Separation and Quantitative Determination of Impurities in Tetracycline

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Abstract □ Anhydrotetracycline, epianhydrotetracycline, epitetracycline, and chlortetracycline are generally found as impurities in tetracycline. A new thin-layer partition chromatographic method is described in which the impurities are separated on a kieselguhr layer impregnated with ethylene glycol–water–acetone–ethyl acetate (2:2:15:15). Compared to previous methods, this determination is easier to perform and more versatile and it prevents rapid epimerization. The quantitative determination of the tetracyclines utilizes spectrophotometry or direct TLC fluorometry.

Keyphrases □ Tetracycline—separation and analysis of impurities □ TLC—separation, tetracycline impurities

The quantitative determination of tetracycline antibiotics by titrimetry (1), polarography (2), colorimetry (3), paper chromatography (4), and column chromatography (5–8) has been reported, but TLC has proved most useful. The identification of three tetracyclines using a kieselguhr layer impregnated with glycerin was reported (9), and several tetracyclines were separated from their epimers using a similar system (10). Three tetracyclines were separated using kieselguhr previously washed several times with 6 *N* HCl to remove the binder (11). Anhydrotetracycline (I), epianhydrotetracycline (II), and epitetracycline (III) were separated from tetracycline (IV) and were quantitated by spectrophotometry (12).

Dijkhuis and Brommet (13) determined I and chlortetracycline (V) in IV on a layer impregnated with a citrate–phosphate buffer containing 10% glycerin. Van den Bulcke (14) studied the influence of the binder and polyalcohols in the stationary phase and of the solvent systems both on the separation of tetracyclines and the detection of their impurities. Microcrystalline cellulose was used to separate I and other impurities from IV (15–18).

Gyanchandani *et al.* (19) first impregnated the plates with the solvent system, a process that conditioned them so that they could be stored for several weeks. Finally, van Hoeck *et al.* (20) separated I, II, and III from IV on a solvent-impregnated plate and determined IV by direct fluorometry. The methods described in Refs. 12–14, 19, and 20 seem to be the most reliable, although they are subject to important criticism.

The use of acid-washed kieselguhr is undesirable (14) because the washing process is lengthy and does not result in reproducible separations. Microcrystalline cellulose is also unsatisfactory because the support, as described by Simmons *et al.* (15–17), is difficult to prepare and does not allow a good separation while the use of citrate and/or phosphate buffers may markedly increase the epimerization of the tetracyclines (19, 21, 22). For the same reason, the temperature should be controlled during the development (21). Most investigators used solutions of IV at concentrations ranging from 0.05% (20) to 0.2% (12, 19), but their procedures failed when higher concentrations of 0.5–1% were used to increase the sensitivity of detection of the impurities to an acceptable level. Failure resulted from the tailing of the IV spot.

Table I—TLC of I–V

Compound	Concentration, μg/μl	$R_f \times 100$ I[a]	II[b]	III[c]
I	0.025	98	86	95
V	0.025	21	32	72
II	0.025	16	27	30
IV	5.0	10	20	50
III	0.025	4	10	28

[a] With 10% VI plates. [b] With 3.7% VI plates used immediately after impregnation. [c] With 3.7% VI plates used 24 hr after impregnation.

The purpose of this research was to develop a TLC procedure allowing separation of traces of I–III and V as impurities in a 0.5% (w/v) solution of IV and their quantitation by means of spectrophotometry or direct TLC fluorometry.

EXPERIMENTAL

Reagents—Disodium ethylenediaminetetraacetate (VI), ethylene glycol, acetone, ethyl acetate, and sodium hydroxide were analytical grade.

Kieselguhr—Both MN-kieselguhr G[1] and kieselguhr G[2] were used. The former was preferred since the slurry was easier to handle and provided a coating with a more homogeneous appearance; it also gave a higher resolution.

Preparation of Plates—Two different supports were prepared using: *(a)* a slurry of 20 g of kieselguhr and 47 ml of 10% VI solution adjusted to pH 8.5 with 20% NaOH, and *(b)* a slurry of 20 g of kieselguhr and 47 ml of 3.7% VI solution. Plates (20 × 20 cm), four at a time, were prepared[3] with a layer thickness of 0.25–0.30 mm. The plates were dried at room temperature (25°) and at a relative humidity of 30–50% for 1 hr. Prior to use, they were developed with the solvent system for 1–2 hr and dried at room temperature for 1 hr.

Solvent System—The system was made up of ethylene glycol–water–acetone–ethyl acetate (2:2:15:15 v/v).

Preparation of Solutions—The substances were dissolved in methanol or a mixture of methanol and 0.1 *N* HCl (9:1). For identification of I–III and V, a concentration of 0.025 μg/μl was used. The solution of IV was used at 5 μg/μl. Calibration curves for di-

[1] Macherey, Nagel & Co, D-516 Düren, Germany.
[2] E. Merck, Darmstadt, Germany.
[3] Desaga applicator, Heidelberg, Germany.

Table II—Absorptivities of I–V Determined on 10% VI Plates

Compound	Wavelength, nm	a [a]	SD	a_w [a]	SD	Recovery, %
I	430	19.32	0.498	17.70	0.796	91
II	430	16.89	0.591	15.16	0.923	90
III	355	32.10	0.169	29.59	0.608	92.5
IV	355	30.79	0.165	27.78	1.454	90.5
V	367.5	20.60	0.500	18.90	1.020	92
	265	35.60	0.670	33.50	0.840	94

[a] Each result is the mean of five determinations.

rect TLC fluorometry were prepared using appropriate concentrations.

Apparatus—A TLC chromatotank (20 × 20 × 8 cm) was lined with filter paper, and 120 ml of solvent was added. The tank was allowed to equilibrate for at least 1 hr. A spectrophotometer[4], a TLC spectrophotometer[5], an electrobalance[6], and a recorder[7] were also used.

Application of Solutions—Volumes of 50 or 100 μl were spotted by syringe[8], and volumes of 1 μl were spotted by disposable pipets[9].

Qualitative Chromatography—One-microliter volumes were applied as spots on a line 2.5 cm above the lower edge of the plate. The plate was placed in the tank and developed for 40 min at 25°. After drying the plate, the spots were observed under UV light (366 nm) and fluorescence was increased by exposure to ammonia vapor. The R_f values observed are listed in Table I. Typical TLC separations on 10% VI plates and 3.7% VI plates are shown in Figs. 1 and 2.

Quantitative Analysis—*Spectrophotometric Assay*—A suitable volume (50–100 μl) of a solution of the test mixture was applied as a 15-cm band on a line 2.5 cm above the lower edge of a 10% VI plate and developed until the solvent front had moved 13–14 cm. After the plate was dried, the fluorescing zones were quickly marked under UV light, removed separately, and extracted with 0.1 *N* HCl. Since centrifuging did not produce a clear solution, the kieselguhr was removed using a porcelain filter A_1, which was washed with 0.1 *N* HCl; the filtrates were combined and adjusted to volume. Further dilutions were prepared using 0.1 *N* HCl.

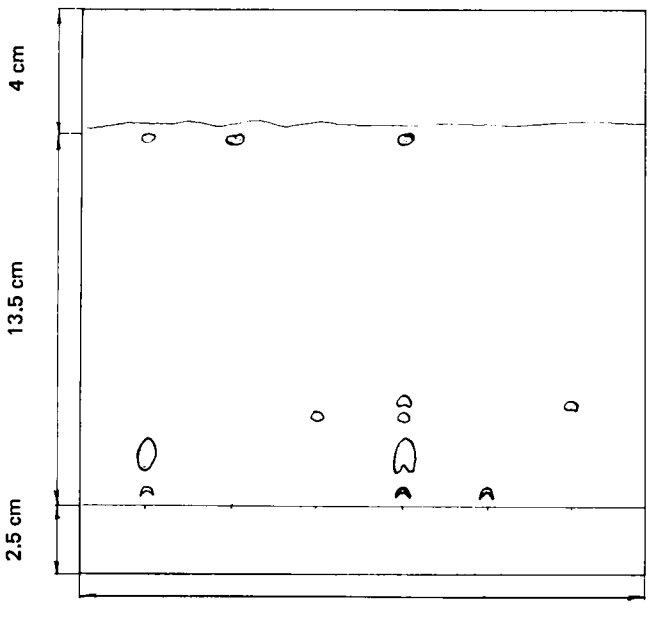

Figure 1—*Example of a typical TLC separation of tetracycline impurities on kieselguhr with 10% VI at pH 8.5. Key (left to right): 5 μg of IV, 0.025 μg of I, 0.025 μg of II, mixture (I–V), 0.025 μg of III, and 0.025 μg of V.*

Compounds I and II were determined using the absorbance at 430 nm, Compounds III and IV were determined at 355 nm, and Compound V was determined at 265 or 367.5 nm against a blank prepared in the same way as the test solutions. The absorbance of the blank at 430 nm was 0.002–0.008 for 20 cm^2 of the kieselguhr layer; at 355 nm, it was 0.050; and at 265 and 367.5 nm, it was 0.240 and 0.038, respectively.

The amount of substance in the mixture was calculated as follows:

$$\% = \frac{(A)(f)100}{(a)(p - l)} \qquad \text{(Eq. 1)}$$

where p is the concentration of sample in grams per liter, l is the loss on drying, f is the dilution factor, a is the absorptivity of the substance being examined, and A is the absorbance measured.

The absorptivities *(a)* were determined for the pure reference substances (Table II). The values a_w were obtained from measurements on the eluates after chromatographing the pure reference substances.

Spectrophotometric determination of the impurities was difficult, because rather large quantities were necessary to produce reliable measurements, *i.e.*, absorbances between 0.150 and 0.750. The final concentration of the extracts from the kieselguhr ranged from 10 to 40 μg/μl. At lower concentrations, the errors increased significantly.

Direct TLC Fluorometry—An irregular distribution of the substances within the spot is unimportant when direct TLC fluorometric evaluation is used. Spots with a regular form are measured with a circular aperture, and the recorded value is proportional to the total fluorescence and, thus, to the concentration of the sample. When using a circular aperture, a slit width of approximately 1.5 mm is used.

With spots of an irregular shape, a rectangular aperture is used. In this case, the area between the curve and the abscissa should be determined on the recorded graph. Disturbing effects such as background, front line fluorescence, and, particularly, dust particles may influence the measurement and are to be avoided. For drawing a calibration curve, the internal standard method is used.

Assay—For the evaluation of II, III, and V, 10% VI plates were used; for the evaluation of I, 3.7% VI plates were used. For each impurity, five solutions with concentrations in geometric progression were prepared; 1 μl of each was spotted at 2.5-cm intervals on the baseline together with 1 μl of the solution of IV. One microliter of the test solution of IV was also applied separately. Two or more impurities were evaluated on a single plate by preparing a combined standard solution of these impurities in a definite proportion and diluting it in a geometric progression.

After separation, the plate was allowed to dry at room temperature for 10 min. The TLC spectrophotometer was adjusted to the spot with the highest concentration, and the transmission scale was adjusted to 100 units. A rectangular aperture was used for all measurements; the other conditions used for each impurity are listed in Table III.

The fluorescence was excited by the 365-nm Hg line and measured by scanning the accurately positioned spots in the x axis or, if necessary, in the y axis direction with a scanning speed of 3 cm/min. The recorded speed was kept at 5 cm/min. The results obtained are reported in Table IV.

Each fluorescent spot was recorded as a peak and was quantitated by measuring the peak area either by triangulation (results by

[4] Zeiss PMQ II.
[5] Zeiss.
[6] Cahn.
[7] Varian A 25.
[8] Hamilton-Bonanduz Schweiz.
[9] Vitrex, Chr. Bardram Birkerod, Denmark.

Table III—Conditions for TLC Spectrophotometric Determination of Each Impurity

Compound	Wavelength, nm	Slit Width, mm	Slit Height, mm	Amplification[a] 2 μg	0.5 μg	0.125 μg	0.0312 μg	0.0078 μg
I	514	0.6	8	0.8/1/II/F	4.1/1/II/F	7.8/1/II/F	1/10/II/F	—
II	533	0.6	6	1.2/1/II/F	4.4/1/II/F	9/1/II/F	3.2/10/II/F	—
III	523	0.6	6	—	5.5/1/II/F	7.5/1/II/F	1.8/10/II/F	6.1/10/II/F
V	528	0.6	6	—	2.2/1/II/F	7/1/II/F	1.6/10/II/F	5.7/10/II/F

[a] Amplification of the TLC spectrophotometric response corresponding to different concentrations of the substances in the spot.

this procedure were unreliable and variable) or, preferably, by cutting out and weighing the peak areas. These weights are a measure of the areas and were used as such in the calculation of the regression line (23) as follows. Let y be the weight of an area corresponding to a concentration x of a spot on the chromatogram and $\tilde{y}$ is the true value of y at any given value of x. Then the equation of the true regression line will be:

$$\tilde{y} = a + bx \qquad \text{(Eq. 2)}$$

where b is the slope and a is the intercept on the y axis at $x = 0$. This equation can be rearranged to:

$$\tilde{y} = \bar{y} + b(x - \bar{x}) \qquad \text{(Eq. 3)}$$

since the point $(\bar{x},\bar{y})$ lies on the estimated regression line. The slope b is given by the following equation:

$$b = \frac{\Sigma xy - \dfrac{(\Sigma x)(\Sigma y)}{n}}{\Sigma x^2 - \dfrac{(\Sigma x)^2}{n}} \qquad \text{(Eq. 4)}$$

where n = sample size.

To draw the estimated regression line, first $(\bar{x},\bar{y})$ is plotted. A convenient amount is added to $\bar{x}$ and b times this amount to $\bar{y}$. Thus, a second point can be plotted. These two points determine the regression line. The standard deviation of the individual y values about the regression line is designated $s_{y.x}$ and is computed by the square root of the following expression:

$$s^2_{y.x} = \frac{1}{n-2}\left\{\Sigma y^2 - \frac{(\Sigma y)^2}{n} - b\left[\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{n}\right]\right\} \qquad \text{(Eq. 5)}$$

The regression was calculated in this way for each impurity and is illustrated for II and V in Fig. 3.

It has been verified that the assay procedure gives a valid estimate. Therefore, the recovery of the concentration of the impurity added in geometrical progression to the spot of the unknown mixture was calculated. The mean of each series of these values was plotted against the corresponding amount of impurity added. An example of the correlation between I added and I recovered is shown in Fig. 4.

RESULTS AND DISCUSSION

Separation of Tetracyclines—Rapid and accurate evaluation of the tetracycline impurities requires a very sensitive separation method. Both the 10% VI method and the 3.7% VI method were suitable for qualitative and quantitative purposes. Figures 1 and 2 show that III, IV, II, V, and I separated in that order. Epichlortetracycline was also separated, with an R_f value between that of III and IV.

Both methods allowed a spectrophotometric determination of the separated substances. However, the 10% VI method gave the best separation; the spots were round and regular in shape with a good resolution appropriate for direct TLC fluorometry. This procedure was adopted for most experiments (Table IV). The spot of I moved so close to the solvent front in this procedure that it could not be evaluated by direct fluorometry and the 3.7% VI method had to be used to estimate this impurity. Before use, the plates were impregnated with the solvent system for at least 1 hr. However, the optimum impregnation time, resolution of the spots, and R_f values varied slightly with the ambient atmospheric conditions such as temperature and relative humidity. Activation of the plates prior to impregnation did not improve the results but resulted in tailing of the spots.

Care was taken to dry layers slowly, preferably at 30–50% relative humidity and a temperature of 25°. Unimpregnated plates prepared in this way could be stored for an unlimited time. The solvent used for impregnation could be used two or three times. Test and standard solutions, when kept at 0° in the dark, were stable for at least 1 week.

Sensitivity—Mixtures of IV with impurities ranging from 4 to 16 mg/ml were adequately resolved by these methods. It was possible to resolve a 16-mg/ml solution of IV containing impurities as follows: IV, 99.78%; I and III, 0.1%; II, 0.07%; and V, 0.5% (Table IV).

Since high concentrations of IV increased tailing, it was preferable to apply the samples as 4–8-mg/ml solutions, using a 1-μl micropipet to prevent overloading of the layer and to keep the spot size small enough to allow estimation by direct fluorometry.

Spectrophotometric Analysis—Spectrophotometry, although an accurate procedure, was less sensitive. If, for instance, 100 μl of a 16-mg/ml tetracycline solution was streaked onto the plate over a distance of 15 cm, the impurities had to be present in quantities of at least 3% of total tetracycline to be detected. Indeed, spectrophotometric determination required a minimum concentration in the eluate of 10 μg/ml of the substance concerned. An impurity percentage less than 3% could be estimated by spotting a greater volume or by increasing the concentration of the solution, but both procedures gave a poor separation.

The absorptivities of I–V are listed in Table II. Each result is the mean of five determinations and the relative standard deviation was less than 3.5% in all instances. The a_w values, however, showed a relative standard deviation up to 6%. Compound V

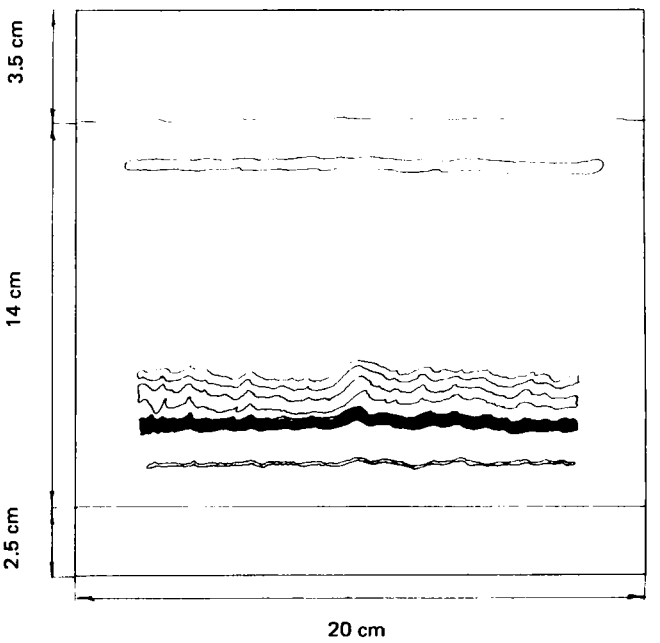

Figure 2—*Example of a typical TLC separation of impurities of tetracycline on kieselguhr with 3.7% VI for spectrophotometric purposes. Bands in ascending order are: 100 μg of III, 5000 μg of IV, 100 μg of II, 100 μg of V, and 100 μg of I.*

Table IV—Recovery of I–III and V Added to Different Amounts of IV by Direct TLC Fluorometry

Compound	Spotted Quantity, μg	Composition, %	Recovery[a], μg	Confidence Limits[a] Maximum	Confidence Limits[a] Minimum	Recovery, %
IV	4.0170	89.60				
I	0.1230	3.06	0.1196	0.1228	0.1165	103
II	0.2219	5.52	0.2163	0.2217	0.2109	97.5
III	0.0458	1.14	0.0454	0.0472	0.0435	99
V	0.0275	0.68	0.0275	0.0283	0.0268	100
IV	4.0170	94.80				
I	0.0615	1.53	0.0691	0.0779	0.0604	112
II	0.1109	2.76	0.1217	0.1319	0.1114	109.5
III	0.0229	0.57	0.0228	0.0260	0.0197	99.5
V	0.0137	0.34	0.0135	0.0148	0.0121	98.5
IV	4.0170	97.41				
I	0.0307	0.76	0.0298	0.0360	0.0236	97
II	0.0555	1.38	0.0588	0.0697	0.0479	106
III	0.0114	0.28	0.0137	0.0178	0.0097	120
V	0.00685	0.17	0.0071	0.0078	0.0064	103.5
IV	4.0170	98.70				
I	0.0154	0.38	0.0144	0.0173	0.0114	93.5
II	0.02775	0.69	0.0227	0.0289	0.0165	82
III	0.0057	0.14	0.0047	0.0072	0.0022	82.5
V	0.0034	0.085	0.0035	0.0043	0.0027	103
IV	4.0170	99.36				
I	0.0077	0.19	0.0061	0.0072	0.0051	79
II	0.0139	0.34	0.0137	0.0164	0.0111	98.5
III	0.00285	0.07	0.0023	0.0035	0.0011	80.5
V	0.0017	0.04	0.0016	0.0021	0.0011	94
IV	8.8658	95.12				
I	0.0825	0.93	0.0819	0.0865	0.0773	99
II	0.1970	2.23	0.1966	0.2020	0.1911	100
III	0.1202	1.36	0.1202	0.1222	0.1200	100
V	0.03216	0.36	0.0323	0.0325	0.0321	100.5
IV	8.8658	97.56				
I	0.0412	0.46	0.0395	0.0432	0.0357	96
II	0.0985	1.12	0.0947	0.1114	0.0781	96
III	0.0601	0.68	0.0591	0.0637	0.0546	98
V	0.01608	0.18	0.0158	0.0166	0.0150	98
IV	8.8658	98.78				
I	0.0206	0.23	0.0199	0.0249	0.0149	96.5
II	0.04925	0.56	0.0525	0.0598	0.0452	106.5
III	0.03005	0.34	0.0324	0.0357	0.0290	108
V	0.00804	0.09	0.0079	0.0087	0.0070	98
IV	8.8658	99.39				
I	0.0103	0.12	0.0086	0.0113	0.0058	83.5
II	0.02462	0.28	0.0248	0.0374	0.0153	101
III	0.01502	0.17	0.0145	0.0158	0.0132	96.5
V	0.00402	0.045	0.0043	0.0052	0.0033	107
IV	8.8658	99.69				
I	0.00515	0.06	0.0107	0.0176	0.0039	208
II	0.01231	0.14	0.0054	0.0083	0.0024	44
III	0.00751	0.09	0.0078	0.0090	0.0066	104
V	0.00201	0.022	0.0021	0.0029	0.0013	105
IV	16.0020	99.346				
I	0.0360	0.225	0.0368	0.0382	0.0354	102
II	0.0220	0.137	0.0221	0.0248	0.0195	100.5
III	0.0330	0.206	0.0333	0.0381	0.0284	101
V	0.0138	0.086	0.0141	0.0149	0.0134	102
IV	16.0020	99.672				
I	0.0180	0.113	0.0158	0.0185	0.0130	88
II	0.0110	0.069	0.0101	0.0139	0.0063	92
III	0.0165	0.103	0.0152	0.0197	0.0107	92
V	0.0069	0.043	0.0064	0.0083	0.0046	92.5
IV	16.0020	99.837				
I	0.0090	0.056	0.0082	0.0098	0.0066	91
II	0.0055	0.035	0.0053	0.0080	0.0026	96
III	0.0082	0.051	0.0073	0.0125	0.0021	89
V	0.0034	0.021	0.0029	0.0037	0.0021	85
IV	16.0020	99.918				
I	0.0045	0.028	0.0063	0.0079	0.0046	140
II	0.0028	0.017	0.0031	0.0048	0.0015	111
III	0.0042	0.026	0.0041	0.0050	0.0032	97.5
V	0.0017	0.011	0.0015	0.0018	0.0012	88
IV	16.0020	99.959				
I	0.0022	0.014	0.0027	0.0040	0.0013	123
II	0.0014	0.009	0.0108	0.0196	0.0020	771
III	0.0021	0.013	0.0065	0.0131	0.0000	310
V	0.00085	0.005	0.0014	0.0017	0.0013	165

[a] Each result is the mean of five determinations. [b] $p = 0.05$, 4 *df*, $t^* = 2.78$.

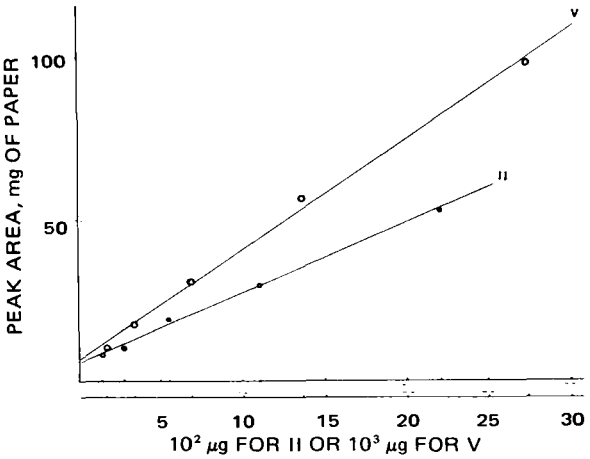

Figure 3—*Example of a calibration graph for II and V separated from a 4-mg/ml tetracycline solution on a 10% VI layer.*

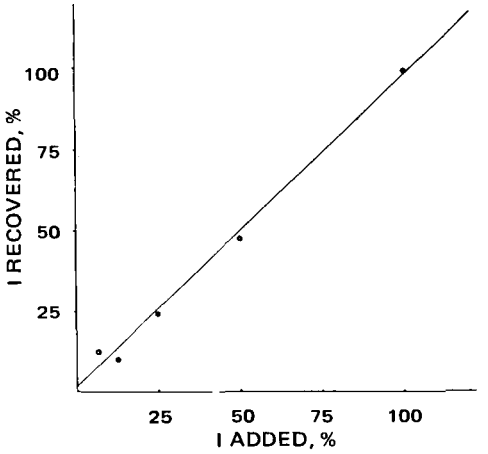

Figure 4—*Example of the correlation between I added and I recovered from an 8.8-mg/ml tetracycline mixture on a 3.7% VI layer by direct TLC fluorometry.*

should be measured at 367.5 nm because the great absorbance of the blank at 265 nm could interfere with an exact evaluation.

Direct TLC Fluorometry—As mentioned previously, a rectangular aperture was used for the measurements since the slit width was much easier to handle and to adjust to the spot concerned; it also allowed a three to fourfold amplification. Since the slit height could not be altered continuously, poor accuracy was obtained where the spots were not completely resolved. Before making quantitative measurements, plates were always inspected under UV light to allow removal of fluorescing dust particles. A chart speed of 5 cm/min was desirable to allow the cutting out of the peak areas. The computation of results by estimation of a regression line and its confidence limits seems somewhat tedious. In cases of routine tetracycline analysis, however, when impurities will occur in quantities of 1% or more and great accuracy is not needed, sufficiently accurate evaluations can be achieved graphically.

The individual regression lines calculated for each impurity separated from 4- and 8-mg/ml tetracycline solutions showed good linearity with a significant correlation coefficient (lowest value 0.9529). Consequently, the mean results of the correlation between the impurity added (%) and the impurity recovered (%) showed good linearity. Compounds I, with a linear equation of $y = 0.27 + 0.99x$ (correlation coefficient 0.9958), and V, with a linear equation of $y = 0.19 + 0.997x$ (correlation coefficient 0.9998), separated from a 4-mg/ml mixture had especially good linearity; $y = 1.68 + 0.962x$ (0.9956) and $y = 0.09 + 0.998x$ (0.998) were obtained, respectively, from an 8.8-mg/ml solution of tetracycline.

The confidence limits (95% confidence interval) of the means are given by $\bar{x} \pm (\sigma/\sqrt{n} \times t^*)$, where $\sigma/\sqrt{n}$ is the standard error and t^* is 2.78 (df 4 and $p = 0.05$)[10]. These values are listed in Table IV and indicate the acceptable accuracy of the direct fluorometric method; each result is the mean of five determinations. For fewer determinations, the critical t^* value becomes much larger and it is better to express the confidence interval as $\bar{x} \pm (1.96 \times \sigma_x)$, where σ_x is the standard deviation. The results, using a 16-mg/ml solution of tetracycline, were not very reliable. Compounds II and III gave regression lines for which the slopes were not significant, although the recoveries were very close to the expected values. For these reasons, the separation of II and III from a 16-mg/ml solution has only a qualitative use. For quantitative purposes, 4–8-mg/ml solutions should be used.

CONCLUSIONS

The TLC method described allows a separation of anhydrotetracycline, epianhydrotetracycline, epitetracycline, and chlortetracycline from a 16-mg/ml solution of tetracycline. Effective measurements of epichlortetracycline at low concentration is impeded by the luminous background left by the spot of tetracycline. A good separation probably can be achieved with other impurities such as doxycycline, rolitetracycline, methacycline, and oxytetracycline.

Direct TLC fluorometry is a very sensitive means of evaluating the impurities in tetracycline, allowing the determination down to a concentration of 0.5% (w/w) in tetracycline. The limit of detection is 50–100 times greater than when spectrophotometry is used.

[10] Table III of Fisher and Yates, "Statistical Tables for Biological, Agricultural and Medical Research."

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GLC Analysis of Caffeine and Codeine Phosphate in Pharmaceutical Preparations

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Abstract □ A procedure for the determination of caffeine and codeine phosphate in pharmaceutical preparations was developed. It depends upon a one-step extraction followed by GLC analysis of the concentrated extract.

Keyphrases □ Caffeine and codeine phosphate—GLC analysis in pharmaceutical products □ Codeine phosphate and caffeine—GLC analysis in pharmaceutical products □ GLC—analysis, caffeine and codeine phosphate

Caffeine and codeine phosphate are used singly or in combination with other drugs in various pharmaceutical preparations. Quality control requirements (1) have made it mandatory to determine both the caffeine and codeine phosphate content uniformity. James (2) reported a procedure for the determination of the codeine and caffeine contents of individual aspirin, phenacetin, caffeine, and codeine phosphate tablets based upon a fluorometric–UV spectroscopic assay. While accurate, this method and other spectroscopic assays (3, 4) involve time-consuming multiple extractions which are liable to inherent errors.

The reported GC methods are either not amenable to single-tablet assay, do not include codeine, or are rather involved (2, 5).

The developed GLC procedure is simple, and the sample preparation involves only two extractions per tablet. This process represents a 77% reduction in the extraction requirements when compared with the compendial method (1) with no loss in accuracy. Caffeine, codeine phosphate, and phenacetin are extracted from the tablet matrix into a common solvent, and no further separation is needed.

EXPERIMENTAL

Equipment—A gas chromatograph[1] equipped with a flame-ionization detector was used. The detector signal was fed into a 1-mv recorder[2] operated with a chart speed of 1.3 cm (0.5 in.)/min. One-microliter samples were injected with a 10-μl syringe[3].

Materials—High purity helium was the carrier gas. Purified hydrogen and compressed air were used in the detector. The stationary phase was 3%, OV-17 on Chromasorb W[4] (80–100 mesh), acid washed and silanized, packed in a 1-m × 0.64-cm o.d. glass column. All chemicals employed were spectrograde. All sample and standard materials were of NF or USP grade.

Operating Conditions—The column was operated isothermally at 240°. Both the detector and the injector port were held at 250°. The carrier gas flow rate was 28 ml/min at an inlet pressure of 40 psig. The initial electrometer range and attenuation settings were 10^2 and 32, respectively. After the elution of the solvent and the first two components, the range setting was 10 and the attenuation setting was 8 for the duration of the run.

Preparation of Standard Solutions—Two standard solutions were prepared, an internal standard and the working standard.

Internal Standard—Three hundred milligrams of nortriptyline hydrochloride was dissolved in 20 ml of 15% sodium chloride solution in a 125-ml separator. The solution was made basic by the addition of 5 ml of 2 *N* NaOH and extracted with 2 × 15 ml of chloroform. The extracts were filtered through filter paper[5] containing 5 g of anhydrous sodium sulfate, and the extracts were combined in a 50-ml volumetric flask. The filter was washed with 15 ml of chloroform, and the washings were added to the combined extracts. Chloroform was used to bring the contents to volume.

Working Standard—In a 125-ml separator, 234 mg of aspirin, 150 mg of phenacetin, 30 mg of caffeine, and 15–60 mg of codeine phosphate were combined. The exact amount of codeine phosphate varied depending upon its concentration in the tablet preparation to be analyzed. The mixture was treated in the same way as the internal standard preparation, except that the extracts and the

Table I—GLC Data

Compound	Retention Time, min	Relative Retention Time, min
Phenacetin	1.2	0.276
Caffeine	1.95	0.448
Nortriptyline	4.35	1.000
Codeine	8.60	1.970

Table II—Standard Samples

Ingredient	Mixture 1, mg	Found, mg	Mixture 2, mg	Found, mg
Aspirin	234	—	234.7	—
Phenacetin	151	—	150.8	—
Caffeine	31.6	31.0	30.6	30.3
Codeine phosphate	15.0	14.9	30.4	30.2

[1] Hewlett-Packard model 402.
[2] Hewlett-Packard model 7127-A.
[3] Hamilton No. 701.
[4] Johns-Manville Corp.
[5] Whatman No. 41.