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Therapeutic drug monitoring for sirolimus in whole blood of organ transplants by high-performance liquid chromatography with ultraviolet detection

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Abstract

We developed and validated an accurate, sensitive, precise and rapid HPLC method with UV detection for the determination of sirolimus in blood samples from renal, cardiac and hepatic transplants. This method overcomes most of the problems related to previously published assays using a narrow-bore column with base deactivated C_{18} reversed phase. Whole blood samples were purified by a combination of a precipitating blood matrix with zinc sulphate and a single step liquid–liquid extraction with acetone and 1-chlorobutane. Calibration curves (range 2.5–150 ng/ml), were linear with coefficients of correlation better than 0.996. The relative standard deviation was determined to be less than 8%. The present method has also been validated by a reference laboratory (St. George's Hospital Medical School, London, UK). More of 300 clinical samples have been analysed with this method.

Keywords: Validation; Sirolimus; Rapamycin; Lactones; Demethoxyrapamycin

1. Introduction

Sirolimus, formerly known as rapamycin, is a macrocyclic lactone obtained by fermentation of *Streptomyces hygroscopicus*. Although first developed as an antifungal agent, the drug was later found to have potent antiproliferation and immunosuppresive properties [1]. It has up to 100-fold greater potency than cyclosporin A and equivalent potency to the structurally similar immunosuppressant tacrolimus. Sirolimus is effective alone or administered in combination with other immunosuppresive agents, such as cyclosporin A. Moreover, this immunosuppresive drug shows amore adequate toxicity profile than other immunosuppresive agents, without nephrotoxicity, neurotoxicity, and hepatoxicity [2]. It was approved in mid-September, 1999, by the US Food and Drugs Administration (FDA) for renal transplants. However, the lower incidence of side effects observed after its administration in comparison with other immunosuppressants brought sirolimus suitable for the treatment of the rejection in other human organ transplants.

As many immunosuppresive agents sirolimus is candidate for therapeutic drug monitoring (TDM), because there are serious consequences of both undermedication (transplant rejection) and overdosing (infection and toxic side effects) situations. TDM of sirolimus acquire relevance for two main reasons: (1) the possibility of pharmacokinetic interactions when it is concomitantly administered with cyclosporine, and (2) intrapatient and interpatient variability in the absorption and metabolism of this drug [3]. In 1995, a panel of experts published consensus guidelines for the TDM of sirolimus. Some of the most important recommendations of the guidelines were: (1) sirolimus metabolites have been shown to have no significant pharmacologic activity and thus should not be included in sirolimus measurements, (2) the detection sensitivity limit must not be over 2 μg/l, (3) a same-day turnaround is optimal, and (4) whole blood must be the matrix of choice for the measurement of sirolimus because around 95% of this drug is bonded to red

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blood cells. Ethylendiaminetetra-acetic acid (EDTA) is the preferred anticoagulant to minimise clotting problem [4].

Chemically, sirolimus is a white crystalline solid readily fat soluble and practically insoluble in water. The ultraviolet absorption spectrum shows a major peak at 277 nm and minor peaks at 267 and 288 nm. The compound is also sensitive to light and temperature [5].

Different analytical approaches were employed in the determination of sirolimus in blood patient samples. In general, for TDM of transplant patients it would be desirable to have a sensitive and specific analytical method that involve an automated and fast sample preparation step and with a high sample turnover. Currently, several commercially available immunoassays such as fluorescence polarization immunoassay, microparticle enzyme immunoassay (MEIA) and enzyme immunoassay technique and immunophilin-binding assay (RRA) fulfil these requirements and are employed for TDM of some immunosuppressants. However, no such method is available for TDM of sirolimus. Preliminary studies showed that MEIA offered inacceptable imprecision and analytical performance with overestimation of 42 and 20%, respectively [6]. On the other hand, RRA showed 10–26% cross-reactivity of the binding assay for sirolimus metabolites [7].

Different chromatographic methods for the quantitation of sirolimus in whole blood have been available for a number of years. These methods require extraction of sirolimus followed by chromatographic separation and detection by either ultraviolet (UV) [8–13] or mass spectrometry (MS) detection (HPLC–MS or HPLC–MS–MS) [14–16]. Although these methods meet many or all generally accepted criteria for validated analysis of immunosuppresive drugs, they are often cumbersome, require glassware preparation (silanized or light protected tubes) or involve equipment (HPLC–MS) not commonly available in the clinical laboratory due to the need of a great investment. Moreover, most of these methods require tedious extraction procedures; with liquid–liquid extraction and washing steps or a combination of liquid–liquid extraction and solid-phase extraction, and very long chromatographic time to resolve sirolimus from several endogenous peaks eluted in the chromatogram. The aim of the present study was to develop a simple HPLC UV assay for sirolimus that was precise and accurate at lower concentrations and was capable of a high throughput.

2. Experimental

2.1. Chemicals, reagents and solutions

Sirolimus (purity > 99.0%) and 32-desmethoxyrapamycin (I.S.) (purity > 99.0%) (Fig. 1) were provided by Wyeth (New York, NY, USA). Acetonitrile, methanol, acetone, *tert*-butyl methyl ether, 1-chlorobutane (HPLC grade), sodium hydroxide and zinc sulfate were obtained from Merck (Darmstadt, Germany).

2.2. Standard solutions and samples

Stock solutions of sirolimus and 32-desmethoxyrapamycin, with a concentration of 1 mg/ml, were prepared separately by dissolving 10 mg of each analyte in methanol. Intermediate stock standards of 100 μg/ml were prepared using 50% methanol in water as solution. Finally, eight standard solutions of sirolimus (125, 250, 500, 750, 1250, 2500, 3750, and 7500 ng/ml) were made by further dilution of the intermediate stock solution with appropriate volumes of 50% methanol in water. The standard solution of 32-desmethoxyrapamycin (1 μg/ml) was similarly prepared. Standard and stock solutions of sirolimus and 32-desmethoxyrapamycin were stored at −80 °C.

Calibration pools of 2.50, 5.00, 10.0, 15.0, 25.0, 50.0, 75.0, and 150 ng/ml were prepared by diluting 0.2 ml of each spiking solution to 10 ml with potassium–EDTA control human whole blood in 10 ml volumetric flasks. Whole blood quality control pools of 3.75, 11,25 and 37.5 ng/ml were prepared by diluting 0.75 ml of 250, 750 and 2500 ng/ml spiking solutions to 50 ml with potassium–EDTA control human whole blood in 50 ml volumetric flasks. Pools were measured into 1.2 aliquots in propylene tubes and frozen at −80 °C until use.

2.3. Sample preparation

Whole-blood samples (1 ml) were transferred to a 13 mm × 100 mm conic tubes and spiked with the internal standard (40 μl of 1 μg/ml of 32-desmethoxyrapamycin). Then, 2 ml of acetone were added to the tubes. After vortex-mixed well for 1 min, 2 ml of zinc sulphate/sodium hydroxide solution pH 6.5 (6.25% of zinc sulphate, 0.5 mol/l of sodium hydroxide) was added to the tubes. The tubes were capped, vortex-mixed for 1 min, and centrifuged at 2600 × *g* and 1 °C for 10 min. The supernatants were transferred to clean 13 × 100 tubes. Next, 1-chlorobutane or *tert*-butyl methyl ether (4 ml) was added to the sample tubes. The tubes were capped, vortex-mixed for 1 min, and centrifuged at 2000 × *g* and 1 °C for 10 min. Afterwards, the obtained supernatant was transferred to clean tubes and dried under vacuum pressure (Vortex evaporator, Labconco) at 40 °C for 10 min. The dried extracts were reconstituted in 200 μl of mobile phase, transferred to limited volume autosampler vials, capped and placed on the HPLC autosampler. A 100 μl aliquot of the supernatant was injected onto HPLC column.

2.4. Apparatus and chromatographic conditions

The apparatus used for the HPLC analysis was a Model 1050 series LC coupled with an UV diode array detector set at 278 nm (Hewlett-Packard, Waldbronn, Germany). Data acquisition and analysis were performed with a Hewlett-Packard computer using the ChemStation G2171 AA programme. Separation was carried out at 50 °C on a

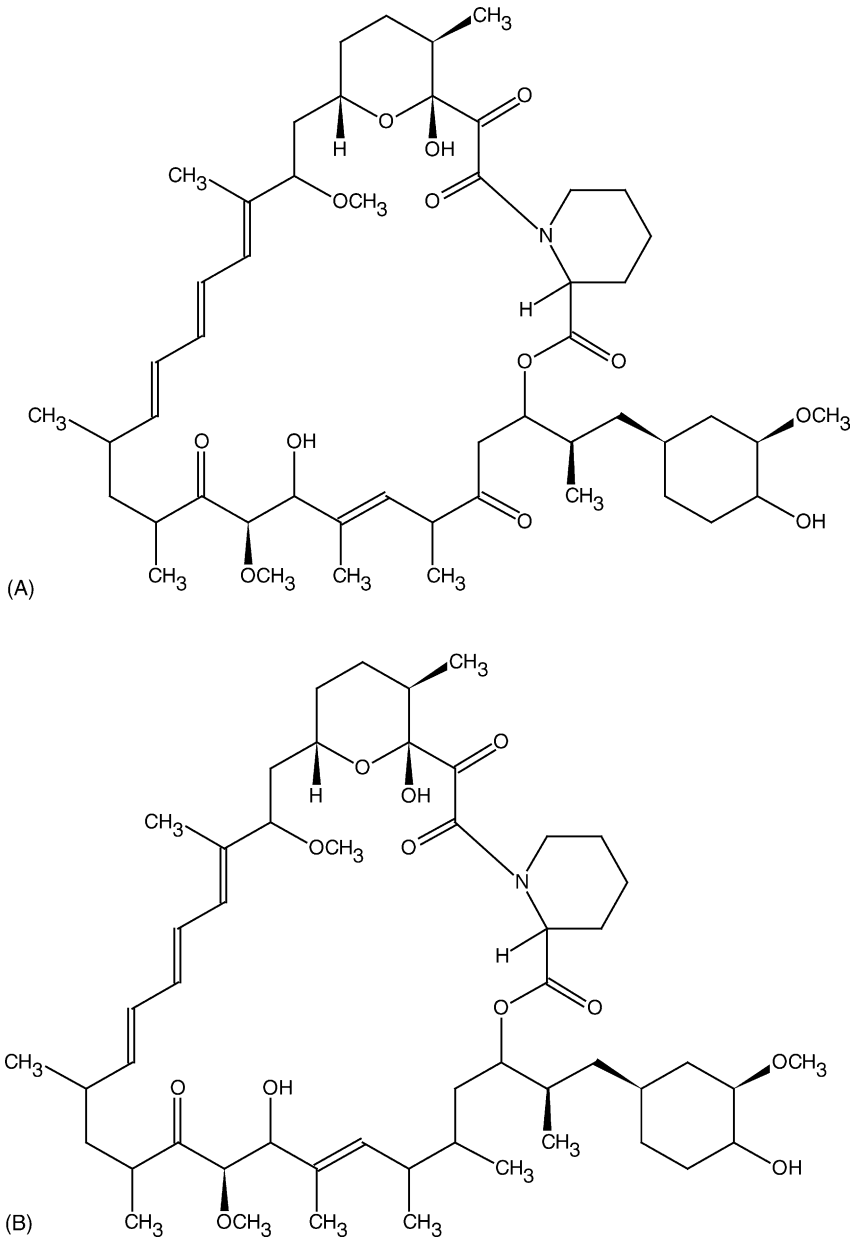

Fig. 1. Chemical structures of sirolimus (A) and 32-desmethoxyrapamycin (B).

reversed-phase, 150 mm × 2.1 mm column packed with C_{18}, 5 μm silica reversed-phase particles (Alltima) obtained from Alltech (Sedriano, Milan, Italy). This column was preceded by a reversed-phase, C_{18}, 5 μm guard column (Kromasil, 20 mm × 4 mm, Symta, Spain). The Mobile phase was a mixture of distilled water methanol acetonitrile (26:50:24, v/v/v). Separation was achieved by isocratic solvent elution at a flow-rate of 0.25 ml/min.

2.5. Quantitation

Each calibration curve consisted of 8 calibration points (2.50, 5.00, 10.0, 15.0, 25.0, 50.0, 75.0, and 150 ng/ml). Calibration curves were determined by least square linear regression analysis (weighting $1/X^2$). Peak area ratio of sirolimus and 32-desmethoxyrapamycin versus the corresponding sirolimus concentration was plotted. The linearity of the method was confirmed by comparing the slopes, the intercepts of calibration curves with zero and the correlation coefficients with 1. Moreover, a Student's *t*-test was used to compare the back-calculated concentrations with each calibration curve versus the nominal ones.

2.6. Validation

The method was validated by analysis of human whole-blood quality control samples prepared at four concentrations spanning the calibration range (2.5, 3.75, 11.25

and 37.5 ng/ml). Three samples of each quality control pool, and calibration samples were analyzed on six different days. On day 1 the number of samples of each quality control pool was five. Precision and accuracy was determined. Precision of the method was expressed as the relative standard deviation of replicate measurements (R.S.D., %). Accuracy was measured according to the following equation:

$$\text{Percentage difference from theoretical value} = \left[\frac{X - C_T}{C_T}\right] \times 100$$

where X is the determined concentration of a quality control and C_T is the theoretical concentration. To be acceptable, the measures should be lower than 15% at all concentrations.

The overall recovery for sirolimus and 32-desmethoxyrapamycin was calculated by comparing the peak-area ratios of spiked samples with those obtained by direct injections of the same amount of both compounds.

The selectivity of the assay was determined by the individual analysis of blank samples. The retention times of endogenous compounds in the matrix were compared with those of sirolimus and 32-desmethoxyrapamycin.

The limit of detection (LOD) was defined as the sample concentration resulting in a peak area of three times the noise level. LOQ was defined as the lowest drug concentration that could be determined with an accuracy and precision <20%. In this work LOD of the assay method was determined by analysis of the peak baseline noise in ten blank samples.

The stability of sirolimus in both frozen whole-blood samples (−80 °C) over 3 months, and in processed samples left at room temperature (20 ± 3 °C) over 24 h, was also studied.

The present method was validated by a reference laboratory in the UK (Dr. D.W. Holt, St. George Hospital Medical School, London, UK) that established an international proficiency testing control system for sirolimus. 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 for the reference laboratory every month.

2.7. Application of the method

To demonstrate the reliability of this method for the study of sirolimus pharmacokinetics, this assay was applied to the TDM of sirolimus in whole-blood samples obtained from a cohort of 100 renal transplant patients treated with a sirolimus-cyclosporin A regimen. Venous blood samples were withdrawn in EDTA tubes immediately prior to dosing and were stored frozen (−20 °C) until analysis. Additionally, a sirolimus pharmacokinetic study was performed in five heart transplant patients after 7 days of treatment. In these patients whole-blood samples were withdrawn at 2, 3, 6, 12 and 24 h postadministration and also store frozen until analysis.

3. Results and discussion

Generally, therapeutic drug monitoring of immunosuppressants in humans involves analysis of a large number of samples and therefore requires simple, rapid and reliable analytical methods. The criteria for an acceptable general performance of sirolimus assays in terms of specificity, accuracy, and precision has been defined by the Lake Louise Consensus Conference [4], and was accomplished by most of previously high-performance liquid chromatographic published methods. However, these present some disadvantages, such as the low degree of automation, the low sample turnover and the high intra-assay variability. Without exception of HPLC–MS methods, sirolimus assays combined the specificity of chromatographic separation with an off-line sample extraction. These off-line sample extraction takes time-consumption and error-prone because of multiple manual, sequential preparation steps, resulting in high inter-assay variability. Quantification of sirolimus in biological matrices is not easy, given the very low dose of the drug employed, and the low concentration levels systemically achieved in blood. Moreover, interfering peaks from endogenous or exogenous sources are common when ultraviolet-visible detection is used. The UV extinction maximum of sirolimus is 277 nm, a wavelength close to he extinction maximum of some proteinaceous derivatives present in blood (280 nm).

Chemically, this immunosuppresive drug is a hydrophobic molecule with very low water solubility (approximately 1 mg/ml) and has no ionizable functional groups between pH 1–10. Sirolimus has been commercialized as a mixture of three isomers. The major component is isomer B, while isomer C accounts for approximately 3–10%. The amount of isomer A in the formulation is generally <0.5%. The substance used as internal standard, 32-desmethoxyrapamycin, also is an isomeric mixture with two isomers B and C. Therefore, care must be taken to ensure that the chromatography conditions separate sirolimus and I.S. isomers. Previously, different reversed-phase columns have been used with this objective. These columns were packed with base-deactivated silica spherical particles. Encapped phases such as Ultrasphere C_{18} and Ultrasphere C_8 have been the most utilized packing in the past. In this paper we developed a quick, robust, and highly reproducible analytical method for sirolimus determination in whole blood samples. This method overcomes the analytical difficulties observed in previously published procedures by means of a substantially improvement of the chromatographic selectivity of the method. We have used a polimerically bounded C_{18} reversed-phase narrow-bore column packed with double encapped spherical silica particles that produce sharp, symmetrically chromatographic peaks (Alltima). This packing also produced an increased detector response with respect to standard columns due to the decrease diffusion of the sample as it goes though a narrower column. In addition, we have introduced methanol in the mobile phase composition, an organic solvent that

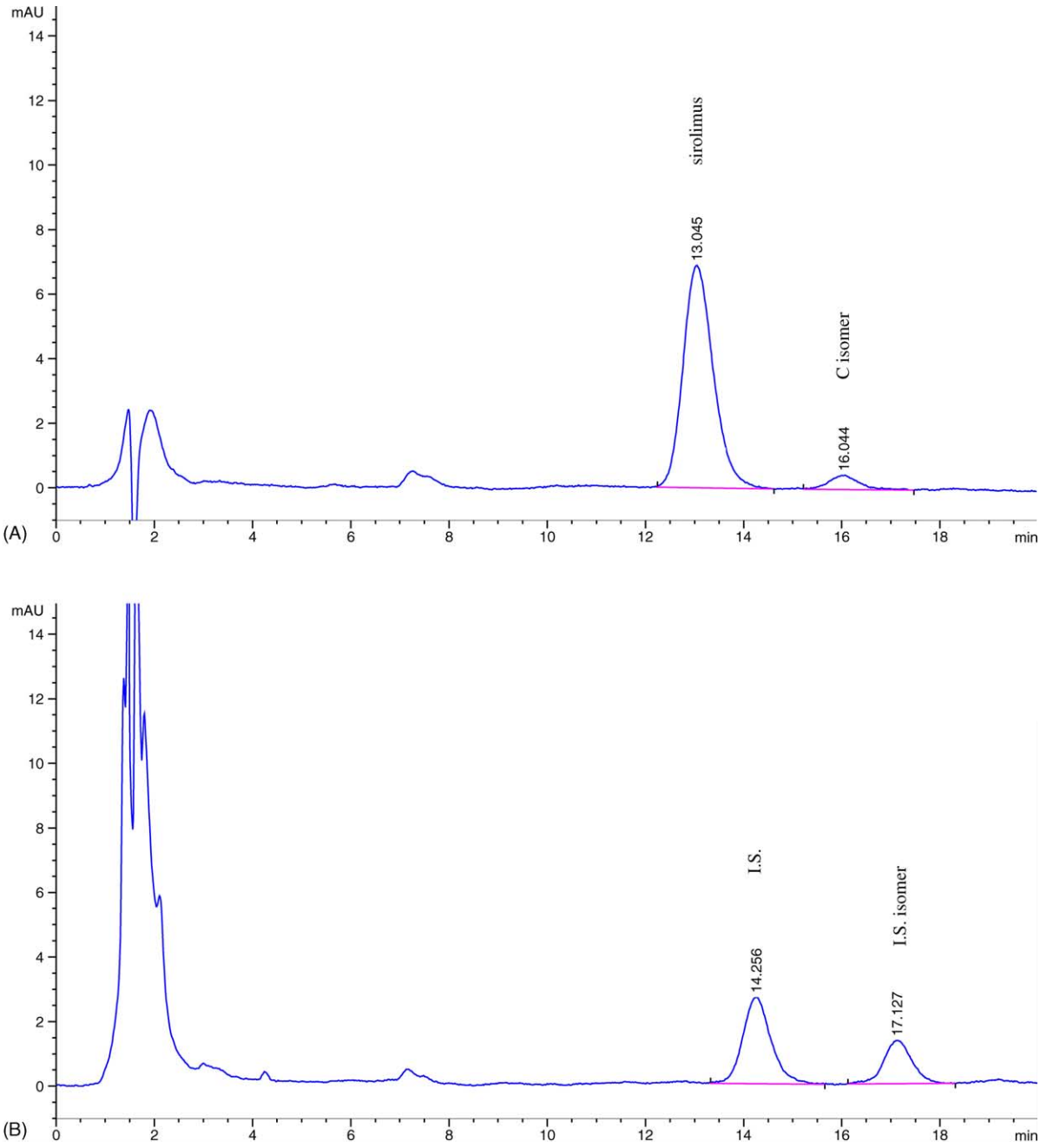

Fig. 2. Chromatograms of (A) a standard solution of sirolimus (50 ng/ml) and (B) a standard solution of 32-desmethoxyrapamicin (40 ng/ml).

yields a selectivity increase over chromatographic separation; and we have reduced the amount of acetonitrile. Fig. 2 displays the chromatograms of standards of sirolimus and 32-desmethoxyrapamycin. The retention time of sirolimus and its isomer C were 13.1 and 16.0 min, respectively, (panel A) while 14.3 and 17.1 were the retention time of I.S. and the corresponding isomer (panel B). As it can be seen, in the chromatographic conditions described above sirolimus and I.S. eluted before their isomers, with R_s value between I.S. and the isomer C of sirolimus > 1.96. The R_s values between sirolimus, I.S., and their respective isomers were 2.84 an 2.98, values that reflect the high degree of selectivity that shows the developed chromatographic method. The symmetry factor of all peaks ranged from 0.8 to 0.9, values near to 1.

The high selectivity degree of the method described here also allowed us to simplify the extensive sample-preparation protocol that must be applied prior to chromatographic analysis. Previously developed techniques involved clean-up techniques with a combination of deproteinization and liquid–liquid extraction with a final purification of the extract with *n*-hexane. Recently, it has been developed a new method for sirolimus determination that involves a solid phase extraction step of blood samples [13]. In this work the

liquid–liquid extraction steps have been substituted by the solid extraction procedure. Although the assay was specific and robust, too many and time-consuming extraction steps have been performed. In our method, the use of *n*-hexane to remove endogenous lipophilic components was unnecessary. Moreover, the high efficiency of the developed method avoided the need to use special or treated glassware, the rinse of extraction tube caps with the appropriate solvent before use, and the post-run flushing steps of the columns with thetrahydrofuran to remove late eluting peaks; procedures described in previously published methods [9,12,17].

Other critical fact in the development of chromatographic methods for the therapeutic drug monitoring of sirolimus was the importance of the maintenance of a high extraction recovery due to the modest UV absorption of sirolimus. The sirolimus trough level of most kidney transplant patients had been maintained between 5 and 15 ng/ml [17]. Therefore, it was important to achieve limits of quantitation

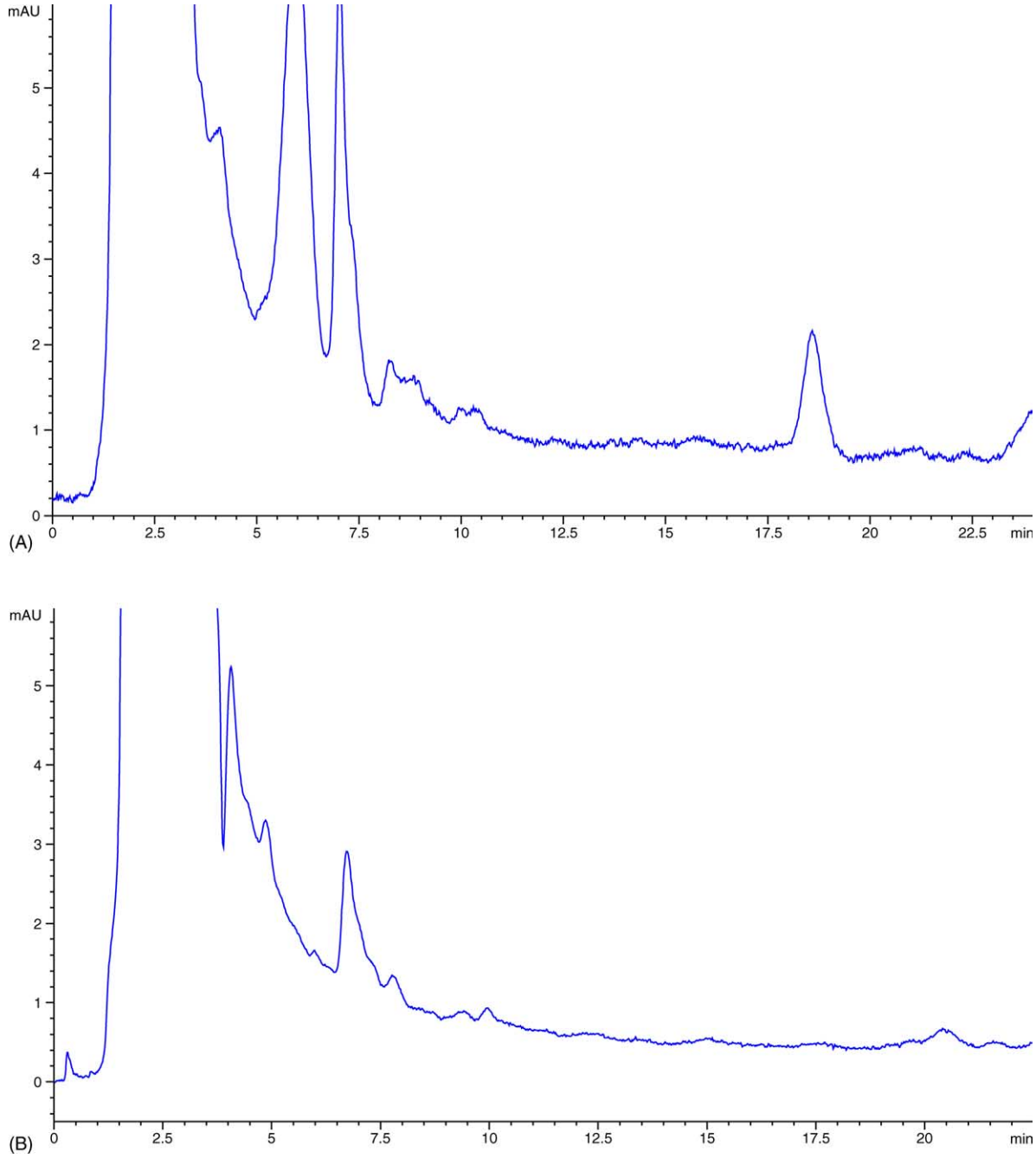

Fig. 3. Chromatograms of (A) blank whole blood sample extracted with *tert*-butylmethylether, (B) whole blood sample extracted with 1-chlorobutane, and (C) resulting from the analysis human blood samples obtained at 24 h (11.06 ng/ml rapamycin) from a subject who received oral doses of Rapamune (2 mg/day).

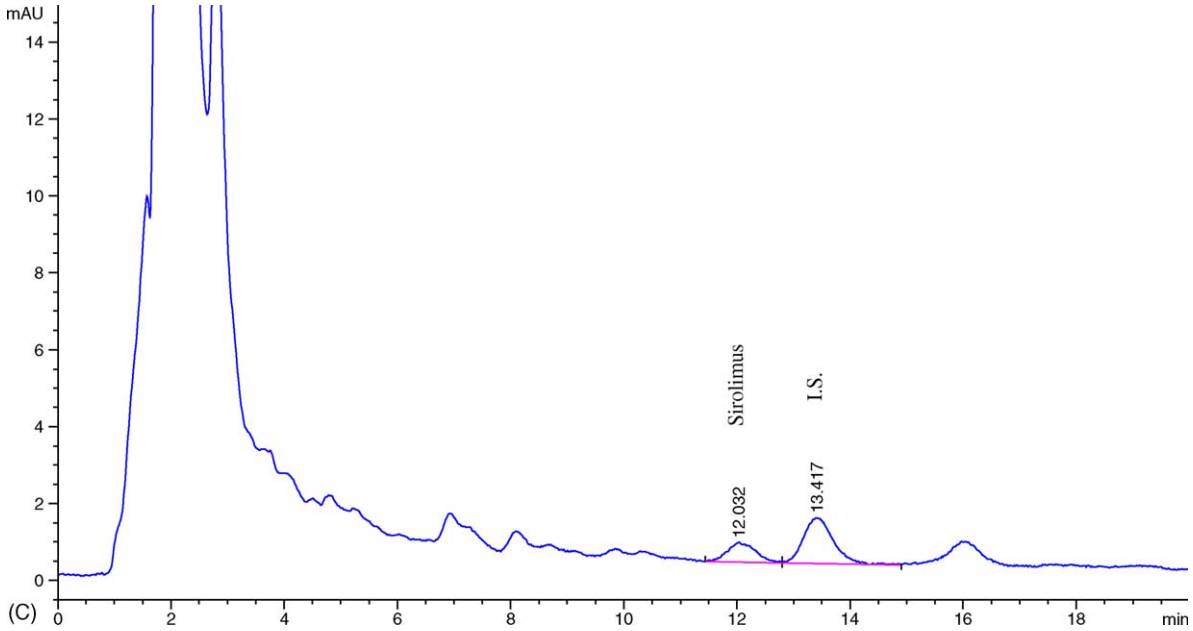

Fig. 3. (*Continued*).

Table 1
Standard calibration curves of sirolimus in whole blood samples

	r	*a*	*b*	Concentration (ng/ml)							
				2.5	5	10	15	25	50	75	150
n	8	8	8	8	8	8	8	8	8	8	8
Mean	0.9963	0.040	−0.041	2.55	5.04	9.34	14.07	25.45	48.15	78.68	159.51
S.D.	0.0011	0.003	0.020	0.10	0.43	0.41	0.58	0.92	2.12	3.34	8.33
R.S.D. (%)	0.11	6.72		4.08	8.54	4.42	4.11	3.61	4.39	4.24	5.22
Accuracy (%)				1.95	0.73	−6.56	−6.19	1.79	−3.70	4.91	6.34

r = correlation coefficient; a = slope; b = intercept.

near to 2–3 ng/ml. To get best recovery we initially employed *tert*-butyl methyl ether, and an efficient extraction solvent. However, despite of the high recoveries achieved (near to 80%), some interference were present in the chromatogram when certain samples were analyzed. In opposite of 1-chlorobutane reached lower recovery (near to 65–70%) but cleaner extracts. Fig. 3 displays the chromatograms of extracts prepared with *tert*-butyl methyl ether and 1-chlorobutane from a blank blood sample; and from blood of a renal transplant patient given sirolimus. In the last case 1-chlorobutane was used as extraction solvent. Blank blood samples did not show peaks corresponding to sirolimus and I.S. retention times. Nevertheless, higher baseline noise was observed when *tert*-butyl methyl ether was used.

No chromatographic interference was found between sirolimus and I.S. and other immunosuppressants such as cyclosphorine, mycophenolic acid and tracolimus. This was confirmed in 35 kidney, hearth and liver transplant recipients treated with sirolimus that received higher doses of these immunosuppressants.

Assay performance of the present method was assessed by all the following criteria: linearity, accuracy, precision, LOD, limit of quantitation (LOQ), stability, applicability to pharmacokinetic studies and to therapeutic drug monitoring of sirolimus in transplant patients. The assay exhibited linearity between the response (y) and the corresponding concentration of sirolimus (x), over the 1–150 ng/ml range in the samples. Results are presented in Table 1. For each point of calibration standards, the concentrations were back-calculated from the equation of the regression curves, and R.S.D. were computed. The obtained values are also reflected in Table 1. Moreover, a linear regression of the back-calculated concentrations versus the nominal ones provided a unit slope and intercept equal to 0. The extraction

Table 2
Accuracy of the method, expressed as relative error in %, for determining sirolimus concentrations

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Accuracy (%, $n = 6$)
2.50	2.42 ± 0.16	−3.34
3.75	3.02 ± 0.25	−9.40
11.25	12.12 ± 0.79	7.74
37.50	39.59 ± 1.60	5.58

Table 3
Between- and within-day variability of the HPLC method for determining sirolimus concentrations

Concentration added (ng/ml)	Between-day variability ($n = 18$)		Within-day variability ($n = 6$)	
	Concentration found (mean ± S.D.) (ng/ml)	R.S.D. (%)	Concentration found (mean ± S.D.) (ng/ml)	R.S.D. (%)
2.50	2.42 ± 0.16	6.61	2.52 ± 0.12	4.88
3.75	3.02 ± 0.25	8.28	3.84 ± 0.30	7.89
11.25	12.12 ± 0.79	5.78	11.05 ± 0.79	7.15
37.50	39.59 ± 1.60	4.04	35.17 ± 1.60	4.56

Table 4
Pharmacokinetic parameters of sirolimus at steady state after oral administration of Rapamune (2 mg/daily) to five heart transplant patients

Patient	Dose (mg)	C_{max} (ng/ml)	C_{min} (ng/ml)	$AUC_{0–24}$ (ng h/ml)	$t_{1/2}$ (h)	V_{ss}/F (l/kg)	CL/F (kg/ml h)
1	2	32.47	10.59	381.8	15.86	0.864	39.51
2	2	13.22	6.69	195.7	30.19	3.000	66.67
3	1	21.39	13.55	371.8	33.30	0.576	11.76
4	2	29.00	13.72	409.1	31.09	2.093	46.51
5	2	10.88	3.14	121.4	10.96	3.635	230.8

C_{max}: maximum concentration of sirolimus in blood after the seventh sirolimus dose administration; C_{min}: minimum concentration of sirolimus in blood after the seventh sirolimus dose administration; $AUC_{0–24}$: area under the blood concentration–time course; $t_{1/2}$: half-life of drug; V_{ss}/F: apparent volume of distribution; CL/F: relative clearance of drug.

recoveries of sirolimus and 32-desmethoxyrapamycin in whole blood samples were 65.7 ± 1.18, 66.2 ± 2.37%, respectively.

Accuracy values were within acceptable limits (Table 2). Less than 20% error of the aforementioned method was observed in the international proficiency testing control system for sirolimus. The results for within- and between-day precision for our sample are presented in Table 3 and the values ranged between 4.04 and 8.28%, 4.56 and 7.89%, respectively. The obtained values for the precision were also acceptable, and similar to obtained in the external proficiency testing for sirolimus measurement by HPLC (within-day precision: 3.3–7.7%, between-day precision: 1.0–2.0%). These results meet with the values internationally accepted for the precision of the analytical techniques developed for therapeutic drug monitoring of sirolimus in transplant patients (RSD < 10% at 5 ng/ml and <5% at 40 ng/ml) [4].

The LOD of sirolimus was 0.68 ng/ml (S/N = 3) and the estimated LOQ was calculated as low as 1.75 ng/ml (S/N = 10) which is similar to described by other authors [13]. The last value was confirmed for our samples. The mean assay result was 1.82 ng/ml ($n = 12$), with R.S.D. <5%. Although the imprecision and inaccuracy are acceptable we set the lower concentration of the drug that could be assayed at 2.5 ng/ml, according with the International Proficiency Testing Scheme.

Sirolimus was stable in whole blood samples stored at −80 °C for at least 6 months [17]. The stability of sirolimus in processed samples left at 4 ± 0.3 °C over 24 h was also studied for our laboratory quality-control set up for the drug at concentrations of 3.75, 11.25 and 37.5 ng/ml, respectively. Sirolimus and the internal standard were also stable in these conditions.

The applicability of this method has been demonstrated in vivo by the determination of sirolimus in blood samples from transplant subjects receiving oral daily doses. A progressive slow reduction of the drug concentration values in blood was found up to the next sirolimus dose. Pharmacokinetic parameters estimated by the noncompartmental approach are listed in Table 4. The observed values of the pharmacokinetic parameters were comparable to those reported for sirolimus in previous studies [3].

Finally, it is interesting to note that under the chromatographic conditions described above we can avoid the extensive sample-preparation protocols and the high turnaround times required for sample analysis in the chromatographic techniques previously developed for the therapeutic drug monitoring of sirolimus in transplant patients. It is possible to produce near to 80 results within the desired 24 h turnaround time using one technician and one HPLC system. Therefore, the developed method is entirely appropriate for therapeutic drug monitoring of sirolimus in a clinical setting.

References

[1] B.D. Kahan, J.Y. Chang, S.N. Sehal, Transplantation 52 (1991) 185.
[2] M.G. Murgia, S. Jordan, B.D. Kahan, Kidney Int. 49 (1996) 209.
[3] A. Johnston, D.W. Holt, Br. J. Clin. Pharmacol. 47 (1999) 339.
[4] R.W. Yatscoff, R. Boeckx, D.W. Holt, et al., Ther. Drug Monit. 17 (1995) 676.
[5] K. Mahalati, B.D. Kahan, Clin. Pharmacokinet. 40 (2001) 573.
[6] P. Salm, P.J. Taylor, P.I. Pillans, Clin. Ther. 45 (1999) 2278.
[7] D.L. Davis, J.N. Murthy, H. Gallant-Haidner, Clin. Biochem. 33 (2000) 1.
[8] S. Maleki, S. Graves, S. Becker, R. Horwatt, D. Hicks, R.M. Stroshane, H. Kincaid, Clin. Ther. 22 (2000) B25.

[9] K.L. Napoli, B.D. Kahan, Clin. Chem. 42 (1996) 1943.
[10] D.W. Holt, T. Lee, A. Johston, Clin. Ther. 22 (2000) B62.
[11] D.C. French, M. Saltzgueber, D.R. Hicks, A.L. Cowper, D.W. Holt, Clin. Chem. 47 (2001) 1316.
[12] J.O. Svensson, C. Brattstrom, J. Sawe, Ther. Drug Monit. 19 (1997) 112.
[13] D. Cattaneo, N. Perico, F. Gaspari, J. Chromatogr. B 774 (2002) 187.
[14] P. Salm, P.J. Taylor, P.I. Pillans, Clin. Ther. 22 (2000) B71.
[15] P.J. Taylor, A.G. Johnson, J. Chromatogr. B 718 (1998) 251.
[16] G.I. Kirchner, C. Vidal, W. Jacobsen, A. Franzke, K. Hallensleben, U. Christian, K.F. Sewing, J. Chromatogr. B 721 (1999) 285.
[17] K.L. Napoli, Clin. Ther. 22 (2000) B14.