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RESEARCH ARTICLE

Spectrophotometric Assay Of Diethylcarbamazine Citrate Using Folin-Ciocalteu Phenol Reagent

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Summary

A rapid and simple spectrophotometric method for the assay of diethylcarbamazine citrate (DEC) is described. The method is based on the formation of blue colored chromogen due to reduction of tungstate and/or molybdate in Folin-Ciocalteu (F-C) reagent by DEC in alkaline medium. The colored species have absorption maximums at 760 nm and the system obeys Beer's law over the concentration range 10-100 μg mL^{-1} DEC. The absorbance was found to increase linearly with increasing concentration of DEC, which is corroborated by the calculated correlation coefficient value of 0.9969. The apparent molar absorption and Sandell sensitivity values were 2.08 × 10^3 L^{-1} mol^{-1} cm^{-1} and 0.188 μg cm^{-2}, respectively. The limits of detection (LOD) and quantification (LOQ) values are also reported. Over the linear range applicable, the accuracy and precision of the method were evaluated on intra-day and inter-day basis; the reported mean accuracy value was found as 100.98 ± 1.69%; the relative error (RE) was ≤ 2.67%, whereas the relative standard deviation (RSD) was ≤ 2.53%. Application of the proposed method to bulk powder and commercial pharmaceutical formulations are also presented.

Key Words: *Diethylcarbamazine citrate, assay, F-C reagent, spectrophotometry, pharmaceuticals.*

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Dietilkarbamazin Sitratın Folin-Ciocalteu Fenol Reaktifi ile Spektrofotometrik Analizi

Özet

Dietilkarbamazin sitrat analizi için hızlı ve basit bir spektrofotometrik yöntem (DEC) anlatılmaktadır. Yöntem, Folin-Ciocalteu (F-C) reaktifi içindeki tungstat ve/veya molibdatın, alkali ortamda DEC ile indirgenmesiyle mavi rengin oluşumu temeline dayanmaktadır. Renkli maddenin maksimum absorbansı 760 nm'dedir ve 10-100 μg mL^{-1} DEC aralığında Beer's kanununa uyum göstermektedir. Absorbansın, DEC'in artan konsantrasyonlarıyla doğrusal olarak artış gösterdiği 0.9969 olarak hesaplanan korelasyon katsayısı ile doğrulanmıştır. Belirgin mol absorbansı ve Sandell hassasiyet değerleri ise sırasıyla 2.08 × 10^3 L^{-1} mol^{-1} cm^{-1} and 0.188 μg cm^{-2} olarak tespit edilmiştir. Algılama (LOD) ve ölçüm (LOQ) değerleri sınırları da bildirilmiştir. Geçerli olan doğrusal aralıkta, yöntemin doğruluğu ve hassasiveti gün içi ve gün arası bazda değerlendirilerek, rapor edilen ortalama doğruluk değeri 100.98 ± 1.69%, bağıl hata (RE)≤2.67% olarak belirlenirken, bağıl standart sapmanın (RSD) ≤2.53% olduğu tespit edilmiştir. Önerilen yöntemin toplu toz ve ticari farmasötik formülasyonlar için uygulanması da sunulmuştur.

Anahtar kelimeler: *Dietilkarbamazin sitrat, analiz, FC reaktif, spektrofotometre, farmasötik.*

INTRODUCTION

Diethylcarbamazine citrate (DEC) (Figure 1), is an anthelmintic agent used in treatment of filarial infections caused by a host of organisms commonly found in the tropics, chemically known as [N, N-diethyl-4-methyl-1-piperazinecarboxamide citrate] [1]. It is also the alternative drug choice in *Onchocerca volvulus* infections and tropical eosinophilia [2]. The drug is present officially in the British Pharmacopoeia (BP) [3], which describes a non-aqueous titration method for its determination and also official in the United States pharmacopoeia (USP), [4] which uses a liquid chromatographic technique with the phosphate buffer system for the assay.

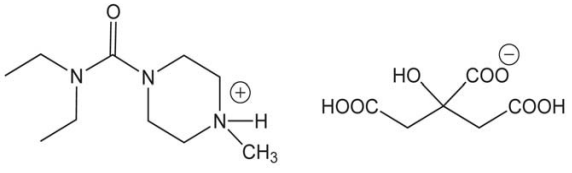

Figure 1. Structure of DEC

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Other than these official methods, a variety of techniques have been reported for the determination of DEC in pharmaceutical dosage forms, which include gas chromatography (GC) [5-8], high performance liquid chromatography (HPLC) [9-11], proton magnetic resonance (PMR) spectroscopy [12, 13], DC polarography analysis [14], ion selective electrode potentiometry [15] and titrimetry [16, 17]. However, in many of the reported methods, particularly for DEC, chromatographic methods are complex, requiring expensive instrumental set up and skilled operator, which are not always found in laboratories of developing and under developed countries. Thus, the need for a simple, selective and low cost method is apparent, especially for routine quality control analysis of pharmaceuticals containing DEC.

Several spectrophotometric methods based on diverse chemical reactions are found in the literature for DEC. Charge-transfer complex formed with iodine was used by Wahbi et al. [18], for the assay of 1-6 μg mL^{-1} DEC in tablets. Chloranilic acid has been employed by two groups of workers [19, 20] as CT complexing agent for the assay of the drug in pharmaceuticals based on the same type of reaction. In a method reported by Basu and Dutta [21], the ion associate formed by DEC with ammonium reineckate at pH 3.5 was filtered, dissolved in acetone and absorbance was measured at 525 nm. The colored condensation product [22] formed by malonic acid with acetic anhydride in the presence of DEC was measured at 333 nm facilitating the assay of the drug in dosage forms. In a similar method [23], the base form of the drug was reacted with malonic acid and acetic anhydride at 80 ^{0}C for 30 min, and the resulting condensation product was measured at 334 nm. The yellow colored condensation product [24] formed by an acetous solution of DEC with acetic anhydride-pyridine mixture was measured at 428 nm and used for the determination of DEC in 10-100 μg mL^{-1} range in commercial tablets.

There are three reports on the use of ion-pair complexation reactions for the spectrophotometric assay of DEC. Rao and Subramanyam [25] employed bromophenol blue at acidic pH as the ion pair complexing agent for the determination of the drug in tablets and biological fluids. The drug in tablets, syrups and parenterals was determined by extracting the ion-pair complex formed with bromocresol green [26] at pH 4.6 with chloroform. The colored complexes of the drug with Fast green FCF at pH 5.0 and orange II in 0.1 M HCl were successfully employed by Sastry et al. [27], for the determination of DEC in bulk drug and pharmaceutical preparations by extractive spectrophotometry.

The reported spectrophotometric methods suffer from one or the other disadvantage such as poor sensitivity and narrow linear range [20], tedious and time consuming steps like precipitation, filtration and washing [21], and heating [22-24]. The extraction methods [25-27] though sensitive, suffer from disadvantages like laborious, tedious and time consuming liquid- liquid extraction step, critical dependence on pH of the aqueous phase and the aqueous-organic phases ratio. Additionally, incomplete extraction of the analyte may lead to erratic results. Hence, there is a need for developing a method free of such disadvantages.

The aim of this investigation was to develop a rapid, simple and selective visible spectrophotometric method for the quantification of DEC in pure drug and in pharmaceutical formulations. The method uses the well known reduction reaction involving Folin-Ciocalteu (F-C) reagent and DEC in basic medium resulting in the formation of a blue chromogen that could be measured at 760 nm. The developed method was successfully applied to the determination of DEC in bulk drug, tablets and in syrup. The proposed method has been demonstrated to be superior to the reported methods with respect to speed, simplicity, sensitivity and cost-effectiveness. The statistical comparison of the proposed method and the official method revealed that there is no significant difference between two methods with respect to accuracy and precision.

MATERIALS AND METHODS

Instruments

A Systronic model 166 digital spectrophotometer (Systronics Ahmedabad, Gujarat, India) was used for absorbance measurements, with matched 1 cm quartz cells.

Chemicals and reagents

The chemicals used were of analytical grade. Distilled water was used throughout the investigation.

Folin-Ciocalteu reagent (Merck, Mumbai, India), sodium carbonate (S.D. Fine Chem. Ltd, Mumbai, India) used were of analytical reagent grade or chemically pure grade and used without further purification. Pharmaceutical grade pure DEC (99.7 per cent) was procured from Inga Laboratories Pvt. Ltd., Mumbai, India, and was used as received. Banocide Forte tablets (Glaxo Smith Kline Pharma. Ltd., Nashik, India) and Banocide syrup (Glaxo Smith Kline Pharma. Ltd., Bangalore, India) were both purchased from local commercial sources.

Standard DEC solution

A stock standard solution of DEC (1000 μg mL^{-1}) was prepared by dissolving 100 mg of pure DEC in water and made up to the volume with water in a 100 mL

volumetric flask. Working concentration of DEC (500 μg mL^{-1}) was prepared by dilution of the above stock solution with water.

F-C reagent (v/v)

A 1:1 aqueous solution was prepared by dissolving accurately measured 50 mL of F-C reagent in 50 mL of water.

Sodium carbonate (Na_2CO_3) (w/v)

A twenty percent (20%) solution was prepared by dissolving 20 g of the pure sodium carbonate in 100 mL of water.

General analytical procedure

Different aliquots of working standard DEC solution (500 μg mL^{-1}) ranging from 0.2-2.0 mL was transferred into a series of 10 mL of volumetric flasks and the total volume was brought to 2 mL with water. To each flask, 3 mL of 1:1 F-C reagent and 2 mL of 20% Na_2CO_3 solution were successively added by means of a microburette. Stoppers were put on the flasks, and the contents were mixed well and kept to room temperature for 15 min. The volume of each flask was made up to the mark with water and the absorbance of each solution was measured at 760 nm against a reagent blank similarly prepared in the absence of DEC.

Standard graph was prepared by plotting the absorbance *versus* drug concentration, and the concentration of the unknown was read from the calibration graph or computed from the respective regression equation derived using the absorbance-concentration data.

Assay procedure for tablets

An amount of finely ground tablet powder equivalent to 5 mg of DEC was accurately weighed into a 100 mL volumetric flask, the flask was shaken with ~60 mL of water for about 20 min; and finally volume was made up to the mark with water. The content was kept aside for 5 min, and filtered using Whatman No. 42 filter paper. The first 10 mL portion of the filtrate was discarded and a suitable aliquot (say 1.5 mL) was used for assay as described earlier.

RESULTS AND DISCUSSION

Folin-Ciocalteu reagent (F-C) is specifically used for the determination of many phenolic compounds utilizing its liability to turn into a blue colored product. Many drug substances such as salbutamol [28] minocycline [29], diclofenac [30], rimetazidine [31], acyclovir [32], methotrexate [33], omeprazole [34], sulphinpyrazone [35], and gliclazide [36], have been determined on this basis. The structural features of DEC allow the use of F-C reagent for its assay. The proposed method is based on the formation of a blue colored chromogen, following the reduction of phospho-molybdo tungsten mixed acid of the F-C reagent [37] by DEC, in the presence of sodium carbonate, which could be measured at 760 nm. The acids mixed in the F-C reagent are the final chromogen and involve the following chemical species:

$3H_2O{\cdot}P_2O_5{\cdot}13WO_3{\cdot}5MoO_3{\cdot}10H_2O$ and
$3H_2O{\cdot}P_2O_5{\cdot}14WO_3{\cdot}4MoO_3{\cdot}10H_2O$

DEC probably effects reduction of oxygen atoms from tungstate and/or molybdate in the F-C reagent, there by producing one or more possible reduced species which have characteristic intense blue color.

METHOD DEVELOPMENT

Optimization of experimental variables

A series of preliminary experiments necessary for rapid and quantitative formation of colored products to achieve the maximum stability and sensitivity were performed. Optimum condition was fixed by varying one parameter at a time while keeping other parameters constant and observing its effect on the absorbance at 760 nm.

Absorption spectra

DEC reacts with F-C reagent in the presence of Na_2CO_3 to form an intensely blue colored product with an absorption maximum at 760 nm. Figure 2 shows the absorption spectra of the reaction product and reagent blank. The colored product showed a maximum absorbance at 760 nm, which was used as the wavelength for determination. Under the same experimental conditions the blank had negligible absorbance.

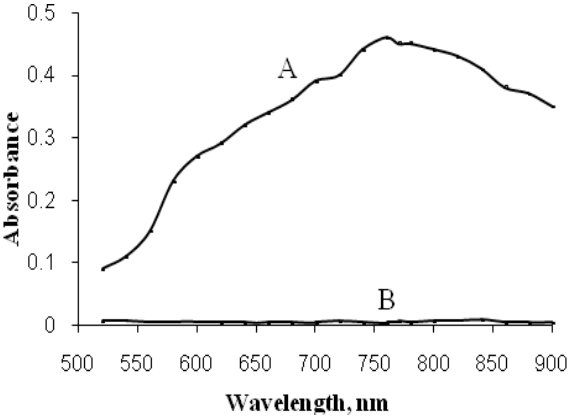

Figure 2. Absorption spectra of: A. reaction product of DEC (75 μg mL^{-1}) with F-C reagent in Na_2CO_3 solution and B. blank

Selection of reaction medium and optimization of the base

To select a suitable medium for the reaction, different aqueous bases such as sodium hydroxide, sodium carbonate or bicarbonate, sodium acetate and sodium hydrogen phosphate were investigated. Better results were obtained with sodium carbonate. In order to determine the optimum concentration of Na_2CO_3, different volumes of 20%

Na_2CO_3 solution (0–5 mL) were attempted at a constant concentration of DEC (75 µg mL^{-1}) and the results of the observation are shown in Fig 3. It was found that different volumes ranging from 1.0 to 3.0 mL of 20% Na_2CO_3 were optimum thus 2.0 mL was used throughout the work.

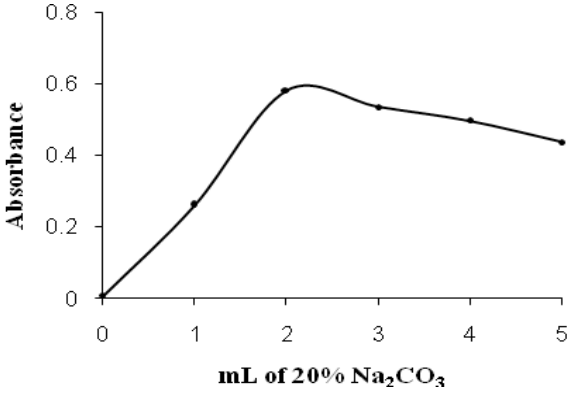

Figure 3. Effect of 20% Na_2CO_3 on color formation (75 µg mL^{-1} DEC)

Effect of concentration of F-C reagent

Several experiments were carried out to study the influence of F–C reagent concentration on the color development and the obtained results are shown in Figure 4. It is apparent that 3.0 mL of reagent gave the maximum color intensity, thus 3.0 mL of reagent was used throughout the investigation.

Effect of reaction time and stability of the color

Maximum color development was obtained in 10-20 min after mixing the reactants, hence the absorbance was measured after 15 min and the color was stable for at least 60 min thereafter.

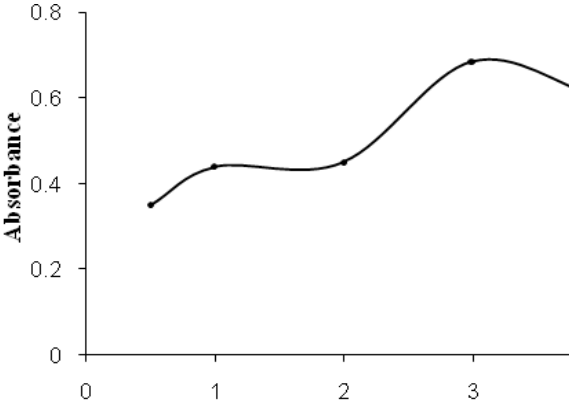

Figure 4. Effect of different volumes of F-C reagent (1:1) on the reaction product with DEC (75 µg mL^{-1}) in Na_2CO_3 solution

Effect of order of addition of reactants

The sequence of order of addition of the reactants had significant effect on the absorbance value. So, the order used in the general procedure should be followed for maximum absorbance.

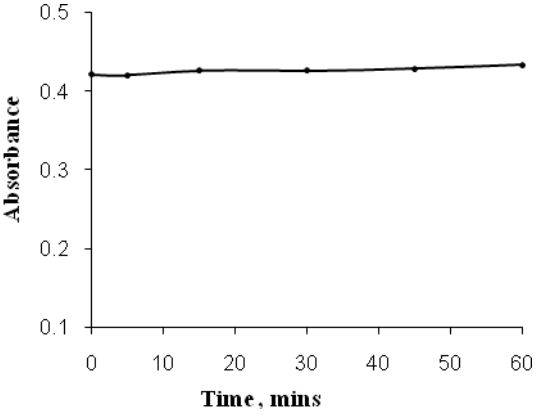

Figure 5. Plot for color stability

Table 1. Sensitivity and regression parameters.

Parameter	Value
λ_{max}, nm	760
Linear range, µg mL^{-1}	10-100
Molar absorption (ε), L mol^{-1} cm^{-1}	2.08×10^3
Sandell sensitivity*, µg cm^{-2}	0.188
Limit of detection (LOD), µg mL^{-1}	1.06
Limit of quantification (LOQ), µg mL^{-1}	3.20
Regression equation, Y**	
Intercept (a)	0.0283
Slope (b)	0.0046
Standard deviation of a (S_a)	0.0998
Standard deviation of b (S_b)	1.02×10^{-3}
Variance (Sa^2)	9.9×10^{-3}
Regression coefficient (r)	0.9969

*Limit of determination as the weight in µg mL^{-1} of solution, which corresponds to an absorbance of A = 0.001 measured in a cuvette of cross-sectional area 1 cm^2 and l = 1 cm. **Y=a+bX, Where Y is the absorbance, X is concentration in µg mL^{-1}, a is intercept, b is slope.

Table 2. Evaluation of intra-day and inter-day accuracy and precision**.**

DEC taken, μg mL^{-1}	Intra-day accuracy and precision (n=7)			Inter-day accuracy and precision (n=5)		
	DEC found μg mL^{-1}	%RE	RSD%	DEC found, μg mL^{-1}	%RE	RSD%
30.0	30.50	1.67	0.95	30.8	2.67	0.86
60.0	60.63	1.05	1.23	60.9	1.50	1.19
90.0	89.20	0.89	2.34	91.2	1.33	1.24

%RE-percent relative error, %RSD-relative standard deviation

VALIDATION OF METHOD

Linearity, sensitivity, limits of detection and quantification

A linear correlation was found between absorbance at λ_{max} and concentration of DEC in the ranges given in Table 2. The graph is described by the linear regression equation: Y = a + bX (where Y-absorbance of 1 cm layer of solution; a-intercept; b-slope and X-concentration in μg $mL^{-1)}$. Linear regression analysis of the Beer's law data incorporating the method of least squares was used to evaluate the slope (b), intercept (a) and correlation coefficient (r) for each system and the values are presented in Table 1. The optical characteristics such as Beer's law limits, molar absorption and Sandell sensitivity values [38] of the method are also given in Table 1. The limits of detection (LOD) and quantification (LOQ) calculated according to ICH guidelines [39] using the formulae: LOD = 3.3 S/b and LOQ = 10 S/b, (where S is the standard deviation of blank absorbance values, and b is the slope of the calibration plot) are also presented in Table 2. The high value of ε and low value of Sandell sensitivity and LOD indicate the high sensitivity of the proposed method.

Accuracy and precision

The assay described under "general procedure" was repeated seven times within the day to determine the repeatability (intra-day precision) and five times on different days to determine the intermediate precision (inter-day precision) of the method. The assay was performed for three levels of analyte. The results of this study are summarized in Table 2. The percentage relative standard deviation (RSD%) values were ≤ 1.56% (intra-day) and ≤ 2.53% (inter-day) indicating high precision of the method. Accuracy was evaluated as percentage relative error (RE) between the measured mean concentrations and taken concentrations for DEC. Bias {bias % = [(Concentration found - known concentration) × 100 / known concentration]} was calculated at each concentration and these results are also presented in Table 2. Percent relative error (RE%) values of ≤ 2.67% demonstrate the high accuracy of the proposed method.

Selectivity of the method

A systematic study was performed to determine the effect of matrix by analyzing the placebo blank and synthetic mixture containing DEC. A placebo blank of the composition: starch (10 mg), acacia (15 mg), hydroxyl cellulose (10 mg), sodium citrate (10 mg), talc (20 mg), magnesium stearate (15 mg) and sodium alginate (10 mg) was made and its solution was prepared as described under 'tablets', by taking 20 mg of the placebo and then subjected to analysis. The absorbance of the placebo solution was almost equal to the absorbance of the blank which revealed no interference. To assess the role of the inactive ingredients on the assay of DEC, a synthetic mixture was separately prepared by adding 10 mg of DEC to 20 mg of the placebo mentioned above. The drug was extracted and the solution prepared as described under the general procedure for tablets. The solutions after appropriate dilution wherever necessary were analyzed following the recommended procedure. The absorbance resulting from 30, 60 and 90 μg mL^{-1} DEC solution were nearly the same as those obtained for pure DEC solutions of identical concentrations. This unequivocally demonstrated the non-interference of the inactive ingredients in the assay of DEC. Further, the slopes of the calibration plot prepared from the synthetic mixture solutions were about the same as those prepared from pure drug solutions.

Robustness

The robustness of the method was evaluated by making small incremental changes in the volume of the F-C reagent or Na_2CO_3 and reaction time, and the effects of the changes were studied by calculating the mean RSD values. The changes had negligible influence on the results as revealed by small intermediate precision values expressed as RSD% (≤ 2.08%).

Table 3. Method robustness and ruggedness expressed as intermediate precision (RSD%).

DEC taken, μg mL^{-1}	Robustness			Ruggedness	
	Parameter altered				
	Volume of F-C reagent * (RSD%)	Volume of Na_2CO_3 * (RSD%)	Reaction time# (RSD%)	Inter-analysts, (RSD%) (n=4)	Inter-cuvettes, (RSD%) (n=4)
30.0	1.25	0.85	2.09	1.50	1.02
60.0	0.98	1.98	1.05	1.99	2.45
90.0	1.54	2.08	1.54	0.57	1.63

*Volumes of F-C reagent added were 1.8, 2.0 and 2.2 mL and volumes of Na_2CO_3 added were 2.8, 3.0 and 3.2 mL.

#The reaction time studied were13, 15 and 17 min.

Table 4. Results of analysis of formulations by the proposed method and statistical comparison of the results with the reference method.

Name of DEC formulation	Nominal amount,	Found* (Percent of label claim ± SD)	
		Reference method	Proposed method
Banocide forte tablets	100 mg per tablet	98.54±1.09	99.62±0.89 t = 1.71 F = 1.50
Banocide forte syrup	120 mg per 5 mL	100.06±0.62	101.36±1.23 *t* = 2.12 *F* = 3.94

*Average of five determinations.

Tabulated *t* value at the 95% confidence level is 2.77.

Tabulated *F* value at the 95% confidence level is 6.39.

Ruggedness

Method ruggedness was expressed as the RSD of the same procedure applied by four different analysts as well as using four different cuvettes. The inter-analysts RSD were within 2.5% whereas the inter-cuvettes RSD for the same DEC amount were less than about 2.65% suggesting that the developed method was rugged. The results are shown in Table 3.

Analysis of pharmaceutical formulations

The described procedure was successfully applied to the determination of DEC in its pharmaceutical formulations. The results obtained (Table 4) were statistically compared with the official BP method [3], which describes a non-aqueous titration for its determination for the assay. The results obtained by the proposed method agreed well with those of reference method and with the label claim. The results were also compared statistically by a Student's *t*-test for accuracy and by a variance *F*-test for precision [40] with those of the reference method at 95 % confidence level as summarized in Table 4. The results showed that the calculated *t*-and *F*-values did not exceed the tabulated values, inferring that proposed method is as accurate and precise as the reference method.

Recovery study

To further assess the accuracy of the method, recovery experiments were performed by applying the standard-addition technique. The recovery was assessed by determining the agreement between the measured standard concentration and added known concentration to the sample. The test was completed by spiking the pre-analyzed tablet powder with pure DEC at three different levels (50, 100 and 150 % of the content present in the tablet powder (taken) and the total was found by the proposed method. Each test was repeated three times. In all the cases, the recovery percentage values ranged between 98.98 and 102.9% with relative standard deviation in the range 0.56-1.35%. Closeness of the results to 100% showed the fairly good accuracy of the method. The results are shown in Table 5.

Table 5. Results of recovery study using standard addition method.

Formulation studied	DEC in formulation, μg mL^{-1}	Pure DEC added, μg mL^{-1}	Total DEC found, μg mL^{-1}	Pure DEC recovered (Percent±SD*)
Banocide forte tablets	29.89	15	44.43	98.98±1.03
	29.89	30	60.25	100.6±0.89
	29.89	45	76.61	102.3±1.22
Banocide forte syrup	30.41	15	45.24	99.62±0.56
	30.41	30	61.13	101.2±1.26
	30.41	45	77.59	102.9±1.35

*Mean value of three determinations.

Table 6. Comparison of performance of the present methods with the existing methods

Sl. No.	Reagent/s used	Methodology	λ_{max} (nm)	Linear range (μg mL^{-1}) ε (L mol^{-1} cm^{-1})	Remarks	Ref. No.
1	*CAA	Measurement of purple color CT complex in dioxane-$CHCl_3$	540	10-400	Mixture of organic solvents used	21
2	Picric acid	Yellow color CT complex measured in alcohol	-	-	-	22
3	Ammonium reineckate	Absorbance of red color product at pH=3.5 in acetone measured	525	-	Tedious & time consuming, precipitation washing and dissolution steps involved	23
4	Malonic acid-acetic anhydride	Measurement of absorption of condensation product	333	-	Heating step and longer contact time involved	25
5	HOAc-Ac_2O and pyridine	Absorbance of yellow color product measured	428	10-110	Heating step and longer contact time involved	26
6	*BPB	Extracted ion-pair complex measured	-	-	Tedious and time consuming extraction step and critical pH adjustment involved	27
7	*BCG	Yellow ion-pair complex measured in chloroform	-	-	Tedious and time consuming extraction step and critical pH adjustment involved	28
8	a)Fast green FCF b)orange- II	Ion-pair complex extracted into chloroform and measured	-	-	Tedious and time consuming extraction step and critical pH adjustment involved	29
9	F-C reagent	Redox reaction, blue colored chromogen measured	760	10-100 2.08×10^3	Rapid, extraction-free, no heating or extraction step involved, sensitive, wide linear dynamic range	Present work

* CAA-chloranilic acid, BCG-bromocresol green, BPB-bromophenol blue, BCP-bromocresol purple

CONCLUSION

In the present work, a new, rapid, simple, and selective spectrophotometric method has been developed, optimized and validated for the determination of DEC in bulk drug and in formulations. Optimization showed that none of the experimental variables is critical for the reproducible and quantitative assay of DEC. The method was found to be linear over an analytical range of 10–100 μg mL^{-1}, demonstrating that the method is applicable over a wide linear dynamic range with better selectivity than most of the published methods. Besides, as can be seen from Table 6, the proposed method is simpler than the reported methods in terms of the optimum conditions since it does not require either heating or extraction with organic solvents. Additionally, since the measurement is made at larger wavelength (760 nm) the interference by the tablet excipients is far less compared to the shorter wavelength used in almost all published methods. The results of *t*- and *F*- tests applied to accuracy and precision data enabled the conclusion that an excellent accuracy and high precision was achieved. Selectivity of the method was demonstrated by the absence of interferences by the co-formulated substances.

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