Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 852 (2007) 674–678
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Relationship between MPA free fraction and free MPAG concentrations in heart transplant recipients based on simultaneous HPLC quantification of the target compounds in human plasma

Xavier Cussonneau [a], Magali Bolon-Larger [a,b], Céline Prunet-Spano [a], Olivier Bastien [c], Roselyne Boulieu [a,b,*]

[a] *Université de Lyon, Université Lyon1, ISPB, Département de Pharmacie Clinique, de Pharmacocinétique et d'Evaluation du Médicament, INSERM ERI 22, Lyon F-69373, France*
[b] *Hospices Civils de Lyon, Hôpital Cardio-vasculaire et Pneumologique Louis Pradel, Laboratoire de Pharmacocinétique Clinique, Bron F-69677, Université de Lyon, INSERM ERI 22, Lyon F-69373, France*
[c] *Hospices Civils de Lyon, Hôpital Cardio-vasculaire et Pneumologique Louis Pradel, Pôle Médico-chirurgical de Transplantation Cardiaque Adulte, Bron F-69677, Université de Lyon, INSERM ERI 22, Lyon F-69373, France*

Received 14 December 2006; accepted 16 February 2007
Available online 28 February 2007

Abstract

A simple and sensitive HPLC method for the simultaneous analysis of free MPA and free MPAG was developed. Separation was achieved on a X-Terra RP18 column with acetonitrile–40 mM orthophosphoric acid as eluents using a gradient elution mode over 35 min at a flow rate of 1.5 ml/min. The assay was linear in the range 0.005 mg/L (LOQ) to 5 mg/L for free MPA and 0.05 mg/L (LOQ) to 200 mg/L for free MPAG. Isolation of free MPA and free MPAG was done by ultrafiltration and the ultrafiltrate was directly injected. A positive correlation between MPA free fractions and free MPAG concentrations was found. Likewise, free MPAG was related to total MPAG concentrations in the seven heart transplant patients.

Keywords: Mycophenolic acid; HPLC; Free fraction; Ultrafiltration

1. Introduction

Mycophenolic acid (MPA), the active metabolite of the prodrug mycophenolate mofetil (MMF) is an immunosuppressive agent that inhibits inosine monophosphate dehydrogenase (IMPDH). MPA is metabolised to phenolic glucuronide (MPAG), which is the major metabolite. MPA is highly bound to albumin (97%), and only the free fraction is able to inhibit IMPDH [1]. Reported protein binding is 82% for MPAG [1]. Significant variations in free MPA concentrations have been observed in patients with uremia, hypoalbuminemia, hyperbilirubinemia [2–4]. MPAG was also found to contribute to modification in MPA protein binding by competition for albumin sites [5] and Kuypers et al. [6] have reported a positive correlation between total MPAG concentration and free MPA. Besides, free MPA $AUC_{0–12\,h}$ was identified as a significant risk factor for leukopenia and/or infection in the initial phase after paediatric renal transplantation with a decision level of 0.4 mg h/L, above which there is an enhanced risk [7]. Considering data on MPAG metabolite, free fraction of MPAG was highly variable and seems to be influenced by total MPAG concentration [8] and a greater MPAG $AUC_{0–12\,h}$ was found in patients who developed leukopenia [6]. These observations emphasize the relevance of the simultaneous determination of free MPA and free MPAG.

Several techniques have been described for the isolation of free fraction of MPA in plasma samples. Most of free MPA quantification methods [9–14] were based on the utilisation of ultrafiltration described by Nowak and Shaw [15] because of its rapidity and relative simplicity in comparison to other tech-

* Corresponding author at: Université Lyon1, ISPB, Département de Pharmacie Clinique, de Pharmacocinétique et d'Evaluation du Médicament, 8 avenue Rockefeller, Lyon F-69373, France. Tel.: +33 4 72 35 73 04; fax: +33 4 78 77 72 03.
E-mail address: roselyne.boulieu@sante.univ-lyon1.fr (R. Boulieu).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.02.038

niques like ultracentrifugation [16] or automated sequential trace enrichment of dialysates (ASTED) [8].

Among the chromatographic methods described for the analysis of free MPA [8,10,12–14,17], only two methods have been reported for the simultaneous determination of free MPA and free MPAG in a single run [8,17]. The first one, described by Aresta et al. [17], has a quantification limit (LOQ) of 26 μg/L for MPA and the second one, described by Mandla et al. [8], has a recovery after dialysis estimated to 52%. However, free MPA determination requires more sensitive method to quantify very low concentrations, which could be found in plasma samples due to its high protein binding. LC–MS methods for the determination of only free MPA have been reported with limit of quantification of 2.5, 0.5 and 1.0 μg/L [12,14,18] but such expensive equipment is not available in all clinical laboratories.

The aim of this study was to develop a rapid and sensitive HPLC-UV method for the simultaneous quantification of free MPA and free MPAG using ultrafiltration as sample pretreatment. The method was applied to the determination of free MPA and free MPAG in heart transplant recipients.

2. Materials and methods

2.1. Chemicals and reagents

MPA was generously supplied by Hoffman Laroche (Basel, Switzerland). MPAG was obtained from Analytical Services International Ltd. (Catersham, Great Britain). Acetonitrile Uvasol (spectrometric grade), orthophosphoric acid 85% Suprapur were purchased from Merck (Fontenay sous bois, France). The sodium chloride solution (9 g/L, pH = 7.4) was obtained from Fresenius Kabi (Sèvres, France).

2.2. Sample preparation

Stock solutions of MPA and MPAG at a concentration of 50 and 2000 mg/L respectively, were prepared with an acetonitrile–water mixture (80:20, v/v) and stored at −80 °C in polystyrene tubes (Elvetec, Genas, France). Standards and quality control samples were not prepared in plasma ultrafiltrate due to the very large volumes of ultrafiltrate required, but in isotonic sodium chloride solution, which has previously been validated as a suitable matrix for such samples [10,12–14].

Isolation of free MPA and free MPAG was done by ultrafiltration using a disposable centrifugal filter device Centricon Ultracel YM-10 (Millipore Corporation, Bedford, USA) equipped with a regenerated cellulose membrane (10 kDa MW cut-off). Plasma samples (500 μL) were placed in the reservoir and centrifuged at 6300 × *g* in a Jouan fixed rotor centrifuge (45 min, 20 °C) to achieve about 300 μL of ultrafiltrate. 100 μL of ultrafiltrate was directly injected into the column.

2.3. Chromatographic conditions

The chromatographic system consisted of Hewlett-Packard 1050 series using HPChem software (Agilent Technologies, Massy, France). Separation of MPA and MPAG was achieved using X-Terra RP18 column (150 × 3.9 mm, 5 μm, Waters, Saint Quentin en Yvelines, France) at room temperature (20 °C).

The chromatographic conditions were those previously described [19] with minor modifications. The mobile phase consisted of acetonitrile–40 mmol/L orthophosphoric acid as eluents, at a flow rate of 1.5 mL/min. The gradient conditions were the following: (A) 40 mmol/L H_3PO_4, (B) 40 mmol/L H_3PO_4/CH_3CN, 55/45 (v/v), with the gradient: 0–4 min (53.3% B), 4–17 min (53.3–100% B), 17–20 min (100–53.3% B), 20–35 min (53.3% B). The detection wavelength was set at 215 nm using a photodiode array detector.

2.4. Assay validation

As previously reported [10,12–14], a protein free isotonic sodium chloride solution (pH = 7.4) was used for calibration curve, accuracy and precision. The linearity assay was assessed using an unweighted linear regression method between the limits of quantification and concentrations of 0.05, 0.125, 0.5, 5 mg/L of free MPA and 2, 5, 20, 200 mg/L of free MPAG. The intra-day and inter-day accuracy and precision were determined by assaying five replicate of low (0.05 mg/L) and high (0.5 mg/L) concentration of MPA and five replicate of low (2 mg/L) and high (100 mg/L) concentration of MPAG.

Selectivity was evaluated using plasma samples from healthy subjects and was also assessed by spiking plasma samples with drugs widely used in transplant recipient such as ciclosporine, tacrolimus, ganciclovir, acyclovir, cotrimoxazole, erythromycin, simvastatin, enalapril, amphotericin B and methylprednisolone. All plasma samples were ultrafiltered prior to be analyzed.

Sorption of the compounds on the ultrafiltration system's membrane was studied by submitting a protein free isotonic sodium chloride solution of MPA/MPAG to the ultrafiltration procedure following by the determination of MPA and MPAG concentrations in the initial solution and in the ultrafiltrate. Sorption of the analytes on polypropylene containers using sodium chloride solution as solvent being previously reported [20], the same methodology was applied to the sample vial of the disposable centrifugal filter device made of polypropylene with a sodium chloride solution containing 0.1/1 mg/L of MPA/MPAG.

2.5. Patient study

The pharmacokinetics of free MPA and free MPAG were investigated in seven heart transplant recipients.

The determination of the total MPA and MPAG concentrations was performed using the same chromatographic system and conditions using a previously described sample pretreatment [19]. After approval by the university hospital ethic committee, and informed consent, the study proceeded within the framework of the clinical practice. Patients who underwent heart transplantation were males and were aged from 21 to 63 years, and treated for at least 1 week with MMF (1 or 1.5 g bid, oral route) in addi-

tion to cyclosporine and corticosteroids as immunosuppressants. The pharmacokinetic study was performed in a time ranging from 6 to 23 days after transplant.

Blood samples were collected in Vacutainer tubes containing EDTA as anticoagulant, at time pre-dose and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 6, 8, 10 and 12 h after dosing. After transport in the ice, the plasma was immediately isolated by centrifugation at 3940 × *g*, 4 °C, for 10 min, and plasma samples were frozen at −20 °C in polystyrene tubes until analysis.

3. Results

3.1. HPLC-UV assay validation for the determination of free MPA and free MPAG

The retention times of MPA and MPAG were 12 and 3.2 min, respectively. Fig. 1 shows typical chromatograms for free MPA and free MPAG in blank ultrafiltrate (A), compared to the standard spiked with 0.05 mg/L of MPA and 2 mg/L of MPAG (B) and a patient ultrafiltrate with determined concentrations of 0.07 mg/L for MPA and 8.0 mg/L for MPAG (C).

Calibration curves fitted by plotting peak area versus concentration were linear over the range 0.005–5 mg/L for MPA and 0.05–200 mg/L for MPAG. The correlation coefficient was higher than 0.999 for the two compounds. Typical regressions equations were $y = 756.8x + 1.1$ for MPA (CV = 1.9%, $n = 5$) and $y = 242.8x + 123.1$ for MPAG (CV = 2.2%, $n = 5$). Intra-day and inter-day precision, accuracy for the two compounds are summarized in Table 1. The lower limit of quantification was 0.005 mg/L for MPA and 0.05 mg/L for MPAG, for a sample volume of 100 μL ultrafiltrate.

The percentage of added MPA and MPAG to a protein free isotonic sodium chloride solution recovered in the ultrafiltrate was 101.5 ± 0.8% and 102.5 ± 0.7%, respectively. These data demonstrate that no adsorption of MPA and MPAG to the ultrafiltration membrane occurred. Likewise, no loss of MPA (recovery of 100.0 ± 1.3%) and MPAG (recovery of 102.4 ± 0.5%) onto the wall of the vial made of polypropylene occurred during ultrafiltration.

Assay selectivity was demonstrated by the absence of interfering peaks at the retention times of MPA and MPAG in plasma samples from healthy subjects and plasma spiked with drugs usually administered to heart transplant patients.

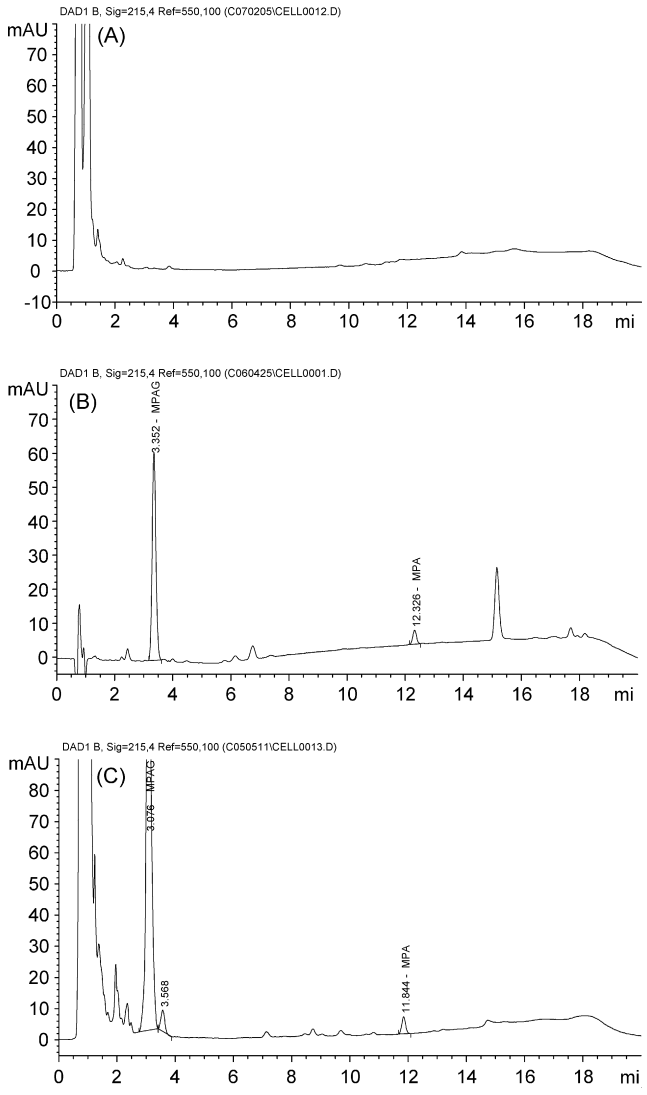

Fig. 1. Representative chromatograms for free MPA and free MPAG of a blank ultrafiltrate (A) compared to the standard spiked with 0.05 mg/L of MPA and 2 mg/L of MPAG (B) and a patient ultrafiltrate with determined concentrations of 0.07 mg/L for MPA and 8.0 mg/L for MPAG (C).

3.2. Pharmacokinetic study of free MPA and free MPAG

The free MPA (A) and free MPAG (B) plasma concentration–time profile of the seven heart transplant patients is illustrated

Table 1
Intra- and inter-assay precision, accuracy and limit of quantification

Accuracy and precision	MPA				MPAG			
	Theoretical concentration (mg/L)	Mean (mg/L)	CV (%)	PE (%)	Theoretical concentration (mg/L)	Mean (mg/L)	CV (%)	PE (%)
Intra-day ($n = 5$)	0.005[a]	0.005	1.9	3.2	0.05[a]	0.06	1.9	12.6
	0.050	0.045	3.1	9.4	5.00	5.47	0.7	9.4
	0.500	0.500	0.7	0.0	100.00	99.46	0.5	0.5
Inter-day ($n = 5$)	0.005[a]	0.005	5.1	3.3	0.05[a]	0.06	8.9	17.8
	0.050	0.052	6.8	4.0	5.00	5.50	2.1	10.0
	0.500	0.501	2.1	0.2	100.00	99.86	0.2	0.1

[a] Limit of quantification.

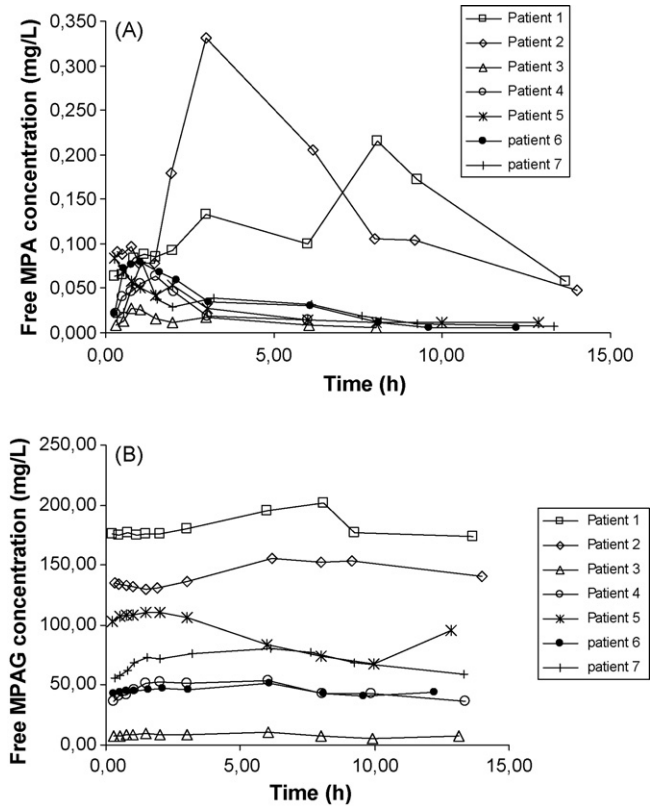

Fig. 2. Free MPA (A) and free MPAG (B) plasma concentration–time profile of the seven heart transplant patients.

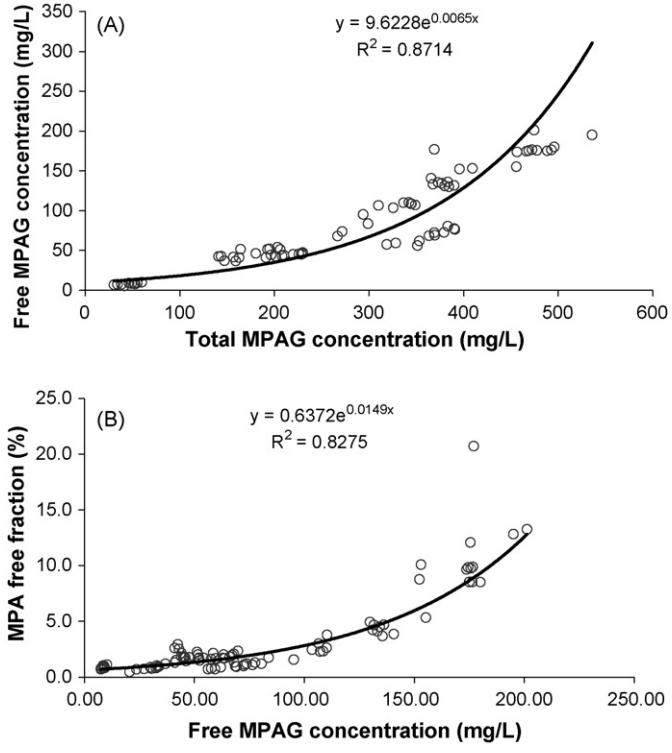

Fig. 3. Correlation between total and free MPA concentration (A) correlation between free fraction of MPA and free MPAG concentration (B) determined in the seven heart transplant patients.

in Fig. 2. Most of the concentrations of free MPA (93.5%), measured in the seven heart transplant patients studied, were higher than the limit of quantification of the method, and ranged from 0.006 to 0.331 mg/L. Free MPAG concentrations ranged from 5.6 to 201.3 mg/L. Two patients exhibit free MPA concentrations higher than the others with maximum value of 0.216 and 0.331 mg/L. This could be explained by hyperbilirubinemia recovered in two patients as previously described [2–4] and by the very low creatinine clearance (respectively 16 and 6 mL/min) observed. Moreover, in these patients, the total and free MPAG concentrations were highest leading to a displacement of the MPA from the plasma protein binding site.

The mean ± SD free fraction was 3.6 ± 3.9% for MPA and 26.0 ± 8.0% for MPAG in the seven heart transplant patients. Very significant correlations were found between free MPAG and total MPAG concentration ($R^2 = 0.8714$, $y = 9.6228e^{0.0065x}$) (Fig. 3A), and between free fraction of MPA and free MPAG concentration ($R^2 = 0.8275$, $y = 0.6372e^{0.0149x}$) (Fig. 3B). A poor correlation between free MPA and total MPA concentration ($R^2 = 0.2015$) was recovered.

4. Discussion

The present HPLC-UV method allows the simultaneous determination of free forms of MPA and its major metabolite MPAG in human plasma in a single run. The method exhibits a quantification limit of 5 μg/L for MPA, with a sample volume of 100 μL, which is lower than the previously published techniques [8,17] using ion-pair reversed-phase LC-UV method (26 μg/L) and automated method (6 μg/L) with an easier sample pretreatment procedure. The LOQ observed in the present method compares favourably with the HPLC-UV method (LOQ = 5 μg/L) of Shipkova et al. [13], the HPLC-fluorescence method (LOQ = 5 μg/L) of Shen et al. [10]. For free MPAG, the limit of quantification was defined at 50 μg/L, which is lower than the LOQ of previous reported HPLC-UV methods with values of 60 μg/L [17], 900 μg/L [8] and 2500 μg/L [19].

Neither adsorption of MPA and MPAG to the filtering membrane nor to the sample vial of the disposable centrifugal filter device was observed. The sample pretreatment described here is rapid and simple, without a solid-phase extraction step following the ultrafiltration as reported in previous publications [12,16,17,21].

As reported previously in renal transplant patients [8,22], our results show a wide interindividual variability in free MPAG concentration in the seven heart transplant recipients. Likewise, our data confirm the existence of a positive correlation ($R^2 = 0.8714$) between free MPAG and total MPAG level.

The mean free fraction of MPA recovered in our pharmacokinetic study confirms those previously observed [5,8–10], but is smaller than those observed by Atcheson et al. who found in patients with impaired renal function mean values of 7.3% [18] and 5.8 ± 3.7% early after renal transplant [22]. This discrepancy may be explained by renal impairment, which affects MPA binding. The mean free fraction of MPAG observed in the seven heart transplant recipients was in the range, 17.4 to 54.1%, reported by Atcheson et al. [22].

From our data, we only found a poor correlation between free MPA and total MPA concentration ($R^2 = 0.2015$) as observed by Mandla et al. [8] in renal transplant recipients. Besides, a significant positive correlation was observed between free fraction of MPA and free MPAG concentration ($R^2 = 0.8275$). This observation, which has not been reported previously, may be of interest in drug monitoring in transplant recipients. Further investigations via the analysis of supplemental data from patients are required to explore more closely the relationship between MPA and MPAG free fraction.

In conclusion, considering the characteristics; simple, sensitive, selective, the HPLC-UV method described is reliable and suitable for the simultaneous analysis of free MPA and free MPAG in solid organ transplant recipients.

References

[1] R.E.S. Bullingham, A.J. Nicholls, B.R. Kamm, Clin. Pharmacokinet. 34 (1998) 429.
[2] L.M. Shaw, B. Kaplan, D. DeNofrio, M. Korecka, K.L. Brayman, Ther. Drug Monit. 22 (2000) 14.
[3] B.A. Atcheson, P.J. Taylor, C.M. Kirkpatrick, S.B. Duffull, D.W. Mudge, P.I. Pillans, D.W. Johnson, S.E. Tett, Ther. Drug Monit. 26 (2004) 284.
[4] H.U. Meier-Kriesche, L.M. Shaw, M. Korecka, B. Kaplan, Ther. Drug Monit. 22 (2000) 27.
[5] B. Kaplan, H.U. Meier-Kriesche, G. Friedman, S. Mulgaonkar, S. Gruber, M. Korecka, K.L. Brayman, L.M. Shaw, et al., J. Clin. Pharmacol. 39 (1999) 715.
[6] D.R.J. Kuypers, Y. Vanrenterghem, J.P. Squifflet, M. Mourad, D. Abramowicz, M. Oellerich, V. Armstrong, M. Shipkova, J. Daems, Ther. Drug Monit. 25 (2003) 609.
[7] L.T. Weber, M. Shipkova, V.W. Armstrong, N. Wagner, E. Schutz, O. Mehls, L.B. Zimmerhackl, M. Oellerich, B. Tonshoff, J. Am. Soc. Nephrol. 13 (2002) 759.
[8] R. Mandla, P.D. Line, K. Midtvedt, S. Bergan, Ther. Drug Monit. 25 (2003) 407.
[9] M.H. Ensom, N. Partovi, D. Decarie, R.J. Dumont, G. Fradet, R.D. Levy, Ther. Drug Monit. 24 (2002) 310.
[10] J. Shen, Z. Jiao, Y.Q. Yu, M. Zhang, M.K. Zhonq, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 817 (2005) 207.
[11] D. Cattaneo, F. Gaspari, S. Ferrari, N. Stucchi, L. Del Priore, N. Perico, E. Gotti, G. Remuzzi, Clin. Transplant. 15 (2001) 402.
[12] C. Willis, P.J. Taylor, P. Salm, S.E. Tett, P.I. Pillans, J. Chromatogr. B Biomed. Sci. Appl. 748 (2000) 151.
[13] M. Shipkova, P.D. Niedmann, V.W. Armstrong, E. Schutz, E. Wieland, L.M. Shaw, M. Oellerich, Clin. Chem. 44 (1998) 1481–1488.
[14] F. Streit, M. Shipkova, V.W. Armstrong, M. Oellerich, Clin. Chem. 50 (2004) 152.
[15] I. Nowak, L.M. Shaw, Clin. Chem. 41 (1995) 1011.
[16] L.J. Langman, D.F. LeGatt, R.W. Yatscoff, Ther. Drug Monit. 16 (1994) 602.
[17] A. Aresta, F. Palmisano, C.G. Zambonin, P. Schena, G. Grandaliano, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 810 (2004) 197.
[18] B. Atcheson, P.J. Taylor, D.W. Mudge, D.W. Johnson, P.I. Pillans, S.E. Tett, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 799 (2004) 157.
[19] M. Bolon, L. Jeanpierre, M. El Barkil, K. Chelbi, M. Sauviat, R. Boulieu, J. Pharm. Biomed. Anal. 36 (2004) 649.
[20] X. Cussonneau, M. Bolon-Larger, R. Boulieu, Clin. Chem. 52 (2006) 904.
[21] E.K. Cho, D.J. Han, S.C. Kim, G.J. Burckart, R. Venkataramanan, J.M. Oh, J. Clin. Pharmacol. 44 (2004) 743.
[22] B. Atcheson, P.J. Taylor, D.W. Mudge, D.W. Johnson, C.M. Hawley, S.B. Campbell, N.M. Isbel, P.I. Pillans, S.E. Tett, Br. J. Clin. Pharmacol. 59 (2004) 271.