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Determination of Phenoxymethyl Penicilloic Acid and Phenoxyethyl Penicilloic Acid in Urine in the Presence of the Parent Penicillins

JAN BIRNER

Abstract □ Penicillin and the corresponding penicilloic acid are extracted by chloroform from an acidified sample of urine which has been partly saturated by ammonium sulfate. A portion of the extract is dried and redissolved in a small volume of acetone. A measured aliquot is chromatographed on a silica gel thin-layer plate by acetone–acetic acid (19:1). The separated penicilloic acid is detected by starch–iodine spraying. The zones are transferred into glass tubes, nitrated, and neutralized by ammonia. The absorbance of the yellow supernatant, which is proportional to the concentration of penicilloic acid, is measured spectrophotometrically.

Keyphrases □ Penicillin, penicilloic acid in urine—penicilloic acid determination □ Phenoxymethyl penicilloic acid—determination in urine □ Phenoxyethyl penicilloic acid—determination in urine □ TLC—analysis □ Starch–iodine spray—TLC spot identification □ Colorimetric analysis—spectrophotometer

Due to their instability in solution, penicillins taken orally or administered parenterally undergo chemical changes, and the breakdown products are excreted from the body in the urine.

Walkenstein *et al.* (1) found that between 30 and 60% penicillin G in urine remains biologically active, the major degradation product being penicilloic acid. The data were obtained by comparison of radioassays and bioassays of urine. Penicillins can be estimated directly by several methods such as iodometric, hydroxylamine, or biological, but penicilloic acid in the presence of penicillin is usually found by difference. Pan (2) gives a method for the determination of penicilloic acid in penicillin G fermentation broth, which is based on extraction at different pH values and subsequent colorimetric determination. Phenoxymethyl penicillin (penicillin V) and phenoxymethyl penicilloic acid can be separated by paper chromatography; Roehr (3) gives R_f values in four different systems.

McGilveray and Strickland (4) use TLC for identification of about 10 penicillins in four systems.

It was observed that using silica gel G and acetone–acetic acid (19:1) in TLC penicillin V and phenoxyethyl penicillin (phenethicillin) can be separated from the corresponding penicilloic acids. The separated penicilloic acids, containing a phenolic group in the side chain, may be determined subsequently by a colorimetric method (5) by nitration, neutralization by ammonia, and spectrophotometric measurement. Based on this observation, a method has been developed that permits direct determination of penicilloic acid, in the presence of penicillin, in urine.

The sample, partially saturated by ammonium sulfate, is extracted by chloroform at pH 2, using a 1:5 ratio of water to chloroform. The chloroform extract is dried and redissolved in acetone, and an aliquot is taken for chromatography on silica gel using acetone–acetic acid (19:1). The zones of separated penicilloic acid, located by starch–iodine spraying, are transferred into glass tubes and nitrated in the presence of the silica to form quinoid radicals, which are yellow in color in the presence of ammonia. The silica, which does not interfere in this process, is separated by centrifugation, and the supernatant is used for spectrophotometric measurements.

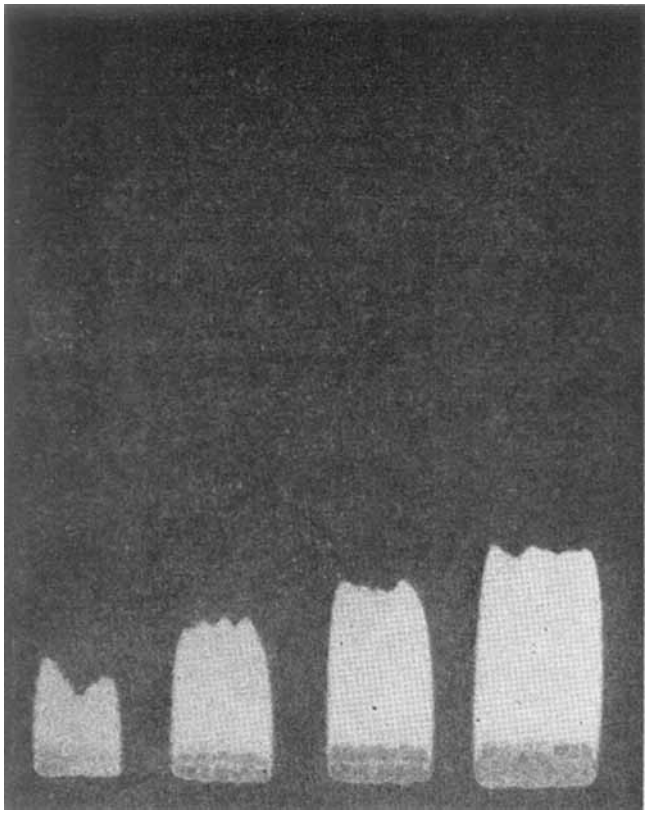

Figure 1—*Starch–iodine-sprayed chromatogram of phenoxymethyl penicilloic acid.*

EXPERIMENTAL

Reagents—Ammonium sulfate A.R.; sodium sulfate anhydrous A.R.; chloroform B.P.; sulfuric acid, 12 *N* A.R.; acetone A.R.; silica gel G according to Stahl for TLC; glacial acetic acid; sodium hydroxide, 2.5 *N;* phosphate buffer, 1%, pH 7; *n*-butanol; 10% potassium nitrate in concentrated sulfuric acid; ammonia 0.880 sp. gr. A.R.–water (1:1); starch, soluble A.R.; 0.1 *N* iodine in 4% potassium iodide A.R.

Procedure—*A. Preparation of Thin-Layer Plates and Starch–Iodine Spray*—Using CAMAG hand-operated apparatus, glass plates, 20 × 10 cm., were coated with silica gel slurry sufficient to produce a thickness of 250–300 μ after drying. The plates were dried in an oven at 80° and finally kept at room temperature exposed to the atmosphere. The starch–iodine solution was prepared by mixing 50 ml. of 1% soluble starch solution, 3 ml. of glacial acetic acid, and 1 ml. of 0.1 *N* iodine.

B. Preparation of Penicilloic Acid—1. Prepare phenoxymethyl penicilloic acid by dissolving 8.5 g. of penicillin V in 80 ml. of water and 9 ml. of 2.5 *N* sodium hydroxide. Keep the solution overnight at 4–8°. After addition of 14 ml. of *n*-butanol, neutralize the solution by addition of dilute sulfuric acid, while mixing and cooling, until a precipitate forms. Filter off the precipitate, wash several times with water, and dry for 4 hr. at 36° and then in a desiccator over P_2O_5; m.p. 121–122° (dec.).

2. Prepare phenoxyethyl penicilloic acid by dissolving 8.5 g. of phenethicillin in 80 ml. of water and 9 ml. of 2.5 *N* sodium hydroxide. Stir the solution for 0.5 hr.; then add 14 ml. of *n*-butanol. Add slowly, while mixing and cooling, 2 *N* sulfuric acid, until the pH reaches approximately 2.8 (approximately 25 ml.). Filter off the precipitate, wash several times with water, and dry at 36° overnight and then in a desiccator over P_2O_5; m.p. 113–114°.

C. Preparation of a Sample—Pipet 2 ml. of urine into a suitable glass-stoppered separator, add 1.5 g. of powdered ammonium sulfate, and mix by swirling to dissolve. Add 10 ml. of chloroform by means of pipet and 0.12 ml. of 12 *N* sulfuric acid which results in a pH of approximately 2. Stopper, and shake immediately for 1 min. After standing for a few minutes to allow the layers to separate, draw off the chloroform layer into a dry test tube, filtering through a small glass funnel containing filter paper on which has been placed about 1 g. of anhydrous sodium sulfate. Transfer 5 ml. of the chloroform extract into a 10–15-ml. test tube fitted with a ground-glass stopper. Remove the chloroform by warming the test tube in a 30–40° water bath while blowing a gentle air current into the tube. Add 0.2 ml. of acetone to the dry residue, stopper immediately with a glass stopper, and gently agitate to dissolve the residue.

D. Separation of Penicilloic Acid from Penicillin and Spectrophotometric Determination of Penicilloic Acid—Stock solutions of the prepared penicilloic acids are made by dissolving in 1% phosphate buffer, pH 7, to furnish a concentration of 10 mg./ml. From these, aliquots are added to normal urine to furnish concentrations ranging from 0.2 to 1.0 mg. acid/ml. Two-milliliter aliquots of these standard solutions are submitted to Procedure C. Using a microsyringe, apply the standards and samples, usually 20-μl. aliquots, each equivalent to 0.1 ml. of original sample or standard, to the plate in duplicate using four application sites for one plate. Develop without delay the plates containing samples and standards by acetone–acetic acid (19:1) in a covered jar lined with filter paper until the solvent front advances 12–14 cm. After drying, spray the plates with starch–iodine solution. The penicilloic acid appears as an elongated spot starting near the point of application, being more elongated at a higher concentration. The penicillin spots, less marked, appear near the liquid front, and the separation is complete. The amount of the sample applied to the plate may be varied depending upon the concentration.

Scrape the white zones containing penicilloic acid onto a square of glossy paper and quantitatively transfer into 11.5 × 1.5-cm. glass centrifuge tubes. Add 0.5 ml. of 10% potassium nitrate in concentrated sulfuric acid to each tube. Immerse the unstoppered tubes in boiling water for 15 min., stirring occasionally by rotation. After cooling, run 1.5 ml. of distilled water into each tube. Mix the solutions by swirling and cool the tubes in ice water. Add 5 ml. of

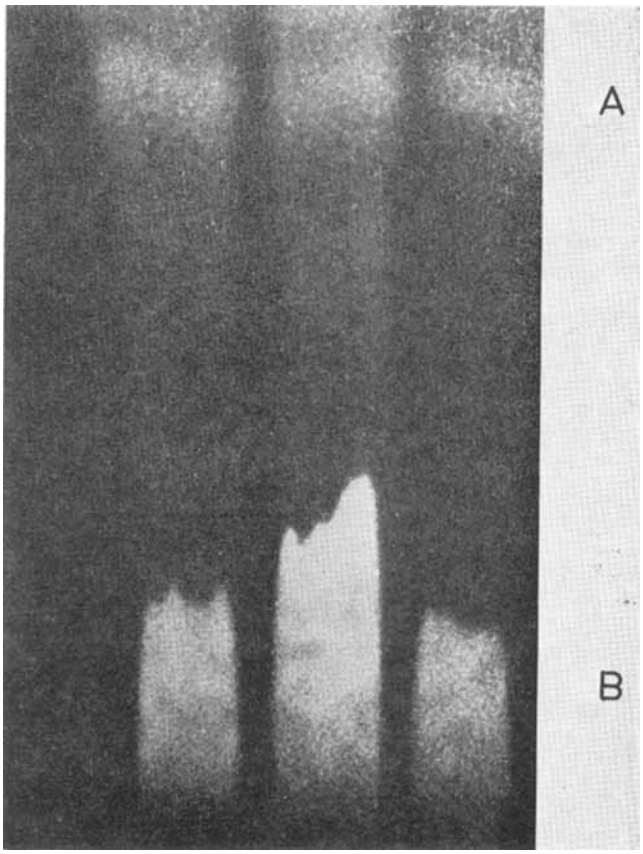

Figure 2—*Chromatogram for chloroform extract from 0.1 ml. urine from Subject 2, phenoxyethyl penicillin-treated. Key: A, phenoxyethyl penicillin; B, phenoxyethyl penicilloic acid.*

Table I—Penicillin and Phenoxymethyl Penicilloic Acid in Urine (mcg./ml.)

Hr.	Donor 1 (79.7 kg.)[a] Pen. V[b]	Pav.[c]	Total	% Pav. in Total	Donor 2 (78.8 kg.) Pen. V	Pav.	Total	% Pav. in Total
1	214	45	259	17.4	308	40	348	11.5
2	214	50	264	18.9	471	160	631	25.4
3	372	290	662	43.8	564	385	949	40.6
4	118	160	278	57.6	322	320	644	49.7
6	48	95	143	66.4	45	60	105	57.1

Hr.	Donor 3 (50.7 kg.) Pen. V	Pav.	Total	% Pav. in Total	Donor 4 (78.8 kg.) Pen. V	Pav.	Total	% Pav. in Total
1	1375	900	2275	39.6	348	250	698	35.8
2	468	850	1318	64.5	855	620	1575	39.4
3	228	540	768	70.3	434	440	874	50.3
4	57	280	337	83.1	224	370	594	62.3
6	22	150	172	87.2	69	320	389	82.3

[a] Numbers in parentheses refer to body weight. [b] Pen. V = phenoxymethyl penicillin. [c] Pav. = phenoxymethyl penicilloic acid.

half-strength ammonia (ammonia 0.880 sp. gr. mixed with water, 1:1) gradually from a buret and again mix the contents of the tubes by swirling and cool. After cooling, occlude the tubes with a thumb protected by rubber and shake several times. Centrifuge the tubes for 10–15 min. at 2000 r.p.m. Remove and measure spectrophotometrically at 420 mμ the yellow supernatant, using 10-mm. cells and setting the instrument on a clear blank obtained by boiling 0.5 ml. of nitrating mixture diluted with 1.5 ml. of water and 5 ml. of diluted ammonia. From the undeveloped part of the glass plate, scrape off, in duplicate, areas of surface equal to the areas extracted for penicilloic acid and put them through the same procedure as the samples to serve as a blank. Any value obtained from this latter is subtracted from the values for samples and standards.

From the values of the standards, plot a curve and obtain data for samples.

To provide typical natural samples for illustration of the method, an experiment was carried out to determine the amount of penicilloic acid present in samples of urine in a group of four volunteers taking penicillin orally. Two penicillins were used in the experiment—phenoxymethyl penicillin (penicillin V) and phenoxyethyl penicillin (phenethicillin). The amount of penicillin taken orally was 0.5 g. of the potassium salt. The urine samples were collected at 0, 1, 2, 3, 4, and 6 hr. and immediately kept in an ice water bath. All volunteers were adult males, coded 1, 2, 3, and 4, weighing 79.7, 78.8, 50.7, and 78.8 kg., respectively. The amount of penicillin in each sample was determined by biological assay. The result for zero-hour samples was nil both for penicilloic acid and penicillin. From the data given by

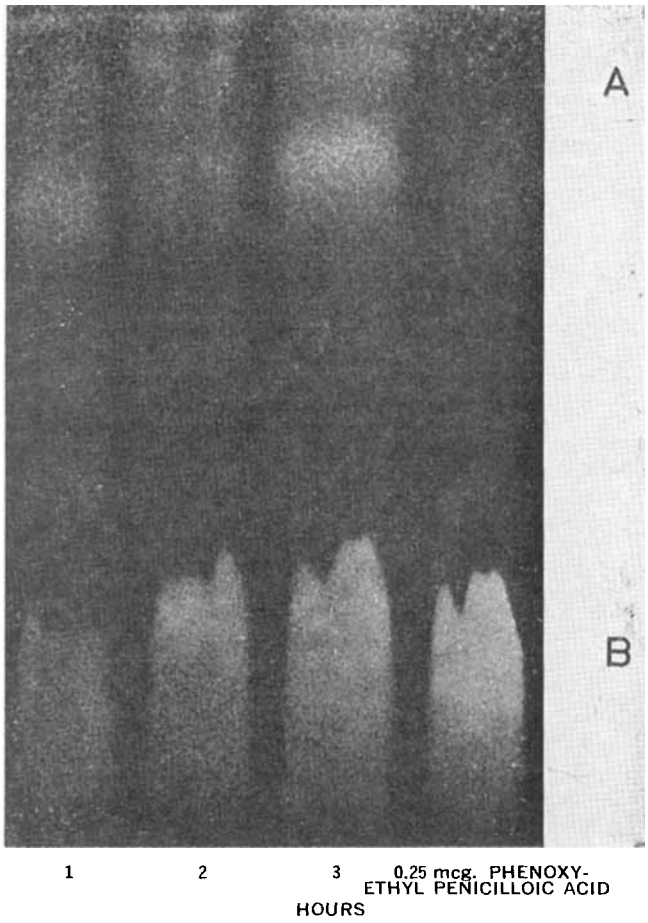

Figure 3—*Chromatogram of chloroform extract from 0.1 ml. urine from Subject 2, phenoxyethyl penicillin-treated. Key: A, phenoxyethyl penicillin; B, phenoxyethyl penicilloic acid.*

assays of penicillin and penicilloic acid, the percentage of the latter was calculated. The data for penicillin V are presented in Table I; the data for phenethicillin are presented in Table II.

A photograph of a developed starch–iodine sprayed plate containing a set of standards of phenoxymethyl penicilloic acid is shown (Fig. 1).

A photograph of a similar chromatogram of samples for phenethicillin from Donor 2 at 0, 1, 2, and 6 hr. is shown (Fig. 2). Samples from this same donor at 1, 2, and 3 hr. and a 25-mcg. standard of penicilloic acid are shown in Fig. 3.

Accuracy of Method—To aliquots of normal urine to which penicillin V had been added to a concentration of 2.5 mg./ml.,

Table II—Phenethicillin and Phenoxyethyl Penicilloic Acid in Urine, mcg./ml.

Hours	Donor 1 (79.7 kg.) Pheneth-icillin	PHA[a]	Total	% of PHA	Donor 2 (78.8 kg.) Pheneth-icillin	PHA	Total	% of PHA
1	254	280	534	52.4	428	170	598	28.4
2	369	380	749	50.7	417	300	717	41.8
3	369	345	714	48.3	494	280	774	36.2
4	204	160	364	44.0	374	220	594	37.0
6	77	40	117	34.2	145	70	215	32.5

Hours	Donor 3 (50.7 kg.) Pheneth-icillin	PHA	Total	% of PHA	Donor 4 (78.8 kg.) Pheneth-icillin	PHA	Total	% of PHA
1	2414	1300	3714	35.0	390	370	760	48.7
2	963	850	1813	46.9	902	550	1452	62.1
3	277	380	657	57.8	471	770	1241	62.0
4	131	200	331	60.4	390	360	750	48.0
6	41	50	91	55.0	101	80	181	44.2

[a] PHA = Phenoxyethyl penicilloic acid.

phenoxymethyl penicilloic acid was added to furnish concentrations of 0, 0.25, and 0.5 mg./ml. These solutions, together with standard phenoxymethyl penicilloic acid preparations as in Procedure D, were submitted to Procedures C and D. Recoveries ranged from 92 to 100%. Similar results were obtained with phenethicillin and phenoxyethyl penicilloic acid.

Extraction Efficiency—Under the described conditions the extraction of phenoxymethyl and phenoxyethyl penicilloic acids from aqueous solutions by chloroform was between 88 and 92% complete. This was confirmed by assaying, by nitration, samples containing 0.5 mg./ml. and 1 mg./ml. directly and after extraction.

DISCUSSION

The yellow quinoid radical produced by nitration of these compounds that originate from the phenolic group could be produced if phenol or its derivatives were present in the sample used for nitration.

During numerous assays of urine samples without penicillin or penicilloic acid, the blank value of the chromatographed chloroform extract was close to or similar to the normal blank value, indicating that phenolic compounds present in urine do not interfere with this assay. The difference in R_f values for penicillins and for the corresponding penicilloic acids probably derives from the fact that the former are monobasic acids, which generally have high values compared with dibasic acids to which class the latter belong. The R_f value of phenoxyacetic acid in the method used is higher than that of penicillin; possible interference by this acid, if present in the extract, is thus eliminated.

The data given in Tables I and II provide an example only of the information obtainable using this method. No attempt has been made to correlate the results with liquid intake, excretion volumes, or other controllable factors.

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Kinetics and Mechanisms of Drug Action on Microorganisms X: Action of Spectinomycin on *Escherichia coli* by Microbial Kinetics

JOBST B. MIELCK and EDWARD R. GARRETT*

Abstract □ The steady-state growth of *Escherichia coli* cultures, $N = N_0 e^{k_0 t}$, is slowly inhibited by spectinomycin to a new steady state with a new rate constant, k_{app}. The $k_{app.}$ is linearly dependent on drug concentration, S, above a certain minimum concentration of spectinomycin, S^*; *i.e.*, $k_{app.} = k_0 - k_S(S - S^*)$. This minimum concentration is a function of the concentration of the media and can be assigned to binding or removal of microbiologically effective spectinomycin as protonated material by the components of the media. The logarithm of the inhibitory constant, k_S, linearly increases with the pH of the media to pH 7.6, and this implies that only uncharged material is biologically active. The slow rate of achievement of a drug-equilibrated, steady-state, microbial generation rate can be reconciled with a relatively rapid reequilibrated rate on dilution with fresh media by postulating depletion of a cell-generated vital metabolite linked to the growth rate of the microorganism.

Keyphrases □ Microorganisms—mechanism, kinetics, drug activity □ Kinetics, mechanism—spectinomycin activity □ Spectinomycin action, *E. coli*—kinetics, mechanism □ pH, organism population, nutrient concentration, effects—spectinomycin-affected generation rates □ *E. coli* generation rates—spectinomycin concentration

The aminoglycoside spectinomycin has antibacterial activity against a variety of Gram-positive and Gram-negative microorganisms (1). The inhibitory action is bacteriostatic and is reversed by washing the drug-affected cells (2). The antibiotic forms a stoichiometric 1:1 complex with the 30-S ribosomal subunit extracted from *Escherichia coli*. The formation of this complex blocks some steps in the translocation of the peptidyl-RNA from the acceptor site for aminoacyl *t*-RNA to the peptidyl donor site. Protein synthesis is thus inhibited (2).

The determination of the generation rates of *E. coli* as a function of the concentrations of a variety of antibiotics and chemotherapeutic agents has established a useful procedure to quantify the effects of drugs on the time course of bacterial generation (3–8).

The purposes of this study are to determine kinetically the inhibitory effects of spectinomycin on the generation rate of *E. coli* in defined concentration ranges and to evaluate the effects of variable inoculum size, concentration, and pH of the culture medium on drug activity.

EXPERIMENTAL

Test Organism—Replicate slants of *E. coli* strain B/r were used in all experiments. The slants had been prepared from a single colony and were stored in a refrigerator at 4°.

Culture Media—Antibiotic medium 3[1] was rehydrated according to the specifications of the manufacturer. The media were filtered twice through Millipore 0.45 μ HA filters and autoclaved at 120° for 15 min. The pH of the media was 7.05 ± 0.05. Various amounts of Millipore-filtered 1.7 *N* HCl and 2.0 *N* NaOH, respectively, were added aseptically after the sterilization to obtain pH values within a range of 7.50 to 5.87 for the investigation of the anti-

[1] Bacto Antibiotic Medium 3, Difco Laboratories, Detroit, Mich.