Simultaneous Separation and Quantitative Determination of Tetracycline, Anhydrotetracycline, 4-Epitetracycline, and 4-Epi-anhydrotetracycline in Degraded Tetracyclines by Thin-Layer Chromatography

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Abstract □ A rapid procedure is described which permits the quantitative separation and determination of tetracycline (TC), 4-epitetracycline (ETC), anhydrotetracycline (ATC), and 4-epi-anhydrotetracycline (EATC) on the same plate by thin-layer chromatography, on kieselguhr impregnated with EDTA at pH 7.5 with ethyl acetate–acetone–water (10:20:3) as solvent, or on kieselguhr with EDTA at pH 9 and water–acetone (1:10) as solvent. The method has an incalculable value in the diagnosis of degraded tetracyclines, it permits the determination of 0.5% of each one of the above-mentioned products in the presence of tetracycline, and it detects quantities of about 0.1% in degraded tetracyclines.

Keyphrases □ Tetracycline, anhydrotetracycline, and 4-epi derivatives—simultaneous determination □ TLC—separation, analysis □ UV light—chromatographic spot visualization □ UV spectrophotometry—analysis □ Colorimetric analysis—spectrophotometer

Following the works of Gross and others (1–7) on reversible renal dysfunction (Fanconi-type syndrome) observed in some patients who have ingested degraded tetracycline, other works have appeared in the literature dealing with the identification and determination of these products of degradation (ATC and EATC) in tetracyclines.

Novelli *et al.* (8) describe a method for the separation and determination by radial chromatography on paper of TC, ATC, and EATC in fermentation substances or in powder. The authors point out the need of applying experimental correction factors in the spectrophotometric determination, which are different for each component, so that the results coincide with the theories and with the microbiological determinations.

Kelly (9) determined ATC and EATC by column chromatography on diatomaceous earth.[1] The process requires a strict pH control and the separation of the two anhydrous forms from each other, and the separation of them from tetracycline does not offer any discontinuation.

In 1956, Rustici and Ferapi (10) separated ATC and EATC by circular TLC on silica gel impregnated with EDTA. The process does not separate TC from ETC, and the authors do not give any quantitative application of the method.

Simmons *et al.* (11) described the separation of ATC and TC on microcrystalline cellulose. Finally, Simmons *et al.* (12) separated TC, ATC, and EATC by a two-dimensional TLC on microcrystalline cellulose.

The authors have considered it of great interest to find a rapid process which allows the simultaneous separation of TC, ETC, EATC, and ATC, and their quantitative determination in the same chromatogram.

Simmons *et al.* (12) worked with final concentrations of ATC and EATC from 1–5 mcg./ml., finding absorbances ranging between 0.020 and 0.100. The authors have preferred to work with greater final concentrations (10–40 mcg./ml.) in order to obtain absorbances between 0.150 and 0.700 which to a high degree limit the errors of interpolation in the absorption readings and the parasite absorbances, and also allow a greater precision in the determinations.

EXPERIMENTAL

Apparatus and Reagents—Spectrophotometer,[2] TLC equipment,[3] and kieselguhr, acetone, ethyl acetate, and EDTA reagent quality.

Standards—Tetracycline hydrochloride USP reference standard; 4-epitetracycline, ammonium salt [prepared by employing tetracycline hydrochloride according to McCormick (13)] with a UV-absorbing spectrum in values of a = 35.2 to 255 mμ; a = 30.4 to 358 mμ; A_{254}/A_{267} = 1.06, and an IR spectrum in accordance with Kaplan *et al.* (14).

After chromatography by the method described in this work (in order to separate traces of tetracycline which it contains as an impurity), its microbiological activity was tested against that of *B. cereus* (variant, mycoides), and activity values were found at about 12% in accordance with Kaplan (14).

Anhydrotetracycline was prepared by employing tetracycline hydrochloride according to Simmons *et al.* (11), and its purity was determined by the chromatographic method described in this work by its absorptivity to 430 mμ (1.698×10^{-2}) and by its UV spectrum in accordance with McCormick *et al.* (13). 4-Epi-anhydrotetracycline was prepared according to McCormick (13) by employing the ammonium salt of the 4-epitetracycline with an absorptivity of 1.611×10^{-2} to 430 mμ in accordance with Kelly (9).

Kieselguhr Purification—Five-hundred grams of cold kieselguhr was stirred with 3 l. of hydrochloric acid (1:2) for 2 hr., decanted, filtered by means of suction, and repeated three times. For decanting it was washed with water until the chlorides were eliminated and dried at 100°, and sifted through sieve No. 200 ASTM.

Preparation of the Plates—Two series of plates were prepared, one with EDTA to pH 9 and another with EDTA to pH 7.5. A slurry of 40 g. of kieselguhr purified with 80 ml. of aqueous solution of EDTA at 5% (neutralized to pH 7.5 or pH 9 with sodium hydroxide at 20%) was poured into the applicator and coated on several 0.3 mm. thick plates. It was allowed to dry at room temperature for 1 hr. and then dried in an oven at 100° for 1 hr.

Solvents—Acetone–water (10:1) was used for the plates at pH 9, and acetone–ethyl acetate–water (20:10:3) was used for the plates at pH 7.5.

Determination of R_f Values—A mixture of TC, ATC, EATC, and ETC in equal quantities weighing 10 mg. was prepared and dissolved in 5 ml. of 0.1 *N* HCl. One microliter of this solution was

[1] Trademarked as Celite 545, Johns-Manville, New York, N. Y.

[2] Perkin-Elmer, model 137 UV.

[3] Shandon.

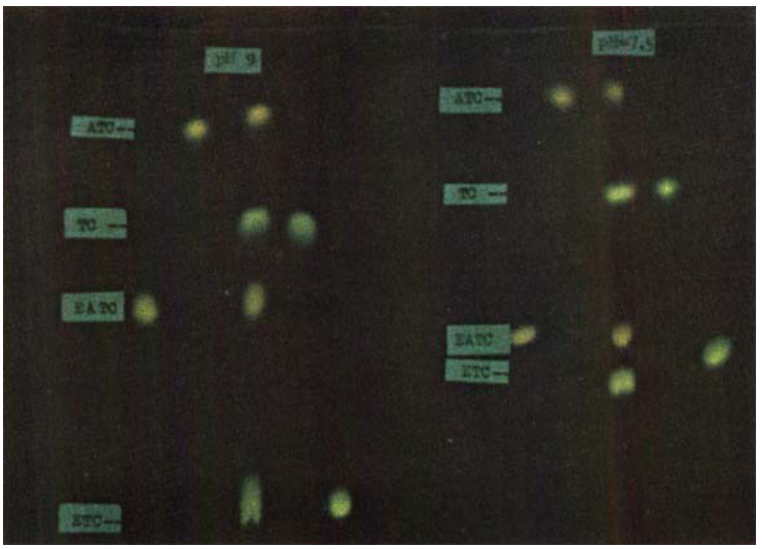

Figure 1—*Left side, left to right: epi-anhydrotetracycline; anhydrotetracycline; mixture (TC, ATC, ETC, and EATC); tetracycline; epi-tetracycline; on kieselguhr and 5% EDTA at pH 9. Right side, left to right; epi-anhydrotetracycline; anhydrotetracycline; mixture (TC, ATC, ETC, and EATC); tetracycline; epitetracycline; on kieselguhr and 5% EDTA at pH 7.5.*

Table I—R_f Values at pH 7.5 and at pH 9 of TC, ATC, ETC, and EATC

Component	pH 7.5	pH 9
Anhydrotetracycline	0.88	0.84
Tetracycline	0.71	0.69
Epi-anhydrotetracycline	0.46	0.55
Epitetracycline	0.38	0.22

placed on plates at pH 7.5 and on plates at pH 9. One microliter of the solution at the same concentration of each one of the pure substances was also placed on the same plates. The plates were then placed into the chromatographic chamber containing 150 ml. of the corresponding solvent (the inner walls were covered with impregnated filter paper from the solvent). The plates were developed at pH 9 with acetone–water (10:1), and plates at pH 7.5 with acetone–ethyl acetate–water (20:10:3).

The R_f values found are shown in Table I, and correspond to Fig. 1.

PROCEDURE

Quantitative Analysis—Along a horizontal line at 2 cm. from the lower edge of each plate, 50 μl. of a solution to be determined was dried in cool air and placed in the chromatographic chamber containing 150 ml. of the corresponding solvent.

The inner walls of the chamber were covered with filter paper impregnated with the solvent in order to assure the saturation of the internal atmosphere of the chamber. When the front reached an approximate distance of 2 cm. (about 20 to 30 min.) from the upper edge of the plate, it was taken from the chamber and dried for 2 min. at 100° and observed under UV light of 366 mμ (the bands delimited).

The bands were separated independently, and the powder was taken into 20-ml. centrifuge tubes with ground-glass stoppers; 2 *N* hydrochloric acid was added and stirred for 5 min.

The tubes were heated during 5 min. over hot water in order to transform tetracycline and 4-epitetracycline to anhydrotetracycline and 4-epi-anhydrotetracycline, allowed to cool, centrifuged, and diluted with water conveniently. Thus, a final concentration of 10 to 40 mcg./ml was obtained.

The absorption was recorded at 430 mμ, and the contents of each

Table II—Results Obtained in the Quantitative Determination of Each of the Four Components of Six Mixtures of TC, ATC, ETC, and EATC of Variable Composition on Kieselguhr at pH 7.5 and Acetone–Ethyl Acetate–Water (20:10:3)

Mixture No.	Composition, %	mcg./Plate Theoretical	mcg./Plate Found	Component, %	Mixture, %
1	25.00 TC	250	243	97.1	24.30
	25.00 ATC	250	250	100.0	25.00
	25.00 ETC	250	235	94.0	23.50
	25.00 EATC	250	239	95.6	23.90
2	55.00 TC	550	533.5	97.0	53.30
	15.00 ATC	150	145.5	97.0	14.50
	15.00 ETC	150	141.0	94.0	14.10
	15.00 EATC	150	144.0	96.0	14.40
3	70.00 TC	700	665.0	95.2	66.50
	10.00 ATC	100	101.0	101.0	10.10
	10.00 ETC	100	92.0	92.0	9.20
	10.00 EATC	100	93.7	93.7	9.37
4	85.00 TC	1,700	1,640.0	96.5	82.00
	5.00 ATC	100	103.0	103.0	5.10
	5.00 ETC	100	101.0	101.0	5.05
	5.00 EATC	100	96.3	96.3	4.80
5	92.50 TC	1,850	1,757.0	95.0	87.80
	2.50 ATC	50	47.0	94.0	2.35
	2.50 ETC	50	50.0	100.0	2.50
	2.50 EATC	50	46.0	92.0	2.30
6	96.25 TC	3,850	3,657.0	95.0	91.40
	1.25 ATC	50	50.0	100.0	1.25
	1.25 ETC	50	48.5	97.2	1.21
	1.25 EATC	50	48.2	96.5	1.20

Table III—Results Obtained in the Quantitative Determination of Each of the Four Components of Four Mixtures of TC, ATC, ETC, and EATC of Variable Composition, on Kieselguhr with EDTA at pH 9 and Acetone–Water (10:1)

Mixture No.	Composition, %	mcg./Plate Theoretical	mcg./Plate Found	Component, %	Mixture, %
1	25.00 TC	250	230.0	92.0	23.00
	25.00 ATC	250	235.0	95.0	23.50
	25.00 ETC	250	241.0	96.5	24.10
	25.00 EATC	250	230.0	92.0	23.00
3	70.00 TC	700	654.5	93.5	65.40
	10.00 ATC	100	102.0	102.0	10.20
	10.00 ETC	100	98.5	98.5	9.85
	10.00 EATC	100	95.5	95.5	9.55
5	92.50 TC	1,850	1,730.0	93.5	86.50
	2.50 ATC	50	51.0	102.0	2.55
	2.50 ETC	50	51.0	102.0	2.55
	2.50 EATC	50	51.0	102.0	2.55
6	96.25 TC	3,850	3,565.0	93.0	89.11
	1.25 ATC	50	48.5	97.0	1.21
	1.25 ETC	50	48.6	97.0	1.21
	1.25 EATC	50	47.3	94.6	1.18

Table IV—Results of the Spectrophotometric and Microbiological Analysis of Four Samples of Tetracycline, Before and After Chromatography, in mg./g. of Tetracycline Hydrochloride

Sample	Method	After Chromatography					Before Chromatography
		TC	ETC	ATC	EATC	Total	
X	Spectrophotometric	933	126	2.6	<1	1,062	1,048
	Microbiological	913	17			930	960
L	Spectrophotometric	890	187	2.1	<1	1,079	1,070
	Microbiological	890	23			913	924
D	Spectrophotometric	905	160	3.0	<1	1,066	1,054
	Microbiological	904	18			922	969
M	Spectrophotometric	990	6			996	1,005
	Microbiological	1,000				1,000	1,000

of the components were calculated by using the values of absorptivity 1.698×10^{-2} for anhydrotetracycline and 1.611×10^{-2} for epi-anhydrotetracycline.

Results are shown for six solutions of known composition in Tables II and III. An example of this separation can be seen in Fig. 2.

DISCUSSION AND APPLICATIONS

The results obtained clearly show that the separation and quantitative determination of TC, ATC, ETC, and EATC is possible at both values of pH.

Although the authors have worked with final solutions containing 20–80 mcg./ml., the best final concentration for reading would be from 20–40 mcg./ml. The separation of the four components are even better at lower concentration, but the quantitative determination depends on the absorbance readings which are under 0.150, where very weak absorbances or interpolations estimated in the readings scale of the apparatus could lead to great errors.

When it would be necessary to use concentrations higher than 40 mcg./μl., because of the low concentration in ATC, ETC, and EATC in the product to be examined, either plates at pH 7.5 should be used or more than one plate should be used for each test.

This method was applied during the analysis of three samples (*X*, *L*, *D*) of tetracycline of the same manufacturer and another (*M*) from a second manufacturer by spectrophotometric and microbiological processes.

The same samples were chromatographed by the process described by separating and estimating the TC and ETC bands by spectrophotometric and microbiological methods, and bands of ATC and EATC spectrophotometrically. The results are shown in Table IV.

The values of Table IV are the average of three tests, and the high

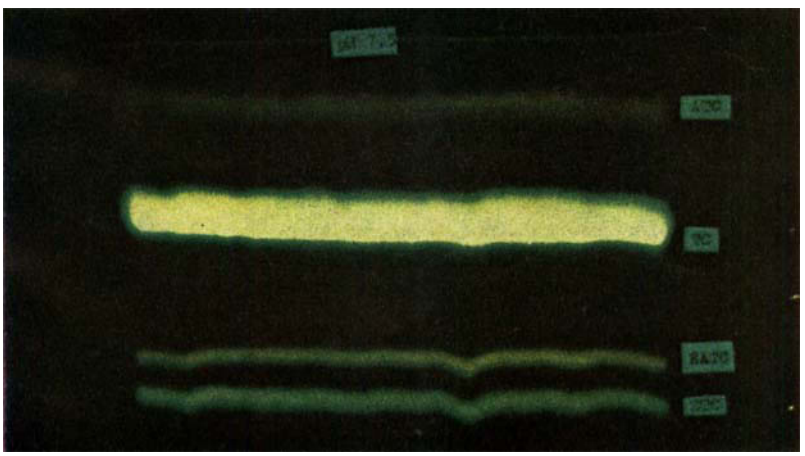

Figure 2—*Chromatogram of a mixture of 925 mcg. of TC, 25 mcg. of ATC, 25 mcg. of ETC, and 25 mcg. of EATC on kieselguhr with 5% EDTA at pH 7.5.*

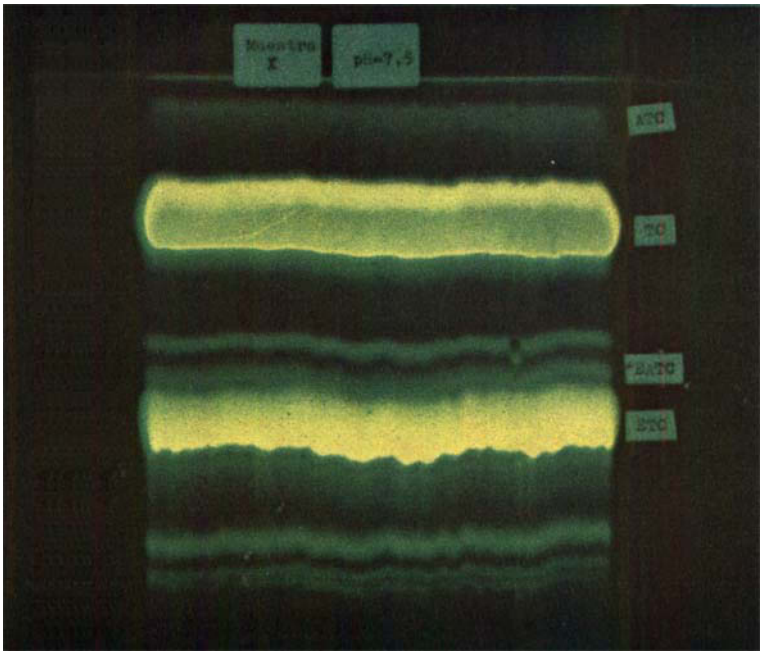

Figure 3— *Chromatogram of 4,000 mcg. of tetracycline, Sample X on kieselguhr with 5% EDTA at pH 7.5. Besides the four bands of TC, ATC, ETC, and EATC, other bands can be observed which are not identified.*

content in 4-epitetracycline in Samples *X*, *L*, and *D* can be observed as well as a good concordance of the spectrophotometric results before and after chromatography. It can also be observed that the microbiological analyses before and after chromatography agree within the 5% limits and that the microbiological activity of the 4-epitetracycline is about 12%.

The method has an incalculable value in the diagnosis of tetracycline as can be seen in Fig. 3 which corresponds to Sample *X* in Table IV.

In this photograph, bands which have not been identified can be observed, besides the four known bands corresponding to TC, ATC, ETC, and EATC.

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