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**A RAPID AND SIMPLE METHOD FOR  
DETECTION OF  $\beta$ -LACTAMASE  
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(Received for publication May 1, 1978)

Following the discovery of staphylococcal  $\beta$ -lactamases<sup>1)</sup> and the subsequent clinical importance of these enzymes<sup>2,14)</sup>, search for  $\beta$ -lactamase inhibitors was initiated. This search included approaches such as (a) testing of synthetic chemicals of diverse structure<sup>4,13,17)</sup>; (b) using penicillinase-neutralizing antisera<sup>4,7,11)</sup>; (c) synthesizing new  $\beta$ -lactams with increased stability to  $\beta$ -lactamases and; (d) searching for  $\beta$ -lactamase inhibitors of natural origin. The first two approaches were only of academic interest, whereas the new semisynthetic penicillins and cephalosporins virtually solved the clinical problem created by  $\beta$ -lactamase-producing staphylococci. The therapeutic problems created by the  $\beta$ -lactamase-producing Gram-negative bacteria have only partly been solved by the introduction of the newer  $\beta$ -lactams<sup>5)</sup>. More recently, much interest has been generated by the discovery of potent  $\beta$ -lactamase inhibitors of biological origin<sup>6,8,9,13)</sup>, of which clavulanic acid has been best characterized<sup>3,12)</sup>. There still exists strong interest in the search for inhibitors of  $\beta$ -lactamases, therefore, we wish to describe a simple and rapid method for the primary screening of such materials.

The procedure presented here utilizes the unique property of nitrocefim<sup>10)</sup>, a weakly-active cephalosporin whose color changes from light yellow to red with  $\beta$ -lactamase hydrolysis of the amide bond of the  $\beta$ -lactam ring (Glaxo chromogenic cephalosporin compound 87/312). The color change is a result of an electron shift along the molecule that contains at the 3'-position a highly conjugated 2,4-dinitrostyryl moiety. The method is facilitated by the development of a colorless semisynthetic peptone-glucose-buffered (PGB) medium allowing for optimal visualiza-

tion of color change (purple-red<sup>16)</sup>). Clavulanic acid was employed to standardize the test procedure.

The most sensitive test system utilizes an 18-hour culture of *Klebsiella pneumoniae* 1200 grown in the above-mentioned colorless medium; 0.5 ml of this bacterial suspension is distributed in small test tubes to which are added in 0.05 ml volumes serial dilutions of the test inhibitor. Inhibitor is not added to a control tube. After mixing, the tubes are held for five minutes at room temperature and one drop of a 0.05% nitrocefim solution is added to each tube. The nitrocefim solution was prepared by first dissolving 2.5 mg nitrocefim in 0.25 ml dimethyl-sulfoxide followed by the addition of 4.75 ml 0.1 M phosphate buffer, pH 7.0. In the control tube, a purple-red color develops within a few minutes. The tubes containing inhibitors (eg. clavulanic acid) remain colorless at concentration capable of inhibiting the available  $\beta$ -lactamase. In this system, a final concentration of  $\leq 1 \mu\text{g/ml}$  of clavulanic acid completely inhibits the development of color (Table 1), consistent with results obtained with biologic methods.<sup>12)</sup>

The method was developed by examining 14  $\beta$ -lactamase-producing strains, as well as a series of crude  $\beta$ -lactamase preparations obtained by rupture of bacterial cells. Table 1 shows the results obtained with some of these enzyme sources with respect to the onset and degree of color change over a one-hour time period. The sensitivity of the method can be quite variable depending upon the  $\beta$ -lactamase-producing organism or crude enzyme preparation employed in the test and thus the results obtained are obviously influenced by the quality and quantity of the  $\beta$ -lactamase. Thus, manipulation of these parameters could result in marked changes in the sensitivity of the test. This also would be true with respect to the specificity of the inhibitors employed.<sup>10)</sup> The grown cultures could be stored in the cold for long periods of time without significant loss of enzyme activity as was true for the crude frozen enzyme preparations.

A rapid, simple method is described that can be employed for the screening of specific, broad-spectrum  $\beta$ -lactamase inhibitors. This test appears to be as sensitive as the biological test for

Table 1. Inhibitory effect of clavulanic acid on the color-development of nitrocefin in the presence of beta-lactamase

Origin of beta-lactamase	Onset of color	Color with sodium clavulanate (Final conc. $\mu\text{g/ml}$ )					
		0(Control)	1	2	5	10	20
<i>Klebsiella pneumoniae</i> 1200 (grown culture)	Immediate	Pink	No	No	No	No	No
	10 minute	Purplish	No	No	No	No	No
	30 minute	Purple	No	No	No	No	No
	60 minute	Purple	No	No	No	No	No
<i>Proteus mirabilis</i> 346 (grown culture)	Immediate	Pink	Yellow	No	No	No	No
	10 minute	Purplish	Yellow	No	No	No	No
	30 minute	Purple	Purplish	No	No	No	No
	60 minute	Purple	Purplish	Yellow	No	No	No
<i>E. coli</i> 804 (grown culture)	Immediate	Purple	Pink	Pink	Yellow	No	No
	10 minute	Purple	Purplish	Pink	Pink	Yellow	No
	30 minute	Purple	Purplish	Purplish	Pink	Pink	No
	60 minute	Purple	Purple	Purple	Purplish	Pink	Yellow
<i>E. coli</i> 804 (lysed culture diluted $\times 100$ )	Immediate	Pink	Pink	No	No	No	No
	10 minute	Pink	Pink	No	No	No	No
	30 minute	Purplish	Pink	No	No	No	No
	60 minute	Purple	Pink	No	No	No	No
<i>Staphylococcus aureus</i> 671 (grown culture)	Immediate	Pink	Pink	No	No	No	No
	10 minute	Purplish	Purplish	Yellow	No	No	No
	30 minute	Purple	Purple	Purplish	Yellow	No	No
	60 minute	Purple	Purple	Purple	Pink	Pink	No

detection of clavulanic acid, a known  $\beta$ -lactamase inhibitor.<sup>12)</sup> This "color-development inhibition" test can also be used for studying the enzyme kinetics as well as enzyme-substrate-inhibitor relationships.

#### Acknowledgment

The authors express their sincere thanks to Glaxo Research Ltd. (Dr. P. W. MUGGLETON) and Beecham Pharmaceuticals Research Division (Drs. F. P. DOYLE and M. COLE) for the generous donation of the research samples of nitrocefin and clavulanic acid, respectively.

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