ESTIMATION OF CHLORAMPHENICOL CINNAMATE

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Chloramphenicol cinnamate is a yellowish-white crystalline powder free from the bitter taste of chloramphenicol, and therefore of particular value for administration to young children. It may be prepared by reacting chloramphenicol with cinnamyl chloride. It is almost insoluble in water, but readily soluble in ethanol and in ethyl acetate. It has a melting point of 115° to 116°, and an optical rotation $[\alpha]_D$ of +55·0° to +58·0°. It is without antibacterial action *in vitro* and cannot therefore be assayed microbiologically.

Chloramphenicol cinnamate is administered in the form of an aqueous suspension ("Alficetyn" Suspension) and although the potency of this preparation when freshly made can be satisfactorily controlled by chemical methods, for example by the polarographic method of Hess[1], or by the colorimetric method of Bessman and Stevens[2], these may give misleading results when used to determine the potency of samples after storage, since certain degradation products containing a nitro group, such as 1-(*p*-nitrophenyl)-2-amino-propane-1 : 3-diol, are indistinguishable polarographically from chloramphenicol itself. Furthermore, chloramphenicol cinnamate cannot be converted into free chloramphenicol as a preliminary to microbiological assay by chemical methods of hydrolysis, as the reaction does not cease with the formation of chloramphenicol, the dichloracetyl radical also being eliminated, giving 1-(*p*-nitrophenyl)-2-aminopropane-1 : 3-diol. It seemed probable, therefore, that only an enzymic method of hydrolysis would be likely to convert chloramphenicol cinnamate quantitatively to chloramphenicol.

Similar difficulties have been reported in the assay of chloramphenicol palmitate and stearate, two other tasteless chloramphenicol derivatives which cannot be assayed microbiologically until converted into chloramphenicol. Glazko, Edgerton, Dill and Lenz[3] found that the palmitate could be quantitatively converted into chloramphenicol by incubation with a bacterial lipase preparation. Trolle-Lassen[4], using a similar method, obtained satisfactory results with the stearate. Both groups of workers reported incomplete hydrolysis with pancreatic and intestinal enzyme preparations, whilst Glazko and others[3] obtained the following figures for the percentage of chloramphenicol palmitate hydrolysed after incubation with different rat tissue extracts: liver, 10; kidney, 8; spleen, 5; duodenal contents, 95·6.

We examined the effect on chloramphenicol cinnamate of several enzyme preparations, namely, saliva, pancreatin, soya bean lipase, castor oil bean lipase, and rat liver extract. When incubated for 4 or 24 hours at 37° with saliva or pancreatin the resulting solution gave no zones of inhibition in the cup plate test, using *E. coli* or *Sarcina lutea* as test

organism. Treatment with ground soya bean powder produced only partial hydrolysis, 10 mg. of chloramphenicol cinnamate after incubation with 5 g. of soya bean meal in 100 ml. of water for 24 hours at 37° with stirring, giving a response equivalent to only 30 per cent of the theoretical amount of chloramphenicol when estimated by means of *Sarcina lutea*. Castor oil bean powder gave somewhat better results, 10 mg. of chloramphenicol cinnamate after incubation with 2 g. of castor oil bean powder in 100 ml. of water for 24 hours at 37° giving a response equivalent to 50 per cent of the theoretical amount of chloramphenicol. When the amount of castor oil bean powder was doubled the value was increased to 60 per cent after 24 hours incubation, to 80 per cent after 48 hours incubation, and to 90 per cent after 72 hours incubation. Thus chloramphenicol cinnamate would not appear to be so readily hydrolysed by lipase as are chloramphenicol palmitate and stearate.

On the other hand, chloramphenicol cinnamate, unlike the palmitate or stearate, is readily hydrolysed by rat liver extract, and satisfactory results were eventually obtained by this means. An ethanolic solution of chloramphenicol cinnamate was added to the rat liver extract, and the mixture was stirred continuously at 37°. Lower results were obtained if the stirring was only intermittent. Incomplete hydrolysis was also obtained with livers from rats that had been used for determining the toxicity of drugs, so that only livers from healthy rats should be used.

Assay of Chloramphenicol Cinnamate

One gram of fresh liver from a healthy rat (Wistar strain) was ground in a pestle and mortar and extracted with sterile distilled water to give a volume of 94 ml. Ten mg. of chloramphenicol cinnamate were dissolved in 4 ml. of absolute ethanol and the solution was added to the rat liver extract giving a colloidal solution. After adjusting the pH to 7·2 with 0·1N sodium hydroxide, the volume was made up to 100 ml. with more sterile distilled water, and 1 ml. of chloroform was added as a preservative. The mixture was immersed in a water bath at 37° and stirred continuously for 24 hours. At the end of this time the solution was assayed by the cup-plate method using *Sarcina lutea* as the test organism, and chloramphenicol as a standard.

Method

Test organism. *Sarcina lutea* NCTC 8340 was grown for 24 hours at 30° on nutrient agar slopes (Medium I) stored at 5° and renewed at monthly intervals.

Inoculum. The growth from a slope culture (Medium I) incubated at 30° for 24 hours was washed into quarter-strength Ringer solution and the suspension was adjusted to an opacity equivalent to the Wellcome Standard opacity tube No. 4. A 0·6-ml. portion was then added to 250 ml. of plate medium (Medium II).

Preparation of plates. The required amount of medium was melted by steaming, cooled to 50° and the inoculum was then added. Fifteen ml. quantities were transferred to standard 4-inch Petri dishes resting on a

level surface; when the agar had set, the plates were removed to a refrigerator. When sufficiently cooled, six 5-mm. diameter discs were cut out of each plate using a sterile cork-borer, and the plates were then returned to the refrigerator until required.

Standard and test solutions. Twenty mg. of pure chloramphenicol were dissolved in 2 ml. of ethanol and the solution was made up to 25 ml. with a phosphate buffer solution (Medium III). Dilutions were made with phosphate buffer solution to give solutions containing 20 and 40 μg. per ml. of chloramphenicol. The test solution was diluted 1:2 and 1:4 with buffer solution to give a "2 and 2" dose assay[5].

Media

Medium I. Nutrient Agar

Eupepton No. 2 (A & H)	10 g.
Sodium chloride	5 g.
Lab Lemco	10 g.
Agar	15 g.
Distilled water to	1000 ml.

Dissolve by steaming and adjust the pH to 7·2. Sterilise in 2-oz. McCartney bottles by autoclaving for 30 minutes at 10 lb. pressure.

Medium II. Plate Agar

Eupepton No. 2 (A & H)	6 g.
Lab Lemco	1·5 g.
"Yeastrel"	3 g.
"Cerelose" (Dextrose)	1 g.
Agar	2·0 g.
Distilled water to	1000 ml.

Dissolve by steaming, adjust the pH to 7·0 and autoclave for 30 minutes at 10 lb. pressure.

Medium III. Phosphate Buffer Solution

Dipotassium hydrogen phosphate ..	7·3 g.
Dihydrogen potassium phosphate ..	3·4 g.
Distilled water to	1000 ml.

Adjust to pH 7·0 and sterilise by autoclaving for 30 minutes at 10 lb. pressure.

Filling the plates. A "set" of four plates was removed from the refrigerator, and any fluid in the holes was removed by suction. Sufficient of the diluted standard and test solutions were put into the holes to fill each to the same level, with a slightly concave meniscus. The filler used for this purpose consisted of a 3-inch length of narrow bore glass tubing, with a $\frac{1}{4}$-inch platinum tube, internal diameter 0·0295 inch, external diameter 0·0365 inch, fused into the tip at an angle of 130°, and a rubber teat at the other end. The tube was rinsed between each change of sample, first with phosphate buffer solution and then with the next

sample to be filled. After being filled, each "set" of plates was incubated for 18 to 20 hours at 30°.

Evaluation of Results

After incubation, the diameters of the zones of inhibition were measured by means of calipers, and the amount of chloramphenicol, in μg. per ml., in the test solution was calculated.

The method of calculation is illustrated by the following example, in which solutions of chloramphenicol cinnamate containing approximately 10 mg. per 100 ml. were assayed (Samples P and Q). The zone diameters, less 12 mm. to simplify the arithmetic, of each sample are recorded, as in Table I (A), S_L and S_H representing the low and high doses of standard,

TABLE I

POTENCY AND FIDUCIAL LIMITS OF CHLORAMPHENICOL CINNAMATE SOLUTION SAMPLES P AND Q

The solutions were diluted 2- and 4-fold, and the standard solutions contained 40 and 20 μg. per ml. of chloramphenicol

(A)

S_L	P_H	Q_L	S_H	P_L	Q_H	Total
1·5	5·0	0·5	5·5	0·5	5·5	18·5
1·0	5·0	1·0	5·5	0·5	6·0	19·0
0·5	5·0	0·0	5·5	0·5	5·0	16·5
1·5	5·0	0·5	5·5	0·5	5·0	18·0
4·5	20·0	2·0	22·0	2·0	21·5	72·0

(B)

	L	H	Total	L_1	L_p
S	4·5	22·0	26·5	17·5	
P	2·0	20·0	22·0	18·0	−4·5
Q	2·0	21·5	23·5	19·5	−3·0
Total	8·5	63·5	72·0	55·0	

and P_L, P_H, Q_L and Q_H the low and high doses of the two samples P and Q. The sums of the responses are then computed and used to calculate L_1, the differences between the high and low dose totals, and L_P, the values of $\Sigma P - \Sigma S$ and $\Sigma Q - \Sigma S$. These values are recorded in Table I (B).

TABLE II

ANALYSIS OF VARIANCE

Correction Term: 216·0

Source	S. of S.	d.f.	M.S.	F.	P.
Regression	126·0417	1			
Preparations	1·3125	2	0·65625	7·62	<0·01 H.S.
Parallelism	0·2708	2	0·1354	1·57	>0·05 N.S.
Doses	127·6250	5			
Plates	0·5833	3			
Error	1·2917	15	0·0861		
Total	129·5	23			

An analysis of variance is then carried out to test the significance of the regression and the absence of lack of parrallelism. The results are given in Table II. The "Preparations" term is normally small, but in the present example was highly significant, indicating that the samples were not diluted to precisely the same range as the standard. Since the

"Parallelism" term was not significant, however, the assays are regarded as valid. The breakdown of the doses sum of the squares into "Regression", "Preparations" and "Parallelism" terms is best accomplished by the use of detached coefficients of orthogonal contrast[6] and the breakdown of the total sum of the squares into "Doses", "Plates" and "Error" terms by the normal rows and columns analysis. The sums of the squares are calculated as follows:

$$\text{Regression} = \frac{(\Sigma L_1)^2}{24} = 126 \cdot 0417$$

$$\text{Preparations} = \frac{(\Sigma S)^2 + (\Sigma P)^2 + (\Sigma Q)^2}{8} - \text{Correction term} = 1 \cdot 3125$$

$$\text{Parallelism} = \frac{(\Sigma L_1 S)^2 + (\Sigma L_1 P)^2 + (\Sigma L_1 Q)^2}{8} - \text{Regression S of S} = 0 \cdot 2708$$

The potency of Sample P and the fiducial limits are calculated as follows:

Log ratio of doses, $I = 0 \cdot 3010$

Error mean square, $s^2 = 0 \cdot 0861$

$$\text{Slope, } b = \frac{\Sigma L_1}{12} = 4 \cdot 583$$

$$\text{For sample P, } M' = \frac{L_P}{8b} = -0 \cdot 12272$$

Log potency ratio, $M = M'.I = -0 \cdot 0369 = \bar{1} \cdot 9631$

Ratio of potencies, $R = \text{antilog } M = 0 \cdot 9185$

The approximate 95 per cent fiducial limits are given by $M \pm t.s_M$ (t has 15 d.f. and equals 2·131) and $t.s_M = \frac{t.I}{b}\sqrt{S^2\left(\frac{1}{4} + \frac{M'^2}{6}\right)} = 0 \cdot 0206$

(The index of significance (g) of the slope b is invariably less than 0·1 in this assay and can be ignored)

whence log potency ratios, M_L and $M_U = \bar{1} \cdot 9425$ and $\bar{1} \cdot 9837$
and ratios of potencies, R_L and $R_U = 0 \cdot 8760$ and $0 \cdot 9632$
equivalent to 95·4 and 104·8 per cent.
Potency $= R \times S_H \times$ dilution of $P_H = 73 \cdot 5$ μg. of chloramphenicol per ml. with limits of 70·1 to 77·0 μg. of chloramphenicol per ml.

The potency of sample Q and the fiducial limits, similarly computed, were found to be 75·6 μg. of chloramphenicol per ml. with limits of 72·1 to 79·2 μg. of chloramphenicol per ml.

Assay of Suspension of Chloramphenicol Cinnamate containing 4·5 *per cent w/v*

To 5 g. of the suspension were added 50 ml. of absolute ethanol and the mixture was thoroughly shaken and allowed to stand. Two ml. of the clear supernatant liquid were then removed and added to 90 ml. of

an extract prepared from 1 g. of finely ground rat liver. The pH was adjusted to 7·2 and the volume was made up to 100 ml. with sterile distilled water. Two ml. of chloroform were added as preservative and the suspension was immersed in a water bath at 37° and stirred continually for 24 hours. The solution was assayed as described above against *Sarcina lutea.*

SUMMARY

1. Chloramphenicol cinnamate cannot be assayed microbiologically until converted into free chloramphenicol. Most hydrolytic agents examined either give incomplete hydrolysis or hydrolyse the substance beyond the chloramphenicol stage. Quantitative conversion to chloramphenicol was however achieved by incubation with rat liver extract.

2. A method of assaying chloramphenicol cinnamate and its suspensions is described.

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