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Analysis of Tetracycline in Pharmaceutical Preparations by Improved High-Performance Liquid Chromatographic Method

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Abstract □ The analysis of tetracycline in pharmaceutical preparations by an improved high-performance liquid chromatographic (HPLC) method is described. The improved method uses a 30-cm long stainless steel column packed with octadecylsilane bonded on 10-μm silica gel, with a linear gradient from 10 to 60% acetonitrile in pH 2.5, 0.02 *M* phosphate buffer in 11 min at a flow rate of 1.0 ml/min (68 atm). The resolution functions obtained between 4-epitetracycline and tetracycline and between 4-epianhydrotetracycline and anhydrotetracycline were improved 150 and 250%, respectively. The analysis of a tetracycline sample takes approximately 16 min; the original method required more than 25 min. The relative standard deviation for the analysis of tetracycline powder was 0.66%, and the recovery of 4-epianhydrotetracycline added in tetracycline was linear over the 0.3–100% range. Recovery of tetracycline from products was better than 99.6% at label concentration. The drug content of products as calculated from the HPLC data agreed well with those of the microbiological assay methods.

Keyphrases □ Tetracycline—analysis, high-performance liquid chromatography, pharmaceutical preparations □ High-performance liquid chromatography—analysis, tetracycline in pharmaceutical preparations □ Antibiotics—tetracycline, analysis, high-performance liquid chromatography, pharmaceutical preparations

On March 4, 1974, the Food and Drug Administration (FDA) established the limit for 4-epianhydrotetracycline in tetracycline pharmaceutical dosage forms marketed in the United States (1). However, the limit is higher than that outlined in the European Pharmacopoeia (2).

Prior to the development of high-performance liquid chromatographic (HPLC) assay methods for tetracycline (3–6), the methods used for the detection and quantitation of tetracycline impurities (7, 8) were very tedious, and none has been accepted by FDA as an alternative method for potency determination. There is also some indication that degradation compounds may be forming on-column during

chromatography (3) when the method outlined in the "Federal Register" is used (1).

The original HPLC method (3, 4) reported for the analysis of tetracycline took a minimum of 25 min of chromatographic time. Also, the resolution between tetracycline and 4-epitetracycline and between anhydrotetracycline and 4-epianhydrotetracycline peaks was not optimum. The new improved HPLC method for tetracycline has many advantages over other methods of analysis, including high speed of analysis, precise quantitation, and improved resolution between tetracycline and its degradation products. The method eliminates the need to perform separate analyses for identity, potency, and stability.

The purpose of this paper is to describe the improved methodology and its application to the analysis of tetracycline in pharmaceutical preparations.

EXPERIMENTAL

Apparatus—A modular liquid chromatograph[1] equipped with a 280-nm UV monitor was used, along with a high-pressure reciprocating positive displacement pump (maximum of 340 atm) equipped with a pulse dampener.

The column, 4.6 × 300 mm packed with octadecylsilane bonded on 10-μm silica gel[2], was attached to a septumless injector valve[3] and the sample side of the 280-nm UV monitor.

Reagents—*Mobile Phases*—

A. One hundred milliliters of acetonitrile and 800 ml of water were placed into a 1000-ml graduated cylinder. One hundred milliliters of 0.2 *M*, pH 2.5 phosphate buffer was then added and mixed.

B. Six hundred milliliters of acetonitrile and 300 ml of water were placed into a 1000-ml graduated cylinder. One hundred milliliters of 0.2 *M*, pH 2.5 phosphate buffer was then added and mixed.

The 0.2 *M*, pH 2.5 phosphate buffer was prepared as follows. A solution containing a suitable quantity of dibasic sodium phosphate was prepared, and the pH of the solution was adjusted to pH 2.5 with phosphoric acid. Water was then added to the volume for a phosphate concentration of 0.2 *M*.

Column Rinse Solution—An aqueous solution containing 80% methanol was prepared and used for rinsing the column after the end of each assay day to prolong column life. This column packing material is unstable when kept at pH < 2.5 for extended periods.

Gradient Elution—A gradient mixer[4] with a 0.25-ml mixing chamber was used. A programmed linear gradient elution, from Mobile Phase A to B in 15 min, was used.

Chromatographic Conditions—The column temperature was ambient with a recorder chart speed of 6.4 mm/min. The electrometer range setting was 0.16 full scale. The column pressure was 68 atm (1000 psi) with a flow rate of 1.0 ml/min. The standard and sample volume injected on-column was 2 μl.

Sample Injection—Immediately after final dilution of the tetracycline reference standards and samples, they were quantitatively injected on-column with the septumless valve injector.

Preparation of Tetracycline Standard—USP tetracycline hydrochloride reference standard, Issue H, was dried at 60° under less than 5 mm Hg pressure for 3 hr. After drying, approximately 4 mg of the reference standard was accurately weighed using an electromagnetic balance[5] and placed into a 10-ml volumetric flask.

Just prior to analysis, each standard was dissolved with 1 ml of absolute methanol and then diluted to volume with 0.01 *M*, pH 4.5 phosphate buffer. When tetracycline was dissolved in 0.01 *M*, pH 4.5 phosphate buffer, no detectable epimerization took place in 6 hr, as determined by the HPLC method.

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[2] Waters Associates, Framingham, Mass.
[3] Micromeritics, Inc., Norcross, Ga.
[4] Ultragrad, LKB, Stockholm, Sweden.
[5] Cahn Instrument Corp., Paramount, Calif.

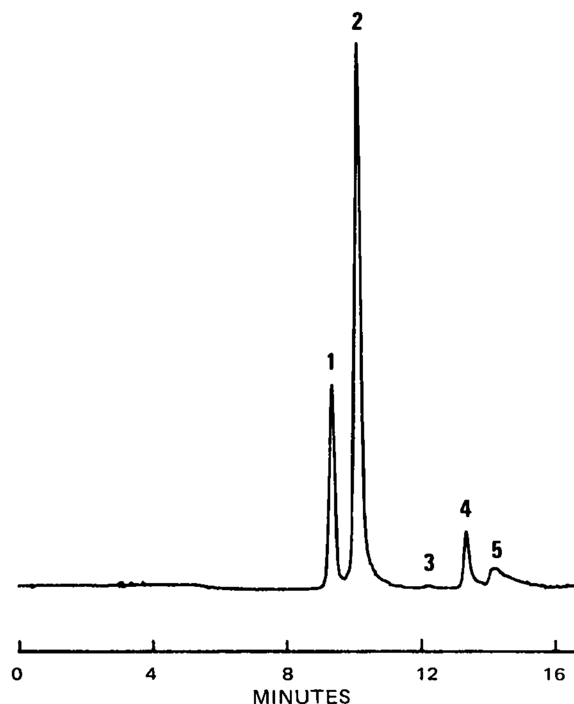

Figure 1—*HPLC of an expired lot of tetracycline phosphate powder indicating improved separation of tetracycline, its epimer, and degradation compounds. A 30-cm column packed with octadecylsilane bonded on 10-μm silica gel was used with 15-min linear gradient elution from 10 to 60% acetonitrile in pH 2.5, 0.02* M *phosphate buffer at a 1.0-ml/min flow rate. Key: 1, 4-epitetracycline; 2, tetracycline; 3, chlortetracycline; 4, 4-epianhydrotetracycline; and 5, anhydrotetracycline.*

Sample Preparation—*Tetracycline Bulk Powder*—Approximately 4 mg of the tetracycline sample was accurately weighed into a 10-ml volumetric flask. Just prior to analysis, each sample was diluted with 1 ml of absolute methanol and brought to volume with 0.01 *M*, pH 4.5 phosphate buffer.

Hard Filled Capsules Containing 250 mg of Tetracycline Hydrochloride—Ten capsules were carefully emptied, and their contents were accurately weighed. From this material, the equivalent of two capsules was then weighed and placed in a 50-ml round-bottom, glass-stoppered, centrifuge tube. Twenty-five milliliters of absolute methanol was added, and the contents were shaken for 5 min. The tubes were then centrifuged for 10 min at 1800 rpm. After centrifuging, 2 ml was removed and placed in a 100-ml volumetric flask. Immediately prior to the analysis of each sample, the flask was diluted to volume with 0.01 *M*, pH 4.5 phosphate buffer.

Hard Filled Capsules Containing 250 mg of Tetracycline Phosphate Complex with Nystatin—With one modification, the same extraction procedure for hard filled capsules containing tetracycline hydrochloride was used. Acidified methanol (2% concentrated hydrochloric acid in absolute methanol) was substituted for absolute methanol. This modification was to convert the tetracycline phosphate complex to the hydrochloride form in order to solubilize the tetracycline in methanol.

Hard Filled Capsules Containing Tetracycline Phosphate Complex with Novobiocin—Ten capsules were weighed for content uniformity. The equivalent weight of three capsules was then weighed and placed in a 50-ml ground-glass-stoppered, round-bottom centrifuge tube. Twenty-five milliliters of acidified methanol (2% concentrated hydrochloric acid in absolute methanol) was volumetrically added, and the contents were shaken for 5 min on a reciprocating shaker. After shaking, the samples were centrifuged for 5 min at 1800 rpm.

Four milliliters of acidified methanol extract was then volumetrically transferred to a 100-ml volumetric flask and diluted to volume with 15% methanol in 0.02 *M*, pH 7.0 phosphate buffer. The pH 7.0 buffer was needed to solubilize novobiocin in an alco-

Table I—Precision of the Tetracycline Determination by HPLC

Weight of Tetracycline, mg/ml	Area of Tetracycline, mm^2	Area–Weight Ratio
0.3896	180.5	463.3
0.4000	182.1	455.3
0.3931	181.1	460.7
0.3948	181.7	460.2
0.3980	181.5	456.0
0.3946	181.9	461.0
0.3942	180.0	456.6
	RSD	0.66%

holic solution. Recovery of tetracycline was reduced when novobiocin was allowed to precipitate.

Film-Coated Tablets Containing 250 or 500 mg of Tetracycline Hydrochloride—To minimize tablet weight variation, 10 film-coated tablets were weighed accurately and then finely ground using a Wiley mill with a 60-mesh screen. A portion of the powder equivalent to the weight of two tablets for the 250-mg tablets and of one tablet for the 500-mg formulation was weighed accurately and placed in a 50-ml ground-glass-stoppered, round-bottom centrifuge tube.

Twenty-five milliliters of methanol was added, and the tube was tightly stoppered and then shaken vigorously on a reciprocating shaker for 5 min. The samples were then centrifuged at 1800 rpm for 10 min. Two milliliters of the methanol extract was then pipetted into a 100-ml volumetric flask. Immediately prior to analysis, the volumetric flask was diluted to volume with 0.01 *M*, pH 4.5 phosphate buffer.

Syrup Containing 125 mg of Tetracycline Base/5 ml—Three milliliters of thoroughly mixed syrup was volumetrically transferred to a 200-ml volumetric flask and diluted to volume with absolute methanol. The contents were thoroughly shaken, and then about 30 ml was decanted into a 50-ml glass-stoppered, round-bottom centrifuge tube and centrifuged at 1800 rpm for 5 min.

Oral Suspension Containing 100 mg of Tetracycline Base/ml and Saccharin—One milliliter of the product was volumetrically pipetted into a 50-ml volumetric flask. One milliliter of 0.2 *M*, pH 2.5 phosphoric acid buffer was added, and the contents were diluted to volume with dimethylformamide. Approximately 30 ml of this solution was transferred into a 50-ml glass-stoppered, round-bottom centrifuge tube. The tube was centrifuged at 2500 rpm for 5 min. This procedure was necessary to remove saccharin, which interferes with the chromatography. After centrifugation, 2 ml of the supernate was pipetted into a 10-ml volumetric flask and diluted to volume with Mobile Phase A.

Calculations—The areas under the peaks of tetracycline, its degradation compounds, and chlortetracycline were measured. The percent composition of these compounds was calculated using the following formula:

$$(A_1 + A_2 + A_3 + A_4 + A_5)/A_s \times W_s/W \times 100 \qquad \text{(Eq. 1)}$$

where:

A_1 = area of tetracycline peak in sample
A_2 = area of 4-epitetracycline peak in sample
A_3 = area of chlortetracycline peak in sample
A_4 = (area of 4-epianhydrotetracycline peak in sample) × 0.76
A_5 = (area of anhydrotetracycline in sample) × 0.76
W_s = weight of tetracycline reference standard in milligrams
W = weight of tetracycline sample in milligrams
0.76 = response factor (The response factor was calculated from the molar absorptivity at 280 nm in the mobile phase, chromatographic peak area, and purity of each component preparation)

Microbiological Assay Method—The methods described in the "Code of Federal Regulations" (9) were used. The pharmaceutical preparations containing tetracycline phosphate complex were analyzed by an agar diffusion cylinder cup method using *Bacillus cereus* (ATCC 11778), and the remaining preparations were analyzed by a turbidimetric method using *Staphylococcus aureus* (ATCC 6538P) as the test microorganism.

Table II—Recovery of 4-Epianhydrotetracycline Added in Tetracycline by HPLC

Weight of Tetracycline, mg/ml	Weight of 4-Epianhydrotetracycline (Corrected), mg/ml	4-Epianhydrotetracycline Added, %	4-Epianhydrotetracycline Recovered, %
0.4000	0	0	0
0.3942	0.00145	0.368	0.34
0.3946	0.00364	0.914	0.98
0.3980	0.01456	3.53	3.5
0.3948	0.02913	6.88	6.2
0.3931	0.05826	12.9	12.3
0	0.07282	100	99.0

RESULTS AND DISCUSSION

The major modifications made to the original HPLC method for tetracyclines (3) were the use of a different reverse-phase column packing material, which necessitated a change in the composition of the mobile phases, and incorporation of a gradient elution system. The column packing material selected was octadecylsilane bonded on 10 μm of silica gel, which was successfully applied for the analysis of bacitracin (10). This packing material is far more stable and efficient than the originally used hydrocarbon polymer. The gradient elution system was used to shorten the chromatographic time and to make the detection of trace quantities of degradation compounds easier. The addition of ethylenediaminetetraacetic acid in the mobile phase was not essential for the analysis of tetracycline with this new system.

Separation of Tetracyclines—As may be seen in Fig. 1, tetracycline, its epimer, and degradation compounds were well separated. The resolution function (R_s) between 4-epitetracycline and tetracycline was improved from 0.93, the original method (3), to 2.29. Between 4-epianhydrotetracycline and anhydrotetracycline, it was improved from 0.43 to 1.50. The R_s was calculated using the formula: $R_s = 2(t_2 - t_1)/(W_1 + W_2)$, where $t_2 - t_1$ equals the difference in retention time of the two adjacent peaks, and $(W_1 + W_2)/2$ equals the average baseline peak width.

The chromatography of a tetracycline sample was shortened to approximately 16 min from the 25 min required by the original method (3).

Quantitative Determination of Tetracycline—The precision of the improved HPLC method was determined by comparing several replicate preparations of the USP reference standard. Table I indicates that the relative standard deviation of the HPLC method

Table III—Recovery of Tetracycline from Pharmaceutical Preparations

Hard Filled Capsules Containing Tetracycline Hydrochloride, 250 mg/Capsule			
Percent of Label Dosage	Tetracycline Added, mg[a]	Tetracycline Recovered, mg[a]	Recovery, %
80 (200 mg/capsule)	401.68	396.14	98.6
100 (250 mg/capsule)	500.62	499.98	99.9
120 (300 mg/capsule)	600.84	584.57	97.3
Syrup Containing Tetracycline Base, 125 mg/5 ml			
Percent of Label Dosage	Tetracycline Added, mg/5 ml	Tetracycline Recovered, mg/5 ml	Recovery, %
90 (112.5 mg/5 ml)	115.17	113.77	98.8
100 (125 mg/5 ml)	128.10	127.54	99.6
120 (150 mg/5 ml)	153.83	152.93	99.4

[a] Weight equivalent to two capsules.

Table IV—Relative Standard Deviation Using a Lot of Hard Filled Capsules Containing 250 mg of Tetracycline Hydrochloride

Weight of Capsules, mg	Area, mm^2	Area–Weight Ratio	Tetracycline/ Capsule Recovered, mg
947.67	166.5	0.17669	265.3
944.92	164.3	0.17388	262.5
950.46	164.0	0.17255	260.6
946.87	164.0	0.17322	261.6
946.82	160.0	0.16989	256.5
947.00	160.7	0.17141	258.8
948.03	163.0	0.17351	262.0
		$\bar{x}$ =	261.0
		RSD =	1.07%

for tetracycline was 0.66%. Thus, good quantitation can be obtained without the use of an internal standard with a valve injector system. However, when an internal standard is preferred, tryptophan at 0.7 mg/ml may be used. Tryptophan elutes earlier, is well separated from 4-epitetracycline, and is not interfered with by incipients in pharmaceutical dosage forms.

To correlate the HPLC data with the microbiological potencies, the microbiological responses of tetracycline, 4-epitetracycline, chlortetracycline, anhydrotetracycline, and 4-epianhydrotetracycline were determined. Five concentrations of each component were prepared and assayed against *B. cereus* (ATCC 11778) and *S. aureus* (ATCC 6538P) using the cylinder cup agar diffusion assay method and the turbidimetric assay method, respectively (9).

The HPLC data indicated that the USP tetracycline hydrochloride reference standard, Issue H, contained 0.25% 4-epitetracycline; 4-epitetracycline powder contained 3.0% tetracycline and 0.34% 4-epianhydrotetracycline; chlortetracycline powder contained 0.98% tetracycline; 4-epianhydrotetracycline powder contained 0.5% chlortetracycline and 8.0% anhydrotetracycline; and anhydrotetracycline powder contained 2.2% 4-epianhydrotetracycline. Therefore, the microbiological responses of these compounds were corrected to compensate for these impurities. The corrected microbiological response factors for 4-epitetracycline, chlortetracycline, 4-epianhydrotetracycline, and anhydrotetracycline are 0.29, 5.92, 0.22, and 0.03 for *B. cereus* and 0.10, 3.26, 0.09, and 0.04 for *S. aureus*, respectively. Therefore, the following equation was devised to make the HPLC data comparable with that of the microbiological assay data:

$$\text{HPLC calculated biopotency } (\mu g/mg) = (A_1 + \alpha A_2 + \beta A_3 + \gamma A_4 + \delta A_5)/A_s \times (W_s/W) \times F_1 \times F_2 \quad \text{(Eq. 2)}$$

where:

F_1 = assigned value of the tetracycline reference standard
F_2 = dilution factor
$\alpha, \beta, \gamma, \delta$ = microbiological response factors for 4-epitetracycline, chlortetracycline, 4-epianhydrotetracycline, and anhydrotetracycline

The sensitivity of the HPLC method to tetracycline remained at approximately 10 ng/sample injected. The method was successfully applied to the determination of tetracycline in clinical samples and in fermentation broth with a minimum of sample preparation.

Determination and Quantitation of 4-Epianhydrotetracycline in Tetracycline—To examine the detection limit and quantitation of 4-epianhydrotetracycline in a tetracycline sample by the HPLC method, USP reference standard solutions containing 0, 0.37, 0.91, 3.5, 6.9, 13, and 100% of 4-epianhydrotetracycline were prepared and analyzed. The purity of 4-epianhydrotetracycline was determined by molar absorptivity (20.08) at 438 nm using the method outlined in the "Federal Register" (1) and the HPLC method. The purity thus determined (71.9%) was used to correct the weight of 4-epianhydrotetracycline added in tetracycline samples.

Table V—Analysis of Pharmaceutical Preparations Containing Tetracycline Base and Tetracycline Hydrochloride by HPLC

Lot	Microbiological Potency	Calculated HPLC Potency	Percent Composition by HPLC[a]				
			I	II	III	IV	V
Hard filled capsules:							
A	254	237.8	95.8	2.7	<0.3	<0.3	<0.3
B	251	253.7	96.8	2.4	<0.3	<0.3	<0.3
C	247	246.6	95.4	2.7	<0.3	<0.3	<0.3
D	255	246.3	94.7	4.3	<0.3	<0.3	<0.3
E	251	254.2	96.1	2.8	<0.3	<0.3	<0.3
F	246	252.3	94.7	3.8	<0.3	<0.3	<0.3
G	250	255.4	92.8	5.1	<0.3	<0.3	<0.3
H	249	260.6	98.9	1.1	<0.3	<0.3	<0.3
I	253	261.0	95.0	2.0	<0.3	<0.3	<0.3
Film-coated tablets:							
A	274	273.5	98.1	1.9	<0.3	<0.3	<0.3
B	259	253.1	97.1	2.2	<0.3	<0.3	<0.3
C	252	258.5	97.4	2.6	<0.3	<0.3	<0.3
D	248	256.8	96.7	3.2	<0.3	<0.3	<0.3
E	252	267.1	97.9	2.1	<0.3	<0.3	<0.3
F	253	258.5	97.6	2.3	<0.3	<0.3	<0.3
G	253	249.5	97.3	2.7	<0.3	<0.3	<0.3
Film-coated tablets:							
A	521	531.5	97.6	2.4	<0.3	<0.3	<0.3
B	490	522.6	97.5	2.5	<0.3	<0.3	<0.3
C	493	509.6	97.5	2.5	<0.3	<0.3	<0.3
Syrup:							
A	137	135.9	93.1	3.9	<0.3	<0.3	<0.3
B	138	136.6	92.8	4.5	<0.3	<0.3	<0.3
C	139	134.9	93.3	4.4	<0.3	<0.3	<0.3
D	131	138.3	92.8	5.3	<0.3	<0.3	<0.3
E	133	135.1	93.6	3.9	<0.3	<0.3	<0.3
F	129	129.9	92.2	4.2	<0.3	<0.3	<0.3
G	133	123.2	92.9	3.4	<0.3	<0.3	<0.3
Oral suspension:							
A	109	108.7	95.6	4.4	<0.3	<0.3	<0.3
B	102	102.7	96.5	3.5	<0.3	<0.3	<0.3
C	112	103.1	96.5	3.5	<0.3	<0.3	<0.3
D	112	104.6	96.8	3.2	<0.3	<0.3	<0.3
E	102	103.1	96.5	3.5	<0.3	<0.3	<0.3

[a] I = tetracycline, II = 4-epitetracycline, III = chlortetracycline, IV = 4-epianhydrotetracycline, and V = anhydrotetracycline.

Table VI—Analysis of Expired Lots of Pharmaceutical Preparations Containing Tetracycline Phosphate Complex by HPLC

Lot	Microbiological Potency	Calculated HPLC Potency	Percent Composition by HPLC[a]				
			I	II	III	IV	V
Hard-filled capsule[b]:							
A	61.6	64.0	76.2	19.9	<0.3	1.9	2.0
B	56.3	58.3	72.3	21.8	<0.3	3.2	2.7
Hard-filled capsule[c]:							
A	241	220	78.3	16.5	<0.3	1.6	3.5
B	219	240	78.3	17.2	<0.3	2.4	2.2
C	226	203	75.5	19.2	<0.3	2.9	2.4
D	226	196	71.1	24.3	<0.3	2.8	1.8

[a] I = tetracycline, II = 4-epitetracycline, III = chlortetracycline, IV = 4-epianhydrotetracycline, and V = anhydrotetracycline. [b] Containing tetracycline phosphate complex with novobiocin. [c] Containing tetracycline phosphate complex with nystatin.

The results shown in Table II indicate that the recovery of 4-epianhydrotetracycline was linear (r = 1.000) over the 0.3–100% range with a linear regression of $y = x - 0.201$. The relative standard deviation of the 4-epianhydrotetracycline determination, performed using a tetracycline sample containing approximately 3% 4-epianhydrotetracycline, was approximately 6%.

Analysis of Pharmaceutical Preparations—Since tetracycline base and tetracycline hydrochloride are readily soluble in methanol, this solvent has been used for extraction of tetracycline from pharmaceutical formulations. The methanol extraction procedure has a distinct advantage over aqueous systems since tetracycline hydrochloride is unstable and the base form is insoluble in water. Both of these tetracycline forms, however, are soluble and stable in methanol for at least 8 hr, with no increase in 4-epitetracycline (3). Also, many of the incipient formulation components are insoluble in methanol and are separated from the tetracycline with a simple centrifuging procedure.

Tetracycline recovery studies were performed using hard filled capsules containing tetracycline hydrochloride and a syrup containing tetracycline base. Recovery was 99.9% for the capsules and 99.6% for the syrup, both at label concentration (Table III). Recovery studies were performed on these two products, since the composition of most formulations is similar.

The relative standard deviation of the method was determined by using eight separate weighings and extractions on one lot of hard filled capsules containing 250 mg of tetracycline hydrochloride. The relative standard deviation of the method was 1.1% (Table IV).

Excellent agreement was obtained between the HPLC calculated biological equivalence and the data obtained by the microbiological method using *S. aureus* (9) for products containing tetracycline base and tetracycline hydrochloride (Table V).

Two products containing tetracycline phosphate complex were then assayed; expired lots were used (Table VI). These expired lots contained less than 3% 4-epianhydrotetracycline, with the exception of one lot containing 3.2%. The limit of 4-epianhydrotetracycline in capsule products is 3% (1). Thus, the HPLC method would be of value as a stability-indicating assay method. The agreement between the HPLC calculated values and values obtained by the agar diffusion assay method using *B. cereus* (9) were satisfactory, although considerable variations in assay values were noted. The variation may be due to the agar diffusion assay method using *B. cereus*, since the method is infrequently performed in our laboratory. We are converting the agar diffusion assay method to the more reliable turbidimetric assay method using *S. aureus* (9).

The high speed of analysis, the precision, and the good sensitivity to minor quantities of degradation compounds make the improved HPLC method ideal for the analysis of pharmaceutical preparations containing tetracycline to determine compliance with the requirements of the FDA (1) and the European Pharmacopoeia (2).

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