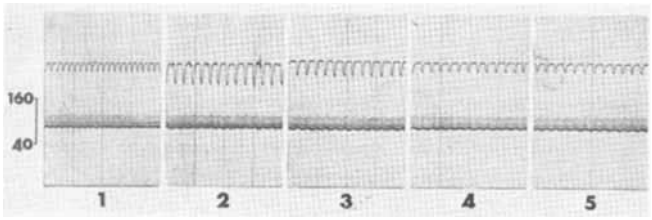

Figure 3—*Action of ampicillin i.v. on the ventilation of a dog* in situ. *From top to bottom: tracing recorded by a balloon inserted into the bronchus; blood pressure (mm. Hg); 1 = control condition; 2 = 2 min. after injection i.v. of 10 mg./kg. of ampicillin; 3 = 30 min. later; 4 = 1 hr. later; 5 = 2 hr. later.*

are only in part associated with changes in bronchial tone. In fact, it should be noted that: (*a*) the baseline of bronchial balloon pressure did not change appreciably in response to ampicillin, indicating that the resting tone of bronchial muscle was slightly or not affected by the drug; (*b*) the ampicillin action was antagonized by the high respiratory depression by morphine; and (*c*) the antibiotic action was unaffected after treatment with atropine, dibenamine, INPEA, chlorpheniramine, cyproheptadine, and hexamethonium.

In conclusion, only *in vitro* is it possible to postulate the ability of ampicillin to relax directly the bronchial musculature. *In vivo* the pressure fluctuations noted with each breath were probably not caused by rapid breath-to-breath changes in bronchial tone. It is more likely that ampicillin increased the activity of the respiratory center, causing an increase in ventilation and a subsequent increase in anatomic deadspace.

REFERENCES

(1) G. Benzi, A. Crema, F. Berté, G. M. Frigo, and E. Arrigoni, *Arch. Int. Pharmacodyn.*, **174,** 74(1968).

(2) G. Benzi, E. Bermudez, E. Arrigoni, and F. Berté, to be published.

(3) J. C. Castillo and E. J. De Beer, *J. Pharmacol Exp. Ther.*, **90,** 104(1947).

(4) D. F. Hawkins, *Brit. J. Pharmacol.*, **10,** 230(1955).

(5) M. Mc Dougal and G. B. West, *ibid.*, **8,** 26(1953).

(6) D. F. Hawkins and H. O. Schild, *ibid.*, **6,** 682(1951).

(7) H. Konzett and R. Rössler, *Arch. Exp. Pathol. Pharmakol.*, **195,** 71(1940).

(8) J. Mead, *Physiol. Rev.*, **41,** 281(1961).

(9) J. A. Nadel and J. G. Widdicombe, *J. Physiol.*, **163,** 13 (1962).

(10) K. H. Kilburn, *J. Appl. Physiol.*, **15,** 229(1960).

(11) J. T. Litchfield, Jr., and F. Wilcoxon, *J. Pharmacol. Exp. Ther.*, **96,** 99(1949).

(12) G. Chen, R. Portman, D. Russel, and C. R. Ensor, *J. Amer. Pharm. Ass., Sci. Ed.*, **40,** 273(1951).

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Determination of Epitetracycline and Chlortetracycline in Tetracycline by Quantitative Thin-Layer Chromatography

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Abstract □ Small amounts of epitetracycline and chlortetracycline in tetracycline were determined by quantitative thin-layer chromatography on kieselguhr layers, impregnated with a citrate–phosphate solution, pH 5.5, containing 10% glycerin. The method involves chromatography under sharply defined conditions (relative humidity, temperature, and rapidity of spotting) in order to obtain a good separation of the different zones and to prevent rapid epimerization during development. After elution with 0.1 *N* HCl, epitetracycline was measured at 356 mμ, while for chlortetracycline the fluorimetric method of Chicarelli was used. The possible identities of three other impurities—with R_f values between epitetracycline and tetracycline—are discussed; their percentages were calculated as epitetracycline.

Keyphrases □ Epitetracycline—analysis, separation in tetracycline □ Chlortetracycline—analysis, separation in tetracycline □ TLC—analysis □ Spectrophotometry—analysis

In 1963, Remmers *et al.* (1) described a spectrophotometric determination of 4-epitetracycline (epi-TC) in tetracycline (TC), based on the absorbance-ratio difference at 254 and 267 mμ. However, this method is not suitable for measuring small amounts of epi-TC in commercial samples; accurate measurements at those wavelengths are disadvantageously affected by the presence of anhydrotetracyclines (ATC, epi-ATC). The paper chromatographic method, reported by Addison and Clark (2), gives better information about the percentage of epi-TC.

When small amounts of chlortetracycline (CTC) are to be determined, the spectrophotometric procedure, given by Woolford *et al.* (3), is not preferable. It is also not possible to apply the fluorimetric procedure [Chicarelli (4)] because the fluorescence of the CTC is quenched by the TC solution.

In 1964, Sonanini and Anker (5) described the identification of three tetracyclines on kieselguhr layers, impregnated with a solution (pH 3.7) containing 5% glycerin. In 1968, Ascione *et al.* (6) reported the separation of some tetracyclines on the same support, impregnated with a EDTA–PEG 400–glycerin solution, pH 7.0.

The present authors used the idea of Sonanini and Anker as a starting point for a quantitative determination of epi-TC and CTC present in commercial samples of TC.

Table I—Percentages of epi-TC and CTC and Compounds A, B, and C in Three Commercial Samples of TC

Sample. No.	epi-TC, %	CTC, %	A, %	B, %	C, %
I	2.4	0	0.3	0.6	0.6
	2.4	0	0.3	0.7	0.7
	2.5	0	0.2	0.9	0.7
II	1.1	3.4	0.3	0.5	0.5
	1.2	3.4	0.2	0.5	0.5
	1.4	3.5	0.3	0.6	0.5
III	5.8	0.1	0.4	0.5	0.4
	5.6	0.1	0.4	0.4	0.5
	5.5	0.1	0.4	0.5	0.6

EXPERIMENTAL

Materials—*Kieselguhr, Purified*—One-hundred grams of Kieselguhr-G (Merck) is suspended in 500 ml. of 4 *N* HCl, boiled, and filtered; this procedure is done three times to remove the binder ($CaSO_4$) and most of the iron. After washing with distilled water, the product is suspended in 500 ml. of a 1% aqueous EDTA solution, pH 3, and boiled again to eliminate traces of Fe^{+++} and Ca^{++} ions. The purified kieselguhr is finally washed with distilled water and methanol and dried at 100°. As a control for purity, 500 mg. is suspended in 5 ml. of 0.1 *N* HCl and centrifuged; the absorbance of the clear solution at 356 mμ is <0.005.

Solution pH 5.5—Forty-three milliliters of citric acid (0.1 *M*), 57.0 ml. of $Na_2HPO_4 \cdot 2H_2O$ (0.2 *M*), and 10 ml. of glycerin (sp. gr. 1.23) were used.

Tetracycline Hydrochloride (USP Reference Standard, Packaged July 1968)—Dried over P_2O_5 at 60° *in vacuo*. Absorbance of a 1% solution in 0.1 *N* HCl in a 1-cm. cell at 356 mμ is 308. Impurities: ATC, 0.1% [spectrophotometric (7)]; CTC, 0.3%; epi-TC, 0.5%; Compound A, 0.1%; Compound B, 0.2%; Compound C, 0.2%.

Epitetracycline Ammonium Salt[1]—Absorbance of a 1% solution in 0.1 *N* HCl in a 1-cm. cell at 356 mμ is 316. Impurities: epi-ATC, 0.3% [spectrophotometric (7)]; TC, 0.5%. Because of its instability, epi-TC was only used in calculating the recoveries of epi-TC and TC after spotting and elution.

Chlortetracycline Hydrochloride—From capsules (Aureomycin), dried over P_2O_5 at 60° *in vacuo*. Absorbance of a 1% solution in 0.1 *N* HCl in a 1-cm. cell at 269 mμ is 194; this value has been corrected for the absorbance of TC present in CTC (0.6%, determined by quantitative TLC).

Oxytetracycline (OTC), 2-acetyl-2-decarboxamido-tetracycline (ADTC),[1] methacycline (MTC), anhydrotetracycline (ATC),[1] and epi-anhydrotetracycline (epi-ATC)[1] were used for the thin-layer chromatographic identification of other impurities in tetracycline.

Kieselguhr Layers—Layers (0.25 mm.) were prepared with the Desaga apparatus by application of a slurry of 4 g. purified kieselguhr in 8 ml. of the solution, pH 5.5, per 20 × 20-cm. glass plate. The plates were stored at 15° and a relative humidity of 65–75% during 2 days before use.

All chemicals used were of analytical grade.

Microliter pipets, 5-μl., 55-mm. length (microcaps, Drummond Scientific), were used.

Apparatus—Beckman DU spectrophotometer. Filter-fluorimeter, primary filter 364 mμ, secondary filter 414 mμ. TLC-tank, 20 × 20 × 8 cm., saturated with dichloromethane–ethanol 95% (9:1) at 15°.

PROCEDURES

Chromatography—The sample of TC is dissolved in methanol at 0° in a concentration of 20 mg. per ml. Approximately 1 mg. of the sample (= *p* mg. dried substance) is spotted rapidly over 15 cm. and the plate is placed immediately in the developing solvent [dichloromethane–ethanol 95% (9:1) at 15°]. The time between spotting and developing must not exceed 3 min. The fluorescing zones are marked under longwave UV light.

[1] The authors are grateful to Dr. J. Keiner (Jena, D.D.R.) for his gift of ADTC and to Mr. J. Ribbers (Organon, Holland) for the preparation of highly purified epi-TC, ATC, and epi-ATC.

R_f values are: epi-TC, 0.1; Compound A, 0.17; Compound B, 0.30; Compound C, 0.38; TC, 0.55–0.65; CTC, 0.80; epi-ATC, 0.85; ATC, 1.

If necessary, the fluorescence of CTC can be activated by spraying a few milliliters of 0.01 *N* NaOH on the plate. Activation of the fluorescence of epi-TC, by spraying with NaOH or by holding the plate above ammonia vapor, is not recommended because of rapid decomposition of this compound.

Assay of Epitetracycline (Hydrochloride)—The epi-TC zone is scratched off, transferred into a stoppered centrifuge tube, and extracted with 5.00 ml. of 0.1 *N* HCl. After centrifuging, the absorbance (*A*) of the clear solution is determined at 356 mμ. To eliminate inaccuracies in spotting and elution, 50 mcg. of the TC standard (concentration 1 mg. per ml.) is spotted over 15 cm.; after elution with 5.00 ml. of 0.1 *N* HCl, the absorbance at 356 mμ is determined (= $A_{stand.}$).

$$\% \text{ epi-TC} = \frac{A \times 5}{A_{stand.} \times p} \qquad \text{(Eq. 1)}$$

In this laboratory the $A_{stand.}$ for TC was 0.277 ± 0.003 (recovery 90%), while for epi-TC with the same procedure a recovery of 91% was obtained. The absorbance of the blank—250 mg. of developed kieselguhr per 5.00 ml. of 0.1 *N* HCl—was negligible; also the background absorbance between epi-TC and Compound A was very small (about 0.005), and therefore no correction had to be made. This background absorbance, due to epimerization during development, increased at higher R_f values; the values of the percentages of Compounds A, B, and C in Table I were corrected with 0.1, 0.2, and 0.2, respectively.

Assay of Chlortetracycline (Hydrochloride)—The CTC zone is scratched off, transferred into a stoppered centrifuge tube, and extracted with 5.00 ml. of 0.2 *N* NaOH. After centrifuging, the fluorescence of the clear solution is determined about 15 min. after NaOH addition, adjusting the reading of the galvanometer at a suitable value. The percentage of CTC (hydrochloride) is calculated from the standard curve.

CTC Standard Curve—To eliminate inaccuracies in spotting and elution, 5, 10, 15, and 20 mcg. of the CTC standard (concentration 0.5 mg. per ml.) are spotted over 15 cm. After elution with 5.00 ml. of 0.2 *N* NaOH the fluorescence is measured. In this laboratory the recovery of the CTC standard was 97 ± 3%, while Beer's law was obeyed. The fluorescence of the blank was nil.

DISCUSSION

Before good results could be obtained in the determination of epi-TC and CTC in TC, many problems had to be solved. While the qualitative examination of these substances was easy—because only 1–5 mcg. of a sample of TC can be spotted on the plate—it was rather difficult to separate them when 1–1.5 mg. had to be examined. (These great quantities were necessary for spectrophotometric measurements.) Moreover, epi-TC and CTC also had to be separated from five other impurities present in most commercial samples of TC: ATC, epi-ATC, and the Compounds A, B, and C. Finally, chromatography on one plate and with one developing sol-

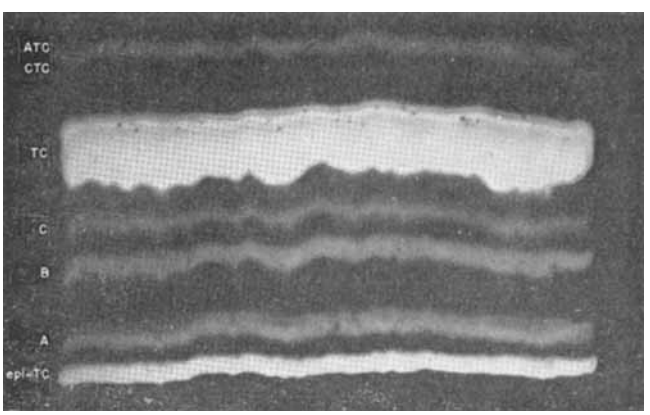

Figure 1—*The quantitative TLC separation of the impurities in commercial tetracycline hydrochloride.*

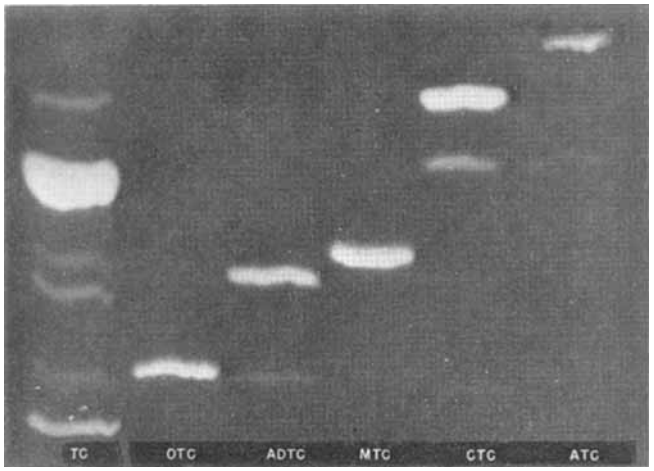

Figure 2—*Chromatogram of five possible impurities in commercial tetracycline (OTC, ADTC, MTC, CTC, and ATC are standard substances).*

vent was tried. After rejecting ethyl acetate and chloroform–acetone (1:1) and kieselguhr layers pH 3.7 (5), dichloromethane–ethanol 95% (9:1) was selected, in combination with kieselguhr impregnated with a buffer solution pH 5.5, containing 10% glycerin. In order to obtain reproducible values in the determination of epi-TC, the influence of the chemicals used and the influence of small variations in the technique were studied. It was found that the separation of the zones depended greatly on the amount of purification of the kieselguhr and on the condition of the layer before and during development (relation of relative humidity to temperature). Further, it was observed that the partial conversion of TC into epi-TC during the determination was strongly influenced by the amount of time between spotting and development and by the temperature of the methanolic TC solution, Working under the conditions described under *Procedures*, the epimerization is negligible and the chromatogram shows sharply defined zones of about 0.5 cm. width. Because the recoveries of epi-TC and TC are practically equal, TC is preferred as a standard substance for epi-TC determinations, being more stable than epi-TC.

As to CTC, the fluorimetric procedure is preferable because of its high sensitivity. Only very high percentages of epi-ATC (R_f 0.85) might quench the fluorescence.

In nearly all samples of TC, three yellow fluorescing zones between epi-TC and TC were observed (Fig. 1) After elution with 0.1 *N* HCl, maximum absorbances were found at about 355 mμ. Two-dimensional TLC using methyl ethyl ketone saturated with the buffer solution, pH 5.5, as second phase suggests that their identities might be oxytetracycline (R_f 0.17), 2-acetyl-2-decarboxamido-tetracycline (R_f 0.30), and methacycline (R_f 0.38) (Fig. 2). Keiner *et al.* (8) used this developing solvent, pH 4.7, to separate ADTC from epi-TC and TC. However, the authors did not succeed in a definite identification by IR, mainly due to the large quantity of eluted glycerin and to the decomposition of these three compounds during the isolation. They were determined quantitatively—calculated as epi-TC—to give an idea of the possible errors in epi-TC determination if no sharply defined zones can be obtained.

The methods for the determination of epi-TC and CTC are not limited to tetracycline substances but can also be performed in some TC formulations. For aqueous solutions and suspensions of TC, it is recommended in most cases to freeze-dry the preparation and to spot after dissolving in methanol.

Finally, this quantitative TLC method can also be applied to determine impurities in other tetracyclines like chlortetracycline, demethylchlortetracycline, and methacycline. In oxytetracycline studies, however, the solvent system used in development should be replaced by another one.

REFERENCES

(1) E. G. Remmers, G. M. Sieger, and A. P. Doerschuk, *J. Pharm. Sci.*, **52,** 752(1963).

(2) E. Addison and R. G. Clark, *J. Pharm. Pharmacol.*, **15,** 268 (1963).

(3) M. H. Woolford and F. S. Chicarelli, *J. Amer. Pharm. Ass., Sci. Ed.*, **45,** 400(1956).

(4) F. S. Chicarelli, P. van Gieson, and M. H. Woolford, *ibid.*, **45,** 418(1956).

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

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

(7) I. C. Dijkhuis, *Pharm. Weekbl.*, **102,** 1308(1967).

(8) J. Keiner, R. Hüttenrauch, and W. Poethke, *Arch. Pharm.*, **300,** 840(1967).

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