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Fluorimetric assay of tetracycline mixtures

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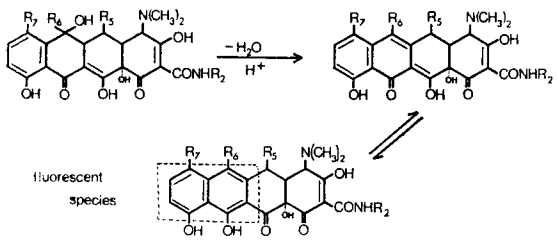
A simple and precise fluorimetric method is described for the simultaneous assay in plasma of a mixture containing chlortetracycline, demethylchlortetracycline and tetracycline. Assay within the therapeutic ranges of 0–5 mg litre^{-1} is achieved by formation of strongly fluorescent aluminium/tetracycline complexes, without prior extraction or separation of the individual antibiotics. This is performed by determinations at the peak excitation and fluorescence emission wavelengths of each tetracycline chelate. The method can be similarly applied to the assay of oxytetracycline, rolitetracycline and minocycline.

A number of fluorimetric procedures have been described for the assay in biological fluids of single members of the tetracycline series (Hayes & DuBuy, 1964; Kohn, 1961; Ibsen, Saunders & Urist, 1963; Scales & Assinder, 1973). Kelly & Hoyt (1969) described a method for a triple mixture containing chlortetracycline (CTC), demethylchlortetracycline (DMCTC) and tetracycline (TC); in which CTC and TC were measured individually and DMCTC concentration was calculated by the difference from a 'total tetracycline' estimation using Kohn's method. This paper describes a simple, more precise and sensitive method for the quantitative estimation in plasma of the individual members of this triple mixture. Oxytetracycline, rolitetracycline and minocycline can also be estimated using the same method.



Tetracycline	$R_2, R_5, R_7 = H$	$R_6 = CH_3$	
Demethylchlortetracycline	$R_2, R_5, R_6 = H$		$R_7 = Cl$
Chlortetracycline	$R_2, R_5 = H$	$R_6 = CH_3$	$R_7 = Cl$
Oxytetracycline	$R_2, R_7 = H$	$R_6 = CH_3$	$R_5 = OH$
Rolitetracycline	$R_5, R_7 = H$	$R_6 = CH_3$	$R_2 = CH\,N$

FIG. 1. Formation of anhydrotetracyclines.

MATERIALS AND METHODS

The hydrochlorides of chlortetraycline, demethylchlortetracycline, tetracycline and minocycline were gifts from Cyanamid Ltd., and were used without further purification. Oxytetracycline hydrochloride was obtained from Pfizer Ltd., and rolitetracycline hydrochloride from Bristol Laboratories Ltd.

Reagent grade anhydrous $AlCl_3$ (BDH) was dissolved to give a 0·75 M aqueous solution and was filtered before use. β-Thiopropionic acid was obtained from Pfaltz and Bauer Inc. All other chemicals were of analytical reagent grade.

Sørensens citrate buffer (pH 6·0) contained 125 g citric acid and 160 ml 10 M NaOH litre^{-1}. Double glass-distilled water was employed throughout for fluorimetric determinations.

Apparatus Uncorrected fluorescence spectra were recorded using a Baird Atomic Fluoripoint spectrofluorimeter at 5 nm resolution, using 1 cm^2 silica cells.

Fluorescence spectra

For each individual tetracycline, fluorescence spectra were obtained as follows:

1, Solutions of 5 mg litre^{-1} in 0·1 M HCl were studied to observe any natural fluorescence properties of unchanged tetracyclines. 2, Measurements were made after heating in a boiling water bath at 100° to produce their respective anhydro-salts, and 3, after complexation with aluminium ions to both the free bases and anhydro-salts, by addition of aluminium chloride solution to buffered solutions of the appropriate tetracycline or tetracycline anhydro-salt. 4, Spectra of chlortetracycline were obtained after addition of 6 M NaOH to produce iso-chlortetracycline.

The qualitative effects on spectra of the tetracyclines in admixture were studied by examining 5 mg litre^{-1} solutions of each tetracycline and subsequently adding various concentrations (0–10 mg litre^{-1}) of the other tetracyclines and comparing

the resultant spectra. TC, DMCTC and CTC were studied in this manner both as their anhydro-salts and after aluminium complexation. CTC was also studied in admixture as the alkaline degradation product iso-chlortetracycline.

Standard curves

Standard curves relating fluorescence intensity to concentration were prepared for each tetracycline in plasma and for the contributive effect of CTC to the fluorescence emission of DMCTC and TC when simultaneously present. The latter were prepared by the addition of varying concentrations of CTC to fixed concentrations of DMCTC or TC and measuring fluorescence emission at the described wavelengths for DMCTC or TC respectively. Linearity of contribution was confirmed by repeated determinations at different fixed concentrations of DMCTC and TC.

Using the experimental conditions described, the relative fluorescence of the three tetracyclines, obtained from the gradients of their plots of concentration versus fluorescence in plasma, were in the ratio of 101:47·6:23·6 for CTC:TC:DMCTC respectively. On the same scale of fluorescence the 'contributive fluorescence' of CTC to DMCTC was 8·2 fluorescence units mg^{-1} $litre^{-1}$ (quinine sulphate in 0·1M H_2SO_4 $4{\cdot}5 \times 10^3$ units mg^{-1} $litre^{-1}$), and for CTC to TC, 10·4 fluorescence units mg^{-1} $litre^{-1}$.

Plasma preparation

4·0 ml of plasma was required for the individual estimation of the three components present. Duplicate results from a single 2·0 ml sample can be obtained using a quarter of the quantities and 0·5 mm^2 microcuvettes for measurement.

The plasma sample was divided into two 2·0 ml aliquots which were analysed separately for CTC and for DMCTC and TC. Each aliquot was deproteinized by the addition of 0·6 ml 1·5 M trichloroacetic acid solution and vortexed to produce thorough mixing with plasma. Samples were then centrifuged at 3500 rev min^{-1} for 5 min to produce 1·6 ml of clear supernatant which was transferred to a separate glass tube.

CTC estimation

HCl (1·2 ml, 0·1M) containing β-thiopropionic acid (β-TPA) (1·0 g $litre^{-1}$) was added to 1·6 ml of supernatant, followed by NaOH (1·2 ml 6 M). Samples were shaken to mix the reagents and allowed to stand for 20 min before fluorimetric measurements were made at 355 nm excitation and 415 nm emission.

TC and DMCTC estimations

To the second 1·6 ml of supernatant HCl (0·5 ml 0·1M) containing β-TPA (1·0 g $litre^{-1}$) was added before heating at 100° for 10 min (polythene balls over the top of each tube were used to minimize loss by evaporation during heating). The tubes were then cooled by immersion in cold water. Sørensens citrate buffer (pH 6·0, 1·0 ml) was added followed by aluminium chloride solution (0·5 ml, 0·75 M). The samples were shaken and allowed to stand for 15 min at room temperature. TC was estimated by measuring fluorescence at 465 nm excitation and 555 nm fluorescence emission. DMCTC was estimated at 385 nm and 485 nm on the same sample.

Before measurement of each batch of samples, readings were standardized against quinine sulphate (0·0167 mg $litre^{-1}$) in 0·1M H_2SO_4 to give a scale deflection of 75% at 350 nm excitation, 455 nm emission.

RESULTS AND DISCUSSION

Although the tetracyclines are only weakly fluorescent in 0·1M HCl, conversion of OTC, TC and CTC to their anhydro-salts (Boothe, Morton & others, 1953) leads to an increase in fluorescence emission. An increase can also be observed in certain members of the series when they are chelated with calcium ions in the presence of barbitone to form calcium-barbitone-tetracycline complexes (Kohn, 1961). However, since all the calcium-barbitone complexes examined emitted fluorescence at the same wavelengths, this technique was unsuitable for the analysis of individual tetracyclines in multi-component mixtures.

Complexation of aluminium to tetracyclines in the absence of barbitone has been reported (Mitscher, Bonacci, & others 1969) and it was observed that such complexes have a stoichiometric ratio of one metal ion per tetracycline molecule at pH 4·9. In these laboratories the addition of aluminium ions to weakly fluorescent tetracyclines and anhydro-tetracyclines at pH 5 led to large increases in fluorescence intensity (Table 1).

Table 1. *Increase in fluorescence produced by addition of Al^{3+} to tetracycline solutions.*

Compound	Anhydro-salt λe	Anhydro-salt λf	Al^{3+} complex λe	Al^{3+} complex λf	% increase in fluorescence
TC	465	555	465	555	4800
CTC	360	420	390	490	3300
OTC	390	510	390	460	1400

The most strongly fluorescent species was chosen for each tetracycline for the purpose of fluorimetric assay, whether it was the anhydro-salt chelate or the free base chelate, as larger detectable changes in concentration permitted greater assay sensitivity. Fluorescence produced by such aluminium/tetracycline complexes is stable for at least 60 min under the experimental conditions described. This is achieved by the use of β-TPA, an antioxidant compound used previously to stabilize tetracycline solutions against photodecomposition (Scales & Assinder, 1973; Hall, O'Grady & Turner, 1974).

Of the tetracyclines examined in the present work, all responded to aluminium complexation to produce an increase in measurable fluorescent intensity. TC and rolitetracycline were found to fluoresce more intensely if heated to produce the anhydro-salts before complexation, and were therefore measured as anhydro-TC/aluminium and anhydro-rolitetracycline/aluminium complexes. The fluorescence intensities of DMCTC, CTC and minocycline were found to remain the same with or without acid hydrolysis procedures before complexation and could therefore be measured as free base complexes. However, it was found that by adding strong alkali (6·0 M NaOH) to CTC, to produce iso-chlorotetracycline (iso-CTC) (Levine, Garlock & Fischbach, 1949) a more intensely fluorescent species than the CTC/Al^{3+} complex was obtained. As the fluorescence emission of iso-CTC lacked any spectral interference between its emission peak and those of DMCTC and TC in admixture, it was advantageous to measure CTC as iso-CTC rather than as the CTC/Al^{3+} complex. By doing so, a greater assay sensitivity for low concentrations of CTC was achieved.

Fluorescence interactions

Peak excitation and emission wavelengths were obtained for each tetracycline species chosen for measurement. For the members of the triple mixture under study these were as follows:

	λe nm	λf nm
iso-CTC	355	415
Al^{3+}/anhydro-TC	465	555
Al^{3+}/DMCTC	385	485

However, due to the formation of CTC/Al^{3+} during the metal complexation procedures for DMCTC and TC in the triple mixture, allowance must be made for the presence of CTC/Al^{3+}. This is due to spectral overlap of the fluorescence of this species with the emission peaks of DMCTC/Al^{3+} and anhydro-TC/Al^{3+}, and is caused by a bathochromic shift in excitation and emission wavelengths of CTC upon complexation from 360, 420 to 390, 490 nm (Fig. 2). The resultant peaks virtually coincide with those of DMCTC/Al^{3+} (385, 485 nm) and separate determinations of DMCTC and CTC in the same sample would not be possible if the alkaline degredation method for CTC estimation was not available.

Calculation of results

Correction of background fluorescence at each set of wavelengths used for measurement was made from duplicate estimations on a drug-free plasma sample obtained from each patient. Where such a plasma

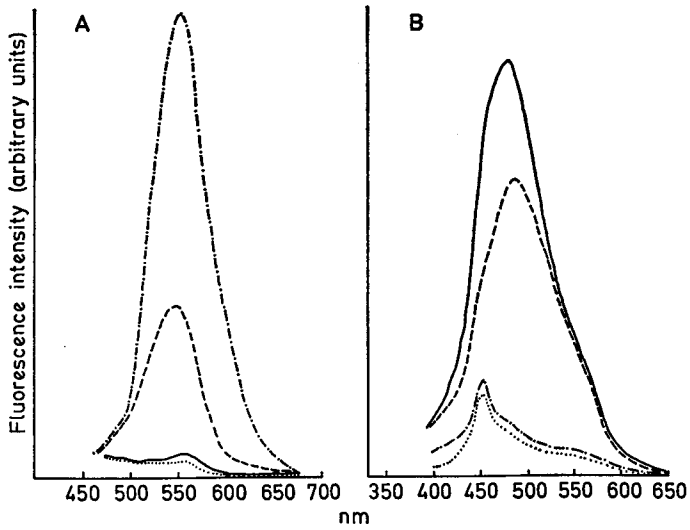


FIG. 2A. Emission spectrum of TC/Al^{3+} with superimposed spectra of DMCTC/Al^{3+} and CTC/Al^{3+}. Excitation wavelength 465 nm. B. Emission spectrum of DMCTC/Al^{3+} with superimposed spectra of CTC/Al^{3+} and TC/Al^{3+}. Excitation wavelength 385 nm. ——DMCTC/Al^{3+}, – – – CTC/Al^{3+}, – – – TC/Al^{3+}, · · · · Blank.

'blank' is not available, mean values can be used from a pooled drug-free plasma sample from other sources. In these laboratories, background fluorescence was not found to differ significantly between patients. The mean of 30 'blank' values were as follows:

CTC	31·5	s. error = 0·9
DMCTC	35	s. error = 1·4
TC	15	s. error = 0·5

(Quinine sulphate standard (0·0167 mg litre^{-1}) = 75

Plasma concentrations were obtained from results using the following notations:

(1) CTC Plasma concn =

$$\frac{\text{Fluorimeter reading—'blank' value}}{\text{gradient of CTC standard curve}} \text{ mg litre}^{-1}$$

(2) DMCTC Plasma concn =

$$\frac{\text{Fluorimeter reading—'blank' value—(CTC mg litre}^{-1} \times \text{DMCTC contributive fluorescence gradient)}}{\text{gradient of DMCTC standard curve}} \text{ mg litre}^{-1}$$

(3) TC Plasma concn =

$$\frac{\text{Fluorimeter reading—'blank' value—(CTC mg litre}^{-1} \times \text{TC contributive fluorescence gradient)}}{\text{gradient of TC standard curve}} \text{ mg litre}^{-1}$$

Assay test

The precision and accuracy of the method were evaluated by repeated estimations performed on prepared triple tetracycline plasma standards containing CTC, DMCTC and TC, of known concentrations. The results of six estimations of each of these test solutions are given in Table 2.

Although the coefficients of variation were higher for measurement of low concentrations of DMCTC, the 20% variation on a 0·3 mg litre^{-1} sample was accepted rather than complicate the general method with specific measures to improve the complexation conditions for DMCTC. Even this variation represents a deviation of only ± 0·06 mg litre^{-1} on the true value, and this compares most favourably with the results at this concentration using microbiological techniques.

Table 2. *Assay test results.*

Plasma standard	Added mg litre^{-1}	Mean found mg litre^{-1}	% Recovery	c.v.
Chlortetracycline				
A	1·4	1·382	98·7	3·2
B	0·2	0·165	82·5	5·0
C	0·7	0·715	102·1	1·2
Demethylchlortetracycline				
A	0·3	0·313	104·3	20·3
B	1·4	1·385	98·9	5·8
C	0·7	0·69	98·6	10·1
Tetracycline				
A	0·8	0·776	97	3·7
B	0·4	0·383	95·7	2·0
C	1·6	1·512	94·5	2·1

Conclusion

In practice the method was easily and rapidly performed, completed results from a batch of ten samples being obtainable within 2 h. The method reliably determines individual plasma tetracycline concentrations down to 0·1 mg litre^{-1} with acceptable accuracy, and fluorescence-concentration relations are linear over the range 0–5 mg litre^{-1}. As metal ion complexation is a common characteristic of the tetracycline antibiotics, the method can be adapted to the assay of other fluorescent members of the tetracycline group, either singly or in combination, in plasma or serum samples.

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REFERENCES

Boothe, J. H., Morton, II, J., Petisi, J. P., Wilkinson, R. G. & Williams, J. H. (1953). *J. Am. chem. Soc.*, **75**, 4621.

Hall, D., O'Grady, F. & Turner, P. (1974). *J. Pharm. Pharmac.*, **26**, *Suppl.*, 117P.

Hayes, J. E. & Dubuy, H. G. (1964). *Analyt. Biochem.*, **7**, 322–327.

Ibsen, K. H., Saunders, R. L. & Urist, M. R. (1963). *Analyt. Biochem.*, **5**, 505–514.

Kelly, R. G. & Hoyt, K. D. (1969). *Z. klin. Chem. U. klin. Biochem.*, **7**, 152–154.

Kohn, K. W. (1961). *Analyt. Chem.*, **33**, 862–866.

Levine, J., Garlock, Jr., E. A. & Fischbach, H. (1949). *J. Am. pharm. Assoc., Sci. Ed.*, **38**, 473–475.

Mitscher, L. A., Bonacci, A. C., Slater-eng, B., Hacker, A. K. & Sokoloski, T. D. (1969). *Antimicrob. Ag. Chemother.*, 111–115.

Scales, B. & Assinder, J. (1973). *J. pharm. Sci.*, **62**, 913–917.