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# Spectrophotometric Determination of 6-Aminopenicillanic Acid Using Bromophenol Blue and Bromothymol Blue

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**Abstract.** Two simple, selective and sensitive spectrophotometric methods are described for the determination of 6-aminopenicillanic acid (6-APA). The methods are based on the reaction of 6-APA with either bromophenol blue (BPB) or bromothymol blue (BTB), to give orange-red and green species, respectively. The coloured products are quantified spectrophotometrically at 625 and 616 nm for BPB and BTB, respectively. The optimization of the different experimental conditions is described. No interferences from different $\beta$-lactams and common degradation products were observed in the determination of 6-APA using BTB, while flucloxacillin, dicloxacillin, adrenaline, vitamin C, urea and common degradation products in any percentage interfere on using BPB only. The BTB method was better than the BPB method, because of its wider range of determination (0.4–20 $\mu g\,ml^{-1}$ vs. 0.4–7.2 $\mu g\,ml^{-1}$ on using BPB), higher molar absorptivity and Sandell sensitivity ($3.27 \times 10^3\,l\,mol^{-1}\,cm^{-1}$ and $0.099\,\mu g\,cm^{-2}$ vs. $2.82 \times 10^3\,l\,mol^{-1}\,cm^{-1}$ and $0.115\,\mu g\,cm^{-2}$), greater stability (3 and 10 days on using BTB and BPB, respectively) and better selectivity. The results were compared with those given by the Official United States Pharmacopeial XXI method.

**Key words:** 6-aminopenicillanic acid, bromophenol blue, bromothymol blue, spectrophotometry.

Bromocresol purple is known to yield charge transfer complexes which are applied for the determination of heparin [1] and erythromycin [2]. Bromocresol green is also known to yield a charge transfer complex, which is applied in the determination of phenytoin sodium [3].

The importance of penicillins as broad spectrum antibiotics is well known. 6-aminopenicillanic acid [551-16-6] is one of the penicillin group of antibiotic

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drugs. Several spectrophotometric and potentiometric procedures for the determination of 6-APA have been reported. The ultraviolet and visible methods are mainly indirect [4–9], since the penicillin nucleus itself has no absorption maximum in these ranges. Potentiometric studies on complex formation of $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ with 6-APA have been reported [10]. In the present work, the application of the $\pi$-acceptors BPB and BTB to the spectrophotometric determination of 6-APA in pure authentic samples is described.

## Experimental

*Apparatus*

A Perkin-Elmer Lambda 3B spectrophotometer with matched 1-cm quartz cells was used for absorbance measurments and an Orion Research Model 601A/digital Ionalyzer pH-meter was used to check the pH of the acetate buffer solutions.

*Materials*

Bromophenol blue (BPB) and bromothymol blue (BTB) from Aldrich were used without further purification. $2 \times 10^{-3} M$ BPB and BTB solutions were prepared by dissolving the appropriate weights in 100 ml of dioxane and acetone, respectively.

A 100 mg $l^{-1}$ solution of 6-APA (Aldrich) were prepared by dissolving 0.025 g of pure sample in 5 ml of methanol and diluting to 250 ml with water.

Acetate buffer solution of pH 1.09–6.9 and universal buffer of pH 2.1–8.6 were prepared as previously recommended [11]. Doubly distilled water was used throughout.

*General Procedure*

*BPB method.* Place 0.1–1.8 ml of 6-APA solution in a 25-ml measuring flask, add 2.5 ml of BPB solution in dioxane, 2.5 ml of dioxane to achieve 20% (v/v) dioxane and 15 ml of acetate buffer solution of pH 4.3, and then complete to the mark with water. Allow to stand at 20–25 °C for about 5 min and measure the absorbance of the orange-red complex at 625 nm against a reagent blank.

*BTB method.* Place 0.1–5 ml of 6-APA solution in a 25-ml measuring flask, add 2.5 ml of $2 \times 10^{-3} M$ BTB solution, 5 ml of acetone to achieve 30% (v/v) acetone and 12.5 ml of acetate buffer solution of pH 5.6, and then complete to the mark with water. Allow to stand at 20–25 °C for about 20 min, then measure the absorbance of the green complex at 616 nm against a reagent blank.

## Results

Several parameters such as pH, amount of buffer added, sequence of addition, reagent concentration, temperature and time were optimized to achieve high sensitivity, low blank readings and high stability.

### *Effect of pH*

In a trial to elucidate the optimum medium for the quantitative determination of 6-APA in aqueous solution, acetate and universal buffer of different pH values were examined. The absorbance readings were maximal with pH 4.3 acetate buffer for BPB and at pH 5.6 and 7.0 for BTB using both acetate and universal buffers. However, acetate buffers of pH 4.3 and 5.6 were used with BPB and BTB,

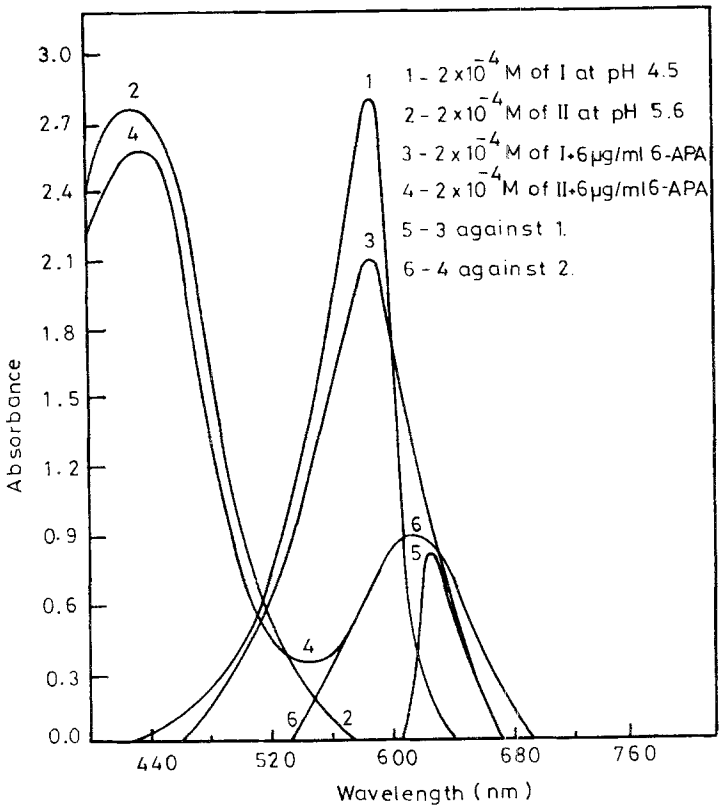


**Fig. 1.** Absorption spectra of 6-APA complexes with BPB (I) and BTB (II)

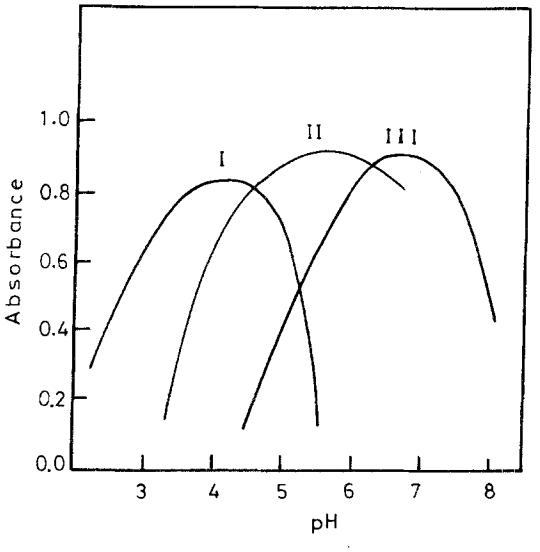


**Fig. 2.** *I* BPB complex in both acetate and universal buffers. *II* BTB complex in acetate buffer. *III* BTB complex in universal buffer. [6-APA] = 150 µg/25 ml and [BPB] or [BTB] = $2 \times 10^{-4} M$

respectively, since the universal buffer affected the colour stability. The amount of buffer added per 25 ml of solution was also investigated and it was found that 15 ml gave marginally the highest absorbance in the case of BPB, whereas for BTB 12.5 ml gave the highest absorbance.

*Effect of Sequence of Addition*

The most favourable sequence is 6-APA – reagent – buffer for the highest colour intensity and the least time for developing maximum absorbance. All other sequences needed longer times and gave lower absorbances.

*Effect of Reagent Concentration*

The effects of BPB and BTB were investigated by taking various amounts of each. 2.5 ml of $2 \times 10^{-3}$ *M* was sufficient in each case to produce maximum absorbance in the reaction with 6-APA, as recorded in Table 1.

*Effect of Time and Temperature*

The optimum reaction time was determined by following the colour development at ambient temperature (20–25 °C). Complete colour intensity was attained after 5 and 20 min for BPB and BTB, respectively, with 6-APA.

The colour of the BPB complex remained stable for 3 days, then the absorbance decreased gradually with a blue shift in $\lambda_{max}$ until the colour returned to that of BPB after 10 days. Using BTB, the absorbance remained stable for more than 10 days without change. No change in the absorbance occurred on increasing the temperature up to 65 °C, above which the absorbance began to fade slowly.

*Effect of Solvent*

As an assay solvent, dioxane afforded maximum sensitivity with BPB whereas acetone was found to be the best for BTB. For the other solvents tested (methanol,

**Table 1.** Effects of BPB and BTB concentration

| | Reagent used | |
|---|---|---|
| Material | BPB | BTB |
| 6-APA | 1.5 ml of 100 mg $l^{-1}$ | 2.5 ml of 100 mg $l^{-1}$ |
| Acetate buffer | 15 ml of pH 4.3 | 12.5 ml of pH 5.6 |
| Time (min) | 5 | 20 |
| Temperature (°C) | 20–25 | 20–25 |
| $\lambda_{max}$ (nm) | 625 | 616 |
| Reagent volume[a] (ml) | *A* | *A* |
| 0.5 | 0.22 | 0.30 |
| 1.0 | 0.37 | 0.57 |
| 1.5 | 0.54 | 0.75 |
| 2.0 | 0.70 | 0.86 |
| 2.5 | 0.72 | 0.87 |
| 3.0 | 0.73 | 0.87 |
| 3.5 | 0.73 | 0.88 |
| 4.0 | 0.74 | 0.88 |

[a] $2 \times 10^{-3}$ *M*.

ethanol, propan-1-ol, and dimethylformamide), the colour development took longer times to achieve the same $\lambda_{max}$ and with lower absorbances than with the best solvent. In addition, it required longer heating times to form the complexes in a water bath at 40–50 °C.

*Nature of the Complexes*

The stoichiometries of the complexes formed between 6-APA and BPB or BTB were investigated at pH 4.3 or 5.6, respectively, by the molar ratio [12] and continuous variation [13] methods. The results indicated the existence of 1:1 complexes for both BPB and BTB.

*Analytical Data*

Beer's law limits, molar absorptivities, regression equations, standard deviations and correlation coefficients obtained by linear least squares treatment of the results are given in Table 2. For more accurate analysis, Ringbom [14] optimum concentration ranges were evaluated, as shown in Table 2. The performance of the present method was assessed by comparison with the pharmacopoeial method [16]. Mean values obtained in Student's *t*- and *F*-tests [15] showed the absence of any systematic error in the method (Table 2).

*Sensitivity, Accuracy and Precision*

The mean Sandell sensitivities as calculated from Beer's law are presented in Table 2. In order to determine the accuracy and precision of the methods, solutions containing three different concentration of 6-APA were prepared and

**Table 2.** Quantitative parameters for the reaction of 6-APA with BPB and BTB

| Parameters | BPB | BTB |
|---|---|---|
| Beer's law limit, $\mu g\,ml^{-1}$ | 0.4–7.2 | 0.4–20 |
| Molar absorptivity, $1\,mol^{-1}\,cm^{-1}$ | $2.82 \times 10^3$ | $3.27 \times 10^3$ |
| Sandell sensitivity, $\mu g\,cm^{-2}/0.001\,A$ | 0.115 | 0.099 |
| Regression equation[a] | | |
| Slope (*b*) | 0.53 | 0.68 |
| Intercept (*a*) | 0.027 | 0.018 |
| Correlation coefficient (*r*) | 0.9986 | 0.9994 |
| Standard deviation, % | 0.7 | 0.55 |
| Range of error, % | ±0.60 | ±0.33 |
| Student $t$[b] (2.310)[c] | 1.728 | 1.473 |
| $F$[b] (2.450)[c] | 1.573 | 1.286 |
| Ringbom optimum range, $\mu g\,ml^{-1}$ | 1–6 | 1–17 |

[a] $A = a + bc$ where $c$ is the concentration in $\mu g\,ml^{-1}$.

[b] Comparison with pharamacopoeial method [16].

[c] Values in parenthesis are the theoretical *t*- and *F*-values for five degrees of .. freedom at $P = 0.05$.

analysed in quintuplicate. The standard deviations and relative standard deviations (Table 3) can be considered satisfactory, at least for the level of concentrations examined.

*Interferences*

No interferences (< 2% is considered non-interferent) were observed in the determination of 6-APA with BPB or BTB from the presence of amoxycillin, ampicillin, cloxacillin, penicillin G, penicillin V, streptomycin sulphate, neomycin sulphate, phenylacetic acid, phenylacetamide, nicotinamide, ephedrine hydrochloride, glucose, fructose and starch when present in 50-fold molar excess, whereas flucloxacillin, dicloxacillin, adrenaline, vitamin C and urea interfered when present in any percentage on using BPB only. Also there was no interference from penicilloic acid, penilloic acid, penillic acid, penaldic acid, benzylpenicilloic acid and ultimately penilloaldehyde, which are the degradation products of 6-APA resulting from thermal and hydrolytic degradation, on the determination of 6-APA using BTB, whereas on using BPB interference occurred.

*Analysis of Authentic Samples*

Synthetic mixtures were prepared from pure standard materials representing the actual situation in several formulations (due to the absence of such drug formulations in the local market in Egypt) and analysed by the general method. The results are listed in Table 4 and show that the proposed methods are accurate and precise.

## Discussion

It is clear that BTB and BPB are highly superior compared with other reagents [4–9], with very rapid formation of the chromophores (5 and 20 min for BPB and BTB, respectively in the cold) and high stability of colour (3 and 10 days for BPB and BTB, respectively) in addition to small levels of interference. BTB is better than BPB in the determination of 6-APA due to the wider range of determination,

**Table 3.** Evaluation of accuracy and precision of the proposed method

| Reagent | 6-APA (μg/25 ml) Added | Found[a] | S.D. | RSD (%) | Confidence limits ($P = 0.05; n = 5$) |
|---|---|---|---|---|---|
| BPB | 75 | 74.7 | 0.06 | 0.55 | 0.07 |
| | 125 | 125.4 | 0.08 | 0.39 | 0.094 |
| | 175 | 174.0 | 0.12 | 0.67 | 0.014 |
| | mean | | | 0.54 | |
| BTB | 140 | 139.5 | 0.03 | 0.54 | 0.035 |
| | 280 | 281.0 | 0.05 | 0.28 | 0.06 |
| | 440 | 441.5 | 0.06 | 0.59 | 0.07 |
| | mean | | | 0.43 | |

[a] Average of five determinations.

**Table 4.** Analysis of authentic sample of 6-APA by the proposed and official methods

| | BTB method | | | BPB method | | |
|---|---|---|---|---|---|---|
| Taken ($\mu g\,ml^{-1}$) | Found ($\mu g\,ml^{-1}$) | Recovery (%) | SD[a] (%) | Found ($\mu g\,ml^{-1}$) | Recovery (%) | SD[a] (%) |
| 2.0 | 2.00 | 100.0 | 0.07 | 2.01 | 100.5 | 0.26 |
| 3.8 | 3.81 | 100.3 | 0.11 | 3.78 | 99.5 | 0.33 |
| 5.4 | 5.38 | 99.6 | 0.25 | 5.43 | 100.5 | 0.37 |
| 7.0 | 7.02 | 100.3 | 0.18 | 6.94 | 99.1 | 0.52 |
| 8.8 | 8.77 | 99.7 | 0.31 | | | |
| 10.4 | 10.44 | 100.4 | 0.24 | | | |
| 12.0 | 12.00 | 100.0 | 0.04 | | | |
| 13.6 | 13.55 | 99.6 | 0.31 | | | |
| 15.4 | 15.35 | 99.7 | 0.27 | | | |
| 17.0 | 17.05 | 100.3 | 0.21 | | | |
| 18.6 | 18.55 | 99.7 | 0.39 | | | |
| 20.0 | 19.90 | 99.5 | 0.33 | | | |

| | Official method [16] | | |
|---|---|---|---|
| Taken ($\mu g\,ml^{-1}$) | Found ($\mu g\,ml^{-1}$) | Recovery (%) | SD[a] (%) |
| 2.0 | 1.98 | 99.0 | 0.29 |
| 3.8 | 3.83 | 100.8 | 0.35 |
| 5.4 | 5.44 | 100.7 | 0.41 |
| 7.0 | 6.95 | 99.3 | 0.28 |
| 8.8 | 8.85 | 100.6 | 0.22 |
| 10.4 | 10.50 | 101.0 | 0.48 |
| 12.0 | 11.90 | 99.2 | 0.51 |
| 13.6 | 13.70 | 100.7 | 0.34 |
| 15.4 | 15.25 | 99.0 | 0.43 |
| 17.0 | 16.90 | 99.4 | 0.23 |
| 18.6 | 18.70 | 100.5 | 0.36 |
| 20.0 | 20.20 | 101.0 | 0.62 |

[a] $n = 5$.

higher molar absorptivity and Sandell sensitivity, stability and lower interference levels. Compared with the official method [16] (procedure for iodometric assay), the proposed methods are more sensitive and in good agreement for accuracy ($t$-test) and reproducibility ($F$-test). The present method is more accurate, with low errors amounting to $\pm 0.60$ and 0.33% using BPB and BTB, respectively, compared with 8% for 1,3-dibromo-5,5-dimethyldantoin [17].

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