(2) J. Swarbrick, *J. Pharm. Sci.*, **54**, 1229(1965).
(3) E. A. Swinyard and S. C. Harvey, "Remington Pharmaceutical Sciences," E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1965.
(4) R. W. Applewhite, A. P. Buckley, and L. W. Nobles, *J. Am. Pharm. Assoc., Pract. Pharm. Ed.*, **15**, 164(1954).
(5) V. P. Gusiakov, N. M. Likholet, and I. M. Kutnaya, *Farm. Zh.*, **22**, 34(1967); through *Intern. Pharm. Abstr.*, **4**, 1074(1967).
(6) D. Küttel, *Pharm. Zentralhalle*, **102**, 115(1963).
(7) *Ibid.*, **103**, 10(1964).
(8) J. J. Walker, R. S. Fischer, and J. J. McHugh, *Am. J. Clin. Pathol.*, **18**, 451(1948).
(9) M. R. V. Paruta and L. D. King, *J. Pharm. Sci.*, **53**, 1217 (1964).
(10) S. G. Bjaastad, N. A. Hall, and A. L. Thakkar, *ibid.*, **54**, 1529(1965).
(11) A. L. Thakkar and N. A. Hall, *ibid.*, **56**, 1121(1967).
(12) F. W. Goodhart and A. N. Martin, *ibid.*, **51**, 52(1962).
(13) C. K. Bahl and H. B. Kostenbauder, *ibid.*, **53**, 1027(1964).
(14) A. G. Mitchell and L. S. C. Wan, *ibid.*, **53**, 1467(1964).
(15) H. B. Klevens, *J. Am. Oil Chemists Soc.*, **26**, 456(1949).
(16) H. B. Klevens, *Chem. Rev.*, **47**, 9(1950).
(17) M. E. L. McBain and E. Hutchinson, "Solubilization and Related Phenomena," Academic, New York, N. Y., 1955.
(18) T. R. Bates, M. Gibaldi, and J. L. Kanig, *J. Pharm. Sci.*, **55**, 191(1966).
(19) D. J. Lamb and L. E. Harris, *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 583(1960).
(20) C. Hansch and S. M. Anderson, *J. Med. Chem.*, **10**, 745 (1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 17, 1969, from the *College of Pharmacy, University of Alexandria, Alexandria, U.A.R.*

Accepted for publication September 19, 1969.

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Improved Thin-layer Chromatographic Identification of Tetracyclines and Their Degradation Products: Application to an Epimerization Study

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Abstract □ An improved thin-layer chromatographic method for the identification of pharmaceutically important tetracyclines and their degradation products is presented. A process is used which conditions the plates with optimum moisture content so that they may be stored without special precautions for as long as 4 weeks before use. Two solvent systems and four spray reagents for the separation and detection of tetracyclines and their degradation products are also presented. Tetracycline hydrochloride and chlortetracycline hydrochloride were allowed to epimerize in phosphate and acetate solutions, respectively. With the aid of UV spectroscopy and TLC analysis, it is demonstrated that epimerization is accompanied by extensive degradation.

Keyphrases □ Tetracyclines, degradation products—identification □ Epimerization, tetracyclines—reaction products separation, identification □ TLC—separation, identification □ UV light—TLC spot visualization

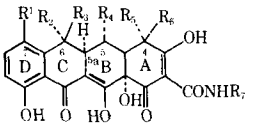
Thin-layer chromatography (TLC) has been used by a number of workers to separate and characterize certain tetracyclines of pharmaceutical importance (Table I) using adsorbent layers of silica gel (1, 2), kieselguhr (3–5), and microcrystalline cellulose (6). Difficulties encountered in these separations have been attributed to the property of tetracyclines to form chelate complexes with metallic ions and to lack of moisture in the support. Hence, sequestering agents (1–5) and glycerin (7) or mixtures of glycerin with polyethylene glycol 400 (PEG 400) (4) have been added to the support.

Of the various methods reported, two (3, 4) are most useful since they can resolve two or more tetracyclines in a single system on one chromatogram. Of these, the one employing the coating of kieselguhr containing EDTA and a developing solvent consisting of methyl ethyl ketone saturated with McIlvaine's buffer (pH 4.7) (3) was especially good for the separation of various degradation products of the tetracyclines. The other method (4), which employs acid-washed diatomaceous earth impregnated with EDTA at pH 7.0 and a glycerin–PEG 400 mixture, and which uses ethyl acetate as the developing solvent, was found satisfactory for the resolution of tetracyclines, though often it was necessary to chromatograph two to four times. In both cases, however, problems in their application were encountered. The most serious difficulty with the former method (3) was the frequent and erratic splitting and streaking of the tetracycline spots. The latter method (4) involves rigid adherance to a lengthy procedure in which the diatomaceous earth is repeatedly washed to remove binder and other acid-soluble materials. This was found not to be reproducible without considerable experience. Furthermore, the plates must be freshly prepared and used immediately. The method is, therefore, particularly inappropriate in situations where a large number of chromatograms must be run in a short time.

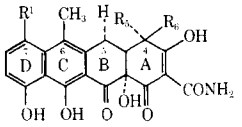
The tetracyclines have most commonly been detected on chromatograms by their fluorescence under long-wave UV light, either with or without exposure of the chromatograms to ammonia vapor (1, 3–5, 8). A few chromogenic spray reagents were described in the earlier literature (1) but have been largely abandoned in recent publications.

This communication describes a simple, rapid, and reproducible method for the separation of six tetracyclines presently marketed in numerous dosage forms.

Table I—Structures of Tetracyclines and Some of Their Degradation Products



(I)



(II)

Compound	Structure	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Tetracycline antibiotics								
Tetracycline (TC)	(I)	H	CH_3	OH	H	$N(CH_3)_2$	H	H
Chlortetracycline (CTC)	(I)	Cl	CH_3	OH	H	$N(CH_3)_2$	H	H
Oxytetracycline (OTC)	(I)	H	CH_3	OH	OH	$N(CH_3)_2$	H	H
Demethylchlortetracycline (DMCTC)	(I)	Cl	H	OH	H	$N(CH_3)_2$	H	H
Methacycline (MC)	(I)	H	CH_2		OH	$N(CH_3)_2$	H	H
Doxycycline (DOXY)	(I)	H	CH_3	H	OH	$N(CH_3)_2$	H	H
Rolitetracycline (RTC)	(I)	H	CH_3	OH	H	$N(CH_3)_2$	H	CH_2N
Degradation products								
4-Epitetracycline (4 epi-TC)	(I)	H	CH_3	OH	H	H	$N(CH_3)_2$	H
4-Epichlortetracycline (4 epi-CTC)	(I)	Cl	CH_3	OH	H	H	$N(CH_3)_3$	H
Anhydrotetracycline (ATC)	(II)	H	—	—	—	$N(CH_3)_2$	H	—
Epianhydrotetracycline (EATC)	(II)	H	—	—	—	H	$N(CH_3)_2$	—
Anhydrochlortetracycline (ACTC)	(II)	Cl	—	—	—	$N(CH_3)_2$	H	—

Various degradation and reaction products are also well separated, even when chromatographed on 4-week old plates. Four spray reagents for the detection of these antibiotics are presented. This TLC method has been used in a preliminary qualitative study of degradation products, other than epimers, formed when tetracycline hydrochloride (TC·HCl) and chlortetracycline hydrochloride (CTC·HCl) (see Table I) were allowed to epimerize at three different pH values at room temperature.

EXPERIMENTAL

Reagents—EDTA disodium salt, glycerin, PEG 400, methyl ethyl ketone, dichloromethane, ethyl formate, sodium phosphate monobasic, sodium acetate, all analytical grade reagents.

Preparation of Plates—With the aid of an applicator, five glass plates (20 × 20 cm.) were coated with 0.25-mm. layers of a slurry of 50 g. kieselguhr G (Merck) in a homogeneous mixture consisting of 95 ml. 0.1 *M* EDTA in water and 5 ml. 20% v/v PEG 400 in glycerin. The plates were allowed to dry at room temperature for at least 4 hr. (preferably overnight), then developed in the Solvent System I (see below) up to the top of the plate. They were then dried and stored in a dust-free atmosphere with no drying agent.

Spotting the Solution—Two microliters of 2 mg./ml. methanolic solutions of the tetracyclines [aqueous solutions in case of rolitetracycline(RTC)] were spotted as usual, 2 cm. from the edge of the plate.

Solvent Systems—*I*—Methyl ethyl ketone saturated with McIlvaine's buffer (pH 4.7) (3).

II—Dichloromethane–ethyl formate–ethanol (9:9:2) saturated with McIlvaine's buffer (pH 4.7).

Spray Reagents—*I—Fast Blue B(Diazo-Reagent)*—Spray Solution A: 0.5% aqueous, freshly prepared solution of fast blue B; Spray Solution B: 0.1 *N* NaOH (aqueous) (1*a*).

II—Diazotized p-Nitroaniline (1*b*)—Spray Solution A: just before spraying, 5% aqueous sodium nitrite solution (1.5 ml.) is added to 0.3% *p*-nitroaniline in 8% HCl (25 ml.); Spray Solution B: 20% aqueous sodium carbonate solution. After spraying with Solution A, the developed chromatogram is sprayed with Solution B, taking care not to make the plate transparent with an excess of the sprays.

III—Modified Sakaguchi Reagent (9)—Boric acid (5 g.) is dissolved in water (150 ml.) and concentrated sulfuric acid (350 ml.). The reagent is stored in a glass-stoppered bottle in a refrigerator and is used cold.

IV—Diphenylpicrylhydrazyl (DPPH) Reagent (10)—Solution A: methanolic solution of DPPH (~1 mg./2 ml.); Solution B: 25% aqueous NaOH solution.

Detection—All the tetracyclines and many of their degradation products could be detected by their fluorescence in longwave UV light (Black Ray UVL-22). For the visualization of the spots in daylight the dried chromatogram was sprayed with one of the spray reagents I–IV. With the first three reagents, colored spots are observed on a nearly white background but with spray reagent IV yellow spots on a bluish white background are obtained (Table II).

Epimerization Experiments—Five milliliters each of 1% aqueous solutions of TC·HCl and CTC·HCl were separately diluted to 25 ml. with distilled water (reference solution), 0.1 *M* sodium dihydrogen phosphate (aqueous) (pH 4.1), and 0.1 *M* sodium acetate (aqueous) (pH 7.3). The solutions were kept at room temperature in 25-ml. volumetric flasks and wrapped in aluminum foil for protection from light. At convenient time intervals, 2 μl. of each solution was examined by TLC using Solvent Systems I and II. Simultaneously, 0.2 ml. of each solution was diluted to 25 ml. with 0.1 *N* H_2SO_4 and immediately scanned between 210 and 400 mμ,[1] noting the absorbance at 254, 267, 298, and 355 mμ. The UV spectra (210–400 mμ) of the three TC·HCl solutions at 0 and 620 hr. are shown in Fig. 1. The corresponding spectra for CTC·HCl are presented in Fig. 2. The TLC of these solutions at 0, 24, and 620 hr. are schematically represented in Fig. 3.

[1] Beckman DB spectrophotometer.

Table II—TLC of Tetracyclines and Some Degradation Products

Compound	$R_f \times 100$[a] System I	$R_f \times 100$[a] System II	Color[b] (and Limit of Detection, mcg.) Reagent I	Reagent II	Reagent III[c]	Reagent IV	Limit of Detection (mcg.) under Longwave UV With Ammonia	Without Ammonia
Tetracycline antibiotics								
TC	53	36	Pk(0.1)	Y(1.2)	Y(0.8)	Pk(1.2)	0.005	0.05
CTC	76	60	Pk(0.16)	Y(1.2)	Y(0.8)	Y-Pk(1.2)	0.0032	0.064
DMCTC	73	44	Pk(0.16)	Y(1.2)	Y(0.8)	Pk-Y(1.2)	0.0032	0.064
OTC	60	20	Y-Br(0.16)	Pk-Y(1.2)	Y(0.8)	Br-Y(1.2)	0.0024	0.064
MC	44	29	Pk-Br(0.16)	Y(1.2)	Y(0.8)	Y(1.2)	0.0024	0.064
DOXY	53	57	Pk-Br(0.16)	Y(1.2)	Y(0.8)	Pk(1.2)	0.0032	0.064
Some Degradation Products of Tetracyclines[d]								
4 epi-TC	20	12	Y-Pk	Y	Y	Pk-Y	—	—
ATC	93	83	Pk	Y	Y	Y-Pk	—	—
EATC	47	50	Y-Pk	Y	Y	Y-Pk	—	—
4 epi-CTC	33	21	Y-Pk	Y	Y	Pk-Y	—	—
ACTC	83	57	Pk	Y	Y	Y	—	—

[a] R_f values vary considerably with tank temperature, especially in case of stored plates. If very low R_f values are obtained, the chromatograms, after brief drying may be rechromatographed in the same solvent system. [b] Pk = pink, Y = yellow, Br = brown. [c] Colors change with excess of spray reagent and with time. [d] Limit of detection is not given since very pure samples were not available.

RESULTS AND DISCUSSION

In the procedure of Ascione *et al.* (4) the support of diatomaceous earth is washed with hot 6 *N* HCl until the washings no longer show the presence of calcium or iron. It is then washed with water until a neutral pH is obtained and dried at 105°. This somewhat tedious process required considerable experience to obtain an acid-washed neutral support, and some batches, perhaps less well washed or dried, gave chromatograms with streaks and tails. Thus, it was decided to use as support the commercially available kieselguhr G (Merck) without treatment other than impregnation with EDTA and glycerin–PEG 400 mixture. Though the tetracylcines with R_f 0.4 and lower gave well-shaped spots, those with higher R_f values showed irregular zig-zag patterns, which appeared to be due to the glycerin–PEG mixture which was also moving with the solvent. The same results were obtained when the amount of glycerin–PEG mixture in the preparation of the slurry was greatly reduced. This defect was, however, completely remedied when the plates were first developed in Solvent System I and allowed to dry before spotting. This process also conditioned the plates with optimum moisture content and allowed a satisfactory resolution of the tetracycline spots, even after the plates were stored up to 4 weeks. In this system, one run with the solvent gave satisfactory resolution of the various spots, in contrast to the published method (4) where it is almost always necessary to chromatograph two to four times. It was ascertained by two-dimensional TLC that no degradation of the tetracyclines occurred on the plates.

Though a few spray reagents for the detection of tetracyclines have been reported by earlier authors (1), they proved unsatisfactory under chromatographic conditions in this laboratory. Recent

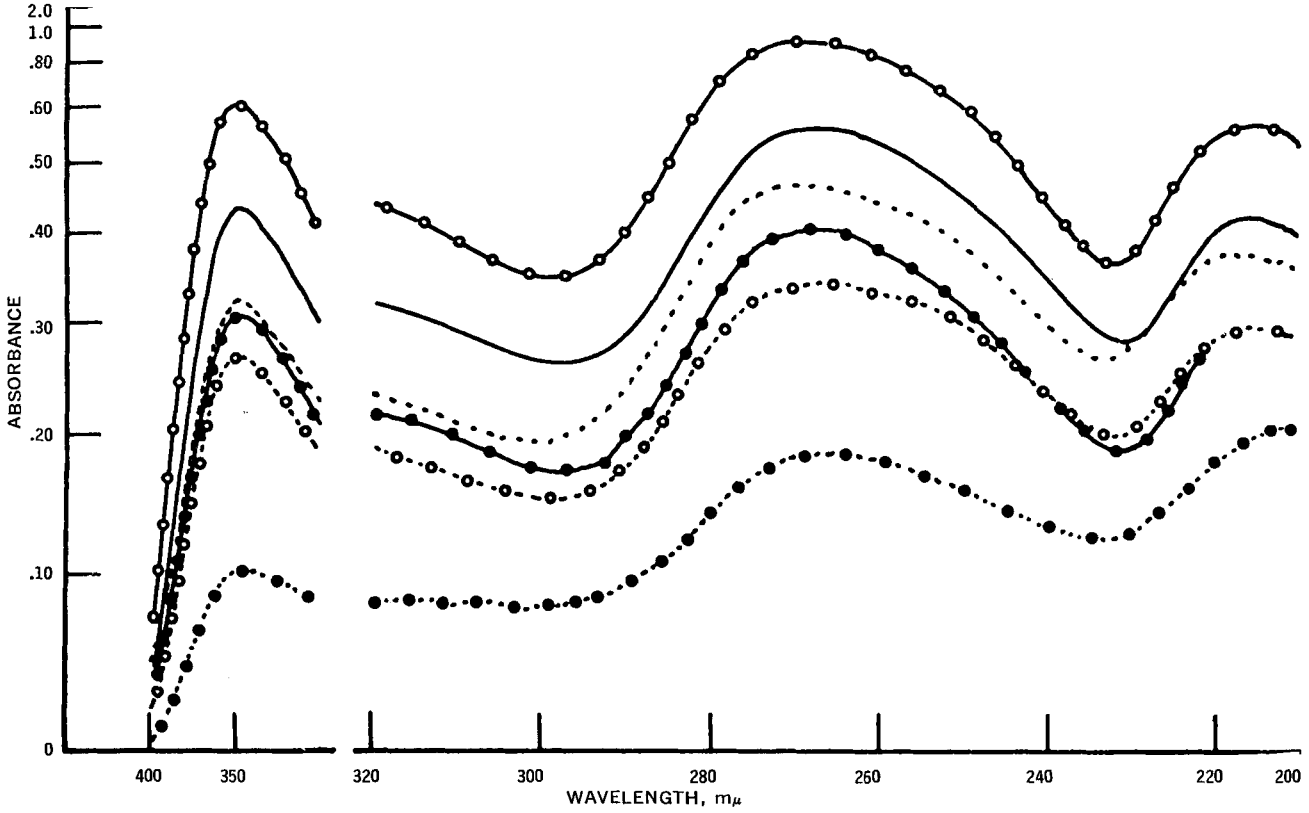


Figure 1—*UV spectra of tetracycline hydrochloride in three solutions (aqueous, phosphate, and acetate). Key: ——, T-0 hr. = aqueous solution at 0 hr.; - - -, T-620 hr. = aqueous solution after 620 hr.;* O—O, *TP-0 hr. = phosphate solution at 0 hr.;* O· · ·O, *TP-620 hr. = phosphate solution after 620 hr.;* ●—●, *TA-0 hr. = acetate solution at 0 hr.;* ●· · ·●, *TA-620 hr. = acetate solution after 620 hr.*

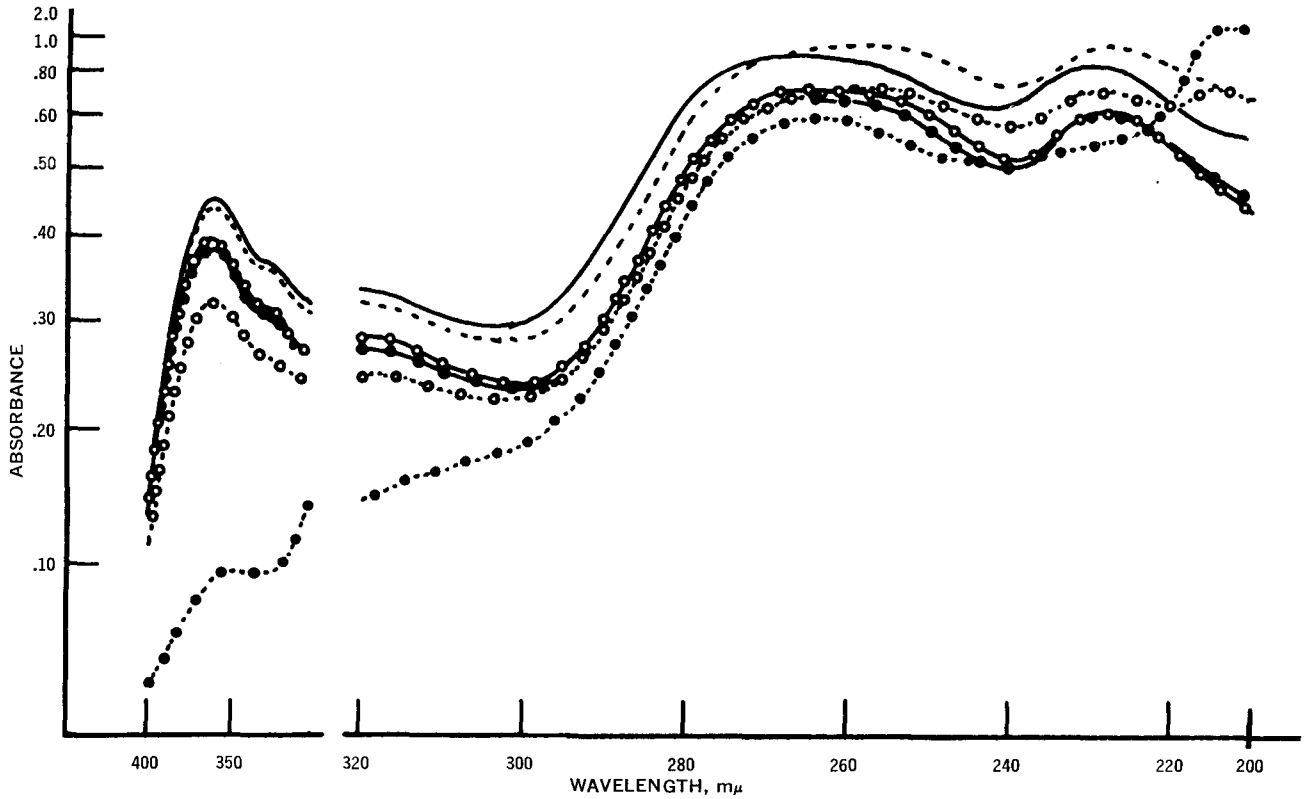


Figure 2—*UV spectra of chlortetracycline hydrochloride in three solutions (aqueous, phosphate, and acetate). Key: ——, C-0 hr. = aqueous solution at 0 hr.; - - -, C-620 hr. = aqueous solution after 620 hr.; O—O, CP-0 hr. = phosphate solution at 0 hr.; O···O, CP-620 hr. = phosphate solution after 620 hr.; ●—●, CA-0 hr. = acetate solution at 0 hr.; ●···●, CA-620 hr. = acetate solution after 620 hr.*

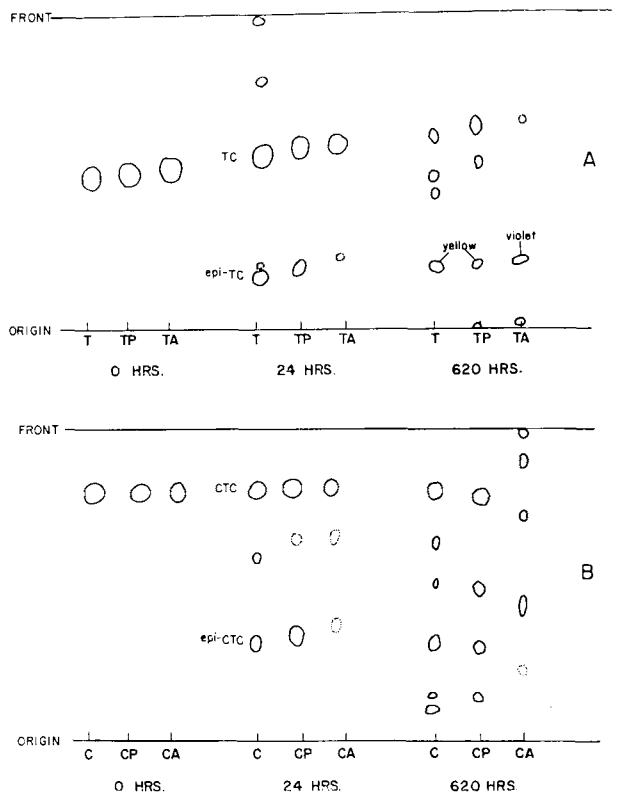


Figure 3—*Schematic TLC of the degradation products of* (A) *tetracycline hydrochloride and* (B) *chlortetracycline hydrochloride in three solutions (aqueous, phosphate, and acetate) at 0, 24, and 620 hr.; Solvent System I. T = TC·HCl in aqueous solution; TP = TC·HCl in phosphate solution; TA = TC·HCl in acetate solution; C = CTC·HCl in aqueous solution; CP = CTC·HCl in phosphate solution; CA = CTC·HCl in acetate solution.*

authors have exclusively made use of longwave UV light for the detection of these compounds (2–8). In an attempt to find a spray reagent which might be useful for the quantitation of tetracyclines by densitometric methods, several sprays used for the detection of phenols and amines (1*c*) were tried. Of these, the four described in the experimental section were found to give colored spots with various tetracyclines and their degradation products (Table II). Though these spray reagents are not as sensitive as the use of UV light, they are satisfactory for most detection purposes.

TLC of Tetracyclines—The chromatography of tetracyclines in Solvent Systems I and II is schematically represented in Fig. 4 and a compilation of approximate R_f values obtained with both the solvent systems is shown in Table II. With the exception of CTC and doxycycline (DOXY) all other tetracyclines are well separated

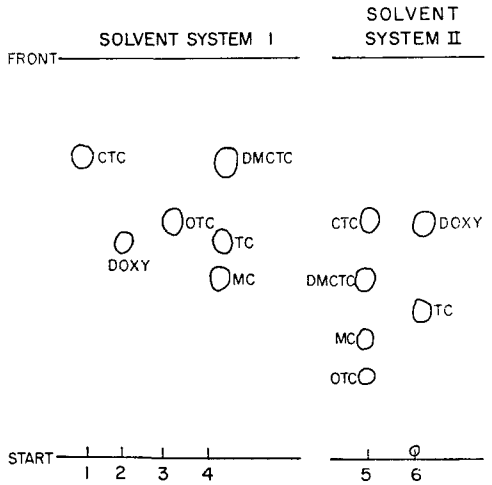


Figure 4—*Schematic chromatogram of tetracyclines in Solvent Systems I (left) and II (right). Samples: 1, CTC; 2, DOXY; 3, OTC; 4, DMCTC, TC, and MC; 5, CTC, DMCTC, MC, and OTC; 6, DOXY and TC.*

from one another in Solvent System II. CTC and DOXY can also be separated from each other in a system composed of dichloromethane–ethyl alcohol (18:1) saturated with the above-mentioned McIlvaine buffer. Though oxytetracycline (OTC) moves higher than TC or methacycline (MC) in System I, it has an R_f value lower than these compounds in System II. Rolitetracycline (RTC), which carries the pyrrolidinomethyl moiety in Position 2 (Table I), did not move in these systems but did so when the alcohol content in System II was increased [dichloromethane–ethylformate–ethanol (2:2:1), R_f = 0.15]. Furthermore, any tetracyclines formed by hydrolysis of RTC can be easily detected by employing System I or II.

TLC of Degradation Products—Following the reports of a Fanconi-like syndrome that developed in patients after the ingestion of outdated or degraded tetracycline capsules (6, 10, and references contained therein), concern was expressed over impurities and degradation products in tetracyclines. 4-Epi-, anhydro-, and 4-epianhydro-derivatives of tetracycline (Table I) have been implicated as the most toxic degradation products accompanying tetracycline (11). 4-Epianhydrotetracycline in relatively large doses was the causative agent of renal tubular damage in the rat and the dog, producing urinary findings suggestive of Fanconi-type syndrome (11). Furthermore, the epimers have been found to possess less than 5% of the antibiotic activity of the parent tetracycline (11).

Solvent Systems I and II are suitable for the separation of epitetracyclines from their parents and of epianhydrotetracycline from tetracycline. Anhydrotetracycline and anhydrochlortetracycline both move to the solvent front in System I but have reasonable mobilities in System II (see Table II).

Epimerization Experiments—The TLC method was successfully applied to a qualitative study of degradation products other than the epimers that may be formed in solutions of tetracyclines. Previously, the process of epimerization has been studied spectroscopically (12–14) and use has been made of 254 mμ/267 mμ and 355 mμ/298 mμ absorbance ratios (14) to follow the epimerization. The decrease in absorbance in the 300–380 mμ region in the spectra of tetracyclines has been regarded as an indicator for degradation other than epimerization.

In this study, TC·HCl and CTC·HCl were separately allowed to epimerize in phosphate and acetate solutions side by side with the corresponding aqueous solutions at room temperature, and the reaction products were studied at suitable time intervals both spectroscopically and by TLC.

The results obtained by spectrophotometric study were in accordance with the earlier epimerization studies (12–14). The UV spectra of TC·HCl and CTC·HCl in the three systems (aqueous, phosphate, and acetate) are shown in Figs. 1 and 2, respectively, and the corresponding TLC results are represented schematically in Figs. 3A and 3B, respectively. It was evident from both the chromatographic and spectroscopic evidence that there was considerable degradation other than epimerization occurring in each system. TLC analysis, however, appears capable of giving more detailed information on the excessive degradation than does the absorbance ratio analysis or the UV absorbance in the 300–380-mμ region.

In addition to the gradual change in color of the phosphate solutions, yellowish-gray deposits also formed and were collected at the end of about 620 hr. They were dissolved in mixtures of ethyl acetate–methanol (~1:3) and on TLC each showed at least four minor spots, beside the one major spot corresponding in R_f values (Systems I and II) to that of the parent tetracycline. Though no further study of these spots was undertaken, it seemed probable that during the process of epimerization, some of the antibiotic was precipitated out of the solution perhaps in the form of phosphate complex. In the acetate system the corresponding deposit was gray in each case and was dissolved in formic acid–methanol (~1:3). On TLC it showed at least seven spots, none of them corresponding to those reported earlier.

The TLC methods described here thus indicated the extensive degradation which occurs in solutions of tetracyclines and may be of value in studying the stability of liquid pharmaceutical preparations containing tetracyclines. If products similar to those detected in the epimerization study are found, it will be important to determine their nature and toxicity.

REFERENCES

(1) E. Stahl, "Dünnschicht-Chromatography: ein Laboratoriumshandbuch," 2nd ed., Springer-Verlag, Berlin, Heidelberg, New York, 1967, p. 547; (*a*), *ibid.*, p. 829; (*b*), *ibid.*, p. 842; (*c*), *ibid.*, p. 815.

(2) Y. Nishimoto, E. Tsuchida, and S. Toyoshima, *Yakugaku Zasshi*, **87,** 516(1967).

(3) J. Keiner, R. Huttenrauch, and W. Poethke, *Pharm. Zentralhalle*, **105,** 705(1966).

(4) P. P. Ascione, J. B. Zagar, and G. P. Chekian, *J. Pharm. Sci.*, **56,** 1393(1967).

(5) I. C. Dijkhius, *Pharm. Weekblad*, **102,** 907(1967).

(6) D. L. Simmons, C. K. Koorengevel, and R. Kubelka, *J. Pharm. Sci.*, **55,** 219(1966).

(7) D. Sonanini and L. Anker, *Pharm. Acta Helv.*, **39,** 518(1964).

(8) "British Pharmacopoeia," 1968, pp. 215, 697, and 1001.

(9) C. T. Smith, *J. Assoc. Offic. Anal. Chemists*, **51,** 750 (1968).

(10) S. R. Sulkowski and J. R. Haserick, *J. Am. Med. Assoc.*, **1964,** 152.

(11) K. F. Benitz and H. F. Diermeier, *Proc. Soc. Exptl. Biol. Med.*, **115,** 930(1964).

(12) J. R. D. McCormick, S. M. Fox, L. L. Smith, B. A. Bitler, J. Reichenthal, V. E. Origoni, W. A. Muller, R. Winterbottom, and A. P. Doerschuck, *J. Am. Chem. Soc.*, **79,** 2849(1957).

(13) E. G. Remmers, G. M. Seiger, and A. P. Doerschuck, *J. Pharm. Sci.*, **52,** 752(1963).

(14) D. A. Hussar, P. J. Niebergall, E. T. Sugita, and J. T. Doluisio, *J. Pharm. Pharmacol.*, **20,** 539(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 14, 1969, from the *Research Laboratories, Food and Drug Directorate, Ottawa, Canada.*

Accepted for publication September 17, 1969.

The authors are indebted to Dr. D. L. Simmons (Frank W. Horner Ltd., Montreal, P. Q.), Dr. R. G. Kelly (Lederle Laboratories, New York), and Mr. R. B. Taylor (Pfizer Company Ltd., Montreal) for gifts of certain reference substances used in this study, and to Mr. J. R. Fooks for technical assistance.

* National Research Council of Canada Postdoctoral Fellow, 1968–1969.