Colorimetric Assay of Tetracycline Antibiotics

L. G. CHATTEN and S. I. KRAUSE

Abstract □ A sensitive colorimetric method of analysis for seven compounds of the tetracycline series was developed, based on the formation of a colored complex between the tetracyclines and quadrivalent thorium. The effects of pH and time upon the stability of the complexes were observed. The method was applied to 29 different pharmaceutical dosage forms, and satisfactory results were obtained for most of these.

Keyphrases □ Tetracycline dosage forms—analysis □ Thorium nitrate–tetracyclines, complex formation—tetracycline analysis □ pH, time, effects—thorium nitrate–tetracycline complex □ Colorimetric analysis—spectrophotometer

Maintained by the BP 1968 (1) and the USP XVII (2) as the official method for the assay of antibiotics, the microbiological assay serves as the standard method employed by pharmaceutical manufacturers to ascertain the potency of their products. However, numerous shortcomings of this method have stimulated many attempts to develop more rapid and precise techniques for the analysis of tetracyclines. Among these have been several paper chromatographic procedures (3–5) and, more recently, a column chromatographic technique using edetic acid (EDTA)-impregnated earth (6).

In addition to chromatographic procedures, numerous other methods including nonaqueous titrimetry (7, 8), UV spectroscopy (9), and fluorometry (10) have exhibited some usefulness.

Colorimetry is yet another technique that lends itself to the quantitative analysis of tetracyclines. In 1955, Sakaguchi and Taguchi (11) noted the formation of a yellow chelate between chlortetracycline and thorium and subsequently developed a colorimetric method for the determination of chlortetracycline in an acetate buffered system of pH 4.0. This method was later applied also to the quantitative estimation of tetracycline and oxytetracycline.

In the present study, efforts were made to investigate the nature and stability of the complexes formed between the tetracyclines and quadrivalent thorium. The purpose of this work was to develop a simple but efficient colorimetric procedure for the quantitative analysis of all the tetracyclines presently marketed and their dosage forms.

EXPERIMENTAL

Apparatus—The following were used: conventional laboratory glassware, Beckman model B spectrophotometer, Beckman zeromatic II pH meter fitted with glass-calomel electrode system, magnetic stirring apparatus, and Büchner funnels (5 cm. diameter).

Reagents and Solutions—All chemicals employed were ACS or reagent grade in quality. The following were used: ethanol, chloroform, glacial acetic acid, 0.05 *N* perchloric acid in dioxane (standardized against primary standard potassium acid phthalate), 0.5% crystal violet in glacial acetic acid, 0.5% thorium nitrate (aqueous), and 0.5% thorium nitrate (ethanolic).

The tetracycline reference compounds used in this investigation were as follows: tetracycline HCl, chlortetracycline HCl, oxytetracycline HCl, demeclocycline HCl, tetracycline phosphate complex, doxycycline monohydrate, and doxycycline HCl.

Methods—*Determination of Reference Standard Purity*—The purities of the crystalline reference compounds were determined by the nonaqueous titrimetric method of Sideri and Osol (7). The progress of all titrations was followed potentiometrically with a Beckman zeromatic II pH meter. In all instances the purity of the standard was found to be not less than 94.8%. This information was taken into account in subsequent calibration curve preparation for the colorimetric assays.

Preparation of Calibration Curves for Colorimetric Procedure—A 25-mg. sample of the crystalline reference compound was dissolved in 100 ml. of distilled water. Into five separate 10-ml. volumetric flasks, aliquots of 0.2, 0.4, 0.6, 0.8, and 1.0 ml. of the 0.25-mg./ml. stock solution were pipeted. To each of these was added 0.5 ml. of 0.5% thorium nitrate. The content of each flask was

Table I—Data Pertaining to Tetracyclines Employed in Study

Name of Drug	Supplier	$\lambda_{max.}$ Complex, nm.	Molar Absorbance of Complex	Time Required for Stable Color, min.
Tetracycline HCl	Upjohn	395	23,404	20
Oxytetracycline HCl	Pfizer	395	14,784	20
Chlortetracycline HCl	Cyanamid	405	12,714	20
Demeclocycline HCl	Cyanamid	405	12,709	20
Tetracycline phosphate complex	Bristol	395	—	20
Doxycycline HCl	Pfizer	395	19,936	35
Doxycycline monohydrate	Pfizer	395	14,506	35

Table II—Results of Thorium Nitrate Colorimetric Method

Commercial Product	Manufacturer	Labeled Drug Content	Labeled Strength, %: Colorimetric	Labeled Strength, %: Manufacturer's Result[a]
Achromycin capsules	Lederle	250 mg. Tetracycline HCl/ capsule	99.6	99.84
Albamycin T capsules	Upjohn	125 mg. Tetracycline HCl/ capsule	99.9	100.00
Aureomycin capsules	Lederle	Chlortetracycline HCl 250 mg./capsule	101.1	Not available
Declostatin capsules	Lederle	Demeclocycline HCl 250 mg./capsule	97.7	99.90
Resteclin capsules	Squibb	250 mg. Tetracycline HCl/ capsule	101.5	118.00[b] 101.20[c]
Signemycin capsules	Roerig	167 mg. Tetracycline HCl/ capsule	99.5	108.90[c]
Vibramycin Scripak capsules	Pfizer	Doxycycline hyclate equivalent to 100 mg. doxycycline base/capsule	100.7	Not available
Achrocidin tablets	Lederle	Tetracycline HCl 125 mg./tablet	100.1	102.91
Aureomycin soluble tablets	Lederle	Chlortetracycline HCl 50 mg./tablet	100.2	Not available
Declomycin tablets	Lederle	Demeclocycline HCl 300 mg./tablet	96.3	100.79
Achrocidin Compound syrup	Lederle	Tetracycline HCl 25 mg./ml.	99.7	111.72
Declomycin syrup	Lederle	Demeclocycline HCl 15 mg./ml.	98.4	121.83
Resteclin syrup	Squibb	25 mg. Phosphate-potentiated tetracycline/ml.	99.4	116.80[b] 136.80[c]
Signemycin syrup	Roerig	Tetracycline HCl 16.6 mg./ml.	101.2	119.70[c]
Aureomycin ear solution	Lederle	Chlortetracycline HCl 50 mg./vial	99.7	Not available
Terra-Cortril eye/ear suspension	Pfizer	Oxytetracycline HCl equivalent to 5 mg. oxytetracycline base/ml.	98.9	100.00[c]
Resteclin aqueous drops	Squibb	Tetracycline HCl 100 mg./ml.	100.00	109.10[b]
Terra-Cortril ointment	Pfizer	Oxytetracycline HCl equivalent to 30 mg. oxytetracycline base/g.	91.6	Not available
Aureomycin ophthalmic ointment	Lederle	Chlortetracycline HCl 10 mg./g.	89.6	Not available
Vibramycin oral suspension	Pfizer	Doxycycline monohydrate equivalent to 5 mg. doxycycline base/ml. (when reconstituted)	105.7	Not available
Aureomycin surgical powder	Lederle	Chlortetracycline HCl 200 mg./g.	99.6	Not available
Terra-Cortril spray	Pfizer	Oxytetracycline HCl equivalent to 60 mg. oxytetracycline base/ml. (300 mg./can)	88.2	106.33[c]
Tetrex capsules	Bristol	Tetracycline phosphate complex = 250 mg. tetracycline HCl/capsule	107.1	104.40[c]
Tetrex-bid capsules	Bristol	Tetracycline phosphate complex = 500 mg. tetracycline HCl/capsule	106.7	104.00[c]
Tetrex-APC capsules	Bristol	Tetracycline phosphate complex = 125 mg. tetracycline HCl/capsule	110.8	110.40[c]
Azotrex capsules	Bristol	Tetracycline phosphate complex = 125 mg. tetracycline HCl/capsule	107.7	112.00[c]
Azotrex syrup	Bristol	Tetracycline phosphate complex = 125 mg. tetracycline HCl/5 ml.	96.8	108.00[c]
Tetrex syrup	Bristol	Tetracycline phosphate complex = 125 mg. tetracycline HCl/5 ml.	103.9	100.00[c]
Tetrex-AP syrup	Bristol	Tetracycline phosphate complex = 125 mg. tetracycline HCl/5 ml.	99.3	104.00[c]

[a] Data supplied by manufacturer. [b] Microbiological assay. [c] Chemical assay method of manufacturer.

then brought to volume with distilled water, and the flasks were well shaken. After 20–35 min., absorbances were determined in a Beckman model B spectrophotometer against a blank prepared identically to the samples but with the exclusion of the thorium nitrate. The wavelengths employed varied from 395 to 405 nm., depending upon the particular tetracycline under investigation. This information, together with the time required to develop a stable color, is given in Table I. For doxycycline hyclate, a calibration curve was

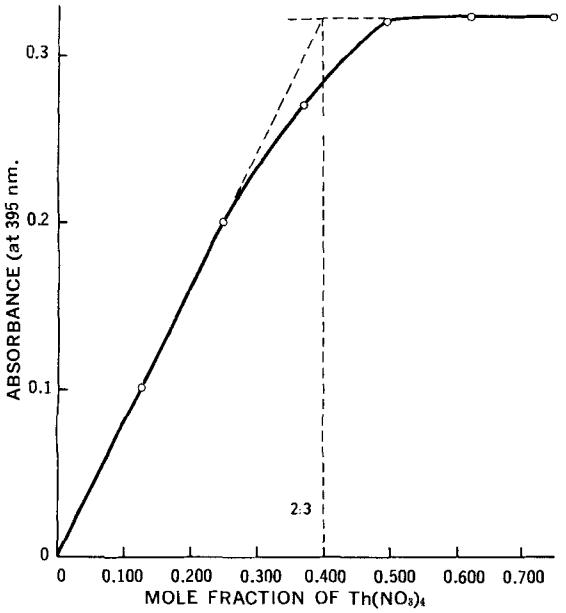

Figure 1—*Molar ratio study of tetracycline–thorium complex system.*

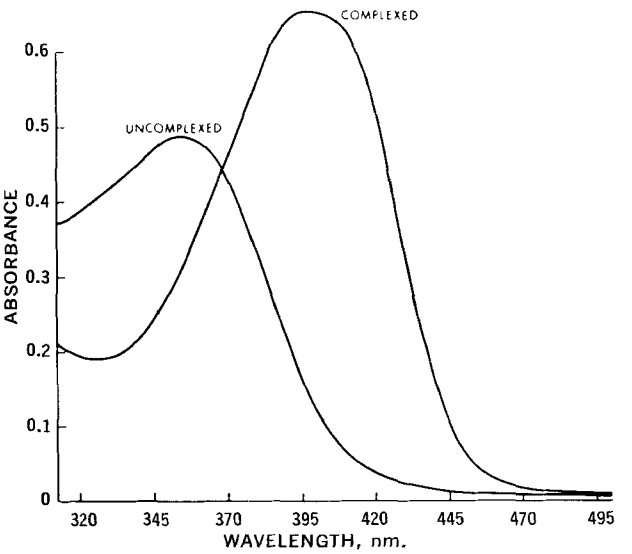

Figure 2—*Absorption spectrum of tetracycline hydrochloride before and after complexation with thorium.*

derived by extrapolation of the data obtained with doxycycline HCl. A calibration curve for an oxytetracycline–thorium complex in chloroform was prepared for the assays of ear/eye suspensions, ointments, and sprays. Good linear curves of absorbance *versus* concentration were obtained in all instances.

Assays of Tablets, Capsules, Syrups, Solutions, and Surgical Powder—Where tablets and capsules were being assayed, samples were taken from the contents of 10 capsules or from the powder obtained by crushing 10 tablets. Solutions containing approximately 25 mg./100 ml. of the particular tetracycline were prepared by placing an approximate quantity of the powdered material or syrup in 100 ml. of distilled water. For tablets and capsules, filtration through Whatman No. 1 filter paper was required. Aliquots of 0.6 ml. of this solution were placed in each of two 10-ml. volumetric flasks. To one of these was added 0.5 ml. of 0.5% thorium nitrate, and the contents of both flasks were brought to volume with distilled water. After a suitable period of time (Table I), the absorbance of the solution containing tetracycline and thorium nitrate was read against a blank (the solution to which thorium nitrate had not been added) at the optimum wavelength.

Assay of Ear/Eye Suspensions—The same procedure was used except that chloroform was employed as the solvent and 0.5% thorium nitrate (ethanolic) was the color-inducing reagent.

Assay of Ointments—The initial 0.025 mg./ml. solution was prepared by heating and stirring a suitable weight of ointment with 90 ml. of distilled water prior to filtration and dilution to an accurate volume. The previous procedure for solutions was then applicable.

Assay of Antibiotic Sprays—A short length of Tygon tubing was attached to the spray nozzle of the can, and the entire contents were expelled into a beaker. The propellant was boiled away, and the remaining liquid was brought to volume with chloroform in a 1000-ml. volumetric flask. Suitable aliquots were than transferred to 10-ml. volumetric flasks for subsequent treatment as with eye/ear preparations.

RESULTS

A large variety of pharmaceutical dosage forms were assayed for their tetracycline content. All figures given in Table II for the colorimetric assay are averages of five determinations. From these data it can be seen that results obtained by the thorium complexation method agree favorably with the manufacturer's results for all tablets and capsules investigated. However, a few discrepancies appear in the comparison of results for some syrups and drops. These differences can be attributed generally to a small degree of chemical alteration and degradation of the constituents between the time of the manufacturers' assays and the thorium complexation assays.

Attempts to achieve good agreement with the manufacturer's results for the antibiotic spray investigated were unsuccessful, probably due to the extreme difficulty in expelling the entire contents of the spray can. Since the method employed was based upon the use of the total can contents, any residual liquid in the can would necessarily lead to low results.

DISCUSSION

The electronic structure of thorium has not yet been fully established to the satisfaction of all physical chemists concerned. Dissent has arisen in the elucidation of the ground-state electronic configuration in the levels outside the radon structure. The two most generally accepted theories are that these outer levels are either occupied as $6d^2$, $7d^2$ or as 5f, 6d, $7s^2$. In which of the actinide elements the first development of a 5f level occurs is not known.

Thorium has been shown to be capable of valences of 2, 3, or 4. In the present study, Th^{+4} is made available for complexation with the tetracyclines as a result of ionic release in aqueous solution from its salt complex $Th(NO_3)_4$. The complexing ability of the tetracyclines is generally attributed to the availability of oxygen for bond formation. The presence of hydroxyl groups in the ligand leads to the ready formation of a species containing thorium as a complex cation.

The stoichiometry of the complex formed between thorium and the tetracyclines is uncertain. However, data obtained from a molar ratio study (Fig. 1) suggest two possibilities: (*a*) that a 2:3 thorium–tetracycline complex is formed, and (*b*) that in the presence of ex-

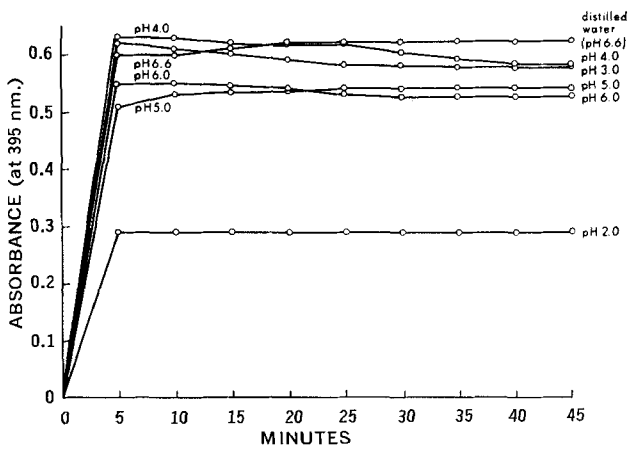

Figure 3—*Effect of pH on color development for tetracycline HCl.*

cess thorium, an equilibrium mixture is formed containing a constant proportion of complexes having differing stoichiometry. If, in fact, only one complex is being formed, its formation constant can be derived from the same data and is 7.7×10^4.

In the series of tetracyclines investigated, absorption maxima of the uncomplexed drugs in aqueous solution were found to range from 345 to 365 nm. Complexation with thorium invariably resulted in a bathochromic shift of 40–50 nm. This can be attributed to the formation of single covalent and dative bonds between oxygen and thorium in the complexed species, leading to further delocalization of electrons from the conjugated portion of the tetracycline molecules and, hence, to increased stability of the system.

In all experimental work conducted, a large excess of thorium nitrate was used so that, theoretically, the tetracycline compound in solution should be 100% complexed. Figure 2 shows only the effect of complexation with thorium on the absorption spectrum of tetracycline itself. However, similar observations were made for the remainder of the compounds studied. It is apparent that if any uncomplexed antibiotics were present, a slight error might be introduced owing to a small absorbance by this material at the optimum wavelength for the complex. To compensate for this possible error, all blanks contained the uncomplexed tetracycline at a suitable concentration. This procedure deviates from the usual method of blank preparation.

Studies undertaken with two of the degradation products of tetracycline, epianhydrotetracycline and anhydrotetracycline, showed that complexation with thorium led to color development in the same manner as with undegraded tetracycline. However, the complexed tetracycline derivatives exhibited maximal absorbance at 485 nm. and, in fact, showed no absorbance at the wavelength used in the colorimetric assay procedure.

In determining the most suitable pH and minimum time for stable color development, it was found that near-maximum absorbances, accompanied by a high degree of stability, were obtained within 20–35 min. using a nonbuffered aqueous system. A large number of buffers appeared to interfere in the complexation process. When attempts were made to obtain quantitative readings in systems above pH 7.0, results were adversely affected by the appearance of a white, flocculent precipitate. Although complexation between thorium and tetracycline still occurs above this point, the presence of $Th(NO_3)_4$ in large excess at this pH leads to the formation of insoluble hydroxides, primarily in the form of $Th(OH)_4$. Thus, distilled water ($pH \simeq 6.6$) was selected as a very convenient and suitable experimental medium. Figure 3 illustrates the effect of pH on color development for tetracycline HCl. Similar curves were obtained for the other tetracyclines in this study, although the levels of absorbance were not always identical. Once maximum color development was achieved, the color of the complexes remained stable for at least 30 min.

Good linear relationships between absorbance and concentration were obtained for all tetracyclines studied.

CONCLUSIONS

1. The proposed colorimetric procedure exhibits a high degree of accuracy and precision which makes it superior to many of the procedures presently being employed for the analysis of the tetracyclines.

2. The structure of the complex formed between the tetracyclines and quadrivalent thorium cannot be precisely postulated at this time. Experimental evidence suggests that a coordination compound containing two thorium to three tetracycline moieties may be formed.

REFERENCES

(1) "The British Pharmacopoeia," The Pharmaceutical Press, London, England, 1968.

(2) "The United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965.

(3) G. B. Selzer and W. W. Wright, *Antibiot. Chemother. (Washington, D. C.)*, **7,** 292(1957).

(4) R. G. Kelly and D. A. Buyske, *ibid.*, **10,** 604(1960).

(5) E. Addison and R. G. Clark, *J. Pharm. Pharmacol.*, **15,** 268 (1963).

(6) P. P. Ascione, J. B. Zagar, and G. P. Chrekian, *J. Pharm. Sci.*, **56,** 1396(1967).

(7) C. N. Sideri and A. Osol, *J. Amer. Pharm. Ass., Sci. Ed.*, **42,** 688(1953).

(8) F. Yokoyama and L. G. Chatten, *ibid.*, **47,** 548(1958).

(9) F. S. Chiccarelli and M. H. Woolford, Jr., *ibid.*, **45,** 400 (1956).

(10) K. W. Kohn, *Anal. Chem.*, **33,** 862(1961).

(11) T. Sakaguchi and K. Taguchi, *Pharm. Bull., Japan*, **3,** 166 (1955).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 29, 1970, from the *Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton 7, Canada.*

Accepted for publication August 6, 1970.

The authors thank the Medical Research Council of Canada for financial assistance during this investigation.

The reference materials and their dosage forms were generously supplied by the following pharmaceutical manufacturers: Bristol Laboratories of Canada Limited (tetracycline HCl, tetracycline phosphate complex, Azotrex capsules, Tetrex-APC capsules, Tetrex-bid capsules, Tetrex syrup, and Tetrex-AP syrup); Cyanamid of Canada Limited [demeclocycline HCl, chlortetracycline HCl, Achrocidin tablets, Achrocidin Compound syrup, Achromycin capsules, Aureomycin capsules, Aureomycin ophthalmic ointment, Aureomycin surgical powder, Aureomycin ear solution, Aureomycin soluble tablets, Declomycin syrup, Declomycin tablets (300 mg.), and Declostatin capsules]; Pfizer Company Limited (oxytetracycline HCl, doxycycline HCl, doxycycline monohydrate, Signemycin capsules, Signemycin syrup, Terra-Cortril spray, Terra-Cortril ear/eye suspension, Terra-Cortril ointment, Vibramycin oral suspension, and Vibramycin Scripak capsules); E. R. Squibb and Sons, Limited (Resteclin aqueous drops, Resteclin capsules, and Resteclin syrup); and The Upjohn Company of Canada (Albamycin T capsules).