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A new spectrophotometric method for the selective determination of ampicillin, amoxycillin and cyclacillin in the presence of polymers and other degradation products

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Abstract: A rapid and convenient spectrophotometric method is described for the quantitative determination of ampicillin and other amino-penicillins. The method involves conversion of the penicillins to the corresponding piperazine-2,5-dione derivatives by heating in an alkaline sorbitol–zinc ion solution for 10 min at 60°C and subsequent treatment of these derivatives with 1 M sodium hydroxide to give a highly absorbing product with λ_{max} at 322 nm. Since an intact β-lactam moiety and a free amino group in the side-chain of the penicillin molecules are required for the piperazinedione formation, the method is highly selective. The method was found to be free of interference from polymerization and other degradation products and its application to assess the stability of the amino-penicillins was demonstrated.

Keywords: *Spectrophotometric assay*; *ampicillin*; *amoxycillin*; *cyclacillin stability*; *piperazine-2,5-dione*.

Introduction

In a recent investigation of the stability of ampicillin (I) in aqueous solutions containing various carbohydrates or polyhydric alcohols the formation of a piperazine-2,5-dione derivative (V) as a major degradation product was demonstrated [1]. The product was shown to result from an intramolecular aminolysis of the α-aminobenzylpenicilloyl esters (IV) initially formed from reaction of ampicillin with the hydroxy compounds [1] (Scheme 1). In subsequent studies [2, 3] the piperazine-2,5-dione derivative was also found to be a major product of degradation of ampicillin in phosphate buffers or solutions containing imidazole or phenol. It was observed that on treatment with 0.5–2 M sodium hydroxide, the derivative rapidly transformed to a product showing a strong absorption with maximum at 322 nm [2].

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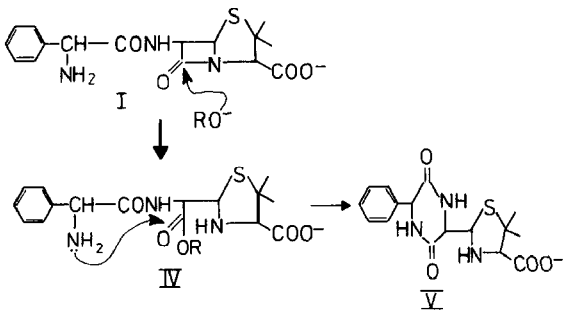
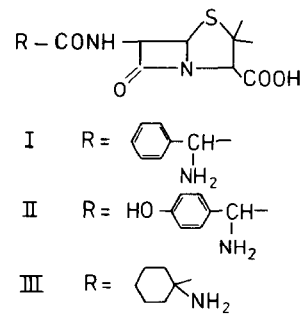
On the basis of these findings a convenient and rapid spectrophotometric method for the determination of ampicillin and other clinically used amino-penicillins (such as amoxycillin (II) and cyclacillin (III)) has been developed. Since both an intact amino side-chain and an intact β-lactam ring are required for the piperazinedione formation, the method is highly specific and has been shown to be useful as a means of assessing the stability of the amino-penicillins.



Scheme 1



Scheme 2

Experimental

Apparatus

Ultraviolet spectroscopic measurements were performed with a Shimadzu UV-190 spectrophotometer using 1 cm silica cuvettes. The pH measurements were made using a Radiometer Type PHM 26 instrument. High-performance liquid chromatography (HPLC) was carried out with a Spectraphysics model 3500 B instrument equipped with a 10 μl loop injection valve. The column used (100 × 4.7 mm i.d.) was packed with LiChrosorb RP-8 (5 μm particles).

Materials and reagents

Ampicillin sodium, amoxycillin trihydrate and cyclacillin, all with a purity better than 97% as determined by TLC [4], were commercial products or from lots used in a previous study [5]. The piperazine-2,5-dione derivative (V) of ampicillin was available from a previous study [1], as were ampicillin di- and polymers [6, 7].

Sorbitol reagent

A 20% m/v solution of sorbitol in a 0.2 M carbonate buffer containing 15 μg/ml of Zn^{2+} was used, the pH of the final solution being 9.25 ± 0.05. Zinc(II) ions were added in the form of zinc(II) sulphate.

Analytical procedure

The aqueous solution of the amino-penicillin sample to be analysed was prepared to contain about 0.8 mg/ml of the penicillin. Two equal samples of 500 μl (A and B) of this solution were pipetted into separate test tubes. To A 5.00 ml of the sorbitol reagent was added, the tube was stoppered and placed in a water bath at 60°C for 10 min. Then 500 μl of the solution was pipetted into a cuvette containing 2000 μl of 1 M sodium hydroxide and the absorbance was monitored at 322 nm using a recorder in series with the

spectrophotometer, until maximum absorbance was reached, in 1–3 min as discussed below. A mixture of one part of water and four parts of 1 M sodium hydroxide was used as the reference solution. Sample B was mixed with 5.00 ml of water and treated with sodium hydroxide as described for sample A. The difference in absorbance between A and B was calculated, and the penicillin concentration of the original sample determined by reference to a standard curve or to a standard sample analysed simultaneously. It should be noted that in those cases where the penicillin solution to be analysed contained no piperazinedione product, solution B could be omitted from the procedure.

Results and Discussion

The method developed for the determination of ampicillin and other amino-penicillins involved the following steps: (a) reaction of the penicillins with sorbitol to produce the corresponding piperazinedione derivative via a penicilloyl ester intermediate, and (b) treatment of this derivative with 1 M sodium hydroxide to give a highly absorbing product with λ_{max} at 322 nm.

Various carbohydrates (e.g. glucose and fructose) and polyhydric alcohols (e.g. sorbitol and glycerol) have previously [1, 8–10] been shown to react with several penicillins to produce penicilloyl esters, which subsequently undergo hydrolysis to produce penicilloic acid or which, in the case of penicillins containing an amino group in the side-chain, undergo a fast intramolecular aminolysis to yield a piperazine-2,5-dione derivative. Sorbitol was selected for the analytical method as the hydroxy compound necessary to produce the intermediate penicilloyl ester because of its high reactivity with penicillins [10], but other hydroxy compounds may also be useful. Fructose and glucose, however, are not suitable because high blank absorbance values are obtained on treatment with 1 M sodium hydroxide.

Optimum conditions for the assay imply a rapid and reproducible conversion of the amino-penicillins to the corresponding piperazinedione derivatives. The following factors were found to affect the conversion and the stability of the piperazinedione derivative as investigated for ampicillin: pH, sorbitol concentration, zinc(II) ion concentration and temperature.

Effect of sorbitol concentration

The rate of reaction of penicillins with sorbitol and, accordingly, the rate of piperazinedione formation increases linearly with sorbitol concentration in neutral and alkaline solutions [10]. A concentration of 20% m/v in the reagent solution was therefore selected as optimum.

Effect of pH

The rate of penicilloylation of sorbitol and similar alcohols by penicillins has been found to be directly proportional to the hydroxide ion concentration up to a pH of about 11 [8, 10]. Therefore a high pH ensures rapid reaction. The yield of the piperazinedione product was, however, found to decrease with increasing pH at pH values greater than 9 (Fig. 1). The decreasing yield at higher pH values may be due to the fact that the hydroxide ion-catalysed hydrolysis of the intermediate penicilloyl ester becomes increasingly predominant in relation to the spontaneous cyclization of ester to piperazinedione

[1]. Furthermore, it was observed that at a pH value greater than about 9.6 the piperazinedione showed a slow degradation after its formation (Fig. 2). Consequently, a pH of 9.25 was chosen, as this appears to be optimal as regards yield and time of reaction.

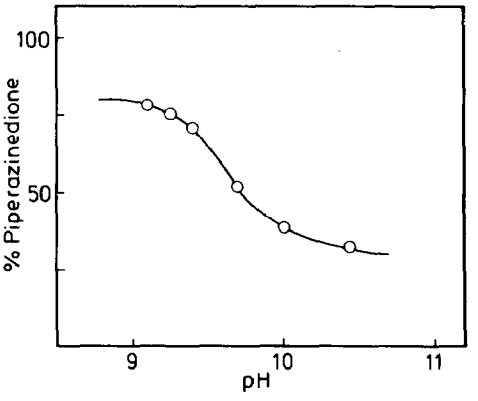


Figure 1
Effect of pH on the yield of the piperazinedione derivative (V) formed by reaction of ampicillin sodium with the sorbitol reagent at 60°C.

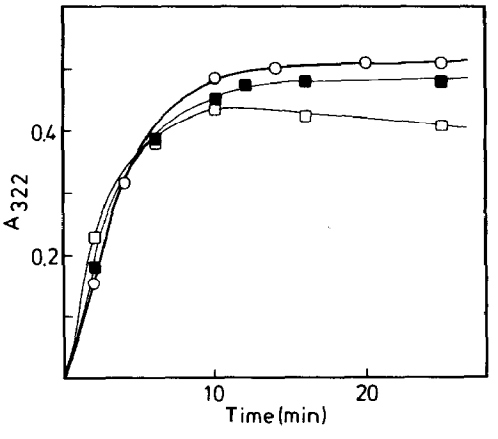


Figure 2
Effect of pH on the formation of the piperazinedione derivative (V) by reaction of ampicillin sodium with the sorbitol reagent at 60°C. Key: ○ pH 9.25; ■ pH 9.50; □ pH 9.80.

Effect of zinc(II) ions

Various metal ions, including iron(II), copper(II) and zinc(II), ions have previously been found to catalyse the degradation of penicillins in methanol [11] and in solutions containing fructose [9, 12, 13], lactate [14] and tris(hydroxymethyl)aminomethane [15]. In the case of fructose the iron(II) ion catalysis has been demonstrated to be a catalysis of penicilloyl ester formation [9]. As shown in Fig. 3, zinc ions were found to be an efficient catalyst for the penicilloylation of sorbitol and, accordingly, for the conversion of the amino-penicillins to the piperazinedione products. In Fig. 4 the half-life for the piperazinedione formation from ampicillin is plotted as a function of zinc(II) ion concentration in the sorbitol reagent. It can be seen that the maximal catalytic effect is reached at a metal ion concentration of about 8 μg/ml. The zinc(II) ion concentration selected for the final reagent composition was 15 μg/ml rather than a lower figure, because preferably the method should also be applicable to the analysis of solutions in

the presence of small amounts of metal-complexing agents such as citrate. Thus at the stated zinc concentration, addition of citrate to the test solution to give a concentration of 0.02 M was found to have no effect. As can be seen from Fig. 3, the overall formation of piperazinedione does not follow strict first-order kinetics, but is characterized by a marked induction period. This arises from the initial formation of a penicilloyl ester of sorbitol as previously described [1].

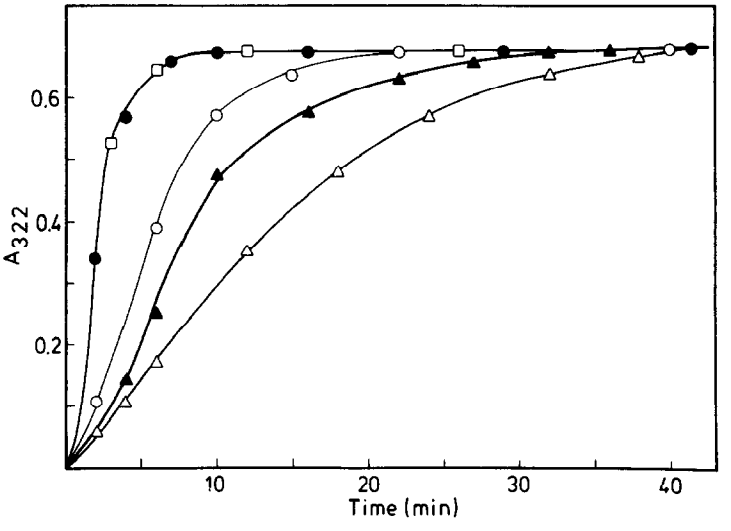


Figure 3
Time-course of the conversion of ampicillin sodium to the piperazinedionc derivative (V), as a function of the concentration of zinc(II) ions in the sorbitol reagent (at 60°C). Key: △ none; ▲ 1 μg/ml; ○ 2.5 μg/ml; ● 10 μg/ml; □ 15 μg/ml.

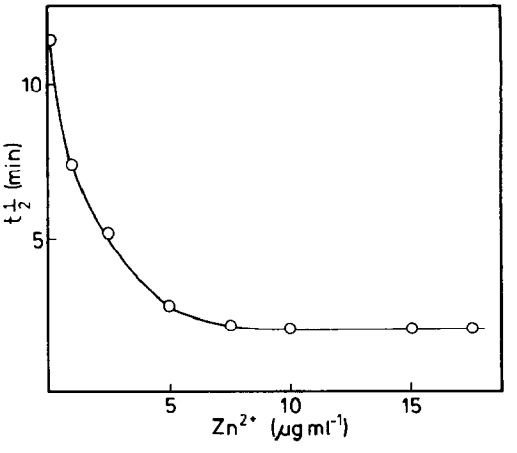


Figure 4
Effect of zinc(II) ion concentration in the sorbitol reagent on the half-life of conversion of ampicillin to the corresponding piperazinedione derivative.

Effect of temperature and time of heating

At 60°C the conversion of both ampicillin, amoxycillin and cyclacillin was found to be complete after 10 min (cf. Fig. 3). Inconveniently long reaction times are needed at room

temperature. As seen from Fig. 3, it is not critical to stop the reaction after 10 min since the piperazinedione remains stable for at least an hour at 60°C.

Figure 5 shows the time-course of absorbance increase at 322 nm for solutions of ampicillin, amoxycillin and cyclacillin treated according to the procedure described. For cyclacillin the absorbance reaches a rapid maximum (1 min) whereas the other amino-penicillins require 3 min. Using 2 M sodium hydroxide instead of 1 M gave similar results whereas concentrations less than 0.5 M resulted in lower final absorbances.

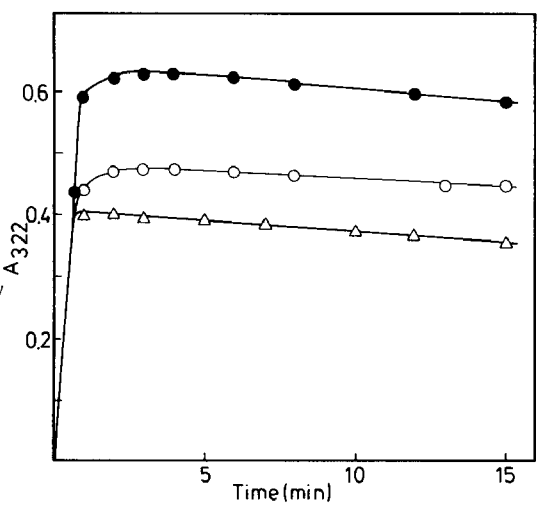


Figure 5
Time-course of absorbance increase at 322 nm during treatment of piperazinedione derivatives, generated from ampicillin (○), amoxycillin (●) and cyclacillin (△), with 1 M sodium hydroxide as described in the text. The concentrations of penicillin in the final assay solutions were 3.2×10^{-5} M (ampicillin), 3.7×10^{-5} M (amoxycillin) and 2.5×10^{-5} M (cyclacillin).

A linear relationship between absorbance and concentration of each penicillin, passing through or close to the origin, was observed within the range of 0–2 mg ml^{-1} for the compounds in the test solution, indicating adherence to the Beer-Lambert law. From the slope of such plots the following molar absorptivities were found: 1.50×10^4 (ampicillin), 1.61×10^4 (cyclacillin) and 1.72×10^4 (amoxycillin). The molar absorptivity at 322 nm of an isolated sample of the piperazinedione derivative of ampicillin [1] was found to be 1.88×10^4 after treatment with 1 M sodium hydroxide or after passing through the entire procedure. Thus, the extent of conversion of ampicillin to this product under the stated assay conditions amounts to 80%.

Precision and sensitivity

To examine the precision of the procedure, 10 determinations were made on the same penicillin solutions. Relative standard deviations between 0.5 and 0.8% for the three amino-penicillins studied were obtained. The method permits determination of the penicillins in concentrations down to 50 μg/ml in the test solution.

Influence of degradation products and applications of the method

Since the formation of the piperazinedione derivative is dependent on both an intact β-lactam ring and an intact amino side-chain group in the penicillin molecule, no interference in the method by degradation products should be expected. This was confirmed in various ways. When aqueous solutions of the penicillins (0.2% at pH 1 and 11) have been stored at 60°C for 24 and 4 h respectively, no penicillin could be detected by the method. Different amounts of freshly prepared 1% m/v penicillin solutions were

then added to these degraded solutions and the mixtures analysed. The percentage recoveries of added penicillin were within the range 99.2–101.0.

Whereas degradation of ampicillin and other penicillins in dilute aqueous solutions (< 1%) leads to the formation of products which lack an intact β-lactam ring [16, 17] and thus are unable to react with sorbitol to produce a piperazinedione, neutral and alkaline solutions of the amino-penicillins at concentrations greater than 1% m/v yield polymers containing an intact β-lactam ring (Fig. 6) [18, 19 and references cited therein]. As can be

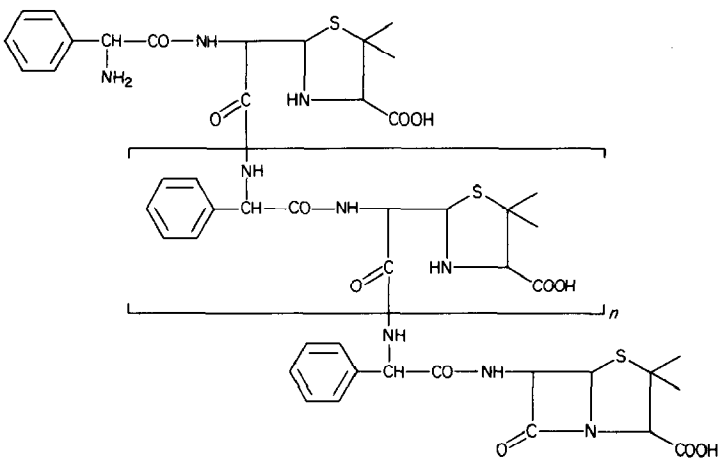


Figure 6
Structure of polymers of ampicillin with an intact β-lactam ring in the terminal unit.

seen from Fig. 6, however, such products do not contain adjacent free side-chain amino groups and intact β-lactam rings and therefore should not be expected to produce any interference. To verify the presumed non-interference of dimers and polymers, solutions of ampicillin sodium with various added amounts of the substances were analysed using the procedure described. The results obtained (Table 1) substantiate the selectivity of the procedure for determination of ampicillin in the presence of its polymerization products.

Table 1
Recoveries of ampicillin sodium from solutions containing the penicillin, dimer and polymers

Sample composition (mg/ml)	Recovery of ampicillin sodium (%)
Ampicillin sodium (1) Ampicillin dimer (1)	99.6
Ampicillin sodium (1) Ampicillin tetramer (1)	100.7
Ampicillin sodium (1) Ampicillin hexamer (1)	100.5

As noted in the Introduction, the piperazinedione derivative may be a major degradation product of the respective amino-penicillin under various conditions. Thus, on degradation of the penicillins in 0.5 M phosphate buffer solutions of pH 6.3–7.4, the

yield of piperazinedione formed was 90–99% [2]. However, despite its efficiency, this phosphate-catalysed piperazinedione formation was not suitable as a basis for the spectrophotometric assay, because of the inconveniently long reaction times required. Any piperazinedione already present in the test solution will, of course, respond in the assay but since the product remains unchanged during the time of heating in the sorbitol reagent, the amount of piperazinedione formed, and from this the amount of intact penicillin, is readily compensated by calculating the difference in absorbances described in the procedure. As already demonstrated by comparison with other procedures [2], the method involving treatment with 1 M sodium hydroxide is capable of determining directly the content of the piperazinedione product in solutions of amino-penicillins. It should also be noted that penicilloylamides formed by aminolysis of amino-penicillins are unable to rearrange into a piperazinedione and thus do not interfere in the assay [3].

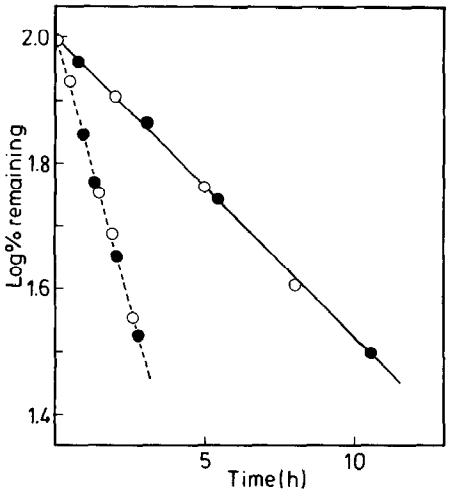


Figure 7
First-order plots for the degradation of ampicillin sodium in 0.05 M hydrochloric acid at 60°C (– – –) and 0.5 M phosphate buffer solution, pH 7.43, at 37°C (——). The residual concentrations of ampicillin were determined by the proposed spectrophotometric method (○) and by an HPLC procedure (●).

The applicability of the proposed method to assess the stability of amino-penicillins was further investigated using ampicillin sodium as test substance. The residual ampicillin was analysed by a stability-indicating HPLC method [1] and by the spectrophotometric procedure. Various buffer solutions containing ampicillin sodium at an initial concentration of 0.05% m/v were kept at a constant temperature and 500 µl aliquots were withdrawn at appropriate intervals for analysis after suitable dilution with water. The results obtained (Fig. 7) show perfect agreement between the two methods in determining concentrations of undegraded ampicillin during degradation up to at least two half-lives.

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