Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 1308–1312

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Short communication

Rapid method for the quantitative determination of efavirenz in human plasma

Seloi Mogatle, Isadore Kanfer*

Faculty of Pharmacy, Rhodes University, Artillery Road, Grahamstown 6140, South Africa

ARTICLE INFO

Article history:
Received 7 November 2008
Received in revised form 10 February 2009
Accepted 9 March 2009
Available online 20 March 2009

Keywords:
Rapid
Cost-effective
Efavirenz
HPLC-UV
Human plasma

ABSTRACT

A pharmacokinetic interaction study between efavirenz (EFV), a non-nucleoside reverse transcriptase inhibitor used in the treatment of HIV-1 infection, and an African traditional medicine, African potato in human subjects was undertaken. This necessitated the development and validation of a quantitative method for the analysis of EFV in plasma. A simple mobile phase consisting of 0.1 M formic acid, acetonitrile and methanol (43:52:5) was pumped at a low flow rate of 0.3 ml/min through a reverse phase Phenomenex® Luna C_{18} (2) (5 μm, 150 mm × 2.0 mm i.d.) column maintained at 40 °C. Diclofenac sodium was used as an internal standard (IS) and EFV and IS were monitored at 247 nm and 275 nm, respectively. A simple and rapid sample preparation involved the addition of mobile phase to 100 μl of plasma to precipitate plasma proteins followed by direct injection of 10 μl of supernatant onto the column. The procedures were validated according to international standards with good reproducibility and linear response ($r = 0.9990$). The intra- and inter-day accuracies were between 12.3 and 17.7% at the LLOQ and between −5.8 and 9.1% for the QC samples. The intra- and inter-day precision of EFV determinations were 5.1 or less and 7.2% RSD or less, respectively across the entire QC concentration range. Mean recovery based on high, medium and low quality control standards ranged between 92.7 and 94.1% with %RSD values better than 3%. Plasma samples were evaluated for short-term (ambient temperature for 6 h) and long-term (−10 ± 2 °C for 60 days) storage conditions and were found to be stable. The method described is cost-effective and has the necessary accuracy and precision for the rapid quantitative determination of EFV in human plasma.

1. Introduction

Efavirenz (EFV) is a non-competitive inhibitor of the reverse transcriptase enzyme in HIV-1. It is effective against HIV-1 but has no efficacy against HIV-2 and human cellular DNA polymerase α, β, γ and δ [1]. The elimination half-life of EFV after single and multiple oral doses is 55–76 h and 40–55 h, respectively, the latter being because EFV induces its own metabolism via induction of CYP3A4 [2]. The drug's relatively long half-life permits a once daily dose in the treatment of HIV-1 infection which can help improve adherence and hence clinical outcomes.

Peak plasma concentrations are reached within 5 h of dosing. The apparent volume of distribution (Vd/F) has been reported to be between ~150–250 l [3,4]. Clearance of EFV is higher in Caucasians than Blacks and Asians [5] and EFV exhibits high intra- and inter-patient variability in plasma EFV concentrations with inter patient variability reaching 84% [6].

Several analytical methods for the analysis of EFV either alone or in combination with other drugs in plasma/serum have been published using reversed-phase HPLC-UV [7–23], LC/MS [24] and HPLC with fluorescence detection [25] and a capillary electrophoresis method [26]. Sample volumes used have ranged from 200 to 900 μl plasma [7,10,11,14–18] whereas a smaller sample size of 100 μl was used by Kappelhoff [9]. Various methods such as liquid–liquid extraction [10,11,14], solid phase extraction [7,15–18] and a protein precipitation method followed by a dilution step with distilled water have been used to extract EFV from plasma [9]. A rapid, accurate, precise, selective, sensitive and cost-effective bioanalytical method for the determination of EFV in human plasma is reported. The method was validated in accordance with FDA guidelines [27] using HPLC-UV. Low injection volumes (10 μl) were used and small plasma samples (100 μl) were processed using a simple protein precipitation step where the supernatant after centrifugation was injected directly on-column without dilution.

2. Experimental

2.1. Chemicals and reagents

EFV (99.8% purity) was donated by Pharmacare Ltd. trading as Aspen Pharmacare (Port Elizabeth, South Africa) and diclofenac sodium used as an internal standard (IS) was obtained from the

* Corresponding author. Tel.: +27 46 603 8399; fax: +27 46 636 1205.
E-mail address: izzy.kanfer@gmail.com (I. Kanfer).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.03.008

Biopharmaceutics Research Institute (Rhodes University, Grahamstown, South Africa). HPLC grade acetonitrile and methanol were purchased from Romil Ltd., Cambridge, United Kingdom while formic acid (99.9%) was sourced from Associated Chemical Enterprises, Pty Ltd., (Johannesburg, South Africa). Water was purified by reverse osmosis and filtration through a Milli-Q purification system (Millipore, Milford, MA, USA). Human plasma with potassium edentate (K-EDTA) as an anticoagulant was obtained from South African National Blood Services, Eastern Cape Headquarters (Port Elizabeth, South Africa) and was stored at $-10 \pm 2\,^{\circ}C$.

2.2. Chromatographic conditions

The HPLC system consisted of a model 2695 Alliance HPLC system and a model 2995 PDA UV detector (Waters, Milford, MA, USA). The eluent was monitored at 247 nm for EFV and 275 nm for diclofenac sodium. Chromatographic separation was achieved at $40 \pm 2\,^{\circ}C$ using a reverse phase Phenomenex Luna® C_{18} (2) (5 μm, 150 mm × 2.0 mm i.d.) together with a Phenonenex Luna® guard column (4 mm × 3.0 mm i.d). The mobile phase consisted of 0.1 M formic acid (pH 2.2), acetonitrile and methanol (43:52:5 v/v) pumped at a flow rate of 0.3 ml/min.

2.3. Preparation of calibration standards and quality control (QC) samples

Stock solutions were prepared by dissolving an accurately weighed amount of EFV (1 mg/ml) in mobile phase. A spiked parent plasma standard was used to prepare calibration standards by serial dilution with blank plasma to provide plasma concentrations in the range 0.20–10 μg/ml. All plasma calibration standards were weighed and converted to volumes using a plasma specific gravity of 1.025 g/ml [28]. The IS, diclofenac sodium solution (4 μg/ml) was prepared by dissolving an accurately weighed amount and dissolving it in mobile phase. All stock solutions were stored in the fridge at $4 \pm 2\,^{\circ}C$. Quality control (QC) plasma samples at low (∼0.30 μg/ml), medium (∼1.5 μg/ml) and high (∼7.5 μg/ml) concentrations were prepared in the same manner but from a separate stock solution.

2.4. Sample preparation

Plasma samples (100 μl) spiked with EFV were transferred to 1.5 ml centrifuge tubes (Eppendorff AG, Hamburg, Germany) and the plasma was precipitated with 200 μl of IS solution. The resulting mixture was vortexed for 50 s and centrifuged at 10 000 × *g* for 10 min. One hundred microlitres of the supernatant was transferred to a micro-insert (Separation Scientific SA (Pty) Ltd., Johannesburg, South Africa), vortexed for 10 s and 10 μl injected directly onto the HPLC column.

2.5. Validation procedures

2.5.1. Calibration curve

Calibration curves were constructed by least-squares linear regression analysis without weighting. The range of the calibration curves was 0.2–10 μg/ml and each of the seven standards was extracted in triplicates and the mean was used to construct the calibration curve.

2.5.2. Lower limit of quantitation (LLOQ)

The LLOQ [27] was determined as 0.2 μg/ml based on a signal to noise ratio of 5:1 from the determination of 5 replicate spiked plasma samples.

2.5.3. Accuracy and precision

Accuracy was defined as percent difference between the mean and target concentration. Intra-day assay accuracy was determined from the analysis of five replicate samples at each of the low, mid and high QC standards, whereas, inter-day accuracy involved the calculation of the mean values of fifteen samples at each of the low, mid and high QC standards over three different days. The percent RSD was used to report precision which was accordingly determined using five replicate extractions of each low, mid and high concentrations, within and between days. Both the intra- and inter-day accuracies and precisions for the LLOQ concentration should be within ±20% and for all other concentrations should be within ±15%.

2.5.4. Selectivity

Blank plasma samples from six different batches of plasma were extracted and inspected for interference from endogenous compounds. Selectivity was determined both for EFV and IS.

2.5.5. Recovery

Extraction efficiency was determined by comparing the peak area of the QC samples in the low, medium and high concentration to those of EFV in mobile phase.

2.5.6. Stability

Plasma QC samples at low, medium and high concentrations were assayed in triplicate. Plasma samples were assessed for freeze-thaw stability, after exposing to three freeze-thaw cycles. Stock solutions of EFV in mobile phase were assessed for stability at ambient temperature for 6 h and following storage at $5 \pm 3\,^{\circ}C$ for

Table 1
Assay performance data.

Run	Replicate	Low QC	Mid QC	High QC
Run 1 (day 1) target values (μg/ml)		0.2956	1.4888	7.4261
	1	0.3254	1.4724	7.1163
	2	0.3270	1.4734	7.5261
	3	0.3368	1.4854	7.4939
	4	0.3169	1.5207	7.5409
	5	0.3065	1.4886	7.3916
Run 2 (day 2) target values (μg/ml)		0.3118	1.5627	7.8119
	1	0.3098	1.4732	7.4138
	2	0.2727	1.4393	7.6667
	3	0.3035	1.4605	7.5816
	4	0.3093	1.4822	7.5645
	5	0.3001	1.5194	7.3239
Run 3 (day 3) target values (μg/ml)		0.3171	1.5787	7.9156
	1	0.3343	1.5134	7.9770
	2	0.3362	1.5557	8.5306
	3	0.3694	1.5302	7.9946
	4	0.3470	1.5295	8.1297
	5	0.3376	1.4978	7.8233
Mean concentration for day 1 (μg/ml)		0.3225	1.4881	7.4138
Mean concentration for day 2 (μg/ml)		0.2991	1.4749	7.5101
Mean concentration for day 3 (μg/ml)		0.3449	1.5253	8.0910
Mean concentration for all days (μg/ml)		0.3222	1.4961	7.6716
Intra-assay accuracy for day 1 (%)		9.1	0.0	−0.2
Intra-assay accuracy for day 2 (%)		−4.1	−5.6	−3.9
Intra-assay accuracy for day 3 (%)		8.8	−3.4	2.2
Inter-assay accuracy (%)		4.5	−3.1	−0.6
Intra-assay precision for day 1 (%RSD)		3.5	1.3	2.4
Intra-assay precision for day 2 (%RSD)		5.1	2.0	1.8
Intra-assay precision for day 3 (%RSD)		4.2	1.4	3.3
Mean inter-assay precision (%RSD)		7.2	2.1	4.5

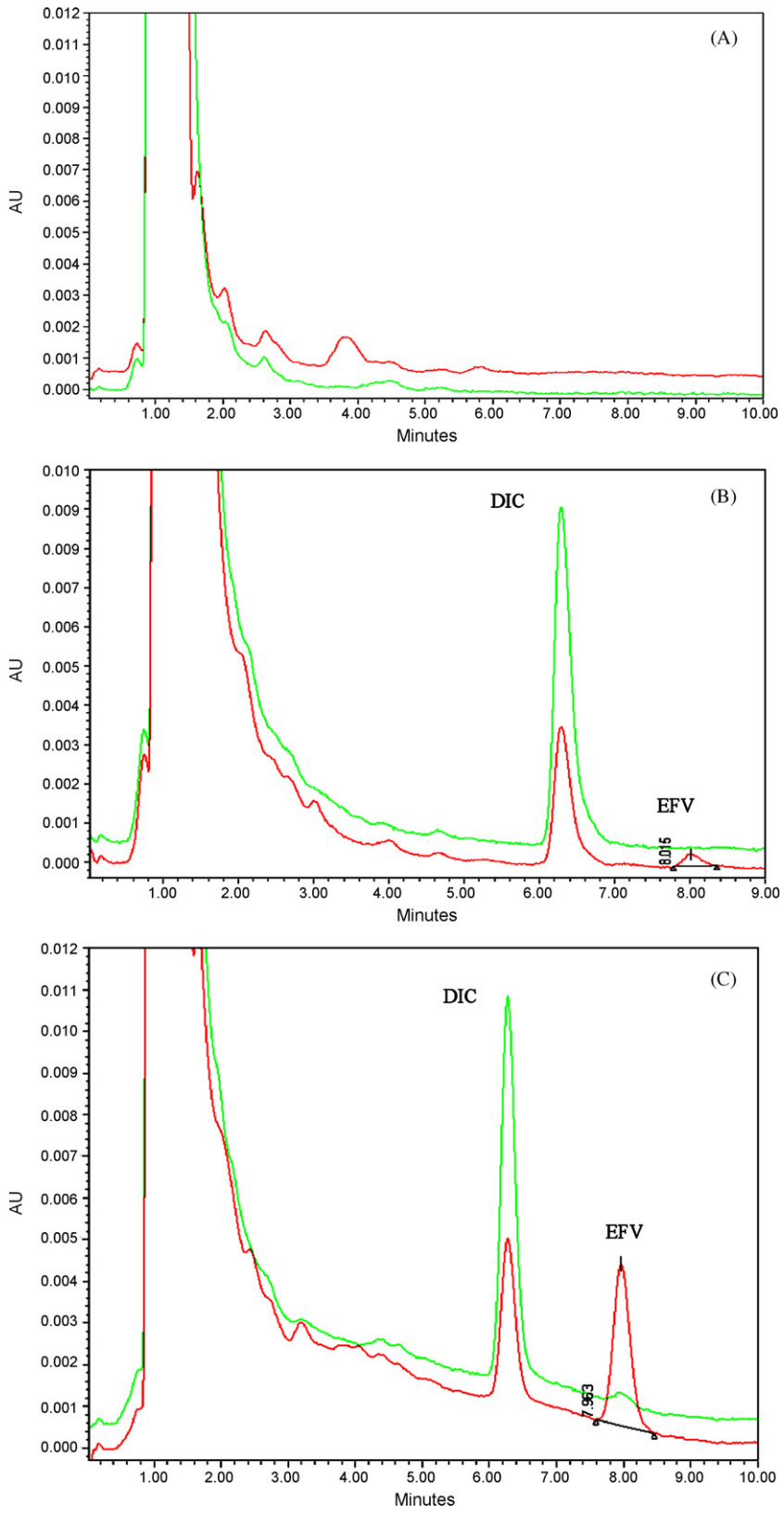

Fig. 1. A blank plasma extract (red: 247 nm and green: 275 nm); B = spiked plasma sample containing EFV (red: 247 nm) at the LLOQ and IS (green: 275 nm) and (C) plasma extract of a volunteer 2 h after the concurrent administration of a 600 mg EFV tablet and AP to a human subject. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

1 week. Unextracted plasma samples were also assessed for short-term stability on the bench-top (ambient temperature) for 4 h and long-term stability at $-10 \pm 2\,^{\circ}C$ for 1 and 2 months. Extracted samples were tested for stability after being stored in the autosampler pending analysis at ambient temperature ($22 \pm 3\,^{\circ}C$) for 24 h. Previously injected samples were re-assayed 24 h after initial injection.

The difference between freshly prepared solutions and the stored samples should be within 5–7% [29]. Bench top stability was

Table 2
Stability of EFV.

Storage condition	QC	Concentration (μg/ml)	Recovery (%)	RSD (%)	*n*
Plasma					
4 h at ambient temperature	Low	0.3100	91.4	3.5	3
	Medium	1.5542	100.4	5.4	3
	High	7.7705	97.3	3.4	3
Three freeze-thaw cycles	Low	0.3095	90.2	4.7	3
	Medium	1.5466	98.7	11.3	3
	High	7.7434	97.2	4.8	3
30 days at −10 °C	Low	0.3082	93.4	7.4	3
	Medium	1.5390	84.5	1.1	3
	High	7.6994	95.5	8.9	3
60 days at −10 °C	Low	0.3133	109.4	7.2	3
	Medium	1.5612	98.3	7.3	3
	High	7.8553	99.4	4.4	3
Final extract					
24 h at ambient temperature	Low	0.3095	98.7	4.9	3
	Medium	1.5466	100.9	4.9	3
	High	7.7434	101.0	5.8	3
Reinjection/reanalysis	Low	0.3095	94.8	2.2	3
	Medium	1.5466	102.1	6.1	3
	High	7.7434	107.2	1.7	3
Stock solutions					
6 h at ambient temperature		9.677	97.2	0.7	3
24 h in fridge		9.677	99.2	0.8	3
1 week in fridge		9.677	95.5	0.8	3

assessed by comparing the results from plasma samples stored on the bench to those of freshly thawed plasma samples where the difference should be within 15% and they should also be within 15% of nominal concentration. For freeze-thaw samples, stability is confirmed if both the mean concentrations of samples exposed to three freeze-thaw cycles and those exposed to one freeze-thaw cycle are found to be within 15% of nominal concentrations.

2.5.7. Application of the analytical method

The method was applied to investigate the effect of an African traditional medicine, African potato (*Hypoxis hemerocallidea*), on the pharmacokinetics of EFV in human subjects [30]. Plasma samples collected over a series of time intervals following the oral administration of 600 mg EFV tablets with and without the administration of African potato were analysed. The main purported active component in African potato is the norlignan, hypoxoside.

3. Results and discussion

3.1. Linearity

The calibration plots were linear and the concentrations of the calibration standards were back calculated using the peak area ratios of EFV/IS. The data were analysed by linear regression (unweighted) analysis, the correlation coefficient was ≥0.9994 and the %RSD for the slopes was 2.3.

3.2. Accuracy and precision

The intra- and inter-day accuracies were within the set criteria (Table 1) and found to be between 12.3 and 17.7% at the LLOQ and between −5.8 and 9.1% for the QC samples. The intra- and inter-day precision of EFV determinations were 5.1 or less and 7.2% RSD or less, respectively across the entire QC concentration range.

3.3. Selectivity

Fig. 1 depicts chromatograms showing a blank plasma extract (A) and the response from a spiked plasma sample containing EFV (λ = 247 nm) at the LLOQ (B) and the IS (λ = 275) nm and (C) plasma samples of a subject collected 2 h following the oral administration of a 600 mg EFV tablet. There were no interfering compounds in any of the different batches of human plasma obtained from six different sources.

3.4. Recovery

Extraction efficiencies of EFV from plasma were 94.1, 92.7 and 94.1 for the low, mid and high QC samples, respectively. The %RSD values ranged from 1.4 to 2.1.

3.5. Stability

EFV was found to be stable under all conditions evaluated as shown in Table 2.

3.6. Application of analytical method to human samples

Following concurrent administration of African potato with EFV, no interference from hypoxoside was seen (Fig. 1C). The plasma concentration profile of a human subject is depicted in Fig. 2 and shows a peak EFV plasma concentration (C_{max}) of 2.49 μg/ml occurring at a corresponding time (T_{max}) of 2 h.

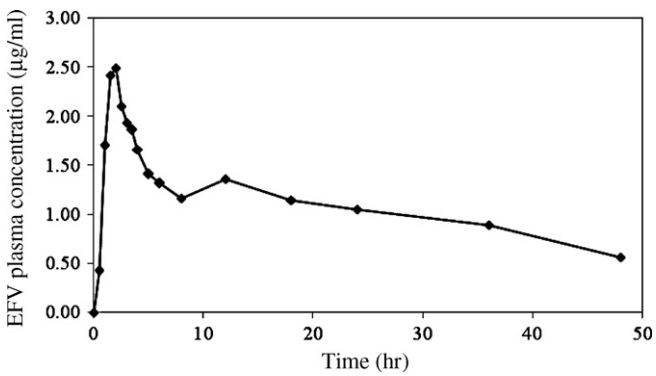

Fig. 2. EFV plasma concentration vs. time profile following the oral administration of a 600 mg EFV tablet to a human subject.

4. Conclusions

A rapid, accurate and precise method with the requisite sensitivity for the quantitative determination of EFV in plasma has been developed and validated. A previously published HPLC method [9] used a similar sample preparation but necessitated a 15 min shaking period following protein precipitation and centrifugation for 10 min and also included a dilution step followed by further centrifugation for an additional 10 min prior to injection. That method utilized an injection volume of 25 μl compared to our method where only a 10 μl injection volume sufficed. Hence our method has improved sample preparation efficiency and has the potential for increased sensitivity and also can be adapted for smaller plasma sample volumes if and when such a situation may arise, such as for paediatric use. Previous methods utilized various organic modifiers and/or ion pairing reagents at relatively high pH (>10 and greater) or even complicated and time consuming gradient elution. The mobile phase used in this study consisted of a simple mixture of a low concentration of formic acid (0.1 M) solution together with acetonitrile and a small amount of methanol (5%) at a pH of 2.2. This system is cost-effective and in addition provides protection of the silica column stationary phase from high pH damage thereby ensuring that the column life is not shortened.

Acknowledgements

The authors greatly acknowledge financial support from the Medical Research Council of South Africa, Botswana government and Ontario HIV/AIDS Treatment Network.

References

[1] S.D. Young, S.F. Britcher, L.O. Tran, L.S. Payne, W.C. Lumma, T.A. Lyle, J.R. Huff, P.S. Anderson, D.B. Olsen, S.S. Carroll, D.J. Pettibone, J.A. O'Brien, R.G. Ball, S.K. Balani, J.H. Lin, I. Chen, W.A. Schleif, V.V. Sardana, W.J. Long, Antimicrob. Agents Chemother. 39 (1995) 2602–2605.
[2] Bristol Myers Squibb Company. SUSTIVA package insert (2008).
[3] J.S. Barrett, A.S. Joshi, M. Chai, T.M. Ludden, W.D. Fiske, H.J. Pieniaszek Jr., Int. J. Clin. Pharmacol. Therap. 40 (2002) 507–519.
[4] C. Csajka, C. Marzolini, K. Fattinger, L.A. Décosterd, J. Fellay, A. Telenti, J. Biollaz, T. Buclin, Clin. Pharmacol. Therap. 73 (2003) 20–30.
[5] D. Burger, I. van der Heiden, C. la Porte, M. van der Ende, P. Groeneveld, C. Richter, P. Koopmans, F. Kroon, H. Sprenger, J. Lindemans, P. Schenk, R. van Schaik, Br. J. Clin. Pharmacol. 61 (2006) 148–154.
[6] L. Ståhle, L. Moberg, J.-O. Svensson, A. Sönnerborg, Therap. Drug Monit. 26 (2004) 267–270.
[7] G. Aymard, M. Legrand, N. Trichereau, B. Diquet, J. Chromatogr. B: Biomed. Sci. Appl. 744 (2000) 227–240.
[8] S.O. Choi, N.L. Rezk, A.D.M. Kashuba, J. Pharm. Biomed. Anal. 43 (2007) 1562–1567.
[9] B.S. Kappelhoff, H. Rosing, A.D.R. Huitema, J.H. Beijnen, J. Chromatogr. B 792 (2003) 353–362.
[10] S.A. Lakshmi, K.K. Kishore, K.D.V.R. Ravi, K.C. Mohan, N.M. Yugandhar, G. Srinubabu, Chromatographia 65 (2007) 359–361.
[11] P. Langmann, D. Schirmer, T. Vath, M. Zilly, H. Klinker, J. Chromatogr. B: Biomed. Sci. Appl. 755 (2001) 151–156.
[12] C. Marzolini, A. Telenti, T. Buclin, J. Biollaz, L.A. Decosterd, J. Chromatogr. B: Biomed. Sci. Appl. 740 (2000) 43–58.
[13] S. Notari, A. Bocedi, G. Ippolito, P. Narciso, L.P. Pucillo, G. Tossini, R.P. Donnorso, F. Gasparrini, P. Ascenzi, J. Chromatogr. B 831 (2006) 258–266.
[14] G. Ramachandran, A.K.H. Kumar, S. Swaminathan, P. Venkatesan, V. Kumaraswami, D.J. Greenblatt, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 835 (2006) 131–135.
[15] N.L. Rezk, R.R. Tidwell, A.D.M. Kashuba, J. Chromatogr. B 805 (2004) 241–247.
[16] M. Sarasa-Nacenta, Y. López-Púa, L.F. López-Cortés, J. Mallolas, J.M. Gatell, X. Carné, J. Chromatogr. B: Biomed. Sci. Appl. 763 (2001) 53–59.
[17] V.A. Simon, M.D. Thiam, L.C. Lipford, J. Chromatogr. A 913 (2001) 447–453.
[18] K. Titier, F. Lagrange, F. Péhoureq, L. Edno-McHeik, N. Moore, M. Molimard, Therap. Drug Monit. 24 (2002) 417–424.
[19] M.L. Turner, K. Reed-Walker, J.R. King, E.P. Acosta, J. Chromatogr. B 784 (2003) 331–341.
[20] Y. Usami, T. Oki, M. Nakai, M. Sagisaka, T. Kaneda, Chem. Pharm. Bull. 51 (2003) 715–718.
[21] A.I. Veldkamp, R.P.G. Van Heeswijk, P.L. Meenhorst, J.W. Mulder, J.M.A. Lange, J.H. Beijnen, R.M.W. Hoetelmans, J. Chromatogr. B: Biomed. Sci. Appl. 734 (1999) 55–61.
[22] P. Villani, M. Pregnolato, S. Banfo, M. Rettani, D. Burroni, E. Seminari, R. Maserati, M.B. Regazzi, Therap. Drug Monit. 21 (1999) 346–350.
[23] D.R. Weller, R.C. Brundage, J. Balfour, H. Henry, H.E. Vezina, J. Chromatogr. B 848 (2007) 369–373.
[24] A. Rouzes, K. Berthoin, F. Xuereb, S. Djabarouti, I. Pellegrin, J.L. Pellegrin, A.C. Coupet, S. Augagneur, H. Budzinski, M.C. Saux, D. Breilh, J. Chromatogr. B 813 (2004) 209–216.
[25] C.Z. Matthews, E.J. Woolf, R.S. Mazenko, H. Haddix-Wiener, C.M. Chavez-Eng, M.L. Constanzer, G.A. Doss, B.K. Matuszewski, J. Pharm. Biomed. Anal. 28 (2002) 925–934.
[26] E.A. Pereira, G.A. Micke, M.F.M. Tavares, J. Chromatogr. A 1091 (2005) 169–176.
[27] U.S. Department of Health and Human Services. FDA. Guidance for Industry Bioanalytical Method Validaton (2001) http://www.fda.gov/CDER/GUIDANCE/4252fnl.htm.
[28] R.J. Trudnowski, C.R. Rico, Clin. Chem. 20 (1974) 615–616.
[29] W. Nowatzke, E. Woolf, AAPS J. 9 (2007) E117–E122.
[30] S. Mogatle, I. Kanfer, SAMJ 98 (2008) 945–949.