A new fluorometric method for simultaneous determination of penicillins and penicilloic acids

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Analysis of penicillins has been directed principally to methods of quantifying the intact molecules. Recent interest in their pharmacokinetics, however, requires an analytical method quantifying both the intact molecules and metabolites in biological fluids simultaneously. Fluorometric methods developed for ampicillin (Miyazaki, Ogino & others, 1974) and amoxicillin (Miyazaki, Ogino & others, 1977) satisfy this requirement but these methods are essentially sensitive only to α-amino-penicillins and not applicable to many other penicillins.

This communication describes a new and sensitive fluorometric method for the analysis of both the intact penicillin and its biotransformation product, penicilloic acid. This method is based on the formation of a fluorescent Schiff-base resulting from the reaction between a certain hydrazine and penilloaldehyde which can be produced (Levine, 1960) from the reaction of penicillioc acid and mercuric chloride in acidic medium.

Materials. Penicillins (cloxacillin sodium, propicillin potassium, phenethicillin potassium, penicillin V potassium, penicillin G potassium and cyclacillin anhydrate) were kindly supplied by Meiji Seika Kaisha, Ltd., and Takeda Chemical Ind. Ltd., Japan. All other chemicals used were of analytical grade.

Instrument. JASCO FP-4 fluorescence spectrophotometer, Japan.

Reagents. DNSH reagent: a solution of CH_2Cl_2 containing 10% 5-dimethylaminonaphthalene-1-sulphonylhydrazine (DNSH, Nakarai Chemicals, Ltd., Japan) and 10% acetic acid. pH 2·5-buffer solution: 0·8% aqueous solution of citric acid adjusted to pH 2·5 and containing 0·03% $HgCl_2$. A reagent for the standardization of the spectrofluorometer: 0·4 μg ml^{-1} of quinine sulphate solution prepared with 0·05 M sulphuric acid.

Method in aqueous solution and urine sample. All experiments were carried out at room temperature (25°). One ml of sample diluted, if necessary, was placed in a 12 ml glass-stoppered centrifuge tube. For total penicillin (treatment 1), 0·5 ml of 1 M NaOH was added to the sample solution, the mixture was allowed to stand for 20 min, and then neutralized with 0·5 ml of 1 M HCl. To this mixture, 1 ml of pH 2·5-buffer solution was added. After exactly 30 min, 5 ml of CH_2Cl_2 was added, the mixture was shaken vigorously for 10 min, centrifuged at 3000 rev min^{-1} for 5 min, and the aqueous phase was discarded. Four ml of the organic phase was pipetted into a suitable tube, 0·5 ml of the DNSH reagent was added, and the mixture was allowed to stand for 90 min in the dark. Addition of 4 ml of 2 M HCl was followed by shaking for exactly 3 min to remove excess DNSH and centrifugation, and the aqueous phase was discarded. Four ml of the organic phase was then added to 4 ml of 0·2 M NaOH and the mixture was shaken for 10 min and centrifuged. The fluorescence intensity of this aqueous layer of the sample solution was measured, with excitation at 320 and emission at 510 nm, setting the intensity of the fluorescence standard solution to 100 units. The true intensity, F_1, of the sample was calculated by subtraction of the intensity of the blank obtained from a simultaneous experiment with a solution not containing the penicillin.

In the measurement of penicilloic acid (treatment 2), the sample was treated by the same procedure as described above for total penicillin, except for the use of 1 ml distilled water instead of NaOH and HCl. The fluorescence intensity, F_2, was calculated by subtracting the intensity of the blank, which was obtained by treatment 2, from that of each sample.

The standard solution of an intact penicillin was prepared with the same buffer solution or urine by dissolving the weighed penicillin which does not contain the corresponding penicilloic acid. From the slopes of the plots of F_1 and F_2, obtained for the standard solution in exactly the same manner as for the sample solutions *vs* the concentration, the values of the fluorescence coefficients I_{P_1} and I_{P_2} were calculated.

Analysis of mixture of intact penicillin and penicilloic acid. With respect to F_1 and F_2 obtained for the mixture solution, the following equations can be written:

$$F_1 = I_{P_1}C_P + I_{A_1}C_A \quad .. \quad .. \quad .. \quad (1)$$

$$F_2 = I_{P_2}C_P + I_{A_2}C_A \quad .. \quad .. \quad .. \quad (2)$$

where C is the concentration, I_{P_1} and I_{A_1} are the fluorescence coefficients obtained from the two treatments described above for the standard penicillin and penicilloic acid solutions of known concentration, and subscripts, P and A, stand for intact penicillin and penicilloic acid, respectively. The contamination of the standard penicillin by penicilloic acid was checked by penamaldate assay (Schwartz & Delduce, 1969) to avoid possible error caused in the determination of I_{P_2}. The contents of penicilloic acid were negligible in all the penicillins used. Since the value of I_{P_1} for penicillin itself and the values of I_{A_1} and I_{A_2} for the corresponding penicilloic acid prepared according to Schwartz & Delduce (1969) were identical, these

* Correspondence.

equations can be simplified and the concentrations, C_P and C_A, can be calculated from:

$$C_P = \frac{F_1 - F_2}{I_{P1} - I_{P2}} \quad .. \quad .. \quad .. \quad .. \quad (3)$$

$$C_A = \frac{F_2 I_{P1} - F_1 I_{P2}}{(I_{P1} - I_{P2}) I_{P1}} \quad .. \quad .. \quad .. \quad (4)$$

In routine experiments, therefore, this method requires only the values of I_{P1} and I_{P2} to be determined under the experimental conditions used for the sample analyses.

Plots for various penicillins of relative fluorescence determined by treatment 1 *vs* the concentration between 5 and 20 or 40 μg ml^{-1} are shown in Fig. 1, all give a good linear relation through the origin with slopes equal to I_{P1}. The fluorescence intensity varied among penicillins depending on the extraction efficiency of penilloaldehyde between CH_2Cl_2 and aqueous acidic solution, rather than the difference in the reactivities of Schiff-base formation in acetic acid-CH_2Cl_2 solution. The ratio, I_{P1}/I_{P2}, was 0·02 for penicillin V and 0·14 for propicillin. The resulting fluorescence is reasonably stable at least for 5 h.

Aqueous mixtures of penicillin V and its penicilloic acid of three different compositions were analysed by this method and the average recoveries (Table 1) were 100·0 ± 4·8% (n = 9) for C_P and 98·1 ± 3·3% (n = 9) for C_A. To obtain reliable results, special care should be taken in the reaction with pH 2·5-buffer solution. Because of the possible degradation of the intact penicillin during the analytical procedure, both the sample and standard solution should be treated for exactly the same time in this process.

A typical result from the application of this method to urine assay after intravenous injection of penicillin

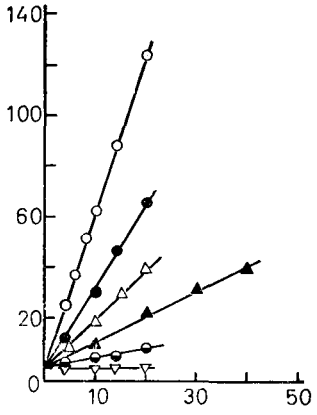

FIG. 1. Plots of concentration *vs* intensity, F_1, of various penicillins. (○) propicillin, (●) cloxacillin, (△) phenethicillin, (▲) penicillin V, (◒) penicillin G, (▽) cyclacillin. Ordinate: Fluorescent intensity. Abscissa: Penicillin concn (μg ml^{-1}).

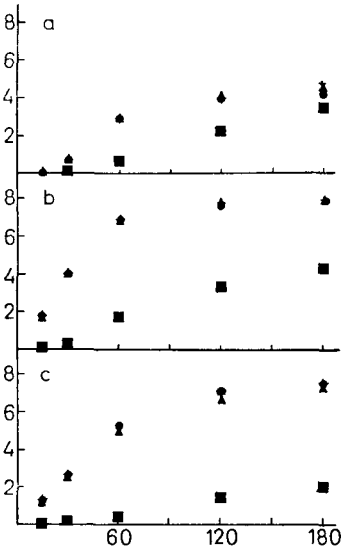

FIG. 2. Cumulative excretion in urine of unchanged penicillin V and its penicilloic acid after single dose intravenous administration of penicillin V potassium (100 mg kg^{-1}). Intact penicillin V by fluorometry (▲); by bioassay (+); by imidazole method (●); penicilloic acid by fluorometry (■). Rat weight a: 248 g; b: 288 g; c: 213 g. Ordinate: Cumulative amount excreted in urine (mg). Abscissa: Time (min).

V in the rat is shown in Fig. 2. It can be seen that the results from the microbiological assay (paper disk diffusion method) and the imidazole method developed by Bundgaard & Ilver (1972) of the same sample were in good agreement with those determined for unchanged penicillin V by the present fluorometric assay. About 9–15% of the dose administered was excreted in urine as penicilloic acid after 3 h, indicating that penicillins undergo metabolism to a significant extent, possibly in the liver, as demonstrated previously in rats by Ryrfeldt (1973) and by Kind, Beaty & others (1968).

The assay method described is simple, precise and reproducible and its speed, specificity, and its capability of measuring simultaneously penicillin and its meta-

Table 1. *Recovery of penicillin V (C_P) and its penicilloic acid (C_P) from synthetic mixtures by fluorometric determination.*

n = number of experiments.

added μg ml^{-1}		found μg ml^{-1} (s.d.)		
C_P	C_A	C_P	C_A	n
25·0	75·0	24·6 (1·6)	72·4 (0·7)	3
50·0	50·0	50·1 (3·0)	49·3 (2·1)	3
75·0	25·0	76·1 (1·5)	24·7 (1·2)	3

bolite, are advantages over microbiological assay procedures.

It may be applied with some modification to the determination of a series of penicillins in serum samples.

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The pH dependent absorption of propranolol and indomethacin by Parafilm, a stimulant of salivary secretion

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The possible use of drug concentration in saliva as a means of predicting plasma concentrations of the drug has attracted attention (Spiers, 1977). To obtain a sample of suitable volume for analysis it is sometimes necessary to stimulate salivary flow, the type of stimulus depending on the investigator's preference. One particular stimulus is the chewing of a waxy strip (Parafilm sealing tissue, Gallenkamp), by the subject, this is then discarded when sufficient saliva has been produced. Because the concentrations of drugs in biological fluids may be affected by the materials they come into contact with on collection and storage (Cotham & Shand, 1975; Rosseel & Bogaert, 1976) the possibility of uptake of drugs by Parafilm has been examined. For this purpose two lipid-soluble drugs, propranolol (a weak base) and indomethacin (a weak acid) were used (Study 1). The pH of saliva increases as salivary flow increases, independently of the stimulus (Dawes & Jenkins, 1964). We have therefore measured the changes in pH associated with increased salivary volume as a result of chewing Parafilm in 10 subjects (Study 2). We also wished to discover if Parafilm absorbed propranolol from the mouth and if the predicted reduction in saliva/plasma drug ratio occurred when salivary flow was stimulated by chewing the material (Study 3).

Study 1. Strips of Parafilm, 10 × 5 cm (each cut into 32 equal pieces about 1 cm^2) were added to 5 ml aliquots of Sörensen's phosphate buffer at pH 5·0, 6·0, 7·0 and 8·0 containing propranolol (200 ng ml^{-1}) or indomethacin (50 $\mu g\ ml^{-1}$). These were mechanically shaken at room temperature (25°) in 30 ml stoppered tubes for 3, 6, 9 or 12 min. Immediately after shaking the film was removed and the solution frozen to −20°. Propranolol was assayed fluorimetrically (Shand, Nuckolls & Oates, 1970) and indomethacin spectrophotometrically (Hvidberg, Lausen & Jansen, 1972). The procedure was repeated at each pH with buffer and film without drugs and the same results were obtained as with buffer alone. During the first 3 min of shaking there was a pH-dependent absorption of both drugs into the film; shaking for a further 9 min produced little change. At higher pH the absorption of propranolol was greater and that of indomethacin smaller (Table 1).

Study 2. The saliva produced by each subject over 2 min was collected by continually drawing the expelled samples into a pre-weighed 5 ml syringe. Air was excluded from the sample since the pH of saliva rises rapidly as carbon dioxide is lost into air (Dawes & Jenkins, 1964). The weight and volume of saliva were noted and its pH measured. After a further 5 min, saliva production was stimulated by the chewing of a

Table 1. *Percent (mean ± s.e., n = 4) of propranolol and indomethacin remaining in solution after shaking with Parafilm for 3 and 12 min at pH 5·0, 6·0, 7·0 and 8·0.*

	Propranolol		Indomethacin	
Time (min) pH	3	12	3	12
5·0	100	100	78 ± 4	71 ± 3
6·0	91 ± 3	86 ± 3	95 ± 1	90 ± 1
7·0	80 ± 2	71 ± 2	94 ± 4	97 ± 2
8·0	63 ± 3	61 ± 1	97 ± 3	96 ± 3

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