Talanta, Vol. 41, No. 1, pp. 21–30, 1994
Printed in Great Britain.

0039-9140/94 $6.00 + 0.00

DETERMINATION OF NAFCILLIN AND METHICILLIN BY DIFFERENT SPECTROFLUORIMETRIC TECHNIQUES

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(*Received* 15 *March* 1993. *Revised* 24 *June* 1993. *Accepted* 24 *June* 1993)

Summary—In order to explore the possibilities of combining synchronous fluorescence and derivative spectrometry and to establish a methodology for this type of technique, nafcillin and methicillin were determined using these techniques.

Several methods for resolving binary mixtures of these penicillins using first derivative spectrofluorimetry, first derivative constant wavelength synchronous luminescence spectrometry and constant energy synchronous luminescence spectrometry are described.

The analyses were performed in aqueous medium at pH 6.20 provided by the addition of phosphate buffer solution.

A complete and exhaustive statistical analysis of the experimental data was performed to demonstrate the validity of these methods, which obtained good results when applied for determining nafcillin and methicillin synthetic and real mixtures.

Fluorescence spectrometry is widely used in quantitative analysis because of its great sensitivity and selectivity as well as its relatively low cost. The technique has not, however, been widely applied to the simultaneous determination of several fluorescent components in mixtures, mainly because the fluorescence spectra of individual substances contain broad bands which may easily overlap. Generally, these compounds are determined by using a prior separation step, which is rather time-consuming for routine analysis and in some cases requires special, expensive instrumentation. For this reason, the development of techniques allowing the direct determination of related compounds through careful selection of instrumental variables, is of great interest. Among these techniques, synchronous and derivative fluorescence spectrometry are the most popular.

Synchronous luminescence spectrometry involves the simultaneous scanning of both the excitation and emission monochromators, synchronized in such a way that a well-defined relationship is maintained between the wavelengths of the monochromators. Conventionally, this relationship is a constant wavelength difference, $\Delta\lambda$, which is called constant wavelength synchronous luminescence spectrometry. When a constant energy difference is maintained between the monochromators, the technique is called constant energy synchronous luminescence spectrometry.

The advantages of synchronous techniques include a reduction in spectral complexity, in peak bandwidths, in Rayleigh scattering and even a reduction in Raman scattering.

The application of derivative techniques to luminescence spectrometry was first suggested by Green and O'Haver.[1] Differentiation narrows spectral bandwidths and enhances minor spectral features, thus improving the selectivity of multi-component spectra.

The use of derivative synchronous fluorescence spectrometry was first proposed by John and Soutar.[2]

Recently, a first study appeared where constant energy synchronous luminescence spectrometry and the derivative technique are combined to determine four polynuclear hydrocarbons.[3]

In this paper we apply these techniques to resolve the mixture of methicillin and nafcillin.

Methicillin and nafcillin are semi-synthetic penicillins in which resistance to β-lactame activity is mainly a result of steric hindrance.

Although in general penicillins are not fluorescent compounds, methicillin and nafcillin exhibit native fluorescence due to the nature of the group bound in the six-position.

As these antibiotics present strongly overlapping spectra, it is not possible to resolve this mixture by conventional fluorescence.

This paper describes three methods to resolve the mixture without using a separation step, by employing first derivative emission spectrometry and first derivative synchronous fluorescence spectrometry (both constant wavelength and constant energy).

In the first method proposed it was necessary to scan two emission spectra at different excitation wavelengths to determine methicillin and nafcillin, respectively.

In the other methods the mixture was resolved by scanning one spectrum, obtaining a higher response with constant energy fluorescence spectrometry than when using constant wavelength fluorescence spectrometry.

EXPERIMENTAL

Reagents

Stock solutions of 250 μg/ml nafcillin and 250 μ/ml methicillin were prepared by dissolving the respective sodium salts in Milli-Q water. Both sodium salts were obtained from the Sigma Chemical Company Products.

Buffer solution 0.05*M*. Buffer solution of pH 6.20 was prepared by mixing suitable amounts of sodium dihydrogen phosphate with sodium hydroxide.

Stock solutions were stored protected from light and maintained below 5°C. Under these conditions, the methicillin solution remained stable for 7 days and the nafcillin solution remained stable for 15 days.

The working samples of both penicillins remained stable for at least 4 hr at room temperature.

Injectable dosages of Staphacillin and Nafcil were supplied by Bristol-Mayers Squibb Company (U.S.A.).

Physiological serum and glucosed physiological serum: Apiroserum (Inst. de Biología y Sueroterapia, S.A.), Pérez Jiménez (Lab. Pérez Jiménez) and Grifols (Lab. Grifols) were randomly purchased from local pharmacies.

Apparatus

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

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

Sample preparations and procedure

Determination of methicillin and nafcillin by first derivative of emission spectra. Transfer aliquots of a stock solution containing suitable amounts of each penicillin into a 25-ml volumetric flask and add 5.0 ml of pH 6.20 buffer solution to every volumetric flask, together with a suitable volume of the Milli-Q water up to the mark, and so prepare working solutions in the range 0.10–1.0 μg/ml. Prepare a reagent blank in a similar way.

Record the emission spectra, thermostating the samples at 20°C, with excitation at 226 nm to determine nafcillin in the presence of methicillin and with excitation at 279 nm to determine methicillin in the presence of nafcillin.

Then correct from the blank signal and calculate the first derivative. The absolute value of the first derivate was measured at 379 and 366 nm for the determination of nafcillin and methicillin, respectively, in the corresponding derivative spectra described above.

Determination of methicillin and nafcillin by first derivative of constant wavelength synchronous spectra. Using this technique we proposed two procedures. One of them enables both penicillins to be determined by recording only one synchronous spectrum when these penicillins are found in the range 0.10–1.0 μg/ml.

Sample preparation was the same as in the first method.

Record the synchronous spectra, thermostating the samples at 20°C, by scanning both monochromators together, while maintaining a constant of 120 nm difference between both wavelengths. Then, correct for the blank signal and calculate the first derivative. The absolute values of the first derivative were measured at 246 and 257 nm for the determination of nafcillin and methicillin, respectively.

The other procedure enables nafcillin to be determined in the presence of methicillin and vice versa, by recording two synchronous spectra.

To determine nafcillin in the range 0.050–1.0 μg/ml in the absence and in the presence of methicillin, record the synchronous spectra maintaining a constant difference of 130 nm between both wavelengths. Calculate the first derivative and measure the absolute value of the derivative at 243 nm.

To determine methicillin in the range 0.050–1.0 μg/ml in the absence and in the presence of nafcillin, record the synchronous spectra maintaining a constant difference of 160 nm between both wavelengths. Calculate the first derivative and measure the absolute value of the derivative at 224 nm.

Determination of methicillin and nafcillin by first derivative of constant energy synchronous spectra. After preparing the samples as described above, record the synchronous spectra maintaining a constant difference of −15,200/cm between their wave numbers. Calculate the first derivative and measure the absolute value at 239 and 231 nm for the determination of nafcillin and methicillin, respectively. This method is valid when nafcillin concentration varies between 0.050 and 1.0 μg/ml and methicillin concentration varies between 0.10 and 1.0 μg/ml.

Simultaneous determination of nafcillin and methicillin in serum by the proposed methods described above. Take a suitable volume of physiological serum containing nafcillin and methicillin and dilute with Milli-Q water to obtain a solution where the concentration of these penicillins is within the range studied. Then complete the assay as described for calibration. The recovery percentage of the two antibiotics was computed from regression equations for pure drugs.

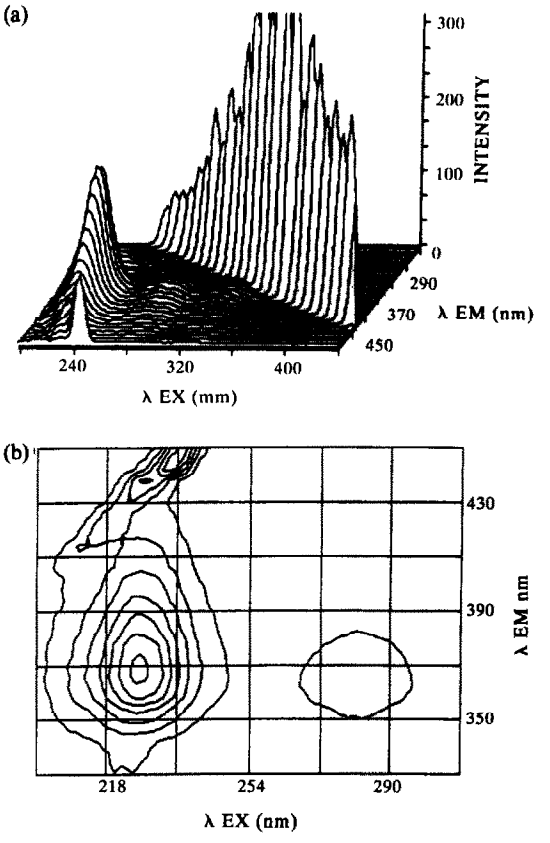

Fig. 1. Isometric plot of the emission–excitation matrix (reversed projection), (a) and in form of contour plot (b) of 0.50 μg/ml of nafcillin.

RESULTS AND DISCUSSION

Spectral characteristics

The best characterization of the compound's fluorescence can be obtained from its three-dimensional spectrum generated with a suitable computer program.[4] 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 (Figs 1(a) and 2(a)). Alternatively, the three-dimensional 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 Figs 1(b) and 2(b), where Rayleigh scattering 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 can be seen, nafcillin presents two fluorescence maxima located at different excitation wavelengths (226 and 279 nm) and the same emission wavelength, 366 nm. Similarly, methicillin exhibits two fluorescence maxima located at different excitation wavelengths (204 and 279 nm) and the same emission wavelength, 379 nm. For nafcillin, the working wavelengths chosen were 226 and 366 nm for excitation and emission, respectively, where the highest intensity is obtained. For methicillin, the excitation and emission wavelengths selected were 279 and 379 nm, respectively. In spite of the fact that we do not obtain maximum sensitivity with these, the inner filter effect, that occurs at 204 nm, is avoided.

Due to strong overlapping, it is not possible to resolve this mixture by conventional fluorescence, and it is necessary to apply other techniques.

Factors affecting fluorescence intensity

The effect of the ethanol content in the medium was investigated. An increase of

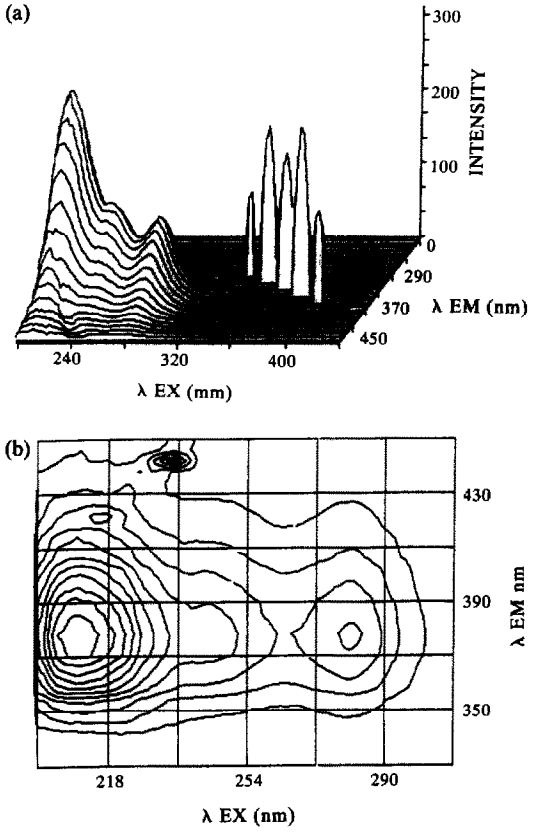

Fig. 2. Isometric plot of the emission–excitation matrix (reversed projection), (a) and in form of contour plot (b) of 0.90 μg/ml of methicillin.

ethanol in the medium causes a small increase in the fluorescence intensity of nafcillin while the fluorescence intensity of methicillin decreases. We chose to work with aqueous solutions where methicillin fluorescence is favoured, since under the working conditions this is the less fluorescent of the two penicillins.

The influence of pH on the fluorescence spectrum of these antibiotics was studied, changing the pH by adding $HClO_4$ or NaOH. The fluorescence of methicillin slightly increases with pH, while the fluorescence of nafcillin does not vary significantly. We selected the value of pH = 6.20, close to neutrality, so as to avoid the possibility of hydrolysis, as the most suitable. This is attained by the addition of phosphate buffer solution with pH = 6.20.

The variation in the fluorescence of these penicillins was investigated using phosphate buffer solutions of different concentrations at pH 6.20. The fluorescence intensity of nafcillin is not affected by the concentration of the buffer used, and although the methicillin maximum located at 204 nm is strongly modified due to the inner filter effect, the maximum located at 279 nm is not affected by the buffer concentration. A 0.05*M* concentration of buffer of pH 6.20 was found to be the best for an adequate buffering capacity.

The dependence of the fluorescence of these penicillins on temperature is critical, with the fluorescence emission of methicillin and nafcillin decreasing by 0.97 and 0.96%/°C, respectively, when the temperature increases from 3 to 70°C. This effect can be explained by higher internal conversion as temperature increases, facilitating nonradiative deactivation of the excited singlet state. Thus, it is advisable to use a thermostat, choosing a measurement temperature of 20°C, close to normal room temperature.

The influence of the penicillin concentration on fluorescence intensities was studied under similar conditions to those in the recommended procedure. The results obtained are the following.

The fluorescence intensity of nafcillin increases with penicillin concentration up to 5.0 μg/ml, remaining constant between 5.0 and 8.0 μg/ml. For penicillin concentrations higher than 8.0 μg/ml, intensity decreases because of fluorescence inversion phenomena.

The fluorescence intensity of methicillin is proportional to the penicillin concentration up to 35 μg/ml. Higher values were not investigated.

Resolution of the mixture of methicillin and nafcillin

As can be seen in the contour plots of these antibiotics (Figs 1(b) and 2(b)) neither excitation nor emission spectra exist where the bands are separated to resolve this mixture by conventional fluorescence due to strong overlapping. For this reason, the use of other fluorescent techniques is necessary.

At the beginning, the first derivative of three-dimensional spectra was calculated, but this technique does not separate the bands either. For this reason, the zero-crossing technique was applied. (This technique involves the measurement of the absolute value of the total derivative spectrum at an abscissa (wavelength) corresponding to the "zero-crossing" point of the derivative spectrum of the interfering component. At this wavelength, the width of the derivative signal of one of the two components passes through zero; measurement of the value of the derivative spectrum of mixture, made at the "zero-crossing" point of the derivative

spectrum of one of the two components, is, therefore, a function only of the concentration of the other component.) The following conclusions were obtained.

- It is not possible to resolve the mixture by the first derivative of any of the excitation spectra because these do not have any adequate zero-crossing point.
- Methicillin and nafcillin are determined by recording two emission spectra and calculating their first derivative.

Figure 3 shows the first derivative emission spectra ($\lambda_{ex} = 226$ nm) of methicillin and nafcillin and their mixture. We selected 379 nm as the zero-crossing wavelength for nafcillin. In Fig. 4 the first derivative emission spectra ($\lambda_{ex} = 279$ nm) is presented and the zero-crossing wavelength for methicillin is selected as 366 nm.

To obtain the first derivative spectra, a scan speed of 240 nm/min was selected after verifying that this parameter has practically no effect on the derivative signal obtained.

Five was chosen as the most suitable width factor (equivalent to 25 points convoluting according to Savitsky and Golay algorithm)[5] for calculation of the derivative since it provides the best signal to noise ratio.

Subsequently, we attempted to observe whether the results obtained would be better if another fluorescent technique was used.

Although we did not observe any 45° trajectory on the contour plots (conventional synchronous spectrum), that would enable these antibiotics to be determined by direct measurement of intensity, sequential scans of the synchronous spectra (40 scans) of two samples containing 0.50 μg/ml of nafcillin and 0.90 μg/ml of methicillin were carried out between 200 and 440 nm at different wavelength intervals (from 5 to 200 nm) (Figs 5(a) and (b) respectively).

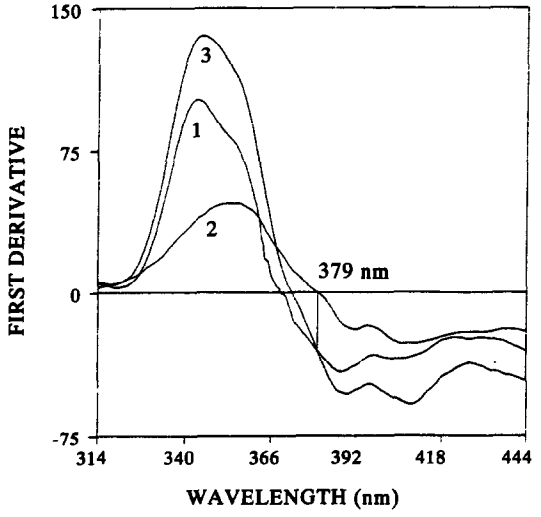

Fig. 3. First derivative emission spectra ($\lambda_{ex} = 226$ nm) of 0.75 μg/ml of nafcillin (1), 0.75 μg/ml of methicillin (2) and their mixture (3).

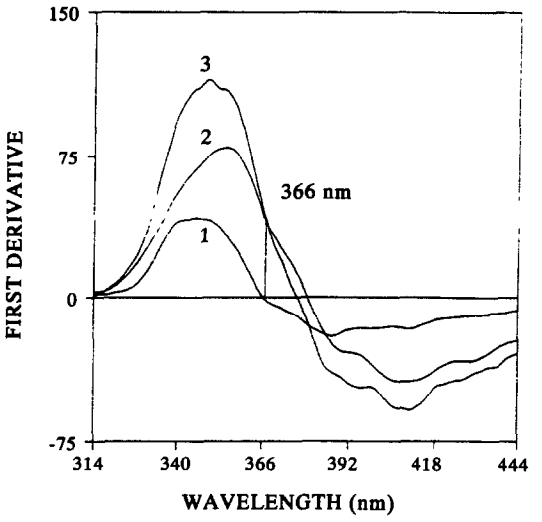

Fig. 4. First derivative emission spectra ($\lambda_{ex} = 279$ nm) of 0.75 μg/ml of nafcillin (1), 0.75 μg/ml of methicillin (2) and their mixture (3).

The first derivative of these spectra was obtained, but as this is not sufficient to resolve the mixture, the zero-crossing technique was applied and we deduced that: it is possible to determine nafcillin and methicillin by the first derivative of synchronous spectra using $\Delta\lambda = 120$ nm.

Figure 6 shows the first derivative spectra of nafcillin and methicillin and their mixture. We selected 246 and 257 nm as the best zero-crossing wavelengths for nafcillin and methicillin, respectively.

As the signals obtained were small, we decided to obtain the first derivative of synchronous spectra with wavelength intervals of 130 nm to determine nafcillin in the presence of methicillin, measuring at 243 nm, and the first derivative of synchronous spectra, recorded with $\Delta\lambda = 160$ nm, to determine methicillin in the presence of nafcillin, measuring at 224 nm. In this way, higher signals were attained than in the previous assay.

In all cases, a scan speed of 240 nm/min and a width factor of 4 (equivalent to 19 points convoluting according to Savitsky and Golay algorithm)[5] were selected to obtain the first derivative of synchronous spectra for the same reasons described above.

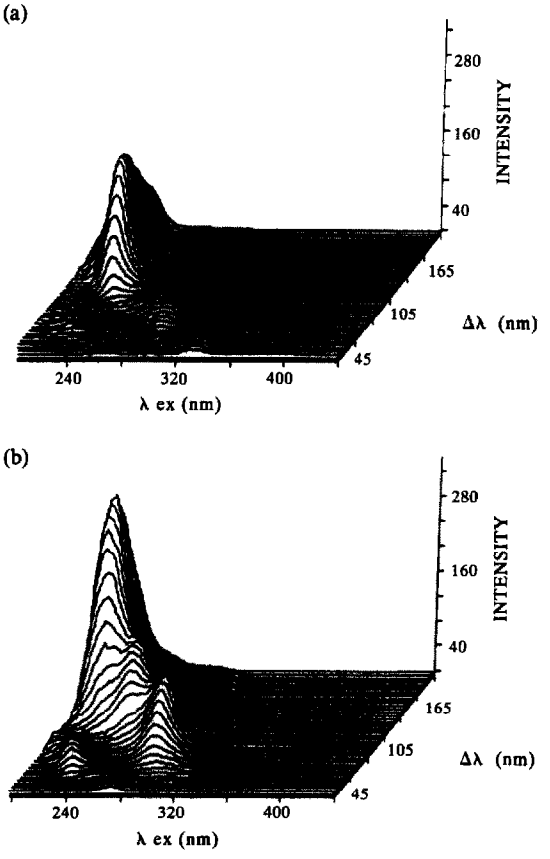

Fig. 5. Isometric plot of the synchronous spectra (forward projection) of 0.50 μg/ml of nafcillin (a) and 0.90 μg/ml of methicillin (b) ($\Delta\lambda = \lambda_{em} - \lambda_{ex}$, from 5 to 200).

Finally, to complete the work, a series of constant energy synchronous spectra of two samples of nafcillin and methicillin (36 scans) were recorded between 200 and 320 nm at different wave number intervals ranging from -4000 to 18,400/cm (Figs 7(a) and (b)).

It was not possible to resolve the mixture even when the first derivative was applied. For this reason, it was necessary to use the zero-crossing technique.

The best sensitivity was achieved for both penicillins when they were resolved by recording the constant energy synchronous spectra with a wave number interval of $-15{,}200$/cm. The nafcillin and methicillin concentrations are proportional to the signal measured at 239 and 231 nm, respectively.

The first derivatives were calculated with a scan speed of 240 nm/min and a width factor of 4 (equivalent to 19 points convoluting according to Savitsky and Golay algorithm)[5] to provide a suitable signal to noise ratio.

Statistical analysis of results

In order to test the mutual independence of the analytical signals of methicillin and nafcillin, *i.e.* to show that zero-crossing wavelengths selected in every method proposed are independent of the other penicillin present, the following experiments were performed.

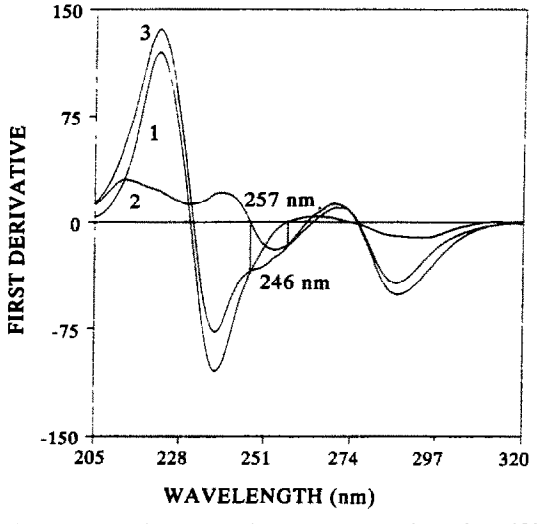

Fig. 6. First derivative synchronous spectra ($\lambda_{em} - \lambda_{ex} = 120$ *nm*) of 0.75 μg/ml of nafcillin (1), 0.75 μg/ml of methicillin (2) and their mixture (3).

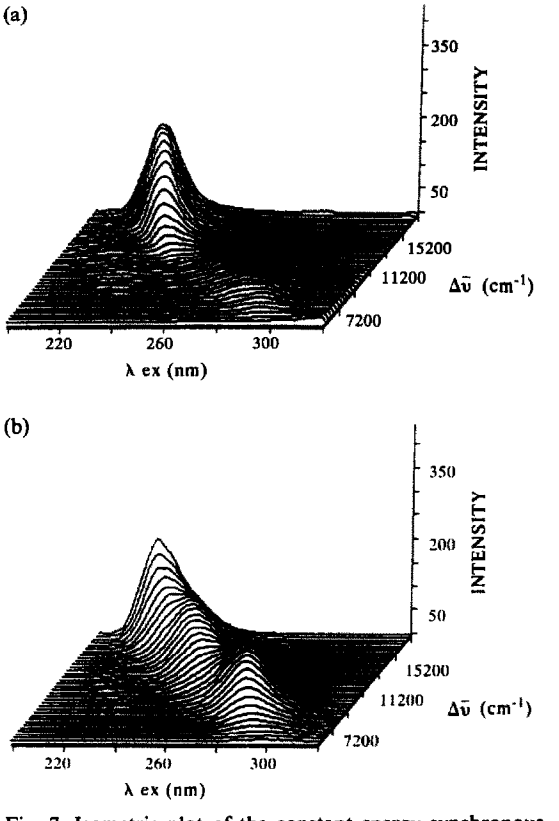

Fig. 7. Isometric plot of the constant energy synchronous spectra (forward projection) of 0.50 μg/ml of nafcillin (a) and 0.90 μg/ml of methicillin (b).

Determination of methicillin and nafcillin by first derivative of emission spectra. Calibration graphs were constructed from first derivative signals for standards containing between 0.10 and 1.0 μg/ml of nafcillin in the absence of methicillin and in the presence of 0.25 and 0.75 μg/ml of methicillin. Similarly, three calibration graphs were prepared for standards containing between 0.10 and 1.0 μg/ml of methicillin in the absence of nafcillin and in the presence of 0.25 and 0.75 μg/ml of nafcillin.

The slope, intercept, correlation coefficient and standard deviations obtained are summarized in Table 1. The linearity of the calibration graphs is validated by the high value for the correlation coefficient of the regression equation. To verify whether the intercept on the ordinate is negligible, its significance was studied applying the Student t-test[6] at 95% confidence level and the respective degrees of freedom. If the intercept on the ordinate is negligible it is necessary to calculate the new value of the slope. As can be seen in Table 2, in all the calibration graphs, the intercept on the ordinate is negligible, since the experimental t-value is smaller than the critical t-value, and therefore the new slope is calculated.

In order to calculate the accuracy of the method a critical evaluation of the experimental was performed. Accuracy is a complex property to which the variation between samples and the calibration graphs–sample interaction all contribute. The statistical technique which enables the total variation to be broken down into these components is called "analysis of variance".

The validity of the analysis of variance assumes that the residual error variance does not change from one sample to another or from one calibration graph to another.

To carry out an analysis of variance, the variance ratio (experimental F) must be calculated and compared to a critical value of F, for adequate degrees of freedom at 95% confidence level.[7,8]

Applying the analysis of variance calculations to the experimental data we obtain the results which are shown in Table 3.

Table 1. Statistical data for calibration graphs in the determination nafcillin and methicillin

Method proposed*	Antibiotic determined	Other antibiotic present: Antibiotic	Concentr. ($\mu g/ml$)	Slope	Intercept	Correlation coefficient	Standard deviation: Slope	Intercept
1*	Nafcillin	—	—	38.60	−0.17	0.9997	0.50	0.31
		Methicillin	0.25	33.40	0.97	0.9891	2.87	1.76
			0.75	36.95	0.04	0.9999	0.31	0.19
	Methicillin	—	—	18.19	0.32	0.9980	0.64	0.41
		Nafcillin	0.25	19.47	0.19	0.9999	0.19	0.12
			0.75	18.58	0.62	0.9992	0.43	0.26
2*	Nafcillin	—	—	45.96	0.85	0.9997	0.64	0.39
($\Delta\lambda = 120$ nm)		Methicillin	0.25	46.35	0.61	0.9995	0.85	0.52
			0.75	46.52	0.47	0.9995	0.89	0.55
	Methicillin	—	—	22.89	−0.40	0.9981	0.82	0.51
		Nafcillin	0.25	21.26	0.85	0.9962	1.07	0.66
			0.75	22.02	0.49	0.9994	0.43	0.26
($\Delta\lambda = 130$ *nm*)	Nafcillin	—	—	93.22	0.97	0.9998	0.94	0.53
		Methicillin	0.25	92.27	1.34	0.9991	1.95	1.09
			0.75	91.61	0.82	0.9997	1.08	0.60
($\Delta\lambda = 160$ *nm*)	Methicillin	—	—	118.91	0.75	0.9988	2.91	1.63
		Nafcillin	0.25	116.30	0.87	0.9996	1.57	0.87
			0.75	116.24	1.34	0.9991	2.50	1.40
3*	Nafcillin	—	—	191.27	2.77	0.9995	3.11	1.75
($\Delta\nu = -15200\ cm^{-1}$)		Methicillin	0.25	192.46	2.44	0.9997	2.36	1.32
			0.75	192.41	2.00	0.9996	2.84	1.25
	Methicillin	—	—	63.94	0.53	0.9989	1.70	1.04
		Nafcillin	0.25	63.78	0.89	0.9985	1.99	1.22
			0.75	64.08	0.76	0.9992	1.51	0.92

1: First derivative spectrofluorimetry. 2*: First derivative constant wavelength synchronous luminescence. 3*: First derivative constant energy synchronous luminescence.

Table 2. Statistical parameters used in Student's t-test

Method used*	Calibration graphs	Experimental "t" value	Critical "t" value	New slope
1*	Nafcillin	0.550	2.776	38.37
	Naf. + 0.25 $\mu g/ml$ Met.	1.686	2.776	37.49
	Naf. + 0.75 $\mu g/ml$ Met.	0.191	2.776	37.00
	Methicillin	0.775	2.776	18.63
	Met. + 0.25 $\mu g/ml$ Naf.	1.346	2.776	19.20
	Met. + 0.75 $\mu g/ml$ Naf.	2.346	2.776	19.44
2*	Nafcillin	2.159	2.776	47.13
($\Delta\lambda = 120$ nm)	Naf. + 0.25 $\mu g/ml$ Met.	1.170	2.776	47.19
	Naf. + 0.75 $\mu g/ml$ Met.	0.845	2.776	47.17
	Methicillin	0.789	2.776	22.34
	Met. + 0.25 $\mu g/ml$ Naf.	1.293	2.776	22.43
	Met. + 0.75 $\mu g/ml$ Naf.	1.874	2.776	22.69
($\Delta\lambda = 130$ nm)	Nafcillin	1.843	2.571	94.58
	Naf. + 0.25 $\mu g/ml$ Met.	1.229	2.571	94.15
	Naf. + 0.75 $\mu g/ml$ Met.	1.355	2.571	92.76
($\Delta\lambda = 160$ nm)	Methicillin	0.457	2.571	119.96
	Met. + 0.25 $\mu g/ml$ Naf.	0.986	2.571	117.52
	Met. + 0.75 $\mu g/ml$ Naf.	0.952	2.571	117.52
3*	Nafcillin	1.554	2.571	195.15
($\Delta\nu = -15{,}200$ cm^{-1})	Naf. + 0.25 $\mu g/ml$ Met.	1.842	2.571	195.92
	Naf. + 0.75 $\mu g/ml$ Met.	1.253	2.571	195.21
	Methicillin	0.506	2.776	64.67
	Met. + 0.25 $\mu g/ml$ Naf.	0.729	2.776	65.00
	Met. + 0.75 $\mu g/ml$ Naf.	0.819	2.776	65.12

1: First derivative spectrofluorimetry. 2*: First derivative constant wavelength synchronous luminescence. 3*: First derivative constant energy synchronous luminescence.

In nafcillin calibration graphs and methicillin graphs the experimental value of F is smaller than the critical value of F, at 95% confidence level, thus the source of variation is not significant. Therefore, it can be deduced that the signal height of the mixture, measured at the zero-crossing point of the derivative spectrum of one of the two components, is a function only of the concentration of the other component, in accordance with the theoretical predictions.

For a series of five standard samples containing 0.75 μg/ml of nafcillin and 0.75 μg/ml of methicillin a relative error of 3.10 and 2.76% and standard deviation of 1.92×10^{-2} and 1.67×10^{-2} μg/ml were obtained for nafcillin and methicillin, respectively (95% confidence level).

Determination of methicillin and nafcillin by first derivative of constant wavelength synchronous spectra. As in the previous case, three

Table 3. Statistical parameters used in the analysis of variance

Method proposed*	Calibration graphs	Experimental "F" value	Critical "F" value
1*	Nafcillin	2.98	4.46
	Methicillin	3.74	4.46
2*	Nafcillin	1.01	4.46
($\Delta\lambda = 120$ nm)	Methicillin	1.59	4.46
($\Delta\lambda = 130$ nm)	Nafcillin	3.78	4.10
($\Delta\lambda = 160$ nm)	Methicillin	1.61	4.10
3*	Nafcillin	1.06	4.10
($\Delta\nu = -15{,}200$ cm^{-1})	Methicillin	3.63	4.46

1: First derivative spectrofluorimetry. 2*: First derivative constant wavelength synchronous luminescence. 3*: First derivative constant energy synchronous luminescence.

calibration graphs were constructed from first derivative signals corresponding to the synchronous spectra with wavelength intervals of 120 nm, for standards containing between 0.10 and 1.0 μg/ml of nafcillin in the absence of methicillin and in the presence of 0.25 and 0.75 μg/ml of methicillin. The same calibration graphs were constructed with methicillin.

Three calibration graphs were constructed from first derivative signals corresponding to the synchronous spectra with wavelength intervals of 130 nm for standards containing between 0.050 and 1.0 μg/ml of nafcillin in the absence of methicillin and in the presence of 0.25 and 0.75 μg/ml of methicillin. From the synchronous spectra recorded with wavelength intervals of 160 nm we constructed three calibration graphs varying the methicillin concentration between 0.050 and 1.0 μg/ml in the absence of nafcillin and in the presence of 0.25 and 0.75 μg/ml of nafcillin.

A similar statistical analysis as described above was performed. The slope, intercept, correlation coefficient and standard deviations obtained are summarized in Table 1. In all cases the intercept on the ordinate is negligible as Table 2 shows.

As can be seen in Table 3 the experimental values of F are smaller than the critical values of F, at 95% confidence level, and so it can be deduced that the presence of nafcillin does not interfere in the measurement of methicillin and vice versa.

For a series of five standard samples containing 0.75 μg/ml of nafcillin and 0.75 μg/ml of methicillin a relative error of 1.65 and 1.90% and standard deviation of 8.37×10^{-3} and 1.14×10^{-2} μg/ml were obtained for nafcillin and methicillin, respectively (95% confidence level) when synchronous spectra with wavelength intervals of 120 nm are used.

When synchronous spectra with wavelength intervals of 130 and 160 nm are used, a relative error of 1.65 and 1.38% and standard deviation of 1.00×10^{-2} and 8.37×10^{-3} μg/ml were obtained for nafcillin and methicillin, respectively (95% confidence level).

Determination of methicillin and nafcillin by first derivative of constant energy synchronous spectra. Finally three nafcillin calibration graphs were constructed from first derivative of constant energy synchronous spectra, varying the concentration between 0.050 and 0.10 μg/ml in the absence of methicillin and in the presence of 0.25 and 0.75 μg/ml of methicillin. In the same way, a further three calibration graphs were prepared for standards containing between 0.10 and 1.0 μg/ml of methicillin in the absence and in the presence of nafcillin.

Table 1 presents the slope, intercept, correlation coefficient and standard deviations obtained.

After carrying out the analysis of variance (Table 3) and the Student t-test (Table 2) it can be deduced that the height of the derivative signal of the mixture, measured at the zero-crossing point of the derivative spectrum of one of the two components, is a function only of the concentration of the other component, in accordance with the theoretical predictions.

For a series of five standard samples containing 0.75 μg/ml of nafcillin and 0.75 μg/ml of methicillin a relative error of 1.50 and 1.90% and standard deviation of 8.94×10^{-3} and 1.14×10^{-2} μg/ml were obtained for nafcillin and methicillin, respectively (95% confidence level).

The validity of the proposed methods was tested by successive determinations of nafcillin and methicillin in synthetic mixtures. The recoveries for methicillin in the concentration range 0.10–1.0 μg/ml added to standards of nafcillin containing 0.25–0.75 μg/ml were 98.1–102.9%, while recoveries for nafcillin (0.10–1.0 μg/ml) added to standards of methicillin containing 0.25–0.75 μg/ml were 98.3–102.7% when the first derivative spectrofluorimetric method was applied. When the first derivative of constant wavelength synchronous luminescence spectrometric method ($\Delta\lambda = 130$ and 160 nm) was used to determine nafcillin and methicillin in the presence of each other in the concentration range 0.25–1.0 μg/ml, the recoveries were 99.0–103.6% and 98.3–102.7%, respectively. For the simultaneous determination of nafcillin and methicillin in the concentration range 0.25–1.0 μg/ml for both compounds, by first derivative of constant wavelength synchronous luminescence spectrometry ($\Delta\lambda = 120$ nm), the recoveries were 97.4–103.9% and 98.4 and 102.8%, respectively. When this determination was obtained by constant energy synchronous luminescence spectrometry (wave number intervals of $-15{,}200$/cm), the recoveries for nafcillin (0.05–0.75 μg/ml) and methicillin (0.25–1.0 μg/ml) were 98.0–101.2% and 98.8–102.3%, respectively. As can be seen, satisfactory results were obtained for recovery percentage of both compounds in all the cases discussed.

APPLICATIONS

In order to study the validity of the method, and because there are no medicines commercially available which contain these two penicillins, the methods proposed were applied to their determination in physiological serum and glucosed physiological serum, since intravenous injection is the preferred administration route in severe infections.[9] The determination of these pencillins was carried out with three serums from different commercial companies: "Apiroserum" (Inst. de Biología y Sueroterapia, S.A.), "Pérez Jiménez" (Lab. Pérez Jiménez) and "Grifols" (Lab. Grifols). Three determinations were carried out for each of these serums and using nafcillin and methicillin medicines ("Nafcil" and "Staphacillin", respectively, Bristol-Mayers Squibb Company, U.S.A.). Recoveries achieved by means of all the methods proposed are in accordance with the real content of nafcillin and methicillin in both the physiological serum and the glucosed physiological serum. Recovery percentages achieved in all cases vary between 98.5% and 102.6% for nafcillin and between 98.3% and 102.9% for methicillin.

CONCLUSIONS

In this study we attempt to explore the possibilities of combining synchronous luminescence spectrometry (both constant wavelength and constant energy) and derivatives techniques and to establish a methodology for this kind of technique, by using it to determine nafcillin and methicillin in synthetic and real mixtures.

Due to the strong overlapping of excitation and emission spectra it is impossible to resolve this mixture by conventional fluorescence. Although application of derivative techniques to the excitation spectra does not resolve this problem, it is possible to determine nafcillin and methicillin in mixtures by the first derivative of two emission spectra using the zero-crossing technique.

By derivative constant wavelength synchronous luminescence spectrometry simultaneous nafcillin and methicillin can be determined using two spectra where the differences between the emission and excitation wavelengths are 130 and 160 nm. It is possible to determine both penicillins simultaneously by obtaining the first derivative of one spectrum ($\Delta\lambda = 120$ nm), but the signals obtained are smaller. In both cases it is necessary to use the zero-crossing technique.

Applying first derivative constant energy synchronous fluorescence, nafcillin and methicillin are determined without the need to obtain two spectra. With this technique the detection limit of nafcillin is improved.

The determination of these penicillins has already been described in the literature. Thus, nafcillin and methicillin have been determined by HPLC, but with limits of detection higher than those of the proposed method.[10] Meetschen and Petz[11] determined these penicillins by gas chromatography following chemical reaction by means of the programmed temperature method. This method is, logically, slower than the proposed method.

Acknowledgement—The authors gratefully acknowledge financial support from the "Dirección General de Investigación Científica y Técnica" (Project No PB 88-0365).

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