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Kinetic fluorimetric determination of the antineoplastic methotrexate (MTX) in human serum

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Abstract

A kinetic study of the oxidation of methotrexate (MTX) in acidic medium and in the presence of potassium permanganate has been made on the basis of the fluorescence–time curves. A kinetic method for the determination of MTX was developed with a range of application between 0.22 and 3.30 μM. The proposed kinetic method permits us to determine MTX in human serum and to avoid the natural fluorescence of the serum. A detection limit of 0.18 μM was calculated in the presence of ascorbic acid as activator. Only 100 s per sample is necessary for the analysis. The interference of pteridin derivatives and the rescue agent folinic acid (leucovorin) was tested.

Keywords: Methotrexate; Kinetic–fluorimetric; Antineoplastic; Human serum

1. Introduction

Methotrexate (MTX, amethopterin, 2,4-diamine-N10-methylpteroyl-glutamic acid) is an antifolate (Scheme 1) that shows significant antitumor activity in acute leukaemia and other neophastic diseases [1,2]. As an analog of folic acid, MTX inhibits the enzyme dihydrofolate reductase causing a lack of reduced folates which are donors of one-carbon units in the biosynthesis of nucleic acids and some amino acids. Like the folates, MTX is intracellularly converted to polyglutamates, which inhibit further enzymes of the folate metabolism and thus take part in the cytostatic action of the drug [3]. The inherent risk of toxicity from high dose therapy, patient management requires plasma or serum MTX monitoring [4] and the coadministrated folinic acid (leucovorin) which is a like folate derivative used as rescue agent. Several methods [5–7] for the determination of MTX in biological samples have been reported, but the majority of those were established for pharmacokinetic studies in anti-cancer therapy using high dose but in rheumatoid therapy a low dose is necessary which requires a

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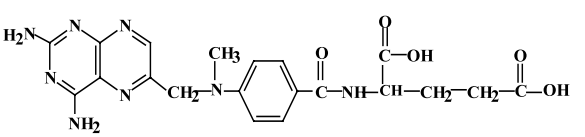
sensitivity of less than 10 ng/ml in plasma [4]. High sensitivity methods have been reported for the determination of MTX based in different techniques and applied to several biological fluids, such as enzyme multiplied immunoassay [8], fluorescence polarization immunoassay [9], radioimmunoassay [3], capillary zone electrophoresis [10,11] and HPLC [12–14].

MTX can be transformed in highly fluorescent derivatives using hydrogen peroxide and potassium permanganate. Several procedures using HPLC with online oxidation using hydrogen peroxide in the mobile phase as fluorogenic reagent at high temperature [15], by using a similar way at ambient temperature [16] and with a post column photochemical induction [17]. The fluorescent generate product is described as similar in all cases but the kinetic of this reaction are not really established. The fluorescence properties of the generated product after oxidation of MTX with potassium permanganate have been established [18]. In the above mentioned procedure, strict control of the reaction time, temperature and HPLC process is required with the object being to separate other fluorescent products present in the samples or others generated in the established chemical conditions.

In this paper, the oxidation of MTX by potassium permanganate has been kinetically studied. The aim of this work is to combine the sensitivity of the fluorescence with the selectivity and speed of the kinetic measurements in the analysis of MTX in human serum. Data about kinetic methods have not been found in the literature. The influence of the physical chemical conditions in the kinetic reaction and the influence of foreign species, as pteridins and like folate rescue agent leucovorin, have been established. An advantage of the proposed kinetic method is the speed in the analysis of MTX in human serum because only



MTX

Scheme 1.

100 s is the time consumed per sample. Also, an increment of the selectivity is observed with respect to the equilibrium method in the presence of some like-folates compounds, such as pteridins. Pteridins derivatives are naturally present in serum samples. HPLC method for determining MTX in the presence of pteridins have not been described in the bibliography. On the other hand, folinic acid (leucovorin), which is a rescue agent, is tolerated at 2:1 ratio.

2. Experimental

2.1. Reagents

All experiments were performed with analytical reagent grade chemicals. MTX was prepared by dissolving 0.010 g of 4-amino-10-methylfolic acid (Sigma, St. Louis, MO, USA) in 100 ml of alkalinized ultrapure-grade water obtained from Water Pro PS (Labconco, Kansas City, Mo, USA). Appropriate MTX solutions of different concentrations were prepared by dilution in ultrapure-grade water. Pteridin derivatives were purchased from Sigma and standard solutions were prepared by dilution with ultrapure water (to avoid the direct exposure to the sunlight).

2.2. Apparatus

Acquisition of kinetic data and fluorescent measurements were made on a Perkin Elmer LS 50 luminescence instrument, equipped with a 150 W continuous xenon lamp, interfaced by a GPIB card and driver with a PC 486 microcomputer. The kinetic curves were registered with $\lambda_{exc} = 380$ nm and $\lambda_{em} = 457$ nm, and taking one experimental point per second. Excitation and emission spectra were recorded in a 10-mm quartz cell at 25 °C, by use of a thermostatic cell holder and a Selecta Unitronic 320 OR thermostatic bath.

2.3. Procedures for the kinetic fluorimetric determination of MTX

2.3.1. General procedure

Place an aliquot of MTX equivalent to 9.9 ×

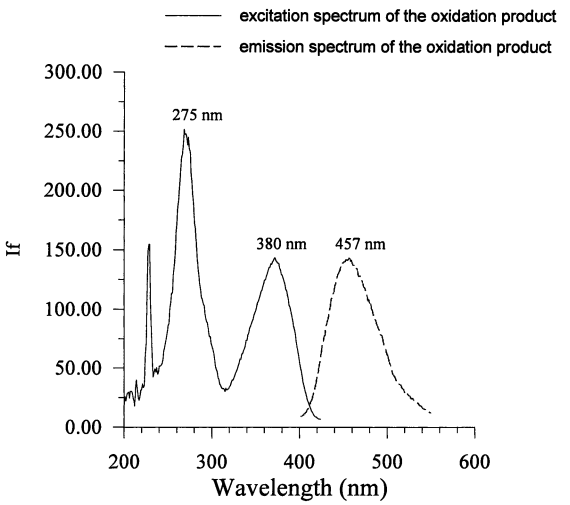


Fig. 1. Fluorescence spectra obtained for the oxidation product of MTX in acidic medium and in the presence of potassium permanganate, [MTX] = 0.97 μM; reaction time = 500 s; pH 5.0; $T^a = 25$ °C.

10^{-4}–6.6×10^{-3} μmol of MTX, in a 3 ml quartz cell, 0.6 ml of pH 5 acetic acid/sodium acetate buffer solution ($C = 0.5$ M), 0.3 ml of 1×10^{-3} M potassium permanganate solution, 0.10 ml of 60 μg/ml ascorbic acid solution and dilute with ultrapure water to a final volume of 3 ml. Scan the evolution of the fluorescence intensity with the time at $\lambda_{exc} = 380$ nm and $\lambda_{em} = 457$ nm during 100 s, maintaining the temperature at 25 °C. Finally, determine the MTX content by measuring the reaction rate as the tangent in the linear period of the kinetic curve obtained by linear regression of ten experimental points and using the appropriate calibration graph.

2.3.2. Analysis of MTX in human serum

Take up to 2 ml of a previous diluted serum (3 ml/100 ml), containing between 9.9×10^{-4} and 6.6×10^{-3} μmol of MTX, 0.6 ml of pH 5 acetic acid/sodium acetate buffer solution ($C = 0.5$ M), 0.3 ml of 1×10^{-3} M potassium permanganate solution, 0.10 ml of 60 μg/ml ascorbic acid solution and dilute with ultrapure water to a final volume of 3 ml, and then follow the general procedure.

3. Results and discussion

MTX solution in acid medium, and in the presence of potassium permanganate, generates a fluorescent derivative with excitation maxima located at 275 and 380 nm, and an emission maximum at 457 nm. In Fig. 1, the excitation and emission spectra, obtained at 500 s of reaction time, using $\lambda_{exc} = 380$ nm and $\lambda_{em} = 457$ nm can be observed. The kinetic curves were obtained at $\lambda = 457$ nm (excitation at 380 nm) during 700 s of reaction time, and a linear relationship between emission intensity and the reaction time was observed.

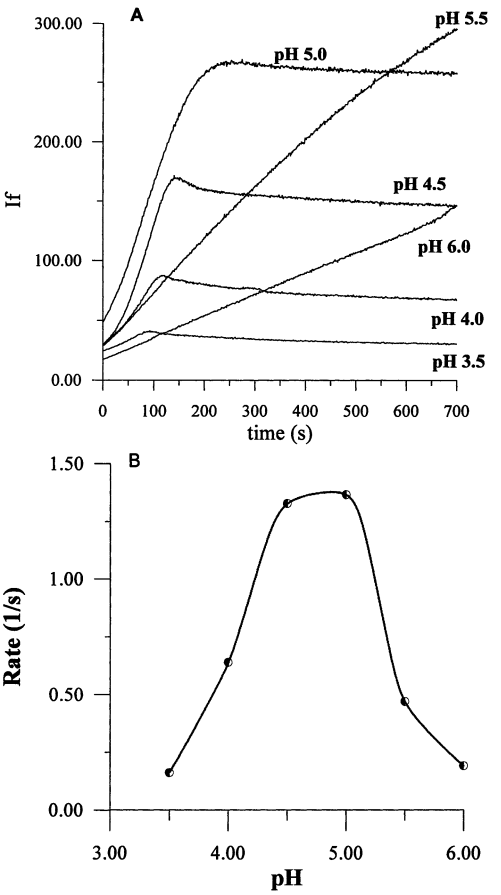


Fig. 2. (A) Influence of pH in the kinetic of the oxidation reaction. (B) Influence of pH on the rate of the reaction (temperature 25 °C; period of measurement: 10 s; [MTX] = 2.20 μM; [$KMnO_4$] = 10^{-4}M).

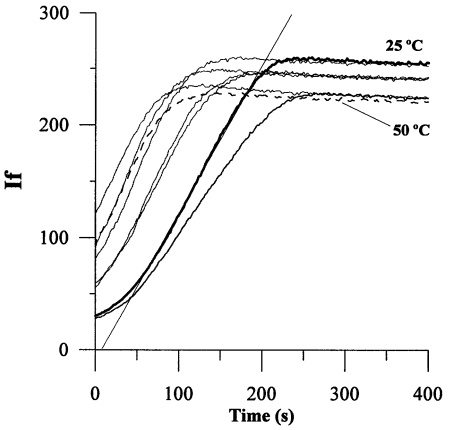


Fig. 3. Influence of the temperature in the kinetic of the reaction (pH 5.0; [MTX] = 2.20 μM; [$KMnO_4$] = 10^{-4} M).

3.1. Influence of chemical variables

Several acetic acid/sodium acetate buffers were tested in order to obtain the optimum pH value to develop the MTX oxidation. The pH value was varied between 3.5 and 6.0. In Fig. 2A, the kinetic curves obtained are shown. As can be observed, the oxidation of MTX is strongly influenced by the acidity of the medium. The fluorescence intensity increases with the increase of the pH. For pH 5, the equilibrium is obtained for 200 s. In the range between 3.5 and 4.3, the rate of the reaction increases with increase of the pH value (Fig. 2B). For a narrow range between 4.3 and 5.0, the rate of reaction remains about constant, but the intensity of fluorescence drastically increasing, with pH increase. For pH > 5, the rate of the reaction decreases with the increasing pH. For obtaining the highest sensitivity, a pH 5 was selected as optimum.

The effect of the temperature on the reaction rate was examined between 20 and 55 °C and the results obtained are represented in Fig. 3. A very short increase in the rate of reaction with the temperature is observed up to 45 °C and the rate of the reaction decreases for higher temperatures. Also, the linear period decreases with the increase in the temperature. 25 °C was selected as optimum value because the intensity of fluorescence is maximum, and in addition, a well-defined kinetic curve is obtained in these conditions.

The influence of potassium permanganate concentration was studied in the range between 1×10^{-5} and 5×10^{-4} M. The rate of reaction was found to increase with a concentration up to 1×10^{-4} M and then intensity of fluorescence is maxima for reaction time higher than 200 s. For potassium permanganate concentrations higher than 1×10^{-4} M, the rate of reaction remains constant, however the intensity of fluorescence decreases with increasing concentrations of the oxidative agent. This fact indicates to us that in presence of high concentration of oxidative agent,

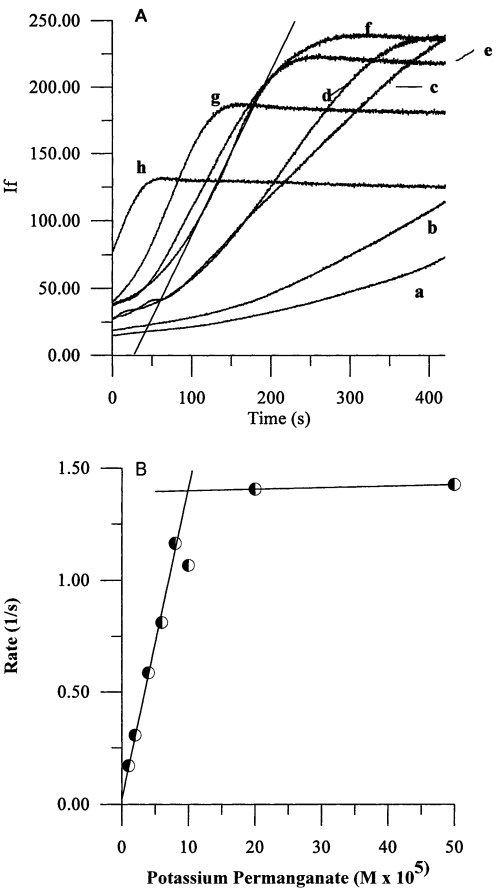


Fig. 4. (A) Influence of the concentration of the oxidative reagent ($KMnO_4$) in the kinetic of the oxidation of MTX: (a) 10^{-5} M; (b) 2×10^{-5} M; (c) 4×10^{-5} M; (d) 6×10^{-5} M; (e) 8×10^{-5} M; (f) 10^{-4} M; (g) 2×10^{-4} M; (h) 5×10^{-4} M. (B) Influence of the potassium permanganate concentration on the rate of reaction ([MTX] = 2.20 μM; temperature: 25 °C; pH 5.0).

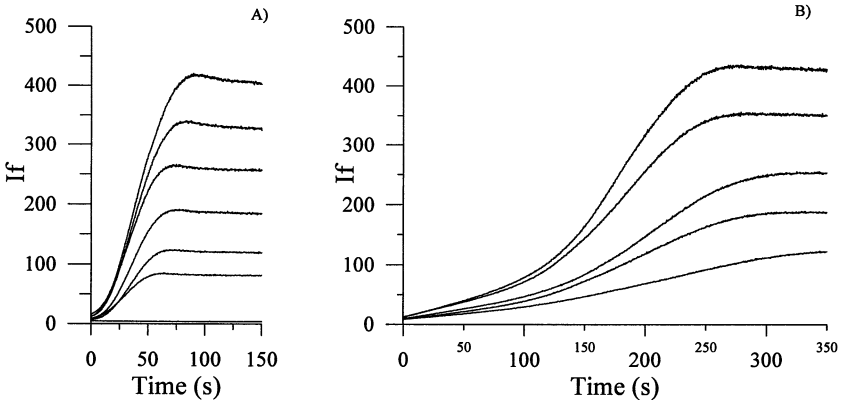


Fig. 5. Kinetic curves obtained for samples containing several concentrations of MTX (A) between 0.22 and 3.30 μM in the presence of ascorbic acid (2 μg/ml) and (B) between 0.88 and 7.15 μM in the absence of ascorbic acid.

the formation of the fluorescent specie is competitive, with other processes giving rise to a non-fluorescent specie. A potassium permanganate of 1×10^{-4} M was selected as optimum (Fig. 4).

In the previous studies of this reaction we have observed that the oxidation reaction is improved by the presence of low amounts of ascorbic acid. The slopes and the shape of the kinetic curves were compared, in the presence and in the absence of ascorbic acid, using several concentrations of MTX. In the presence of ascorbic acid, the kinetic curves present a higher slope, and a similar small induction period for all concentrations of MTX, as well as a higher stability of the product of the oxidation. The equilibrium was obtained in only 50 s. The ascorbic acid seems to act as activator of the process of oxidation, stabilizing the formation of the fluorophoro. In Fig. 5 the kinetic curves obtained in the presence of 2 μg/ml and in its absence are shown. A 2 μg/ml ascorbic acid was selected as optimum to obtain the maxima sensitivity.

3.2. Calibration curves and analytical parameters

Under the optimum physical–chemical conditions, the fluorescence–time signals at $\lambda_{ex} = 380$ nm and $\lambda_{em} = 457$ nm were recorded between 0 and 400 s for solutions containing different amounts of MTX, in the absence and presence of 2 μg/ml of ascorbic acid. In both cases, the analytical signal was the tangent to the kinetic curve in the linear period obtained by application of the least-squares method in a range of ten experimental points. In Table 1, analytical and statistical parameters are summarized. The rate of the reaction was found to be first order with respect to MTX concentration in the range 0.88–7.15 μM in the absence of ascorbic acid and in the range 0.20–3.30 μM in the presence of 2 μg ml^{-1} of ascorbic acid. As can be observed, the sensitivity of the kinetic procedure is strongly increased by the presence of ascorbic acid. The coefficients of correlation (R^2) were 0.9971 and 0.9943, respec-

Table 1
Analytical and statistical parameters for kinetic–fluorimetric determination of MTX

	In the absence of ascorbic acid	In the presence of 2 μg/ml of ascorbic acid
Slope $\left(\frac{1}{\text{s.}\mu\text{M}}\right)$	1.032	2.613
Range of application[a]	0.88–7.15	0.20–3.30
Detection limit: Winefordner criterium, $k = 3$ [19]	0.44	0.17
Clayton criterium $\alpha = \beta = 0.05$, $n = 3$ [20]	0.33	0.18
Residual deviation	0.126	0.32

[a] Concentrations are expressed in μM.

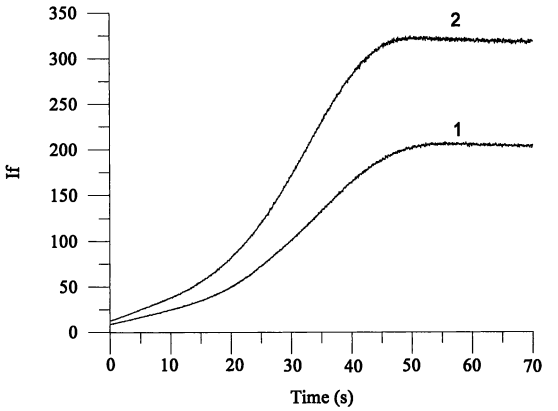


Fig. 6. Kinetic curves obtained for serum samples containing 1.69 μM (1) and 2.53 μM (2) of MTX in the presence of ascorbic acid (2 μg/ml).

tively. For a series of 11 samples containing 1.10 μM of MTX, the relative standard deviation (RSD) was 7% and the relative error was 5% (confidence level, 95%).

3.3. Applications

The proposed kinetic method, in the presence of ascorbic acid, has been tested in the analysis of a human serum spiked with MTX. The concentration range of MTX was between 0.83 and 3.36 μM. Several dilutions of serum were assayed and adequate recovery for MTX was only obtained when a dilution of 1/50 of the serum was assayed. Low recovery values are calculated when higher amounts of serum were used. In Fig. 6, the kinetic curves obtained for serum samples containing 1.69 and 2.53 μM of MTX are shown. Statistical parameter for calibration in serum are summarized in Table 2. The slope of the calibration curve in the presence of serum is similar to the reference slope (external standard method). No significant statistical difference ($P > 0.05$) was found when a comparison between the external standard and the standard addition calibration was made [21]. Recovery values were between 93 and 104%. This fact shows that the serum matrix does not interfere with the kinetic determination in the established chemical conditions.

Table 2
Determination of MTX in human serum using the proposed kinetic procedure and the standard addition methodology

Sample	[MTX][a]		Recovery (%)
	Added	Found[b] (RSD)	
Human serum	0	0.099 (5.20)	–
	0.836	0.845 (4.03)	101
	1.188	1.105 (4.20)	93
	1.694	1.765 (5.10)	104
	2.531	2.588 (3.20)	103
	3.367	3.248 (3.50)	96
Statistical parameter for calibration in serum:			
Slope of calibration $\left(\frac{1}{\text{s.}\mu\text{M}}\right)$:		2.501	
Deviation of the slope:		0.099	
Intercept:		0.350	
Deviation of the intercept:		0.211	
Coefficient of correlation:		0.9944	
Detection limit:			
Winefordner criterium: $k = 3$ [19]		0.316	
Clayton criterium: $\alpha = \beta = 0.05$, $n = 3$ [20]		0.520	

[a] In μM.

[b] Mean of three replicates; RSD: relative standard deviation for $n = 3$.

Reference of slope of calibration: 2.610 $\left(\frac{1}{\text{s.}\mu\text{M}}\right)$

3.4. Influence of foreign species

The effect of the presence of compounds naturally existing in the urine such as pteridins were tested, also the rescue agent leucovorin (folinic acid) was assayed. A 2% relative error was selected as the tolerated limit. Neopterin, isoxanthopterin and folinic acid are tolerated in a 2/1, interferent/MTX ratio, and biopterin at 1/1 interferent/MTX ratio. Xanthopterin strongly interferes in the kinetic determination of MTX. It is necessary to indicate that pteridins are present in concentrations smaller than MTX in human serum.

4. Conclusions

The proposed kinetic–fluorimetric method based in oxidation of MTX allows the determination of MTX in human serum without pretreatment. This procedure combines the selectivity of the fluorescence detection with the speed of the kinetic measurements. The determination can be performed by measuring the tangent to the kinetic curves at 457 nm (exciting at 380 nm) in a range of 10 s (ten experimental points). The total analysis time per sample is lower than 100 s. An exhaustive statistical analysis was applied to the calibration graph in human serum including least square regression. The model chosen is an adequate description of the true relationship between evolution of the fluorescence with the time and the MTX concentration.

The proposed method can be easily applied to the routine analysis and the procedure could be semiautomatized by the use of a stopped flow mixing module. This possibility is actually being tested. The interference due to the natural fluorescence of the serum matrix can be avoided by using kinetic measurements and this fact could be considered as an advantage against the equilibrium methods.

The proposed method allows us the determination of MTX in the presence of other like folates compounds such as pteridines, and this possibility has been not found in the bibliography. The determination of MTX in the presence of leucovorin has only been reported by mean HPLC, but methods without a separation process have not been described.

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References

[1] W.A. Bleyer, Cancer 41 (1978) 36–51.
[2] K. Kimura, Y.M. Wang, Methotrexate in Cancer Therapy, Raven Press, New York, 1986, pp. 213–223.
[3] M. Fleisher, Therap. Drugs Monitoring 15 (1993) 521–526.
[4] T. Okuda, M. Motohashi, I. Aoki, T. Yashiki, J. Chromatogr. B 662 (1994) 79–84.
[5] R. Gahdhi, A.J. Khopade, N.K. Jain, Indian Drugs 34 (1997) 455–458.
[6] C.S.P. Sastry, J.S.V.M. Lingeswara-Rao, Anal. Lett. 29 (1996) 1763–1778.
[7] A.J. Miranda, A. Costa, S. Arribas, P. Tuñón, Anal. Quim. 83 (1987) 342–346.
[8] C. De Porcer-Morton, J. Vhuang, M. Specker, R. Bastiani, Performance Evaluation of EMIT Methotrexate Assay, Syva, Palo Alto, CA, 1978.
[9] M.A. Pesce, S.H. Bodourian, Therap. Drugs Monitoring 8 (1986) 115–121.
[10] Y. Mrestani, R.N.H. Neubert, Electrophoresis 19 (1998) 3022–3025.
[11] F. Sczesny, G. Hempel, J. Boos, G. Blaschke, J. Chromatogr. B 718 (1998) 177–185.
[12] R.M. Mader, B. Rizovski, G. Steger, H. Rainer, R. Proprentner, R. Kotz, J. Chromatogr. Biomed. Appl. 124 (1993) 311–316.
[13] S. Belz, C. Frickel, C. Wolfrom, H. Nau, G. Henze, J. Chromatogr. Biomed. Appl. 661 (1994) 109–118.
[14] S. Chen, Y. Liu, D.W. Armstrong, J.I. Borrell, B. Martinez-Teipel, J.L. Matallana, J. Liq. Chromatogr. 18 (1995) 1495–1507.
[15] H. Kubo, Y. Umiguchi, M. Fukumoto, T. Kinoshita, Anal. Sci. 8 (1992) 789–792.
[16] G. Lu, H.W. Jun, J. Liq. Chromatogr. 18 (1995) 155–171.
[17] J. Salamoun, M. Smrz, F. Kiss, A. Salamounova, J. Chromatogr. Biomed. Appl. 63 (1997) 213–223.
[18] T. Suzuki, H. Hashimoto, N. Ichinose, Fresenius J. Anal. Chem. 351 (1995) 806–807.

[19] G.L. Long, J.D. Winefordner, Anal. Chem. 55 (1983) 712A–714A.
[20] C.A. Clayton, J.W. Hines, P.D. Elkins, Anal. Chem. 59 (1987) 2506–2514.
[21] M. Valcarcel, A. Rios, 1992. La calidad en los Laboratorios Analíticos, Reverté Edit., Madrid.