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Automated, fast and sensitive quantification of drugs in blood by liquid chromatography–mass spectrometry with on-line extraction: immunosuppressants

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Abstract

We developed a universal LC–mass spectrometry assay with automated online extraction (LC/LC–MS) to quantify the immunosuppressants cyclosporine, tacrolimus, sirolimus and SDZ-RAD alone or in combination in whole blood. After protein precipitation, samples were loaded on a C_{18} extraction column, were washed and, after activation of the column-switching valve, were backflushed onto the C_8 analytical column. $[M+Na]^+$ ions were detected in the selected ion mode. For tacrolimus, sirolimus and SDZ-RAD, the assay was linear from 0.25 to 100 μg/l and for cyclosporine from 7.5 to 1250 μg/l (all $r^2>0.99$). Analytical recovery was >85% and, in general, inter-day, intra-day variability for precision and accuracy were <10%. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Sample preparation; Cyclosporine; Tacrolimus; Sirolimus; SDZ-RAD

1. Introduction

Today, the calcineurin inhibitors cyclosporine and tacrolimus are the basis of most immunosuppressive protocols after organ transplantation. In addition, they are used in the therapy of autoimmune diseases [1–3]. Sirolimus and SDZ-RAD have immunosuppressive mechanisms distinct from those of cyclosporine and tacrolimus. Sirolimus and SDZ-RAD inhibit interleukin-2-stimulated cell cycle progression at the G_1–S interface [4–6]. Co-administration of cyclosporine with sirolimus or SDZ-RAD results in synergistic immunosuppression [6–9] and significantly less rejection of transplant organs [10,11]. The combination of tacrolimus and sirolimus is also beneficial for transplant patients [12,13]. Sirolimus has recently been approved in the US [14] and SDZ-RAD is in phase III of its clinical development.

Cyclosporine is a cyclic undecapeptide (molecular mass, 1203.6 Da). Tacrolimus, sirolimus and SDZ-RAD have macrolide backbone structures (Fig. 1). Tacrolimus (molecular mass, 803.5 Da) is a macrolide lactone with a hemi-ketal masked α,β-diketoamide functionality in a 23-membered ring.

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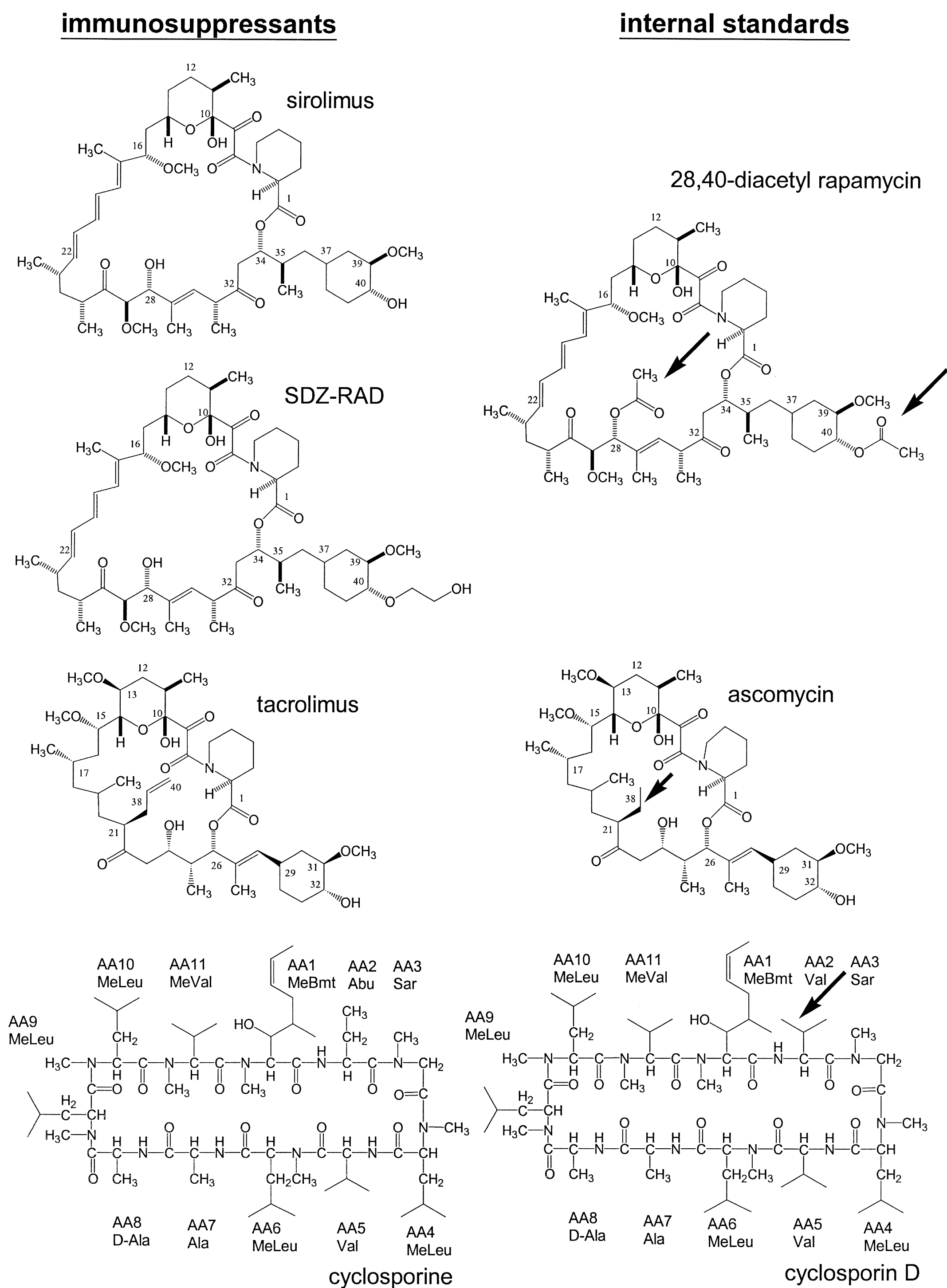

Fig. 1. Structures of the immunosuppressants and their internal standards. Numbering of the macrolide immunosuppressants sirolimus, SDZ-RAD, and tacrolimus and their internal standards follows the IUPAC guidelines [30]. The arrows indicate the structural differences between the internal standards and the corresponding immunosuppressant(s). AA, amino acid.

Sirolimus (rapamycin, molecular mass, 913.6 Da) is a 31-membered triene macrolide lactone with a hemiketal-masked α,β-dioxocarboxamide. SDZ-RAD (molecular mass, 957.2 Da) is the semi-synthetic 40-*O*-(2-hydroxyethyl) derivative of rapamycin [15]. The study drugs have in common that they are soluble in alcohols, acetonitrile, ethers and halogenated hydrocarbons and they are practically insoluble in water and aliphatic hydrocarbons.

The clinical management of cyclosporine and tacrolimus is complicated by their narrow therapeutic indices, intra- and inter-individually highly variable pharmacokinetics, and the lack of a reliable correlation between dose and drug exposure. The four immunosuppressants are mainly metabolized by cytochrome P4503A in the liver and small intestine [16–18] and are substrates of the ATP-binding cassette transporter P-glycoprotein [19]. Several drugs commonly used after transplantation, which are cytochrome P4503A and/or P-glycoprotein substrates, inhibitors and/or inducers, affect blood concentrations of immunosuppressants with the requirement for dose adjustments [20]. Therefore, regular therapeutic drug monitoring and blood concentration guided dosing regimens have been recommended [21–25]. These are general clinical practice for cyclosporine and tacrolimus [21–24] and are discussed for sirolimus [25,26] and SDZ-RAD [11]. For therapeutic drug monitoring of cyclosporine, tacrolimus, sirolimus and SDZ-RAD, several assays are available [23,24]. In clinical routine monitoring immunoassays are mostly used. However, these have the disadvantage that the antibodies used cross-react to a varying extent with metabolites, the immunosuppressants cannot be measured simultaneously and immunoassays are either not available or approved for all immunosuppressants, for example sirolimus [23] and SDZ-RAD.

Increasingly, immunosuppressive drug regimens used after transplantation are based on a combination of two and more immunosuppressants, which require blood level-guided dosing [23,24]. In the near future, more novel immunosuppressive drugs will become available and immunosuppressive drug regimens will be even more individualized. Analytical laboratories will consequently be challenged with blood samples containing a variety of different immunosuppressants and their combinations with relatively few samples containing the same drugs [24]. It was therefore our goal, to develop a single analytical assay for the automated, specific and sensitive measurement of immunosuppressants alone and in combination. Based on our previous work [27–29], we used LC–MS in combination with a rapid automated online extraction procedure (LC/LC–MS).

2. Experimental

2.1. Chemicals

Cyclosporine, SDZ-RAD (40-(2-hydroxyethyl)-rapamycin) and cyclosporin D were kind gifts of Novartis Pharma (Basel, Switzerland) and tacrolimus the kind gift of Fujisawa Healthcare (Deerfield, IL, USA). Sirolimus (rapamycin) and ascomycin were purchased from Sigma (St. Louis, MO, USA). 28,40-*O*-Diacetyl rapamycin was synthesized, purified, the structure verified and purity established as described by Streit et al. [31]. Methanol and water were of HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid and zinc sulfate were from Sigma, and were of reagent grade.

2.2. Equipment

Samples were analyzed on a Hewlett-Packard (Palo Alto, CA, USA) LC/LC–mass selective detector system consisting of the following series 1100 HPLC components. HPLC I: G1311A quarternary pump, G1322A degasser and G1329A autosampler equipped with a G1330A thermostat. HPLC II: G1312A binary pump, G1322A degasser, G1316A column thermostat and G1946A mass selective detector. The two HPLC systems were connected via a 7240 Rheodyne six-port switching valve mounted on a step motor (Rheodyne, Cotati, CA) (see Fig. 2). The system was controlled and data were processed using ChemStation Software Revision A.06.01 (Hewlett-Packard).

2.3. Stock solutions

Stock solutions were prepared from three independent weighings. The immunosuppressants (cyclosporine, tacrolimus, sirolimus and SDZ-RAD) and the

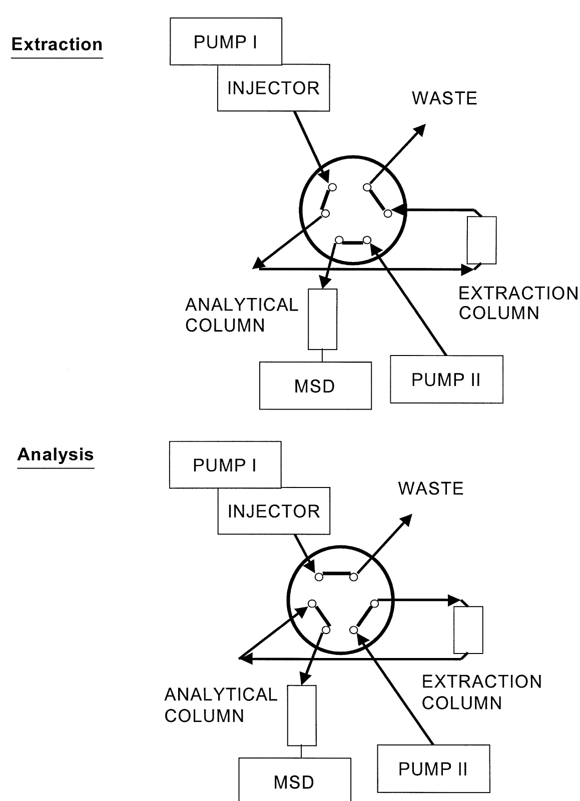

Fig. 2. Connection of the column switching valve and valve positions. HPLC 1, pump I, injector, extraction column; HPLC 2, pump II, analytical column, mass selective detector. MSD, mass-selective detector.

internal standards (cyclosporin D, ascomycin and 28,40-*O*-diacetyl rapamycin; structures see Fig. 1) were dissolved in methanol–0.1% formic acid (9/1, v/v), resulting in a concentration of 1 g/l. For the final preparation of calibration and quality control samples as well as internal standard solutions, stock solutions were diluted using methanol–0.1% formic acid. Due to the instability of sirolimus, SDZ-RAD and 28,40-*O*-diacetyl rapamycin, stock solutions had to be stored at −80°C [31,32].

2.4. Blood samples

Blood for development and validation of the assay was drawn from healthy volunteers. EDTA was used as anticoagulant. Patient samples were drawn during various clinical studies. Collection of blood samples and quantification of immunosuppressants were part of the study protocols. The studies were approved by either the Stanford University or the University of California, San Francisco, ethics committees. All subjects gave their written consents and the studies were carried out in compliance with the Declaration of Helsinki and its amendments following good clinical practice (GCP) guidelines. Blood samples were stored at −80°C.

2.5. Sample preparation (see Table 1)

As a first step, the internal standards were added to the protein precipitation reagent (methanol–0.4 *M* $ZnSO_4$, 4/1, v/v) resulting in final concentrations of 250 μg/l cyclosporin D and 50 μg/l ascomycin and 28,40-*O*-diacetyl rapamycin. Due to the instability of 28,40-*O*-diacetyl rapamycin [31,32], the protein precipitation/internal standard solution had to be freshly prepared before extraction. The internal standard concentrations correspond to addition of 500 μg

Table 1
Comparison of sample preparation steps of blood samples for LC/LC–MS and LC–MS analysis[a]

LC/LC-MS	LC-MS
0.1 ml blood + internal standard + 0.2 ml 0.2 mol/l $ZnSO_4$/ methanol (30/70 v/v)	1 ml blood + internal standard + 2 ml 0.2 mol/l $ZnSO_4$/ methanol (30/70 v/v)
↓	↓
vortex 20 s centrifugation 8000 ·g, 5 min	vortex 20 s centrifugation (2000 ·g, 2 min)
↓	↓ Column preparation: + 2 ml methanol + 2 ml 0.1% formic acid
	draw supernatant through column (-15 mmHg vacuum)
	+ 2 ml 0.1% formic acid
	↓ dry column
	elution with 1.5 ml dichloromethane
	↓
	evaporate to dryness
	↓
↓	reconstitute in 120 μl methanol / 0.1% formic acid
	↓
inject 100 μl into LC/LC-MS	inject 100 μl into LC-MS system

[a] The LC–MS sample preparation procedure was used for SDZ-RAD [32], and with small modifications for tacrolimus [33,34] and sirolimus [31].

cyclosporin D and 100 μg ascomycin and 28,40-diacetyl rapamycin to a 1-ml blood sample.

One hundred μl of the blood sample were transferred into an Eppendorf cup and 200 μl of the protein precipitation reagent were added (Table 1). Samples were vortexed for 20 s and centrifuged at 8000 *g* for 5 min. Two-hundred μl of the supernatant were transferred into HPLC screw cap vials with 250-μl inserts (Hewlett-Packard).

2.6. LC/LC–MS analysis (Table 2)

One hundred μl of the samples were injected onto a 10×2-mm extraction column (Keystone Scientific, Bellefonte, PA) filled with Hypersil ODS-1 of 10 μm particle size (Shandon, Chadwick, UK). Samples were washed with a mobile phase of 40% methanol and 60% 0.1% formic acid supplemented with 1 μmol/l sodium formate. The flow was 5 ml/min and the temperature for the extraction column was set to 65°C. After 0.75 min, the switching valve was activated (Fig. 2) and the analytes were eluted in the backflush mode from the extraction column onto the 50×4.6-mm C_8, 3.5 μm analytical column (Zorbax XDB C_8, Hewlett-Packard, Palo Alto, CA). The mobile phase consisted of methanol and 0.1% formic acid supplemented with 1 μmol/l sodium formate. The following gradient was run (Table 2): time 0 min, 65% methanol; 9 min, 95% methanol. The flow-rate was 0.4 ml/min. The analytical column was also kept at 65°C. Two minutes after sample injection, the mass-selective detector was activated. Settings of the mass selective detector are listed in Table 3. The next sample was injected after 9.5 min.

Table 2
Time programs for solvent delivery pumps (HPLC I and HPLC II), column switching valve and mass selective detector (MSD)[a]

Time	HPLC I	HPLC II	Column switch
0.00 min	40% methanol 5 ml/ min	65% methanol 0.4 ml/ min	Valve in extraction position ↓
0.70 min	40% methanol 5 ml/ min	↓	Valve switches to analysis position
0.80 min . . .	95% methanol 0.1 ml/ min . .	↓	↓ 2.00 min MSD on
6.80 min	95% methanol 0.1 ml/ min	↓	Valve switches back to extraction position
6.81 min	95 % methanol 5 ml/ min	↓	↓
8.00 min	40% methanol 5 ml/ min	95% methanol 0.4 ml/ min	↓
9.00 min	↓	95% methanol 0.4 ml/ min	↓ 9.00 min: MSD off
9.50 min	↓	65% methanol 0.4 ml/ min	↓

[a] Columns for the solvent delivery pumps (HPLC I and HPLC II) show the solvent composition (percent organic solvent, other solvent: 0.1% formic acid+1 μmol/l sodium acetate) and the flow-rate.

2.7. Method validation

2.7.1. Calibration and calibration control samples

Precision control samples (concentrations see Table 4) and calibration control samples (cyclosporine: 5, 7.5, 10, 25, 50, 100, 250, 500, 750, 1000, 1250, 1500 μg/l; tacrolimus, sirolimus, SDZ-RAD: 0.1, 0.25, 0.5, 1, 5, 1, 20, 50, 75 and 100 μg/l) as well as blank samples were prepared in bulk using freshly drawn blood. To allow distribution, samples were incubated at 37°C in a water bath for 30 min. Then 1-ml aliquots were transferred into screw-cap glass tubes and either immediately analyzed or stored at −80°C.

2.7.2. Acceptance criteria

The assay was considered acceptable if precision (% C.V.) at each concentration was less than 15% for intra- and day-to-day variability. The accuracy compared with the nominal value had to be within ±15% for both intra- and day-to-day variability. The calibration curve had to have a correlation coefficient r^2 of 0.99 or better. The absolute recovery had to exceed 60%.

2.7.3. Calibration curve

Six samples of each concentration were measured. Linearity was assessed using the regression analysis implemented in the Microcal Origin software (version 3.5, Microcal Software, Northampton, MA, USA).

2.7.4. 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 variation had to be less than 20%.

Table 3
Mass selective detector settings[a]

Parameter	
Capillary exit voltage (fragmentor)	+160 V
Capillary voltage (V_{cap})	−4000 V
Ion energy (octopole)	+5 V
Nebulizer gas	Nitrogen, purity 5.0; pressure, 40 p.s.i. (1 p.s.i.=6.894.76 Pa)
Drying gas	Nitrogen, purity 5.0; temperature, 300°C; flow, 10 l/min
Quadrupole temperature	100°C
Selected ions	*m/z*
Cyclosporine	1224: cyclosporine, 1238: cyclosporin D (internal standard)
Tacrolimus	826: tacrolimus, 815: ascomycin (internal standard)
Rapamycin, SDZ-RAD	936: sirolimus, 980: SDZ-RAD, 1020: 28,40-diacetyl rapamycin (internal standard)
Dwell time/ion	124 ms

[a] Positive ions $[M+Na]^+$ were measured in the selected ion mode. The nomenclature follows that used in the ChemStation software (revision A.06.01, Hewlett-Packard).

2.7.5. 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 ($n=10$ for each concentration). Day-to-day variability was assessed by analysis of five sets of quality control samples on three different days.

Table 4
Validation results

	Cyclosporine		SDZ-RAD		Sirolimus		Tacrolimus	
Lower limit of quantitation	7.5 μg/l		0.25 μg/l		0.25 μg/l		0.25 μg/l	
Upper limit of quantitation	1250 μg/l		100 μg/l		100 μg/l		100 μg/l	
Regression analysis	$y=0.93(\pm0.03)+15.1(17.4)x$ $r^2=0.995$		$y=0.96(\pm0.03)+0.05(\pm0.04)x$ $r^2=0.999$		$y=0.99(\pm0.02)+0.09(\pm0.08)x$ $r^2=0.990$		$y=0.94(\pm0.01)+0.06(\pm0.06)x$ $r^2=0.999$	
Intra-day precision	7.5 μg/l:	2.5%	1 μg/l:	2.5%	1.5 μg/l:	7.2%	0.25 μg/l:	12.3%
	125 μg/l:	3.9%	5 μg/l:	3.9%	15 μg/l:	5.5%	1 μg/l:	3.7%
	375 μg/l:	0.9%	25 μg/l:	0.9%	40 μg/l:	6.2%	25 μg/l:	1.5%
	1250 μg/l:	2.6%	100 μg/l:	2.6%			100 μg/l:	15.8%
Day-to-day precision	75 μg/l:	2.5%	1 μg/l:	6.5%	1.5 μg/l:	7.1%	5 μg/l:	4.4%
	200 μg/l:	3.6%	25 μg/l:	5.5%	15 μg/l:	9.8%	20 μg/l:	0.7%
	700 μg/l:	2.7%	100 μg/l:	9.1%	40 μg/l:	6.7%	70 μg/l:	1.6%
Accuracy	75 μg/l:	−0.9%	1 μg/l:	−7.1%	1.5 μg/l:	+3.7%	5 μg/l:	−3.4%
	200 μg/l:	−4.1%	25 μg/l:	+2.6%	15 μg/l:	+7.4%	20 μg/l:	+8.6%
	700 μg/l:	+2.6%	100 μg/l:	−3.8%	40 μg/l:	+8.0%	70 μg/l:	+3.6%

2.7.6. 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 standard solutions of the immunosuppressants (in methanol–0.1% formic acid, 9:1, v/v) directly on the analytical column, bypassing the extraction column.

2.7.7. Matrix interferences and carry-over effects

The lack of matrix interferences was established by analysis of blank blood samples ($n=6$). The lack of carry-over effects was assessed by alternately analyzing blank blood samples ($n=6$) and blood samples containing concentrations of the immunosuppressants at the upper limit of quantitation (100 μg/l, $n=6$).

2.7.8. Within-batch stability

Stability of the immunosuppressants and their internal standards after protein precipitation in the autosampler was established for 48 h. Ten sets of quality control samples were prepared as described in Section 2.5, and placed into the autosampler adjusted to +10°C. Five sets were analyzed at once (controls) and five sets 48 h later.

3. Results

The recoveries of the study drugs after protein precipitation and column switching were: cyclosporine, 91.3±9.8%; tacrolimus, 96.2±5.6%; sirolimus, 88.0±12.1%; and SDZ-RAD, 86.1±9.9% (means±standard deviation). The recoveries of the internal standards were not significantly different from those of the immunosuppressants. Comparison of peak areas after injection of immunosuppressant solutions (10 μl of 1 mg/l in methanol–0.1% formic acid, $n=5$) into the LC/LC–MS system with those after injection of the same solution directly onto the analytical column showed that no drug was lost during the online extraction procedure (cyclosporine, 101±3.2%; tacrolimus, 99.6±5.5%; sirolimus, 102.3±3.4%; SDZ-RAD, 98.8±5.0%) and indicated that losses and most of the variability during extraction had to be attributed to the protein precipitation step.

Under the conditions described, in the positive mode, the immunosuppressants and their internal standards were mainly detected as sodium adducts $[M+Na]^+$ (Fig. 3). The intensities of $[M+H]^+$ and $[M+K]^+$ combined were less than 10% of the $[M+Na]^+$ signals.

The lower limit of quantitation of cyclosporine was 7.5 μg/l, and 0.25 μg/l for the macrolides. Although peaks were detected at lower concentrations with a signal-to-noise ratio above 3, more than 20% (two of six) of the samples were outside the predefined acceptance limits. The upper limit of quantitation was 1250 μg/l for cyclosporine. Higher concentrations of cyclosporine gave results more than 15% below the nominal concentration in four of six samples. For tacrolimus, sirolimus and SDZ-RAD, the LC/LC–MS assay was linear up to the highest concentration tested (100 μg/l). Intra-day, day-to-day precision and accuracy were within the pre-defined acceptance limits (Table 4). No matrix interferences or carry-over effects were seen. Within-batch stability was at least 48 h, and thus exceeded the autosampler capacity: It took less than 17 h to run 100 samples with a sample turnover rate of 10 min/sample.

Representative ion chromatograms of patient samples are shown in Figs. 4 and 5.

The methanol–formic acid gradient used to elute the analytes from the analytical column also allowed for separation and simultaneous quantification of the major metabolites of the immunosuppressants. As found for the parent compounds, electrospray ionization of the metabolites yielded >90% as sodium adducts $[M+Na]^+$: cyclosporine: $m/z=1256$: dihydroxy cyclosporine, $m/z=1240$: hydroxy cyclosporine (AM1 and AM9 as one peak, AM1c), $m/z=1210$: AM4N; tacrolimus: $m/z=812$: 13-*O*-, 15-*O*-, and 31-*O*-desmethyl tacrolimus (Fig. 4C); sirolimus: $m/z=952$: hydroxy sirolimus, $m/z=968$: dihydroxy sirolimus, $m/z=922$: desmethyl sirolimus; SDZ-RAD: $m/z=996$: hydroxy SDZ-RAD, $m/z=966$: desmethyl SDZ-RAD. Potential differences of the detector responses of the metabolites and the parent compounds and/or internal standards were assessed by comparison of the peak area ratios (internal standard/metabolite and parent compound/metabolite) after UV and MS detection. The ratios calculated after UV and MS detection were not significantly different from each other (paired *t*-test, $n=10$ sam-

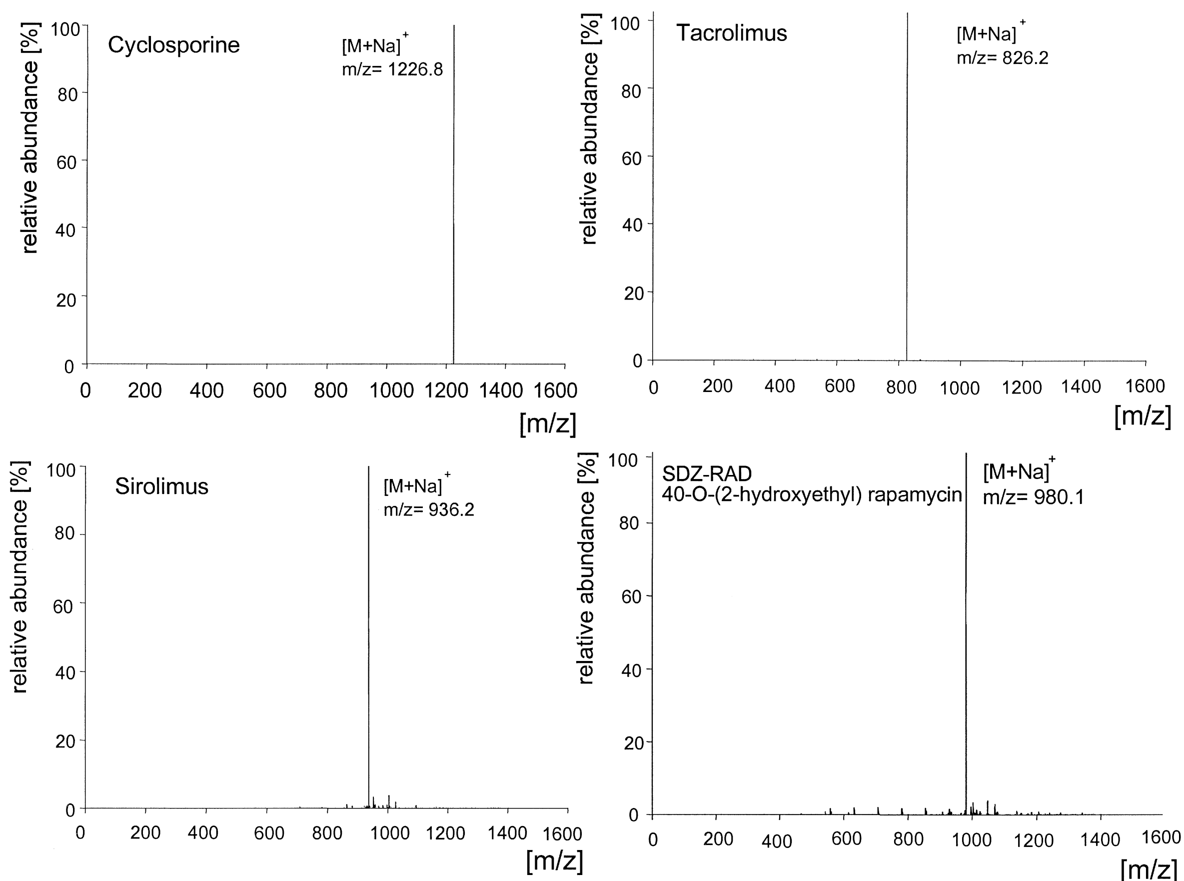

Fig. 3. Full scan mass spectra after injection of the study drugs into the mass spectrometer. Five-hundred ng of the study drugs in 50 μl methanol–0.1% formic acid (9/1, v/v) were injected into the LC/LC–MS system.

ples), indicating that the internal standards, parent compounds and metabolites resulted in similar detector responses.

As of today, we have used our LC/LC–MS for the measurement of more than 10 000 samples for therapeutic drug monitoring as well as animal and clinical studies. The extraction column was changed every 500 samples. More than 2500 samples were run on an analytical column without loss of sensitivity, accuracy or precision.

4. Discussion

Previously described HPLC-UV assays of immunosuppressants are specific and are generally considered the gold standard in the quantification of immunosuppressants. However, they suffer from poor precision, are vulnerable to interferences from matrix and co-administered drugs and require tedious and time-consuming extraction procedures [24]. Even when HPLC was combined with mass spectrometry detection, extensive multi-step extraction procedures (see Table 1) resulting in unacceptable variability were required [31–34]. Most LC–MS/MS assays [35–39] also use multi-step external column extraction procedures, and only LC–MS/MS assays for the quantification of single immunosuppressants have been reported. Here, we describe an LC/LC–MS assay, utilizing automated online sample extraction and a mass-selective detector, that

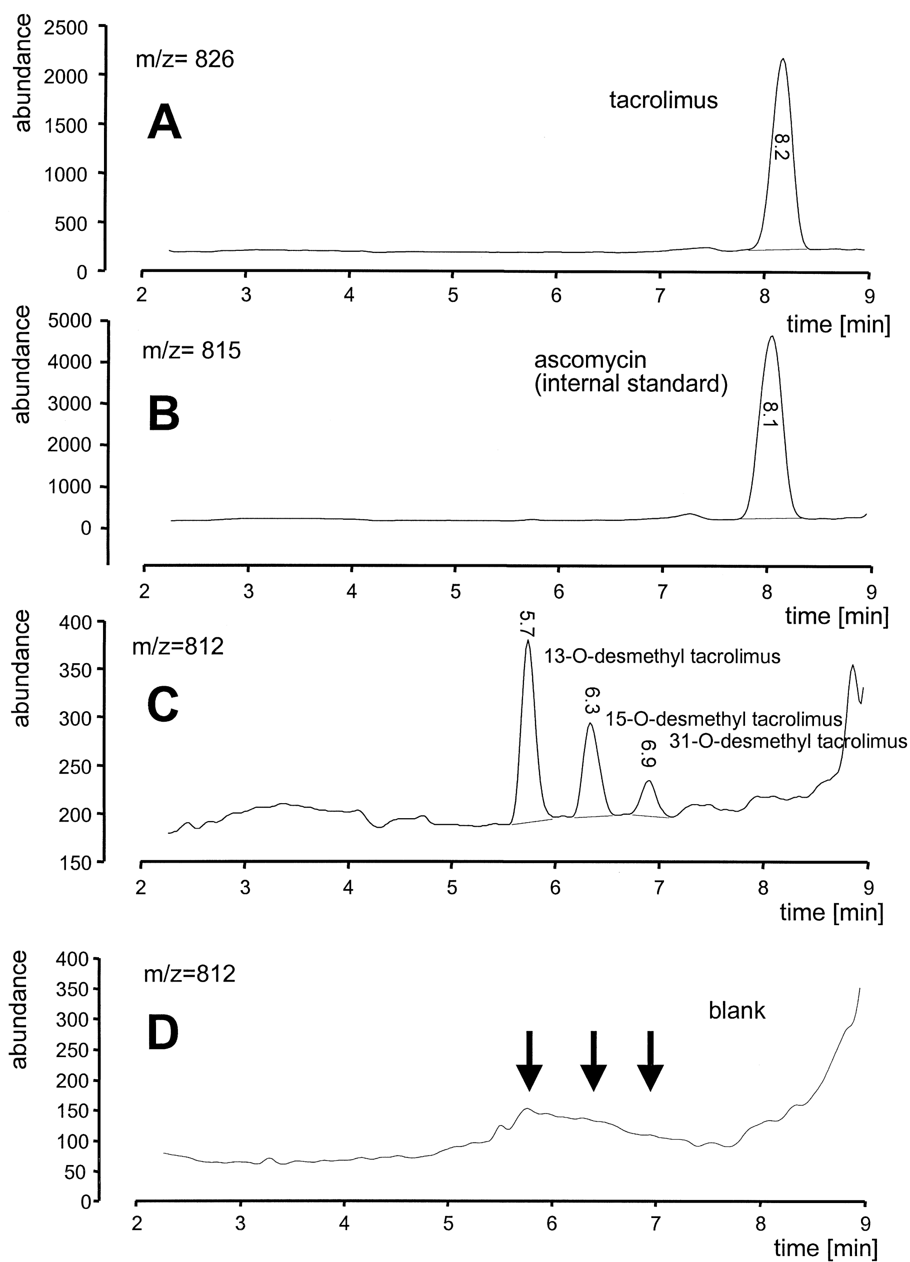

Fig. 4. Representative ion chromatograms of tacrolimus blood samples. Blood samples were taken before and after a healthy volunteer took a single oral 5-mg tacrolimus dose. Ion chromatogram (A) shows tacrolimus (38.6 μg/l, 2.5 h after tacrolimus administration), (B) the internal standard ascomycin (100 μg/l) for the analyses in (A) and (C), (C) the metabolites 13-*O*-desmethyl (2.2 μg/l), 15-*O*-desmethyl (1.4 μg/l) and 31-*O*-desmethyl tacrolimus (0.73 μg/l, same sample as (A) and (B)), and (D) the ion chromatogram (m/z=812, desmethyl tacrolimus) of a blank sample of the same pharmacokinetic profile drawn before tacrolimus administration. The arrows mark the retention times of the tacrolimus metabolite peaks.

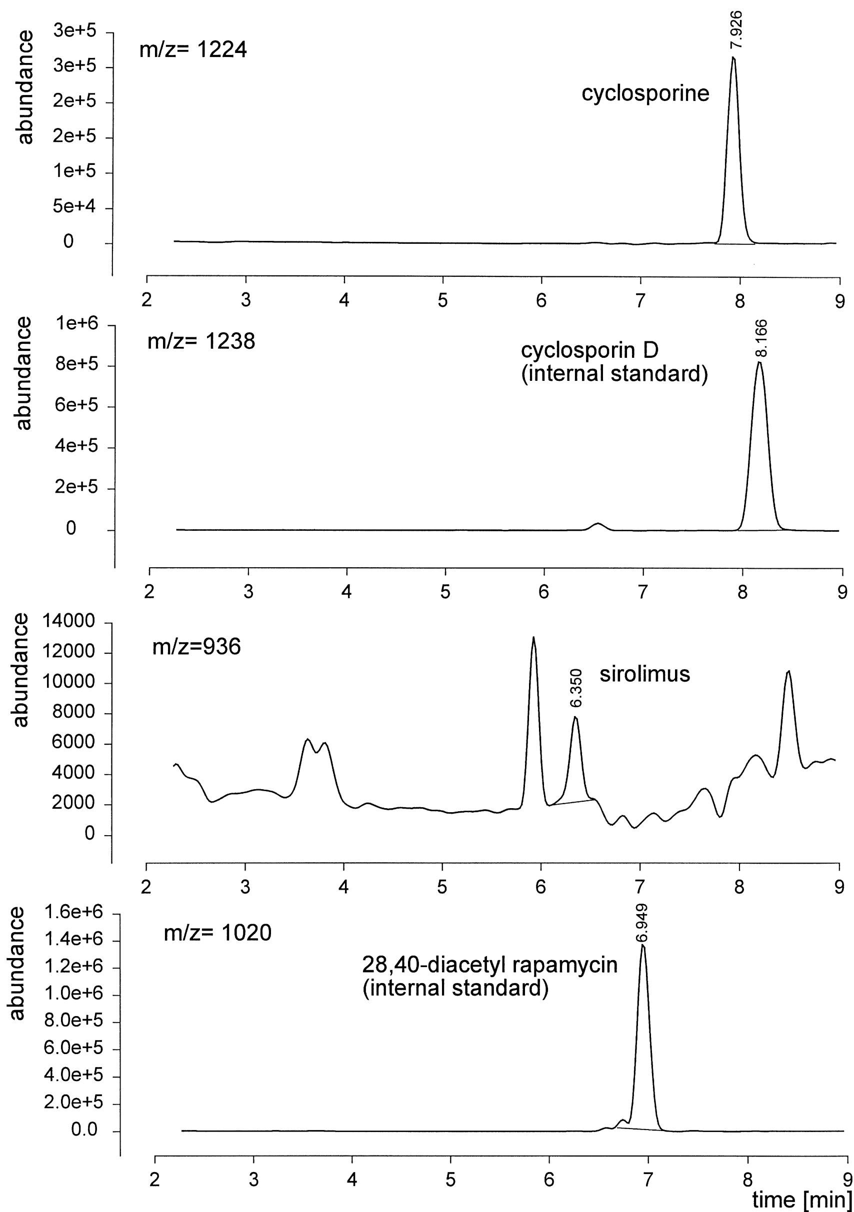

Fig. 5. Representative ion chromatograms of a blood sample from a kidney graft patient 12 h after oral administration of cyclosporine and 8 h after oral administration of sirolimus. Five hundred μg/l cyclosporin D and 100 μg/l 28,40-*O*-diacetyl rapamycin were added as internal standards for cyclosporine and sirolimus, respectively. The concentration of cyclosporine was 162 μg/l and that of sirolimus was 0.55 μg/l.

is integrated into the analytical system as an HPLC component.

Although LC–MS assays have been reported for cyclosporine, tacrolimus, sirolimus and SDZ-RAD, development of an assay to quantify all four drugs in one assay involved identification of conditions during extraction and HPLC analysis that accommodated the quantification of all study drugs. Potential

problems involved the 10-fold higher therapeutic concentration range of cyclosporine in comparison to that of the macrolide immunosuppressants and the chromatographic conditions necessary to avoid broadening of cyclosporine peaks. As shown, the extraction and LC–MS procedure described above resulted in a lower limit of quantitation that was 5-fold lower than the lower limit of the clinical target concentrations for each of the immunosuppressants, while the linear range of the assay included the concentration ranges relevant for patients.

Although good precision and accuracy of the measurement of immunosuppressant in blood in combination with automated online-extraction can be achieved without addition of internal standards [27–29], we used internal standards for the reasons discussed in detail elsewhere [31–33]. In comparison to other internal standards used for LC–MS quantification of tacrolimus [33,34], ascomycin has the advantage that it is generally available from a commercial source. As an alternative to 28,40-*O*-diacetyl rapamycin, we used 32-desmethoxy rapamycin (Wyeth-Ayerst Research, Pearl River, NY, USA) or 40-*O*-(3-hydroxypropyl) rapamycin (Novartis Pharma, Basel, Switzerland) with similar results. Sirolimus cannot be used as an internal standard for quantification of SDZ-RAD since blood of SDZ-RAD treated patients may contain traces of sirolimus, as a minor SDZ-RAD metabolite [18].

Our assay uses only 100 μl of blood and therefore allows for quantification of immunosuppressants in small animal studies and pediatric patients [37]. In addition, we avoided the large injection volumes necessary in previously described LC/LC–MS assays [27–29]. The extensive sample preparation procedures used in previously described LC–MS assays for immunosuppressive drugs were necessary since the immunosuppressants distribute mainly into the corpuscular blood components, and whole blood is the recommended matrix for therapeutic drug monitoring [21,22,25]. As for our assay, most other LC–MS assays include a protein precipitation step with $ZnSO_4$ [27–29,31–34,38]. The automated column extraction procedure was necessary to remove residual blood components and high concentrations of inorganic salt to reduce contamination of the MS source.

Column-switching techniques for automated online sample preparation are well established in HPLC analysis [40]. In general, these have worked well with plasma samples and drugs with specific UV-absorption maxima. In comparison to plasma, blood sample preparation is much more complex [41] and both cyclosporine and tacrolimus have UV absorption maxima $<$200 nm. Although HPLC-UV assays in combination with column-switching on-line sample preparation have been described for cyclosporine [42–44], the extraction procedures were complicated and time-consuming and those assays have never widely been used. In comparison to HPLC-UV, LC–MS due to its selectivity is more robust against interferences. In our assay, the supernatants after protein precipitation were loaded onto the extraction columns and washed at a high solvent flow of 5 ml/min for 0.7 min. This was a significant improvement over previously published LC–MS assays for immunosuppressants using online sample preparation [27–29] that required an extraction wash step of several minutes. In our experience, column switching sample preparation has not been a significant source of analytical failures.

One of the problems with the HPLC analysis of cyclosporine is peak broadening due to incomplete separation of conformers [45]. Peak broadening is reduced by column temperatures of 65°C and above, and mobile phase pH$<$5 [41,45]. An additional reason for the low pH of the mobile phase was the stability of sirolimus, SDZ-RAD and 28,40-*O*-diacetyl rapamycin [32,46].

We took advantage of the automatic bypass valve in the mass selective detector. The mass-selective detector was activated 2 min after the column switch (Fig. 2). Before the bypass valve was activated, material not retained on the column such as inorganic salt was flushed into waste without getting into contact with the electrospray source.

Sodium adduct ions, $[M+Na]^+$, gave the strongest signals. Even if 2 m*M* ammonium acetate was added to the loading buffer and mobile phase to induce formation of $[M+NH_4]^+$ at the expense of other ion species, $[M+Na]^+$ still gave a significant signal. Addition of sodium ions to the loading buffer and mobile phase, however, almost completely suppressed formation of other ions (Fig. 3). Therefore,

as described previously [27–29,31,32], we decided to focus the mass spectrometer on $[M+Na]^+$ and added sodium formate to the HPLC solvents. Measurement of negative ions was not an option due to the low pH of the mobile phase required.

Our assay separately quantified the major metabolites of the four drugs. Due to the unavailability of authentic metabolite standard materials, no attempt was made to validate quantification of the metabolites. Similar detector responses of the corresponding parent compounds, metabolites and internal standards have been described before [31–34], and indicated that the metabolite concentrations can be estimated by extrapolating from the calibration curves of the parent compounds. This approach is supported by previously reported studies [29,31]. If quantification of the metabolites is not required, the methanol–0.1% formic acid gradient (Table 2) can be replaced by an isocratic elution with methanol–0.1% formic acid (9/1, v/v). This modification results in a 2-min shorter analysis time and a higher sample turn-over.

Our LC/LC–MS assay can easily be adapted to analyze novel immunosuppressants with structures related to cyclosporine, tacrolimus or sirolimus by addition of the corresponding masses to the list of selected ions. In our laboratory, the LC/LC–MS assay served as a platform for the development of assays for other groups of drugs such as HIV protease inhibitors, HMG-CoA reductase inhibitors, azole antifungals, mycophenolic acid and metabolites, as well as losartan and its active major metabolite EXP3174. In most cases, the adaptation involved modification of the loading buffer and focussing the mass spectrometer on the corresponding selected ions. The set up of the LC/LC–MS system as described above, allows us to analyze different drug classes in one automated sequence.

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References

[1] B.D. Kahan, New Engl. J. Med. 321 (1989) 1725.
[2] D. Faulds, K.L. Goa, P. Benfield, Drugs 45 (1993) 953.
[3] D.H. Peters, A. Fitton, G.L. Plosker, D. Faulds, Drugs 46 (1993) 746.
[4] S.L. Schreiber, Science 251 (1991) 283.
[5] 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.
[6] T. Boehler, J. Waiser, K. Budde, S. Lichter, A. Jauho, L. Fritsche, A. Korn, H.H. Neumayer, Transplant. Proc. 30 (1998) 2195.
[7] H.-J. Schuurman, S. Cottens, S. Fuchs, J. Joergensen, T. Meerloo, R. Sedrani, M. Tanner, G. Zenke, W. Schuler, Transplantation 64 (1997) 32.
[8] S.M. Stepkowski, L. Tian, K.L. Napoli, R. Ghobrial, M.E. Wang, T.C. Chou, B.D. Kahan, Clin. Exp. Immunol. 108 (1997) 63.
[9] S.M. Stepkowski, K.L. Napoli, M.E. Wang, X. Qu, T.C. Chou, B.D. Kahan, Transplantation 62 (1996) 986.
[10] B.D. Kahan, B.A. Julian, M.D. Pescovitz, Y. Vanrenterghem, J. Neylan, Transplantation. 68 (1999) 1526.
[11] B. Hausen B, T. Ikonen, N. Briffa, G.J. Berry, U. Christians, R.C. Robbins, L. Hook, N. Serkova, L.Z. Benet, W. Schuler, R.E. Morris, Transplantation 69 (2000) 76.
[12] D.M. Vu, S. Qi, D. Xu, J. Wu, W.E. Fitzsimmons, S.N. Sehgal, L. Dumont, S. Busque, P. Daloze, H. Chen, Transplantation 64 (1997) 1853.
[13] V.C. McAlister, Z.H. Gao, K. Peltekian, J. Domingues, K. Mahalati, M.S. MacDonald, Lancet 355 (2000) 376.
[14] J.L. Miller, Am. J. Health Syst. Pharm. 56 (1999) 2177.
[15] R. Sedrani, S. Cottens, J. Kallen, W. Schuler, Transplant. Proc. 30 (1998) 2192.
[16] T. Kronbach, V. Fischer, U.A. Meyer, Clin. Pharmacol. Ther. 43 (1998) 630.
[17] M. Sattler, F.P. Guengerich, C.H. Yun, U. Christians, K.F. Sewing, Drug Metab. Dispos. 20 (1992) 753.
[18] W. Jacobsen, N. Serkova, K. Hallensleben, K. Sewing, L.Z. Benet, U. Christians, ISSX Proc. 15 (1999) 117.
[19] A. Lo, G.J. Burckhart, J. Clin. Pharmacol. 39 (1999) 995.
[20] J.F. Trotter, Semin. Gastroenterol. Dis. 9 (1998) 147.
[21] M. Oellerich, V.W. Armstrong, B.D. Kahan, L. Shaw, D.W. Holt, R.W. Yatscoff, A. Lindholm, P. Halloran, K. Gallicano, K. Wonigeit, E. Schuetz, H. Shran, T. Annesley, Ther. Drug Monit. 17 (1995) 643.
[22] W.J. Jusko, A.W. Thomson, J.J. Fung, P. McMaster, P.H. Wong, E. Zylber-Katz, U. Christians, W. Fitzsimmons, R. Lieberman, J. McBride, M. Kobayashi, S.J. Soldin, Ther. Drug Monit. 17 (1995) 606.
[23] L.M. Shaw, D.W. Holt, P. Keown, R. Venkataramanan, R.W. Yatscoff, Clin. Ther. 21 (1999) 1632.
[24] A. Johnston, D.W. Holt, Br. J. Clin. Pharmacol. 47 (1999) 339.
[25] R.W. Yatscoff, R. Boeckx, D.W. Holt, B.D. Kahan, D.F.

LeGatt, S. Sehgal, S.J. Soldin, K. Napoli, C. Stiller, Ther. Drug Monit. 17 (1995) 676.
[26] K.L. Napoli, B.D. Kahan, Transplant. Proc. 30 (1998) 2189.
[27] C. Vidal, G.I. Kirchner, G. Wuensch, K.F. Sewing, Clin. Chem. 44 (1998) 1275.
[28] G.I. Kirchner, C. Vidal, W. Jacobsen, A. Franzke, K. Hallensleben, U. Christians, K.F. Sewing, J. Chromatogr. B 721 (1999) 285.
[29] G.I. Kirchner, C. Vidal, M. Winkler, L. Mueller, W. Jacobsen, A. Franzke, K.F. Sewing, Ther. Drug Monit. 21 (1999) 116.
[30] IUPAC Nomenclature of Organic Chemistry. A Guide to IUPAC Nomenclature of Organic Components (recommendations 1993). Blackwell Scientific Publications, Boston, MA, 1993.
[31] 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.
[32] I. Segarra, T.R. Brazelton, N. Gutermann, B. Hausen, R.E. Morris, L.Z. Benet, U. Christians, J. Chromatogr. B 720 (1998) 179.
[33] U. Christians, F. Braun, M. Schmidt, N. Kosian, H.M. Schiebel, L. Ernst, M. Winkler, C. Kruse, A. Linck, K.F. Sewing, Clin. Chem. 38 (1992) 2025.
[34] A.K. Gonschior, U. Christians, M. Winkler, H.M. Schiebel, A. Linck, K.F. Sewing, Ther. Drug Monit. 17 (1995) 504.
[35] P.J. Taylor, A. Jones, G.A. Balderson, S.V. Lynch, R.L. Norri, S.M. Pond, Clin. Chem. 42 (1996) 279.
[36] A.M. Alak, S. Moy, M. Cook, P. Lizak, A. Niggebiugge, S. Menard, A. Chilton, J. Pharm. Biomed. Anal. 16 (1997) 7.
[37] P.J. Taylor, C.E. Jones, P.T. Martin, S.V. Lynch, A.G. Johnson, S.M. Pond, J. Chromatogr. B 705 (1998) 289.
[38] P.J. Taylor, A.G. Johnson, J. Chromatogr. B 718 (1998) 251.
[39] Q. Zhang, J. Simpson, H.I. Aboleneen, Ther. Drug Monit. 19 (1997) 470.
[40] R. Huber, K. Zech, in: R.W. Frei, K. Zech (Eds.), Selective Sample Handling and Detection in High-Performance Liquid Chromatography, J. Chromatogr. Library, Vol. 39A, Elsevier, Amsterdam, 1988, pp. 81–144.
[41] U. Christians, K.F. Sewing, in: K. Zech, R.W. Frei (Eds.), Selective Sample Handling and Detection in High-Performance Liquid Chromatography, J. Chromatogr. Library, Vol. 39B, Elsevier, Amsterdam, 1989, pp. 82–132.
[42] D.J. Gmur, P. Meier, G.C. Yee, J. Chromatogr. 425 (1988) 343.
[43] H.T. Smith, W.T. Robinson, J. Chromatogr. 305 (1984) 353.
[44] P.E. Wallemacq, M. Lesne, J. Chromatogr. 413 (1987) 131.
[45] L.D. Bowers, S.E. Mathews, J. Chromatogr. 333 (1985) 231.
[46] P.C. Wang, K.W. Chan, R.A. Schiksnis, J. Scatina, S.F. Sisenwine, J. Liq. Chromatogr. 17 (1994) 3383.