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Assessment of sirolimus concentrations in whole blood by high-performance liquid chromatography with ultraviolet detection

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Abstract

A novel, reversed-phase high-performance liquid chromatographic (HPLC) method is described for the analysis of sirolimus (SRL) in whole blood. The samples were purified by precipitating blood matrix with zinc sulfate, SRL was then extracted with acetone followed by solid-phase extraction. The method was linear over a range of 1–100 ng/ml and the lower limit of quantification was 2.5 ng/ml. The coefficient of variation (within day) was below 8.0% for the lowest SRL concentration. The day-to-day coefficient of variation was below 6.6%. The assay did not show interference peaks with immunosuppressive drugs commonly given to transplant patients. With the simplified extraction procedure described, 60 samples (including controls and calibration curve) can be quantified in a day. The sensitivity and rapidity of this analytical procedure makes it useful for routine therapeutic monitoring of SRL.

Keywords: Sirolimus

1. Introduction

Sirolimus (Rapamycin, SRL), a 31-membered triene macrolide lactone with a hemiketal-masked α,β-dioxocarboxamide and a molecular mass of 913.6 Da, is a novel antirejection drug with potent immunosuppressive activity both in vitro and in vivo [1–5]. Studies in humans have shown a relationship between trough blood SRL concentrations and immunosuppressive efficacy and toxicity of the drug [6–8]. This suggests that monitoring of the SRL concentrations is advisable to optimize the drug dosing regimen. Since the drug has a narrow therapeutic index, it is important to develop an adequate method to quantify SRL in biological matrices. SRL is extensively distributed in red blood cells, independently of concentration and temperature, so whole blood should be the preferred matrix for therapeutic drug monitoring [5,9].

Several high-performance liquid chromatographic (HPLC) methods have been developed so far for quantifying SRL in whole blood with extraction steps using a variety of solvents and mixtures [10–13], or with liquid–liquid and subsequent solid-phase extraction [14]. These HPLC methods used both ultraviolet (UV) [10–14] and mass spectrometry

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(MS) detection (HPLC–MS or HPLC–MS–MS) [15,16].

Although these methods meet many or all generally accepted criteria for validated analysis of immunosuppressive drugs, they are often cumbersome, have a very long sample preparation and/or chromatographic time, require glassware preparation (silanized or light protected tubes), use toxic solvents (i.e. 1-chlorobutane) or involve equipment (HPLC–MS) not commonly available in the clinical laboratory that need great investment. Therefore, given the increasing use of SRL in clinical transplantation, there is now need for more feasible methods to be applied in the routine clinical practice which also would allow simultaneous measurement of a large number of samples. Furthermore, for optimal patient management, it is advisable to reduce the time between drawing a blood sample and the availability of the final drug concentration value.

Here we describe an accurate, sensitive, precise and rapid HPLC method that overcomes the problems related to previously published assays.

2. Experimental

2.1. Reagents

SRL and 32-*O*-desmethoxyrapamycin (internal standard, I.S.) were generous gifts from Wyeth-Ayerst Research Laboratories (Princeton, NJ). Stock solutions containing 50 and 100 μg/ml were appropriately prepared in methanol for SRL and I.S., respectively. SRL working solutions of 100, 500, and 2000 ng/ml were prepared in 50/50 methanol–water and for I.S. a working solution of 1000 ng/ml was prepared in methanol. All the solutions were stored at −20 °C; stock solutions were stable for at least 24 months and working solutions for at least 2 months. Acetonitrile and methanol (BDH, Milan, Italy) were HPLC grade; acetone and hexane HPLC grade were supplied by Fluka (Milan, Italy). Zinc sulfate heptahydrate, analytical grade, was purchased from Fluka and a 5% solution was prepared in distilled water. All other chemicals were analytical grade.

Bond-Elut C_{18}, 200 mg, 3-ml cartridges were obtained from Varian (Leinì, Italy).

2.2. Sample preparation

One millilitre volume of whole blood sample was pipetted into disposable polypropylene tubes and added to with 50 μl of I.S. 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. The tubes were vortex-mixed for a further 50–60 s and centrifuged at 3000 *g* for 5 min at room temperature. The clear supernatant was poured into another polypropylene tube, added to with 2 ml distilled water, mixed and loaded onto a Bond-Elut cartridge (preconditioned with 1 ml acetonitrile followed by 1 ml methanol and finally by 1 ml distilled water) placed on a Vac Elut 20 Manifold (Varian). The Bond-Elut cartridges were washed with 1.5 ml of 70% methanol–30% water. In each step the solvent was allowed to drop out from the cartridge. Then 500 μl hexane were added and the column was allowed to go dry under vacuum. SRL and I.S. 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) and the residue was dissolved in 150 μl of water–methanol–acetonitrile (40/30/30) and transferred in a polypropylene vial. Internal calibration curves for SRL were prepared for each set of samples.

At least 60 samples (including controls and calibration curve) can be extracted in 4 h and processed by HPLC in less than 20 h.

2.3. Chromatographic equipment

A System Gold HPLC equipped with a model 166 UV detector set at 278 nm and a model 508 autosampler (Beckman, Fullerton, CA) with the sample tray kept at 4 °C, were used. A 90-μl aliquot of sample was injected onto reversed-phase C_{18}, 5 μm, guard column (Alltima, 7.5×4.6 mm, Alltech, Sedriano, Milan, Italy) connected to a 75×4.6 mm column packed with Ultrasphere C_8, 3 μm (Beckman) heated at 50 °C by a Model 880 oven (Spark-Holland, Emmen, The Netherlands) and was eluted by a mixture of distilled water–methanol–acetonitrile (34/30/36) pumped at a rate of 1 ml/min. Due

to the high percentage of the organic phase that may dry off, resulting in increased retention time, the mobile phase was prepared every 1 or 2 days before analysis, filtered and degassed under vacuum using a polycarbonate 0.4-μm membrane. An in-line filter (0.5 μm) was placed between the autosampler and the column.

2.4. Method validation

The linearity of our method was tested by constructing a curve from 1 to 100 ng/ml (adding 1, 2.5, 5, 10, 15, 20, 40, 60, 80 and 100 ng of SRL, followed by 1 ml each of SRL-free whole blood). The linearity was established by linear regression of the peak height ratio of the drug/I.S. versus the concentration.

Taking into account the therapeutic range of SRL trough levels, we used a calibration curve of seven points ranging from 2.5 to 60 ng/ml. This calibration curve was routinely used in any instance the assay was performed. Calibrator pools of 2.5, 5, 10, 15, 20, 40 and 60 ng/ml were prepared by diluting each spiking solution to 10 ml with K_3EDTA control human whole blood in 10-ml volumetric flasks. The flasks were stoppered and shaken to mix. Pools are measured into 1-ml aliquots in polypropylene tubes and frozen at −80 °C until use. The overall recovery for SRL and I.S. was calculated by comparing the peak-height ratios of spiked samples with those obtained by direct injections of the same amount of both compounds. The intra-assay precision was verified at five concentrations (2.5, 5, 10, 20, and 40 ng/ml) for five analyses. The inter-day precision was determined at the same five concentrations in five replicates. The inaccuracy was calculated as the percentage error from the true value.

The present method has been validated by a reference laboratory in the UK (Dr. D.W. Holt, St. George's Hospital Medical School, London) that has established an international proficiency testing control system for sirolimus [17]. According to this centre, less than 20% error of the aforementioned method is considered acceptable. For the proficiency test, initially 78 blinded samples, packaged as five batches of samples each, were analyzed. In addition, ongoing proficiency was tested by analyzing three blinded samples from the Reference Laboratory every month.

3. Results

3.1. Chromatographic separation, calibration and recovery

Fig. 1 displays the chromatograms of extracts prepared from a blank blood spiked with known amounts of SRL and from blood of a transplant patient given SRL. The retention time of SRL and I.S. were 13.1 and 14.5 min, respectively, and the chromatographic step required 18 min per sample. No late eluting peaks were found either in blank blood specimens or in blood taken from kidney, liver, and heart transplanted patients. Both SRL and I.S. eluted as symmetrical and relatively sharp peaks (panel A). Blank blood samples did not show peaks corresponding to SRL and I.S. retention times. This was confirmed in 50 healthy volunteers as well as in five kidney and in five liver transplant recipients in an SRL-free regimen. Representative chromatograms of these blank samples are shown in Fig. 1 panel B. On the other hand, a concentration-dependent increase in the SRL peak height was documented when known amounts of the drug were added to blood samples to achieve a final concentration of 2.5, 5, and 40 ng/ml, respectively (panels C–E). Moreover, a distinct peak of SRL was found in the blood sample collected from a kidney transplant patient given SRL as a part of the immunosuppressive therapy. Furthermore, no chromatographic interference was found between SRL or I.S. and other immunosuppressants such as cyclosporine, mycophenolic acid, azathioprine and steroids as shown when blood samples from transplanted patients were added in vitro with SRL, or when blood from healthy subjects was spiked in vitro with SRL and immunosuppressants. Even high concentration of the above mentioned drugs did not affect the SRL chromatographic profiles.

Occasionally a sharp peak occurred in some chromatograms at about 10.5–11 min—which did not affect the assay's performance—probably dependent on the different lot of Bond-Elut cartridges used.

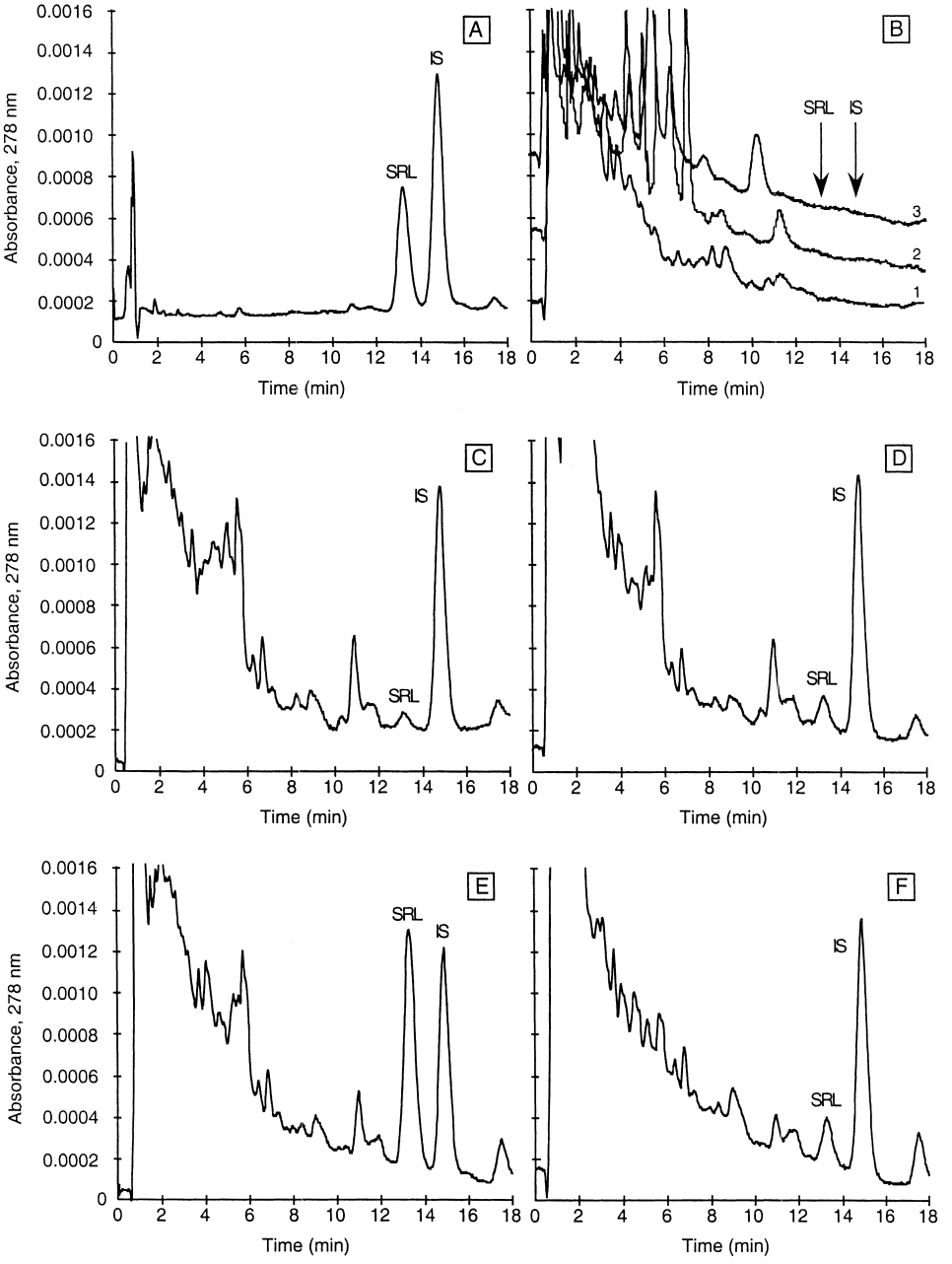

Fig. 1. Typical chromatograms of (A) sirolimus (SRL) and internal standard (I.S.); (B) drug-free blood taken from a healthy volunteer (1), a kidney (2) and a liver (3) transplant patient; (C, D, E) blood spiked with 2.5, 5, 40 ng/ml SRL, respectively; (F) a trough blood sample (7.1 ng/ml) taken from a kidney transplant patient immediately before the next oral dose of SRL.

Linearity was determined by least-squares linear regression analysis of the peak height of SRL/I.S. versus SRL concentration. The method demonstrated excellent linearity over the range of 1–100 ng/ml ($y = 0.02474x + 0.00053$, $r = 0.999$).

Lower limit of detection (LLOD), defined as a

signal-to-noise ratio ≥3, was 1 ng/ml (mean 1.2±0.3, C.V. 19.6%, inaccuracy 22.7%, $n=5$). Although the imprecision and inaccuracy are acceptable according to the International Proficiency Testing Scheme (a value lower than 25% is accepted for samples ≤7.5 ng/ml), we decided to set the lower limit of quantification (LLOQ)—defined as the lowest concentration of the drug that could be assayed with a good level of precision and inaccuracy—at 2.5 ng/ml. This is a suitable value to detect accurately the expected low SRL trough concentration in transplant patients.

Linear regression of the peak height ratio of the drug/I.S. versus the concentration for calibration curves (seven points ranging from 2.5 to 60 ng/ml) produced a correlation coefficient greater than 0.998. A typical equation describing the calibration curve was $y = 0.02358$ (SE: 0.00015)$x + 0.02075$ (SE: 0.00313).

The overall recovery, calculated by comparing the peak-height ratios of spiked samples with those obtained by direct injections of the same amount of SRL and I.S., was checked at 5 and 20 ng/ml. The recovery from blood precipitation with zinc sulfate was 71.1±2.9% for SRL and 70.0±3.3% for I.S., whereas that from reversed-phase extraction by using Bond-Elut cartridges was 86.0±3.2% for SRL and 85.9±2.8% for I.S. Thus, the overall extraction recovery was 61.1±3.1% for SRL and 60.1±2.1% for I.S.

3.2. Precision and inaccuracy of the assay

The within-day and between-day coefficients of variation for SRL in whole blood are reported in Table 1. The within-day precision of the assay, as estimated by the coefficient of variation of the measurement, was below 8.0%. The between-day coefficient of variation was still low (6.6%).

The inaccuracy of the method was low and, as expected, data were better for the highest SRL concentration tested (40 ng/ml). The result of inaccuracy obtained with the lowest concentration of the drug (2.5 ng/ml) was still acceptable (8.8%). The precision of our method was good also when replicates from a patient sample were considered (mean 7.7±0.4 ng/ml; C.V. 5.7%, $n=5$).

The minimal overlapping between SRL and I.S. peaks observed when concentrations higher than 40 ng/ml were analyzed did not influence the accuracy of the analysis. This was confirmed by injecting a lower volume (40 μl) of the same sample at two concentration levels. The complete separation between the analytes was restored and results were similar (40 μl injection: 39.4±1.1 ng/ml; 90 μl injection: 39.8±1.0 ng/ml; 40 μl injection: 80.7±5.5 ng/ml; 90 μl injection: 82.5±4.7 ng/ml).

The extraction procedure of the samples was tested with different blood volumes (ranging from 300 to 600 μl) at 7.5 ng/ml (mean 7.9±0.4 ng/ml; $n=5$) and 22.5 ng/ml (mean 22.0±0.7; $n=5$). In each instance we obtained an acceptable precision (C.V. less than 6.0%). These results suggest that there is no need for additional changes in the method protocol when a small volume of samples are available.

3.3. Application to human pharmacokinetic study

The assay was then assessed in vivo on a full SRL

Table 1
Reproducibility of the HPLC method

	SRL concentration (ng/ml)				
	2.5	5	10	20	40
Within-day assay					
Mean±SD (ng/ml)	2.7±0.2	5.0±0.4	10.3±0.2	19.1±0.7	39.3±0.6
Precision (C.V., %)	7.4	8.0	1.9	3.7	1.5
Inaccuracy (%)	8.0	0.0	3.0	−4.5	−1.8
Between-day assay					
Mean±SD (ng/ml)	2.7±0.2	5.0±0.2	10.3±0.3	18.8±0.5	42.3±0.8
Precision (C.V., %)	6.6	3.1	2.7	2.7	1.8
Inaccuracy (%)	8.8	−0.8	3.0	−5.8	5.7

Each observation $n=5$.

pharmacokinetic profile after the morning oral administration of 2 mg/day of the drug in a kidney transplanted patient with stable renal function (serum creatinine 1.4 mg/dl) and on triple immunosuppressive therapy with cyclosporine, SRL and steroid (Fig. 2). He was shifted for safety reasons from azathioprine to SRL just a week before the pharmacokinetic analysis. From a trough level of 4.5 ng/ml, the SRL blood concentration rapidly increased to reach a peak value at 2 h after dosing. Thereafter, a progressive slow reduction of the drug concentration value in the blood was found up to the next SRL dosing 24 h later.

4. Discussion

Pharmacokinetic studies in humans often involve analysis of a large number of samples and therefore require simple, rapid and reliable analytical methods. Furthermore, daily analysis of an increasing number of drugs as part of the routine drug monitoring activity, urges the need for HPLC methods that can be easily run and rapidly set up. Quantification of SRL in biological matrices is not easy, given the very low dose of the drug routinely employed that reflects its high potency. Moreover, the assay may be affected by several variables including the recovery of the analyte during sample processing and potential interfering peaks in the chromatograms. This explains the quite high inaccuracy value (20%) for the assay which is accepted, even by the international reference laboratory for proficiency testing of SRL [17]. Moreover, the ''gold standard'' HPLC–MS methods are superior to HPLC–UV methods, but however do come at a prohibitive financial cost.

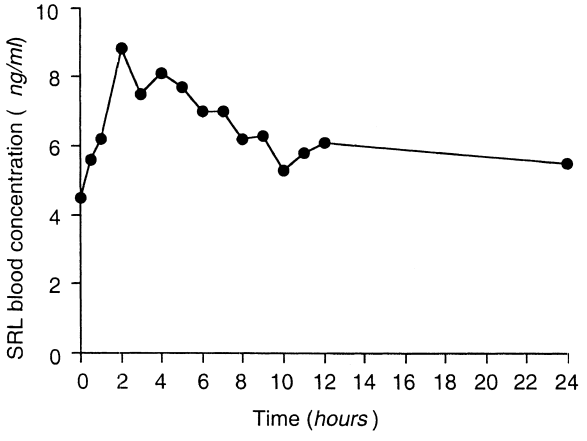

Fig. 2. Pharmacokinetic profile in whole blood of a kidney transplant recipient given SRL (2 mg/day) as a part of the immunosuppressive therapy.

Here, we developed a new method for SRL determination in whole blood specimens, which is robust enough to be run on standard HPLC equipment, using only basic analytical reagents. The assay has adequate sensitivity, precision and accuracy for therapeutic monitoring of the drug in transplant patients. Taking into account the sample preparation step and the chromatographic time, 60 samples (including controls and calibration curve) per day can be extracted and analyzed by a single technician, using two Vac Elut Manifold chambers.

This method overcomes the analytical difficulties and the long time required for sample preparation described in the previously published procedures [10,11,14,18] together with the need for use special (light protected) or treated (silanized) glassware [11,14,18].

Extraction at the high pH used in the previously published methods [12,13] gave isomerization of SRL and internal standard [19]. This could be a problem because chromatography must separate SRL isomer C from internal standard isomer B. Our method works in mild acidic condition (pH 6) and isomerization is negligible at this pH [14]. An additional advantage is the limited use of toxic organic solvents such as 1-chlorobutane, methyl *tert*.-butyl ether [12,13,16], that are also not commonly available in the laboratory basic armamentarium.

Bond-Elut cartridges with 200 mg of sorbent allowed the use of very small volumes for either cleaning-up and elution steps, thus speeding up the solid–liquid extraction step and further reducing the time of the SRL measurement. The extraction procedure of the samples can also be successfully applied to blood volumes ranging from 300 μl to 1 ml, without any additional change in the method protocol.

Extracted samples need to be kept at 4 °C in the autosampler tray for analysis. Indeed when samples were allowed to stand at room temperature for 12–20 h, a partial degradation of the I.S. became evident.

The mobile phase of the chromatographic step included methanol which contributes to sharpen the peaks, and the use of the C_8 column substantially shortened the time of analysis. Although a complete chromatographic separation between SRL and internal standard (I.S.) peaks was obtained only for blood concentrations lower than 40 ng/ml (which largely cover the proposed SRL trough level of 5–15 ng/ml [5,8,20]), concentration values at least up to 80 ng/ml—which encompass the range of expected concentration for complete pharmacokinetic profile—were also accurately determined.

Analytical C_8 columns can derive from different lots that theoretically could affect the reproducibility of the method. To exclude this possibility, the chromatographic run was also confirmed using a C_8 column belonging to different lots, providing evidence of the validity and robustness of our method. A single C_8 column did not lose its efficiency after injecting more than 400 samples.

In the chromatographic apparatus a short guard column (7.5×4.6 mm) was included that markedly improved the chromatographic run. Both the C_8 and C_{18} guard column types were tested, but overall the best chromatographic results were achieved with the latter. Indeed, despite a similar separation of SRL and I.S. peaks with the two types of guard column, the less polar C_{18} gave a retention time for both components of about 1 min longer than the C_8 type column.

Sirolimus is metabolized by CYP3A4 to demethylated and hydroxylated metabolites, which might interfere with the peak of the parent compound during the chromatographic run. However, these metabolites are much more polar and have a shorter retention time on reverse phase HPLC (Wyeth Lederle Italia, personal communication) which excludes this possibility.

The proposed HPLC method has been also successfully used to determine the whole blood pharmacokinetic profile in a kidney transplant patient receiving SRL as a part of the immunosuppressive therapy with cyclosporine and prednisone. The whole pharmacokinetic profile was consistent with that previously obtained with other proposed, but more complex HPLC methods [14,18]. However, the quite low trough and maximum peak blood SRL concentration we found in this particular patient, despite the therapeutic dose of the drug employed (2 mg/day), can be due to the poor absorption of the drug in the first days after starting SRL treatment as previously documented [9,20].

In summary, this report represents a step forward for a simple, rapid and sensitive HPLC method to determine SRL concentration in the whole blood. The simplicity and rapidity of sample handling, and the analytical specificity for SRL make this procedure useful for routine therapeutic monitoring of the drug as well as for pharmacokinetic studies, even in particularly not well equipped institutions.

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