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

G CHANDRA SEKHAR*

Dept. of Chemistry, Government degree college, Jammalamadugu, Kadapa(Dt.), AP, India.

****E-mail:csgandii3@gmail.com***

Abstract:

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 based on oxidative coupling reaction with 2,2-Bipyridine reagent at pH-4.5 which is extractable at 520 nm. The Beer's law was obeyed in the concentration range 0.4 ml to 2.4 ml (4 to 24 μg ml^{-1}). The RSD was found to be RSD is 0.1847% 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.

Keywords: Spectrophotometry, Chloramphenicol, 2,2-BP, Oxidative coupling

INTRODUCTION

Several analytical methods have been reported for the determination of Chloramphenicol in various samples, such as shrimp,[1-11] seafood, food,[12–15] urine, serum [14–16] and pharmaceutical formulations [17–22] based on liquid chromatography (LC),[5,12] liquid chromatography–mass spectrometry (LC-MS),[3,7–11,14,15] gas chromatography (GC), gas chromatography–mass spectrometry (GC-MS),[3,12,14] capillary zone

electrophoresis,[16,17]enzyme-linked immunosorbent assay (ELISA),[3,13] spectrophotometry,[18,19] and chemiluminescence.[20–22] LC-MS is a common method that is used to determine Chloramphenicol, because of its high sensitivity, and low limit of detection. However, it needs expensive apparatus and reagents, and is time-consuming. A sensitive, rapid and cheap method for analysis is still needed. Electrochemical methods are widely used in many applications because they are simple, and involve no more reagents for derivatization and low cost. Several methods have been developed for the determination of Chloramphenicol using electrochemical detection, such as voltammetry at electrochemically activated carbon fiber microelectrodes4 and capillary-zone electrophoresis with amperometric detection at a carbon disk electrode[17] and a carbon fiber micro-disk array electrode.[16] Boron-doped diamond thin film (BDD) electrodes have many advantages for electro analytical applications, due to their unique characteristics, which include a very low background current,[23,24] a wide electrochemical potential window in aqueous solutions,[25,26] a long-term stability of response,[27–30] a slight adsorption of polar organic molecules[28] and low sensitivity to dissolved oxygen.[31] Because of these attractive properties, BDD electrodes have been successfully used for the determination of various compounds, such as tiopronin,[30]acetaminophen,[32] D-penicillamine,[33] captopril,[34] lincomycin,[35] sulfonamides,[36]malachite green and leucomalachite green.[37] Sensitive voltammetric determination of Chloramphenicol by using single-wall carbon nanotube–gold nanoparticle–ionic liquid composite film modified glassy carbon electrodes was developed by Wuhan et al [38,39].

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.

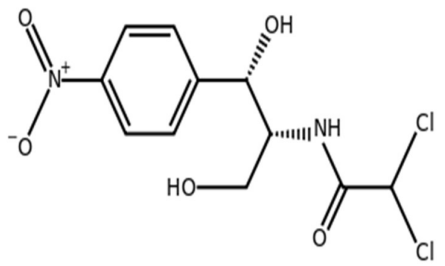


Fig: 1. Chemical Structure of Chloramphenicol

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 simple UV-Visible spectrophotometric method for the quantitative determination of Chloramphenicol. Functional group used for color development of Chloramphenicol was primaryamine group. The results obtain in this method was based on oxidative coupling reaction with 2,2-Bipyridine..

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.

1. MATERIALS AND METHODS

a. Pure sample

The pure sample was collected from CIPLA pharmaceuticals. Avalahalli, Vigro agar, Bangalore-560 049.

A. 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 pipette 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)

B. Preparation of standard calibration curve of pure drug

1. **Solvent**

Methanol was used as solvent in the present investigation.

2. **Preparation of calibration curve**

Fresh aliquots of Chloramphenicol ranging from 0.4 to 2.4 ml were transferred into a series of 10 ml volumetric flasks to provide final concentration range of 4 to 24 µg ml^{-1}.To each flask 1ml of (0.01M) 2, 2-Bipyridine solution was added followed by 1ml of (0.2%) Ferric chloride solution and resulting solution was heated and finally 1ml (0.2M) orthophosphoric acid solution was added. The solutions were cooled at room temperature and made up to mark with distilled water. The absorbance of orange red colored chromogen was measured at 520 nm against the reagent blank. The color species was stable for 24 hours. The amount of

Chloramphenicol present in the sample solution was computed from its calibration curve.

3. **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 minutes 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 pipette out into 100 ml volumetric flask and the volume was made up to the mark with methanol to obtained the final concentration of 100 to 24 µg ml^{-1} (Stock solution). Subsequent dilutions of this solution were made with methanol to get concentration of 4 to 24 µg $ml^{-1}$and were prepared as above and analyzed at the selected wavelength, 520 nm and the results were statistically validated.

4. **Procedure for blood sample**

After collection of blood sample it will be centrifuged. For isolation of Chloramphenicol from 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 dry residue 100 mg was dissolved in 100 ml of methanol (1000 to24 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 to24 µg ml^{-1}).

From the above solution ranging from 0.5-3ml (5-30 µg /ml) were transferred in to 10 ml volumetric flask and to the each flask 1ml of (0.01M) 2, 2-Bipyridine solution was added followed by 1ml of (0.2%) Ferric chloride solution and made up to the mark with methanol. Then the resulting solution

was heated for 15 minutes and finally 1ml (0.2M) orthophosphoric acid solution was added. The solutions were cooled at room temperature and made up to the mark with distilled water. The absorbance of orange red colored chromogen was measured at 520 nm against the reagent blank. The color species was stable for 24 hours. The amount of Chloramphenicol present in the sample solution was computed from its calibration curve.

2. RESULTS AND DISCUSSIONS

1. Optical parameters

In order to ascertain the optimum wavelength of maximum absorption (λ_{max}) formed in UV-Visible spectrophotometric method (Reference method – A) and of the colored species formed in each so the four visible spetrophotometric methods, specified amount of Chloramphenicol in final solution 4 $\mu g\ ml^{-1}$ was taken and the colors were developed following the above mentioned procedure. The absorption spectra was scanned on spectrophotometer in the wavelength region of 200-400 nm (for method A) and 380-800 nm for method against corresponding reagent blanks. The regent blank absorption spectrum of each method was also recorded against distilled water/methanol. The obtained results were graphically represented in figure 1.

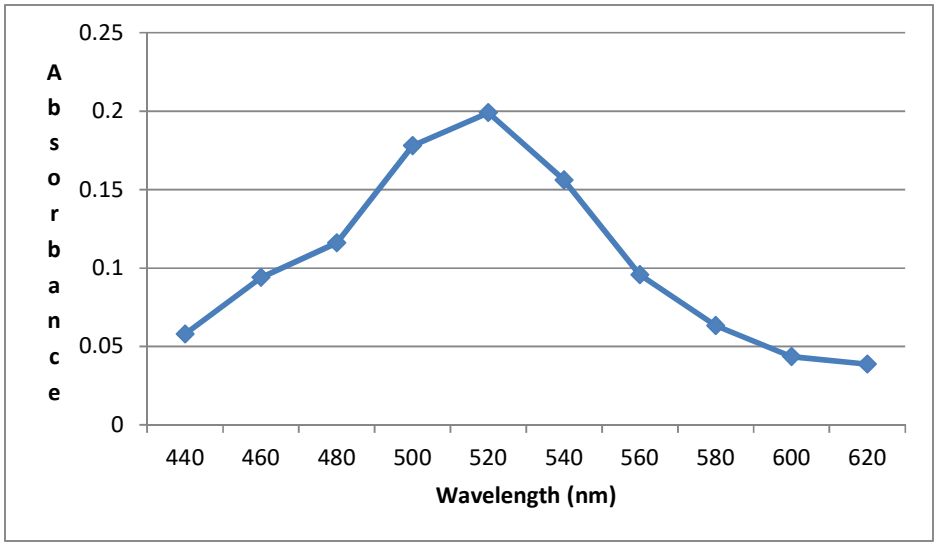


Fig-1: Absorption spectrum of Chloramphenicol with 2,2-Bipyridine/$FeCl_3$

2. Parameters fixation

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

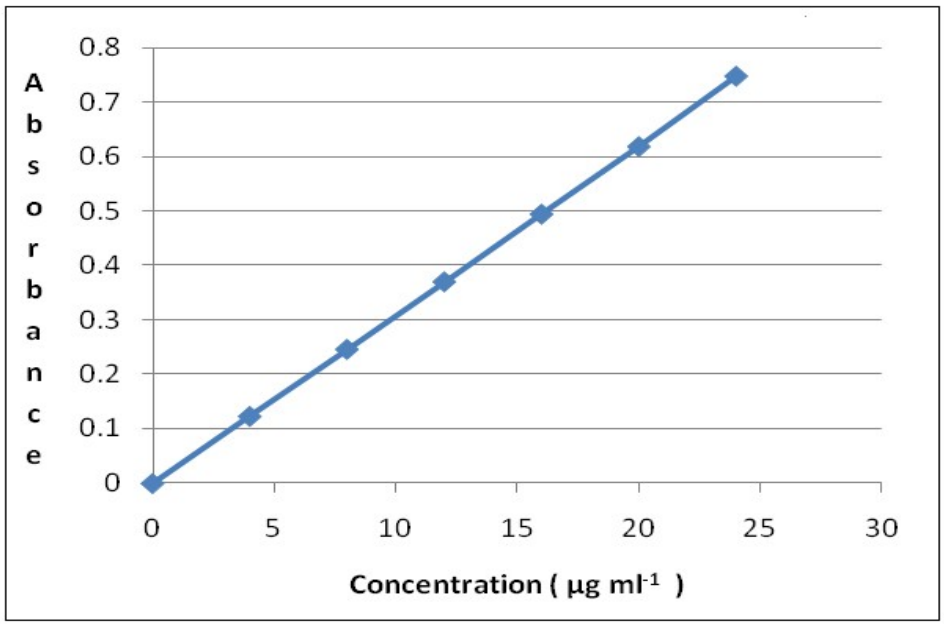


Fig-2: Beer's law plot of Chloramphenicol with 2, 2-Bipyridine/$FeCl_3$

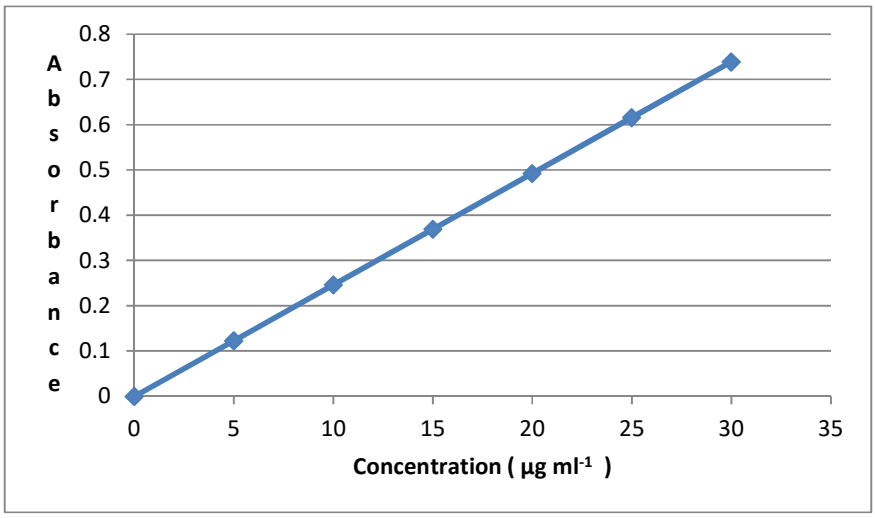


Fig-2.3: Beer's law plot for Chloramphenicol in blood sample

3. Method

The results obtained in this method were based on oxidation followed by complex formation reaction of Chloramphenicol with 2,2-Bipyridine, Ferric chloride and orthophosphoric acid to form an orange red colored chromogen that exhibited maximum absorption at 520 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 2, 2-Bipyridine reagent was shown in figure. The effect of various parameters such as concentration and volume of 2,2-Bipyridine and strength of acid order of addition of reagents, solvent for final dilution were studied by means of control experiments varying one parameters at a time.

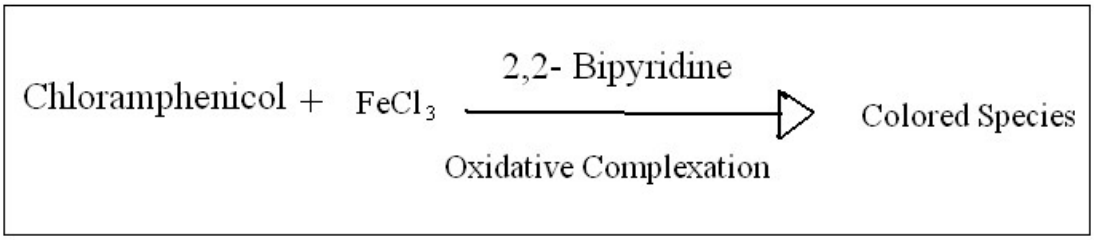
4. Optical characteristics

The reference method adhere to beer's law the absorbance at appropriate wave length 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.

The Beer's law plot of the system illustrated graphically least square regression analysis was carried out for the slope. Intercept and correlation coefficient, Beer's law limits, molar absorptivity & Sandells sensitivity for Chloramphenicol with each of mentioned reagents was calculated. The optical characteristics were present in the Table.

In order to test whether the colored species formed in the method adhere the beer's law the absorbance at 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 Beer's law plots of the system illustrated graphically least square regression analysis was carried out for the slope, intercept and correlation coefficient, Beer's law limits molar absorptivity Sandells sensitivity for Chloramphenicol with each of mentioned reagents were calculated. The optical characteristics are represented in the Table 1.



Parameter	Visible method
Absorption maxima(nm)	520
Beer's law limits (µg ml^{-1})	4-24
Molar absorptivity (l $mol^{-1}cm^{-1}$)	1.0024×10^4
Sandell's Sensitivity (µg cm^{-2})	0.0322
Regression equation (Y*)	---
Slope (b)	0.0311
Intercept(a)	0.003
Standard deviation(SD)	0.00086
Correlation coefficient (r^2)	0.9999
%RSD (Relative Standard deviation)*	0.1847
Range of errors	---
Confidence limits with 0.05 level	0.00064
Confidence limits with 0.01 level	0.00084
Limits of detection (LOD)(µg ml^{-1})	0.0771
Limits of quantification (LOQ) (µg ml^{-1})	0.2572

*RSD of six independent determinations.

Table-1: Optical characteristics and precision by (2, 2-B.P)

4. Precision

The precision of each one among the five proposed spectrophotometric methods were ascertained separately from the absorbance values obtained by actual determination of a fixed amount of Chloramphenicol 4 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 2.

5. 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 Tables.. The proposed methods also applied for Biological Samples (Blood) for good recoveries are obtained which were recorded in Table 2.

6. 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 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 flask and dissolved in solvent. Finally the volume was made up to the mark with solvent. The solution was filtered through Whatman No. 41 filter 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 Tables 3 to 5. The results obtained were compared with expected results and were statistically validated in Tables 4 to 9.

7. Linearity and Range

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

8. 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 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

9. 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 measure five times and standard deviation was calculated and represented in Table 9.

10. 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 excess fold than anticipated in formulations.

11. Solution Stability

The stability of the solutions under study was established by keeping the solution at room temperature for 48 hours. The results indicate no significant change in assay values indicating stability of Drug in the solvent used during analysis. The results were recorded in Tables 1 to 9.

Name of the Formulation	Formulation in (mg)	Amount found by the proposed method (mg)	Amount found by the reference method (mg)	% Recovery
Ocupol-D	250	249.16 t=0.00309* F=7.1723*	248.19	99.60
Phenicol	250	249.37 t=0.00316* F=7.1678*	247.98	99.43

*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-2:Assay results of Chloramphenicol in formulations by UV-Visible method

Amount of Chloramphenicolin formulation (mg)	Amount of Standard Chloramphenicol added (mg)	Total amount found (mg)	% Recovery
249.26 249.16 248.74	200 200 200	448.66 448.48 447.73	99.70 99.66 99.49
248.83	250	497.66	99.53

248.75	250	497.5	99.5
248.75	250	497.5	99.5
249.16	300	548.15	99.66
249.37	300	548.61	99.74
249.02	300	547.84	99.6

Table-3: Determination of accuracy of Chloramphenicol

Total amount found (mean)	Standard deviation	% RSD
249.05	0.2759	0.1107
348.77	0.0461	0.0132
249.18	0.1761	0.0706

The results are the mean of five readings at each level of recovered

Table-4: Statistical data for accuracy determination

Conc. (µg ml^{-1})	Abs 1	Abs 2	Abs 3	Mean	Std. deviation	(%) RSD*
4	0.123	0.124	0.125	0.124	0.001	0.806
8	0.246	0.245	0.247	0.246	0.001	0.406
12	0.370	0.376	0.372	0.372	0.003	0.806
16	0.494	0.496	0.497	0.495	0.0015	0.3030
20	0.618	0.617	0.619	0.618	0.001	0.1618
24	0.741	0.744	0.744	0.743	0.0017	0.2288

*RSD of six independent determinations

Table-5: Repeatability data for Chloramphenicol at 520 nm

Conc. in µg/ml	Time in hours							
4	4	8	12	16	20	24	28	32
	0.123	0.145	0.168	0.175	0.183	0.1987	0.156	0.095

Table-6: Color stability data for 2, 2- Bipyridine method

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	3.98 F=9.8364* t=0.0029*	3.88	97.42
Phenicol	5	3.97 F=9.8356* t=0.00278*	3.87	97.41

*t and F values refer to comparison of the proposed method with reference method.

*Theoretical values at 95% confidence limits t=0.00196 and F=9.7866.

Table-7: Assay results of Chloramphenicol in blood sample

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	7.99	79.90
5	3.98	5	7.98	79.80

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

Table-8: Determination of accuracy of Chloramphenicol

Concentration in (µg ml^{-1})	Abs 1	Abs 2	Abs 3	Mean	Std. Deviation	(%) RSD
5	0.1231	0.1232	0.1232	0.1231	0.0005	0.0406
10	0.2463	0.2465	0.2467	0.2465	0.0001	0.0405
15	0.3695	0.3696	0.3697	0.3696	0.0001	0.02705
20	0.4926	0.4924	0.4924	0.4924	0.0001	0.0203
25	0.6158	0.6157	0.6155	0.6156	0.0001	0.01624
30	0.7390	0.7391	0.7392	0.7391	0.0001	0.01352

*RSD of six independent determinations

Table-9: Repeatability data for Chloramphenicol at 520 nm

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 summery 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 hours 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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