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Area Under Curve and Zero Order UV-Spectrophotometric Methods: Development and Validation for the Simultaneous Estimation of Caffeine and Metformin in the Formulation

Sushmita I. Hiremath
Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Vidya Nagar, Hubli 580031, Karnataka, India

AHM Viswanathaswamy*
Department of Pharmacy Practice, KLE College of Pharmacy, Vidya Nagar, Hubli 580031, Karnataka, India
[*Corresponding author]

Shazia Mulla
Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Vidya Nagar, Hubli 580031, Karnataka, India

Shreyas P. Kulkarni
Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Vidya Nagar, Hubli 580031, Karnataka, India

Sagar Y. Patil
Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Vidya Nagar, Hubli 580031, Karnataka, India

Nikhil Gawas
Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Belagavi 590010, Karnataka, India

Mahesh S. Palled
Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Belagavi 590010, Karnataka, India

Suresh S. Honnalli
Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Vidya Nagar, Hubli 580031, Karnataka, India

Pooja Koganole
Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Vidya Nagar, Hubli 580031, Karnataka, India

Pooja Gouda
Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Vidya Nagar, Hubli 580031, Karnataka, India

Abstract
Caffeine (CAF) and Metformin (MET), two well-known drugs for CNS stimulation and Antidiabetic activity respectively when taken in combination can create wonders for treatment of fibrosarcoma and type-2 diabetes. We have carried out this research to provide a simple, safe, precise, accurate and inexpensive method for simultaneous estimation of CAF and MET in bulk using Area Under Curve (AUC) and Zero Order UV-Spectroscopic (ZOS) methods. The developed method was also validated as per ICH guidelines. Distilled water is chosen as the solvent for both the methods. For Caffeine, area between 251-289.5 nm was employed to measure the AUC and 273 nm was employed to measure the absorbance. For Metformin, area between 224.5-239.5 nm was employed to measure the AUC and 233 nm was employed to measure the absorbance. In both AUC and ZOS method, CAF and MET showed linearity in the concentration range of 4-20 µg/ml and 0.6-3.0 µg/ml respectively. All the validation parameters for CAF and MET by both the methods exhibited % RSD less than 2% which is well within the acceptance range. Overall, the developed and validated Spectroscopic methods can be effectively exercised for the quality control of CAF and MET in formulation.

Keywords
Area Under Curve method, Caffeine, Metformin, ICH guidelines, Zero Order Spectroscopic method

INTRODUCTION

Caffeine (CAF) is a central nervous system (CNS) stimulant belonging to methylxanthine class. Caffeine is a type of drug that is restorative for brain and the nervous system. Caffeine is found in many drinks such as coffee, tea, soft drinks and energy drinks. Chocolate also contains caffeine. Energy drinks often have more caffeine and sugar than soft drinks. IUPAC name of caffeine is 1, 3, 7-Trimethyl-3,7-dihydro-1H-purine-2,6-dione (Figure 1). Caffeine is present in coffee, black and green tea, cocoa, cola soft drinks and energy drinks. It may also be in chocolate bars, energy bars and some non-prescription medications, such as cough syrup and slimming tablets. Caffeine stimulates the activity in your brain and nervous system and it also increases the circulation of chemicals such as cortisol and adrenaline in the body. In large doses of caffeine, it makes people anxious and cause difficulty in sleeping but in short doses, it makes the brain more refreshed and focused. Caffeine is found in the seeds, fruits, nuts, or leaves of a number of plants native to Africa, East Asia and South America and the top known source of caffeine is the coffee bean, the seed of the Coffee plant. Caffeine has bioavailability of 99%, protein binding of 25-36%, onset of action is within 45 min to 1 hour and has a duration of action of 3 to 4 hours. Caffeine increases intracellular concentrations of cyclic adenosine monophosphate (cAMP) by inhibiting phosphodiesterase enzymes in skeletal muscle and adipose tissues. These actions promote lipolysis via the activation of hormone-sensitive lipases with the release of free fatty acids. Caffeine is used to control fatigue, drowsiness and also used in the treatment of apnea of prematurity and prevention and treatment of Broncho pulmonary dysplasia of premature infants, migraine headaches and post-dural puncture headaches, improve athletic performance, treat depression, treatment of Alzheimer's and Parkinson disease, orthostatic hypotension, asthma, improves muscular strength and power, and may enhance muscular endurance [1, 2].

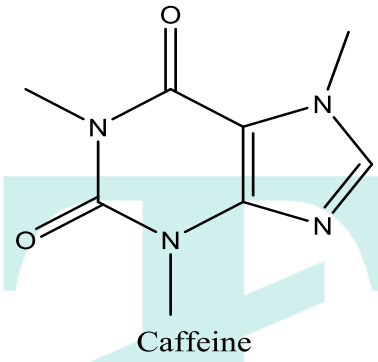


Fig. 1 Chemical Structure of CAF

Metformin is a biguanideantihyperglycemic agent. IUPAC name of metformin (MET) is N, N-Dimethylimidodicarbonimidicdiamide (Figure 2). It reduces the glucose production in the liver, rising the insulin sensitivity of body tissues and increasing GDF15 secretion, which reduces appetite and caloric intake. This potent drug was discovered in 1922. Metformin was originally developed from natural compounds found in the plant Galegaofficinalis, known as French lilac or goat's rue. The preparation method of metformin is as follows: dicyandiamide and dimethylamine hydrochloride are used as raw materials and heated by utilizing microwave. The two raw materials are heated till 100-160°C to acquire a product system consisting of metformin hydrochloride. The mechanism of action of metformin is the modification of energy metabolism of the cell. Metformin strives its glucose lowering effect by facing the action of glucagon and inhibiting hepatic gluconeogenesis. The route of administration of metformin is by mouth. Metformin has bioavailability of 50-60%, has minimal protein binding,not metabolized by liver, has an elimination half -life of 4 to 8.7 hours and excretion is done by urine and has a molar mass of 129.167 g/mole.Metformin is used to lower the blood sugar level especially in type 2 diabetes and it is also used for polycystic ovariansyndrome. Metformin is used to control high blood sugar, helps prevent kidney damage, blindness, nerve problems, loss of limbs, and sexual function problems. Metformin works by helping to restore body's proper response to the insulin that is produced naturally. It also decreases the amount of sugar that the liver makes and that the stomach or intestines absorb [3, 4, 5].

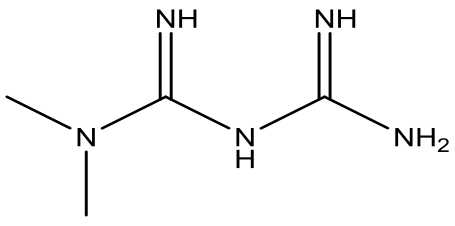


Fig. 2 Chemical Structure of MET

After administering BHK-21/C13 cells subcutaneously to hamsters, all injected animals developed fibrosarcomas at the injection site. The peroral administration of caffeine and metformin dramatically reduced tumor development. This was confirmed by noticeably reduced tumor weight and volume as well as by reduced tumor cell proliferation as demonstrated

by Ki-67 staining on hamster tumor sections. Streptozotocin-induced hyperglycemia is significantly reduced by Metformin plus black coffee therapy [6, 7].

As the major portion of the population consumes caffeine daily while metformin in taken in tablet dosage form, quality monitoring of these substances is crucial in pharmaceutical industry. Numerous techniques have been published by researchers for analyzing the medicine and formulations. Few analytical techniques are reported in scientific papers and literature for estimating caffeine and metformin in various forms. Fewer of these analytical techniques were emphasized and examined.

The literature search is that there many papers on estimation and analysis of the drugs under study i.e., CAF and MET separately by different analytical techniques such as UV-Spectrophotometry [8-29], HPLC [30], HPTLC-UV [31], GC-MS [32], RP-HPLC [24] [33], TLC [9] and conductometrictitrations [34].CAF was estimated in marketed tea by HPTLC method where they used ethyl acetate: Methanol as solvent phase and UV method using water as solvent at 274 nm [31]. CAF content in soft and energy drinks was also evaluated using TLC and UV Spectroscopic method at 273 nm which was linear in the concentration range of 4-12 µg/ml [9]. CAF was also simultaneously estimated with some other drugs such as paracetamol, chlorogenic acids, aspirin, codeine and ibuprofen [8, 10, 14, 15]. MET in bulk and tablet dosage form was estimated by UV-spectroscopic method at 234 nm [16] and 232 nm [18] using water as solvent. MET in combination with some other drugs such as glimepiride [17], dapagliflozin [19], pioglitazone [33], amlodipine, glibenclamide [30], and sitagliptin phosphate [29] was also simultaneously estimated using UV Spectrophotometric and RP-HPLC methods. On the other hand, there are hardly few papers that give us information on simultaneous estimation of Caffeine and Metformin in combination. This combination has anticancer activity in hamsters [6] and synergistic effect in treatment of type-2 diabetes in mice model [7]. In addition to that caffeine also enhances the hypoglycemic effect of both gliciazide and metformin HCl in healthy rats [35].

To fill in the gaps that we come across during the literature review, the current research particularly focuses on developing and validating the UV-Spectrophotometric method as per ICH guidelines for the simultaneous estimation of CAF and MET so that a complete meaning can be attached to the discoveries done using these drugs.

MATERIALS AND METHODS

Instruments, Reagents and Chemicals

For weighing the drugs, Shimadzu Electronic balance was used. Shimadzu UV-1900i UV-Vis spectrophotometer was used to measure absorbance and AUC. The software used for carrying out our study is UV Probe and LabSolutions. The reagent solutions and chemicals were collected from the store of KLE College of Pharmacy, Hubballi.

Optimization of Methods

Preparation of stock solution

The standard stock solution-I (SS-I) of caffeine was prepared in 10 ml volumetric flask (VF) by dissolving 10mg of caffeine in distilled water and diluting it up to the 10 ml mark .Later, 1 ml from SS-I was pipetted out in a 10 ml VF to form stock solution-II (SS-II). From SS-II, serial dilution were prepared in the concentration range of 4 µg/ml to 20 µg/ml. Similarly the standard stock solution-I (SS–I) of metformin was prepared in 10 ml VF by dissolving 10 mg of metformin in distilled water and diluting it up to the 10 ml mark. Later, 1 ml from SS-I was pipetted out in a 10 ml VF to form stock solution-II (SS-II). From SS-II, serial dilution were prepared in the concentration range of 0.6 µg/ml to 3.0 µg/ml.

Selection of solvent and wavelength of analysis

The solubility analysis and literature Survey revealing that caffeine was less soluble in distilled water at room temperature whereas metformin was highly soluble in distilled water. In order to recognize the wavelength for analysis, solution containing 10 µg/ml of CAF and MET were analyzed in the UV region of 200-400 nm and spectrum was obtained. The wavelength for maximum absorbance (λ_{max}) of caffeine was found to be 273 nm and wavelength for maximum absorbance (λ_{max}) of metformin was found to be 233 nm.

Measurement of area under curve for AUC and Absorbance for ZOS methods

For Caffeine, the AUC between these two wavelengths 251 – 289.5 nm were used for measurement in AUC method and 273 nm was used to measure the absorbance of solutions in ZOS method. For Metformin, the AUC between these two wavelengths 224.5 – 239.5 nm were used for measurement in AUC method and 233 nm was used to measure the absorbance of solutions in ZOS method.

Validation

The developed techniques were validated as per ICH guidelines in terms of linearity, accuracy, specificity, LOQ, LOD, selectivity, precision, assay, robustness and ruggedness.

Selectivity and Specificity

The word selectivity allude to a method that generates a response for a single analyte only, whereas the term specificity makes reference to a method that issues response for a number of chemical entities that may or may not be prominent from each other [36].

Standard calibration curve

The series of dilutions of 4,8,12,16 and 20 µg/ml were made from the standard stock solution (SS-II) of caffeine and 0.6, 1.2 , 1.8 , 2.4 and 3.0 µg/ml were the series of dilutions made from the standard stock solution (SS-II) of metformin. For AUC method, 251–289.5 nm was selected for the determination of caffeine and 224.5–239.5 nm was selected for the determination of metformin. For ZOS method, absorbance of Caffeine and Metformin were measured at 273 nm and 233 nm respectively. The calibration curves of concentration vs. absorbance for CAF and MET were prepared and the r^2values were calculated.

Detection and Quantification Limits

The Limit of Detection (LOD) of an analytical method is the lowest amount of an analyte in a sample that can be discovered, but not necessarily quantitated.

The limit of quantitation is the lowest concentration of analyte in a sample that can be insistent on with allowable accuracy and precision under the affirmed operational conditions of the method [36].

Precision

Precision was studied to assess the preciseness of the technique. Precision of a method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings. The system precision, interday and intraday precision were used [37].

Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same sample under a variety of normal test conditions, such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, etc. [38].

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. [38].

Accuracy

The accuracy of the developed method was evaluated at three different levels i.e., 50, 100 and 150%. The mixtures of standard solution and sample solution of both Caffeine and Metformin at the above mentioned levels were analysed and the %RSD were calculated.

Assay

For assay procedure, about twenty tablets of Caffeine and Metformin in combination were weighed, crushed to fine powder and used for the assay procedure. Equivalent weight of 10 mg of both the drugs were taken in separate 10 ml volumetric flasks and made-up to the 10ml mark using distilled water to prepare stock solution followed by sonication for 3 minutes. Series of dilutions were within beer's range and the absorbance was calculated and reported.

Preparation of tablets

400 mg tablets of Caffeine and Metformin in combination were prepared in-house using wet granulation method. The ingredients used in the tablet are depicted in Table 1.

Table 1 Tablet Formulation

Ingredient	Role of ingredient	Quantity
Metformin	API	250 mg
Caffeine	API	25 mg
Lactose	Diluent	125 mg
Polyvinyl alcohol	Binder	q.s.
Isopropyl alcohol	Granulating agent	q.s.
Talc	Glidant	q.s.
Magnesium stearate	Lubricant	q.s.

RESULTS

Development

Distilled water was selected as the solvent in which Caffeine (CAF) and Metformin (MET) were soluble and the spectrum obtained showed maximum absorbance (λ_{max}) at 273nm and 233nm respectively. The other specifications are depicted in Table 2.

Table 2 Specifications of AUC and ZOS Spectroscopic Method

No.	Parameters	Specification (Caffeine)	Specification (Metformin)
1	Method	Spectrophotometric	Spectrophotometric
2	Instrument	UV-Spectrophotometer	UV-Spectrophotometer
3	Model	Shimadzu	Shimadzu
4	Make	UV-1900i	UV-1900i
5	Software	LabSolutions UV-Vis	LabSolutions UV-Vis
6	Analyte	Caffeine	Metformin
7	Solvent	Distilled water	Distilled water
8	Lambda Max.	273 nm	233 nm

Validation

Specificity and Selectivity

The solvent spectrum did not show any interference at the wavelengths273nm and 233nm which are the λ_{max} of CAF and MET respectively. This clearly shows the specificity and selectivity of the method used. The solvent spectrum of solvent and the drugs CAF and MET are depicted in Figure 3 and Figure 4.

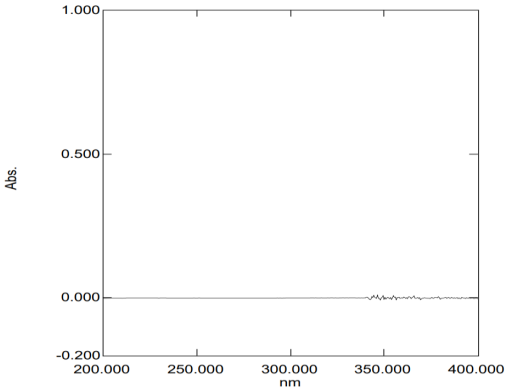


Fig. 3 Spectrum of Blank Solvent

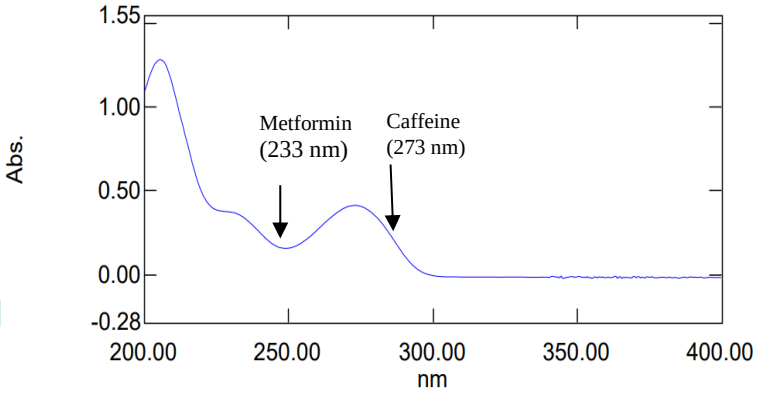


Fig. 4 Spectrum of Caffeine and Metformin

Linearity and Range

The standard calibration curve for ZOS method was plotted with absorbance on y-axis and concentration on x-axis while for AUC method the standard calibration curve was plotted with AUC on y-axis and concentration on x-axis by using linear dilution of CAF and MET. For CAF, the calibration curve was linear in the serial dilution of 4, 8, 12, 16 and 20 µg/ml with a correlation coefficient (r^2) of 0.9945 in ZOS method and 0.999 in AUC method.. For MET, the calibration curve was linear in the serial dilution of 0.6, 1.2, 1.8, 2.4 and 3.0 µg/ml with a correlation coefficient (r^2) of 0.9983 in ZOS method and 0.9921 in AUC method. The linearity data of CAF and MET are depicted in Table 3 and Table 4. The standard calibration curves of CAF and MET are shown in Figure 5,6 ,7 and 8. Overlay spectrum is shown in Figure 9 and AUC graphs are shown in Figure 10.

Table 3 Linearity and range data of Caffeine

No.	Concentration (µg/ml)	Area Under Curve (251-289.5nm)	Absorbance at 273 nm
1	4	3.055	0.23
2	8	6.03	0.43
3	12	9.239	0.65
4	16	12.31	0.86
5	20	15.317	0.998
r^2		0.9999	0.9945
LOD		0.19	0.09
LOQ		0.58	0.27

Table 4 Linearity and range data of Metformin

No.	Concentration (µg/ml)	Area Under Curve (224.5 - 239.5 nm)	Absorbance at 233 nm
1	0.6	0.171	0.21
2	1.2	0.349	0.373
3	1.8	0.539	0.562
4	2.4	0.639	0.704
5	3	0.825	0.897
r^2		0.9921	0.9983
LOD		0.08	0.05
LOQ		0.24	0.17

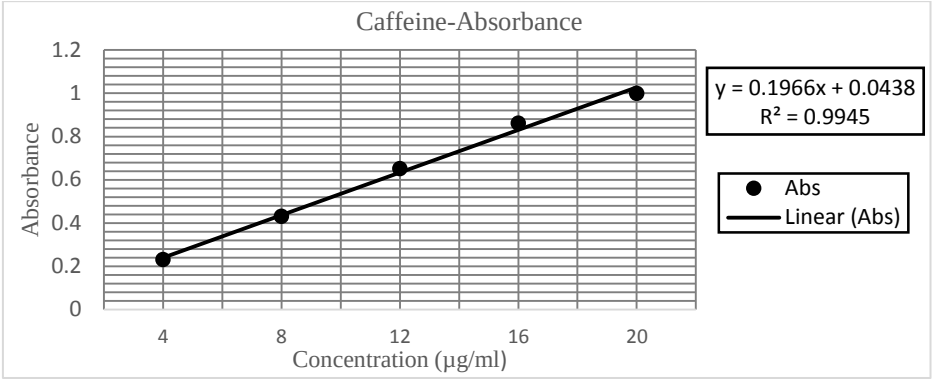


Fig. 5 Standard calibration curve of Caffeine for ZOS method

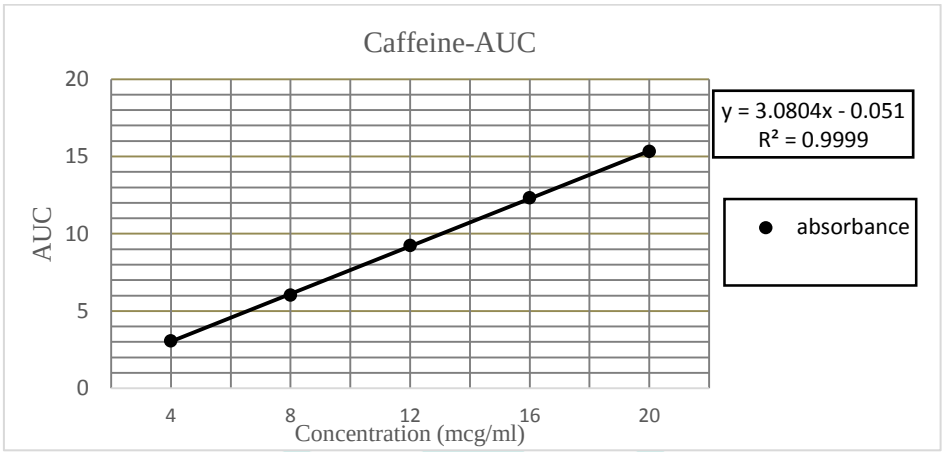


Fig. 6 Standard calibration curve of Caffeine for AUC method

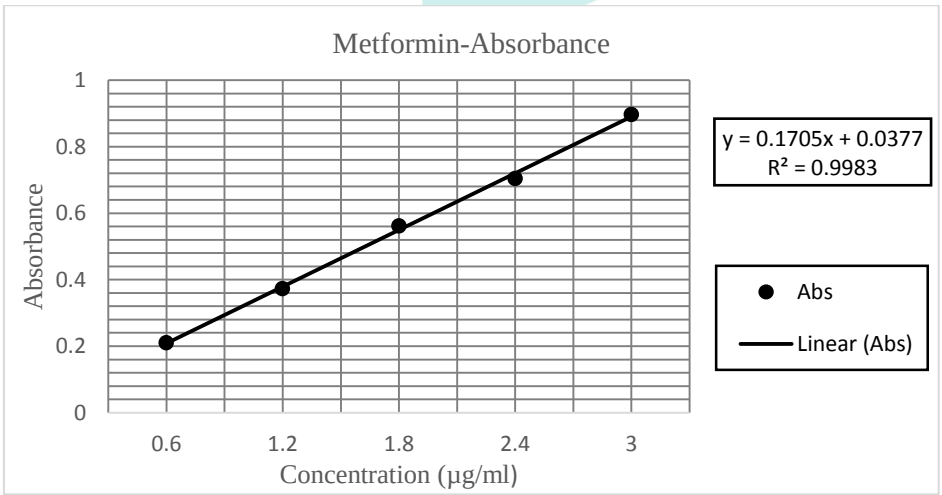


Fig. 7 Standard calibration curve of Metformin for ZOS method

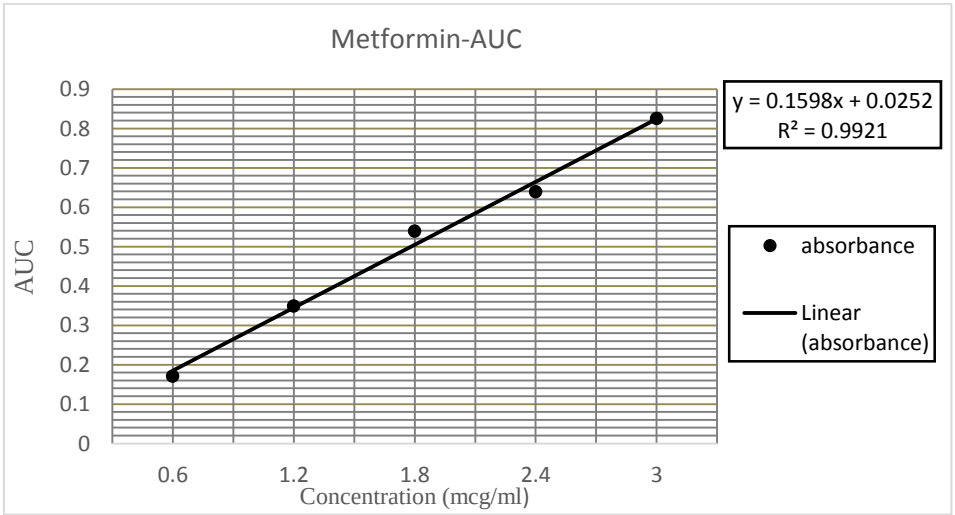


Fig. 8 Standard calibration curve of Metformin for AUC method

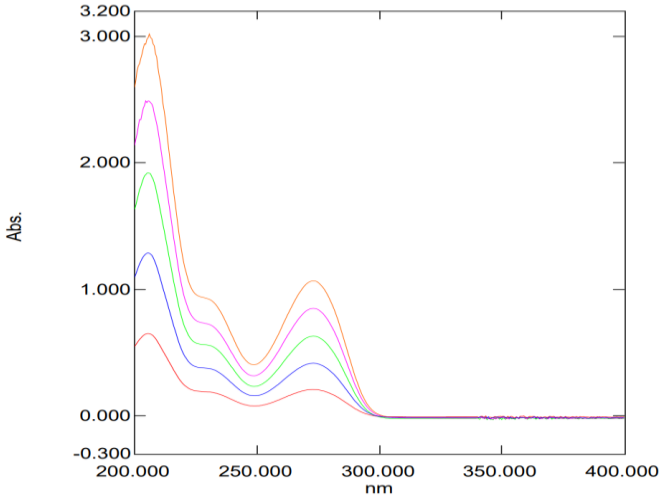
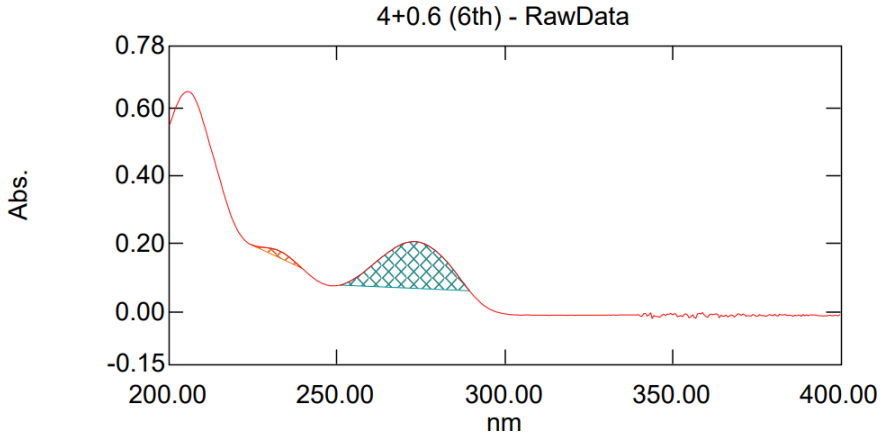
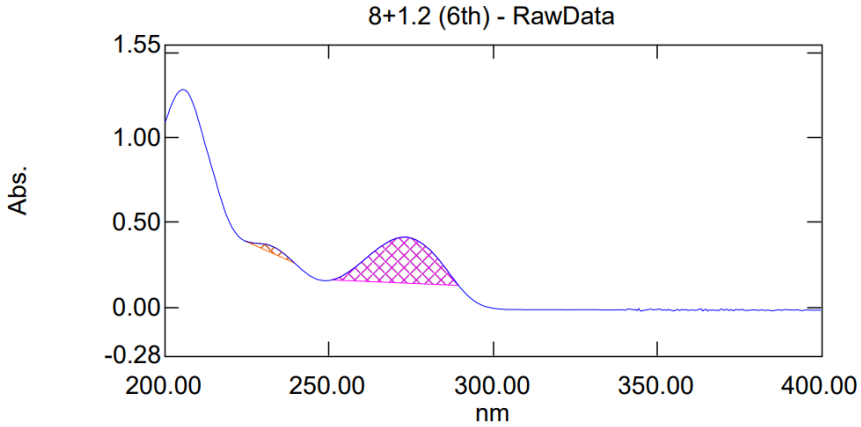


Fig. 9 Linearity overlay spectrum of Caffeine and Metformin

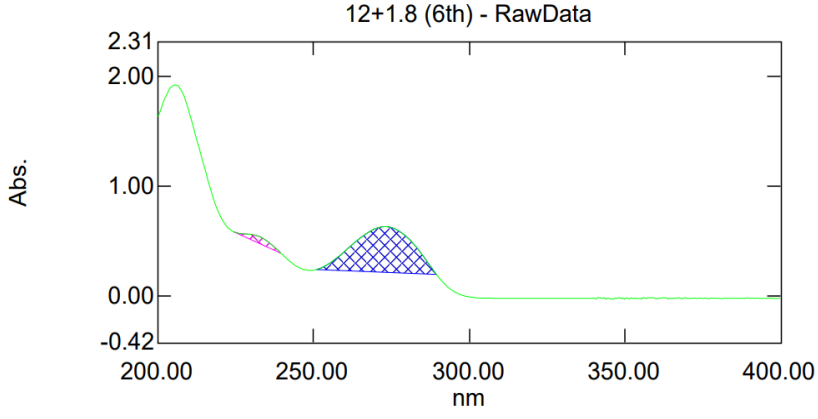
AUC of Caffeine 4 µg/ml and Metformin 0.6 µg/ml



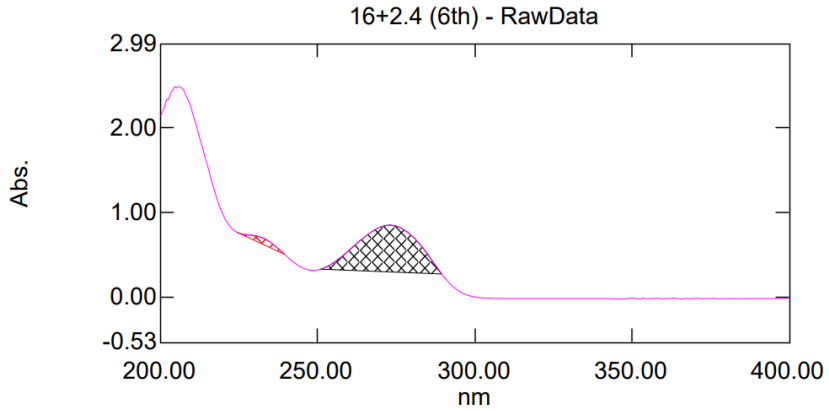
AUC of Caffeine 8 µg/ml and Metformin 1.2 µg/ml



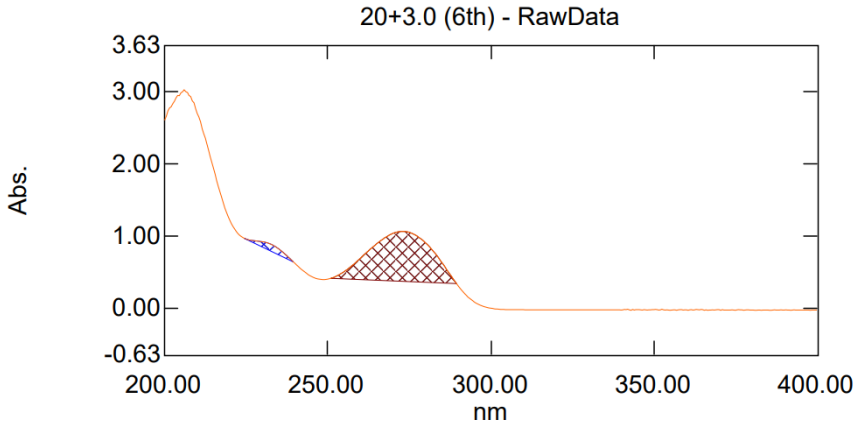
AUC of Caffeine 12 µg/ml and Metformin 1.8 µg/ml



AUC of Caffeine 16 µg/ml and Metformin 2.4 µg/ml



AUC of Caffeine 20 µg/ml and Metformin 3.0 µg/ml



UV-Spectrum of Blank

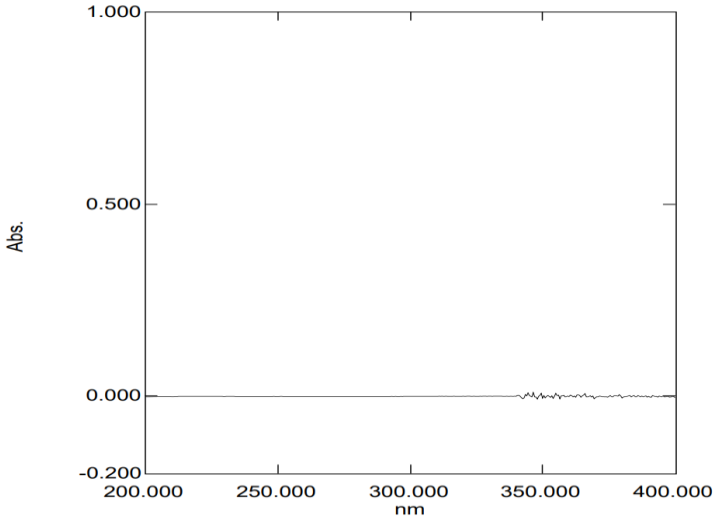


Fig. 10 AUC spectrum of Caffeine and Metformin

Precision

As the %RSD of both CAF and MET solutions in system, interday and intraday precision at different levels for both ZOS and AUC method were found to be less than 2%, hence the developed method is considered to be precise. The precision data of CAF ad MET are depicted in Table 5 and Table 6.

Table 5 Precision Data of Caffeine

Precision	%RSD of AUC Method			%RSD of ZOS Method		
Concentration	4µg/ml	12µg/ml	20µg/ml	4µg/ml	12µg/ml	20µg/ml
System precision	1.857	-	-	1.682	-	-
Interday 1	1.802	1.465	1.306	1.981	1.011	1.207
Interday 2	1.365	1.076	0.812	0.907	1.519	0.870
Interday 3	1.823	0.953	0.482	1.343	1.290	1.613
Intraday-1 (1 Hr)	1.253	1.549	0.988	1.449	1.903	1.666
Intraday-2 (4 Hr)	1.101	0.829	0.613	1.763	1.169	0.856
Intraday-3 (8 Hr)	1.787	1.699	0.488	0.638	1.858	0.450

Table 6 Precision Data of Metformin

Precision	%RSD of AUC Method			%RSD of ZOS Method		
Concentration	0.6µg/ml	1.8µg/ml	3.0µg/ml	0.6µg/ml	1.8µg/ml	3.0µg/ml
System precision	1.347	-	-	1.988	-	
Interday 1	0.864	0.938	1.831	1.492	1.908	1.769
Interday 2	1.197	1.642	1.395	1.935	1.134	0.928
Interday 3	1.487	1.658	0.464	1.724	1.333	1.267
Intraday-1 (1 Hr)	1.161	1.342	1.059	1.209	1.341	1.370
Intraday-2 (4 Hr)	1.161	1.428	1.036	0.935	1.017	0.844
Intraday-3 (8 Hr)	1.788	1.683	1.154	1.493	1.707	0.946

Robustness

The %RSD values of CAF and MET were found to be less than 2% for both ZOS and AUC method which indicates the method is robust as well as rugged with small change in nanometer. The Robustness data of CAF and MET are depicted in Table 7 and Table 8.

Table 7 Robustness Data of Caffeine

Robustness	%RSD of ZOS Method		
Conc. (µg/ml)	4	12	20
271 nm	0.526	0.855	1.645
272 nm	1.08	0.296	1.684
273 nm	1.195	1.136	1.465
274 nm	0.794	1.002	1.574
275 nm	0.803	1.807	1.705

Table 8 Robustness Data of Metformin

Robustness	%RSD of ZOS Method		
Conc. (µg/ml)	0.6	1.8	3
231 nm	1.667	0.543	1.364
232 nm	1.786	1.446	0.822
233 nm	1.887	0.841	1.178
234 nm	1.17	0.559	0.774
235 nm	1.302	1.457	1.189

Ruggedness

The %RSD values for CAF and MET drug solution with change in analyst and the instrument for both ZOS and AUC method were found to be in the acceptance range, thus the method is considered to be rugged. The Ruggedness data of CAF and MET are depicted in Table 9 and Table 10.

Table 9 Ruggedness Data of Caffeine

Ruggedness	% RSD of AUC Method			% RSD of ZOS Method		
Concentration (µg/ml)	4	12	20	4	12	20
Change Analyst-1	1.331	1.362	1.357	1.449	1.848	1.385
Change Analyst-2	1.101	0.289	0.661	0.982	0.878	0.363

Table 10 Ruggedness Data of Metformin

Ruggedness	% RSD of AUC Method			% RSD of ZOS Method		
Concentration (µg/ml)	0.6	1.8	3.0	0.6	1.8	3.0
Change Analyst-1	1.586	1.342	1.250	1.702	1.341	1.332
Change Analyst-2	1.525	1.389	1.014	0.935	1.017	0.844

Accuracy

Accuracy of CAF and MET was well within the acceptance range for both ZOS and AUC method. The Accuracy data of CAF and MET are depicted in the Table 11 and Table 12.

Table 11 Accuracy of Caffeine

Level	Standard added (µg/ml)	Sample added (µg/ml)	Total Conc. (µg/ml)	%Recovery of AUC	%Recovery of ZOS
50%	7	1	8	95.9-110.3%	107.50- 114.8%
100%	7	5	12	107.02-107.72%	102.20-103.32%
150%	7	9	16	104.24-108.72%	101.24-102.49%

Table 12 Accuracy of Metformin

Level	Standard added (µg/ml)	Sample added (µg/ml)	Total Conc. (µg/ml)	%Recovery of AUC	%Recovery of ZOS
50%	0.5	0.7	1.2	102.86-108.57%	92.86-104.29%
100%	0.5	1.3	1.8	93.85-108.46%	97.69-102.31%
150%	0.5	1.9	2.4	96.32-99.47%	98.95-103.16%

Assay

For Caffeine, the assay values by AUC and ZOS method were found to be 98.33-99.93% and 95.65-98.99% respectively while for Metformin, the assay values by AUC and ZOS method were found to be 94.15-99.52% and 94.76-98.22% respectively. The Assay data of CAF and MET are depicted in the Table 13 and Table 14.

Table 13 Assay data of Caffeine

Drug Name	Label Claim (mg/tablet)	Average Weight (mg)	Conc. (µg/ml)	%Purity of AUC	%Purity of ZOS
Caffeine	400 mg	393 mg	4	98.33%	95.65%
			12	99.80%	97.54%
			20	99.93%	98.99%

Table 14 Assay data of Metformin

Drug Name	Label Claim (mg/tablet)	Average Weight (mg)	Conc. (µg/ml)	%Purity of AUC	%Purity of ZOS
Metformin	400 mg	393 mg	0.6	94.15%	94.76%
			1.8	97.77%	98.04%
			3.0	99.52%	98.22%

DISCUSSION

The current research was focused on development of a simple, accurate, precise, economical, sensitive, specific, validated UV-Spectrophotometric method for simultaneous estimation of Caffeine (CAF) and Metformin (MET) in bulk form using Area Under Curve (AUC) and Zero Order Spectroscopic (ZOS) methods. In case of caffeine, the AUC was measured in the range of 251-289.5 nm for the first method, and for the second method, the absorbance was measured at 273 nm. While in case of Metformin, the AUC was measured in the range of 224.5-239.5 nm for the first method, and for the second method, the absorbance was measured at 233 nm. Since both CAF and MET are soluble in distilled water, it was used as the solvent for UV-spectrometric estimation in both the methods. As per ICH guidelines, the developed method was optimized and standardised with regard to linearity, specificity, selectivity, precision, robustness, ruggedness, limit of detection (LOD), limit of quantification (LOQ), and accuracy. Both the methods showed linearity between the quantity ranges of 4 – 20 µg/ml and 0.6 – 3.0 µg/ml for CAF and MET respectively. All the validation parameters for the developed method exhibited %RSD below 2%. In terms of recovery values, both the methods were determined to be accurate.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no competing financial interests.

CONCLUSION

On reviewing the literature survey, we got to know that there are no relevant papers on simultaneous estimation of CAF and MET by AUC and ZOS UV-Spectrophotometric methods. Therefore, in the present research work we have developed new UV-Spectrophotometric methods using distilled water as solvent which was really cost-efficient and easy to work with. This study concludes that the developed and validated Area Under Curve and Zero Order UV spectroscopic method for simultaneous estimation of Caffeine and Metformin in bulk is simple, accurate, precise, inexpensive, and can be used for further studies in the research field. The results were upto the mark and at satisfactory level. Thus these methods can be useful in pharmaceuticalresearch sector for quality control analysis.

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