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RESEARCH ARTICLE

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

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ABSTRACT

A simple, précis, rapid sensitive and accurate spectrophotometric methods have been developed for the estimation of Chloramphenicol UV in pure form and its pharmaceutical formulations based on oxidative coupling reaction UV with MBTH reagent at P ^{H} -4 which is extractable at 620 nm. Beer's law is obeyed in the concentration range 1-6 ml (10-60 µgml^{-1}) .The developed method was applied directly and easily for the analysis of the pharmaceutical formulations. RSD was found to be 0.0194% and recovery 99.73%. The method was completely validated and proven to be rugged. The interferences of the ingredients and excipients were not observed. The repeatability and the performance of the proved method were established by point and internal hypothesis and through recovery studies.

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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, ast, 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 Ametoctradin UV is $C_{11}H_{12}Cl_2N_2O_5$and the molecular weight is 323.13 grams. It has the following structure.

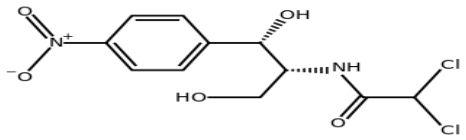


Fig: 1. Chemical Structure of Chloramphenicol

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 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 all methods 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 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)

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 1ml (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 pipette out into 100 ml volumetric flask and the volume was made up to the mark with methanol to obtain the final concentration of 100µg ml^{-1} (Stock solution).

Subsequent dilutions of this solution were made with methanol to get concentration of10 to 60 µg $ml^{-1}$and were prepared as above and analyzed at the selected wavelength,620nm and the results were statistically validated

Procedure for blood sample

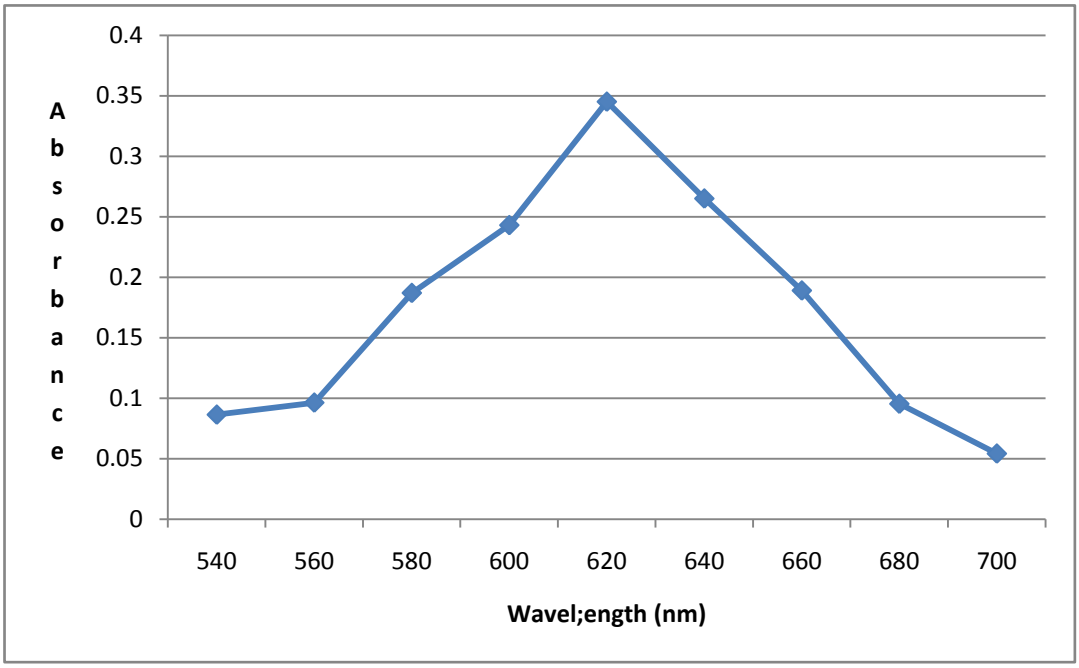
After collection of blood sample it will be centrifuged. For isolation of Chloramphenicol from plasma sample, Methanol was used for protein precipitation. Liquid- Liquid extraction was performed with plasma by alkalinization with 1M NaOH, followed by extraction with 30% dichloromethane in Hexane. The upper organic

layer was evaporated to dryness, and reaming dry residue 100 mg was dissolved in 100 ml of Methanol (1000µgml^{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) were transferred in to 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 1ml (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 DISCUSSIONS

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 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 200-400nm (for method A) and 380-800 nm (for this Method) against corresponding reagent blanks. The regent blank absorption spectrum of each method was also recorded against distilled water /methanol. The results are graphically represented in fig-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 this method reproducibility and reasonable period of stability of final colored species formed. The following studies were conducted.

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

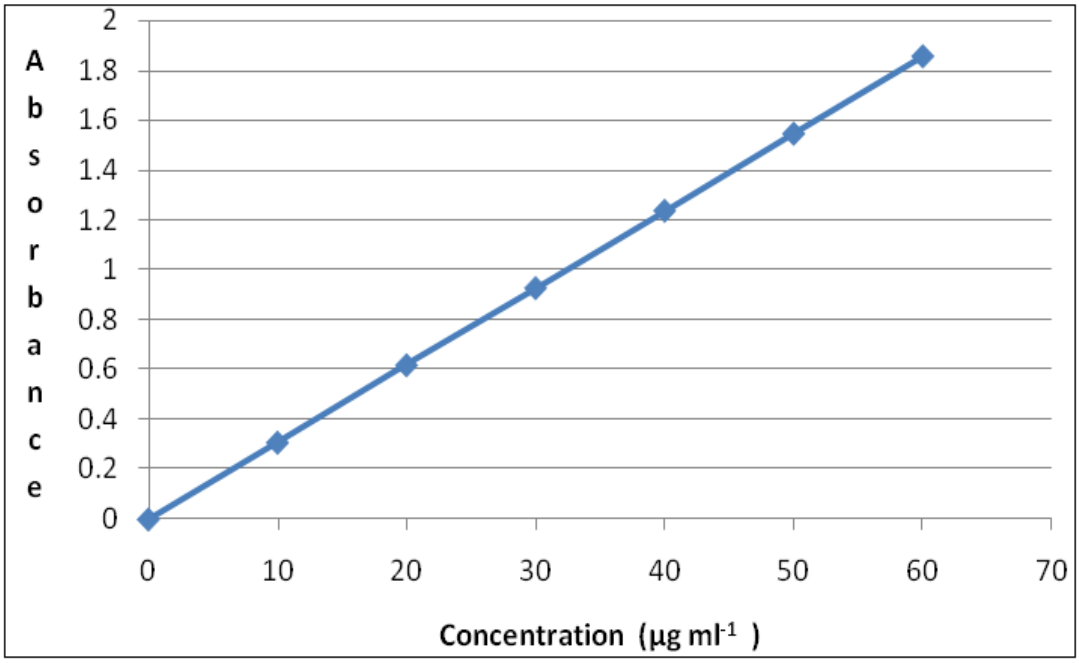
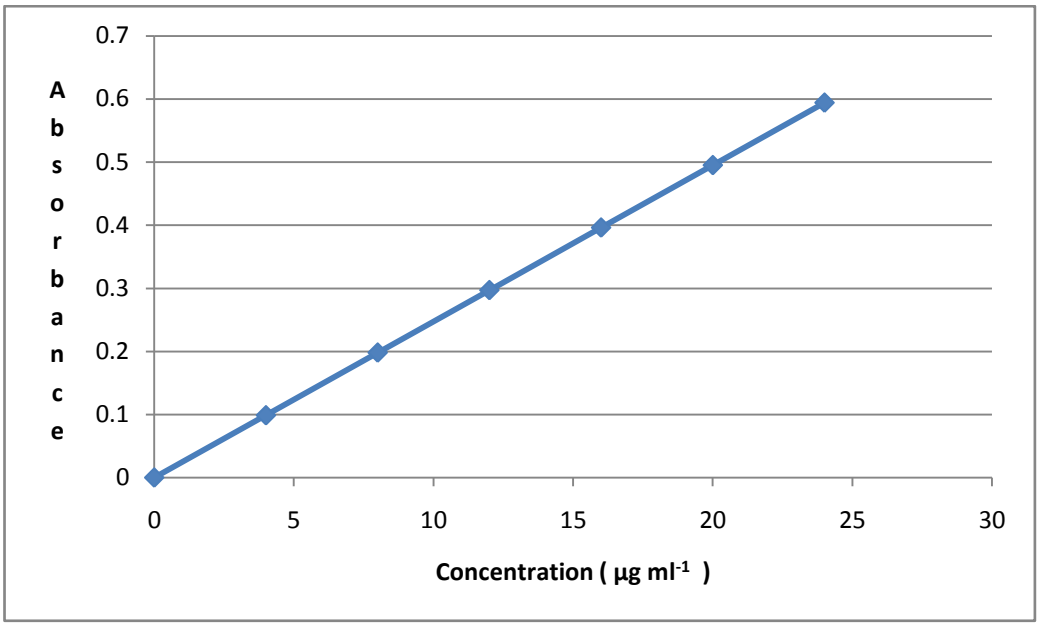


Fig-4: Beer's law plot for MBTH in blood sample



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 acid order of addition of reagents, solvent for final dilution were studied by means of control experiments varying one parameters at a time.

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. Least square regression analysis was carried out for the slope. Intercept and correlation coefficient, Beer's law limits, molar absorptivity & Sandell'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 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&4) 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 presented in the Table-1.

Fig-5: A Schematic reaction Mechanism of Chloramphenicol with MBTH

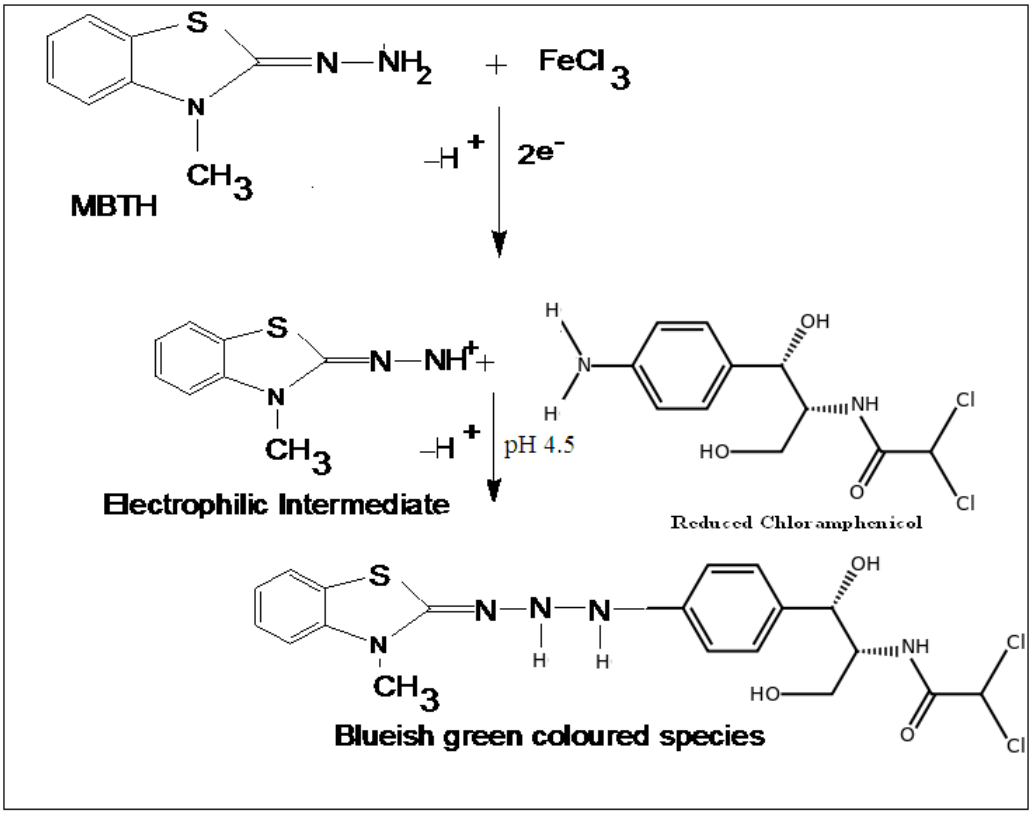


Table-1: Optical characteristics and precision by (MBTH)

Parameter	Visible method
Color	Green
Absorption maxima (nm)	620
Beer's law limits (µg ml^{-1})	10-60
Molar absorptivity (l $mol^{-1}cm^{-1}$)	1.0032×10^{4}
Sandell'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

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 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 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 solvent. Finally the volume was made up to the mark with solvent. The solution was filtered through Whatman 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 analytical method is its ability to elicit test results that are directly proportional to the concentration of analyze in sample within 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

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.

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 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 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 Hours. 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.

Table-2: Assay results of Chloramphenicol in formulations by U.V-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 t=0.0031* F=7.07714*	248.19	99.53
Phenicol	250	249.56 t=0.0032* 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
249.33 249.44 248.75	200 200 200	448.79 448.99 447.75	99.73 99.77 99.5
248.66 247.5 248.19	250 250 250	497.32 495.00 496.38	99.46 99.00 99.27
249.45 249.54 249.34	300 300 300	548.79 548.98 548.54	99.78 99.81 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 recoverey.

Table-5: Repeatability data for Chloramphenicol at 620 nm

Conc. ($\mu g\ ml^{-1}$)	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	Time in Hours							
30	4	8	12	16	20	24	28	32
	0.927	0.972	0.928	0.928	0.929	0.929	0.812	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 t=0.0029* F=1.0091*	3.88	97.16
Phenicol	5	3.89 t=0.0028* F=1.0089*	3.87	99.48

*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-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 620nm

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

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, Comercial 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 Hrs 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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