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Fluorimetric Determination of Ampicillin by use of Non-toxic Catalysts. Estimation of β-Lactamase Activity and Parameters

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Abstract

A fluorescence assay based on the use of biological reducing agents as catalysts rather than heavy metal ions has been developed for estimation of ampicillin concentrations.

The assay is based on the conversion of ampicillin to its penicilloate, by treatment with sodium hydroxide, then neutralization, dilution with 0·5 M acetate buffer at pH 4 and heating for 30 min at 100°C in the presence of ascorbic acid (0·5 mg) and EDTA (50 μM). Reduced glutathione, cysteine and *N*-acetylcysteine also catalysed the development of fluorescence. A practical sensitivity range of 0·5–50 μM ampicillin was used. The assay was used to estimate ampicillin in some biological solutions to which the antibiotic has been added. Milk, blood serum, trypticase soy broth and nutrient broth containing 25 μM antibiotic assayed at 18·5, 21·7, 16·5 and 14·7 μM, respectively, with standard deviations between 1·2 and 0·7%. The low results were attributed to binding of some ampicillin by proteins or peptides which were removed by pretreatment. Urine containing 5 mM ampicillin assayed at 4·97 mM with a standard deviation of 3%. A modification of the procedure was used to measure β-lactamase activity against ampicillin in several organisms in a fixed time assay. Kinetic parameters of a commercial β-lactamase preparation from *Bacillus cereus* could also be determined by an additional modification. In both instances a correction was required for the intrinsic fluorescence of ampicillin remaining in the solution. The preparation examined had a Michaelis constant (K_m) of 0·32 mM, a maximum velocity (V_{max}) of 5398 μmol ampicillin hydrolysed mg^{-1} min^{-1}, an apparent catalytic constant (K_{cat}, turnover number) of 20 220 s^{-1} and a K_{cat}/K_m ratio of $0{\cdot}63 \times 10^7$ M^{-1} s^{-1}.

The major advantage of using this assay technique is that toxic metals are not used in the development of fluorescence so it is more environmentally acceptable. The technique is useful for examining β-lactamase activity against ampicillin and might be useful for pharmaceutical products—for determining available therapeutic levels and for monitoring the activity of penicillin acylase against the penicilloate of ampicillin.

Ampicillin concentrations in biological fluids can be measured by several colorimetric procedures (Smith et al 1967; Bundgaard 1974), HPLC (Tsiju & Robertson 1975) and fluorimetry (Jusko 1971; Miyazaki et al 1974; Barbhaiya & Turner 1977). Fluorimetric methods for analysis of ampicillin are based on treatment to form its penicilloate (ampicilloate) then heating with formaldehyde (Jusko 1971; Barbhaiya & Turner 1977) or mercuric chloride (Miyazaki et al 1974). Some colorimetric methods use copper (Smith et al 1967) or mercuric chloride (Bundgaard 1974). These catalysts are very toxic and their continued use, even in small amounts, is now environmentally undesirable. This investigation was undertaken to develop a fluorimetric assay for ampicillin which eliminated such toxic reagents from analytical solutions. Special objectives of the work were the development of an analytical technique suitable for estimation of ampicillin in biological fluids and for measurement of β-lactamase enzyme activity.

Materials and Methods

Instruments

A Shimadzu SP 500 spectrophotofluorimeter was used for fluorescence readings and a Metrohm AG 396 pH meter for pH readings. Absorbance measurements were obtained by means of a Cary 3E spectrophotometer.

Chemicals

Ampicillin, reduced glutathione, cysteine, *N*-acetylcysteine and β-lactamase (from *Bacillus cereus* EC 3.5.2.6) were obtained from Sigma (St Louis, MO) and extra pure ascorbic acid from Merck (Darmstadt, Germany). Amoxycillin was donated by Fawn and MacCallum of Croydon and disodium ethylenediaminetetraacetic acid (EDTA) was purchased from May and Baker (Footscray, Melbourne, Australia). Ampicilloate and the penicilloate of amoxycillin (amoxycilloate) were prepared by treatment with alkali then neutralization.

Procedure

In the basic method ampicilloate was formed by treatment of 1 vol. ampicillin solution with 1 vol. 0·1 M NaOH for 10 min. The solution was neutralized with 1 vol. 0·1 M HCl (or a volume containing the equivalent amount of hydrogen ion) and then diluted to 20 vols with 0·5 M buffer pH 4. Ampicilloate solution (concentration range 0·05–50 μM; 2 mL) was treated with reagent containing ascorbic acid (0·5 mg mL^{-1}) and EDTA (50 mM) in acetate buffer pH 4 (0·5 M; 1 mL). The mixture was heated at 100°C for exactly 30 min, cooled, and the fluorescence read using an excitation wavelength of 344 nm and an emission wavelength of 450 nm.

Deproteination of solutions

Ampicillin was estimated in blood serum, milk, trypticase soy broth or nutrient broth (1 mL of each), containing proteins or peptides, to which ampicillin (25 nmol mL^{-1}) had been added. Solutions were treated with NaOH (1 M; 0·1 mL) and left at room temperature. After 10 min a mixture (0·2 mL) of HCl (0·5 M) and trichloroacetic acid (26%; final concentration 4%) was added, and the solution mixed and centrifuged at 10 000 *g* for 10 min. Quadruplicate samples of the supernatant fluid (0·1 mL) were diluted to 2 mL with 0·5 M acetate buffer at pH 4, treated with ascorbic acid/EDTA reagent, and heated as described above. A standard calibration curve for ampicilloate was prepared in the concentration range 0·5–5 μM. Unheated solutions were used to correct for any observed intrinsic fluorescence of the proteins and peptides present.

β-Lactamase activity

β-Lactamase preparations and bacterial suspensions (0·4 mL) in phosphate buffer (0·05 M; pH 7) were mixed with ampicillin (6·25 mM; 1·6 mL) in the same buffer. Samples (0·1 mL) were assayed for ampicilloate after mixing with trichloroacetic acid (8%; 0·1 mL) at 4°C, diluting to 20 mL with acetate buffer (0·5 M; pH 4), centrifuging, and adding 2 mL (duplicates) to ascorbic acid/EDTA solution (1 mL) and heating as described above. When calculating the amount of ampicilloate formed the contribution of the intrinsic fluorescence of ampicillin was taken into account by use of equation 1:

$$[\text{Ampicilloate}] = [F_{\text{test}} - F_{\text{ampicillin}}] \times 50/[F_{\text{ampicilloate}} - F_{\text{ampicillin}}] \quad (1)$$

where F_{test} was the fluorescence of the test solution, $F_{\text{ampicillin}}$ was that of 50 nmol of ampicillin in 2 mL of diluted substrate which was compared with $F_{\text{ampicilloate}}$ the arbitrary relative fluorescence value 100 given by 50 nmol of ampicilloate in 2 mL.

The Michaelis constant for β-lactamase activity on ampicillin was determined by mixing enzyme solution (50 μL) with ampicillin solutions (50 μL containing 0·4, 0·133, 0·08, 0·057, 0·044, 0·036 and 0·031 mM ampicillin) in phosphate buffer (pH 7; 0·05 M). After 5 min incubation at 37°C, cold trichloroacetic acid (8%; 0·1 mL) was added. This solution (0·2 mL) was diluted to 2 mL with acetate buffer (pH 4; 0·5 M) and ascorbic acid/EDTA solution (1 mL) was added before heat treatment. The fluorescence of 0·2 mM ampicilloate was used as the 100% relative fluorescence reference point for the experiment. The relative fluorescence of different concentrations of ampicillin, used as substrates, and the total ampicilloate formed therefrom, were determined. These values were substituted in equation 1. The corresponding and respective concentrations of substrate used were also substituted in the equation (replacing the "50") to enable accurate calculation of the concentration of ampicilloate formed in each reaction mixture. Three experiments were performed (using 7·5, 14·9 and 47 ng protein and substrate concentrations in quadruplicate) and results were treated statistically (Wilkinson 1961), using a weighting factor of 1 for each point.

Results

Development of fluorescence

Maximum fluorescence was obtained after heating ampicilloate for 20 min (Figure 1) but 30 min heating was used routinely to allow for any possible variations which might occur in the rate of development of the fluorescence, and to give a standard heating time. Fluorescence was relatively stable after cooling but ca 7·5% of the fluorescence was lost during the next hour. A small amount of

fluorescence developed slowly in a reaction at room temperature but there was a very long lag phase.

Fluorescence spectra

Excitation peaks occurred at 344 nm and 379 nm and the emission spectrum peaked at 450 nm with a small shoulder at 425 nm. Omission of ascorbic acid resulted in only one excitation and one emission peak with maxima at 330 nm and 435 nm, respectively.

Concentration of ascorbic acid

Maximum fluorescence was obtained by use of 0·3 mg mL^{-1} ascorbic acid in 0·5 M acetate buffer pH 4 (Figure 2) but a concentration of 0·5 mg mL^{-1} was used routinely to ensure an adequate concentration in the event of any extraneous oxidation of some of the ascorbic acid. Maximum fluorescence also required EDTA which would chelate trace metal ions to prevent them oxidizing ascorbic acid (Taqui-Khan & Martel 1967).

Standard curves

The standard curves for ampicilloate were linear between 0·5 and 50 μM (Figure 3) and the intrinsic fluorescence of the same concentration of untreated ampicillin was also linear, but lower. Readings from solutions containing less than 0·5 μM ampicilloate were inconsistent and blanks were unacceptably high. A linear spectrophotometric standard curve for ampicilloate was obtained by reading absorbance at 379 nm. A sensitivity range of 50–300 μg mL^{-1} was obtained.

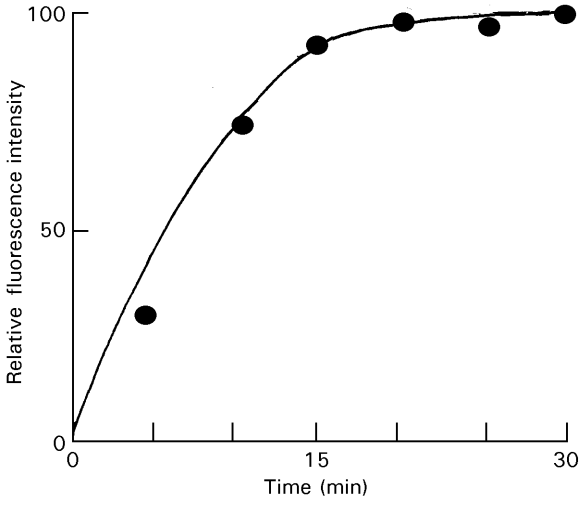

Figure 1. Effect of heating time at 100°C on the development of fluorescence of 50 nmol ampicilloate in the presence of ascorbic acid and EDTA.

Precision

The mean of 30 estimates of 25 μM ampicilloate (the mid point range) was 25·75 μM with a standard deviation of 0·14 μM (1·1%). The line of best fit to the points for ampicilloate on the standard curve (10 points), within the concentration range 5–50 μM was $y = 4{\cdot}8x - 1{\cdot}4$. The correlation coefficient (r)

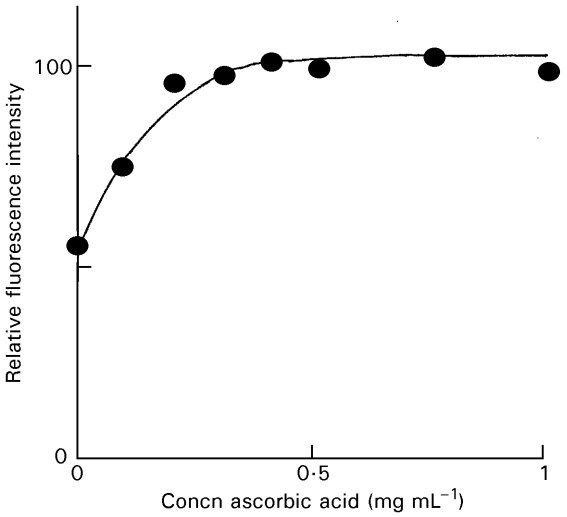

Figure 2. Effect of ascorbic acid concentration on the fluorescence obtained after heating 50 nmol ampicilloate at 100°C for 30 min.

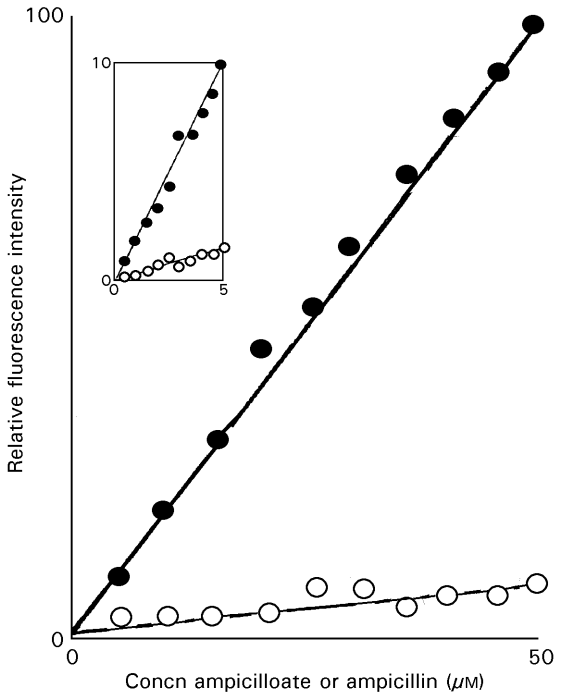

Figure 3. Standard calibration curve for ampicilloate (●) within the concentration range 5–50 μM. The open symbols (○) indicate the fluorescence obtained after heating intact ampicillin under the same conditions. The inset shows results obtained from standards within the concentration range 0·5–5 μM.

was 0·95. The stability and level of precision is quite acceptable for many purposes, especially enzyme kinetics.

Required reactants and interference

Fluorescence was maximum in 0·5 M acetate buffer between pH 4 and pH 5 and pH 4 was used routinely. It depended on the presence of both EDTA and ascorbic acid (Table 1). Reduced glutathione, at normal serum levels (2 mM; Meister & Anderson 1983), also catalysed development of strong fluorescence but cysteine and *N*-acetylcysteine were less effective. Amoxicilloate fluoresced at longer wavelengths (excitation 386 nm; emission 476 nm) and any potential interference from this substance could be minimized by exciting the ampicilloate at the shorter of the two wavelengths.

Ampicillin in biological fluids

Results from analyses of several biological fluids to which ampicillin had been added at levels which might be encountered during therapy, are shown in Table 2. Results from water and urine are close to the amounts added but the amounts found in the other solutions were lower, suggesting the probable binding of some ampicillin to the precipitated proteins or peptides, with only free ampicillin being measured. Binding effects would be observed with most chemical analytical methods of similar sensitivity so the assay might be useful for determining available therapeutic levels. The microbiological media both showed a small amount of intrinsic fluorescence which required correction.

β-Lactamase activity

The β-lactamase activity determined in uninduced cultures of the Gram-positive *Bacillus subtilis* was low in comparison with the enzyme activity found in *Bacillus cereus* (Table 3). The activity of the Gram-negative *Escherichia coli* was low in comparison with that of *Pseudomonas aeru-ginosa*. Enzyme was inhibited by treatment with trichloroacetic acid.

Rates of formation of ampicilloate by a commercial enzyme preparation from *B. cereus* (Sigma) and the enzymes in cells of *Alcaligenes faecalis* ATCC 15246 are shown in Figure 4; these indicate that the technique is useful for fixed time-rate studies.

Enzyme kinetics

Determination of the Michaelis constant for the commercial enzyme preparation from *B. cereus* was simple. The apparent Michaelis constant (K_m) was 0·32 mM (standard error 37·3%). This result is close to a range reported for some other preparations of *B. cereus* β-lactamases and obtained by use

Table 1. Effect of added compounds on the development of fluorescence.

Reaction mixture	Relative fluorescence
Ampicillin	16·8
Ampicilloate + no additions	50
Ampicilloate + EDTA	70
Ampicilloate + ascorbic acid	82
Ampicilloate + ascorbic acid + EDTA	100
Ampicilloate + reduced glutathione + EDTA	92
Ampicilloate + cysteine + EDTA	77
Ampicilloate + *N*-acetylcysteine + EDTA	83
Amoxycillin + ascorbic acid + EDTA	0
Amoxycilloate + ascorbic acid + EDTA	6·6

Each reaction mixture (2 mL) contained ampicillin, ampicilloate, amoxycillin or amoxycilloate (50 nmol). Solution containing the different additives (1 mL) was then added and the mixture was heated. Additions were 0·5 mg ascorbic acid, 50 μmol EDTA, or 2 μmol of thiol.

Table 2. Estimation of ampicillin in biological fluids.

Sample	Concentration added (nmol mL^{-1})	Analytical result (nmol mL^{-1})	Relative standard deviation (%)
Water	25	25·2	0·7
Urine	5000	4970	3
Blood serum	25	18·5	1·1
Milk	25	21·7	1·2
Trypticase soy broth	25	16·5	1·2
Nutrient broth	25	14·7	0·7

Ampicillin was added to each solution at the concentration shown. The solution (1 mL) was taken, NaOH (1 M; 0·1 mL) was added, and the solution was left to stand for 10 min to form the ampicilloate. Trichloroacetic acid solution (26%; 0·2 mL) containing HCl (0·5 M) was added to the solutions and these were mixed, left to stand for 10 min, and then centrifuged. The supernatant fluid (quadruplicate 0·1-mL samples) was added to acetate buffer (pH 4, 0·5 M; 1·9 mL) before addition of ascorbic acid/EDTA solution and further treatment. Before analysis urine was diluted with buffer such that the ampicilloate concentration was within the range 0·5–5 μM. A standard curve within the concentration range 0·5–5 μM was used.

Table 3. Estimation of β-lactamase activity in bacteria.

Organism	Activity (mM ampicilloate formed mL^{-1})
Bacillus cereus	5
Bacillus subtilis	0·52
Escherichia coli K12	0·48
Pseudomonas aeruginosa	3·3

The reaction temperature was 37°C and the reaction time 14 h. The reaction mixture contained ampicillin (6·25 mM; 1·6 mL; final concentration 5 mM) and bacterial cell suspension (0·4 mL). Samples (0·1 mL) were treated with cold trichloroacetic acid (8%, 0·1 mL), left for 1 min and then diluted to 20 mL with acetate buffer (pH 4; 0·5 M), with mixing. Duplicate samples (2 mL) were treated with ascorbic acid/EDTA solution and heated. A standard calibration curve within the concentration range 5–50 μM was used for the experiments.

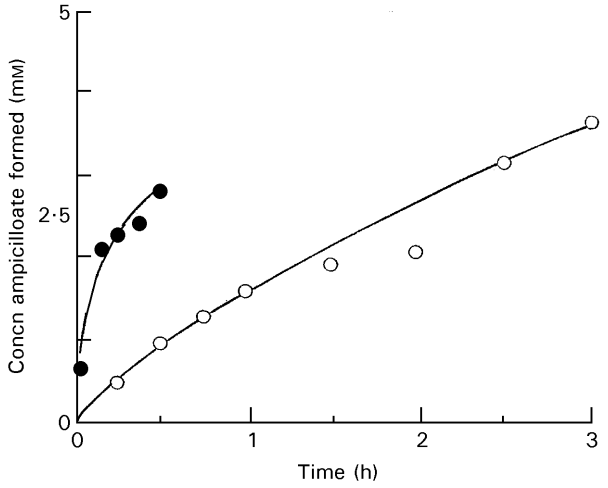

Figure 4. Rate of formation of ampicilloate by treatment of ampicillin (5 mM) with commercial β-lactamase (10 μg) (●) and with a cell suspension of *A. faecalis* ATCC 15246 (○). The reaction temperature was 37°C.

of different analytical techniques and different conditions (Kuwabara & Abraham 1967; Waley 1975). Sodium dodecylsulphate polyacrylamide gel electrophoresis SDS–PAGE (Laemmli 1970) showed that the preparation used also contained other protein matter. The apparent V_{max} was 5398 μmol ampicillin hydrolysed $(mg\,protein)^{-1}$ min^{-1} (standard error 25·9%), the apparent turnover number (K_{cat}) was 20 220 s^{-1} (average M_r 28 kDa) and the K_{cat}/K_m value for ampicillin was $0{\cdot}63 \times 10^7\,M^{-1}\,S^{-1}$. The technique was suitable for a fixed-time assay for enzyme assays and kinetics and obviated any need to ensure that the rate of ampicilloate formation and the rate of production of indicator product reached a steady state, as is necessary in the microiodometric procedure (Sykes & Nordstrom 1972).

Discussion

This simple fluorimetric procedure is sensitive, relatively specific and obviates the need for sample clean-up before analysis. Analysis can be performed over a wide range of concentrations at pH 4. Toxic chemicals are not used as catalysts, and thus not discarded as wastes, making the procedure more environmentally acceptable than other fluorescence methods used for analysis of ampicillin (Jusko 1971; Miyazaki et al 1974; Barbhaiya & Turner 1977). The fluorescent product(s) had spectral properties different from those reported by these earlier workers and is probably a different compound, because it is formed with reducing agents and could not be extracted with ethyl acetate. In the presence of methanol some metal ions catalyse the formation of fluorescent derivatives of ampicillin (Navarro et al 1998). These have fluorescent properties slightly different from those of the product formed in this study but the different solvent and catalysts limit comparisons until the fluorescent compounds are purified.

This analytical procedure might be useful for pharmaceutical preparations containing excipients. Differences between the fluorescence of ampicilloate and ampicillin suggest that little ampicillin is converted to its penicilloate at pH 4 under the conditions which normally favour formation of penicillenates (oxazolones) from intact penicillins (Herriott 1946). This fluorimetric enzymic assay is particularly suitable for reactions of β-lactamase with ampicillin and possibly amoxicillin and some cephalosporins. 6-Aminopenicillanic acid and its open β-lactam form (penicic acid) does not react, which might also be useful in investigation of the effect of different penicillin acylases on ampicilloate.

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