THE DETERMINATION OF EPITETRACYCLINE AND TETRACYCLINE BY ION-EXCHANGE PAPER CHROMATOGRAPHY AND ITS APPLICATION TO HUMAN URINE AND SERUM

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A simple quantitative chromatographic method is described for the separation of epitetracycline and tetracycline in pharmaceutical preparations. The method is applicable qualitatively to human serum and quantitatively to the biological determination of tetracycline in urine. Whatman's modified cellulose phosphate cation-exchange paper is developed by the descending technique using 0·1 per cent w/v ammonium chloride solution. The spots are located as yellow fluorescent spots under ultra-violet light and ammonia vapour, eluted in 10 per cent w/v ammonium chloride, and the extinction determined at 356 mμ. The method is not suitable for the separation of chlortetracycline or oxytetracycline from tetracycline.

THE tetracyclines undergo a reversible isomerisation between the pH range 2 to 6 (Doerschuk, Bitler and McCormick, 1955). This has been shown to be an epimerisation at Carbon 4, giving rise to a new series of compounds, the epitetracyclines or quatrimycins. Their preparation and properties have been described by McCormick and others (1957).

Commercial tetracycline normally contains 5 per cent or less of its epimer. Liquid suspensions and solutions of tetracycline may contain appreciable amounts of epitetracycline. A simple quantitative method for its estimation is desirable, since epitetracycline has only about 5 per cent of the *in vitro* biological activity of tetracycline.

Previous methods of chromatographic separation involve the use of paper saturated with McElvaine's buffer at pH 3·5 and developing with nitromethane-chloroform-pyridine (Selzer and Wright, 1957), or nitromethane-benzene-collidine mixtures (Coppi, 1960). These solvent systems are toxic and unpleasant to use and in both, a third band between the epi-band and the main band has been noted on standard preparations of tetracycline. This, on rechromatographing, is resolved into epitetracycline and tetracycline, showing that some conversion of tetracycline to epitetracycline may take place on the paper. In addition, variable results can be obtained due to the difficulty of standardising the degree of dampness of the buffered paper. These systems proved unsatisfactory for resolving epitetracycline from tetracycline in urine and serum samples.

Kelly and Buyske (1960) described a method for the separation of epitetracycline in urine using Whatman No. 1 paper impregnated with ethylenediaminetetra-acetic acid, and developing with the organic phase of n-butanol:ammonium hydroxide:water system, in the ratio 4:1:5. At the loading necessary for the quantitative determination of epitetracycline in bulk tetracycline and broths, appreciable quantities of

anhydrotetracycline are formed. This does not occur with the other systems mentioned.

The present paper describes a simple system for the separation of epitetracycline from tetracycline. It is suitable for quantitative determinations in tetracycline destined for therapeutic use, for quantitative determination in urine and qualitative separation in blood serum samples.

Experimental and results

General

All tetracyclines used were commercial samples with the exception of the epitetracycline ammonium salt, and the anhydrotetracycline, which were prepared in this laboratory according to the method of McCormick and others (1957). Other reagents were of analytical grade.

Whatman modified cellulose phosphate cation-exchange paper has many of the characteristics of a strongly acidic cation-exchange resin. In the acid form it is essentially cellulose dihydrogen phosphate and thus contains both strong and very weak acidic groups. Using this paper and water as developing solvent, epitetracycline and tetracycline gave variable R_F values. Anhydrotetracycline was not separated from epitetracycline. The former compound is the product of the acidic degradation of tetracycline. As acidic conditions prevail at various stages in the production of tetracycline, anhydrotetracycline is a possible impurity. Satisfactory separation with reproducible R_F values could be achieved by development of the chromatogram with 0·1 per cent w/v ammonium chloride solution. The R_F values of various tetracyclines in this system are given in Table I.

TABLE I

R_F VALUES OF TETRACYCLINE ON MODIFIED CELLULOSE PHOSPHATE CATION EXCHANGE PAPER USING 0·1 PER CENT W/V AMMONIUM CHLORIDE FOR DEVELOPMENT

Sample	R_F value
Tetracycline	0·59
Epitetracycline	0·36
Chlortetracycline	0·61
Oxytetracycline	0·61
6-Demethylchlortetracycline	0·53
Epi-6-demethyl-chlortetracycline	0·32
6-Demethyl-6-desoxy-tetracycline	0·42
Epi-6-demethyl-6-desoxy-tetracycline	0·21
6-Methylene oxytetracycline	0·46
Anhydrotetracycline	0·14

Attempts to elute epitetracycline and tetracycline from the paper with 0·01N hydrochloric acid gave low recoveries. Better recoveries were obtained if the acid strength was increased to 0·5N, but the formation of anhydrotetracycline during elution rendered this procedure unacceptable. Elution with 10 per cent w/v sodium chloride had a similar disadvantage due to the formation of isotetracycline. 10 per cent w/v ammonium chloride was finally selected for the elution. Tetracycline was stable in this solution over several hours.

Recovery from the paper was 93·1 $\pm$ 1·5 per cent based on 26 experiments using commercial samples of tetracycline base and hydrochloride.

This is comparable with previous methods described for this estimation. A typical series of results is shown in Table II.

TABLE II

TETRACYCLINE AND EPITETRACYCLINE CONTENTS OF COMMERCIAL SAMPLES OF TETRACYCLINE

Tetracycline	Sample	Total tetracyclines by direct U.V. measurement, per cent	Tetracycline ex chromatography, per cent	Epitetracycline ex chromatography, per cent	Recovery total tetracyclines, per cent	Moisture, per cent
Base	1	90·2	80·8	2·9	92·8	9·1
	2	90·3	82·0	2·9	94·0	9·1
Base	1	91·9	82·4	2·4	92·3	7·5
	2	92·0	84·1	2·5	94·1	7·5
Base	1	89·5	80·1	2·3	92·1	9·7
	2	89·6	80·7	2·8	93·2	9·7
Hydrochloride	1	99·5	90·5	2·2	93·2	0·15
	2	99·6	89·6	2·5	92·5	0·15
Hydrochloride	1	99·7	92·1	2·4	94·8	0·28
	2	99·5	91·9	2·3	94·7	0·28
Hydrochloride	1	99·4	90·2	2·7	93·5	0·31
	2	99·3	90·1	2·6	93·4	0·31

Tetracycline hydrochloride (125 mg.) was dissolved in 0·01N hydrochloric acid to 25 ml.

Tetracycline base (125 mg.) was dissolved in a minimum of 0·1N hydrochloric acid (not greater than 5 ml.) and diluted to 25 ml. with water.

A starting line was marked on a sheet of Whatman modified cellulose phosphate cation-exchange paper about 10 cm. from one end of the sheet and 2 cm. from the edges. 0·2 ml. (≡1 mg.) of the tetracycline solution, or a suitably diluted aliquot of other preparations was streaked on the line. The sheet was placed, together with a blank, in a chromatography tank and developed with 0·1 per cent w/v ammonium chloride by the descending technique. After the solvent front had travelled about 25 cm. the papers were air dried for some hours, preferably overnight.

The yellow fluorescent bands were located under Wood's light in the presence of ammonia vapour. They were cut into 1 cm. squares, taking equivalent areas from the "blank sheet" for the blank determinations. The main band (tetracycline) was eluted by shaking in 100 ml. of 10 per cent w/v ammonium chloride solution and the epitetracycline band in 20 ml. of 10 per cent w/v ammonium chloride solution for 30 min. Alternatively the mixtures could be stood for 2 hr. with occasional shaking. If only small amounts of epitetracycline were present the bands from two or more sheets were combined in one elution. The suspensions were filtered and the optical densities measured in a 1 cm. cell at 356 mμ. E (1 per cent, 1 cm.) at 356 mμ for tetracycline and epitetracycline in 10 per cent w/v ammonium chloride was 325.

Separation in Urine

Initial experiments were designed to demonstrate any interference that urine constituents might exert on the chromatographic system.

Tetracycline and epitetracycline, both separate and in admixture, were dissolved in urine (100 μg./ml.), a level comparable to that found in urine during tetracycline therapy. The solutions were spotted on the paper in 0·02 ml. aliquots (≡2 μg. antibiotic) and run according to the described method. The chromatogram, inspected under Wood's light and ammonia vapour, showed that tetracycline and epimer could be separated under these conditions with no interference from urine salts. The separation was confirmed by bio-autography.

To show that the method would separate the epi-compound in urine of patients taking tetracycline, three groups of patients were dosed with tetracycline tablets at low, medium, and high dose levels, and the urine collected for 4 hr. after dosing. In all instances, compact spots of tetracycline were revealed by Wood's light and ammonia, and at the highest dose, a faint epitetracycline spot was detectable. Bio-autographs showed the same picture as those obtained when the tetracycline and epitetracycline were added to the urine, and R_F values were comparable.

Kelly and Buyske (1960) reported that a particular failure of many of the earlier chromatographic systems was their tendency to give very diffuse spots at loadings greater than 5 μg. Using the described method, tetracycline, 100 μg. in 0·02 ml., gave a main spot 3 cm. diameter and a spot of epitetracycline 1·5 cm. diameter without streaking.

Spectrophotometric estimation of eluates was not possible due to the high urine blank. Using a microbiological assay (Grove and Randall, 1955) tetracycline was estimated in samples of urine containing 100 μg./ml. of added tetracycline. 0·04 ml. of the sample was chromatographed and the tetracycline spot eluted in 10 ml. of 10 per cent w/v ammonium chloride solution, centrifuged, and determined by a microbiological plate assay using *Bacillus cereus* as the test organism. Recovery was comparable to that obtained for the standard procedure described above. Results are shown in Table III.

TABLE III

ESTIMATION OF TETRACYCLINE IN URINE MICROBIOLOGICALLY AFTER CHROMATOGRAPHY AND ELUTION

Urine sample	Added tetracycline, μg./ml.	Recovered tetracycline after chromatography, μg./ml.
blanks	Nil	Nil
1	100	91·3
2	100	101·3
3	100	88·7
4	100	88·8
5	100	92·6
6	100	93·7

Separation in Blood Serum

The normal level of tetracycline in blood serum after tetracycline therapy is about 1–1·5 μg./ml., but preliminary experiments were made on

samples of serum containing added tetracycline and epitetracycline (100 μg./ml.), to detect the spots by visual location. Serum (0·01 ml.) $\equiv$ 1 μg. of epitetracycline and 1 μg. of tetracycline gave a satisfactory separation. At loadings up to 5 μg. separation was still satisfactory and no streaking occurred. The R_F values were: epitetracycline, 0·31; tetracycline, 0·46.

Serum from each of the 3 groups of patients in the experiment described above was examined by the described method. In each instance, 0·1 ml. was streaked on the paper and 0·02 ml. on a thin strip suitable for bio-autography. No bands were detected by visual examination, but a zone of epitetracycline was observed on the bio-autographs at the medium and high dose levels and a zone for tetracycline in all dose levels. The R_F values were comparable to those quoted above.

Discussion

In the separation and estimation of epitetracycline in tetracycline where admixture with chlortetracycline or oxytetracycline is not important, the above method has several advantages over previous methods described. No buffering of the chromatographic paper is required. Simple aqueous non-toxic reagents are used. Saturation of the tank is not critical. No detectable epimerisation takes place on the paper. The method is applicable to urine and serum, and, coupled with a microbiological assay after elution, can be used for the quantitative determination in urine.

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