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Use of post-column fluorescence derivatization to develop a liquid chromatographic assay for ranitidine and its metabolites in biological fluids

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Abstract

Ranitidine and its main metabolites, ranitidine N-oxide and ranitidine S-oxide, were determined in plasma and urine after separation using reversed-phase liquid chromatography. The mobile phase consisted of an initial isocratic step with 7:93 (v/v) acetonitrile–7.5 m*M* phosphate buffer (pH 6) for 8 min, followed by a linear gradient up to a 25:75 (v/v) mixture over 1 min. Detection was carried out by a post-column fluorimetric derivatization based on the reaction of the drugs with sodium hypochlorite, giving rise to primary amines that reacted with *o*-phthalaldehyde and 2-mercaptoethanol to form highly fluorescent products. The calibration graphs, based on peak area, were linear in the range 0.1–4 μg/ml for all drugs. The detection limits were 30, 41 and 32 ng/ml (8.6, 12.5 and 9.1 pmol) for ranitidine S-oxide, ranitidine N-oxide and ranitidine, respectively. Chromatographic profiles obtained for plasma and urine samples showed no interference from endogenous compounds.

Keywords: Ranitidine; Ranitidine oxide

1. Introduction

The clinical use of ranitidine is based on its inhibitory effect on gastric acid secretion, the degree of inhibition being directly related to the plasma concentration of the drug. Thus, an inhibition of approximately 50% in acid secretion has been achieved with plasma concentrations of 100 ng/ml [1]. The drug is primarily excreted in urine and 60% or more may appear in the urine without modification, with most of the remaining products being oxidation metabolites. Different methods for detecting ranitidine and its metabolites in human fluids have been developed. These are frequently based on liquid chromatography with spectrophotometric detection [2–9]. For the fluorimetric analysis of secondary amines, a very common reaction consists of an initial oxidation step using hypochlorite to oxidize the secondary to a primary amine [10,11]. Subsequently, the primary amine reacts with *o*-phthalaldehyde (OPA) in combination with a thiol compound, usually 2-mercaptoethanol (ME), to produce fluorescent species [12–14]. Using this reaction, we recently developed a highly sensitive automated method, using flow injection analysis (FIA), for the determination of ranitidine in pharmaceutical preparations [15]. However, this procedure was not suitable for analysing the drug in human samples, due to

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the interference of ranitidine metabolites, which also gave fluorescent products, and matrix constituents.

In this study, the separation of ranitidine and its main metabolites, ranitidine S-oxide and ranitidine N-oxide, was optimized using reversed-phase liquid chromatography. Detection was performed by post-column derivatization using a FIA system involving the prior oxidation of the drug with sodium hypochlorite and then reaction with OPA and ME to produce fluorescence. In this way, the proposed procedure shows a good sensitivity and a better selectivity than other methods because the interferences caused by other compounds using the UV detection method are avoided with the fluorimetric derivatization reaction. The method allowed both the determination of ranitidine and its metabolites in physiological samples such as plasma and urine and the evaluation of the kinetics of the drugs in urine.

2. Experimental

2.1. Apparatus

The HPLC system consisted of a Kontron 325 liquid chromatograph operating at room temperature and with a flow-rate of 1.0 ml/min, and a Kontron SFM 25 fluorescence detector set at wavelengths of 350 and 450 nm (excitation and emission). Samples (100 μl) were injected manually using a Model 7125-075 Rheodyne injection valve. The analytical column (Supelco) was 15×0.4 cm I.D. stainless steel packed with Spherisorb ODS-2 with a particle size of 5 μm. A Supelco guard column packed with the same stationary phase was also used. The void volume was measured by injecting a stronger solvent that absorbed at the wavelength used. The post-column flow injection system consisted of a Gilson Minipuls HP4 peristaltic pump, a Hellma 176.052-QS fluorimetric flow cell, 0.5 mm I.D. PTFE tubing and various end-fittings and connectors (Omnifit). Thermostating of the reactor coils was carried out using a laboratory-made electronic device. A PC integration pack (Kontron) was used to record the chromatograms and integrate the areas under the peaks.

2.2. Reagents

Acetonitrile (Romil, Loughborough, UK) was of liquid chromatographic grade. Doubly distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). The solvents were degassed by purging with helium gas. A 0.02 *M* OPA (Fluka) solution was prepared in 2% ethanol and 0.5 *M* borate buffer (pH 10.5). A 0.1 *M* 2-mercaptoethanol (Sigma) solution was prepared in 0.5 *M* borate buffer (pH 10.5). Solutions were kept in dark bottles at 4°C. The 5 m*M* sodium hypochlorite was diluted from the commercial product (Probus, 12%) in 0.05 *M* sodium acetate–acetic acid buffer (pH 4.5). A 500 μg/ml stock solution of ranitidine hydrochloride (Sigma) was prepared in water. Ranitidine N-oxide and ranitidine S-oxide were kindly supplied by Glaxo (Barcelona, Spain); solutions (500 μg/ml) were also prepared in water and kept at 4°C. Working standard solutions were prepared by dilution with water just before use.

2.3. Mobile phase and post-column FI system

Separation was carried out with an initial isocratic mobile phase of 7:93 (v/v) acetonitrile–7.5 m*M* phosphate buffer (pH 6) for 8 min followed by a linear gradient from 7:93 (v/v) to 25:75 (v/v) over 1 min, this mixture being held for 6 min. Finally, the initial conditions were re-established in 1 min and held for 10 min. The flow-rate was 1.0 ml/min. The analytical system is shown in Fig. 1. The post-column flow manifold consisted of a T-piece in which the separated drugs are mixed with the sodium hypochlorite solution; this stream was then passed through a thermostated reaction coil (0.8 m×0.5 mm I.D.) at 25°C. The oxidized effluent was mixed with the fluorogenic reagent in another T-piece. This reagent was obtained by merging the 0.02 *M* solution of OPA in 0.5 *M* borate buffer (pH 10.5) with a stream of 0.1 *M* ME in 0.5 *M* borate buffer. The resulting solution flowed through a second reactor coil (2.5 m×0.5 mm I.D.) thermostated at 25°C and then passed into the flow cell for fluorescence recording. All carrier streams were pumped at the same flow-rate by means of a peristaltic pump with a total flow-rate of 1.0 ml/min.

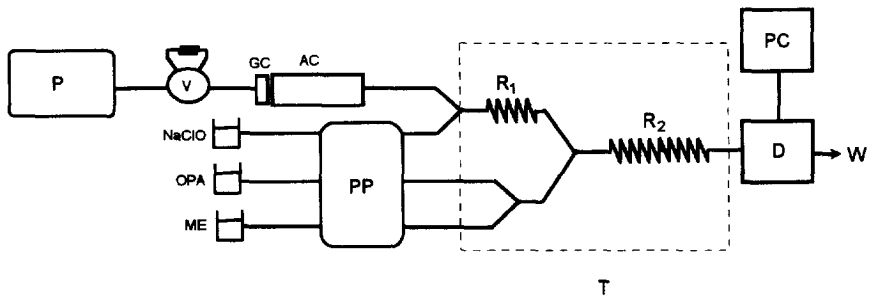

Fig. 1. Manifold for the liquid chromatographic analysis of ranitidine and its metabolites with post-column fluorescence derivatization. (P) HPLC pump, flow-rate, 1 ml/min; (V) injection valve, sample-loop, 100 μl; (GC) guard column; (AC) analytical column; (PP) peristaltic pump, total flow-rate, 1 ml/min; (R_1) reactor coil, 0.8 m long and 0.5 mm I.D.; (R_2) reactor coil, 2.5 m long and 0.5 mm I.D.; (T) thermostat at 25°C; (D) fluorimeter 350/450 nm (excitation/emission); (PC) personal computer; (W) waste.

2.4. Sample preparation

Human plasma (500 μl) was made alkaline with 100 μl of 0.1 *M* NaOH. Methylene chloride (3 ml) was added. The tube was shaken for 10 min and then centrifuged for 10 min at 2000 *g*. The aqueous upper layer was recovered and re-extracted with a second 3 ml volume of methylene chloride. After a similar treatment, the aqueous layer was discarded. The organic phases were combined and evaporated to dryness under argon. The residue was dissolved in 500 μl of mobile phase and analyzed by HPLC. Human urine samples (1 ml) were diluted up to 25 ml with water to decrease the ranitidine concentration within the range of the calibration graph. An aliquot was filtered through a 0.2-μm nylon Millipore chromatographic filter and analyzed. After each day, the column was washed with acetonitrile and then water before re-equilibration with the mobile phase.

3. Results and discussion

3.1. Selection of the flow-rate

The experimental conditions for the fluorescence reaction have been studied previously [15] using FIA and were again checked by replacing the buffer carrier by the effluent from the analytical column. The optimized diagram for the post-column derivatization is shown in Fig. 1. The flow-rates of the chromatographic mobile phase and the post-column derivatization reagents strongly affected the sensitivity of the reaction. Thus, the chromatographic mobile phase flow-rate was varied between 0.5 and 2.0 ml/min, while the total flow-rate delivered by the peristaltic pump was kept constant at 1 ml/min. As expected, retention times decreased for higher flow-rates, particularly in the case of ranitidine, which was more retained in the column than the metabolites. When the mobile phase flow-rate decreased, both the peak area and the peak width obtained were considerably increased. Therefore, a value of 1 ml/min for the mobile phase flow-rate was selected as a compromise between optimal separation of the peaks, sensitivity, adequate peak shape and sampling frequency.

3.2. Optimization of the mobile phase

Acetonitrile–water mobile phases were inadequate because ranitidine and its metabolites were not eluted from the column. Fig. 2A shows the variation in the capacity factors (k') of the drugs when the percentage of acetonitrile was varied from 5 to 20% using 7.5 m*M* phosphate buffer (pH 6) as the aqueous modifier. Ranitidine was slightly retained by the stationary phase, while the metabolites were eluted at the void volume for high percentages of acetonitrile (>20%). With a mixture containing 7% acetonitrile, the metabolites gave adequate k' values but ranitidine was strongly retained in the column. For this reason, a percentage of 10% acetonitrile was

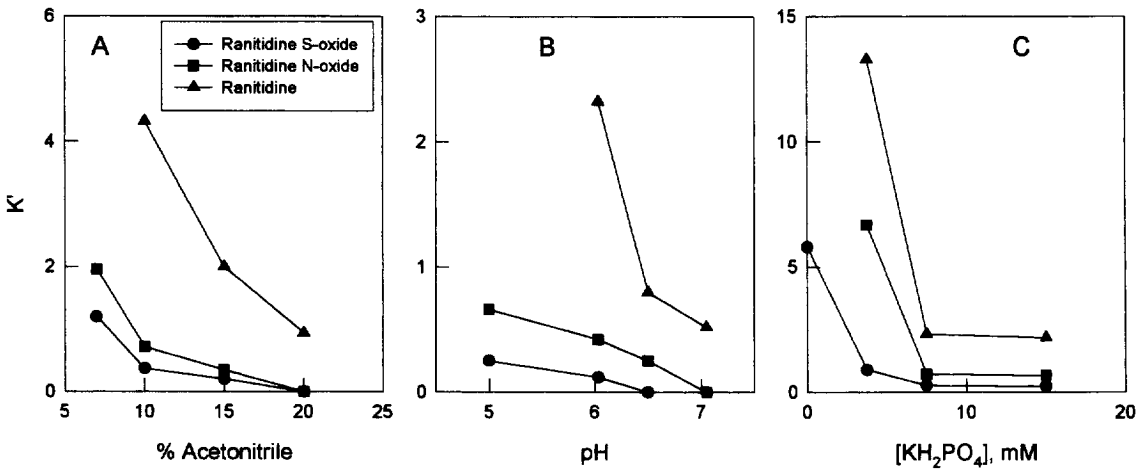

Fig. 2. Influence of the percentage of acetonitrile on the capacity factors of ranitidine and its metabolites when using different acetonitrile–7.5 m*M* phosphate buffer (pH 6) mixtures as mobile phases (A). Effect of the pH (B) and the phosphate concentration (C) when using acetonitrile–phosphate buffers (10:90, v/v) as mobile phases.

selected. The pH of the buffer was varied between 5 and 7 (Fig. 2B). At pH 5, ranitidine was markedly retained and no elution was detected after 20 min. When the pH was higher, the retention time of ranitidine rapidly decreased, while the metabolites eluted near the void time. Consequently, a pH of 6 was selected. Fig. 2C shows the effect of varying the concentration of phosphate in the 0–15 m*M* range. Neither ranitidine nor ranitidine N-oxide were eluted in the absence of phosphate, the capacity factors for all species decreasing when the phosphate concentration was higher. A 7.5-m*M* concentration was chosen as optimal. Once these experimental variables were studied, the percentage of acetonitrile in the mobile phase was reconsidered and, finally, an 8% value was selected as being adequate.

In summary, isocratic elution of ranitidine and its metabolites would be possible using a mobile phase of 8:92 (v/v) acetonitrile–7.5 m*M* phosphate buffer (pH 6). The elution order was ranitidine S-oxide (5.0

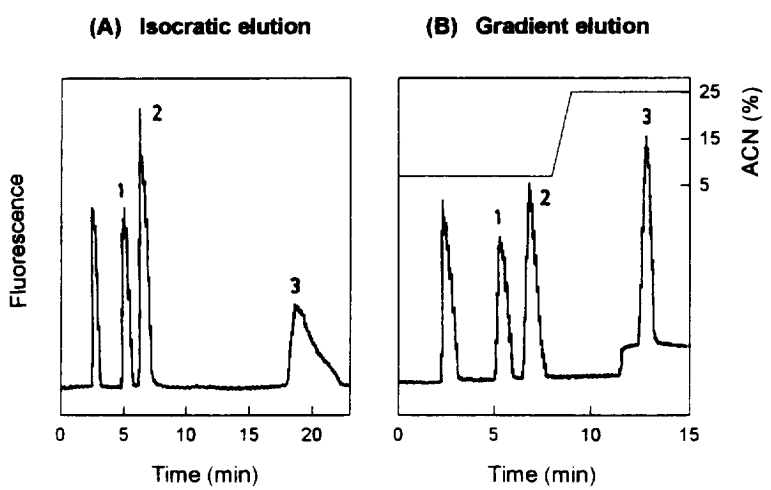

Fig. 3. Chromatographic profiles using isocratic elution (A) and gradient elution (B). In both cases, the peaks corresponded to: 1, ranitidine S-oxide (0.65 μg/ml); 2, ranitidine N-oxide (1.0 μg/ml) and 3, ranitidine (1.0 μg/ml). The first peak corresponded to the void time.

Table 1
Calibration parameters

	Ranitidine S-oxide	Ranitidine N-oxide	Ranitidine
Slope (ml/μg)	84.92	69.4 6	78.09
Intercept	−6.26	−4.98	0.24
Correlation coefficient	0.9998	0.9995	0.9999
Detection limit (ng/ml)	30	41	32
Quantitation limit (ng/ml)	98	138	106
Precision within-run (R.S.D.) (0.1; 0.5; 1.0 μg/ml levels)	6.2; 4.7; 3.1	6.5; 4.4; 3.4	7.2; 5.0; 4.1
Precision between-run (R.S.D.) (0.1; 0.5; 1.0 μg/ml levels)	8.8; 6.3; 4.4	8.3; 6.4; 4.8	8.9; 7.6; 5.2

min), ranitidine N-oxide (6.8 min) and ranitidine (19.3 min). Although the metabolites were eluted with this mobile phase, ranitidine eluted with a high capacity factor, giving a tailed peak (Fig. 3A) and so another elution programme was tried. This consisted of an initial isocratic step with a 7:93 (v/v)

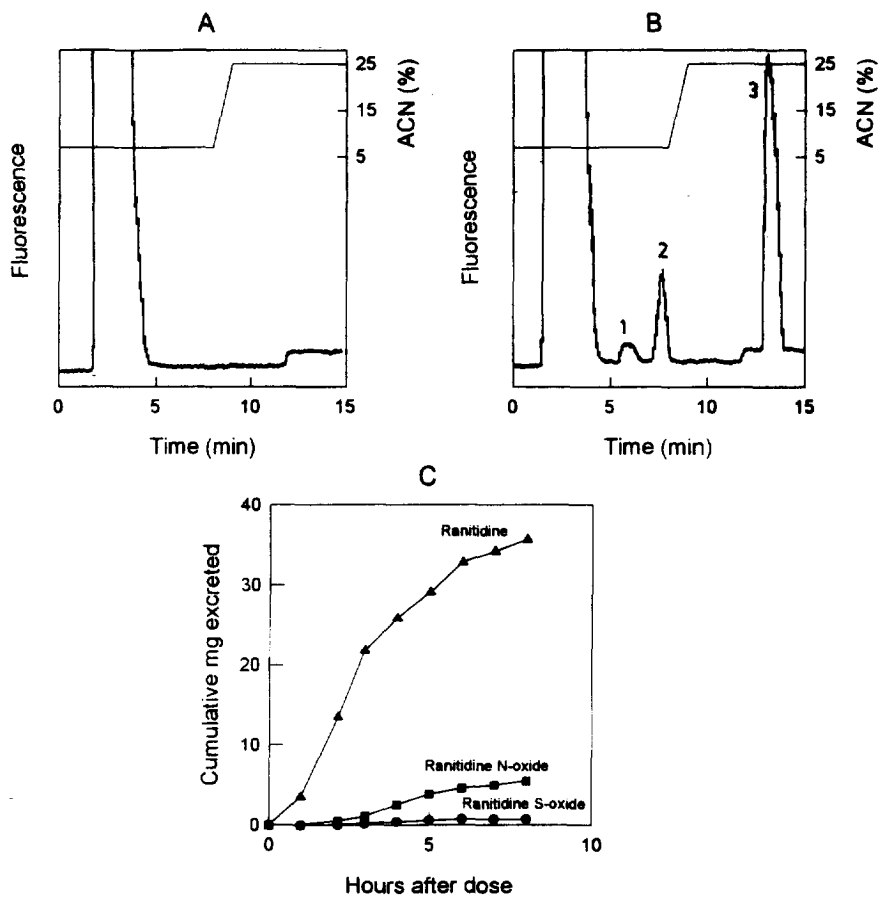

Fig. 4. Chromatographic profiles corresponding to (A) a blank urine sample and (B) a urine sample containing ranitidine S-oxide (1, estimated concentration of 0.6 μg/ml), ranitidine N-oxide (2, 1.2 μg/ml) and ranitidine (3, 2.8 μg/ml). (C) Plot of the cumulative excretion of ranitidine and its metabolites in urine after an oral dose of 150 mg of ranitidine.

acetonitrile–7.5 m*M* phosphate buffer (pH 6) mixture for 8 min followed by a linear gradient from 7:93 (v/v) to 25:75 (v/v) over 1 min, this mixture being held for 6 min. Finally, the initial conditions were re-established in 1 min and held for 10 min. The chromatographic profile obtained using this programme is shown in Fig. 3B; the total analysis time was lower and the ranitidine peak shape was considerably improved.

3.3. Calibration and precision

Calibration graphs were performed by plotting concentration against peak area and were linear over the range 0.1–4 μg/ml (10–400 ng) for all the drugs tested. Table 1 shows the equations obtained for the calibration graphs and the regression coefficients. The detection limits were calculated on the basis of 3σ and the quantitation limits on the basis of 10σ, according to Miller and Miller [17]. The precision of the method was demonstrated by repetitive analyses, calculating the average relative standard deviation (R.S.D.) for ten replicate determinations at different concentrations (0.1, 0.5 and 2.0 μg/ml) of each drug. Table 1 also shows the values obtained for the precision within-run, with the experiments carried out in rapid succession, and for the precision between-runs, with the experiments carried out on different occasions [17].

3.4. Analysis of physiological samples and recovery study

Fig. 4 shows the chromatograms corresponding to a blank urine sample (A) and a urine sample containing ranitidine and its metabolites (B) using the gradient elution procedure. The method was selective and there was no interference from matrix compounds because no overlapping between peaks appeared in the samples from three individuals. After the oral administration of a 150-mg dose of ranitidine to a volunteer, a pharmacokinetic study was carried out. Serial samples of urine were collected from 0 to 8 h after administration of the drug and these were analyzed using the proposed method. Fig. 4C shows a plot of the cumulative urinary excretion of ranitidine and its metabolites. The percentages of the oral dose excreted during the first 8 h after administration of ranitidine were 23.8, 3.7 and 0.5% for ranitidine, ranitidine N-oxide and ranitidine S-oxide, respectively. These values are in good agreement with previous studies [4,16] and demonstrate the applicability of the method.

A sample of human plasma was also analyzed and Fig. 5 shows typical chromatograms of extracts from drug-free (A) and from spiked (B) plasma. Again, no interfering peaks with either ranitidine or its metabolites were observed in the plasma samples investigated. The absolute recoveries were evaluated by comparing the concentrations found in plasma sam-

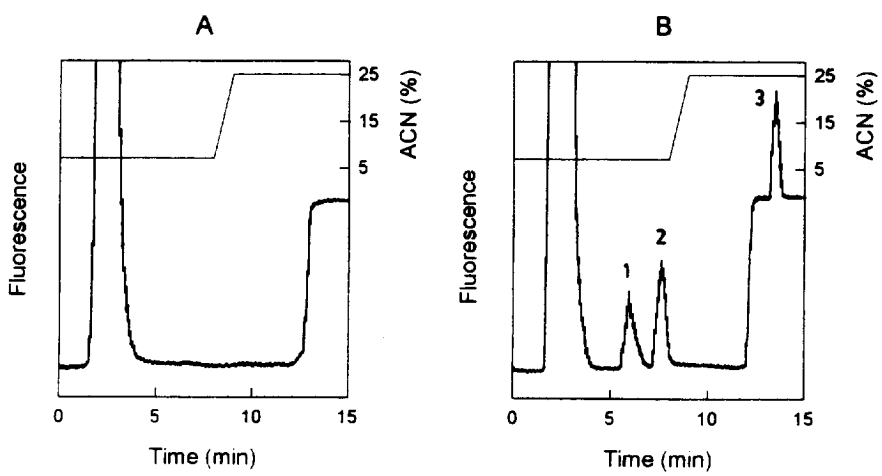

Fig. 5. Chromatographic profiles corresponding to (A) a blank plasma sample and (B) a plasma sample spiked with 100 ng/ml each of ranitidine S-oxide (1), ranitidine N-oxide (2) and ranitidine (3).

ples spiked with known amounts of each drug (100 and 200 ng/ml) and submitted to the extraction procedure with the concentrations obtained by direct injection of standard solutions at each concentration level. When all spike and recovery data were combined, an average recovery±R.S.D. (n=6) of 97.9±6.4% was obtained. This average value indicates that recovery was essentially quantitative.

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References

[1] G.A. Goodman, L.S. Goodman, T.W. Rall and F. Murad (Editors), The Pharmacological Basis of Therapeutics, MacMillan, New York, 7th ed., 1985.
[2] P.F. Carey and L.E. Martin, J. Liq. Chromatogr., 2 (1979) 1291.
[3] H.M. Vandenberghe, S.M. MacLeod, W.A. Mahon, P.A. Lebert and S.J. Soldin, Ther. Drug Monit., 2 (1980) 379.
[4] P.F. Carey, L.E. Martin and P.E. Owen, J. Chromatogr., 225 (1981) 161.
[5] P.F. Carey, L.E. Martin and M.B. Evans, Chromatographia, 19 (1984) 200.
[6] T. Prueksaritanont, N. Sittichai, S. Prueksaritanont and R. Vongsaroj, J. Chromatogr., 82 (1989) 175.
[7] C.A. Lau-Cam, M. Rahman and R.W. Roos, J. Liq. Chromatogr., 17 (1994) 1089.
[8] M.S. Smith, J. Oxford and M.B. Evans, J. Chromatogr. A, 683 (1994) 402.
[9] G.L. Hoyer, J. LeDoux and P.E. Nolan, Jr., J. Liq. Chromatogr., 18 (1995) 1239.
[10] A. Himuro, H. Nakamura and Z. Tamura, J. Chromatogr., 264 (1983) 423.
[11] A. Himuro, H. Nakamura and Z. Tamura, Anal. Chim. Acta, 147 (1983) 317.
[12] N. Ichinose, G. Schwedt, F.M. Schnepel and K. Adachi, Fluorometric Analysis in Biomedical Chemistry, Wiley, New York, 1991.
[13] M. Roth, Anal. Chem., 43 (1971) 880.
[14] S. Allenmark, S. Bergström and L. Enerbäck, Anal. Biochem., 144 (1985) 98.
[15] C. López-Erroz, P. Viñas, N. Campillo and M. Hernández-Córdoba, Analyst, 121 (1996) 1043.
[16] G. Mullersman and H. Derendorf, J. Chromatogr., 381 (1986) 385.
[17] J.C. Miller and J.N. Miller, Statistics for Analytical Chemistry, Ellis Horwood, Chichester, 3rd ed., 1993.