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DIRECT DENSITOMETRIC DETERMINATION OF TETRACYCLINE IN PHARMACEUTICAL PREPARATIONS

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SUMMARY

A direct densitometric method for the assay of tetracycline after thin-layer chromatography is described. Fast Blue Salt B spray reagent was used to detect the spots prior to densitometric scanning and automatic integration of the spot areas. The method was applied to five representative pharmaceutical formulations and the results were compared with those obtained using a published chromatographic–spectrophotometric method. This latter method was also modified to account for recovery losses and adapted to the assay of tetracycline in dosage forms. The densitometric method is more accurate, rapid and simple than the official microbiological assay.

INTRODUCTION

Tetracyclines have been analyzed by titrimetric[1,2], polarographic[3,4], chromatographic[5–12], spectrophotometric[13–18] and microbiological[19,20] methods. The precision of the latter method is rarely better than ±15% (ref. 9) and accuracy is limited by the presence of biologically active tetracycline-like substances in the sample being analyzed[8]. The microbiological method nevertheless remains the official assay of the USP and BP.

Spectrophotometric procedures tend to be more accurate but are based on a prior separation by column[5,21,22] or thin-layer[6,9,23] chromatography or on a conversion of the antibiotic to its more stable anhydro compound[7,8,18].

Analysis involving elution before measurement, however, is time consuming and problematic[24,25]. The direct quantitation of compounds separated on TLC plates has the obvious advantage that in most cases the preliminary treatment required is minimal. Direct densitometry has already seen extensive application in the analysis of alkaloids[26], steroids[27–29] and pharmaceuticals[30,31] and its successful application to the assay of antibiotics would be a marked improvement over the present microbiological procedures which are particularly time consuming.

This paper presents a sensitive, specific and rapid method for the assay of tetracycline by direct densitometry after TLC and its application to five pharmaceutical formulations. The results are compared with those obtained using the

chromatographic–spectrophotometric method of FERNANDEZ *et al.*[7] which, in addition, we have modified for the assay of tetracycline in pharmaceutical dosage forms.

EXPERIMENTAL

Preparation of solutions for chromatography

Preparation of standard solutions

Tetracycline hydrochloride USP reference standard (previously dried at 60° *in vacuo*) was made up to an appropriate concentration in 0.05 *N* HCl. Solutions suitable for TLC were usually prepared to contain 1–8 $\mu g/\mu l$ for the densitometric method and 10–50 $\mu g/\mu l$ for the spectrophotometric procedure.

Sampling and preparation of assay solutions

Tablets and capsules. Twenty tablets or capsules of tetracycline HCl selected at random were combined and finely powdered when required. An amount of powder equivalent to the average weight of one tablet or capsule was dissolved in the calculated volume of 0.05 *N* HCl (to give the above quoted concentrations based on the label claims) measured from a burette into a glass stoppered 15-ml centrifuge tube. The sample solutions were then centrifuged for 10 min and the supernatant pipetted quickly into another stoppered test tube.

Syrups. A convenient volume was diluted to the indicated concentration levels with 0.05 *N* HCl, the solution centrifuged and the supernatant liquid treated in the same manner as for the tablets and capsules.

Thin-layer chromatography

TLC plates (20 × 20 cm, 0.3 mm thickness) were prepared by standard equipment using a slurry of 40 g of commercial Kieselguhr (E. Merck) with 80 ml of a 5% aqueous solution of reagent grade EDTA neutralized to pH 7.5 (with either 20% NaOH or conc. NH_4OH). The plates were air-dried overnight. The solvent system was acetone–ethyl acetate–water (80:40:12) and the chromatographic chamber; lined with filter paper, was saturated with the solvent vapor for 1 h before use.

Hamilton microsyringes attached to a repeating dispenser were used for application of samples to the plates.

Chromatographic–spectrophotometric method

The method of FERNANDEZ *et al.*[7] was used with the following modifications. The plates were prepared with commercial grade Kieselguhr and buffered at pH 7.5. Solutions of tetracycline hydrochloride containing 10–50 $\mu g/\mu l$ were prepared as above and 100–300 μg applied along a horizontal line 2 cm from the lower edge. A clear margin of 4–5 cm was left on both sides of the plate. After development to a height of 15 cm (30–35 min), the plate was air dried and the band corresponding to tetracycline isolated. The amount of tetracycline in the sample was determined by the procedure of FERNANDEZ *et al.*; the absorbance of the resulting anhydrotetracycline solution was measured at 430 $m\mu$ on a Beckman DK2 spectrophotometer.

Recovery losses using this method were estimated by treating a freshly prepared solution of USP reference standard of tetracycline HCl (by microbiological assay, 99.5%) of known concentration (1 $\mu g/\mu l$) in the same manner as was the sample.

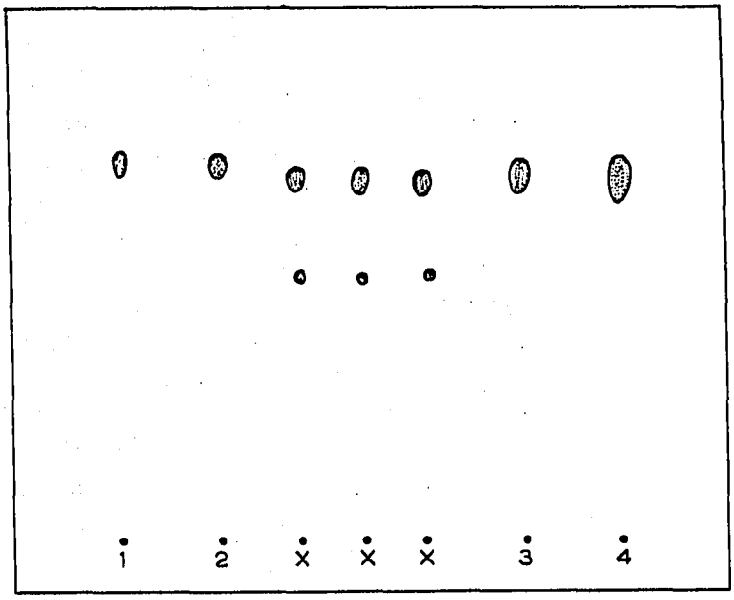

Fig. 1. Representative chromatogram for densitometry. 1 = 1λ tetracycline HCl standard, 2 = 2λ, x = unknown sample, 3 = 3λ, 4 = 4λ. Lower spot in sample is *epi*-tetracycline. Kieselguhr plate with 5% EDTA, pH 7.5.

Over several determinations the absorptivity of anhydrotetracycline was found to be 16.00 $\pm$ 0.25 (94.7% recovery) *versus* a theoretical absorptivity[7] of 16.90. The absorbance of the blank-Kieselguhr in 2 *N* HCl was subtracted from the measured value in each case.

Chromatographic–densitometric method

Three identical aliquots of the sample to be analyzed were applied to the starting line in the central region of the plate at a distance of 2 cm above the lower edge. Four different concentrations of reference sample were applied as spots in increasing concentration from left to right across the plate, two spots being on each side of the three unknowns (Fig. 1). The plate was developed to about 15 cm (30–35 min), removed from the tank, uniformly sprayed with a freshly prepared 0.5% aqueous solution of Fast Blue Salt B and heated for 3 min at 110°. The characteristic purple-pink spots were analyzed within 1 h by direct densitometry.

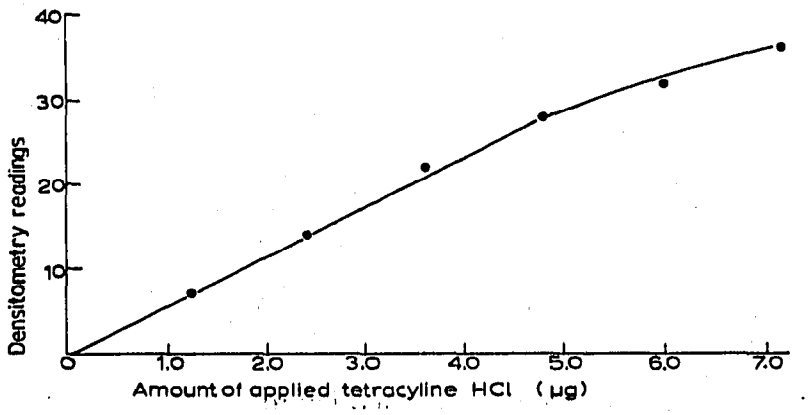

Fig. 2. Representative standard curve (Fast Blue spray).

Densitometry

A Photovolt Densitometer (Model 520M; Photovolt Corp., New York) equipped with a motor driven TLC stage (2.0 in./min), a Varicord 42B recorder (2.0 in./min), a search unit C and an Integraph integrator Model 49 was employed. The colored spots were scanned in the direction of solvent flow. The aperture (0.1 mm width) was adjusted so that it was just long enough to encompass the largest spot in any given run. The search-head was adjusted to about 2 mm above the upper surface of the chromatographic plate. All readings were taken in a darkened room.

Standard curves were prepared for each individual plate by plotting the integrals of the observed peaks (integraph readings) for the four spots of reference standard *versus* the amounts (μg) spotted on the plate (Fig. 2). The values for the unknown sample, applied in triplicate and prepared in such a manner that the approximate concentration (label claim) was between the values of reference standards, were found from the curve and the results expressed as a percentage of label claim.

RESULTS AND DISCUSSION

One of the major difficulties in a chromatographic–spectrophotometric assay is the inherent loss on recovery of the material from the chromatographic plates. DIJKHUIS AND BROMMET[6] found that they could recover only 90% of the applied tetracycline and accordingly corrected their assay to compensate for this. FERNANDEZ *et al.*[7] reported recoveries of 95–97% of known amounts of tetracycline in six mix-

TABLE I

RESULTS OF ASSAY OF TETRACYCLINE HCl IN FIVE AGED PHARMACEUTICAL PREPARATIONS IN PERCENT OF LABEL CLAIM

Dosage form	*Method of assay*[a]	*Percent tetracycline HCl found*	*Number of determinations*	*Standard deviation*
(1) Capsule	A	88.4	6	3.7
	B	93.3	6	3.7
	C	91.8	14	5.8
(2) Capsule	A	90.8	6	1.1
	B	95.8	6	1.1
	C	93.1	22	4.3
(3) Capsule	A	70.0	15	1.9
	B	73.8	15	1.9
	C	74.6	20	3.4
(4) Tablet	A	86.5	10	4.4
	B	91.0	10	4.4
	C	90.4	16	5.4
(5) Syrup	A	86.9	9	5.7
	B	91.7	9	5.7
	C	91.8	9	2.9

[a] Method A, method of FERNANDEZ *et al.*[7]; method B, chromatographic–spectrophotometric method corrected for recovery losses; method C, densitometry.

tures, however, no estimation as to the number of determinations for each sample nor the precision in their values is given.

In our laboratory the method of FERNANDEZ *et al.* using a fresh sample of tetracycline HCl USP reference standard (which was found by microbiological assay to contain 995 μg/mg tetracycline HCl) consistently led to recoveries of 94.7%. These results were based on the absorptivity of anhydrotetracycline found for the USP reference sample treated in an identical manner as the pharmaceutical samples. It would seem appropriate then that a correction factor should be applied if this method is to be used for an accurate assay of tetracycline in pharmaceutical formulations.

In Table I are listed the results of the determination of tetracycline content in five aged pharmaceutical preparations by three methods. The first method is that of FERNANDEZ *et al.*[7] and shows the values and the standard deviation that were found when their procedure was applied to the assay of tetracycline in dosage forms. The second row of results were obtained using the chromatographic–spectrophotometric method; however, this method was modified by using the corrected absorptivity value of anhydrotetracycline to account for the estimated recovery losses on chromatography. These values should more closely resemble the true tetracycline content compared to the results obtained by the original spectrophotometric method.

The third set of results were obtained by direct densitometry on the thin-layer chromatogram. In general, although the densitometric method was less precise, it was much more rapid and gave results well within the accuracy obtained by the official microbiological assay.

The purple coloration formed on reaction of the tetracyclines with Fast Blue Salt B reagent has already been used for detection after chromatography, its limit of detection reportedly[32] being 0.1 μg. The spots were found to be stable for several hours although readings were normally taken within 1 h.

Using this reagent, standard curves could be obtained with concentrations from 1 to 30 μg per spot, although amounts from 1 to 7 μg were used since larger quantities led to deviations from linearity (see Fig. 2). Within this concentration range the tetracycline formed well-rounded spots and no tailing was observed. Product excipients normally present in tetracycline preparations were found not to interfere with the chromatographic assay. Methyl paraben, salicylamine, salicylamide, aspirin, caffeine, ascorbic acid, phenacetin, diphenylpyraline and pyrilamine maleate were clearly separated from the tetracycline band on the thin-layer plate.

Attempts to standardize satisfactorily conditions of chromatography from plate to plate were not successful, coefficients of variation for integraph readings between plates being as high as 20%. It was necessary therefore to follow the above described procedure in which unknown and standard in a range of concentrations are run on the same plate and a curve plotted for each plate. Standard deviations for readings taken this way are then within practical limits (see Table I).

Although less precise than the spectrophotometric method, the assay of tetracycline by densitometry on thin-layer chromatograms is more sensitive and less time consuming. Because the amount of tetracycline is read directly from the plate after separation of impurities and the value interpolated from standard treated in an identical manner, the densitometric method is more accurate than the official microbiological assay in which biologically active tetracycline-like substances interfere.

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