found as impurities with or decomposition products of chloral hydrate, will not interfere with its analysis.

The speed, accuracy, and specificity of the NMR method make it a useful procedure which can provide a rapid assay with an accuracy of about 1–2%. This method can be very useful for stability studies of chloral hydrate alone or in the capsule dosage form. It could also be utilized for individual capsule analysis.

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Modified Method for Determining Tetracycline, 4-Epitetracycline, and Anhydrotetracyclines in Tetracycline Base or Hydrochloride

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Abstract □ A partition column chromatographic procedure was developed for the assay of tetracycline samples for tetracycline (I), 4-epitetracycline (II), anhydrotetracycline (III), and 4-epianhydrotetracycline (IV). Two separate portions of a sample are used, one for the assay of I and II and the other, after a concentration step, for the assay of III and IV. Both portions are assayed with the same chromatographic system. The compounds are eluted from a column of diatomaceous earth coated with a pH 7.0 buffer containing disodium ethylenediaminetetraacetate, glycerin, and polyethylene glycol 400. The individual eluates are assayed by absorption spectroscopy. The precision of the assay for I, $SD \pm 0.5\%$, suggests that a chemical method such as this should be considered as the official assay method for tetracycline samples.

Keyphrases □ Tetracycline base or hydrochloride—column chromatographic analysis for tetracycline and impurities, evaluated as potential compendial method □ 4-Epitetracycline—column chromatographic analysis, tetracycline base or hydrochloride □ Anhydrotetracyclines—column chromatographic analysis, tetracycline base or hydrochloride □ 4-Epianhydrotetracycline—column chromatographic analysis, tetracycline base or hydrochloride □ Column chromatography, partition—analysis, tetracycline base or hydrochloride, evaluated as potential compendial method

The analysis of tetracycline samples for tetracycline (I) and three associated impurities, 4-epitetracycline (II), anhydrotetracycline (III), and 4-epianhydrotetracycline (IV), has been carried out by various chromatographic procedures (1–8). Only two of these procedures (7, 8) include methods for the assay of all four compounds. A TLC procedure (7) gave recoveries of I lower than those desirable for accurate quantitation. The second method (8) extended earlier work (6) to include the determination of III and IV.

The chromatographic system of Ascione *et al.* (6) has been used in this laboratory for the analysis of tetracycline raw materials and for stability assays of syrups and tablets. The method, as extended (8), does not give accurate assay values for the amounts of impurities in actual samples. The synthetic mixtures analyzed (8) contained about 10% each of III and IV. Work in this laboratory and in that of others (9) has shown that samples of tetracycline hydrochloride or base usually contain a few tenths of a percent or less of each impurity. These low amounts require a concentration step prior to the assay of III and IV; this, in turn, requires two samples for a complete analysis. The method given here is applicable to actual samples of tetracycline base and hydrochloride and has been so used (Table I). The method should be equally applicable to tetracycline phosphate.

EXPERIMENTAL

Materials and Reagents—Benzene, chloroform, *n*-butanol, methanol, acetic acid, ammonium hydroxide, and disodium ethylenediaminetetraacetate (V) were reagent grade.

For the 20% polyethylene glycol 400 in glycerin solution, add polyethylene glycol 400 to 80 ml. of glycerin USP to make 100 ml.

For the glycol buffer solution, adjust a 0.1 *M* solution of V to pH 7.0 with concentrated ammonium hydroxide. To 95 ml. of this solu-

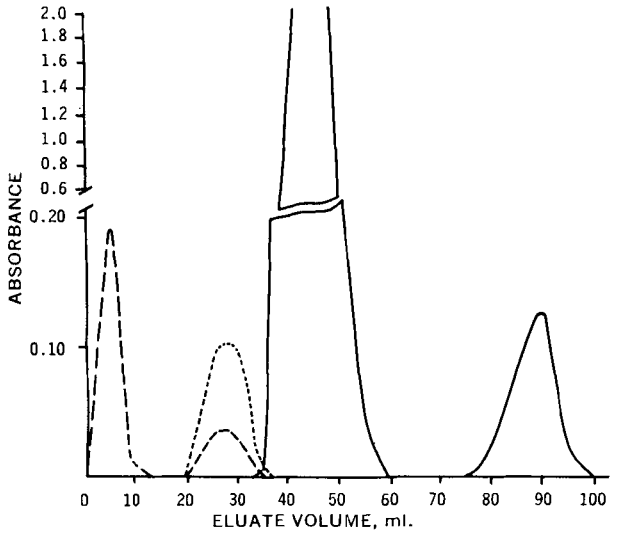

Figure 1—*The curves show the elution of the compounds from the partition column by this procedure. Key: – –, elution of III and IV, absorbance at 435 nm.; ——, elution of I and II, absorbance at 366 nm.; and ---, elution of VI, absorbance at 380 nm. The elution curve of III and IV represents the equivalent of 20 mg. of sample added to the column. The elution curve of VI was determined by using 0.05 mg. VI.*

tion, add 20% polyethylene glycol 400 in glycerin to make 100 ml.

For the acetic acid solution, use 1 *M* acetic acid in methanol; use freshly prepared reagent.

For the ammonia solution, dilute 1 ml. of concentrated ammonia to 17 ml. with methanol.

The chromatographic column used, 1.5-cm. diameter by 30-cm. length, had a Teflon stopcock. Acid- and solvent-washed diatomaceous earth[1] was prepared as described by Ascione *et al.* (6). The column packing and columns were also prepared as described by Ascione *et al.* (6).

Standards—The I-hydrochloride used was USP Tetracycline Hydrochloride Reference Standard. The III-base was prepared by the method of Simmons *et al.* (1). The IV-hydrochloride was prepared by the method of Simmons *et al.* (2) and recrystallized by dissolving in warm methanol, adding hydrochloric acid, and cooling. The II-ammonium salt was prepared by the method of McCormick *et al.* (10).

Reference Solutions—Weigh accurately about 100 mg. each of I-hydrochloride, II-ammonium salt, and IV-hydrochloride and dissolve each separately in 10 ml. of methanol. Add 0.4 ml. of 1 *M* ammonium hydroxide in methanol to the I and IV standards. Dilute with chloroform to give solutions containing 0.02 mg. I/ml., 0.004 mg. II/ml., and 0.004 mg. IV/ml. The final dilutions are made to contain 2 ml. of 1 *M* acetic acid solution in methanol/50 ml.; the final dilution of II is made to contain, in addition, 17 ml. of *n*-butanol/50 ml. Weigh accurately about 100 mg. of III-base. Dissolve in benzene and dilute with benzene to give a solution containing 0.004 mg./ml.

Determination of I and II—*Sample Preparation and Elution*—Weigh accurately about 25 mg. of I-base or I-hydrochloride into a 25-ml. volumetric flask. Dissolve in 2 ml. of 0.1 *M* hydrochloric acid. Add 15 ml. of the glycol buffer and then 2 ml. of 0.1 *M* sodium hydroxide; dilute to 25 ml. with the glycol buffer. The remaining sample preparation, elution, and the fractions taken for assay were the same as those of Ascione *et al.* (6).

Assay of Column Eluates—Add 2.0 ml. of 1 *M* acetic acid in methanol to the I fraction and dilute to 50 ml. with chloroform. Determine the absorbance of this solution against chloroform in a 1-cm. cell at the absorbance maximum at 366 nm. and compare to the absorbance of the standard solution of I.

Add 2.0 ml. of 1 *M* acetic acid in methanol to the II fraction and dilute to the nearest milliliter mark with chloroform. Determine the absorbance of this solution against a 35% *n*-butanol in chloroform solution in 5-cm. cells at the absorbance maximum at 366 nm. Compare the absorbance of this solution to the absorbance of the standard solution of II.

Determination of III and IV—*Sample Preparation*—Carry out the dissolution, pH adjustment, and extraction as quickly as possible to avoid sample degradation.

Weigh accurately about 100 mg. of I-base or I-hydrochloride into a 100-ml. beaker. Add 5 ml. of 0.1 *M* hydrochloric acid and swirl until the solid dissolves. Add 25 ml. of a 0.1 *M* solution of V and adjust to pH 4.5 with 1 *M* hydrochloric acid or 1 *M* sodium hydroxide as necessary. Transfer the solution to a separator with the aid of a little water and extract the solution with three 15-ml. portions of chloroform. Combine the chloroform extracts in a 50-ml. volumetric flask and dilute to volume with chloroform. Pipet 10.0 ml. of this solution into a 50-ml. beaker. Evaporate the chloroform at room temperature with a stream of nitrogen. Pipet 1.0 ml. of the glycol buffer solution into the beaker and dissolve the residue. Add 2.0 g. diatomaceous earth and proceed as for the sample preparation in the determination of I.

Elution of III and IV—Elute the column with 20 ml. of benzene followed by 40 ml. of chloroform after the benzene has drained to the top of the column. Collect the first 15 ml. of eluate in a 25-ml. glass-stoppered, graduated cylinder (III fraction). Collect the next 20 ml. of eluate in another 25-ml. glass-stoppered, graduated cylinder (IV fraction). Discard the remainder of the eluate.

Assay of Column Eluates—Dilute the III fraction to 20 ml. with benzene. Determine the absorbance of this solution against benzene in a 5-cm. cell at the absorbance maximum at 435 nm. Compare the absorbance of this solution to the absorbance of the standard solution of III.

To the IV fraction, add 2.0 ml. of 1 *M* acetic acid in methanol; dilute with chloroform to 25 ml. Determine the absorbance of this solution against chloroform in a 5-cm. cell at the absorbance maximum at 435 nm. Compare the absorbance of this solution to the absorbance of the standard solution of IV. Detection of chlortetracycline (VI) is made by determining the absorbance of this solution at 380 nm. (See *Results and Discussion.*)

RESULTS AND DISCUSSION

Both I and II are determined by application of 1 mg. of sample to the partition column. The amounts of III and IV eluted from this column are very low and cannot be determined in this eluate. The amount of II eluted is also small but is limited by the I elution. If more than 1 mg. of sample is put on the column, the I tails into the II fraction. The amount of II eluted is sufficient for an accurate assay only when a 5-cm. cell is used for the determination of the UV absorbance; the absorbance in a 1-cm. cell would be 0.04 for the eluate of a sample containing 5% II. In an earlier paper (6), a 5-cm. cell was used to measure the absorbance of the II eluate; more recently (8), a 1-cm. cell was reported.

Since the anhydro compounds are present in very small amounts in most samples of I, 20 mg. of the sample is required to provide a sufficient amount for an accurate determination. Since 20 mg. of I will not dissolve in the 1 ml. of pH 7.0 buffer needed for the chromatography, a concentration step is required. Chloroform selectively, although not exclusively, extracts the anhydro compounds from the pH 4.5 buffer. Evaporation of an aliquot of the extract equivalent to 20 mg. of I leaves a residue which is readily soluble in 1 ml. of the pH 7.0 buffer.

The extraction of III and IV from aqueous buffer solutions into chloroform was shown to be maximal in the pH range of 4.0–5.5 (4). A phosphate buffer, an acetate buffer, and the V buffer as prepared in this study, all at pH 4.5, were tested for recovery of the two anhydro compounds. Only 94% recovery was obtained with the phosphate and acetate buffers, but 100% recovery of the anhydrotetracyclines was obtained with the V buffer when the extraction was carried out as in the described procedure.

Figure 1 shows the elution curve of all four compounds. The data were obtained from two different sample sizes as already discussed. Five-milliliter fractions were taken throughout each elution. Because absorbance readings of the fractions were made in 1-cm. cells, they were much lower than they would be for the total eluate of each impurity, which was made in 5-cm. cells. The elution curve for VI is also given in Fig. 1.

I-hydrochloride (Sample 4, Table I), III-base, and IV-hydrochloride were analyzed by the described procedure to determine the

[1] Celite 545, Johns-Manville Corp., New York, NY 10016

Table I—Analysis of Tetracycline Samples

Sample	Form	Percent Determined I	II	III	IV	Water[a]
1	Base	80.0	8.0	0.9	0.2	9.5
2	Base	80.0	5.9	0.3	0.03	11.1
3	Base	80.8	7.6	0.3	0.05	12.1
4	Hydrochloride salt	94.4	4.1	0.4	0.1	—[b]
USP reference standard[c]	Hydrochloride salt	98.0	1.9	0.1	0.03	—[d]
USP reference standard[c]	Hydrochloride salt	97.6	1.3	0.03	0.02	—[d]

[a] Water determined by loss on drying in a vacuum oven at 60° for 8 hr. [b] Not determined. [c] Two different samples of the USP reference standard were used. The first was packed July 1967, the second July 1968. [d] The USP samples were dried prior to use according to label instructions.

amounts of the two anhydro compounds present in each. The I-hydrochloride was found to contain 0.4% III and 0.1% IV. The III-base contained 99% III and 1% IV, and the IV-hydrochloride contained 93% IV and 7% III. These values were used in subsequent recovery experiments.

III and IV were added to the I-hydrochloride sample and recovered by the method described. Average recoveries of 93% of the III and 92% of the IV were obtained over the range of 0.5–3% of the compounds added as impurities to I.

USP Tetracycline Hydrochloride Reference Standard was used to study the precision and accuracy of the I determination by this method. The amounts of I and II in the sample were determined by the present procedure, and the sum of these two values was compared to the amount of total tetracycline taken to obtain the recovery of I. Five samples gave an average recovery of 99.1 ± 0.5%. II was added as an impurity in amounts of 5 and 10% to the USP reference standard. The average recovery of II was 99%. Several tetracycline samples were analyzed by this method, and the results obtained are summarized in Table I. Simmons *et al.* (5) reported a I content of 97.8% for the USP reference standard, which is in good agreement with the values reported here.

Compound I in 0.03 *M* hydrochloric acid has been reported as being stable over long periods of time without any acid-catalyzed dehydration to III (10). It was found that a solution of I-base in 0.03 *M* hydrochloric acid increases in total anhydro compounds from 0.3 to 0.5% in 6 hr. and to 1.1% in 24 hr.; work with any acid solution should be carried out promptly to avoid this degradation.

Compound VI is sometimes present in I samples as an impurity. Its elution from the column coincides with that of IV. If present, VI can be readily detected in amounts of 0.25% or more by its absorbance maximum at 380 nm. Since the extraction of VI by the procedure given is not quantitative, no attempt was made to include VI in the assay procedure. Synthetic solutions were prepared containing either VI or IV or both. The amounts of each were varied and were equivalent to those amounts which, if present in I samples, would cover the range from 0 to 5%. The solutions were quantitatively analyzed for both components with absorbance measurements at 380 and 450 nm. where VI has no absorbance. The maximum relative error in the determination of either compound was 3%.

Absorption spectra were measured on a recording spectrophotometer[2]. The spectra of all the compounds varied with the solvent and the ionic form of the molecule. Each compound is eluted from the column as the free base, while only the III reference standard is in this form. Since suitable standard samples of the free bases of the other three compounds are not readily available, some adjustments of solution conditions in the eluates and the standard solutions are necessary.

Previously, a methanolic ammonia solution was used by Ascione *et al.* (6) to produce the same molecular species in both sample and standard solutions of I and II. Its use was based partly on the increased absorbance that resulted, although the solutions produced were somewhat unstable. This instability led to the investigation of the use of acetic acid to produce the same molecular species in both sample and standard solutions. Prior to the addition of acetic acid, the protonated species, when present in standard solutions, must be neutralized with a slight excess of ammonia.

The stabilities of the solutions containing either ammonia or acetic acid are sufficient for analytical purposes for both I and II. The most stable solution of I is that of the free base in chloroform without any added reagent; its absorbance remained constant for 24 hr. The solution of the ammonium salt of II is likewise more stable than those solutions with added reagent. The difficulty of routinely producing a solution containing the precise equivalent of the free base of I in its standard solution and of the ammonium salt of II in the sample eluate eliminates these species for reference purposes.

Solutions of VI containing ammonia are quite unstable; a steady decrease in absorbance was noted by others (6) and confirmed by the authors. Solutions of VI containing acetic acid show a slight rise in absorbance in the first 30 min. and then are stable up to 5 hr.

The absorbances of I and II solutions containing acetic acid are constant with varying amounts of the reagent. The absorbances of ammoniacal solutions increase as the concentration of the reagent increases. The absorbance increase noted by others (6) occurs, for the most part, when the protonated species is converted to the free base. The following absorbances were noted for solutions containing identical concentrations of about 2 mg./100 ml. of I in chloroform: hydrochloride, 0.510; free base, 0.637; base plus acetic acid, 0.646; and base plus ammonia, 0.655; all were prepared from the same stock solution. The reagents in the last two cases were added in the amounts described herein and by others (6), respectively.

The official methods for the analysis of tetracycline raw material do not include chemical methods for the assay of the I content or of the individual impurities, particularly for the potentially harmful 4-epianhydrotetracycline (11–14). The authors recommend consideration of an assay method, such as the one presented here, for official adoption. The accuracy of determination of I by this method is superior to the present microbiological method used as an official assay. The latter method has an uncertainty of 10–15%, an order of magnitude less precise than the chemical method given here.

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[2] Cary model 14.