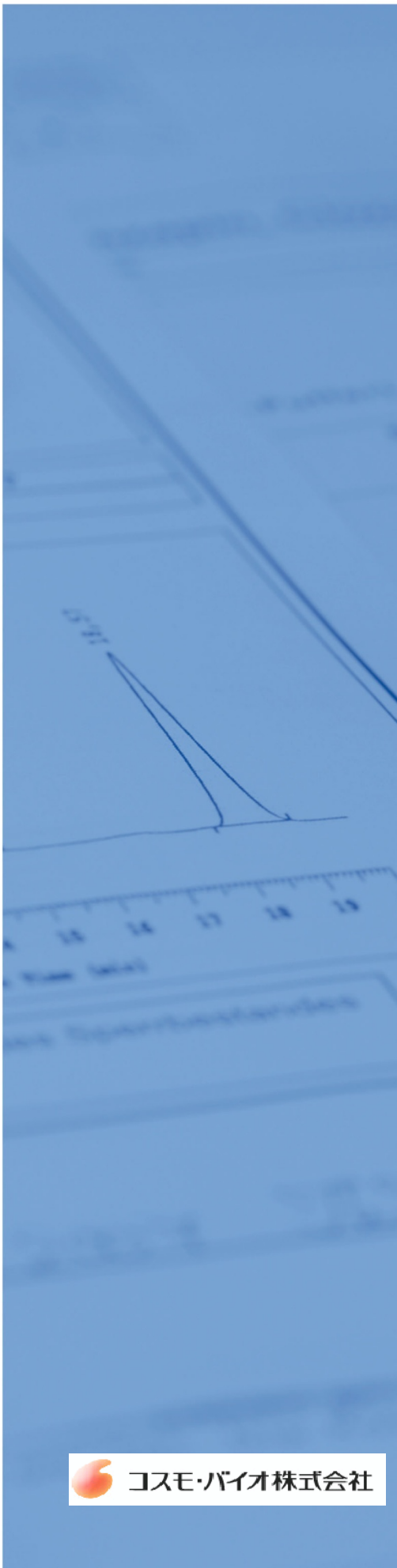
RECIPE®

Instruction Manual

ClinMass® LC-MS/MS Complete Kit

Immunosuppressants in Whole Blood – On-Line Analysis



REF MS1000

IVD For in vitro diagnostic use

CE IVDD, 98/79/EC

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1 Introduction

1.1 Intended use

This ClinMass® Complete Kit is intended for the determination of Cyclosporine A, Tacrolimus, Sirolimus, and Everolimus from human whole blood with LC-MS/MS.

The kit components have to be used in accordance with this user manual. The kit is not designed for combination with components by other manufacturers.

1.1.1 IVD symbols

Symbols according to EU directive 98/79/EC for in vitro diagnostic medical devices (IVDD), which are used on the product labels and in this user manual:

IVD	For in vitro diagnostic use	REF	Order number
	Manufacturer	LOT	Lot number
			Upper temperature limit: … °C
			Temperature limits: … °C to …°C
			Expiry date: …
			See instructions for use

1.2 Clinical background

Cyclosporine A, Tacrolimus, Sirolimus, and Everolimus (see figure 1) are immunosuppressive drugs used after organ transplantation. The goal of the therapy is to prevent an acute allograft rejection by inhibition of the immunological defence of the recipient with, as far as possible, minimal effect on the immunological resistance towards infections [1-3].

Immunosuppressive drugs function through various mechanisms. Cyclosporine A and Tacrolimus are calcineurin inhibitors and block the interleukin-2 production, leading to a decrease in T lymphocyte proliferation. Sirolimus and Everolimus act at a later stage than the calicineurin inhibitors by inhibiting the interleukin-2-stimulated cell cycle progression [4]. Due to the complementary mechanisms of action, these two classes of agents are often combined in patient treatment, whereby taking advantage of the synergistic effects [5].

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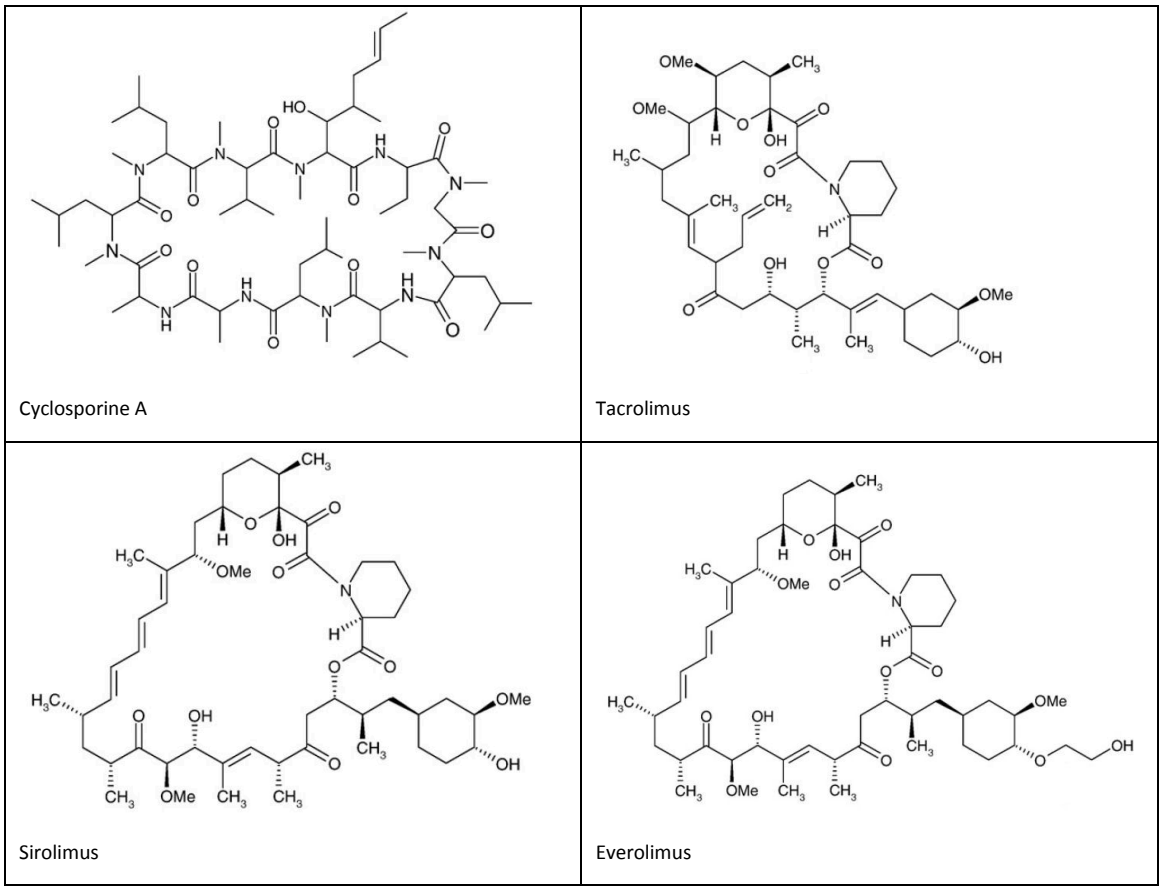

Figure 1: Structural formulas of Cyclosporine A, Tacrolimus, Sirolimus, and Everolimus

The administration of immunosuppressants requires accurate therapeutic drug monitoring (TDM) within a narrow therapeutic concentration range. Overdose of these drugs increases the risks of severe side effects, whilst underdose can result in immunological rejection and organ loss or damage. Both can significantly reduce the lifespan of the organ recipients.

The pharmacokinetics of immunosuppressants, in general, characteristically shows poor bioavailability and a large intra- and inter-individual variation. Consequently, the correlation between drug dosage and blood concentration is poor and results in a need to individualise the dose regimen for different recipients.

To avoid over- or under-administration, dosage regimens are usually adjusted according to the whole blood concentration. For the dose adjustment, the trough concentrations (termed as C-0) are often used, i.e. the values, which are measured before the next medication. For Cyclosporine A, the C-2 level (blood level measured 2 hours after dose administration) is preferred, due to a better correlation with the pharmacological effect [2, 6].

For the therapeutic drug monitoring, liquid chromatography (LC) based methods are considered the methodology of choice. Immunological methods, although widely used in clinical laboratories, lack in analytical specificity. Cross reactions between drug and drug metabolites can result in an overestimation of the measured drug, with unacceptable biases in some clinical situations (see e.g. [2]). The use of liquid chromatography with tandem mass spectrometry (LC-MS/MS) allows a highly selective quantification of the main drug independently from its metabolites (see section 1.3).

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1.3 General description of the analytical procedure

In this analytical method, Cyclosporine A, Tacrolimus, Sirolimus and Everolimus are determined from human whole blood by on-line SPE HPLC with electrospray-tandem mass spectrometry.

Prior to the on-line analysis a short sample clean-up is performed in order to remove the sample matrix and to spike with the internal standards (sample pretreatment, see section 5.2).

Conventional HPLC methods require manual sample preparation, performed prior to the sample injection. With on-line HPLC methods, the sample preparation is performed within the LC-MS/MS configuration by column switching, using a multiple port valve and a SPE-column (on-line sample preparation).

After chromatographic separation on the second, the analytical column within the HPLC system, the analytes are ionised by electrospray ionisation (ESI) and detected by the tandem mass spectrometer (MS/MS).

In electrospray ionization, the sample components are ionized and then transferred to the gas phase, where they subsequently pass into the MS/MS, which is composed of two quadrupoles, connected through a collision cell.

In the present analytical method, the MS/MS measurement of the analytes is performed in the MRM (Multiple Reaction Monitoring) mode. In this mode only selected ions (known as the 'precursor ions') with a defined mass/charge ratio (m/z) are isolated in the first quadrupole and subsequently are transferred into the collision cell. These ions are then fragmented by impact with an inert gas (argon or nitrogen) at selectively appropriate voltage settings. Among the fragments generated (known as the 'product ions'), only those with a defined m/z ratio are isolated in the final quadrupole for subsequent detection. Thus, measurement in MRM mode ensures identification and quantification with high selectivity and sensitivity, with the analyte identification based on highly characteristic mass transitions for the compound of interest.

ClinMass® Optimisation Mixes are provided for the optimisation of the MS/MS parameters (see section 5.3.1) and for the test run of the analytical system (see section 5.3.2).

The calibration of the analytical system is performed by use of ClinCal® Multi-Level Calibrators (see section 5.3.3). For this purpose a 4-level calibrator set (level 0-3, order no. 9033) as well as a 7-level calibrator set (level 0-6, order no. 9933) are available. For an extended calibration range with an additional, high calibration point (level 7), the whole blood calibrator with order no. 9028 is optionally available.

Quality control is performed by use of ClinChek® Whole Blood Controls. These controls are available in five different concentrations (see section 5.3.4).

2 Components of the complete kit and accessories

2.1 Ordering information

Order No.	Description	Quantity
MS1000	**ClinMass® Complete Kit for Immunosuppressants in Whole Blood** for 400 assays	1 pce.
	Contents:	
	Autosampler Washing Solution	1 x MS1005
	SPE - Buffer	2 x MS1009
	Mobile Phase	1 x MS1010
	IS Internal Standard, lyophil.	3 x MS1212
	Sample Pretreatment Vials	4 x MS1020
	P Precipitant	1 x MS1021
	Manual	
	Separately available components:	
MS1005	Autosampler Washing Solution	1000 ml
MS1006	Reagent MP	8 ml
MS1009	SPE - Buffer	1000 ml
MS1010	Mobile Phase	800 ml
MS1014	Optimisation Mix 1, lyophil.	2 ml
MS1015	Optimisation Mix 2, lyophil.	3 ml
MS1020	Sample Pretreatment Vials	100 pcs.
MS1021	P Precipitant	80 ml
MS1212	IS Internal Standard, lyophil.	3 ml
5013	Whole Blood Calibrator, lyophil. (single point)	5 x 2 ml
9028	Whole Blood Calibrator, lyophil. (additional level for order nos. 9033 and 9933)	2 x 2 ml
9033	Whole Blood Calibrator Set, lyophil. (Level 0 - 3)	4 x 1 x 2 ml
9933	Whole Blood Calibrator Set, lyophil. (Level 0 - 6)	7 x 1 x 2 ml
	Start Accessories:	
MS1030	Analytical Column with test chromatogram	1 pce.
MS1031	SPE - Column	1 pce.
MS1032	Guard Column Holder incl. 1 Guard Column	1 pce.
MS1033	Guard Column	5 pcs.
FK7400	Inline-Filter (stainless steel sieve, free of dead volume)	1 pce.
	Accessories:	
FK1102	Switching valve Sykam (6-port / 3-channel incl. electr. drive, PEEK)	1 pce.
FK7330	Endfittings	2 pcs.
FK7340	Sealings and sieves	4 pcs. each
FK7350	Endstoppers	2 pcs.
	ClinChek® Controls:	
8830	Whole Blood Control, lyophil. Level I	5 x 2 ml
8831	Whole Blood Control, lyophil. Level II	5 x 2 ml
8832	Whole Blood Control, lyophil. Level III	5 x 2 ml
8833	Whole Blood Control, lyophil. Level I, II, III	3 x 2 x 2 ml
8903	Whole Blood Control, lyophil. Level IV, V	2 x 2 x 2 ml

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2.1.1 Safety information

Several of the kit components (e.g. mobile phases and reagents) are chemical preparations and thus may contain hazardous substances. For safety information, please consult the appropriate Material Safety Data Sheet (MSDS) for each component.

The calibrator and control materials are prepared from human whole blood. Although the products are tested for the absence of common infection markers, they should still be considered as potentially infectious. For this reason we recommend the product to be handled with the same precautions as patient samples. Detailed safety information is given in the appropriate Material Safety Data Sheet (MSDS).

2.1.2 Storage conditions and lifetime of kit components

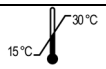
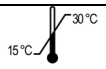
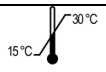
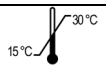
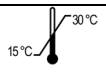
Please unpack the kit components from the transport packaging **immediately upon receipt** and follow the instructions for the storage conditions given on the product labels and table 1.

Unused components, stored under appropriate conditions can be used until the expiry date given on the product label.

After use of ClinMass® Reagents and ClinMass® Mobile Phases, the bottles must be closed tightly and stored immediately under the required conditions. Provided proper use and storage procedures are followed, the lifetime of the reagents is the same as for the unused products.

For storage conditions and life times of ClinMass® Internal Standard and Optimisation Mixes as well as for ClinCal® Calibrators and ClinChek® Controls (lyophilised / after reconstitution) please also refer to the appropriate product data sheets.

Table 1: Storage conditions of kit components

Order no.		Product description	Storage conditions	
REF	MS1005	Autosampler Washing Solution		Store at 15 - 30 °C
REF	MS1006	Reagent MP		Store at 15 - 30 °C
REF	MS1009	SPE-Buffer		Store at 15 - 30 °C
REF	MS1010	Mobile Phase		Store at 15 - 30 °C
REF	MS1014	Optimisation Mix 1, lyophil.		Store below - 18 °C*
REF	MS1015	Optimisation Mix 2, lyophil.		Store below - 18 °C*
REF	MS1020	Sample Pretreatment Vials	Store at ambient temperature	
REF	MS1021	P Precipitant		Store at 15 - 30 °C

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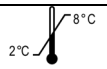
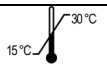
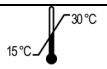
REF	MS1212	IS Internal Standard, lyophil.		Store below - 18 °C*
REF	5013	Whole Blood Calibrator, lyophil.		Store at 2 - 8 °C*
REF	9028	Whole Blood Calibrator (additional level for order nos. 9033 and 9933), lyophil.		Store at 2 - 8 °C*
REF	9033	Whole Blood Calibrator Set (Level 0-3), lyophil.		Store at 2 - 8 °C*
REF	9933	Whole Blood Calibrator Set (Level 0-6), lyophil.		Store at 2 - 8 °C*
REF	MS1030	Analytical Column		Store at 15 - 30 °C
REF	MS1031	SPE-Column		Store at 15 - 30 °C
REF	MS1032, MS1033	Guard Column		Store at 15 - 30 °C
REF	FK7400	Inline-Filter	Store at ambient temperature	
REF	FK1102	Switching valve Sykam (6-port / 3-channel)	Store at ambient temperature	
REF	FK7330	Endfittings	Store at ambient temperature	
REF	FK7340	Sealings and sieves	Store at ambient temperature	
REF	FK7350	Endstoppers	Store at ambient temperature	
REF	8830 - 8833	Whole Blood Controls, Level I, II, III, lyophil.		Store at 2 - 8 °C*
REF	8903	Whole Blood Controls, Level IV, V, lyophil.		Store at 2 - 8 °C*

*Refers to the lyophilised product. For storage conditions after reconstitution, please refer to the product data sheet.

2.1.3 Disposal of laboratory waste

For disposal, laboratory waste should be collected separately with regard to its different chemical properties. Recommendations for the disposal of the product and of the packaging are given in section 13 of the appropriate Material Safety Data Sheet (MSDS).

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3 Required instruments

Using this test kit requires a LC system with tandem mass spectrometer (LC-MS/MS) and evaluation software.

Requirements for the tandem mass spectrometer:

The tandem mass spectrometer should be of comparable (or higher) sensitivity as the instruments described in section 4.4.2.

Required LC modules:

- Autosampler (with cooling function, 4 °C)
- Isocratic HPLC pump 1 (SPE-buffer)
- Isocratic HPLC pump 2 (mobile phase)
- 6-port-3-channel-switching valve (e.g. RECIPE, order no. FK1102)
- Column heater (60 °C)
- Degasser (optional)

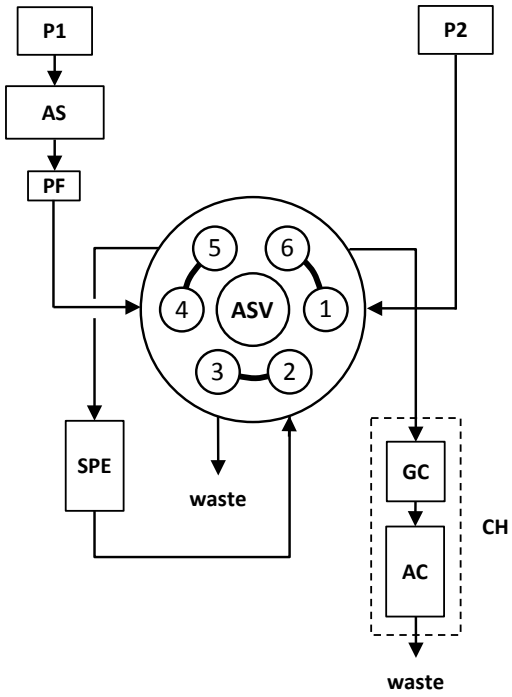
For sample pretreatment the following laboratory instruments are required:

- Pipettes, pipette tips
- Tabletop centrifuge
- Vortex mixer

4 Operation of the analytical system

4.1 Configuration of the LC system

Connect the LC modules (P1, P2, AS) and the automatic switching valve (ASV) as shown in the figure below, **with exception** of the columns (SPE, GC, AC). Put the outlet capillaries (from ASV to waste) into a safe waste container.



Abbreviations:
P1: HPLC pump 1; P2: HPLC pump 2; AS: autosampler; PF: inline-filter (order no.: FK7400); ASV: automatic switching valve (high pressure switching valve, 6-port/3-channel, order no.: FK1102); SPE: SPE-column; GC: guard column; AC: analytical column; CH: column heater

Figure 2: Configuration of the LC system

4.1.1 Reagent MP

For operation with an Agilent MS/MS system, Reagent MP (order no. MS1006) must be added to the mobile phase.

Add the whole amount of Reagent MP (8 ml) to a freshly opened bottle of mobile phase.

4.2 Flushing of the LC system

Set both HPLC pumps (P1, P2) at a flow rate of 1 ml/min and flush the LC system with 10 ml SPE-buffer and mobile phase, respectively.

Afterwards connect the SPE-column (SPE), the guard column (GC), and the analytical column (AC) as shown in figure 2. The guard column and the analytical column have to be installed within the column heater (CH).

When connecting the SPE-column and the analytical column, please take care that the flow direction follows the arrow marking on the columns!

4.3 Equilibration of the LC system

After flushing the system (see section 4.2) the equilibration is performed as follows:

- Set both HPLC pumps (P1, P2) to a flow rate of 0.5 ml/min, set the column heater to 60 °C, and allow approximately 10 ml SPE-buffer and mobile phase, respectively, to flow through the columns.
- After this, **stop the HPLC pumps** and connect the outlet capillary of the analytical column (AC) with the tandem mass spectrometer.

4.4 Starting the analytical system

The following sections provide the parameters for the LC system (see section 4.4.1) and the tandem mass spectrometer (see section 4.4.2). For optimisation, equilibration and testing, as well as for calibration of the LC-MS/MS system please refer to section 5.3.

Please consult the user manual of the tandem mass spectrometer to ensure appropriate usage. User trainings, provided by the instrument manufacturer, may also be advisable.

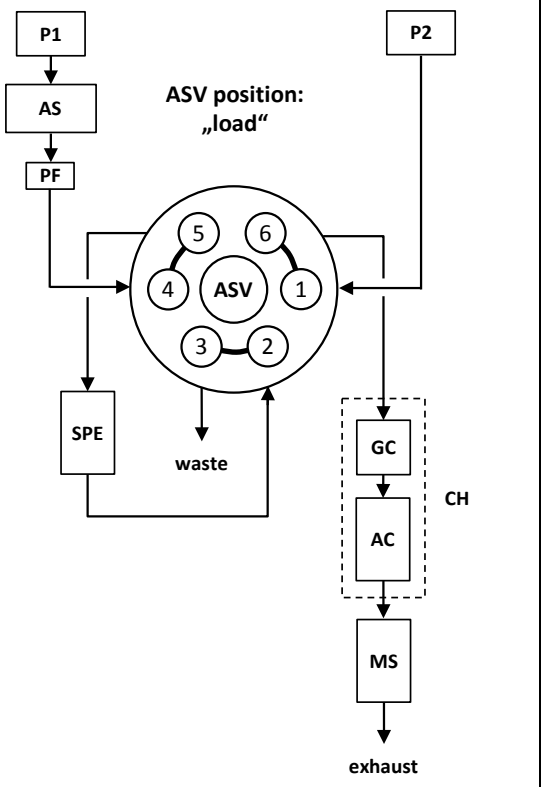
4.4.1 LC parameters

Table 2: LC parameters

Isocratic HPLC pump 1 (SPE-buffer):	Flow rates: 0.1 ml/min, 2.5 ml/min; see section 4.4.1.1, table 3
Isocratic HPLC pump 2 (mobile phase):	Flow rates: 0.5 ml/min, 1.0 ml/min; see section 4.4.1.1, table 3
SPE-buffer / mobile phase:	Make sure that the bottles are closed well to avoid alteration of the retention times through evaporation of components of the SPE-buffer and mobile phase.
Reagent MP:	For operation with an Agilent MS/MS system, Reagent MP must be added to the mobile phase (see section 4.1.1).
Columns:	The analytical column (AC) and the guard column (GC) are installed within the column heater (60° C). At a flow rate of 0.5 ml/min, the backpressure of the analytical column should not exceed 80 bar. At a flow rate of 2.5 ml/min, the backpressure of the SPE column should not exceed 150 bar. For the complete HPLC system, the backpressure should not exceed 250 bar. The sealings and frits of the inline filter (PF) and the guard column should be replaced after every 500 injections. These parts should also be replaced, if the backpressure of the inline filter exceeds 10 bar (at a flow rate of 2.5 ml/min) or if that of the guard column exceeds 10 bar (at a flow rate of 0.5 ml/min).
Column heater:	60 °C
Autosampler:	Set the autosampler cooling function to 4 °C. The autosampler washing solution (order no. MS1005) is available for the flushing of the injection system. Please also refer to the user manual of the autosampler manufacturer. Injection volume: 50 µl Injection interval: 2 min
Automatic switching valve:	See section 4.4.1.1

4.4.1.1 Automatic switching valve (on-line analysis)

The SPE sample clean up is performed on-line by use of a 6-port-3-channel automatic switching valve. A description of the working principle is given in figure 3:



Abbreviations:
P1: HPLC pump 1; P2: HPLC pump 2; AS: autosampler; PF1: inline-filter (order no.: FK7400); ASV: automatic switching valve (high pressure switching valve, 6-port/3-channel, order no.: FK1102); SPE: SPE-column; GC: guard column; AC: analytical column; CH: column heater; MS: tandem mass spectrometer

ASV position "load": switching interval: 0 - 0.50 min:

The sample is injected into the LC system and is transferred to the SPE column, which extracts the analytes selectively from the sample matrix. The matrix components are washed through the SPE column and thus are eluted to the waste. Meanwhile, the analytical column is reequilibrated from the previous injection cycle.

ASV position "inject": switching interval: 0.50 - 1.65 min:

The automatic switching valve switches to the "inject" position. The extracted analytes are eluted from the SPE column with mobile phase under backflush mode. They are transferred to the analytical column, on which they are chromatographically separated.

ASV position "load": switching interval: 1.65 - 2 min:

The automatic switching valve switches back to the "load" position. Both columns (SPE- and analytical column) are reequilibrated for the next injection.

Figure 3: Automatic switching valve and configuration of the LC system

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The switching times and positions of the automatic switching valve (ASV) and the flow rates of the HPLC pumps P1 and P2 are programmed according to the following table.

Table 3: Switching times and positions (ASV), flow rates of the HPLC pumps (P1, P2)

Time [min]	ASV position	Pump P1 flow rate [ml/min]	Event SPE-column	Pump P2 flow rate [ml/min]	Event analytical column
0.00	load	0.1		0.5	
0.01		2.5	loading		equilibration
0.50	inject	2.5	elution		loading
0.51		0.1			separation
1.30				0.5	
1.35				1.0	
1.50		0.1			
1.51		2.5			
1.55				1.0	
1.65	load		equilibration	0.5	equilibration
1.99		2.5			
2.00		0.1		0.5	

4.4.2 MS/MS parameters

The MS/MS parameters, indicated in the tables of sections 4.4.2.1 to 4.4.2.4 are recommended values only. This particularly applies to the mass transition specific parameters. The values should be regarded as starting points for optimisation. The optima vary between different MS/MS systems and therefore should be optimised for the system to be used ("compound optimisation", see section 5.3.1).

For analyte / internal standard assignments see the table below.

Table 4: Analyte / internal standard assignments and corresponding LC retention times (RT)

Analyte	RT [min]	Internal Standard IS (order no. MS1212)	RT [min]
Cyclosporine A	1.45	Cyclosporine D	1.50
Tacrolimus	1.26	Ascomycine	1.25
Sirolimus	1.34	d^4-Everolimus	1.35
Everolimus	1.35	d^4-Everolimus	1.35

4.4.2.1 Agilent

The following parameters refer to the MS/MS system Agilent 6430/6410 with Hotbox and Agilent 6460.

Table 5: MS/MS parameters

	Agilent 6430/6410 w. Hotbox	**Agilent 6460**
Ion Source	ESI, positive	ESI+Agilent Jet Stream, positive
Resolution MS1 and MS2	unit (0.7 amu)	unit (0.7 amu)
Gas Temperature	275 °C	225 °C
Gas Flow	9 l/min	9 l/min
Nebulizer	35 psi	35 psi
Sheath Gas Temperature	---	325 °C
Sheath Gas Flow	---	12 l/min
Capillary Voltage	4000 V	4000 V
Nozzle Voltage	---	300 V
Mass Transitions	see table 6	see table 7

Table 6: Mass transitions, Agilent 6430/6410 with Hotbox

Substance	**Precursor [amu]**	**Product [amu]**	**Dwell time [ms]**	**Fragmentor [V]**	**CE [V]**	**Cell Accelerator Voltage [V]**
Cyclosporine A (Quantifier)	1219.9	1202.9	35	200	13	7
Cyclosporine A (Qualifier)	1219.9	1184.9	35	200	33	7
Cyclosporine D (Quantifier)	1233.8	1216.6	35	220	16	7
Cyclosporine D (Qualifier)	1233.8	1198.8	35	220	28	7
Tacrolimus (Quantifier)	821.5	768.4	35	110	17	7
Tacrolimus (Qualifier)	821.4	786.4	35	110	12	7
Ascomycine (Quantifier)	809.6	756.6	35	120	15	7
Ascomycine (Qualifier)	809.6	774.4	35	120	12	7
Sirolimus (Quantifier)	931.5	864.4	35	160	13	7

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Sirolimus (Qualifier)	931.5	846.5	35	160	21	7
Everolimus (Quantifier)	975.5	908.4	35	180	13	7
Everolimus (Qualifier)	975.5	858.5	35	180	24	7
d_4-Everolimus (Quantifier)	979.6	912.5	35	154	12	7
d_4-Everolimus (Qualifier)	979.6	894.5	35	154	20	7

Table 7: Mass transitions, Agilent 6460

Substance	**Precursor [amu]**	**Product [amu]**	**Dwell time [ms]**	**Fragmentor [V]**	**CE [V]**	**Cell Accelerator Voltage [V]**
Cyclosporine A (Quantifier)	1219.9	1202.9	35	200	13	7
Cyclosporine A (Qualifier)	1219.9	1184.9	35	200	33	7
Cyclosporine D (Quantifier)	1234	1216.8	35	177	16	7
Cyclosporine D (Qualifier)	1234	1198.8	35	177	28	7
Tacrolimus (Quantifier)	821.6	768.4	35	140	17	7
Tacrolimus (Qualifier)	821.6	786.4	35	140	12	7
Ascomycine (Quantifier)	809.5	756.4	35	154	16	7
Ascomycine (Qualifier)	809.5	774.4	35	154	12	7
Sirolimus (Quantifier)	931.7	864.5	35	160	13	7
Sirolimus (Qualifier)	931.7	846.5	35	160	21	7
Everolimus (Quantifier)	975.6	908.6	35	190	13	7
Everolimus (Qualifier)	975.6	858.5	35	190	24	7
d_4-Everolimus (Quantifier)	979.6	912.5	35	154	12	7
d_4-Everolimus (Qualifier)	979.6	894.5	35	154	20	7

4.4.2.2 Applied Biosystems

The following parameters refer to the MS/MS systems API 3000 and API 4000.

Table 8: MS/MS parameters

	API 3000	**API 4000**
Ion Source	Turbolonspray ESI positive	Turbolonspray ESI positive
Resolution Q1 and Q3	unit (0.7 amu)	unit (0.7 amu)
Nebuliser Gas/GS 1	14	65
GS 2	---	60
Curtain Gas	14	30
Collision Gas	10	6
Ion Spray Voltage	5000 V	5000 V
Source Temperature	500 °C	350 °C
Interface Heater	---	ON
Mass transitions	see table 9	see table 10

Table 9: Mass transitions, API 3000

Substance	**Precursor [amu]**	**Product [amu]**	**Dwell time [ms]**	**DP [V]**	**FP [V]**	**CE [V]**	**CXP [V]**	**EP [V]**
Cyclosporine A (Quantifier)	1219.9	1203.2	35	41	260	23	54	10
Cyclosporine A (Qualifier)	1219.9	1184.6	35	41	310	45	30	10
Cyclosporine D (Quantifier)	1233.9	1217.2	35	46	300	49	30	10
Tacrolimus (Quantifier)	821.6	768.4	35	51	280	29	20	10
Ascomycine (Quantifier)	809.5	756.6	35	51	330	29	18	10
Sirolimus (Quantifier)	931.7	864.4	40	46	290	23	22	10
Everolimus (Quantifier)	975.6	908.4	40	46	260	23	24	10
d_4-Everolimus (Quantifier)	979.6	912.6	40	46	280	25	24	10

Table 10: Mass transitions, API 4000

Substance	Precursor [amu]	Product [amu]	Dwell time [ms]	DP [V]	CE [V]	CXP [V]	EP [V]
Cyclosporine A (Quantifier)	1219.7	1202.8	25	66	27	42	10
Cyclosporine A (Qualifier)	1219.7	1184.8	25	66	49	40	10
Cyclosporine D (Quantifier)	1233.8	1216.9	25	66	37	22	10
Cyclosporine D (Qualifier)	1233.8	1198.7	25	66	49	22	10
Tacrolimus (Quantifier)	821.5	768.4	25	61	31	26	10
Tacrolimus (Qualifier)	821.5	786.3	25	61	25	26	10
Ascomycine (Quantifier)	809.4	756.3	25	66	39	26	10
Ascomycine (Qualifier)	809.4	774.4	25	61	25	26	10
Sirolimus (Quantifier)	931.5	864.5	30	66	25	16	10
Sirolimus (Qualifier)	931.5	882.5	30	66	19	30	10
Everolimus (Quantifier)	975.6	908.4	30	66	33	16	10
Everolimus (Qualifier)	975.6	926.7	30	66	20	16	10
d_4-Everolimus (Quantifier)	979.6	912.5	30	61	37	16	10
d_4-Everolimus (Qualifier)	979.6	930.5	30	66	21	16	10

4.4.2.3 Thermo

The following parameters refer to the MS/MS system TSQ Quantum Ultra.

Table 11: MS/MS parameters, TSQ Quantum Ultra

Ion Source	H-ESI II, positive
Resolution Q1 and Q3	0.7 amu
Spray Voltage	3000 V
Vaporiser Temperature	300 °C
Sheath Gas Pressure	40
Ion Sweep Gas Pressure	2.0
Aux Gas Pressure	15
Capillary Temperature	200 °C
Skimmer Offset	-5 V
Collision Pressure	1.5 mTorr (argon)
Probe Position	C
Mass Transitions	see table 12

Table 12: Mass transitions, TSQ Quantum Ultra

Substance	**Precursor [amu]**	**Product [amu]**	**Dwell time [ms]**	**Collision Energy**	**Tube Lens Offset [V]**
Cyclosporine A	1220.0	1203.3	50	17	190
Cyclosporine D	1234.0	1217.0	50	17	190
Tacrolimus	821.6	768.4	50	18	190
Ascomycine	809.5	756.6	50	18	190
Sirolimus	931.7	864.6	75	15	190
Everolimus	975.7	908.8	75	16	190
d_4-Everolimus	979.7	912.6	75	16	190

4.4.2.4 Shimadzu

The following parameters refer to the MS/MS system LCMS-8030.

Table 13: MS/MS parameters, LCMS-8030

Ion Source	ESI, positive
Resolution Q1 and Q3	0.7 amu
Interface Voltage	2500 V
Conversion Dynode	10000 V
Desolvation Line	220 °C
Heat Block	400 °C
Nebulizer Gas	3 L/min
Drying Gas	15 L/min
Mass Transitions	see table 14

Table 14: Mass transitions, LCMS-8030

Substance	Precursor [amu]	Product [amu]	Dwell time [ms]	Collision Energy
Cyclosporine A	1219.9	1203.0	30	-21
Cyclosporine D	1233.9	1216.9	30	-20
Tacrolimus	821.5	768.6	30	-22
Ascomycine	809.3	756.4	30	-20
Sirolimus	931.6	864.7	30	-18
Everolimus	975.8	908.7	30	-19
d_4-Everolimus	979.5	912.6	30	-20

4.5 Standby mode

When the analytical system is not in use, the pumps have to be switched off. The SPE-buffer and the mobile phase can be left within the LC system.

The vacuum pumps of the tandem mass spectrometer (MS/MS system) should be in permanent operation. In order to protect the ion source and multiplier, the MS/MS system should be switched into the standby mode.

For a longer operation pause, the SPE-Column and the analytical column should be disconnected and stored tightly closed. The LC system should then be flushed with a water/methanol mixture (1:1).

5 Implementation of the analytical procedure

5.1 Collection and storage of whole blood samples

The analysis is performed from EDTA whole blood.

If the determination is performed within the same day, the samples may be stored at room temperature (15 - 30 °C). At temperatures between 2 - 8 °C, the samples may be stored for up to 7 days. If the samples shall be stored for a longer period of time, the samples must be stored at temperatures below - 18 °C (multiple freeze-thaw cycles should be avoided).

5.2 Sample pretreatment

5.2.1 Reconstitution of the lyophilised whole blood calibrators / controls

ClinCal® Whole Blood Calibrators and ClinChek® Whole Blood Controls (see section 2.1) are lyophilised and thus must be reconstituted before use. Information regarding reconstitution, along with analyte concentrations and information about storage and stability, is given in the appropriate product data sheets.

5.2.2 Work flow

Sample pretreatment:

Precipitation:

200 µl P Precipitant	20 µl IS Internal Standard	100 µl whole blood (calibrator, control, patient)

mix for 30 sec (vortex mixer), ⇩ incubate (5 min, room temp.)

mix for 10 sec (vortex mixer), ⇩ centrifuge (5 min, 10000 x g)

LC-MS/MS analysis:

inject 50 µl of the supernatant

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5.2.2.1 Precipitation

Pipette 200 µl Precipitant P, 20 µl Internal Standard IS and 100 µl of the whole blood sample (calibrator, control, patient) into a sample pretreatment vial. Mix for 30 sec on a vortex mixer and afterwards incubate for 5 min at room temperature (15 - 30 °C). After this, mix for 10 sec again (vortex mixer) and centrifuge for 5 min with a rotation speed of 10000 x g.

N.b.: Precipitant and internal standard may be pre-mixed (mixing ratio: 200+20) in accordance to the actual daily amount required. 220 µl* of this mixture is then mixed with 100 µl whole blood sample.

* Multipettes: with some multipettes the setting of 220 µl may not be possible. In these cases a volume between 200 - 240 µl can be alternatively set.

5.2.2.2 LC-MS/MS analysis

Transfer the centrifuge supernatant to a sample vial, which is suitable for the autosampler in use (brown glass vials are recommended). Inject 50 µl of the supernatant into the LC-MS/MS system.

5.2.2.3 Stability of the pretreated samples

Stored in the dark, at temperatures between 2 - 8 °C, pretreated samples are stable for at least 12 hours.

5.3 LC-MS/MS analysis

Independent from the analytical method, the mass accuracy of the tandem mass spectrometer (MS/MS) should be checked at regular intervals. A mass calibration may be required.

For information regarding the check-up of the MS/MS system, please refer to the documentation provided by the instrument manufacturer.

5.3.1 Compound optimisation (MS/MS)

For the optimisation of the MS/MS system parameters Optimisation Mix 1 and 2 (order nos. MS1014 and MS1015) are provided (“compound optimisation”).

Optimisation Mix 1 contains a preparation of the analytes, i.e. Cyclosporine A, Tacrolimus, Sirolimus, and Everolimus. Optimisation Mix 2 contains a preparation of the internal standards, i.e. Cyclosporine D, Ascomycine, and d_4-Everolimus.

Optimisation Mix 1 and 2 are lyophilised and thus have to be reconstituted before use. Information regarding the reconstitution is given in the appropriate product data sheets. If necessary, Optimisation Mix 1 and 2 should be diluted with mobile phase according to the sensitivity of the MS/MS system in use.

The compound optimisation procedure for the MS/MS system in use should then be followed in order to optimise the ionisation source parameters and the compound specific mass transition parameters.

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5.3.2 Equilibration of the analytical system and test run

Equilibrate the entire analytical system for at least 30 min before injecting samples.

In order to confirm the performance of the analytical system, repeatedly inject a mixture of the Optimisation Mix 1 and 2 (see preparation below), until two consecutive chromatograms, comparable in retention times and peak areas, are obtained.

The mixture is prepared from:

- 50 µl Optimisation Mix 1 (order no. MS1014)
- 100 µl Optimisation Mix 2 (order no. MS1015)
- 850 µl Mobile Phase (order no. MS1010)

A further dilution of the mixture with mobile phase may be required, depending on the sensitivity of the MS/MS system in use.

5.3.3 Calibration run

For calibration, a ClinCal® 4-Level Whole Blood Calibrator Set (level 0 - 3, order no. 9033) and a ClinCal® 7-Level Whole Blood Calibrator Set (level 0 - 6, order no. 9933) are available. For an extended calibration range with an additional, high calibration point (level 7), the whole blood calibrator with order no. 9028 is optionally available.

After reconstitution (see section 5.2.1), the calibrators must be pretreated as described for the patient samples (see section 5.2).

For each analytical series, freshly prepared calibrators are required.

5.3.4 Accuracy control

For the quality control of the analytical measurements, ClinChek® Whole Blood Controls are available in five different concentrations (level I, order no. 8830; level II, order no. 8831; level III, order no. 8832; level I - III, order no. 8833 as well as level IV - V, order no. 8903).

Please note:
The usage of control levels IV and V (order no. 8903) requires an extension of the calibration range with level 7 of the whole blood calibrator with order no. 9028 (see section 5.3.3).

These controls are lyophilised and, subsequently to reconstitution, must be pretreated as described for the patient samples (see section 5.2).

For each analytical series, freshly pretreated controls must be used. In case of large analytical series, we recommend to inject these controls additionally at the end of the series.

5.3.5 Example chromatogram

Example chromatogram of the ClinCal® Whole Blood Calibrator (order no. 9933), level 4, recorded with the MS/MS system API3000:

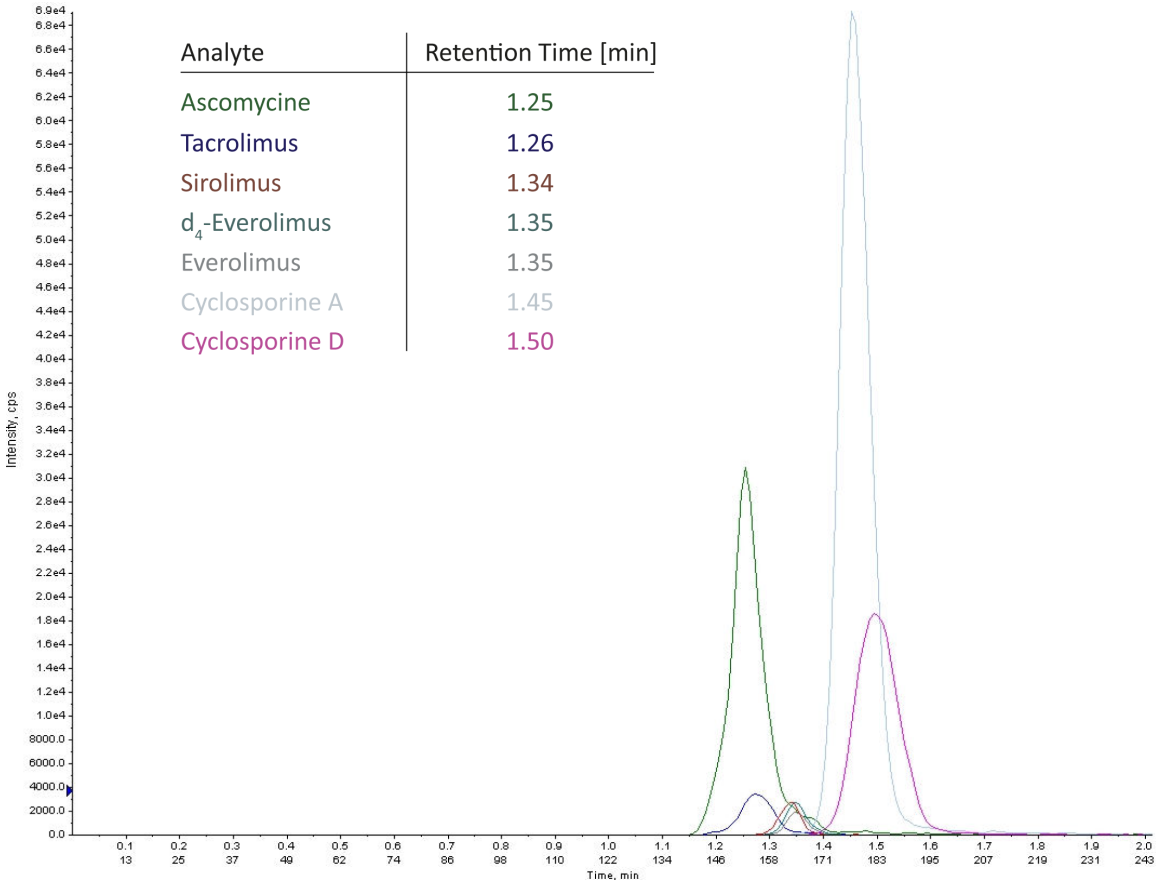

Figure 4: Chromatogram of the ClinCal® Whole Blood Calibrator, level 4

6 Evaluation

The analyte detection is achieved using compound specific mass transitions (see section 4.4.2)

The evaluation of the analyte concentration is performed with the internal standard method using the peak areas.

Calibration curves are achieved for the calibrators by plotting the ratio *analyte peak area/internal standard peak area* against the ratio *analyte concentration/internal standard concentration*.

The analyte concentrations for samples and controls are calculated from the calibration curve.

Please consult the software user manual of the MS/MS manufacturer in order to ensure correct evaluation of the results.

For the calculation of mass concentrations [µg/l] into molar concentrations [µmol/l], and vice versa, the analytical results have to be multiplied with the factors shown in table 15.

Table 15: Conversion factors

Analyte	**Molecular weight [g/mol]**	**Conversion factor : µmol/l --> µg/l**	**Conversion factor: µg/l --> µmol/l**
Cyclosporine A	1202.63	1202.63	8.315×10^{-4}
Tacrolimus	804.15	804.15	1.244×10^{-3}
Sirolimus	914.17	914.17	1.094×10^{-3}
Everolimus	958.24	958.24	1.044×10^{-3}

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7 Test data

7.1 Test performance

The results were obtained with the MS/MS-system Agilent 6460.

7.1.1 Linearity, quantitation limit, detection limit

	Cyclosporine A	Tacrolimus	Sirolimus	Everolimus
LLOD [µg/l]*	0.011	0.004	0.073	0.126
LLOQ [µg/l]**	0.037	0.014	0.244	0.420
Linearity [µg/l]	0.037 - 2000	0.014 - 80	0.244 - 80	0.420 - 80

*LLOD: Lower limit of detection, S/N=3, **LLOQ: Lower limit of quantitation, S/N=10; S: Signal, N: Noise

7.1.2 Recovery

For Cyclosporine A, Tacrolimus, Sirolimus, and Everolimus, mean recovery rates between 90 - 100 % were obtained.

7.1.3 Precision

For the evaluation of the intra- and interassay precision, 3 samples with the following concentrations were used:

	Cyclosporine A [µg/l]	Tacrolimus [µg/l]	Sirolimus [µg/l]	Everolimus [µg/l]
Sample 1	62.5	3.28	3.64	3.34
Sample 2	132	6.67	11.2	10.6
Sample 3	258	13.3	18.9	18.2

7.1.3.1 Intraassay

For the determination of the intraassay precision the samples were measured in 3 analytical series, each by 6-fold determination (n = 18; n: number of values per sample). The following coefficients of variation (CV) were obtained (mean values):

	Cyclosporine A CV [%]	Tacrolimus CV [%]	Sirolimus CV [%]	Everolimus CV [%]
Sample 1	3.98	6.57	6.01	6.95
Sample 2	1.51	3.40	4.49	3.59
Sample 3	1.47	4.34	3.84	4.37

7.1.3.2 Interassay

For the determination of the interassay precision the samples were measured in 8 analytical series, each by 2-fold determination (n = 16; n: number of values per sample). The following coefficients of variation (CV) were obtained:

	Cyclosporine A CV [%]	Tacrolimus CV [%]	Sirolimus CV [%]	Everolimus CV [%]
Sample 1	8.88	4.28	5.65	4.48
Sample 2	7.82	4.78	4.00	3.85
Sample 3	6.26	1.63	3.54	3.83

7.2 Reference ranges

The therapeutical ranges depend on several factors, such as the type of transplantation, time after graft and co-medication with other immunosuppressive agents.

For this reason, general therapeutical ranges cannot be given but must be established individually for each patient.

8 References

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9 Troubleshooting

Problem	Possible cause	Corrective measure
Gradient profile cannot be generated	Defective HPLC pump	Check the pumps
	Air within the system	Degas the mobile phases and flush and purge the HPLC system thoroughly
	Fluctuation of the flow rate	Check the pumps
Interference signals	Injection system contaminated	• Rinse needle with methanol or inject 10 x mobile phase • Check flushport solvent level • Clean/exchange needle seat assembly and/or injection valve
	Sample vials contaminated	Use new vials
	Vial septum contaminated	Use another septum
	Mobile phase contaminated	Change the mobile phases and flush the system
	Column(s) (guard / analytical column) contaminated	Change the guard / analytical column
	Mass resolution too low	Optimise mass resolution
	System not correctly configured	Check all connections
No signals	Injector defect	Check injector
	Defective HPLC pump	Check the pumps
	MS/MS system not ready for operation	Check the MS/MS system

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Problem	Possible cause	Corrective measure
Decrease of sensitivity	Ion source contaminated	Clean the ion source
	Mass spectrometer contaminated	Clean the mass spectrometer
	Leakage of injection valve	Check the injector
	Shift of mass calibration	Recalibrate MS/MS system
	MRM transitions not optimal	Optimise MRM transitions
	Ionisation conditions not optimal	Optimise the parameters of the ion source
	Mass resolution too high	Optimise the mass resolution
High fluctuations of signals	Spray instable	Check the spray needle capillary and clean or exchange, if necessary
	Fluctuation of the flow rate	Check the HPLC pumps
	Gas flow rate instable	Check the gas pipes
No vacuum	Defective vacuum pumps	Check the pre- and high-vacuum pumps
	Leakage within the vacuum system	Check the vacuum tubes and fittings
No gas supply	Defective of nitrogen generator	Check the nitrogen generator
	Defective compressor	Check the compressor
	Gas bottle is empty	Replace the gas bottle
	Inlet gas pressures are not within the specified range	Regulate the inlet gas pressures

10 Appendix: EC-Declaration of Conformity

Declaration of Conformity

for in-vitro diagnostic medical devices, acc. to article 9 (1) of the directive 98/79/EC

The company

RECIPE Chemicals + Instruments GmbH
Dessauerstraße 3
80992 Munich / Germany

declares, that the CE labelled product

ClinMass® Complete Kit for Immunosuppressants (order no. MS1000)

meets all applicable provisions of the directive on in vitro diagnostic medical devices 98/79/EC. The conformity assessment was performed according to annex III. The technical documentation is held according to annex III no. 3.

Munich, 15.03.2013

Alfred Bauer
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