DEVELOPMENT OF REFERENCE MEASUREMENT PROCEDURES WITH LC-MS IN CLINICAL CHEMISTRY

PHD THESIS

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and

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THIS THESIS IS DEDICATED TO MY PARENTS

"WE MUST HAVE PERSEVERANCE AND ABOVE ALL CONFIDENCE IN OURSELVES.

WE MUST BELIEVE THAT WE ARE GIFTED FOR SOMETHING,

AND THAT THIS THING, AT WHATEVER COST, MUST BE ATTAINED."

/MARIE CURIE/

Contents

TABLE OF CONTENTS

LIST OF PUBLICATIONS

LIST OF FULL PAPERS DIRECTLY RELATED TO THE SUBJECT OF THE THESIS:

I. Modified HPLC-Electrospray Ionization/Mass Spectrometry Method for HbA_{1c} Based on IFCC Reference Measurement Procedure
Kaiser P, Akerboom T, **Molnar P**, and Reinauer H
Clin. Chem. 54(6):1018-1022 (2008)
IF 6.886

II. Procedure for Determination of Immunosuppressive Drugs in Whole Blood with Liquid Chromatography-Isotope Dilution Mass Spectrometry
Molnár PM, Dux L, Reinauer H, Kress M, Akerboom T, Szederkényi E, Kaiser P
Clin. Lab. 57(11-12):983-992 (2011)
IF 0.827 (Scopus)

LIST OF FULL PAPERS INDIRECTLY RELATED TO THE SUBJECT OF THE THESIS:

I. Quinidine as an ABCB1 Probe for Testing Drug Interactions at the Blood–Brain Barrier: An In Vitro In Vivo Correlation Study
Sziráki I, Erdő E, Beéry E, **Molnár PM**, Fazakas Cs, Wilhelm I, Makai I, Kis E, Herédi-Szabó K, Abonyi T, Krizbai I, Tóth GK, Krajcsi P
J. of Biomol. Screen. 16(8):886-894 (2011)
IF 2.500 (Scopus)

LIST OF ABBREVIATIONS

ACN - ACETONITRILE

AMU - ATOMIC MASS UNIT

CEN - EUROPEAN COMMITTEE FOR STANDARDIZATION

CMIA - CHEMILUMINESCENT MICROPARTICLE IMMUNOASSAY

CPS - COUNT PER SECOND

CV - COEFFICIENT OF VARIATION

DCCT - DIABETES CONTROL AND COMPLICATIONS TRIAL

EDTA - ETHYLENEDIAMINETETRAACETIC ACID

EN - EUROPEAN STANDARD

EQAS - EXTERNAL QUALITY ASSESSMENT SCHEMES

ESI - ELECTROSPRAY IONIZATION

HbA_{1C} - HAEMOGLOBIN A_{1C}

HPLC - HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC-CE - HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY CAPILLARY ELECTROPHORESIS

HPLC-MS/MS - HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

IFCC - INTERNATIONAL FEDERATION OF CLINICAL CHEMISTRY AND LABORATORY MEDICINE

ISO - INTERNATIONAL ORGANIZATION FOR STANDARDIZATION

LC-IDMS/MS - LIQUID CHROMATOGRAPHY–ISOTOPE DILUTION– TANDEM MASS SPECTROMETRY

LC-MS - LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

LLOQ - LOWER LIMIT OF QUANTITATION

LOQ - LIMIT OF QUANTITATION

MEOH - METHANOL

MS - MASS SPECTROMETRY

Q1 – QUADRUPOLE 1

Q3 - QUADRUPOLE 3

SRM – SELECTED REACTION MONITORING

TFA - TRIFLUOROACETIC ACID

TDM – THERAPEUTIC DRUG MONITORING

UKPDS - UNITED KINGDOM PROSPECTIVE DIABETES STUDY

1. INTRODUCTION

In Hungary the participation in external quality assessment schemes (EQAS) is mandatory for the medical laboratories, but there is no regulation how the target values and acceptability criteria in the EQAS are determined. Therefore the Hungarian EQAS-organization QualiCont In Vitro Diagnostic Quality Control Nonprofit Public Utility Ltd. is following the international development, and follows the regulation of the IVDMD Directive 98/79/EC (1), ISO 17043:2009 (2) and the mandated and harmonized standard EN 14136:2004 (3). In accordance with these regulations QualiCont sets the target values in the EQA samples for many schemes by reference measurement procedures, by which the best accuracy of measurement with traceability according to ISO 17511 (4) can be achieved. By this way the general basis for the evaluation of external quality assessment schemes (EQAS) is given.

For routine analyses high-throughput procedures are developed for determination of HbA_{1c} and for therapeutic drug monitoring (TDM) in medical laboratory. The most frequent principles used for these analyses are immunological procedures with specific antibodies directed against the analytes. The immunological procedures have different calibrations and use different antibodies and measurement principles. They sometimes have poor comparability of values, which is documented in many external quality assessment schemes.

To overcome the problem of poor comparability reference measurement procedures and certified reference materials with the best accuracy possible have been developed for the calibration of the routine tests by the manufacturers. At CEN and at ISO appropriate written standards had been issued demonstrating the state of the art in the development of reference measurement procedures and of certified reference materials.

The development of reference measurement procedures should follow the requirements of ISO 15193:2009 (5) . The reference measurement procedures of this study have considered the recommendations of the relevant ISO standard (ISO 15193:2009). The status of the presented procedures shall be classified as “candidate reference measurement procedures”. Only after confirmation of the procedure and the reference measurement values by an authorized and competent international organization a reference measurement procedure is acknowledged and by this way internationally established. The permanent improvement of

existing reference measurement procedures and establishment of new reference measurement procedures is a common activity of an EQAS organization.

PATHOBIOCHEMICAL ASPECTS OF HBA$_{1C}$

Glucose binds non-enzymatically to the N-terminal valine residue of the β-chain of the hemoglobin A_0 in the red blood cells. In the first step a labile Schiff base intermediate is formed. After spontaneous isomeric modification (Amadori rearrangement) the irreversible product HbA_{1c} is formed. The development of the Schiff base is a fast and reversible process. The formation of the Amadori product from the Schiff base is slow, but much faster than the reverse reaction, so that the glycation product tends to accumulate on proteins.

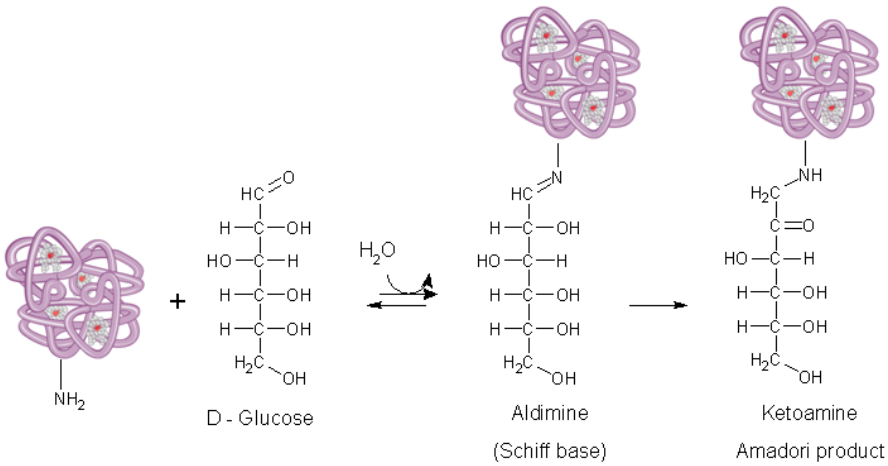


Figure 1-1 Non-enzymatic glycation of hemoglobin

The HbA_{1c} level of the blood depends on the concentration of the blood glucose and on the lifespan of red blood cells, reflecting the mean blood glucose concentration over the last 2-3 months. The percentage of the HbA_{1c} in diabetic patient may rise up to about 16%. HbA_{1c} is a well-accepted measure of the quality of diabetes management and meanwhile used for the diagnosis of diabetes mellitus, because the analyte is independent of the present food intake (6). The amount of the HbA_{1c} is an important factor when deciding the therapy of the single patient. (7). Long-term complications (microvascular and macrovascular complications) of the diabetic disease are correlated with the HbA_{1c} blood levels (Table 1-1) (8-11). This has been demonstrated in the clinical studies Diabetes Control and Complications Trial (DCCT) and United Kingdom Prospective Diabetes Study (UKPDS). In these studies it was shown that

a decrease of abs.1% HbA_{1c} results in a reduction of long-term diabetic complications of rel. 35 % (12).

	Minor risk	Risk of macroangiopathic complications	Risk of microangiopathic complications
HbA_{1c} [%]	≥ 6.5	> 6.5	> 7.5
Fasting/preprandial plasma glucose [mmol/L]	≤ 6.0	> 6.0	≥ 7.0
Self-monitoring of fasting glucose level via patient testing [mmol/L]	≤ 5.5	> 5.5	> 6.0
Peak postprandial glucose level [mmol/L]	< 7.5	≥ 7.5	> 9.0

Table 1-1 Risk levels based on the carbohydrate metabolism of the Guideline of the Hungarian Diabetes Association 2009 (13)

There are several methods (HPLC, electrophoresis, affinity chromatography, isoelectric focusing, various immunoassays and mass spectrometry) for the determination of the HbA_{1c} level in blood. Routine laboratories usually use immunochemical or chromatographic techniques to measure HbA_{1c} (14-16). The first strategy to standardize HbA_{1c} measurements goes back to the DCCT study. The basis of the standardization effort was the development of an HPLC method. This standardization was continued by NGSP in the USA and was the basis for calibration for the manufactures. In parallel standardization for HbA_{1c} measurements was performed in Sweden (Mono-S) (15) and Japan (JDS) (17). The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) established the *Working Group on standardization of HbA_{1c}*. The basis of the calibration of the reference measurement procedure was pure HbA_0 and HbA_{1c} as the primary reference materials (18), and was used as calibrators in CE and LC-MS procedures (19). To avoid confusion between the NGSP calibration (units in %) and IFCC calibration (mmol/mol) the patient values must be expressed together with the appropriate units. For conversion of IFCC values to NGSP derived values (and vice versa) for HbA1c the master equation can be used (20).

$$\textbf{IFCC } HbA_{1c} \textbf{ (mmol/mol) = [DCCT } HbA_{1c} \textbf{ (\%) – 2.15] * 10.929}$$

PATHOBIOCHEMICAL ASPECTS OF IMMUNOSUPPRESSIVE DRUGS

Aim of the immunosuppressive therapy is to prevent rejection of transplanted organs, in which case the immunologic defense mechanisms of the recipient will be inhibited. Patients after organ transplantation are forced to take lifelong immunosuppressive drugs to prevent rejection of transplanted tissues and organs.

The aim of an effective therapeutic drug monitoring is to develop an individualized immunsupressive therapy, which reduces the acute and chronic rejection reactions (21). The reduction of anti-rejection therapy decreases opportunistic infections and development of malignant tumor (22) (23).

Newly developed and clinically available immunosuppressive drugs are used alone or in combinations after organ transplantation (24, 25). Immunosuppressive drugs show a very narrow therapeutic range and have both different intra- and interindividual pharmacokinetic and pharmacodynamic properties (26-28). Overdosage of immunosuppressive drugs have to be avoided because of very serious side effects (Table 1-2). Therefore, the exact dosage of immunosuppressive therapy, and monitoring of the therapy are of importance for long-term transplant success. (29-31)

Drugs	Year of introduction	IS type	Targets	Mode of action (inhibition)	Side effects
Cyclosporin A (Sandimmun®)	1984	Calcineurin inhibitors (CNI)	Calcineurin phosphatase enzyme	IL-2 synthesis T-cell activation	nephrotoxicity, TMA, HUS, hypertrichosis, gum hypertrophy, hyperlipidaemia, hypertension, tremor, post-transplant diabetes mellitus, neuro-toxicity
Tacrolimus (Prograf®)	2000				
Sirolimus (Rapamune®)	2002	mTOR inhibitor	T-cells, Endothelial cells	T-cell proliferation	hyperlipidaemia, anemia, thrombocytopenia
Everolimus (Certican®)	2005				

Table 1-2 Characteristic of four immunosuppressive drugs (32)

The available analytical methods for therapeutic drug monitoring can divided into two groups such as immunological (33-35) and chromatographic (HPLC) procedures. (34, 36-45). The HPLC methods are coupled with UV detectors or with mass spectrometry. The most commonly used methods in routine laboratories are the immunological procedures (46). These methods detect not only the parent substances but also active and inactive metabolites (47, 48). Therefore a reference measurement procedure shall be developed to analyze the parent drugs (Figure 1-2).

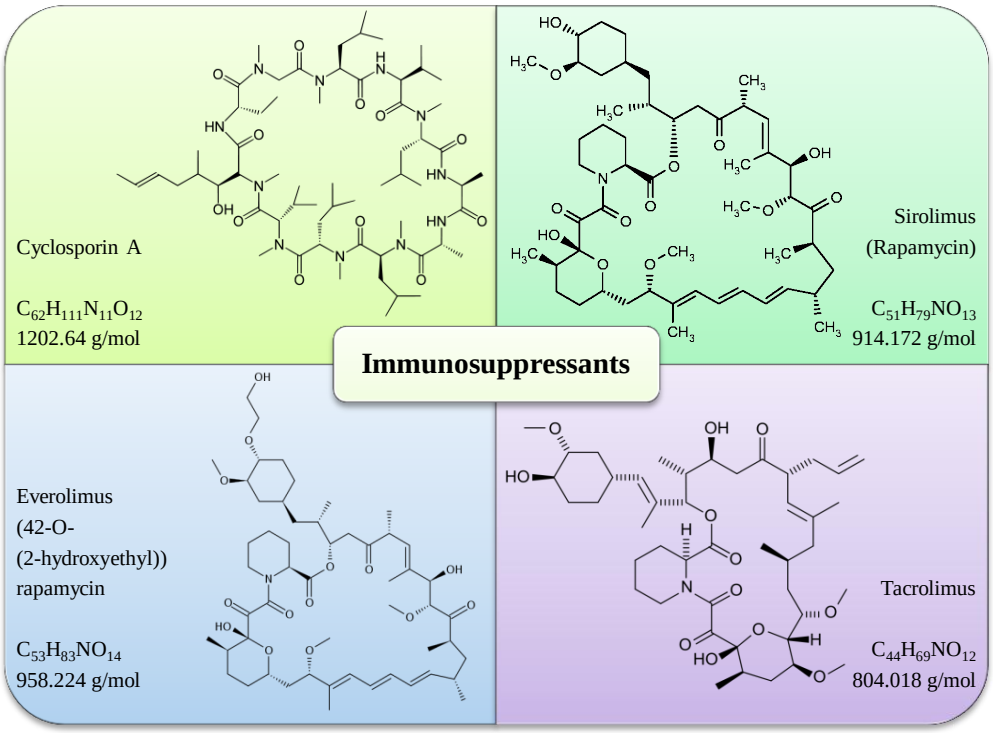


Figure 1-2 Chemical structures of immunosuppressive drugs used in this study

2. AIMS

The aim of the study was focused on improving patient care through the implementation of reference measurement procedures of highest metrological order to promote the standardization of analytical procedures in medical laboratories.

In this study two LC-MS candidate reference measurement procedures are presented.

For HbA_{1c} a reference measurement procedure has already been published, but there was a need for more robustness of this procedure.

No reference measurement procedure existed so far for immunosuppressive drugs. By the use of isotopic labeled internal standards reference measurement procedures for these drugs are developed.

3. MATERIALS AND METHODS

3.1. CHEMICALS AND REAGENTS

Substance	Supply source
Trifluoroacetic acid (25% solution in water)	Merck
Acetonitrile	Merck
Methanol	Merck
Formic acid (98%, puriss. p.a.)	Sigma-Aldrich
Cesium hydroxide (99.97 % purity)	Sigma-Aldrich
Ethyl acetate	Sigma-Aldrich
Tert.-butyl methyl ether	Sigma-Aldrich
Ammonium acetate	Fluka Analytical
Endoproteinase Glu-C (seq. grade, EC 3.4.21.19)	Roche Diagnostics
Jupiter™ Proteo column	Phenomenex, Germany
Water (Direct-Q™)	Millipore GmbH, Germany
Calibration material and secondary reference material for HbA_{1c}	IFCC Working Group on HbA1c Standardization
Lyophilized whole blood samples	Recipe
Cyclosporin A (>99%)	LC Laboratories, USA
Sirolimus /rapamycin/ (>99 %)	LC Laboratories, USA
Tacrolimus /FK-560/ (>98 %)	LC Laboratories, USA
Everolimus /42-O-(hydroxyethyl)rapamycin/ (>99 %)	LC Laboratories, USA
$[^{2}H_{12}]$-cyclosporin A	AlsaChim, France
$[^{13}C,^{2}H_{3}]$-rapamycin	AlsaChim, France
$[^{13}C,^{2}H_{2}]$-tacrolimus	AlsaChim, France
$[^{13}C_{2},^{2}H_{4}]$-42-O-(2-hydroxyethyl)rapamycin	AlsaChim, France

3.2. EQUIPMENTS

Substance	Supply source
LaChrom L 7100 quaternary pump	Hitachi-Merck
LaChrom L 7400 autosampler	Hitachi-Merck
LaChrom D 7000 interface	Hitachi-Merck

L 7350 column oven	Hitachi-Merck
Finnigan-MAT TSQ® 7000 triple stage quadrupole tandem mass spectrometer with ESI	Finnigan-MAT
Thermo Finnigan Xcalibur™ software (version 1.3)	Finnigan-MAT
SCL-10A system controller	Shimadzu Corporation
3 LC-10ADvp pumps (A, B, C),	Shimadzu Corporation
DGU-14A degasser	Shimadzu Corporation
SIL-10AD autoinjector	Shimadzu Corporation
CTU-10AS column oven	Shimadzu Corporation
FCU-12A flow switch (rotary valve)	Shimadzu Corporation
API 4000 equipped with a TurboV™ ESI	Applied Biosystems

3.3. MEASUREMENT CONDITIONS FOR DETERMINATION OF HbA_{1c}

3.3.1. ELUTION SOLUTIONS

A binary gradient elution system consisting of eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in acetonitrile) was applied.

3.3.2. PREPARATION OF CALIBRATORS AND WHOLE BLOOD SAMPLES

The samples were prepared following the IFCC reference measurement procedure for HbA_{1c} (19). The principle of this reference measurement procedure for HbA_{1c} is the determination of the ratio of glycated to nonglycated β-N-terminal hexapeptide of hemoglobin. Sample preparation was performed by the following three-step procedure (Figure 3-1).

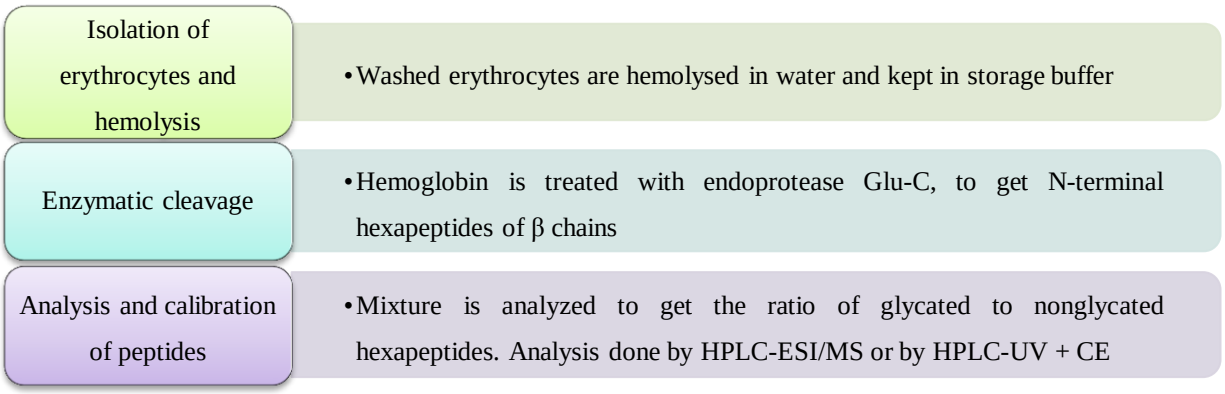


Figure 3-1 Sample preparation for HbA_{1c} determination

3.3.3. HPLC-MS/MS MEASUREMENT CONDITIONS

SETTING OF THE HPLC SYSTEM

Elution was performed on the C12 reversed-phase Jupiter Proteo column with a binary gradient elution and a flow rate of 300 µL/min, a column temperature of 50 °C, and an injection volume of 1 µL. The elution profile is given in Table 3-1.

Time	Module	Events	Settings
0.01	pumps	%B	0
0.02	subcontroller	rotary valve A	1
3.00	pumps	%B	0
5.00	subcontroller	rotary valve A	0
9.00	pumps	%B	6
16.40	subcontroller	rotary valve A	1
16.50	pumps	%B	6
16.60	pumps	%B	100
19.50	pumps	%B	100
19.60	pumps	%B	0
27.00	subcontroller	rotary valve A	0
28.00	system controller	stop	

Table 3-1 HPLC conditions. Rotary valve A switches from binary system pump A and B (position 1) to auxiliary pump C (position 0).

SETTING OF THE MASS SPECTROMETER

A postcolumn flow splitting system (1:6 split ratio) was installed. To avoid contamination of the ESI source, a switching valve system (rotary valve A) was introduced as described in (49).

The ESI/MS measurements settings are shown in Table 3-2.

The detected masses are shown the in Table 3-3.

API 4000 Event (program abbreviation)	Settings
Scan type	Q1 Multiple Ions
Polarity	positive
Resolution Q1	0.3 amu
CUR	50.00
GS1	40.00
GS2	70.00
IS	5000.00
TEM	150.00
ihe	ON
DP	30.00
EP	10.00

Table 3-2 Settings of the API 4000 mass spectrometer for Q1 multiplie ion mode

Analyte	Q1 multiple ion, m/z
	348.3
	429.3

Table 3-3 Detected masses – theoretical masses of doubly protonated hexapeptides. The doubly protonated β-N-terminal hexapeptides were monitored at m/z 348.3 for HbA_0 and m/z 429.3 for HbA_{1c}.

3.3.4. METHOD VALIDATION

LINEARITY

For calibration, we used calibrators containing mixtures of chromatographically purified HbA_{1c} and HbA_0 obtained from the IFCC Working Group on HbA_{1c} Standardization (18). After

digestion, we analyzed the calibrators by HPLC-ESI/MS and plotted the peak area ratios of the β-N-terminal hexapeptides against the ratios of HbA_{1c} and HbA_0 concentrations.

IMPRECISION AND INACCURACY

For internal quality control, we used hemolysate samples with target values assigned by the IFCC Working Group on Standardization of HbA_{1c}. We determined the target values of lyophilized whole blood samples with the original IFCC reference measurement procedure. Following the IFCC regulations for HbA_{1c} determination, 2 digestions were performed and 2 sequences were measured for each sample. The 4 values obtained were averaged and the results presented as statistical means (19).

3.4. MEASUREMENT CONDITIONS FOR DETERMINATION OF CYCLOSPORIN A, SIROLIMUS, TACROLIMUS, AND EVEROLIMUS

3.4.1. ELUTIONS AND STANDARD STOCK SOLUTIONS

ELUTION SOLUTIONS

For the chromatographic separation a ternary gradient was used for elution consisting of eluent A: 0.1 % v/v formic acid in water + 0.1 mmol/L Cs formate; eluent B: 0.1 % v/v formic acid in methanol + 0.1 mmol/L Cs formate and eluent C: 0.1 % v/v formic acid in acetonitrile + 0.1 mmol/L Cs formate. Cs formate was prepared by neutralising CsOH with formic acid to pH 7.

STANDARD STOCK SOLUTIONS

Standard stock solutions of cyclosporin A, sirolimus, tacrolimus, and everolimus were prepared by dissolving about 5 mg drug in 5 mL methanol each. The stock solutions were diluted for LC-MS/MS analysis with MeOH/H_2O 1:1 consisting 0.1 % v/v formic acid and 0.1 mmol/L Cs formate. Analytes and solvents were weighed on a calibrated precision balance. Aliquots were stored in 1.5 ml glass vial with screw cap and Silicone/PTFE seal below - 20 °C up to three months.

3.4.2. WHOLE BLOOD SAMPLES AND CALIBRATORS

DRUG FREE WHOLE BLOOD SAMPLES

Drug free EDTA whole blood for preparation of spiked control samples were collected by the Department of Biochemistry, Faculty of Medicine, University of Szeged. The samples were stored at -20 ^{o}C.

WHOLE BLOOD CALIBRATORS

MassCheck® Immunosuppressants Whole Blood Multilevel Calibrators (Chromsystems, Munich, Germany) were used as controls. The lyophilized calibrators were stored at -20 ^{o}C.

DRUG-TREATED PATIENT WHOLE BLOOD SAMPLES

Whole blood samples from drug-treated kidney transplant patients were collected in the Transplant Center, Department of Surgery, University of Szeged. 2 mL of venous blood was collected from each patient using BD Vacutainer® Plus Plastic K_2EDTA Tubes. Samples were obtained in a clinical research protocol approved by the Ethic Committee by University of Szeged. The samples were immediately analyzed or stored at -20 ^{o}C.

3.4.3. PREPARATION OF CALIBRATORS AND WHOLE BLOOD SAMPLES

PREPARATION OF CALIBRATORS

For the LC-ID-MS/MS measurements for each analyte three calibrators with isotope ratios of 1.00 were prepared by weighing and mixing 50 µL containing 10 ng labeled analyte and 50 µL containing 10 ng unlabeled analyte in 85 µL methanol/water (1:1 v/v) consisting 0.1 % v/v formic acid and 0.1 mmol/L Cs formate. 40 µL of the calibrator solutions were injected into the LC-MS/MS system resulting in an absolute amount of about 5 ng on column. These calibrators were prepared freshly for each campaign.

PREPARATION OF CONTROLS

Lyophilised whole blood controls were reconstituted with water as indicated by the manufacturer. Aliquots were stored in CryoPure tube (Sarstedt, Nümbrecht, Germany) with screw caps below -20°C up to three months.

PREPARATION OF WHOLE BLOOD SAMPLES

For the recovery studies drug free EDTA whole blood samples were spiked and processed for each immunosuppressant separately. For the spiking procedure different working solutions of each immunosuppressant were prepared in methanol/water (1:1 v/v) consisting of 0.1 % v/v formic acid and 0.1 mmol/L Cs formate from the stock solutions of the unlabeled compounds by weighing. The resulting concentrations are indicated in Table 3-4. The samples were spiked with maximally 30 µL of the immunosuppressant standard solution per 1 mL whole blood and were equilibrated at room temperature on a roller mixer for 40 minutes.

Spiked concentration [µg/L]	Cyclosporin A	Sirolimus	Tacrolimus	Everolimus
LLOQ	8.07	2.14	2.04	2.09
low	38.7	4.19	4.03	3.98
intermediate	397	12.7	12.6	12.0
medium	979	33.2	30.8	31.4
high	2109	84.3	81.2	81.1

Table 3-4 Concentration of the spiked whole blood samples

3.3.5. LIQUID-LIQUID EXTRACTION PROCEDURE OF THE WHOLE BLOOD SAMPLES

Whole blood (patient samples, controls and spiked samples) containing absolute approximately 20 ng cyclosporin A, 5 ng sirolimus, 10 ng tacrolimus, or 2 ng everolimus respectively were used for extraction. Therefore each sample was prequantified by an iterative approach to determine the approximate analyte concentration. The samples were spiked with the corresponding labeled standard to result in an isotope ratio of 1:1 for each analyte. The spiked samples were equilibrated on a roller mixer for 40 minutes at room temperature. Liquid-liquid extraction was performed as described previously (50). The respective internal standard [$^{2}H_{12}$]-cyclosporin A, [^{13}C,$^{2}H_{3}$]-rapamycin, [^{13}C, $^{2}H_{2}$]-tacrolimus and [$^{13}C_{2}$,$^{2}H_{4}$]-42-O-(2-Hydroxyethyl)rapamycin was added to 200 µl sample (spiked sample or patients sample) in a labeled/unlabeled ratio of 1:1 for each analyte. A preparation method for

lyophilized calibrators and whole blood sample was developed. The liquid-liquid extraction procedure is shown in Figure 3-2.

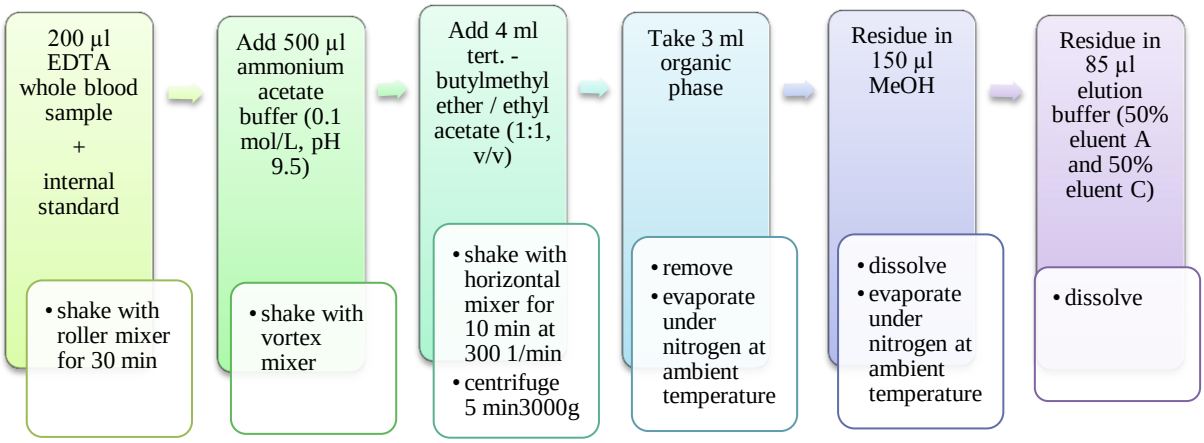


Figure 3-2 Liquid – liquid extraction procedure

3.3.6. HPLC-MS/MS MEASUREMENT CONDITIONS

SETTING OF THE HPLC SYSTEM

A phenyl-hexyl-RP column (Luna®, 2 x 150 mm, 5 µm, Phenomenex, Aschaffenburg, Germany) was used for the chromatographic separation.

The mobile phase was delivered using a ternary gradient elution profile. The flow rate was 300 µL/min. The column temperature was set to 50 °C. Table 3-5 shows the gradient profile.

SETTING OF THE MASS SPECTROMETER

The MS detection was performed in the positive ion mode. The settings of the instrument and mass transitions were determined by direct infusion of each immunosuppressant drug and its isotope labeled internal standard solution separately. The mass transitions (m/z) for MS detection are given in Table 3-6. The m/z of 132.9 represents the Cs^+ ion.

Time	Module	Events	Setting
0.01	pumps	%B	50
0.02	subcontroller	rotary valve A	1
1.00	pumps	%B	78
6.00	subcontroller	rotary valve A	0
16.00	pumps	%B	78
16.05	pumps	%C	0
20.00	pumps	%B	33
20.01	pumps	%C	39
30.05	pumps	%B	33
30.10	pumps	%C	39
31.00	pumps	%C	100
37.05	pumps	%C	100
37.10	pumps	%B	0
38.00	pumps	%C	0
38.05	pumps	%C	0
38.05	pumps	%B	50
42.00	pumps	Stop	

Table 3-5 Gradient profile for HPLC separation

Analyte	Precursor ion, m/z	Product ion, m/z
Cyclosporin A	1335.0	132.9
[$^{2}H_{12}$]-cyclosporin A	1347.3	132.9
Sirolimus (Rapamycin)	1046.5	132.9
[^{13}C, $^{2}H_{3}$]-rapamycin	1050.6	132.9
Tacrolimus	936.4	132.9
[^{13}C,$^{2}H_{2}$]-tacrolimus	939.2	132.9
Everolimus (42-O-(2-hydroxyethyl)rapamycin)	1090.5	132.9
[$^{13}C_{2}$,$^{2}H_{4}$]-42-O-(2-hydroxyethyl)rapamycin	1096.6	132.9

Table 3-6 Mass transitions of the four immunosuppressive agents cyclosporin A, sirolimus, tacrolimus and everolimus and the internal standards

TSQ 7000 measurements were acquired in the selected reaction monitoring mode (SRM). The scan time was 0.2 s and the scan width was 0.6 m/z for the Q1 and Q3 quadrupoles. MS acquisitions were done in centroid mode. The settings of the TSQ 7000 and API 4000 mass spectrometers are given in Table 3-7.

TSQ 7000 Event (program abbreviation)	Setting
Capillary Temperature	270°C
ESI Spray Voltage	4.5 kV
Sheath Gas	60 psi
Auxiliary Gas	17 ml/min
CID	1.9 mT
Offset	-75 eV
Emult	1600 kV

API 4000 Event (program abbreviation)	Setting
CAD	medium
CUR	15.00
GS1	60.00
GS2	30.00
IS	5500.00
TEM	350.00
ihe	ON
DP	140.00
EP	10.00
CE	100.00
CXP	14.00

Table 3-7 Instrument settings for the TSQ 7000 in SRM and API 4000 in MRM mode

3.3.7. IMMUNOASSAYS

Analysis was performed by chemiluminescent immunoassay (CMIA) with the Abbott Architect i1000 analyzer as described in (33).

3.3.8. METHOD VALIDATION

RECOVERY AND INTRA-ASSAY PRECISION

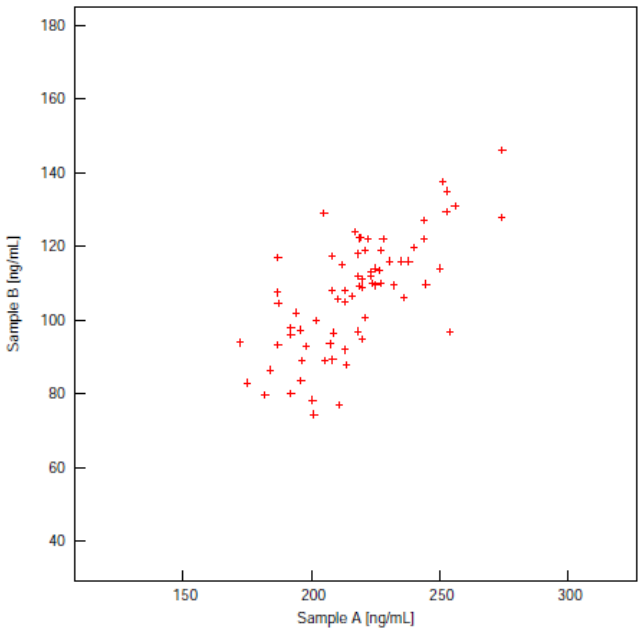
For quality control drug-free human EDTA blood was spiked with standard solutions at concentrations of. 8.07, 38.7, 397, 979, and 2109 µg/L for cyclosporin A, 2.14, 4.19, 12.7, 33.2 ,and 84.3 µg/L for sirolimus, 2.04, 4.03, 12.6, 30.8, and 81.2 µg/L for tacrolimus, 2.09, 3.98, 12.0, 31.4, and 81.1 µg/L for everolimus. The recoveries and intra-assay precision were determined by measurements in duplicate on 5 consecutive days.

Commercially available Multilevel Calibrators at three different levels were measured in duplicate on 3 consecutive days.

4. RESULTS AND DISCUSSION

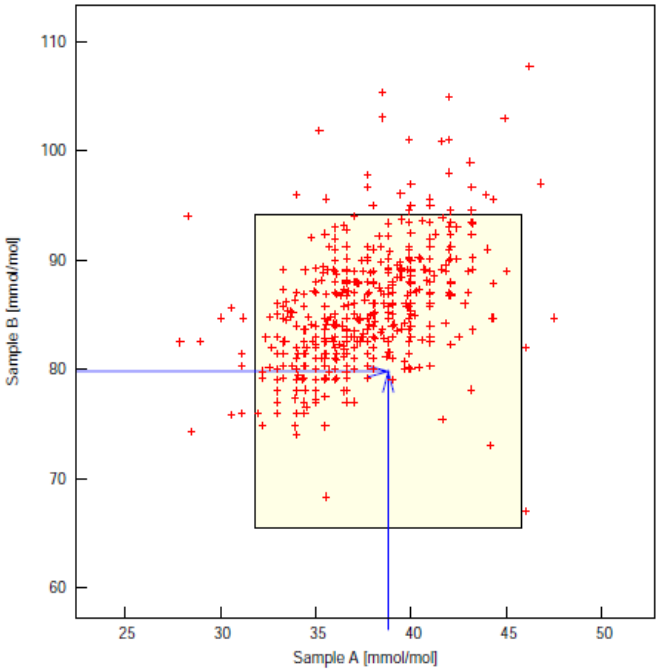
The main progress in the quality of laboratory analyses for patient care arrives from the development of reliable analytic procedures, which are accurate, precise and specific. By this way they promote the comparability of values independent of tests and the laboratories where the analyses have been performed. Following these procedures the health care system saves money and improves patient care (33)(39).

The existing poor comparability of values of some analytical systems is best demonstrated by the evaluation in interlaboratory surveys. In these schemes the laboratories analyse the same samples with their instruments or kits originating from different manufacturers. In these external quality assessment schemes dispersion of values between the different laboratories and between the analytical tests is well demonstrated as shown in Figure 4-1 and Figure 4-2.



Cyclosporin A	Median (ng/mL)	Mean (ng/mL)	CV%	n
Sample A	218.25	217.30	10.10	76
Sample B	109.50	107.14	14.53	76

Figure 4-1 Youden-plot of the values in an External Quality Assessment Scheme for Cyclosporin A run by INSTAND e.V. (2011)



HbA_{1c}	Median (mmol/mol)	Mean (mmol/mol)	CV%	n
Sample A	37.30	37.59	8.08	632
Sample B	84.70	85.26	6.59	632

Figure 4-2 Youden-plot of the values in an External Quality Assessment Scheme for Cyclosporin A run by INSTAND e.V. (2011). The reference measurement values for the two samples are identified by the arrows.

The reasons for the different values are mainly the different calibration of the test systems, the different specificity of the antibodies used and the unspecific effect of matrix components. The existing problem can be solved by developing reference measurement procedures which are independent of matrix effects and by using certified reference materials as calibrator by the manufacturers.

In this study the analytical performance of important analytes in patient care have been examined and reference measurement procedures were developed to set target values in EQAS and working standards with best accuracy and precision possible.

4.1. DETERMINATION OF HbA_{1c}

The original IFCC reference measurement procedure has been established in the IFCC Working Group on HbA_{1c} Standardization using LC-ESI-MS and HPLC-CE (19).

In the original IFCC reference method calibration was performed using external standards (51). For external calibration a long-term stability in the measurement system with stabile electrospray ionization is required.

The original IFCC method using a cyano-propyl column and TFA for elution had some limitations, because of a poor robustness. This has been already discussed in a recent study (49). The long-term evaluation showed peak tailing, irregular peak profiles and instability of retention time in the chromatographic separation. Additionally, a lack of reproducibility of the elution profile on columns with different batches was observed (Figure 4-3).

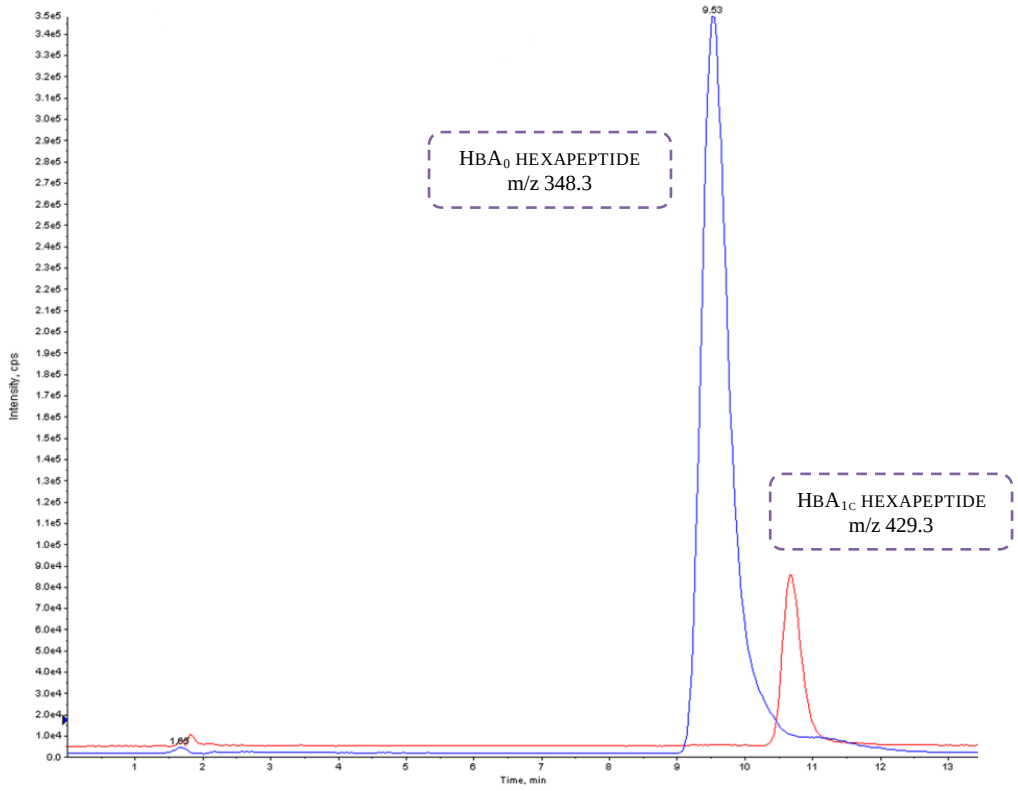


Figure 4-3 HPLC-ESI/MS chromatogram of a digested mixture of HbA_{1c} and HbA_0 using a cyano-propyl column, following the original IFCC reference measurement procedure.

In a first step the analytical column was replaced by a C12 reversed phase column. The effect on the elution profile is shown in Figure 4-4. The C12 reversed-phase Jupiter Proteo column gives better peak shapes of the HbA_0 and HbA_{1c} derived β-N-terminal hexapeptides and a better separation performance.

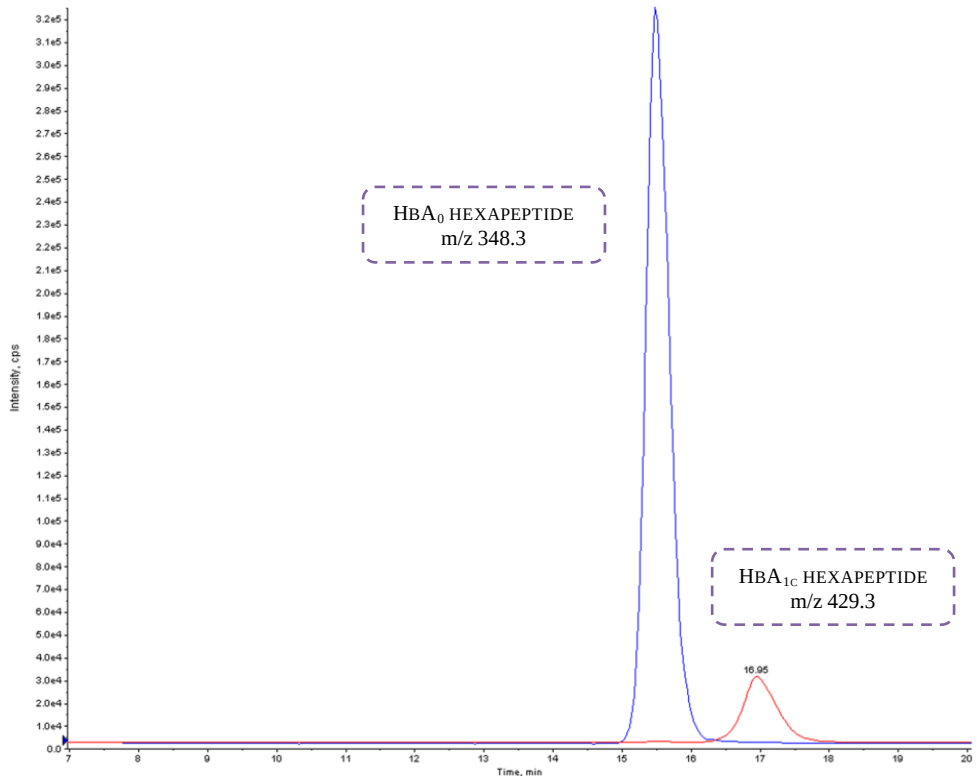


Figure 4-4 HPLC-ESI/MS chromatogram of digested mixture of HbA_{1c} and HbA_0 using a C12-reversed phase column

In a second modification TFA was replaced by formic acid in the elution buffer (Figure 4-5). At 0.1% formic acid, peak sharpness is enhanced and signal intensities highly improved. This allows a dilution of the sample of 1:5 and a reduction of the injection volume from 3 to 1 µL. Approximately 15-fold higher absolute signal intensities are obtained with formic acid compared with TFA.

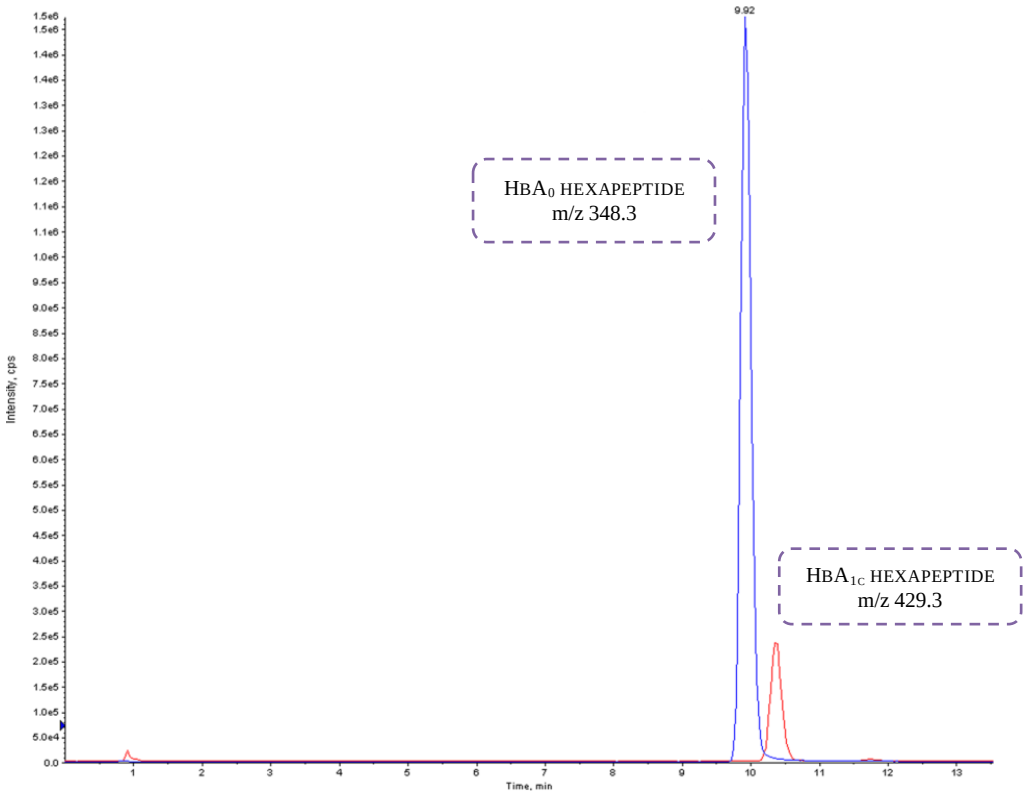


Figure 4-5 HPLC-ESI/MS chromatogram of digested mixture of HbA_{1c} and HbA_0 using a C12-reversed phase column and formic acid in the elution buffer

HPLC conditions	Original IFCC	Modified
HPLC elution buffer	A:0.025% TFA in water B:0.023% TFA in ACN	A:0.07% FA in water B:0.07% FA in ACN
Analytical column	Zorbax SB-CN, 5 µ; 2.1mm x 150mm	Jupiter Proteo 4 µm, 2.0 x 50 mm
Flow rate	350 µl/min	300 µl/min
Injection volume	10 µl	1 µl
Column temperature	50 °C	50 °C
Post column splitting	none	1:6
Switching off ESI source of the HPLC flow	14.5-18.0 min	0-5.0 min 13.4-27.0 min

Table 4-1 Comparison of the original IFCC and the modified measurement methods

4.1.1. VALIDATION

LINEARITY AND CHROMATOGRAPHIC REPRODUCIBILITY

For the test of the system stability the peak area ratios and the retention times of the β-N-terminal hexapeptides of HbA_{1c} and HbA_0 were determined in a digested hemolysate sample by repeated measurements. The C12 reversed-phase column showed a highly reproducible elution profile for the two peptides as shown Table 4-2.

	n	Within-run coefficient of variation CV %
Peak area ratio	48	1.34
Retention time	41	0.17

Table 4-2 System stability parameters

As shown in Figure 4-7 linear calibration curves for the IFCC calibrators are obtained in the concentration range between 10 and 130 mmol/mol HbA_{1c}. Based on the IFCC protocol for HbA_{1c} measurements (19) the analysis of samples has to be performed in a defined sequence (Figure 4-6). The calibrators have to be determined before and after controls and the samples.

The analysis sequence requires long-term stability of the analytical system. The congruence of the calibration curves (Figure 4-6) at the beginning and the end of one measuring sequence, demonstrates the system stability (49).

Figure 4-6 Set-up analysis sequence for one digestion

The target values set by the IFCC Working Group on HbA_{1c} Standardization of the IFCC calibrators (expressed as ratio concentration of HbA_{1c} vs HbA_0) are plotted against the peak area ratios of the glycated and nonglycated β-N-terminal hexapeptides of HbA_{1c} and HbA_0.

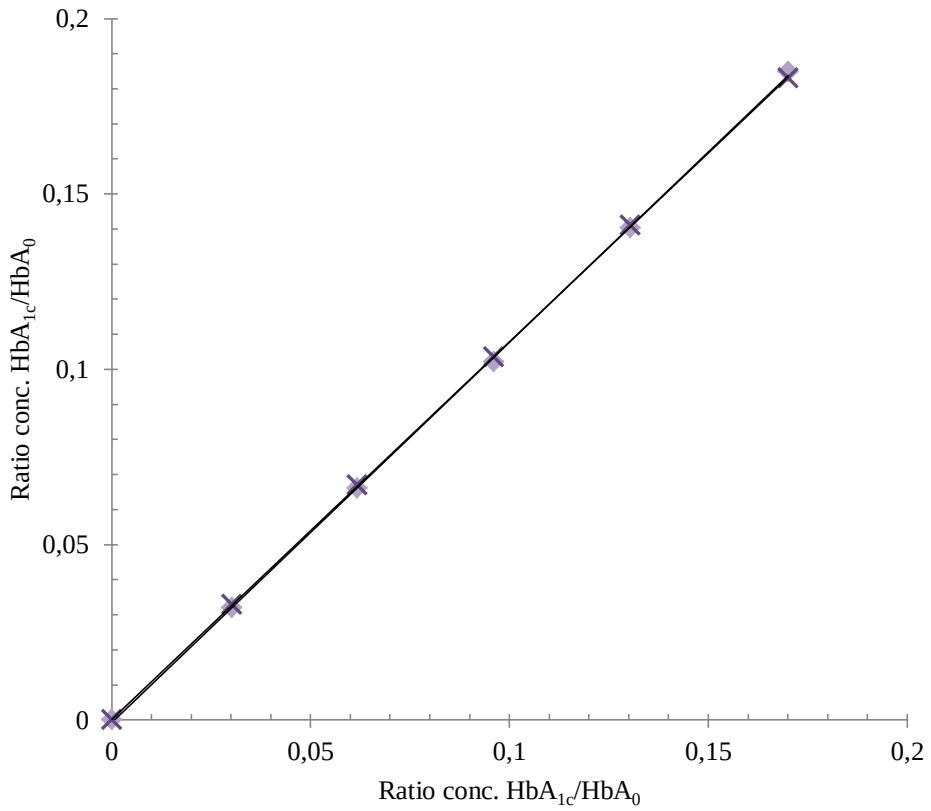


Figure 4-7 Calibration curves of 6 digested IFCC calibrators, measured at the beginning (♦) and the end (X) of one measuring sequence. The regression equation for calibration curve 1 (♦) is: y = 1,0844x - 0,0007 R^2 = 0,9998, and for calibration curve 2 (X) is: y = 1,076x + 0,0003 R^2 = 1.

IMPRECISION AND INACCURACY

In Table 4-3 the values for the between-run coefficient of variation (CV %) and deviation from the target values (bias %) are given for IFCC quality control samples and lyophilized whole blood samples obtained by the modified procedure.

For the IFCC control samples the values for the between-run coefficient of variation were between 0.71% and 1.86% and deviations from the IFCC target values were between -0.87 and 1.00 relative %.

For lyophilized whole blood samples the between- run coefficient of variation values were between 1.08% and 1.90% and the deviations from the target values were between -1.45 and 1.41 relative %.

	Target HbA_{1c} [mmol/mol]	Mean HbA_{1c} [mmol/mol]	CV [%]	Bias [%]
IFCC quality control samples	32.1	31.9	1.86	-0.78
	32.4	32.7	0.84	1.00
	84.8	84.7	0.71	-0.09
	85.8	85.1	1.41	-0.87
Lyophilized whole blood samples	32.8	32.3	1.08	-1.45
	54.3	55.1	1.34	1.41
	78.7	77.6	1.90	-1.40

Table 4-3 Imprecision and inaccuracy of measurements (n=4) of IFCC quality control samples and lyophilized whole blood samples

Though a reference measurement procedure for HbA_{1c} was published by the IFCC-Group the performance of the method had some limitations (19). The main deficiencies were the instability of retention times, peak-tailing and by this way a poor reproducibility of measurement values. By changing the column and the elution gradient on the special column the peak shapes became symmetric and more reproducible. These improvements were accepted in the IFCC-working group as an essential modification of the original reference measurement procedure.

In the original IFCC reference measurement procedures an external calibration of the analytical systems were used. In a further development based on this modified analytical method an LC-ID/MS procedure was established.

Thus, the procedure developed in this study became an essential part of a new calibration system using LC-IDMS (52), which is now traceable to SI units according to ISO 17511. This new reference measurement procedure fulfills the requirements of traceability to SI-units.

On the basis of the recommendations of the Hungarian Society of Laboratory Medicine and the Hungarian Diabetes Association from 1 April 2011, patient results shall be provided in Hungary both as IFCC standardized units (mmol/mol) and DCCT aligned units (%). From 1 April 2013, results will be reported only in the new IFCC units (53).

4.2. DETERMINATION OF CYCLOSPORIN A, SIROLIMUS, TACROLIMUS AND EVEROLIMUS

The analysis of the concentrations of immunosuppressants in blood is an effective procedure to monitor the therapeutic range and to avoid both overdose and underdose. By this way the risk for a possible rejection of the transplanted organs or tissues and strong side effects during the therapy can be reduced. Therefore the drug monitoring in this field is of outstanding clinical interest. The internal and external quality control of the analyses in transplantation medicine is a basic requirement in the national and international clinical practice guidelines (54). These analyses shall be performed with reliable procedures and appropriate calibration.

Therapeutic monitoring of immunosuppressive drugs is performed routinely with immunological or chromatographic procedures (35, 55-57). The most commonly used immunosuppressive drugs for maintenance therapy are cyclosporin A, sirolimus, tacrolimus and everolimus (Table 1-2 and Figure 1-1).

The analyses of many immunoassays have the disadvantage of interfering metabolites. The available analytical routine procedures of the market give different results when analysing the same samples as shown in external quality assessment schemes. The reasons for the dispersion of the observed values analysed by routine methods are probably different calibration and specificity of the antibodies used (34, 36). Immunologic procedures detect not only the parent substances but also metabolites (58). LC/MS measurement procedures routinely used in drug monitoring without separation of the single drugs and their metabolites may also give erroneous results (44, 59-62). To overcome the dispersion of values analysed with different routine procedures and to get the intended comparability of patient values standardization of these measurements shall be realised (63). From clinical point of view there is a need for reference measurement procedures and certified reference materials as required in the “Directive 98/79/EC” on in vitro diagnostic medical devices. For the transferability of patient values between clinics and general practitioners the comparability of the results shall be given at the best accuracy possible.

In previous studies it has been shown that immunosuppressive drugs can be determined by LC-MS/MS as Cs^+ adducts (64). Based on this principle and by introducing labeled internal standards a reference measurement procedure based on LC-ID/MS for

immunosuppressant drugs was developed. Figure 4-8 shows the separation of the four immunosuppressive drugs tacrolimus, sirolimus, everolimus, and cyclosporin A in spiked whole blood sample after liquid-liquid extraction.

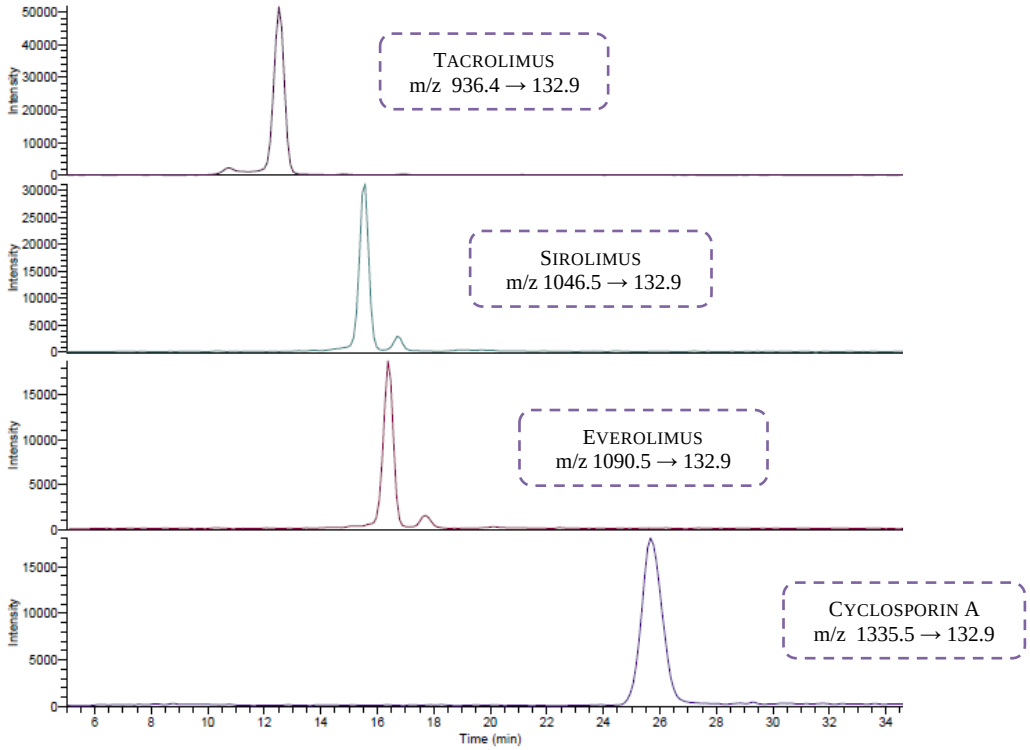


Figure 4-8 HPLC-MS/MS chromatogram of Cs-adducts of immunosuppressive drugs in the SRM mode. The abscissa shows the retention time in minutes, the ordinate the peak height in arbitrary units (counts per second, cps)

Defined amount of synthesized labeled immunosuppressants were added to the samples. The chemical structures of isotope-labeled analytes are shown the Figure 4-9. The corresponding ^{2}H or ^{13}C labeled internal standard was used for each analyte. The native standards are demonstrated in Figure 1-1. Figure 4-10 demonstrates the chromatogram of a whole blood sample after liquid-liquid extraction. Native and isotope-labeled standards can be identified with the same retention time because they have very similar physicochemical properties.

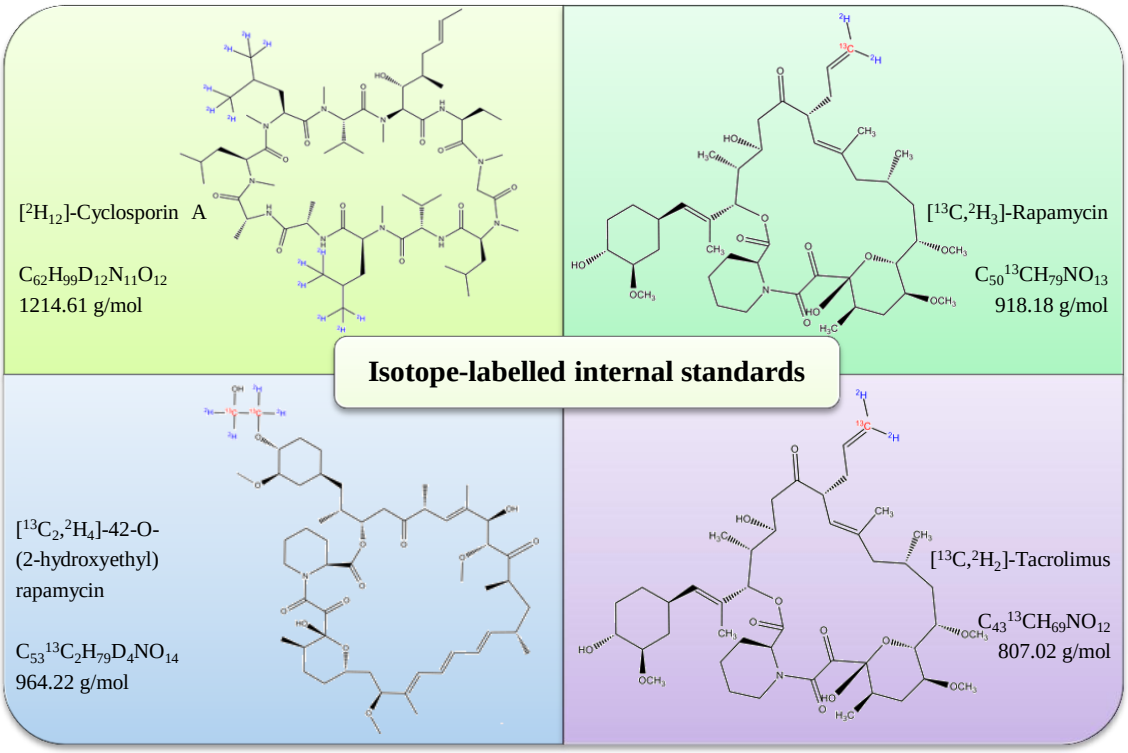


Figure 4-9 Chemical structure of isotope-labeled internal standards

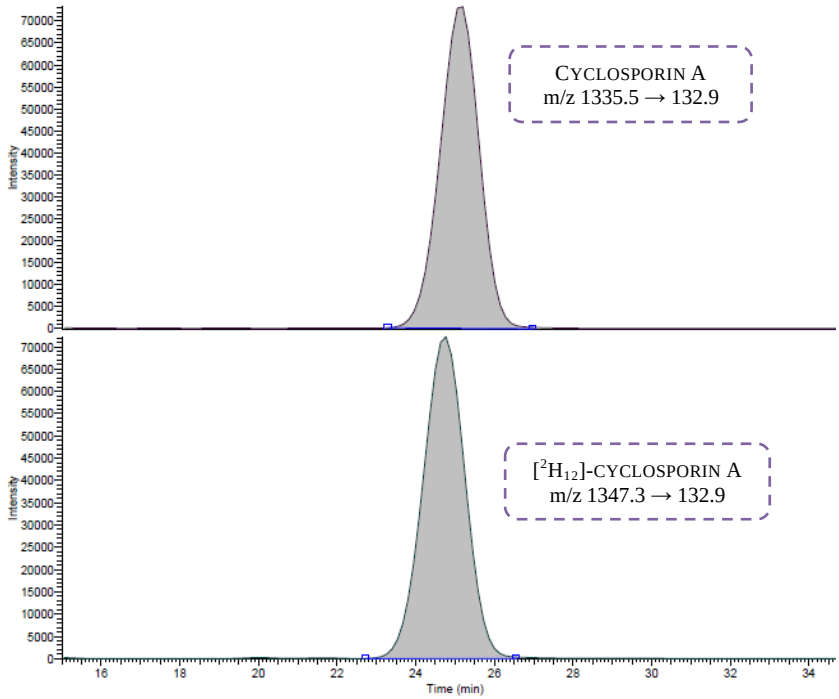


Figure 4-10 HPLC-MS/MS chromatogram of native cyclosporin A and the internal standard [$^2H_{12}$]-cyclosporin A in EDTA whole blood sample. The abscissa shows the retention time in minutes, the ordinate the peak height in arbitrary units (counts per second, cps)

4.2.1. VALIDATION

LINEARITY AND RECOVERY

The linearity of the analytic procedure in serum samples can be demonstrated for each analyte by a linear regression plotting the theoretical spiked concentrations against the mean values of the measured concentrations (Figure 4-11 and Table 4-4).

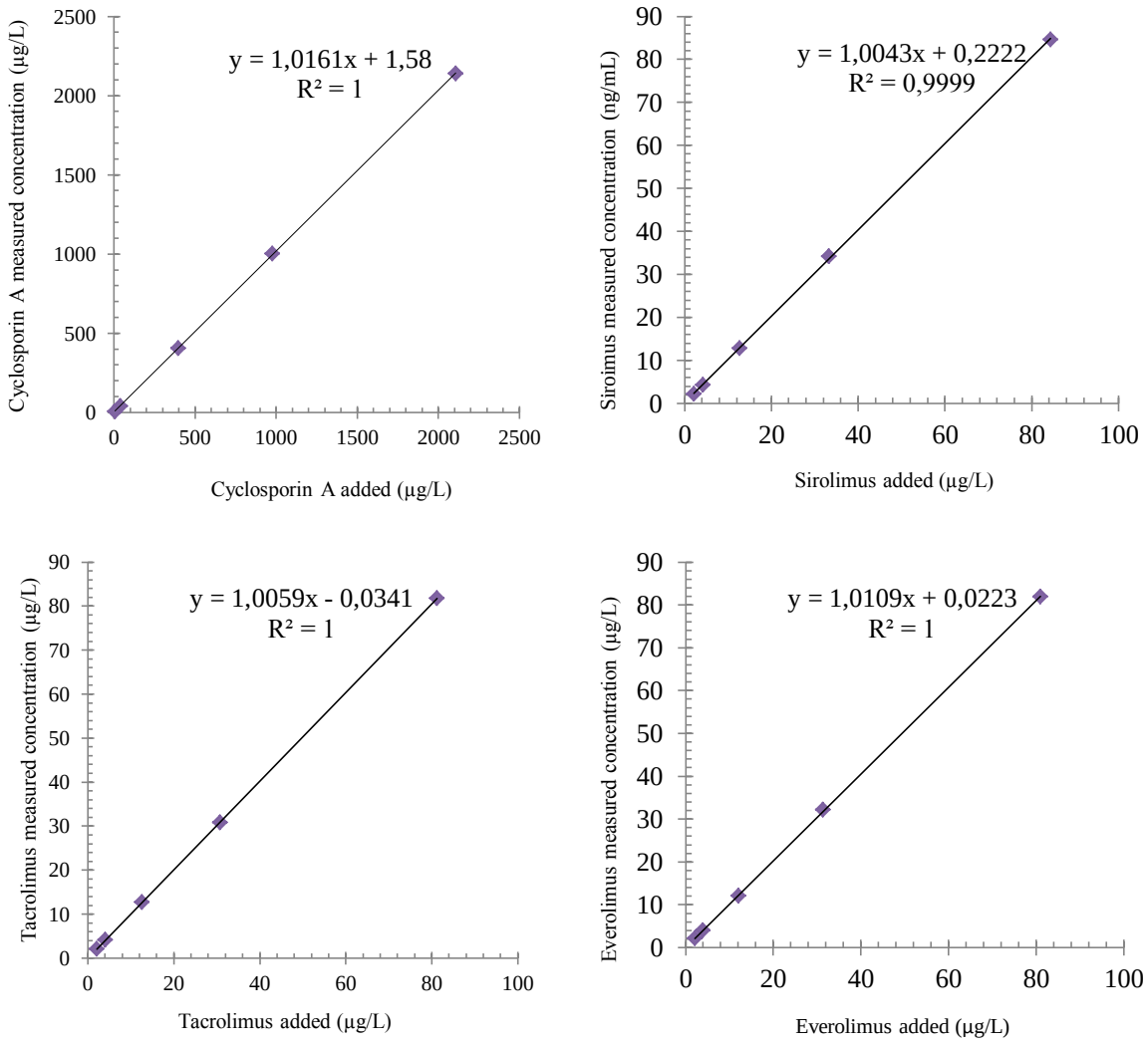


Figure 4-11 Recovery and linearity data for immunosuppressive drugs

Drug free EDTA whole blood samples were spiked with the four immunosuppressive drugs and their corresponding isotope-labeled forms at different concentrations (Table 3-4) covering the therapeutic ranges. The results of the recovery are shown in Table 4-4.

Immunosuppressant	Spiked concentration [µg/L]	Mean value measured by LC-IDMS/MS [µg/L] n=5	Recovery [%]	CV [%]
Cyclosporin A				
LOQ	8.07	8.13	100.8	2.60
low	38.7	39.6	102.3	1.45
intermediate	397	404	101.8	1.17
medium	979	1003	102.5	2.30
high	2109	2141	101.5	2.37
Sirolimus				
LOQ	2.14	2.20	103.0	0.92
low	4.19	4.29	102.3	1.31
intermediate	12.7	12.9	101.6	1.72
medium	33.2	34.2	102.9	1.23
high	84.3	84.7	100.4	0.81
Tacrolimus				
LOQ	2.04	2.07	101.2	1.06
low	4.03	4.04	100.2	0.53
intermediate	12.6	12.7	100.6	0.95
medium	30.8	30.8	99.98	0.44
high	81.2	81.7	100.6	0.53
Everolimus				
LOQ	2.09	2.08	99.8	1.70
low	3.98	3.96	99.5	4.34
intermediate	12.0	12.1	100.3	0.82
medium	31.4	32.1	102.4	1.61
high	81.1	81.8	100.9	1.12

Table 4-4 Recoveries of immunosuppressants spiked in EDTA whole blood at therapeutic ranges

The mean recovery of the added immunosuppressants in whole blood was 101.8 % for cyclosporin A, 102.4 % for sirolimus, 100.5 % for tacrolimus, and 100.5 % for everolimus.

Imprecision is expressed as the coefficient of variation (CV), 1.17-2.60 % for cyclosporin A, 0.92 -1.72 % for sirolimus, 0.44 - 1.06 % for tacrolimus and 0.82 - 4.34 % for everolimus.

Commercially available Multilevel Calibrators were used as controls and their concentrations were compared with values obtained by the LC-ID-MS/MS method. The assigned values of these lyophilized whole blood samples were obtained as a consensus value from the data of an international network of laboratories. Calibrators were used as whole blood control samples at three different concentration levels (Table 4-5).

Immunosuppressant	**Assigned value [µg/L]**	**LC-IDMS/MS value [µg/L]**	**CV [%]**	**Deviation from assigned value [%]**
Cyclosporin A				
Low	23.5	22.6	1.51	-3.83
Medium	127	116	1.82	-8.66
High	484	472	0.87	-2.48
Sirolimus				
Low	2.60	2.38	1.60	-8.46
Medium	6.60	6.46	0.99	-2.12
High	20.0	19.5	1.31	-2.50
Tacrolimus				
Low	2.10	2.24	1.83	6.67
Medium	5.80	6.26	1.64	7.93
High	17.3	18.9	1.41	9.25
Everolimus				
Low	2.20	2.50	0.49	13.6
Medium	5.90	6.58	0.88	11.5
High	17.0	19.3	0.52	13.5

Table 4-5 Comparison with commercially available lyophilized control sample (n=6).

Differences between -8.46 and +13.6 % were observed at high precision of measurement.

DRUG-TREATED PATIENT WHOLE BLOOD SAMPLES

Additionally the values in whole blood samples obtained with the newly developed reference measurement procedure were compared with those obtained with a routine immunoassay (CMIA) (43). The correlation between the methods is presented in Figure 4-12.

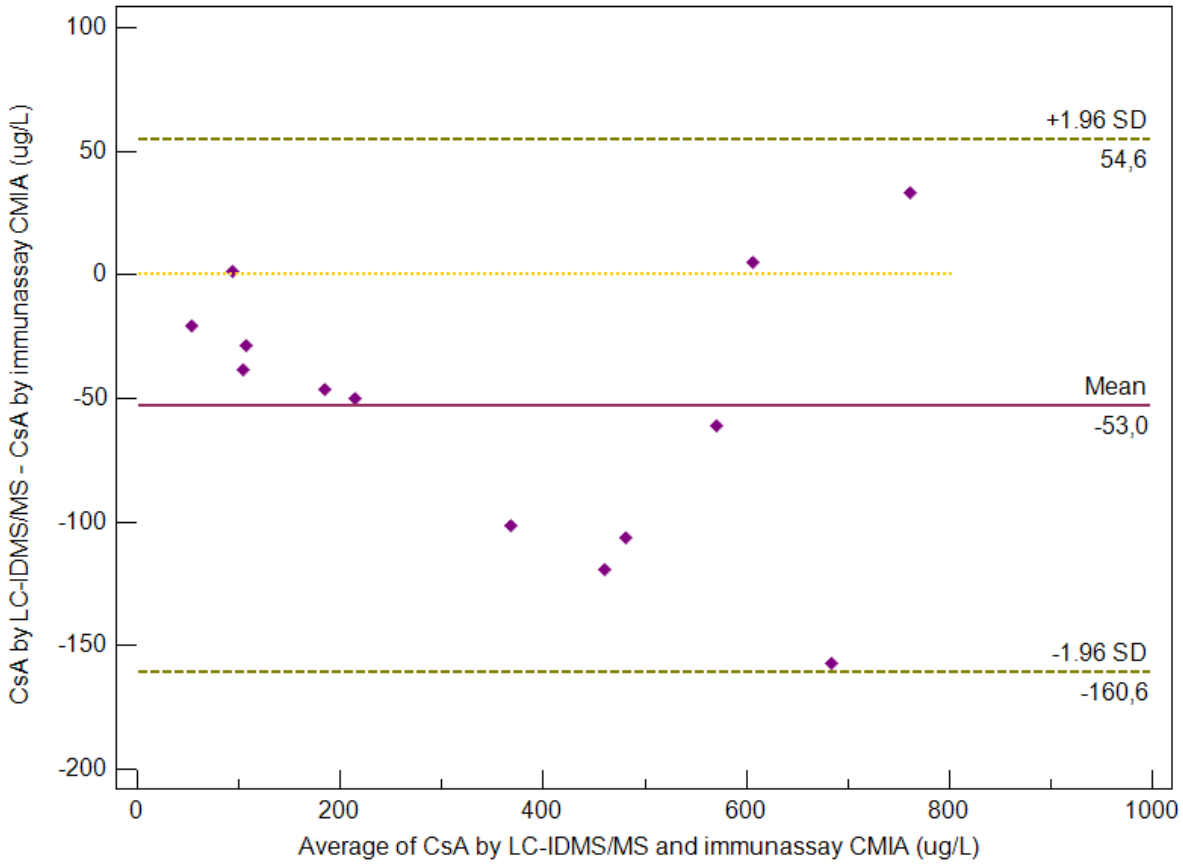


Figure 4-12 Bland-Altman regression of cyclosporin A concentrations measured with immunoassay CMIA and LC-ID-MS/MS (n=20). ♦ cyclosporin A whole blood concentrations in samples from renal transplant recipients

5. SUMMARY

For managing diseases like diabetes mellitus and for monitoring therapeutic levels of immunosuppressive drugs analytical procedures have been developed with high accuracy and precision as required in ISO 15193:2009 for reference measurement procedures. The common principle of these analytic procedures is LC-MS.

For HbA_{1c} an analytic procedure based on LC-MS was improved to a more robust, highly accurate and precise method, which meanwhile has been accepted by the IFCC Working Group on HbA_{1c} standardization as the new and common reference measurement procedure.

A reference measurement procedure for four immunosuppressive drugs was developed based on LC-IDMS/MS. This new procedure was realized in several steps by the development of an extraction procedure for serum samples and the development of an isotope dilution procedure by using labeled internal standards.

The evaluation of all this procedure was performed following ISO 15193:2009.

Reference measurement procedures have been established for HbA_{1c} and immunosuppressive drugs to improve analytical results in patient samples, to recalibrate analytical systems, and to evaluate EQA schemes with target values of the best accuracy possible.

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