AN H.P.L.C. ASSAY FOR CEPHALOSPORINASE ACTIVITY

R. J. MEHTA, M. K. FOX*, D. J. NEWMAN and C. H. NASH

Microbiology and Analytical Chemistry*
Departments, Smith Kline & French Laboratories
Philadelphia, Pennsylvania 19101, U.S.A.

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The β-lactamases (E. C. 3.5. 2.8) are a heterogeneous group of enzymes that have the capability of cleaving the β-lactam ring of penicillin and cephalosporin antibiotics. These enzymes are commonly found in various clinically important Gram-positive and Gram-negative microorganisms. Many techniques including hydroxylamine, microbiological, iodometric, acidimetric/alkalimetric and spectrophotometric have been used for the assay of β-lactamases because no single technique has proven ideal for all situations (SYKES & MATTHEW, 1976)[1].

Recently, reverse phase high performance liquid chromatography (HPLC) has been used for the detection and separation of the cephalosporin antibiotics (WHITE *et al.*, 1975;[2] MILLER & NEUSS, 1976[3]). In this paper we wish to report how this technique can be used for a rapid stoichiometric assay of cephalosporinases, simply by measurement of the substrate concentration remaining after the reaction.

The culture used in this study was a clinical isolate of *Bacteroides fragilis* subsp. *fragilis* (strain H-154) and was grown under anaerobic conditions in SCHAEDLER's medium (BBL) at 37°C. Log phase cells (7 hours) were harvested in air by centrifugation at $20{,}000 \times g$ for 15 minutes at 4°C and washed twice with 20 volumes of ice-cold deionized water.

Five grams (wet weight) of the washed cells were mixed with 5 g of ballotini (Superfine, 3 M Company, St. Paul, Minnesota) in 50 ml of 10 mM potassium phosphate buffer, pH 7.0 containing 5 mM dithioerythritol (Pierce Chemical Co., Rockford, Illinois). The ice-cold suspension(0°C) was sonicated using a Branson Sonifier, Model #J-17A, with a 3-cm probe at a power setting of 100 watts for a total of 5 minutes in 30-second bursts with 2-minute cooling intervals. During the sonication, the temperature was not allowed to rise above 8°C. The cell debris were removed by centrifugation at $20{,}000 \times g$ for 15 minutes at 4°C followed by a further centrifugation of the supernatant solution at $100{,}000 \times g$ (Beckman L2) for 90 minutes at 4°C. The clear supernatant solution from this treatment was used as the enzyme source after assaying for protein concentration by the LOWRY[4] and/or the WARBURG & CHRISTIAN[5] techniques, taking care to compensate for the effects of the dithioerythritol.

All reagents used were of at least "reagent grade" quality, and except where noted, were obtained from Fisher Scientific Co., Pittsburgh, Pennsylvania. The HPLC system was built by the Analytical and Physical Chemistry Department, and comprised a 15×0.46 cm column of C18 bonded Lichrosorb (Applied Science Inc. State College, Pennsylvania) coupled to a pump (model # 26980-4, Haskel Engineering & Supply, Burbank, California), a UV detector (model #153, Altex Scientific Inc., Berkeley, California) and a 20 μl injection loop (cat. #190A48; Chromatronix Inc., Berkeley, California). This system was run at room temperature, at a pump pressure of 55 atm. and at a flow rate of 1.4 ml·min^{-1}.

The enzyme assay was run at 37°C with a 5-ml reaction mixture containing 50 mM potassium phosphate, pH 7.0; 2.5 mM dithioerythritol and different concentrations of cephalosporin C (Eli Lilly and Company, Indianapolis, Indiana). The reaction was started by the addition of the enzyme ($0 \sim 60$ μg of protein), and at different time intervals, 500 μl aliquots were withdrawn, frozen at -78°C and stored at -70°C until needed.

Just prior to HPLC assay, the frozen aliquots were diluted to a suitable known volume with 2.2 mM potassium phosphate, pH 4.0, and 20 μl of this solution was injected into the column *via* a loop system. The column was developed with 2.2 mM potassium phosphate, pH 4.0, and the amount of cephalosporin C remaining was determined by comparison with standards run under the same conditions in the absence of enzyme. Replicates were within $\pm 5\%$ once amplification and dilution factors were taken into account.

The linearity of the assay under the conditions described is shown in Fig. 1 (with respect to enzyme concentration) and Fig. 2 (with respect to time). In both cases, the points are means of replicates and the initial substrate concentration was 5.7 mM. In Fig. 3, we show a representative LINEWEAVER-BURK plot obtained with this assay

Fig. 1. β-Lactamase assay with respect to enzyme concentration

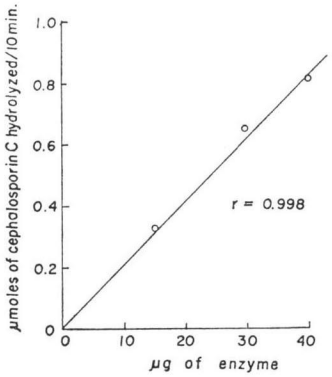


Fig. 2. β-Lactamase assay with respect to time

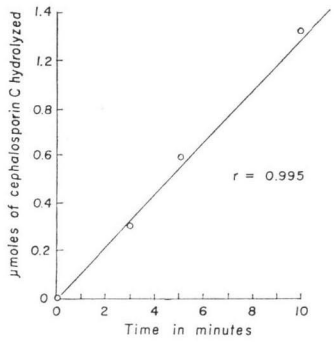
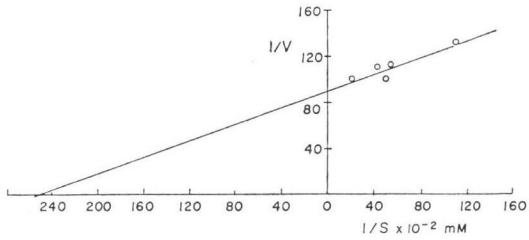


Fig. 3. LINEWEAVER-BURK plot of β-lactamase
Cephalosporin C HPLC. pH 5.5, 37°C, Km=0.4 mM



and the β-lactamase from *B. fragilis*, thus giving an indication of the range of substrate concentration over which the assay can be effectively used.

The HPLC assay is relatively fast and very reproducible. In addition, it may be used at substrate concentrations and conditions well outside of those permissible with the UV or biological techniques. Finally, the separation of the products of the hydrolysis from the remaining substrate during the assay allows one to investigate the hydrolysis pattern of the substrate under varying conditions if so desired.

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