ELSEVIER

Journal of Chromatography B, 720 (1998) 179–187

JOURNAL OF CHROMATOGRAPHY B

Development of a high-performance liquid chromatographic–electrospray mass spectrometric assay for the specific and sensitive quantification of the novel immunosuppressive macrolide 40-*O*-(2-hydroxyethyl)rapamycin

Ignacio Segarra[a], Timothy R. Brazelton[b], Natalya Guterman[a], Bernard Hausen[b], Wolfgang Jacobsen[a], Randall E. Morris[b], Leslie Z. Benet[a], Uwe Christians[a, b,*]

[a]*Department of Biopharmaceutical Sciences, University of California at San Francisco, San Francisco, CA 94143-0446, USA*
[b]*Laboratory of Transplantation Immunology, Department of Cardiothoracic Surgery, Stanford University Medical School, Stanford, CA 94305-5247, USA*

Received 12 March 1998; received in revised form 6 August 1998; accepted 10 September 1998

Abstract

It was our objective to develop a rapid, sensitive and specific assay to quantify the immunosuppressive macrolide 40-*O*-(2-hydroxyethyl)rapamycin (SDZ-RAD) in blood of transplant patients. SDZ-RAD was extracted from blood by solid–liquid extraction. SDZ-RAD and its internal standard 28,40-diacetyl rapamycin were quantified using HPLC–electrospray MS. The assay was linear from 0.1 to 100 μg/l (r^2=0.99). The mean recovery was 83% for SDZ-RAD and 80.5% for the internal standard. The mean day-to-day precision was 8.0%. Extracted samples were stable at 20°C for at least 48 h and SDZ-RAD blood samples at −80°C for at least six months.

Keywords: 40-*O*-(2-Hydroxyethyl)rapamycin

1. Introduction

SDZ-RAD [40-*O*-(2-hydroxyethyl)-rapamycin, structure see Fig. 1A) is a semi-synthetic derivative of the macrolide immunosuppressant sirolimus. It has been demonstrated that SDZ-RAD is a potent immunosuppressant in solid organ transplant and autoimmune disease models and inhibits proliferation of vascular smooth muscle cells [1,2]. The molecular mechanism underlying its immunosuppressive activity is similar to that of sirolimus [3] and different from that of the immunosuppressants tacrolimus and cyclosporine. In vitro in antigen driven human T-cell proliferation assays, the half-maximal inhibition concentration (IC_{50}) of SDZ-RAD was in the sub-nanomolar range and, depending on the assay, two- to three-times higher than that of sirolimus [1]. However, in vivo SDZ-RAD was at least as active as sirolimus [1]. Synergistic immunosuppressive activity of cyclosporine and SDZ-RAD was demonstrated in vitro and in animal transplant models in vivo [2]. SDZ-RAD is presently in phase II of its clinical development as an immunosuppressant in combina-

*Corresponding author. Tel: +1 415 5024968; Fax: +1 415 5028139. e-mail: uwec@itsa.ucsf.edu

0378-4347/98/$19.00
PII: S0378-4347(98)00426-5

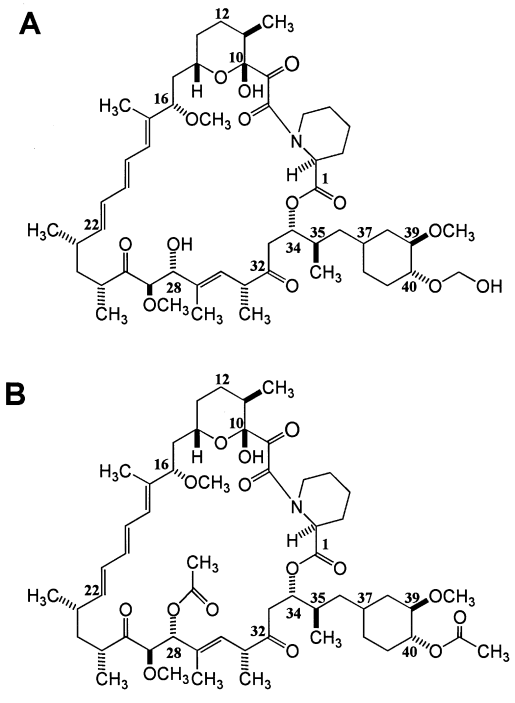

Fig. 1. Structures of SDZ-RAD [40-*O*-(2-hydroxy-ethyl) rapamycin] and the internal standard 28,40-diacetyl rapamycin. Numbering of the C-atoms follows the IUPAC guidelines [4].

tion with cyclosporine after solid organ transplantation.

SDZ-RAD is soluble in alcohols, acetonitrile, ethers, and halogenated hydrocarbons and practically insoluble in water and aliphatic hydrocarbons. Its triene group is responsible for an UV absorption maximum at 276 nm. SDZ-RAD doses effective to suppress an immune response against transplant organs are similar to those of the macrolide immunosuppressants tacrolimus and sirolimus, and blood concentrations in the low μg/l range can be expected.

Because of the low concentrations of macrolide immunosuppressants in blood of transplant patients, several high-performance liquid chomatography–mass spectrometry (HPLC–MS) assays have been developed for the quantification of sirolimus and tacrolimus in blood [5–8]. HPLC–UV assays have been described for sirolimus but their lower limits of quantitation are close to the concentrations expected in blood of patients 24 h after dosing (trough concentrations) [9–13]. In comparison to the HPLC–MS assays, HPLC–UV assays require extensive extraction procedures and relatively long run times to separate sirolimus from interferences.

Therefore, it was our objective to develop and validate a HPLC–MS assay for the quantification of SDZ-RAD in blood of transplant patients undergoing pharmacokinetic studies and for therapeutic drug monitoring during clinical trials.

2. Experimental

2.1. Materials and equipment

SDZ-RAD was the kind gift of Novartis Pharma AG (Basle, Switzerland) and rapamycin was purchased from Sigma (St. Louis, MO, USA). Stock solutions were prepared using acetonitrile–sulfuric acid, pH 3 (75:25, v/v) and stored at −80°C until use. Sulfuric acid (ACS grade) and HPLC-grade acetonitrile, methanol and methylene chloride were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid, sodium formate, and zinc sulfate (all ACS grade) were purchased from Sigma. Extraction columns (bonded phase C_{18}, 3 ml) were from Varian (Harbor City, CA, USA) and 250×4 mm analytical columns filled with Hypersil (Shandon, Chadwick, UK) C_8, 3 μm material from Keystone Scientific (Bellefonte, PA, USA). HPLC vials, 100-μl inserts and PTFE screw caps were obtained from Hewlett-Packard (Palo Alto, CA, USA).

Samples were analyzed on a Hewlett-Packard HPLC–electrospray MS system. The series 1100 HPLC system consisted of a G1322A degasser, a G1312A binary pump, a G1313A autosampler, and a G1316A column thermostat. The 59987A electrospray interface was equipped with an Iris Hexapole Ion Guide (Analytica of Branford, Branford, CT, USA) and connected to a 5989B mass spectrometer. The HPLC–MS system was controlled and data were processed using ChemStation software revision A04.02 for the HPLC system and C.03.00 for the electrospray interface and mass spectrometer (all Hewlett-Packard).

2.2. Blood samples

Blood for development and validation of the assay was drawn from healthy volunteers. EDTA was used as anticoagulant. Patients samples were obtained from stable lung transplant patients. These patients were enrolled in a SDZ-RAD pharmacokinetic study at Stanford Medical Center. The study and additional blood draws for the present study were approved by the Stanford University ethical committee. All subjects gave their written consent. The study was carried out following the good clinical practice (GCP) guidelines. During this study, stable lung transplant patients, whose immunosuppressive drug regimens were based on cyclosporine, received single SDZ-RAD doses of 0.1 and 0.35 mg/kg/d separated by a one week wash-out phase.

2.3. Internal standard

The internal standard, 28,40-diacetyl rapamycin (see Fig. 1B) was synthesized following the procedure described by Streit et al. [5]. The structure was verified using HPLC–MS–MS analysis in combination with collision induced dissociation and analysis of the fragment pattern [5]. Purity of the internal standard was established by HPLC–UV and HPLC–MS analysis.

2.4. Sample preparation

To 1-ml blood samples, 20 μl of 28,40-diacetyl sirolimus internal standard solution (2.25 mg/l in acetonitrile–sulfuric acid, pH 3, 75:25, v/v) and 2 ml methanol–0.2 mol/l $ZnSO_4$ (80:20, v/v) for protein precipitation were added. Samples were vortexed for 30 s and centrifuged at 1500 *g* for 3 min. The supernatant was loaded on C_{18} extraction cartridges by drawing the samples through the columns using −10 mmHg vacuum (1 mmHg=133.322 Pa). The extraction columns had previously been primed with 3 ml methanol and 3 ml sulfuric acid, pH 3. Samples were washed with 3 ml sulfuric acid pH 3 and dried by drawing air through the columns for 5 min. SDZ-RAD and its internal standard were eluted with 1.5 ml methylene chloride. Methylene chloride was evaporated under a stream of nitrogen and samples were reconstituted in 120 μl acetonitrile–0.1% formic acid (75:25, v/v). Samples were transferred into HPLC vials with conical 100-μl inserts and sealed with PTFE screw caps.

2.5. HPLC–MS analysis

One hundred μl of the sample was injected into the HPLC–MS system. SDZ-RAD and its internal standard were isocratically eluted from the analytical column. The mobile phase consisted of methanol–0.1% formic acid supplemented with 1 μmol/l sodium formate. The flow-rate was 0.4 ml/min and the column temperature was 45°C. The total run time was 16 min.

The electrospray source was set to the following parameters (parameter names as used in the ChemStation software): VCap: −5000 V, VEnd: −4500 V, VCyl: −6000 V, capillary exit voltage: 175 V. The drying gas was adjusted to a value of 42 (arbitrary units) and heated to 350°C. The pressure of the needle spray gas was 80 p.s.i. (1 p.s.i.=6894.76 Pa). The quadrupole was heated to 120°C. The mass spectrometer was run in the positive mode and the multiplier voltage was set to 1750 V and the X-ray voltage to 10 000 V. The mass spectrometer was run in the single ion mode. Since sodium adducts gave the strongest signals, the mass spectrometer was focused on m/z=980 ($[M+Na]^+$ of SDZ-RAD) and m/z=1020 ($[M+Na]^+$ of the internal standard) with a dwell time of 500 ms per ion. The RTE integrator option was used for quantification. Concentrations of SDZ-RAD were corrected using the internal standard and calculated using an SDZ-RAD calibration curve.

2.6. Method validation

2.6.1. Samples

Quality control (1.5, 15 and 40 μg/l), calibration samples (0.1, 0.25, 0.5, 1, 5, 10, 20, 50, 75 and 100 μg/l) and blank samples were prepared in bulk using freshly drawn blood. To allow distribution of SDZ-RAD, blood samples were incubated at 37°C in a water bath for 30 min. Hereafter, 1-ml aliquots were transferred into 10-ml glass centrifuge tubes and either immediately analyzed or kept frozen at −80°C.

2.6.2. Acceptance criteria

The assay was considered acceptable if precision (% C.V.) at each concentration was less than 15% for intra-day and day-to-day variability. The accuracy compared with the nominal value had to be within ±15% for both intra- and day-to-day. The calibration curve had to have a correlation coefficient r of 0.99 or better. The lower limit of quantitation had to be 0.25 μg/l or better. The absolute recovery had to exceed 60%.

2.6.3. Linearity

Six calibration samples of each concentration, prepared with blood as described above, were measured. Linearity was assessed using the regression analysis implemented in the Microcal Origin (version 3.5) software.

2.6.4. Lower limit of quantitation

The lowest concentration that met the following criteria was accepted as the lower limit of quantitation: 80% of the samples analyzed had to be within ±20% of the nominal value, and precision and accuracy had to be better than 20%.

2.6.5. Day-to-day and intra-day precision, accuracy

Intra-day precision and accuracy were evaluated from the results of the quality control samples processed the same day (n=9 for each concentration). Day-to-day variability was assessed by analysis of five sets of quality control samples (one set=three samples containing 1.5, 15 or 40 μg/l) on three different days (total: 15 sets of quality controls). Differences between the results of samples analyzed at different days were assessed using analysis of variance (GLM procedure, SAS version 6.05).

2.6.6. Recovery

Recoveries were calculated from the quality control samples (n=6 for each concentration). The mass spectrometer responses of the extracted samples were compared with the response after injection of respective amounts of internal standard or SDZ-RAD standard solutions (in acetonitrile–sulfuric acid. pH 3, 75:25, v/v). Recoveries of SDZ-RAD at various concentrations and the internal standard were compared using analysis of variance (GLM procedure, SAS).

2.6.7. Dilution integrity and partial volume verification

To establish dilution integrity, five blood samples containing 40 μg/l SDZ-RAD were diluted two-fold and five-fold using blood from healthy volunteers. Partial volume verification was conducted by extracting the partial volumes of 0.2 ml and 0.5 ml of six blood samples containing 40 μg/l SDZ-RAD. The deviations of the results from the nominal values in percent were calculated.

2.6.8. Specificity and interferences

The lack of matrix interferences was established by analysis of blank blood samples (n=12). To investigate a possible interference with SDZ-RAD metabolites or cyclosporine and its metabolites (AM1, AM9, AM1c, AM4N), these were added to blank blood samples before extraction resulting in a final concentration of 500 μg/l (n=4). SDZ-RAD metabolites (three hydroxylated, one demethylated metabolite) were generated by human liver microsomes following the procedure described by Christians et al. [14] for the in vitro generation of sirolimus metabolites. Cyclosporine metabolites were generated and isolated using a previously described method [15]. Extracted blood samples containing an SDZ-RAD concentration at the upper limit of quantitation (100 μg/l, n=6) and blank blood samples were alternately injected to exclude carry-over effects.

2.6.9. Stability

To establish benchtop stability, ten sets of quality control samples were prepared. Five sets were extracted and immediately analyzed (controls), the other five sets were stored at 20°C and analyzed 24 h later. In addition, samples containing 70 μg/l SDZ-RAD were prepared and analyzed immediately (controls) and after storage for 3 d and 7 d at 20°C (n=5). The results were compared with those of the controls by calculating the % deviation. Twenty-four sets of quality control samples were prepared to test freeze–thaw stability of SDZ-RAD. Six sets served as controls and were analyzed immediately. Six sets of samples were subjected to one freeze–thaw cycle,

six sets were subjected to two freeze–thaw cycles and another six samples to three freeze–thaw cycles. Samples were stored at −80°C and thawed by leaving them at room temperature for 2 h. During validation, the stability of the extracted quality control samples at −20°C was established for one week. Twelve sets of control samples were prepared. Six sets were extracted and analyzed immediately (controls). Six sets were kept at −20°C and analyzed one week later. Stability of SDZ-RAD and its internal standard in the autosampler (within-batch stability) was evaluated for 48 h. Ten sets of quality control samples were extracted and placed in the autosampler (+20°C). Five sets of samples were analyzed at once (controls), the other five sets 48 h later.

3. Results

SDZ-RAD and the internal standard were mainly detected as the sodium adduct $[M+Na]^+$. The relative intensities of $[M+H]^+$ and $[M+K]^+$ were less than 5% of the sodium adduct (Fig. 2). This ratio was independent from whether or not sodium formate was added to the mobile phase. The relative intensity of the $[M-H]^-$ using a mobile phase of pH 8.5 (0.0005% triethylamine) was five-fold less than that of the $[M+Na]^+$. When the capillary exit voltage was varied between 50 and 300 V in 25-V steps, the $[M+Na]^+$ was detected with the highest sensitivity at 175 V. Only little fragmentation was detected at this voltage (Fig. 2). SDZ-RAD eluted from the analytical column with a retention time of 9.85 min and the internal standard with a retention time of 10.5 min (Fig. 3).

3.1. Linearity, precision and accuracy

In blood, the assay was linear from 0.1 to 100 μg/l SDZ-RAD with $y=0.993\ (\pm 0.017)x+0.637\ (\pm 0.712)$ [mean (±standard deviation), $n=6$] with $r^2=0.99$. The lowest concentration matching the limit of quantitation (LOQ) acceptance criteria was 0.1 μg/l: 5/6 samples were within ±20% of the

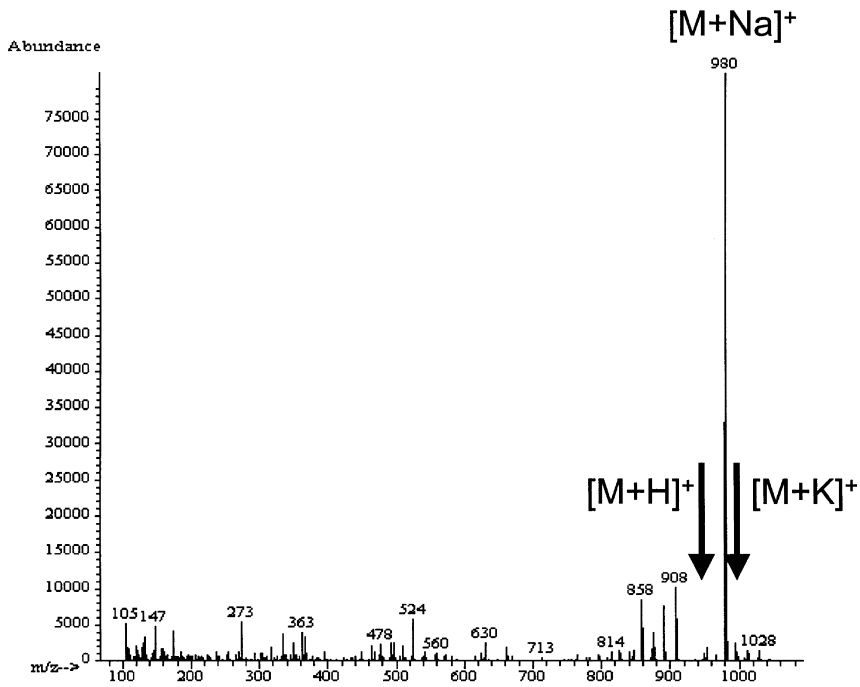

Fig. 2. Mass spectrum ($m/z=100$ to 1100) of SDZ-RAD. One μg SDZ-RAD in 10 μl methanol–formic acid, pH 4 (9:1, v/v) was injected onto the analytical column. The chromatographic and mass spectrometry conditions were the same as described in Section 2.5.

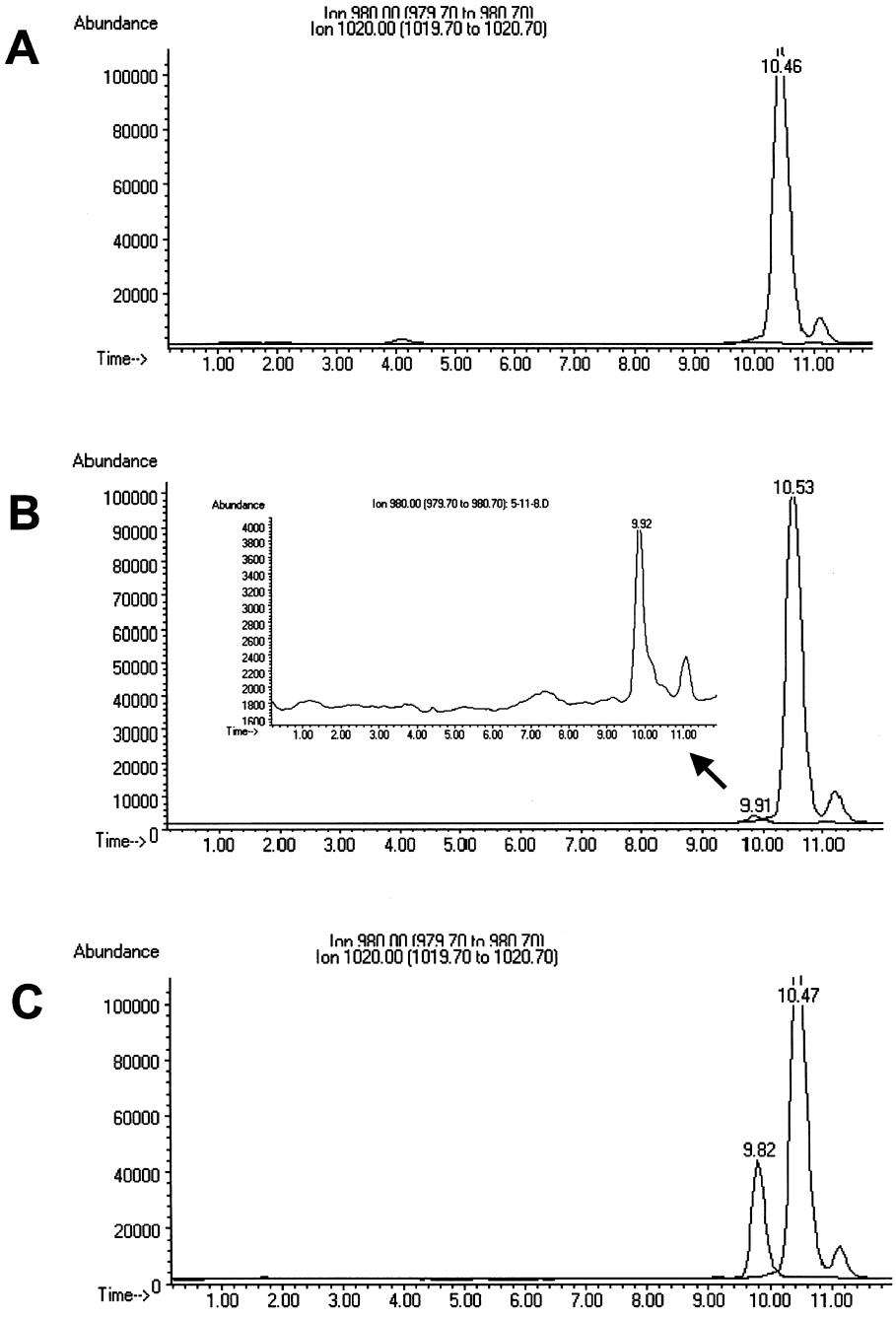

Fig. 3. Representative ion chromatograms of a blank blood sample (A), a blood sample to which 0.5 μg/l SDZ-RAD was added (B) and a blood sample from a lung transplant patient enrolled in a pharmacokinetic trial 24 h (trough concentration) after a single 0.01 mg/kg SDZ-RAD dose (C). The *x*-axes of the ion chromatograms show the time (min). The ion chromatograms of SDZ-RAD (m/z=980, mean retention time: 9.85 min) and the internal standard 28,40-diacetyl rapamycin (m/z=1020, mean retention time: 10.5 min) are overlaid. The concentration of the internal standard added to the blood samples was 45 μg/l. The insert in (B) shows an amplification of the SDZ-RAD ion chromatogram (m/z=980). The SDZ-RAD concentration in (C) is 13.3 μg/l.

nominal value. The C.V. (n=6) was 16.8% and accuracy+10%. A representative ion chromatogram of a blood sample containing an SDZ-RAD concentration at the LOQ is shown in Fig. 3B. Precision at the upper LOQ (100 μg/l) was 11.7% and the mean deviation from the nominal value −0.7%. The intra-day precision for blood samples containing 1.5 μg/l SDZ-RAD was 7.2% (n=9), for 15 μg/l 5.5%, and for 40 μg/l 6.2%. The mean deviation from the nominal value (accuracy) was +3.7% for 1.5 μg/l samples, +7.4% for 15 μg/l samples and +8% for 40 μg/l samples (n=9). On three different days (n=5 for each day and concentration) the results varied by 7.2% for 1.5 μg/l samples, by 10.8% for 15 μg/l samples and by 6.7% for 40 μg/l samples (day-to-day precision). Analysis of variance did not show any differences among the results obtained on different days.

3.2. Recovery

The mean (±standard deviation) recovery from blood was 91.7±6.4% (n=6) for 1.5 μg/l samples, 77.7±8.7% for 15 μg/l samples and 79.5±6.0% for 40 μg/l samples. The recovery of the internal standard from blood was 80.5±4.7% (n=18). Although analysis of variance showed a statistically significant difference (p<0.02) with the recoveries higher for the 1.5 μg/l samples than for the other SDZ-RAD concentrations and the internal standard in this batch of samples, this had no practical value as reflected by the acceptable accuracy in other batches of samples (vide supra). The mean recovery of SDZ-RAD, including all concentrations, of 83.0% was equal to that of the internal standard.

3.3. Dilution integrity and partial volume verification

The mean deviation from the nominal value of the two-fold dilution was −3.5±4.0% (mean±standard deviation, n=5), and that of the five-fold dilution −6.8±6.5%. The mean deviation from the nominal value of the 0.2 ml partial volume samples was −5.7±5.2% (mean±standard deviation, n=6), and that of the 0.5 ml samples −4.6±9.8%.

3.4. Specificity and interferences.

No peaks interfering with SDZ-RAD or the internal standard were present in blank blood samples (n=12, Fig. 3A). The SDZ-RAD metabolites were detected at m/z=996 ($[M+Na]^+$ hydroxy-SDZ-RAD) and at m/z=966 ($[M+Na]^+$ desmethyl-SDZ-RAD), had shorter retention times than SDZ-RAD or its internal standard and thus, did not interfere with the quantification of SDZ-RAD (m/z=980) or its internal standard (m/z=1020). Cyclosporine (m/z=1224, $[M+Na]^+$) and its metabolites AM1, AM9, AM1c (all m/z=1240 $[M+Na]^+$) and AM4N (m/z=1210, $[M+Na]^+$) did also not interfere. No carry-over effects were detected when alternately extracts from blank blood samples and blood samples containing 100 μg/l SDZ-RAD and its internal standard were analyzed.

3.5. Stability

SDZ-RAD and internal standard stock solutions (in acetonitrile–sulfuric acid, pH 3, 75:25, v/v) could not be stored long-term in a refrigerator (+4°C). After two weeks, the concentrations of a 0.1 mg/l SDZ-RAD stock solution was 15.3%, that of a 1 mg/l SDZ-RAD stock solution 13.5% and that of the internal standard solution (2.25 mg/l) 15.2% lower than those of freshly prepared solutions (n=3). Stock solutions were stable for at least six months when stored at −80°C.

After storage at room temperature for 24 h, the mean deviation of 1.5 μg/l blood samples from their immediately extracted and analyzed controls was +2.7% (n=6), that of the 15 μg/l samples −11.5% and that of the 40 μg/l samples −11.6%. When blood samples containing 70 μg/l were kept at 20°C, after two days the mean deviations from the controls were −0.9% and after seven days+3.3% (n=5).

After one freeze–thaw cycle, the mean deviations of 1.5 μg/l samples from unfrozen controls were +6.3% (n=6), −7.8% (two freeze–thaw cycles), and −4.8% (three freeze–thaw cycles), that of the 15 μg/l samples +3.0% (one freeze–thaw cycle), −2.4% (two freeze–thaw cycles), and −5.7% (three freeze–thaw cycles), and that of the 40 μg/l samples −4.0% (one freeze–thaw cycle), −0.6% (two freeze–thaw cycles), and −14.0% (three freeze–

thaw cycles). SDZ-RAD blood samples could be exposed to at least three freeze–thaw cycles. Blood samples stored at −80°C were stable for at least six months.

After storage of extracted samples at −20°C for one week, the mean deviations from immediately analyzed controls were −1.3% for the 1.5 μg/l, +10.5% for the 15 μg/l, and −4.1% for the 40 μg/l samples ($n=6$). Extracted samples in the autosampler (20°C) were stable for at least 48 h. The mean deviation of the 1.5 μg/l samples from their controls was −8.6%, that of the 15 μg/l samples −8.7% and that of the 40 μg/l samples −14.4% ($n=6$).

As of today, this HPLC–MS assay was used to quantify SDZ-RAD in more than 2000 blood samples. A representative ion chromatogram of a blood sample from a lung transplant patient enrolled in a SDZ-RAD single dose pharmacokinetic study is shown in Fig. 3C.

4. Discussion

For measurement of rapamycin in blood, several HPLC–UV assays have been described [9–12]. We developed an HPLC–MS rather than an HPLC–UV assay for the following reasons: the expected concentrations in pharmacokinetic studies, especially at later times after drug administration which are critical for determination of the terminal half-life, are below the LOQ of HPLC–UV assays. Our SDZ-RAD HPLC–MS assay met all predefined acceptance criteria. Following the recommendations of most recent consensus documents on therapeutic drug monitoring of macrolide immunosuppressants [13,16], blood rather than plasma was chosen as the matrix for our SDZ-RAD assay. The more specific detection by a mass spectrometer compared with UV detection allowed for simpler and less time-consuming sample preparation, and, since separation of SDZ-RAD ($m/z=980$) and its internal standard ($m/z=1020$) was not required, significantly shorter run times and a higher sample turn-over. The composition of the protein precipitation reagent was critical for recovery. Reduction of the methanol contents by as little as 10% resulted in unacceptably low recoveries <50%. SDZ-RAD and its internal standard were eluted from the extraction columns using methylene chloride. Due to the relative instability of SDZ-RAD and its internal standard, fast evaporation within a few minutes without application of elevated temperatures was critical. Elution of SDZ-RAD using the mobile phase avoiding an evaporation step as described for a sirolimus HPLC–MS assay [5] resulted in significantly lower recoveries of 50% and less. Although addition of sodium ions had no effect on the relative intensity of $[M+Na]^+$, 1 μmol/l sodium formate was added to the mobile phase to keep the sodium concentration constant. The use of an internal standard was necessary to compensate for losses during extraction, in-process instability of SDZ-RAD and for decreased sensitivity of mass spectrometric detection due to contamination of the electrospray target.

Stability has been a major problem of rapamycin [17] and, due to structural similarities, could also be expected to be critical for SDZ-RAD sample handling and measurement. From our results, it was concluded that SDZ-RAD blood samples are stable for more than six months if stored at −80°C and can be thawed and frozen at least three times. Extracted samples during analysis were stable for at least 48 h and extracted samples stored at −20°C for at least two weeks. SDZ-RAD and the internal standard were most stable in a solvent containing more than 10% water with a pH<5. In pure organic solvents, both compounds had a significantly shorter half-life. However, for long-term storage, stock solutions had to be kept at −80°C. SDZ-RAD was more stable in blood samples, up to one week at +20°C. It can be hypothesized that in blood samples binding to immunophilins stabilized SDZ-RAD. Thus, SDZ-RAD blood samples do not have to be frozen when shipped from the clinical sites to analytical laboratories for therapeutic drug monitoring.

More than 80 samples were extracted and analyzed a day. The HPLC column was stable for more than 1000 samples. Emergency samples were analyzed within 1 h and in a concentration-controlled trial, results were returned the same day. Our method can easily be automated by using column switching for sample preparation [18] after the protein precipitation step as described by Vidal et al. [19]. However, this latter assay [19] does not use an internal standard, which, for the reasons discussed above, is a serious disadvantage.

With a few modifications, our HPLC–MS assay can be used to also quantify SDZ-RAD metabolites and, as described for sirolimus [20], concomitantly quantify cyclosporine and its metabolites in the same run.

Acknowledgements

This study was supported by NIH grant GM 26691 (I.S.), and the Deutsche Forschungsgemeinschaft grant Ch95/6-1 (U.C.).

References

[1] 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.
[2] H.-J. Schuurman, S. Cottens, S. Fuchs, J. Joergensen, T. Meerloo, R. Sedrani, M. Tanner, G. Zenke, W. Schuler, Transplantation 64 (1997) 32.
[3] T.R. Brazelton, R.E. Morris, Curr. Opinion Immunol. 8 (1996) 710.
[4] IUPAC Nomenclature of Organic Chemistry, A Guide To IUPAC Nomenclature of Organic Components (recommendations 1993, Blackwell Scientific Publications, Boston, MA, 1993.
[5] F. Streit, U. Christians, H.M. Schiebel, K.L. Napoli, L. Ernst, A. Linck, B.D. Kahan, K.F. Sewing, Clin. Chem. 42 (1996) 1417.
[6] P.J. Taylor, N.S. Hogan, S.V. Lynch, A.G. Johnson, S.M. Pond, Clin. Chem. 43 (1997) 2189.
[7] A.K. Gonschior, U. Christians, M. Winkler, H.M. Schiebel, A. Linck, K.F. Sewing, Ther. Drug Monit. 17 (1995) 504.
[8] A.M. Alak, S. Moy, M. Cook, P. Lizak, A. Niggebiugge, S. Menard, A. Chilton, A. Chilton. J. Pharm. Biomed. Anal. 16 (1997) 7.
[9] J.O. Svensson, C. Brattström, J. Säwe, Ther. Drug Monit. 19 (1997) 112.
[10] K.L. Napoli, B.D. Kahan, Clin.Chem. 42 (1996) 1943.
[11] R.W. Yatscoff, C. Faraci, P. Bolingbroke, Ther. Drug Monit. 14 (1992) 138.
[12] K.L. Napoli, B.D. Kahan, J. Chromatogr. B 654 (1994) 111.
[13] R.W. Yatscoff, R. Boeckx, D.W. Holt, B.D. Kahan, D.F. Legatt, S. Sehgal, S.J. Soldin, K.L. Napoli, C. Stiller, Ther. Drug Monit. 17 (1995) 676.
[14] U. Christians, M. Sattler, H.M. Schiebel, C. Kruse, H.H. Radeke, A. Linck, K.F. Sewing, Drug Metab. Dispos. 20 (1992) 186.
[15] U. Christians, S. Strohmeyer, R. Kownatzki, H.M. Schiebel, J. Bleck, K. Kohlhaw, R. Schottmann, K.F. Sewing, Xenobiotica 21 (1991) 1199.
[16] W. Jusko, A.W. Thomas, J. Fung, P. McMaster, S.H. Wong, E. Zylber-Katz, U. Christians, M. Winkler, W.E. Fitzsimmons, R. Lieberman, J. McBride, M. Kobayashi, S.J. Soldin, Ther. Drug Monit. 17 (1995) 606.
[17] P.C. Wang, K.W. Chan, R.A. Schiksnis, J. Scatina, S.F. Sisenwine, J. Liq. Chromatogr. 17 (1994) 3383.
[18] R. Huber, K. Zech, in: R.W. Frei, K. Zech (Eds.), Selective Sample Handling and Detection in High-Performance Liquid Chromatography (Journal of Chromatography Library, Vol. 39A), Elsevier, Amsterdam, 1988, pp. 81–144.
[19] C. Vidal, G. Kirchner, K.F. Sewing, Ther. Drug Monit. 19 (1997) 557.
[20] I. Segarra, L.C. Floren, K.L. Napoli, B.D. Kahan, L.Z. Benet, U. Christians, Pharm. Res. 14 (1997) S268.