Volume 15 Number2
Journal of Advances in Chemistry

Novel Analytical Study For The Charge-Transfer Reactions Of Omeprazole With 2,3-Dichloro-Naphthoquinone And 2,3,5,6-Tetrabromo-1,4-Benzoquinone: Application For The Development Of Microwell Assay Of Omeprazole

Saad A. Alkahtania, Ashraf M. Mahmoudb, C*, And Samer S. Abu Al-Rubb
[a] Department of Clinical Pharmacy, College of Pharmacy, Najran University, P.O. Box 1988, Najran 11001, Kingdom of Saudi Arabia.
[b] Department of Pharmaceutical Chemistry, College of Pharmacy, Najran University, P.O. Box 1988, Najran 11001, Kingdom of Saudi Arabia.
[c] Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Assiut University, Assiut 71526, Egypt.
* Correspondence:
E-mail: a.mahmoud2007@yahoo.com
Current address: Department of Pharmaceutical Chemistry, College of Pharmacy, Najran University, Najran 11001, Kingdom of Saudi Arabia

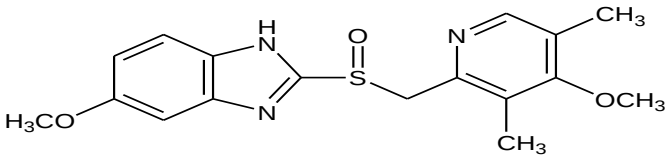
Abstract

Novel analytical study was performed in order to develop and validate new high-throughput microwell-based spectrophotometric assays for determination of omeprazole (OMZ) in its pharmaceutical formulations. The proposed assays were based on the charge-transfer (CT) reaction of OMZ with 2,3-dichloronaphthoquinone (DCNQ) and 2,3,5,6-tetrabromo-1,4-benzo-quinone (BROM). In the present study, the CT reactions was carried out in microwell plates as reaction vessels in order to increase the automation of the assays and the efficiency of its use in quality control laboratories (QCLs). All factors affecting the CT reactions were carefully studied, and the conditions were optimized. Kinetics and stoichiometry of the CT reactions were investigated, and the mechanism was postulated. Activation energy of the CT reactions was determined and found to be 13.87 and 16.27 Kcal mol^{-1} for the reaction of OMZ with DCNQ and BROM, respectively. The initial rate and fixed time methods were applied for generating the calibration graphs for determination of OMZ concentrations. Under the optimum conditions, the linear range was 0.145 – 1.45 x 10^{-4} and 1.45 – 7.25 x 10^{-4} M with LOD of 0.6 and 6.0 μg ml^{-1} for DCNQ and BROM, respectively. Analytical performance of the proposed methods, in terms of accuracy and precision, was statistically validated and the results were satisfactory; RSD was <2.8% for both repeatability and reproducibility. The proposed methods were successfully applied to the analysis of OMZ in its dosage forms and the recovery results (98.64 – 100.6 ± 0.25 -2.74 %) were comparable with those of the reported method. The developed method may provide a safer and economic tool for the analysis of OMZ in QCLs.

Keywords: *Omeprazole; 2,3-dichloronaphthoquinone; bromanil; Charge-transfer complexes; Kinetics.*

1. Introduction

Omeprazole (OMZ) is a selective and irreversible proton pump inhibitor. It blocks the stomach acid release via specific inhibition of the H+/K+-ATPase enzyme system present at the secretory surface of gastric parietal cells. Omeprazole is listed in the WHO's list of essential medicines, the most effective and safe medicines needed in a health system. Therefore, OMZ is widely used for treatment of peptic ulcers, gastroesophageal reflux, and Zollinger–Ellison syndrome [1-2]. Recently, OMZ has been used as a potential anti-inflammatory agent [3] to protect the gastric mucosa from the inflammation caused by Helicobacter pylori infection (in a triple therapy combination with amoxicillin, and clarithromycin) or by long-term therapy with aspirin and non-steroidal anti-inflammatory agents. This therapeutic importance of OMZ was behind the growing interest in the development of many analytical methods for its determination in the pharmaceutical formulations and/ or biological samples. Omeprazole is a lipophilic base with pKa1=4.2 and pKa2= 9.0.



Omeprazole

Literature survey showed that several techniques have been reported for assay of OMZ in pharmaceutical preparations or biological fluids. The reported techniques are liquid chromatography with either UV [4-9] or Mass spectrometric [10-12] detection, electrophoresis [13, 14], electrochemistry [15, 16], fluorimetry [17-19] and spectrophotometry [2, 20-25]. The sophisticated instrumentation, high analysis cost and long time required to obtain the most suitable conditions limit the routine use of the separation-based techniques in quality control laboratories (QCLs) where higher sensitivity is not required. Among these techniques, spectrophotometry stands out, as the most effective and convenient analytical technique as compared to the others with respect to simplicity, cost, and wide availability in most QCLs. However, matrix interference disadvantage the direct UV spectrophotometric analysis of OMZ [3]. In addition, other interferences resulting from non-selective oxidation of OMZ may affect some reported methods [20]. Today, Kinetic spectrophotometric methods have gained great interest in the area of pharmaceutical analysis. Kinetic methods can improve the selectivity owing to the possible avoiding of the matrix interferences in the commercial products if they can withstand the reaction conditions established for the proposed kinetic method [26].

Nowadays, molecular charge–transfer (CT) reactions have been widely used in different areas of pharmaceutical and medical sciences. The importance and widespread of CT reactions are attributed to their presence in many biological systems [27], their use in studying the thermodynamics and pharmacodynamics of medical substances [28], and in explaining how the drugs and the receptors interact [29]. In the area of pharmaceutical analysis, their great importance comes from the rapid formation of intensely colored CT complexes characterized by new absorption band of longer λmax than that of their components [30]. The higher λmax of the new band is a decisive advantage since the interference from the associated ingredients shall be far less at higher wavelengths. The formation of new absorption bands in CT reactions resulted in development of many spectrophotometric assays for pharmaceutical compounds [31-33]. CT reactions were reported for OMZ using both iodine as σ-acceptors [25] and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) [19, 25], 7,7',8,8'-tetracyanoquinodimethane (TCNQ) [32] and p-chloranilic acid [33] as π-acceptors. Yet, no attempt has been carried out for using both 2,3-dichloronaphthoquinone (DCNQ) and 2,3,5,6-tetrabromo-1,4-benzoquinone (BROM) reagents for CT complexation of OMZ. The use of micro-well plates as reaction vessels instead of normal volumetric flasks can reduce both consumption and cost of organic solvents, and decrease the risk of human exposure to toxic solvents [19].

These facts promoted our interest in utilizing DCNQ and BROM reagents for developing novel, simple and low-cost kinetic spectrophotometric methods for the determination of OMZ in QCLs.

2. Experimental

2.1. Apparatus

A Shimadzu model UV-1601 PC (Japan) UV-VIS double beam spectrophotometer with matched 1-cm quartz cells was used for recording the electronic absorption spectra. BioTek ELx 808 ELISA reader (BioTek, USA) was used for all measurements. UltraCruz® polypropylene microplates, 96 well with U bottom (were obtained from Santa Cruz Biotechnology, Inc. (USA). Finn pipette adjustable 8 channel-pipettes were purchased from Sigma Chemical Co., USA.

2.2. Chemicals and reagents

OMZ (Hetero Drugs Ltd, Hyderabad, India) was used as working standard. 2,3,5,6-Tetrabromo-1,4-benzoquinone (bromanil; BROM, Hopkin & Williams Ltd, UK) was 2.36×10^{-3} M (0.1%) in acetonitrile. 2,3-Dichloro-naphthoquinone (DCNQ; Merck, Schuchardt, Munich, Germany) was 3.52×10^{-3} M (0.08%) in acetonitrile. BROM and DCNQ solutions were freshly prepared daily. All solvents and other chemicals used throughout this study were of analytical grade.

2.3. Pharmaceutical formulations

Gastrazole capsules (Riyadh Pharma, Saudi Arabia) and Losec tablets (AstraZeneca, Sweden) were labeled to contain 20mg OMZ per capsule or tablet.

2.4. Preparation of standard and sample solutions

2.4.1. Preparation of stock standard solution

One hundred twenty five mg of OMZ was accurately weighed, transferred into a 50-ml calibrated flask, dissolved in 20 ml acetonitrile, and completed to volume with the same solvent to obtain a stock solution of 2.5 $mg.ml^{-1}$. This stock solution was further diluted with acetonitrile to obtain suitable concentrations that lie in the linear range of each particular assay method.

2.4.2. Preparation of tablets or capsules solution

Twenty tablets or the contents of twenty capsules of each formulation were finely powdered and/or weighed. A quantity of the powder equivalent to 75 mg of OMZ was transferred into a 25-ml calibrated flask, dissolved in 20 ml of acetonitrile, swirled and sonicated for 5 min. The flask content was completed to volume with acetonitrile, shaken well for 15 min and filtered. The first portion of the filtrate was rejected and a measured volume of the filtrate was diluted quantitatively with acetonitrile to yield suitable concentrations that lie in the linear range of each particular assay method.

2.5. General analytical procedure

2.5.1. Initial rate method

Hundred microliters of the standard or sample solution of OMZ (12.0 – 500.0 μg ml^{-1}) was transferred into the wells of 96-microwell plate. Hundred microliters of DCNQ ($3.52x10^{-3}$ M) or BROM ($2.36x10^{-3}$ M) solution was added to each well. ELISA microplate reader measured the absorbance of the resulting solutions at 416 and 490 nm for DCNQ and BROM, respectively. The CT reaction was monitored at 45 ± 2 °C and the absorbance was recorded as a function of time each 5 min for 40 and 25 min for DCNQ and BROM, respectively. Blank wells were treated similarly using 100 µl of acetonitrile instead of OMZ. The absorbance of blank wells were subtracted from those of standard or sample wells.

2.5.2. Fixed time method

Hundred microliters of the standard or sample solution of OMZ (12.0 – 500.0 μg ml^{-1}) was transferred into the wells of 96-microwell plate. Hundred microliters of DCNQ ($3.52x10^{-3}$ M) or BROM ($2.36x10^{-3}$ M) solution was added to each well. The CT reaction was allowed to proceed at 45 ± 2 °C for 40 and 25 min for DCNQ and BROM, respectively. ELISA microplate reader measured the absorbance of the resulting solutions at 416 and 490 nm for DCNQ and BROM, respectively. Blank wells were treated similarly using 100 µl of acetonitrile instead of OMZ. The absorbance of blank wells were subtracted from those of standard or sample wells.

2.6. Association constant and free energy change

Series of OMZ solutions (4×10^{-5} - 6×10^{-2} M) in acetonitrile were prepared. These solutions and DCNQ (3.6×10^{-3} M) and BROM (2.4×10^{-3} M) solutions in acetonitrile were equilibrated for 30 min in a thermostatically controlled water bath at 45±2 ^{o}C. Five milliliters of each acceptor solution was mixed rapidly with 5 ml of OMZ solution in 10-ml calibrated flasks. The absorbance of the solutions was measured immediately at the corresponding maxima against reagent blanks treated similarly.

2.7. Molecular modeling for the CT complexes

Molecular modeling for the CT complexes were carried out using the MOPAC package in the CHEM 3D Ultra, version 9.0 (ChemOffice software, CambridgeSoft Corporation, Cambridge, MA, USA) implemented with molecular dynamics computations software (MM2). OMZ and the acceptor (bromanil as representative examples) were energy-minimized alone and both together to obtain the most energy-minimized conformation of OMZ CT complexes. Furthermore, the total charges on all nitrogen atoms were calculated.

2.8. Validation of the proposed methods

The following validation parameters were assessed and evaluated according to ICH guidelines [34]. The standard curve for determination of OMZ was constructed by plotting the color intensities obtained by using the general procedures of the proposed methods as a function of the corresponding concentrations. The least squares method was used for getting the regression equation. The limits of detection (LOD) and limits of quantitation (LOQ) were determined [34] using the formula: LOD or LOQ = κ.SDa/b, where κ = 3.3 for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope (sensitivity) of the proposed method. Intra- and inter-day accuracy and precision of the proposed method were assessed by recovery studies at three (low, medium and high) concentration levels. Recovery was determined by the standard addition method. Known amounts of OMZ were added to the pre-determined drug-containing dosage forms, and then determined by the proposed methods. The mean recovery was determined by dividing the measured concentrations to the concentrations taken for analysis, expressed in percentages. The intra-day precision and accuracy were determined by carrying out the assay of six replicate samples of each concentration as one batch in a single run, while the inter-day precision and accuracy were determined by repeating the assays for the same samples at three different days. The selectivity of the proposed method was studied by testing the interferences liability from the excipients of the dosage forms. Common excipients such as starch, glucose, lactose, acacia, and magnesium stearate were used in this study. The recovery was calculated each time. Ruggedness was also tested by two different analysts at two different elapsed times. Moreover, robustness was evaluated at small variation in the π-acceptor concentration, reaction temperature and reaction time

3. Results and discussion

3.1. Spectral characteristics of the formed complexes, design and strategy for the assay development

OMZ has an absorption spectrum in the UV range of 200-340 nm with maximum absorption at 278 and 298 nm. However, it has no considerable absorption band in the visible range of 400–600 nm. Addition of OMZ to both polycyanoquinone (DCNQ) and polyhaloquinone (bromanil) π-acceptors in polar solvents such as acetonitrile or methanol resulted in colored CT complexes characterized by considerable absorption bands in the visible range of 400–600 nm with high ε-values. This was attributed to complete electron transfer from OMZ to the π-acceptor moiety (A) accompanied by the formation of colored radical ions [30], according to Scheme I. The high ionizing power of the polar solvent is the driving force for dissociation of the (OMZ-A) CT complex to form the colored radical ion. Spectral features of OMZ CT complexes with the studied π-acceptors were similar to those observed for their interaction with other donors [29, 35-36], and the reported values of the radical anions of these acceptors obtained by the iodide reduction method [36]. As shown in **Figure 1.**

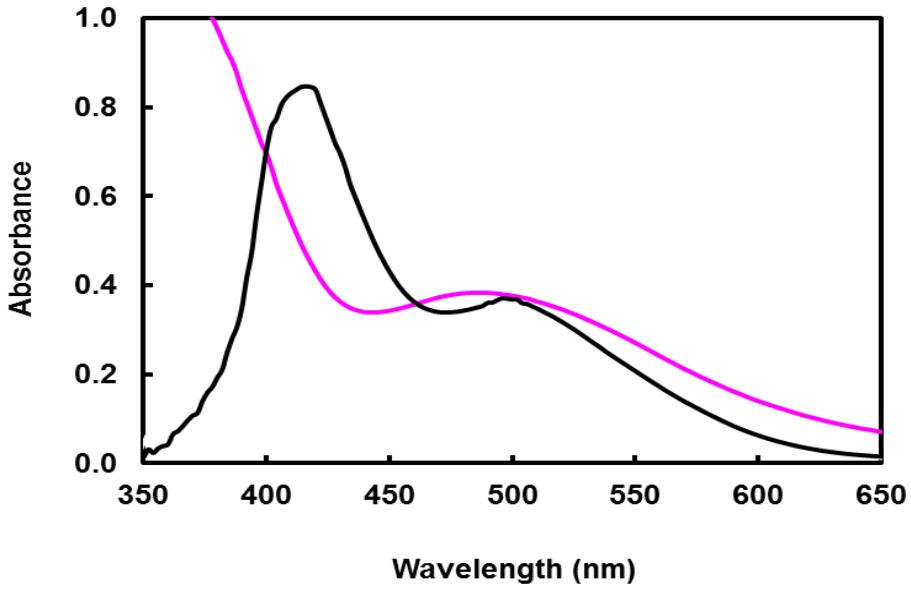


Figure 1. Absorption spectra of OMZ (50 μg ml^{-1}, 1) and its reaction products with DCNQ (1.76 x 10^{-2} M, 2), and BROM (1.18 x 10^{-2} M, 3) in acetonitrile. Concentrations of OMZ were 50 and 100 μg ml^{-1} for the reaction with DCNQ and BROM, respectively.

The OMZ-DCNQ complex exhibited two absorption bands at 416 and 496 nm (intensity of the 416 nm band is 2.2fold more than the 496 nm band), while OMZ-BROM exhibited only one absorption band at 490 nm. The measurements of OMZ-DCNQ complex were carried out at 416 nm to increase the sensitivity of the assay. The interaction of OMZ with both DCNQ and BROM was found to be slow at room temperature (25±2°C). Therefore, they were carried out at elevated temperatures.

$$\mathrm{OMZ} + \mathrm{A} \rightleftharpoons \underset{\text{Complex}}{[\mathrm{OMZ}\text{-}\mathrm{A}]} \xrightleftharpoons{\text{Polar solvent}} \underset{\text{Radical ions}}{\mathrm{OMZ}^{\bullet+} + \mathrm{A}^{\bullet-}}$$

Scheme I

Our previous success in increasing the automation, economy and safety of the CT-reactions [19, 37] encouraged us to design a micro-well-based CT assays and measuring the signal with an ELISA reader.

3.2. Optimization of the reaction conditions

The factors influencing the reaction of OMZ with both DCNQ and BROM in the 96-microwell designed format and hence the color intensity (the reagent concentration, reaction time, reaction temperature, and the diluting solvent) were carefully studied by altering each variable in turn while keeping the others constant.

3.2.1. Effect of temperature on the color intensity

Preliminary experiments for studying the reaction of OMZ with both DCNQ and BROM π-acceptors indicated very slow reaction for both studied acceptors with OMZ. Therefore, it seemed better to investigate the effect of temperature on the reaction of OMZ with both acceptor firstly through following up the color development at different temperatures using fixed concentrations of OMZ and the acceptors. Different temperatures: 25, 30, 35, 40, 45, 50, 60, 70 and 80 °C were tried to reach the optimum reaction temperature for OMZ with the investigated acceptors. The results showed that complete color development was reached at 45°C and becomes nearly stable until reached 70°C. However, higher temperatures than 70°C was found to decrease the color intensity. In order to suit for measuring the absorbance using ELISA reader, a temperature of 45 ± 2°C was found to be the best choice for the investigated π-acceptors (**Figure 2**). Therefore, further experiments were carried out at 45 ± 2°C.

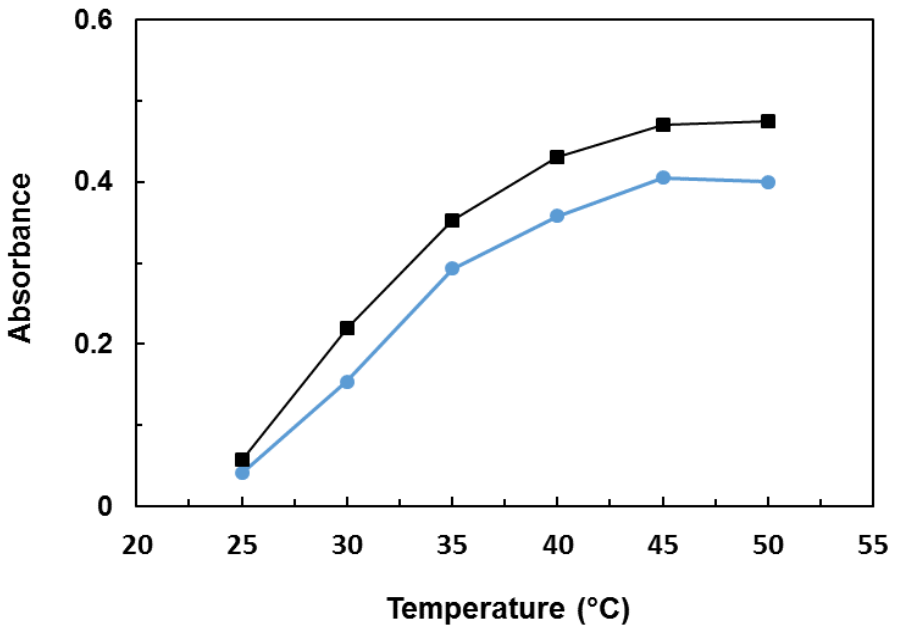


Figure 2. Effect of reagent concentration on the color intensity of the CTC of OMZ with DCNQ (●) and BROM (◆) reagents in acetonitrile at 45±2°C. OMZ concentration was 55 and 200 μg ml^{-1} for DCNQ and BROM, respectively.

3.2.2. Effect of π-acceptor concentration on the color intensity

The CT complex formation reaction was studied as a function of π-acceptor concentration. The results indicated that 100 µl of DCNQ (0.08%) and BROM (0.1%) working solution were the optimum reagent concentrations, and higher blank readings with decreased absorbance intensities were obtained if higher amounts of π-acceptors were used (**Figure 3**).

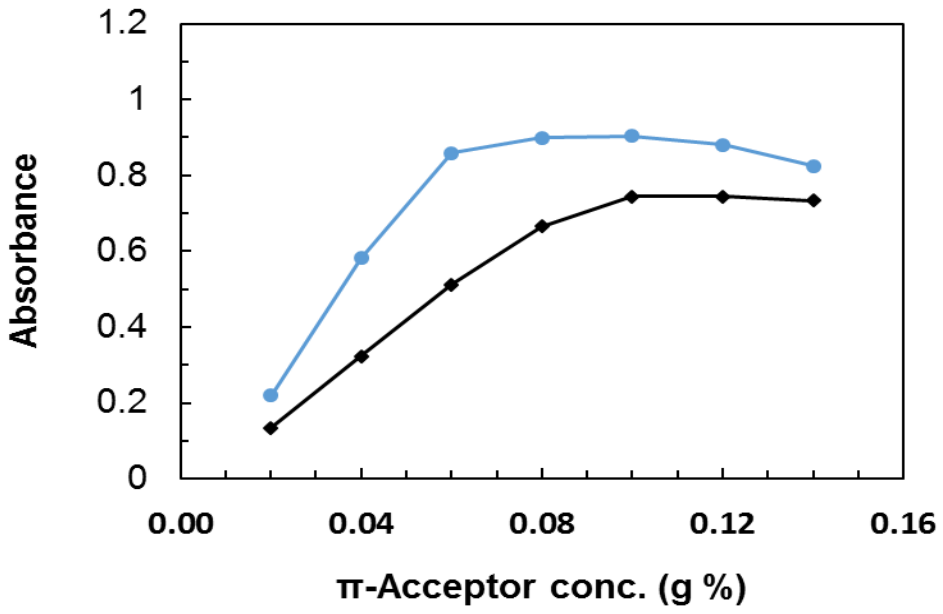


Figure 3. Effect of temperature on the color development of the CTC of OMZ with DCNQ (1.76 x 10^{-2} M (◆)) and BROM ((1.18 x 10^{-2} M (●)) reagents in acetonitrile. OMZ concentration was 30 and 100 μg ml^{-1} for DCNQ and BROM, respectively.

3.2.3. Effect of time on the color intensity

Since the colored complex formation increases with time, it was deemed useful to generate absorbance-time curve in order to determine the reaction kinetics and the optimum time for obtaining the highest absorbance intensity. This was performed by following up the color development at 45 ± 2°C using fixed amounts of OMZ and studied acceptors. The results revealed that complete color development was attained after 25 min and 40 min for BROM and DCNQ, respectively (**Figure 4**). The developed colors remained stable for at least a further 15 min, and then decreased.

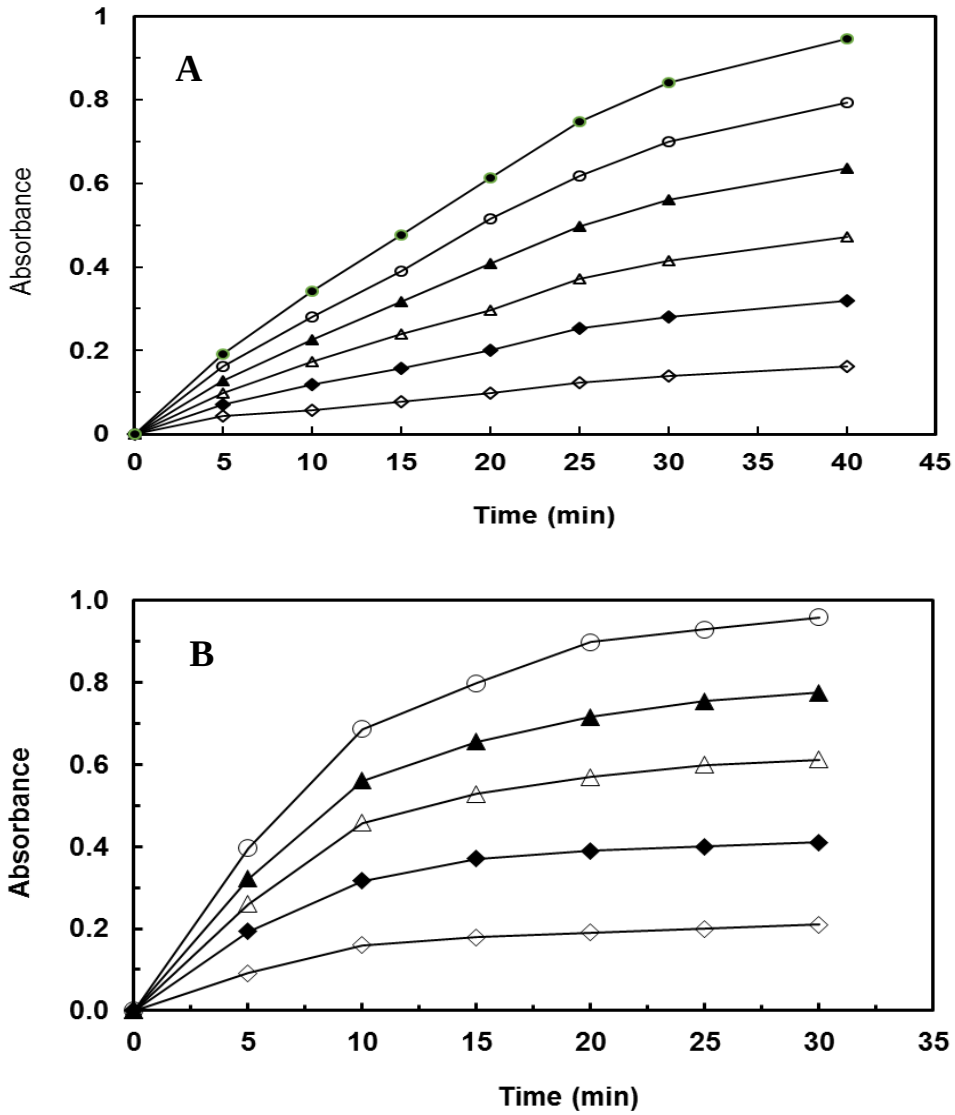


Figure 4. The absorbance-time curve for the reaction of OMZ at room temperature with: (A) DCNQ (1.76 x 10^{-2} M); the concentrations of OMZ were 2.9×10^{-5} (◇), 5.8×10^{-5} (◆), 8.69×10^{-5} (Δ), 11.6×10^{-5} (▲), 14.5×10^{-5} (O), and 17.4×10^{-5} M (●), and

(B) BROM (1.18 x 10^{-2} M); the concentrations of OMZ were 1.45×10^{-4} (◇), 2.9×10^{-4} (◆), 4.35×10^{-5} (Δ), 5.8×10^{-5} (▲), 7.25×10^{-5} M (O)

3.2.4. Effect of solvent on the color intensity

In order to select the most appropriate solvent, the reactions were carried out in different solvents. Small shifts in the position of the maximum absorption peaks were observed, and the absorption intensities were influenced. Acetonitrile was found to be an ideal solvent because it resulted in the highest sensitivity. This was attributed to its high dielectric constant that promotes the highest yield of radical anions, in addition to its high solvating power for the π-acceptors [38].

The optimum conditions for obtaining the highest sensitivity for both π-acceptors were summarized in Table 1.

Table 1: Optimum conditions for the charge-transfer reaction of OMZ with DCNQ and BROM at the specific λmax and reaction temperature.

Kinetic parameters	DCNQ method	BROM method
λ_{max} (nm)	416	490
Solvent	Acetonitrile	Acetonitrile
Acceptor conc. (%)	0.08	0.10
Time (min)	40	25
Temperature (°C)	45	45

The relative sensitivity of the investigated π-acceptors employed in the present analytical study may be attributed to their difference in electron affinities, as well as the conditions employed in the reaction (π-acceptor concentration and reaction time).

3.3. Conductivity studies

In the present investigation, the resulting OMZ–acceptor reaction product in acetonitrile exhibited appreciable conductivities which may be due to the formation of CT complex. As a representative example, OMZ-DCNQ complex yielded a conductivity–mole fraction plot with maximum at OMZ: DCNQ molar ratio of 1:1 as indicated in **Figure 5**.

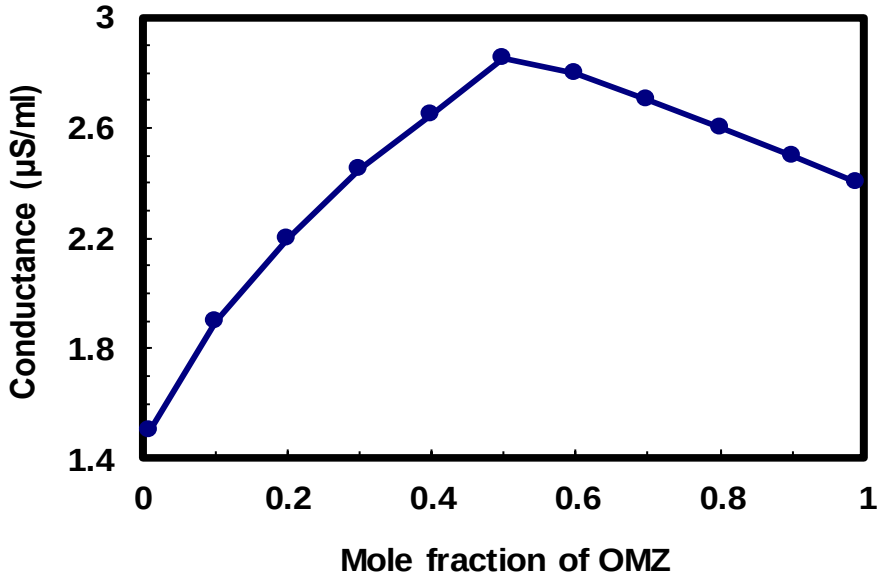


Figure 5. Conductivity of OMZ-DCNQ complex vs. mole fraction of OMZ plot in acetonitrile at room temperature.

The increase in the observed conductivity upon complex formation is attributed to the ionization of the formed OMZ-DCNQ complexes into radical ions in polar solvents giving rise to appreciable conductivity according to Scheme II [39]. The value of the conductivity above a base line connecting the conductivities of pure OMZ and DCNQ solutions is a measure of the excess conductivity caused by the formation and subsequent ionization of the CT complex.

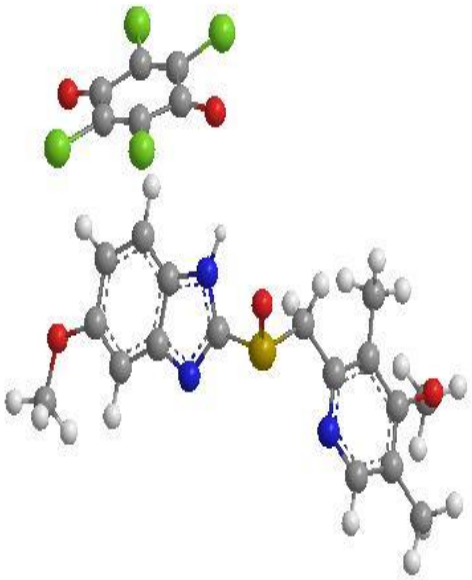
3.4. Molar ratio of the CT complex reaction and the proposed site of interaction

Job's method [40] was carried out using master equimolar solutions of OMZ and acceptors; (BROM; 1 $\times 10^{-3}$ M), and (DCNQ; 3 $\times 10^{-4}$ M) for determining the molar ratio of OMZ to each of BROM or DCNQ in the CT reactions. The results showed 1:1 ratio in both CT reactions confirming that OMZ has only one site for the reaction with each of BROM or DCNQ.

3.5. Molecular modeling and the suggested mechanism of the CT reaction

The results of studies of both conductivity and Job's method of continuous variation as well as the molecular modeling for OMZ-BROM complex confirmed that the reaction could occur only at the benzimidazole moiety of OMZ. (At the benzimidazole nitrogen in the para-position of OCH3-group since it has the highest electron density as compared to the other nitrogen atoms). **Figure 6** shows the most energy-minimized conformation of OMZ-BROM complex.

Figure 6. The most energy-minimized conformation of OMZ-BROM charge-transfer complexes.



This is due to the fact that, the negative inductive effect of SO group decreases the charge densities (basicity) at the tertiary nitrogen atom. Thus, the suggested mechanism for the CT OMZ - DCNQ reaction was postulated and assembled by the scheme shown in

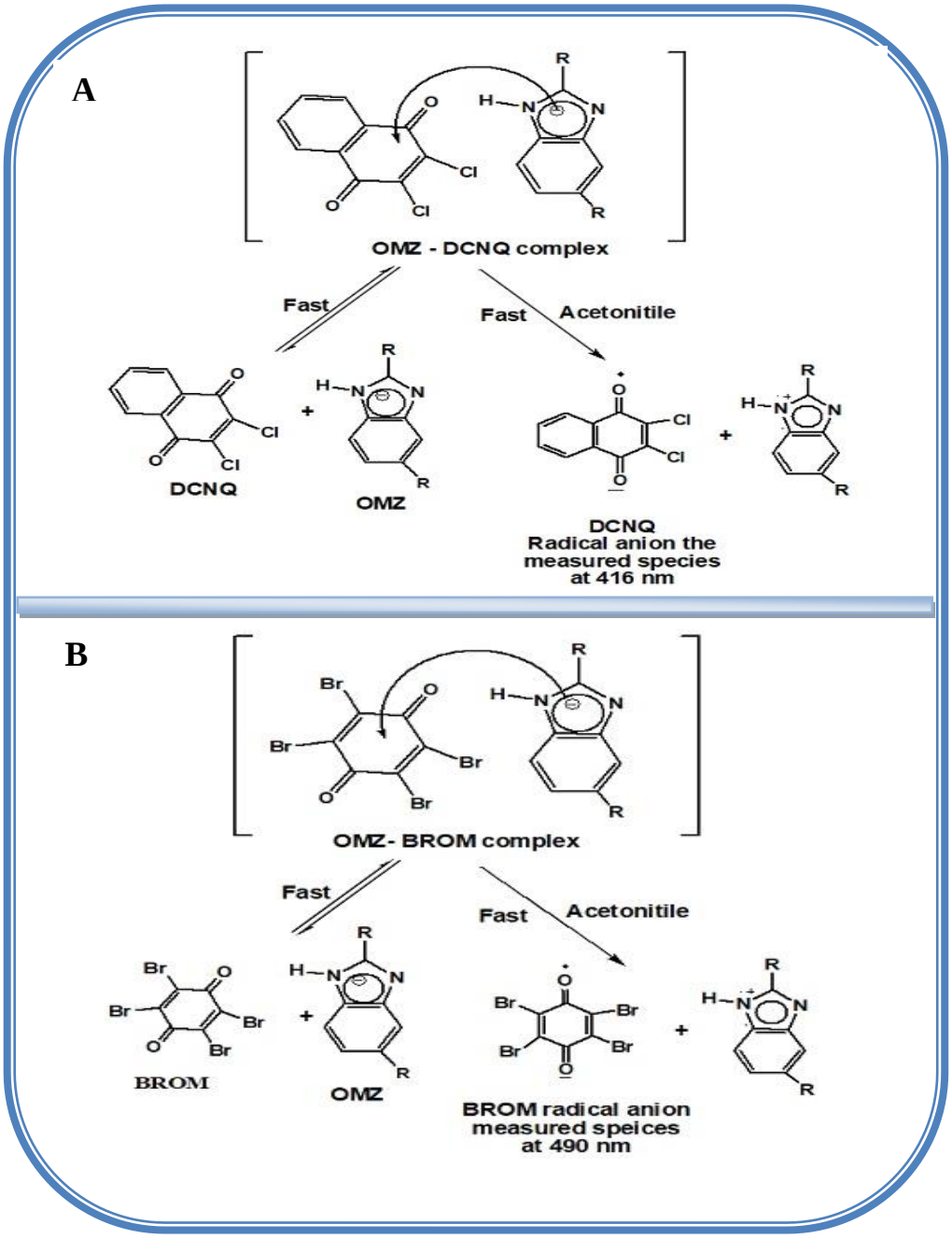


Figure7. A scheme for the charge-transfer reaction of OMZ with both (A) DCNQ and (B) BROM reagents.

3.6. Kinetic studies for the formation of OMZ-DCNQ and OMZ-BROM complexes

3.6.1. Order of the proposed CT reactions

Under the described optimum conditions, the absorbance-time curves for the reaction of OMZ with both DCNQ and bromanil acceptors were constructed. **Figure 4** shows the absorbance-time curves for the reaction of varying OMZ concentrations (0.29- 1.74 x 10^{-4} M) with fixed concentration of BROM (1.18 $\times 10^{-2}$ M) or DCNQ (1.76 $\times 10^{-2}$ M). The initial reaction rates (K) of the reactions were determined from the slopes tangents of these curves. Regression analysis was performed by plotting log K (at different OMZ concentrations) vs. log C by fitting the results to the following equation [35]

$$\text{Log K} = \log K' + n \log C$$

Where: K is the initial reaction rate, *K'* is the rate constant, *C* is the molar concentration of OMZ, and *n* (slope) is the order of the reaction. The results obtained in Table 2 for both reactions confirmed that the both reactions were first order with respect to OMZ, where the value of (n) were 1.03 and 1.02 (≈ 1) for both BROM and DCNQ, respectively.

Table 2: Kinetic parameters obtained from Benesi-Hildebrand, Arrhenius and first-order linear plots of OMZ CT complexes with DCNQ and BROM at the specific λmax and reaction temperature.

Kinetic parameters	DCNQ method	BROM method
Correlation coefficient (r)	0.9998	0.9991
Order of CT reaction (n)	1.03	1.02
Specific rate constant, sec^{-1} (k) x 10^4	5.63	4.53
Activation energy, Kcal mol^{-1} (Ea)*	13.87	16.27
Activation entropy change, Kcal mol^{-1} deg (ΔS^o)	-49.03	-48.90
Molar absorptivity, l. mol^{-1} cm^{-1} (ε) x 10^3	2.82	1.34
Standard free energy change, Kcal mol^{-1} (ΔG^o)♠	- 3.88	- 3.80
Association constant, $l.mol^{-1}$ (Kc) x 10^3	0.69	0.62

♠ Negative sign of ΔG^o indicates endothermic reaction

However, under the optimized experimental conditions, both bromanil and DCNQ were used in relative excess amount as compared to OMZ in the reaction mixture. Thus, both reactions were considered as pseudo-first order reactions.

3.6.2. Activation energy and entropy of activation of the CT complexes

The activation energy, the minimum kinetic energy that a molecule must possess in order to undergo a reaction, can be determined from Arrhenius equation [41]:

$$\text{Log } k = \log A - Ea / 2.303\ RT$$

Where: k is the apparent rate constant, *A* is a constant known as Arrhenius frequency factor, Ea is the activation energy, T is the absolute temperature (273+ °C), and R is the gas constant; 1.987 Calories deg^{-1} $mole^{-1}$. This was done by generation of absorbance-time curves for each reaction at different temperatures; 25, 30, 35, 40, 45, 50, and 60 °C using fixed concentrations of OMZ and the acceptors. The values of Log K were plotted vs 1/T

The activation energy of the reaction of OMZ with both acceptors determined from the slope; (– Ea / 2.303 R) of the straight lines were 13.87 and 16.27 Kcal mol^{-1} (Table 2) for both DCNQ and BROM, respectively. These high activation energies obtained for both the investigated reactions explained that the reaction between OMZ and both acceptors was strongly dependent on the temperature and both acceptors could be used as useful reagents for determination of OMZ. Furthermore, the change in the entropy of activation of the transition state of the complexes was determined using the following equation [41]:

$$A = (RT/Nh).e^{\Delta S^*/R}$$

Where: A is the Arrhenius frequency factor, T is the absolute temperature, and R is the gas constant, N is Avogadro's number (6.62 x 10^{-27} erg sec $mole^{-1}$), h is Planck's constant (6.02 x 10^{23} molecule $mole^{-1}$) and ΔS^* is the change in the entropy of activation (Cal $mole^{-1}$ deg^{-1}). The obtained large negative entropies of activation of the complexes (Table 2) support the formation of more polar transition state in the polar solvent

3.6.3. Association constant and standard free energy change of the CT complexes

The association constant was evaluated at the corresponding λmax for each OMZ–acceptor complex using the Benesi–Hildebrand equation [42]:

$$\frac{[A_\circ]}{A^{AD}} = \frac{1}{\varepsilon^{AD}} + \frac{1}{K_c^{AD}.\varepsilon^{AD}} \times \frac{1}{[D_\circ]}$$

where $[A_\circ]$, the concentration of the acceptor; $[D_\circ]$, the concentration of the donor; A^{AD}, the absorbance of the complex formed at the specific wavelength; ε^{AD}, the molar absorptivity of the complex formed at the specific wavelength; K_c^{AD}; the association constant of the complex (l mol^{-1}). On plotting the values $[A_\circ]/A^{AD}$ versus $l/[D_\circ]$, straight lines were obtained, from which the association constant, correlation coefficient and the molar absorptivity of OMZ–acceptor complexes were calculated (Table 2). The relative low values of the association constants of both BROM-OMZ and DCNQ-OMZ complexes (Table 2) may be attributed to the common feature of CT complexes with π–acceptors and dissociation of OMZ–acceptors complexes to their radical anions.

The standard free energy change of the complex is related to the association constant by the following equation [30]:

$$\Delta G^0 = - 2.303\ RT \log Kc$$

where ΔG^0 is the standard free energy change of the complex; R is the gas constant (1.987 cal mol^{-1} °C); T is the temperature in Kelvin degrees (273°C), and Kc is the association constant of OMZ-acceptor complex (l mol^{-1}). OMZ formed complexes with very low association constant values (Table 2) with the investigated π-acceptors due to the dissociation of the original donor–acceptor complex to the radical anion [30].

3.7. Validation of the proposed kinetic methods

In the present study, since the color formation of the CT complexes increases with time, it was utilized to elaborate kinetic-based methods for the determination of OMZ. Kinetic spectrophotometric methods are becoming of great interest in pharmaceutical analysis [26].

3.7.1. Linearity and limits of detection

3.7.1.1. Initial rate method

Under the above described optimum conditions, summarized in Table 1, the initial rates of the CT reaction of OMZ with the acceptors would follow a pseudo-first order kinetic, and were found to obey the following equation:

$$\text{Log } K = \log K' + n \log C$$

Regression analysis using the method of least square was performed to evaluate the slope, intercept and correlation coefficient. The analytical parameters and results of regression analysis are given in Table 3.

Table 3. Analytical parameters for the initial rate method of the proposed kinetic spectrophotometric methods for determination of OMZ

Method	Linear range (M)	Least square equation (Log V = log K' + n log C) [a]		Correlation coefficient (r)	LOD (M)
		Intercept (log K')	Slope (n)		
DCNQ	1.45×10^{-5}-1.45×10^{-4} M	2.1993	1.0230	0.9996	0.17 x10^{-5} M
BROM	1.45×10^{-4}-7.25×10^{-4} M	1.5140	1.0220	0.9991	1.7 x10^{-5} M

[a] V is the reaction rate, K' is the conditional rate constant, n is the order of reaction, and C is the molar concentration of OMZ.

The limits of detection (LOD) were calculated and found to be 0.174 and 1.74x10^{-5} M for DCNQ and BROM, respectively. These low values confirmed the good sensitivity of the initial rate method and consequently its capability to determine low amounts of OMZ.

3.7.1.2. Fixed time method

In this method, the absorbance of the reaction solutions containing varying amounts of OMZ was measured at a pre-selected fixed time. Calibration plots of absorbances versus the concentrations of OMZ were established at fixed periods of time for the reactions (Table 4). The regression equations, correlation coefficients, and the limits of detection and quantification are given in Table 4.

Table 4: Analytical parameters for the fixed time method of the proposed kinetic methods for determination of OMZ

Method	Time (min)	Range □□g ml^{-1})	R	Slope (b) ± SD	Intercept (a) ± SD	LOD □□g ml^{-1})	LOQ □□g ml^{-1})
DCNQ	5	30.0 -280	0.9988	0.0030 ± 0.0001	0.0105 ± 0.0029	3.27	9.92
	10	20.0 -150	0.9996	0.0056 ± 0.0011	0.0029 ± 0.0030	1.79	5.43
	20	10.0 - 90	0.9997	0.0104 ± 0.0006	-0.0081 ± 0.0047	1.50	4.55
	30	6.0 - 60	0.9996	0.0141 ± 0.0007	-0.0029 ± 0.0025	0.58	1.76
	40	5.0 - 50	0.9997	0.0158 ± 0.0005	0.0031 ± 0.0027	0.56	1.69
BROM	5	50.0 -600	0.9985	0.0017 ± 0.0001	0.0118 ± 0.0083	16.06	48.67
	10	30.0 -300	0.9996	0.0030 ± 0.0003	0.0127 ± 0.0069	7.45	22.57
	15	25.0 - 275	0.9970	0.0034 ± 0.0005	0.0184 ± 0.0079	7.76	23.51
	20	25.0 - 250	0.9997	0.0037 ± 0.0006	0.0108 ± 0.0077	6.9	20.91
	25	20.0 -225	0.9998	0.0039 ± 0.0004	0.0135±0.0072	6.21	18.82

The lowest limits of detection and quantification were obtained with fixed times of 40 and 25 min, for DCNQ and BROM, respectively. However, the fixed times of 10 min showed wider concentration range for quantification. According

to the ICH guidelines for validation of analytical procedures [34], the detection limit is not required to be part of the validation. Therefore, based on wider concentration range and less time of analysis, the reaction time of 10 min was recommended for DCNQ and BROM for the determination of OMZ by the fixed time method, if the sensitivity is not required.

3.7.2. Accuracy and precision

The accuracy and precision of the proposed kinetic spectrophotometric methods were determined [43] at three concentration levels of OMZ by analyzing five replicate samples of each concentration by both the initial rate and fixed time methods. The relative standard deviations (RSD) for the results did not exceed 2.04 % (Table 5), proving the high reproducibility of the results and the precision of the method. This good level of precision was suitable for quality control analysis of OMZ in its pharmaceutical tablets.

Table 5: Evaluation of the accuracy and precision of the initial rate and fixed time methods of the proposed methods for determination of OMZ

Method	Amount taken ($\mu g\ ml^{-1}$)	Recovery (% ± RSD) [a]	
		Initial rate method	Fixed time method
DCNQ	5	101.5 ± 1.90	101.1 ± 1.85
	25	99.5 ± 1.85	98.5 ± 0.68
	50	99.6 ± 0.96	99.2 ± 0.45
BROM	25	100.1 ± 1.97	100.6 ± 2.04
	100	98.9 ± 1.02	99.9 ± 1.65
	200	101.1 ± 1.25	101.3 ± 1.12

[a] Recovery was calculated as the amount found / amount taken ×100. Values are mean ± RSD for five determinations.

3.7.3. Specificity and interference

The proposed kinetic methods have the advantage of that all measurements in both DCNQ and BROM methods are performed in the visible region, away from the UV-absorbing interfering substances that might be co-extracted from OMZ-containing dosage forms. Potential interferences by the excipients in the dosage forms were studied. Samples were prepared by mixing known amount (20 mg) of OMZ with various amounts of the common excipients such as starch, glucose, lactose, acacia, and magnesium stearate. The results (Table 6) showed that no interference was observed from any of these excipients with the proposed methods.

Table 6: Analysis of OMZ in presence of common excipients by the proposed fixed-time method

Ingredient	Recovery (% ± SD) [a]	
	DCNQ	BROM
Starch (50)[b]	99.77 ± 0.98	98.95 ± 1.75
Glucose (10)	99.12 ± 1.15	98.59 ± 1.87
Lactose (10)	98.45 ± 0.58	99.71 ± 1.42
Acacia (10)	100.07 ± 0.97	97.96 ± 0.85
MS[c] (10)	99.63 ± 1.13	99.02 ± 1.21
Average ± SD	99.41 ± 0.64	98.85 ± 0.64
Pool SD	0.96	1.42

[a] Values are mean of three determinations.

[b] Figures in parenthesis are the amounts in mg added per 20 mg of OMZ.

[c] MS = Magnesium stearate.

3.7.4. Ruggedness and robustness

The ruggedness of the proposed methods was assessed by applying the procedures using two different instruments in two different laboratories at different elapsed time. Results obtained from lab-to-lab and day-to-day variation was found to be reproducible, as RSD did not exceed 3%. Robustness of the procedures was assessed by evaluating the influence of small variation of experimental variables [44], e.g. concentrations of acceptor reagents on the analytical performance of the proposed methods. In these experiments, one experimental parameter was changed while the other parameters were kept unchanged, and the recovery percentage was calculated each time. The small variations in any of the variables did not significantly affect the results; recovery percentages were 97.3 – 102.1% ± 1.62 - 2.37. This provided an indication for the reliability of the proposed methods during routine work.

3.8. Application of the proposed methods to the analysis of pharmaceutical tablets

Depending on the obtained validation results, the proposed procedures were found to be suitable for the routine quality control analysis of OMZ. The proposed and the reported methods [25] were applied to the determination of OMZ in its dosage forms. The results obtained by the proposed methods were statistically compared with those obtained by the reported method. The recovery of the labeled amount was 98.6 – 100.5 ± 0.88 -1.57% (Table 7).

Table 7: Analysis of OMZ in its dosage forms by the proposed kinetic spectrophotometric methods

Dosage form		**Label claim (% ± SD) [a]**		
		DCNQ	**Bromanil**	**Reported [c]**
1. Gastrazole capsules			Initial rate method	
	Recovery (% ± SD)	99.1 ± 1.09	99.5 ± 0.88	100.4 ± 1.34
	t-value[b]	1.68	1.26	
	F-value[b]	1.51	2.32	
			Fixed-time method	
	Recovery (% ± SD)	99.6 ± 1.45	100.6 ± 1.17	
	t-value[b]	0.91	0.25	
	F-value[b]	0.85	1.31	
2. Losec tablets			Initial rate method	
	Recovery (% ± SD)	98.7 ± 1.35	98.6 ± 1.57	100.9 ± 1.18
	t-value[b]	2.74	2.61	
	F-value[b]	1.31	1.77	
			Fixed-time method	
	Recovery (% ± SD)	100.5 ± 1.35	99.1 ± 1.07	
	t-value[b]	0.12	1.7	
	F-value[b]	0.76	1.22	

[a] Values are mean of five determinations.

[b] The tabulated values of t and F at 95% confidence limit are 2.78 and 6.39, respectively.

[c] Reference 25.

The results of *t*- and F-tests revealed that no significant differences were found between the proposed and reported methods at 95% confidence level with respect to precision and accuracy proving that all the proposed methods are applicable to the analysis of OMZ with comparable analytical performance.

4. Conclusions

The charge-transfer complexes of OMZ with both π-acceptors BROM and DCNQ have been investigated by UV-VIS spectrophotometry, conductimetry, and by computational molecular modeling. A suggested mechanism for these CT reactions based on the spectroscopic study was postulated. The obtained colored complexes were utilized in the development of simple and accurate kinetic spectrophotometric methods for the analysis of OMZ in QCLs. The described methods was fully validated according to ICH guidelines. The proposed methods are superior to the previously reported spectrophotometric methods, because of many advantages:

1. It used new (BROM and DCNQ) π-acceptors for determination of OMZ.
2. The present method used 96-microwell plate as a reaction vessel and the ELISA reader to increase the automation and the high-throughput of the proposed methods to suit for the analysis of OMZ in QCLs.
3. The use of micro-well plates provides a safer and economic tool for determination of OMZ contents in pharmaceutical dosage forms through decreasing the analysis cost and the risk of human exposure to toxic solvents.
4. The proposed method is superior to the previously reported spectrophotometric methods for determination of OMZ in the terms of safety, improved selectivity, and high-throughput property.
5. The use of both kinetic and automated methodology has overcome the short stability time of the signal (only 15 min)

Acknowledgment

The authors highly acknowledged the financial support of this research from the Deanship of Scientific Research, Najran University for funding the work: Project Code [NU/Mid/15/063].

Also, the authors are grateful to Prof. Hamdy M. Abdel-Rahman (Department of Pharmaceutical Medicinal Chemistry, Faculty of Pharmacy, Assiut University) for carrying out the molecular modeling.

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