Use of Circular Dichroism in Analysis of Mixtures of Tetracycline and 4-Epitetracycline and Its Application to Assay of Commercial Products

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Abstract □ Mixtures of tetracycline and 4-epitetracycline were assayed utilizing the large difference in their circular dichroism spectra at 262 nm. The assay was evaluated using synthetic mixtures and applied to the analysis of commercial tetracycline hydrochloride capsules. The results obtained with the newly developed procedure compare favorably with a published spectrophotometric method. In comparison with other techniques, the proposed method has several advantages. It is specific in that it permits differentiation between tetracycline and its 4-epimer, permitting calculation of the actual level of each epimer. It is accurate and precise, having an average error of 2% for tetracycline detection and 3.3% for 4-epitetracycline. It involves a simple procedure that requires no prior separation of degradation products under usual use conditions. Furthermore, it is very rapid, requiring only the experimental measurements of ellipticity at 262 nm. and absorbance at 356 nm. The disadvantages of the assay are its inability to determine analytically the content of anhydrotetracyclines and the possible interference of these materials if they are present in large amounts. However, levels of 10% anhydrotetracyclines affect the assay for tetracycline by only 1%. In the analysis of eight commercial products, the tetracycline content varied from 92 to 111% of the labeled amount and 4-epitetracycline was present in amounts of 3–17%. When compared with a spectrophotometric assay that required prior separation of anhydro compounds, the method introduced here gave the same analytical results for tetracycline content in the various products.

Keyphrases □ Tetracycline–epitetracycline mixtures—analysis by circular dichroism, determination in commercial products, compared with spectrophotometric method □ Epitetracycline–tetracycline mixtures—analysis by circular dichroism, determination in commercial products, compared with spectrophotometric method □ Degradation products—circular dichroism analysis of tetracycline–epitetracycline mixtures, applied to commercial products □ Circular dichroism—used to analyze tetracycline–epitetracycline mixtures, applied to commercial products

Tetracyclines may degrade through at least four possible mechanisms: epimerization, dehydration, hydrolysis, and oxidation. The first two mechanisms are the most commonly encountered reactions, so their degradation products are of specific interest in this study. Epimerization at carbon-4 leads to an inactive and nontoxic drug form (1). The kinetics of this reaction were studied by several workers (2, 3), showing that epimerization follows a reversible first-order process occurring most rapidly between pH 3 and 5. Dehydration and aromatization of the C-ring of tetracycline lead to anhydro derivatives at low pH. Anhydrotetracycline appears to be inactive *in vivo*, but its 4-epimer has been implicated in the toxic effects following use of outdated tetracycline products (4). The potential severity of this hazard provided the impetus to develop practical analytical procedures to detect and quantify both inactive and toxic tetracycline derivatives.

Titrimetric, polarographic, chromatographic, spectral, and microbiological methods have been used to analyze the tetracyclines. The latter three techniques seem to have received the most attention. The most widely used assay is the official microbiological method (5, 6). The disadvantages inherent in this method are that it has an accuracy of ±15% (7) and it is of limited specificity since several tetracycline-like materials have biological activity. Furthermore, the assay is carried out under conditions where some *in situ* epimerization is possible (pH 4.5 and incubation at 32–35° for 3–4 hr.). Among the several spectral methods used, absorbance ratios at two wavelengths have received the most attention. McCormick *et al.* (8) introduced an absorbance ratio method for tetracycline and 4-epitetracycline mixtures where ratios at 254 and 267 nm. are utilized. The sensitivity of the method is expected to be low, since the absorbance ratios range from 0.86 for tetracycline to 1.07 for epitetracycline. Because anhydrotetracycline and 4-epianhydrotetracycline absorb strongly in the 250–275-nm. range, the absorbance ratio technique by itself is not useful for samples containing these decomposition products. Pernarowski *et al.* (9) used an absorbance ratio at 357 and 391 nm. to detect tetracycline in the presence of anhydrotetracycline and epianhydrotetracycline. The assay, however, does not distinguish between epimers and essentially measures total content of tetracycline and epitetracycline when epitetracycline is present, as it usually is.

The number and variety of chromatographic methods applied to the analysis of tetracyclines probably exceed those of any other general assay method investigated. The inadequate and inconsistent recovery of tetracycline and degradation products by paper chromatography (10) is improved by using column chromatography. Griffiths (11), for example, made use of a column[1] that provided reproducibility but lacked specificity; tetracycline and epitetracycline elute in one fraction and anhydrotetracycline and epianhydrotetracycline elute in another. Ascione *et al.* (7, 12) reported both column chromatographic and TLC procedures for tetracyclines. Although these methods provide separation of tetracycline and its degradation products, detailed requirements for column preparation and repeated plate development in the TLC method make the techniques tedious. An automated procedure was recently introduced based on this technique (13), but it does not provide separate analysis for decomposition products. The procedure reported by Fike and Brake (14) does apparently separate and analyze degradation products. TLC separation followed by a conversion to anhydrotetracyclines serves as the basis for

[1] Sephadex G-25.

additional methods of analysis of mixtures of tetracyclines and their degradation products (15, 16). The methods appear useful for assay of commercial samples, but success requires strict attention to procedural detail (17) and they are unsuited for routine analysis of large numbers of samples.

In studies involving the solution conformation and sites of metal-ion binding of tetracycline derivatives (18–20), note was made of the large difference in the circular dichroism spectra of acidic solutions of tetracycline and epitetracycline at 262 nm. It appeared that this large difference could be used to analyze tetracycline in the presence of epitetracycline. The approach seemed feasible because circular dichroism measurements are rapid and relatively sensitive and require small samples; moreover, they are selective for tetracycline and epitetracycline. Anticipating that such an approach would offer advantages over existing techniques and because circular dichroism has yet to realize its potential for analysis of natural product mixtures, we developed and evaluated an analytical method to determine tetracycline content in the presence of its degradation products. Eight different brands of tetracycline hydrochloride capsules were analyzed by both circular dichroism and traditional UV chromatographic methods to determine the practicality of the method and the significance and incidence of tetracycline epimerization in finished products. All of these products had passed FDA batch certification but were intercepted at the point of dispensing. Therefore, they are representative of what the patient is receiving rather than what the pharmacist receives.

EXPERIMENTAL[2]

The ellipticity and absorbance of pure tetracycline, epitetracycline, anhydrotetracycline, and epianhydrotetracycline were determined as a function of concentration at 262 nm. (ellipticity, circular dichroism), and 356, 267, and 254 nm. (absorbances, UV). UV scans were made between 450 and 240 nm. All solutions were made in 0.03 *N* HCl where epimerization and dehydration are minimized (1). The concentration range for circular dichroism measurements was generally 0.001–0.005% and, when necessary, from 2 to 5 times more dilute for UV measurements.

Known mixtures of tetracycline and epitetracycline were made where the fraction of tetracycline ranged from 0.2 to 0.9. The total concentration of the mixture was about 9×10^{-5} *M* in the circular dichroism measurements and, when necessary, from 2 to 5 times more dilute to obtain measurable UV absorbances. The blank in all cases was 0.03 *N* HCl.

The commercial samples were analyzed in the following manner. The contents of 10 capsules from each product were pooled. The samples were then analyzed for total anhydrotetracycline content using a method based on the FDA screening procedure[3]. An amount of the pooled sample containing 250 mg. of tetracycline was mixed with 50 ml. of 0.15 *N* HCl for 5 min. The mixture was then vacuum filtered, the residue was washed with water, and the filtrate was diluted to 250 ml. with water. The resulting solution contained 0.1% tetracycline in 0.03 *N* HCl. This stock solution was then used to prepare 0.04 and 0.001% solutions. The absorbance of the 0.04% solution was read at 430 nm. and the 0.001% solution at 356 nm. These two measurements were used in the calculation of total anhydro content. For application to the new method of analysis, the stock solution was diluted to 0.005% and its circular dichroism spectrum was measured. The absorbance of a 0.0014% solution (1:4 dilution of the circular dichroism solution) was measured at 356 nm.

For comparative purposes, a spectrophotometric assay was carried out on three sample products. In this method, it is necessary to separate anhydrotetracyclines from the mixture. The chromatographic technique reported by Griffiths (11) was used for this purpose. A resin column[1] preswollen in 0.03 *N* HCl was used. A portion of commercial sample containing 200 mg. of tetracycline was diluted to 10 ml. with 0.03 *N* HCl and shaken in a glass-stoppered centrifuge tube for 5 min. The suspension was centrifuged, and 0.5 ml. of the supernate was applied to the column. The column was eluted with 0.03 *N* HCl, and 5-ml. fractions were automatically collected. Fractionation was followed by measuring absorbance at 273 nm. Tubes containing the tetracycline–epitetracycline fraction (approximately the first 70–80 ml. eluted) were carefully combined and diluted to 250 ml. with 0.03 *N* HCl. The absorbance of this solution was measured at 267 and 254 nm. for spectrophotometric analysis of tetracycline and epitetracycline.

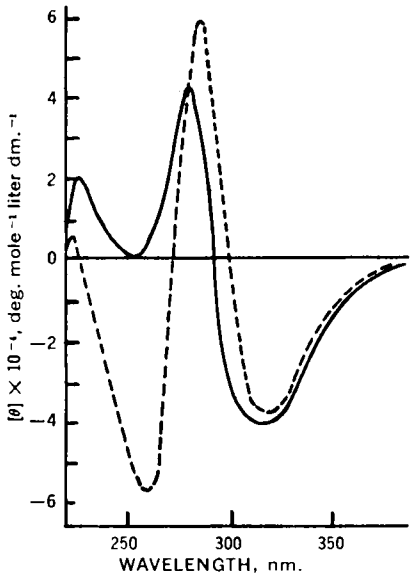

Figure 1—*Molar ellipticity* [θ] *of tetracycline hydrochloride* (- - -) *and 4-epitetracycline ammonium salt* (— —) *as a function of wavelength in 0.03* N *HCl.*

RESULTS AND DISCUSSION

The circular dichroism curves of tetracycline and epitetracycline (Fig. 1) show that there is a large difference in ellipticity between the two epimers at 262 nm. At this wavelength a mixture of the two compounds would give an ellipticity that is a sum of the ellipticities for each species:

$$\psi_{MIX} = k_{TC}C_{TC} + k_{ETC}C_{ETC} \qquad \text{(Eq. 1)}$$

where ψ_{MIX} is the observed ellipticity; and k_{TC} and k_{ETC} are the proportionality constants defining the linear relation between ellipticity and molar concentration, C_{TC} and C_{ETC}, for tetracycline and epitetracycline, respectively, where the path length of the cell

[1] Circular dichroism spectra were determined using a Durrum-Jasco ORD/UV-5 instrument with the Sprowl Scientific SS 20-2 circular dichroism modification. Samples were run in a 1-cm. path length cell at ambient temperature (29°). UV and visible light absorption were determined in a Cary model 15 or a Gilford model 240 spectrophotometer at ambient temperature. A Warner-Chilcott model 1205 D3 automatic fraction collector was used in conjunction with column chromatographic procedures.

The following materials were donated by Lederle Laboratories and used as standards: tetracycline hydrochloride (CL-13554; 4574B-1296), 4-epitetracycline ammonium salt (CL-16956; 705B-133-9), anhydrotetracycline hydrochloride (CL-17901; 8412 B97-A), and 4-epianhydrotetracycline (CL; 27212-7117B-93-5).

The several samples of commercial tetracycline capsules were acquired from the active stock of various retail and hospital pharmacies. They were collected at random without conscious regard for expiration date; however, none of the samples had expired.

Sephadex G-25, Medium (Pharmacia), was used in the chromatographic procedure. All other chemicals were of analytical grade quality.

[3] Personal communication with Dr. William W. Wright, Acting Director, National Center for Antibiotics and Insulin Analysis, Food and Drug Administration, Washington, D.C.

Table I—Circular Dichroism (CD) and UV Constants for Various Tetracyclines in 0.03 *N* HCl

Compound	Mode	Wavelength, nm.	Constant, deg./*M* (CD), *A*/*M* (UV) ± Average Percent Error	Symbol
Tetracycline hydrochloride	CD	262	−579.9 ± 3.8[a]	k_{TC}
	UV	356	14,495 ± 0.3[a]	a_{TC}
	UV	267	18,367 ± 0.42[b]	a_{TC}^{267}
	UV	254	15,571 ± 0.24[b]	a_{TC}^{254}
Epitetracycline hydrochloride	CD	262	+133.4 ± 9.2[a]	k_{ETC}
	UV	356	13,833 ± 1.2[a]	a_{ETC}
	UV	267	14,477 ± 0.39[c]	a_{ETC}^{267}
	UV	254	15,467 ± 0.32[c]	a_{ETC}^{254}
Anhydrotetracycline hydrochloride	UV	356	928 ± 5.8[b]	
	CD	262	+864 ± 8.2[d]	
Epianhydrotetracycline hydrochloride	UV	356	736 ± 4.3[c]	
	CD	262	+904 ± 1.7[d]	

[a] Average of 9–10 values. [b] Average of five values. [c] Average of four values. [d] Average of three values.

Table II—Results of the Analysis of Known Mixtures of Tetracycline and Epitetracycline by Circular Dichroism Method

Sample Number	Concentration Added, $M \times 10^5$		Ellipticity, ψ_{MIX}	Absorbance × Dilution Factor, nA_{MIX}	Concentrations Found, $M \times 10^5$		Percent Recovery	
	Tetracycline	Epitetracycline			Tetracycline	Epitetracycline	Tetracycline	Epitetracycline
1	8.32	0	−0.0488°	1.395	8.65	—	104.0	—
2	8.15	0	−0.0499°	1.180	8.52	—	104.5	—
3	7.49	0.90	−0.0433°	1.235	7.67	0.885	102.4	98.3
4	6.66	1.80	−0.0373°	1.245	6.85	1.82	102.9	101.1
5	6.24	2.25	−0.0323°	1.225	6.13	2.43	98.2	108.0
6	6.11	2.24	−0.0346°	1.190	6.40	1.89	104.7	84.4
7	4.16	4.50	−0.0180°	1.225	4.14	4.51	99.5	100.2
8	4.08	4.48	−0.0171°	1.187	3.97	4.42	97.3	98.7
9	2.08	6.75	−0.00375°	1.210	2.14	6.50	102.9	96.3
10	2.04	6.72	−0.00315°	1.198	2.04	6.52	100.0	97.0
11	0	9.00	+0.0155°	1.210	0	9.31	—	103.4
12	0	8.96	+0.0125°	1.215	0	8.90	—	99.3
					Average percent recovery		101.8	98.7
					Average percent error[a]		2.0	3.3

[a] Calculated as the average absolute difference between the percent recovered in each mixture and the average percent recovery for all 10 samples.

is 1 cm. Although it is possible to use a second equation involving ellipticity at another wavelength to calculate the content of tetracycline and epitetracycline in a sample, the greater precision that can be achieved (Table I) by using absorbance measurements to determine total tetracyclines led to the use of a second equation based on UV analysis. As discussed later, a UV analysis of a sample should be made to determine the content of anhydrotetracyclines since they can interfere with the precision of the circular dichroism measurements if they are present in significant amounts. Absorbance at 356 nm., where both tetracycline and epitetracycline show an absorption peak, is proportional to the total tetracycline content. The solutions used to measure Ψ are usually too concentrated for direct UV measurement; hence, they are diluted by a factor, n, prior to analysis. Thus, the absorbance of the solution used in determining ψ is related to the molar concentration, C, of tetracycline and epitetracycline by:

$$nA_{MIX} = a_{TC}C_{TC} + a_{ETC}C_{ETC} \qquad \text{(Eq. 2)}$$

where nA_{MIX} is the absorbance of the circular dichroism solution, diluted n-fold, using a cell with a 1-cm. path length, and a_{TC} and a_{ETC} are the molar absorbances for each species. Applying Cramer's rule (21) to Eqs. 1 and 2 gives:

$$C_{TC} = \frac{a_{ETC}\psi_{MIX} - nA_{MIX}k_{ETC}}{k_{TC}a_{ETC} - k_{ETC}a_{TC}} \qquad \text{(Eq. 3)}$$

and:

$$C_{ETC} = \frac{nA_{MIX}k_{TC} - a_{TC}\psi_{MIX}}{k_{TC}a_{ETC} - k_{ETC}a_{TC}} \qquad \text{(Eq. 4)}$$

The various constants involved in Eqs. 3 and 4 were determined from known concentrations of pure materials and are listed in Table I. In this study, we found that the constants generated for tetracycline may depend on the source of tetracycline and vary slightly from time to time; thus, they should be verified periodically to ensure the highest accuracy. Substitution of the constants from Table I into Eqs. 3 and 4 leads to:

$$C_{TC} = (103.7\psi_{MIX} - A_{MIX})(-1.340 \times 10^{-6}) \qquad \text{(Eq. 5)}$$

$$C_{ETC} = (-A_{MIX} - 25.0\psi_{MIX})(-5.824 \times 10^{-6}) \qquad \text{(Eq. 6)}$$

Analysis of known mixtures of tetracycline and epitetracycline using Eqs. 5 and 6 gave the results listed in Table II. Recovery of tetracycline averaged 101.6% with an average error of 2.0%, while epitetracycline gave a recovery of 98.7% with an average error of 3.3%. A comparison of these results with a spectrophotometric assay was made. Absorbance of these same mixtures at 267 and 254 nm. was used and the fraction of tetracycline, F_{TC}, present was calculated using the mathematical form of the equation used by Pernarowski *et al.* (22):

$$F_{TC} = \frac{a_{ETC}^{254}Q_0 - a_{ETC}^{267}}{(a_{TC}^{267} - a_{ETC}^{267}) - (a_{TC}^{254} - a_{ETC}^{254})Q_0} \qquad \text{(Eq. 7)}$$

where Q_0 is the absorbance ratio A_{267}/A_{254}; and a_{TC} and a_{ETC} are the absorbances of tetracycline and epitetracycline, respectively, at wavelengths designated by the superscripts. The values for these constants are given in Table I and their substitution into Eq. 7 gives:

$$F_{TC} = \frac{15{,}467Q_0 - 14{,}477}{3890 - 104Q_0} \qquad \text{(Eq. 8)}$$

By using the mixtures listed in Table II, the fraction of tetracycline in each was determined using Eq. 8. The results of the analysis (Table III) show that an average recovery of 102.2% with an average error of 3.4% was obtained. This compares favorably with the results using the circular dichroism method.

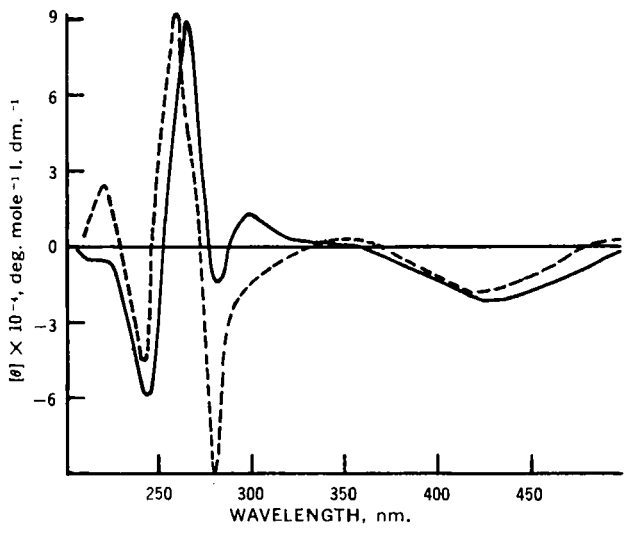

Figure 2—*Molar ellipticity [θ] of anhydrotetracycline hydrochloride (——) and 4-epianhydrotetracycline (- - -) as a function of wavelength in 0.03* N *HCl.*

Eight commercial samples of tetracycline hydrochloride capsules were then analyzed using the circular dichroism method *without* prior separation of anhydro compounds. The circular dichroism spectra of epianhydrotetracycline and anhydrotetracycline (Fig. 2) show that these compounds may interfere with the circular dichroism assay if present in significant amounts. When using the circular dichroism and UV constants for epianhydrotetracycline and anhydrotetracycline listed in Table I, it is possible to calculate the error that would be introduced in this assay if a solution of 1×10^{-4} *M* tetracyclines contained 90% tetracycline and 10% anhydrotetracycline. [Pernarowski *et al.* (9) reported that the maximum amount of anhydrotetracycline present in six commercial samples tested by them was 10%; in the present study the maximum percentage of anhydrotetracyclines in eight samples was 5.3%.] The theoretical ellipticity that would be observed at 262 nm. would be −0.0513°, and the absorbance of the solution at 356 nm. would be 1.314 for the tetracycline–anhydrotetracycline mixture. Using the new assay, Eqs. 5 and 6 would predict 88.9% tetracycline and 1.69% epitetracycline (rather than 90% tetracycline and 0% epitetracycline). Thus, the method would be low with respect to tetracycline recovery and predict the presence of a small amount of epitetracycline even though none were present. Analysis of the eight commercial products studied showed that the products contained from 2 to 5% anhydro species. This amount would not appreciably affect the tetracycline and epitetracycline calculation. Determination of the anhydro content was based on the FDA screening procedure using the formula:

$$\text{percent anhydrotetracyclines} = \frac{(A_{430} - A_{356} \times 0.0019)100}{180} \qquad \text{(Eq. 9)}$$

where A_{430} is the absorbance of the 0.04% solution at 430 nm. times 25, A_{356} is the absorbance of the 0.001% solution at 356 nm. times 1000, 0.0019 is the absorbance ratio (430 nm./356 nm.) for tetracycline, and 180 is the absorptivity (1%, 1 cm.) of anhydrotetracycline at 430 nm. The first column of Table IV lists the total percent of anhydrotetracyclines present in each product as determined by Eq. 9. Columns 2 and 3 (Table IV) give the percentage of tetracycline and epitetracycline in each product using the new assay. All samples contain tetracycline within accepted limits but also contain amounts of epitetracycline which vary from 3 to 17%. The values were not corrected for the small amount of anhydrotetracycline present. Column 5 of Table IV gives the fraction of tetracycline hydrochloride determined by the absorbance ratio assay (Eq. 7) where anhydrotetracyclines were first removed by resin column chromatography. The fraction of tetracycline present determined by the absorbance ratio method compares quite well with that obtained by the new technique (Table IV, Column 6).

Since limits have not been set on permissible levels of epitetracycline in commercial products, it is difficult to discuss the significance of the amounts found. Perhaps the most significant result of this analysis of commercial samples is the prevalence of capsule overfilling (in seven of eight products). Sample 8 would pass certification, showing 95–97% tetracycline, but it constitutes a poorer grade of drug because of the amounts of epitetracycline and anhydro compounds present. There is no specified upper limit on the amount of tetracycline that may be present in the standards set forth in the "Code of Federal Regulations," nor is any limit set for anhydrotetracycline and epianhydrotetracycline.

When Pernarowski *et al.* (9) examined six commercial tetracycline products, using absorbance ratios at 357 and 391 nm., they reported the samples as having 90–94% of the labeled tetracycline content, the remainder being only anhydro compounds as seen by TLC. Results of the present study indicate small amounts of anhydrotetracyclines and from 3 to 17% epitetracycline. There is no reason to expect that the samples used previously (9) were the same as those used in the present study, nor is it reasonable to expect large errors in epitetracycline content in the present method even in the presence of 5% anhydrotetracycline.

To determine further the accuracy with which epitetracycline could reasonably be estimated when present at low concentrations, eight known mixtures containing 4–17% epitetracycline in the presence of tetracycline were analyzed using the circular dichroism method and Eqs. 5 and 6. In this study, the concentration of epitetracycline in the samples varies from 3.82×10^{-6} to 1.58×10^{-5} *M* while the tetracycline was held constant at approximately 8×10^{-5} *M* (Table V). When the absolute difference between the percent found and the percent added is taken, the average error in detecting percent tetracycline is found to be 1.5%. Similar analyses of the results show that the percent of epitetracycline relative to the percent added was low by an average of 2.9% in all mixtures studied. Be-

Table III—Results of Known Mixtures of Tetracycline and Epitetracycline by the Spectrophotometric Method

Sample Number[a]	Q_0, A_{267}/A_{254}	Fraction Tetracycline Added	Fraction Tetracycline Found[b]	Percent Recovery
1	1.176	1.00	0.985	98.5
2	1.184	1.00	1.018	101.8
3	1.170	0.893	0.960	107.5
4	1.136	0.787	0.820	104.2
5	1.122	0.735	0.763	103.8
6	1.119	0.732	0.750	102.5
7	1.051	0.480	0.471	98.1
8	1.045	0.477	0.446	93.5
9	0.9964	0.236	0.245	103.8
10	0.9981	0.233	0.253	108.6
		Average percent recovery		102.2
		Average percent error[c]		3.4

[a] Same samples as that listed in Table II. [b] Calculated using Eq. 8. [c] Calculated as the average absolute difference between the percent recovered in each mixture and the average percent recovery (102.2%).

Table IV—Analysis of Commercial Tetracycline Capsules

Product	Percent Total Anhydrotetracycline[a]	Percent Tetracycline Hydrochloride[b]	Percent Epitetracycline Hydrochloride[b]	Fraction Tetracycline —Hydrochloride— UV Method[c]	Fraction Tetracycline —Hydrochloride— Circular Dichroism Method
C-1	2.4	92.1	8.6	—	—
C-2	2.2	97.6	8.7	—	—
C-3	2.4	84.2	9.6	—	—
C-4	2.2	92.7	10.9	—	—
C-5	2.6	95.5	11.2	—	—
C-6	3.3	111.4	3.0	0.97	0.97
C-7	3.0	104.9	3.4	0.96	0.97
C-8	5.3	95.3	17.4	0.86	0.86

[a] Determined by the FDA screening procedure for total anhydrotetracyclines. [b] Determined using the circular dichroism method. [c] Determined by the column chromatography–absorbance ratio (267/254 nm.) *via* Eq. 7.

Table V—Results of Circular Dichroism Analysis of Known Mixtures Containing Low Percentages of Epitetracycline

Percent Added[a]		Percent Found[b]	
Tetracycline	Epitetracycline	Tetracycline	Epitetracycline
82.7	17.3	84.4	15.0
84.2	15.8	84.3	13.3
86.3	13.7	88.4	11.1
87.2	12.8	86.8	9.3
90.6	9.4	93.2	6.3
91.0	9.0	91.3	5.9
95.2	4.8	99.7	2.1
95.4	4.6	96.0	1.3

[a] Calculated as: C_{TC} added or C_{ETC} added/C_{TC} added + C_{ETC} added × 100. [b] Calculated as: C_{TC} found or C_{ETC} found/C_{TC} added + C_{ETC} added × 100.

cause of the small amounts of epitetracycline present and the concomitant small changes in the ellipticity of mixtures of it and tetracycline, the assay for epitetracycline was predictably subject to error, but the error involved would not explain the amounts of epitetracycline found in commercial products in this study.

It appears that no presently existing assay will by itself completely quantify a mixture of tetracycline, epitetracycline, anhydrotetracycline, and epianhydrotetracycline. Absorbance ratios at 357 and 391 nm. permit the calculation of the fraction of total tetracycline (tetracycline and epitetracycline). Absorbance ratios at 254 and 267 nm. will predict tetracycline content providing anhydro compounds are not present. The new technique will predict tetracycline and epitetracycline levels if large amounts of anhydrotetracyclines are not present. Thus, it would appear that a recommended procedure for the analysis of tetracycline products would be to determine the total anhydro content by the FDA screening method. If more than 5% anhydrotetracyclines are present, the anhydro compounds should be separated by the described resin chromatography and the tetracycline–epitetracycline fraction should be analyzed using this method. Alternatively, mathematical corrections for the increase in epitetracycline can be made without prior chromatographic separation.

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