CHROM. 4143

Quantitative determination of tetracycline hydrochloride by thin-layer chromatography

Commercial grades of tetracycline (TC) and its salts may contain up to 10% of other tetracyclines as impurities, *e.g.* 4-*epi*-tetracycline (ETC), 7-chlortetracycline (CTC), anhydrotetracycline (ATC) and 4-*epi*-anhydrotetracycline (EATC). Various analytical procedures are available in the literature for the determination of TC in pharmaceutical preparations but their major drawback is the inherent lack of specificity. The official methods of the U.S.P. and B.P. rely on microbiological assays for estimating TC content but these methods are not reliable since most tetracyclines possess some degree of antimicrobial activity.

In a recent stability investigation in these laboratories it was necessary to use an assay which was specific for following TC behaviour. Since a TLC procedure had proven successful for the quantitative determination of anhydrotetracyclines in degraded tetracycline products[1], the authors decided to extend the system to include the separation and determination of tetracycline. Resolution of ATC (R_F 1.0) and EATC (R_F 0.52) had been achieved by partition TLC on microcrystalline cellulose in which the stationary and mobile phases were buffered 0.1 *M* disodium EDTA–0.1% ammonium chloride solution and buffer-saturated chloroform, respectively. In this system, TC remained with ETC at or near the origin. The addition of 5% *n*-butanol to the solvent system resulted in the migration and complete separation of TC and contaminants. Tetracycline (R_F 0.25) was recovered as ATC from the chromatogram by extraction with 5 *N* hydrochloric acid and determined spectrophotometrically at 434 nm.

A spectrophotometric method based on the acid-catalyzed conversion of the tetracyclines to their anhydro components was first described by LEVINE *et al.*[2] and officially adopted by GROVE AND RANDALL[3]. The procedure was later modified by CHICCARELLI *et al.*[4] to compensate for the possible presence of CTC in TC, or *vice versa*, but unfortunately no provisions were made for the contaminants ETC, ATC or EATC.

This chromatographic method permitted the authors to investigate the purity of a U.S.P. Reference Standard and three commercial grades of tetracycline hydrochloride obtained from European and American suppliers.

Experimental and results

Preparation of plates. Microcrystalline cellulose* (30 g) was mixed rapidly in a mortar and pestle with water (108 ml). A layer (0.50 mm) of this homogeneous slurry was then applied to five clean glass plates (20 × 20 cm) with a suitable TLC spreader. Prior to spotting the plates were permitted to air dry at room temperature for 10 min and then heated in an oven at 90° for 30 min.

Procedure. Sample solutions (10.0 mg/ml) of tetracycline hydrochloride** (U.S.P. reference sample and three commercial grades from European and American suppliers) were prepared in 0.05 *N* hydrochloric acid and aliquots (20 µl) removed and

* Marketed as Avicel by American Viscose Corp., Marcus Hook, Pa., U.S.A.

** All samples were dried *in vacuo* (0.05 mm) for 12 h at room temperature prior to assay.

TABLE I

PURITY OF TETRACYCLINE HYDROCHLORIDE SAMPLES

Sample	*Absorbance* × 10^3[a]		*Pure tetracycline (%)*
	Direct dilution	*Dilution after TLC*	
A	356 ± 2.9	348 ± 2.5	97.8
B	356 ± 3.0	328 ± 2.8	92.1
C	358 ± 1.5	349 ± 2.0	97.4
D	357 ± 2.0	341 ± 1.6	95.5

[a] Sample concentration of 20 μg/ml, where A = U.S.P. reference standard, B and C = North American sources, and D = European source.

diluted to volume with 5 *N* hydrochloric acid in 10 ml volumetric flasks. Under these acidic conditions, TC, ETC and CTC are readily and quantitatively converted to their corresponding anhydro derivatives[4]. On the basis of reproducible absorbance readings, these yellow-colored solutions were found to be stable for more than 30 min. Dilution absorbances (Table I) were determined at 434 nm *versus* 5 *N* hydrochloric acid in 1 cm cells on a suitable spectrophotometer.

Identical aliquots of the foregoing TC sample solutions were manually applied in bands (2–3 cm) on microcrystalline cellulose plates. Better resolution is achieved with more concentrated spot loads if the samples are applied in bands or streaks. The plates were uniformly sprayed with approximately 10 ml of buffer solution (0.1 *M* disodium EDTA–0.1% ammonium chloride) from a nitrogen-pressurized chromatographic sprayer* (10 ml capacity) and immediately permitted to develop in a chamber containing 100 ml of buffer saturated *n*-butanol–chloroform (5:95). The technique of buffer-spraying the chromatoplates provided an immediately operative partition system which did not require repeated chromatography for resolution[5]. The effect of increasing butanol concentration on the R_F values of the various tetracyclines is illustrated in Fig. 1.

After development to a distance of 16 cm (45 min) the plates are removed, dried in a cool stream of air and examined for TC (R_F 0.25) under short wavelength

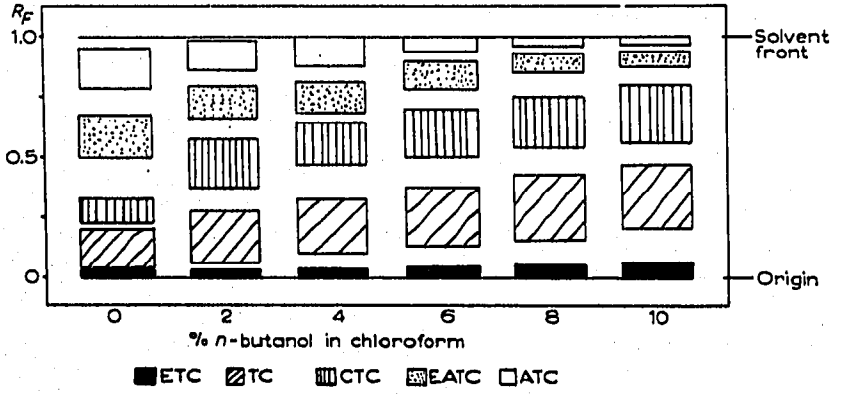

Fig. 1. Effect of increasing butanol concentration on the R_F values of tetracyclines.

* Marketed by Kensington Scientific Corp., Berkeley, Calif., U.S.A.

UV light. TC spot areas (about 4 × 4 cm) were scraped into 3 ml fine sintered glass funnels and vacuum extracted with 5 *N* hydrochloric acid (about 6 ml) into 10 ml volumetric flasks. Dilution absorbances (Table I) were determined at 434 nm *versus* 5 *N* hydrochloric acid in 1 cm cells on a suitable spectrophotometer.

The quantitative nature of the recovery procedure was assured by combining all spot areas from a chromatogram into a filter funnel and extracting with 5 *N* hydrochloric acid. The absorbance value of the extract was identical to that obtained from direct dilution.

The nature of the impurities was determined by utilizing appropriate reference samples and examining an ammonia-exposed chromatoplate under UV light. ETC was the major contaminant (about 2–6%) in the four samples with appreciable quantities of CTC, EATC and ATC (about 2–3%) found only in commercial sample B (Table I). A trace spot area with R_F 0.20 was not identified in the latter sample but its presence between the TC and ETC spots suggested *epi*-chlortetracycline (ECTC) or 2-acetyl-2-decarboxamidotetracycline[6]. Impurity contents were estimated by determining the differences in absorbance readings between direct acid dilutions and acid extracts of TC and combined TC impurity spot areas. TC and ETC accounted for 99.5% of the absorbance reading in the U.S.P. reference sample. The remaining absorbance contribution was attributed to anhydro components.

Epimerization of TC to ETC did not appear to be a contributing factor during the chromatographic operations since similar absorbance readings were obtained on acid extraction of TC spots after single- and two-dimensional development. In the latter case the TC spot area was treated with 15–20 μl of 0.05 *N* hydrochloric acid following the primary development and the chromatoplate resprayed with buffer. After two-dimensional development using the same solvent systems, the TC was again located and recovered according to the foregoing procedure.

Discussion

As evidenced by the purity of the U.S.P. reference sample (Table I) the major difficulty in this investigation was obtaining an analytically pure TC sample which was chromatographically devoid of impurities. This absence of a suitable working standard and recovery difficulties[7,8] ruled out the feasibility of isolating intact TC from thin-layer or paper chromatograms. An additional disadvantage in isolating TC was the large differences in extinction values at 356 nm between tetracyclines

TABLE II

EXTINCTION VALUES FOR TETRACYCLINES

Tetracyclines	*Extinction value*	
	356 nm in 0.01 N HCl	*434 nm in 5 N HCl*
TC	314	178
ETC	300	178
ATC	17	185
EATC	17	187
CTC	180	128

and anhydrotetracyclines (Table II). A significant quantity of anhydrotetracyclines in the samples would not be accounted for in direct dilution absorbance readings at 356 nm.

The major impurities found in the TC samples were ETC, ATC and EATC. In view of the expected similarities in extinction values for EATC and ATC, the absorbance reading at 434 nm after acid-catalyzed transformation of the TC samples to total anhydro components should be comparable to the value obtained from an equivalent concentration of pure ATC. In actual fact, the $E^{1\%}_{1\,cm}$ values for the four acid-treated TC samples are in complete agreement with the values (185–187) reported by LEESON[9] for both highly purified ATC and EATC.

$$E^{1\%}_{1cm}\,\mathrm{ATC} = \frac{A_{434} \times 10^4}{C} \times \frac{480}{462}$$

where C = concentration of TC in μg/ml.

Since the absorbance values from direct dilutions can be interpreted as representing pure ATC, the prior separation of TC by chromatography and recovery as ATC reflects the actual content or contribution of pure TC in the TC samples. This method was utilized to follow the stability pattern of TC in various buffered formulations and will be reported in a future paper.

Research Laboratories, Frank W. Horner Ltd.,
Montreal, Quebec (Canada)

D. L. SIMMONS
R. J. RANZ
H. S. L. WOO
P. PICOTTE

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