Quantitative Analysis of Anhydrotetracycline on Microcrystalline Cellulose

By D. L. SIMMONS, C. K. KOORENGEVEL, R. KUBELKA, and P. SEERS

A quantitative TLC method for the determination of anhydrotetracycline alone and in the presence of tetracycline has been developed on microcrystalline cellulose. It is a rapid and precise method for determining small amounts of this compound in tetracycline samples, provided one works within the range of 10–40 mcg. of anhydrotetracycline per spot.

In general, the separation of tetracycline antibiotics has been performed by chromatographic methods based on pH control of the adsorbent. Early investigators employed complex solvent systems with buffered paper (1, 2) or paper impregnated with sequestering agents (3). The quantitative separation of these antibiotics using a column of powdered cellulose impregnated with tartrate buffer was reported in 1961 by Hrdy and Vesely (4). In 1962 Addison and Clark (5) described a method for the separation of tetracycline from epitetracycline and anhydrotetracyline using a modified Whatman cellulose phosphate cation exchange paper. Kelly (6) employed a column of silicate containing a buffered EDTA solution to effect the resolution of anhydrotetracycline and epianhydrotetracycline in the presence of large quantities of tetracycline.

The application of microcrystalline cellulose to thin-layer chromatography has only recently been investigated (7, 8). Wolfrom and co-workers (9) reported that TLC on a microcrystalline form of cellulose[1] has been more efficient than paper chromatography in resolving certain water-soluble sugars, sugar derivatives, amino acids, and related compounds. This material has almost completely displaced the papergram in their laboratory, and in most cases was found to be superior to silica gel where both systems were applicable. These results combined with the published data on the resolution of tetracycline on paper led the authors to investigate the behavior of anhydrotetracycline on microcrystalline cellulose.

EXPERIMENTAL

Preparation of Anhydrotetracycline.—A solution of tetracycline hydrochloride (15 Gm.) in 0.1 *N* hydrochloric acid (500 ml.) was warmed on a steam bath at 70° for 1 hr. After cooling, the precipitate was collected by filtration and suspended in water (500 ml.). The suspension was adjusted to pH 4.5 with 5 *N* sodium hydroxide solution and extracted with ethyl acetate (2 × 400 ml.). The dried organic extracts were concentrated to approximately 50 ml. and cooled. The product (5.0 Gm.) was collected by filtration and recrystallized from benzene as yellow needles, m.p. 217.5°. [Lit. m.p. 215–220° (10).]

Anal.—Calcd. for $C_{22}H_{22}NO_7$: C, 61.96; H, 5.19. Found: C, 62.18; H, 5.08.

Preparation of Plates.—Microcrystalline cellulose (50 Gm.) was passed through a 100 mesh sieve and mixed in a mortar and pestle for 2 min. with 0.05% ammonium chloride solution (180 ml.). Since microcrystalline cellulose is commonly used in the pharmaceutical industry as a gelling agent, it was decided to employ a dilute salt solution such as ammonium chloride instead of water alone so that the gelling process was minimized. This modification permitted easier manipulation of the cellulose slurry. A 0.25-mm. layer of this homogeneous slurry was then applied to 5 clean glass plates (20 × 20 cm.) with a Desaga variable applicator. Prior to spotting, the plates were allowed to dry at room temperature for 10 min. and then heated in an oven at 90° for 30 min. Unlike silica gel plates, no special storage conditions were necessary.

Plate Development and Recovery of Anhydrotetracycline.—The plates were spotted by applying aliquots (10, 15, 20. . . 60 μl.) of a freshly prepared standard solution of anhydrotetracycline in methanol (1 mcg./μl.) approximately 3 cm. from the edge of each chromatoplate. These quantities are equivalent to 10–60 mcg. of anhydrotetracycline per spot. The plates were then placed in a developing chamber containing 0.1% aqueous ammonium chloride solution (160 ml., pH 5.6); filter paper saturated with the eluant lined the interior chamber walls. The plates were allowed to develop for 20 min., removed from the chamber, and dried for 10 min. under ambient conditions. An area (4 × 6 cm.) was marked off around the visible yellow spot (R_f 0.35), and the surrounding adsorbent was removed by a straight edge spatula and discarded. Individual spots were scraped off and collected in 3-ml. sintered-glass filter funnels, and the anhydrotetracycline was recovered from the adsorbent by washing the contents of the funnel with hot methanol directly into a 10-ml. volumetric flask under vacuum in a vacuum bell jar. The absorbances of the resulting filtrates were determined at 428 mμ against a methanol blank on a Beckman DU spectrophotometer. It was not necessary to employ a methanolic extract of cellulose for blank determinations at this wavelength. The procedure was repeated 5 times for each concentration.

Similar aliquots of the standard solution were transferred directly to 10-ml. volumetric flasks, adjusted to volume with methanol, and the absorbances read at 428 mμ.

Results are summarized in Table I.

Estimation of Anhydrotetracycline in Tetracycline Standard Mixture.—Tetracycline hydrochloride (80 mg.) was dissolved in 8.0 ml. of anhydrotetracycline standard solution in a 10-ml. volumetric flask, and the contents were adjusted to volume with methanol. An aliquot (0.04 ml., equivalent to 32 mcg. of anhydrotetracycline and 320 mcg. tetracycline hydrochloride) was removed by micrometer

Received October 15, 1965, from the Research Laboratories, Frank W. Horner Ltd., Montreal, Quebec, Canada.
Accepted for publication November 30, 1965.

[1] Marketed as Avicel by the American Viscose Co.

TABLE I.—ANALYSIS OF ABSORBANCES[a] FOR DILUTIONS OF ANHYDROTETRACYCLINE STANDARD SOLUTION (1 mcg./μl.)

Concn., mcg./10 ml.	Absorbances Direct Dilution	Dilution After TLC
10.0	0.020 ± 4.4%	0.021 ± 4.0%
15.0	0.029 ± 4.9	0.031 ± 3.2
20.0	0.041 ± 1.5	0.041 ± 1.9
25.0	0.052 ± 1.2	0.052 ± 1.4
30.0	0.062 ± 1.2	0.063 ± 2.1
35.0	0.072 ± 2.4	0.071 ± 2.4
40.0	0.082 ± 0.54	0.081 ± 1.8
45.0	0.093 ± 0	0.088 ± 0.71
50.0	0.102 ± 0.98	0.096 ± 0.78
60.0	0.122 ± 0.82	0.116 ± 2.0

[a] ± = per cent standard deviation.

syringe and spotted on a cellulose coated plate. The development and recovery of anhydrotetracycline from the mixture was performed in triplicate according to the procedure described previously. Anhydrotetracycline was visible as a yellow spot at R_f 0.35, whereas tetracycline required ultraviolet light for detection at the solvent front. The quantity of anhydro-derivative recovered from the 0.04-ml. application was obtained by multiplying the observed absorbance (A_0) by the concentration of standard (C_{st}) over the corresponding absorbance of standard (A_{st}).

$$\frac{A_0 \times C_{st}}{A_{st}}$$

The total anhydrotetracycline in the sample was then determined by accounting for dilutions and weight conversion (Table II).

RESULTS AND DISCUSSION

Results indicate that quantitative isolation of anhydrotetracycline by microcrystalline cellulose TLC is possible over the 10–40 mcg. range, but deviations from the standard occur at quantities above 40 mcg. (Table I). Fairly extensive tailing of the chromatogram was observed in the 45–60 mcg. range; this is probably due to overloading of the film with anhydrotetracycline and/or its solvent. The anhydrotetracycline can only be recovered quantitatively in this range by extracting the entire cellulose area between origin and spot.

TABLE II.—RECOVERY OF ANHYDROTETRACYCLINE FROM TEST SAMPLE (32 mcg./SPOT)

Absorbance	mcg. Anhydro/Spot
0.064	31.2
0.065	31.7
0.065	31.7

An interesting feature of the procedure described is that significant changes to the pH (1.5–7.8) of the solvent system did not alter the behavior of the chromatogram. This is in contrast to the exacting pH requirements of previous methods.

Initially this chromatographic procedure helped to demonstrate the purity of the anhydrotetracycline. After each recrystallization from benzene, a standard solution (1 mcg./μl.) was prepared and subjected to TLC on cellulose. When the absorbance readings of the direct dilutions of the standard were identical to the dilutions obtained following TLC, the compound was judged to be of analytical purity. Subsequent elemental analysis substantiated this judgment.

Additional evidence for the purity of the anhydrotetracycline standard was obtained by demonstrating the absence of epianhydrotetracycline, an anticipated contaminant, by subjecting the compound to a chromatographic method established for this purpose (3). This method also showed that epimerization of the anhydrotetracycline did not take place under the TLC conditions described in this paper and is in agreement with the findings of Addison and Clark (5). These investigators found that tetracycline in solution in aqueous ammonium chloride remained stable over several hours.

A mixture of anhydrotetracycline and tetracycline did not have an adverse effect on the chromatogram. The authors' demonstrate that the anhydro-derivative can be quantitatively isolated and determined from a test mixture containing anhydrotetracycline and a large quantity of tetracycline.

Since the foregoing method for the quantitative determination of anhydrotetracycline in tetracycline mixtures was established, the authors have developed procedures for the separation and determination of anhydro- and epianhydrotetracyclines in standard tetracycline mixtures and degraded tetracycline products. These procedures will be reported in a future paper.

REFERENCES

(1) Coppi, G., *Farmaco Pavia, Ed. Prat.*, **15**, 407(1960).
(2) Selzer, O. B., and Wright, W. W., *Antibiot. Chemotherapy*, **7**, 292(1957).
(3) Kelly, R. G., and Buyske, D. E., *ibid.*, **10**, 604(1960).
(4) Hrdy, O., and Vesely, P., *Cesk. Farm.*, **10**, 126(1961).
(5) Addison, E., and Clark, R. G., *J. Pharm. Pharmacol.*, **15**, 268(1963).
(6) Kelly, R. G., *J. Pharm. Sci.*, **53**, 1551(1964).
(7) Wolfrom, M. L., Patin, D. L., and de Lederkremer, R. M., *Chem. Ind. London*, **1964**, 1065.
(8) Grippo, P., Iaccarino, M., Rossi, M., and Scarano, E., *Biochim. Biophys. Acta*, **95**, 1(1965).
(9) Wolfrom, M. L., Patin, D. L., and de Lederkremer, R. M., *J. Chromatog.*, **17**, 488(1965).
(10) Stephens, C. R., Conover, L. H., Pasternack, R., Hochstein, F. A., Moreland, W. T., Regna, P. P., Pilgrim, F. J., Brunings, K. J., and Woodward, R. B., *J. Am. Chem. Soc.*, **76**, 3568(1954).