Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 5375–5380

BIOORGANIC & MEDICINAL CHEMISTRY

Ultrasensitive Assay of Azithromycin in Medicine and Bio-Fluids Based on Its Enhanced Luminol–H_2O_2 Chemiluminescence Reaction Using Flow Injection Technique

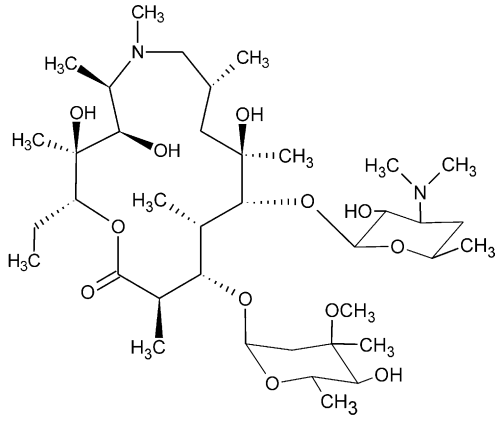
Zhenghua Song* and Changna Wang

Department of Chemistry, Northwest University, Xi'an, 710069, China

Received 28 May 2003; revised 13 September 2003; accepted 22 September 2003

Abstract—A simple flow injection chemiluminescence method with synergistic enhancement has been investigated for the rapid and sensitive determination of azithromycin. The synergistic action was significant in the chemiluminescence system of luminol–hydrogen peroxide with azithromycin as an enhancer. The enhanced chemiluminescence intensity was linear with the concentration of azithromycin over the range from 0.1 pg mL^{-1} to 1.0 ng mL^{-1} (r^2=0.9988) with a detection limit (3σ) of 0.04 pg mL^{-1}. At a flow rate of 2.0 mL min^{-1}, a complete analytical process could be performed within 0.5 min, including sampling and washing, with a relative standard deviation of less than 3.0%. The proposed method was applied successfully in the assay of azithromycin in pharmaceutical preparations, human urine and serum without any pre-treatment procedure.

Introduction



Anthromycin (AZM), a white crystalline powder as dihydrate with a chemical formula of $C_{38}H_{72}N_2O_{12}{\cdot}2H_2O$,[1] is an acid stable orally administered macrolide antimicrobial drug. Although derived from erythromycin, AZM differs chemically from erythromycin in that a methyl-substituted nitrogen atom is incorporated into the lactone ring, which enhances permeation in gram-negative bacteria.[2] As one of the best antibiotics available today, AZM is an effective therapeutic agent for the treatment of certain infections caused by bacteria, such as pneumonia,[3] venereal disease (VD)[4] and ear, lung, skin, and throat infections.[5,6] In acquired immunodeficiency syndrome (AIDS) patients, AZM is used to prevent pneumonia, called disseminated *Mycobacterium avium* complex (MAC) infection.[7]

Among the methods available, high-performance liquid chromatography (HPLC) coupled with different types of detectors, involving electrochemical detection,[8–11] UV,[12,13] MS[14] and fluorescence detection,[15] were widely used. And several other analytical approaches, including spectrophotometry,[16] fluorimetry[17] and bioassay[18,19] also performed in the determination of AZM. However, the batch methods are time consuming and laborious; on the other hand, the chromatographic methods are slow and require expensive instrumentation. Compared to these techniques, chemiluminescence (CL) method combined with flow injection (FI) technique, an attractive alternative for pharmaceutical analysis,[20,21] is superior in many ways: the sensitivity is high, linear dynamic range is wide, measurements can be made extremely rapidly and the equipments are inexpensive

*Corresponding author. Tel.: +86-2-9830-2456; fax: +86-2-9830-3798; e-mail: songzhenghua@hotmail.com

0968-0896/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2003.09.029

and simple to operate. We have currently reported on the luminol–ferricyanide and luminol–periodate CL systems for the determination of berberine[22] and analgin[23] coupled with FI technique. However, there has been no report dealing with a CL method for the assay of AZM. Thus, a simple, fast, precise and accurate FI–CL method is described in this paper, which is ideal for the determination of AZM. Additionally, the proposed procedure is the most sensitive method reported so far. In this work, it was observed that AZM could sharply intensify the CL derived from the luminol–hydrogen peroxide system, and the CL intensity responded to the concentration of AZM linearly ranging from 0.1 pg mL^{-1} to 1.0 ng mL^{-1} with a relative standard deviation (RSD) of less than 3.0%. Moreover, at a flow rate of 2.0 mL min^{-1}, a complete determination of AZM, including sampling and washing, could be accomplished in 0.5 min, offering the sampling efficiency of 120 h^{-1} accordingly. The method was applied successfully in an assay of AZM for pharmaceutical preparations, human urine and serum without any pretreatment with recovery from 93.5 to 113.3% and RSDs of less than 4.0%.

Experimental

Reagents

All chemicals used were of analytical reagent grade. Water purified in a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout. AZM standard solution (1.029 mg mL^{-1}) was obtained from Shaanxi Institute for Drug Control, and the working strength solutions were prepared freshly from the above stock standard solutions as required. Hydrogen peroxide was purchased from Xi'an Chemical Reagent Plant. Luminol (Fluka, Biochemika) was obtained from Xi'an Medicine Purchasing and Supply Station, China.

Luminol was used as supplied to prepare a 2.5×10^{-2} mol L^{-1} stock standard solution by dissolving 4.40 g of luminol with 0.1 mol L^{-1} sodium hydroxide to 1.0 L in a brown calibrated flask. Hydrogen peroxide (33.3%) was diluted by pure water to give a final concentration of 0.1 mol L^{-1}.

Apparatus

The schematic diagram of the FI system employed is illustrated in Figure 1. A peristaltic pump (Shanghai meter electromotor plant, model ND-15, 15 rpm) was utilized to pump each of all flow streams at a flow rate of 2.0 mL min^{-1}. PTFE tubing (1.0 mm i.d.) was used throughout the manifold for carrying the CL reagents. A six-way valve with loop of 100 μL was employed for sampling. The CL emission cell is a spiral glass tube (1.0 mm i.d., 15 cm length) producing a large surface area exposed to the adjacent photomultiplier tube (PMT) (Hamamatsu, model IP28). Extreme precautions were taken to ensure that the cell compartment and the photomultiplier tube were light-tight. The CL signal produced in the CL emission cell was detected without wavelength discrimination and the PMT output was amplified and quantified by a luminosity meter (Xi'an Keri Electron Device Ltd., model GD-1) connected to a recorder (Shanghai Dahua Instrument and Meter Plant, model XWT-206).

Procedures

The carrier water and the solutions (sample, hydrogen peroxide, luminol and sodium hydroxide) were propelled at a constant flow rate of 2.0 mL min^{-1}. The pump was started to wash the whole flow system until a stable baseline was recorded. One hundred microlitres of luminol was injected into the carrier stream by a six-way valve quantitatively, which was then merged with the AZM and hydrogen peroxide stream. The mixed solution was delivered to the CL cell in an alkaline medium, which was therefore detected with the PMT and luminometer. The concentration of the sample was quantified by the increment of CL intensity ($\Delta I = I_s - I_o$), where I_o and I_s are CL signals in the absence and in the presence of AZM, respectively.

Determination of AZM in pharmaceutical preparations

The different preparations were purchased from the local market. The injection containing 0.125 g AZM was diluted to 5.0, 10.0 or 25 mL with water, respectively. After appropriate dilution, the content of AZM was quantified directly by the proposed method.

Determination of AZM in spiked human urine and serum samples

The urine samples collected from three volunteers and the serum samples supplied by the Hospital of Northwest University were spiked before determination. To prepare the spiked samples, known quantities of AZM (1.0, 1.5 or 2.0 μg for urine, 5.0 or 7.5 μg for serum) were spiked into 1.0 mL of urine or serum. After homogenization, 0.1 mL aliquot of the spiked sample was diluted to 50 mL. After dilution with a factor of 1.0×10^2 for urine samples and 1.0×10^3 for serum samples, the samples were determined by the proposed method directly.

Results and Discussion

CL intensity–time profile

Before the FI method was carried out, the kinetic curve was examined by static method. The kinetic profile for

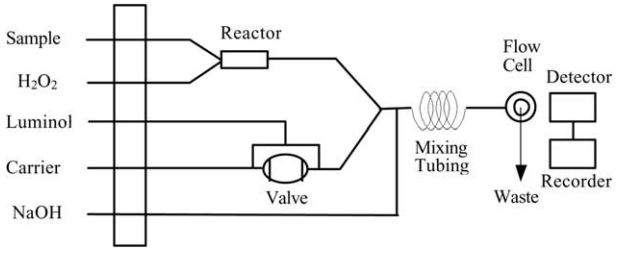


Figure 1. Schematic diagram of the flow injection system for AZM determination.

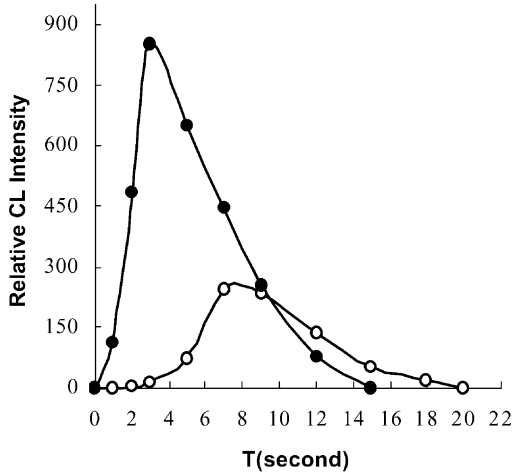


Figure 2. Kinetic CL intensity–time profile in static system. ○: CL intensity in the absence of AZM ●: CL intensity in the presence of AZM (10 pg mL^{-1}).

CL intensity of luminol-hydrogen peroxide reaction versus time was tested using 1.0×10^{-7} mol L^{-1} luminol and 1.0×10^{-5} mol L^{-1} hydrogen peroxide in 0.025 mol L^{-1} sodium hydroxide solution. As Figure 2 shown, the mixed solution of luminol and hydrogen peroxide gave an evident CL signal in alkaline medium, which reached a maximum at 7 s after the mixing of reactants, and became extinguished within 20 s thereafter. The CL signal increased amazingly in the present of AZM (10 pg mL^{-1}). Only in 3 s, the CL intensity approached the maximum, then tended to vanish in the following 15 s, giving a maximum intensity 3.5-fold as that in the absence of AZM.

Selection of oxidant

The characteristics of several oxidants, including permanganate, periodate, ferrocyanide, dichromate and hydrogen peroxide of the same concentration reacting with luminol in the presence of AZM were evaluated, and the results were summarized in Table 1. It was found that AZM as an enhancer exhibiting an enhancement only for luminol–hydrogen peroxide system. In other tested systems, however, no obvious CL increment (ΔI) could be measured, which suggested that the luminol-hydrogen peroxide CL system was more sensitive and selective for the determination of AZM. Therefore, luminol–hydrogen peroxide system was suitable for subsequent experiment.

Table 1. Characteristics of different oxidants[a]

Types of CL intensity	Relative CL intensity[b] ($n=3$)				
	$KMnO_4$	KIO_4	$K_3Fe(CN)_6$	$K_2Cr_2O_7$	H_2O_2
I_o	34	50	25	14	245
I_s	31	50	25	16	850
I_s-I_o	−3	0	0	2	605

[a]The concentrations for AZM, luminol, oxidants and NaOH were 10.0 pg mL^{-1}, 1×10^{-7} mol L^{-1}, 1×10^{-5} mol L^{-1}, and 0.025 mol L^{-1}, respectively.
[b]HV = −650 V.

Effects of luminol and hydrogen peroxide concentration

The effect of luminol and hydrogen peroxide concentration on the CL intensity was investigated in the presence of 10 pg mL^{-1} AZM over the ranges of 1.0×10^{-10}–1.0×10^{-6} mol L^{-1} and 1.0×10^{-7}–1.0×10^{-4} mol L^{-1}, respectively. In order to compare the effects, the relative CL intensity (ΔI) was plotted against concentration of luminol and hydrogen peroxide. With respect to luminol, at concentrations lower than 1.0×10^{-10} mol L^{-1} no CL was detected, above which CL intensity gradually increased and reached a maximum at 1.0×10^{-7} mol L^{-1}. Above this concentration the intensity became constant. As regards to hydrogen peroxide, with the increasing concentration of hydrogen peroxide, the CL intensity increased steeply and reached a maximum intensity at 1.0×10^{-5} mol L^{-1} of hydrogen peroxide. Then the intensity decreased considerably with hydrogen peroxide concentrations higher than 1.0×10^{-5} mol L^{-1}. Therefore, 1.0×10^{-7} mol L^{-1} luminol and 1.0×10^{-5} mol L^{-1} hydrogen peroxide were chosen as the optimum concentration and used in subsequent experiment.

Effect of sodium hydroxide concentration

Owing to the nature of luminol CL reaction, which is much favored under alkaline condition, sodium hydroxide was introduced into the CL cell through a flow line to improve the sensitivity of the system. The effect of sodium hydroxide concentration on CL was tested by measuring the CL intensity with a series of sodium hydroxide solutions from 0.01 to 0.25 mol L^{-1}. The CL intensity approached its maximum value at 0.025 mol L^{-1} of sodium hydroxide. At concentrations above or below this, the CL intensity decreased dramatically. Thus, 0.025 mol L^{-1} sodium hydroxide was selected as optimum for the present system.

Effect of flow rate and the length of mixing tubing

The signal-to-noise ratio was greatly influenced by the flow rate. A rate of 2.0 mL min^{-1} was chosen as a suitable condition with superior sensitivity, precision and reducing reagent consumption. The length of the mixing tube was also adjusted to yield maximum light emission in the CL cell. It was observed that a 5.0 cm of mixing tube afforded the best results with regards to sensitivity and reproducibility. Accordingly, 5.0 cm was then selected as the optimum length of mixing tube.

Performance of proposed method for AZM measurements

Under the optimized conditions, the increment of CL intensity was found linear with the concentration of AZM over a range from 0.1 pg mL^{-1} to 1.0 ng mL^{-1}. The regression equation was $\Delta I = 1.817\ C_{AZM} + 32.489$, $r^2 = 0.9988$, with a detection limit of 0.04 pg mL^{-1} (3σ, $n=5$). The RSDs of five determinations were 4.97, 3.86, 2.20 and 1.86% with AZM concentration of 0.5, 5.0, 50.0 and 500.0 pg mL^{-1}, respectively. At a flow rate of 2.0 mL min^{-1}, a determination for analyte including

Table 2. Tolerable concentration with respect to 10 pg mL^{-1} AZM for some interfering species (<5% error)

Species	Tolerable Conc. (μg mL^{-1})
Glutin, barbiturate, oxalic acid, urea, acetone	>30
Starch, lactose, cellulose, stearic acid, agar, talc, citric acid, fructose, sucrose	>10
Methanol, ethanol	7.0
Cl^-, NO_3^-, Ac^-, I^-, SO_4^{2-}, PO_4^{3-}, $Cr_2O_7^{2-}$, borate, oxalate, tartrate, citrate, malic acid	1.0
Globulin	0.7
NH_4^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Zn^{2+}, Ag^+, myoglobin,	0.5
Uric acid	0.3
Co^{2+}, Fe^{3+}, Fe^{2+}, Mn^{2+}	5×10^{-4}

Table 3. Results of AZM in pharmaceutical preparations[a]

Sample no.	Added pg mL^{-1}	Found pg mL^{-1}	RSD%	Recovery%	By proposed method mg mL^{-1}	By UV mg mL^{-1}
1	0	4.9±0.1	1.86	95.0	24.63±0.31	24.75
	10	14.4±0.2	1.26			
2	0	5.0±0.1	1.85	104.9	12.50±0.17	12.74
	15	20.7±0.3	1.39			
3	0	5.0±0.1	1.86	109.6	4.96±0.10	4.94
	20	26.3±0.3	0.93			
4	0	5.1±0.1	1.88	98.0	25.51±0.42	25.49
	10	14.9±0.2	1.64			
5	0	5.0±0.1	2.01	96.3	12.50±0.15	12.63
	15	19.4±0.2	1.18			
6	0	5.1±0.2	2.42	106.3	5.05±0.12	4.98
	20	26.3±0.5	1.95			

[a]The average of five determinations.

sampling and washing could be accomplished in 0.5 min, giving a throughput of 120 h^{-1} with a RSD of less than 3.0%.

Interference studies

The interference of foreign substances was investigated by analyzing a standard solution of 10 pg mL^{-1} AZM to which increasing amounts of interfering species were added. The tolerable concentration of a foreign species was taken as a relative error <5% and the results were listed in Table 2. Compounds abundant in human urine and serum such as salt, lipid and proteins caused no obvious interference for the determination of AZM.

Applications

Determination of AZM in pharmaceutical preparations. Following the procedure described above, six pharmaceutical injections were determined for AZM by standard addition method, and the results were listed in Table 3. To verify the results obtained by the proposed method, spectrometric method (UV-1100, Beijing Rayleigh Analytical Instrument Corporation) was applied to determine the samples at 205 nm and the correlationship between the results of two methods was shown as the following regression equation:

$$C_{CL} = 0.9973\ C_{UV} + 0.0323, \text{ and } r^2 = 0.9998.$$

Determination of AZM in spiked human urine and serum samples

The proposed CL method was also applied to determine AZM in spiked human urine and serum at picogram level. Three spiked urine samples and six spiked serum samples were determined for AZM by the standard addition method. The results of determination were listed in Tables 4 and 5, with recovery from 93.5 to 113.3% and *t*-test from 0.74 to 2.53. It was reported[24] that the final concentrations in urine and serum of patients were 22.5 and 0.05–0.41 μg mL^{-1} after oral administered 500 mg AZM. It is obvious that the proposed method is very sensitive for AZM determination in biological fluids, especially in serum sample; smaller volume of serum is enough.

Conclusions

The presented CL method combined with FI technique offered prominent advantages including instrumental simplicity, high sampling efficiency, reducing reagents consumption, analytical sensitivity and selectivity compared with the existed methods. The satisfactory performance in an assay of AZM in pharmaceutical preparations and biological fluids demonstrated that the method was practical and suitable not only for quality control analysis but also for complex biological samples, confirming the promise for pharmacological and clinical researches.

Table 4. Results of AZM in spiked human urine[a]

Sample no.	Added pg mL^{-1}	Found pg mL^{-1}	RSD %	Recovery %	t $t_{0.05,\ 5}=2.57$	Content in urine μg mL^{-1} By proposed method /spiked
1	0	10.3±0.3	2.86	105.8	2.23	1.03/1.0
	10	20.9±0.3	1.31			
2	0	9.9±0.3	3.01	110.1	1.09	0.99/1.0
	20	31.9±0.6	1.83			
3	0	9.8±0.2	2.01	113.3	2.32	0.98/1.0
	30	43.8±0.9	1.96			
4	0	20.3±0.4	2.13	105.1	1.33	2.03/2.0
	10	30.8±0.4	1.22			
5	0	19.7±0.4	2.12	97.3	1.44	1.97/2.0
	20	39.2±0.6	1.54			
6	0	19.8±0.4	2.10	94.9	0.91	1.98/2.0
	30	48.3±0.7	1.40			
7	0	14.6±0.4	2.47	102.6	2.38	1.46/1.5
	10	24.9±0.3	1.30			
8	0	15.3±0.3	1.93	95.1	2.34	1.53/1.5
	20	34.3±0.5	1.56			
9	0	14.9±0.4	2.55	99.2	0.74	1.49/1.5
	30	44.6±0.4	0.81			

[a]The average of five determinations.

Table 5. Results of AZM in spiked human serum[a]

Sample no.	Added pg mL^{-1}	Found pg mL^{-1}	RSD %	Recovery %	*t*-test $t_{0.05,}=2.57$	Content in serum μg×mL^{-1} By proposed method/spiked
1	0	10.2±0.2	2.34	95.9	1.91	5.10/5.0
	10	19.8±0.3	1.61			
2	0	9.8±0.2	2.37	102.0	1.78	4.91/5.0
	15	23.9±0.5	2.21			
3	0	10.1±0.2	2.34	112.8	0.77	5.05/5.0
	20	31.5±0.4	1.20			
4	0	9.8±0.2	2.87	102.0	1.43	4.90/5.0
	10	20±0.3	0.50			
5	0	9.9±0.1	1.23	106.3	1.94	4.95/5.0
	20	31.2±0.4	1.20			
6	0	9.8±0.2	2.00	106.4	2.43	4.89/5.0
	30	41.7±0.5	1.16			
7	0	10.2±0.2	2.18	109.5	2.38	5.12/5.0
	10	21.2±0.3	1.32			
8	0	10.1±0.2	1.85	93.5	1.08	5.05/5.0
	20	28.8±0.3	1.16			
9	0	10.1±0.2	1.48	97.4	1.98	5.07/5.0
	30	39.5±0.4	1.11			
10	0	9.8±0.2	1.85	107.1	2.20	4.91/5.0
	10	20.5±0.2	1.12			
11	0	9.8±0.2	1.83	104.1	2.04	4.92/5.0
	20	30.7±0.3	1.03			
12	0	10.2±0.2	1.81	96.9	2.53	5.10/5.0
	30	39.3±0.5	1.34			
13	0	14.9±0.2	1.16	103.0	1.97	7.42/7.5
	10	25.2±0.2	0.90			
14	0	15.3±0.3	1.83	99.24	2.42	7.65/7.5
	20	35.2±0.4	1.09			
15	0	15.1±0.2	1.48	96.5	0.75	7.54/7.5
	30	44.0±0.6	1.26			
16	0	15.2±0.2	1.20	93.9	1.94	7.58/7.5
	10	24.9±0.2	0.90			
17	0	15.2±0.3	1.70	106.8	1.32	7.58/7.5
	20	36.5±0.4	0.95			
18	0	15.2±0.2	1.60	99.5	2.06	7.61/7.5
	30	45.1±0.7	1.50			

[a]The average of five determinations.

References and Notes

1. Denniston, P. L., Jr. *Physicians GenRx*, 5th ed.; Mosby-Year Book, Inc: St. Louis, 1995.
2. Peters, D. H.; Friedel, H. A.; McTavish, D. *Drugs* **1992**, *44*, 750.
3. Kinasewitz, G.; Wood, R. G. *Eur. J. Clin. Microbiol. Infect. Dis.* **1991**, *10*, 872.
4. Steingrimsson, O.; Olafsson, J. H.; Thorarinsson, H. *J. Antimicrob. Chemother.* **1990**, *25*, 109.
5. Lassus, A. *J. Antimicrob. Chemother.* **1990**, *25*, 115.
6. Bradbury, F. *J. Antimicrob. Chemother.* **1993**, *31*, 153.
7. Young, L. S.; Wiviott, L.; Wu, M.; Kolonoski, P.; Bolan, R.; Inderlide, C. B. *Lancet* **1991**, *338*, 1107.
8. Taninaka, C.; Ohtani, H.; Hanad, E.; Kotaki, H.; Sato, H.; Iga, T. *J. Chromatogr. B: Biomed. Appl.* **2000**, *738*, 405.
9. Raines, D. A.; Yusuf, A.; Jabak, M. H.; Ahmed, W. S.; Karcioglu, Z. A.; El-Yazigi, A. *Ther. Drug Monit.* **1998**, *20*, 680.
10. Kirchhoff, R. M.; Laufen, H. *Int. J. Clin. Pharmacol. Ther.* **1999**, *37*, 361.
11. Gandni, R.; Kaul, C. L.; Panchagnula, R. *J. Pharm. Biomed. Anal.* **2000**, *23*, 1073.
12. Kamau, F. N.; Chepkwony, H. K.; Ngugi, J. K.; Debremaeker, D.; Roets, E.; Hoogmartens, J. *J. Chromatogr. Sci.* **2002**, *40*, 529.
13. Zubata, P.; Ceresole, R.; Rosasco, M. A.; Pizzorno, M. T. *J. Pharm. Biomed. Anal.* **2002**, *27*, 833.
14. Fouda, H. G.; Schneider, R. P. *Ther. Drug Monit.* **1995**, *17*, 179.
15. Sastre, T. J.; Guchelaar, H. J. *J. Chromatogr. B* **1998**, *720*, 89.
16. Kelani, Khadiga; Bebawy Lories, L. *Anal. Lett.* **1997**, *30*, 1843.
17. Khashaba, P. Y. *J. Pharm. Biomed. Anal.* **2002**, *27*, 923.
18. Breier, A. R.; Garcia, C. V.; Oppe, T. P.; Steppe, M.; Schapoval, E. E. *J. Pharm. Biomed. Anal.* **2002**, *29*, 957.
19. Jacks, S.; Giguère, S.; Gronwall, P. R.; Brown, M. P.; Merritt, K. A. *Am. J. Vet. Res.* **2001**, *62*, 1870.
20. Economou, A.; Themelis, D. G.; Theodoridis, G.; Tzanavaras, P. D. *Anal. Chim. Acta* **2002**, *463*, 249.
21. Song, Z. H.; Hou, S. *Anal. Chim. Acta* **2003**, *488*, 71.
22. Song, Z. H.; Zhao, T. Z.; Wang, L.; Xiao, Z. *Bioogan. Med. Chem.* **2001**, *9*, 1701.
23. Song, Z. H.; Zhang, N. *Bioogan. Med. Chem.* **2002**, *10*, 2091.
24. http://www.rxlist.com/cgi/generic/trovazit_cp.htm