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Chemical Assay of Thiamphenicol and Chloramphenicol

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Spectrophotometric and colorimetric methods for the assay of thiamphenicol (TP) and their application to that of chloramphenicol (CP) are described.

The spectrophotometric method is based upon the reaction of TP with alkali to produce hydrolyzed TP, D-*threo*-2-amino-3-*p*-methylsulfonylphenyl-1,3-propanediol, which then converted to *p*-methylsulfonylbenzaldehyde by periodate oxidation.

The other is a colorimetric determination of the aldehyde with APHS reagent (diethanolamine salt of azobenzenephenylhydrazinesulfonic acid). The new reagent, in comparison to the free acid, has the advantages in the stability on storage both in crystalline and solution state and in the solubility in some solvents.

Thiamphenicol(TP), D-*threo*-2,2-dichloro-N-[β-hydroxy-α-(hydroxymethyl)-*p*-(methylsulfonyl)phenethyl]acetamide, is a potent antibacterial agent.[2]

Relatively little is known about the biotransformation of this drug. In view of the clinical importance of TP, the authors have been studying on its biotransformation.

In this paper, a rapid ultraviolet (UV) method and a colorimetric method for the assay of TP and chloramphenicol(CP) in biological materials are described.

In the UV method, TP and CP can be very simply and rapidly converted into *p*-methylsulfonylbenzaldehyde(MSBA) and *p*-nitrobenzaldehyde (NBA) respectively by a method similar to that described by McChesney, *et al.*[3] These aldehydes are extracted with ethylene dichloride and measured spectrophotometrically.

The colorimetric assay is based on the measurement of the color produced by the reaction of the aldehyde with azobenzenephenylhydrazinesulfonic acid.[4]

The standard procedures of these methods were established by investigating some factors which were concerned with the formation of the aldehydes and the development of the final color.

Experimental

Materials and Reagents——TP, kindly supplied by Eisai Co., Ltd., was recrystallized from water, mp 165—166°. CP having mp of 150—151° was used. MSBA was prepared from TP[3] and recrystallized from EtOH, mp 160°. Azobenzenephenylhydrazinesulfonic acid diethanolamine salt (APHS) was prepared as follows: Azobenzenephenylhydrazinesulfonic acid (0.01 mole), freshly prepared from aniline by the method of Troger, *et al.*,[5] and diethanolamine (0.01 mole) were dissolved in 10 ml of hot 90% (v/v) MeOH, and rapidly filtrated. On cooling, yellow crystals separated; mp 151—152°. *Anal.* Calcd. for $C_{16}H_{23}O_5N_5S$: C, 48.35; H, 5.83; N, 17.62. Found: C, 48.00; H, 5.75; N, 17.44.

APHS Reagent——0.025% (w/v) solution of APHS in MeOH (reagent grade). It should be kept in a cold and dark place, and is valid for one week.

H_2SO_4 Reagent——12% (v/v) solution of H_2SO_4 (reagent grade) in MeOH.

HCl Reagent——MeOH is saturated with dry HCl gas at 0°, and the solution is then diluted to the concentration of 10% (w/v).

1) Location: *a*) *1–35, Nozawa, Setagaya-ku, Tokyo*; *b*) *Kita-12, Nishi-6, Sapporo.*
2) J. Laplassotte and M. Brunaud, *Thérapie*, **16**, 101 (1961).
3) E.W. McChesney, J.M. Shekosky, H.W. Eckert, and R.F. Koss, *J. Pharm. Sci.*, **49**, 28 (1960).
4) F. Feigle, "Spot Test in Organic Analysis," 6th ed., Elsevier Publishing Company, 1960, p. 228.
5) J. Tröger and W. Hille, *Arch. Pharm.*, **244**, 309 (1906).

Result

Methods

UV Method——In a glass-stoppered test tube, 1 ml of sample solution and 1 ml of 1N NaOH solution are placed. The tube is heated in a boiling water bath for 10 min, and then cooled to room temperature. One ml of 1M NaH_2PO_4, 5 ml of ethylene dichloride and 3 ml of 0.5% $NaIO_4$ solution are added to the mixture. In this procedure, the $NaIO_4$ solution should be added as quickly as possible. The mixture is shaken mechanically for 10 min and then centrifuged for 2 min. The absorbance of the clear organic phase is measured at 244 mμ.

For the assay CP, 1 ml of 0.5N NaOH solution is used instead of 1N NaOH solution, and the reaction mixture is neutralized with 1 ml of 0.5M NaH_2PO_4 solution. The absorbance of the organic phase is measured at 266 mμ.

The reagent blanks are run through the same procedure. As Fig. 1 shows, the standard curves were prepared with their solutions ranging from 5 to 50 μg. Conformity to Beer's law over the entire range of the concentration was obtained.

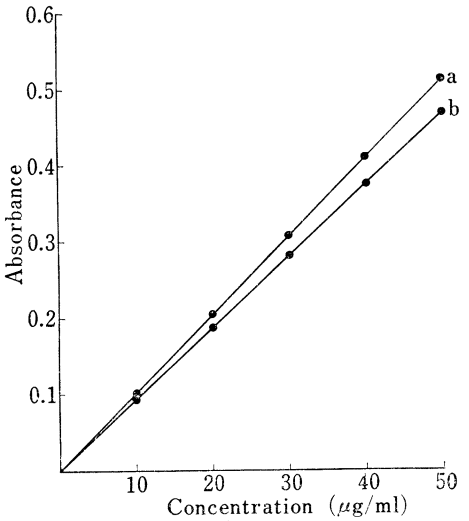

Fig. 1. Relationship between Absorbance and Concentration of Drugs

a: TP (at 244 mμ), b: CP (at 266 mμ)

Colorimetric Method——In a 10 ml measuring flask, 4 ml of the organic phase obtained from the procedure of the UV method, 3 ml of APHS reagent and 1 ml of H_2SO_4 reagent are placed. The mixture is heated in a water bath at 70° for 45 min. After the reaction mixture has been cooled to room temperature, the produced pigment is dissolved in 0.5 ml of MeOH, and the solution is made up to 10 ml with ethylene dichloride. Five ml of the violet solution is transferred into a glass-stoppered test tube containing 4 ml of 5N HCl, and the mixture is shaken mechanically for 10 min. After centrifugation, the absorbance at 415 mμ of the organic phase is measured against a blank prepared through the same procedure. For the assay of CP the absorbance at 438 mμ is measured.

If the more sensitivity is required, 0.5 ml of HCl reagent is added to 4 ml of the yellow organic phase. Then the absorbance of the resulting blue solution is measured at 590 mμ. Both the blue dyes obtained from TP and CP have the same absorption maximum at 590 mμ, as shown in Fig. 2.

The standard curve of each drug was prepared with the solution in concentration from 5 to 50 μg/ml. Conformity to Beer's law over the entire range of the concentration was obtained as shown in Fig. 3.

Establishment of Conditions for the Assay of TP and CP by the UV Method

The procedures described above were obtained after the investigation of some factors which were concerned in the formation of MSBA and NBA and in the development of the final color. The present paper describes mainly the investigation for the assay of TP, since the conditions for the assay of CP, with the exception of the hydrolysis, are substantially the same as TP.

Conditions for the Hydrolysis of TP and CP——Conditions for the hydrolysis of TP and CP were established by varing the time and temperature of the reaction and the concentration of alkali or acid solution.

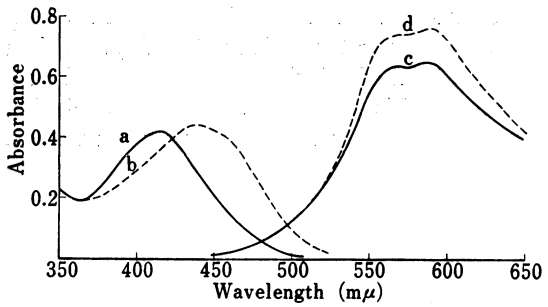

Fig. 2. Absorption Spectra of the Coloring Matters from TP and CP

a : yellow color (TP, 40 μg)
b : yellow color (CP, 40 μg)
c : blue color (TP, 40 μg)
d : blue color (CP, 40 μg)

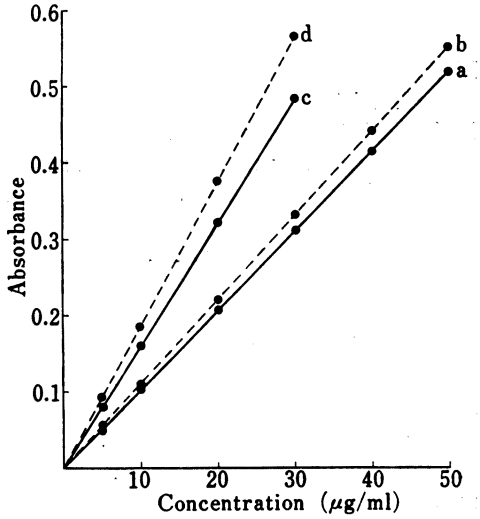

Fig. 3. Relationship between Absorbance and Concentration of Drugs

a: TP (at 415 mμ), b: CP (at 438 mμ)
c: TP (at 590 mμ), d: CP (at 590 mμ)

One ml of samle solution containing 50 μg of TP or CP is pipetted into a glass-stoppered test tube. To the tube is added 1 ml of NaOH solution or HCl solution, and the tube is stood at room temperature or heated in a boiling water bath. After the desired time the reaction solution is treated under the same condition as described in the standard procedure of the UV method. The hydrolysis of TP proceeds completely in 5 min in a boiling water bath with alkali as shown in Fig. 4.

Consequently, the standard procedure adopted 1 N NaOH as the reagent and the reaction time of 10 min in a boiling water bath.

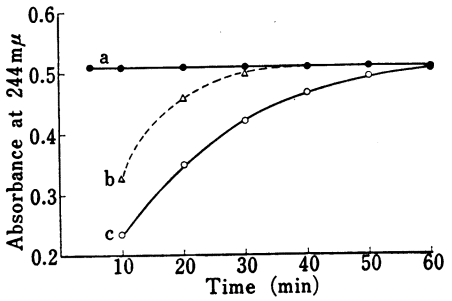

Fig. 4. Hydrolysis of TP

a : 0.25, 0.5, 1.0 and 5.0N NaOH (at 100°)
b : 1.0N NaOH (at room temp.)
c : 5.0N HCl (at 100°)

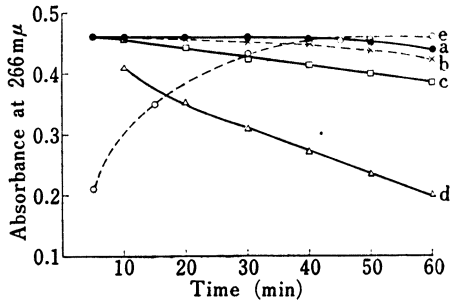

Fig. 5. Hydrolysis of CP

a : 0.25N NaOH (at 100°)
b : 0.5 N NaOH (at 100°)
c : 1.0 N NaOH (at 100°)
d : 5.0 N NaOH (at 100°)
e : 1.0N NaOH (at room temp.)

As Fig. 5 shows, CP is also rapidly hydrolyzed by alkali. However, the continuous decrease in the absorbance of NBA at 266 mμ is observed as the time proceeds. This may be attributed to the decomposition of the hydrolyte of CP to *p*-nitrophenol.[6]

6) W. Doll, *Arzneimittel-Forshung*, **5**, 97 (1955).

In the standard procedure, therefore, 0.5N NaOH solution was used as the reagent for the hydrolysis, and the reaction time for 10 min in a boiling water bath was adopted.

Conditions for Periodate Oxidation——The optimum condition for the periodate oxidation of the hydrolyte of TP, D-*threo*-2-amino-1-*p*-methylsulfonylphenyl-1,3-propanediol, were established by making investigation into the concentration of the reagent, the reaction time and the effect of pH.

Firstly, the effect of the concentration of periodate on the reaction was studied. For this investigation, the hydrolyzed TP solution was prepared as follows. In a 100 ml measuring flask, 10 ml of TP solution (0.4 mg/ml) and 10 ml of 1N NaOH solution are placed. The mixture is heated in a boiling water bath for 30 min. After cooling, 10 ml of 1M NaH_2PO_4 is added to the mixture and then the mixture is made up to 100 ml with distilled water. The pH of the solution was about 7.5 to 8.0.

One ml of the hydrolyzed TP solution is pipetted into a glass-stoppered test tube containing 5 ml of ethylene dichloride, then 3 ml of periodate solution is added into the tube. At the desired time, the mixture is shaken mechanically for 10 min to extract MSBA occurred. After centrifugation, the absorbance of the clear organic phase is measured at 244 mμ.

As Table Ia shows, the oxidation proceeds rapidly in all experiments. However, the continuous decrease in the absorbance at 244 mμ is observed as the time proceeds. This may be due to the oxidation of MSBA with the excess of periodate.

Table Ib shows the results obtained from the experiment in which the oxidation and the extraction were done simultaneously.

Three ml of the periodate solution is added as quickly as possible into the reaction tube containing 1 ml of the hydrolyzed TP solution and 5 ml of ethylene dichloride. And then the tube is shaken mechanically. At the desired time, the reaction mixture is centrifuged, and the absorbance of the clear organic phase is measured at 244 mμ. Under this condition, the absorbance is constant for 40 min in both concentrations of the periodate solution, 1.25 and 0.01%(w/v).

Table Ia. Effect of Periodate Concentration on the Reaction Time

Time (min)	Concentration of $NaIO_4$ solution, % (w/v)			
	1.25	0.50	0.10	0.01
5	0.409	0.409	0.409	0.409
15	0.405	0.405	0.407	0.409
20	0.403	0.403	0.405	0.407
25	0.401	0.401	0.403	0.405

Table Ib. Effect of Periodate Concentration on the Reaction Time

Time (min)	Concentration of $NaIO_4$ solution, % (w/v)	
	1.25	0.01
5	0.410	0.410
10	0.410	0.410
10	0.410	0.409
20	0.410	0.410
30	0.409	0.410
40	0.410	0.410

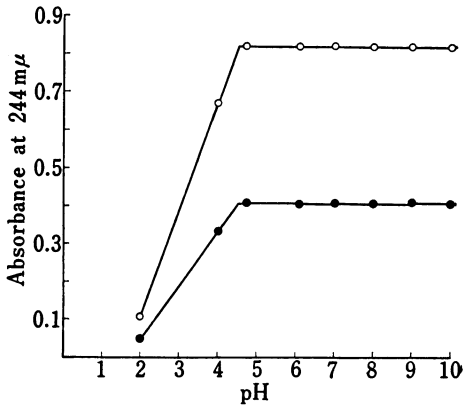

Fig. 6. Effect of pH on the Periodate Oxidation of Hydrolyzed TP

potassium chloride buffer for pH 2.01; phthalate buffer for pH 4.0 and 4.7; phosphate buffer for pH 6.1, 7.01 and 8.0; borate buffer for pH 9.0 and 10.0

—●—: 40 μg (TP), —○—: 80 μg (TP)

In consideration of practical application in biological materials the standard procedure adopted 0.5% periodate solution and the reaction time for 10 min.

Effect of pH on Periodate Oxidation of Hydrolyzed TP——For this study, the test solution was prepared as follows. Four ml of the hydrolyzed TP solution in concentrations from 1 mg/ml to 2 mg/ml as TP is diluted to 100 ml with various buffer solutions.

One ml of the test solution is placed in the reaction tube containing 5 ml of ethylene dichloride. Three ml of 0.5% periodate solution is then added to the mixture, and the experiment is carried out under the same conditions as the standard procedure. The pH of the solution is substantially constant before and after the reaction.

As Fig. 6 shows, at low pH the reaction did not proceed fully, and did at pH higher than 4.7.

Establishment of the Conditions for the Assay of TP by the Colorimetric Method

This method is based on the reaction of the aldehyde, obtained by the UV method, with APHS reagent in the presence of H_2SO_4 reagent to form a violet dye which is turned to a stable yellow color by treatment with 5 N HCl solution. The visible light absorption spectrum of the yellow color is shown in Fig. 2.

The yellow color is turned to a stable blue color again by treatment with HCl reagent. As Fig. 2 shows, its absorbance is about 50% higher than that of the yellow color.

Effect of Temperature and Time of Reaction and Concentration of H_2SO_4 Reagent on Color Development——Firstly, the correlation of temperature and time in the color development was studied.

As Fig. 7 shows, a plateau in the absorbance at 415 mμ existed between 40 and 60 min at 70±1°.

Fig. 8 shows the relationship between the concentration of H_2SO_4 reagent and the reaction time under the standard condition of the colorimetric method. A plateau in the absorbance at 415 mμ existed between 40 and 50 min in the concentration of 12%. Thus, in order to obtain the best reproducibility, the reaction was carried out for 45 min at 70±1°, and 12% (v/v) H_2SO_4 reagent was employed.

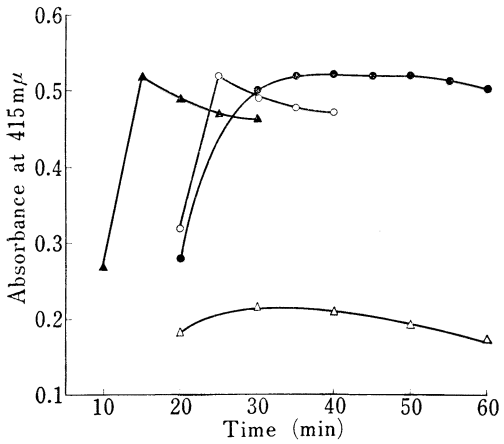

Fig. 7. Effect of Reaction Temperature on Color Development

The test solution is prepared from TP solution (50 μg/ml) by the standard procedure of the UV method. Three ml of 0.025% APHS reagent and 1 ml of 12% (v/v) H_2SO_4 reagent are used.
—△—: 65±1°, —●—: 70±1°,
—○—: 75±1°, —▲—: 80±1°

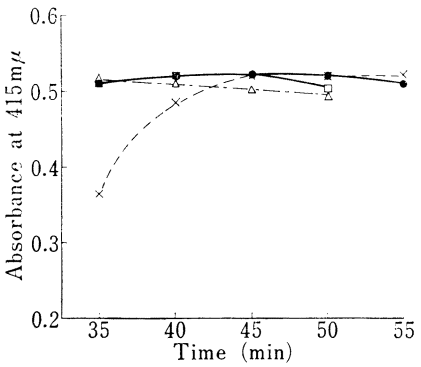

Fig. 8. Relationship between Concentration of H_2SO_4 Reagent and Reaction Time

The test solution containing 50 μg of TP is treated by the same condition as the standard procedure of the colorimetric method except the concentration of H_2SO_4 reagent.
——△——: 5% (v/v), ——□——: 10% (v/v),
——●——: 12% (v/v), ——×——: 15% (v/v)

Concentration of APHS Reagent——The effect of the concentration of APHS reagent on the color development under the same condition as the standard procedure is shown in Fig. 9.

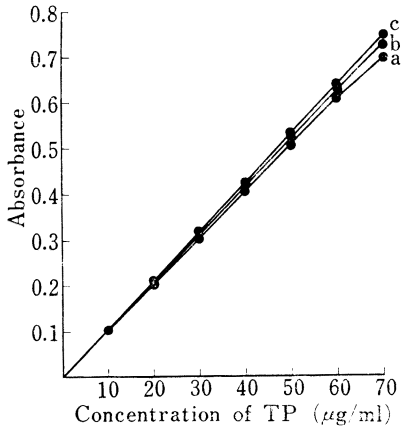

Fig. 9. Relationship between APHS Concentration and Absorbance at 415 mμ

a: 0.01%, b: 0.015%, c: 0.02, 0.025 and 0.03%

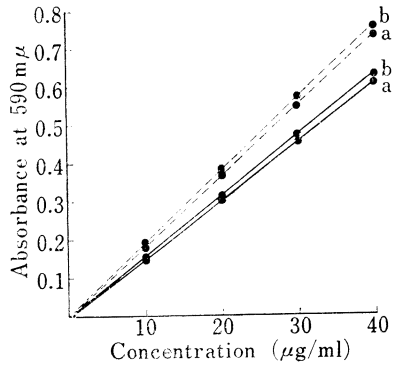

Fig. 10. Relationship between Concentration of HCl Reagent and Absorbance at 590 mμ

—●—: TP, ---●---: CP

a: 5% (w/v), b: 7.5, 10, 15 and 20% (w/v)

It indicates that 0.02% APHS reagent is sufficient for the color development. From the practical view point, therefore, the standard procedure adopted 0.025% APHS reagent.

Relationship between Absorbance at 590 mμ and Concentration of HCl Reagent——The effect of the concentration of HCl reagent on the absorbance at 590 mμ under the same condition as the standard procedure is shown in Fig. 10.

In order to obtain a sufficient color development, the concentration of HCl reagent is required above 7.5%(w/v). Thus the standard procedure adopted 10%(w/v) HCl reagent.

Stability of Colors——Table IIa shows that the absorbances at 415 mμ for TP and at 438 mμ for CP are constant during the observation period, when the colored solutions are allowed to stand for 60 min after the color formation.

Table IIb shows that the absorbances at 590 mμ for TP and CP could be kept in nearly constant readings during the observation period of 60 min. Therefore, the absorbances of the colors were measured within 60 min after the color formation.

Table IIa. Stability of Color

	Time after the color formation, min					
	10	20	30	40	50	60
Absorbance at 415 mμ[a)]	0.415	0.414	0.415	0.415	0.415	0.415
Absorbance at 438 mμ[b)]	0.440	0.441	0.440	0.399	0.440	0.440

a): TP, 40 μg *b*): CP, 40 μg

Table IIb. Stability of Color[a)]

	Time after the color formation, min					
	10	20	30	40	50	60
TP [20 μg]	0.302	0.302	0.302	0.303	0.303	0.302
CP [20 μg]	0.376	0.376	0.376	0.376	0.376	0.376

a) The absorbances at 590 mμ were measured.

Discussion

Hitherto two methods have been described in the literature for the chemical assay of TP.

TP is degraded by alkali hydrolysis, followed by periodate oxidation to give *p*-methylsulfonylbenzaldehyde(MSBA). In the method of McChesney, *et al.*,[3] this aldehyde is measured colorimetrically as the alkali salt of its *p*-nitrophenylhydrazone. The procedure, however, is complicate and requires too much time for the assay.

On the other hand, the method of Forist and Madden, depending on the determination of dichloroacetohydroxiamic acid produced by the reaction of TP with alkaline hydroxylamine,[9] is not adequate for the assay in biological materials because of its insufficient sensitibity.

In this paper, the UV and colorimetric methods for the assay of TP and CP are described.

In the two methods, the UV method is suitable for the rapid determination of TP or CP in some pharmaceuticals, while the colorimetric method is suitable for the assay of these in biological fluids. Generally, there are normal blanks in the estimation of drugs in biological materials. When the normal blanks interfer with the color development, these should be removed prior to the periodate oxidation by extracting with a polar organic solvent such as ethyl acetate.[8]

Azobenzenephenylhydrazinesulfonic acid has been used for the detection of aldehydes.[4] This reagent is, however, considerably unstable even in crystalline state. The present paper proposes its diethanolamine salt(APHS) which is easily obtained in pure crystalline state from each mole of the two components. This new reagent is considerably stable when it is kept in dry state. Its solution in MeOH is also stable at least for one week.

The yellow dye obtained from APHS reagent and MSBA, or APHS reagent and NBA is turned to the blue color which has absorption maximum at 590 mμ by adding HCl reagent. Its absorbance is about 50% higher than that of the yellow color. However, in consideration of practical application in biological materials, the yellow color may be quite enough for these assay.

The formation of the blue color may be also applied to separable determination of TP and CP. That is, the sum of TP and CP is determined by the colorimetric method described in this paper, and the assay of CP in the mixture may be made by the method of Glazko, *et al.*[9]

7) A.A. Forist and S.T. Madden, *J. Pharm. Sci.*, **50**, 269 (1961).
8) T. Arita, R. Holi, and T. Uesugi, *Chemotherapy*, **19**, 843 (1971).
9) A.J. Glazko, L.M. Wolf, and W.A. Dill, *Arch. Biochem. Biophys.*, **23**, 411 (1949).