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Simultaneous on-line extraction and analysis of sirolimus (rapamycin) and ciclosporin in blood by liquid chromatography–electrospray mass spectrometry

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Abstract

We developed a sensitive and specific semi-automated liquid chromatography–electrospray mass spectrometric (HPLC–ESI-MS) assay for the simultaneous quantification of sirolimus and ciclosporin in blood. Following a simple protein precipitation step, the supernatants were injected into the HPLC system and extracted on-line. After column switching, the analytes were backflushed from the extraction column onto the analytical narrow-bore column and eluted into the ESI-MS system. The assay was linear from 0.4 to 100 μg/l sirolimus and from 2 to 1500 μg/l ciclosporin. The mean recoveries of sirolimus and ciclosporin were 98 and 96%, respectively. The mean interday precision/accuracy was 8.6%/−4.8% for sirolimus and 9.3%/−2.9% for ciclosporin.

Keywords: Sirolimus; Rapamycin; Ciclosporin

1. Introduction

Sirolimus (rapamycin) is a 31-membered triene macrolide lactone with a hemiketal-masked α,β-dioxocarboxamide and has a molecular weight of 913.6 Da (Fig. 1). It is currently in phase III of its clinical development as an immunosuppressant after organ transplantation [1–5]. Because of its synergistic immunosuppressive activity [6–8] and different spectrum of potential side effects [9–15], sirolimus is co-administered with the undecapeptide immunosuppressant ciclosporin (cyclosporin A, cyclosporine, Fig. 1). However, clinical management of both immunosuppressants is complicated by their highly variable pharmacokinetics in combination with a narrow therapeutic index. It can be expected that, as for ciclosporin [16], sirolimus dosing must be guided by therapeutic drug monitoring [17]. In addition, both drugs are mainly metabolized by cytochrome P450 3A4 in the liver and small intestine and are substrates of the ATP-binding cassette transporter p-glycoprotein [18–21]. Ciclosporin is known to interact with several drugs, which are cytochrome P450 3A and p-glycoprotein substrates, and such interactions can also be anticipated for sirolimus.

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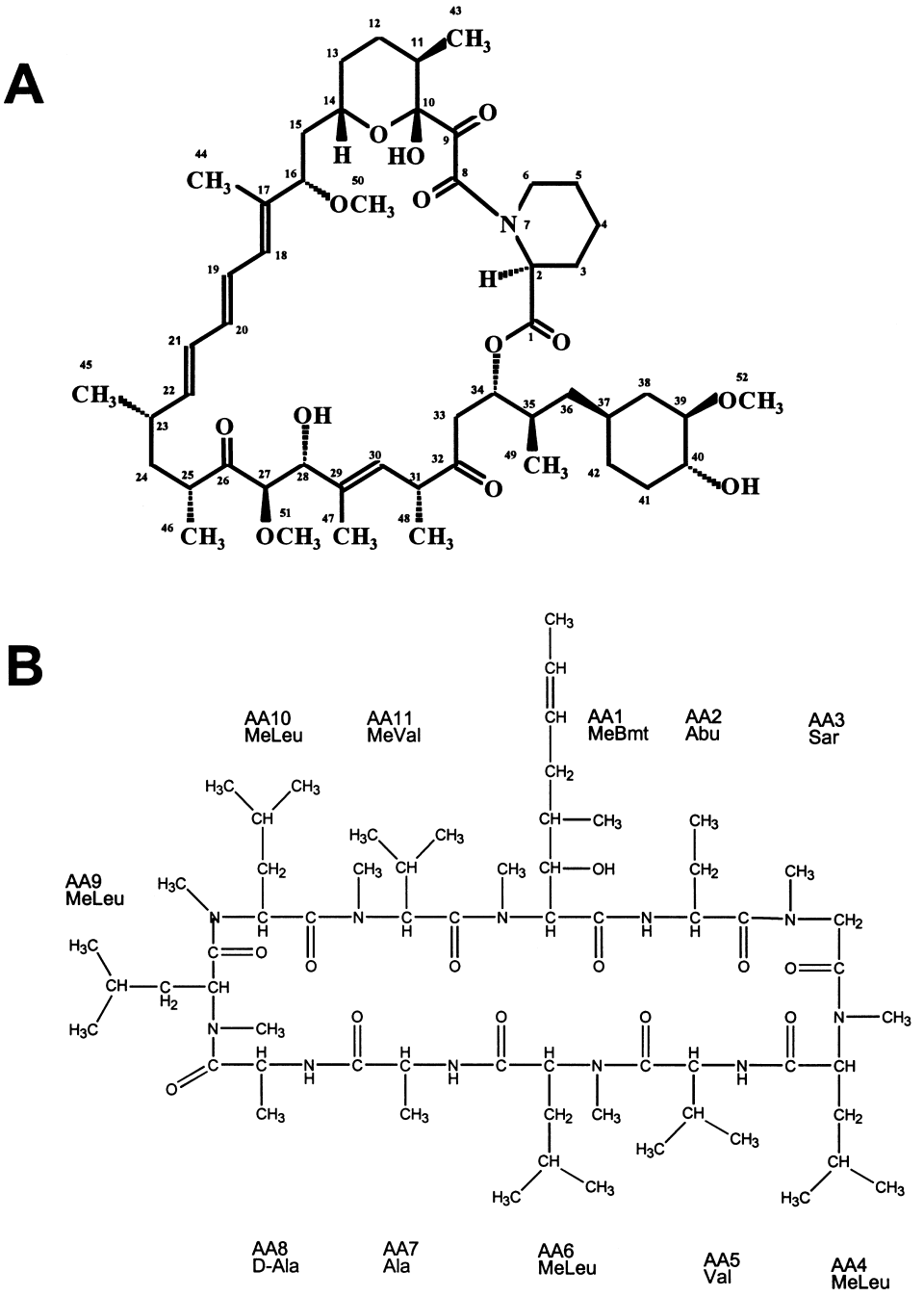

Fig. 1. Chemical structures of sirolimus (A) and ciclosporin (B). Numbering of the atoms of the sirolimus molecule follows the IUPAC guidelines [33]. AA, amino acid number.

For therapeutic drug monitoring of transplant patients who concomitantly receive the immunosuppressants ciclosporin and sirolimus, it would be desirable to have an analytical method that measures both drugs in the same sample in one analytical run, that is sensitive and specific, involves an automated

and fast sample preparation step and has a high sample turnover. Currently no such method is available. Ciclosporin immunoassays, although labeled as specific, still significantly crossreact with ciclosporin metabolites [16]. Since ciclosporin lacks a chromophor, HPLC–UV assays are vulnerable to interferences and require tedious extraction procedures and long HPLC run times [22,23]. Several HPLC–UV methods have been developed for sirolimus [24,25]. In addition to the requirement of extensive sample preparation and relatively long analytical run times, the lower limit of quantitation of these assays of 2 μg/l is probably not sufficient to measure the blood trough concentrations ($C_{24\ h}$) of individual patients [24,25]. Although immunoassays have not yet been described for sirolimus, they can also be expected to significantly crossreact with sirolimus metabolites. In comparison with HPLC–UV, HPLC–electrospray mass spectrometry (ESI-MS) assays have several advantages. ESI-MS detection is more than 10-fold more sensitive than HPLC–UV [26]. Due to the specificity of ESI-MS detection, complete HPLC separation of compounds with different molecular weights is unnecessary and therefore run times are usually considerably shorter than for HPLC–UV allowing for a significantly higher sample turnover.

We describe here the development of a novel sensitive and specific HPLC–ESI-MS method for simultaneous detection of sirolimus and ciclosporin involving a simple and fast semi-automated on-line sample extraction procedure.

2. Experimental

2.1. Reagents

HPLC-grade methanol, acetonitrile and water were purchased from J.T. Baker (Deventer, The Netherlands). All solvents were degassed before use in the HPLC system. Zinc sulphate was from Sigma Aldrich (Deisenhofen, Germany). The nitrogen used for mass spectrometry had a purity of 99.99%. Sirolimus was purchased from Sigma (Deisenhofen, Germany) and ciclosporin was kindly provided by Novartis Pharmaceuticals Inc. (Nürnberg, Germany).

2.2. On-line sample preparation and chromatographic separation

The semi-automated sample preparation consisted of a manual deproteinization step and automatic column-switching on-line HPLC extraction. To 1 ml human blood, 1 ml protein precipitation solution which consisted of a mixture of methanol and 0.4 mol/l zinc sulphate (4:1, v/v) was added. Samples were vortexed for 15 s and centrifuged at 1500 *g* for 6 min. Supernatants were transferred into 1.8 ml brown glass vials (Hewlett-Packard, Waldbronn, Germany) and placed into the autosampler of a HP 1090 series II liquid chromatograph (Hewlett-Packard). The on-line sample extraction equipment included an autoinjector fitted with a Hamilton 500 μl syringe, a Rheodyne 7010 six-port high-pressure switching valve with a two-position Rheodyne 5701 pneumatic actuator and a solenoid valve (Rheodyne, Cotati, CA), which was controlled by the HP ChemStation software (version 04.02, Hewlett Packard). Samples were concentrated and washed on a 30×4 mm extraction guard column filled with C-18 Nucleosil® 100 (Macherey-Nagel, Düren, Germany) of 10 μm particle size (Schambeck SFD, Bad Honnef, Germany). The solvent for sample cleaning on the extraction column was delivered by HP 1090 HPLC pumps. The extraction procedure consisted of three steps (Fig. 2). Step A (valve position A): 400 μl of the supernatants were injected into the HPLC system and loaded onto the extraction column (mobile phase, water pH 7.0; flow-rate, 0.35 ml/min). During this step sirolimus and ciclosporin were concentrated on the column, and potentially interfering material was washed into waste. In parallel, the analytical 250×2 mm Hypersil® ODS column (Shandon, Chadwick, UK), particle size 5 μm, was equilibrated with methanol–water 90/10 (v/v, flow-rate 0.2 ml/min), which was delivered by an additional external HPLC pump (WellChrom Micro-Star® K-100, Knauer, Berlin, Germany). After 4 min, the switching valve changed position. Step B (valve position B): the extract was eluted in the back-flush mode onto the analytical column. After another 4 min, the valve switched back. Step C (valve position A): sirolimus and ciclosporin were eluted from the analytical column into the ESI-MS system using isocratic elution with methanol–water

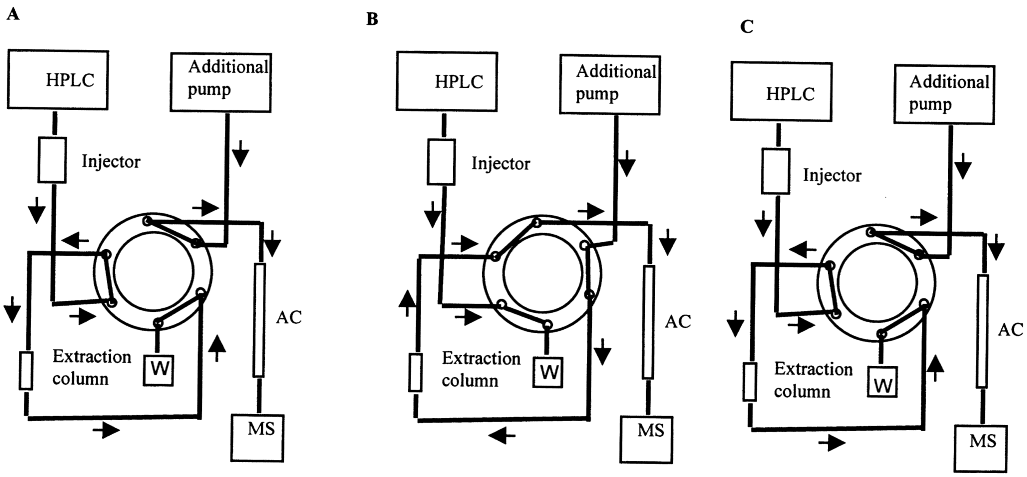

Fig. 2. On-line-extraction. W, waste; AC, analytical column. (A) Loading of the extraction column; (B) sample elution from the extraction column; (C) separation on the analytical column.

(90/10, v/v). The flow-rate was 0.2 ml/min, and the column temperature was 35°C. During this step the extraction column was washed with methanol for 2 min and subsequently re-equilibrated with water (flow-rate 0.35 ml/min). The total run time between injections was 15 min.

2.3. Electrospray mass spectrometry

The HPLC system was linked to an HP59987A electrospray interface, which was connected to an HP5989B mass spectrometer (all Hewlett-Packard). The mass spectrometer was controlled and data were recorded and analyzed using ChemStation software revision 01.04 (Hewlett-Packard).

The following electrospray parameters were used: spray gas, nitrogen (pressure 540 kPa); drying gas, 10 l/min; temperature, 350°C; capillary voltage, −4000 V; end-plate voltage, −3500 V; and cylinder electrode voltage, −6000 V. Positive ions were recorded. The mass analyzer was focused on the sodium adduct ions $[M+Na]^+$ of sirolimus (m/z 936.6) and ciclosporin (m/z 1224.9) with a dwell time of 0.5 s for each mass. The capillary exit voltage was adjusted to result in the best sensitivity for the individual compounds: 280 V for m/z 936.6 (sirolimus) and 300 V for m/z 1224.9 (ciclosporin). The multiplier voltage was 1890 V.

2.4. Method validation

Sirolimus (in acetonitrile) and ciclosporin (in acetonitrile–water 50/50, v/v, pH 3) were added to EDTA-anticoagulated blood from healthy volunteers. The linearity of the method was assessed over the concentration range 0.4–100 μg/l for sirolimus and 2–1500 μg/l for ciclosporin by Spearman regression analysis of triplicate standard curves. According to the clinical dosing regimens, the concentrations of ciclosporin were chosen 10- or 20-fold higher than those of sirolimus. The calibration curves consisted of 15 data points at the following sirolimus/ciclosporin concentrations: 0.2/4, 0.4/8, 0.5/10, 0.75/15, 1/20, 1.5/30, 3/60, 5/100, 7.5/150, 10/200, 15/300, 20/400, 35/700, 50/1000, 75/1500 and 100/2000 μg/l. Recoveries were determined at sirolimus/ciclosporin concentrations of 6/60, 15/150, 30/300 and 70/700 μg/l ($n=6$) and were calculated as percent ratios of the peak areas after protein precipitation of spiked 1 ml blood samples and on-line extraction and the peak areas after direct injection of respective amounts of ciclosporin and sirolimus (in acetonitrile–water pH 3, 50/50, v/v) onto the ana-

lytical column. Accuracy and precision were studied from replicate sets of samples containing known concentrations corresponding to low, middle, high and very high concentrations within the linear range. Accuracy was determined by calculating the mean % ratio of the concentration measured and the nominal concentration. Intra-day precision was calculated based on the measurements of six samples of the same concentration analyzed in one batch. Inter-day accuracy and precision were assessed by analysing six replicates of the same quality control samples with four different concentrations on three subsequent days. Based on FDA guidelines [27], ≤15% error of accuracy and precision was considered acceptable. The lower limit of quantification was measured using blood samples containing 0.1, 0.15, 0.2, 0.4, 0.5, 1 and 2 μg/l sirolimus and ciclosporin ($n=5$). 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%. Stability of sirolimus and ciclosporin in blood was studied at concentrations of 5/100, 15/300 and 35/700 μg/l for 4 days at room temperature, for 14 days at +4°C and for 4 month at −20°C. Three replicates were analyzed for each concentration. The stability of sirolimus and ciclosporin in the supernatants after protein precipitation during storage in the autosampler was studied at concentrations of 5/100, 15/300 and 35/700 μg/l over a time period of 72 h.

3. Results

In the positive ion mode, both sirolimus and ciclosporin were mainly detected as their sodium adduct ions $[M+Na]^+$. For sirolimus, the relative intensities of $[M+H]^+$ and $[M+K]^+$ were <5% of $[M+Na]^+$ (Fig. 3A). For ciclosporin, the relative intensity of $[M+H]^+$ was <20% and the relative intensity of $[M+K]^+$ was <5% of $[M+Na]^+$ (Fig. 3B). The ratios of sodium adducts versus potassium adducts were equal in all calibration samples. Detection of positive ions was 10-fold more sensitive than detection of negative ions. Therefore, the mass spectrometer was focused on the sodium adducts. Influence of the capillary exit voltage on signal intensity was evaluated. A capillary exit voltage of 280 V for sirolimus and of 300 V for ciclosporin resulted in the most intense signals and only little fragmentation (Fig. 3). Addition of sodium acetate, formic acid, acetic acid and triethylamine in combination with negative ion monitoring as well as the use of acetonitrile or propanol resulted in equal or lower signal intensities compared with the water–methanol mobile phase used. The retention times of sirolimus and ciclosporin were 9.7±0.03 min ($n=40$, mean±standard deviation) and 11.5±0.07 min ($n=40$), respectively. As described previously, sirolimus eluted in a double peak pattern due to the separation of *cis*- and *trans*-isomers [26,28,29]. Representative ion chromatograms are shown in Fig. 4. Protein precipitation and on-line sample extraction yielded a mean recovery of 98% for sirolimus and 96% for ciclosporin (Table 1). The lower limit of quantification was 0.4 μg/l for sirolimus and 2 μg/l for ciclosporin. Calibration curves were linear from 0.4 to 100 μg/l for sirolimus and from 2 to 1500 μg/l for ciclosporin. The following regression equations were calculated for sirolimus: $y = 89.6x - 33$ ($r^2 = 0.998$) and for ciclosporin: $y = 86.6x + 2142$ ($r^2 = 0.987$). The presence of high ciclosporin concentrations did not influence sirolimus quantification and vice versa. Intra-day and inter-day variability and accuracy of sirolimus and ciclosporin are listed in Table 2. All values met the pre-defined acceptance criteria of ±15%. A carry-over effect was excluded by alternately analyzing samples containing the highest concentration of the calibration curve and blank samples. Sirolimus and ciclosporin were stable in blood for at least 4 months at −20°C (mean: 98% of controls for both compounds). Sirolimus and ciclosporin concentrations were unchanged after storage of blood at +4°C for 1 week. After 14 days at +4°C, sirolimus concentrations had decreased by a mean 4%. During storage at room temperature, concentrations were stable for at least 4 days. Supernatants after protein precipitation containing ciclosporin and sirolimus were stable in the autosampler at room temperature over a period of 36 h. After 72 h, sirolimus concentrations had decreased to 81±5%.

Our assay allowed for analysis of 96 blood

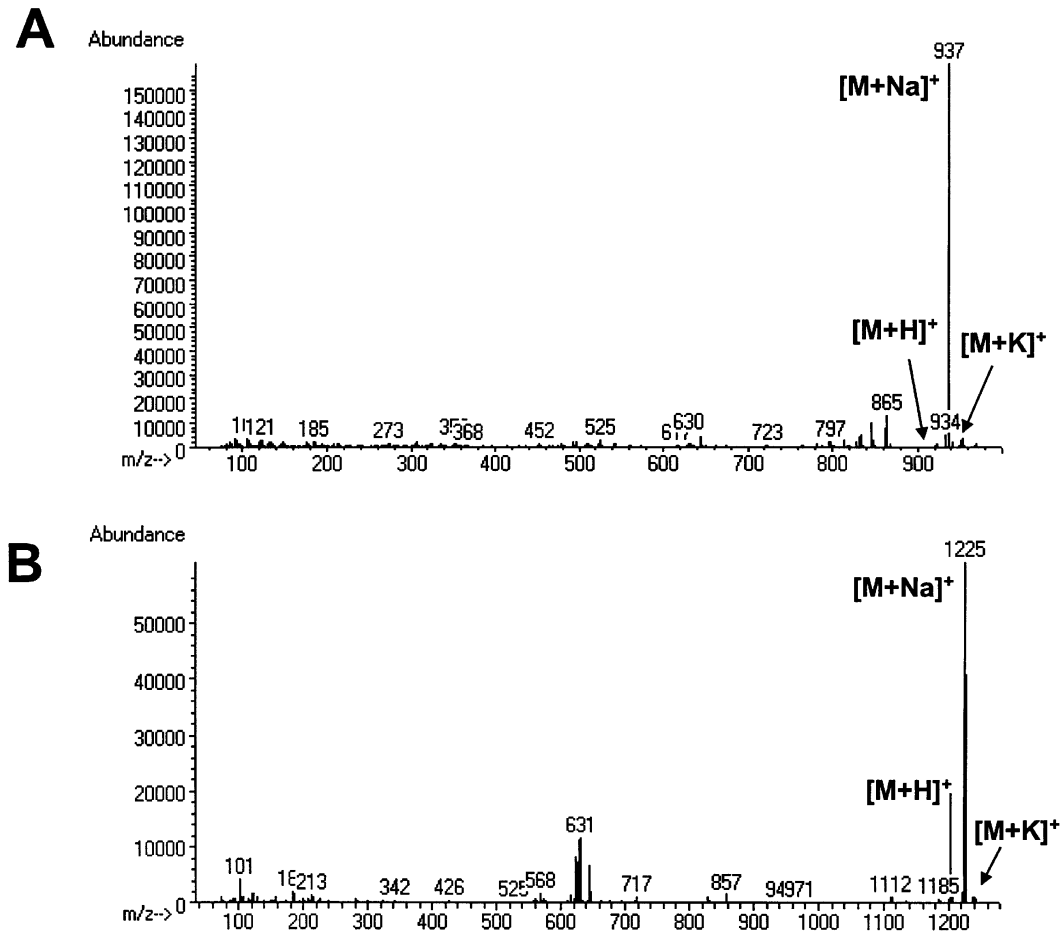

Fig. 3. Mass spectra of sirolimus (A) and ciclosporin (B). One microgram sirolimus or ciclosporin (acetonitrile–water 50/50, v/v) was injected into the HPLC–ESI-MS system. The chromatographic and mass spectrometry conditions were the same as described in the Experimental section including the column switch procedure.

samples/day. At least 500 sirolimus/ciclosporin blood samples were analyzed using the same extraction guard column and analytical column with no loss in sensitivity, accuracy or precision.

4. Discussion

The criteria for an acceptable general performance of ciclosporin and sirolimus assays in terms of specificity, accuracy, and precision has been defined by the Lake Louise Consensus Conference [16,17]. These criteria are generally not met by immunoassays, which are mostly used in the clinical practice of ciclosporin therapeutic drug monitoring. Since sirolimus is still under clinical development, commercial immunoassays have not been introduced. However, it can be expected that the antibodies of such assays will, like in the case of ciclosporin, also cross-react to variable extents with sirolimus metabolites [17] which have been shown to be present in blood at similar concentrations as the parent compound [26]. Although without exception non-specific, ciclosporin immunoassays have the advantage of a high degree of automation, higher sample turnover and low intra-assay variability [16].

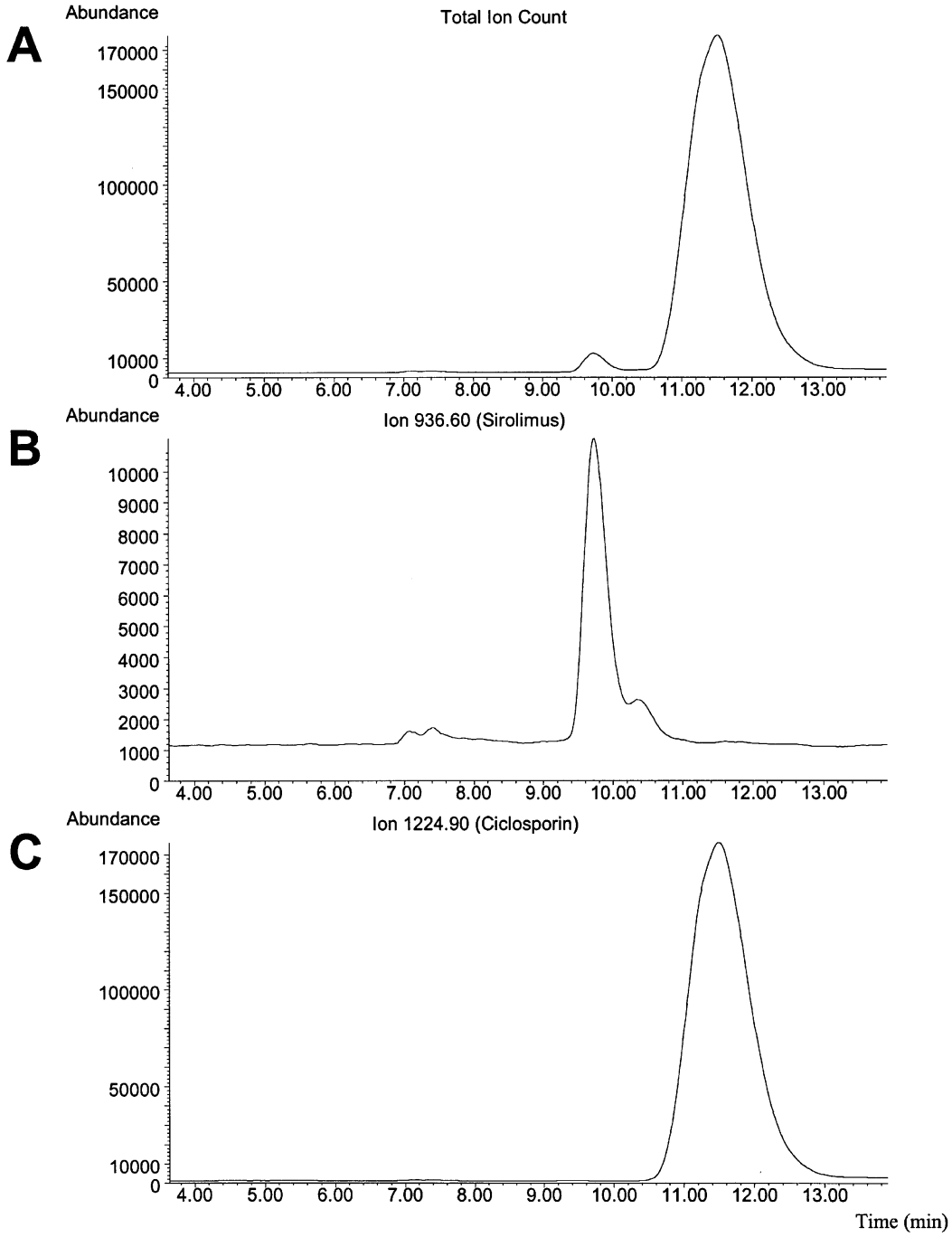

Fig. 4. Representative HPLC–electrospray ion chromatograms of sirolimus and ciclosporin. (A) Total ion count of blood spiked with 20 μg/l sirolimus and 400 μg/l ciclosporin; (B) ion trace of $m/z = 936.6$ (sirolimus); (C) ion trace of $m/z = 1224.9$ (ciclosporin).

Table 1
Recovery (%) of sirolimus and ciclosporin ($n=6$) from blood spiked with both compounds

	Concentration (μg/l)	%±SD
Sirolimus	6	87.3±8.9
	15	96.5±7.7
	30	102.5±4.6
	70	106.2±6.2
Ciclosporin	60	93.6±9.1
	150	103.8±8.5
	300	98.4±3.6
	700	89.6±5.0

By combining the specificity and sensitivity of mass spectrometry detection and on-line sample extraction, our method is significantly less laborious, faster, more robust against interferences such as co-administered drugs, more sensitive, and more reproducible than HPLC–UV. The degree of automation of our assay, which requires only a fast manual protein precipitation step, is comparable to most immunoassays. However, our method has the advantage of being a specific assay and to be able to measure two drugs at the same time in one assay.

Sample throughput and automation for HPLC–MS has become a major concern in the pharmaceutical and clinical laboratory. All previously published methods for quantification of sirolimus [24–26] or the structurally related macrolide immunosuppressant tacrolimus [30,31] were combined with an off-line sample extraction. These off-line sample extraction methods have several disadvantages. They are very time-consuming and error-prone because of multiple manual, sequential preparation steps resulting in high inter-assay variability [32]. During method development, initially cyclosporin D for ciclosporin and 28-*O*-acetyl- or 28,40-*O*-acetyl sirolimus for sirolimus were used as internal standards. The sirolimus internal standards are not commercially available and had to be synthesised as described by Streit et al. [26]. However, the synthesis procedure described by Streit et al. [26] yields only small amounts of the internal standard in solution and

Table 2
Intra-day and inter-day variability of sirolimus and ciclosporin concentrations in blood samples ($n=6$) measured by HPLC–electrospray mass spectrometry

	Theoretical conc. (μg/l)	Mean found conc.±SD (μg/l)	CV (%)	Deviation (%)
Intra-day				
Sirolimus	5	4.6±0.2	4.6	−7.2
	25	25.6±1.0	3.9	+2.6
	50	47.3±1.4	2.9	−5.4
	100	93.3±3.0	3.2	−6.7
Ciclosporin	50	52.9±2.8	5.2	+5.8
	250	239.0±6.0	2.5	−4.4
	500	481.3±20.7	4.3	−3.8
	1000	907.6±50.8	5.6	−9.3
Inter-day				
Sirolimus	5	4.3±0.4	9.5	−13.8
	25	23.8±2.0	8.3	−5.0
	50	47.5±4.2	8.9	−5.0
	100	94.4±7.4	7.8	−5.7
Ciclosporin	50	48.3±4.7	9.7	−3.5
	250	263.1±24.1	9.1	+5.2
	500	473.8±40.3	8.5	−5.2
	1000	910.3±92.9	10.2	−9.0

requires extensive structural identification. Acetyl sirolimus derivatives as internal standards bear the potential risk of contamination with sirolimus due to degradation and require constant control of their purity. The acetyl sirolimus derivatives are impractical as internal standards for routine analysis, since they are unstable in solution and frequent synthesis is required. Not surprisingly, initial validation data actually showed better linearity and inter- and intraday variability for sirolimus without than with internal standard. Since the use of an internal standard did not make a difference for ciclosporin either, we decided against the use of internal standards. Retrospectively, this was supported by the fact that our validation data without internal standards was well within the acceptance limits and compared favourably with those of other previously described assays for sirolimus or ciclosporin [24–26,30,31]. It can be expected that the lower variability compared with other sirolimus or ciclosporin assays was mostly due to the lower variability of our automated on-line extraction procedure compared with manual off-line extraction procedures. In our case, addition of sodium to the mobile phase did not change signal intensities and was considered unnecessary. In another laboratory environment, however, addition of sodium to the mobile phase may be required.

Since ciclosporin and sirolimus distribute mainly into blood cells, whole blood has been recommended as matrix of choice [16,17] and was also used as matrix in our assay. By focusing the mass analyzer on ions of the major metabolites [26], concomitant quantification of metabolites and parent compounds can easily be achieved with our method.

In conclusion, our semi-automated HPLC–ESI-MS method met all pre-defined validation acceptance criteria. Our method allows for a higher sample turnover, is more sensitive and less time-consuming than previously described HPLC–UV and HPLC mass spectrometry methods. In addition, it has the same degree of automation as the immunoassays currently used for clinical drug monitoring, is more specific and, therefore, gives more reliable results. Recent developments indicate a tendency to the use of more effective immunosuppressive drug regimens in transplantation involving more than one drug that requires blood level guided dosing and therapeutic drug monitoring such as the combination of ciclosporin and sirolimus. In these patients, our assay has the advantage over all other assays of simultaneously measuring ciclosporin and sirolimus.

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