Journal of Chromatography B, 878 (2010) 492–496

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Performance of tiloronoxim and tilorone determination in human blood by HPLC–MS/MS: Method validation, uncertainty assessment and its application to a pharmacokinetic study

Xianhua Zhang[a,b], Jingli Duan[a], Suodi Zhai[a,b], Yiheng Yang[a], Li Yang[a,*]

[a] Department of Pharmacy, Peking University Third Hospital, 49 North Garden Rd., Beijing 100191, PR China
[b] Center for Therapeutic Drugs Monitoring and Clinical Toxicology, Peking University, Beijing 100191, PR China

ARTICLE INFO

Article history:
Received 21 October 2009
Accepted 10 December 2009
Available online 21 December 2009

Keywords:
Tiloronoxim
Tilorone
HPLC–MS/MS
Blood
Total error
Measurement uncertainty
Pharmacokinetics

ABSTRACT

A highly sensitive and selective HPLC–MS/MS method is presented for the quantitative determination of tiloronoxim and its metabolite tilorone in human blood. An aliquot of 200 μl human blood was extracted with a mixture of chloroform/ethyl ether (1/2, v/v), using metoprolol as the internal standard (the IS). Separation was achieved on an Xterra MS C18 column (50 mm × 2.1 mm, 5 μm) with a gradient mobile phase of methanol/water containing 15 mM ammonium bicarbonate (pH 10.5). Detection was performed using positive MRM mode on a TurboIonSpray source. The mass transitions monitored were m/z 426.3 → 100.0, m/z 411.3 → 100.0 and m/z 268.3 → 116.1 for tiloronoxim, tilorone and the IS, respectively. The method was fully validated using total error theory, which is based on β-expectation tolerance intervals and include trueness and intermediate precision. The method was found to be accurate over a concentration range of 1–100 ng/ml for both compounds. The measurement uncertainty based on β-expectation tolerance intervals was assessed at each concentration level of the validation standards. This method was successively applied to a pharmacokinetic study of tiloronoxim in healthy volunteers.

1. Introduction

Tiloronoxim dihydrochloride, a newly synthesized tilorone analog, is a small molecular interferon inducer [1]. Its bis-basic tricyclic structure (see Fig. S1) exhibits antiviral, anti-tumor activity based on its immunomodulating properties. It is rapidly and widely distributed in the body and is excreted in urine. Pre-clinic study has demonstrated the efficacy of tiloronoxim with low chronic toxicity [2]. It is metabolized to several metabolites in human blood and among these metabolites, tilorone is a pharmacologically active one [3] (Fig. S1). Both tiloronoxim and tilorone can stimulate Ab production of the IgM, IgG, and IgE classes and modulates the humeral and cell mediated immune responses. They also suppress a wide variety of immune responses and inhibit paralysis [1]. The concentration level of tiloronoxim and its metabolite in blood is very low after oral administration, which challenges the development of analytical methods.

A few papers have been published for the determination of tiloronoxim or tilorone in biofluids. Determination of tiloronoxim and tilorone in human urine using HPLC–MS/MS method has been reported and the lower quantification limit was 1 ng/ml [4]. But this method has not been applied to plasma or blood samples. For blood or plasma samples, only a few publications have been reported using HPLC/UV methods [5–7]. Among these methods, the reported lowest quantification limit in blood was 40 ng/ml and this is not likely to provide sufficient sensitivity for the measurement of tiloronoxim and tilorone at low levels. In the pharmacokinetic study of tiloronoxim, the expected C_{max} was only about 20 ng/ml with a dose of 50 mg, which requires a lower limit of quantification of no more than 2 ng/ml. Furthermore, lack of specificity in biological fluids and long analysis time with HPLC/UV methods do not meet the high throughout needs of pharmacokinetic studies, which requires a rapid feedback of analytical information. Therefore, the development of more sensitive and specific methods is of great importance.

The present paper describes a sensitive, specific and rapid HPLC–MS/MS method for the simultaneous determination of tiloronoxim and tilorone in an aliquot of 0.2 ml human blood using metoprolol as the IS. This method was fully validated to the criteria of FDA [8].

In the present days, measurement uncertainty has gradually become an important parameter of analytical performance besides the validation criteria [9,10]. For that purpose, an original strategy proposed by the Societe Francaise des Sciences et Techniques Pharmaceutiques (SFSTP) was applied which was based on accuracy profiles as a decision tool [11–17]. Using this validation strategy,

* Corresponding author. Tel.: +86 10 82266673; fax: +86 10 82266673.
E-mail address: yangli432@yahoo.com.cn (L. Yang).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.12.016

it was possible to assess the uncertainty of measurements without any further experiments. In this paper, we assessed the measurement uncertainty of the developed method using total error theory. And this method was also applied to a pharmacokinetic study of tiloronoxim.

2. Experimental

2.1. Chemicals

Tiloronoxim dichloride and tilorone dichloride were provided by Chinese Center for Disease Control and Prevention (Beijing, China). Metoprolol fumarate was purchased from National Institute for the Control of Biological Products (Beijing, China).

HPLC grade methanol (Fisher Chemical, USA) and ammonium bicarbonate (Sigma) were used in the experiments. NaOH (Beijing Chemical Reagents Company, China), chloroform (Beijing Chemical Reagents Company, China) and ethyl ether (Tianjin Chemical Reagents Factory, China) were of analytical grade. Ultrapure water was prepared with a Milli-Q purification system (Bedford, USA).

Drug free blood was kindly provided by Clinical Laboratory of Peking University Third Hospital.

2.2. Apparatus

An Agilent 1100 HPLC system (Agilent Technology, Boeblingen, Baden-Wuerttemberg, Germany) coupled to an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) was employed, run by Analyst software (version 1.4, Applied Biosystems, Concord, Ontario, Canada).

The validation results were computed with Microsoft Excel 2003.

2.3. Preparation of stock solutions, calibration standards and validation standards

An appropriate amount of tiloronoxim, tilorone and metoprolol (the IS) were dissolved in methanol to prepare stock solutions. Working solutions were prepared from the stock solutions with methanol. All the stock solutions and working solutions were stored at 4 °C. Details about the preparation of calibration standards and validation standards are shown in supplementary note 1 (Supplementary Information, SI).

2.4. Sample extraction

Frozen blood samples were extracted with a mixture of chloroform and ethyl ether (1/2, v/v), details about extraction procedures are described in supplementary note 2 in SI.

2.5. HPLC–MS/MS conditions

The separation was carried out on an Xterra MS C18 column (50 mm × 2.1 mm, 5 μm, Waters, Santry, Dublin, Ireland). The gradient mobile phase consisted of ammonium bicarbonate (15 mM, pH 10.5, A) and methanol (B). More details about chromatographic conditions are presented in supplementary note 3 in SI. The mass spectrometer was operated on positive MRM mode with a TurboIonSpray source, details about MS/MS conditions are given in supplementary note 4 in SI.

2.6. Validation procedures

The method was validated for selectivity, linearity, recovery, trueness, precision and accuracy. The lower limit of quantification (LLOQ), matrix effect and stability of the analytes in blood were also tested. More details about validation standards are described in supplementary note 5 in SI.

2.7. Pharmacokinetic study

The developed method was applied to a human pharmacokinetic study of tiloronoxim. Experimental details are depicted in supplementary note 6 in SI.

3. Results and discussion

3.1. Method development

Both tiloronoxim and tilorone are strong polar compounds. In preliminary experiments, methanol and acetonitrile were used to precipitate proteins, but the chromatograms were rather poor due to serious matrix effect and high noise. Cost of solid phase extraction (SPE) method with cartridges was too high and was not considered. To obtain "clean" samples, liquid–liquid extraction method was used. Different kinds of organic solvents were tried, such as ethyl acetate, methyl tert-butyl ether and ethyl ether. But the recoveries were rather low (<20%) with these solvents even after basification due to the strong polarity of the analytes. Higher recovery (about 60–65%) was obtained when using chloroform as solvent. However, the operation was not convenient because the organic layer was at the bottom layer. Taking all these factors into account, a mixture of chloroform and ethyl ether (1/2, v/v) was employed as the extraction solvent. The liquid–liquid extraction procedures were performed two times to improve the recovery.

Different types of columns, such as Zorbax XDB C18, Restek Ultra C18, Sunfire C18 and Xterra MS C18 were tried, using mobile phases at low pH. But the retention was weak and the matrix effect was serious. Therefore, a mobile phase with high pH was employed. Only Xterra MS C18 can endure mobile phases at high pH and hence it was used in the following experiments. With a gradient mobile phase containing methanol/ammonium bicarbonate (15 mM, pH 10.5), sharp peaks and strong retentions were obtained with little matrix effect. Under these conditions, the retention times of tiloronoxim, tilorone and the IS were typically 6.3, 6.5 and 4.4 min (see Fig. 1), respectively. The total analysis time was 8 min for each run.

The compounds were detected with MRM mode and the MS/MS parameters were optimized. The precursor ions were m/z = 426.3 (tiloronoxim) and m/z = 411.3 (tilorone). The MS/MS spectra are showed in Fig. S2. The quantification transitions were m/z 426.3 → 100.0 for tiloronoxim, m/z 411.3 → 100.0 for tilorone and m/z 268.3 → 116.1 for the IS.

3.2. Method validation

3.2.1. Selectivity

No endogenous inferences were observed in extracts from drug free human blood. The miniatures in Fig. 1 show the representative chromatograms of a drug free blood sample, demonstrating that the developed LC–MS/MS method is highly selective.

3.2.2. Matrix effect and extraction recovery

Post-column infusing method was used to check the matrix effect and we adjusted the retention of the analytes and the IS away from the suppression regions to minimize the matrix effect. Further experiments were performed to evaluate the matrix effect and extraction recovery. The results are shown in Table S1. As can be seen, the absolute matrix effect ranged from 88.19% to 100.60% for tiloronoxim and 98.11% to 106.90% for tilorone under current conditions. The relative matrix effect was less than 10.57% for both analytes (Table S1). It indicated that significant matrix

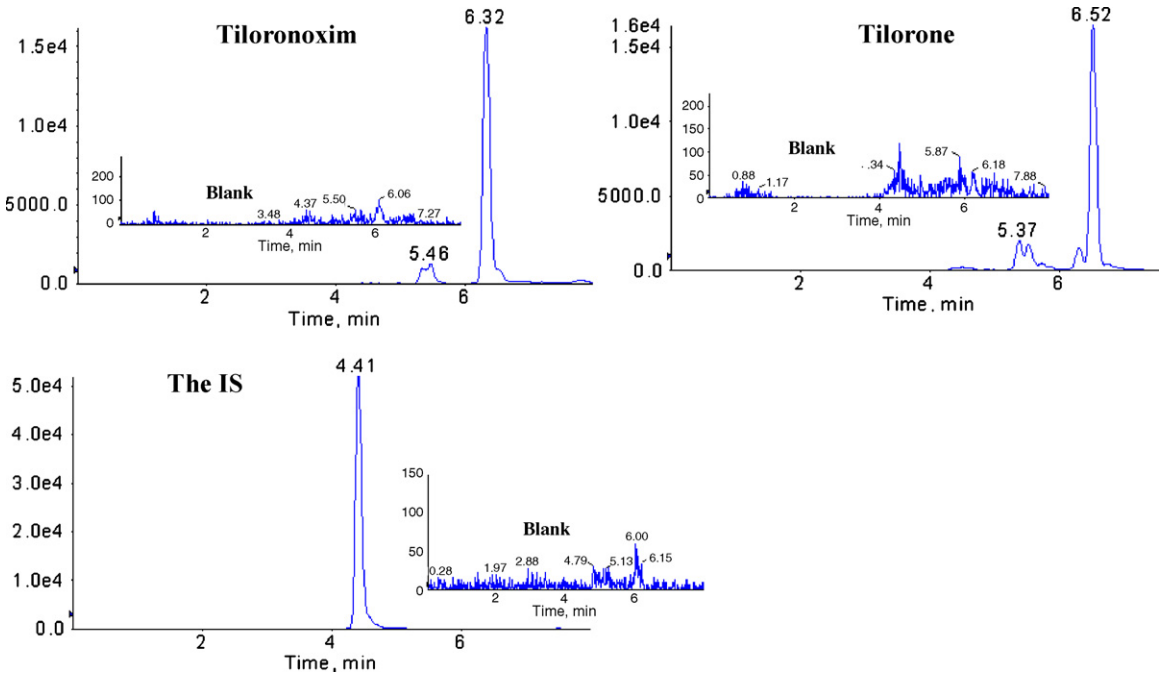

Fig. 1. Representative chromatograms of a spiked blood sample and a drug free blood sample (tiloronoxim conc.: 50 ng/ml; tilorone conc.: 50 ng/ml).

effect was absent. The recoveries were calculated from the response ratio of the validation standards to drug free samples spiked post-extraction at the same concentration. The mean recoveries for tiloronoxim and tilorone were 52.80% and 55.20%, respectively.

3.2.3. *Response functions*

To determine the most suitable regression mode for the method, different calibration models have been evaluated. Seven calibration standards were prepared and analyzed in duplicates on three days. Independent validation standards were analyzed at three levels in five replicates on three days.

From the data obtained, the concentrations of the validation standards were back-calculated to determine the mean relative bias, the standard deviation for intermediate precision and finally the upper and lower β-expectation tolerance limits at 95% level.

Different accuracy profiles were plotted and the weighted ($1/X$) linear regression was found to be the most suitable one. The acceptance limits were set at ±15% at mid- and high-validation levels and ±20% at low-validation levels. The accuracy profiles are shown in Fig. S3. The response functions obtained by applying these regression models are presented in Table 1.

Table 1
Method validation results of tiloronoxim and tilorone in human blood.

Response function		Tiloronoxim	Tilorone
Calibration model		Calibration range (7 levels) Weighted $1/X$ linear regression	Calibration range (7 levels) Weighted $1/X$ linear regression
Trueness	Spiked conc. (ng/ml)	Relative bias (%)	Relative bias (%)
	1	6.67	0.53
	2	−2.53	3.60
	10	5.90	1.96
	50	1.75	3.60
Precision	Spiked conc. (ng/ml)	Repeatability/intermediate precision (RSD%)	Repeatability/intermediate precision (RSD%)
	1	3.76/8.77	8.18/13.38
	2	4.56/11.65	5.41/9.93
	10	4.52/7.97	5.56/7.79
	50	4.97/10.09	7.39/7.45
Accuracy	Spiked conc. (ng/ml)	Lower/upper β-expectation limits (ng/ml)	Lower/upper β-expectation limits (ng/ml)
	1	0.96/1.18	0.86/1.16
	2	1.94/2.46	1.84/2.30
	10	9.94/11.82	9.33/11.07
	50	47.62/59.14	47.77/55.83
	Spiked conc. (ng/ml)	β-Expectation lower and upper tolerance limits of the relative error (%)	β-Expectation lower and upper tolerance limits of the relative error (%)
		−3.24/16.58	−14.43/15.49
	1	−14.37/9.31	−7.73/14.93
	2	−2.71/14.51	−6.52/10.44
	10	−9.05/12.55	−4.18/11.38
	50	1–100	1–100
Linearity	Range (ng/ml)	0.0308	0.0303
	Slope	0.00113	−0.00892
	Intercept	0.9953	0.9990
LLOQ (ng/ml)	r	1	1

Table 2
Estimates of measurement uncertainties using the selected regression models.

Spiked conc. (ng/ml)	Uncertainty of bias (ng/ml)	Uncertainty (ng/ml)	Expanded uncertainty $k=2$ (ng/ml)	Relative uncertainty (%)
Tiloronoxim				
2	0.12	0.26	0.52	13.36
10	0.41	0.94	1.87	8.84
50	2.63	5.76	11.53	11.33
Tilorone				
2	0.10	0.23	0.47	11.33
10	0.35	0.87	1.73	8.48
50	1.16	4.03	8.06	7.78

Table 3
Pharmacokinetics parameters of three groups of healthy volunteers after single doses of tiloronoxim ($n=12$).

Parameter	50 mg	100 mg	150 mg	Relative coefficient (r)
T_{max} (h)	1.9 ± 1.0	1.8 ± 0.9	2.3 ± 0.8	
C_{max} (ng/ml)	23.4 ± 11.1	57.4 ± 22.4	90.2 ± 26.5	0.9892
AUC_{0-t} (ng/ml/h)	327.0 ± 125.7	718.5 ± 256.0	1056.0 ± 389.8	0.9991
$AUC_{0-\infty}$ (ng/ml/h)	392.5 ± 150.5	822.5 ± 304.5	1195.6 ± 452.8	0.9991
$t_{1/2(ke)}$ (h)	9.7 ± 1.4	9.1 ± 1.2	9.4 ± 1.2	
K_e (1/h)	0.077 ± 0.009	0.073 ± 0.009	0.074 ± 0.008	

3.2.4. Trueness

Trueness in terms of relative bias (%) was assessed from the validation standards of tiloronoxim and tilorone at three concentration levels, as can be seen from Table 1. The results were acceptable according to the FDA criteria.

3.2.5. Precision

The precision of the method was determined by computing the relative standard deviation for repeatability and intermediate precision at each validation levels for both compounds. The results are presented in Table 1. As can be seen, the repeatability and intermediate precisions of tiloronoxim ranged over 4.52–4.97% and 7.79–11.65%, respectively. For tilorone, repeatability and intermediate precisions were 5.41–7.41% and 7.45–9.93%, respectively.

3.2.6. Accuracy and LOQ

Accuracy refers to the closeness of agreement between the test results and the nominal values [17]. It takes into account the total error, i.e. the sum of systematic and random errors, related to the test results. As presented in Table 1, the upper and lower relative β-expectation tolerance limits of the mean bias (%) did not exceeded the acceptance limit at each validation level. Therefore, the method was accurate over the concentration range investigated. The lower limits of quantification (LLOQ) were 1 ng/ml for both compounds.

3.2.7. Linearity of the results

To evaluate the linearity of the method, a linear regression curve was fitted on the back-calculated concentrations of the calibration standards. The equations obtained and the coefficients of the determination were shown in Table 1. As shown in Table 1, the regression fitted well with coefficients >0.99 for tiloronoxim and tilorone. The linearity was demonstrated using β-expectation tolerance interval approach (Fig. S4). The results showed that the upper and lower β-expectation tolerance limits were included inside the acceptance limits over the whole range of linear levels for both compounds.

3.2.8. Stability

The results of stability evaluation in human blood are summarized in Table S2. The mean recoveries of tiloronoxim ranged from 104.13% to 108.67% after 3 freeze–thaw cycles. For tilorone, mean recoveries ranged between 101.23% and 115.00% after 3 freeze–thaw cycles. The results demonstrate that tiloronoxim and tilorone were stable in blood after enduring three freeze–thaw cycles. And there is no degradation for both compounds in human blood after storage at room temperature for up to 12 h. The reconstitute samples can endure 12 h in auto sampler at room temperature for both compounds.

3.2.9. Uncertainty assessment

As a parameter of performance, the uncertainty characterizes the dispersion of the measurand. The measurement uncertainty was evaluated at each validation level according to the demonstration of Feinberg et al. [18] and the results are summarized in Table 2. The expanded uncertainty was computed using a coverage factor (k) of 2, representing a 95% confidence level. As shown in Table 2, the relative uncertainty of each compound at all validation levels did not exceed 15%, which demonstrates that with a confidence level of 95%, the unknown true value located at maximum ±15% around the measured result.

3.3. Pharmacokinetics

The developed HPLC–MS/MS method was applied to a pharmacokinetic study of tiloronoxim after single doses of tiloronoxim tablets. Fig. 2 shows the blood concentration–time profiles of three doses.

The pharmacokinetic parameters were calculated with 3P97 software. The data fitted well by two-compartment model with a weighting factor of $1/c^2$. Main pharmacokinetic parameters are shown in Table 3.

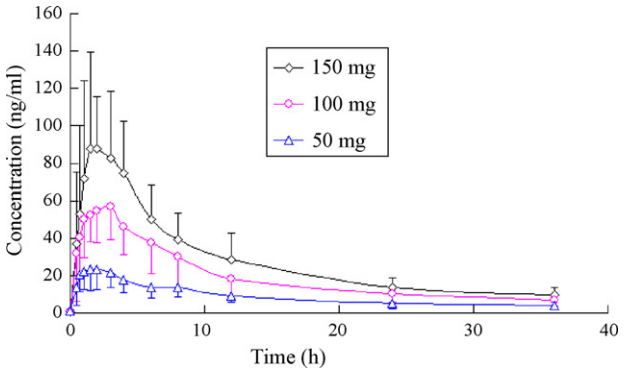

Fig. 2. Blood concentration–time profiles of different doses of tiloronoxim ($n=12$).

4. Conclusions

The developed HPLC–MS/MS method for the simultaneous quantification of tiloronoxim and tilorone in human blood was rapid, selective and highly sensitive with a LLOQ of 1 ng/ml for both compounds. Only 0.2 ml of blood was needed, which greatly facilitated the collection of samples. The method covered a broad concentration range of 1–100 ng/ml for both compounds. The validation approach using accuracy profiles based on β-expectation tolerance intervals for the total measurement error allowed evaluating the capacity of the method to give fit for the purpose results. The measurement uncertainty was estimated without any additional experiments. The uncertainty results demonstrate that with a confidence level of 95%, the true value of measurand located at maximum ±15% around the measured result. The validated method was successively applied to a pharmacokinetic study of tiloronoxim.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.12.016.

References

[1] K.B. Dasari, T. Srikrishnan, J. Chem. Crystallogr. 30 (2000) 269.
[2] Y.H. Cheng, J.Y. Zhang, L.H. Qin, S.J. Zhang, S.L. Han, Q.C. Du, R.H. Li, P.P. Yao, X.Y. Qin, J. Hyg. Res. 19 (1990) 1.
[3] Y.H. Cheng, L.H. Qin, X. Xu, J.Y. Zhang, R.H. Huang, J. Hyg. Res. 21 (1992) 1.
[4] X. Zhang, L. Yang, S. Zhai, J. Duan, J. Chromatogr. B 875 (2008) 349.
[5] J.Y. Zhang, Y.H. Cheng, J. Hyg. Res. 24 (1995) 195.
[6] L. Yang, S.D. Zhai, X. Zhao, H. Wu, Chin. J. New Drugs 15 (2006) 891.
[7] J.M. Wu, H.Y. Li, Chin. J. Pharm. Anal. 23 (2003) 477.
[8] FDA Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), 2001, http://www.fda.gov/cder/guidance/index.htm.
[9] M. Burns, Trac.-Trends Anal. Chem. 23 (2004) 393.
[10] R.D. Marini, A.C. Servais, E. Rozet, P. Chiap, B. Boulanger, S. Rudaz, J. Crommen, Ph. Hubert, M. Fillet, J. Chromatogr. A 1120 (2006) 102.
[11] Ph. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.-A. Compagnon, W. Dewe, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, STP Pharma Pratiques 13 (2003) 101.
[12] Ph. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.-A. Compagnon, W. Dewe, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, J. Pharm. Biomed. Anal. 36 (2004) 579.
[13] E. Rozet, R. Morello, F. Lecomte, G.B. Martin, P. Chiap, J. Crommenc, K.S. Boosb, Ph. Hubert, J. Chromatogr. B 844 (2006) 251.
[14] M. Feinberg, M. Laurentie, Accredit. Qual. Assur. 11 (2006) 3.
[15] E. Cavalier, E. Rozet, N. Dubois, C. Charlier, Ph. Hubert, J.P. Chapelle, J.M. Krzesinski, P. Delanaye, Clin. Chim. Acta 396 (2008) 80.
[16] Ph. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, N. Cohen, P.-A. Compagnon, W. Dewe, M. Feinberg, M. Laurentie, N. Mercier, G. Muzard, L. Valat, E. Rozet, J. Pharm. Biomed. Anal. 48 (2008) 760.
[17] R.D. Marini, P. Chiap, B. Boulanger, S. Rudaz, E. Rozet, J. Crommen, Ph. Hubert, Talanta 68 (2006) 1166.
[18] M. Feinberg, B. Boulanger, W. Dewe, Ph. Hubert, Anal. Bioanal. Chem. 380 (2004) 502.