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QUANTITATIVE THIN LAYER CHROMATOGRAPHIC ESTIMATION OF TETRACYCLINE ANTIBIOTICS*†

SYED FAROOQ ALI

Kurram Chemical Co., Rawalpindi

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Abstract. Determination of tetracycline antibiotics by spectroscopic methods is erroneous, The TLC method with 1, 1-dichloroethane-chloroform-ethylacetate-methanol system was found most suitable for the separation of these antibiotics.

Spectrophotometric determination of tetracycline antibiotics is erroneous in the presence of their degradation products, such as anhydrotetracycline, 4-epitetracycline and 4-epianhydrotetracycline, and in the presence of colour additives, for example in the coloured preparations of oral drops and syrups.

Several methods have been reported for the determination of tetracycline antibiotics and their degradation products in presence of each other. Work in this field was intensified after the work of Gross[1] on reversible renal dysfunction (Fanconi type syndrome). In 1969 a method was reported based on the application of absorbance ratio to the determination of tetracycline in presence of its degradation products, The ratio was determined at 357 and 391 nm respectively and following a regression line the relative amount of tetracycline was determined by the formula.

$$\text{Tetracycline } \% = \frac{Q\ 357 : 391 - 0.23}{0.0367}$$

where Q 357 : 391 is the absorbance ratio value at 357 to that of at 391 nm.[2]

Quantitative TLC methods for the determination of tetracycline and its degradation products have been reported by Simmons *et al.*,[3] who determined the degradation products of tetracycline by using a two-dimensional technique. After extraction in vacuum with hot methanol, they determined these spectrophotometrically by reading at 428 nm. Fernandez *et al.*,[4] obtained an excellent separation of these products on kieselghur impergnated with EDAT at pH 7.5, and using ethyl acetate–acetone water (10:20:3) system. After elution and extraction these substances were determined by hydrolysing with HCl and reading the yellow colour thus developed at 430 nm.

Dijkhuis *et al.*,[5] determined small amounts of epitetracycline and chlortetracycline in tetracylcine using the plates impergnated with citrate phosphate buffer at pH 5.5, and glycerin. The chromatography was carried out under sharply defined conditions of moisture and pH, and the plates were developed with dichloromethane–ethanol 95% (9:1). After elution and extraction the epitetracycline was determined at 356 nm and a fluorometric method was used to determine chlortetracycline.

Column chromatographic method was also reported by Ascione *et al.*,[6] for the determination of tetracycline and its degradation products.

Walton *et al.*,[7] studied various market products for the presence of degradationn products of tetracycline and determined them by column and paper chromatography.

Experimental

Anhydrotetracycline was prepared by the method of Simmons *et al.*,[3] and its purity was ascertained by TLC and by checking its absorptivity at 428 nm.

TLC equipment of Shandon and spectrophotometer models DU and DBG of Beckman were used.

Kieselguhr GF Merck, and all reagents of analytical grade were used.

Preparation of Thin Layer Plates. Kieselguhr (100 g) was boiled with 500 ml 10% w/w HCl, washed with distilled water till it is free of traces of HCl, and dried at 100°C.[8] For the control of its purity 500 mg was suspended in 5 ml methanolic HCl, centrifuged, filtered and its absorbance determined at 380 nm and 428 nm. At these wavelengths the impurity should not exceed 0.005.(5).

Slurry was made by mixing the kieselguhr with M/10 EDTA (37.225 g disodium edetate was dissolved and made up to 1000 ml with water, whose pH was previously adjusted to 7.0 with dilute NH_3 solution) and 5% glycerin. 20 × 10 cm glass plates were coated to a thickness of 0.25 mm using a Shandon TLC coater, and the plates were dried at room temperature.

Preparation of the Developing Solvent. Equal amounts of each of 1,1-dichloroethane, ethyl acetate, chloroform and methanol were mixed, shaken with about one-third of its volume of M/10 EDTA at pH 7.0, and filtered. Simultaneously ethyl acetate–chloroform–acetone (2:2:1) was also prepared exactly in the same manner.

Preparation of the TLC Chamber. Glass chamber, 27 cm height and 15 cm dia, was brought in equilibration with a saturated solution of ammonium chloride at least 24 hr before use. Thin layer plates were then allowed to equilibriate with the same ammonium chloride solution at least for 24 hr. Developing solvent was then added to the chamber and filter paper lining was applied to the walls in order to obtain a chamber saturation state.

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Chromatography. Working standards of tetracycline hydrochloride, demethylchlortetracycline hyhydrochloride, doxycylcine hydrochloride, methacycline hydrochloride and anhydrotetracycline hydrochloride were accurately weighed in amounts ranging between 10–30 mg and were dissolved in 0.01N methanolic HCl and made up to 10 ml in a volumetric flask. Samples of degraded tetracycline antibiotics and different dosage forms were also prepared in the same manner, so as to contain in each 10 ml between 10–30 mg of the antibiotic. Using a micropipette of 10 micron accuratly measured amounts of each were applied to thin layer plate about 2 cm above from the lower layer and were allowed to dry at the room temperature. Care was taken to involve minimum time between spot application and extraction.

First development was given with the system 1, 1–dichloroethane–chloroform–ethyl acetate–methanol (1:1:1:1) followed by another with chloroform ethyl acetate–acetone (2:2:1) and the spots were located under long wave UV light.

The hR_f values obtained with these systems in this laboratory are anhydrotetracycline hydrochloride, 50; demethylchlortetracycline hydrochloride, 16; doxycycline hydrochloride, 14; tetracycline hydrochloride, 11; and methacycline hydrochloride, 04.

Quantitative Estimation. Spots were located in long-wave UV light, and were scraped carefully in wide-mouth, stoppered, graduated centrifuge tube. The volume was made up to 10 ml with the aid of 0.01N methanolic HCl the tubes were shaken for few minutes, and centrifuged at about 2000 rev/min for 10 min.

The clear supernatant (5–7 ml) was transferred to a 10-ml volumetric flask, 0.5 ml 20% sodium hydroxide was then added and the volume made to 10 ml with methanolic HCl. Exactly 6 min after the addition of NaOH, the absorbance was recorded on a Beckman DU spectrophotometer at 380 nm. A blank prepared in the same manner was run omitting only the antibiotic under examination.

In some cases it was observed that a slight turbidity developed after the addition of NaOH, however, this was removed by rapidly filtering the solution, and reading thereafter.

Estimation of Anhydrotetracycline Hydrochloride. Anhydrotetracycline was determined after extracting in the same manner as above, using the methanolic HCl only.

After extraction and subsequent dilution the solution was read at 428 nm in a Beckman DU spectrophotometer and compared to a blank which omitted the anhydrotetracycline.[3]

Results and Discussions

Spectrophotometric method of Woolford and Chiecarelli[9] was used for the determination of these antibiotics and their degradation products after TLC and subsequent extraction. The method recommends determination of tetracycline at 380 nm after addition of sodium hydroxide, so that the final solution is 0.25N.

It was found that after and subsequent extraction, the colour developed with the NaOH was stable for several minutes and excellent for the determination of doxycylcine and methacycline hydrochloride as well.

For anhydrotetracycline due to its intense yellow colour the method of direct spectrophotometric reading at 428 nm in a methanolic HCl solution was adopted.[3]

The TLC in strictly defined conditions of moisture, pH etc. and with the system 1,1-dichloroethane–chloroform–ethyl acetate–methanol, was found to be excellent for the separation of these antibiotics from their degradation products and from the colour additives they contain.

Tailing and poor resolution was observed in some cases when the conditions of moisture and pH were abnormal.

The colour developed by the addition of sodium hydroxide with doxycycline and methacycline was not so intense as the one developed with tetracycline and demethylchlortetracycline, and therefore it was required to take relatively larger amounts for the examination of these two antibiotics.

TABLE 1. RESULTS OF A SERIES OF DETERMINATIONS OF VARIOUS DOSAGE FORMS.

Antibiotic	Theoretical amount (mg)	Determined amount (mg)	Per cent	Variation
Tetracycline hydrochloride	250/Cap	253·75	101·5	+1·5
	250/Cap	247·25	98·9	—1·1
	125/5 ml	123·75	99·0	—1·0
	100/ml	100·80	100·8	+0·8
Demethylchlortetracycline hydrochloride	150/Cap	151·95	101·3	+1·3
	60/ml	60·90	101·5	+1·5
	60/ml	60·84	101·4	+1·4
	60/ml	58·92	98·9	—1·1
Methacycline hydrochloride	150/Cap	152·55	101·7	+1·7
	150/Cap	151·48	100·9	+0·9
	80/100 mg	78·56	98·2	—1·8
	80/100 mg	79·36	99·2	—0·8
Doxycycline hydrochloride	100/Cap	98·20	98·2	—1·8
	60/100 mg	60·78	101·3	+1·3
	70/100 mg	70·91	101·3	+1·3
	100/Cap	98·40	98·4	—1·6

Table 2. Comparison of the Accuracies of the Comparative Absorbance Method[2] and the Method of Quantitative TLC (sample. tetracycline hydrochloride contaminated with known amounts of anhydrotetracycline).

Observations (x)	Average (Ex)	Per cent	Deviations (d)	Square of d (d²)	Average deviation $\frac{Ed}{n}$	Standard deviation $s=\frac{Ed^2}{n-1}$
Comparative absorbance method						
8256		101·1	+1·1	1·21		
8446		103·4	+3·4	11·56		
7884	8166	96·5	−3·5	12·25	7·24	2·87
8080		98·9	−1·1	1·21		
6666		95·2	−4·8	23·04		
7060		100·8	+0·8	0·64		
7170	7001	102·4	+2·4	5·76		
7110		101·5	+1·5	2·25		
				$Ed^2=57·92$		
Quantitative TLC method						
463		101·3	+103	1·69		
468		102·4	+2·4	5·76		
470		102·8	+2·8	7·84		
458		100·2	+0·2	0·04		
451	457	98·7	−1·3	1·69	3·32	1·93
443		97·0	−3·0	9·00		
449		98·2	−1·8	3·24		
461		100·8	+0·8	0·64		
458		100·2	+0·2	0·04		
				$Ed^2=29.94$		

Good separation and resolution on the TLC plates was achieved with the quantities up to 1 mg, however, amounts ranging between 0.1–0.2 mg were found to be excellent for separation and quantitative estimation, and the amounts as low as 0.75 mg were determined accurately.

Table 1 shows the results of a series of determination, samples used for the determination of antibiotics were deliberately contaminated with the known amounts of anhydrotetracycline.

In Table 2 the method is compared to the method of comparative absorbance[2] and is found to be superior. However, this particular portion of this study was carried out on a Beckman DBG spectrophotometer.

The method for the quantitative estimation of anhydrotetracycline had an accuracy of ±1.5%; with minimum amounts up to 0.05 mg were estimated accurately. Preparations containing as low as 4% anhydrotetracycline can, therefore, be determined.

It was also observed that these antibiotics after elution and extraction with the methanolic HCl can be determined quantitatively by direct spectrophotometric reading at the following wave lengths: tetracycline hydrochloride, 357; demethylchlortetracycline 340; methacycline hydrochloride, 340; and doxycycline hydrochloride, 349 nm.

However, the accuracy of such a method was less than the one discussed.

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