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A novel application of ratio spectra derivative spectroscopy for the determination of amphotericin in highly icteric plasma

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Abstract

Derivative spectroscopy has been utilised for the determination of amphotericin in various biological matrices including plasma, serum, urine and brain tissue. Whilst these methods have all been shown to be suitable for the determination of the drug in these matrices it has been reported that the application fails in the case of highly icteric plasma, this being due to the presence of high concentrations (>50 μM) of bilirubin. This paper details the application of ratio spectra derivative spectroscopy to overcome the interference of bilirubin with amphotericin in such situations.

Keywords: Ratio spectra derivative spectra; Amphotericin; Bilirubin; Plasma; Icteric

1. Introduction

Amphotericin B [1] (Fig. 1) is a polyene macrolide antifungal agent used in the treatment of systemic fungal infections especially in immunocompromised patients and for oral and perioral infections. Various methods for the quantification of amphotericin B in biological matrices using HPLC, LC/MS/MS, ELISA and UV–vis spectrophotometry have been described [1–12]. The UV–vis methods are all based on derivative spectra and have been applied to the determination of amphotericin B in brain tissue [10], serum and urine [11] and plasma [12], respectively. Monteil et al. [12] in describing the use of second order derivative spectroscopy for the measurement of this drug in plasma report excellent results with the exception of samples obtained from highly icteric patients. In these samples the authors reported that the high levels of bilirubin [2] (Fig. 1) (≥50 μM) give rise to interferences that affect the determination of amphotericin. The problem arises from the fact that bilirubin has an absorbance in the region of 400 nm which is the region where amphotericin absorbances occur.

Salinas et al. [13] described the development of ratio spectra derivative spectroscopy. In this methodology the spectra of various solutions containing different concentrations of two components [M+N] are obtained and these spectra are divided by a spectrum of one of the components [say N]. The "ratio spectra" thus obtained are then differentiated with respect to wavelength and the derivative spectra amplitudes for a given wavelength are plotted against the concentration of M C_M to give a calibration graph for that component. Application of the method to an unknown sample containing both M and N, and use of the calibration graph, will then enable the determination of C_M in the unknown mixture. The reverse procedure (division by M) can be used to determine C_N. This method was successful and permitted the resolution of binary mixtures of compounds having overlapping spectra. (The full mathematical basis of this method is described in detail in ref. [13] and is not presented here). The method has been applied successfully to the determination of co-formulated pharmaceuticals in a number of instances [14–17].

The application of derivative spectroscopy has often been used for the determination of one compound in the presence of a second interfering compound. However, in the case of the interference of bilirubin in plasma interfering with the determination of amphotericin this was reported to be unsuccessful. We have therefore investigated the possible application of ratio spectra derivative spectroscopy to the determination of amphotericin B in the plasma to eliminate this interference due to bilirubin.

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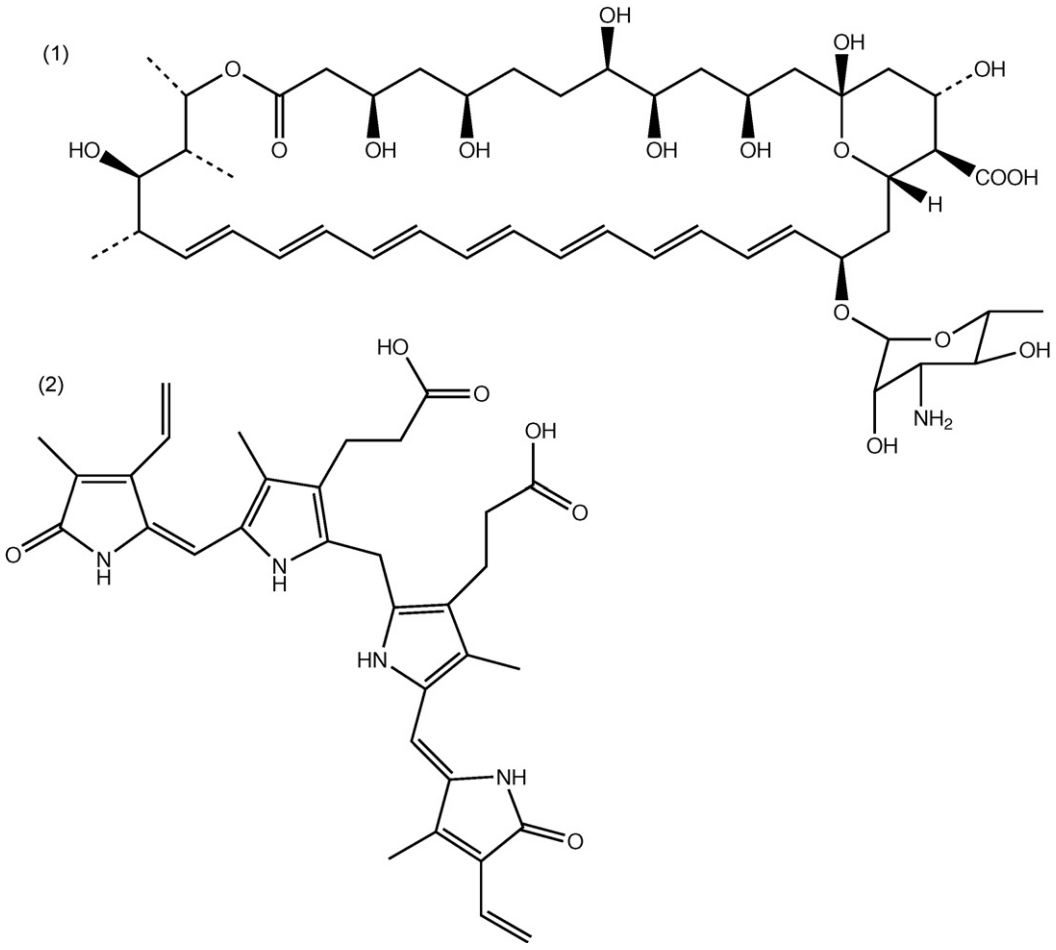

Fig. 1. The structures of amphotericin (1) and bilirubin (2).

2. Experimental

2.1. Instrumentation

Two spectrophotometers were used in this investigation:

- Cary 50 Scan UV–vis Spectrophotometer (Varian).
- Philips PU 8720 UV–vis Scanning Spectrophotometer.

UV scans were performed between 300 and 500 nm using a 10 mm path-length quartz cuvette purchased from Strana (Essex, England).

2.2. Materials

Amphotericin B (Amphotericin B from *Streptomyces* sp. ~80% (HPLC)) and bilirubin (≥98%) were purchased from Sigma (Poole, England). Amphotericin B was stored in refrigerator until required. Bilirubin was stored at −20 °C until required. Horse plasma (Heparinised) was purchased from TCS Biosciences Ltd (Buckingham, UK). The plasma was stored at −20 °C until required. Dimethylsulfoxide (DMSO) and methanol were Laboratory Reagent Grade, purchased from Fisher Scientific UK Ltd (Loughborough, Leicestershire, UK). Potassium dihydrogen phosphate and disodium hydrogen orthophosphate were purchased from Fisher Scientific UK Ltd (Loughborough, Leicestershire, UK). All water was processed using a Millipore-Q Reagent System (Waters, England).

2.3. Methodology

2.3.1. Preparation of solutions

A stock solution of amphotericin B was prepared (500 mg/l) by dissolution in approximately 70 ml of DMSO/methanol (50/50, v/v). This was stirred for approximately 2 h at room temperature protected from light. The mixture was transferred into a 100 ml volumetric flask and made up to 100 ml with the DMSO/methanol mixture. The flask was covered in aluminum foil and stored in a refrigerator. The stock solution of amphotericin was diluted with de-ionised water to produce a 25 mg/l solution and then further dilutions were prepared again using the de-ionised water. All solutions were protected from light.

The stock solution of amphotericin was diluted with horse plasma to produce a 25 mg/l solution and then further dilutions were prepared again using the plasma. All solutions were protected from light.

An alkaline solution of bilirubin 10 mg/100 ml was prepared in phosphate buffer pH 7.4 which was treated 5 drops of NaOH

(2 M). The flask was covered in aluminum foil and stored in a refrigerator.

2.3.2. Determination of solutions of amphotericin B in de-ionised water

Solutions of amphotericin (1.25–5 mg/l) in de-ionised water were prepared using the 25 mg/l solution and the zero order spectra determined between 300 and 500 nm; a scan rate of 200 nm/min was used. The second order derivative spectra [with smoothing] were generated using the "Maths" function of the WinCary software. A calibration curve was constructed using the zero to trough amplitude corresponding to the absorbance at 409 nm in the zero order spectra. Three test solutions of amphotericin in de-ionised water (4.375, 3.000 and 1.875 mg/l) were determined as above and the accuracy and bias of the measured concentrations of amphotericin in these samples calculated. In all experiments each solution sample was prepared and analysed in triplicate.

The above procedure was repeated but with the use of an acetonitrile pretreatment step. To 1 ml of each solution was added 2 ml of acetonitrile and the mixture vortexed for 30 s. The samples were then processed as above as were test samples.

Solutions of amphotericin (1.25–5 mg/l) in de-ionised water containing bilirubin (51.3 μM) were prepared and the zero order spectra determined between 300 and 500 nm, a scan rate of 200 nm/min was used. These zero order spectra were then divided by spectra of standard bilirubin solutions (51.3 μM or 25.7 μM) and the second order derivative spectra (with smoothing) of these ratio spectra were generated using the "Maths" function of the WinCary software. A calibration curve was constructed using the peak to trough amplitude corresponding to the absorbance at 409 nm in the zero order spectra. Three test solutions (QC samples) of amphotericin in de-ionised water (4.375, 3.000 and 1.875 mg/l) were determined and the accuracy and bias of the measured concentrations of amphotericin in these samples calculated.

The above procedure was repeated but with the use of an acetonitrile pretreatment step. To 1 ml of each solution was added 2 ml of acetonitrile and the mixture vortexed for 30 s. The samples were then processed as above along with the test samples.

2.3.3. Determination of solutions of amphotericin B in plasma

Solutions of amphotericin (1.25–5 mg/l) in plasma were prepared using the 25 mg/l solution and the zero order spectra determined between 300 and 500 nm; a scan rate of 200 nm/min was used. The procedure undertaken in this section was repeated on these samples as well as three test samples to determine bias and accuracy.

The procedure described above was repeated but with the use of an acetonitrile pretreatment step. To 1 ml of each solution was added 2 ml of acetonitrile and the mixture vortexed for 30 s. The samples were then processed as above as were test samples.

Solutions of amphotericin (1.25–5 mg/l) in plasma containing bilirubin (51.3 μM) were prepared and the zero order spectra determined between 300 and 500 nm, a scan rate of 200 nm/min was used. These zero order spectra were then divided by those of standard bilirubin solutions (51.3 μM or 25.7 μM) and the second order derivative spectra (with smoothing) of these ratio spectra were generated using the "Maths" function of the WinCary software. A calibration curve was constructed using the peak to trough amplitude corresponding to the absorbance at 409 nm in the zero order spectra. Three test solutions of amphotericin in plasma (4.375, 3.000 and 1.875 mg/l) were determined and the accuracy and bias of the measured concentrations of amphotericin in these samples calculated.

The procedure described above in 2.3.8 was repeated but with the use of an acetonitrile pretreatment step. To 1 ml of each solution was added 2 ml of acetonitrile and the mixture vortexed for 30 s. The samples were then processed as above as were test samples.

The above procedure was repeated using two concentrations of amphotericin (4.375 and 1.875 mg/l) and three concentrations of bilirubin (64.1, 51.3 and 26.6 μM).

3. Results and discussion

These studies were concerned with establishing the feasibility of using ratio spectra derivative spectroscopy for the determination of amphotericin B in plasma in the presence of the bilirubin, however, preliminary investigations (described in outline only for the sake of brevity) involved establishing the procedure in aqueous solution and in plasma without bilirubin interference. Spectral investigations of both zero, first and higher order spectra of amphotericin in water confirmed the findings reported by previous workers [10–12]. The zero order spectra of amphotericin B, bilirubin and a mixture of amphotericin B and bilirubin in de-ionised water are presented in Fig. 2. The second derivative spectrum of amphotericin B and the ratio spectra second derivative spectrum of amphotericin B in the presence of bilirubin in de-ionised water (with bilirubin as the divisor) are presented in Fig. 3. The zero order spectra of solutions of amphotericin B, bilirubin and amphotericin B in the presence of 51.3 μM bilirubin clearly demonstrates the interference of bilirubin at the concentration Monteil et al. [12] had suggested led to difficulties. Based on our initial investigations we decided to proceed with investigations using second order derivative spectroscopy as had Monteil et al. [12]. When investigating the procedure for the determination amphotericin B in de-ionised water in the absence of bilirubin the generated data were excellent in terms the correlation coefficient of the calibration curve and the bias and %CV of the QC samples investigated. This was as expected, based on the previous literature. Investigations then focused on aqueous samples containing varying concentrations of amphotericin in the presence of 51.3 μM bilirubin. The data generated for amphotericin B in de-ionised water in the presence of bilirubin are based on the second derivative of the ratio spectra. The division by bilirubin solutions (51.3 μM or 25.7 μM) to obtain the ratio spectra indicated that the 51.3 μM solution resulted in better data. Statistical data on calibration solutions and test solutions (correlation coefficients, bias and %CV) were inferior to those obtained in the absence of bilirubin. In the analytical procedures detailed by Monteil et al. [12] and Botslogu et al.

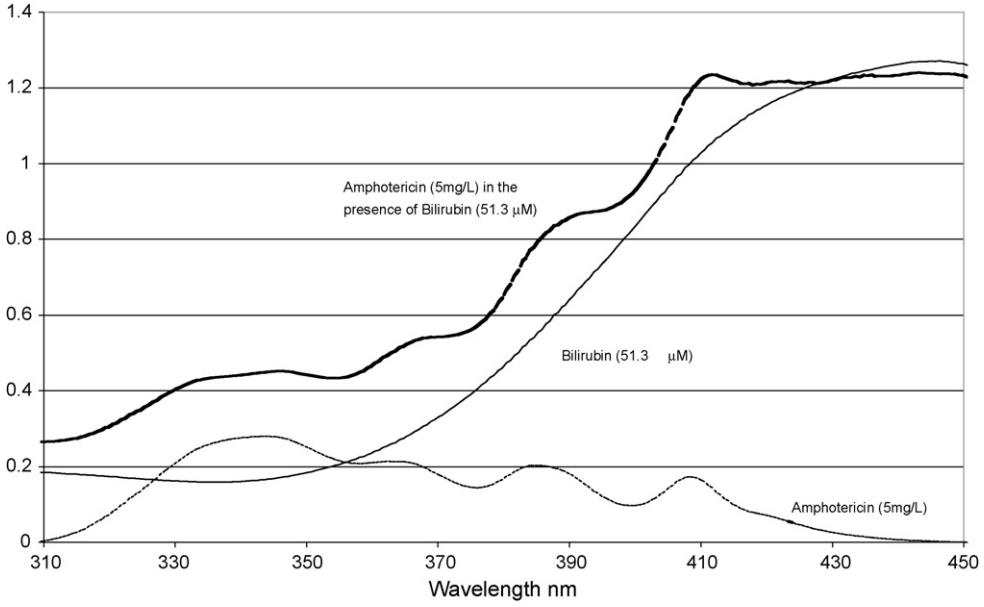

Fig. 2. Zero order spectra of solutions of amphotericin B (5 mg/l), bilirubin (51.3 μM) and amphotericin B (5 mg/l) plus bilirubin (51.3 μM) in de-ionised water.

[11] when determining the drug in the biological matrices a pre-treatment step involving addition of acetonitrile was included, this procedure is presumably a protein precipitation step. We therefore investigated this again in samples of amphotericin B dissolved in de-ionised water. Once again satisfactory analytical data were obtained, both in the absence and presence of bilirubin, which suggested that the second derivative of the ratio spectra (using a bilirubin solution as the divisor) was capable of removing the interference of bilirubin. From these data we also concluded that the use of the acetonitrile pre-treatment step resulted in more reliable analyses.

Our work then focused on the application of this method to the analysis of amphotericin B in plasma samples. Due to the recent implementation of the Human Tissue Act in the UK in this preliminary study we have utilised horse plasma in place of human plasma (Fig. 4).

Once again sets of standard samples of amphotericin were prepared in plasma in presence of bilirubin (51.3 μM), calibration curves were constructed and then QC solutions analysed. These procedures were then repeated with the inclusion of the acetonitrile step. These results are presented in Table 1 and these data clearly indicate the feasibility of determining amphotericin in plasma in the presence of bilirubin using ratio second derivative spectra. The results also indicate the superiority of the inclusion of the acetonitrile pre-treatment step. In order to investigate the applicability of the method we also investigated its applica-

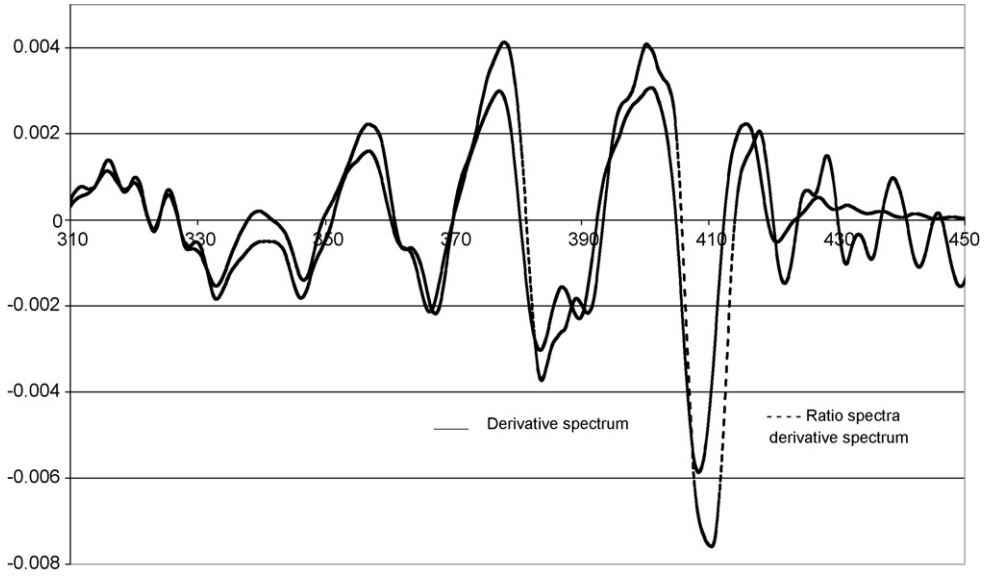

Fig. 3. The second derivative spectrum of amphotericin B in de-ionised water and the ratio spectra second derivative spectrum of amphotericin B in the presence of bilirubin (51.3 μM) in de-ionised water using bilirubin (51.3 μM) as the divisor solution.

Table 1
Statistical data for analysis of amphotericin B in plasma in the presence of bilirubin (51.3 μM) with and without an acetonitrile pre-treatment step using the ratio spectra second derivative spectrum method

	With no acetonitrile step		With acetonitrile step	
Equation	$y = -0.00255x - 0.00015$		$y = -0.00163x - 0.00048$	
Correlation coefficient (r)	0.9965		0.9915	
Concentration of amphotericin (mg/l)	Accuracy (bias %)	Precision (%CV)	Accuracy (bias %)	Precision (%CV)
4.375	91.80	2. 77	96.00	2.94
3.125	113.02	1.35	106.20	1.89
1.875	102.20	4.07	98.90	1.18

Table 2
Statistical data for analysis of amphotericin B in plasma in the presence of bilirubin (64.1, 51.3 and 26.6 μM) with an acetonitrile pre-treatment step using the ratio spectra second derivative spectrum method

Equation		$y = -0.00171x - 0.00049$	
Correlation coefficient (r)		0.9915	
Concentration of amphotericin (mg/l)	Concentration of bilirubin (μM)	Accuracy (bias %)	Precision (%CV)
4.375	64.1	102.9	2.7
4.375	51.3	105.2	1.2
4.375	26.6	107.8	1.2
1.875	64.1	99.3	0.8
1.875	51.3	98.1	6.3
1.875	26.6	105.5	1.4

tion with solutions containing constant amphotericin concentrations with variable bilirubin concentrations. The results are presented in Table 2. These data again clearly demonstrate the applicability of the method. In the work by Monteil et al. [12] they had initially indicated the use of amphotericin concentrations in the range 1.25–5 mg/l which we have repeated, however, they do report a linear range for their method of 0.05–5 mg/l and that the normal therapeutic range is 0.5–3.5 mg/l. In the present study we were able to demonstrate a linear response in the range 0.5–5 mg/l ($y = 0.0018x + 0.0005$; $r = 0.9734$), which covers the full therapeutic range. The inability to extend the lower range of this calibration curve is possibly related to the extra step involved in the production of the ratio spectra.

The basis for this study was the use of derivative spectroscopy for the determination of amphotericin in biological matrices and the reported problem that in plasma from highly icteric patients

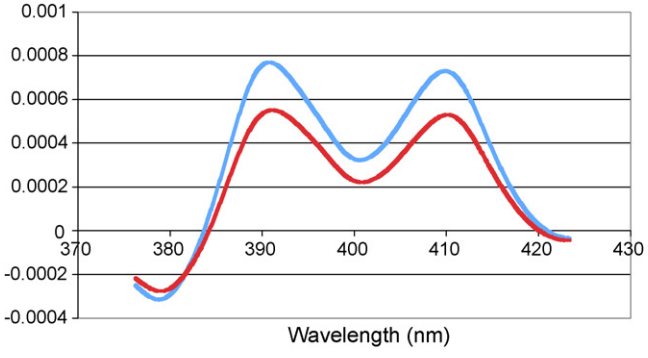

Fig. 4. The second derivative ratio spectra of amphotericin (4.00 and 5.00 mg/l) in horse plasma containing 51.3 μM bilirubin.

the bilirubin present caused interference in this determination. The results presented in this work suggest that ratio spectra derivative spectroscopy is capable of removing the interference of bilirubin in such circumstances. Actual patient samples could not be determined at this time due to procedural difficulties, however, the possible interference from metabolites of amphotericin B should not be a problem due to the reported lack of metabolism of amphotericin B [18].

References

[1] G.G. Granich, G.S. Kobayashi, D.J. Krogstad, Antimicrob. Agents Chemother. 29 (1986) 584–588.
[2] T. Eldem, N. Arican-Cellat, J. Pharm. Biomed. Anal. 25 (2001) 53–64.
[3] P. Egger, R. Bellmann, C.J. Wiedermann, J. Chromatogr. B 760 (2001) 307–313.
[4] M. Lambros, S. Abbas, D. Bourne, J. Chromatogr. B Biomed. Appl. 685 (1996) 135–140.
[5] R. Lopez, L. Pou, I. Andres, V. Monteforte, A. Roman, C. Pascual, J. Chromatogr. A 812 (1998) 135–139.
[6] T. Eldem, N. Arican-Cellat, I. Agabeyoglu, M. Akova, E. Kansu, Int. J. Pharm. 213 (2001) 153–161.
[7] G. Wurthwein, A.H. Groll, G. Hempel, F.C. Adler-Shohet, J.M. Lieberman, T.J. Walsh, Antimicrob. Agents Chemother. 49 (2005) 5092–5098.
[8] J.W. Lee, M.E. Petersen, P. Lin, D. Dressler, I. Bekersky, Ther. Drug Monit. 23 (2001) 268–276.
[9] J.D. Cleary, S.W. Chapman, J. Deng, C.J. Lobb, Antimicrob. Agents Chemother. 40 (1996) 637–641.
[10] P. Casaccia, A. Ladogana, Y.G. Xi, L. Ingrosso, M. Pocchiari, M.C. Silvestrini, A. Cittadini, Antimicrob. Agents Chemother. 35 (1991) 1486–1488.
[11] N.A. Botsoglou, D.J. Fletouris, G.E. Papageorgiou, P. Florou-Paneri, A.J. Mamtis, J. Pharm. Sci. 85 (1996) 402–406.
[12] C.G. Monteil, M.F. Kergueris, P. Looss, L. Thomas, C. Larousse, J. Pharm. Biomed. Anal. 17 (1998) 481–485.

[13] F. Salinas, J. Berzas Nevado, A. Espinosa Mansilla, Talanta 37 (1990) 347–351.
[14] F.H. Metwally, Y.S. El-Saharty, M. Refaat, S.Z. El-Khateeb, J. AOAC Int. 90 (2007) 391–404.
[15] H.E. Abdellatef, M.M. Ayad, S.M. Soliman, N.F. Youssef, Spectrochim. Acta A: Mol. Biomol. Spectrosc. 66 (2007) 1147–1151.
[16] Y.S. El-Saharty, F.H. Metwally, M. Refaat, S.Z. El-Khateeb, J. AOAC Int. 90 (2007) 102–112.
[17] S.E. Vignaduzzo, R.M. Maggio, P.M. Castellano, T.S. Kaufman, Anal. Bioanal. Chem. 386 (2006) 2239–2244.
[18] R. Bellmann, Curr. Clin. Pharmacol. 2 (2007) 37–58.