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New rapid assay for methicillin by spectrofluorimetry in pharmaceutical dosage forms

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Abstract: A method for the spectrofluorimetric determination of methicillin is proposed (λ_{ex} = 279 nm, λ_{em} = 379 nm), for concentrations between 0.030 and 10.0 μg ml^{-1}. The method was performed in water, at pH 6.20 provided by addition of phosphate buffer solution. The values obtained for detection and determination limits were 0.0188 and 0.063 μg ml^{-1}, respectively. The method was successfully applied to assay a commercial injection containing methicillin sodium monohydrate.

Keywords: *Methicillin; spectrofluorimetry; pharmaceutical dosage.*

Introduction

Methicillin was the first semisynthetic penicillin highly effective in penicillinase-producing staphylococcal infections in which activity is conferred mainly by steric hindrance. At doses of penicillinase activity which would completely destroy benzylpenicillin, serum concentrations of methicillin appear to be minimally affected.

At pH 2 biological activity of methicillin is quickly destroyed, therefore it cannot be given orally.

Although the antibacterial spectrum of methicillin is similar to that of penicillin G, its general potency is lower. Its therapeutic indication is the treatment of staphylococcal infections, especially when penicillinase production is suspected or proved [1].

Newer compounds, synthesized following the same structure–activity consideration were found to have therapeutic advantages over methicillin, Nafcillin, oxacillin, cloxacillin, dicloxacillin and flucloxacillin are not only resistant to the degradation of staphylococcal penicillinase but they are acid-stable and well absorbed from the gastrointestinal tract (except nafcillin) and possess greater intrinsic activities [1].

The majority of penicillins are not fluorescent compounds because the association of β-lactam and thiazolidine rings does not exhibit fluorescence by itself.

The methods described in the bibliography are based on derivatization reactions with a β-lactamic ring, therefore none of those is specific.

Jusko *et al.* [2] developed the first fluorimetric determination of penicillins based on the formation of a strongly fluorescent yellow product during acid hydrolysis at elevated temperature of ampicillin and other α-aminopenicillins [3–5].

Other described methods involve basic hydrolysis [6] in the presence of 2-methoxyethanol, or acid hydrolysis in the presence of Hg(II) [7–9].

Baker and Havlicek [10] used the fluorescamine derivative of penicillins and Mori *et al.* [11] developed a method for penicillin determination based on a quenching reaction.

Only one direct fluorimetric assay for nafcillin [12] has been described but a preliminary extraction is necessary.

Methicillin contains a bound group in the 6-position, which can be used as a source of fluorescence emission when excited at appropriate wavelengths. Using this property, a rapid spectrofluorimetric assay method for the determination of methicillin has been developed.

Experimental

Reagents

A stock solution of methicillin, 250 μg ml^{-1}

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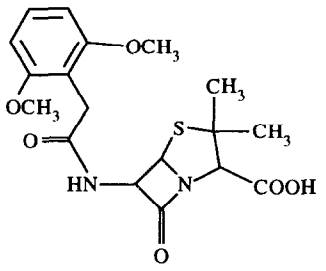

Methicillin

was prepared by dissolving the standard methicillin (Sigma) in Milli-Q water. A 0.05 M buffer solution of pH 6.20 was prepared by mixing adequate amounts of sodium dihydrogen phosphate with sodium hydroxide. A stock solution of methicillin was stored protected from light and maintained at 5°C. Under these conditions, the solution of methicillin was stable for 7 days. The working sample of methicillin was stable for at least 3 h at room temperature. Staphacillin injections were supplied by Bristol-Myers Squibb Company (USA).

Apparatus

All fluorimetric measurements were performed on a Perkin–Elmer LS-50 equipped with a Xenon lamp, connected to Ataio S 3000 ST 386 computer fitted with the Perkin–Elmer FL Data Manager software (designed for handling fluorescence data on a personal computer) and Epson FX-850 printer.

Thermostatic equipment and Crison, model 2001, pH-meter with a glass-saturated calomel combination electrode were also used.

Sample preparation and procedure

Calibration graph. A suitable aliquot containing 0.75–250 μg of methicillin was transferred into a 25 ml volumetric flask, 5.0 ml of buffer solution pH 6.20 was added and the solution diluted to volume with water. The fluorescence intensity was measured at 379 nm, with excitation at 279 nm against a reagent blank prepared in a similar way but without methicillin.

Procedure for injections. The sample solution was prepared by dissolving the contents of the vial in a 1000 ml volumetric flask and diluted to volume with Milli-Q water. A suitable volume of the sample solution was diluted with Milli-Q water to obtain a solution where the concentration of the methicillin was within the range studied (0.70 μg ml^{-1}). The assay was completed as described for calibration. The percentage of recovery of methicillin was calculated from the calibration graph of pure drug.

Results and Discussion

Spectral characteristics

The best characterization of the penicillin fluorescence is obtained from the emission–excitation matrix derived from a suitable computer program [13]. This spectrum can be obtained and presented as the isometric projection, where the emission spectra at stepped increments of excitation wavelength are recorded and plotted. A reversed projection of the data can sometimes indicate emission peaks hidden by the foreground. Alternatively, the tridimensional spectra can be effectively transformed to a plot in the two dimensions of excitation and emission wavelength by linking points of equal intensity to form contours, as shown in Fig. 1 where Rayleigh scatter has been removed. This contour presentation has generally been found to be more useful than the isometric projection for indicating the presence of hidden emission peaks.

As it can be seen, the methicillin presents two excitation maxima localized at 204 and 279 nm and a shoulder at 245 nm, and one emission maximum at 379 nm. The wavelengths chosen were 279 and 379 nm for excitation and emission, respectively, although we do not obtain the maximum sensitivity, the inner filter effect, that occurs at 204 nm, is avoided.

Factors affecting fluorescence intensity

Since the luminiscence behaviour of molecules is affected by the dielectric constant of the medium [14], the effect of ethanol content in the medium was investigated by preparing samples of penicillin varying only the ethanol percentage between 10 and 90%, v/v. The results are shown in Fig. 2; the relative intensity of fluorescence decreases with the ethanol content in the medium, for that we have selected an aqueous medium.

The influence of pH on the fluorescence spectrum was studied changing the pH by adding $HClO_4$ or NaOH. The fluorescence

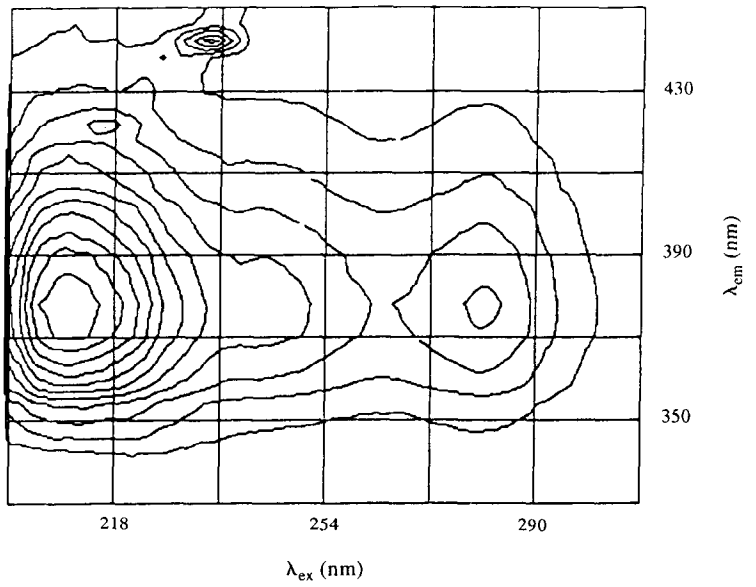

Figure 1
Contour plot of methicillin fluorescence (0.90 μg ml^{-1}) pH 6.20.

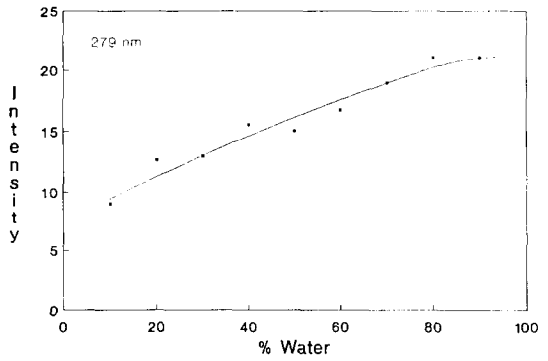

Figure 2
Influence of the ethanol content on the fluorescence intensity. [Methicillin] = 0.80 μg ml^{-1}, pH 6.20.

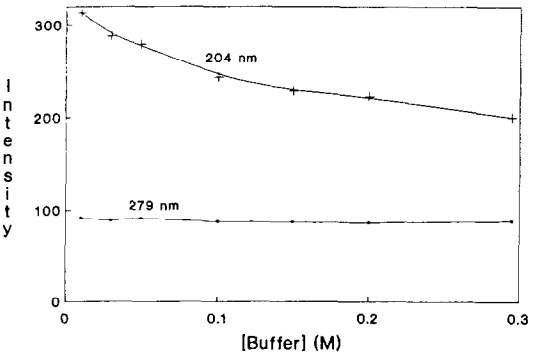

Figure 3
Influence of buffer concentration on the fluorescence intensity. [Methicillin] = 0.80 μg ml^{-1}, pH 6.20.

intensity suffers a slight increase with pH. A pH of 6.20 was selected, close to neutrality, to avoid the possibility of hydrolysis. This was achieved by adding phosphate buffer solution at pH 6.20.

A study was made of the influence of phosphate buffer, pH 6.20, on the fluorescence spectrum, with the conclusion that the buffer concentration has a considerable effect on the excitation maximum appearing at 204 nm, which is reduced in intensity and at the same time is displaced towards higher wavelengths as the buffer concentration is increased (Fig. 3); while the excitation maximum appearing at 279 nm suffers hardly any appreciable loss of intensity or displacement. A buffer concentration of 0.05 M was chosen as that providing the most suitable buffering capacity.

The dependence of the fluorescence intensity on temperature is critical, decreasing the fluorescence emission by 0.96% $°C^{-1}$ when the temperature increases from 3 to 70°C (Fig. 4). This effect can be explained by the higher internal conversion as temperature increases, facilitating non-radiative deactivation of the excited singlet state. Thus, it is recommended to use a thermostat, and control the temperature at 20°C, i.e. close to room temperature.

The fluorescence intensity of methicillin is proportional to the pencillin concentration up to 35 μg ml^{-1}. Concentration values higher than 35 ppm were not investigated.

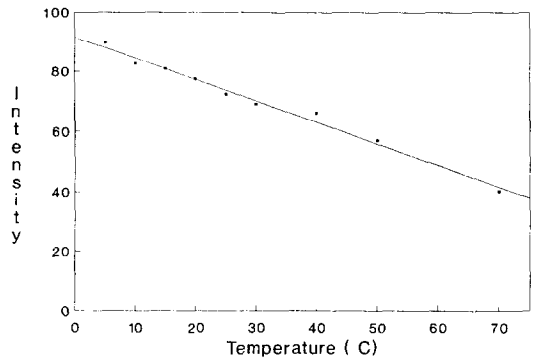

Figure 4
Influence of the temperature on the fluorecence intensity. [Methicillin] = 0.70 μg ml^{-1}.

Spectrofluorimetric determination of methicillin

Under the operating conditions outlined above, a method is proposed to determine methicillin by the direct measurement of fluorescence intensity at 379 nm, with excitation at 279 nm for two ranges of concentration covering a total interval of 0.030 to 10.0 μg ml^{-1}.

The proposed method was evaluated by a statistical analysis of experimental data by fitting the least-squares line according to $y = a + bx$. The results are presented in Tables 1 and 2.

To verify if the intercepts on the y-axis were negligible, significances were studied by applying Student's t-test at 95% confidence level and suitable degrees of freedom [15]. If the intercepts on the y-axis for the lines calculated by the least-squares technique are negligible, it is necessary to perform it again according to the function $y = b'x$, and therefore the new value of the slopes of graphs calibration (b') may be calculated. As it can be seen in Tables 1 and 2, the intercepts on the y-axis were negligible in both ranges of concentrations, since experimental t is smaller than theoretical t and therefore the new slopes were calculated.

Table 1
Statistical parameters to the first range of concentrations (0.03–1.0 μg ml^{-1})

Intercept on the y-axis	0.17
SD of intercept	1.03
Slope	107.93
SD of slope	1.90
Correlation coefficient	0.9989
Theoretical t value	2.306
Experimental t value	0.161
New slope	108.16

Table 2
Statistical parameters to the second range of concentrations (1.0–10.0 μg ml^{-1})

Intercept on the y-axis	1.82
SD of intercept	2.67
Slope	32.56
SD of slope	0.48
Correlation coefficient	0.9992
Theoretical t value	2.306
Experimental t value	0.680
New slope	32.83

The high value for the correlation coefficient of the regression equation and a negligible value for the intercept on the y-axis demonstrate the satisfactory linear relationship between fluorescence intensity and methicillin concentration in the ranges studied.

For two series of 10 standard samples at 0.30 and 5.0 μg ml^{-1} of methicillin, relative errors of 2.6 and 0.17% and standard deviations of 0.0107 and 0.0116 μg ml^{-1}, respectively, were obtained (95% confidence level).

The detection limit was 0.0188 μg ml^{-1} when defined as the analyte concentration leading to a luminiscence intensity that is three times the blank standard deviation and determination limit is 0.063 μg ml^{-1} when defined as the analyte concentration leading to a luminiscence intensity that is 10 times the blank standard deviation [16, 17].

Applications

Methicillin is not commercially available in Spain and we have only been able to obtain one injection product (Staphacillin) supplied by Bristol-Myers Squibb Co. (USA).

Table 3 shows the results, expressed as a percentage of the nominal content resulting from the average of three determinations of three different vials. To determine the ability of the spectrofluorimetric assay to accurately determine the methicillin in pharmaceutical preparations, these were also analysed by turbidimetry, iodometry and HPLC. As it can be seen, there was an excellent agreement between values obtained by previous techniques.

To test whether methicillin fluorescence is affected by excipients the spectrum of 'Stapha-

Table 3
Results obtained in assay

Method	% R
HPLC	102.4
Turbidimetric assay	103.1
Iodometric assay	102.7
Spectrofluorimetric assay	101.9

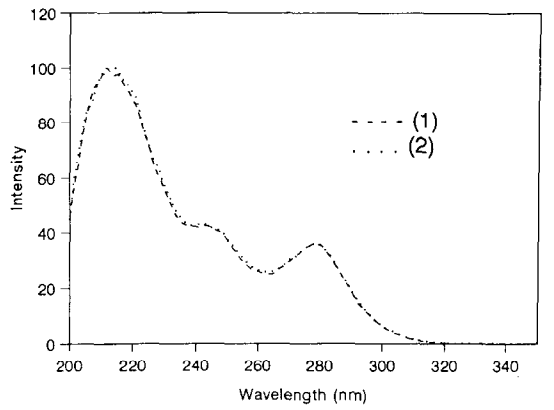

Figure 5
Normalized excitation spectra of Staphacillin (1) and standard methicillin (2), pH = 6.20.

cillin' solution was recorded in a similar way to the proposed method and this was compared with the spectrum of standard methicillin solution (Fig. 5). The shape of both spectra are equal in the range of wavelengths tested. This means that the only fluorescent compound in the pharmaceutical dosage is the methicillin and so the presence of excipients are compatible with this method. This fact was corroborated by means of the Standard Additions method.

Conclusions

A method for the direct fluorimetric determination of methicillin is described. The fluorescence is monitored at 379 nm (wavelength of excitation 279 nm) and the range of application of the method is between 0.030 and 10.0 μg ml^{-1}. The detection and determination limits obtained are 0.0188 and 0.063 μg ml^{-1}, respectively.

The method has been satisfactorily applied to the determination of methicillin in pharmaceutical dosage.

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