Assay of Chloramphenicol and Its Esters in Formulations

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Abstract □ A method for the estimation of chloramphenicol and its esters in some pharmaceutical preparations is described. The method is based on measuring the violet color produced by the interaction of chloramphenicol with hydroxylamine and ferric chloride in an alcoholic medium.

Keyphrases □ Chloramphenicol, esters in dosage forms—analysis □ Colorimetric analysis—spectrophotometer □ Hydroxylamine HCl, ferric chloride—color formation, chloramphenicol

The estimation of chloramphenicol and its esters has been, and is still, the subject of much investigation. The need for further investigation is evidenced by the fact that most of the methods reported for the estimation of chloramphenicol are subject to difficulties during their application. These difficulties may be attributed to the unfavorable solubility of chloramphenicol or its derivatives in the solvent of the experiment, as in case of titrimetric (1) and polarographic methods (2). Most color reagents used in colorimetric assays (3–7) may interact also with the decomposition products of chloramphenicol, thus giving erroneous results. These decomposition products may also interfere in spectrophotometric methods (8). Aihara *et al.* (4) proposed a method based on measuring the color developed from the action of ferric chloride on the hydroxamic acid which is produced by the interaction of chloramphenicol and hydroxylamine in aqueous alkaline medium. The method, however, is applicable only to free chloramphenicol and not to its water-insoluble esters.

In the present investigation, the authors used the hydroxylamine reaction in establishing another colorimetric method suitable for estimating chloramphenicol and its esters in various pharmaceutical forms.

EXPERIMENTAL

Reagents—The following were used: (*a*) authentic samples of chloramphenicol, chloramphenicol palmitate, chloramphenicol stearate, and chloramphenicol succinate; (*b*) standard test solution of chloramphenicol or its esters: a 0.2% solution of chloramphenicol or any of the mentioned derivatives was prepared by dissolving 100 mg. of the sample in absolute ethanol and adjusting to 50 ml. with the same solvent; (*c*) ethanolic hydroxylamine hydrochloride reagent: a saturated solution of hydroxylamine hydrochloride in absolute ethanol; (*d*) ethanolic sodium hydroxide solution: a saturated solution of sodium hydroxide in absolute ethanol; (*e*) ethanolic hydrochloric acid: 20 ml. of hydrochloric acid (35% w/v) completed to 100 ml. with absolute ethanol; (*f*) ferric chloride solution: 1% w/v in distilled water; and (*g*) ethanolic succinic acid solution: 91.3 mg. of succinic acid dissolved in absolute ethanol and adjusted to 50 ml. with the same solvent.

Analytical grade reagents were used whenever possible.

In a spectrophotometric study of the violet color resulting from the action of hydroxylamine hydrochloride and ferric chloride on chloramphenicol, a Prolabo spectrophotometer was used. The optimum wavelength for the measurement was found to be 505 mμ.

Procedure—Pipet a volume of the ethanolic solution of chloramphenicol or its esters, equivalent to 1–5 mg., into a small conical flask. (In the case of free chloramphenicol, add 1 ml. of succinic acid solution.) Add 1 ml. of hydroxylamine hydrochloride solution to the solution in the flask, and then add 1 ml. of sodium hydroxide solution. Complete to 5 ml. with absolute ethanol and heat on a water bath at 85–95° for 5 min. Transfer the flask to a refrigerator freezer and allow to stand for 15 min. Transfer the contents of the flask quantitatively to a 10-ml. measuring tube with the aid of absolute ethanol (previously cooled to the same temperature as the mixture) and adjust the volume to approximately 7.5 ml. Add to the mixture, in the following succession, 1 ml. of distilled water (cooled as described), 1 ml. of 20% hydrochloric acid in absolute ethanol, and 0.5 ml. of ferric chloride solution; mix well. Measure the violet color after 2–5 min. in a 1-cm. cell at 505 mμ against a blank made simultaneously, omitting the addition of hydroxylamine reagent. The results are deduced from absorbance–concentration curves of chloramphenicol esters according to the sample analyzed. In the case of the chloramphenicol base, the results are deduced by comparing with standard preparations containing the same amount of succinic acid treated simultaneously.

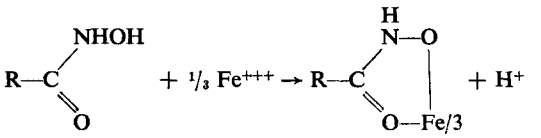
Precision of the Method—Absorbance *versus* concentration of chloramphenicol palmitate was measured and the deviation was 1.5%.

Mechanism of Reaction—It has been suggested (4) that hydroxylamine reacts with the amide group of chloramphenicol to give a hydroxamic acid. The addition of ferric ion to the hydroxamic acid solution produces a color (9–11) which could be utilized in the quantitative measurement. Feigl *et al.* (12) suggested that a carboxylic acid cannot be converted into a hydroxamic acid by the action of hydroxylamine and sodium hydroxide, while its ester can be converted under the same conditions (Scheme I).

$$\mathrm{RCOOR' + NH_2OH + NaOH \rightarrow RCO(NHONa) + R'OH + H_2O}$$
$$\mathrm{RCO(NHONa) + HCl \rightarrow RCONHOH + NaCl}$$

Scheme I

The hydroxamic acid formed gives a violet color with ferric chloride due to the formation of a water-soluble inner complex with ferric ion (Scheme II).



Scheme II

Accordingly, the esters of chloramphenicol would behave similarly.

Range—The color produced with chloramphenicol esters obeys Beer's law in the range of 300–500 mcg./ml. for the palmitate, 300–575 mcg./ml. for the stearate, and 300–485 mcg./ml. for the succinate.

Reproducibility—The reproducibility of the results is measured by applying the proposed method on solutions of known concentrations of chloramphenicol and its esters. The results are shown in Table I.

Application of the Method on Pharmaceutical Preparations—*Extraction of Chloramphenicol and Its Derivatives*—With a formulated preparation, it is necessary first to isolate chloramphenicol from the other accompanying ingredients. This can be accomplished by extracting the chloramphenicol or its derivatives by means of a suitable organic solvent as follows.

Chloramphenicol eye drops contain chloramphenicol, boric acid, borax, and phenyl mercuric nitrate. A volume equivalent to 50 mg. of chloramphenicol is mixed with an equal volume of phosphate buffer at pH 6.5 in a separating funnel and shaken with successive portions of chloroform (4 × 20 ml.) until complete

Table I—Assay of Solutions of Known Concentration of Chloramphenicol or Its Derivatives

Chloramphenicol			Chloramphenicol Palmitate			Chloramphenicol Succinate		
Added Amount, mcg.	Found Amount, mcg.	Error, %	Added Amount, mcg.	Found Amount, mcg.	Error, %	Added Amount, mcg.	Found Amount, mcg.	Error, %
250	255.7	2.3	300	305.7	1.9	300	308.1	2.7
275	277.0	0.70	350	353.5	1.0	325	325.0	0.0
300	301.0	0.30	375	375.0	0.0	380	351.0	0.3
350	351.0	0.30	400	401.0	0.25	375	374.0	0.3
400	401.5	0.40	425	424.0	−0.2	400	398.0	−0.5
425	424.0	−0.2	475	471.0	−0.8	425	419.0	−1.4
450	440.5	−2.1	500	491.5	−1.7	450	436.5	−3.0

extraction is effected. The chloroform is evaporated, and the residue is dissolved in 50 ml. of ethanol.

Chloramphenicol ear drops contain chloramphenicol and propylene glycol. A volume equivalent to 50 mg. chloramphenicol is directly extracted with chloroform (4 × 15 ml.), and the procedure described for eye drops is followed.

Chloramphenicol suppositories contain chloramphenicol in a suppository base. A weight of the suppositories equivalent to 50 mg. chloramphenicol is shaken with hot water and allowed to cool; the base is extracted with petroleum ether (3 × 10 ml.). The chloramphenicol remaining in the liquid is then extracted with chloroform (4 × 15 ml.) and treated as previously described.

Chloramphenicol palmitate suspension contains chloramphenicol palmitate and suspending, coloring, and flavoring agents.

Chloramphenicol palmitate and streptomycin suspension contains chloramphenicol palmitate, streptomycin sulfate, and suspending, coloring, and flavoring agents.

Chloramphenicol stearate suspension contains chloramphenicol stearate and suspending, coloring, and flavoring agents.

A volume of each of these three suspensions equivalent to 50 mg. of chloramphenicol is treated as described for eye drops.

Chloramphenicol succinate injection contains chloramphenicol succinate with a diluent in a powder form. An equivalent to 50 mg. of chloramphenicol is dissolved in 50 ml. ethanol.

All of the formulations were supplied from freshly prepared batches. The resulting ethanolic solutions of chloramphenicol or its esters were then assayed as described under *Procedure*. The results obtained were compared with those of the Aihara *et al.* (4) method, the spectrophotometric method (1), and the α-naphthol method (6). They are compiled in Table II.

Estimation of Chloramphenicol in Degraded Samples—The estimation of chloramphenicol in degraded samples of palmitate suspension, eye drops, and ear drops was carried out to examine the potentialities of the proposed method.

Degradation of chloramphenicol was achieved by incubating the preparation at different temperatures for 2 months. The suspension darkened in color and acquired a caramel-like odor. The eye drops and ear drops acquired a yellow color. The results were also compared with those of the spectrophotometric (1) and α-naphthol methods (6) and are represented in Table III.

Table II—Percentage of Chloramphenicol and Its Esters with Respect to the Labeled Amounts in Different Formulations

Samples	Proposed Method	Aihara *et al.* Method	Spectrophotometric Method	α-Naphthol Method
Chloramphenicol	99	99	98	99.9
Ear drops	97.5	98	98.2	97
Eye drops	96	97	96.2	96
Capsules	99	100	99.5	101
Suppositories	101	100	103	102
Chloramphenicol palmitate	105	Negative	102	97
Suspension	107	Negative	105	102
Suspension and streptomycin	103	Negative	107	101
Chloramphenicol stearate	98	Negative	100	98
Suspension	102	Negative	105	101
Chloramphenicol succinate	99.8	Negative	101	97
Injection	101	Negative	102	98

RESULTS AND DISCUSSION

The ethanol used in the experiment was found advantageous due to its solubilizing effect and for increasing the sensitivity of the reaction. Minimum sensitivity is given by 70% ethanol and maximum sensitivity by 90% ethanol. The performance of the interaction in absolute ethanol is 5 times as sensitive as in ethanol 70%. However, the presence of about 15% water is necessary to dissolve the resulting sodium chloride and ferric hydroxamate. To choose the optimum pH for the hydrolysis step, many alkalies and buffer solutions were tried. When using boric acid buffer (pH 10), a faint green, fluorescent, unstable color was produced. The addition of 1 ml. of phosphate buffer (pH 8) together with 1 ml. of sodium hydroxide solution stabilized the final violet color, but the sensitivity of the reaction decreased. The use of higher concentrations of phosphate buffer inhibited the resulting color. Higher and lower concentrations of sodium hydroxide solution and hydroxylamine reagent were tried, and 1 ml. of saturated ethanolic solutions of each was found most convenient for the color formation. At concentrations below 300 mcg./ml. of chloramphenicol, another grade of color is formed and this does not obey Beer's law.

The interaction was favored by heating. The time and temperature of heating affected the sensitivity of the reaction; heating for 5 min. at a temperature of 85–95° was found most suitable for the completion of the reaction. In the acidification step, the optimum pH was found in the range of 2.1–2.3; this was obtained by employing 1 ml. of ethanolic 20% hydrochloric acid. Hydrochloric acid buffer and organic acids were found to lessen the sensitivity of the color.

The intensity of the color increases greatly by cooling and reaches its maximum at 0° and falls to a minimum at 15°. The time of cooling affects the intensity of the produced color; the maximum color is obtained after 15 min. of cooling.

It is of great importance to adhere to the specified amount and concentration of ferric chloride (0.5 ml. of 1% solution); lower

Table III—Percentage of Chloramphenicol with Respect to Labeled Amounts in Degraded Samples

	Proposed Method	Naphthol Method	UV Method	Proposed Method	Naphthol Method	UV Method
	37°			45°		
Palmitate suspension	102	101	102	90	104	110
Eye drops	97	96	98	91	96.9	101
Ear drops	100	99	99.5	90	99.5	107
	55°			60°		
Palmitate suspension	82	110	140	76	130	[a]
Eye drops	80	101	110	50	[a]	[a]
Ear drops	64	104	130	35	[a]	[a]

[a] Discordant results.

concentrations gave incomplete reactions, and higher concentrations vitiated the violet color and changed it to yellow. When impure ferric chloride or an unfresh solution of it was employed, the addition of 2 drops of hydrogen peroxide (10 vol.) restored and potentiated the violet color. However, excess hydrogen peroxide was found unfavorable as the color changed to orange. The violet color, produced by the action of ferric chloride, develops within 2 min. and should be measured within 2–5 min. The color is not affected by light but is very sensitive to temperature.

Free chloramphenicol gave the same reaction as the salts, but the sensitivity was 5 times lower ($\leq$15 mg.) and the results were not reproducible. The addition of a carboxylic acid, however, was found to increase the sensitivity of the reaction. Many acids were tried, and succinic acid gave the highest sensitivity ($\leq$3 mg.). Although succinic acid alone gave negative reactions with the reagent, its presence affected the sensitivity of the reaction. Therefore, equal amounts of succinic acid were added to both test and standard preparations.

Due to the slight yellowish color produced by the action of sodium hydroxide on chloramphenicol or its esters, the blank experiment was done exactly like the experiment, omitting the addition of hydroxylamine hydrochloride reagent.

Data presented in Table I indicate the accuracy of the proposed method and the reproducibility of the results. The percentage of error with respect to the added and found amounts (Table I) ranges from $\pm$2.1 to 2.3 in chloramphenicol, from $\pm$1.7 to 1.9 in chloramphenicol palmitate, and from $\pm$2.7 to 3 in chloramphenicol succinate. The method was found applicable to different freshly formulated preparations, and the results (Table II) are comparable with those of the Aihara *et al.* method (4), the spectrophotometric method, and the α-naphthol method (6). The method of Aihara *et al.* (4), however, was not applicable to chloramphenicol esters. The comparison of the proposed method to the α-naphthol method and the spectrophotometric method on degraded samples of chloramphenicol and its palmitate produced varying results (Table III). The chloramphenicol analysis of the heated preparation decreased with an increase in temperature in the case of the proposed method and increased with the other two methods. This fact indicates that by means of the hydroxylamine method, chloramphenicol and its esters can be determined, while some of the degradation products interfere in the results of the other two methods. In addition, the color produced by the α-naphthol method was not the same in all cases; it ranged from greenish to bluish.

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Application of Absorbance Ratios to Analysis of Pharmaceuticals VI: Analysis of Binary Mixture Using a Reference Spectrum

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Abstract □ Binary mixtures may be resolved by using absorbance ratio values and a reference spectrum for one component in the mixture. The method is based on the differences between the absorbance values for the mixture at any two wavelengths and the values for a solution containing only one component in the mixture. Three constants are required to resolve the mixture, but only one of these is an absorptivity value. Unlike the absorbance ratio method described in the literature, this method does not depend on the use of a wavelength at which the two components in the mixture have identical absorptivity values.

Keyphrases □ Binary mixture analysis—reference spectrum □ Caffeine–Na benzoate—spectral characteristics □ Absorbance ratios—analysis □ UV spectrophotometry—analysis, absorbance ratios

The ratio of two absorbance values determined on the same solution at two different wavelengths is a constant. These ratios may be used to determine both the relative and absolute concentrations of the components in a binary mixture (1). However, absolute concentrations (w/v) cannot be determined unless one of the two wavelengths chosen for the analysis represents an isoabsorptive point.

Isoabsorptive points (those wavelengths at which the two components in a mixture have identical absorptivity values) are difficult to isolate. The mathematical derivations in the next section show that absolute concentration values can be obtained by analyzing the mixture at wavelengths that do not represent isoabsorptive points, and that the number and nature of the constants in the derived equations are the same as those associated with the absorbance ratio method of analysis (1). This method of analysis is based, therefore, on the use of two absorbance ratio values (Q values), one absorptivity value, and the differences, at two wave-