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A new spectrophotometric method for the determination of penicillins

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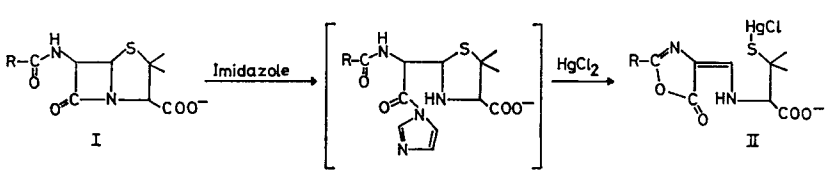
A rapid and convenient method is described for the determination of nine commonly used penicillins. This is based on the spectrophotometric measurement at 325–345 nm of penicillenic acid mercuric mercaptides of the penicillins, formed in quantitative yield on heating at 60° or on standing at 20° in a 1·2 M imidazole and 10^{-3} M mercuric chloride solution at pH 6·8. The method, which permits detection of concentrations of penicillins down to 0·5 μg/ml, is reproducible. Since the intact penicillin molecule is required for the penicillenic acid formation, the method is highly specific and useful as a means of assessing stability.

Isomerization of penicillins into the corresponding penicillenic acids has been the basis of spectrophotometric methods for the quantitative determination of this group of drugs. The penicillenic acids, which have a strong ultraviolet absorption with maximum at 320–360 nm, are formed by degradation of the penicillins in acidic aqueous solution (Krejci, 1956; De Weck, 1962). They are labile, especially in acidic solution (Longridge & Timms, 1971), but they can be stabilized to some extent by addition of a copper salt (Stock, 1954; Holbrook, 1958; Smith, De Grey & Patel, 1967; Saccani & Pitrolo, 1969) or a mercuric salt (Brandriss, Denny & others, 1963). The foregoing methods have the disadvantage that the conversion has to be made in an acidic solution, where penicillin undergoes other degradation reactions (Bundgaard, 1971a) and where the penicillenic acids have limited stability. Furthermore, the method involving the addition of a mercuric salt cannot be applied for the determination of acid-stable penicillins, such as ampicillin (Tutt & Schwartz, 1971), phenoxymethylpenicillin and phenethicillin (our own observation), because the rate of formation of the penicillenic acid is much slower than the rate of its degradation.

Recently it was found that imidazole in neutral aqueous solutions with a content of mercuric chloride reacts with benzylpenicillin and several semi-synthetic penicillins (I) with the formation of the mercuric mercaptides of the corresponding penicillenic acids (II) in a quantitative yield (Bundgaard, 1971 b; 1972a, b). The catalytic reaction proceeds via a self-catalysed nucleophilic attack of the imidazole base upon the β-lactam bond with the intermediate formation of *N*-penicilloylimidazole (see below).

A specific, sensitive, and rapid spectrophotometric method for the determination of a series of penicillins based on this observed reaction has been developed.



MATERIALS AND METHODS

Apparatus.

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

Materials and reagents

Oxacillin sodium and dicloxacillin sodium were obtained from H. Lundbeck & Co. A/S, Copenhagen; benzylpenicillin sodium from Leo Pharmaceutical Ltd., Copenhagen; phenoxymethylpenicillin potassium from Bayer, Germany; cloxacillin sodium, methicillin sodium, carbenicillin sodium, phenethicillin potassium, propicillin potassium, and ampicillin sodium from AB Astra, Sweden. The dicloxacillin sodium was recrystallized from 2-propanol-water (Doyle, Hanson & others, 1963); the other compounds were used as received. Using the alkaline titrimetric method described by Patterson & Emery (1948), the content of the respective penicillin in the products was determined as 97·0–100·2%.

All the chemicals used were of reagent grade. Imidazole (E. Merck AG, Darmstadt) was recrystallized twice from benzene and washed with ether (25 g of imidazole in 80 ml of benzene) to remove an ultraviolet-absorbing impurity present.

Imidazole reagent. This was 1·2 M aqueous imidazole solution containing mercuric chloride in a concentration of 10^{-3} M, pH 6·8. The reagent is prepared as follows: Dissolve 8·25 g imidazole in 60 ml of water, add 10 ml of 5 M hydrochloric acid and thereafter, add 10 ml of a solution of mercuric chloride (0·27 g dissolved in 100 ml of water). Adjust the pH to 6·80 $\pm$ 0·05 with 5 M hydrochloric acid (about 4 ml is required) and dilute to 100 ml with water.

General procedure

Prepare a solution in distilled water of the sample of the penicillin at a concentration of 40–50 μg/ml. Pipette two equal samples of 1000 μl, *A* and *B*, of this solution into separate test tubes. To *A*, add 5·00 ml of the imidazole reagent, stopper the tube and stand it in a water bath at 60° for the time interval specified in Table 1,

Table 1. *Absorptivities and absorption maxima of penicillenic acid mercuric mercaptides formed by heating of the penicillins in the imidazole reagent for the specified time at 60°.*

Compound	Side chain, R in structure I	λ_{max} of penicillenic acid mercaptide (II) (nm)	Molar absorptivity at λ_{max} of II	Time of heating at 60° (min)
Benzylpenicillin sodium	Benzyl	325	$26{\cdot}6\times10^3$	25
Phenoxymethylpenicillin potassium	α-Phenoxymethyl	325	$26{\cdot}5\times10^3$	20
Phenethicillin potassium	α-Phenoxyethyl	325	$26{\cdot}8\times10^3$	25
Propicillin potassium	α-Phenoxypropyl	325	$26{\cdot}6\times10^3$	25
Oxacillin sodium	3-Phenyl-5-methyl-4-isoxazolyl	343	$27{\cdot}6\times10^3$	25
Cloxacillin sodium	3-(2-Chlorophenyl)-5- methyl-4-isoxazolyl	343	$28{\cdot}0\times10^3$	25
Dicloxacillin sodium	3-(2,6-Dichlorophenyl)-5-methyl-4-isoxazolyl	345	$28{\cdot}0\times10^3$	20
Methicillin sodium	2,6-Dimethoxyphenyl	335	$26{\cdot}5\times10^3$	30
Carbenicillin sodium	α-Carboxybenzyl	325	$26{\cdot}7\times10^3$	25

then remove the tube and cool it to 20°. To sample *B*, add 5·00 ml of distilled water. Measure the absorbance in a 1 cm cell of *A* and *B* at the wavelength indicated in Table 1 for the particular penicillin using a solution of one part of distilled water and five parts of the imidazole reagent as reference solution for *A*, and distilled water for *B*. Calculate the difference in absorbance between *A* and *B* and determine the penicillin concentration of the original sample by reference to a standard curve. A straight-line relation between absorbance and concentration of each penicillin was observed within a range of 0–20 μg ml^{-1} of the penicillin in reaction solution, indicating adherence to Beer's law. The calibration curve can be used over 24 h.

RESULTS AND DISCUSSION

Optimal conditions for the assay imply a rapid and quantitative conversion of the penicillins into the stable mercuric mercaptides of the corresponding penicillenic acids. The rate of the imidazole-catalysed isomerization is a function of pH, concentration of imidazole, and temperature, whereas the mercuric chloride has no effect (Bundgaard, 1972 a,b).

Effect of pH. For benzylpenicillin the rate of the isomerization at any total imidazole concentration is maximal at pH 7·4 at 37°, but the dependence of pH is not very great within the range pH 6·7–8 (Bundgaard, 1972 a). A pH of 6·8 (23°) was selected for the assay as the penicillenic acids have a greater stability at this than at higher pH values.

Effect of imidazole concentration. The imidazole concentration has a pronounced effect on the rate of the isomerization as the rate increases with the square on the concentration of imidazole (Bundgaard, 1972 a,b). A molar concentration of imidazole in the reaction solution was selected.

Effect of temperature and time of heating. The effect of temperature on the time course of formation of benzylpenicillenic acid is shown in Fig. 1. Although the rate is greatest at 95°, the penicillenic acid formed is not so stable at this temperature as at lower temperatures. At 60° and below, the yield of penicillenic acid is unchanged. Table 1 indicates the time necessary to complete the isomerization at 60° of the various penicillins into the corresponding penicillenic acids. The molar absorptivity

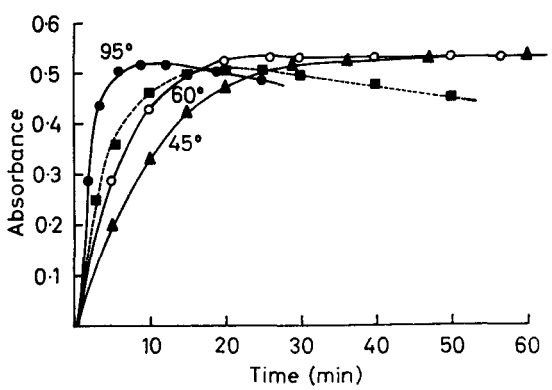


FIG. 1. The effect of temperature on time course of formation of benzylpenicillenic acid mercuric mercaptide [concentration of benzylpenicillin sodium in the reaction solution (1·0 M imidazole, pH 6·8) is 2·00 × 10^{-5} M, absorbance at 325 nm] and the formation of a product with an absorption maximum at 311 nm obtained by heating ampicillin sodium (2·56 × 10^{-5} M absorbance at 311 nm at 60° in the imidazole reagent (broken line).

of the various penicillenic acids indicated in Table 1 was determined from the slopes of straight-line plots of absorbance as a function of the concentration of penicillin in the reaction solution. As a molar absorptivity of $26{\cdot}6 \times 10^3$ (322 nm, ethanol) has been reported for synthetic benzylpenicillenic acid (Livermore, Carpenter & others, 1948), the isomerization of the penicillins under the assay conditions is virtually quantitative. The stability of the penicillenic acids at 60° is good (for benzylpenicillin, see Fig. 1). After a period twice that required to complete the reaction, the absorbance decreases by only 0–0·3% for each of the penicillins. This behaviour has application in the assay procedure of degraded penicillin samples with a content of penicillenic acid or of penicillenic acid disulphide which is far more stable than penicillenic acid (Longridge & Timms, 1971). These degradation products remain unchanged during the time of heating and therefore, the measured absorbance after the heating will be a sum of formed and pre-formed penicillenic acid. The amount of the penicillenic acid formed, and from this the amount of intact penicillin, is determined by making the subtraction described under general procedure.

The spectrophotometric assay can also be performed at other temperatures; for benzylpenicillin the reaction is complete within 3 h at 21°, for the most reactive penicillin, dicloxacillin, the reaction takes about 2 h, and for the least reactive, methicillin, the period required is 4 h. At room temperature all the completed reaction solutions are stable for more than 24 h.

Precision of the assay procedure

To determine the precision of the procedure, 20 assays were made on the same solution. For benzylpenicillin and phenoxymethylpenicillin the standard deviation as percentages, were 0·45% and 0·51%, respectively.

Influence of degradation products and pharmaceutical adjuvants

Aqueous solutions of benzylpenicillin (1%) at pH 3 and 11 were kept at room temperature for 24 h. No undegraded penicillin could be found by the method. Different amounts of these solutions were mixed with a freshly prepared 1% benzylpenicillin solution and a sufficient amount of 0·1 M phosphate buffer pH 6·5 to obtain mixtures with a total concentration of undegraded penicillin equal to 0·1%. Aliquots of the mixtures were analysed by the general procedure. The percentage recovery of added benzylpenicillin was within the range 99·0–101·2, thus showing no interference in the method by degradation products. As 6-aminopenicillanic acid also was not found to interfere, the method has obvious applications for stability studies of penicillins.

Pharmaceutical products of the penicillins often contain sodium citrate and various carbohydrates. On analysis of 1% benzylpenicillin sodium and 1% phenoxymethylpenicillin potassium solutions containing sodium citrate (5%), glucose or lactose (5%) it was found that these adjuvants caused no interference.

Analysis of ampicillin

Reaction of ampicillin (α-aminobenzylpenicillin) with imidazole at pH 6·5–7·5 in the presence of mercuric chloride was found to differ from the reaction of the other penicillins. In the cited pH range an unstable product with an absorption maximum at 311 nm was formed. Apparently, the product is not the mercuric mercaptide of α-aminobenzylpenicillenic acid (expected λ_{max}: 325 nm) but the reaction can be utilized as a basis for analysis of ampicillin. When the general procedure was per-

formed on ampicillin sodium the product with λ_{max} at 311 nm was formed as a function of time as shown in Fig. 1. The maximum absorbance of the chromophore formed occurs after 20–25 min, and, based on the ampicillin concentration, this absorbance corresponds to a molar absorptivity of $19{\cdot}8 \times 10^3$. Beer's law was obeyed within a range of 0–30 μg/ml of ampicillin sodium.

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