

## Stability of Amoxicillin-Clavulanate in BACTEC Medium Determined by High-Performance Liquid Chromatography and Bioassay

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**The stabilities of amoxicillin (16 µg/ml) and clavulanate (8 µg/ml), alone and in combination in BACTEC medium (Middlebrook 7H12B medium), were determined by high-performance liquid chromatography (HPLC) and bioassay. By HPLC, the half-life of amoxicillin (trihydrate and sodium) in combination with clavulanate in nonradiolabelled 7H12B medium was 6.7 days, whereas the half-life of clavulanate in combination with amoxicillin was 2.0 days. By bioassay, the half-lives of amoxicillin trihydrate and clavulanate in radiolabelled 7H12B medium were comparable (7 and 2 days, respectively) to those determined by HPLC. When clavulanate was tested alone, the half-life was determined to be 1.88 days by HPLC and 1.87 days by bioassay. The relatively short half-life of clavulanate can be adjusted by a procedure of "topping up," or adding one-half the concentration of clavulanate every second day, in order to allow accurate amoxicillin-clavulanate MIC testing with the BACTEC mycobacterial susceptibility system.**

The BACTEC system for radiometric detection of mycobacterial growth is often used to determine the drug susceptibilities of slowly growing mycobacteria (3). Although this system is currently one of the most rapid methods of determining susceptibility, it often requires an incubation period of 7 to 10 days. Susceptibility testing for evaluating the effectiveness of amoxicillin-clavulanate against slowly growing organisms may be hampered by the relative instability of clavulanate, which can result in decreased activity as the incubation period progresses. High-performance liquid chromatography (HPLC) and bioassay methods have been used to determine the half-lives of amoxicillin trihydrate and amoxicillin sodium in combination with clavulanate (16 and 8 µg/ml, respectively) in BACTEC media (with and without the radiolabelled substrate) for a 7-day incubation period at 37°C. Clavulanate alone was also tested under these same conditions. On the basis of the present study, a "topping-up" procedure is recommended when testing the slowly growing mycobacteria against amoxicillin-clavulanate in the BACTEC system.

Amoxicillin trihydrate, amoxicillin sodium, and lithium clavulanate were obtained as reference standards from SmithKline Beecham Pharmaceuticals, Philadelphia, Pa. BACTEC medium without the radiolabelled substrate (BWR) was prepared in-house and consisted of Middlebrook 7H9 broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.), casein hydrolysate, bovine serum albumin, and catalase (Sigma Chemical Co., St. Louis, Mo.) at a pH of 6.8 ± 0.2. Amoxicillin in combination with clavulanate (16 and 8 µg/ml, respectively) was prepared in 30 ml of BWR on 4 separate trial days. Eight milliliters was distributed into three separate test tubes, and the test tubes were incubated at 37°C for 7 days. A 0.5-ml aliquot was removed from each tube at 0, 6, 12, 24, 30, 36, and 48 h and at 3, 4, 5, 6, and 7 days. Samples were stored at -70°C until the day of analysis. For the HPLC assay, concentrations of 2 and 1 to 64 and 32 µg of amoxicillin and clavulanate per

ml, respectively, in BWR served as standards on each day of analysis. BWR was used as a drug-free control.

HPLC analysis was performed with an Ultrasphere octyldecyl silane, 5-µm, C<sub>18</sub>, 4.6-by-2.5-cm column (Beckman Instruments Inc., Fullerton, Calif.). The mobile phase was 92% 0.1 M phosphate buffer (pH 3.2) with 8% (vol/vol) HPLC-grade methanol, and the flow rate was 1.5 ml/min. The UV detector (Shimadzu Corp., Kyoto, Japan) wavelength was 227 nm, and the injection volume was 0.1 ml. The retention times for amoxicillin and clavulanate were approximately 8.9 and 3.7 min, respectively. These retention times were comparable to those described in previous studies (2, 4), which analyzed both components by HPLC in biological fluids. Samples were maintained at 5°C within the Waters model 717 autosampler (Millipore Waters Chromatography, Milford, Mass.) throughout the analysis. All peak heights were recorded with a Shimadzu CR501 Chromatopac integrator (Shimadzu Corp., Kyoto, Japan). The half-life of each of the compounds was determined by using the least-squares regression of log concentration versus time. Determination of the 95% confidence intervals was calculated in Microsoft Excel version 4.0 by using the following formula (1):

$$\frac{t \cdot s}{|\hat{\beta}_1|} \sqrt{\left(\frac{1}{m} + \frac{1}{n}\right) + \frac{(\bar{x}_0 - \bar{x})^2}{S_{xx}}}$$

where  $m$  is the slope,  $n$  is the number of sample concentrations, and  $t$  is the tabled  $t$  value at 95% confidence,  $\hat{\beta}_1$  is the least squares estimator of  $\beta_1$ , and  $S$  is the sum of squares of the distance between  $x$  measurement and its mean.

The bioassay method (5) for amoxicillin used *Micrococcus luteus* ATCC 9341 in antibiotic medium 2 (Difco Laboratories, Detroit, Mich.) and an inoculum of 0.6 ml of a 48-h nutrient broth culture per 100 ml of agar.

Clavulanate was assayed by using *Klebsiella pneumoniae* ATCC 29665 in nutrient agar containing 60 µg of benzyl penicillin per ml and an inoculum of 3 ml of an 18-h tryptone soy broth culture per 100 ml of agar.

Preparation of standards and dilution of aliquots within the assay range (amoxicillin, 0.02 to 1.0 µg/ml; clavulanate, 0.08 to

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TABLE 1. Half-life of amoxicillin (trihydrate and sodium) in combination with clavulanate and clavulanate alone determined by HPLC and bioassay

Agent(s)	Half-life (days) <sup>a</sup>	95% Confidence intervals
Amoxicillin trihydrate in combination with clavulanate (16/8 µg/ml)	6.65 (7.07) <sup>b</sup>	±0.8 (0.53)
Amoxicillin sodium in combination with clavulanate (16/8 µg/ml)	6.41	±0.4
Clavulanate in combination with amoxicillin trihydrate (16/8 µg/ml)	2.00 (1.89)	±0.5 (0.19)
Clavulanate in combination with amoxicillin sodium (16/8 µg/ml)	1.99	±0.8
Clavulanate alone (8 µg/ml)	1.88 (1.87)	±0.5 (0.40)

<sup>a</sup> Half-lives are averages of four trials for HPLC and three trials for bioassay.

<sup>b</sup> Values in parentheses are results determined by bioassay.

5.0 µg/ml) were performed in pH 6.5 phosphate buffer. Holes of 7 mm were cut into the agar in large assay plates (32 by 40 mm), and the 80-µl volumes of the solutions were filled into them in duplicate in a pattern designed to minimize bias. The plates were incubated overnight at 30°C (amoxicillin) and 37°C (clavulanate), and the diameters of the zones of inhibition were read to the nearest 0.1 mm. Drug concentrations were calculated from a regression of zone diameter versus log concentration.

The half-lives of amoxicillin (trihydrate and sodium) and clavulanate in combination and the half-life of clavulanate alone are presented in Table 1. The half-life of amoxicillin trihydrate in combination with clavulanate determined by HPLC was  $6.65 \pm 0.8$  days (average of four trials), and the half-life determined by bioassay was  $7.07 \pm 0.53$  days (average of three trials). When amoxicillin sodium was tested under the same conditions, the half-life of amoxicillin was  $6.41 \pm 0.4$  days. The half-lives of clavulanate in combination with amoxicillin trihydrate and amoxicillin sodium determined by HPLC were  $2.00 \pm 0.5$  and  $1.99 \pm 0.8$  days (average of four trials), respectively. The half-life of clavulanate in combination with amoxicillin trihydrate determined by bioassay was  $1.89 \pm 0.19$  days

(average of three trials). When clavulanate was tested independently by HPLC and bioassay, the half-lives were  $1.88 \pm 0.5$  and  $1.87 \pm 0.4$  days, respectively.

The results of the present study indicate that the concentration of amoxicillin in BACTEC medium will decrease to one-half the initial concentration within a 7- to 10-day incubation period, whereas clavulanate will reduce to one-half the initial concentration in 2 days.

Watt et al. (6) described a method for replenishing antibiotic concentrations for unstable agents in the BACTEC radiometric susceptibility test system. They referred to this procedure as "topping up." The data from the present study support a topping-up procedure in which half the initial concentration of amoxicillin and clavulanate is added every 6 and 2 days, respectively, to the BACTEC susceptibility test vials. Since the half-life of amoxicillin is approximately 6 to 7 days and the incubation period for mycobacteria in the BACTEC system is often 7 to 10 days, it probably is not crucial that the amoxicillin concentration be increased or topped-up. Further studies involving topping up with clavulanate are necessary to determine if this procedure will make a significant difference in MICs when testing amoxicillin-clavulanate with slowly growing mycobacteria in the BACTEC susceptibility test system.

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