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Original Article

# VALIDATED SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF CHLORAMPHENICOL IN PURE AND IN ITS DOSAGE FORM

**P. SUGUNA*, B. SATHYANARAYANA, N. V. S. NAIDU**

**Department of Chemistry, S. V. University, Tirupati 517502, A. P., India**
**Email: pydalasuguna@gmail.com**

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**ABSTRACT**

**Objective:** A simple, economic, selective, precise, and accurate UV-Visible spectrophotometric method for the analysis of Chloramphenicol in bulk drug and pharmaceutical formulations was developed and validated in the present study.

**Methods:** Based on oxidative coupling reaction with MBTH reagent at $P^{H}$-4.5, which is extractable at 620 nm. The Beer's law is obeyed in the concentration range 1-6 ml (10-60 µg $ml^{-1}$).

**Results:** The RSD was found to be 0.0194%, and recovery is 99.73%. The method was completely validated and proven to be rugged. The interferences of the ingredients and recipients were not observed. The repeatability and the performance of the proved method were established by point and internal hypothesis and through recovery studies.

**Conclusion:** The method was found to be accurate and precise, as indicated by recovery studies close to 100 and % RSD is not more than 2. The summary of validation parameters of proposed UV-Visible method is given.

**Keywords:** Spectrophotometry, Chloramphenicol, MBTH, Oxidative coupling



## INTRODUCTION

Several analytical methods have been reported for the determination of Chloramphenicol in various samples, such as Chloramphenicol (CAP) is 2,2 dichloro-N-[(1R,2R)-2-hydroxy-1-hydroxymethyl-2-(4-nitrophenyl)ethyl]acetamide, C11H12Cl2N2O5, whereas its chemical structure is: Its molecular weight is 323.1 g mol-1, It is a white, grayish-white or yellowish-white, fine crystalline powder or fine crystals, needles or elongated plates, freely soluble in methanol, ethanol, butanol, ethyl acetate, acetone, and in propylene glycol, slightly soluble in water, and ether, insoluble in benzene, and petroleum ether, it melts at 150.5–151.5 °C [1].

Chloramphenicol is a bacteriostatic antimicrobial. It is considered a prototypical broad-spectrum antibiotic, alongside the tetracycline. Chloramphenicol is effective against a wide variety of Gram-positive and Gram-negative bacteria, including most anaerobic organisms. It is widely used because it is inexpensive and readily available [2]. The most serious adverse effect associated with chloramphenicol treatment is bone marrow toxicity, which may occur in two distinct forms: bone marrow suppression, which is a direct toxic effect of the drug and is usually reversible, and aplastic anemia, which is idiosyncratic (rare, unpredictable, and unrelated to dose) and generally fatal. CAP is a non-irritant and is used by the local application for the treatment of a variety of infections of the skin, ear, and eye including trachoma [3]. Various methods have been reported for the determination of CAP in pharmaceutical preparations, including HPLC [4], LC-Mass spectrometry [5-7], Polarographic [8], electro generated chemiluminescence [9], Fluorescent [10], enzymatic method [11], colorimetric and spectrophotometric methods [12-19]. The literature is still poor in analytical procedures based on kinetics, especially for drugs in pharmaceuticals or biological fluids. However, some specific advantages in the application of kinetic methods can be expected such as, selectivity due to the measurement of the evolution of the absorbance with the time of the reaction instead of the measurement of absorbance value. Potassium permanganate has frequently been utilized for kinetic measurements in the field of pharmaceutical analysis. Many pharmaceutical compounds have been determined kinetically through this approach such as tetracycline hydrochloride [20], cephalosporins [21]. A norfloxacin [22] was determined by its reaction with acetaldehyde and 2,3,5,6–tetrachloro–1, 4-benzoquinone to give a colored product. Ketoprofen [23] was determined kinetically by oxidative coupling reaction of the drug with Hind S. Al-Ward2 3MBTH reagent in the presence of Ce (IV) in acidic medium. Ramipril has also determined kinetically based on the reaction of the carboxylic group of the drug with a mixture of potassium iodate and potassium iodide and the reaction was followed spectrophotometrically [24]. The empirical formula for Chloramphenicol is $C_{11}H_{12}Cl_2N_2O_5$ and the molecular weight is 323.13 grams. It has the following structure.

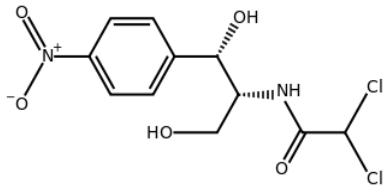


**Fig. 1: Chemical structure of chloramphenicol**

There is, however, no reported UV-Visible spectrophotometric method for the analysis of Chloramphenicol in its technical grade and formulations. In the present study, an attempt has been made to develop a simple UV-visible spectrophotometric method for the quantitative determination of Chloramphenicol. Functional group used for color development of Chloramphenicol was primary amine group. The results obtain in this method was based on oxidative coupling reaction with MBTH.

An attempt has been made to develop and validate the method to ensure their accuracy, precision, repeatability, reproducibility and other analytical method validation parameters as mentioned in the various guidelines

## MATERIALS AND METHODS

### Pure sample

The pure sample was collected from CIPLA pharmaceuticals. Avalahalli, Vigro agar, Bangalore, 560049.

**Preparation of standard stock solution**

Accurately weighed 100 mg of Chloramphenicol was dissolved in 40 ml of methanol in 100 ml volumetric flask and volume was made up to the mark with methanol. i.e. 1000 µg $ml^{-1}$ (Stock solution A)

From the above stock solution, A 10 ml of solution was pipetted out into 100 ml volumetric flask and the volume was made up to the mark with methanol to obtain the final concentration of 100 µg $ml^{-1}$ (Stock solution B)

**Preparation of calibration curve**

Fresh aliquots of Chloramphenicol ranging from 1 to 6 ml were transferred into a series of 10 ml volumetric flasks to provide final concentration range of 10 to 60 µg/ml. To each flask, 1.5 ml of (0.2%) MBTH solution was added followed by 2 ml of (0.7%) Ferric chloride solution and resulting solution was heated for 15 min and finally 1 ml (0.5N) HCl solution was added. The solutions were cooled at room temperature and made up to mark with distilled water. The absorbance of Green colored chromogen was measured at 620 nm against the reagent blank. The color species was stable for 24 h. The amount of Chloramphenicol present in the sample solution was computed from its calibration curve.

**Procedure for formulations**

Twenty tablets containing Chloramphenicol were weighed and finely powdered. An accurately weighed portion of the powder equivalent to 100 mg of Chloramphenicol was dissolved in a 100 ml of methanol and mixed for about 5 min and then filtered. The methanol was evaporated to dryness. The remaining portion of solution was diluted in a 100 ml volumetric flask to the volume with methanol up to 100 ml to get the stock solution A. 10 ml of aliquots was pipetted out into 100 ml volumetric flask and the volume was made up to the mark with methanol to obtain the final concentration of 100 µg $ml^{-1}$ (Stock solution).

Subsequent dilutions of this solution were made with methanol to get concentration of 10 to 60 µg $ml^{-1}$and were prepared as above and analyzed at the selected wavelength, 620 nm and the results were statistically validated

**Procedure for blood sample**

After collection of a blood sample, it will be centrifuged. For isolation of Chloramphenicol from a plasma sample, Methanol was used for protein precipitation. Liquid-Liquid extraction was performed with plasma by alkalization with 1M NaOH, followed by extraction with 30% dichloromethane in Hexane. The upper organic layer was evaporated to dryness, and reaming dry residue 100 mg was dissolved in 100 ml of Methanol (1000 µg $ml^{-1}$). From the above solution, 10 ml is taken into a 100 ml of volumetric flask and made up to the mark with methanol (100 µg $ml^{-1}$). From the above solution ranging from 0.4-2.4 (4-24 µg $ml^{-1}$) were transferred into 10 ml volumetric flask and to the each flask 1.5 ml of (0.2%) MBTH solution was added followed by 2 ml of (0.7%) Ferric chloride solution and made up to the mark with methanol. Then the resulting solution was heated for 15 min and finally 1 ml (0.5N) HCl solution was added. The solutions were cooled at room temperature and made up to the mark with distilled water. The absorbance of Green colored chromogen was measured at 620 nm against the reagent blank. The color species was stable for 24 h. The amount of Chloramphenicol present in the sample solution was computed from its calibration curve.

**RESULTS AND DISCUSSION**

**Validation reports**

**Optical parameters**

In order to ascertain the optimum wavelength of maximum absorption ($\lambda_{max}$) formed in UV-visible spectrophotometric method (Reference method–A) and of the colored species formed in each so the four visible spectrophotometric methods, specified amount of Chloramphenicol in final solution 10 µg $ml^{-1}$(method A), 10 µg $ml^{-1}$ for this method were taken and the colors were developed following the above mentioned procedures individually. The absorption spectra were scanned on spectrophotometer in the wavelength region of 200-400 nm (for method A) and 380-800 nm (for this Method) against corresponding reagent blanks. The reagent blank absorption spectrum of each method was also recorded against distilled water/methanol. The results are graphically represented in fig 2.

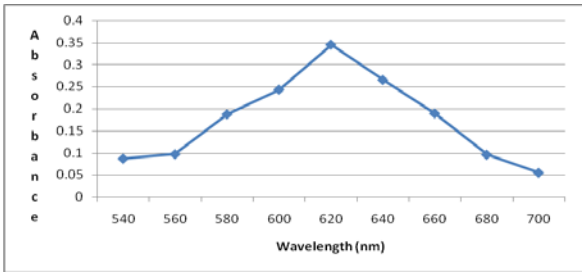


**Fig. 2: Absorption spectrum of Chloramphenicol with MBTH/$FeCl_3$**

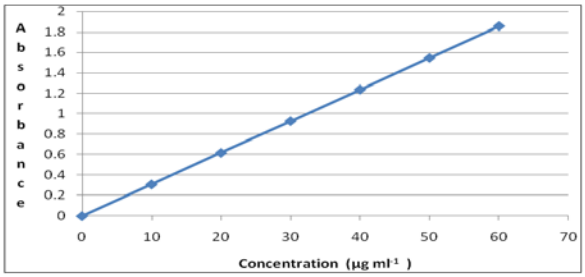


**Fig. 3: Beer's law plot of Chloramphenicol with MBTH/$FeCl_3$**

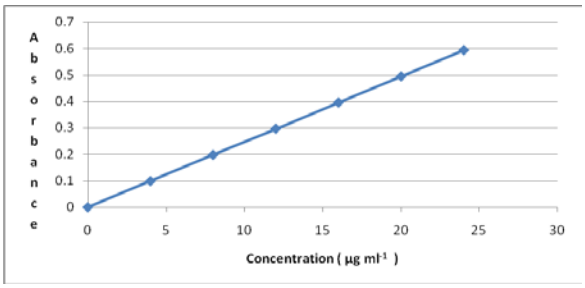


**Fig. 4: Beer's law plot for MBTH in blood sample**

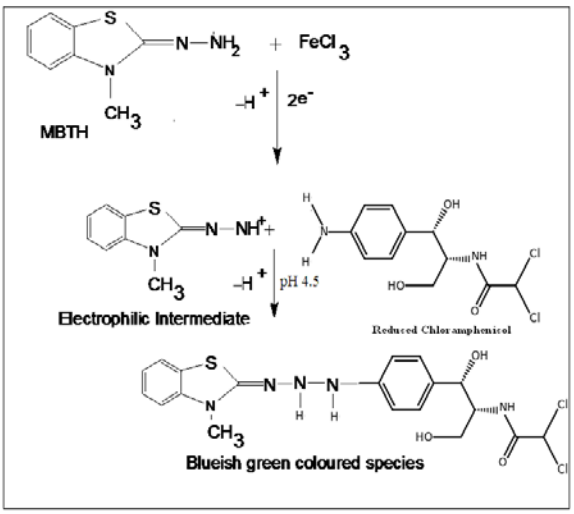


**Fig. 5: A schematic reaction mechanism of chloramphenicol with MBTH**

**Table 1: Optical characteristics and precision by (MBTH)**

| Parameter | Visible method |
|---|---|
| Color | Green |
| Absorption maxima (nm) | 620 |
| Beer's law limits (µg $ml^{-1}$) | 10-60 |
| Molar absorptivity (l $mol^{-1}$ $cm^{-1}$) | $1.0032\times10^{4}$ |
| Sandal's Sensitivity (µg $cm^{-2}$) | 0.0322 |
| Regression equation (Y*) | |
| Slope (b) | 0.0309 |
| Intercept (a) | 0.0014 |
| Standard deviation (SD) | 0.00021 |
| Correlation coefficient ($r^2$) | 0.9999 |
| % RSD (Relative Standard deviation)* | 0.0194 |
| Range of errors | |
| Confidence limits with 0.05 level | 0.00016 |
| Confidence limits with 0.01 level | 0.00021 |
| Limits of detection (LOD) (µg $ml^{-1}$) | 0.01941 |
| Limits of quantification (LOQ) (µg $ml^{-1}$) | 0.06472 |

*RSD of six independent determinations

**Table 2: Assay results of chloramphenicol in formulations by UV-visible method**

| Name of the Formulation | Formulation in (mg) | Amount found by the proposed method (mg) | Amount found by the reference method [40, 41] (mg) | % recovery |
|---|---|---|---|---|
| Ocupol-D | 250 | 249.34<br>t=0.0031*<br>F=7.07714* | 248.19 | 99.53 |
| Phenicol | 250 | 249.56<br>t=0.0032*<br>F=7.0664* | 247.98 | 99.36 |

*t and F-values refer to comparison of the proposed method with reference method, *Theoretical values at 95% confidence limits t= 0.0029 and F= 6.5594

**Table 3: Determination of accuracy of Chloramphenicol**

| Amount of CP in formulation (mg) | Amount of standard CP added (mg) | Total amount found (mg) | % recovery |
|---|---|---|---|
| 249.33 | 200 | 448.79 | 99.73 |
| 249.44 | 200 | 448.99 | 99.77 |
| 248.75 | 200 | 447.75 | 99.5 |
| 248.66 | 250 | 497.32 | 99.46 |
| 247.5 | 250 | 495.00 | 99.00 |
| 248.19 | 250 | 496.38 | 99.27 |
| 249.45 | 300 | 548.79 | 99.78 |
| 249.54 | 300 | 548.98 | 99.81 |
| 249.34 | 300 | 548.54 | 99.73 |

**Table 4: Statistical data for accuracy determination**

| Total amount found (mean) | Standard deviation | % RSD |
|---|---|---|
| 249.17 | 0.370 | 0.148 |
| 248.11 | 0.583 | 0.234 |
| 249.44 | 0.100 | 0.0400 |

*The results are the mean of five readings at each level of recovery.

**Table 5: Repeatability data for Chloramphenicol at 620 nm**

| Conc. (µg $ml^{-1}$) | Abs 1 | Abs2 | Abs3 | Mean | Std. deviation | (%) RSD* |
|---|---|---|---|---|---|---|
| 10 | 0.308 | 0.307 | 0.309 | 0.308 | 0.001 | 0.324 |
| 20 | 0.618 | 0.617 | 0.614 | 0.616 | 0.002 | 0.324 |
| 30 | 0.927 | 0.928 | 0.928 | 0.927 | 0.0005 | 0.053 |
| 40 | 1.237 | 1.235 | 1.236 | 1.236 | 0.001 | 0.0809 |
| 50 | 1.546 | 1.547 | 1.548 | 1.547 | 0.001 | 0.0646 |
| 60 | 1.856 | 1.858 | 1.857 | 1.857 | 0.001 | 0.0538 |

*RSD of six independent determinations

**Table 6: Color stability data for MBTH method**

| Conc. in µg ml$^{-1}$ | Time in hours | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 30 | 4 | 8 | 12 | 16 | 20 | 24 | 28 | 32 |
| | 0.927 | 0.927 | 0.927 | 0.927 | 0.927 | 0.927 | 0.910 | 0.809 |

**Table 7: Assay results of Chloramphenicol in blood sample**

| Name of the formulation | Formulation in (mg) | Amount found by the proposed method in (mg) | Amount found by the reference method [40, 41] (mg) | % recovery |
|---|---|---|---|---|
| Ocupol-D | 5 | 3.99<br>t=0.0029*<br>F=1.0091* | 3.88 | 97.16 |
| Phenicol | 5 | 3.89<br>t=0.0028*<br>F=1.0089* | 3.87 | 99.48 |

*t and F values refer to the comparison of the proposed method with the reference method, *Theoretical values at 95% confidence limits t=0.00196 and F=9.7866.

**Table 8: Determination of accuracy of Chloramphenicol**

| Name of the formulation in (mg) | Amount of drug in blood sample (mg) | Amount of standard drug added in (mg) | Total amount found (mg) | % recovery |
|---|---|---|---|---|
| 5 | 3.99 | 5 | 7.98 | 79.80 |
| 5 | 3.89 | 5 | 7.99 | 79.90 |

*The results are the mean of five readings at each level of recovery.

**Table 9: Repeatability data for Chloramphenicol at 620 nm**

| Concentration in (µg ml$^{-1}$) | Abs1 | Abs2 | Abs3 | Mean | Std. deviation | (%) RSD* |
|---|---|---|---|---|---|---|
| 4 | 0.0987 | 0.0986 | 0.0989 | 0.0987 | 0.0001 | 0.1013 |
| 8 | 0.198 | 0.197 | 0.196 | 0.197 | 0.0001 | 0.0507 |
| 12 | 0.297 | 0.296 | 0.297 | 0.296 | 0.0005 | 0.1689 |
| 16 | 0.3961 | 0.3959 | 0.3968 | 0.396 | 0.0004 | 0.1010 |
| 20 | 0.495 | 0.494 | 0.496 | 0.495 | 0.0001 | 0.0202 |
| 24 | 0.594 | 0.595 | 0.593 | 0.594 | 0.0001 | 0.0168 |

*RSD of six independent determinations

**Parameters fixation**

In developing these methods, a systematic study of the effects of various relevant parameters in the methods concerned were undertaken by verifying one parameter at a time and controlling all another parameter to get the maximum color development for this method reproducibility and reasonable period of stability of final colored species formed. The following studies were conducted.

**Method**

The results obtained in this method were based on oxidation followed by coupling reaction of Chloramphenicol with MBTH, ferric chloride and orthophosphoric acid to form a green colored chromogen that exhibited maximum absorption at 620 nm against the corresponding reagent blank. The functional group used for the color development for this method was primary amine group. A schematic reaction mechanism of Chloramphenicol with MBTH reagent was shown in (fig. 5). The effect of various parameters such as concentration and volume of MBTH and strength of the acid order of addition of reagents, a solvent for final dilution were studied by means of control experiments varying one parameter at a time.

**Optical characteristics**

The reference method adheres to beer's law the absorbance at appropriate wavelength of a set of solutions contains different amounts of Chloramphenicol and specified amount of reagents (as described in the recommended procedure) were noted against appropriate reagent blank. Least square regression analysis was carried out for the slope. Intercept and correlation coefficient, Beer's law limits, molar absorptivity & Sandal's sensitivity for Chloramphenicol with each of mentioned reagents was calculated. In order to test whether the colored species formed in the method adhere the beer's law the absorbance at an appropriate wavelength of a set of solutions contain different amounts of Chloramphenicol and specified amount of reagents (as described in the recommended procedure) were noted against appropriate reagent blanks or distilled water. The beers law plots of the system illustrated graphically (fig. 3 and 4) least square regression analysis was carried out for the slope, intercept and correlation coefficient, beer's law limits molar absorptivity Sandals sensitivity for Chloramphenicol with each of mentioned reagents were calculated. The optical characteristics are presented in the table 1.

**Precision**

The precision of each one among the five proposed spectrophotometric methods was ascertained separately from the absorbance values obtained by actual determination of a fixed amount of Chloramphenicol 10 µg ml$^{-1}$in final solution. The percent relative standard deviation and percent range of error (at 0.05 and 0.01 confidence limits) were calculated for the proposed methods and presented in table 1.

**Analysis of formulations**

Commercial formulations of Chloramphenicol were successfully analyzed by the proposed methods. The values obtained from the proposed and reference methods were compared statistically by the t and F tests and were found that those proposed methods do not differ significantly from the reported methods and they were

presented in table 2. The proposed methods also applied for Biological Samples (Blood) for good recoveries are obtained which were recorded in table 7.

**Accuracy**

Recovery studies were carried by applying the method to drugs sample present in formulations to which known amount of Chloramphenicol of label claim was added (standard addition method). The recovery studies were carried by applying the method to a biological sample (Blood) to which known amount of Chloramphenicol correspond to 2 mg formulations taken by the patient. By the follow of standard addition method, 2 mg of label claim was added. After the addition of these standards, the contents were transferred to 100 ml volumetric flash and dissolved in a solvent. Finally, the volume was made up to the mark with solvent. The solution was filtered through what man No. 41filter paper. The mixed sample solutions were analyzed, and their absorbance value was determined. At each level of recovery five determinations were performed and present in table 3. The results obtained were compared with expected results and were statistically validated in table 4.

**Linearity and range**

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analyzing in the sample within a given range. The range of analytical method is the interval between the upper and lower levels of analyzing that have been demonstrated within a suitable level of precision, accuracy and linearity

**Specificity and selectivity**

Specificity is a procedure to detect quantitatively the analyze in the presence of components that may be expected to the present in the sample matrix. While selectivity is a procedure to detect the analyze qualitatively in the presence of components that may be expected to present in the sample matrix. The excipient in formulations was spiked in a pre-weighed quantity of drugs, and then absorbance was measured, and calculations were done to determine the quantity of the drugs.

**Repeatability**

Standard solutions of Chloramphenicol were prepared, and absorbance was measured against the solvent as the blank. The observance of the same concentration solution was measured five times and the standard deviation was calculated and presented in tables 9.

**Interferences studies**

The effect of wide range of inactive, ingredients usually present in the formulations for the assay of Chloramphenicol under optimum conditions was investigated. None of them interfered in the proposed methods even when they are present in the excess fold than anticipated in formulations.

**Solution stability**

The stability of the solutions under study was established by keeping the solution at room temperature for 48 H. The results indicate no significant change in assay values indicating stability of Drug in the solvent used during analysis. The results are recorded in table 6.

**CONCLUSION**

The method was found to be accurate and precise, as indicated by recovery studies close to 100 and % RSD is not more than 2. The summary of validation parameters of proposed UV-Visible method is given. The simple, accurate and precise UV-Visible method for the determination of Chloramphenicol as bulk, Commercial samples and Blood samples has been developed. The method may be recommended for routine and quality control analysis of the investigated pure in bulk and samples. The analytical solution is found to be stable up to 48 H at room temperature. Hence, it is concluded that the analytical method is validated and can be used for routine analysis and for stability study.

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**CONFLICT OF INTERESTS**

Declare none

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