at Y does affect activity, with the more lipophilic and electron-withdrawing groups being classified as active.

The detection of the role of lipophilicity is consistent with the report of Hansch *et al.* (3), who found that the activity of analogs of II was a function of the lipophilicity of the substituents at the 3-nitrogen.

The interpretation of these results in terms of the mode of action of the triazenes can only be viewed as suggestive at this point. It was suggested previously that the triazenes must be demethylated to form an active metabolite (13). The compounds in this study are subject to such activation, but the physicochemical effect on metabolism of substituents removed from the site of *N*-demethylation has not been studied for triazenes. Therefore, further work is necessary before conclusions can be drawn.

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Rapid and Sensitive Direct TLC Fluorometric Method for Evaluation of Impurities in Oxytetracycline

GILBERT J. WILLEKENS

Abstract □ A rapid partition TLC method for the determination of oxytetracycline and its degradation products is described. Quantitative determination of the substances is performed by direct TLC fluorometry in 2 hr using plates prepared previously.

Keyphrases □ Oxytetracycline and impurities—TLC fluorometric analyses in prepared solutions □ TLC fluorometry—analyses, oxytetracycline and impurities in prepared solutions □ Fluorometry, TLC—analyses, oxytetracycline and impurities in prepared solutions □ Antibacterials—oxytetracycline and impurities, TLC fluorometric analyses in prepared solutions

Tetracyclines are sensitive to both acidic and alkaline degradation media (1). Storage under adverse moisture and temperature conditions gives rise to acidic degradation products. The first stage of acidic degradation is the formation of anhydro compounds, which have attracted a great deal of attention because of their potential toxicity (2, 3).

Although the most likely degradation products of oxytetracycline (I) are not toxic, an accurate and convenient analytical method is needed to investigate the purity of commercial I samples. Anhydrooxytetracycline (II), epioxytetracycline (III), α-apooxytetracycline (IV), and β-apooxytetracycline (V) occur as impurities in I, but little information has been published about their quantitative evaluation.

Compound I was determined by UV spectrophotometry (4) and fluorometry (5). Degradation products were separated from I by paper chromatography (6, 7). Some acidic degradation products of I were determined chromatographically using paper impregnated with 10% (w/v) urea in pH 5.0 McIlvaine's buffer (8). After elution, I was quantitated by spectrophotometry.

Compounds II, IV, V, and terrinolide (VI) were separated on a kieselguhr layer (9). Compound I also was separated from its impurities on a diatomaceous earth column, and I and V were evaluated by spectrophotometry (10).

The use of paper chromatography is undesirable (6–8) because the procedure is lengthy and not reproducible. A TLC separation of II, IV, and V from I on kieselguhr (9) could not be achieved in this laboratory.

The TLC procedure presented is rapid and allows the quantitation of traces of II–V in I by direct TLC fluorometry. The method is reproducible and is sensitive to 10^{-2} μg of II–V in a 1% (w/v) solution of I.

EXPERIMENTAL

Reagents—Disodium ethylenediaminetetraacetate (VII), ethylene glycol, acetone, ethyl formate, ethyl acetoacetate, and sodium hydroxide were analytical grade. Kieselguhr G[1] was used.

Preparation of Plates—A slurry of 20 g of kieselguhr and 47 ml of 5% (w/v) aqueous VII, previously adjusted to pH 9 with 20% NaOH, was prepared[2] and spread on glass plates at a thickness of 0.25–0.30 mm. The plates were dried in air for a minimum of 2 hr at room temperature (25°); a transverse channel of approximately 1-mm width was drawn in the layer

[1] Macherey, Nagel & Co, D-516 Düren, Germany.

[2] Desaga applicator, Heidelberg, Germany.

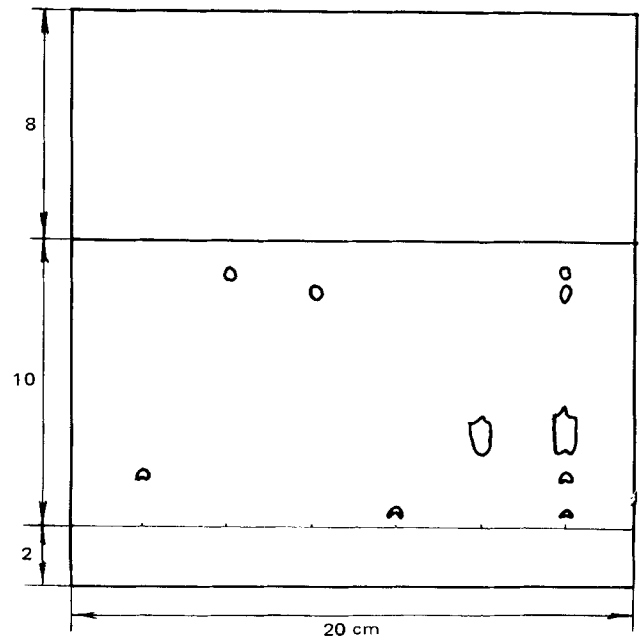

Figure 1—*Typical TLC separation of oxytetracycline impurities on kieselguhr with 5% VII at pH 9. Key (left to right): 0.1 μg of IV (greenish-blue), 0.1 μg of V (blue), 0.1 μg of II (brown), 0.1 μg of III (yellow), 5 μg of I (yellow), and a mixture of I–V.*

12 cm from the one edge of the plate to limit the movement of the solvent front.

The plates were stored in a drying chamber[3] at 30°. Prior to use, they were developed with the solvent system up to the transverse channel (20 min) and dried for 5 min in air and for another 15 min in the drying chamber at 30°. Unimpregnated plates could be stored for an unlimited time.

Solvent System—The system consisted of ethylene glycol–water–acetone–ethyl formate–ethyl acetoacetate (3:6:45:30:15 v/v).

Preparation of Solutions—Samples were dissolved in absolute methanol. For II–VI, a concentration of 0.1 μg/μl was used; I was used at 5 μg/μl. For quantitative TLC fluorometric evaluation, geometric dilutions of impurities were used to define the standardization curves.

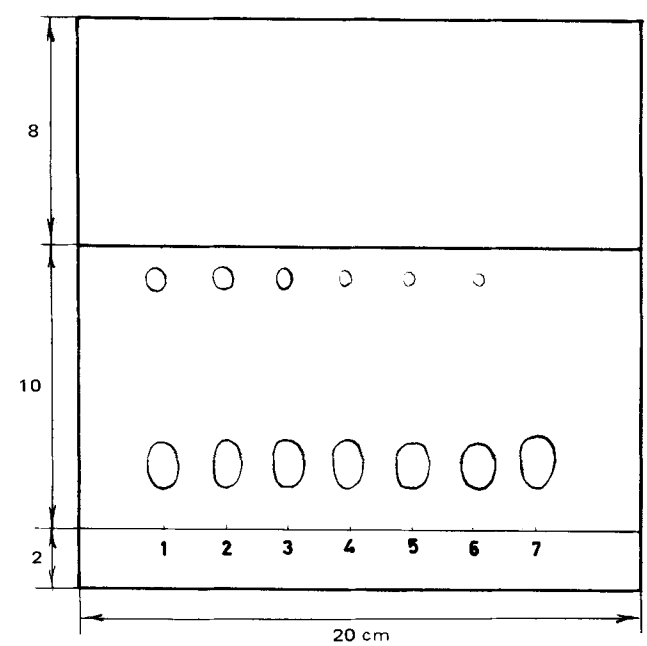

Figure 2—*TLC separation of V from I. Key: 1–7, 8.1 μg of I; 1 and 2, 0.154 μg of V; and 3–6, 0.077, 0.0385, 0.01925, and 0.00963 μg of V, respectively.*

[3] Bekso, Brussels, Belgium.

Table I—Conditions for TLC Fluorometric Determination of Each Impurity

Compound	Fluorescence Emission Wavelength, nm	Slit Width, mm	Slit Height, mm
II	516	0.5	6
III	526	0.3	3.5
IV	500	0.5	6
V	477	0.5	6

Apparatus—A TLC tank (20 × 20 × 8 cm) was lined with filter paper, and 100 ml of solvent was added. The tank was allowed to equilibrate for 1 hr before use. A TLC spectrophotometer[4], an electrobalance[5], and a recorder[6] also were used.

Application of Solutions—Volumes of 1 μl were spotted with disposable pipets[7].

Qualitative Chromatography—Immediately after a plate was removed from the drying chamber, all adsorbent above the origin was covered by a clean glass plate in close contact with the layer while 1-μl volumes were applied as spots on a line 2 cm above the lower edge of the plate. The loaded plate was returned to the drying chamber for 3 min and then placed in the tank and developed at room temperature (25°) in the dark until the solvent front traveled approximately 7 cm (7–10 min).

After development, the plate was dried in air. The spots were visualized under UV light (366 nm). The hR_f values observed for I–VI were 31, 83, 5, 18, 90, and 76, respectively. A typical TLC separation is shown in Fig. 1.

Quantitative Analysis—For each impurity, five reference solutions with concentrations in geometric progression were spotted (1 μl) on the baseline 2 cm above the lower edge of the plate and 2 cm apart, together with 1 μl of the test solution of I. The solution with the highest concentration was spotted twice, and 1 μl of the test solution of I also was applied separately. An example of this spotting procedure is shown in Fig. 2.

After development, the plate was dried in the air and inspected under UV light to remove dust particles in or near the spots under test. The TLC spectrophotometer was adjusted to the first spot with the highest concentration, and the transmission scale and recorder scale were adjusted to 100 units. A rectangular aperture was used for all measurements; the other conditions used for each impurity are listed in Table I.

The fluorescence was excited by the 365-nm mercury line and measured by scanning the accurately positioned spots in the x-axis direction with a scanning speed of 3 cm/min. The recorder speed was kept at 6 cm/min. The peak areas were cut out (11) and weighed to quantitate the spots (Table II).

RESULTS AND DISCUSSION

Separation of Oxytetracyclines—Several TLC systems were examined to find a separation method with sufficient resolution and sensitivity to determine the most likely degradation products of I by direct TLC fluorometry at the 10^{-2} μg level. The presented procedure separated V, II, VI, I, IV, and III quantitatively in that order. Figure 1 shows the arrangement of the spots.

The time required for a complete analysis of one oxytetracycline sample with two impurities was 2 hr using previously prepared plates. The solvent system could be used four times. Test and standard solutions, when kept at 0° in the dark, were stable for at least 3 weeks. However, solutions of II were not stable; II was exceptionally labile and was isomerized to a mixture of IV and V. Ambient atmospheric conditions such as temperature and relative humidity influenced impregnation and development markedly, and care was always taken to dry layers as described.

Optimization of Relative Humidity—The optimum water content in the support was determined by varying the drying conditions. All impurities were well separated on plates equilibrated at a relative humidity of less than 30%; at humidities above 50%, tailing of the spots resulted. For practical reasons, the use of a drying chamber was preferred to equilibration over appropriate saturated salt solutions or sulfuric acid dilutions.

Standard Curve—The relationship between fluorescence and quantity of a substance in a spot on the chromatogram is very complicated, and the shape of the standard curve was dependent upon many factors. Although a straight line passing through the origin might be

[4] Zeiss.
[5] Cahn.
[6] Varian A 25.
[7] Vitrex, Chr. Bardram Birkerod, Denmark.

Table II—Analysis of Known Mixtures of Oxytetracycline and Its Impurities by TLC and Direct Fluorometry

Compound	Spotted Quantity, μg	Recovery[a], μg	Confidence Limits		Recovery, %
			Maximum	Minimum	
I	2.066				
III	0.13630	0.13393	0.13561	0.13225	98.2
	0.06815	0.07273	0.07708	0.06838	106.7
	0.03408	0.03540	0.03802	0.03278	103.9
	0.01704	0.01657	0.01818	0.01496	97.3
	0.00852	0.00544	0.00716	0.00372	63.9
I	4.184				
III	0.13630	0.13527	0.13663	0.13391	99.2
	0.06815	0.07039	0.07323	0.06755	103.3
	0.03408	0.03375	0.03560	0.03190	99
	0.01704	0.01778	0.02048	0.01508	104.3
	0.00852	0.00689	0.00898	0.00480	80.9
I	9.668				
III	0.13630	0.13361	0.14081	0.12641	98
	0.06815	0.07499	0.09212	0.05786	110
	0.03408	0.03189	0.03498	0.02880	93.6
	0.01704	0.01613	0.02028	0.01198	94.6
	0.00852	0.00746	0.01136	0.00356	87.5
I	5.712				
IV	0.11930	0.12015	0.12184	0.11846	100.7
	0.05965	0.05766	0.06137	0.05395	96.6
	0.02983	0.03000	0.03283	0.02717	100.6
	0.01491	0.01569	0.01979	0.01159	105.2
	0.00746	0.00769	0.00991	0.00547	103.1
I	8.100				
IV	0.11930	0.11193	0.11495	0.10891	93.8
	0.05965	0.05994	0.06617	0.05371	100.5
	0.02983	0.02948	0.03426	0.02470	98.8
	0.01491	0.01410	0.01673	0.01147	94.6
	0.00746	0.00837	0.01210	0.00464	112.2
I	5.712				
V	0.15410	0.15320	0.16276	0.15188	99.4
	0.07704	0.07147	0.08239	0.06055	92.8
	0.03852	0.03507	0.03912	0.03102	91.0
	0.01926	0.02032	0.02456	0.01608	105.5
	0.00963	0.01437	0.01920	0.00954	149.2
I	8.100				
V	0.15410	0.15691	0.16309	0.15073	101.8
	0.07704	0.07229	0.08524	0.05934	93.8
	0.03852	0.03527	0.03912	0.03142	91.6
	0.01926	0.02016	0.02429	0.01603	104.7
	0.00963	0.01392	0.01935	0.00849	144.5
I	17.736				
V	0.15410	0.15470	0.15566	0.15374	100.4
	0.07704	0.07450	0.07785	0.07115	96.7
	0.03852	0.03670	0.04113	0.03227	95.3
	0.01926	0.01857	0.01929	0.01785	96.4
	0.00963	0.01308	0.01534	0.01082	135.8

[a] Mean of five determinations.

expected, the line was usually curved. The irregular distribution of a substance in the spot, the uneven thickness of a layer, the position of the origin, and the immersion depth of the plate were relatively unimportant, but the degree of curvature increased with increasing spot size, the amount of material spotted onto the plate, and the direction of scanning.

With a load not greater than 0.5 μg and with the origin spot area less than 5 mm^2, satisfactory results were obtained. Standard curves prepared using vertical scanning did not pass through the origin and were not reproducible. Horizontal scanning gave better results and was used. The movement of the solvent front was kept to a minimum to avoid unnecessary diffusion of spots.

Compounds II and V were sufficiently separated when the solvent front had moved 5–6 cm, and III and IV were separated with 8–9 cm of movement. The quantitation of IV was somewhat complicated by streaking of the I spot.

Reproducibility and Accuracy—The reproducibility was determined over a range of loads and proportions of impurities (Table II). Except for very low concentrations, the recovery was always better than 90%. The high percent recovery of V was due to demixing of the solvent at that part of the chromatogram. A part of the subfront became visible and fluorescent because of the irregular and insufficient drying of the plates. The V spot was accidentally hammered to this subfront. Despite the high recovery, the results conform to the 95% confidence limits, except for the last one. However, at the very low concentration levels (5–10 × 10^{-3} μg), a greater error of 30–40% is acceptable.

The individual regression lines calculated for each impurity when separated from 4-, 8-, and 16-mg/ml oxytetracycline solutions showed good linearity with a significant correlation coefficiënt (lowest value 0.9458). Also, the correlation between the impurity added (x_{th}) and the impurity recovered (x_{exp}) showed good linearity and was statistically significant (lowest value 0.9971). 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 $p = 0.05$). Each result is the mean of five determinations and illustrates the reproducibility and accuracy of the method.

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Kinetics and Mechanisms of Drug Action on Microorganisms XXIII: Microbial Kinetic Assay for Fluorouracil in Biological Fluids and Its Application to Human Pharmacokinetics

EDWARD R. GARRETT ˣ, GAIL H. HURST, and J. RUSSELL GREEN, Jr. *

Abstract □ The apparent first-order generation rate constant, k_{app}, of *Escherichia coli* is a function of fluorouracil concentration, C, *i.e.*, $1/(k_0 - k_{app}) = k_1(1/C) + k_2$, where k_0 is the constant in the absence of drug. A routine procedure, using only six counts to establish a daily calibration curve (at 125 and 225 min after medium inoculation and with 0, 15, and 30 ng of fluorouracil/ml), assayed unknown drug concentrations in plasma or urine diluted to 15–30 ng/ml with standard deviations of 12%. Uracil completely antagonized the inhibitory action of fluorouracil but not of 5-fluorouridine, 5-fluorodeoxyuridine, chloramphenicol, or tetracycline, so fluorouracil could be assayed in the presence of the latter compounds by kinetic studies with and without uracil. Fluorouracil degraded in generating cultures of *E. coli*, and the drug effects on filtered organisms persisted on their inoculation into drug-free medium. Potential products of fluorouracil solvolysis, barbituric and isobarbituric acids, had no significant effect on *E. coli* generation. Human plasma protein binding of fluorouracil averaged 10%. The pharmacokinetics of intravenously administered fluorouracil to cancer patients showed a dose-dependent two-compartment body model with a faster terminal phase of elimination at the lower dose. Total and metabolic plasma clearances increased with a decreasing intravenous dose and exceeded hepatic plasma flow to indicate extrahepatic metabolism. The initial distribution phase and the renal clearance did not appear dose dependent, and the terminal semilogarithmic plots of plasma levels against time were linear for both 0.5- and 1.0-g doses. A possible explanation is product- or metabolite-inhibited metabolism. Infusion studies of 1.0 g of fluorouracil showed increased metabolic clearances to confirm dose dependency and to be consistent with this postulate. The oral absorption of unchanged fluorouracil was highly variable between 1 and 15% of the administered dose, showing a large first-pass effect.

Keyphrases □ Fluorouracil—microbial kinetic analysis in biological fluids, pharmacokinetics in humans □ Microbial kinetics—analysis, fluorouracil in biological fluids □ Pharmacokinetics—fluorouracil in humans, studied using microbial kinetic analysis □ Antineoplastic agents—fluorouracil, microbial kinetic analysis in biological fluids, pharmacokinetics in humans

Facile, sensitive, and nontedious assays for fluorouracil in biological fluids are needed for the proper and routine evaluation of its pharmacokinetics and for the evaluation of proper dosage regimens. The bioavailability on oral administration also needs to be assessed. This paper reports the development of a microbial kinetic assay of fluorouracil in biological fluids and its application to human pharmacokinetics.

The GLC analysis of fluorouracil in biological fluids requires its separation and derivatization. Separation by 22-hr dialysis with subsequent trimethylsilylation permitted GLC analysis sensitive to drug concentrations down to 1 μg/ml (1). Separation for a similar GLC analysis was also effected by an 80% extraction from biological fluids buffered at pH 6 into 16% 1-propanol in ether (2). A sensitivity to drug concentrations down to 0.5 μg/ml was shown (2), and a reproducibility of ±5% was claimed. An ion-exchange recovery of the drug, with a subsequent GLC flash-methylation technique using trimethylanilinium hydroxide, also was reported; the apparent sensitivity was 1.0 μg/ml of urine (3). A recently published isotope dilution mass fragmentographic assay (4) claimed a standard deviation of ±6% for 10 ng/ml extracted from plasma.

A sensitive classical disk–agar plate microbiological assay based on the correlation of zones of inhibition of *Streptococcus faecalis* with fluorouracil was developed and applied to the time course of the drug in plasma and urine; the estimated error was ±25%/zonal measurement (5).

Fluorouracil lessens the generation rate of logarithmic phase microorganisms, and the degree of decrease can be related to the drug concentration (6). This ability provided the basis for a sensitive assay of fluorouracil in biological fluids below the drug levels necessary for complete inhibition of generation. The tedious and time-consuming separation procedures vital for GLC assays and the time lag necessary for organism incubation in the disk–agar plate studies were avoided. Also, since the inhibitory effect of fluorouracil on microbial generation can be reversed by the addition of excess uracil, fluorouracil can be specifically assayed in the presence of other antibacterial agents that do not show this reversibility. The assay was applied to the study of fluorouracil pharmacokinetics in humans.

EXPERIMENTAL

Organism—Replicate slants of *Escherichia coli* (ATCC 12407) were prepared from a single colony and stored at 4°.