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

SUMMARY OF PHD THESIS

PETRA MAGDOLNA MOLNÁR MSC

INSTAND E.V. GESELLSCHAFT ZUR FÖRDERUNG DER QUALITÄTSSICHERUNG IN MEDIZINISCHEN LABORATORIEN E.V. DÜSSELDORF, GERMANY

AND

DEPARTMENT OF BIOCHEMISTRY, FACULTY OF MEDICINE UNIVERSITY OF SZEGED, HUNGARY

2012.

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 Nonprofit Public Utility Ltd. is following the international development, and follows the regulation of the IVDMD Directive 98/79 EC., ISO 17043:2009 and the mandated and harmonized standard EN 14136:2004. 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 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 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. 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 procedure 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}

The amount of HbA_{1c} is an important measure in managing the care of diabetic patients Long-term complications (microvascular and macrovascular complications) of the diabetic disease are correlated with the quality of diabetes management. This has been demonstrated in the clinical studies Diabetes Control and Complications Trial (DCCT) and United Kingdom Prospective Diabetes Study (UKPDS). The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) established a 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, and was used as calibrators in CE and LC-MS procedures.

PATHOBIOCHEMICAL ASPECTS OF IMMUNOSUPPRESSIVE DRUGS

Aim of the immunosuppressive therapy is to prevent rejection of transplanted organs, in which case the defence mechanisms of the patient will be inhibited. Immunosuppressive drugs show a very narrow therapeutic range and both intra- and interindividual pharmacokinetic and pharmacodynamic variability. Therefore, the exact dosage of immunosuppressive therapy, and monitoring of the drug level in blood are of high importance for long-term transplant success. The most commonly used methods in routine laboratories are immunological procedures. The antibodies used detect not only the parent substances but cross-reactive metabolites also. Therefore a reference measurement procedure shall be developed to analyze the parent drugs only.

AIMS AND SCOPE

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.

MATERIALS AND METHODS

HPLC-MS/MS SYSTEMS FOR DETERMINATION HbA_{1c} AND IMMUNOSUPPRESSIVE DRUGS

a. The HPLC system was a Merck-Hitachi LaChrom® HPLC system. The mass spectrometer was a Finnigan-MAT TSQ® 7000 triple stage quadrupole tandem mass spectrometer equipped with an electrospray ionization interface.
b. The HPLC system was a Shimadzu HPLC system. The mass spectrometer was an API 4000 equipped with a TurboV™ ESI source with TurboIonSpray™ probe.

MEASUREMENT CONDITIONS FOR DETERMINATION OF HbA_{1c}

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

The samples were prepared in accordance with the IFCC reference measurement procedure for HbA_{1c}. The principle of the reference measurement procedure for HbA_{1c} was the determination of the ratio of glycated to nonglycated β-N-terminal hexapeptide of hemoglobin.

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 doubly protonated β-N-terminal hexapeptides were monitored at m/z 348.3 for HbA_0 and m/z 429.3 for HbA_{1c}.

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

A ternary gradient was used for the chromatographic separation 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.

Standard stock solutions of cyclosporin A, sirolimus, tacrolimus, and everolimus were prepared by dissolving about 5 mg drug in 5 mL methanol each. Drug free EDTA whole blood for preparation of spiked control samples were donated from Department of Biochemistry, Faculty of Medicine, University of Szeged. MassCheck® Immunosuppressants Whole Blood Multilevel Calibrators were used as controls. Whole blood samples from drug-treated kidney transplant patients were collected in the Transplant Center, Department of Surgery, University of Szeged.

For each analyte three calibrators with isotope ratios of 1.00 were prepared for LC-ID-MS/MS measurements by weighing and mixing 50 µL working solution containing 10 ng labeled analyte and 50 µL containing 10 ng unlabeled analyte in 85 µL methanol/water (1:1 v/v) consisting of 0.1 % v/v formic acid and 0.1 mmol/L Cs formate.

Recovery studies were performed in drug free EDTA whole blood samples, which 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 0.1 % v/v formic acid and 0.1 mmol/L Cs formate from the stock solutions of the unlabeled compounds by

weighing, resulting in spiked concentrations which were within the therapeutic ranges. Whole blood (patient samples, controls and spiked samples) containing an absolute amount of approximately 20 ng cyclosporin A, 5 ng sirolimus, 10 ng tacrolimus, or 2 ng everolimus respectively were used for extraction. The respective isotope labeled 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 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 shows the Figure 1.

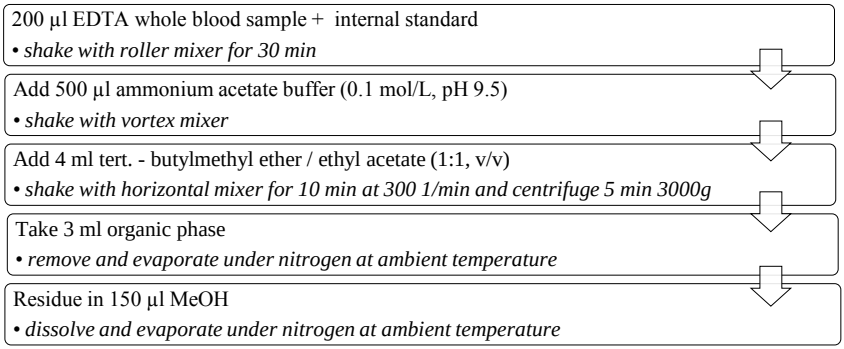

Figure 1: Liquid – liquid extraction procedure

A phenyl-hexyl-RP column (Luna®, 2 x 150 mm, 5 µm, Phenomenex, Aschaffenburg, Germany) was used for the chromatographic separation. The flow rate was 300 µL/min. The column temperature was set at 50 °C.

The precursor ions (m/z) of the native and the isotopic labeled internal standards are as follows: Cyclosporin A 1335.0, [$^{2}H_{12}$]-cyclosporin A 1347.3, Sirolimus (Rapamycin) 1046.5, [^{13}C,$^{2}H_{3}$]-rapamycin 1050.6, Tacrolimus 936.4, [^{13}C,$^{2}H_{2}$]-tacrolimus 939.2, Everolimus (42-O-(2-hydroxyethyl)rapamycin) 1090.5, [$^{13}C_{2}$,$^{2}H_{4}$]-42-O-(2-hydroxyethyl)rapamycin 1096.6.The product ion of m/z 132.9 represents the Cs^{+} ion. TSQ 7000 acquisition was performed in selected reaction monitoring (SRM).

RESULTS AND DISCUSSION

DETERMINATION OF HBA_{1C}

The original IFCC method had some limitations because of the poor robustness. 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. Based on these experiences, TFA was replaced by formic acid in the elution buffer and the column was replaced by a C12 reversed phase column. Using these modifications stabile retention time, higher peak symmetry, higher signal intensities and a good column batch-to-batch reproducibility were achieved.

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 2: Differences between the original IFCC and the modified measurement me

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 for HbA_{1c} and HbA_0 were determined in a digested hemolysate by repeated measurements. The C12 reversed-phase column showed a highly reproducible elution profile for the two peptides. Linear calibration curves for the IFCC calibrators are obtained in the concentration range between 9 and 130 mmol/mol $HbA_{1c.}$ The external calibration requires long-term stability of the analytical system.

Imprecision and inaccuracy: The values for the between-run coefficient of variation (CV %) and the deviation from the target values (bias %) were calculated in IFCC quality control samples and also from lyophilized whole blood samples. The between-run coefficient of variation was between 0.71% and 1.86% and the deviation from the IFCC target values was between -0.87 and 1.00 relative %. In lyophilized whole blood samples the values for the between- run coefficient of variation were between 1.08% and 1.90% and the values for the deviation from the target values were between -1.45 and 1.41 relative %.

These improvements were accepted in the IFCC-working group as an essential modification of the original reference measurement procedure.

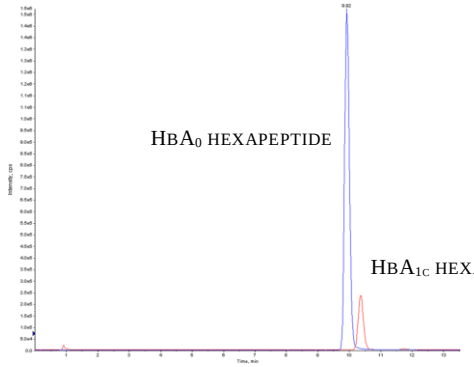

Figure 2: HPLC-ESI/MS chromatogram of digested mixture of HbA_{1c} and HbA_0 using a C12-reversed phase column and formic acid in the elution buffer

DETERMINATION OF CYCLOSPORIN A, SIROLIMUS, TACROLIMUS AND EVEROLIMUS

Therapeutic drug monitoring of immunosuppressive drugs is performed routinely with immunological and chromatographic procedures. The available analytical routine procedures of the market give different results when analysing the same samples as shown in external quality assessment schemes. Immunologic procedures detect not only the parent substances but also metabolites. LC/MS measurement procedures routinely used in drug monitoring without separation of the single drugs and their metabolites may also give erroneous results. 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 we could show that immunosuppressive drugs can be determined by LC-MS/MS as Cs^+ adducts.

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 3 shows the separation of the four immunosuppressive drugs tacrolimus, sirolimus, everolimus, and cyclosporin A in spiked whole blood sample after liquid-liquid extraction.

Defined amounts of synthesized immunosuppressants were added to the samples.The corresponding ^2H or ^{13}C labeled internal standard was used for each analyte. After prequantification a ratio of 1:1 between the analyte and labeled compounds was adjusted.

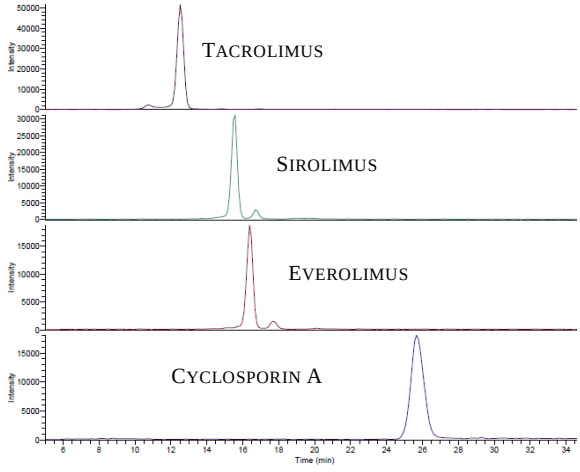

Figure 3: HPLC-MS/MS chromatogram of Cs-adducts of immunosuppressive drugs in the SRM mode. The abscissa shows the retention time in minutes, the ordinate the peak height in arbitrary units (counts per second, cps)

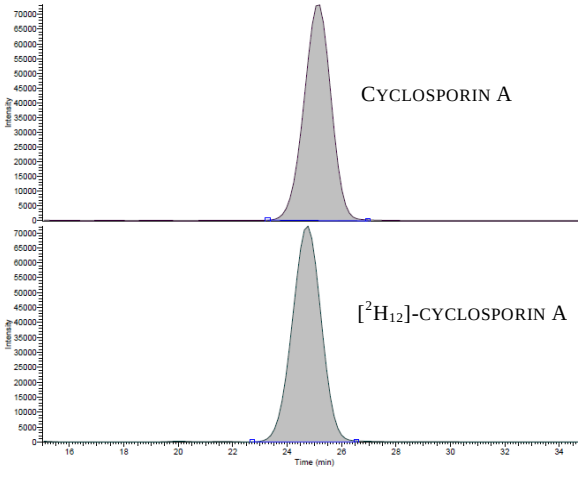

Figure 4: HPLC-MS/MS chromatogram of native cyclosporin A and the internal standard [$^2H_{12}$]-cyclosporin A in EDTA whole blood sample. The abscissa shows the retention time in minutes, the ordinate the peak height in arbitrary units (counts per second, cps)

Drug free EDTA whole blood samples were spiked with the four immunosuppressive drugs and their corresponding isotope-labeled compounds at different concentrations covering the 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.The imprecision, expressed as the coefficient of variation was 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. Calibrators were used as whole blood control samples at three different concentration levels. Differences between -8.46 and +13.6 % were observed at high precision of measurement.

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).

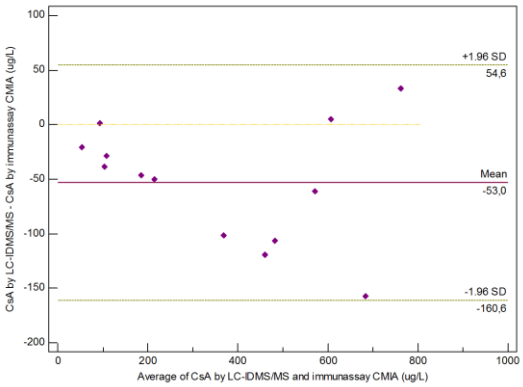

Figure 5: Bland-Altman regression of cyclosporin A concentrations measured with immunoassay CMIA and LC-ID-MS/MS (n=20). ♦ cyclosporin A whole blood concentrations in samples from renal transplant recipients

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.

New results:

Modification of the reference measurement procedure for the determination of HbA_{1c}

For HbA_{1c} an analytic procedure based on LC-MS was improved to a more robust, highly accurate and precise method.

- ✓ Improvement of the peak shapes by using of C12 reversed-phase Jupiter Proteo column.
- ✓ Replacement of TFA by formic acid in the elution buffer resulting in a better peak sharpness and in improved signal intensities.
- ✓ Reduction of injection volume to 1 µL.
- ✓ The newly developed method meanwhile has been accepted by the IFCC Working Group on HbA_{1c} standardization as the new and common reference measurement procedure.

New procedure for the determination of immunosuppressive drugs

A reference measurement procedure for four immunosuppressive drugs was developed based on LC-IDMS/MS.

- ✓ The use of stable isotopes as internal standards ([$^{2}H_{12}$]-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) is considered as the most accurate quantification technique and preferred for reference measurement procedures.
- ✓ For mass spectrometric detection the Cs^{+} adducts formation of the cyclosporin A, sirolimus, tacrolimus and everolimus were used in SRM mode.
- ✓ Liquid-liquid extraction method was developed for preparation of EDTA whole blood samples.

Following ISO standard 15193 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.

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

I. Modified HPLC-Electrospray Ionization/Mass Spectrometry Method for HbA1c 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 POSTER PRESENTATIONS RELATED TO THE SUBJECT OF THE THESIS:

I. **Molnár PM**, Kaiser P, Dux L, Reinauer H: Quantification of immunsuppressive drugs by liquid chromatography-isotope-dilution mass spectrometry (LC-IDMS/MS) with Cs^+ adduct

8th Balaton Symposium on High-Performance Separation Methods and 15th International Symposium on Separation Sciences, Siófok

II. **Molnár PM**, Kaiser P, Dux L, Reinauer H: Quantification of immunsuppressive drugs by liquid chromatography-isotope-dilution mass spectrometry (LC-IDMS/MS) with Cs^+ adduct

Magyar Transzplantációs Társaság XI. Kongresszusa, Galyatető

III. **Molnár PM**, Dux L, Reinauer H, Szederkényi E, Szenohradszky P, Kaiser P: Quantification of immunsuppressive drugs by liquid chromatography-isotope-dilution mass spectrometry (LC-IDMS/MS) with Cs^+ adduct

11th International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analyzers and International Symposium on Hyphenated Techniques for Sample Preparation, Brugge, Belgium

IV. **Molnár PM**, Dux L, Reinauer H, Szederkényi E, Szenohradszky P, Kaiser P: Candidate reference measurement procedure for determination of

immunosuppressive drugs with liquid chromatography-isotope dilution mass spectrometry

Magyar Laboratóriumi Diagnosztikai Társaság 55. Nagygyűlése, Pécs

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 POSTER PRESENTATIONS INDIRECTLY RELATED TO THE SUBJECT OF THE THESIS:

I. **Molnár PM**, Kaiser P, Dux L: Determination of antiretroviral drugs with HPLC-MS/MS using Cs^+ adducts

Symposium of Separation Sciences 2008, Sárvár, Hungary

ACKNOWLEDGMENTS

First, I would like to thank **Professor Dr. László Dux**, head of the Department of Biochemistry and my supervisor for providing excellent opportunity to carry out the work at the Department and at the INSTAND e.V. (Düsseldorf, Germany). I would like to give the expression of my sincere gratitude for his support, patience and encouragement during my PhD studies.

I would like to thank to my supervisor, **Dr. Patricia Kaiser**, head of Reference Laboratory of INSTAND e.V. for her excellent leadership, supervision and guidance throughout the project, for introducing me to reference measurement procedures and HPLC-mass spectrometry fields.

I sincerely thank **Professor Dr. Hans Reinauer**, honorary president of INSTAND e.V. for giving me the opportunity to work at Reference laboratory, where I started my research work. I would like to express my thanks for all the facilities required to carry on my project and correction of my manuscript.

I am also much indebted to all members of the staff of the Departments of Biochemistry and the INSTAND e.V. for their remarks, suggestions and for the helpful and warm atmosphere during my PhD years.

I would also like to thank Dr. Zoltán Kele and Dr. Tamás Janáky (Department of Medical Chemistry, University of Szeged) for professional advices and the technical assistance in HPLC and mass spectrometric measurements. I am grateful to Dr. Edit Szederkényi and Dr. Pál Szenohradszky (Department of Surgery, University of Szeged) for the collaboration and for providing us the samples from kidney graft patients. I wish to acknowledge to QualiCont Nonprofit Public Utility Ltd., particularly to Dr. Erika Sárkány managing director for the collaboration and professional advices. I would like to express my thanks to Dr. Árpád Kallai director and Dr. Antónia Német, the head of the Central Laboratory for their support at my current job in Elisabeth Hospital (Hódmezővásárhely).

Above all, I would like to express my warmest and heartfelt thanks to **my Parents** for their love and encouragement. I wish to thank for their endless support

and help whenever needed. I thank also to my other relatives for support, and all my friends inside and outside the scientific world, for your interest in my work and for being great friends.