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Note

Determination of *p*-hydroxypenicillin V, *p*-hydroxyphenoxyacetic acid, phenoxyacetic acid, and penicillin V in production fermentation broth

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The analytical methodology for penicillin V (phenoxymethylpenicillin, or 6-phenoxyacetamidopenicillanic acid) includes a large number of techniques. High-performance liquid chromatographic (HPLC) methods are replacing many of the older procedures because of selectivity advantages. There is in the literature HPLC methodology for penicillin V in production intermediates[1], in degradation product mixtures[2], in suspensions[3], and in solid dose forms[4]. *p*-Hydroxyphenoxymethylpenicillin was first detected in phenoxymethylpenicillin fermentation broth samples in 1957[5]. Paper chromatographic and thin-layer chromatographic methods exist in the literature[6,7] for both *p*-hydroxyphenoxyacetic acid and *p*-hydroxyphenoxymethylpenicillin. In addition, phenoxyacetic acid, the precursor for the synthesis of penicillin V is a frequently mentioned substance in the previously enumerated references. As of this writing, however, no report exists which comprehensively treats the precise quantitation of all the significant components found in penicillin broths.

In order to understand the mechanism of formation of *p*-hydroxyphenoxymethylpenicillin, knowledge of the levels of hydroxylated precursor and the precursor (phenoxyacetic acid) at different fermentation times is required. In our analytical laboratory, the availability of the electrochemical detector has greatly improved the selectivity and sensitivity of assays for broth constituents. A recent article[8] has already illustrated the advantages of the electrochemical detection of phenol substituted penicillins. This report also describes a proposal for the mechanism of the oxidation of the phenol-bearing side chain. We have used a standard 5.0 μl electrochemical detector connected in series behind a low-volume (2.4 μl) ultraviolet detector cell. In this manner, the two hydroxylated species (*p*-hydroxypenicillin V and *p*-hydroxyphenoxyacetic acid) and the two non-hydroxylated species (phenoxyacetic acid and penicillin V) can all be detected on the same chromatogram from a single injection and without the need of a gradient.

Our report compares the new combination detector system to a two injector, two mobile phase–HPLC procedure that had been in previous use. The older procedure required approximately three times as much instrument time per sample as the newer procedure but worked reliably for several years.

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EXPERIMENTAL

Apparatus

A modular liquid chromatograph was equipped with a variable-wavelength ultraviolet spectrophotometer (Model 85; Perkin-Elmer, Norwalk, CT, U.S.A.) set at 220 nm, a pump (Model M45; Waters Assoc., Milford, MA, U.S.A.), an electrochemical detector (Model LC 4A; Bioanalytical Systems, West Lafayette, IN, U.S.A.), a strip-chart recorder with two inputs (Model 291MM; Linear Instrument Corp., Irvine, CA, U.S.A.), an autoinjector (Model LC-420, Perkin-Elmer), and associated on-line computers (Model 21 MX mini; Hewlett-Packard, Palo Alto, CA, U.S.A.). The ultraviolet detector measured penicillin V and phenoxyacetic acid at 220 nm. Effluent from the ultraviolet detector was routed in series to the electrochemical detector where oxidation currents were monitored at +0.8 V with respect to a silver/silver chloride reference.

The detector signal was sampled by the on-line computer at a rate of 200 points/min. The chart speed was set at 1.5 min/cm and the upper pen used for the electrochemical signal while the lower pen was employed for the ultraviolet signal. A 3-mm offset in the trace of the signals occurs on the chart even though the same substance can be detected nearly simultaneously on both detectors.

The HPLC column packing was an Altex Ultrasphere ODS, 5 μm material (Catalogue 256-05; Altex Scientific, Berkeley, CA, U.S.A.). The mobile phase was acetonitrile–ammonium phosphate buffer (prepared from a 1.5% phosphoric acid solution, buffered to pH 3.0 with ammonium hydroxide) (30:70). All injections were made on stream with a 20-μl sample loop. The flow-rate was set to 1.0 ml/min.

Reagents

An HPLC-grade acetonitrile was employed. All other chemicals were reagent-grade.

Standard preparation

Corporate reference standard phenoxymethylpenicillin potassium salt, *p*-hydroxyphenoxymethylpenicillin, phenoxyacetic acid, and *p*-hydroxyphenoxyacetic acid (received from the Lilly Research Labs.) were prepared in pH 6.0 phosphate buffer 0.1 *M*, to contain 50 μg/ml, 10 μg/ml, 10 μg/ml, and 2 μg/ml, respectively. Solutions with each standard substance at twice and one-half the previously described respective concentrations were also used to verify linearity of response on a periodic basis.

Sample preparation

Penicillin broth was filtered through a No. 1 Whatman paper, and appropriate serial dilutions were made with pH 6.0, 0.1 *M* potassium phosphate buffer. The solution was additionally micro-filtered prior to injection.

RESULTS AND DISCUSSION

Typical chromatograms are shown in Fig. 1. Chromatogram A displays the ultraviolet response (bottom trace) and the electrochemical response (upper trace)

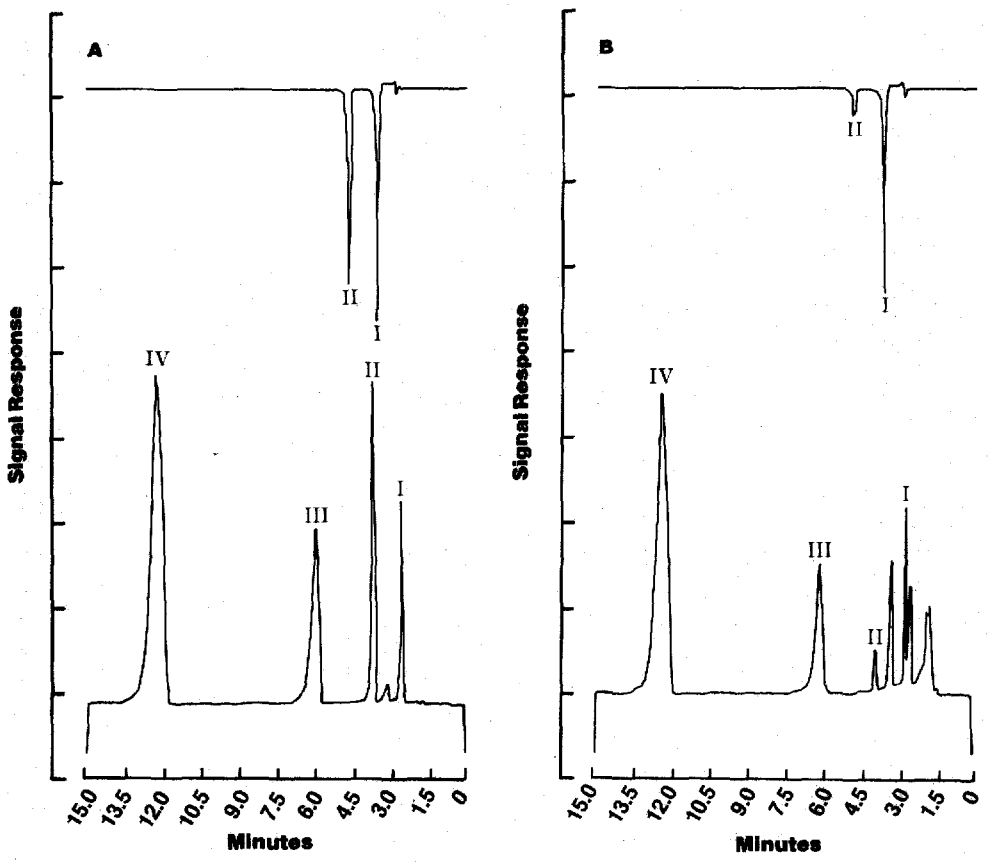

Fig. 1. HPLC separations. Chromatogram A displays the ultraviolet responses (lower trace) and the electrochemical responses (upper trace) for a four-component standard mixture. Chromatogram B displays the corresponding peak responses from a sample of preharvest broth. The retention times are measured from the time of injection. Peaks: I = *p*-hydroxyphenoxyacetic acid; II = *p*-hydroxypenicillin V; III = phenoxyacetic acid; IV = penicillin V.

for the four standards. Chromatogram B displays the same responses for a preharvest broth. All four substances respond to ultraviolet detection, but only the hydroxylated materials respond to the electrochemical detector. As can be seen, there are virtually no other electrochemically active substances other than the two expected compounds.

Linearity of response was evaluated. For the ultraviolet detector, the usual linear curve with no appreciable intercept was observed. The linear correlation coefficient was 0.999 and 0.999 for phenoxyacetic acid and penicillin V, respectively. For the electrochemical detector a correlation coefficient of at least 0.999 was also obtained for both the *p*-hydroxyphenoxyacetic acid and *p*-hydroxypenicillin V at the periodic intervals of test. Again, a negligible intercept was found. The concentration ranges were those described in the standard preparations.

The precision of the method was determined by the analysis of 19 aliquots of a lyophilized harvested broth. A relative standard deviation of 1.19% was obtained for the assay of the penicillin V. Because of the very low levels of other impurities

in the lyophilized harvest broth, the relative standard deviations for these impurities were necessarily higher. A relative standard deviation of 2.91% was obtained for the *p*-hydroxypenicillin V, 3.20% for the *p*-hydroxyphenoxyacetic acid, and 5.0% for the phenoxyacetic acid. The latter deviation was high because the level was close to its detection limit. Re-evaluation on a broth containing higher levels of phenoxyacetic acid gave a lower deviation (0.95%).

The newer method being reported was also compared to an older corporate procedure. The older established procedure consisted of an assay in two parts with two mobile phases, two injectors, and a chemically different HPLC packing. The new method was applied to several fermentation broths over a predetermined period of time. Table I shows the comparison, and the data agree well. In order to avoid stating the details of the fermentation process, times have been indicated as fractions of harvest time, and only the ratio of the old assay to new assay has been shown. In the case of the *p*-hydroxyphenoxyacetic acid, one could conclude that some false high

TABLE I

COMPARATIVE ASSAY RESULTS BASED ON RATIO OLD TO NEW ASSAY

Lot	*p-Hydroxyphenoxyacetic acid*	*Phenoxyacetic acid*	*p-Hydroxypenicillin V*	*Penicillin V*
616-1	0.81	1.19	1.06	1.08
617-1	1.00	1.02	1.10	1.01
618-1	0.86	0.82	1.03	1.00
619-1	0.79	0.96	1.35	1.03
L1	0.95	2.12	1.06	0.96
629-.3	2.70	0.94	0.96	1.20
634-.2	0.50	0.75	0.45	1.03
L2	0.94	0.95	1.14	0.95
629-.5	2.60	0.97	1.09	0.91
634-.3	8.00	1.06	0.65	0.74
635-.7	1.02	1.06	1.06	1.02
636-.7	0.93	1.08	1.17	1.00
637-.7	0.96	1.12	1.09	1.02
638-.6	0.86	1.09	1.09	0.99
639-.6	0.91	1.15	1.05	1.04
L3	0.81	0.99	1.07	1.01
629-1	0.86	0.89	1.04	1.02
634-.9	0.82	1.00	0.87	1.03
640-.7	1.38	0.99	*	*
643-.6	1.32	1.00	0.96	0.99
L4	1.26	0.72	1.00	1.00
633-1	1.03	1.04	1.06	1.02
634-1	0.75	1.03	0.96	1.03
636-1	0.59	1.70	0.88	1.06
L5	0.97	1.63	1.04	1.03
635-.9	0.91	1.10	0.98	1.10
636-.7	1.14	1.08	1.44	0.98
632-.7	0.95	1.00	1.41	0.96
T15-.4	2.50	9.50	1.09	1.05
L6	0.89	0.97	1.18	0.95

* Insufficient sample for comparison assay.

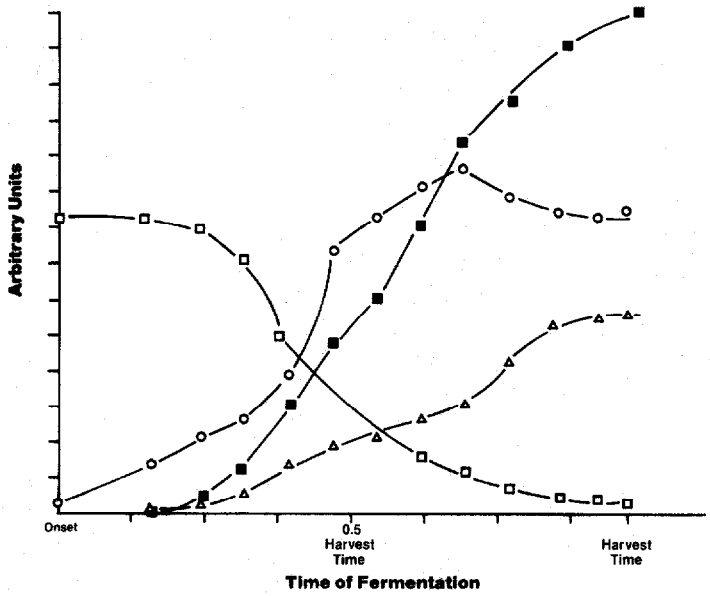

Fig. 2. Consumption and formation plots. The four major components in a penicillin broth, *p*-hydroxyphenoxyacetic acid (○), *p*-hydroxypenicillin V (△), phenoxyacetic acid (□), and penicillin V (■) are indicated by relative concentration as a function of fermentation time.

data were produced by the older procedure, especially in the early fermentation periods before harvest (when peaks were small and baselines high). With the basis of good agreement and shorter assay time now established, data were collected on a number of fermentations so that an investigation of the kinetics of the formation and removal of impurities could be carried out. Fig. 2 shows a characteristic, but not necessarily process-definitive, demonstration of how the four measured species vary with fermentation time. The concentrations are displayed relative to each maximum concentration rather than to their absolute values, and the time is shown as a fraction of the harvest time. Plots such as seen in this figure have much value in determining process strategy and in process control.

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