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Spectrophotometric determination of ampicillin sodium in the presence of its degradation and polymerization products

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A spectrophotometric method is described for the quantitative determination of ampicillin sodium [sodium salt of 6-(D(−)-phenylacetamido) penicillanic acid]. The method involves acetylation of ampicillin with acetic anhydride in aqueous solution at pH 9 to yield α-acetamido benzylpenicillin and subsequent measurement at 325 nm of α-acetamidobenzylpenicillenic acid mercuric mercaptide, formed in a quantitative yield on heating for 30 min at 60° in a 1·0M imidazole and 8×10^{-4}M mercuric chloride solution at pH 6·8. It has been demonstrated that degradation products do not interfere in the method whereas those di- and polymerization products of ampicillin which contain an intact β-lactam ring are capable of reacting with imidazole with the formation of penicillenic acid. A technique involving acid-catalysed opening of the β-lactam ring of these products under conditions where ampicillin is degraded to only a minor extent has been developed, and besides permitting a selective determination of ampicillin the technique permits the determination of the polymeric substances.

In neutral aqueous solution benzylpenicillin and several semi-synthetic penicillins undergo an imidazole–catalysed rearrangement into the corresponding penicillenic acids which yield the corresponding stable mercuric mercaptides in the presence of mercuric chloride (Bundgaard, 1971, 1972 a, b). This has been made the basis of a spectrophotometric method for the quantitative determination of a series of penicillins (Bundgaard & Ilver, 1972). Ampicillin (D(−)-[α-aminobenzylpenicillin]), however, differs from the other penicillins in that it reacts with imidazole in the presence of mercuric chloride to form an unstable product with an absorption maximum at a different wavelength (311 nm rather than 325 nm).

The present paper shows that preliminary acetylation of the side chain amino-group allows ampicillin to be assayed by the same method as other penicillins. In addition, a technique by which ampicillin can be assayed in the presence of its polymerization products is reported. Such products are formed when concentrated aqueous solutions of ampicillin sodium are stored for a short time at room temperature (Stewart, 1968, 1969; Grant, 1970; Butcher, Stanfield & others, 1971; Smith & Marshall, 1971; Dewdney, Smith & Wheeler, 1971; Smith, Dewdney & Wheeler, 1971; Ottens, De Haan & Sengers, 1971; Kuchinskas & Levy, 1972). Finally, a method for the detection and determination of ampicillin polymers containing an intact β-lactam ring has been worked out. Such substances (as well as polymers without intact β-lactam rings) have been shown to be strongly antigenic and are considered to play a part in eliciting some allergic reactions to penicillins (Dewdney & others, 1971; Smith & others, 1971).

MATERIALS AND METHODS

Apparatus

A Zeiss PMQ II spectrophotometer and a Radiometer model PHM 26 pH meter were used for the measurements.

Materials and reagents

Ampicillin sodium (Doktacillin) (AB Astra, Sweden) was examined by thin-layer chromatography [silica gel G; ethyl acetate–acetic acid–water (70:15:15)]. 5 μl of ampicillin sodium solutions (50% v/v aqueous ethanol) equivalent to 0·2, 0·3, 0·4, 1, 10, 20 and 30 μg of the sample were spotted on two chromatoplates. After development and subsequent drying in air, one chromatoplate was sprayed with chloroplatinic reagent (Pokorny, Vitezic & Japelj, 1973) the other exposed to iodine vapour; both procedures visualize ampicillin sodium equivalent to 0·2 μg. The 30 μg sample of ampicillin sodium showed two impurity spots which were less intense in both detection procedures than the spot corresponding to 0·2 μg of ampicillin sodium. Hence, the purity of ampicillin sodium was assumed to be more than 98·7%; this sample was used throughout this work. For the calculation of the molar absorptivity no correction was made with respect to the purity.

All chemicals and solvents were of analytical grade. Imidazole (E. Merck AG, Darmstadt) was recrystallized twice from benzene and washed with ether (Bundgaard & Ilver, 1972).

Imidazole reagent. This is a 1·2M aqueous imidazole solution containing mercuric chloride 10^{-3}M, pH 6·8 (Bundgaard & Ilver, 1972).

0·2M *acetic anhydride solution.* Acetic anhydride (1·0 ml) and acetonitrile (to 50 ml). This solution is stable for at least one month at room temperature.

0·1M *borate buffer pH 9·0.* Boric acid (1·24 g), 1N sodium hydroxide (8·3 ml) and water (to 200 ml).

0·2M *borate solution pH 12·0.* Boric acid (2·5 g), 1N sodium hydroxide (35 ml) and water (to 200 ml).

Analytical procedure I (polymerization products not present)

Prepare a solution in water of ampicillin sodium (or ampicillin) at a concentration of 50–60 μg ml^{-1}. Pipette 500 μl into a test tube, add 500 μl of the 0·1M borate buffer pH 9·0, then 50 μl of the 0·2M acetic anhydride solution. After 1–5 min, add 5·00 ml of the imidazole reagent, stopper the tube and allow to stand at 60° for 30 min. Cool the solution to room temperature, and measure the absorbance at 325 nm (1 cm cell) using solution of one part of water and five parts of the imidazole reagent as reference solution. Determine the ampicillin concentration of the original sample by reference to a standard curve. A straight-line relation between absorbance and concentration of ampicillin sodium was observed within the range of 0–10 μg ml^{-1} of penicillin in the reaction solution.

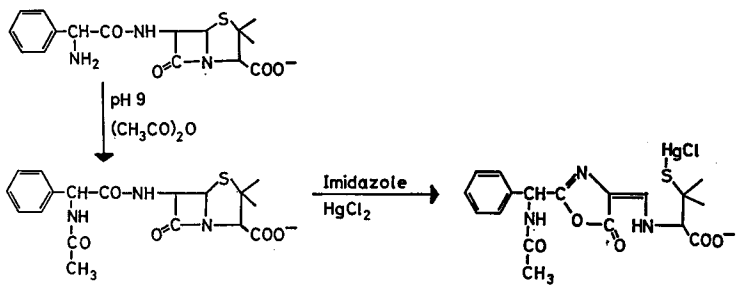
Analytical procedure II (polymerization products present)

Prepare a solution in water of the ampicillin sample at a concentration of 1–1·5 mg ml^{-1}. Pipette 500 μl into a test tube containing 4·50 ml of standardized 0·1N hydrochloric acid pre-equilibrated at 60°. Thermostat immediately at 60° and after exactly

30 min transfer 500 μl of the solution to a tube containing 500 μl of the 0·2M borate solution pH 12·0. Mix, and add 50 μl of the 0·2M acetic anhydride solution and proceed as described in the analytical procedure I. Determine the ampicillin concentration of the original sample by reference to a standard curve prepared by carrying out the same procedure on ampicillin sodium solutions of various known concentrations or, preferably, by reference to a suitable standard preparation assayed simultaneously with the sample analysed. A straight-line relation between absorbance and concentration of ampicillin sodium was observed within the range of 0–2 mg ml^{-1} of the compound in the original sample solution.

RESULTS AND DISCUSSION

The quantitative determination of ampicillin (D(−)-[α-aminobenzylpenicillin]) involves initial acetylation of the side chain amino-group to give α-acetamidobenzylpenicillin and subsequent spectrophotometric measurement at 325 nm (λ_{max}) of α-acetamidobenzylpenicillenic acid mercuric mercaptide, formed by a nucleophilic imidazole-catalysed rearrangement of the α-acetamidobenzylpenicillin (Scheme 1). The conditions of the rearrangement used for the present analysis are those of Bundgaard & Ilver (1972).



Scheme 1

Treatment of ampicillin at pH 9 and room temperature (20–25°) with acetic anhydride in acetonitrile and at the final acetic anhydride concentration of 10^{-2}M leads to complete acetylation instantaneously. A constant absorbance was observed for ampicillin solutions treated with acetic anhydride for ½ to 15 min. Changing the concentration of acetic anhydride in the acetonitrile solution to 0·1 or 0·3M produced no changes in the measured absorbance compared with that observed for the 0·2M solution.

The time course of the imidazole–catalysed rearrangement (Scheme 1) at 60° is shown in Fig. 1. After about 25 min at 60° the reaction is complete whereas at 20–25° 3·5 h is required. At room temperature the penicillenic acid mercuric mercaptide is stable for more than 24 h.

From the slope of a straight-line plot of absorbance *vs* concentration of acetylated ampicillin, a molar absorptivity of the penicillenic acid mercuric mercaptide of 26·7 × 10^3 was found. This value is the same as the molar absorptivity of other penicillenic acid mercuric mercaptides with absorption maximum at 325 nm (Bundgaard & Ilver, 1972), thus indicating that both the acetylation and the subsequent imidazole–catalysed rearrangement under the assay conditions proceed quantitatively.

When ampicillin sodium is treated with imidazole itself it rearranges, at least partly, to the corresponding penicillenic acid (λ_{max} 322 nm). In presence of mercuric chloride an unstable product with λ_{max} 311 nm (Fig. 1) is formed. This product is probably the α-aminobenzylpenicillenic acid in which both the thiol and the side chain amino-group have complexed with mercuric chloride.

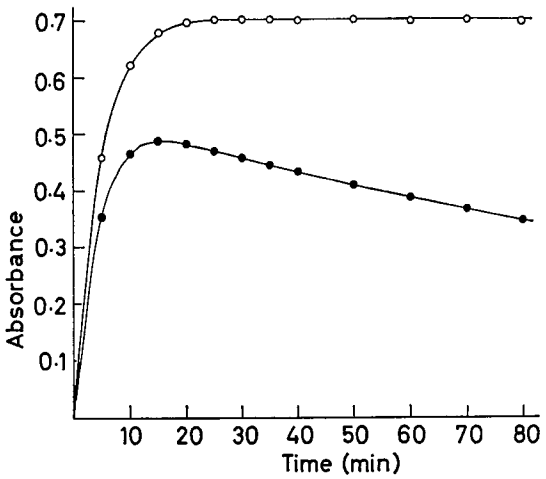


FIG. 1. The time course of formation of α-acetamidobenzylpenicillenic acid mercuric mercaptide (○) [concentration of *N*-acetylampicillin sodium in the reaction solution (1·0M imidazole, pH 6·8) is $2{\cdot}6 \times 10^{-5}$M, absorbance at 325 nm] and of a product with an absorption maximum at 311 nm (●) obtained by heating ampicillin sodium ($2{\cdot}6 \times 10^{-5}$M) in the same imidazole solution (absorbance at 311 nm). The temperature is in both cases 60°.

Influence of degradation products

When aqueous solutions of ampicillin sodium (0·2%) at pH 1 and 11 have been stored at 60° for 24 h and 3 h, respectively, no ampicillin could be detected by the method (procedure I). If different amounts of a freshly prepared 1% ampicillin sodium solution are then added to these degraded solutions the percentage recovery of added ampicillin sodium is within the range 99·4–100·8, thus showing no interference in the method by degradation products.

Influence of polymerization products

Degradation of ampicillin sodium in dilute aqueous solutions (<1% ampicillin sodium) leads to the formation of products which are without an intact β-lactam ring (Hou & Poole, 1969); they are thus unable to react with imidazole to produce penicillenic acid. Ampicillin solutions >1% w/v yield polymers (Fig. 2) (Grant, 1970; Smith & Marshall, 1971; Kuchinskas & Levy, 1972). Such polymers represent a linear polymerization of ampicillin units with opening of individual β-lactam rings in all but the terminal unit. As the presence of an intact β-lactam ring in the dimer and polymers makes these products capable of forming the corresponding penicillenic acids through reaction with imidazole, they may be expected to interfere in the assay of unpolymerized ampicillin. Procedure II, designed to avoid this, makes use of the different acid-stabilities of ampicillin and the di-and polymeric substances which in fact can be viewed as real penicillins (*N*-penicilloylated ampicillins).

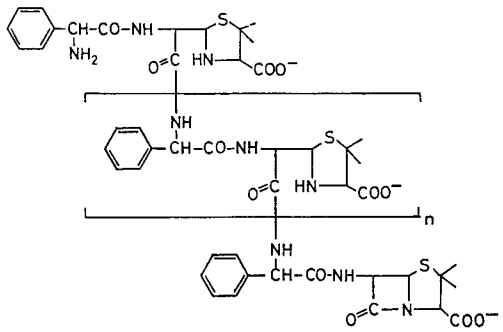


FIG. 2. Polymers of α-aminobenzylpenicillin (ampicillin).

It has been recognized (Doyle, Nayler & others, 1961, 1963) that the stability of penicillins in acidic solutions depends greatly on the nature of the side-chain and that the strong inductive effect of the protonated α-amino-group in ampicillin makes this penicillin especially acid-stable compared with e.g. benzylpenicillin. Penicilloylation or another form of acylation of the amino-group should remove this inductive effect and transform the ampicillin into a more acid–unstable penicillin. Thus, *N*-benzoylated ampicillin has been shown to be more labile in acidic aqueous solutions than ampicillin (Tutt & Schwartz, 1971). The determination of the acid–stability of *N*-acetylampicillin and ampicillin gave apparent first-order rate constants for the degradation in 0·090N hydrochloric acid at 60° of 0·275 min^{-1} for *N*-acetylampicillin and of 0·0115 min^{-1} for ampicillin.

On the assumption that the ampicillin dimer and polymers have an acid–stability which like that of *N*-acetylampicillin is considerably lower than that of ampicillin, then it would be possible to open completely the β-lactam ring of these products under conditions where the β-lactam ring of ampicillin is affected to but a minor extent. To test this assumption (the dimer and polymers were not available) the following experiment was made. A solution of ampicillin sodium (25% w/v) in water was allowed to stand at room temperature for 22 h and an aliquot (1·00 ml after appropriate dilution with water) was poured into a test tube containing 9 ml of 0·100N hydrochloric acid pre-equilibrated and maintained at 60°. Aliquots of 500 μl were periodically withdrawn and transferred to tubes containing 500 μl of the 0·2M borate solution pH 12·0; these were assayed by Method I. The 0·2M borate solution pH 12·0, described under reagents, contains sufficient sodium hydroxide to adjust the pH of the solution after the addition of an equal part of 0·090N hydrochloric acid to 9.

A plot of the logarithm of the measured absorbance at 325 nm against time (Fig. 3) shows that the partially decomposed ampicillin sodium solution contains β-lactam substances which are degraded more rapidly in 0·090N hydrochloric acid than is ampicillin, and which also react with imidazole, thus contributing to the measured absorbance. After a short time, however, the β-lactam bond of these products (ampicillin di- and polymers) is broken completely and thereafter only the β-lactam ring of ampicillin remains. The linear portion of the curve in Fig. 3 represents the degradation of ampicillin, and from the slope of this line an apparent first-order rate constant of 0·0117 min^{-1} was calculated. This agrees with the rate constant (0·0115 min^{-1}) for the degradation of ampicillin determined under the same conditions

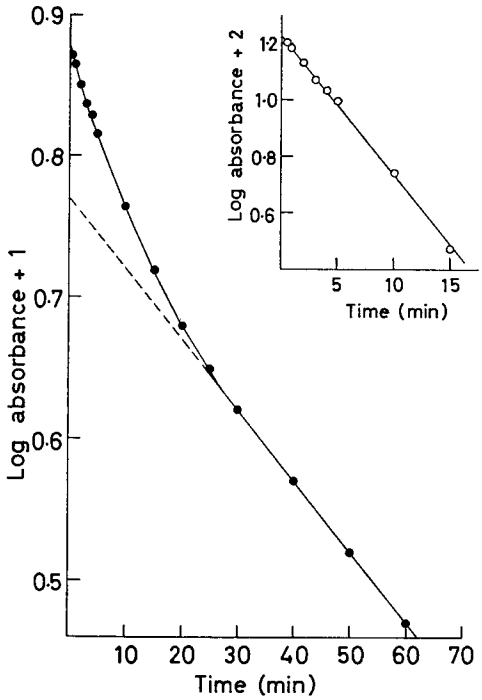


FIG. 3. Rate of degradation of ampicillin polymers and ampicillin in admixture in 0·090N hydrochloric acid at 60°. The inset is a first-order plot for the degradation of the polymers. Absorbance is measured at 325 nm.

using a pure ampicillin sodium solution. When the antilogarithms of the values along the extrapolated line (broken line in Fig. 3) representing the contribution of ampicillin to the total absorbance are subtracted from this total the absorbance due solely to di- and polymers is obtained. Plotting the logarithms of these absorbance values against time results in a straight line (inset of Fig. 3) from the slope of which an apparent first-order rate constant for the degradation of di- or polymers of 0·112 min^{-1} was calculated. This is ten times greater than the corresponding value for degradation of ampicillin, thus confirming the assumption of lower acid-stability of ampicillin di- or polymers compared with ampicillin. That the inserted plot in Fig. 3 is a straight line means either that one polymer is present in a much greater amount than others (most likely the initially formed dimer) or that dimer and other polymers degrade with similar rates in hydrochloric acid.

The concentration of the ampicillin sodium in the original solution can be determined by extrapolating the linear portion of the curve in Fig. 3 to zero time. The absorbance value thus obtained represents the ampicillin concentration in the sample before treatment with acid and is without contribution from di- and polymers. Another and more simple way to determine the concentration of ampicillin without interference from di- and polymers is to pretreat the ampicillin solution with hydrochloric acid (normality after dilution: 0·090) at 60° for exactly 30 min. As seen from Fig. 3 such a treatment destroys all imidazole-reactive compounds with the exception of ampicillin which degrades only partly (30·0%). To obtain greatest precision when such a procedure is used it is recommended that an ampicillin sodium standard is treated with acid at the same time as the sample to be assayed.

The stability of ampicillin sodium in aqueous solution has been reported to be highly dependent upon concentration (Savello & Shangraw, 1971; Hiranaka, Frazier & Gallelli, 1972) and since the lower stability of the more concentrated solutions is most likely due to the polymerization reaction (Schwartz & Hayton, 1972), and since the analytical methods used in these investigations, iodometric and hydroxamic acid assays, do not distinguish between ampicillin and di- and polymers containing an intact β-lactam ring (which really are formed as shown in Fig. 3), the stability of ampicillin sodium in aqueous solutions should be re-examined using e.g. the method described here.

Precision of the analytical procedure

Ten determinations made on the same ampicillin sodium solution gave relative standard deviations for procedures I and II of 0·47 and 1·04%, respectively. The determinations based on procedure II were not carried out simultaneously and the relative standard deviation obtained thus shows that, in spite of being dependent upon a reaction sequence in which 30% degradation occurs, the method is reliable. A relative standard deviation of 1·12% was obtained from 6 determinations (procedure II) of the content of ampicillin sodium in the partially (60·5%) decomposed solution mentioned above. From analysis of a commercial ampicillin sodium sample, which in proportion to the commercial reference sample used otherwise in this work, showed a content of ampicillin sodium of 89·5% (procedure I) and 89·4% (procedure II), the s.d. was 0·41% for procedure I and 1·13% for procedure II ($n = 6$). The identical results of analyses obtained from use of procedures I and II show that the sample concerned contains no detectable dimers and polymers with intact β-lactam rings.

Analysis of ampicillin dimer and polymers

The method for the analysis of ampicillin sodium in the presence of polymeric compounds can also be used for the determination of these products. If the sample to be analysed is treated with hydrochloric acid as described above, and the absorbance-time data are plotted as shown in Fig. 3, then at zero time the difference between total absorbance and absorbance due to ampicillin (equal to the intercept of the extrapolated linear portion of the plot) represents absorbance due to the faster reacting di- and polymeric substances. For the calculation of the concentration of these compounds a molar absorptivity of $26·7 \times 10^3$ for the mercuric mercaptides of the corresponding penicillenic acids may be used. This value is considered as being correct within ±5% (cf. Bundgaard & Ilver, 1972).

The data used in Fig. 3 are for a 25% (0·647M) ampicillin sodium solution which has been kept at room temperature for 22 h. In this the molar concentrations of undegraded ampicillin sodium and of β-lactam containing dimer and higher polymerization products are 0·266 and 0·076, respectively. It is only those di- and polymers whose terminal unit contains an intact β-lactam ring which can be determined in this way.

REFERENCES

BUNDGAARD, H. (1971). *Tetrahedron Lett.*, 4613–4616.
BUNDGAARD, H. (1972a). *Dansk Tidsskr. Farm.*, **46**, 29–40.
BUNDGAARD, H. (1972b). *Ibid.*, **46**, 85–91.
BUNDGAARD, H. & ILVER, K. (1972). *J. Pharm. Pharmac.*, **24**, 790–794.

BUTCHER, B. T., STANFIELD, M. K., STEWART, G. T. & ZEMELMAN, R. (1971). *Molecular Crystals and Liquid Crystals*, **12**, 321–333.

DEWDNEY, J. M., SMITH, H. & WHEELER, A. W. (1971). *Immunology*, **21**, 517–525.

DOYLE, F. P., NAYLER, J. H. C., SMITH, H. & STOVE, E. R. (1961). *Nature*, **191**, 1091–1092.

DOYLE, F. P., NAYLER, J. H. C., WADDINGTON, H. R. J., HANSON, J. C. & THOMAS, G. R. T. (1963). *J. chem. Soc.*, 497–506.

GRANT, N. H. (1970). In *Peptides: chemistry and biochemistry*, pp. 487–498. Editors: Weinstein, B. & Lande, S. New York: Marcel Dekker.

HIRANAKA, P. K., FRAZIER, A. G. & GALLELLI, J. F. (1972). *American Journal Hospital Pharmacy*, **29**, 321–322.

HOU, J. P. & POOLE, J. W. (1969). *J. pharm. Sci.*, **58**, 447–454.

KUCHINSKAS, E. J. & LEVY, G. N. (1972). *Ibid.*, **61**, 727–729.

OTTENS, H., DE HAAN, E. & SENGERS, C. H. J. (1971). *Int. Arch. Allergy*, **41**, 575–591.

POKORNY, M., VITEZIC, N. & JAPELJ, M. (1973). *J. Chromatog.*, **77**, 458–460.

SAVELLO, D. R. & SHANGRAW, R. F. (1971). *American Journal Hospital Pharmacy*, **28**, 754–759.

SCHWARTZ, M. A. & HAYTON, W. L. (1972). *J. pharm. Sci.*, **61**, 906–909.

SMITH, H., DEWDNEY, J. M. & WHEELER, A. W. (1971). *Immunology*, **21**, 527–533.

SMITH, H. & MARSHALL, A. C. (1971). *Nature*, **232**, 45–46.

STEWART, G. T. (1968). *Antimicrobial Agents and Chemotherapy*, **1967**, 543–549.

STEWART, G. T. (1969). *Ibid.*, **1968**, 128–135.

TUTT, D. E. & SCHWARTZ, M. A. (1971). *Analyt. Chem.*, **43**, 338–342.