Stability Determination of 3-Bromo-2-hydroxy-*N*-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinon-4-imine in Ethanol by First-Derivative Spectrophotometry

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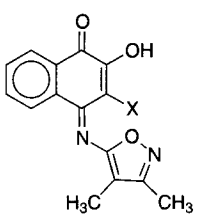
Abstract □ The degradation kinetics of a new potential tripanocidal and antibacterial agent, 3-bromo-2-hydroxy-*N*-(3,4-dimethyl-5- isoxazolyl)-1,4-naphthoquinon-4-imine (**2**), in 95% ethanol, was investigated between 35 and 50 °C under room-light and light-protected conditions. The decomposition product was isolated and identified as 2-hydroxy-*N*-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinon-4-imine (**1**). A simple, rapid, and stability-indicating method for the determination of **2** in the presence of **1** using "*zero crossing*" first-derivative spectrophotometry is reported. The validity of this method was proved using synthetic mixtures of the intact drug with its decomposition product and by statistical analysis of the calibration data. Pseudo-first-order constants for the degradation reaction of **2**, obtained from linear plots of the residual concentration logarithms vs time, the calculated activation parameters E_a, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ were similar under room-light and light-protected conditions. The *in vitro* antibacterial activity of **2** was also evaluated.

Introduction

The isoxazolylnaphthoquinone compounds[1,2] are a family that shows pharmacological interest as potential tripanocidal[3–8] and antibacterial agents,[9,10] where the best results were obtained with 2-hydroxy-*N*-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinon-4-imine (**1**).[3,4,9,10]

Taking into account these important biological results, our interest was focused on 3-bromo-2-hydroxy-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinon-4-imine (**2**), a new bromine derivative of **1**.[11]



1: X = H
2: X = Br

The derivative UV spectrophotometry method[12,13] has been successfully applied in pharmaceutical analysis, particularly for stability studies.[14,15] In previous papers, we have described the analysis of degradation reactions of some isoxazolylnaphthoquinone derivatives by second-derivative spectrophotometry.[16,17]

Continuing our investigations on analytical procedures for these potentially active compounds, this paper presents a simple, rapid, and sensitive method for the determination of **2**, in 95% ethanol, under room-light and dark conditions between 35 and 50 °C, in the presence of its corresponding degradation product by first-derivative spectrophotometry using a "*zero crossing*" technique of measurement.[12,18–20] The statistical analysis of the data yielded accurate and reproducible results.

Experimenal Section

Apparatus—Ultraviolet spectrophotometric (UV) studies were carried out on a Shimadzu UV-260 spectrophotometer using 1 cm quartz cuvettes. The mass spectra were recorded on a Finnigan Model 3300 F-100 quadrupole mass spectrometer. Melting points were determined on a Büchi 510 apparatus and were uncorrected. A Chromatotron Model 7924T was used for preparative radial chromatography (PRC). The coating rotor was prepared with Silicagel 60 PF 254 with calcium sulfate (Merck). For kinetic measurements, the constant-temperature bath was regulated by a Haake D_8 thermostat with ±0.1 °C precision.

Materials—All chemicals and reagents were of analytical grade. Ethanol was treated with sulfanilic acid and potassium hydroxide/Zn according to the literature procedure.[21] Compound **2** was prepared from **1** and *N*-bromosuccinimide as previously reported.[11]

Separation and Identification of Degradation Product—In 4.5 mL of 95% ethanol was dissolved 11.1 mg (0.03 mmol) of **2**. The solutions were stored at 50 °C under room-light and light-protected conditions in a constant-temperature bath for 24 h. Then, the flasks were withdrawn and immediately cooled in an ice bath, and the solvent was evaporated to dryness under reduced pressure. The residues were separated by PRC with an appropriate gradient of solvents, giving **1** as only product (58% for room light, 46% for dark). Mp: 214–5 °C (lit.[1] mp 215–6 °C). MS (%): 268 (13.5) (M^+), 130 (67.2), 105 (11.8), 96 (86.1), 68 (86.5), 43 (98.0). UV (ethanol): λ_{max} 234.4, 300.5, 441.6 nm.

Spectrophotometric Analysis—Normal and first-derivative absorption spectra of standard and sample solutions of **1** and **2** were recorded between 220 and 350 nm against ethanol as a blank. Suitable settings were a slit width of 1 nm and a fast scan speed. The recorder scale expansion was also optimized to facilitate reading the recorder tracing.

Calibration Graphs—For the calibration graph of compound **1**, two stock solutions were prepared in dichloromethane (1.39×10^{-3} M). Aliquots were taken in duplicate and diluted with ethanol to obtain final concentrations in the range of $(1.39–4.16) \times 10^{-5}$ M. The heights h_1 (mm) at 238.5 nm were measured and normalized as follows:[18,19] $H_1 = h_1$ (mm) × scale expansion/153 mm full scale, and a linear relation between H_1 vs C (mol/L) was established using regression analysis. The same procedure was carried out for compound **2** and the stock solutions were 3.50×10^{-4} M. The final concentrations were in the range of $(1.40–4.19) \times 10^{-5}$ M. The heights h_2 at 301.0 nm were measured and normalized. The plot of H_2 vs C (mol/L) was linear at the concentration range examined, showing agreement with Beer's law.

Preparation of Standard Mixtures—Solutions of **1** (1.33×10^{-4} M) from the stock solution and **2** (1.51×10^{-4} M) were prepared in ethanol. Aliquots were taken and diluted with ethanol to obtain final concentrations in the range of $(1.05–3.49) \times 10^{-5}$ M for **1** and $(1.06–3.50) \times 10^{-5}$ M for **2**. The absorption and first-derivative spectra for different mixtures of **1** and **2** were recorded between 220 and 350 nm in order to measure the peak height at 301.0 nm. Compound **2** was finally quantified by comparing the measured signals with the

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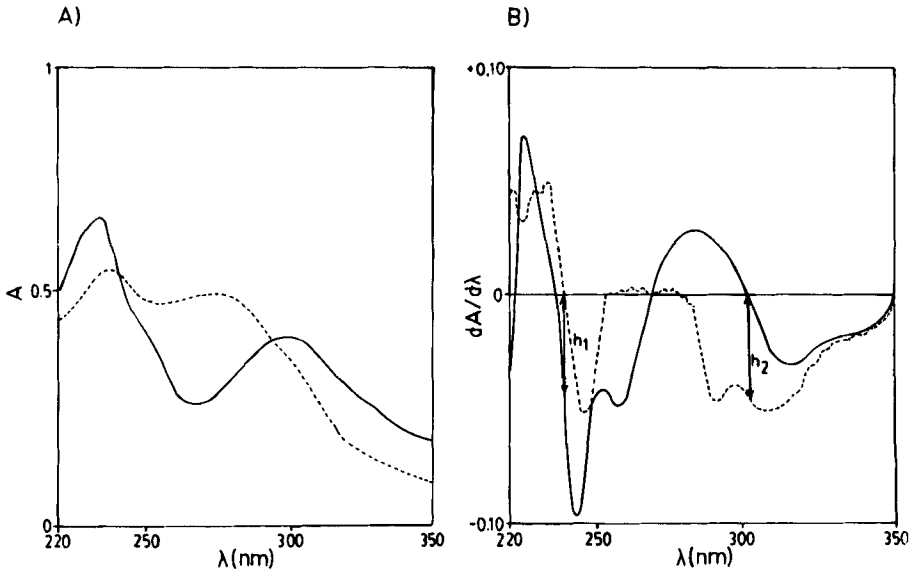

Figure 1—(A) Zero-order absorption spectra of 3.50×10^{-5} M solutions of compounds **1** and **2**. Key: —, **1**; - -, **2**. (B) First-derivative spectra of 3.50×10^{-5} M solutions of compounds **1** and **2**. Key: —, **1**; - -, **2**.

corresponding calibration run. Repeatability assays were measured by replicate analysis of a 3.50×10^{-5} M solution on the same day. Reproducibility assays were measured for three successive days by the same analyst using the same spectrophotometer.

Kinetic Studies—A stock solution of **2** (3.50×10^{-4} M) was prepared in ethanol. Aliquots were taken from the stock solution and diluted with 95% ethanol to produce a final concentration of 3.50×10^{-5} M. The sample solutions were transferred to 5 mL glass ampules and 5 mL amber glass ampules, flame sealed, and then stored at the appropriate temperature in a constant-temperature bath, while the *"zero time"* samples were maintained at −20 °C. Ampules were withdrawn at suitable time intervals, immediately cooled in an ice-bath, and stored in a −20 °C freezer until analyzed. All transfers and dilutions were carried out in the dark. These kinetic studies were done in duplicate. Upon removal of the last samples, the stored solutions were equilibrated to room temperature and then analyzed by first-derivative spectroscopy.

***In Vitro* Antibacterial Activity**[22]—The MIC (μg/mL) of **2** was determined in a Mueller–Hinton medium by using the standard tube dilution method according to the procedures of the NCCLS[23] (same experimental conditions of **1**[9]) against *Staphylococcus aureus* ATCC (American Type Culture Collection) 29213, *S. aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 and clinical strains of *Serratia spp.* and *Salmonella spp.* The stock solution was prepared by dissolving 0.0101 g of **2** in 0.1 mL of dimethyl sulfoxide (DMSO) and dilution to 5 mL with Mueller–Hinton medium. The DMSO was assayed to determine its antibacterial properties.

Results and Discussion

Since isoxazolylnaphthoquinone compounds are sparingly soluble in water and ethanol is usually used in formulations, we determined the stability of **2** in ethanolic solutions. Compound **2** decomposed to compound **1** under room-light and light-protected conditions. The R_f value, melting point, UV spectra, and MS of **1** were identical with those of an authentic sample.[1]

Figure 1A shows the zero-order absorption spectra of 3.5×10^{-5} M solutions of **1** and **2**. It is evident that an accurate direct absorption measurement of **1** or **2** could not be achieved because of their spectral overlapping. To overcome this problem first-derivative spectra were recorded. Examination of the first-derivative spectra (Figure 1B) of **1** and **2** reveals that the two compounds can be determined in presence of each other by direct measurement of the first derivatives at two different wavelengths using the *"zero crossing"* technique.[12,18–20] Two different wavelengths were used, 238.5 nm (*zero crossing* wavelength of **2**) for the determination of compound **1** and 301.0 nm (*zero crossing* wavelength of **1**) for the determination of compound **2**.

Good isosbestic points at 234.0, 266.5, and 301.0 nm for **1** (Figure 2A) and 238.5 nm for **2** (Figure 2B) were obtained when the first-derivative spectra of solutions are taken at different concentration values of **1**, over the range $(1.39–4.16) \times 10^{-5}$ M (Figure 2A), and of **2**, over the range $(1.40–4.19) \times 10^{-5}$ M (Figure 2B), which were employed in the calibration graph.

The heights at 238.5 and 301.0 nm are denoted as h_1 and h_2 and these heights are proportional to **1** and **2** concentrations, respectively (Figure 2A,B). The ordinate values of H_1 and H_2 were obtained from the h_1 and h_2 (mm) measurements and normalized as described in the Experimental Section.

Calibration Graphs and Statistical Analysis of Results—The H_1 and H_2 values at the selected wavelengths are linearly correlated to the concentrations over the range $(1.39–4.16) \times 10^{-5}$ M for **1** and $(1.40–4.19) \times 10^{-5}$ M for **2**. The regression equations derived from the least-squares method, the correlation coefficients, variance, and detection limits are shown in Table 1. The values of correlation coefficients and variances indicate good linearity of the calibration graphics and negligible scatter of the experimental points.

Tests of the significance of the regression line intercepts were performed to verify if the intercept (a) differed significantly from the theoretical value of zero.

One method for estimating the difference $a - 0$ is based on the determination of the quantities $t = a/s_a$ and their comparison with the corresponding tabular data for t distribution.[18–20] The calculated value for compound **2** ($t = 0.36$) is smaller than that tabulated at a 95% significance level with Student's t, which means that the intercept is not significantly different from zero. Conversely, the calculated value for compound 1 ($t = 4.57$) is higher than the tabulated $t = 2.31$ for 95% significance level, which means that the intercept is significantly different from zero. Thus, the method under these conditions presents constant errors.

However, a more rigorous test was carried out, too. It requires the construction from calibration data of a *"joint of confidence region"* for slope b and intercept a, which is an ellipse having as its center the least-squares estimates of a and b.[24]

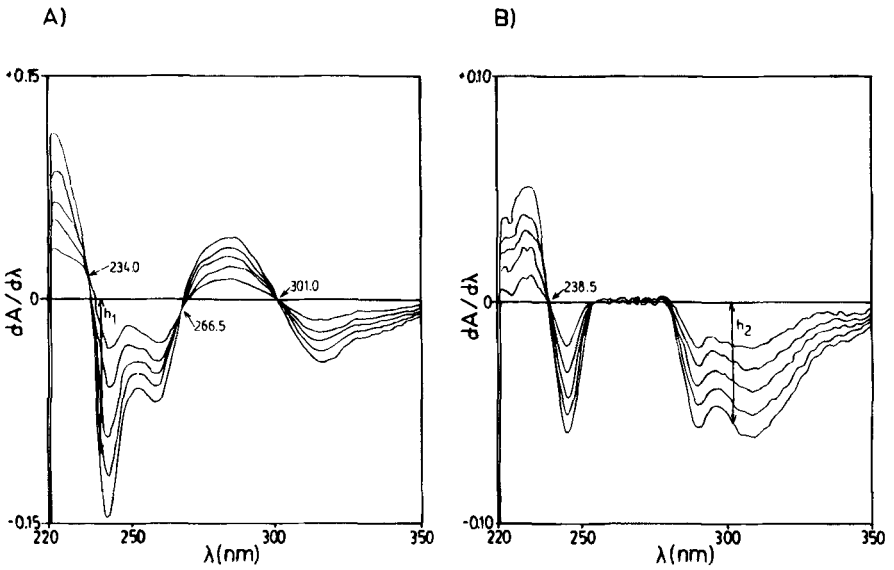

Figure 2—(A) Set of first-derivative spectra of (1.39–4.16) × 10^{-5} M **1**. (B) Set of first-derivative spectra of (1.40–4.19) × 10^{-5} M **2**. The reference was ethanol.

Table 1—Statistical Analysis for Compounds 1 and 2

	λ (nm)	Regression Equation[a]	r	V[b]	DL[c] (mg/L)
1	238.5	$H_1 = 2.37 \times 10^{-2} + 3.74 \times 10^3 X$	0.9982	4.90×10^{-6}	1.10
2	301.0	$H_2 = 8.57 \times 10^{-4} + 1.31 \times 10^3 X$	0.9979	6.92×10^{-7}	1.40

[a] X is the drug concentration (mol/L). [b] Variance. [c] Detection limit; number of standard specimens, $n = 10$; level of significance, $p = 0.05$.

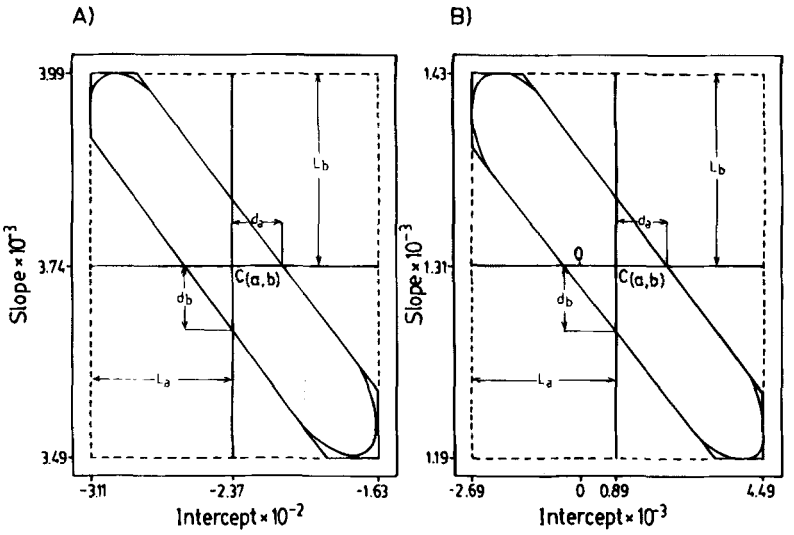

Figure 3—Joint confidence regions at a $p = 0.05$ level of significance for the slope and intercept of regression equations of compounds **1** (A) and **2** (B).

The ellipses constructed with a 95% criterion of confidence are shown in Figure 3. It can be clearly observed for compound **2** (Figure 3B), that the ellipse contains the point for which the intercept is zero, confirming the hypothesis that a is equal to zero. In the ellipse constructed for **1** (Figure 3A), it is clearly observable that the point for which the intercept is zero falls outside the ellipse, discarding thus the hypothesis that $a = 0$.

These results led us to use the *"zero crossing"* first-derivative method at 301.0 nm for determining the disappearance kinetics of compound **2**.

In order to confirm the validity and applicability of the proposed method, six synthetic mixtures of **1** and **2** were prepared and analyzed by *"zero crossing"* first-derivative spectrophotometry at 301.0 nm. The concentrations of the standard mixtures were chosen to give similar conditions to the stability studies. The recoveries obtained are presented in Table 2.

As it can be seen, the results indicate that the recoveries obtained are in good agreement with the theoretical amounts of the compounds.

The precision of the results for the standard 3.50 × 10^{-5} M solution in terms of repeatability [$n = 10$, 100.5 ± 0.82% (percent found ± SD)] and reproducibility [$n = 10$, 100.5 ± 0.10% (percent found ± SD)] shows that the method has satisfactory precision.

Reaction Order and Rate Constants—The H_2 values for each sample were measured at the specified wavelength, and concentrations of the intact drug were calculated using a linear regression equation (Table 1).

The linear relationship between the logarithmic concentra-

Table 2—Recovery of 2 in the Presence of Its Degradation Product 1 by Using the "*Zero Crossing*" First-Derivative Method

1 (mg/L)	2 (mg/L)	% Recovered[a]
	12.1	100.5
	8.35	100.1
2.81	8.35	100.8
4.67	6.25	100.5
2.81	5.85	100.6
6.47	3.65	99.9
	Mean ± SD	100.4 ± 0.33

[a] Average of three determinations.

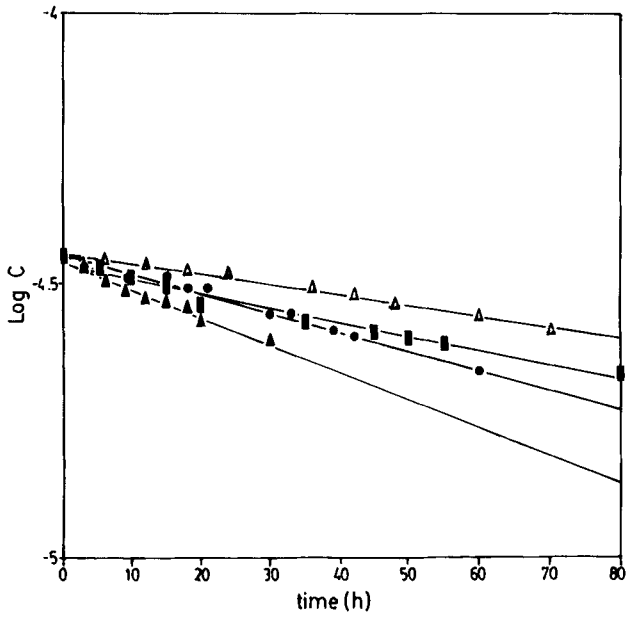

Figure 4—Effect of temperature on the pseudo-first-order rate constants for the degradation of **2** in 95% ethanol under room-light conditions. Key: ▲, 50 °C; ● 45 °C; ■, 40 °C; △, 35 °C.

tion of the intact drug and the storage time at 35–50 °C indicates a pseudo-first-order degradation kinetics for **2** under room-light and light-protected conditions (Figures 4 and 5).

The experimental rate constants together with the values obtained by extrapolation from the Arrhenius equation[25] are listed in Table 3. The values of E_a, log A, and the activation parameters[26] are given in Table 4.

Inspection of Tables 3 and 4 shows that light has practically no effect on the degradation rate, in contrast with results observed for other halogen derivatives of isoxazolylnaphthoquinones.[17] However, according to their t_{90} values, solutions of **2** can be stored only around 2 days at room temperature, even if they were conserved in the dark, and no more than 10 days (light-protected conditions) at 5 °C.

Antibacterial Activity—Compound **1** showed *in vitro* and *in vivo* antibacterial activity against *S. aureus* with a MIC of 32 μg/mL. Besides, **1** did not show resistance induction.[9,10] Compound **2** showed antibacterial activity against *S. aureus* with a MIC of 64 μg/mL and showed no antibacterial activity against *E. coli*, *P. aeruginosa*, *Serratia*, and *Salmonella*. Additional studies are in progress[22] to determine the biomedical values of these antibacterial agents.

Conclusions

The experimental results demonstrate that the problem of determining mixtures of isoxazolylnaphthoquinones having close overlapping spectra can easily be solved by using derivative spectrophotometry, since it increases the fine structure of the bands obtained by classical UV spectrophotometry. The determination of **2** in the presence of **1** can be done by "*zero crossing*" first-derivative spectroscopy at 301.0 nm.

The selectivity, accuracy, precision, short analysis time, and wide spread availability of commercial instruments with derivative capability indicate that the proposed stability-indicating method is especially suitable for routine quality control and for stability evaluation of solutions containing **2**.

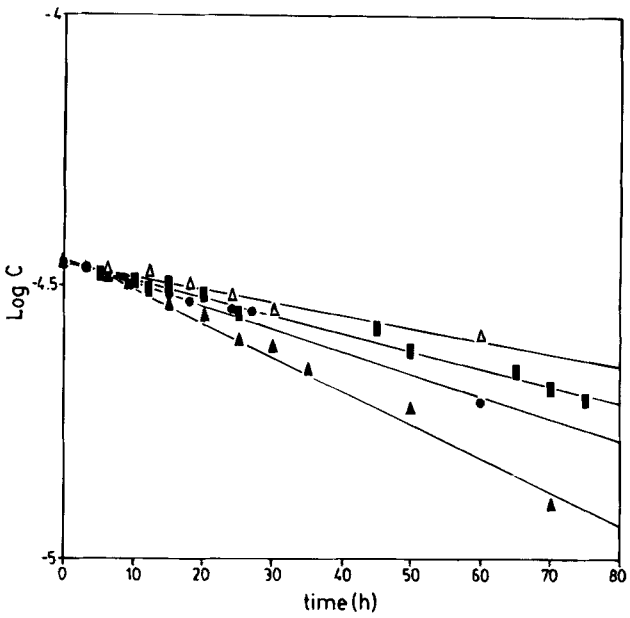

Figure 5—Effect of temperature on the pseudo-first-order rate constants for the degradation of **2** in 95% ethanol under light-protected conditions. Key: ▲, 50 °C; ●. 45 °C; ■, 40 °C; △, 35 °C.

Table 3—Effect of Temperature under Room-Light and Light-Protected Conditions on Pseudo-First-Order Constants for the Degradation of 2 in 95% Ethanol

Conditions	T (°C)	$k_{obs} \times 10^2$ h^{-1}	$t_{1/2}$ (h)	t_{90} (h)
Room	50	1.41	49.2	7.45
light	45	0.942	73.6	11.1
	40	0.722	96.0	14.5
	35	0.529	131	19.8
	25[a]	0.260	266	40.4
	5[a]	0.0558	1242	188
Light	50	1.15	60.3	9.13
protected	45	0.801	86.5	13.1
	40	0.600	115	17.5
	35	0.429	161	24.5
	25[a]	0.210	330	50.0
	5[a]	0.0443	1564	237

[a] Data calculated by extrapolation.

Table 4—Summary of Activation Parameters for the Degradation of 2 in 95% Ethanol[a]

	E_a (kcal/mol)	log A	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (cal/mol)
RL[b]	12.68 ± 0.80	10.27 ± 0.55	12.07 ± 0.16	−13.65 ± 0.21
LP[b]	12.82 ± 0.46	10.28 ± 0.32	12.26 ± 0.10	−13.53 ± 0.11

[a] Expressed as estimate ± SD. [b] RL, room light; LP, light protected.

References and Notes

1. Fernández, A. E.; de Bertorello, M. M.; Manzo, R. H. *Anal. Asoc. Quím. Argent.* **1982**, *70*, 49.

2. Ortiz, C. S.; Longhi, M. R.; de Bertorello, M. M.; Briñón, M. C. *Org. Prep. Proc. Int.* **1991**, *23*, 181.
3. Schwarcz de Tarlovsky, M. N.; Goijman, S. G.; Stoppani, A. O. M. *Rev. Argent. Microbiol.* **1988**, *20*, 183.
4. Pahn, E. M.; Molina Portela, M. P.; Stoppani, A. O. M. *Rev. Argent. Microbiol.* **1988**, *20*, 107.
5. Molina Portela, M. P.; Stoppani, A. O. M. *Rev. Argent. Microbiol.* **1989**, *21*, 102.
6. Schwarcz de Tarlovsky, M. N.; Goijman, S. G.; Molina Portela, M. P.; Stoppani, A. O. M. *Experientia*, **1990**, *46*, 502.
7. Amuchástegui, P.; Moretti, E.; Basso, B.; Sperandeo, N.; de Bertorello, M. M.; Briñón, M. C. *J. Protozool.* **1990**, *37*, 15A.
8. Amuchástegui, P.; Moretti, E.; Basso, B.; Sperandeo, N.; de Bertorello, M. M. *Rev. Argent. Microbiol.* **1990**, *22*, 199.
9. Bogdanov, P.; Albesa, I.; Sperandeo, N. R.; de Bertorello, M. M. *Rev. Argent. Microbiol.* **1992**, *24*, 1.
10. Sperandeo, N. R.; Eraso, A.; Albesa, I.; Bogdanov, P.; de Bertorello, M. M. 2nd Congreso de la Sociedad Argentina de Pedriatría. 1st Simposio de Control de Infecciones; Buenos Aires, April 1992.
11. Dabbene, V. G.; Briñón, M. C.; M. de Bertorello, M. *Org. Prep. Proc. Int.* **1994**, *26*, 6.
12. O'Haver, T. C.; Green, G. L. *Anal. Chem.* **1976**, *48*, 312.
13. O'Haver, T. C., *Anal. Chem.* **1979**, *51*, 91A.
14. Korany, M. A.; Bedair, M.; Mahgoub, H.; Elsayed, M. A., *J. Assoc. Off. Anal. Chem.* **1986**, *69*, 608.
15. Abdellateff, H.; Elbalkiny, M.; Aboulkheir, A. *J. Pharm. Biomed. Anal.* **1989**, *7*, 571.
16. Longhi, M. R.; de Bertorello, M. M.; Briñón, M. C. *J. Pharm. Sci.* **1991**, *80*, 1.
17. Dabbene, V. *Ph.D. Thesis* Facultad Ciencias Químicas, Universidad Nacional de Córdoba, 1994.
18. Morelli, B. *Analyst* **1988**, *113*, 1077.
19. Morelli, B. *J. Pharm. Sci.* **1988**, *77*, 1042.
20. Murillo Pulgarin, J. A.; Alañón Molina, A.; Rodríguez Flores, J. *Anal. Lett.* **1992**, *25*, 1275.
21. Perrin, D. D.; Armanego, W.; Perrin, D. R. *Purification of Laboratory Chemicals*; Pergamon Press: Oxford, England, 1980.
22. Albesa, I.; Bogdanov, P. To be published.
23. National Committee for Clinical Laboratory Standards. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*, 2nd ed; Approved standard, NCCLS Document M7-A-2; NCCLS: Villanova, PA, 1990.
24. Mandel, J.; Linning, F. J. *Anal. Chem.* **1957**, *29*, 743.
25. Connors, K. A. *Chemical Stability of Pharmaceuticals*, 2nd ed John Wiley & Sons: New York, 1968; pp 18.
26. Connors, K. A. *J. Parent. Sci. Technol.* **1981**, *35*, 186.

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