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SPECTROFLUORIMETRIC ANALYSIS OF CEFOXITIN IN PHARMACEUTICAL DOSAGE

J. A. MURILLO, J. M. LEMUS and L. F. GARCÍA

Department of Analytical Chemistry and Food Technology, University of Castilla-La Mancha, 13071 Ciudad Real, Spain

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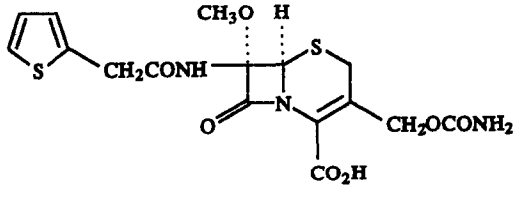
Summary—A fluorescence method involving sample pre-treatment is investigated concerning the determination of cefoxitin. A fluorescent product is formed when samples containing cefoxitin are subjected to alkaline hydrolysis with 1.0*M* sodium hydroxide and heated for 60 min at 90°. The fluorescence is measured in ethanol/water medium (50% v/v) at approximately pH 2.0 provided by adding of 0.1*M* hydrochloric acid. The fluorescence excitation and emission maxima were 317 and 400 nm, respectively. The quantitative range is between 0.020 and 1.40 μg/ml. A detection limit of 2×10^{-3} μg/ml was found. The proposed method has been applied to the determination of cefoxitin in commercial injections, saline and glucosed physiological serum.

Cefoxitin is a semisynthetic cephamycin antibiotic derived from cephamycin C. The drug is a β-lactam antibiotic structurally and pharmacologically related to cephalosporins and penicillins. Cephamycins have the greatest stability to β-lactamases. This striking stability to hydrolysis is related to the methoxy group in the 7-α-position, which is a common feature in all these compounds. Thus, cefoxitin is active against *Bacteroides* because of its stability to practically all β-lactamases, including inducible cephalosporinases.[1,2]



Cefoxitin

Cefoxitin is an important therapeutic antimicrobial agent. This antibiotic has been used largely for the treatment of mixed aerobic–anaerobic infections. In this way, this cephamycin appears to be effective in the initial treatment in intra-abdominal infections[3–5] and respiratory tract infections.[6–10]

Several methods for its determination have been described in the bibliography. The official procedures utilize a spectrophotometric analysis by using the hydroxylamine method.[11–14] Microbiological assay techniques have been applied.[15,16] HPLC has been used to quantify the content of cefoxitin in body fluids.[17–19]

Fluorimetric procedures for other cephalosporins have been developed.[20–22] Cefoxitin has been also determined by using a fluorimetric method which involves complex procedure based on the addition of hydrochloric acid, heating and cooling, followed by addition of sodium hydroxide in order to make the pH 12 and keeping the final solution at room temperature for 60 min. The fluorescence intensity was measured at the excitation and emission wavelengths of 385 nm and 460 nm, respectively. The minimum detectable concentration of cefoxitin was 1.25 μg/ml for aqueous solution.[23]

The purpose of this study was to develop a fluorimetric method less tedious, more rapid and sensitive than the previously reported for estimating cefoxitin. This selective fluorimetric assay is simple and sensitive enough to be useful for determining this antibiotic in commercialized pharmaceutical samples and physiological sera (saline and glucosed).

EXPERIMENTAL

Reagents

A stock solution containing 100 μg/ml of cefoxitin (Sigma R.A.) in Milli-Q water was prepared. This solution was stored protected in the dark and maintained below 4°. All

experiments were performed with analytical-reagent grade chemicals and pure solvents.

Injectable dosage forms of Mefoxitin (MSD de España, S.A.) and Cefaxicina (Comp. Española de la Penicilina y Antibióticos, S.A.), were labelled to contain 1 g of cefoxitin sodium salt per vial in both cases.

Saline physiological serum, 0.9% sodium chloride, was supplied from Apiroserum (Instituto de Biología y Sueroterapia, S.A.) and Grifols (Laboratorios Grifols S.A.). Glucosed physiological serum, 5% anhydre glucose (dextrose), was supplied from Apiroserum (Instituto de Biología y Sueroterapia, S.A.).

Apparatus

Fluorimetric measurements were made 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. A thermostatically controlled bath, Selecta Model Frigiterm, was used for temperature control. A pH-meter, Crison Model 2001, with a glass-saturated calomel combination electrode was also used.

General procedure

A stock solution containing 100 μg/ml drug in water is prepared. This stock solution is four-fold diluted with sodium hydroxide. The resulting alkaline solution (1.0*M*) is heated at 90° for 60 min to produce a fluorescent product. After heating in a water bath, the sample is cooled in an ice water bath. Finally, the solution containing the fluorescent product formed from cefoxitin is adjusted to neutral pH with hydrochloric acid.

Aliquots of this neutralized solution of the hydrolized cephamycin are transferred into a 25-ml volumetric flask to produce working solutions in the range 0.020–1.40 μg/ml. Then, add 2.5 ml of 0.1*M* hydrochloric acid to obtain a pH of 2.0 in the final solution and enough ethanol to ensure a final ethanol content of 50% v/v to every volumetric flask, and dilute to volume with water. Measure the fluorescence intensity at 400 nm with excitation at 317 nm, against a blank sample that had been treated similarly.

Procedure for injections

Cefoxitin powder for injection is used by dilution of the contents of proprietary vials of parenteral material in different media: Milli-Q water, saline physiological serum and glucosed physiological serum. In aqueous medium, the vial content is dissolved in a 100-ml standard flask and diluted to volume with Milli-Q water. The method was applied to the injectable solutions after dilution of the sample. For the clinical use of 100-ml intravenous infusions, a 1-g vial of cefoxitin must be added so that the final solution has a concentration of 10 mg/ml. Therefore, the content of the vial is dissolved in the case of both saline physiological serum and glucosed physiological serum, so that the concentration of antibiotic used clinically is obtained. A suitable quantity of these solutions of cefoxitin in the saline and glucosed solutions was analysed following the general procedure described above, after prior dilution. The percentage of the antibiotic present was calculated using the linear regression equation obtained for pure compound.

RESULTS AND DISCUSSION

Optimization of hydrolysis conditions

The fluorescence intensity produced depends on such factors as the sodium hydroxide concentration, the reaction temperature and the reaction time.

The influence of the sodium hydroxide concentration on the fluorescence intensity of the product formed after heating the cefoxitin at 90° for 60 min can be seen in Fig. 1. As the fluorescence intensity shows considerable variation at the highest and lowest concentrations of the range studied, a sodium hydroxide concentration of 1.0*M* is considered to be optimum, since this lies in the intermediate zone where the effect of change in the sodium hydroxide concentration is minimal.

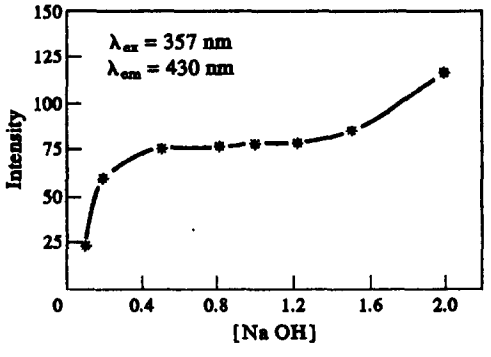


Fig. 1. Effect of sodium hydroxide concentration on the fluorescence of cefoxitin standars after reaction at 90° for 60 min. [Cefoxitin] = 1.0 μg/ml.

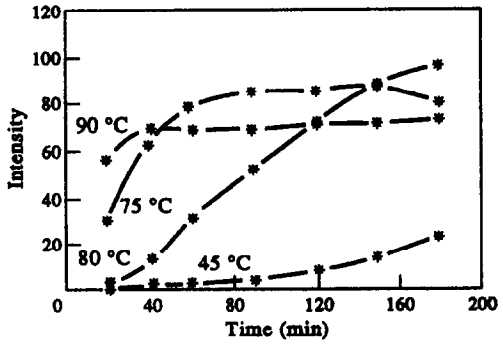


Fig. 2. Effect of temperature and heating time on the fluorescence of cefoxitin standards in 1.0*M* NaOH. [Cefoxitin] = 1.0 μg/ml.

To determine the rate of formation of the fluorescent product, four cefoxitin solutions containing 5.0 μg/ml of the drug were prepared in 1.0*M* sodium hydroxide and heated at different temperatures (45, 60, 75 and 90°) for 150 min. At 15-min intervals, a 5.0-ml aliquot was withdrawn and the volume was adjusted to 25.0 ml in the volumetric flasks and the fluorescence intensity was subsequently measured. As can be observed in Fig. 2, although higher fluorescence signals can be obtained at 60 and 75°, longer reaction times would be necessary to achieve the stabilisation of the signal. When the cefoxitin reacts with 1.0*M* sodium hydroxide at 90°, a constant fluorescence intensity is obtained for between 50 and 180 min, which indicates that in this range the influence of the hydrolysis time is not critical, and easily reproducible signals are obtained. A 60-min period is considered a suitable reaction time for hydrolysis at 90°.

The literature on cephalosporins gives no hint to possible fluorescent products under extremely alkaline conditions. Jusko[24] proposes the formation of a diketopiperazine as a product of ampicillin acid hydrolysis. The presence of an α-aminobencyl group in the penicillins or cephalosporins is necessary for the formation of this product. The first studies on hydrolysis of cephalosporins indicated that oxazolones could not be obtained.[25] However, Yu *et al.*[20] propose the formation of oxazolones as a fluorescent product if the hydrolysis is conducted at high temperatures and under highly alkaline conditions as in our case.

The stability of the fluorescent product formed from cefoxitin was studied. The results showed that in neutral medium, the fluorophor was stable at ambient temperature (20° approximately) for at least 24 hr and at 4° for at least 30 days. The dissolution is less stable in a highly acid and basic medium. For this reason, we propose changing this dissolution to neutral pH after hydrolysis treatment for its storage.

Effect of variables on fluorescence intensity

The fluorescence intensity is generally increased by the presence of ethanol in the final assay sample,[26] as demonstrated by previous experiments. Because of this, the influence of pH on the fluorescence spectrum was studied in an ethanol–water medium (50/50% v/v). To choose the pH at which to perform the assay, the pH of the sample was varied by adding hydrochloric acid or sodium hydroxide. The results show a higher fluorescence intensity at acidic pH and so the solution containing the fluorescent product formed from cefoxitin was adjusted to pH 2.0. The selected pH was provided by adding an adequate amount of 0.1*M* hydrochloric acid.

The influence of the amount of ethanol in the medium on the fluorescence intensity was studied by varying the ethanol percentage between 0 and 96% v/v and keeping the pH at approximately 2.0. The relative intensity of fluorescence increased with the ethanol content in the medium [Fig. 3(a)]. In order to obtain the maximum intensity with regard to the dilution of the aqueous reaction mixture [Fig. 3(b)], the final assay samples were made 50% v/v

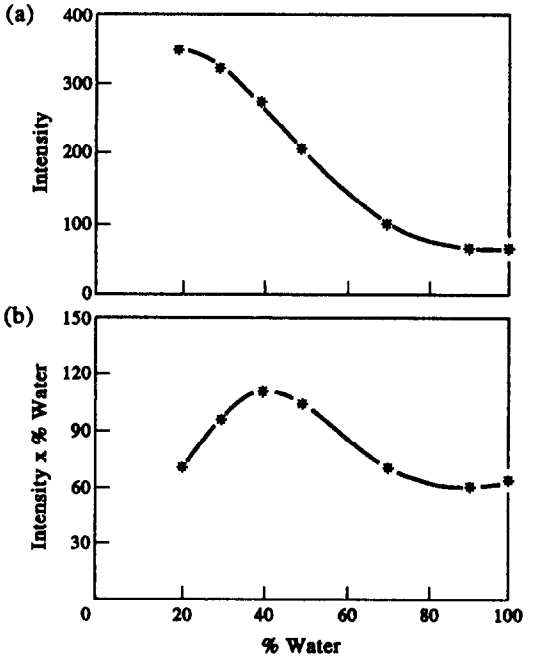


Fig. 3. (a) Influence of the ethanol content. (b) Representation of fluorescence intensity with regard to aqueous fraction. [Cefoxitin] = 0.80 μg/ml. pH = 2.0.

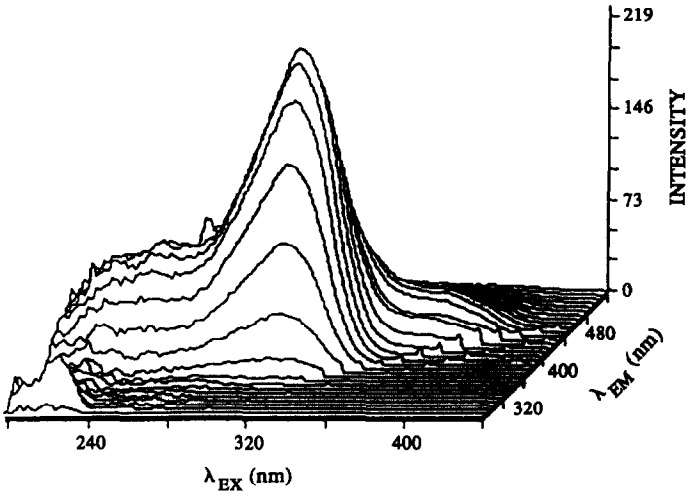


Fig. 4. Isometric plot of the emission-excitation matrix (Forward projection) of cefoxitin after reaction at 90° for 60 min in 1.0*M* NaOH. [Cefoxitin] = 0.80 μg/ml. Ethanol-water content 50/50% v/v, pH = 2.0.

ethanol/water before carrying out the fluorescence measurements. The fluorescent product was satisfactorily stable in these conditions as judged by little change in fluorescence intensity for 180 min.

The dependence of the fluorescence intensity on the temperature of the final reaction mixture is critical, showing a decrease of the fluorescence emission of 0.64%/°*C* when the temperature increases from 3 to 70°. This effect can be explained by the higher internal conversion as temperature increases, facilitating non-radiative deactivation of the excited singlet state.[25] It is therefore recommended that a thermostat with a measurement temperature of 20°, *i.e.*, about room temperature, is used.

The effect of cefoxitin concentration on the fluorescence of the fluorophor derived from this drug is another factor to be considered. The fluorescence intensity increases with concentration up to 10 μg/ml. Inner filter quenching due to high fluorophor concentration becomes significant when the cefoxitin concentration exceeds 10 μg/ml. A constant fluorescence intensity was obtained between 25 and 50 μg/ml and a decrease for cefoxitin concentration higher than 50 μg/ml because of fluorescence inversion phenomena.

Fluorescence spectral properties

An intensely fluorescent product was obtained from cefoxitin in alkaline solutions after heating at 90° for 60 min.

The tridimensional spectrum provides the best characterization of the cefoxitin degradation product fluorescence. This spectrum is obtained with a suitable computer program[27] and presented as the isometric projection, where the excitation spectra at stepped increments of 4 nm emission wavelength are recorded and plotted. Figure 4 shows the tridimensional spectrum of the fluorophor where the light scattering has been removed. In the two-dimensional representation (Fig. 5) it can be observed that the most representative fluorescence maximum of cefoxitin fluorescent product is localized at 317 nm excitation wavelength and 400 nm emission wavelength and so all fluorescence measurements in subsequent studies were carried out at these wavelengths.

Spectrofluorimetric determination of cefoxitin

By using the above technique, a method to determine cefoxitin by the direct fluorescence intensity measurements at constant excitation and emission wavelengths (317 and 400 nm, respectively) is obtained.

A linear regression equation ($y = a + mx$) for cefoxitin over the range 0.020–1.40 μg/ml was obtained. The equation resulting from application of the least square method to the experimental data is displayed in Table 1 together with the statistical parameters. The significance of the intercept on the y-axis of the obtained regression line was evaluated by applying the test of Student's "t" at 95% of confidence level and ten degrees of freedom.[28] If the intercept on the y-axis for the line calculated by the least square method is negligible, it is necessary to perform again the fitting of the data according

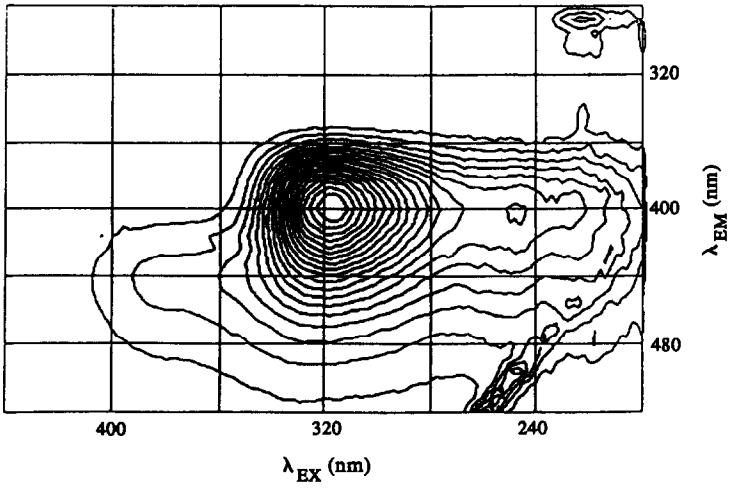


Fig. 5. Contour plot of cefoxitin fluorescence after reaction at 90° for 60 min in 1.0*M* NaOH. [Cefoxitin] = 0.80 μg/ml. Ethanol–water content 50/50% v/v, pH = 2.0.

to a function "$y = m_0 x$" and therefore the new value of the slope (m_0) may be calculated. The results of this study are reported in Table 1. The value calculated for "t" does not exceed the theoretical value and hence intercept on the y axis is negligible. Consequently, the new value of the slope is calculated.

The linearity of standard curve (Fig. 6) in the usable concentration range and the negligible scatter of the experimental points is clearly evidenced by the high value of the correlation coefficient. This together with the negligible value for the intercept on the y-axis showed the excellent linear relationship between fluorescence intensity and cefoxitin concentration.

In order to determine the accuracy and the precision of the recommended procedure, two series of ten standard samples containing 0.20 and 0.80 μg/ml of the antibiotic were prepared and fluorescence measurements were made on each reaction product obtained according to the proposed method. A relative standard deviation of 1.8 and 0.5% and a mean standard analytical error of 1.63×10^{-3} and 1.67×10^{-3} were found for each sample set at 95% confidence level.

The detection limit is 2×10^{-3} μg/ml and the determination limit is 8×10^{-3} μg/ml when they are defined as the analyte concentration leading to a luminescence intensity that is three and ten times the blank standard deviation,[29,30] respectively.

Table 1. Statistical parameters

Intercept on the y-axis (a)	1.97
Standard deviation of intercept	1.38
Slope (m)	252.49
Standard deviation of slope	1.92
Correlation coefficient	0.999
Theoretical "t" value	2.228
Experimental "t" value	1.432
New slope (m_0)	254.55

Determination of cefoxitin in pharmaceuticals

Cefoxitin is commercially available as the sodium salt which is not appreciably absorbed from the gastrointestinal tract and must be given parenterally. Therefore, the proposed method was applied to the determination of cefoxitin in all the pharmaceutical products commercialized in Spain that contain this antibiotic: Mefoxitin and Cefaxicina injections. The assays were carried out as described under the procedure for injections. The Table 2 shows the assay results, expressed as percentage of the contents resulting of the average of three determinations of three different vials of pharmaceutical products. These results were in good agreement with the stated content of the antibiotic.

Since cefoxitin is administered intravenously

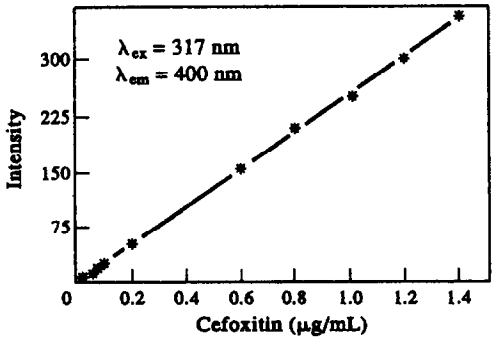


Fig. 6. Fluorimetric standard curve obtained for cefoxitin.

Table 2. Determination of cefoxitin in pharmaceutical dosage

Pharmaceutical products	Cefoxitin Taken ($\mu g/ml$)	Found* ($\mu g/ml$)	Standard Deviation ($\mu g/ml$)	Recovery (%)
Mefoxitin	0.80	0.791	8.74×10^{-3}	98.8
Cefaxicina	0.80	0.794	8.89×10^{-3}	99.2

*Average of three determinations with three replicates each one.

Table 3. Determination of cefoxitin in saline physiological serum and glucosed physiological serum

Medium	Pharmaceutical products: Mefoxitin Taken ($\mu g/ml$)	Found* ($\mu g/ml$)	Recovery (%)	Cefaxicina Taken ($\mu g/ml$)	Found* ($\mu g/ml$)	Recovery (%)
Saline physiol. serum (Apiroserum)	0.80	0.789	98.6	0.80	0.795	99.4
Saline physiol. serum (Grifols)	0.80	0.792	99.0	0.80	0.810	101.2
Glucosed physiol. serum (Apiroserum)	0.80	0.790	98.8	0.80	0.785	98.1

*Average of three determinations with three replicates each one.

in serious infections, the method was applied to the determination of the antibiotic in serum. The intravenous infusion were: saline physiological serum (Apiroserum and Grifols, 0.9% sodium chloride) and glucosed physiological serum (Apiroserum, 5% glucose). The results are shown in Table 3. Since the other components of the pharmaceutical preparations tested showed no fluorescence over the range of wavelengths used, the proposed assay method is specific for the determination of cefoxitin in the indicated pharmaceutical formulations.

CONCLUSIONS

The described method permitted the fluorimetric determination of cefoxitin in the concentration range from 0.020 to 1.40 μg/ml. The measurements of the fluorophor product were carried out at 400 nm with excitation at 317 nm. The method was found to be more sensitive than a previously reported fluorimetric method[23] because a limit of determination smaller (8×10^{-3} μg/ml) was found. Moreover, the present method offers advantages in rapidity and simplicity over the existing method. The method proposed improves on the sensitivity of that described by HPLC by Wheeler *et al.*,[17] which can only detect up to 1 μg/ml, and on that described by Torchia *et al.*,[18] where the detection limit is 0.1 μg/ml. The method was applied to the determination of cefoxitin in pharmaceutical products, in both aqueous medium and physiological serum (saline and glucosed). Satisfactorily recovery for each dosage formulation were found.

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REFERENCES

1. E. O. Stapley and J. Birnbaum, *Chemistry and microbiological properties of the cephamycins*, in Salton and Shockman (eds.) *β-Lactam Antibiotics-Mode of Action, New Developments and Future Prospects*, p. 327. Academic Press, New York, 1981.
2. E. O. Birnbaum, A. K. Stapley, H. Miller, H. Wallick, D. Hendlin and H. B. Woodruff, *J. Antimicrob. Chemother.*, 1978, **4** (Suppl. B), 15.
3. D. L. Hemsell, M. L. Heard, B. Nobles and P. G. Hemsell, *Obstetrics and Gynecology*, 1984, **63**, 285.
4. G. Herman, A. W. Cohen, G. H. Talbot, R. Coghlan, P. Faidley-Mangen and R. R. MacGregor, *ibid.*, 1986, **67**, 371.
5. F. P. Tally, K. McGowan, J. M. Kellum, S. L. Gorbach and T. F. O'Donnell, *Annals of Surgery*, 1981, **193**, 318.
6. D. F. Busch, P. N. R. Heseltine, R. D. Meyer, V. L. Sutter and S. M. Finegold, *J. Antimicrob. Chemother.*, 1978, **4** (Suppl.), 197.
7. J. L. LeFrock, R. F. Schell, B. B. Carr, S. F. Kowalsky and J. R. Tillotson, *Drug Intelligence and Clinical Pharmacy*, 1982a, **16**, 306.
8. J. S. Martínez, I. G. Duran and L. R. Solís, *J. Antimicrob. Chemother.*, 1978, **4** (Suppl.), 189.
9. H. Thadepalli, I. R. Webb and V. Bach, *ibid.*, 1978, **4** (Suppl.), 203.
10. M. Shah, E. B. Helm and W. Stille, *ibid.*, 1978, **4** (Suppl. B), 117.
11. *British Pharmacopoeia 1980*, Her Majesty's Stationery Office, London, 1980.
12. *The United States Pharmacopoeia, XXth Rev.*, United States Pharmacopeial Convention, Easton, PA, 1980.

13. *The National Formulary, XVth Ed.*, American Pharmaceutical Association, Washington, D.C., 1980.
14. *Code of Federal Regulations*, U.S. Government Printing Office, Washington, D.C. Title 21.
15. W. Brumfitt, J. Kosmidis, J. M. T. Hamilton-Miller and J. N. G. Gilchrist, *Antimicrob. Agents Chemother.*, 1974, **6,** 290.
16. W. E. Feldman, S. Moffitt and N. S. Manning, *ibid.*, 1982, **21,** 468.
17. L. A. Wheeler, M. De Meo, B. D. Kirby, R. S. Jerauld and S. M. Finegold, *J. Chromatogr.*, 1980, **183,** 357.
18. M. G. Torchia and R. G. Danzinger, *ibid.*, 1980, **181,** 120.
19. C. E. Fasching and L. R. Peterson, *Antimicrob. Agents Chemother.*, 1982, **21,** 628.
20. A. B. C. Yu, C. H. Nightingale and D. R. Flanagan, *J. Pharm. Sci.*, 1977, **66,** 212.
21. R. H. Barbhaiya and P. Turner, *Br. J. Clin. Pharmacol.*, 1977, **4,** 427.
22. *Idem, ibid.*, 1977, **4,** 734.
23. Z. H. Al-Rawi and S. Tabaqchali, *J. Antimicrob. Chemother.*, 1979, **5,** 81.
24. W. J. Jusko, *J. Pharm. Sci.*, 1971, **60,** 728.
25. J. D'A. Jeffery, E. P. Abraham and G. G. F. Newton, *Biochem. J.*, 1960, **75,** 216.
26. W. R, Seitz, *Luminiscence spectrometry (Fluorimetry and Phosphorimetry)*, in P. J. Elving, E. J. Meehan and I. M. Kolthoff, (eds.), *Treatise Anal. Chem.*, Chap. 4 (2ª edition). Wiley, New York (1981).
27. J. A. Murillo and A. Alañón, *Comp. Chem.*, in the press.
28. P. D. Lark, B. R. Craven and R. C. L. Bosworth, *The Handling of Chemical Data*, Pergamon Press, 1968.
29. *Spectrochim. Acta, B*, 1978, **33B,** 242.
30. *Anal. Chem.*, 1980, **52,** 2242.