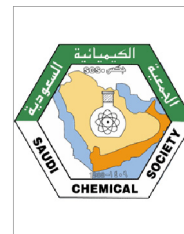King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Simultaneous spectrophotometric determination of trimethoprim and sulphamethoxazole following charge-transfer complexation with chloranilic acid

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Abstract A simple, accurate and precise simultaneous spectrophotometric method has been developed for the analysis of trimethoprim–sulphamethoxazole combination in pure and tablet dosage forms. The method involves direct charge transfer complexation of trimethoprim (TMP) with chloranilic acid (CAA) in acetonitrile–dioxane solvent mixture and complexation of sulphamethoxazole (SMZ) after its hydrolysis in dilute H_2SO_4. Optimization of temperature and time revealed the superiority of room temperature and 20 and 30 min respectively for TMP and SMZ. Optimal detector responses were obtained at 520 and 440 nm and were therefore selected as working wavelength maxima for SMZ and TMP respectively. TMP and hydrolysed SMZ were combined with CAA at mole ratios of 1:1 and 1:3 respectively. Accuracies were generally less than 4% (estimated as degree of inaccuracy or error) with a precision of the order of less than 2% on a three-day assessment. Physicochemical factors responsible for complex stability were estimated and related to the observed data. The method was successfully applied to the determination of TMP and SMZ in tablet dosage forms with accuracies comparable to the official BP method. There were no interferences from common tablet excipients and TMP complex did not interfere with the assay of SMZ. The developed method could find application in routine quality control of TMP–SMZ combination. It is the first reported full simultaneous colorimetric assay of TMP and SMZ using the same analytical reagent.

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1. Introduction

The sulphonamides were the first effective chemotherapeutic agents to be employed systemically for the prevention and cure of bacterial infections in human beings (Mandel and Petri, 1996). Trimethoprim (TMP) is commonly used in combination with sulphamethoxazole (SMZ) as an effective anti-microbial

agent. The combination is used in the treatment of urinary tract infection and as a powerful bacteriostatic agent (Qureshi et al., 1997). The introduction of TMP in combination with SMZ constitutes an important advance in the development of chemically effective antimicrobial agents. The antimicrobial activity of the combination results from the action of the drugs on two steps of the enzymatic pathway for the synthesis of tetrahydrofolic acid (Hitchings, 1961).

Trimethoprim has been determined in pharmaceutical preparations by spectrophotometric methods (Issa et al., 1998), electro-analytical techniques (Aboudon et al., 1994; Ahmed and Elbeshlawy, 1995), liquid chromatography (Hruska and Frye, 2004; Chen et al., 1988) and gas chromatography (Ernemann et al., 1990) among others. SMZ, on the other hand, has been determined singly using spectrophotometry (Mohammed et al., 1988; Nagaraja et al., 2002), fluorimetry (Vree et al., 1994) and HPLC. However, simultaneous determination of both compounds has been carried out by spectrophotometric methods with multi-component analysis based on the use of second derivative (Altesor et al., 1993), first derivative and spectral ratio (Markopoulou et al., 2004), bivariate calibration spectrophotometrics (Lopez-Martinez et al., 2002), HPLC (Gochin et al., 1981) and chemometrics (Goodarzi et al., 2009). Other methods include ratio spectra derivative spectrophotometry (Nevado et al., 1992), diazotization of SMZ and direct UV measurement of TMP (Shamsa and Amani, 2006), multivariate calibration approaches (Ni et al., 2006) and H-point standard additions method (Givinnrad et al., 2011). The USP (2009) suggests HPLC as the official assay procedure for the quality control of TMP–SMZ combination product while the British Pharmacopoeia (2009) utilizes a sequential method based on extraction by an organic solvent. These methods have their peculiar advantages and applicability but many of them are generally complex in nature and need expensive instruments and ultra-pure solvents.

The simultaneous spectrophotometric determination of TMP–SMZ has often proved difficult due to the overlapping nature of their UV absorption spectra. Two methods separately described the spectrophotometric determination of trimethoprim (Zhang et al., 1995) and sulphamethoxazole (Frag and Mohamed, 2010). This has necessitated the development of several chemometric techniques which are often beyond the reach of an average analyst in the developing world. Charge transfer complexation reactions have found wide applicability in quality control of important chemical compounds of pharmaceutical interest. Chloranilic acid has been extensively adopted as a π-electron acceptor for the spectrophotometric determination of several drugs (Mahrous et al., 1986; Zakhari et al., 1986; Okide and Udoh, 1998; Adikwu et al., 1999, 2010, 2011; Basavaiah and Charan, 2002). In this report a simple, accurate and precise simultaneous spectrophotometric method is described for the first time for the determination of TMP and SMZ combination product in bulk samples and tablets following utilization of CAA as a Lewis acid acceptor and TMP–SMZ as n-electron donors. The novelty of this newly developed method lies in its ability to utilize a single reagent for the determination of two-component sample through a simple mechanistic design of the reaction steps. The developed methods were compared with the official BP assay procedures for TMP–SMZ tablets.

2. Experimental

2.1. Apparatus

A UV–VIS spectrophotometer (UNICAM Aurora fitted with Scan software V1.1, Pye Unicam, UK), Mettler analytical balance, Ultrasonic bath (Langford, UK), and Vortex mixer (Griffins and George, UK) were used for this study.

2.2. Materials and reagents

All reagents and solvents were of analytical reagent grade and include methanol, acetonitrile, sodium hydroxide, sulphuric acid, acetone, 1,4-dioxane, ethylacetate, dichloromethane (all from BDH, Poole, England), chloranilic acid (Sigma–Aldrich UK), and precoated thin layer plates GF_{254} 0.2 mm (Merck, Germany). Trimethoprim and Sulphamethoxazole chemical reference substances were gifts from SKG Pharma, Lagos Nigeria.

2.3. Drug formulations

Tablet formulations analysed in this study are Septrin® (Glaxowellcome, Nigeria), Samtrim® (Sam Pharmaceuticals ltd, Nigeria), Rancotrim forte® (Ranbaxy Lab ltd, Nigeria) and Primpex® (SKG Pharma, Nigeria) tablets. They were obtained from Pharmacy retail outlets in Ibadan, Nigeria and were all within their shelf life periods at the time of analysis.

2.4. Preparation of solutions

A standard solution of chloranilic acid (CAA) was prepared by dissolving 0.01 g of CAA in 10 mL of acetonitrile to give a molarity of 4.785×10^{-3} M. Equimolar concentrations of trimethoprim (TMP) and sulphamethoxazole (SMZ) were prepared in 1,4-dioxane by weighing out 0.013 and 0.015 g respectively and dissolving in 10 mL of dioxane solution.

Hydrolysis of SMZ was carried out in 70% H_2SO_4 on a reflux condenser for 1 h, cooled and then neutralized with equimolar concentration of NaOH in an ice bath.

2.5. General procedures

The general procedures carried out in this study include evidence of complexation, selection of analytical wavelength, optimization studies, validation and dosage form analysis.

2.5.1. Evidence of charge transfer complex formation

Equal volumes of TMP and CAA (0.5 mL) were mixed in a test tube with a vortex mixer and the colour produced immediately at room temperature and at 70 °C was observed. The reaction mixture was made up to 10 mL with 1,4-dioxane/acetonitrile mixture (4:6). Similar procedure was carried out for hydrolysed SMZ (HSMZ) and mixed with acetone-distilled water.

Normal phase thin layer chromatographic analysis was carried out on the complexes produced at room temperature (15 min) using mobile phases Ethylacetate: methanol (8:2

and 6:4). The plates were viewed under UV light at 254 and 366 nm and the respective R_f values were calculated.

2.5.2. Selection of analytical wavelength

A test tube containing 0.5 mL of CAA stock solution was prepared. 9.5 mL of 1,4-dioxan/acetonitrile (4:6) mixture was added. Another test tube containing 0.5 mL of TMP stock solution was also prepared. 9.5 mL of 1, 4-dioxan/acetonitrile (4:6) mixture was added to make up 5 mL of the reaction mixture. Then the complex was prepared by placing 0.5 mL of TMP solution as mentioned above in a clean test tube and 0.5 mL of CAA was added. This was allowed to stand for 5 min after which 9 mL of 1,4-dioxan/acetonitrile (4:6) was added. The absorption spectrum of the complex was recorded against the CAA and TMP blanks using a mixture of 1,4-dioxan and acetonitrile (4:6) as the blank solvent. These procedures were repeated for HSMZ using acetone–water as blank solvent. The wavelength of maximum absorption ($\lambda_{\max}$) of the product of the reaction was noted.

2.5.3. Optimization studies

2.5.3.1. Optimization of temperature and time.
Temperature and time were optimized using the method of steepest ascent (Miller and Miller, 1993). Aliquots of TMP stock solution (0.5 mL) were added to the CAA solution (0.5 mL) in a test tube and the reaction mixture was vortex mixed followed by incubation at 30 and 50 °C for 5 and 20 min. The procedure was repeated at 60 and 80 °C. Each determination was done in duplicates. The absorbance readings of the complex were taken at 520 nm after making up to 10 mL with 1,4-dioxane/acetonitrile mixture. The optimum reaction time at the optimum temperature obtained was determined by repeating the above procedure and the reaction was terminated by making up to 5 mL with the solvent mixture at 0, 2, 5, 10, 15, 20, 25 and 30 min respectively. The optimal reaction time was taken as the time corresponding to the maximal absorbance of the samples. The entire optimization procedure for temperature and time was similarly carried out for HSMZ.

2.5.3.2. Optimization of diluting solvent.
The effect of solvents on the absorptivity of the TMP– and HSMZ–CAA complexes was studied by investigating the dilution of the reaction mixture with solvents such as ethylacetate, water, methanol, 1,4-dioxane, acetonitrile, and dichloromethane. Optimization of solvent was thereafter effected using a mixtures of 1,4-dioxane and acetonitrile for TMP–CAA complex and acetone/water for HSMZ–CAA complex. All determinations were done in duplicates and absorbance reading taken at 520 nm.

2.6. Stoichiometric ratio determination

Job's method of continuous variation (Rose, 1964) was employed to determine the stoichiometric ratio in which TMP and HSMZ combined with CAA respectively. Various volumes of TMP and HSMZ were made up to 1 mL with CAA solutions. The tubes were incubated at 30 °C for 15 and 30 min respectively. At the end of the reaction time, 1,4-dioxane/acetonitrile and acetone/water (6:4) were added to each of TMP and HSMZ tubes respectively. Absorbance readings were taken at 520 nm. Blank values were subtracted from those of the reaction mixture. All procedures were carried out in duplicate.

2.7. Validation studies

2.7.1. Preparation of calibration curves

Calibration curves were prepared on each of three days for TMP– and HSMZ–CAA complexes. For TMP, varying concentration of the stock solution was added to 0.5 mL CAA and the reactions were allowed to proceed at the optimal conditions obtained. For HSMZ, varying concentrations ranging from 0 to 10 µg mL^{-1} were prepared from the stock and carried through the optimized procedures. All absorbance readings were recorded and the calibration was assessed using linear regression analysis (Origin ver. 6.1).

The limit of detection (LOD) was computed from the calibration graphs using the equation 3.3 σ/s where σ is the standard deviation of three blank determinations and s is the slope of the calibration curve (ICH, 2011). The limit of quantitation (LOQ) was calculated as 10 σ/s according to ICH guidelines (ICH, 2011). Other validation parameters calculated were Sandell's sensitivities and molar absorptivities.

2.7.2. Assessment of accuracy and precision

The assay precision and accuracy were determined on each of three successive days as documented by the USP (2000). For TMP–CAA complexes, concentrations of 7.5, 30 and 45 µg mL^{-1} were adopted as low, mid and high concentration ranges while 2, 4, and 8 µg mL^{-1} were used for HSMZ–CAA complexes. The intra- and inter-day variations were then estimated.

2.7.3. Physicochemical parametrization

The formation constants were obtained from the equation $[A]_0/[A] = \varepsilon/K[D]_0 + 1/\varepsilon$ using the Benesi–Hildebrand plot, where K is the formation constant, A is the absorbance of the complex and ε is the molar absorptivity. $[A]_0$ and $[D]_0$
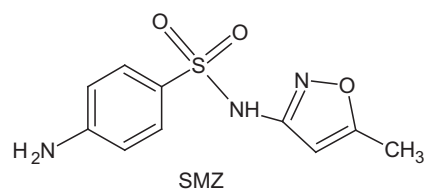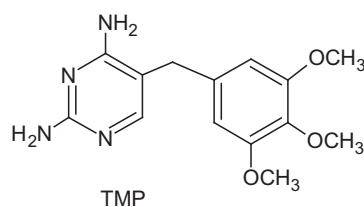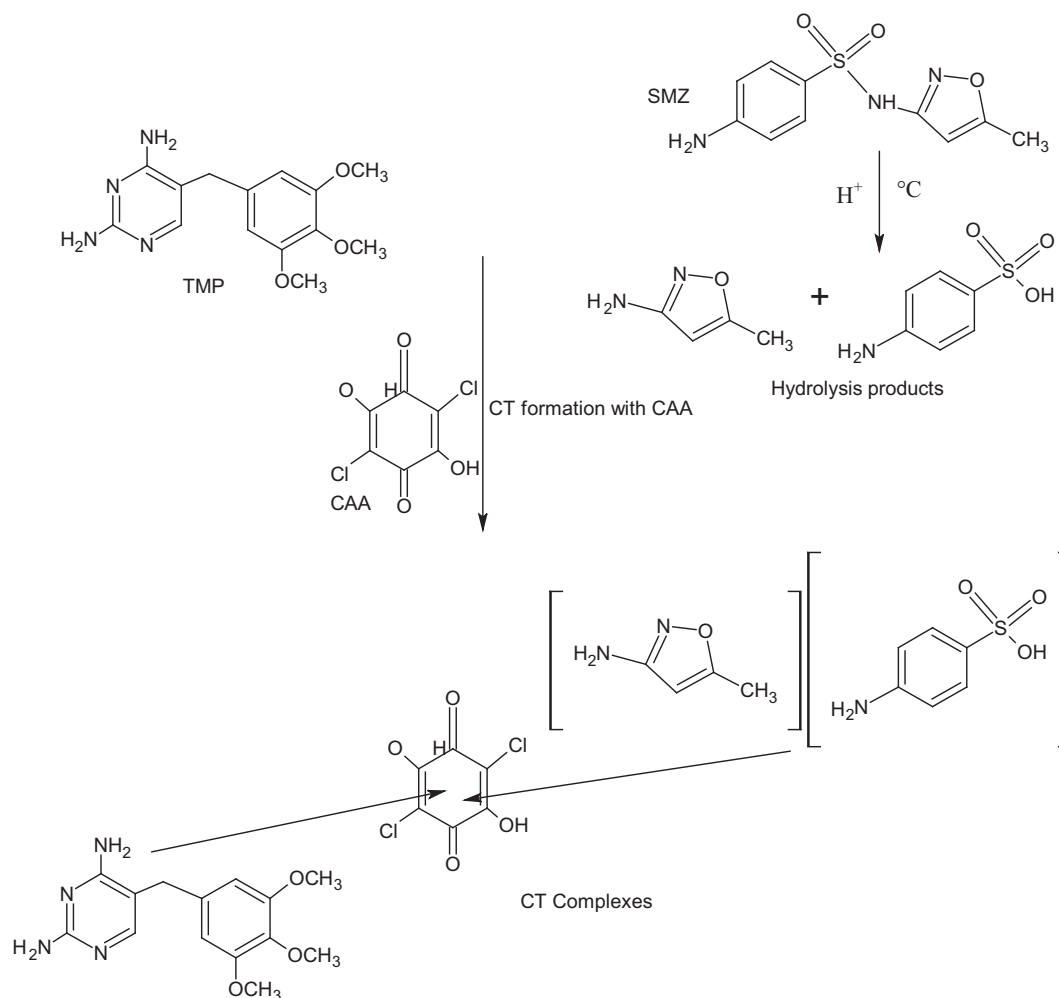

Figure 1 Chemical structures of trimethoprim and sulphamethoxazole.

Scheme 1 Proposed CT complexation between TMP, SMZ and CAA.

Table 1 R_f values for thin layer chromatographic analysis.

Mobile phase systems	TMP	TMP–CAA complex	CAA	SMZ	HSMZ–CAA complex
Ethyl acetate:methanol (8:2)	0.41	0.11	0.09	0.11	0.06, 0.21
Ethyl acetate:methanol (6:4)	0.74	0.51	0.49	0.10	0.07, 0.44

are the initial concentrations of acceptor and donor respectively (Benesi and Hildebrand, 1949). Formation constants were obtained from the ratio of intercept to slope and from the inverse intercept of Benesi–Hildebrand plots. Other physiochemical parameters estimated for both complexes include energy of transition, transition dipole, oscillator frequency, free energy change and ionization energies.

2.8. Tablet analysis

For the analysis of TMP in the tablets, amount equivalent to 0.015 g was weighed and dispersed in 10 mL of acetonitrile. Each sample was processed using the optimized CAA technique as outlined above. For SMZ, amount equivalent to 0.01 g SMZ was hydrolysed for each brand and then processed

as determined for the pure SMZ sample using the charge transfer complexation procedure. The official method adopted for TMP consists of a UV spectrophotometric determination using absorbance measurements at 271 nm against a calibration curve. The content of SMZ was also analysed using sodium nitrite titration with end-point being detected electrometrically (British Pharmacopoeia, 2009).

2.8.1. Interference studies

The effect of commonly used tablet excipients was studied by spiking known amounts of TMP and SMZ into each of magnesium stearate, starch, lactose and gelatin. Recovery studies were carried out using the optimized procedures.

In order to study the influence of trimethoprim on the analysis of SMZ, 0.002 g of TMP (representing one-fifth of the

amount of SMZ analysed) was carried through the hydrolytic process adopted for SMZ and the hydrolysed TMP was subjected to charge transfer complexation reaction with CAA.

2.9. Statistical analysis

Statistical analysis between the results of the assay of TMP and SMZ using the new CAA and the standard methods was done using F-ratio and Student's *t* tests. *P* values less than or equal to 0.05 were considered significant.

3. Results and discussion

3.1. Evidence of complex formation

Trimethoprim formed an immediate purple-coloured solution on reaction with CAA. This is evidence of formation of a charge transfer (CT) complexation. In this regard, CAA has found several applications for the analysis of pharmaceuticals. CAA is an electron deficient molecule and often reacts as a π-electron acceptor. Thus, molecules that can provide electron by atomic orbital overlap will give a brilliantly coloured solution with CAA. Prominent electron donors are species containing nonbonding (*n*-electrons) electrons from functional groups such as $-NH_2$, $-OCH_3$, $-NHR$, and $-NR_2$. The structures of TMP and SMZ are presented in Fig. 1. Both molecules possess NH_2 and are thus likely donors to CAA to form a CT complex. TMP readily formed a CT complex and on TLC analysis yielded adducts that are different in migration pattern from either CAA or TMP in the two chromatographic conditions used.

   The reaction of SMZ did not give any meaningful observable difference in the intensity of colour compared to CAA. This is in spite of the presence of $-NH_2$ group that can serve as an *n*-electron donor. Examining the structure of SMZ (Fig. 1) shows the presence of the sulphamido ($-SO_2NH_2$) group *para* to the amino group. The $-SO_2NH_2$ has an electron-withdrawing effect due to the internal mesomeric property of the sulphonyl residue. Thus the ring and the *para* amino group are deactivated. The lone pair of electron on the benzenoid residue is therefore not available for CT complex formation. An attempt at making this amino group free was the hydrolysis of the SMZ molecule. Upon hydrolysis with H_2SO_4 and neutralization with NaOH, SMZ produced a pinkish to light purple colour on reaction with CAA. The reaction occurring between TMP and hydrolysed SMZ with CAA is presented in Scheme 1. TLC analysis of the SMZ hydrolysis product, SMZ and the complexes produced with CAA is also presented in Table 1. The hydrolysed SMZ–CAA CT complex in the two mobile phases adopted showed the presence of two clearly separated spots showing that the products suggested in Scheme 1 were formed. The novelty in this regard is the quantitative nature of this hydrolysis of SMZ to react with CAA. This is the first reported method for the simultaneous determination of TMP–SMZ combination with CAA and it holds promise for application in quality control of these important antibacterial combination agents.

3.2. Absorption spectra

The electronic absorption spectrum produced by the reaction of TMP with CAA is presented in Fig. 2 while that produced

between HSMZ and CAA is shown in Fig. 3. Both spectra are shown overlaid on that of CAA and the respective drugs. The evidence of the formation of a CT complex presented in Table 1 is clearly justified by the spectra of TMP shown in Fig. 2. TMP has a principal λ_{max} at 220 nm and a minor peak at 260 nm while CAA has absorption maxima at 210, 440 and a shoulder
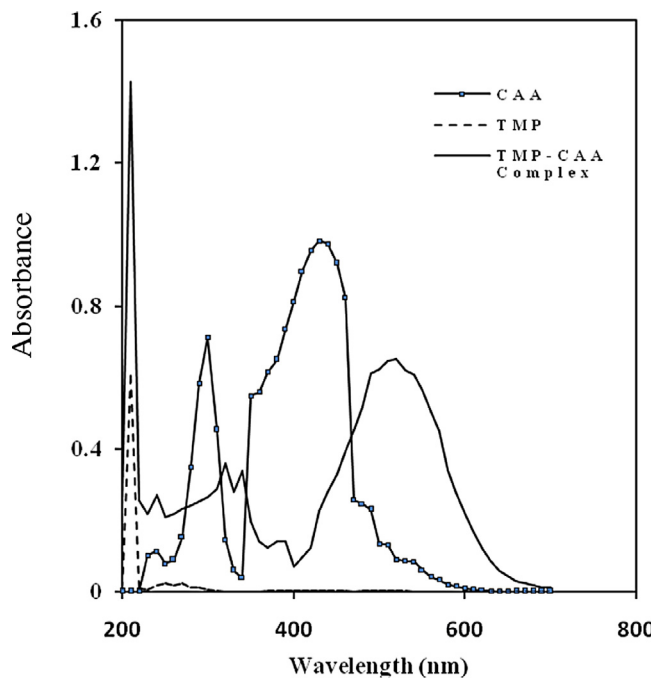
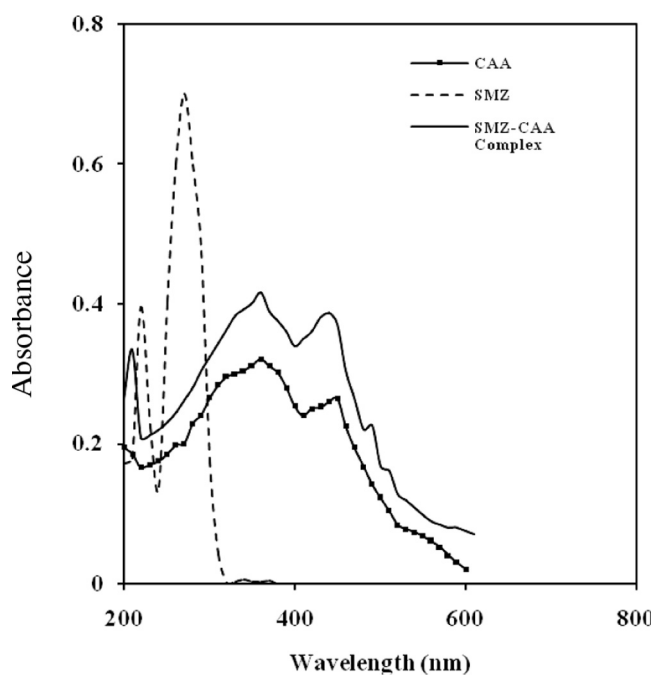**Figure 2** Absorption spectra of TMP, CAA and the CT complex.

Figure 3 Absorption spectra of SMZ, CAA and the CT complex.

at 490 nm. Upon forming a complex a new absorption maximum was observed at 520 nm. This new λ_{max} was adopted as the analytical wavelength for the determination of TMP in this study.

The spectral pattern produced by HSMZ with CAA is presented in Fig. 3. Upon hydrolysis of SMZ and reaction of the products with CAA, a hyperchromic shift of the absorption of the new complex was obtained relative to that of CAA with peaks at 360 and 440 nm and minor shoulders at 490 and 510 nm. The spectral pattern of CAA was also completely altered in the solvent used (acetone/water). On the colorimeter,
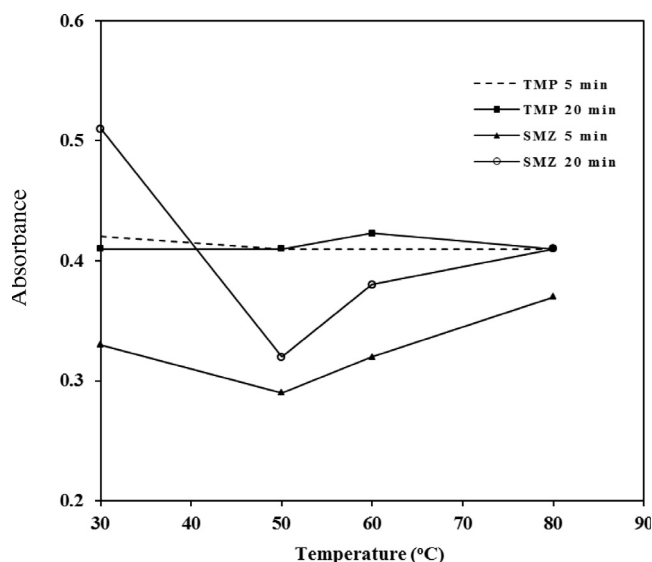
optimal difference in absorptivity between CAA and HSMZ–CAA complexes was found to be 520 nm. The hyperchromic shift produced by HSMZ on reaction with CAA relative to the spectrum of CAA may be due to the lack of extensive chromophoric conjugation in the products of the hydrolytic step. It is also possible that the optimal solvent combination adopted may have significantly affected the spectral pattern of HSMZ–CAA complex.

3.3. Optimization studies

Two parameters that can affect the formation of the CT complex were determined and optimized. These are temperature of reaction and the time allowed for reaction to occur at the optimum temperature. The results are presented for both TMP and HSMZ complexes in Fig. 4.

For the TMP complex, optimum temperature was found to be 30 °C. The differences in the absorption of TMP complex as a function of temperature were not great. However, highest absorptivity was found at 30 °C. Though at 60 °C, a slight increase was observed but the difference is not high enough to justify the adoption of 60 °C. The relative stability of TMP–CAA complex with an increase in temperature is rather anomalous as most CT complexes are not stable at elevated temperatures. Since CT complex formation is an association of some type, increase in temperature expectedly should break down the CT complex. However, the presence of multiple donor groups in the TMP skeleton may have led to the formation of a highly stable CT complex. Since two –NH$_2$ and three –OCH$_3$ groups are present in the molecule; a high electron density is likely that will produce a stable CT complex. This might also explain why the formation was instantaneous.

For HSMZ–CAA complex, the absorbance readings produced at 5 and 20 min following room temperature incubation
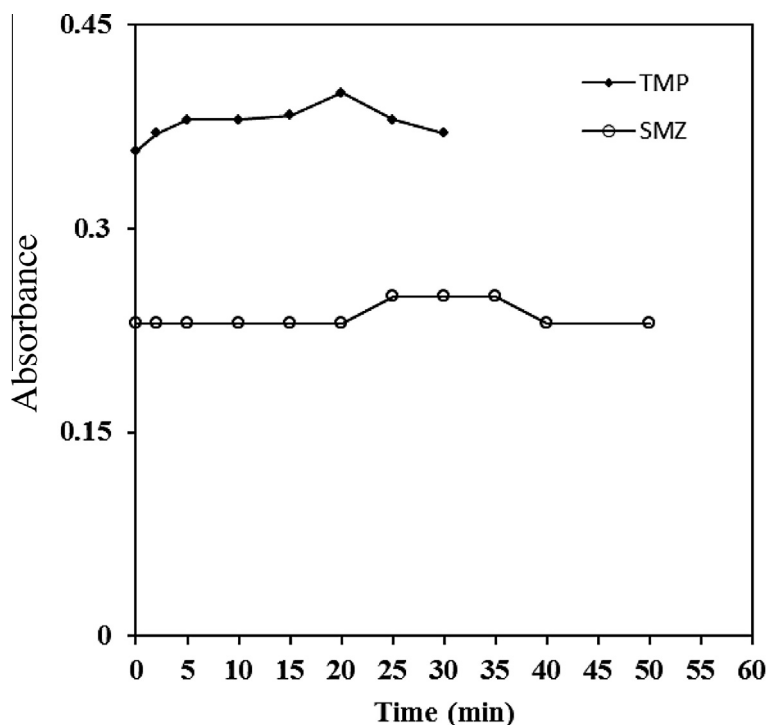

Figure 4   Optimization of reaction temperature.

Figure 5   Optimization of reaction time.

were the highest. At temperatures beyond 30 °C, the absorbance declined sharply and then later rose slightly at 60 and 80 °C. Thus, 30 °C was selected as the optimum temperature for the formation of the CT complex with hydrolysed SMZ. As earlier noted, the two products resulting from the hydrolytic step are not highly conjugated and this may account for the relative lack of stability of the HSMZ–CAA complex as the temperature increases. This is quite the reverse of the effect produced with TMP. Thus HSMZ–CAA obeyed the typical mechanism of CT reactions where increasing temperature
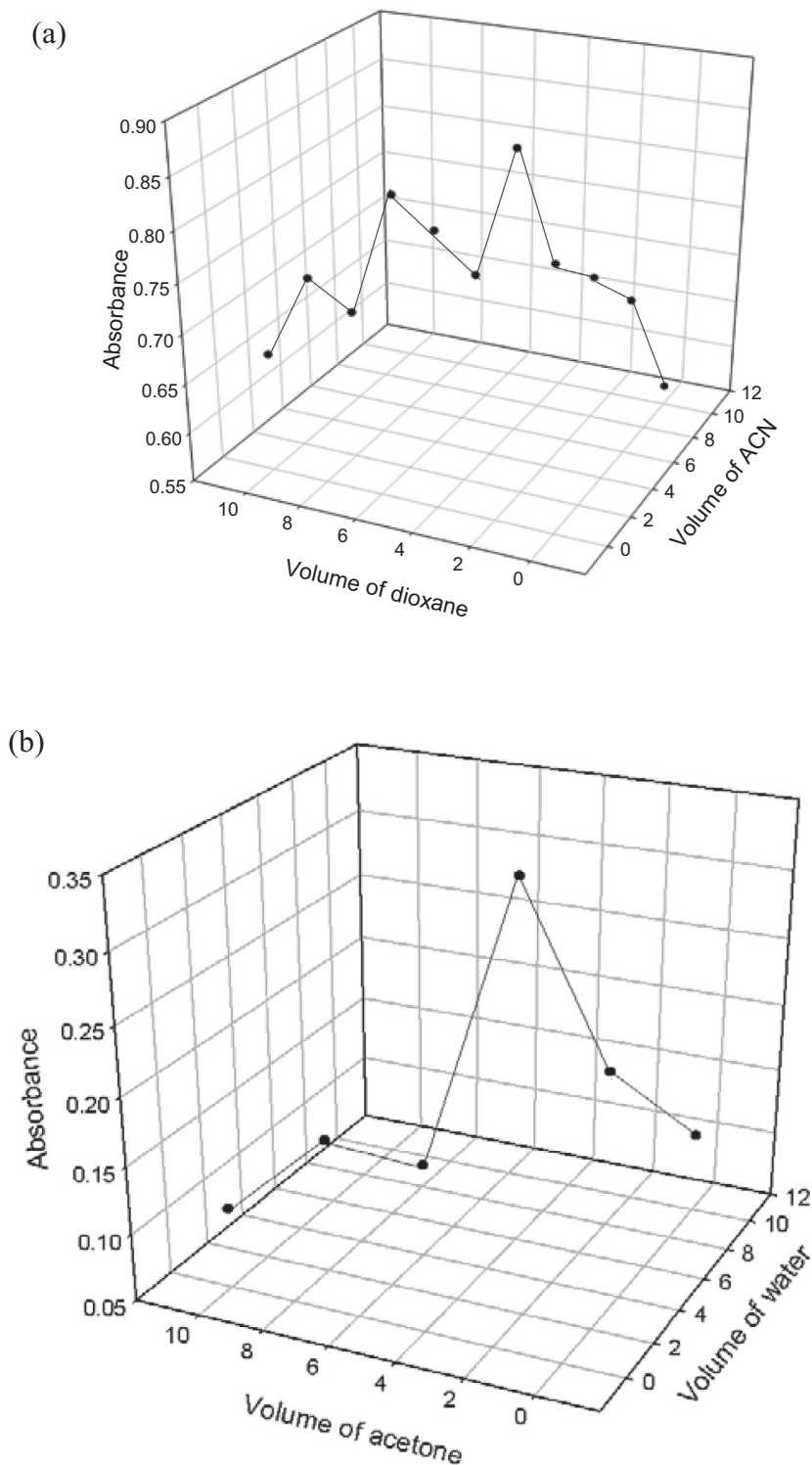

Figure 6   Optimization of diluting solvents (a) TMP–CAA, (b) HSMZ–CAA.
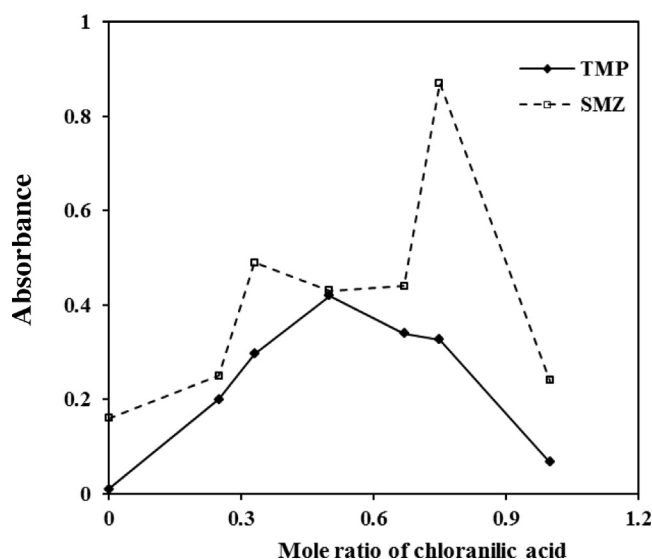
Figure 7   Stoichiometric ratio determination.

breaks down the product of the complexation (Adegoke et al., 2010, 2011).

Optimization of the time required for the complex formation between the two drugs and CAA was thereafter investigated at 0–30 min. The result is presented in Fig. 5. For TMP–CAA complex, the absorbance reading rose gradually from 0 min to a near plateau at 5–15 min and then attained a peak at 20 min. Thereafter, the absorbance values declined and thus 20 min was selected as the optimum time required

for complex formation at 30 °C. For HSMZ–CAA complex, a constant value was obtained from 0 to 20 min, and then a sharp slope which plateaus at 25–35 min before a decline was noticed thereafter time intervals beyond 35 min. Thus, 30 min reaction time was allowed for the HSMZ–CAA complex formation.

The solvent required for the dilution of the reaction mixture after the complex formation was investigated by utilizing solvents such as methanol, chloroform, dichloromethane, acetone, 1,4-dioxane and ethylacetate for the TMP–CAA complex. The non-polar solvents (chloroform, dichloromethane and ethylacetate) were found unsuitable as they formed colloidal particles with the mixture and neither were they able to extract the complex out of the aqueous medium. Best colour stabilizing effects were observed with acetonitrile and 1,4-dioxane. Attempt at optimizing the effect of using acetonitrile-1,4-dioxane produced the best results and a 6:4 ratio was therefore adopted as the optimal solvent mixture for the spectrophotometric determination of TMP with CAA. The result is presented in Fig. 6a. Fig. 6b shows the results for the optimization of diluting solvent for HSMZ–CAA reaction mixture. Optimal absorptivity was found with acetone–water (6:4) mixture. The influence of solvents on coloured solutions is an extensive study and this effect bears a direct relationship to the nature of the colour-absorbing species and the medium they are contained in. Thus, the different media required for the two complexes may not be unconnected with differences in the structures of the CT complexes and the mode of formation of the complexes. While, HSMZ contains H_2SO_4 and NaOH in its medium, solvent mixtures that can further dilute the medium (such as acetone and water) gave the highest

Table 2   Analytical and validation parameters for the CT complexes of TMP and HSMZ with CAA.

Parameter	Trimethoprim	Sulphamethoxazole
Beer's law limits, ($\mu g\,mL^{-1}$)	7.5–60	2–10
Limit of detection, ($\mu g\,mL^{-1}$)	2.69	0.589
Limit of quantitation, ($\mu g\,mL^{-1}$)	8.96	1.964
Molar absorptivity (L $Mol^{-1}\,cm^{-1}$)	2.9816×10^3	2.7929×10^4
Sandell's sensitivity, ($\mu g\,mL^{-1}$ per 0.001 absorbance unit)	0.208	0.0417
Regression equation[a]		
Intercept ± 95%CI	$0.09634 \pm 1.99 \times 10^{-2}$	$0.0035 \pm 7.057 \times 10^{-4}$
Slope ± 95%CI	$0.0048 \pm 7.5 \times 10^{-5}$	$0.024 \pm 2.627 \times 10^{-3}$
Correlation coefficient, r	0.9989	0.9992
Formation constant[b], K	1.007×10^{10}	4.957×10^{11}

[a] $Y = bX + a$, where Y is the absorbance for concentration X $\mu g\,mL^{-1}$.
[b] Estimated from Benesi–Hildebrand plot.

Table 3   Intra-day accuracy and precision of the charge transfer complexation method.

Drug	Amount taken ($\mu g\,mL^{-1}$)	Amount found ($\mu g\,mL^{-1}$)	Recovery (%)[a]	RSD (%)[b]	Relative error (%)
Trimethoprim	7.5	7.57	100.93	1.18	0.88
	30	29.91	99.70	0.89	-0.29
	45	45.13	100.29	1.59	0.30
Sulphamethoxazole	2	2.10	105.0	0.048	4.76
	4	4.075	101.88	0.075	1.84
	8	8.125	101.56	0.025	1.54

[a] Average of six determinations.
[b] RSD, relative standard deviation.

absorbance values compared to TMP–CAA where acetonitrile–dioxane mixture was optimal. Acetonitrile and 1,4-dioxane have found a regular use in most CAA procedures as the solvents are known to stabilize the CT complexes resulting from CAA and other charge donors (Adegoke et al., 2010; Onah and Odeiani, 2002).

3.4. Stoichiometric ratio determination

Using the optimized procedures, equimolar concentrations of both TMP and HSMZ were reacted at mole ratios ranging from 0 to 1.0 relative to the CAA concentration. The result produced is presented in Fig. 7. For TMP, optimal absorptivity was found at mole ratio 1:1. This confirms that under the optimal condition, one mole of TMP combines with one mole of CAA to form a stable CT complex. This uni-molar relationship was confirmed by the formation of a single spot in TLC studies (Table 1). For HSMZ–CAA complex formation, two peaks were observed, one at CAA mole ratio of 0.33 and a major one at mole ratio of 0.75. The major one at 0.75 was adopted as it proves the fact that two hydrolytic products are yielded on hydrolysis of the SMZ molecule while the third mole may be required by intact SMZ which may still be present as a residual in the medium. Thus, subsequent complexation reaction between CAA and HSMZ was carried out using 0.75 mL of CAA.

3.5. Validation studies

Calibration lines were prepared on three successive days and the average absorbance readings were used to describe linear regression line for the assay of both TMP and SMZ by the CT method using their optimized conditions. The assays of TMP were linear over the range 7.5–60 µg mL^{-1} of TMP with a linear regression equation of $Y = 0.0048X + 0.09634$ with a correlation coefficient of 0.9989. The limits of detection (LOD) and quantitation (LOQ) were estimated as stipulated by the ICH guidelines. The LOD and LOQ values obtained respectively are 2.69 and 8.96 µg mL^{-1}. The Sandell's sensitivity obtained is 0.21 µg/mL per 0.001 absorbance units. These

sensitivities stand at par with many other methods that have been reported for the simultaneous determination of TMP and SMZ. Not only does the method has good sensitivities, the initial hydrolytic step did not affect the recovery and neither of the components interfered with the other in the course of the analysis. This stems from the fact that the absorption maxima for either TMP or SMZ were clearly separated from each other. This clear-cut separation in absorption pattern is not common in previously reported simultaneous techniques. The various analytical and validation parameters are presented in Table 2. Table 2 also contains the analytical and validation parameters obtained for the analysis of SMZ by the CT procedure with CAA. The assays were linear over the range 2–10 µg/mL ($r = 0.9992$) with LOD and LOQ of 0.589 and 1.964 µg/mL respectively. The Sandell's sensitivity is 0.0417 µg/mL per 0.001 absorbance units. For both TMP and SMZ, low confidence interval limits (95%) were obtained and a moderately high molar absorptivity was produced.

Accuracy and precision were estimated over three days by recovery studies. The intra- and inter-day data obtained are presented in Tables 3 and 4 respectively. The accuracy of the new spectrophotometric method using CAA for TMP ranged from 0.11 to 0.33% (relative error), while that of SMZ ranged from 1.54% to 4.76%. For SMZ, lower relative errors were obtained with higher concentration ranges than with low concentration values. The recoveries of TMP from quality control samples over three-day assessment ranged from 100.11 to 101.11 with precisions in the order of 1.28– to 1.72 (% RSD). Similar inter-day recovery values obtained for SMZ are in the range 101.63–105% with precisions of 0.024–0.048 (% RSD). The near 100% recoveries and low precision values obtained for both TMP and SMZ in the CT procedures with CAA point to the suitability of this new procedure for the antibacterial agents.

3.6. Physicochemical parametrization of the charge transfer complexation

The physiochemical parameters that will indicate the observed stability of the complexes of TMP and SMZ with CAA were estimated. The results are presented in Table 5.

Table 4 Inter-day accuracy and precision of the charge transfer complexation method.

Drug	Amount taken (µg mL^{-1})	Amount found (µg mL^{-1})	Recovery (%)[a]	RSD (%)[b]	Relative error (%)
Trimethoprim	7.5	7.58	101.11	1.28	1.10
	30	30.03	100.11	1.72	0.11
	45	45.13	100.30	1.59	0.30
Sulphamethoxazole	2	2.10	105.0	0.048	4.76
	4	4.09	102.25	0.048	2.20
	8	8.13	101.63	0.024	1.60

[a] Average of twelve determinations.
[b] RSD, relative standard deviation.

Table 5 Various physicochemical parameters of the CT complex between trimethoprim, sulphamethoxazole and CAA.

Solvent (6:4)	CT λ_{max} (nm)	hv_{CT} (eV)	f	μ_{EN} (Debye)	R_N (eV)	ΔG^0 (KJ Mol^{-1})	I_D (eV)	W (eV)
1,4-Dioxane:ACN	520	2.303	1.288	1.185	7.853	−58.032	6.054	2.651
Acetone:H$_2$O	440	1.949	1.207	3.338	3.114	−67.851	6.108	2.736

Based on the electronic spectra of the CT complex formed between TMP, SMZ and CAA at various concentrations of the donor for a fixed concentration of the acceptor, the formation constant K_{CT} and the molar absorptivity ε_{CT} were estimated using the Benesi–Hildebrand (BH) equation (Eq. (1)). The absorbance values obtained in the calibration curve plot were plotted as a function of ratio of the molar concentration of the donor: acceptor ([D]$_0$: [A]$_0$) according to the Benesi–Hildebrand equation (Benesi and Hildebrand, 1949).

$$\frac{[A]_0}{A} = \frac{1}{K_{CT}\varepsilon_{CT}} \cdot \frac{1}{[D]_0} + \frac{1}{\varepsilon_{CT}} \tag{1}$$

where $[A]_0$ is the initial concentration of the acceptor (CAA), A is the absorbance of the charge transfer band, $[D]_0$ is the initial concentration of the donor (TMP and hydrolysed SMZ), K_{CT} is the formation constant of the new charge transfer band and ε_{CT} is the molar absorptivity. A plot of $[A]_0/A$ against $1/[D]_0$ will yield intercept as $1/\varepsilon$ and the slope as $1/K\varepsilon$ from where the formation constant and the molar absorptivity are obtained. The concentration of the acceptor was kept greater than the donors and fixed so that a wide concentration range could be adopted. The BH plot is presented in Fig. 8 and the two physicochemical parameters were estimated from an assessment of the slope and the intercept and are presented in Table 1. The immediate formation of the CT complexes between CAA and the drugs is evident from the high values of formation constants obtained for both complexes.

Some other physicochemical properties of the charge transfer bands were estimated such as molar transition energy, oscillator strength, transition dipole, resonance energy, standard free energy and the ionization potential of the donor species; in order to establish the stability or otherwise of the formed complex between the two drugs and CAA.

The oscillator strength (f) is a dimensionless quantity used to express the transition probability of the CT band and the transition dipole moment (μ_{EN}) of the CT complex (Tsubomura and Lang, 1961). Both parameters are obtained from Eqs. (2) and (3) respectively.

$$f = 4.32 \times 10^{-9}[\varepsilon \Delta V_{1/2}] \tag{2}$$

$$\mu_{EN} = 0.095 \left[\frac{\varepsilon_{CT} \Delta V_{1/2}}{\Delta V_{1/2}}\right]^{1/2} \tag{3}$$

where $\Delta v_{1/2}$ is the half-width i.e. the width of the band at the half the maximum absorption, and $\Delta v \approx$ wavenumber at the absorption maximum. The oscillator strength, f and the transition dipole moment obtained are 1.288 and 1.185 as well as 1.207 and 3.338 Debye respectively for TMP and SMZ. The standard free energy changes of the complexation (ΔG^0) were calculated from the charge transfer formation constant K_{CT} according to Eq. (4) (Person, 1962),

$$-\Delta G^0 = 2.303 RT \log K_{CT} \tag{4}$$

where ΔG^0 is the free energy of the CT complex (kJ Mol^{-1}), R is the gas constant (8.314 JMol^{-1} K^{-1}) and K is the absolute temperature. ΔG^0 was calculated to be -58.032 and -67.851 kJ Mol^{-1} respectively for TMP and HSMZ CT complexes.

Another physicochemical parameter calculated was the transition energy of the complex which is obtained from the expression hv_{CT} where h is Planck's constant and v_{CT} is the wavenumber of the absorption peak of the CT complex. The

transition energy was found to be 2.303 and 1.949 eV respectively for TMP and HSMZ complexes.

The ionization potential, I_D, of the donor in the charge transfer complex is calculated using the empirical equation derived by Aloisi and Pignatoro (1973) (Eq. (5)).

$$I_D(\text{eV}) = 5.76 + 1.53 \times 10^{-4} V_{CT} \tag{5}$$

where v_{CT} is the wavenumber of the CT band in cm^{-1}. I_D was found to be 6.054 and 6.108 eV respectively for TMP and HSMZ.

The resonance energy of the complex (R_N) in the ground state is obtained from the theoretical equation derived by Briegleb (1961), given in Eq. (6).

$$\varepsilon_{CT} = 7.7 \times 10^4 / [hv_{CT}] / R_N - 3.5 \tag{6}$$

where ε_{CT} is the molar absorptivity of the complex at the maximum of the CT absorption, hv_{CT} is the transition energy of the complex. The resonance energies for TMP and HSMZ were calculated as 7.853 and 3.114 eV, respectively.

The dissociation energy (W) of the formed CT complex between lumefantrine and CAA was calculated from the tran-
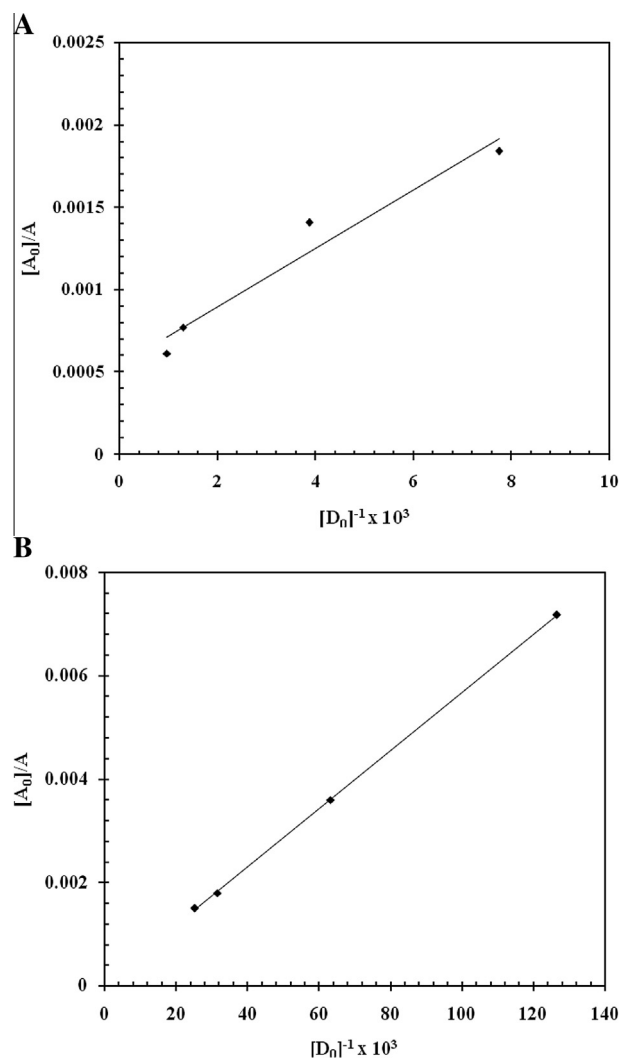

Figure 8 Benesi–Hildebrand plot for the CT complex formation [(a) TMP complex; (b) SMZ complex].

sition energy ($h\nu_{CT}$), ionization potential of the donor (I_D) and the electron affinity of CAA ($E_A = 1.1$) using the relationship in Eq. (7) (McConnell et al., 1953).

$$hv_{CT} = I_D - E_A - W \qquad (7)$$

The dissociation energies for TMP and HSMZ complexes were found to be 2.651 and 2.736 eV, respectively.

A look at the values obtained for the physicochemical parameters points to the good stability of the complex formed between CAA as acceptor and TMP and HSMZ as donors. For TMP, the ionization potential of the donor gave a high value of 6.054 eV showing TMP is a good n-electron donor. The transition energy is about three times less than this ionization energy of TMP, hence, the transition energy is readily surmounted and the complex is produced readily. I_D was also found to be 2.5 times higher than the dissociation energy, W. Thus the spontaneous decomposition of the CT complex will be minimal. Likewise, the high values of the oscillator frequency and the resonance energy point to the good stability observed for the complex. The standard free energy gave a negative value pointing to the exothermic nature of the complex formation. The value was also found to be very high explaining the spontaneity of the formation of the complex. The transition dipole moment having a high value suggests the existence of a good ion pair which was readily solvated and stabilized by the solvent mixture. The respective values obtained for HSMZ also follows a similar trend in terms of the ionization potential being much higher than the dissociation energy and the transition energy. The resonance energy for the HSMZ–CAA complex was two times less than that of the TMP–CAA complex and this will explain the hyperchromic nature of the observed spectra of HSMZ relative to the extended chromophoric elongation obtained for TMP–CAA complex. However, the dissociation energy for HSMZ–CAA is higher than that of TMP–CAA and the standard free energy change is also lower. This will probably account for why increasing temperature led to decreasing absorptivity of the HSMZ–CAA complexes. Generally, the physicochemical parameters assessed in this study have pointed to the suitability of CT complexation reaction of CAA with trimethoprim and hydrolysed sulphamethoxazole.

3.7. Tablet analysis

The optimized procedure developed in this study was adopted for the analysis of TMP and SMZ in tablet formulations. The results of the comparative assessment with those of official methods are presented in Table 6. Statistical analysis was done using F-ratio test and Student's t-test. There were no significant differences found between the contents of two drugs as assessed by both the new method and the official method.

There was also no interference from the commonly utilized excipients except magnesium stearate which gave a high absorbance value probably due to the colloidal solution produced in the laboratory mixture with it that scattered light.

An attempt at investigating the influence of TMP on the absorbance and hence determination of SMZ when present in dosage forms was made. The amount of TMP present in the co-formulation of TMP–SMZ was carried through the process of hydrolysis and the absorbance determined. It was discovered that the absorbance values were near zero values. Thus, the principal objective of a simultaneous determination of TMP and SMZ was accomplished. The first procedure in a dosage form analysis will entail direct determination of TMP (which forms an immediate CT complex) and then hydrolysis of the dosage form for the reaction of SMZ.

Clearly recognizable advantages of this new procedure for the simultaneous determination of TMP and SMZ are simplicity, high accuracy and high precision. When compared with some previously reported methods, some other advantages are recognized. For instance, the new method is more sensitive and utilized a lower calibration range for both drugs and gave better recoveries than the ratio-spectra derivative spectrophotometry reported by Nevado et al., 1992. The new method is also better in terms of simplicity compared with the method reported by Shamsa and Amani (2006) who used two different methods for SMZ (diazotization) and TMP (direct analysis at 274 nm). The newly reported simultaneous spectrophotometric method also utilized lower and more sensitive calibration range than the oxidation method in alkaline medium reported by Qureshi et al., 1997. The utilization of readily available reagents and instrumentation also lends the new method

Table 6 Comparative dosage form analysis using new charge transfer complexation and official methods.

Drug formulations	New method[a]		Official method[a]		Mean recovery ± SD (%)[b]	Error (%)	Statistics (p Values)[c]	
	Amount found (mg)	RSD (%)	Amount found (mg)	RSD (%)			F-test	t-test
Trimethoprim content								
Septrin	79.42	0.98	79.31	0.68	100.14	0.14	0.89	0.48
Primpex	83.15	3.27	80.94	1.19	102.73	2.66	0.25	0.94
Samtrim	78.38	0.31	78.40	3.52	99.97	1.28	0.002	0.13
Rancotrim Forte	81.93	3.43	79.88	1.59	102.57	2.50	0.09	0.72
Sulphamethoxazole content								
Septrin	498.50	0.89	501.24	1.27	99.45	0.55	0.69	0.39
Primpex	509.38	0.86	502.81	1.22	101.31	1.29	0.49	0.19
Samtrim	497.29	0.86	496.34	0.89	100.19	0.19	0.09	0.11
Rancotrim Forte	497.79	0.85	501.54	0.76	99.25	0.75	0.12	0.20

[a] Mean value, $n = 6$. Content of trimethoprim stated by BP ranges from 90% to 110% while for sulphamethoxazole is 92–107%.
[b] % recovery calculated as a ratio of the new method to the official method.
[c] Statistical analyses done between the results obtained from the proposed method and the official method.

reported here to further adaptation especially in poor-resource economies where applications of newer technologies such as capillary electrophoresis and HPLC are limited.

4. Conclusions

The simultaneous spectrophotometric determination of trimethoprim and sulphamethoxazole was successfully carried out by charge transfer complexation reaction using chloranilic acid as a derivatizing reagent. The procedure is simple, accurate and precise and could find application in quality control of these important antibacterial agents.

References

Aboudon, M., Issa, Y.M., Shoukry, A.F., 1994. New platic membrane ion-selective electrode for determination of trimethoprim. J. Chem. Technol. Biotechnol. 61, 31–35.

Adegoke, O.A., Babalola, C.P., Isaac, P.O.K., Kotila, O.A., 2011. Spectrophotometric studies of the charge-transfer complexation between lumefantrine and chloranilic acid in acetonitrile. Acta Pharm. Sci. 53, 135–149.

Adegoke, O.A., Umoh, O.E., Soyinka, J.O., 2010. Spectrophotometric determination of metronidazole and tinidazole via charge transfer complexation using chloranilic acid. J. Iran. Chem. Soc. 7, 359–370.

Adikwu, M.V., Ofokansi, K.C., Attaama, A.A., 1999. Spectrophotometric and thermodynamic studies of the charge-transfer interaction between diethylcarbamazine citrate and chloranilic acid. Chem. Pharm. Bull. (Jpn.) 47, 463–466.

Ahmed, M.A., Elbeshlawy, M.M., 1995. Novel polymeric membrane electrodes for selective determination of trimethoprim. Anal. Lett. 28, 2123–2138.

Aloisi, G., Pignatoro, S., 1973. Molecular complexes of substituted thiophens with σ and π acceptors. J. Chem. Soc. Faraday Trans. 1 (69), 534.

Altesor, C., Corbi, P., Dol, I., Knochen, M., 1993. Application of experimental design to the development of a multicomponent derivative spectrophotometric method: simultaneous determination of sulfamethoxazole and trimethoprim. Analyst 118, 1549–1553.

Basavaiah, K., Charan, V.S., 2002. The use of chloranilic acid for the spectrophotometric determination of three antihistamines. Turk. J. Chem. 26, 653–661.

Benesi, H.A., Hildebrand, J.H., 1949. Spectrophotometric investigation of the interaction of iodine with aromatic hydrocarbons. J. Am. Chem. Soc. 71, 2703–2707.

Briegleb, G., 1961. Elektron-Donator-Acceptor Komplexe. Springer, Berlin.

British Pharmacopoeia, 2009. volume III Her Majesty's Stationery Office Ltd., London.

Chen, G., Tu, Y.H., Allen, L.V., Wang, D.P., 1988. Determination of trimethoprim in rat blood, plasma, prostate gland and seminal vesicles by high-performance liquid chromatography. Int. J. Pharm. 46, 89–93.

Ernemann, U., Schirmer, D., Wober, A., Langmann, P., Warzecha, K., Joeres, R., Klinker, H., 1990. Gas chromatographic determination of trimethoprim in plasma during high-dose trimethoprim–sulfamethoxazole treatment for Pneumocystis carinii pneumonia. J. Chromatogr. B: Biomed. Sci. Appl. 528, 243–249.

Frag, E.Y., Mohamed, G.G., 2010. Electronic, infrared, mass, ^{1}H NMR spectral studies of the charge-transfer complexes of sulphonamide drugs with p-acceptors in acetonitrile. J. Mol. Struct. 979, 46–55.

Givinnrad, M.H., Saber-Tehrani, M., Aberoomand-Azar, P., Mohagheghian, M., 2011. H-point standard additions method for simultaneous determination of sulfamethoxazole and trimethoprim in pharmaceutical formulations and biological fluids with simultaneous addition of two analytes. Spectrochim. Acta Part A 78, 1196–1200.

Gochin, R., Kanfer, I., Haigh, J.M., 1981. Simultaneous determination of trimethoprim, sulphamethoxazole and N[4]-acetylsulphamethoxazole in serum and urine by high-performance liquid chromatography. J. Chromatogr. B: Biomed Sci. Appl. 223, 139–145.

Goodarzi, M., Shahbazikhah, P., Sohrabi, M.R., Fathabadi, M., Nouri, S.H., 2009. Direct orthogonal signal correction-partial least squares for simultaneous spectrophotometric determination of sulfamethoxazole and trimethoprim in pharmaceutical formulation and synthetic samples. J. Chil. Chem. Soc. 54, 309–313.

Hitchings, G.H., 1961. A biochemical approach to chemotherapy. Ann. N. Y. Acad. Sci. 23, 700–705.

Hruska, M.W., Frye, R.F., 2004. Determination of trimethoprim in low-volume human plasma by liquid chromatography. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 807, 301–305.

ICH Topic Q2 (R1), Validation of Analytical Procedures: Text and Methodology (CPMP/ICH/281/95); (accessed 03.06.2011).

Issa, Y.M., El-Ansary, A.L., Selim, W., 1998. Enthalpimetric determination of sulfa drugs in pure form and pharmaceutical formulations. Anal. Lett. 31, 131–146.

Lopez-Martinez, L., Lopez-de-Alba, P.L., de-Leon-Rodriquez, L.M., Yopez-Murrieta, M.L., 2002. Simultaneous determination of binary mixtures of trimethoprim and sulfamethoxazole or sulphamethoxypyridazine by the bivariate calibration spectrophotometric method. J. Pharm. Biomed. Anal. 30, 77–85.

Mahrous, M.S., Abdel Salam, M., Issa, A.S., Abdel-Hamid, M., 1986. Use of p-chloranilic acid for the colorimetric determination of some antimalarials. Talanta 33, 185–186.

Mandel, G.L., Petri, W.A., 1996. Antimicrobial agents (continued): sulfonamides, trimethoprim–sulfamethoxzole, quinolones and agents for urinary tract infections. In: Hardman, J.G., Limbird, L.E. (Eds.), Goodman and Gilman's Pharmacological Basis of Therapeutics, nineth ed. McGraw-Hill, New York, p. 1057.

Markopoulou, C.K., Malliou, E.T., Koundorellis, J.E., 2004. Chemometric and derivative methods as flexible spectrophotometric approaches for dissolution and assaying tests in multicomponent tablets. IL Farmaco 59, 627–636.

McConnell, H.M., Ham, J.J., Platt, J.R., 1953. Regularities in the spectra of molecular complexes. J. Chem. Phys. 21, 66–70.

Miller, J.C., Miller, J.N., 1993. Statistics for Analytical Chemistry, third ed. Prentice Hall, London, UK.

Mohammed, F.A., Mohammed, A.I., El-Shabouri, S.R.E., 1988. Visible spectrophotometric determination of sulphonamides. J. Pharm. Biomed. Anal. 6, 175–183.

Nagaraja, P., Sunitha, K.R., Vasantha, R.A., Yathirajan, H.S., 2002. Iminodibenzyl as a novel coupling agent for the spectrophotometric determination of sulfonamide derivatives. Eur. J. Pharm. Biopharm. 53, 187–192.

Nevado, J.J.B., Gallego, J.M.L., Penalvo, G.C., 1992. Determination of sulphamethoxazole and trimethoprim by ratio spectra derivative spectrophotometry. Fresenius J. Anal. Chem. 342, 723–728.

Ni, Y., Qi, Z., Kokot, S., 2006. Simultaneous ultraviolet–spectrophotometric determination of sulfonamides by multivariate calibration approaches. Chemom. Intell. Lab. Syst. 82, 241–247.

Okide, G.B., Udoh, U.E., 1998. Spectrophotometric determination of ketamine hydrochloride by charge–transfer complexation with p-chloranilic acid. Indian J. Pharm. Sci. 60, 368–370.

Onah, J.O., Odeiani, J.E., 2002. Physico-chemical studies on the charge-transfer complex formed between sulfadoxine and pyrimethamine with chloranilic acid. J. Pharm. Biomed. Anal. 29, 639–647.

Person, W.B., 1962. Thermodynamic properties of donor–acceptor complexes. J. Am. Chem. Soc. 84, 536–540.

Qureshi, S.Z., Helaleh, M.I.H., Rahman, N., Jamhour, R.M.A.Q., 1997. Spectrophotometric determination of trimethoprim by oxidation in drug formulations. Fresenius J. Anal. Chem. 357, 1005–1007.

Rose, J., 1964. Advanced Physicochemical Experiments. Pitman, London, p. 54.

Shamsa, F., Amani, L., 2006. Determination of sulfamethoxazole and trimethoprim in pharmaceuticals by visible and UV spectrophotometry, Iran. J. Pharm. Res. 1, 31–36.

Tsubomura, H., Lang, R., 1961. Molecular complexes and their spectra. XIII. Complexes of iodine with amides, diethyl sulphide and diethyl disulfides. J. Am. Chem. Soc. 83, 2085–2092.

United States Pharmacopoeia 24/National Formulary 19, US Pharmacopoeial Convention, Rockville 2000.

United States Pharmacopoeia 32/National Formulary 27, 2009. United States Pharmacopoeial Convention, Rockville, USA.

Vree, T.B., Van der ven, J.A.M., Verwey-van Wissen, C.P.W.G.M., VanEwijik-Beneken Kolmer, E.W.J., Swolfa, A.E.M., Van Gabn, P.M., Amatdjais-Groenen, H. 1994. J Chromatogr. B: Biomed. Sci. Appl. 658, 327.

Zakhari, N.A., Rizk, M., Ibrahim, F., Walash, M.I., 1986. Determination of phenothiazines by charge-transfer complex formation with chloranilic acid. Talanta 33, 111–114.

Zhang, N., Zhou, X.-G., Guan, R.-C., Zhang, Y.-Q., 1995. Study on charge transfer reaction of trimethoprim with chloranilic acid by spectrophotometry. Chin. J. Pharm. Anal. 15 (5), 38–39.