Determination of *p*-Hydroxyphenoxy Methyl Penicillin in Phenoxymethyl Penicillin Fermentation Samples

By JAN BIRNER

A method based on paper chromatography and biological assay has been developed for the determination of *p*-hydroxyphenoxy methyl penicillin in phenoxy methyl penicillin (penicillin V) fermentation samples. The penicillin broth is extracted into methyl isobutyl ketone. The extract is chromatographed in triplicate using Whatman No. 1 paper treated with sodium citrate at pH 5.5 and ethyl ether saturated with 28 percent ammonium sulfate. The penicillins are located by reference to a strip cut from the chromatogram and developed colorimetrically. The appropriate areas are then cut out, eluted separately with 1 percent phosphate buffer, and the eluates are determined by biological assay. As the R_f values for phenoxymethyl penicillin and *p*-hydroxyphenoxy methyl penicillin under the conditions described are, respectively, 0.60 and 0.13, the separation is complete.

PARA-HYDROXYPHENOXY methyl penicillin was first detected in phenoxymethyl penicillin (penicillin V) fermentation broth samples by de Flines *et al.* (1). According to these authors the mold (*Penicillium chrysogenum*, strain WLS 49-133) is able either to oxidize phenoxyacetic acid to *p*-hydroxyphenoxy acetic acid and utilize this compound as a precursor for the synthesis of *p*-hydroxyphenoxy methyl penicillin or else can hydroxylate phenoxymethyl penicillin directly.

The *p*-hydroxyphenoxy methyl penicillin was isolated by the above-mentioned workers in the form of crystalline free acid.

Although its antibacterial spectrum is similar to that of penicillin V, *p*-hydroxyphenoxy methyl penicillin is much less stable in acid solutions. Vanderhaeghe *et al.* (2) found *p*-hydroxyphenoxy methyl penicillin as a metabolite of penicillin V in urine, and they have separated the two compounds by means of paper chromatography.

During the manufacture of penicillin V by fermentation, the assay of samples by one or other of the commonly used methods (hydroxylamine, iodimetric, or biological) usually furnishes values representing the sum of the penicillins contained in them. If they are to be estimated individually it is necessary that they be first separated. The procedure described shows how this may be done.

EXPERIMENTAL

Reagents, Solutions, and Materials—Sulfuric acid 12 *N* A.R.; ammonium sulfate A.R.; sodium sulfate anhydrous A.R.; methyl isobutyl ketone, redistilled sodium citrate A.R.; ethyl ether redistilled; starch, soluble laboratory reagent; 0.1 *N* iodine in 4% potassium iodide A.R.; glacial acetic acid; ammonia 0.880 sp. gr.; phosphate buffer 1% pH = 7.0; Whatman No. 1 paper for chromatography.

Procedure—Pipet 2 ml. of filtered broth into a suitable centrifuge tube fitted with a glass-ground stopper; add approximately 2 g. of solid ammonium sulfate and 0.1 ml of 12 *N* sulfuric acid. Mix by swirling this solution (pH approx. 2.0), add from a pipet 5 ml. of methyl isobutyl ketone (M.I.B.K.), stopper the tube, shake vigorously for 45 sec., and then centrifuge for 5 min. Transfer a portion of the organic layer into another dry tube by means of a Pasteur pipet, dry, by shaking with a small amount of anhydrous sodium sulfate, and finally transfer it by filtration into a third dry test tube.

Treat Whatman No. 1 chromatography paper by passing through a 10% solution of sodium citrate, adjusted to pH 5.5 by hydrochloric acid, followed by drying. Using a microsyringe (capacity 20–50 µl., in 1 µl. divisions), apply to the paper three aliquots, each 20 µl. of the extract. From a freshly prepared standard solution of penicillin V in methanol containing 1 mg. of the antibiotic per ml., apply 10 µl. as a control. The four spots are placed 5 cm. from the bottom of the paper and 5 cm. apart.

Chromatography is performed by the ascending technique. Redistilled ethyl ether to be used as development solvent is equilibrated at room temperature against an ammonium sulfate solution, prepared by dissolving 40 Gm. of the salt in 100 ml. of water. Suspend the chromatogram in a suitable chromatographic tank (or in a jar lined with filter paper) containing equilibrated ethyl ether and a vessel with water.

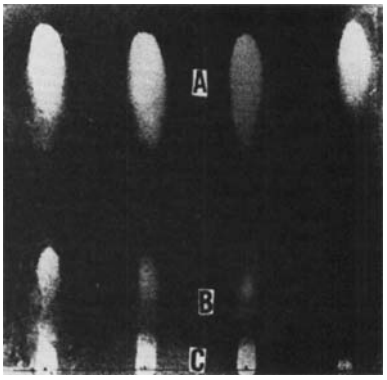

Fig. 1—Chromatogram for methyl isobutyl ketone extract of phenoxymethyl penicillin. Key: A, phenoxymethyl penicillin; B, p-hydroxyphenoxy methyl penicillin; C, biologically inactive.

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TABLE I—RESULTS OF ASSAYS OF FERMENTATION SAMPLES

Fermentation Batch No.	Units of Penicillin Recovered from Chromatogram From 8 μl. of Broth: Pen. V	p-Hydroxy Pen. V	Total	10 mcg., Pen. V[a]	Control Assayed Directly, in Units	p-Hydroxy Pen. V, %
1	58.5	2.20	60.70	16.60	67.20	3.78
2	76.20	3.38	79.58	17.10	87.00	4.24
3	56.00	2.00	58.00	16.50	63.90	3.44
4	66.15	2.76	68.91	16.38	77.40	4.00
5	60.30	2.46	62.76	16.50	66.60	3.90

[a] 1 mcg. pen. V equals 1.695 unit.

After an hour's equilibration, start chromatography from outside by pushing to the bottom of the tank the rod on which the paper is suspended. Following development (up to 16 hr.), cut off the first strip containing 20 μl. of the extract, hold for a few sec. over a jet of steam, then hang for 10 min. in a closed jar containing concentrated ammonia. Spray the strip with starch-iodine solution, prepared by mixing 50 ml. of 1% soluble starch solution, 3 ml. glacial acetic acid, and 1 ml. 0.1 *N* iodine. The white spots on a blue background, which appear after a few min., indicate the position of the corresponding penicillins. A photograph of a chromatogram developed as a whole colorimetrically is shown as Fig. 1. The colored strip is used as a template for locating the penicillins on the remaining part of the chromatogram. Cut out the corresponding areas of the chromatogram (sample in duplicate and standard penicillin V), place in 1.9 cm. × 15.2 cm. test tubes and elute with a measured amount of 1% phosphate buffer pH 7.0. The amount of buffer used for elution per tube is 4 ml. for the *p*-hydroxyphenoxy methyl penicillin and 10 ml. for phenoxymethyl penicillin. Elution is achieved by shaking the stoppered test tubes for 0.5 hr. on a shaking machine. Transfer the same volume of original organic extract as was applied to the chromatogram (20 μl.) directly into a test tube and extract with 10 ml. of buffer to serve as a control. Assay biologically the penicillin content of the solutions as penicillin V and calculate the percentage of *p*-hydroxyphenoxy methyl penicillin.

RESULTS

The data for experiments performed on five samples taken from penicillin fermentation tanks are shown in Table I.

The completeness of extraction of penicillin V (99.82% recovery) under the described conditions was confirmed by biological assay.

The developed method permits the determination of the *p*-hydroxyphenoxy methyl penicillin in the fermented broth during all stages of the fermentation. It was also used in the consequent processes of penicillin extraction and purification and as a qualitative test in the final product.

REFERENCES

(1) de Flines, J., Waisvisz, J. M., Hoette, I., and Struyk, A. P., *Antibiot. Chemotherapy*, **7**, 497(1957).
(2) Vanderhaeghe, H., Parmentier, G., and Evrard, E., *Nature*, **200**, 891(1963).

Keyphrases

p-Hydroxyphenoxy methyl penicillin—analysis
Phenoxymethyl penicillin fermentations—*p*-hydroxyphenoxymethyl penicillin determination
Paper chromatography—separation
Biological analysis—penicillins

Antifertility Factors from Plants I. Preliminary Extraction and Screening

By CHARLES F. BARFKNECHT and HUNG CHIH PENG

Three plants were examined for possible antifertility effects. *Prunus emarginata* and *Lonicera ciliosa* were found to reduce litter production in mice, while *Lysichitum americanum* was not active. The initial extraction and screening procedures are described.

THE ETHNOBOTANICAL literature contains a report of the use of plant teas to affect the reproductive processes by Indians of the Northwest U. S. Specifically, the roots of *Lysichitum americanum*, the leaves of *Lonicera ciliosa*, and the wood of *Prunus emarginata* were used for antifertility and abortifacient effects (1). Generally the women drank the teas prepared from the ground plants, but some tribes made no distinction on the sex of the person. The report also failed to mention a dosage regimen, but apparently more than one dose was necessary to obtain the antifertility effect. It is the purpose of this study to verify the antifertility activity and determine the constituents responsible.

Cranston (2) studied the effect of *Lithospermum ruderale* extracts on mice. *Lithospermum* was used by Indians of the Southwest U. S. to affect reproduction. She prepared an ethanolic extract

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