Spectrophotometric Determination of Chloroquine, Pyrimethamine and Trimethoprim by Ion Pair Extraction in Pharmaceutical Formulation and Urine

PADMARAJAIAH NAGARAJA*, ASHWINEE KUMAR SHRESTHA, ANANTHARAMAN SHIVAKUMAR AND AVINASH K. GOWDA

Department of Studies in Chemistry, University of Mysore, Manasagangotri, Mysore 570006, India

ABSTRACT

A simple and accurate method for the determination of chloroquine diphosphate (CQ), pyrimethamine (PYM) and trimethoprim (TMP) in pure, pharmaceutical and urine samples is described. The method involved an ion pair extraction procedure with chloroform using an acid base indicator, bromocresol purple (BCP), as a counter ion. The ion pair extracted in organic phase was yellow, and the absorbance was measured at λ_{max} = 420 nm. Beer's law was obeyed over the concentration range of 1.25-8.75, 0.62-7.5, and 1.25-10.71 µg/mL, respectively. The Sandell's sensitivity was 0.01258, 0.01102 and 0.01464 µg/cm^2. The Ringbom optimum concentration range, which was 2.5-7.5, 1.25-7.5 and 2.5-10.7 µg/mL for CQ, PYM, and TMP, respectively, exhibited reliability of the method. Stoichiometry and thermodynamic of the complexes were evaluated by Job's method and Benesi-Hildebrand plot. The statistical results of intra- and inter-day estimation of drugs and comparison with the reported methods demonstrated high precision and accuracy of the method. The procedure was rapid, simple and suitable for quality control purpose.

Key words: ion pair complex, bromocresol purple, chloroquine diphosphate, pyrimethamine, trimethoprim

INTRODUCTION

Malaria, which was nearly wiped out on the 60's, is reemerging as the top infectious killer and is currently the highest priority tropical disease of the World Health Organisation (WHO). It is caused by erythrocytic forms of *Plasmodium vivax, P. ovale, P. malariae,* and *P. falciparum,* preferentially affecting children younger than 5 years of age, pregnant women, and non-immune individuals[1]. CQ, PYM and TMP are blood schizontocides[1,2], acting on the blood form of the parasite and thereby terminating clinical attack of Malaria.

CQ, a quinoline-based drug, is highly effective and has been the mainstay of antimalarial chemotherapy. However, the presence of CQ-resistant Malaria has been widely reported. CQ acts by preventing heme polymerization, which kills the parasite via oxidative damage to their critical biomolecules[1]. CQ is also used in arthritis[3] and cancer treatment[4-6].

The major role of diaminopyridine drugs, PYM and TMP, has been attributed to their ability to block the synthesis of tetrahydrofolate by inhibiting dihydrofolate reductase[1,7]. PYM is recommended for use as a suppressive treatment in CQ-resistant area. The drug is always given with a sulfonamide or sulfone[1]. TMP along with sulfamethoxazole, commonly known as co-trimoxazole, a widely used antibacterial agent, is used as a prophylaxis especially in HIV patients[8].

Various methods have been reported for the quantification of these drugs. CQ is analysed by LC/MS[9], HPTLC[10], HPLC[10,11], and spectrophotometric methods[12-14]. PYM is determined by LC/MS[15], HPLC[16,17], and spectrophotometric methods[12,18,19]. TMP is estimated by HPLC[20], voltammetric[21] and spectrophotometric methods[22-25].

In this study, we have explored the use of Bromocresol Purple (BCP), a sulfonephthalein indicator, to establish a simple, reliable, and rapid method for the estimation of these drugs in pure, and pharmaceutical products. The method was also applied in human urine samples as the concentration range that can be estimated by the proposed method lies within the range discussed for CQ[26], PYM[27,28], and TMP[24,29], mainly during the treatment of the patient with acute infection, when the dosage administered is high[30]. In addition, the association constant, molar ration of reactant, and free energy change were also determined.

* Author for correspondence. Tel: +91-821-2412557;
  Fax: +91-821-2421263; E-mail: profpn58@yahoo.com

MATERIALS AND METHODS

I. *Apparatus*

A JASCO (Model UVIDEC-610) UV/VIS spectrophotometer with 1 cm-matched glass cell was used for all absorbance measurements.

II. *Reagents and Solutions*

All chemicals used were of analytical grade; twice distilled water was used throughout the experiment. BCP, CQ, TMP and PYM were obtained from Sigma-Aldrich Co (St. Louis, USA).

(I) *Preparation of BCP*

A 200 μg/mL BCP solution was prepared by dissolving 20 mg of BCP in 100 mL of 10% ethanol.

(II) *Preparation of Standard Solution*

Stock solutions of 200 μg/mL CQ were prepared in water. The same concentration of PYM and TMP were prepared in 10% ethanol. The solutions were further diluted quantitatively according to their linear calibration range.

(III) *Preparation of Tablet Sample Solution*

Twenty tablets of each drug were weighed and finely powdered using mortar and pestle. A quantity equivalent to 20 mg of each drug was transferred to 100-mL volumetric flasks to prepare 200 μg/mL solutions. The mixtures were shaken mechanically with water for 5 min, sonicated in an ultrasonic bath for 30 min, diluted to the volume, mixed and filtered. Appropriate aliquots of the filtrates were further diluted with water to obtain the required concentrations.

(IV) *Preparation of Urine Samples*

Drug-free human urine samples were collected from healthy volunteers and stored in a freezer at -20℃. Urine samples were centrifuged for 5 min, and the clear supernatants were used as stock sample solutions. Stock solutions were spiked with suitable quantity of drugs to prepare urine containing 200 μg/mL of drugs.

III. *Methods*

(I) *Standard Procedure for the Determination of Drugs*

Aliquots of 0.31-2.2 mL of CQ, 0.15-1.87 mL of PYM, and 0.31-2.67 mL of TMP were transferred from standard stock solutions into a series of 10-mL stoppered flasks, followed by addition of 1.5 mL of acetic acid/sodium acetate buffer (0.1 M) of pH 4.5 and 2 mL

of BCP. The reaction mixtures were extracted with 5 mL of chloroform by shaking for 40 seconds at vortex. One milliliter of the organic layer was diluted to 10 mL with chloroform in volumetric flasks. The absorbance of yellow-colored extracts was measured at 420 nm against corresponding blank solutions. All measurements were
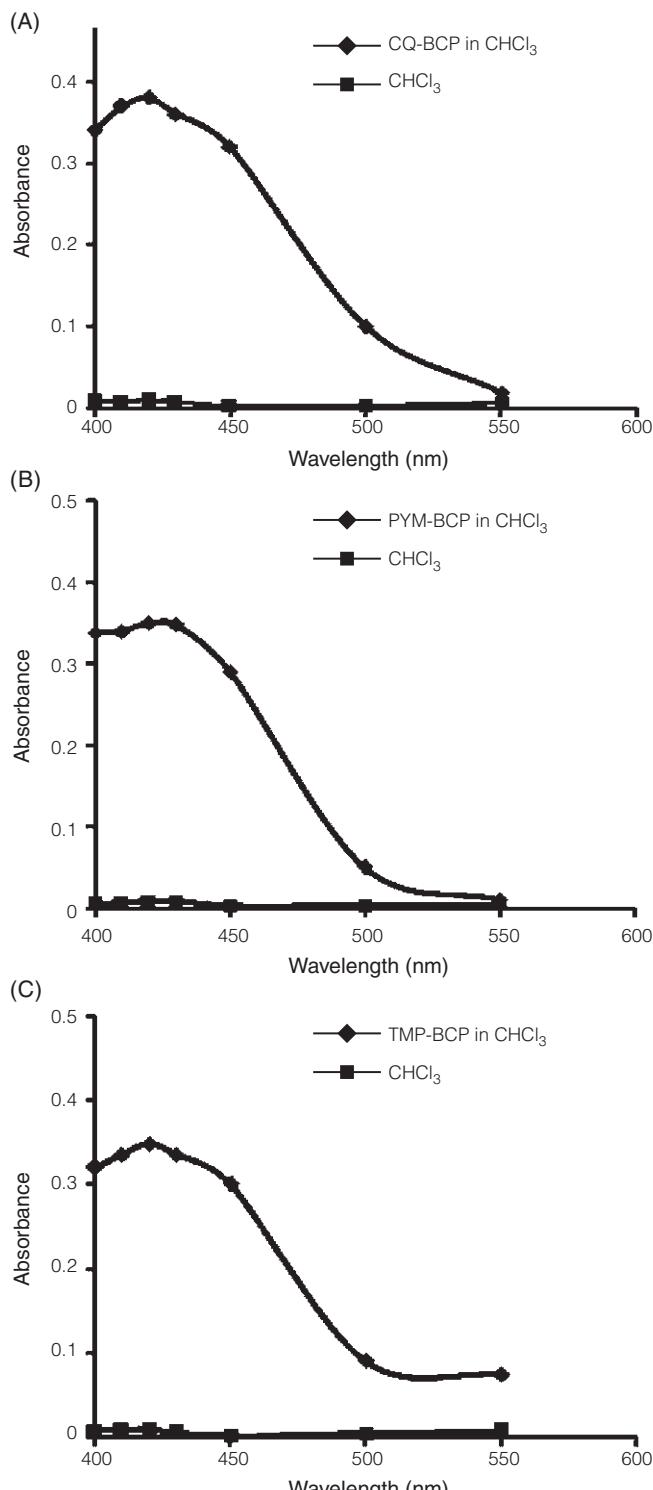
Figure 1. Absorption spectra of (A) CQ (5.0 μg/mL), (B) PYM (3.75 μg/mL), and (C) TMP (5.0 μg/mL).

made at room temperature (25 ± 1°C). The calibration curves were prepared by plotting absorbance *versus* concentration of the respective drugs.

(II) *Application of the Proposed Method to Pharmaceutical Formulations*

The recoveries of the drugs were examined by standard addition method by transferring a known quantity to standard flasks. When the standard solutions with suitable concentrations of drugs were added, the concentrations fell within the respective linear calibration range, except in one. The solutions were treated as mentioned in the "standard procedure section."

(III) *Application of the Proposed Method to the Determination of Drugs in Spiked Human Urine*

The recovery study was carried out in urine samples by standard addition method. As mentioned above, spiked urine in three different concentrations were transferred to a series of standard flasks. Different amounts of drugs were added from the standard stock solution, causing the total concentration fell within the Beer's law range.

RESULTS AND DISCUSSION

I. *Spectral and Elemental Characterization*

The method was based on the ion transfer complex formed by BCP, an acidic dye, and drugs that have basic nitrogen as electron donor to yield ion-pair salts which form color compound extractable from the aqueous solution to organic phase. The absorbance spectra and proposed reaction mechanism are shown in Figure 1 and Scheme 1.
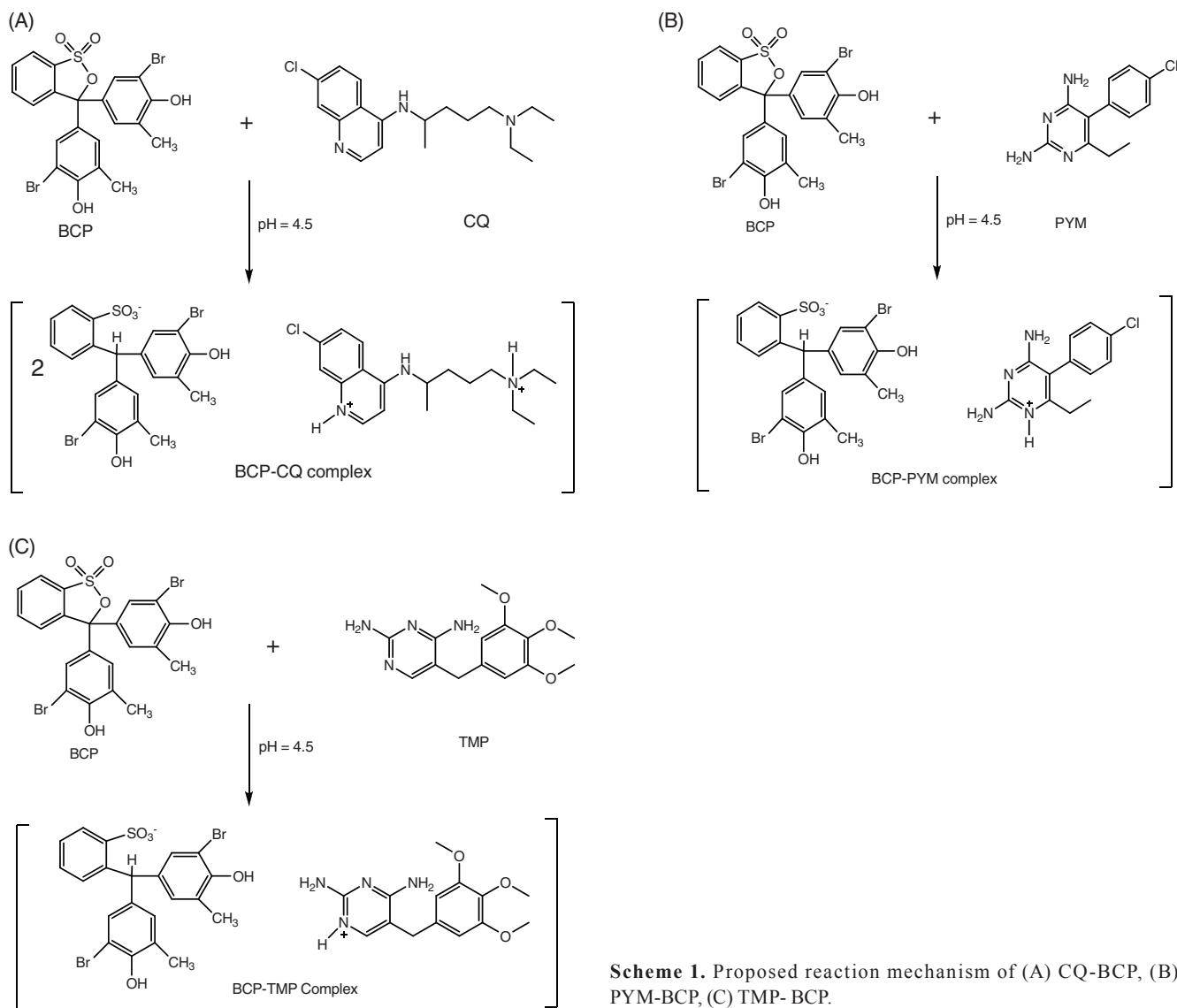
Scheme 1. Proposed reaction mechanism of (A) CQ-BCP, (B) PYM-BCP, (C) TMP- BCP.

II. *Optimization of Reaction Variables*

(I) *Concentration of BCP*

The effect of dye concentration on the intensity of the color developed was tested using different amounts of BCP. The result showed that 1 mL of BCP was optimum for this method.

(II) *Effect of pH Buffer*

A variable relationship between the absorbance at 420 nm and the pH for CQ, PYM and TMP, which was studied in the range of 3-5.5 using acetic acid/sodium acetate buffer (0.1 M), indicated that the reactions took place at optimum pH of 4.5 (Figure 2).

(III) *Selection of Extraction Solvent*

The effect of extracting solvent on the ion pair complex was studied in two organic solvents, dichloromethane and chloroform. Chloroform was selected because of its high color intensity.

(IV) *Optimization of Shaking Time*

Shaking times of 10 seconds to 2 minutes were studied. The optimum shaking time was fixed at 40 seconds.

(V) *Stoichiometric Relationship*

The combination ratio was evaluated by the Job's method of continuous variation (Figure 3), which shows that the complex formed between the drugs, CQ, PYM, and TMP, and BCP were 1 : 2, 1 : 1 and 1 : 1 respectively. The plot of log absorbance against log BCP and the drugs in limiting logarithmic method (Figure 3) shows the slope of 0.459 : 0.232 for CQ : BCP; 0.782 :
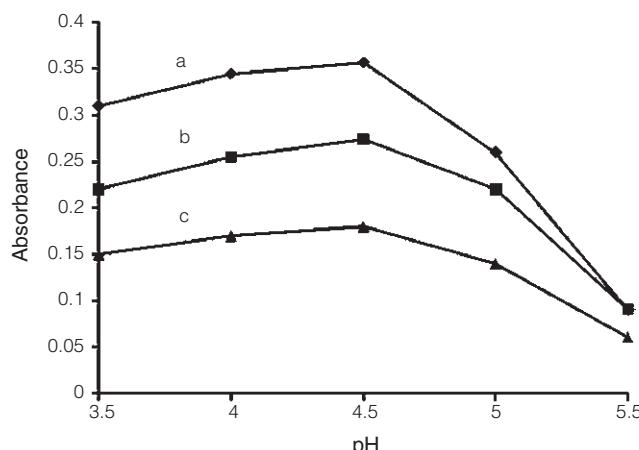
0.781 for PYM : BCP and 0.640 : 0.618 for TMP : BCP which further confirmed the complex ratio at 1 : 2, 1 : 1 and 1 : 1 respectively.

(VI) *Association Constants and Standard Free Energy Changes*

The association constants were calculated for the interaction of each drug with BCP using Benesi-Hildebrand equation:

$$\frac{Ao}{A} = \frac{1}{\varepsilon} + \frac{1}{K\varepsilon D_0}$$

where, A_0 and D_0 are the initial concentration of the acceptor and the donor, respectively. ε is the molar absorptivity of the complex and K is the association constant. The standard free energy changes of complex (ΔG^0) were calculated from the association constant by the following equation:

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

where, R is Gas constant, and T is the temperature in Kelvin. A straight line was obtained by plotting the value of A_0/A against $1/D$ (Figure 4). The intercept of line with the ordinate is $1/\varepsilon$ and the slope equal to $1/K \varepsilon$. The molar absorptivity was 42424, 21192, and 20608 L/mol·cm for CQ, PYM, and TMP, respectively, which correlated with the value obtained from linear calibration curve. The association constant (K) was 3981, 5225, and 4332 M^{-1}, and the standard free energy, ΔG^0, was -4.90, -5.07, and -4.96 kcal for CQ, PYM, and TMP, respectively.

III. *Method Validation*

(I) *Linearity, Detection and Quantification Limits*

The Beer's law range, molar absorptivity, sandell's sensitivity, regression equation, and correlation coefficient were determined for each drug. A linear relationship was found within the range of 1.25-8.75 μg/mL for CQ, 0.625-7.5 μg/mL for PYM, and 1.25-10.7 μg/mL for TMP. Regression analysis of the Beer's Law plots revealed strong correlation. The graph showed a negligible intercept, which was calculated by the least-square method's regression equation:

$$A = a + bc,$$

where, A is the absorbance of solution in a 1-cm cell, a is the intercept, b is the slope, and c is the concentration of the measured solution in μg/mL. The high molar absorptivity of the resulting colored solution indicated high sensitivity of the method. The limit of detection (LOD) and the limit of quantification (LOQ) value were determined using the formula:


Figure 2. Effect of pH on the formation of complex between BCP and (a) CQ (5 μg/mL), (b) PYM (2.5 μg/mL) and (c) TMP (2.5 μg/mL).

LOD or LOQ = K SD/b

where K = 3 for LOD and 10 for LOQ. SD is standard deviation of the response of the blank solution and b is slope of calibration curve. The Ringbom plot demonstrated the range of 2.5-7.5, 1.25-7.5, and 2.5-10.7 μg/mL
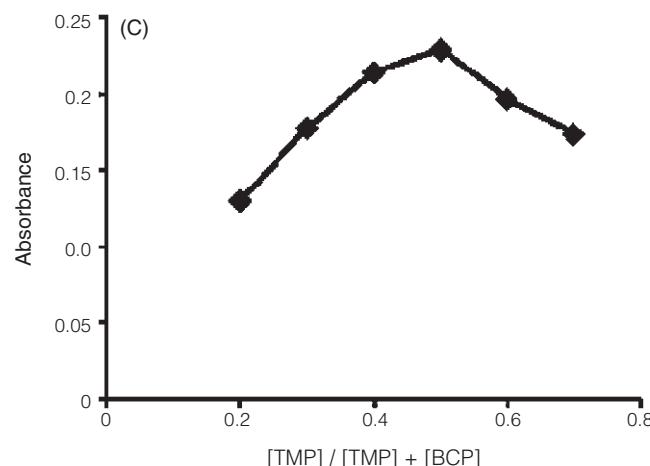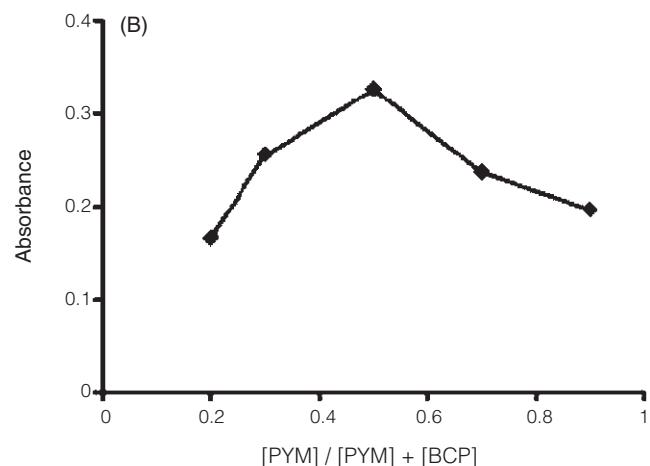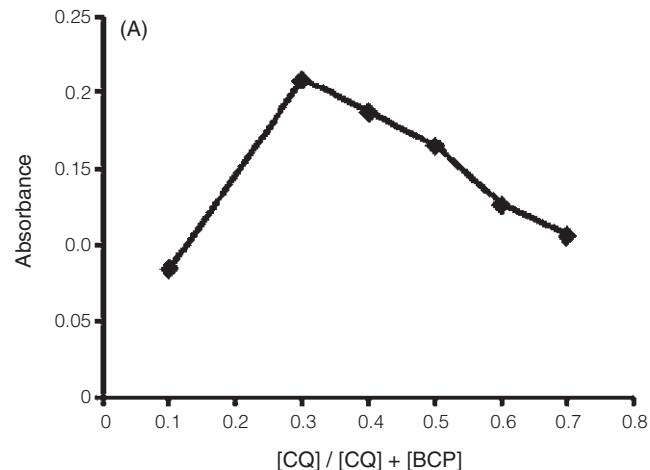
for CQ, PYM, and TMO, respectively as indicated on Table 1. The confidence limits for the slope of line of regression and intercept were computed using the relation, b ± tSb and a ± tSa at 95% confidence level. The results are shown in Table 1.

The error (Sc) in the determination of a given
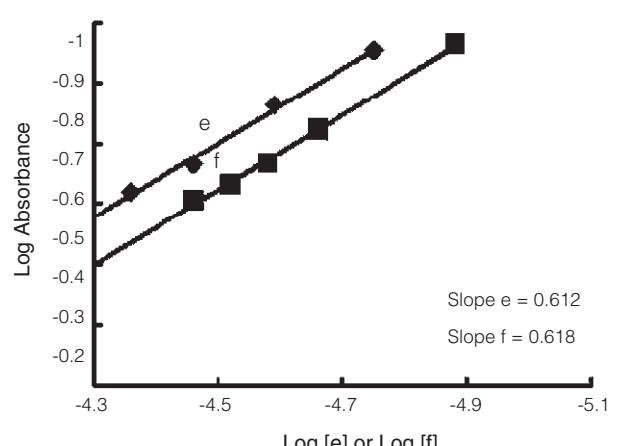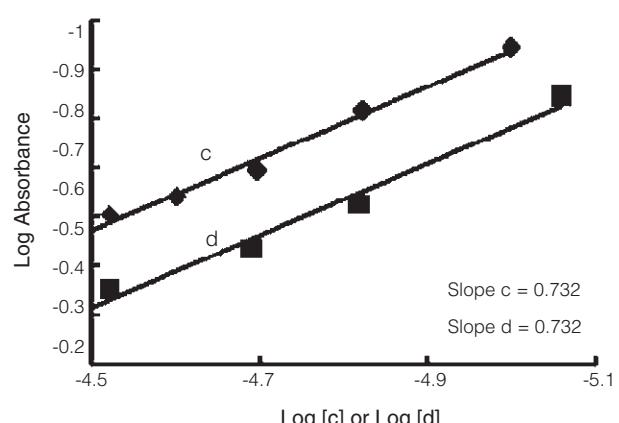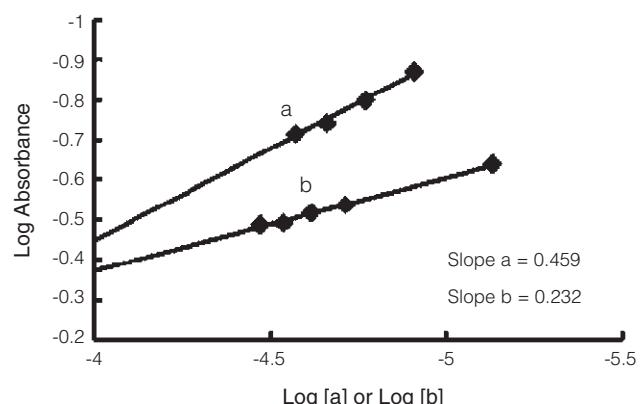
Figure 3. Job's method of continuous variation plots and Limiting logarithmic plots for the molar reactivity for (A) CQ , CQ and BCP = 4.85 × 10^{-5} M, a = Slope for CQ, b = Slope for BCP; (B) PYM, PYM and BCP = 1.0 × 10^{-4} M, c = Slope for PYM, d = Slope for BCP; (C) TMP, TMP and BCP = 8.6 × 10^{-5} M, e = Slope for TMP, f = Slope for BCP.

concentration of drugs was defined by the expression:

$$Sc = \frac{Sy/x}{b}\left[1 + \frac{1}{n} + \frac{(yo - \overline{y})^2}{b^2 \sum (x - \overline{x})^2}\right]^{1/2}$$

where $\overline{y}$ and $\overline{x}$ are the average values of the absorbance and concentration for n standard samples, respectively. Figure 5 shows the graph of the error, Sc *vs.* concentration of respective drugs. Minimum error was reached at absorbance of 5, 3.75, and 5 μg/mL of CQ, PYM, and TMP, respectively.

(II) *Interference Study*

The effect of common excipients used in the pharmaceutical preparation was studied by analyzing synthetic sample solutions containing the quantity of drugs as mentioned on Table 2 with 100-fold more concentration of each excipient. For sulfadoxine and sulfamethoxazole, the study was carried out at 200 times the concentration of PYM and TMP, respectively. The tolerance limit was the concentrations that gave an error of ± 3.0% in the determination of drugs. The results indicated that the excipients studied did not interfere with the quantitative analysis by the present method. The sample

Figure 4. Benesi-Hildbrand plots for (A) CQ-BCP, (B) PYM-BCP, and (C) TMP-BCP.
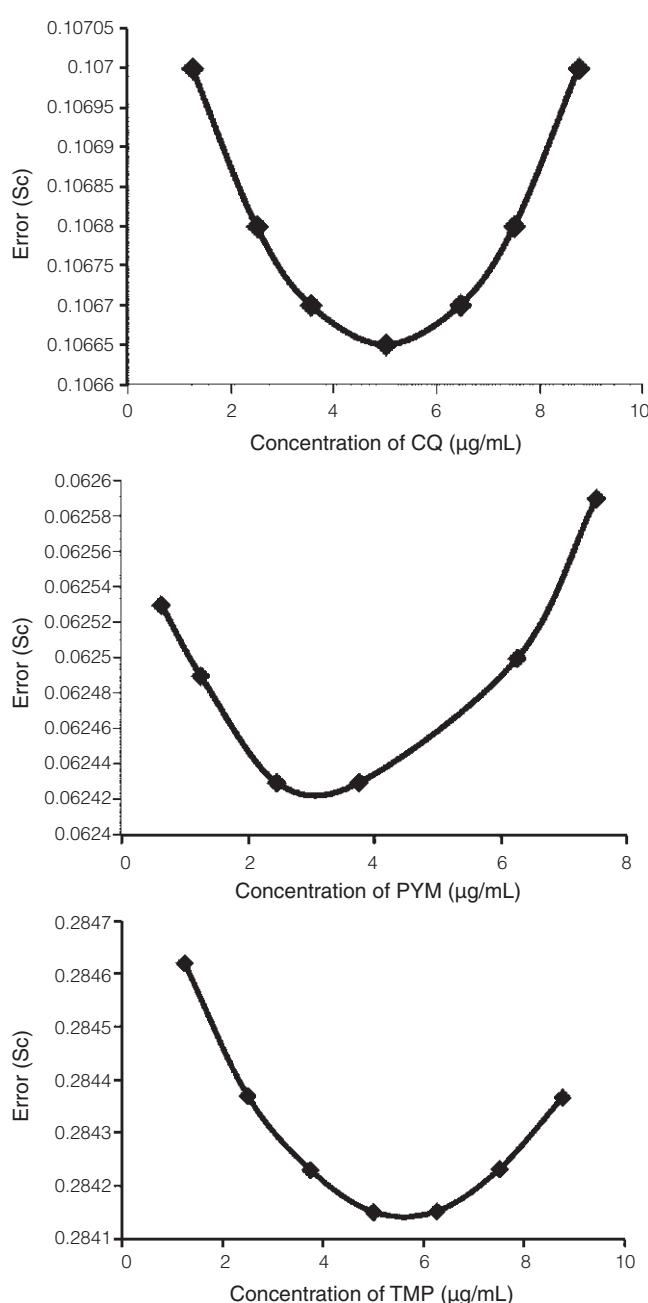
Figure 5. Plots of error in the determination of CQ, PYM, and TMP.

Table 1. Optical characteristics and statistical data of the regression equation for the reaction of the proposed method

Parameters	Optical characteristic		
	CQ	PYM	TMP
Colour	Yellow	Yellow	Yellow
λmax (nm)	420	420	420
Beer's Law range (μg/mL)	1.25-8.75	0.62-7.5	1.25-10.71
Molar absorptivity (L/mol·cm)	4.09×10^4	2.25×10^4	1.98×10^4
Sandell's sensitivity (μg/cm^2)	0.01258	0.01102	0.01464
Limit of Detection (μg/mL)	0.128	0.107	0.154
Limit of Quantification (μg/mL)	0.428	0.356	0.513
Ringbom optimum concentration range (μg/mL)	2.5-7.5	1.25-7.5	2.5-10.7
Regression Equation (Y)[a]			
Slope (B)	0.0793	0.0950	0.0662
Intercept (A)	-0.0026	-0.0134	0.0106
Correlation Coefficient(r)	0.9989	0.9998	0.9991
Relative standard deviation[b]	0.27	0.136	0.902
± tSb[c]	3.05×10^{-3}	1.82×10^{-3}	5.05×10^{-3}
± tSa[d]	0.0172	8.05	0.0325

[a] Y = Bx + A, where x is the concentration of the measured solution in μg/mL.
[b] Average of six determinations (concentration of 5, 3.75 and 5 μg/mL of pure drugs of CQ, PYM, and TMP respectively).
[c] Confidence Interval for Slope at 95% confidence limit for five degree of freedom.
[d] Confidence Interval for Intercept at 95% confidence limit for five degree of freedom.

Table 2. Recovery of drugs from solutions with a 100 fold concentration of various additives* present

Excipients	Recovery[a] ± S.D		
	CQ[b]	PYM[c]	TMP[d]
Dextrose	100.00 ± 0.02	100.02 ± 0.05	99.91 ± 0.05
Lactose	100.00 ± 0.07	99.90 ± 0.06	100.93 ± 0.05
Starch	99.10 ± 0.03	99.84 ± 0.03	99.81 ± 0.10
Sucrose	99.91 ± 0.03	99.85 ± 0.03	100.10 ± 0.07
Carboxymethyl cellulose	99.82 ± 0.04	100.21 ± 0.07	99.96 ± 0.03
Talc	100.04 ± 0.05	99.90 ± 0.06	99.60 ± 0.06
Magnesium Sterate	99.40 ± 0.10	100.04 ± 0.03	100.11 ± 0.07
Sodium Chloride	100.08 ± 0.02	100.04 ± 0.04	100.05 ± 0.10
Sulfadoxine*		100.02 ± 0.02	
Sulfamethoxazole*			99.91 ± 0.02
Urea	100.00 ± 0.01	100.00 ± 0.10	100.03 ± 0.03
Creatinine	99.90 ± 0.02	99.86 ± 0.10	99.90 ± 0.07
Bovine serum albumin	99.91 ± 0.02	99.94 ± 0.08	99.75 ± 0.03

[a] Mean of 3 determinations.
[b] Concentration of CQ used - 5 μg/mL.
[c] Concentration of PYM used - 3.75 μg/mL.
[d] Concentration of TMP used - 5 μg/mL
* Concentrations of additives taken were 200 times more.

containing bovine serum albumin (BSA) was allowed to stand for 30 min before measuring the absorbance.

(III) *Precision and Accuracy*

The short-term precision (intra-day precision) of the drugs was evaluated by measuring 5 independent samples at 3 different concentration levels (2.5, 3.75, 5.0 μg/mL for CQ, 1.25, 2.5, 3.75 μg/mL for PYM, and 3.75, 5.0, 6.25 μg/mL for TMP). Similarly, the assay for

daily precision (inter-day precision) at the same concentration level was repeated for 5 consecutive days. The standard deviations for the intra-day and inter-day assays were between 0.02 - 0.09 and 0.05 - 0.09, respectively, indicating good precision of the proposed method. The results are presented in Table 3.

The proposed method was applied to estimate drugs in pharmaceutical formulations. Precision of the method was checked by taking six replicate measurements. The results were compared statistically with reference methods. The standard deviation of the labeled amount ranged between 0.07 and 0.09. Student's *t*- values ≤ 2.44 for pharmaceutical samples at the 95% confidence limits indicated insignificant difference between the proposed and the reported methods. Analytical results measured for the same pharmaceuticals by the proposed and reference methods were compared using F-test. F-values ≤ 5.05 indicated insignificant difference in precision between both methods at 95% confidence interval. Reliability and accuracy of the proposed method were further ascertained through recovery studies using the standard addition method by adding different amounts of standard drugs to the pre-analyzed dosage forms such that the cumulative amount did not exceed their linearity range. The mean percentage recoveries, relative to the labeled amounts ranged from 99.71 (± 0.21) to 99.99 (± 0.15). Similarly, the recovery study in urine at three different concentration ranges by standard addition method was carried out. The excellent recoveries illustrated absence of interference from the matrix effect in the urine samples. The results are shown on Tables 4 and 5.

Table 3. Intraday and interday precision data

	Amount Taken (μg/mL)	Intraday (%Recovery ± S.D[a])	Intraday (%Recovery ± S.D[b])
CQ	2.5	99.92 ± 0.09	99.81 ± 0.09
	3.75	100.04 ± 0.09	99.91 ± 0.09
	5.0	100.06 ± 0.04	100.05 ± 0.08
PYM	1.25	99.95 ± 0.05	99.62 ± 0.07
	2.5	100.05 ± 0.02	99.84 ± 0.07
	3.75	100.06 ± 0.03	99.91 ± 0.07
TMP	3.75	100.04 ± 0.05	99.63 ± 0.08
	5.0	100.02 ± 0.04	99.96 ± 0.06
	6.25	100.07 ± 0.06	99.50 ± 0.05

[a] Mean of 5 determinations
[b] Mean of 5 determinations performed over a period of 5 days

Table 4. Analysis of drugs in pharmaceutical formulations

Formulation	Labelled (mg)	Proposed method ± S.D (mg)[a]	Reported Method ± S.D(mg)[a*]	%Recovery ± R.S.D[b]
Emquin[c]	250	249.96 ± 0.09	250.01 ± 0.10[18]	99.97 ± 0.21
		F = 1.23		
		t = 0.92		
Amalar[d]	25	24.90 ± 0.07	24.8 ± 0.09[18]	99.71 ± 0.19
		F = 1.65		
		t = 2.17		
Septran[e]	80	80.01 ± 0.09	79.90 ± 0.11[23]	99.99 ± 0.15
		F = 1.49		
		t = 1.88		

[a] Average ± standard deviation of six determinations; the t- and F- values obtained after comparison to the reference methods have following theoretical values at 95% confidence limits; *t* = 2.44 F = 5.05
[b] After adding four different amounts of pure drugs to the fixed concentration of preanalysed pharmaceutical formulations.
[c] CQ equivalent to 250 mg/tablet (E-Merck, India)
[d] PYM equivalent to 25 mg/tablet (Alimbic, India)
[e] TMP equivalent to 80 mg/tablet (GSK, India)
* Numbers inside the bracket indicate reference number of the reported methods given under References Section.

Table 5. Analysis of drugs in urine

Drug	Drug added (μg/mL)	Drug Found (μg/mL) ± SD
CQ	3	3.01 ± 0.02
	4	3.99 ± 0.01
	5	5.01 ± 0.07
PYM	2	1.99 ± 0.02
	3	3.00 ± 0.02
	4	4.01 ± 0.03
TMP	3	2.98 ± 0.05
	4	3.99 ± 0.02
	5	5.02 ± 0.08

CONCLUSIONS

The recommended method using BCP for the analysis of the drugs is simple, inexpensive sensitive and accurate. The optical parameters and statistical comparison have justified application of this method in routine drug estimation in pure and dosage forms. Furthermore, the procedure does not involve any critical reaction conditions or tedious sample preparation steps. Therefore, this method can be recommended for routine analysis of these drugs.

ACKNOWLEDGMENTS

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