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Spectrophotometric determination of diaminopyrimidines using benzoquinone

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Abstract

The diaminopyrimidine derivatives trimethoprim (TMP), pyrimethamine (PMA) and 2,4-diaminopyrimidine (2,4-DAP) are found to react readily and efficiently in an aqueous solution with *p*-benzoquinone (*p*-BQ) to form a colored product with an absorption optimum wavelength of about 500 nm. The optimum reaction time, pH, temperature, solvent, and [*p*-BQ] are determined by separate trials. These conditions are confirmed by a MultiSimplex optimization method. The molar absorptivities of the TMP, PMA, and 2,4-DAP reaction products at 500 nm are 10 830, 10 650, and 9660 l mol^{-1} cm^{-1}, respectively. TMP shows a linear range between 5 and 100 mg/l while PMA and 2,4-DAP exhibit linearity between 15 and 75 mg l^{-1} and 5 and 30 mg l^{-1}, respectively. There is some specificity to this reaction; 2-aminopyrimidine does not react. Under the optimum conditions, sulfamethoxazole (SM) reacts rather poorly with a molar absorptivity of about 110 l mol^{-} cm^{-1}. Using *p*-BQ, TMP in a pharmaceutical sample can be determined in the presence of SM using derivative spectrophotometry. The TMP-*p*-BQ reaction is adaptable for flow injection analysis. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Diaminopyrimidine; *p*-Benzoquinone; Derivative visible spectrophotometry; Flow injection analysis

1. Introduction

The major role of the antibacterial diaminopyrimidine derivatives has been attributed to their ability to block the synthesis of tetrahydrofolate by inhibiting the enzyme dihydrofolate reductase (DHFR). This inhibition action will lead to the blockage of many important cell constituents necessary for bacteria growth [1]. It was found that the activity of the inhibitors was strongly influenced by the chemical structures of the diaminopyrimidines. The antimalaria properties of pyrimethamine (PMA) were suppressed due to the lack of an alkyl group on the pyrimidine ring. This modification, however, increased its activity as an antibacterial drug. Trimethoprim (TMP) was found to act as a strong antibacterial drug due in part to three methoxy groups substituted on the benzene ring (Fig. 1) [2]. In his paper, Then has described in great detail the history and the growing interest of scientists in the synthesis of diaminopyrimidine derivatives and their combina-

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tions with other drugs such as sulfonamides up to 1993.

Controversial ideas have occurred in the literature concerning the efficiency of the TMP–sulfonamide combination. Brumfitt and Hamilton-Miller claimed that the increased cases of bacterial resistance as well as undesirable side effects were reported when co-trimoxazole drugs containing TMP and sulfamethoxazole (SM) were prescribed [3]. Periti [4] argued that co-trimoxazole should only be used when other alternatives were not possible. His opinion was based on the notion that the drug combination efficiency was based only on an in vitro synergism observation [5,6]. However, even with these concerns, the combination of TMP and SM has continued to be prescribed daily worldwide [7].

In recent years, more work has been devoted toward the synthesis of new DHFR inhibitors based on the 2,4-diaminopyrimidine (2,4-DAP) structure. Botta et al. reported the synthesis of isotrimethoprim, 2,4-diamino-(3,4,5-trimethoxybenzyl)pyrimidine, and a few other derivatives [8]. Selassie and coworkers have listed more than 65 derivatives of 2,4-DAPs and studied their inhibition activities of *E. coli* DHFR [9]. Just recently, Lopez and his group published their work on the synthesis of new diaminopyrimidine derivatives and investigated their potential as anti-AIDS drugs [10].

Various spectroscopic techniques have been reported for the quantitation of TMP in simple and complex matrices. A colorimetric method based on measuring the ion-pair formation of TMP with bromophenol blue in the presence of SM was investigated. The formation of a TMP ion-pair was reported to be favorable when the pH of the aqueous phase was 3.2 [11]. However, chloroform, a cancer suspect agent, is used for extraction of the ion-pair before measurement. Korany et al. [12] described the use of first derivative spectrophotometry for the determination of four two-component mixtures in pharmaceutical dosages, one of which contained TMP and SM. Because this work was done in the 240–280 nm UV region, interferences could be a problem. Sigel and Grace [13] discussed the separation of TMP and four of its metabolites using TLC and measuring the fluorescence signal on non-fluorescing silica gel plates. Quantitative measurements were done

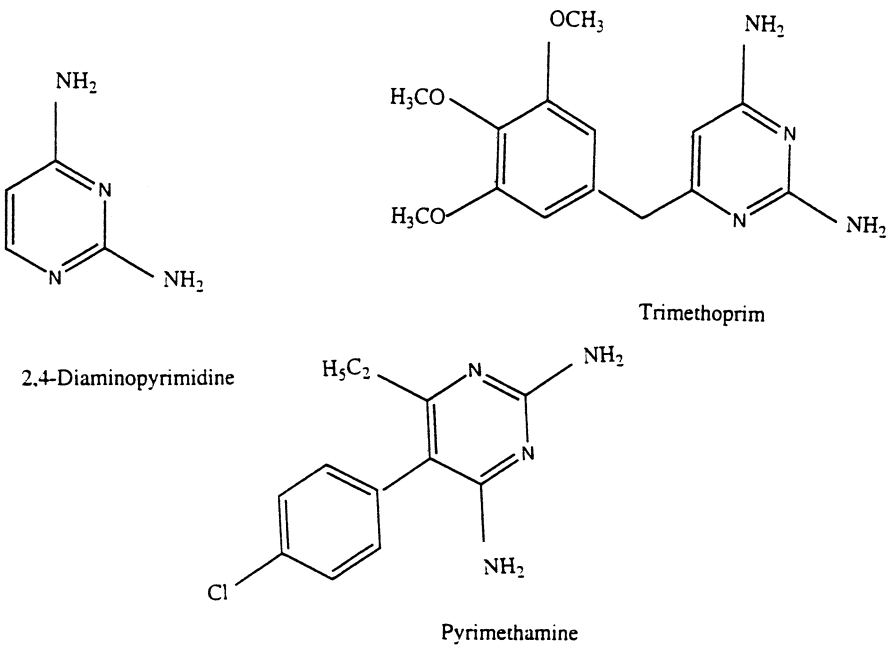

Fig. 1. Chemical structures of the diaminopyrimidine derivatives.

in urine, plasma, and various tissues. However, the method did not show the potential to be applicable for the determination of other diaminopyrimidines and the development time for a measurable fluorescence signal was excessive. In another paper, Sigel et al. [14] reported the use of a spectrophotometric method in combination with TLC for the quantitative determination of TMP and SM in plasma. The method was based on measuring the absorbance of the two compounds at 280 and 265 nm, respectively. A few years later, a liquid chromatographic method with UV–Vis detection at 254 nm was used to analyze a mixture of TMP and SM in dosage forms [15]. The author also separated two degradation products of TMP.

Several reports have appeared in the literature describing the usefulness and the simplicity of using *p*-benzoquinone (*p*-BQ) as a spectrophotometric chromogenic reagent. Hassan and his group used this approach to determine several aliphatic primary and secondary amines such as piperidine [16]. Their method involved the reaction of the amine with an excess of *p*-BQ with the reaction product monitored spectrophotometrically at 510 nm. The elemental analysis and IR data indicated that piperidyl-*p*-benzoquinone was the product when the concentration ratio between *p*-BQ:amine exceeded 30. On the other hand, a different product, 2,5-bis(dipiperidyl)-*p*-benzoquinone with a maximum absorbance at 370 nm was isolated when an equal molar ratio of *p*-BQ and amine was used. The reactivity of the aromatic amines such as aniline, toluidines, and chloroaniline with *p*-BQ was also investigated [17]. Two different products were isolated and identified. The first chromogen, 2-anilino-*p*-benzoquinone showed an absorbance at 505 nm which was formed when the quinone:amine molar ratio exceeded 30. The second isolated product with a maximum absorbance at 370 nm and identified as 2,5-dianilino-*p*-benzoquinone was formed when the quinone:aniline molar ratio was 1:1 and 3:1. A spectrophotometric method for the determination of cysteine in various matrices was also developed using *p*-BQ [18]. This approach did not require any prior derivatization or extraction before the assay was used. The reaction selectivity of *p*-BQ for sulfonamides was investigated by Mohamed and his group [19]. Their work showed that the selective determination of SM or sulfamoxole in the presence of TMP was possible.

The main goal of this study was to explore the potential use of *p*-BQ as a chromogenic reagent for the determination of diaminopyrimidine derivatives. To the best of our knowledge, diamines have not been studied with respect to their reactivity with *p*-BQ. TMP, PMA, and 2,4-DAP were found to react very efficiently with *p*-BQ to generate a red colored product with a maximum absorbance at 500 nm. The optimum reaction conditions were determined by separate trial approach and confirmed by a MultiSimplex optimization method. Pharmaceutical samples for TMP were analyzed by fourth derivative spectrophotometry and application to flow injection analysis was made.

2. Experimental

2.1. Instrumentation

All of the off-line measurements were collected on a Hewlett Packard 8452A diode array spectrophotometer that was interfaced to a HP 486/33N PC computer. Quartz cuvettes with a pathlength of 1 cm were used. The MultiSimplex software was designed by Stenbergsgr.9 (Karlskrona, Sweden) and was distributed by Statistical Designs (Houston TX).

The flow injection system consisted of a Nicolet ternary gradient LC pump model 9560 and a Hitachi L-4500 diode array detector. The detector was controlled by a computer with Hitachi Chromatography Data Station software. A six-port valve (Rheodyne 7161) with an injection loop of 50 μl was used. The reaction took place in a 0.49 ml ETFE tube reactor (2.5 m × 500 μm ID) that was knitted on a 1 × 1 cm plastic grid mesh.

2.2. Reagents

All reagents were of analytical grade. TMP, PMA, and sulfamethaxazole (SM) were received from Sigma (St. Louis, MO). The 2,4-DAP and 2-aminopyrimidine (2-AP) were sent from Aldrich

(Milwaukee, WI). The pharmaceutical tablets, those with TMP (100 mg) only and those containing a mixture of TMP (80 mg) and SM (400 mg), were purchased from a local pharmacy. *p*-Benzoqunione (*p*-BQ) was obtained from Acros (Fair Lawn, NJ). Ammonium acetate and potassium phosphate were from Fisher Scientific (Fair Lawn, NJ). The pH 3, 4, and 5 buffers were based on 10 mM ammonium acetate and the pH 7 buffer was 10 mM phosphate. The HPLC grade solvents, methanol (MeOH), isopropanol (2-PrOH), and acetonitrile (ACN) were obtained from Burdick and Jackson (Muskegon, MI). Ethanol 200 proof (EtOH) was purchased from Quantum Chemical Co. (Newark, NJ). All solutions were prepared using high quality purified water from a Milli-Q System (Millipore, Bedford, MA).

2.3. Procedure

2.3.1. Off-line analysis

The *p*-BQ solution was prepared fresh daily in distilled water and organic solvent. The volumetric flask containing *p*-BQ was wrapped with aluminum foil and stored in a dark place. Each analyte was dissolved in a mixture of distilled water, 10 mM sodium phosphate buffer adjusted to the desired pH, and the organic solvent in appropriate proportions. A 2 ml volume of *p*-BQ was pipetted into a pyrex test tube containing 2 ml of the analyte. The 150 ml pyrex test tube was capped, shaken vigorously, and placed in a Fisher model 900 water bath with a controlled temperature. The starting time of the reaction was the moment when the test tube was placed in the water bath. For the blank, a solution containing only *p*-BQ was used. Upon completion of the reaction, the test tubes were removed from the water bath and placed in a beaker containing cold water. This was done to bring the solutions down to room temperature. Finally, the contents of the test tube were transferred to the quartz cuvette and the absorbance was measured against the blank. A calibration curve was constructed by preparing various concentrations of TMP standards in 47/53 MeOH/H_2O. Each standard was reacted with *p*-BQ under optimum reaction conditions and filtered through a 0.45 μm nylon filter before the absorbance signal was recorded at 500 nm.

Pharmaceutical tablets that contained either TMP with SM or TMP alone were analyzed in the following way. The pharmaceutical tablet was weighed on the analytical balance and then crushed into a fine powder using a mortar and pestle. An appropriate amount of the fine powder corresponding to the desirable amount of TMP was weighed into a 25 ml volumetric flask. The fine powder was suspended in about 15 ml of 47/53 MeOH/H_2O, and sonicated for about 10 min to ensure complete dissolution of the active ingredient. The solution was brought to volume by adding more 47/53 MeOH/H_2O before filtering through a 0.45 μm nylon membrane. The samples were reacted with *p*-BQ under optimum conditions and treated in a similar way as the TMP standards. The recovery of TMP was calculated using the unknown TMP absorbance at 500 nm and the regression equation generated from the standard calibration curve. The quantitation of TMP in the presence of SM was determined as follows. First several standards containing TMP and SM with a respective mg ratio of 1:5 were prepared in 47/53 MeOH/H_2O. The standards were reacted with *p*-BQ prepared at a similar solvent composition. The reactions were performed under the optimum reaction conditions for TMP (Table 1). Fourth derivative spectrophotometric analysis with three smoothing points was acquired for each standard. A peak with positive and negative maxima at 486 and 490 nm, respectively, was generated. The absolute distance between the two maxima was plotted against the TMP concentration.

An experiment was conducted to test the stability of the chromogenic product that was formed by the reaction of 2,4-DAP with *p*-BQ. The absorbance of the product was measured immediately after the reaction was done. The test tube containing the remaining reaction product was wrapped in aluminum foil and stored in the refrigerator at 4 °C for 68 days.

Table 1
Comparison of the optimum conditions obtained by the MultiSimplex optimization method and by separate trials

Analyte	Temperature (°C)	Time (min)	[*p*-BQ] (g l^{-1})	%MeOH in H_2O
TMP	64 (60)	13 (10)	14.85 (12.50)	47 (50)
PMA	76 (80)	12 (10)	14.85 (15)	47 (50)
2,4-DAP	66 (70)	12 (10)	12.35 (12.50)	47 (50)

() Indicates optimum conditions obtained by separate trials. TMP (140 mg l^{-1}), PMA (100 mg l^{-1}), and 2,4-DAP (50 mg l^{-1}), ($n = 3$).

The absorbance of the chromogen was measured against the blank, which was treated the same way.

2.3.2. On-line analysis

The running solvent for the flow injection system was a 47/53 MeOH/H_2O solution of *p*-BQ (14.85 g l^{-1}) that was previously filtered through a 0.45 μm nylon membrane filter. A flow rate gradient of 0.1 ml min^{-1} for 4 min, followed by 1 ml min^{-1} for 2 min was used. To avoid any undesirable dilution of analyte, a sample volume of 150 μl was injected into the 50 μl loop. The knitted ETFE tubing coil, in which the reaction took place, was inserted between the injector and the detector inside the controlled temperature water bath.

3. Results and discussion

3.1. Method optimization (off-line)

The method optimization in this experiment involved testing several parameters, such as pH, temperature, *p*-BQ concentration, and heating time. The optimum conditions for each parameter were investigated separately. A typical absorbance spectrum is shown in Fig. 2 indicating the maximum absorbance is at 500 nm as expected for a red colored product. This spectrum is similar to that previously published for monoamine compounds [19]. The noise below 460 nm is due to the absorbance difference of *p*-BQ in the blank and the sample.

3.1.1. pH effect

A variable relationship between the absorbance at 500 nm and the pH for TMP, PMA, 2,4-DAP and SM is shown in Fig. 3. This relationship indicates that the reaction between the diaminopyrimidine derivatives and *p*-BQ does not occur at pH < 2. The slow reactivity of the diaminopyrimidines with *p*-BQ at acidic pH may be attributed to the low nucleophilicity of the protonated amines. Similar observations are reported for the reaction of primary and secondary aliphatic amines with *p*-BQ [16]. In contrast, the absorbance of the product formed in the reaction of SM and *p*-BQ shows a significant increase at pH < 2, especially in 0.1 M HCl. This was also observed by Mohamad et al. [19]. In the pH range between 2 and 5, the diaminopyrimidine derivatives show a variation in their reactivity with *p*-BQ. For example, it is clear that in the above mentioned pH range, 2,4-DAP is more reactive than TMP. The optimum pH condition for TMP, DMA, and 2,4-DAP is found with 50/50 H_2O/MeOH at the apparent pH 7 (Fig. 3). SM shows some absorbance at that pH but it is not significant. An experiment was performed in which the diaminopyrimidines are reacted with *p*-BQ in 10 mM potassium phosphate buffer (pH 7). The absorbance was found to be much lower than if the reaction takes place in the absence of the buffer.

3.1.2. Solvent

In these experiments, organic solvents including MeOH, EtOH, 2-PrOH, and ACN were examined in various compositions with H_2O. The results for TMP are summarized in Fig. 4. The data indicate

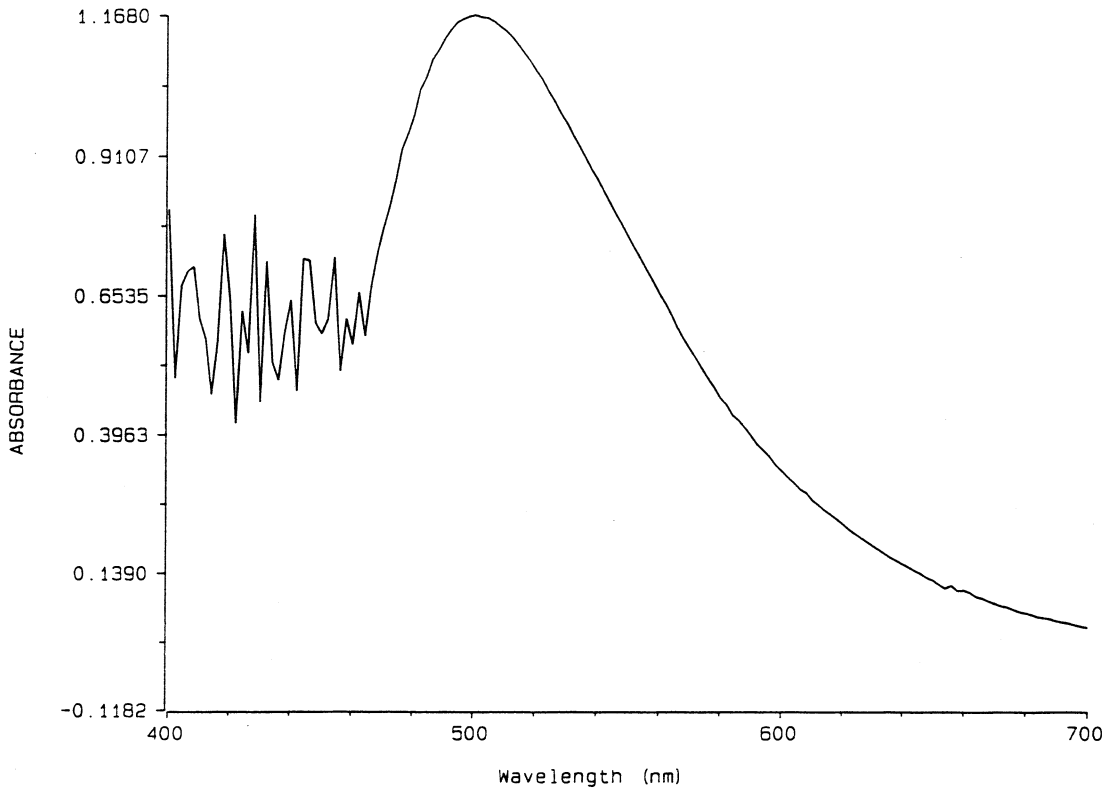

Fig. 2. UV–Vis absorption spectrum of the reaction product of TMP with *p*-BQ. [TMP] = 27 mg l^{-1}, [*p*-BQ] = 14.85 g l^{-1}, Temperature: 64 °C, time: 13 min, solvent: 47/53 MeOH/H_2O.

that under the specified conditions, a 40% solution of MeOH is the best, since the reactivity of diaminopyrimidines is slower (> 15 min) at a higher content of MeOH. In 2-PrOH solution, the water content has to be raised up to a value greater then 40%, in order to obtain a positive result. Thus, the reactivity of the co-organic solvent decreases in the following order MeOH > EtOH > 2-PrOH. This may be attributed to the dielectric constant (MeOH 32.6, EtOH 24.3, 2-PrOH 18.3) and solvent–solute interaction. Interestingly, when ACN is used, there is no reaction observed between TMP and *p*-BQ even in 50% ACN (data not shown). ACN, with dielectric constant of 37.5, does not fit this trend but probably deactivates the pyrimidine ring and makes it less active toward the reaction with *p*-BQ. Similar behavior has been reported by Hassan et al. [16]. Their results suggest that the reactivity of primary and secondary aliphatic amines tends to slow down when a solvent such as ACN or dioxane is used.

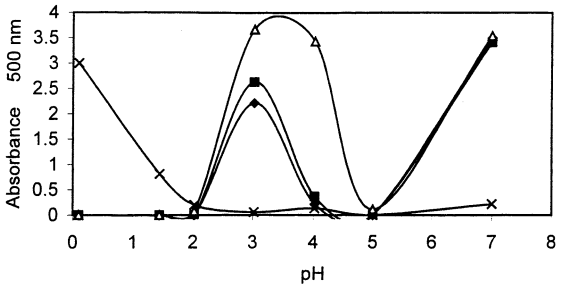

Fig. 3. pH effect on the absorption of the reaction products of diaminopyrimidines with *p*-BQ. TMP (◆), PMA (■), 2,4-DAP (△), SM (×). Time: 15 min, temperature: 60 °C, [*p*-BQ] = 14.75 g l^{-1}, [TMP] = 200 mg l^{-1}, [PMA] = 200 mg l^{-1}, [2,4-DAP] = 200 mg l^{-1}, [SM] = 500 mg l^{-1} ($n = 3$).

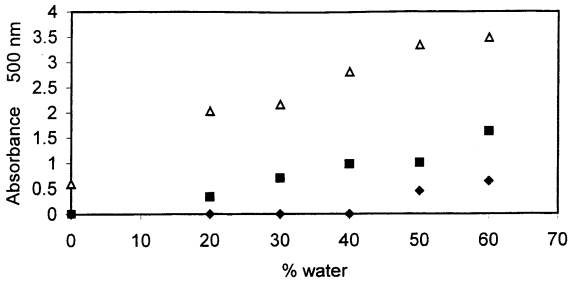

Fig. 4. Effect of the aqueous organic solvent on the reaction of TMP with *p*-BQ. MeOH (△), EtOH (■), 2-PrOH (◆). [TMP] = 140 mg l^{-1}, temperature: 60 °C, time: 15 min, [*p*-BQ] = 12.50 g l^{-1} ($n = 3$)

3.1.3. Temperature effect

The influence of the temperature on the reaction between TMP with *p*-BQ is shown in Fig. 5. The optimum reaction temperature of the water bath for TMP is found to be 60 °C, when the experiment is conducted in 50/50 (v/v) MeOH/H_2O for 15 min. As shown in Fig. 5, a noticeable decrease of the absorbance at 500 nm is associated with the decrease in the water bath temperature, with an exception at pH 5.

The variation in temperature has a less significant impact on the reactivity of PMA with *p*-BQ when a mixture of 50/50 MeOH/H_2O is used as a solvent (data not shown). However, the optimum temperature is chosen to be 80 °C, since a slightly higher absorbance is observed. In 50/50 MeOH/H_2O, a similar strong absorbance to that of TMP but with a small variation is noticed for 2,4-DAP in the temperature range of 50–80 °C with the maximum absorbance observed at 70 °C (data not shown).

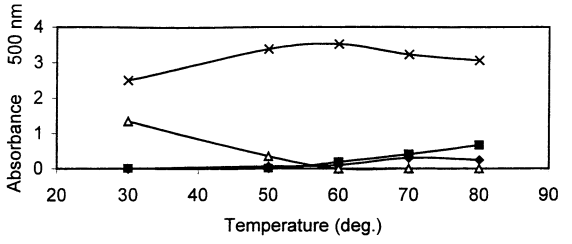

Fig. 5. Temperature effect on the absorbance of TMP (140 mg l^{-1}) reaction product. 50/50 (v/v) of: (×) MeOH/H_2O, (◆) MeOH/acetate buffer (pH 3), (■) MeOH/ acetate buffer (pH 4), (△) MeOH/acetate buffer (pH 5), [*p*-BQ] = 12.50 g l^{-1} ($n = 3$).

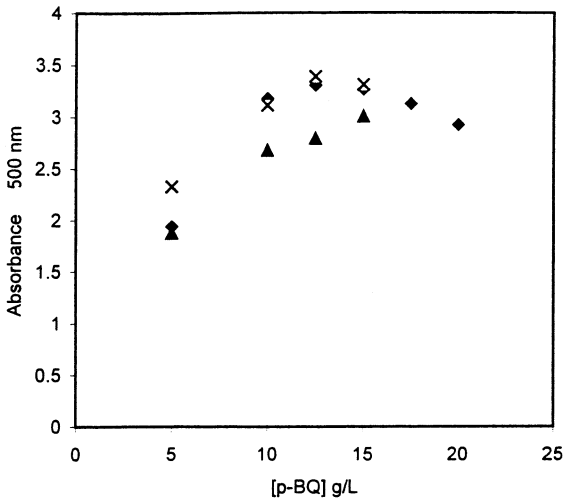

Fig. 6. Reactivity of 140 mg l^{-1} TMP (◆), 100 mg l^{-1} PMA (▲) and 50 mg l^{-1} 2,4-DAP (×) with various concentrations of [*p*-BQ]. Time: 10 min, solvent: 50/50 MeOH/H_2O, temperature: optimum values as determined by separate trails (Table 1) ($n = 3$).

3.1.4. p-Benzoquinone concentration

The effect of *p*-BQ concentration on the development of the red color is tested for all diaminopyrimidine derivatives at their optimum temperature (Fig. 6). As shown for all three diaminopyrimidine products, there is a sharp increase in the absorbance which is associated with the increase in the *p*-BQ concentration up to 10 g l^{-1}. At higher concentrations of *p*-BQ, the blank signal increases as well as the solubility problem of *p*-BQ becomes apparent. From these data, the optimum concentration of *p*-BQ for TMP and 2,4-DAP is thought to be 12.5 g l^{-1}. For PMA, the optimum concentration for *p*-BQ is closer to 15 g l^{-1}.

3.1.5. Heating time

A typical output of these experiments is presented by plotting the absorbance of the red product formed by the reaction of TMP with *p*-BQ versus the heating time. These data indicate that a heating time of 10 min in 50/50 MeOH/H_2O is sufficient to produce the optimum absorbance signal with a minimal interference from the blank (Fig. 7). This result is also applicable to the reaction of PMA and 2,4-DAP with *p*-BQ. In

all cases when the experiment is done in a buffered system, no appreciable signal is noticed (data not shown).

3.2. MultiSimplex optimization

The optimization approach for one variable at a time does not necessarily select the best outcome of the experiment; a MultiSimplex optimization method can be used for confirmation. The MultiSimplex optimization method used in this experiment is described in depth in Ref. [20] and can be summarized in the following sentences. The main principle of the Simplex approach is based on the design $k+1$ trials, where k is the number of variables. In the present study, where four variables, pH, temperature, time, and [p-BQ] are involved, the first Simplex geometry will have five trials. After performing the first Simplex, the Simplex algorithm will sequentially search for the target by varying one variable at a time. The expansion and contraction in the Simplex movement enable the user to reach the target quickly and effectively. The optimization objective of the Simplex is to reach a predefined maximum absorbance value.

Separate sets of experiments aimed to find the optimum conditions for the reaction of TMP, 2,4-DAP, and PMA with p-BQ using the MultiSimplex approach were conducted. Table 1 summarizes the results of the MultiSimplex approach ($\#$ experiments = 8) and compares them to conditions obtained by separate trials. The two sets of data are close to each other, however, the values obtained by the MultiSimplex method are probably more accurate.

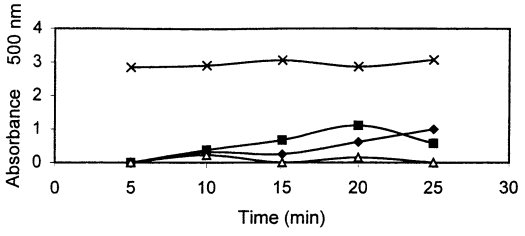

Fig. 7. Dependence of the absorbance of 140 mg l^{-1} TMP on the heating time. Temperature: 60 °C, solvent: 50/50 MeOH/H_2O, [p-BQ] = 12.50 g l^{-1} ($n=3$). (×) MeOH/H_2O, (◆) MeOH/buffer (pH 3), (■) MeOH/buffer (pH 4), (△) MeOH/buffer (pH 5).

A confirmation experiment by separate trials was conducted to test the integrity of the optimum conditions since they were determined at higher absorbance values where a deviation from Beers Law may exist. In this experiment, lower concentrations of analytes (18 mg l^{-1} TMP, 14 mg l^{-1} PMA, and 5 mg l^{-1} 2,4-DAP) were used. Three points were selected for each parameter (temperature, time, [p-BQ] and % MeOH). One point has exactly the same optimum value as that determined previously, one point was lower than the optimum, and one was higher. The results are the same as those determined previously and are summarized in the brackets of Table 1. The data in Table 1 differ from those found previously for aromatic amines [17] with respect to temperature (50 °C) and time (1 h).

3.3. Method linearity, specificity, and stability

The optimum conditions obtained by the MultiSimplex method were used to test the method linearity for the diaminopyrimidine derivatives. For PMA, Beers law is obeyed in the range of 10–75 mg l^{-1} with a correlation coefficient of 0.9915 ($y = 0.0428x - 0.2604$). The relative standard deviation (RSD) for these seven points taken in triplicate generally ranged from 1 to 4%. At higher concentrations, deviation from linearity is taking place, in part because of the high absorbance. The other two analytes show a shorter linear range, extending from 15 to 50 mg l^{-1} for TMP with a correlation coefficient of 0.998 ($y = 0.0373x - 0.189$), and from 5 to 30 mg l^{-1} for 2,4-DAP with a correlation coefficient of 0.9923 ($y = 0.0877x - 0.1923$). The molar absorptivity of TMP, PMA and 2,4-DAP at 500 nm are calculated to be 10 830, 10 650, and 9660 l mol^{-} cm^{-1}, respectively. These molar absorptivity values are about two to three times higher than those for aliphatic amines such as piperidine [16] and aromatic amines such as aniline [17].

The spectrophotometric method for the determination of diaminopyrimidines based on the reaction with p-BQ shows some specificity. An experiment conducted for the reactivity of p-BQ

with aminopyrimidine under the optimum reaction conditions obtained by the MultiSimplex method showed no positive results are observed. This indicates that the presence of at least two amine groups on the pyrimidine ring probably facilitates product formation because the reaction time is quite short at about 12 min.

The product stability data reveal that upon refrigerated storage of the reaction product of 2,4-DAP with *p*-BQ for 68 days, the absorbance of the product at 500 nm increases by more than 100%. This indicates that during the storage, a slow reaction between *p*-BQ and 2,4-DAP is continuously taking place. However, short-term reproducibility on the order of hours was fine. Identification of the diaminopyrimidine-*p*-BQ reaction product by LC/MS was inconclusive. Isolation of the product by crystallization was not obtained. Although the DAP:*p*-BQ reaction ratio is different but the wavelength of maximum absorbance is similar, a 2:1 DAP–*p*-BQ complex is probable, analogous to products previously reported [17]. The possibility of the formation of two different compounds as described in Section 1 cannot be ruled out.

3.4. Method application and derivative spectrophotometry

The applicability of the method to the analysis of two pharmaceutical samples containing either TMP alone or both TMP and SM is described in this section. Pharmaceutical tablets containing 100 mg l^{-1} of TMP only as the active ingredient were first tested. The computed recovery of TMP from two pharmaceutical tablets is found to be 99.3 with an RSD value of 5.8% based on four trials.

Derivative spectrophotometry provides an alternative approach for the quantitative determination of the analyte of the interest in the presence of a spectral interference from the matrix and/or other component spectral overlap. Because the mg ratio of SM to TMP in the second tablet sample is 5:1, SM, despite its 10 fold lower molar absorptivity, is found to react with *p*-BQ to give an appreciable absorbance under the optimum reaction conditions for TMP. In our work, an approach

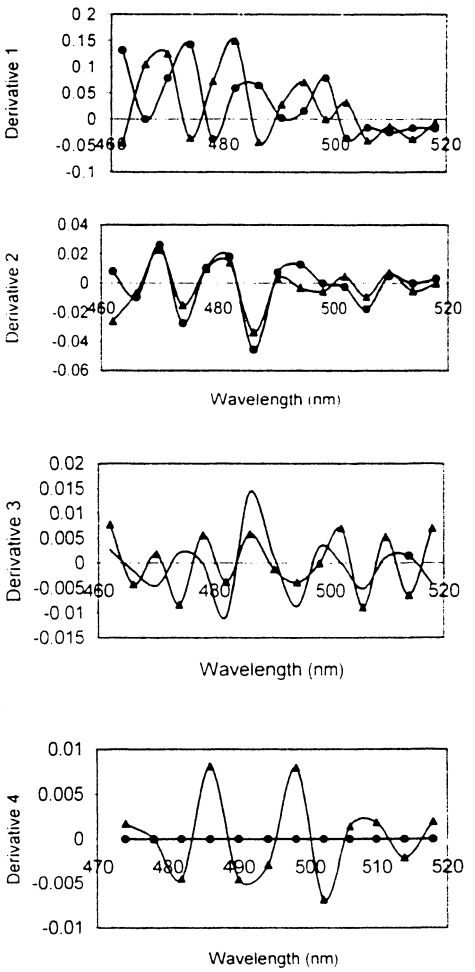

Fig. 8. Different orders of derivative spectrophotometry for a mixture of TMP and SM. [TMP] = 60 mg l^{-1}, [SM] = 300 mg l^{-1}. Other conditions as in Table 1 for TMP. The symbols ▲ and ● refer to TMP and SM, respectively.

similar to the one described by Salinas et al. [21] is taken to quantitatively determine the amount of TMP after its reaction with *p*-BQ in the presence of SM. Although different orders of derivative spectrophotometry were tested, only the fourth derivative with three smoothing points was found to generate reproducible results (Fig. 8). The fourth derivative calibration curve of standard TMP solutions was linear from 60 to 140 mg l^{-1} for four data points taken in triplicate. The linear least-squares regression equation was $y = 7 \times 10^{-5}x + 0.0014$ with $R^2 = 0.9974$. The recovery

calculation of a mock unknown is found to be 101% with a RSD of 3.7% based on three trials. The calculated recovery of the real sample (two tablets) is estimated to be 111% with the corresponding RSD value of 0.4% based on four trials. This recovery is somewhat high as noted on one occasion for the TMP only tablet. Expected excepients such as corn starch, lactose, magnesium stearate, and sodium starch gluconate are not expected to interfere since these compounds lack amine groups. More work to validate this analytical method for tablets is necessary. However the primary goal to characterize *p*-BQ as a chromogenic reagent for diaminopyrimidines has been met.

3.5. Flow injection analysis

The automation of the spectrophotometric procedure developed for the diaminopyrimidines was another challenge. To attain the maximum reasonable reaction time (4 min) possible in the reaction coil without excessive peak broadening, a flow gradient as described in Section 2 has to be used. The other reaction conditions such as temperature, solvent composition, and [*p*-BQ] are taken as optimized by the MultiSimplex experiment (Table 1). The high concentration of BQ in the running solvent is a major concern, because tube plugging and contamination of the detector are possible outcomes. Due likely to the shorter reaction time, the optimum absorbance is observed instead of 476 nm for the lower analyte concentrations (< 60 mg l^{-1}). At higher concentrations, the optimum wavelength shifts back again toward the longer wavelength. Examination of the results reveals that a linear range is only possible between 120 and 300 mg l^{-1} of TMP. The detection limit for TMP, PMA, and 2,4-DAP are 60, 70, and 20 mg l^{-1}, respectively. The better detection limit for 2,4-DAP may be attributed to the lack of steric hindrance on the pyrimidine ring of 2,4-DAP, which facilitates a faster reaction time.

4. Conclusion

We have shown that diaminopyrimidines react readily with *p*-BQ to form colored products with high molar absorbtivities of about 10 000 l mol^{-1} cm^{-1}. Because of the negative intercept, detection limits are only about 5 mg l^{-1}. Application of this method using fourth derivative spectrophotometry permitted the determination of TMP in the presence of SM. It is reasonable to speculate that SM based on Fig. 3 could be determined in the presence of TMP by reaction with *p*-BQ at a very acidic pH (< 1). Therefore this method shows promise as a simpler approach using just one reagent for the colorimetric determination of TMP and SM as compared to two completely different reactions as described previously [11]. Finally, this *p*-BQ prederivatization chemistry in conjunction with HPLC should permit the absorbance determination of both aliphatic and aromatic amines at a single visible wavelength. For example, ophthalmic solutions containing the peptide polymyxin B and TMP could likely be analyzed.

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