Determination of 6-Demethyltetracycline HCl in 6-Demethylchlortetracycline HCl

By FORTUNATO S. CHICCARELLI

6-Demethyltetracycline, when heated with nitric acid, forms a stable colored derivative having an absorption maximum at 425 mμ. 6-Demethylchlortetracycline under the same condition is unstable. When 6-demethylchlortetracycline is heated with nitric acid, the color produced by the 6-demethyltetracycline fraction can be measured directly or extracted with *n*-butanol.

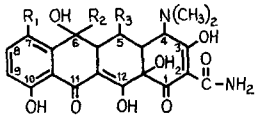
In the production of 6-demethylchlortetracycline (1)[1] small amounts of 6-demethyltetracycline are also formed. An automated method of assay for 6-demethylchlortetracycline based on the difference in the rates of conversion of these components to their respective anhydro forms has been presented (2). A manual procedure (3) based on the same principle has also been developed. To detect the presence of small concentrations of 6-demethyltetracycline in 6-demethylchlortetracycline, a direct analytical procedure was required.

Tetracycline, chlortetracycline, 6-demethyltetracycline, and 6-demethylchlortetracycline all undergo transformation to anhydro compounds when their respective solutions are heated with hydrochloric acid. The order of stability to HCl is 6-demethylchlortetracycline, 6-demethyltetracycline, chlortetracycline, and tetracycline with 6-demethylchlortetracycline being the most stable. Oxytetracycline forms colorless apoxytetracyclines (4).

With this in mind the above antibiotics were heated with other acids, one of which was nitric acid. A relatively stable intense yellow color remained in the 6-demethyltetracycline solution while the other solutions became relatively colorless. It was decided to investigate this difference in behavior.

EXPERIMENTAL

Scheme I shows the structural formula of the antibiotics tested. A diagrammatic scheme has been used to point up the differences and similarities.



	R_1	R_2	R_3
Tetracycline	H	CH_3	H
Chlortetracycline	Cl	CH_3	H
5-Hydroxytetracycline	H	CH_3	OH
6-demethyltetracycline	H	H	H
6-demethylchlortetracycline	Cl	H	H

Scheme I

Figure 1 shows the absorption spectra of tetracycline (TC), chlortetracycline (CTC), 5-hydroxytetracycline (OTC), 6-demethyltetracycline (DMTC), and 7-chloro-6-demethyltetracycline (DMCTC) from 300 mμ to 500 mμ at 25 mcg./ml. in 0.1 *N* hydrochloric acid.

Figure 2 shows the absorption spectra of each of the antibiotics after heating at 100° with nitric acid. Solutions of each were made with 200 mcg./ml. 0.07 *M* H_3PO_4. Forty ml. of the solution plus 20 ml. 4.0 *M* HNO_3 were heated in a water bath for 15 min. After cooling and diluting up to 100 ml., the absorption spectra were determined from 300 mμ–500 mμ. Of the five antibiotics studied, DMTC was unique in that a maximum appearing in the visible range (420–440 mμ) was retained. The product(s) of this reaction is now under study.

The effect of heating time at 100° with DMTC and DMCTC in the presence of nitric acid is shown in Table I. Solutions of each antibiotic were made with 200 mcg./ml. 0.07 *M* H_3PO_4. Forty ml. of this solution plus 20 ml. 4.0 *M* HNO_3 were heated in a boiling water bath for the time specified. At the end of each time interval the flasks were cooled and diluted up to 200 ml. with water. The absorbance of the solutions was determined at 425 mμ. The

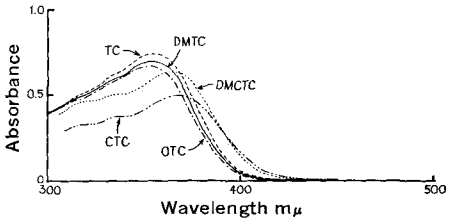


Fig. 1—Absorption spectra of tetracycline (TC), chlortetracycline (CTC), 5-hydroxytetracycline (OTC), 6-demethyltetracycline (DMTC), 6-demethylchlortetracycline (DMCTC) from 300 mμ to 500 mμ at 25 mcg./ml. in 0.1 N HCl.

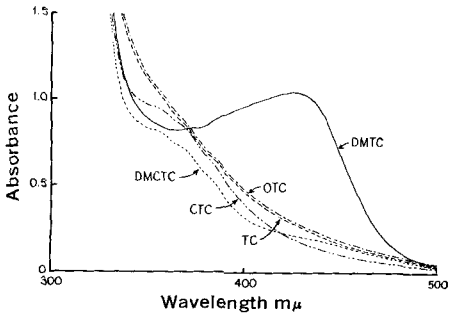


Fig. 2—Absorption spectra of tetracycline (TC), chlortetracycline (CTC), 5-hydroxytetracycline (OTC), 6-demethyltetracycline (DMTC), 6-demethylchlortetracycline (DMCTC), after heating at 100° with HNO_3 for 15 min.

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[1] Declomycin is the registered trademark of American Cyanamid Co. for 6-demethylchlortetracycline HCl.

TABLE I—DIFFERENCE IN ABSORPTION WITH TIME OF HEATING AS A VARIABLE

Time, min.	Absorbance at 425 mμ DMCTC	DMTC
0	0.0	0.0
3	0.345	0.260
6	0.135	0.515
9	0.115	0.505
12	0.110	0.490
15	0.105	0.475
18	0.100	0.460
21	0.095	0.445

high absorbance value at 425 mμ after 3 min. heating suggests the formation of the DMCTC anhydro derivatives. Lower values at the 6-, 9-, 12-min. intervals may be attributed to oxidative cleavage of the anhydro molecule. The high absorbance at 425 mμ of the DMTC after heating in HNO_3 is probably due to a stable 7-nitro-anhydro DMTC.

Figure 3 shows the absorption spectra from 300 mμ to 500 mμ of butanol extracts of DMCTC standard solutions containing 0–8% DMTC. Determinations (*versus n*-butanol) were made with 1-cm. cells.

Figure 4 contains the spectra from 300 mμ to 500 mμ of standard solutions when measured directly with 10-cm. cells. The 0% treated standard solution is used in the reference cell.

Table II contains five separate results when a well-mixed synthetic sample of DMCTC containing 3% DMTC was assayed. The sample was made from standard DMCTC and DMTC.

Application of this procedure to refined DMCTC gave greater precision than the method previously used (5).

METHOD

Apparatus—Beckman spectrophotometer model DU 2400 equipped to handle 1-cm. or 10-cm. quartz cells. Cell correction was applied when necessary. Complete spectra were made with a Cary recording spectrophotometer.

Reagents—Nitric acid, 4.0 *M*, made from reagent grade which is free of yellow oxides of nitrogen; H_3PO_4, 0.07 *M*; *n*-butanol, reagent; NaCl, C.P. 6-demethylchlortetracycline HCl standard, 200 mcg./ml. (100 mg./500 ml.); and 6-demethyltetracycline HCl standard, 200 mcg./ml. (100 mg./500 ml.). Standard solutions are made in 0.07 *M* H_3PO_4.

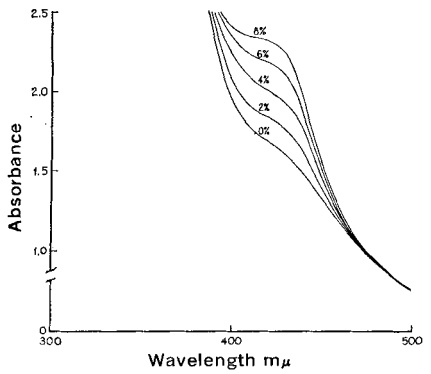

Fig. 3—Absorption spectra of demethyltetracycline (vs. n-butanol) standard solutions when measured after butanol extraction, with 1-cm. cells.

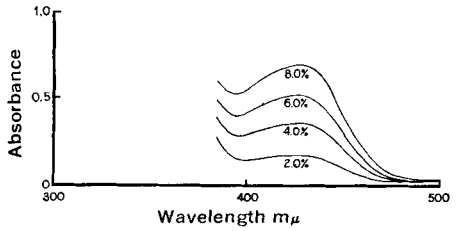

Fig. 4—Absorption spectra of demethyltetracycline standard solutions when measured directly, with 10-cm. cells.

TABLE II—RESULTS WITH SYNTHETIC SAMPLE

Determination	% DMTC
A	3.1
B	2.8
C	2.9
D	3.4
E	3.0

Procedure—Transfer an accurately weighed sample of about 100 mg. DMCTC to a 50-ml. volumetric flask. Add 0.07 *M* H_3PO_4 and mix until solution is complete. Make up to mark with 0.07 *M* H_3PO_4 and mix. Pipet a 40.0-ml. aliquot into a 100-ml. volumetric flask. Add 20 ml. 4.0 *M* HNO_3 and heat in a boiling water bath for exactly 15 min. with occasional swirling.

Standard solutions and samples should be treated at the same time. Pipet 2.0-, 4.0-, 6.0-, and 8.0-ml. aliquots of DMTC standard solution (200 mcg./ml., 0.07 *M* H_3PO_4) into separate 100-ml. volumetric flasks. Make each up to mark with DMCTC standard solution (200 mcg./ml., 0.07 *M* H_3PO_4) and mix. Transfer a 40.0-ml. aliquot of the DMCTC standard solution (0%) and 40.0-ml. aliquots from each of the 2.0, 4.0, 6.0, and 8.0% DMTC standards to separate 100-ml. volumetric flasks. To each add 20 ml. 4.0 *M* HNO_3 and heat in the same manner as the sample.

After heating is complete, remove all flasks, cool, and make to volume with water. Mix. The following alternatives can be used for the absorbance determination.

For 1-cm. cells transfer the solutions to individual separatory funnels containing 40 Gm. NaCl. Shake for 1 min. Add 10 ml. *n*-butanol and shake for 2 min. Collect the butanol extracts and centrifuge. Determine the absorbance (*versus n*-butanol) at 425 mμ using the 0.1 position of the scale. For 10-cm. cells fill the two 10-cm. cells with the 0% standard solution. Using one as the reference, measure the absorbance of the other at 425 mμ. The difference between this is the cell correction. Measure the standard and unknown solutions against the 0% reference solution at 425 mμ.

Plot a concentration curve using the 0, 2, 4, 6, and 8% absorbance values. Before the curve can be used, the sample absorbance must be corrected for the amount of antibiotic in the original weight taken.

Transfer a 5-ml. aliquot of the sample solution to a 100-ml. volumetric flask. Make up to mark with 0.07 *M* H_3PO_4 and mix. Determine its absorbance at 368 mμ. (Acid isobestic point of DMTC and DMCTC.) Repeat the determination with a 5-ml.

portion of the DMCTC standard solution treated in the same manner.

The corrected sample absorbance will give a direct measure of the DMTC when used with the standard curve.

Calculation—(*a*) mg. of antibiotic (DMTC and DMCTC) in sample weight:

$$\frac{\text{sample abs. at 368 m}\mu}{\text{standard abs. at 368 m}\mu} + 10.0 \times \frac{100}{1000} \times \frac{500}{5} = \text{mg.}$$

(*b*) corrected sample absorbance value to be used with concentration curve: $\frac{100}{\text{mg.}} \times$ abs. at 425 mμ (sample) = corrected value

SUMMARY

With the use of nitric acid, an assay for DMTC in DMCTC has been developed. DMCTC is unstable to nitric acid while DMTC is sufficiently stable for purpose of assay. The data presented suggest that DMTC could also be measured in the presence of CTC, TC, and OTC by this procedure.

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Keyphrases

6-Demethyltetracycline in 6-demethylchlortetracycline—analysis

6-Demethylchlortetracycline—nitric acid transformation

Spectrophotometry—analysis

Quantitative Thin-Layer Chromatography of Sympathomimetic Amines

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Thin-layer chromatography for quantitative estimation of sympathomimetic amines has been used. Two methods were performed: the weight/area relationship and the elution of the spots and spectrophotometric determination in the ultraviolet. For the first method, solutions of the amines, in increasing concentration, were applied on cellulose thin layer, and a solution of unknown concentration of the same amines was run parallel to the standards. The spot was compared with the standards using a recording photoelectric densitometer. For the second method the standard spots were eluted from the cellulose plates and their absorption was measured. The absorptivity of a similarly eluted spot of unknown concentration, but parallel running, was measured and compared with the standards. The accuracy of the methods appeared to be between 95–98 percent.

THE APPLICATION of thin-layer chromatography to pharmaceutical analysis has been used increasingly during the past years. A number of methods for quantitative analysis have been employed since the technique was first introduced by Stahl (1) in 1956.

Seher (2) has suggested that in thin-layer chromatography the weight of the material and the spot area are proportional. Purdy and Truter (3) found that quantitation is based on a linear relationship existing between the square root of the area of a component after chromatographing, and the logarithm of the weight of the applied sample. According to them, Seher's data, as well as that of Stahl (4) and Breuner and Niederwieser (5), fit the relationship for loads of 1 to 80 mcg./spot. Pelka and Metcalf (6) examining long-chain tertiary amines, found the method applicable for loads of approximately 200 mcg.

Morrison and Orr (7), in an analysis of selected pharmaceutical mixtures in tablet and capsule forms and by using thin-layer chromatography, obtained quantitative results from the developed chromatograms. A linear relationship between the spot area, in sq. mm., and the weight of the spots was observed.

The method was applicable for concentrations up to 250 mcg. However, this method requires a satisfactory spray reagent, otherwise its use for quantitative work is doubtful.

Ganshirt *et al.* (8) used thin-layer chromatography for the quantitative assay of a number of bile acids, and Zollner *et al.* (9) found the method applicable for the quantitative estimation of cholesterol esters.

Millett *et al.* (10) described other techniques for

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