Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 816 (2005) 99–105
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

# High-performance liquid chromatography with ultraviolet detection for therapeutic drug monitoring of everolimus

Sara Baldelli[a,b], Stefano Murgia[a,b], Simona Merlini[a,b], Stefania Zenoni[a,b], Norberto Perico[a,b], Giuseppe Remuzzi[a,b], Dario Cattaneo[a,b,*]

[a] *Department of Medicine and Transplantation, Ospedali Riuniti di Bergamo–Mario Negri Institute for Pharmacological Research, Via Gavazzeni 11, 24125 Bergamo, Italy*
[b] *Center for Research on Organ Transplantation "Chiara Cucchi De Alessandri & Gilberto Crespi", Mario Negri Institute for Pharmacological Research, Bergamo, Italy*

Received 21 June 2004; accepted 8 November 2004
Available online 10 December 2004

**Abstract**

We developed and validated a high-performance liquid chromatography–ultraviolet (HPLC–UV) method for determining everolimus concentrations in human whole blood. Sample preparation involved a solid-phase extraction after protein precipitation. The separation of everolimus from internal standard (IS) and endogenous components was achieved using an isocratic elution on an octyl column. The method showed a linear relationship between peak height ratios and blood concentrations in the range of 1–200 ng/mL ($r^2 = 0.9997$). The observed intra- and inter-day assay imprecision had a coefficient of variation (CV) = 12.8%, and inaccuracy was 11.4%. The method was found to be precise, accurate, and sensible making it useful for routine therapeutic monitoring of everolimus.


*Keywords:* Everolimus; Validation; High-performance liquid chromatography

## 1. Introduction

Everolimus (Certican®, Novartis Pharmaceuticals, Basel, Switzerland) is a macrolide bearing a stable 2-hydroxyethyl chain substitution at position 40 on the rapamycin structure, rationally developed to improve the pharmacokinetic characteristics of rapamycin and enhance its bioavailability [1]. Experimental studies [2,3] and phase II–III clinical trials [4,5] have shown that everolimus is a potent immunosuppressive agent, and strong correlation between drug trough concentration and clinical outcome has been reported [4,6,7]. Everolimus is metabolized by the intestinal and hepatic cytochrome P450 3A4 [8], a system involved in the metabolism of several other agents commonly used to manage transplant recipients [9], underlying the possibility of many drug-to-drug pharmacokinetic interactions. Moreover, everolimus blood levels may be affected by hepatic insufficiency [10] and/or ethnicity [9]. Together, these factors may contribute to the daily exposure of patients to everolimus, ultimately requiring proper drug dose adjustments. Therefore, therapeutic drug monitoring of everolimus concentrations may be crucial to select the optimal dose of everolimus allowing adequate immunosuppression and minimizing potential drug-related toxicity. Different high-performance liquid chromatography (HPLC) assays have been reported so far for the measurement of everolimus concentrations in the whole blood [11–16]. Although these methods meet the criteria for validated analysis of immunosuppressive drugs, they require mass spectrometric detection, which is not always available in clinical laboratories. Since everolimus is now entering in routine clinical practice medicine, we aimed to set up a simple HPLC method with ultraviolet (UV) detection that was precise and accurate at low concentrations and capable at high throughput. This

* Corresponding author. Tel.: +39 035 319888; fax: +39 035 319331.
*E-mail address:* dcattaneo@marionegri.it (D. Cattaneo).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.11.016

study describes the chromatographic conditions as well as sample extraction and complete validation of the proposed method.

## 2. Experimental

### 2.1. Materials

Everolimus (purity 97%) was generously provided by Novartis Pharma (Basel, Switzerland) and the internal standard (IS; 32-*O*-desmethoxyrapamycin) **(**purity 97%) by Wyeth-Ayerst Research Laboratories (Princeton, NJ) (Fig. 1). Stock solutions containing 50 and 100 μg/mL for everolimus and IS, respectively, were appropriately prepared in methanol. Everolimus working solutions of 100, 500, and 2000 ng/mL were prepared for dilution with methanol/water (50:50) and for IS a working solution of 1000 ng/mL was prepared with methanol. All the solutions were stored at −20 °C. Stock solutions were stable for at least 12 months and working solutions for at least 1 month. Acetonitrile, methanol, heptane (BDH, Milan, Italy), acetone and hexane (Fluka, Milan, Italy) were all HPLC grade. Zinc sulfate heptahydrate, analytical grade, was purchased from Fluka and a 5% solution was prepared in distilled water (2.8% in $ZnSO_4$). All other chemicals were analytical grade quality. Deionized water used in all aqueous solutions was obtained from a Milli-Q water purification system (Millipore, Milan, Italy).

### 2.2. HPLC–UV apparatus and conditions

The HPLC system consisted of an analytical column (Ultrasphere C8 with 3 μm beads; 75 mm × 4.6 mm, Beckman, Fullerton, CA) heated at 60 °C by a Croco-cil oven (Beckman) equipped with an autosampler (model 508, Beckman) set at 4 °C and an UV detector (model 166, Beckman) set at 278 nm. The mobile phase consisted of 56% acetonitrile in water and was delivered at a flow rate of 1 mL/min. Data were collected and processed using a 32 Karat software for HPLC system (Beckman).

### 2.3. Calibration curve and quality control samples

Calibrator samples were prepared mixing appropriate volumes of everolimus from stock working solutions to EDTA anticoagulated human whole blood from healthy volunteers to achieve different concentrations from 1 to 80 ng/mL.

Quality control (QC) samples were prepared spiking known volumes of everolimus from stocked working solutions to drug-free human whole blood to obtain five concentrations at the lower limit of quantification (1 ng/mL), within the linear range at low (1.3 ng/mL), medium (3 and 25 ng/mL), high levels (70 ng/mL) and at the upper limit of quantification (200 ng/mL) for everolimus. The QC samples were stored frozen at −20 °C until analysis.

### 2.4. Sample preparation

Blood samples were collected in EDTA tubes and stored at −20 °C. One milliliter volume of whole-blood sample was pipetted into labeled disposable polypropylene tubes and added with 50 μL of IS solution (1000 ng/mL). The tubes were vortex-mixed for 40 s; 1.5 mL of zinc sulfate solution was first added followed by 1.5 mL acetone for protein precipitation. The tubes were vortex-mixed for further 50–60 s and centrifuged at 3000 × *g* for 10 min at room temperature. The clear supernatant was poured into another polypropylene tube, added with 2 mL distilled water, mixed and loaded onto a preconditioned (3 mL acetonitrile and 1 mL distilled water) Bond-Elut cartridge (C18, 200 mg, 3 mL, Varian, Leinì,

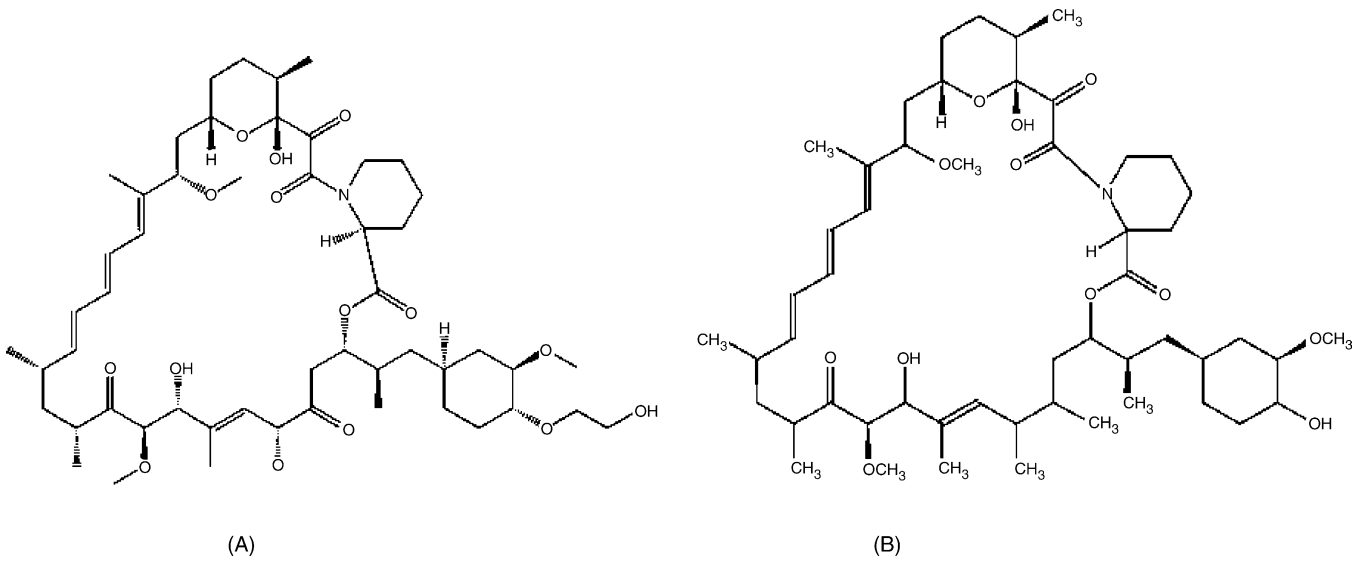


Fig. 1. Chemical structure of everolimus (40-*O*-(2-hydroxyethyl)rapamycin) (A) and of 32-*O*-desmethoxyrapamycin (B).

Italy), on a Vac Elut 20 Manifold (Varian). The Bond-Elut cartridges were washed with 3 mL of a solution methanol/water (30:70). In each step, the solvent was allowed to drip out from the cartridge. One milliliter of hexane was then added and the column was allowed to go dry under vacuum. Everolimus and IS were eluted in polypropylene tubes with 1 mL acetonitrile. In all steps, the flow rate did not exceed 1 mL/min. The eluate was taken to dryness either under a gentle nitrogen stream in a water bath at 37 °C or in a model RC 10.09 centrifugal evaporator (Jouan, Saint-Herblain, France), the residue was dissolved in 150 μL of the mobile phase, and then 1 mL of heptane was added to each tube. The tubes were vortex-mixed for 3 min, centrifuged at 3000 × *g* for 10 min; the heptane layer was removed from each sample and discarded. The extracts were transferred to polypropylene vials, capped and placed on the HPLC autosampler, and 90 μL were injected.

### 2.5. Method validation

Assay performance was determined in accordance with the FDA Guidance for Bioanalytical Methods Validation for Human Studies [17].

#### 2.5.1. Specificity

The specificity of the method was evaluated as lack of matrix interference by analysis of human drug-free whole-blood samples from different volunteers ($n = 15$). To investigate potential endogenous interference, blank samples were spiked with high concentrations of the most common immunosuppressive agents (including cyclosporine, mycophenolate mofetil, mycophenolate sodium, steroids, tacrolimus, and azathioprine) and analyzed.

In addition, to test potential concomitant medication or xenobiotic interference, blood from different transplant patients ($n = 30$) on immunosuppressive therapy and most common antifungal, antihypertensive and hypolipidemic agents were analyzed.

#### 2.5.2. Linearity

The linearity of the method was tested by constructing a standard curve from 1 to 200 ng/mL of everolimus, plotting the peak height ratios of the drug to IS versus the nominal drug concentration, and applying a linear least squares regression analysis without weighing. The method was considered linear if the coefficient of regression ($r^2$) calculated as mean of 10 curves was equal or better than 0.99 [17].

The calibration curve for estimating everolimus concentrations in unknown samples consisted of seven everolimus concentrations (1, 2.5, 5, 10, 20, 40, and 80 ng/mL), and was prepared in every analysis.

#### 2.5.3. Inaccuracy and imprecision

The within- and between-day coefficient of variation (CV) and the inaccuracy of the method were assessed by calculating daily and overall CVs and bias values for QC (five replicates at each concentration per analytical run) that were assayed in five separate analytical runs. According to the guidelines for bioanalytical methods published by FDA [17], the assay was considered acceptable if imprecision at each concentration was less than 15% for both within- and between-day variability. The inaccuracy should be between ±15% [17].

#### 2.5.4. Lower limit of quantification

The lowest identifiable discrete and reproducible concentration that showed an imprecision of 20% and accuracy of 80–120% [17] was accepted as lower limit of quantitation (LOQ).

#### 2.5.5. Recovery

To determine the extraction efficiency, the peak height ratios of spiked blood samples were compared to those obtained from direct injections of the same amount of everolimus and IS. The assay was accepted if recovery exceeded 60% [17].

#### 2.5.6. Dilution integrity

To establish dilution stability, blood samples at concentrations of 100 ng/mL were diluted two-fold (500 + 500 μL) and 200 ng/mL were diluted two-fold (500 + 500 μL) and five-fold (200 + 800 μL) using everolimus free blood. Deviation from the nominal value was determined on three replicates.

#### 2.5.7. Stability studies

Long-term stability of standard working solutions, respectively, at 500 and 2000 ng/mL was evaluated at two temperatures (4 and −20 °C) and over 28 days.

Freeze–thaw, short-term and long-term and autosampler stability of everolimus in the matrix was evaluated at two concentrations (3 and 70 ng/mL).

Analyte stability was determined after three freeze–thaw cycles. Six aliquots each at two concentrations were prepared. Three aliquots were analyzed promptly prepared, the other three were stored at −20 °C thawed at room temperature and refrozen under the same conditions. The cycle was repeated for two more times and analyzed on the third cycle. Comparison between mean results was performed calculating the percentage difference.

Short-term stability of the analyte, simulating work bench conditions, was evaluated on six aliquots, each at two concentrations stored at −20 °C. Three aliquots were thawed at room temperature over a time period of 15 h. After 15 h, the other three aliquots were thawed and both sets were extracted and processed. Means of the response were compared and percentage difference calculated.

Long-term stability has been established storing 18 aliquots of two concentrations at −20 °C and measuring the concentration over a period of 28 days. Mean concentrations obtained have been compared to the mean of back-calculated values for the standards at the same concentrations from the first day of long-term stability.

Stability of everolimus and the internal standard in the autosampler was evaluated for 24 h. Ten sets of quality control

were extract and placed in the autosampler at 4 °C. Five sets of samples were analyzed immediately, the other five sets 24 h later.

## 3. Results

Under the given conditions, everolimus and IS eluted at retention times of 9 and 10.4 min, respectively, and the total analysis time required was 13 min per sample. Peaks were well resolved, symmetrical and relatively sharp with comparable profiles.

Representative chromatograms of extracts from human drug-free whole-blood samples, a control human whole-blood sample spiked with known amount of everolimus (1 and 2.5 ng/mL), and a sample from a patient receiving everolimus as part of his immunosuppressive therapy (9.4 ng/mL) are shown in Fig. 2A–C, respectively. No detectable interfering peak was found with retention times close to those of everolimus and IS due to the matrix in the extracts from human drug-free whole-blood samples (Fig. 2A). Similarly, no interferences were detected due to concomitant medications in sample spiked with different amount of most common immunosuppressive agents (including cyclosporine, mycophenolate mofetil, mycophenolate sodium, steroids, tacrolimus, and azathioprine) or in blood samples from transplant patients on therapy with immunosuppressive agents and the most common antifungal, antihypertensive and lipid lowering medications.

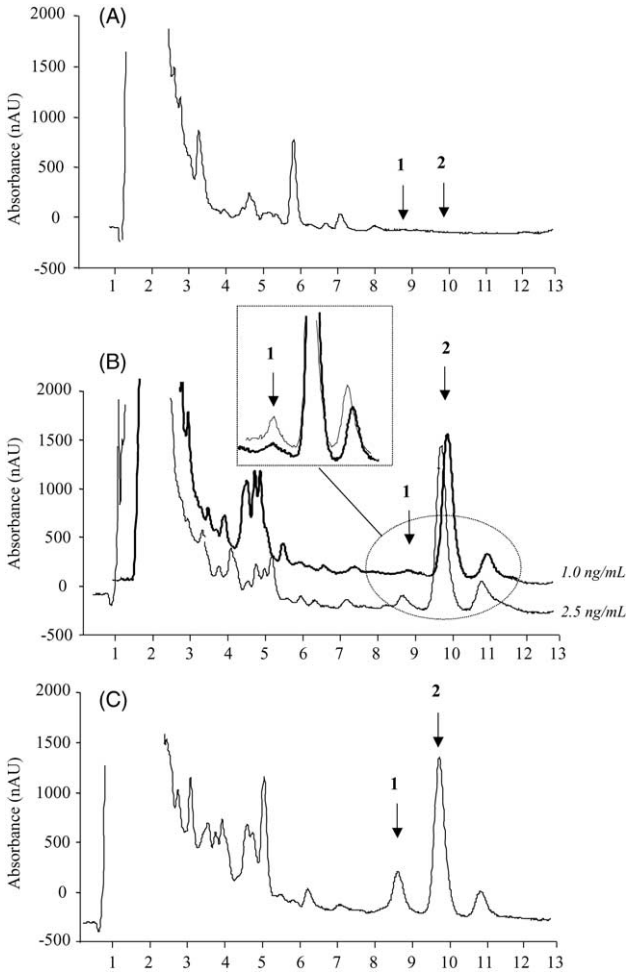


Fig. 2. HPLC chromatograms of extracts from control human whole blood (A), control human whole blood spiked with 1 and 2.5 ng/mL everolimus (B), and whole blood from a patient receiving everolimus (C, everolimus concentration, 9.4 ng/mL): (1) everolimus; (2) IS.

A sharp peak at the retention time of 11 min occurred in all chromatograms. By comparison of chromatograms obtained from extracts with chromatograms of samples of IS at different concentration, the peak was found to be the isomer of the IS. This was confirmed by the fact that IS is commercialized as an isomeric mixture of two isomers B and C [18]. The peak was well separated and did not affect the performance of the method.

A linear equation was judged to produce the best fit for concentration/response relationship in the range of everolimus concentrations of 1–200 ng/mL with an acceptable coefficient of regression ($r^2 = 0.9997$, $n = 10$). The lower limit of quantitation was established at 1 ng/mL with a within-day CV and inaccuracy of 10.7 and −10.6%, respectively, and a between-day CV and inaccuracy of 14.5 and 10%.

The within- and between-day inaccuracy of quality control samples were 0.3–11.4 and 0.8–7.6%, respectively; and the within- and between-day imprecision CVs 3.9–12.8 and 2.4–9.8%, respectively (Table 1).

Mean drug recoveries, calculated by comparing the peak height ratios of extracted blood samples with those obtained from unextracted calibrators with the same amount of everolimus were 76 ± 8% for everolimus and 65 ± 5% for IS.

Dilution stability of 100 ng/mL sample concentration based on a two-fold dilution showed an inaccuracy of −7.5 ± 4.5% (mean ± S.D.), while those of 200 ng/mL concentration based on a two- or five-fold dilution revealed an inaccuracy of −6.8 ± 2.7 and −1.4 ± 8.3%, respectively.

Stock solution of everolimus in MeOH/$H_2O$ (1:1) cannot be stored at a temperature of 4 °C. Indeed, after 7 days, the concentration of the 500 ng/mL everolimus solution increased of 13.6% and the concentration of the 2000 ng/mL solution increased of 13.3%. In contrast, a temperature of −20 °C was found to be more effective, as variation in concentrations over 28 days was limited (+6.01% on the 500 ng/mL solution and +4.0% on the 2000 ng/mL solution).

There was no significant loss in everolimus concentrations (Table 2), as shown by percentage difference concentration values obtained during stability tests on blood samples, freeze–thaw stability after three cycles, short-term stability after 15 h at room temperature and long-term stability after 31 days below −20 °C.

Stability of everolimus and internal standard in post-extracted samples was tested using 10 sets of quality controls (at 3 and 70 ng/mL) placed in the autosampler at 4 °C and analyzed immediately (first 5 sets of samples) or 24 h

Table 1
Performance of the HPLC method for the determination of everolimus (RAD) concentration in whole blood

| | Spiked RAD concentration | | | | | |
|---|---|---|---|---|---|---|
| | 1 ng/mL | 1.5 ng/mL | 3 ng/mL | 25 ng/mL | 70 ng/mL | 200 ng/mL |
| Within-day assay | | | | | | |
| Mean ± S.D. (ng/mL) | 0.9 ± 0.1 | 1.6 ± 0.2 | 3.0 ± 0.1 | 25.2 ± 1.1 | 67.8 ± 2.7 | 219 ± 25 |
| Imprecision (CV%) | 10.7 | 12.8 | 5.0 | 4.5 | 3.9 | 11.6 |
| Inaccuracy (%) | −10.6 | 7.1 | −0.3 | 0.9 | −3.1 | 9.6 |
| Between-day assay | | | | | | |
| Mean ± S.D. (ng/mL) | 1.1 ± 0.2 | 1.6 ± 0.1 | 2.9 ± 0.1 | 23.6 ± 2.3 | 69.5 ± 4.0 | 206 ± 14 |
| Imprecision (CV%) | 14.5 | 7.3 | 2.4 | 9.8 | 5.7 | 7.0 |
| Inaccuracy (%) | 10.0 | 3.5 | −2.8 | −5.7 | −0.8 | 3.0 |

Table 2
Stability data of everolimus (RAD) concentrations in whole blood during validation

| Parameter | Results (%) | |
|---|---|---|
| | 3 ng/mL | 70 ng/mL |
| Long-term stock solution (28 days at −20 °C) | +4.0 | +6.1 |
| Freeze–thaw (three cycles) | −3.1 | +3.1 |
| Short-term blood samples (15 h at room temperature) | +9.8 | +3.9 |
| Long-term blood sample (28 days at −20 °C) | +3.4 | +4.6 |

after sample extraction (last 5 sets of samples). The mean observed difference between the samples was below 5% for both concentrations (2.2% at 3 ng/mL and 1.8% at 70 ng/mL).

Moreover, the suitability of the proposed HPLC method was tested by measuring everolimus pharmacokinetic profiles in whole-blood samples from heart transplant recipients given everolimus as a part of their triple immunosuppressive therapy, including cyclosporine and steroids [19]. A representative blood concentration versus time profile obtained following oral administration of everolimus (1.5 mg) is shown in Fig. 3.

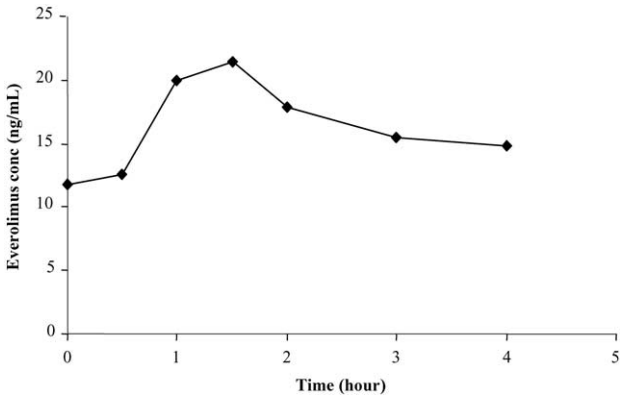


Fig. 3. Representative blood concentration vs. time profile obtained following oral administration of everolimus (1.5 mg) in a heart transplant patient.

## 4. Discussion

The assay described herein is a modification of a previous HPLC method with ultraviolet detection we developed for the measurement of whole blood rapamycin levels [20]. The method is currently used in our laboratory for routine drug analysis and its ongoing proficiency is tested by a reference laboratory in UK every month [21]. In that case, the lower limit of quantification was 2.5 ng/mL, a suitable value to detect accurately the expected rapamycin concentration in transplant patients [22].

Because of close structural formula, the rapamycin analogue everolimus and the parent compound present similar chemical and physical properties and consequently a comparable ultraviolet (UV) spectrum (with an absorption maximum at a wavelength of 278 nm due to the triene group in the molecule), high distribution in red blood cells, as well as similar biological activity and metabolism. For these reasons, the above-mentioned method was firstly applied for everolimus too. The new drug showed retention time and peak heights close to those previously observed with the parent compound. Recently, however, it has been shown that everolimus is effective as immunosuppressant at lower blood concentrations than rapamycin [1], with a proposed therapeutic range of 3–15 ng/mL, instead of 5–15 ng/mL recommended for rapamycin. This implies that the minimum therapeutic concentration suggested for everolimus (3 ng/mL), is too close to the lower limit of quantification (2.5 ng/mL) we achieved with the previous HPLC method suitable for rapamycin monitoring, therefore the procedure set up for rapamycin assay cannot be directly transferred to everolimus measurement.

To get round this shortcoming, some steps of our originally described HPLC method were modified considering the polarity introduced with the new functional group [20]. In particular, the volume used for elution of the interference doubled and a final additional step of purification was introduced with the aim to increase the sensitivity of the method and make it suitable to adequately measure everolimus blood levels. Also the chromatographic conditions were reviewed in order to improve peak shape and to obtain chromatograms in which everolimus peak could be unequivocally detectable and quantifiable.

Moreover, the temperature was increased for shortening the retention time of everolimus, thus reducing time analysis and facilitate obtaining narrow and Gaussian-shaped peaks. A temperature of 60 °C was found to be the most efficient without lowering the column performance. In addition, different mobile phases were studied in order to achieve adequate separation of peaks and the best results were obtained with a binary mobile phase. In these conditions, heights of the peaks were increased, allowing for a limit of quantitation of everolimus of 1 ng/mL, about three times lower than that achieved with rapamycin.

Although lower LOQ have been reported using HPLC–MS [11–16], these methods are limited by expenses and often required technical expertise, eventually restricting their use to very few clinical laboratories. The simple HPLC method with UV detection we described, is suitable to assess quantitative determination of everolimus levels in whole blood even at very low concentrations. At the upper limit of quantification, set at 200 ng/mL, we observed a slight decrease in the within-day performance of the method. It should be mentioned, however, that this value greatly exceeds everolimus concentrations found in different pharmacokinetic studies [9,23].

Furthermore, this assay complies with proposed FDA guidelines for the validation of a bioanalytical method in terms of linearity, precision, accuracy of the method and stability for standards and QC samples in whole blood [17]. This demonstrated that the method proposed is reliable and reproducible for the quantitative measurement of everolimus.

Our proposed HPLC method has been also successfully used to determine the whole blood abbreviated pharmacokinetic profile in heart transplant patients receiving everolimus as part of their antirejection therapy. The observed profiles were in agreement with recently published pharmacokinetic studies [9,23], thus confirming that the assay was unaffected by any interferences due to concomitant medications or drug metabolites and was also able to discriminate between close blood concentrations.

Taking into account the sample preparation step and the chromatographic time, more than 40 samples can be extracted and analyzed in 10 h. However, in case of emergency samples, it is possible to obtain the result in less than 3 h. As suggest by FDA guidelines [17], in order to avoid problems of inaccuracy or imprecision due to the positions of a sample in a run, during each assay run, a number of QC samples are incorporated and analyzed at the beginning, in the middle and at the end of the batch of samples. The results of the QC samples provide the basis of accepting or rejecting the run.

In summary, this report provides a simple, sensitive, and feasible method to assess quantitative determination of everolimus levels in whole blood. To our knowledge, this is the first report on an HPLC assay with UV detection as a useful tool for therapeutic monitoring of this novel immunosuppressant, even in particularly not well-equipped institutions.

## Acknowledgments

Dr. Dario Cattaneo is a recipient of a “Fondazione Monzino” fellowship. The authors thank the Association for Research on Transplantation (ART) for continuous support. We are also very grateful to Dr. Roberto Fiocchi (Cardiovascular Department, Ospedali Riuniti di Bergamo, Bergamo, Italy) for providing us blood samples from heart transplant patients given everolimus as part of their immunosuppressive therapy.

## References

[1] R. Sedrani, S. Cottens, J. Kallen, W. Schuler, Transplant. Proc. 30 (1998) 2192.
[2] O. Viklicky, H. Zou, V. Muller, J. Lacha, A. Szabo, U. Heeman, Transplantation 69 (2000) 497.
[3] W. Schuler, R. Sedrani, S. Cottens, B. Haberlin, M. Schulz, H.J. Schuurman, G. Zenke, H.G. Zerwes, M.H. Schreier, Transplantation 64 (1997) 36.
[4] J.M. Kovarik, B. Kaplan, H.T. Silva, B.D. Kahan, J. Dantal, S. Vitko, R. Boger, C. Rordorf, Transplantation 73 (2002) 920.
[5] R.L. Doyle, M.I. Hertz, J.M. Dunitz, J.E. Loyd, A.A. Stecenko, R.L. Wong, K.A. Chappell, T. Brazelton, J.M. Kovarik, S. Appeldingemanse, L. Dou, H.T. Smith, D. Tudor, R.E. Morris, J. Lung Heart Transplant. 20 (2001) 330.
[6] B.D. Kahan, B. Kaplan, M.I. Lorber, M. Winkler, N. Cambon, R.S. Boger, Transplantation 71 (2001) 1400.
[7] H.H. Neumayer, K. Paradis, A. Korn, L. Fritsche, K. Budde, M. Winkler, V. Kliem, R. Pichlmayr, I.A. Hauser, K. Burkhardt, A.E. Lison, I. Barndt, A.S. Dingermanse, Br. J. Clin. Pharmacol. 48 (1999) 694.
[8] W. Jacobsen, N. Serkova, B. Hausen, R.E. Morris, L.Z. Benet, U. Christians, Transplant. Proc. 33 (2001) 514.
[9] J.M. Kovarik, C.H. Hsu, L. McMahon, S. Bethier, C. Rordorf, Clin. Pharmacol. Ther. 70 (2001) 247.
[10] J.M. Kovarik, H. Sabia, J. Figueiredo, H. Zimmermann, C. Reynolds, S.C. Dilzer, K. Lasseter, C. Rordorf, Clin. Pharmacol. Ther. 70 (2001) 425.
[11] P. Salm, P.J. Taylor, S.V. Lynch, P.I. Pillans, J. Chromatogr. B 772 (2002) 283.
[12] G.I. Kirchner, C. Vidal, M. Winkler, W. Jacobsen, A. Franzke, K.F. Sewing, Ther. Drug Monit. 21 (1999) 116.
[13] I. Segarra, T.R. Brazelton, N. Guterman, B. Hausen, W. Jacobsen, R.E. Morris, L.Z. Benet, U. Christians, J. Chromatogr. B 720 (1998) 179.
[14] K. Hallensleben, M. Raida, G. Habermehl, J. Am. Mass Spectrom. 11 (2000) 516.
[15] C. Vidal, G. Kirchner, G. Wunsch, K.F. Sewing, Clin. Chem. 44 (1998) 1275.
[16] F. Streit, V.W. Armstrong, M. Oellerich, Clin. Chem. 48 (2002) 955.
[17] FDA Guidance for Industry, Center for Drug Evaluation and Research, US Department for Health and Human Services, May 2001.
[18] M.A. Campanero, E. Cardenas, B. Sadaba, E. Garcia-Quetlas, M.J. Munoz-Juarez, I. Gil-Aldea, D. Pazo, J.R. Azanza, J. Honorato, J. Chromatogr. A 1031 (2004) 265.

[19] H.J. Eisen, E.M. Tuzcu, R. Dorent, J. Kobashigawa, D. Mancini, H.A. Valantine-von Kaeppler, R.C. Starling, K. Sorensen, M. Hummel, J.M. Lind, K. Abeywickrama, H.P. Bernhardt, N. Engl. J. Med. 349 (2003) 847.
[20] D. Cattaneo, N. Perico, F. Gaspari, J. Chromatogr. B 114 (2002) 187.
[21] K. Jones, A. Johnston, D.W. Holt, Clin. Ther. 22 (2000) 122.
[22] B.D. Kahan, K.L. Napoli, P.A. Kelly, J. Podbielski, I. Hussein, D.L. Urbauer, S.H. Katz, C.T. Van Buren, Clin. Transplant. 14 (2000) 97.
[23] J.M. Kovarik, B. Kaplan, H.T. Silva, B.D. Kahan, J. Dantal, L. McMahon, S. Berthier, C. Hsu, C. Rodorf, Am. J. Transplant. 3 (2003) 606.