extraction is critical. Recovery studies at pH 4.75 showed complete extraction of anthranilic acid over the effective assay range of 2–10 μg/ml (Table I); but at a slightly higher pH level of 5.0 or at a slightly lower level of 4.5, only about one-half of the anthranilic acid was extracted (Fig. 2).

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Detection of Penicillin G and Ampicillin as Contaminants in Tetracyclines and Penicillamine

DORIS HERBST

Abstract □ A method was developed to detect residual levels of ampicillin and penicillin G in various tetracyclines and penicillamine. Residues are detected by reversed-phase TLC followed by bioautography. The directness of the techniques makes this method a good means of detecting residual contaminants in drugs.

Keyphrases □ Penicillin G—TLC–bioautographic analysis as contaminant in various tetracyclines and penicillamine □ Ampicillin—TLC–bioautographic analysis as contaminant in various tetracyclines and penicillamine □ TLC–bioautography—analysis, penicillin G and ampicillin as contaminants in various tetracyclines and penicillamine □ Tetracyclines, various—TLC–bioautographic analysis of penicillin G and ampicillin as contaminants □ Penicillamine—TLC–bioautographic analysis of penicillin G and ampicillin as contaminants □ Antibacterials—TLC–bioautographic analysis of penicillin G and ampicillin as contaminants in various tetracyclines and penicillamine

The problem of penicillin contamination in nonpenicillin products was recognized as early as 1964. An ad hoc Advisory Committee on Penicillin Contamination composed of experts in the fields of allergy and penicillin therapy (1) was convened by the Commissioner of Food and Drugs to evaluate the potential public health problems. The potential danger of allergic reactions, ranging from minor symptoms to fatal anaphylaxis, and the possibility of sensitizing individuals by repeated trace doses were considered.

Based on the data then available, limits on the amount of penicillin allowable in nonpenicillin drugs were recommended. These limits were less than 0.05 unit/maximum single dose for parenteral drugs and less than 0.5 unit/maximum single dose for oral drugs. Methods to detect penicillin G were specified by the Food and Drug Administration (2).

BACKGROUND

In 1974, a screening study (3) was conducted to ascertain which, if any, of the other penicillins could be detected in erythromycin and tetracycline by existing methods. That study showed that the current methods were less sensitive to residual levels of phenethicillin, methicillin, nafcillin, oxacillin, and cephalothin and were completely incapable of detecting residual ampicillin.

This 1974 evaluation indicated the need for new methods to detect residual ampicillin. Investigations conducted in 1974 resulted in a new bioautographic method (4) to separate and detect as little as 1 ppm of ampicillin from tetracycline hydrochloride bulk powder.

In 1975, new procedures (5) were described for the detection of ampicillin and penicillin G contaminants in demeclocycline and chlortetracycline. Although analytical capabilities had been expanded, additional methods were needed to detect ampicillin and penicillin G in doxycycline, oxytetracycline, and methacycline. Biagi *et al.* (6) described a reversed-phase TLC method to determine partition data for the penicillins. Using various concentrations of acetone in the mobile phase (6) of this system, they varied the R_f values of 11 penicillins and concluded: "The most hydrophilic compounds are the first to reach a maximum R_f value. On the other hand, at 0% acetone in the mobile phase the most lipophilic compounds remained close to the origin" (6).

The possibility that this type of system might be applied to residual contamination of other antibiotics seemed feasible. The techniques described by Biagi *et al.* (6) were applied to the detection of residual ampicillin and penicillin G in the tetracyclines and residual penicillin G in penicillamine.

EXPERIMENTAL

Preparation of TLC Plates—TLC plates[1], 20 × 20 cm, precoated with silica gel GF[2], were impregnated with silicone[3] by developing the plates in 200 ml of silicone–ether[4] (5:95 v/v) in a covered developing chamber. After overnight development, the plates were removed from the chamber and allowed to air dry at room temperature.

Sample Preparation—With glass-stoppered volumetric flasks to prevent evaporation of the solvent, standard solutions of ampicillin were prepared in acetone[5] to contain 0.1, 0.05, 0.025, 0.0125, and 0.00625 μg/ml. A similar set of penicillin G standards was prepared. Portions of 25 mg of chlortetracycline (I), demeclocycline (II), methacycline (III), minocycline (IV), oxytetracycline (V), tetracycline (VI), and doxycycline hyclate (VII) were placed in small (7 ml) glass-stoppered weighing vials. One milliliter of each standard solution was added to the vials containing the tetracyclines; then the vials were stoppered and gently shaken for a few seconds to form a suspension of the drugs.

This same procedure was followed for a second set of samples using the penicillin G standards. In addition, 250-mg portions of penicillamine

[1] Analtech, Inc., Blue Hen Industrial Park, Newark, DE 19711.

[2] Brinkmann Instruments, Westbury, NY 11590.

[3] Silicone DC 200 (350 centistokes), Applied Science Laboratories, State College, PA 16801.

[4] Burdick & Jackson Laboratories, Muskegon, MI 49442.

[5] Fisher Scientific, Fair Lawn, N.J.

Table I—Maximum Volume Spotted and Lower Limits of Detection of Penicillin Residues in Tetracyclines and Penicillamine[a]

Antibiotic	Volume Spotted, μl	Total Units of Suspensions, μg/ml	Lower Detection Limits, ng
Tetracycline	50	0.025	0.75
Methacycline	50	0.025	0.75
Doxycycline	50	0.025	0.75
Demeclocycline	50	0.025	0.75
Minocycline	50	0.025	0.75
Oxytetracycline	20	0.05	0.60
Chlortetracycline	50	0.025	0.75
Penicillamine	60	0.0125	0.45

[a] Suspensions were made by adding 1 ml of acetone containing the levels of penicillin listed above to 25 mg of each tetracycline. A solution was made by adding 10 ml of acetone containing 0.0125 unit of penicillin/ml to the contents of one 250-mg penicillamine capsule. The maximum amount of these samples that exhibited acceptable separation and the lower limits of detection are listed.

capsules were placed in screw-capped glass test tubes (16 × 125 mm) and spiked with 10 ml of each standard solution of penicillin G.

Chromatographic Procedure—The silicone-impregnated plates were marked with a finish line horizontally about 1 cm from the top of the plate. With a 10-μl tip and holder, aliquots of 10–100 μl were spotted on a line 2 cm from the bottom of the plates. Various aliquots were used to determine the maximum amount that could be spotted and still maintain sufficient separation of the contaminant from the antibiotic. These aliquots were delivered 10 μl at a time and dried with a gentle air flow to keep the spots as small as possible.

An aqueous buffer was prepared by placing 19.4 g of sodium acetate[6] (crystal) in 1 liter of distilled water and lowering the pH to 7.4 by adding small amounts of barbital[5] while stirring. This buffer was then filtered through analytical filter paper[7] to remove any undissolved barbital. The impregnated plates were then placed in an unsaturated tank containing 100 ml of barbital acetate buffer–acetone (94:6 v/v). The solvent front was allowed to move to the finish line, and the plates were removed and air dried at room temperature. The drying process was hastened by incubating the plates at 54° for approximately 10 min.

Bioautographic Procedure—The dried chromatographic plates were placed in plastic bioassay trays[8] (23 × 23 cm, with lids) and secured to the bottom of the plastic trays with double-sided tape to prevent the plates from floating when the liquid agar medium was added. Each plate was flooded with 100 ml of antibiotic medium No. 4 (yeast beef agar),

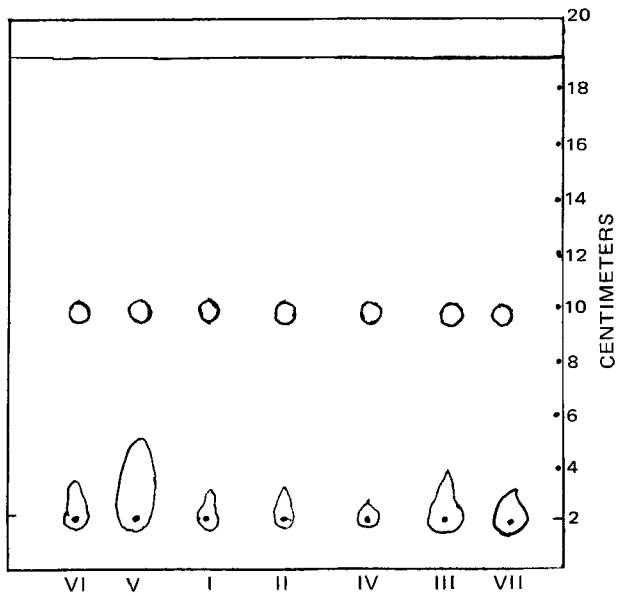

Figure 1—*Sketch of typical plate. Key: circular zones, ampicillin; and bearded zones, various tetracyclines.*

[6] J. T. Baker Chemical Co., Phillipsburg, NJ 08865.
[7] No. 588, Schleicher & Schuell, Keene, NH 03431.
[8] Vanguard International Inc., Red Bank, NJ 07701.

Table II—Maximum Volume Spotted and Lower Limits of Detection of Ampicillin Residues in Tetracyclines[a]

Antibiotic	Volume Spotted, μl	Total Units of Suspensions, μg/ml	Lower Detection Limits, ng
Tetracycline	30	0.025	0.75
Methacycline	30	0.025	0.75
Doxycycline	30	0.025	0.75
Demeclocycline	30	0.025	0.75
Minocycline	30	0.025	0.75
Oxytetracycline	20	0.025	0.5
Chlortetracycline	30	0.025	0.75

[a] Suspensions were made by adding 1 ml of acetone containing 0.025 μg of ampicillin/ml to 25 mg of each tetracycline. The maximum amounts of these suspensions spotted that exhibited acceptable separation and the lower limits of detection are listed.

which had been inoculated with 0.02 ml of a suspension of *Sarcina lutea* (ATCC 9431[9]).

When the agar had set (about 20 min), the bioassay trays were covered, placed in a plastic bag with a piece of damp paper, sealed, and refrigerated for approximately 1 hr. The trays were then incubated with the sealed bag intact at 30° overnight. After 16–18 hr of incubation, the areas of inhibition were visualized by spraying the plates with a solution of 2% 2,3,5-triphenyltetrazolium chloride[5] plus 2% dextrose[10] in 50% methanol[5].

RESULTS AND DISCUSSION

The bioautographic plates exhibited clear areas of inhibition in the presence of the drugs in contrast to a dark-purple background. The tetracyclines tended to produce areas of inhibition that remained at the starting line but bearded upward. Ampicillin migrated an average distance of 8 cm; its R_f value was 0.47 (Fig. 1). Penicillin residues, when separated from the tetracyclines, presented a picture similar to that shown in Fig. 1 but had migrated a distance of 5 cm for an R_f value of 0.29.

To determine the maximum amount of the spiked antibiotic suspensions that could be spotted and still maintain adequate separation of the two drugs, suspensions at levels of 0.2, 0.1, 0.05, 0.025, and 0.0125 μg of ampicillin/ml and unit of penicillin G/ml were spotted in varying amounts (10–100 μl). The amounts spotted and the sensitivities of this system to penicillin G and ampicillin in the various drugs used are shown in Tables I and II.

Penicillamine (250 mg) was spiked with penicillin G (10 ml) at levels of 0.2, 0.1, 0.05, 0.025, and 0.0125 unit/ml, and 60 μl of each solution was spotted. Good separation was effected, the penicillin G migrated 6.5 cm, and an R_f value of 0.38 was obtained.

Although an extensive stability study of the spiked sample suspensions was not conducted, spot checks indicated that penicillin G activity had not diminished appreciably in 6 days when suspensions were stored in stoppered vials at room temperature. Tetracycline, methacycline, and chlortetracycline in suspension with penicillin G solutions, as well as a standard solution of penicillin G, were tested and responded similarly to the original assays.

In conclusion, this method has the capability of screening large numbers of samples for contamination in a relatively short time. Seven samples and one control can be run on each plate. The directness of the procedures and the minimum amount of handling involved substantially reduced the risk of cross-contamination.

Further application of these procedures, now in progress, will be the subject of a future paper.

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[9] American Type Culture Collection, Rockville, MD 20841.
[10] Mallinckrodt Chemical Works, St. Louis, MO 63160.

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Kinetics of Concomitant Degradation of Tetracycline to Epitetracycline, Anhydrotetracycline, and Epianhydrotetracycline in Acid Phosphate Solution

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Abstract □ The concentrations of tetracycline, epitetracycline, anhydrotetracycline, and epianhydrotetracycline in pH 1.5 phosphate solution were followed as a function of time at four temperatures. Separation and quantification of all four species were accomplished using high-pressure liquid chromatography. Through nonlinear regression analysis, rate constants for the reversible first-order epimerization of tetracycline and anhydrotetracycline and for the first-order dehydration of tetracycline and epitetracycline were obtained. Solutions to the differential equations obtained through Laplace transforms successfully predict concentrations found experimentally. The energy of activation for each reaction step was calculated and ranged from 15 to 27 kcal/mole. The rate constants for tetracycline and epitetracycline dehydration conform with those of earlier studies that used different experimental methods. The study shows that epimerization of tetracycline and anhydrotetracycline can take place at a low pH.

Keyphrases □ Tetracycline—kinetics of degradation at pH 1.5, effect of temperature □ Degradation kinetics—tetracycline at pH 1.5, effect of temperature □ Antibacterials—tetracycline, kinetics of degradation at pH 1.5, effect of temperature

Tetracycline degradation to toxic epianhydrotetracycline can take place through tetracycline epimerization to epitetracycline (1, 2) followed by dehydration to epianhydrotetracycline (3) or by dehydration of tetracycline to anhydrotetracycline (4) followed by epimerization to epianhydrotetracycline (5). The kinetics of each individual step have been studied separately under conditions where it was assumed that only the reaction of interest was operative. No reported studies followed all potential reactions simultaneously under the same experimental conditions. The use of high-pressure liquid chromatography (HPLC), which separates all four compounds (tetracycline, epitetracycline, anhydrotetracycline, and epianhydrotetracycline), permits such a study (5). This paper presents the results for the solution degradation of tetracycline at pH 1.5 together with the rate expressions defining these results.

EXPERIMENTAL

Materials—Tetracycline hydrochloride[1] (I), 4-epitetracycline ammonium salt[2] (II), anhydrotetracycline hydrochloride[3] (III), and 4-epianhydrotetracycline[4] (IV) were used as obtained. All other chemicals were reagent grade, and double-distilled deionized water was used to make all solutions.

Apparatus—A high-pressure liquid chromatograph[5] with a multiwavelength detector[6] was used with a 1-m × 2.1-mm strong cation-exchange column[7].

Separation and Quantification—The mobile phase employed in the HPLC separation consisted of 0.07 *M* phosphate–0.0075 *M* ethylenediaminetetraacetic acid adjusted to pH 7.0. To improve the separation for tetracycline and its degradation products, the operating procedure was a slight modification of a previously reported method (5). Specifically, the elution was carried out at a column temperature of 36° and a flow rate of 0.55 ml/min (575 psi). The eluent was monitored at 254 nm at 0.08 absorbance unit full scale (aufs).

Areas under the individual peaks were measured with a polar compensating planimeter[8]. Known injected amounts of I–IV in 0.03 *N* HCl were correlated with the areas under the chromatograms obtained. The slopes of the linear relationship between moles added and area were 2.625×10^{-10}, 2.681×10^{-10}, 1.154×10^{-10}, and 1.377×10^{-10} mole/cm^2 for I, II, III, and IV, respectively.

Kinetic Method—A phosphoric acid stock solution (1 *M*) was adjusted to pH 1.5 with a concentrated potassium hydroxide solution. The solution was scrubbed with nitrogen and allowed to equilibrate at the temperature desired. Appropriate amounts of I were weighed into volumetric flasks and dissolved in the phosphate solution. The pH did not change during the study. The reaction flasks, sealed with a rubber septum, were immediately placed in a water bath[9] that also protected the solution from light.

At appropriate time intervals, samples were withdrawn, placed in vials, and immersed in ice to stop the reaction. A fixed volume of this sample was assayed chromatographically by equating areas found with concentration through a standard curve. The reactions were followed until all I was lost.

RESULTS AND DISCUSSION

A modification of a previously reported HPLC assay for tetracycline and its degradation products (5) results in less overlap between I and its epimer (Fig. 1). The retention times for IV, III, II, and I were 5.5, 8.25, 22.75, and 30.25 min, respectively.

The relationship between the concentration of each of the four species in the reaction as a function of time gave results as shown in Fig. 2 (obtained at 75°). Four temperatures were used (60–80°), and duplicate kinetic experiments were run at each temperature. Studies made at higher temperatures yielded results involving large errors, making data analysis tenuous.

The concentration–time profile found at each temperature was assumed to be a consequence of the reaction illustrated in Scheme I.

[1] Lot 2K030-71 EA, Pfizer.
[2] Batch 430, British Pharmacopoeia Commission.
[3] Batch 428, British Pharmacopoeia Commission.
[4] Lot 3339-99-1, GS-6659, Pfizer.
[5] DuPont model 830.
[6] DuPont model 835.
[7] DuPont Zipax SCX.
[8] Model 62005, Keuffel and Esser Co.
[9] Haake model FS2.