combination of three analytes and dividing it by the total absorption band of VGT and MTH (2.5 µg/ml) as the divisor. After that, the first derivatives of ratio spectra were made. The amount of RGF was then calculated at 257.2 nm in the selected spectral zone. The UV spectra of different amounts of VGT and the ternary combination were documented and divided by RGF and MTH (1 µg/ml each in methanol) to get ratio spectra, which were also recorded.

Then, the ratio spectra 1st derivative was recorded, and the signals at 291.4 nm were used to estimate VGT. To obtain the ratio spectra, UV spectra of analyte solutions using different MTH concentrations, and the mixture of three analytes were recorded and division of these spectra using the sum of the absorption spectra of RGF and VGT (2.5 µg/ml) solutions as the divisor was performed. The first derivative of these ratio spectra was then plotted and the quantity of MTH was found out by utilizing the first derivative signals at 243 nm (Dinç *et al.*, 2002a, 2002b; Gohel *et al.*, 2014; Hajian and Soltaninezhad, 2013; Omer and Fakhre, 2020; Youssef and Maher, 2008).

Assessment of sample solution

The solution was made and diluted, as covered in the earlier section. For SEM, the extent of the analyte was determined by simultaneously calculating a formula based on the absorbances of sample solutions at various wavelengths and standard absorptivity values (Table 1). Peak amplitude was measured for the remaining approaches (RDS, DRZC, and DDRS), and regression equations were employed in order to quantify the extent of the analyte.

Validation of spectroscopic methods

The planned methodologies were authenticated by referring to the regulations of the "International Conference on Harmonization" and some of the reported research articles (Attimarad *et al.*, 2021a, 2022a; Banik *et al.*, 2015; ICH, 2005; Kumari and Khansili, 2020; Sen *et al.*, 2015, 2016, 2022).

Specificity

Tablet excipients included in the formulation were tested for potential interactions with the medication's active ingredient. Each of the tablet excipients was mixed together in the correct proportions, diluted by means of methanol, then filtered utilizing Whatman filter paper (41) to produce the final solution. To further investigate the potential chemical interaction between excipients and analytes, UV scanning was used to compare all dummy and standard solutions.

Linearity and range

Using the SEM method, absorbances were recorded at 228, 210, and 236 nm for RGF, VGT, and MTH in methanol to determine linear correlation and range; using the RDS method, the amplitude difference was determined (RGF: 254.6–251.6 nm; VGT: 260.6–250 nm; MTH: 240–236 nm). Both the DRZC (RGF: 223.2 nm; VGT: 350.4 nm; MTH: 242.6 nm) and DDRS (RGF: 257.2 nm; VGT: 291.4 nm; MTH: 243 nm) methods were used to measure the amplitude. Standard analyte absorbance versus concentration calibration plots was created using the SEM method, reference analyte amplitude difference versus concentration calibration plots using the RDS method, and peak amplitude versus concentration calibration plots using the DRZC and DDRS methods. By employing the least-squares method, we were able to calculate the correlation coefficient, slope, and intercept. The results were calculated as the mean of six separate assessments.

Precision

To assess the accuracy of the procedure, tests were executed to examine its repeatability, intra and inter-day precision. Analyzing sample solutions (RGF, VGT, and MTH: 2 and 8 µg/ml) 6 times and determining the % RSD by evaluating the outcome of each of the medications at numerous wavelengths considering the methodology allowed us to evaluate the repeatability of offered approaches. Testing sample solutions in triplicate on the same day at two different concentrations (RGF, VGT, and MTH: 2 and 8 µg/ml) allowed for an evaluation of intra-day precision within the working range. However, the percentage RSD was calculated throughout 3 days of testing using sample solutions with concentrations at the low and high ends of the range (RGF, VGT, and MTH: 2 and 8 µg/ml, respectively) to assess the consistency between days.

Accuracy

Recovery studies were conducted using the conventional standard addition procedure to verify the viability of the anticipated methods. Standard RGF, VGT, and MTH at the 50%, 100%, and 150% level were added to a sample solution that had already been analyzed (RGF, VGT, and MTH: 2, 4, and 6 µg/ml) to produce a comparable quantity, and the solution was reanalyzed using projected methods before the percentage recoveries were calculated. Results from the accuracy research were analyzed using the following formula, which considers the % of reference RGF, VGT, and MTH recuperated from the pharmaceutical product with the help of the below given formula:

% Recovery = (Total amount of analyte found after adding reference analytes – Amount of analytes found before adding reference analytes)/(Amount of reference analytes added) × 100

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

Through the use of mentioned parameters, the sensitivity of the suggested approaches were evaluated. The LOD and LOQ

of the investigated analytes were assessed using the following formula, which was taken directly from the ICH strategies.

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

$$LOD = 10 \times \frac{\sigma}{S}$$

In the above equation σ = The SD of the response, S = Mean of the slope (standard graph)

Solution stability

We kept medication solutions at ambient environmental setup and in the refrigerator (6°C) and compared their responses and spectral pattern to those of freshly prepared solutions at regular intervals to see how well they held up over time, so analyzing their stability.

Statistical comparison by one-way ANOVA

Assay results were compared using one-way ANOVA, Microsoft 365, Microsoft Corporation, USA.

RESULTS AND DISCUSSION

It is anticipated that the projected spectrophotometric approaches would find widespread application in quality control (QC) settings where both cost and turnaround time for assessments are critical. UV spectroscopic methods are often used to study pharmaceutical preparations because they are easy, quick, cheap, and give consistent results. Compared to other ways of analyzing, these spectrophotometric methods are better and have many benefits. But it is hard to analyze all the analytes without first separating them if the UV spectra of the different components overlap. The proposed work comes up with some simpler and cheaper way to analyze RGF, VGT, and MTH concurrently in combined formulation bearing overlaying spectra.

Simultaneous equation method

The SEM was created and verified for the quantification of RGF, VGT, and MTH in tablet formulation, demonstrating adequate sensitivity and selectivity. The maximum absorbance in the zero-order UV spectra was seen at 228, 210, and 236 nm for RGF, VGT, and MTH, respectively (Fig. 2). As can be seen in Figure 2, the UV spectra of RGF, VGT, and MTH display spectral overlap, which allows for simultaneous estimation of RGF, VGT, and MTH in the ternary mixture. Using a simultaneous equation, the quantity of drugs in the mixture was calculated. Table 1 displays absorptivity data, whereas Table 2 displays the results of the method validation parameters.

RDS method

It has been demonstrated time and again that derivatization of UV spectra results in an increase in both specificity and selectivity of pharmaceuticals in combined dosage form by enhancing the resolution/resolving power of spectra. As a bonus, derivatization eliminates the excipient effects and permits the computation with respect to the existence of another analyte. The ratio spectroscopic method is predicated on the idea that by

Table 2. Summarized data of method validation parameters and linear regression.

Parameters/Drugs	SEM			RDS			DRZC			DDRS		
	RGF	VGT	MTH	RGF	VGT	MTH	RGF	VGT	MTH	RGF	VGT	MTH
Wavelength (nm)	228	210	236	254.6–251.6	250–260.6	240–236	223.2	350.4	242.6	257.2	291.4	243
Linearity range (µg/ml)						2.5–15						
Correlation coefficient	0.9951	0.9945	0.9997	0.9978	0.9942	0.9985	0.9918	0.9934	0.9965	0.9992	0.9913	0.9992
Regression equation	y = 0.0218x − 0.0006	y = 0.0065x + 0.0021	y = 0.0571x 0.0173	y = 0.8498x − 1.7487	y = 0.131x + 2.0449	y = 12.124x − 1.1579	y = 0.0344x − 0.0514	y = 0.0328x + 0.8301	y = 7.0496x − 3.1827	y = 0.4852x − 1.0303	y = 0.0573x + 0.6935	y = 6.9998x − 2.4483
LOD (µg/ml)	0.0574	0.0997	0.0276	0.1270	0.6069	0.0589	0.0847	0.1654	0.3685	0.1604	0.4533	0.3484
LOQ (µg/ml)	0.1739	0.2991	0.0836	0.3849	1.8390	0.1784	0.2566	0.5013	1.1165	0.4861	1.3737	1.0558
Specificity						Specific (No interference)						
Precision (% RSD)												
Repeatability of measurement (n = 6)[a]	0.8234	1.4011	0.6450	0.1128	0.0638	0.2005	0.6087	0.3047	1.0405	0.2257	0.2678	0.6809
Intra-day (n = 3)[a]	1.0646	1.6074	0.9054	0.3653	0.2732	0.7768	0.8754	0.5359	1.2126	0.4874	0.5613	1.1386
Inter-day (n = 3)[a]	1.1551	1.6451	1.0414	1.0101	1.0606	1.0132	1.0221	1.0364	1.4059	1.1030	1.0231	1.1517

[a]n = number of estimations, % RSD.

division of the spectra in combination with one of the spectra of the analyte, one can obtain a spectrum free of interferences from the analyte and the excipient. Using a spectrum that has been selected as a divisor also reduces background noise and scientific errors. Measurements are made in relation to the peaks, which improves precision, sensitivity, and specificity; this is another advantage of the ratio spectra method. Consequently, ratio spectroscopic methods were chosen to develop as it gives superior outcomes in comparison with other UV methods. In order to find the optimal double divisor, various RGF, VGT, and MTH concentrations were tested. In the end, we decided to evaluate the ternary mixture of RGF, VGT, and MTH using 2.5 µg/ml of VGT and MTH, 1 µg/ml of RGF and MTH and 2.5 µg/ml of RGF and VGT. All solutions of double divisors were prepared separately using methanol. The ratio difference spectroscopic approach was accomplished by saving the absorption spectra of solutions produced at several strengths of RGF and of the ternary combination and division of those spectra using UV spectra of 2.5 µg/ml of VGT and MTH, as displayed in Figure 3(A).

The ratio spectrum's peak amplitude at 254.6 nm was deducted from the amplitude at 251.6 nm to find the resulting amplitude. In the next step, we plotted linearity graphs by using the values of the amplitude differences and the amount of RGF to determine the correlation coefficient and the regression equation. As can be seen in Figure 3(B), UV spectra were recorded for solutions made up of varying strengths of VGT and the ternary mixture, and then divided using RGF and MTH (1 µg/ml) as divisors, yielding the resultant ratio spectra. The ratio spectrum

was then created by deducting the amplitude obtained at 250 nm from the amplitude at 260.6 nm. Linearity graphs were then created by using the values of the amplitude differences and the respective amounts of VGT to determine the regression equation and the corresponding correlation coefficient. Figure 3(C) displays the ratio spectra, achieved by the division of absorption spectra produced at several strengths of MTH and the ternary mixture by absorption band of 2.5 µg/ml of RGF and VGT separately in methanol. The amplitude at 236 nm was then deducted from the ratio spectrum's peak amplitude at 240 nm. Afterward, correlation coefficients and equations of regression were derived using linearity graphs constructed with a difference in amplitude and the corresponding quantity of MTH.

Divisor and scaling factor optimization for first derivative of ratio spectra

Different experimental parameter settings were optimized to provide the best possible curve of the 1st derivative of ratio spectra. The most critical parameter involved adjusting the divisor and the scaling factor to maximize efficiency. Differing concentrations of RGF, VGT, and MTH were tested in order to find the optimal concentration to use as a divider. The final decision for RGF and MTH quantification was to use a VGT divisor of 2.5 µg/ml. Similarly, for the estimation of VGT, 2.5 µg/ml of RGF was finalized as a divisor. In order to acquire the first derivative of the ratio spectra, the scaling factor was fixed/tuned at four. First derivative spectra at 2, 4, 8, and 10 nm were explored to determine the optimal wavelength. According to the findings, an
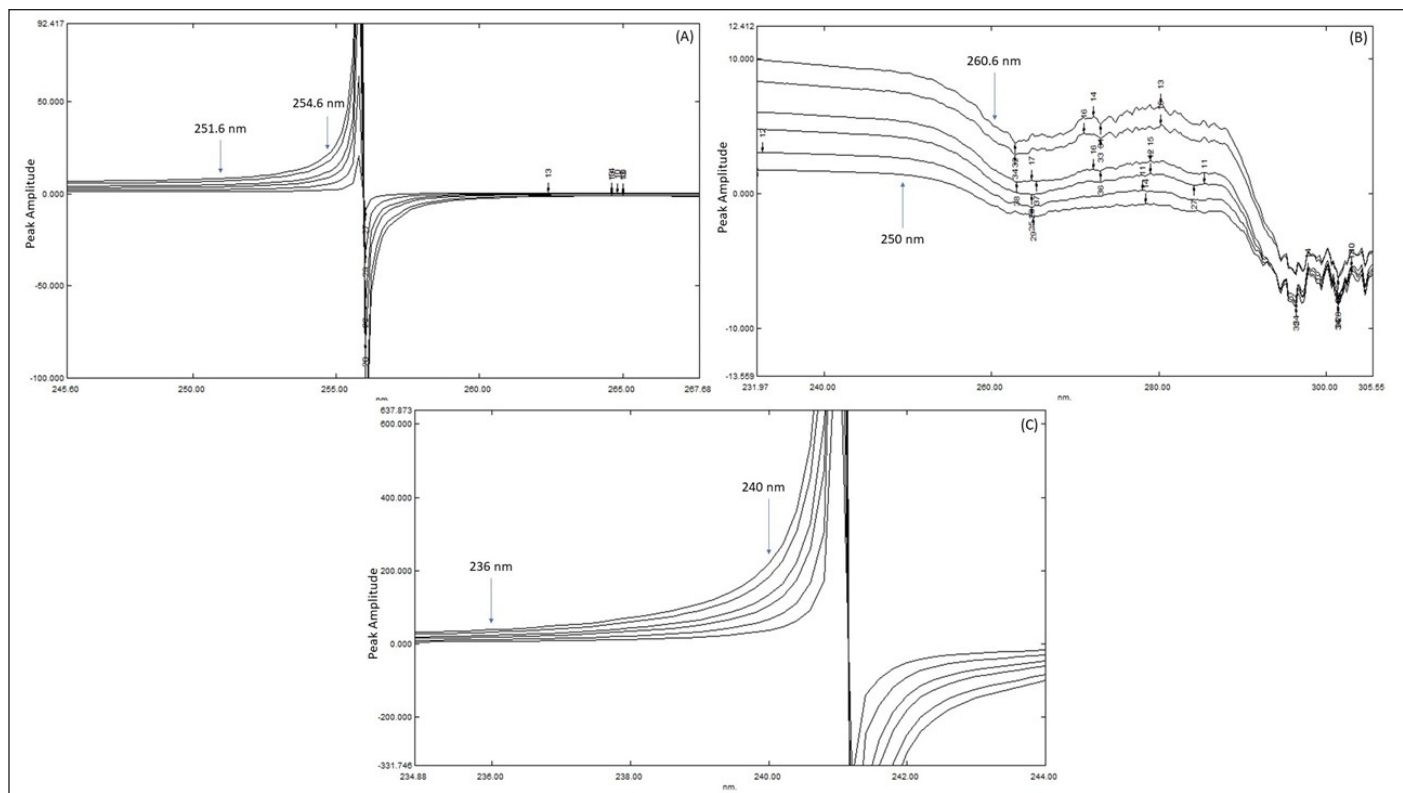

Figure 3. (A) Overlain ratio spectra of RGF utilizing 2.5 µg/ml (VGT + MTH) as divisor; (B) Overlain ratio spectra of VGT utilizing 1 µg/ml (RGF + MTH) as divisor; (C) Overlain ratio spectra of MTH taking 2.5 µg/ml (RGF + VGT) as divisor.

8 nm wavelength was the most effective, thus that is the one that was selected and used, scaled up by a factor of 4.

DRZC approach

In this procedure, solutions with varying concentrations of RGF and MTH were scanned and stored between 200 and 400 nm. Utilizing the spectrum of a standard solution containing 2.5 µg/ml VGT, the recorded spectra were subsequently split. Consequently, the ratio spectra were transformed into their first derivative spectra.

By following the analytical signals in the 1st derivative spectra at 223.2 nm for RGF and 242.6 nm for MTH, as displayed in Figures 4A and C, the strengths of RGF and MTH in the ternary combination were able to estimate. Similar ratio spectra were achieved by division of the absorption spectra of VGT and MTH by the spectrum of a standard solution of 2.5 µg/ml RGF. Using a value of Δλ as 8 nm and a scaling factor of 4, the acquired ratio spectra were transformed into their first derivative. These spectra were then analyzed at 350.4 nm to determine the VGT concentration in the ternary combination (Fig. 4B).

DDRS derivative method

First, ternary combinations of RGF, VGT, and MTH were created at varying concentrations, and scanning was done in the UV spectrum (200–400 nm). Then, to obtain the ratio spectra, we divided each spectrum by 2.5 µg/ml VGT and MTH as the denominator. Further, as can be seen in Figure 5(A), the first derivative of ratio spectra was obtained by setting Δλ = 8.

A calibration graph was produced by plotting the amplitude at 257.2 nm versus the concentration to estimate the occurrence of RGF. A different set of ratio spectra was collected by splitting ternary mixtures of RGF, VGT, and MTH with 1 µg/ml of RGF & MTH as the double divisor. Figure 5(B) also shows the first derivative of ratio spectra that were achieved with the help of Δλ as 8 and a scaling factor of 4. To evaluate the presence of VGT, we measured the amplitude at 291.4 nm and then plotted this value against the concentration at this wavelength to create a calibration graph. The last bunch of ratio spectra was obtained by division of ternary mixtures of RGF, VGT, and MTH by 2.5 µg/ml of RGF and 2.5 µg/ml of VGT, respectively. Figure 5(C) displays the result of taking the first derivative of ratio spectra with a value equal to Δλ as 8 and a scaling factor of 4. A linear graph was created by putting up the amplitude at 243 nm against the concentration to obtain the MTH concentration.

Method validation

All of the planned methodologies were evaluated in agreement with "International Conference on Harmonization" standards. The next section will elaborate on the results of different validation parameters.

Specificity

Excipients and typical pharmaceuticals did not interact while carrying out the specificity study by observing overlapping spectra of placebo (a blend of reported excipients as per the information from the manufacturer) and medication solutions.

Figure 4. (A) First derivative ratio spectra of RGF (a1) 2.5, (a2) 5, (a3), 7.5 (a4) 10, (a5) 12.5, (a6) 15 taking 2.5 µg/ml VGT as divisor (Δλ = 8 nm). (B) First derivative ratio spectra of VGT (b1) 2.5, (b2) 5, (b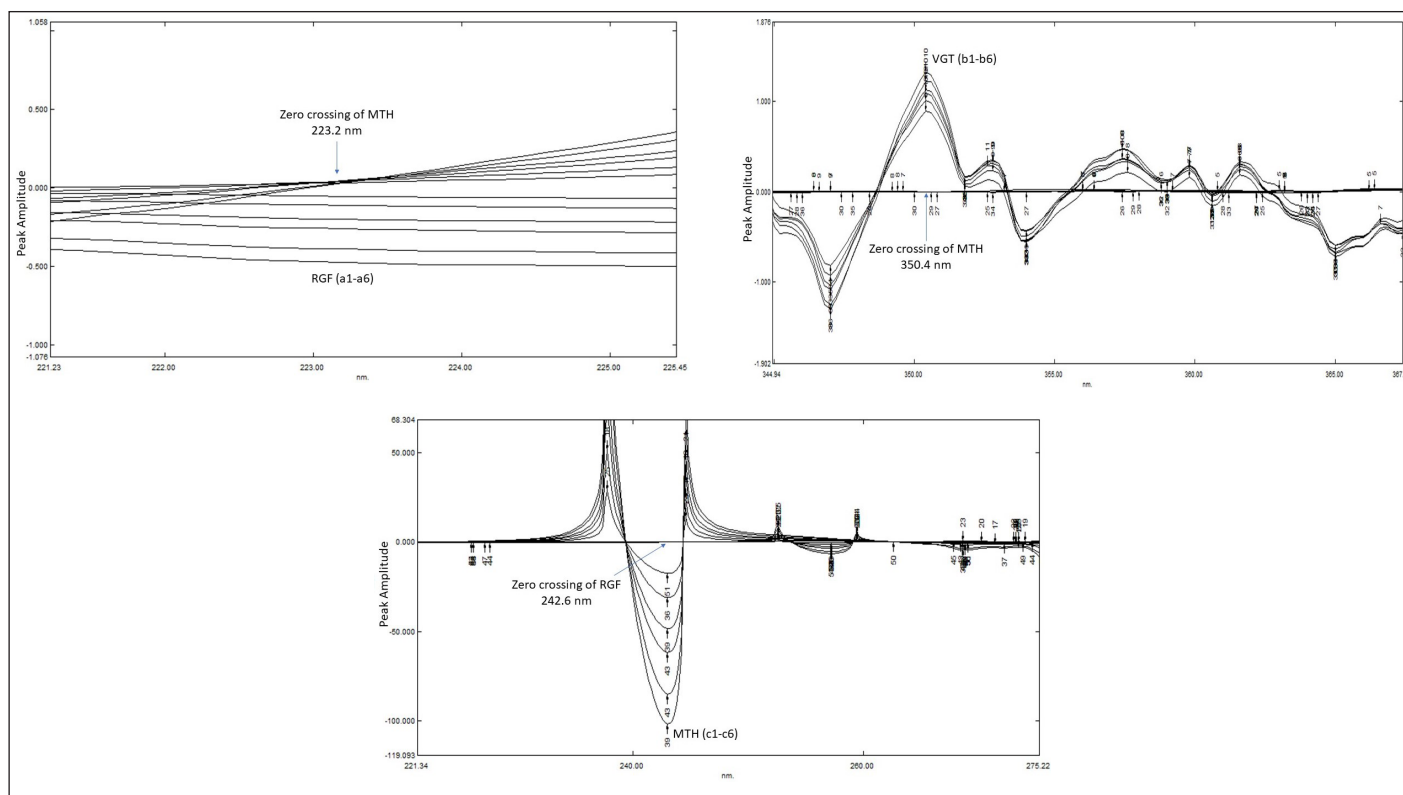3), 7.5 (b4) 10, (b5) 12.5, (b6) 15 taking 2.5 µg/ml RGF as divisor (Δλ = 8 nm). (C) First derivative ratio spectra of MTH (c1) 2.5, (c2) 5, (c3), 7.5 (c4) 10, (c5) 12.5, (c6) 15 taking 2.5 µg/ml VGT as divisor (Δλ = 8 nm).
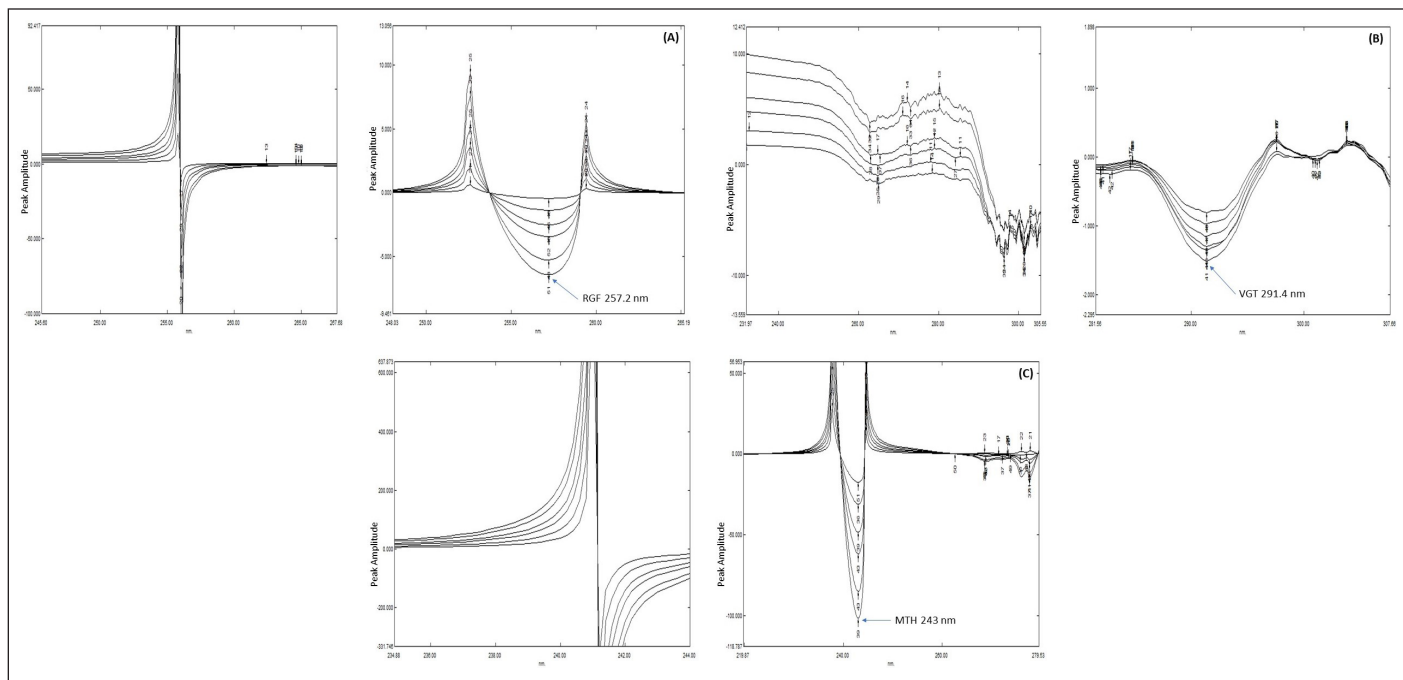
Figure 5. (A) Ratio spectra and corresponding 1st derivative ratio spectra of RGF using 2.5 µg/ml VGT + 2.5 µg/ml of MTH as divisor at $\Delta\lambda = 8$. (B) Ratio spectra and corresponding 1st derivative ratio spectra of VGT using 1 µg/ml RGF + 1 µg/ml of MTH as divisor at $\Delta\lambda = 8$. (C) Ratio spectra and corresponding 1st derivative ratio spectra of MTH using 2.5 µg/ml RGF + 2.5 µg/ml of VGT as divisor at $\Delta\lambda = 8$.

Table 3. Recovery information of the planned approaches.

Drugs	Level (%)	Recovery (%)[a]				RSD (%)			
		SEM	RDS	DRZC	DDRS	SEM	RDS	DRZC	DDRS
RGF	50	99.53 ± 0.79	99.36 ± 1.70	99.66 ± 0.84	99.74 ± 1.62	0.79	1.71	0.85	1.62
	100	98.42 ± 0.65	99.47 ± 0.70	99.40 ± 0.75	98.74 ± 1.10	0.66	0.70	0.76	1.11
	150	99.83 ± 0.23	99.30 ± 0.96	98.56 ± 0.68	100.05 ± 1.12	0.23	0.96	0.69	1.12
VGT	50	98.55 ± 0.78	99.49 ± 0.35	100.17 ± 1.70	98.68 ± 1.04	0.79	0.36	1.70	1.05
	100	99.52 ± 1.25	99.77 ± 1.27	99.29 ± 0.60	100.20 ± 1.05	1.25	1.27	0.60	1.05
	150	99.44 ± 1.91	99.04 ± 0.82	99.75 ± 0.39	98.74 ± 0.76	1.92	0.83	0.39	0.77
MTH	50	100.27 ± 0.93	100.04 ± 1.88	100.67 ± 0.70	99.26 ± 0.35	0.93	1.88	0.70	0.35
	100	100.84 ± 1.47	98.94 ± 0.54	101.00 ± 1.53	99.73 ± 1.07	1.46	0.55	1.52	1.07
	150	98.67 ± 0.18	98.33 ± 0.73	99.74 ± 0.96	98.97 ± 0.70	0.19	0.75	0.97	0.71

[a]Mean ± SD (*n* = 3), SD (Standard deviation), % RSD.

Table 4. Outcome of formulation evaluation by various approaches.

Drugs	Labelled amount (mg/tab)	Amount estimated (mg/tab)				Estimated (%)[a]				RSD (%)			
		SEM	RDS	DRZC	DDRS	SEM	RDS	DRZC	DDRS	SEM	RDS	DRZC	DDRS
RGF	100	99.12	98.81	98.95	99.13	99.12 ± 0.56	98.82 ± 0.89	98.95 ± 1.54	99.13 ± 1.36	0.57	0.90	1.55	1.36
VGT	50	49.89	49.41	49.61	49.85	99.79 ± 1.12	98.82 ± 0.92	99.23 ± 1.72	98.78 ± 1.03	1.12	0.93	1.73	1.03
MTH	500	495.43	498.05	496.35	497.30	99.09 ± 0.21	99.61 ± 0.56	99.27 ± 0.60	99.46 ± 0.55	0.21	0.56	0.61	0.55

[a]Mean ± SD (*n* = 6), SD (Standard deviation), % RSD.

Linearity and range

With the SEM approach, we measured linear association and range by evaluating absorbance at specific wavelengths, while with the RDS method, we measured amplitude difference. The DRZC and DDRS techniques, however, utilized peak amplitude for quantification. All three medicines showed a linear relationship

between 2.5 and 15 µg/ml, regardless of the technique used. Every proposed approaches were linear, as supported by the correlation coefficient's value (Table 2). The results were calculated as the mean of six separate assessments.

Precision

All the predicted methods have great precision as evidenced by the precision experiment results (repeatability, intra,

and inter-day changeability) expressed in % RSD guarantee the ICH acceptable constraints (<2) revealed in Table 2.

Accuracy

The expected procedures' accuracy was determined using the standard addition approach for recovering analytes. The studies' recovery rates ranged from 97% to 103% across all drugs and methods, proving the efficacy of the established approaches (Table 3).

LOD and LOQ

The level of responsiveness of the predicted procedures was demonstrated by the low values of LOD and LOQ (Table 2).

Solution stability

The solution remained unchanged for 2 days at room temperature and for 10 days when chilled to 6°C.

Estimation of RGF, VGT, and MTH in tablet dosage form

Assessments of RGF, VGT, and MTH were conducted with the help of the planned methods. The data set was statistically validated after six replicate measurements, with results ranging from 97% to 102% for all the analytes. Thus, the developed methods can be employed for the concurrent evaluation of RGF, VGT, and MTH in combined tablet (Table 4).

Statistical comparison by one-way ANOVA

An examination of the data from the assays was done using statistical methods to determine the impact of each of the four

Table 5. Statistical comparison of assay results utilizing one way ANOVA.

Groups	Methods	Mean[a]	Variance	F	F crit	p-value
RGF	SEM	99.23	0.3195			
	RDS	98.82	0.7911	0.2961		0.8277
	DRZC	98.81	0.7960			
	DDRS	99.13	1.8395			
VGT	SEM	99.80	1.2570			
	RDS	98.82	0.8423	0.9441	3.0984	0.4380
	DRZC	99.08	2.4932			
	DDRS	98.78	1.0518			
MTH	SEM	99.09	0.0453			
	RDS	99.61	0.3122	1.2245		0.3268
	DRZC	99.27	0.3657			
	DDRS	99.46	0.3023			

[a]$n = 6$ (Number of determination); p value (significant if $p < 0.05$).

Table 6. Comparison of proposed methods with reported methods.

Methods compared	Name of method	Drugs	Range (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)	Specificity
Proposed methods	SEM	RGF		0.0574	0.1739	Specific
		VGT		0.0997	0.2991	
		MTH		0.0276	0.0836	
	RDS	RGF		0.1270	0.3849	
		VGT		0.6069	1.8390	
		MTH	2.5–15	0.0589	0.1784	
	DRZC	RGF		0.0847	0.2566	
		VGT		0.1654	0.5013	
		MTH		0.3685	1.1165	
	DDRS	RGF		0.1604	0.4861	
		VGT		0.4533	1.3737	
		MTH		0.3484	1.0558	
Hydrophilic liquid chromatography (HILIC) (Attimarad *et al.*, 2022)	HILIC	RGF	20–50	4.73	14.34	Not reported
		VGT	10–75	2.81	8.54	
		MTH	50–750	1.48	43.88	
UV spectroscopy and HPLC (Patel and Desai, 2022)	UV	RGF	2–10	0.1016	0.3078	Not reported
		VGT	1–5	0.0437	0.1323	
		MTH	10–50	0.0333	0.1010	
	HPLC	RGF	2–10	0.1998	0.6053	Specific
		VGT	1–5	0.2630	0.7968	
		MTH	10–20	0.0815	0.2471	

Note: Abbreviations are mentioned earlier.

approaches that were projected. One-way ANOVA was employed to compare the statistical significance of the four different approaches. The significance level for all tests was set at $p < 0.05$. Table 5 demonstrates the outcomes of the one-way ANOVA and it was found that the created procedures differed little from each other.

Proposed methods explain the estimation of RGF, VGT, and MTH in the ternary admixture which was compared with two recently published research articles (Attimarad *et al.*, 2022c; Patel and Desai, 2022) where authors elaborated simultaneous estimation of all three drugs under study. But reported methods show some shortcomings in various aspects like range, sensitivity, specificity, etc. Therefore, the proposed methods justify their existence and have been compared with the reported methods (Table 6). The HILIC method (Attimarad *et al.*, 2022c) suffers from low sensitivity in terms of range, LOD, and LOQ; did not adopt specificity parameter which is very crucial for an analytical method when compared with proposed methods. Whereas another published article (Patel and Desai, 2022) did not opt for a specificity parameter, wavelength selection is not appropriate and mismatched throughout the article (UV method). In HPLC method calibration range of metformin is mismatched throughout the manuscript along with many other flaws. Therefore, the proposed methods are found to be superior when compared with the reported methods.

CONCLUSION

For the concurrent evaluation of RGF, VGT, and MTH in the ternary admixture, four spectroscopic methodologies were proposed: SEM, RDS, DRZC, and DDRS. In accordance with ICH guidelines, the established procedures were verified. The suggested procedures worked reliably, were straightforward to implement, quickly responded to user input, were easy to replicate, and cost effective. Moreover, all of the proposed UV-spectrophotometric methods require fewer steps in sample preparation and provide a wider concentration range with acceptable sensitivity and found to be superior when compared with reported methods. In the QC segment, where time and money are of the essence, the proposed spectrophotometric techniques are held to be reliable. Furthermore, these methods are considered more cost-effective than other analytical procedures because they do not call for costly solvents or intricate instruments. As a result, traditional QC research of RGF, VGT, and MTH in combined dosage forms (tablet) can make use of all existing methodologies.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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