REFERENCES

(1) American National Red Cross, "First Aid Textbook," 4th ed., 19th printing, Doubleday, Garden City, N. Y., 1957, pp. 47–54.
(2) Flint, T. J., "Emergency Treatment and Management," 3rd ed., W. B. Saunders Co., Philadelphia, Pa., 1964, p. 411.
(3) Adams, W. C., *Pediat. Clin. North Am.*, **8**, 351(1961).
(4) Dreisbach, R. H., "Handbook of Poisoning, Diagnosis and Treatment," 4th ed., Lange Medical Publications, Los Altos, Calif., 1963, p. 3.
(5) Ferguson, H. C., *Toxicol. Appl. Pharmacol.*, **4**, 759 (1962).
(6) Schanker, L. S., Tocco, D. J., Brodie, B. B., and Hogben, C. A. M., *J. Pharmacol. Exptl. Therap.*, **123**, 81 (1958).
(7) Schanker, L. S., *Pharmacol. Rev.*, **14**, 501(1962).
(8) Schanker, L. S., in Hogben, C. A. M., and Lindregen, P. L., "Drugs and Membranes," Pergamon Press, New York, N. Y., 1963, pp. 121–130.
(9) Wilson, T. H., "Intestinal Absorption," W. B. Saunders Co., Philadelphia, Pa., 1962, pp. 46, 244–245.
(10) Brodie, B. B., in Binns, T. B., "Absorption and Distribution of Drugs," E. & S. Livingston Ltd., Edinburgh and London, 1964, pp. 16–48.
(11) Hillman, E., and Harpur, E. R., *New Engl. J. Med.*, **264**, 138(1961).
(12) Martin, B. K., in Binns, T. B., "Absorption and Distribution of Drugs," E. & S. Livingston Ltd., Edinburgh and London, 1964, pp. 165–172.
(13) Levy, G., in Dixon, A. St. J., Martin, B. K., Smith, M. J. H., and Wood, P. H. N., "Salicylates—An International Symposium," J. & A. Churchill Ltd., London, England, 1963, p. 17.
(14) Snedecor, G. W., "Statistical Methods," 5th ed., Iowa State University Press, Ames, Iowa, 1956, pp. 85–89.
(15) Goldbaum, L. R., *J. Pharmacol. Exptl. Therap.*, **94**, 68(1948).
(16) Picchioni, A. L., Chin, L., Verhulst, L., and Dieterle, B., *Toxicol. Appl. Pharmacol.*, **8**, 447(1966).
(17) Brodie, B. B., and Udenfriend, S., *J. Pharmacol. Exptl. Therap.*, **78**, 154(1943).
(18) Winter, C. A., Risley, E. A., and Nuss, G. W., *Proc. Soc. Exptl. Biol. Med.*, **111**, 544(1962).
(19) Trinder, P., *Biochem. J.*, **57**, 301(1954).
(20) Cotty, V., Zurzola, F., Beezley, T., and Rodgers, A., *J. Pharm. Sci.*, **54**, 868(1965).
(21) Schanker, L. S., Shore, P. A., Brodie, B. B., and Hogben, C. A. M., *J. Pharmacol. Exptl. Therap.*, **120**, 528 (1957).
(22) Hogben, C. A. M., Tocco, D. J., Brodie, B. B., and Schanker, L. S., *ibid.*, **125**, 275(1959).
(23) Goth, A., "Medical Pharmacology Principles & Concepts," 2nd ed., C. V. Mosby Co., St. Louis, Mo., 1964, pp. 21–25.
(24) Truitt, E. B., Jr., and Morgan, A. M., *J. Pharm. Sci.*, **53**, 129(1964).

Quantitative Determination by Thin-Layer Chromatography of Anhydrotetracyclines in Degraded Tetracycline Tablets

By D. L. SIMMONS, H. S. L. WOO, C. M. KOORENGEVEL, and P. SEERS

A two-dimensional thin-layer chromatography (TLC) procedure on microcrystalline cellulose is presented for the quantitative determination of anhydrotetracycline and epianhydrotetracycline in degraded tetracycline tablets. Initial development is performed with 0.1 *M* EDTA–0.1 per cent ammonium chloride solution to separate the anhydrotetracyclines (R_f 0.34–0.38) from the tetracycline (R_f 0.72) and methanol-soluble excipients. Anhydrotetracycline (R_f 1.0) and epianhydrotetracycline (R_f 0.52) are then resolved by developing the chromatogram with chloroform which has been saturated with the same EDTA–ammonium chloride solution. A complete assay for these anhydro compounds can be performed in less than 2 hr.

POOR RESOLUTION and/or excessive development time has characterized previous chromatographic attempts to separate tetracycline (TC) and its major degradation products, anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC). The latter was recently incriminated in the reversible renal dysfunction (Fanconi-type syndrome) caused by the ingestion of degraded TC products (1–7).

In a qualitative examination of TC and its anhydro derivatives by radial chromatography, using water-saturated butanol on silica gel, Rustici and Ferappi (8) obtained the following similar R_f values after 2 hr. of development: TC (0.36), ATC (0.50), and EATC (0.40). When Kelly and Buyske (9) employed paper which had been impregnated with 0.1 *M* EDTA solution and the solvent system *n*-butanol–ammonium hydroxide–water (4:1:5), they obtained the following 16 hr. R_f values: TC (0.39), ATC (0.62), and EATC (0.40). In a recent paper (10) the authors reported the quantitative analysis of ATC from TC test mixtures by TLC on microcrystalline cellulose. This method required only 20 min. development with 0.1% ammonium chloride solution to give R_f values of 0.38 and 0.72 for the ATC and TC, respectively. Subsequent experiments revealed that mixtures of EATC, ATC, and TC are partially resolved by the same chromatographic system, but overlapping of the ATC (0.38) and EATC (0.34) occurred. In order to completely resolve the two anhydro-compounds, two-dimensional chromatography was attempted. The findings of Kelly (11) that ATC and EATC can be separated by partition chromatography employing buffered 0.1 *M* EDTA solution (pH 7.8) as stationary phase and buffer saturated chloroform as moving phase, prompted the authors to utilize these findings in their search for a suitable second solvent system. By developing the chromatogram with 0.1 *M* EDTA (disodium salt)–0.1% ammonium chloride solution (pH 4.5), followed by chloroform which had been saturated with the same EDTA–ammonium chloride solution, complete resolution of the ATC (1.0) and EATC (0.52) occurred. On altering the pH of the aqueous phase between 3.5 and 8.0, R_f values for ATC (1.0) and TC (0.17) were observed to remain constant in the second development;

Received May 13, 1966, from Frank W. Horner Ltd., Montreal, Quebec, Canada.
Accepted for publication July 18, 1966.
The authors thank Mr. M. B. Poole for his valuable assistance with this report.

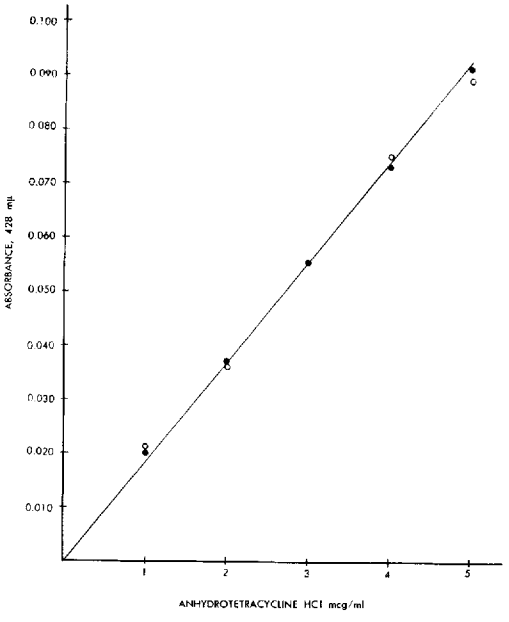

Fig. 1.—Absorbance of ATC as a function of concentration. Key: ●, direct dilutions; ○, dilutions after TLC.

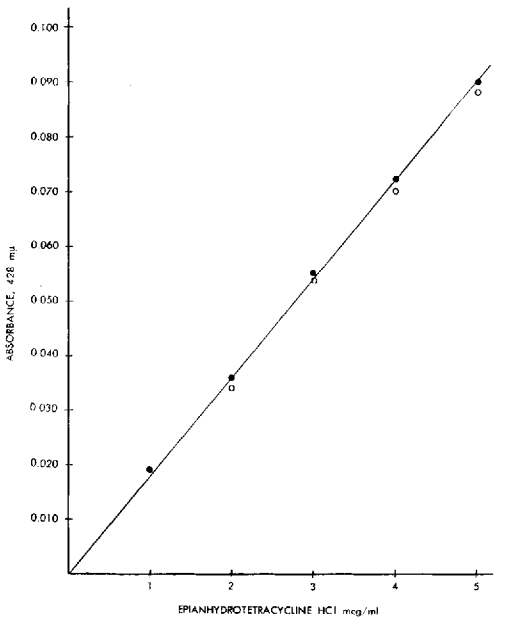

Fig. 2.—Absorbance of EATC as a function of concentration. Key: ●, direct dilutions; ○, dilutions after TLC.

TABLE I.—ANALYSIS[a] OF TETRACYCLINE TABLET TEST MIXTURES

ATC, mg./Tablet			EATC, mg./Tablet			Spot Load, ml.	Dilutions of TLC Extracts, ml.
Added	Found	Recovery, %	Added	Found	Recovery, %		
0	1.08 ± 0.06		0	0.28 ± 0.02		0.2	5
0.5	1.54 ± 0.11	97.4	0.5	0.76 ± 0.13	97.4	0.2	5
1.0	1.99 ± 0.16	99.0	1.0	1.35 ± 0.08	105.5	0.1	5
5.0	6.09 ± 0.10	100.2	5.0	5.16 ± 0.12	97.8	0.1	10
10.0	10.95 ± 0.08	98.8	10.0	10.32 ± 0.24	100.4	0.05	10

[a] ± = standard deviation. The average of five determinations. The blank value for EATC (0.23 mg.) was obtained from the difference between total anhydro content and ATC only.

on the other hand, the R_f value for EATC varied between 0.60 and 0.30. In contrast to Kelly's procedure (11) the results demonstrate that precise pH requirements are not necessary for the system to be operative. Such a large difference between the R_f values for ATC and EATC led to their easy isolation and determination.

EXPERIMENTAL

Preparation of Anhydrotetracycline Hydrochloride.—A modified procedure of McCormick *et al.* (12) was used. Tetracycline hydrochloride was heated in methanol–concentrated hydrochloric acid (1:4) on a steam bath for 30 min. The solution was cooled and diluted with ice water; the resulting precipitate was collected by filtration. Purification was achieved by dissolving the precipitate in hot methanol–concentrated hydrochloric acid (30:1), treating the solution with charcoal, and filtering it through Celite. Crystallization occurred when the filtrate was cooled, and a small amount of concentrated hydrochloric acid was added. By repeating this procedure 6 times, a product was obtained with the melting point and ultraviolet absorption spectrum (0.1 *N* sodium hydroxide solution) of pure anhydrotetracycline hydrochloride.

Preparation of Epianhydrotetracycline Hydrochloride.—By employing the ammonium salt of epi-tetracycline (12) in the foregoing procedure instead of tetracycline, a pure sample of epianhydrotetracycline hydrochloride was prepared.

Preparation of Standards.—Standard solutions (2 mg./ml.) of ATC and EATC in methanol were prepared and aliquots (5, 10, . . . 25 μl.) were removed and diluted to volume with methanol in 10-ml. volumetric flasks. Dilution absorbances were determined at 428 mμ on a Beckman DU spectrophotometer. The results are illustrated in Figs. 1 and 2.

Identical aliquots of the two foregoing standard solutions were subjected to the two-dimensional chromatographic procedures described below for TC tablet test mixtures. The methanolic TLC extracts were diluted to volume with 10 ml. of methanol and dilution absorbances were determined spectrophotometrically at 428 mμ. Standard

TABLE II.—ANALYSIS[a] OF DEGRADED TETRACYCLINE TABLETS

Sample	Spot Load, Vol., ml.	ATC, mg./Tablet	EATC, mg./Tablet
A	0.02	16.23 ± 0.48	15.28 ± 0.66
B	0.04	8.10 ± 0.29	5.89 ± 0.37

[a] ± standard deviation. The average of five determinations.

curves, comparing dilutions after TLC with direct dilutions, are illustrated for ATC and EATC in Figs. 1 and 2, respectively.

Procedure.—*TC Tablet Test Mixtures.*—TC tablets[1] each containing 250 mg. of tetracycline hydrochloride were crushed by mortar and pestle. The resulting powder was deliberately contaminated with known quantities of ATC and EATC. Powder equivalent to one tablet was transferred to a 3-ml. sintered-glass funnel and vacuum extracted with approximately 20 ml. of hot methanol. The extract volumes were adjusted to 25 ml. in volumetric flasks and appropriate aliquots spotted for two-dimensional chromatography (13) on microcrystalline cellulose plates (10). The chromatograms were developed for 20 min. in a chamber containing 0.1 *M* EDTA—0.1% ammonium chloride solution (pH 4.5). Development was followed by drying the plates under ambient conditions for 25 min. The plates were then placed for two-dimensional chromatography in a second chamber which contained chloroform saturated with the EDTA–ammonium chloride solution as used previously. Development for 20 min. was then permitted. R_f values of 1.0 and 0.60 were obtained for ATC and EATC, respectively. After removing and thoroughly drying the plates, the visible spots were scraped into 3-ml. sintered-glass funnels and vacuum extracted with hot methanol. Care was taken to ensure that complete drying of the microcrystalline cellulose occurred prior to extraction with methanol. When the adsorbent was too wet, EDTA was found to be extracted and caused characteristic increases (0.008) in absorbance at 428 mμ. These results were confirmed by deliberate addition of EDTA solution to test samples. A drying period of not less than 5 min. under a gentle stream of air[2] proved to be satisfactory.

[1] Marketed as Tetrosol Tablets by Frank W. Horner Ltd., Montreal, Quebec, Canada (July 1965).

[2] Oster "airjet" hair dryer, John Oster Manufacturing Co., Milwaukee, Wis.

As previously described by the authors (10), quantitative isolation and determination of anhydrotetracycline from microcrystalline cellulose is only possible in the 10–40 mcg./spot range. To keep within this range when working with samples containing unknown quantities of anhydro- compounds, the authors used the separation of the initial plate load as an indication of the quantities involved in the analysis. Suitable absorbance readings for ATC and EATC solutions were obtained by diluting the extracts of the plate scrapings with appropriate quantities of methanol. The spot load volumes and TLC extract dilutions which were used experimentally are indicated in Table I for easy reference.

Degraded Tetracycline Tablets.—Having established the validity of the foregoing procedures in the analysis of TC samples to which anhydrotetracyclines had been added, the authors applied the procedure to the analysis of degraded TC tablets. Sample *A* had been subjected to a 6-week thermal degradation study at 70° (14) followed by a storage period of 4 years at room temperature in screw-cap vials. Sample *B* had been stored in plug-cap glass vials, under tropical conditions for 4 years. The results are given in Table II.

DISCUSSION

A simple, rapid, and quantitative analysis for ATC and EATC has been performed by two-dimensional TLC on microcrystalline cellulose. The procedure is not affected by tablet excipients, and permits easy separation and quantitative recovery of minute (0.5 mg.) quantities of the harmful impurity, EATC. The method can be adopted for routine control quantitative or qualitative analysis of TC raw materials and solid dosage forms.

REFERENCES

(1) Flimpter, G. W., *J. Am. Med. Assoc.*, **184**, 111(1963).
(2) Fulop, M., and Drapkin, A., *New Engl. J. Med.*, **19**, 986(1965).
(3) Gross, J. M., *Ann. Internal Med.*, **58**, 523(1963).
(4) Ehrlich, L. I., and Stein, H. S., *Pediatrics*, **31**, 339 (1963).
(5) *Ibid.*, **31**, 698(1963).
(6) Sulkowski, S. R., and Haserick, J. R., *J. Am. Med. Assoc.*, **189**, 178(1964).
(7) Rosenthal, J. M., *Pediatrics*, **31**, 697(1963).
(8) Rustici, L., and Ferappi, M., *Boll. Chim. Farm.*, **104**, 305(1965).
(9) Kelly, R. G., and Buyske, D. A., *Antibiot. Chemotherapy*, **10**, 604(1960).
(10) Simmons, D. L., Koorengevel, C. M., Kubelka, R., and Seers, P., *J. Pharm. Sci.*, **55**, 219(1966).
(11) Kelly, R. G., *ibid.*, **53**, 1551(1964).
(12) McCormick, J. R. D., Fox, S. M., Smith, L. L., Bitler, B. A., Reichenthal, J., Origoni, V. E., Muller, W. H., Winterbottom, R., and Doerschuk, A. P., *J. Am. Chem. Soc.*, **79**, 2849(1957).
(13) Truter, E. I., "Thin Film Chromatography," Interscience Publishers, John Wiley & Sons, Inc., New York, N. Y., 1963, p. 67.
(14) Tardif, R., Frank W. Horner Ltd., unpublished data.