Colorimetric Assay for Chloramphenicol Using 1-Naphthol

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Chloramphenicol heated in 6 *N* sodium hydroxide with 1-naphthol gives a blue color that is the basis for a quantitative assay method.

ASSAY METHODS for chloramphenicol are many and varied, but most of them have significant restrictions with respect to their applicability. Microbiological methods (1, 2) cannot be used for chloramphenicol palmitate, since the unhydrolyzed ester lacks antibacterial activity. Polarographic analysis is also not applicable to chloramphenicol palmitate because of unfavorable solubility characteristics (3). Reduction and diazotization methods (4, 5) give the same color response with numerous other compounds containing the aromatic nitro substituent and thus cannot specifically identify the reactant as chloramphenicol. In addition, the latter methods, dependent only on the nitrophenyl group of the molecule, will not reflect degradation involving other parts of the molecule. The ultraviolet spectrophotometric procedure (6) for the assay of chloramphenicol, its derivatives, and their many dosage forms, reflects the absorbance, irrespective of its origin, of the test solution at wavelengths from 271 to 278 mμ. Thus, the chloramphenicol ingredient must be separated from other components of its dosage forms, which can give erroneously high results. Also, the spectrophotometric procedure will not reflect degradative modifications not involving the nitrophenyl group.

In the course of his work on a colorimetric test for dihydrostreptomycin, Weiss (7) discovered that chloramphenicol, after acid and alkaline hydrolysis, produced a blue color with 1-naphthol in alkaline solution. This result proved to be unique among all the antibiotics then available. The present work was undertaken to establish the optimum conditions for the utilization of the reaction as an analytical procedure, and to check its specificity among various antibiotics and compounds structurally related to chloramphenicol.

The reduction, diazotization, and coupling method of Glazko (4) as modified by Levine and Fischbach (5), the ultraviolet spectrophotometric assay (6), and the microbiological assay (1, 2) were used as corroborative procedures. Further modification of the reduction-diazotization procedure was found to be of advantage, and the details of the method used are given below.

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EXPERIMENTAL

Preparation of Chloramphenicol Samples

Chloramphenicol Powder—Accurately weigh approximately 50 mg. of the working standard or sample, transfer to a 250-ml. volumetric flask, dissolve in about 5 ml. of methanol, dilute to volume with water, and mix. For the analysis of chloramphenicol palmitate, dilute the standard to volume with chloroform instead of water.

Capsules—Place the contents of five 250-mg. capsules in a 250-ml. volumetric flask, swirl with approximately 25 ml. of methanol, bring to volume with water, and mix. Dilute this solution with water to an estimated concentration of 0.1 mg./ml.

Tablets—Grind five tablets to a fine powder. Accurately weigh approximately 60 mg. (equivalent to 50 mg. of chloramphenicol) and transfer to a 250-ml. volumetric flask. Add 10 ml. of methanol, swirl the mixture for several minutes, bring to volume with water, and mix.

Chloramphenicol Sodium Succinate Powder—Accurately weigh approximately 70 mg. (equivalent to 50 mg. of chloramphenicol), transfer to a 250-ml. volumetric flask, and dissolve in and bring to volume with water.

Chloramphenicol Palmitate Powder—Accurately weigh approximately 87 mg. (equivalent to 50 mg. of chloramphenicol), transfer to a 250-ml. volumetric flask, and dissolve in and bring to volume with chloroform.

Oral Suspension—Measure 1 ml. of suspension, using a syringe fitted with a 13-gauge needle, and place in a 125-ml. separator containing 25 ml. of chloroform. Shake the separator vigorously for

2–3 min., add 10 ml. of water, and shake vigorously for an additional 2–3-min. period. Allow the layers to separate and filter the chloroform layer through absorbent cotton one or more times until the filtrate is clear. Dilute an aliquot of filtrate with chloroform to a concentration of 0.2 mg. of chloramphenicol per ml.

1-Naphthol Method

Reagents—1-Naphthol, 2 mg./ml. in 6 *N* sodium hydroxide, prepared immediately before use by dissolving 1-naphthol in a small amount of methanol, adding 12 *N* sodium hydroxide and diluting 1:1 with water. Sodium hydroxide (12 *N*) prepared from a 50% solution.

Procedure—Into 100-ml. volumetric flasks place 4.0, 6.0, and 8.0 ml. of the standard solution and 6.0, 4.0, and 2.0 ml. of water to give a final volume of 10 ml. Use a flask containing 10 ml. of water as a blank.

Place 5.0 ml. of the 0.2 mg./ml. solutions of chloramphenicol or chloramphenicol sodium succinate into 100-ml. volumetric flasks and add 5.0 ml. of water.

In the case of capsules and aqueous injection, assay 10.0 ml. of solutions containing 0.1 mg./ml.

For chloramphenicol palmitate chloroform solutions, pipet 5.0 or 6.0 ml. into 100-ml. volumetric flasks and evaporate to dryness at room temperature, using a stream of air. Dissolve the residue in 1 ml. of methanol by careful swirling and then dilute with 10 ml. of water.

To each flask add 10 ml. of 12 *N* sodium hydroxide and 10 ml. of 1-naphthol solution. Swirl the flasks and place in a boiling water bath for 30 min.; then cool to room temperature within the next 5 min. in a tap water bath. Add methanol to volume and mix. After a color development period of 1 hr., measure the absorbance at 610 mμ against the reagent blank, using a suitable spectrophotometer.

Precision of the 1-Naphthol Method—A series of 75 absorbance *versus* concentration experiments, each involving from one to four concentrations, was performed and the 200 absorptivity (1 mg./ml., 1 cm.) values ranged from 24.1 to 40.4 (average 33.3), with 95% of them being between 30.0 and 37.3. The deviation of any absorptivity value from the average in those experiments with two or more samples was less than 3% for 158 of the 172 values determined. Because of the large variation in absorptivity values among different groups of two or more as opposed to the good precision obtained within groups, it was concluded that the absorptivity value of the standard should be determined each time samples are assayed. In addition, the absorptivity value obtained varied with different lots of 1-naphthol.

Effect of Changes in Conditions, Reagents, and Procedure—The blue color could not be extracted into chloroform from alkaline solution. However, when the solution was acidified and extracted with chloroform, and the chloroform extract shaken with fresh alkali, the color appeared in the alkaline phase.

When the blue solution was gradually neutralized, the blue color remained visible, though lessened, down to pH 9.6. From 9.6 to 9.1, it gradually disappeared, leaving a pink color. Addition of alkali restored the blue color.

It was found that 1-naphthol may be added to the reaction either before or after the heating step. Prior addition was found to be superior if aqueous solutions of chloramphenicol were employed, but subsequent addition was found to be advantageous when the chloramphenicol was dissolved in methanol, although the latter procedure was not sufficiently reproducible for quantitative applications.

The ability of 1-naphthol to produce the blue color decreased notably when this reagent was prepared in less concentrated (1 *N*) sodium hydroxide. When the heating was done in less concentrated alkali or the alkali was partially neutralized before dilution with methanol, the blue color was less stable, *i.e.*, the final color would be purple or bluish-pink. The same result occurred when in the final step the reaction mixture was diluted in water rather than methanol. When the solution was partially neutralized (pH 11 or 12) following hydrolysis, the blue color appeared more rapidly, but on standing it would not achieve the same final intensity and purity as when 6 *N* sodium hydroxide solution was used.

Low temperature (80–85°) reduced the reaction rate and was impractically slow, while high temperature (120–150°) experiments disclosed no apparent advantage.

In some experiments 10 ml. of water was added prior to dilution with methanol. This was done to eliminate carbonate precipitation which occurred occasionally after the addition of methanol.

Other alcohols, such as ethanol, 1-propanol, or isopropanol, either decreased the blue color (ethanol) or produced a green solution (propanol).

When dissolved in methanol, *p*-nitrobenzaldehyde reacted similarly to chloramphenicol, but when dissolved in water it produced a green color. Dichloroacetic acid, nitrobenzene, and benzaldehyde did not give a color.

2-Naphthol, 1,4-naphthoquinone, and 1-naphthol-3,6-disulfonic acid did not give a color response when substituted for 1-naphthol. When 1-naphthylamine and 1-hydroxy-2-naphthoic acid were substituted, the former gave a pink color of lower intensity and the latter gave an unstable blue color.

Mechanism of the 1-Naphthol Reaction—The determination of the chemical nature of the colored complex and of the products of the heating step was not deemed to be within the scope of this investigation. However, it is notable that *p*-nitrobenzaldehyde produces azoxy compounds (8) and chloramphenicol gives azo compounds (9) when heated in alkaline solution. A coupling reaction between 1-naphthol and azo compounds has been proposed (10). Further, the production of a blue color in concentrated sodium hydroxide by the reaction of 1-naphthol with *p*-nitrodiazobenzaldehyde has been used as a test for 1-naphthol in 2-naphthol (11).

A similar reaction, production of a blue color with thymol and chloramphenicol in alkali, is preceded by reduction of the chloramphenicol by means of aluminum wire (12).

Diazotization Method

Reagents—Five percent aqueous sodium nitrite, 5% aqueous sodium sulfamate, and 0.5% aqueous *N*-(1-naphthyl)ethylenediamine dihydrochloride solutions. Aqueous sodium hydrosulfite containing 25 mg./ml. prepared immediately before use.

Procedure—Into 100-ml. volumetric flasks pipet 1.0, 2.0, and 3.0 ml. of a 0.2 mg./ml. solution of

TABLE I—RESULTS OF FOUR ASSAY METHODS APPLIED TO CHLORAMPHENICOL SAMPLES

Sample	Label Claim	Assay Results Found by Four Methods— 1-Naphthol	Red.-Diaz.	UV	Microbiological
Chloramphenicol-1	1000 mcg./mg.[a]	992, 1016	[b]	989, 981, 981	1012
Chloramphenicol-2		997, 1003	[b]	981, 975, 981	1013
Chloramphenicol-3		1003, 973	[b]	1010, 980, 971	992
Chloramphenicol-4		1003	[b]	977	963
Capsule-1	250 mg./capsule	261, 251	[b]	250	[b]
Capsule-2		266, 257	[b]	250	[b]
Capsule-3		252, 264	[b]	252	[b]
Tablet	250 mg./tablet	250, 254, 245, 251	246, 259 246, 255	242, 249	[b]
Aqueous injection	1 Gm./vial	1.090, 1.071, 1.051 1.062, 1.065, 1.058	[b]	1.020, 1.140 1.130	[b]
Chloramphenicol sodium succinate	726 mcg./1 mg.[a]	710, 736, 746, 722, 710	[b]	747, 743, 745	[b]
Chloramphenicol palmitate-1	575 mcg./mg.[a]	562, 568	571, 577, 578	587, 587, 587	[b]
Chloramphenicol palmitate-2		599, 562	573, 541, 564	560, 560, 560	[b]
Oral suspension-1	31.25 mg./ml.	33.7, 33.2, 34.8 33.3, 33.7, 32.8	33.3, 31.8, 34.5 33.0, 34.4, 34.1	36.8, 35.4	[b]
Oral suspension-2		24.2 25.1	[b] [b]	25.6, 25.6	[b]

[a] Theoretical potency. [b] Not tested.

chloramphenicol standard, 2.0 ml. of 0.2 mg./ml. solutions of chloramphenicol powder, or the volume (1–3 ml.) equivalent to 0.4 mg. chloramphenicol of solutions prepared from other dosage forms as in the 1-naphthol method. Add 5.0 ml. of 0.1 *N* sodium hydroxide. (In the case of chloramphenicol palmitate, the chloroform solutions are evaporated to dryness, the residue is dissolved in 1 ml. of methanol, and 5.0 ml. of 0.1 *N* sodium hydroxide is added.) Place the flasks in a boiling water bath for 15 min., allow the flasks to cool to room temperature, and add 2 ml. of sodium hydrosulfite solution to each. Fifteen minutes later, add 1 ml. of sodium nitrite solution and 1 ml. of concentrated hydrochloric acid. After 3–5 min., add 2 ml. of sodium sulfamate. After another 3–5 min., during which the flasks are occasionally swirled vigorously, apply a stream of air to assure elimination of nitrous fumes. To each flask add 1 ml. of *N*-(1-naphthyl)ethylenediamine dihydrochloride solution, 25 ml. of methanol, and sufficient water to make 100 ml. After 2 hr. record the absorbance between 530 and 570 mμ. Peak absorption is observed at 553 ± 2 mμ.

RESULTS

Application of the 1-Naphthol Assay Method—Samples of chloramphenicol powder, capsules, tablets, and aqueous injection vials, chloramphenicol sodium succinate, and chloramphenicol palmitate powder and oral suspension were tested by the 1-naphthol colorimetric test and the ultraviolet spectrophotometric method, and in some cases by the reduction-diazotization procedure or the microbiological turbidimetric assay. As shown in Table I, results obtained by the 1-naphthol method compare favorably with the results of other procedures.

Tests on Degraded Material—Tests were run on a sample of chloramphenicol palmitate oral suspension which had been frozen in transit and thawed by heating. The suspension had a strong caramel odor and was dark brown (ordinarily suspensions have a pleasant flavory odor and are cream colored). As shown in Table II, there was good agreement between the 1-naphthol method and the reduction-diazotization procedure, while the ultraviolet spectrophotometric method gave considerably higher results. The former methods gave values for chloramphenicol content of a magnitude that would be reasonably expected and indicated the product was low in potency, while the latter method reflected excess nonspecific absorption due to the presence of degradation products.

TABLE II—ASSAY RESULTS ON DEGRADED CHLORAMPHENICOL PALMITATE ORAL SUSPENSION

Bottle	Extract	1-Naphthol[a]	Red.-Diaz.[a]	UV[a]
1	1	27.9	27.4	50.3
	2	27.9	27.1	56.0
2	1	24.0	26.9	41.6
	2	25.9	23.9	42.2
3	1	25.3	24.9	48.6
	2	25.0	23.7	48.4

[a] Milligrams chloramphenicol per ml. of suspension. Label claim 31.25 mg./ml.

Aqueous chloramphenicol standard solutions of various ages ranging up to 9 months were assayed by the various methods used above. The ultraviolet and reduction-diazotization methods indicated all solutions to be 100% potent. The microbiological and 1-naphthol assays reflected continuing loss of potency with age. However, the coincidence of the results produced by these two methods was only qualitative, not quantitative, in that the 1-naphthol assays did not show as much loss as the microbiological assays.

Specificity of the Methods—Several compounds structurally related to chloramphenicol were subjected to 1-naphthol, reduction-diazotization, ultraviolet, and microbiological assays. The results summarized in Table III reveal that all the chemical methods were insensitive to differences in structural configuration. The only other modifications to which the 1-naphthol method was insensitive were those involving the acyl group. The *m*-nitro analog of chloramphenicol did not give a blue color.

TABLE III—ASSAY OF CHLORAMPHENICOL AND STRUCTURALLY RELATED COMPOUNDS

Compd.	1-Naphthol Color	1-Naphthol mcg./mg.	Red.-Diaz. mcg./mg.	UV[a] mμ	UV[a] mcg./mg.	Microbiol. Assay[b] Plate[c]	Microbiol. Assay[b] Turb.[d]
Chloramphenicol FDA working std.	Blue	1000	1000	277	1000	1000	1000
Chloramphenicol project std.	Blue	982	982	276	1017	1000	970
Chloramphenicol sample	Blue	997	985	277	1014	1000	1000
p-Nitrobenzaldehyde	Yellow	0	2032	267	3027	0	12.0
p-Nitro-α-acetamino-β-hydroxypropiophenone	Blue	247	949	268	1916	23.1	11.8
L-Threo-1-*p*-nitrophenyl-2-amino-1,3-propanediol	Blue	1357	1451	275	1549	2.3	5.0
DL-Threo-1-*p*-nitrophenyl-2-amino-1,3-propanediol	Blue	1316	1503	276	1534	4.6	1.0
D-Threo-1-*m*-nitrophenyl-2-amino-1,3-propanediol	Green	0	1286	266	1211	0	8.0
L-Threo-1-*p*-nitrophenyl-2-acetamino-1,3-propanediol	Blue	1214	1288	277	1139	0	0.2
DL-Threo-1-phenyl-2-nitro-1,3-propanediol	Yellow	0	0	250	2317	0.8	2.0
D-Threo-1-phenyl-2-amino-1,3-propanediol	Yellow	0	3.5	No peak	0	50.6	5.6
L-Threo-1-phenyl-2-amino-1,3-propanediol	Yellow	0	0	No peak	0	3.2	0.3
DL-Threo-1-phenyl-2-amino-1,3-propanediol	Yellow	0	0	No peak	0	2.4	0.3
DL-Threo-1-phenyl-2-dichloroacetamido-1,3-propanediol	Yellow	0	2.7	No peak	0	0	21.0
D-Threo-1-*m*-nitrophenyl-2-dichloroacetamido-1,3-propanediol	Yellow	0	849	268	765	0	125

[a] Wavelength at which absorption maximum occurred. [b] In terms of mcg. of chloramphenicol equivalent potency per mg. [c] *S. lutea* (1). [d] *E. coli* (2).

1-*p*-Nitrophenyl-2-amino-1,3-propanediol gave 5.5 times the color response of *p*-nitro-α-acetamino-β-hydroxypropiophenone, while the reduction-diazotization and ultraviolet procedures yielded relatively little difference in response with these compounds. Otherwise, the only structural modification to which these methods responded was the loss of the nitrophenyl function. Some of the compounds structurally related to chloramphenicol possessed an antimicrobial activity ranging up to 12.5% of that of chloramphenicol.

SUMMARY

A new analytical method for chloramphenicol involves the reaction between 1-naphthol and the product of heating chloramphenicol in strong alkali. Certain advantages of specificity and applicability over present methods are obtained.

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Keyphrases

Chloramphenicol formulations
Chloramphenicol assay
Colorimetry—spectrophotometric analysis
1-Naphthol—reagent
Mechanism-1—naphthol reaction
Diazotization—analysis