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# Validated Spectrophotometric Determination of Brinzolamide Using Two Charge Transfer Complexation Reactions.

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**ABSTRACT**

This work presents two simple and direct spectrophotometric methods for determination of the carbonic anhydrase inhibitor brinzolamide (BNZ) through charge transfer complexation reactions. This work is considered the first investigation for colorimetric methods for estimation of BNZ. Method I is based on reaction of the drug with p-chloranilic acid (p-CA) in acetonitrile to give a red colored product with maximum absorbance at 521 nm. Method II is based upon the interaction of BNZ and picric acid (PA) in chloroform resulting in the formation of a yellow complex measured at 407 nm. Factors affecting the color development were studied and optimized. Stoichiometry of the reactions was determined. The proposed colorimetric procedures were validated with respect to linearity, ranges, precision, accuracy, robustness, detection and quantification limits. Regression analysis for the calibration curves of the formed color products with p-CA and PA showed good linear relationships over the concentration ranges of 40–360 and 8–48 µg/mL respectively. The method was successfully applied to the assay of BNZ in its pharmaceutical preparation (ophthalmic suspension) with good accuracy and precision. Assay results were statistically compared to a reference pharmacopoeial HPLC method where no significant differences were observed between the proposed methods and reference method.

**Keywords:** Brinzolamide ; Spectrophotometric Determination ; Charge transfer complex ; p-Chloranilic acid ; Picric acid.

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## INTRODUCTION

Brinzolamide (BNZ) (Figure 1) is a carbonic anhydrase inhibitor. It is used to reduce elevated intra-ocular pressure in the management of open-angle glaucoma and ocular hypertension, either alone or as adjunctive therapy with a topical beta blocker [1]. Literature survey revealed few methods for the analysis of BNZ. The USP 2011 recommended reversed-phase HPLC methods for the assay of BNZ powder and ophthalmic suspension [2]. The simultaneous estimation of BNZ and timolol maleate was carried out using HPLC [3,4], HPTLC [4] and spectrophotometric methods [5]. On the other hand, derivative and derivative-ratio spectrophotometric methods were applied for determination of BNZ-brimonidine tartrate mixture [6]. Recently, determination of BNZ in dried blood spots was reported using HPLC-MS/MS method [7].

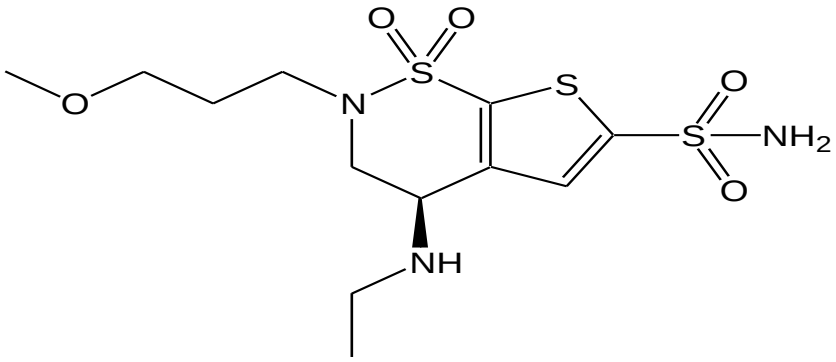


**Figure 1: Chemical structure of brinzolamide (BNZ).**

The interest to establish simple, fast, and adequately sensitive spectrophotometric methods to be suitable for routine analysis in control laboratories has been one of the main targets for analytical chemists. The formation of a charge transfer complex involves transfer of electronic charge from an "electron rich" molecule to an "electron deficient" molecule. As a result, one compound becomes partially positively charged with respect to the other and a weak electrostatic bond is formed. The molecular interactions between electron donors and electron acceptors are generally associated with the formation of intensely colored charge-transfer complexes, which absorb radiation in the visible region. Typically, the electron donor and electron acceptor combine in a 1:1 molar ratio to form the charge transfer complex. The rapid formation of charge transfer complexes leads to their utility in the development of simple and convenient spectrophotometric methods for numerous pharmaceutical compounds [8-13].

To the best of our knowledge, no reports could be found in the scientific literature for the application of charge transfer complexation in the spectrophotometric determination for BNZ; furthermore, only a couple of articles suggest spectrophotometric methods for BNZ and they depend merely on measurement in the UV region [5,6], i.e., there is no evidence for the application of colorimetric methods for the analysis of BNZ. The aim of this work is to develop simple, rapid, adequately sensitive and cost effective spectrophotometric methods to be suitable for routine analysis of BNZ in pure form and in pharmaceutical dosage form. The developed methods are based on the formation of intensely colored charge-transfer complexes with the chromogenic reagents: p-chloranilic acid (p-CA) and picric acid (PA).

## EXPERIMENTAL

### Instrumentation

Spectrophotometric measurements were performed using Shimadzu 1601 PC UV-VIS spectrophotometer with matched 1-cm quartz cells.

### Materials and Reagents

Pure sample of BNZ was kindly obtained from Pharco Pharmaceuticals Co., Alexandria, Egypt. Analytical grade of 2,5-dichloro-3,6-dihydroxy-1,4-benzoquinone (p-chloranilic acid, pCA) and 2,4,6-trinitrophenol (Picric acid, PA) (BDH Chemicals, Poole, UK), HPLC grade acetonitrile and isopropanol (Carbon Group, Ringaskiddy,

County Cork, Ireland), HPLC grade methanol (Lab-Scan Analytical Sciences, Gliwice, Poland), HPLC grade ethanol absolute (Scharlau Chemie S.A., Sentmenat, Spain), laboratory reagent grade chloroform (Fisher scientific, UK), analytical grade acetone (Tedia Company Inc., Fairfield, OH, USA) and sodium sulphate anhydrous (El-Nasr Pharmaceutical Chemicals Co., Egypt) were used. Pharmaceutical preparation assayed in the study was Azopt® ophthalmic suspension labeled to contain 10 mg BNZ per mL (manufactured by S.A. Alcon-Couvreur N.V., B-2870 Puurs, Belgium).

**Preparation of Standard and Reagents' Solutions**

Stock standard solutions of BNZ, 1000 µg/mL and 80 µg/mL, were separately prepared in acetonitrile and chloroform respectively. p-CA solution, 4000 µg/mL (4 mg/mL), was also prepared on acetonitrile while PA solution, 1000 µg/mL (1 mg/mL), was prepared in chloroform. All solutions were freshly prepared.

**General Procedures**

For the reaction with p-CA: aliquots of BNZ stock standard solution (1000 µg/mL) were transferred into a series of 25-mL volumetric flasks, treated with 2 mL of p-CA solution and the volume was completed with acetonitrile. The reaction mixtures were kept at room temperature for 5 min, and then absorbance was measured at 521 nm against reagent blank. For the reaction with PA: aliquots of BNZ stock standard solution (80 µg/mL) were transferred into a series of 5-mL volumetric flasks, treated with 2 mL of PA solution, and the volume was completed with chloroform and the absorbance was measured at 407 nm against reagent blank. In each method, absorbance values were plotted against the corresponding drug concentrations to construct the calibration graph.

**Assay of Pharmaceutical Dosage Form (Ophthalmic Suspension)**

Pharmaceutical preparation assayed in this study is Azopt® ophthalmic suspension labeled to contain 10 mg of BNZ per mL. Sample preparation includes mixing content of ten droppers. Then 5 mL of the mixture equivalent to 50 mg BNZ was transferred into 50 mL volumetric flask. The solution was diluted to volume with acetonitrile to reach a final concentration 1000 µg/mL for BNZ (stock sample solution for reaction with p-CA). Similarly, 5 mL of the mixture equivalent to 50 mg BNZ was transferred into 100 mL-volumetric flask followed by addition of 30 mL acetonitrile and sonication for about 10 minutes, the solution was diluted to volume with chloroform and filtered on anhydrous sodium sulphate. Finally 15 mL of filtrate was transferred to 100 mL volumetric flask and the solution was diluted to volume with chloroform to reach a concentration of 75 µg/mL BNZ (stock sample solution for reaction with PA).

Aliquots of the BNZ stock sample solutions were transferred into a series of 25-mL and 5-mL volumetric flasks, and the general procedures were then followed. Recovery values were calculated from similarly treated standard solutions. For standard addition assay, sample solutions were spiked with aliquots of stock standard solutions of BNZ to obtain final concentrations within the previously specified range then treated as under general procedures. Recovered concentrations were calculated by comparing the analyte response with the increment response attained after addition of the standard.

## RESULTS AND DISCUSSION

**Spectral characteristics and proposed mechanisms for the reactions**

The chemical structure of BNZ contains an aliphatic secondary amino group which can be exploited for the development of several colorimetric methods for analysis of BNZ. Being an electron donor, BNZ reacts with p-CA (as π-acceptor), giving characteristic deep purple colored product which exhibits an absorption maximum at 521 nm (Figure 2). Similarly, BNZ reacts with PA to yield a yellow product which exhibits an absorption spectrum with broad maximum at 407 nm (Figure 3). These colored products can be attributed to the formation of charge-transfer complexes between BNZ, as electron donor, and p-CA or PA, as π-acceptors, followed by subsequent dissociation of the donor-acceptor complexes to form the purple and the yellow radical anions of p-CA and PA respectively. These reactions can be demonstrated by the following scheme:

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$$D + A \rightleftharpoons D{-}A \rightleftharpoons D^{\bullet+} + A^{\bullet-}$$

Donor π-acceptor Complex Radical ions

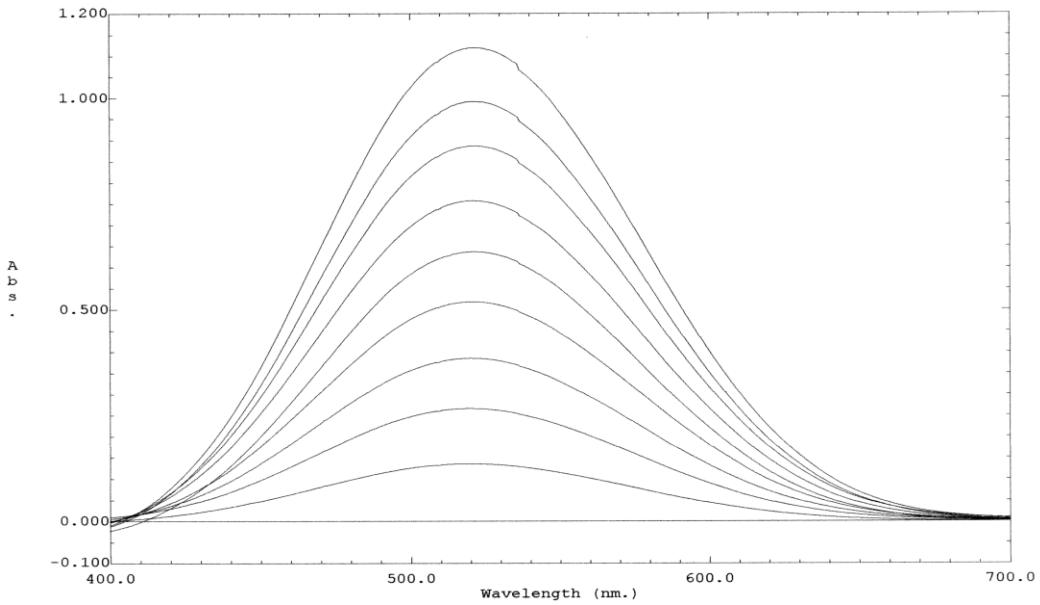


**Figure 2: Absorption spectra of the reaction product of different concentrations of BNZ (40, 80, 120, 160, 200, 240, 280, 320 and 360 µg/mL) with p-CA in acetonitrile.**

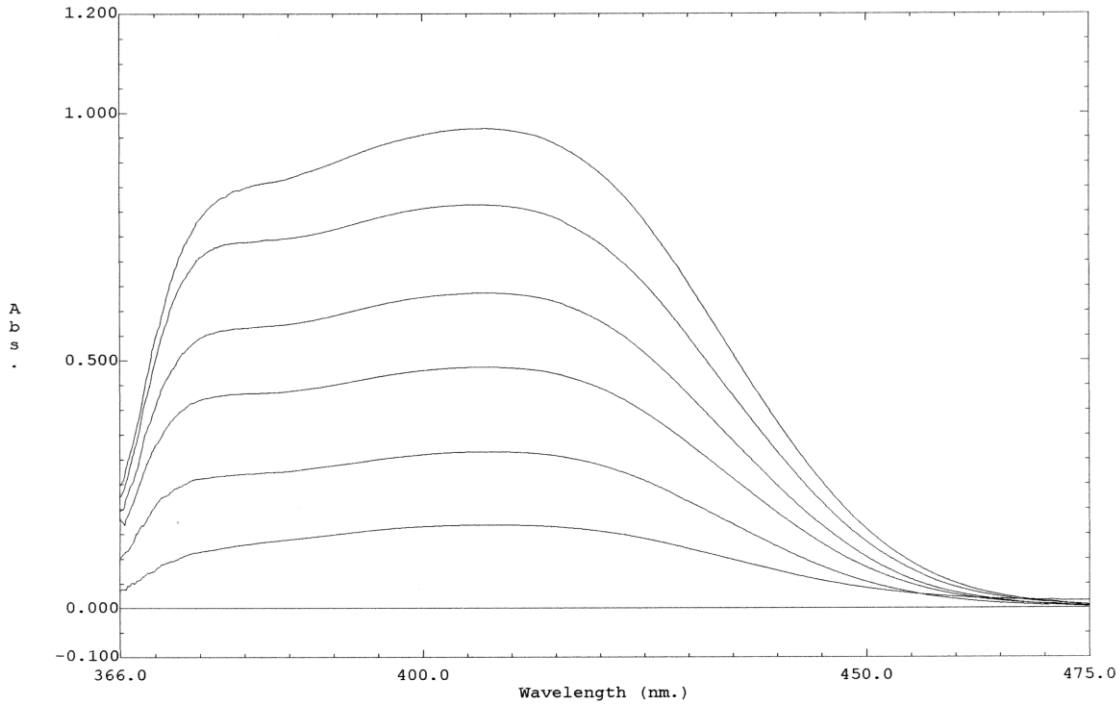


**Figure 3: Absorption spectra of the reaction product of different concentrations of BNZ (8, 16, 24, 32, 40 and 48 µg/mL) with PA in chloroform.**

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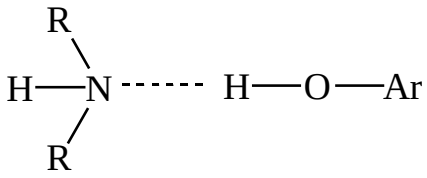
In addition, several previous reports suggest that the reactions of electron rich compounds with PA proceed through intermolecular hydrogen bonding [13-15]. This typically may occur between the basic center on BNZ (the secondary amino group) and the acidic center on PA (the phenolic group).




**Optimization of experimental conditions**

Different factors affecting the color development and its stability were carefully studied and optimized. Such factors were changed individually while keeping the others constant. These factors include reagent concentration, diluting solvent and reaction time. The effect of reagent concentration (in terms of reagent volume) on the absorbance values was tested. It was found that increasing the volume of both p-CA and PA solutions resulted in increasing the color intensity up to 2.0 mL, after which no more increase in absorbance was recorded. Chloroform is considered the ideal diluting solvent for the reaction with PA while in case of p-CA; the reaction was carried out in different organic solvents such as acetone, acetonitrile, ethanol, isopropanol and methanol. Small shifts in the position of the maximum absorption peak were observed, whereas the absorption intensities were clearly influenced. Acetonitrile was found to be the ideal solvent for the reaction with p-CA. The optimum reaction time was determined by monitoring the color development at room temperature (20±2 °C). Maximum color intensity was reached after 5 min with p-CA; accordingly, the reaction was allowed to take place for 5 min before recording the absorbance. In case of PA, complete color development was attained instantaneously and increasing the reaction time did not show any further increase in absorbance. Therefore, the absorbance readings were taken at zero time.

**Stoichiometry of the reactions**

The stoichiometry of the reactions was studied by Job's method of continuous variation [16] using equimolar concentrations of the drug and reagents (0.012 M and $2\times10^{-3}$ M for reactions with p-CA and PA respectively). Job's plot reached a maximum value at a mole fraction of 0.5 for both reactions indicating a molar reaction ratio of 1:1 between the drug and the tested π acceptors (Figure 4).

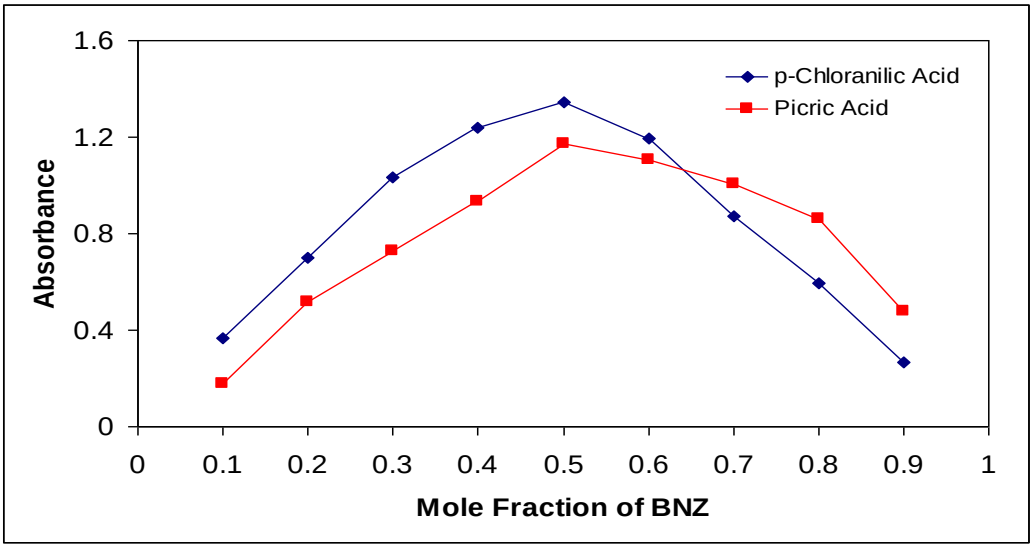



**Figure 4: Continuous variation (Job's method) plots for the reactions of BNZ with p-CA and PA.**

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**Validation of the proposed methods**

***Linearity and concentration range***

Under the optimal experimental spectrophotometric conditions, a linear relationship exists between absorbance readings of each reaction product and the corresponding concentrations of BNZ. Table 1 presents the linearity data and statistical parameters for the studied methods including linear regression equations, concentration ranges, correlation coefficients, molar absorptivity values, standard deviations of the intercept ($S_a$), the slope ($S_b$) and standard deviation of residuals ($S_{y/x}$). Regression analysis shows good linearity as revealed from the correlation coefficient values (r > 0.9997) and RSD% of the slope values which were found less than 1.2 %. The analysis of variance test for the regression lines reveals that, for equal degrees of freedom, an increase in the variance ratio (F values) means an increase in the mean of squares due to regression and a decrease in the mean of squares due to residuals. The greater the mean of squares due to regression, the steeper is the regression line. The smaller the mean of squares due to residuals, the less is the scatter of experimental points around the regression line. Consequently, regression lines with high F values (low significance F) are much better than those with lower ones. Good regression lines show high values for both r and F statistical parameters [17].

***Limits of detection and quantification***

In accordance with the ICH guidelines on validation of analytical procedures [18], the limit of detection, LOD = 3.3 σ/s, where σ is the standard deviation of the intercept of the regression line and s is the sensitivity, namely the slope of the calibration curve. On the other hand, the limit of quantification (LOQ) is defined as 10 σ/s. The LOD and LOQ values were calculated and presented in Table 1.

**Table 1: Analytical parameters for the determination of BNZ using the proposed charge transfer spectrophotometric methods.**

| Parameter | p-CA | PA |
|---|---|---|
| Wavelength (nm) | 521 | 407 |
| Linearity range (µg/mL) | 40 – 360 | 8 – 48 |
| Molar absorptivity (ε) (L $mol^{-1}$ $cm^{-1}$) | 1170 | 7735 |
| Intercept (a) | 0.0255 | -0.0003 |
| Slope (b) | 0.00305 | 0.02017 |
| Correlation coefficient ( r ) | 0.99980 | 0.99975 |
| $S_a$ | 0.00587 | 0.00709 |
| $S_b$ | $2.47 \times 10^{-5}$ | $2.28 \times 10^{-4}$ |
| RSD% of slope | 0.81 | 1.13 |
| $S_{y/x}$ | 0.00639 | 0.00762 |
| F | 15262 | 7856 |
| Significance F | $1.90 \times 10^{-11}$ | $9.71 \times 10^{-8}$ |
| LOD (µg/mL) | 6.35 | 1.16 |
| LOQ (µg/mL) | 19.25 | 3.52 |

***Accuracy and precision***

The accuracy and within-day (intra-day) precision for the proposed procedures were studied at four concentration levels for BNZ using three replicate determinations for each concentration within one day. Similarly, the accuracy and between-day (inter-day) precision were tested by analyzing the same concentrations using three replicate determinations repeated on three days. The recovered concentrations were calculated using the corresponding regression equations and were found to be satisfactory. The percentage relative standard deviation (RSD %) values were less than 1.75 % and the percentage relative error ($E_r$ %) values were less than 1.9 % proving the high precision and accuracy of the developed methods for the estimation of BNZ in bulk form (Table 2).

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**Table 2: Accuracy and precision for the analysis of BNZ in bulk form using the proposed charge transfer spectrophotometric methods.**

| Reagent | Parameter | Nominal value (µg/mL) | Found ± SD[a] (µg/mL) | RSD(%)[b] | $E_r$(%)[c] |
|---|---|---|---|---|---|
| p-CA | Within-day | 80 | 78.62 ± 0.65 | 0.83 | -1.72 |
| | | 120 | 118.39 ± 1.12 | 0.95 | -1.34 |
| | | 200 | 202.49 ± 1.77 | 0.87 | 1.25 |
| | | 300 | 301.85 ± 2.83 | 0.94 | 0.62 |
| | Between-day | 80 | 78.86 ± 0.62 | 0.79 | -1.42 |
| | | 120 | 117.75 ± 1.27 | 1.08 | -1.87 |
| | | 200 | 202.18 ± 2.23 | 1.10 | 1.09 |
| | | 300 | 300.53 ± 2.06 | 0.69 | 0.18 |
| PA | Within-day | 16 | 15.81 ± 0.17 | 1.08 | -1.19 |
| | | 24 | 24.11 ± 0.04 | 0.17 | 0.46 |
| | | 32 | 31.84 ± 0.16 | 0.50 | -0.50 |
| | | 40 | 40.00 ± 0.22 | 0.55 | 0.00 |
| | Between-day | 16 | 15.87 ± 0.27 | 1.70 | -0.81 |
| | | 24 | 23.86 ± 0.24 | 1.01 | -0.58 |
| | | 32 | 31.60 ± 0.35 | 1.11 | -1.25 |
| | | 40 | 39.59 ± 0.29 | 0.73 | -1.02 |

[a] Mean ± standard deviation for three determinations.
[b] % Relative standard deviation.
[c] % Relative error.

***Robustness***

Robustness was examined by making small variations in the working wavelengths (± 3 nm) and volume of reagents (± 10 %) then examining the results. These variations did not have any significant effect on the measured absorbance values of the reaction products. RSD% of the measured absorbance for the studied variations did not exceed 2%. Table 3 shows the effect of the studied variations on the absorbance readings of the reaction products.

**Table 3: Study of robustness of the proposed spectrophotometric methods.**

| Reagent | Parameter | Absorbance |
|---|---|---|
| p-CA | **Reagent volume (mL)** | |
| | 1.8 | 0.6218 |
| | 2.0 | 0.6400 |
| | 2.2 | 0.6377 |
| | **RSD%** | **1.57** |
| | **Working wavelength (nm)** | |
| | 518 | 0.6389 |
| | 521 | 0.6400 |
| | 524 | 0.6387 |
| | **RSD%** | **0.11** |
| PA | **Reagent volume (mL)** | |
| | 1.8 | 0.6321 |
| | 2.0 | 0.6421 |
| | 2.2 | 0.6359 |
| | **RSD%** | **0.79** |
| | **Working wavelength (nm)** | |
| | 404 | 0.6420 |
| | 407 | 0.6421 |
| | 410 | 0.6346 |
| | **RSD%** | **0.67** |

***Stability of solutions***

The stability of the colored products at room temperature was examined. No significant spectrophotometric changes were observed within 30 min after measurement. Also, the stock standard solutions of BNZ were stable for at least 3 days when stored refrigerated at 4 ºC.

**Assay of BNZ ophthalmic suspension**

The proposed colorimetric methods were applied to the determination of BNZ in Azopt® ophthalmic suspension. The assay results revealed satisfactory accuracy and precision as indicated from % recovery, SD and RSD% values (Table 4). No interference could be observed from the common excipients. A previous study described the spectrophotometric determination of the commonly used preservative benzalkonium chloride with PA in the concentration range 2-8 µg/mL [19]. However, benzalkonium chloride did not show any interference in the reaction with PA in our study. The reason for this is that the cited preservative is usually added in ophthalmic preparations in concentrations not more than 0.02%, accordingly, it will be diluted during sample preparation to concentrations far below its linearity range required to react with PA.

The USP reference HPLC method [2] was applied for the estimation of BNZ in its commercial product. The results of the proposed methods were statistically compared with those of the reference method using the one-way analysis of variance test (Single factor ANOVA) [20]. The calculated F-value did not exceed the critical value, indicating that there were no significant differences between the proposed methods together with the reference method (Table 4). Moreover, the proposed spectrophotometric methods were employed for the assay of BNZ dosage form using the standard addition technique. Again, the proposed methods proved to be successful, accurate and precise for the determination of the drug in its formulation (Table 4).

**Table 4: Analysis of BNZ in Azopt® Ophthalmic Suspension using the proposed spectrophotometric methods and the reference method.**

| Using external standard analysis | | | |
|---|---|---|---|
| **Parameters** | **p-CA** | **PA** | **Reference method** |
| **%Recovery ± SD[a]** | 101.29 ± 0.55 | 100.27 ± 1.28 | 101.78 ± 0.61 |
| **RSD(%)[b]** | 0.54 | 1.28 | 0.60 |

**ANOVA (Single Factor)**

| Source of Variation | SS | df | MS | **F** | P-value | **F (critical)** |
|---|---|---|---|---|---|---|
| Between Groups | 3.544609 | 2 | 1.772304 | **2.749403** | 0.104001 | **3.885294** |
| Within Groups | 7.735373 | 12 | 0.644614 | | | |
| Total | 11.279982 | 14 | | | | |

| Using standard addition analysis | | |
|---|---|---|
| **Parameters** | **p-CA** | **PA** |
| **%Recovery ± SD[a]** | 99.84 ± 0.42 | 100.94 ± 1.81 |
| **RSD(%)[b]** | 0.42 | 1.79 |

[a] Mean ± standard deviation for five determinations.
[b] % Relative standard deviation.

## CONCLUSION

This study described two validated spectrophotometric methods based on charge transfer complexation for the determination of BNZ in pure form as well as in ophthalmic suspension dosage form. The proposed methods are simple, direct, inexpensive and reproducible. Obviously, better sensitivity was achieved using picric acid as reagent as indicated from calibration data, molar absorptivity and LOD values. To the best of our knowledge, we could not find any articles in the literature dealing with the application of charge transfer reactions for the quantification of BNZ; moreover, this is the first investigation for a colorimetric assay for the drug. The proposed methods have the advantage that measurements are performed in the visible region, away from any possible interfering UV-absorbing excipients that might be co-extracted from BNZ dosage form.

The statistical parameters and the recovery data reveal good accuracy and precision of the methods. The developed methods do not require elaborate treatment or sophisticated experimental setup usually associated with HPLC methods of analysis. The developed methods used only a spectrophotometer, which is available in all quality control laboratories therefore they can be considered economic, useful and convenient for the routine and quality control assay of the drug in bulk form and in pharmaceutical formulations.

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