DEVELOPMENT AND OPTIMIZATION OF REFERENCE MEASUREMENT PROCEDURES

PhD thesis

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This thesis is dedicated

to my son Marvin

LIST OF PUBLICATIONS RELATED TO THE THESIS

[I] Xie, Y., Zacharias, E., **Hoff, P.**, Tegtmeier, F. Ion channel involvement in anoxic depolarisation induced by cardiac arrest in rat brain. J. Cereb. Blood Flow Metab.,15, 587-594 (1995)

[II] Kress, M., Meißner, D., **Kaiser, P.**, Hanke, R. Wood, W.G. Determination of theophylline by HPLC and GC-IDMS, the effect of chemically similar xanthine derivatives on the specificity of the method and the possibility of paracetamol as interfering substance. Clin. Lab. 48, 541-551 (2002)

[III] Kress, M., Meißner, D., **Kaiser, P.**, Hanke, R., Wood, W.G. The measurement of theophylline in human serum or plasma using gas chromatography and isotope dilution-mass-spectrometry (GC-IDMS) taking other substituted xanthines into consideration. Clin. Lab. 48, 535-540 (2002)

[IV] Kress, M., Meißner, D., **Kaiser, P.**, Wood, W.G. How to make things work again- Troubleshooting using the GC-IDMS determination of triacylglycerols as an example. Clin. Lab. 48, 635-646 (2002)

[V] **Kaiser, P.**, Kramer, U., Meißner, D., Kress, M., Wood, W.G., Reinauer, H. Determination of the cardiac glycosides digoxin and digitoxin by liquid chromatography combined with isotope-dilution mass spectrometry (LC-IDMS) - a candidate reference measurement procedure. Clin. Lab. 49, 329-343 (2003)

[VI] **Kaiser, P.**, Akerboom, T., Wood, W.G., Reinauer, H. A novel LC-IDMS/MS method for the determination of the cardiac glycosides digoxin and digitoxin using caesium adducts. Clin. Lab. 52, 37-42 (2006)

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LIST OF ABBREVIATIONS

amu	atomic mass unit
EN	European Norm
EQA	External Quality Assessment
EQAS	External Quality Assessment Schemes
GC	Gas Chromatography
GC-IDMS	Gas Chromatography-Isotope Dilution Mass Spectrometry
GC-MS	Gas Chromatography- Mass Spectrometry
HbA_{1c}	Haemoglobin A_{1c}
HbA_0	Haemoglobin A_0
HPLC	High Performance Liquid Chromatography
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IQA	Internal Quality Assessment
ISO	International Organisation for Standardization
IVDMD	In Vitro Diagnostic Medical Device
JCTLM	Joint Committee for Traceability in Laboratory Medicine
LC	Liquid-Chromatography
LC-ESI-MS	Liquid-Chromatography-Electrospray Ionisation-Mass Spectrometry
LC-IDMS	Liquid-Chromatography-Isotope Dilution Mass Spectrometry
LC-MS	Liquid-Chromatography-Mass Spectrometry
LC-MS/MS	Liquid-Chromatography-Mass Spectrometry/ Mass Spectrometry
MRM	Multiple Reaction Monitoring
m/z	mass-charge-ratio
RiliBÄK	Richtlinien der Bundesärztekammer Guideline of the Federal Medical Council for Quality Assurance of Quantitative Analyses in Laboratory Medicine
psi	pounds per square inch
SI	International System of Units
SIM	Selected Ion Monitoring
TFA	trifluoroacetic acid

for API 4000 mass spectrometer :

CAD	collisionally activated dissociation
CE	collision energy
CID	collision induced dissociation
CUR	curtain gas
CXP	collision cell exit potential
DP	declustering potential
EP	entrance potential
GS1	nebulizer gas
GS2	heater gas
ihe	interface heater
IS	ion spray voltage
TEM	temperature

TERMS AND DEFINITIONS

analyte

component indicated in the name of a measurable quantity (ISO 18153: 2003)

accuracy of measurement

closeness of the agreement between the result of a measurement and a true value of a measurand (ISO 17511:2003)

assigned value

value attributed to a particular quantity and accepted, sometimes by convention, as having an uncertainty appropriate for a given purpose (EN 14136:2004)

bias

the difference between the expectation of the test results and a true value of the measurand

NOTE: An estimator is the "statistical sample bias of measurements" which is the "average minus its reference value". (ISO 171511:2003)

external quality assessment (=EQA)

determination of individual and collective laboratory performance, and performance characteristics of examination procedures by means of interlaboratory comparison

(EN 14136:2004)

median
one type of average, found by arranging the values in order and then selecting the one in the middle

precision of measurements
closeness of agreement between independent results of measurements obtained under stipulated conditions

NOTE 1 Precision of measurement cannot be given a numerical value in terms of the measurand , only descriptions such as "sufficient" or "insufficient" for a stated purpose.

NOTE 2 The degree of precision is usually expressed numerically by the statistical measures of imprecision of measurements, such as standard deviation and coefficient of variation, that are inversely related to precision.

NOTE 3 "Precision" of a given measurement procedure is subdivided according to the specified precision conditions. "Repeatability" relates to essentially unchanged conditions and is often termed "within-serial" or "within-run precision". "Reproducibility" [between-run precision] relates to changes in conditions, e.g. time, different laboratories, operators, and measuring systems (including different calibrators and reagent batches) (ISO 17511:2003)

primary standard
standard that is designated or widely acknowledged as having the highest metrological qualities and whose value is accepted without reference to other standards of the same quantity (ISO 17511:2003)

reference interval (= biological reference interval)
central 95 % interval of the distribution of reference values (ISO 15189:2003)

reference material
substances which are used to obtain the metrological traceability, both through time, distances, and different measurement procedures (ISO 15195:2005)

reference measurement procedure
thoroughly investigated measurement procedure shown to yield values having an uncertainty of measurement commensurate with its intended use, especially in assessing the trueness of other measurement procedures for the same quantity and in characterizing reference materials (EN 14136:2004)

target value

accepted reference value (EN 14136:2004)

trueness of measurement

closeness of agreement between the average value obtained from a large series of results of measurements and a true value of a measurand (ISO 15189:2003)

true value

value consistent of a definition of a given particular quantity. True values are by nature indeterminate (ISO 17511:2003)

secondary standard

standard whose value is assigned by comparison with a primary standard of the same quantity (ISO 17511:2003)

uncertainty of measurement

parameter associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand (ISO 15189:2003);
the measurement uncertainty is inherent to the measurement procedure and shall be distinguished from effects of mistake, that is a deviation from the prescribed procedure (ISO 15193:2005)
The uncertainty of measurement can be estimated by repeated measurements of a given sample and the standard deviation estimated from the results.

Youden plot

graphical technique for comparing the between laboratory variability including the acceptability criteria

Scan modi used with the mass spectrometer API 4000:

Q1 scan

a full scan using the first quadrupole (Q1); the ion intensity is returned for every requested mass in the scan range

Q1 Multiple Ion scan (= SIM)

a scan using the first quadrupole (Q1); the ion intensity is returned for the specified point mass

MRM (Multiple Reaction Monitoring) scan

mode of operating a triple quadrupole instrument so that an ion of given mass (Q1) must fragment or dissociate to give a product ion of specific mass (Q3) for a response to be detected; it is used for a very specific target compound analysis

Product Ion scan

a MS/MS full scan where the first quadrupole (Q1) is fixed to transmit a specific precursor ion and the third quadrupole (Q3) sweeps a mass range; it is an experiment that will search for all of the products of a particular precursor ion.

Precursor Ion scan

a MS/MS scan where the third quadrupole (Q3) is fixed at a specified mass-to-charge ratio and the first quadrupole (Q1) sweeps a mass range; it is a scan for the ion of a specific mass-to-charge ratio that is generating a specific product ion.

Neutral Loss scan

MS/MS scan where both the first quadrupole (Q1) and the third quadrupole (Q3) sweep a mass range, a fixed mass apart; a response is observed if the ion chosen by the first analyzer fragments by losing the neutral loss specified.

1. INTRODUCTION

In the European Union harmonized regulations and rules for manufacturers and customers of "in vitro diagnostic medical devices (IVDMD)" have been implemented. In this free and common market, basic regulations have been issued in the "Directive 98/79/EC of the European Parliament and of the Council on in vitro Diagnostic Medical Devices" [1]. The Common Market is an "*area without internal frontiers in which the free movement of goods, persons, services and capital is ensured*" [1]. The maintenance or improvement of a high level of health protection is one of the main objectives. The access of products on this market – with the exception of the analytes in Annex II of [1] – is in the hand of the manufactures who have to follow a conformity assessment procedure and the rules for the affixing the CE-label on their products (see Annex III in [1]). The "vigilance of the market" is left mainly to the organizers of external quality assessment schemes. The relevant paragraph (Article 11, paragraph 2) clearly expresses this new function of the External Quality Assessment Schemes (EQAS):

"Where a Member State requires medical practitioners, the medical institutions or the organisers of external quality assessment schemes to inform the competent authorities of any incidents referred to in paragraph 1, it shall take the necessary steps to ensure that the manufacturer of the device concerned, or his authorised representative, is also informed of the incident".

In the "Essential Requirements" (Annex I of [1]) of the IVDMD-directive one regulation is of special importance in the context of performance characteristics of IVDMDs, that is the "*traceability"* of values.

"*The traceability of values assigned to calibrators and/or control materials must be assured through available reference measurement procedures and/or available reference material of a higher order"*.

Another definition of the traceability is given in the VIM [2]: "*A property of the result of a measurement or the value of a measurement standard whereby it can be related, with a stated uncertainty of measurement to stated references, usually national or international measurement standards, through an unbroken chain of comparisons"*.

The whole process of traceability in laboratory analyses is demonstrated in figure 1.

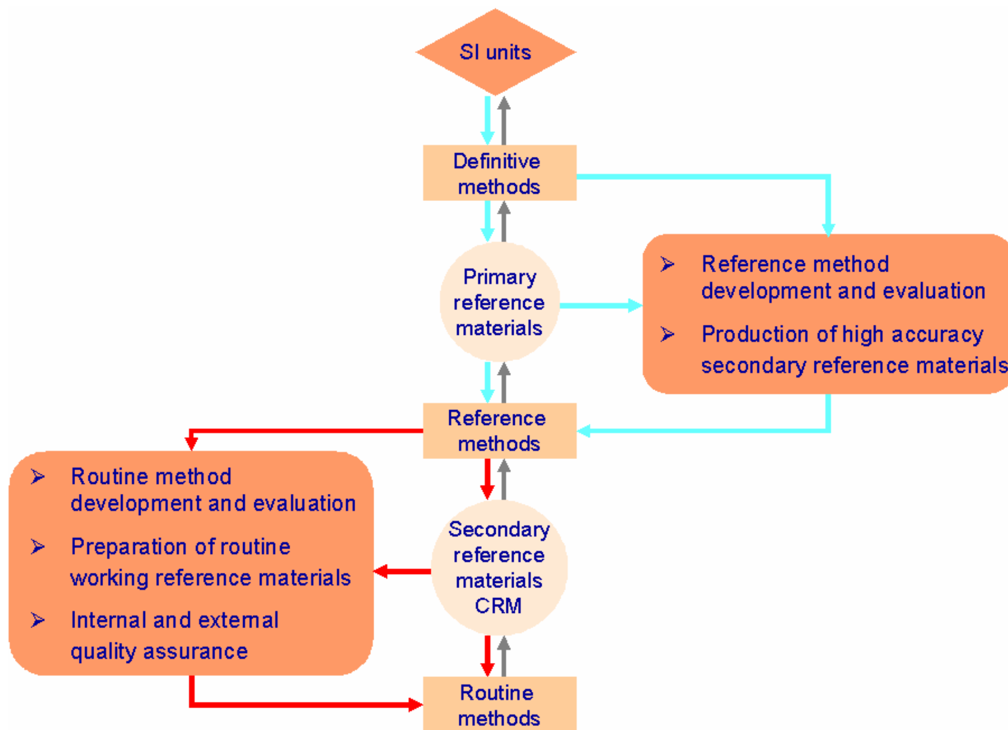


Figure 1 **Flowchart of the traceability of reference methods and routine methods to the highest metrological order that is the SI units**

The traceability of routine methods should be directly related to reference materials and reference measurement procedure and finally to the SI units (figure 1). The traceability concept is applied by using primary and secondary standards, reference measurement procedures and certified reference materials (CRM). The manufacturers should calibrate their analytical systems with reference measurement procedures and/or certified reference materials, thus getting a link between IQA and EQA.

Based on the Article 11 of the IVDMD Directive a mandated European Standard EN 14136 [3] was developed, where again the criteria for the evaluation of kits and instruments in EQAS have been defined. In paragraph 6.1.3 of the EN 14136 the assigning of values in survey samples should be traceable to a specific metrological level (as shown in EN ISO 17511: 2002"In vitro diagnostic medical devices- Measurement of quantities in samples of biological origin- Metrological traceability of values assigned to calibrators and control materials" [4] and EN ISO 18153: 2000 "In vitro diagnostic medical devices- Measurement of quantities in samples of biological origin- Metrological traceability of values for catalytic concentration of enzymes assigned to calibrators and control materials" [5]). The most accurate procedures to assign

common and reliable target values for quality control samples are the reference measurement procedures.
In Germany the "Guideline of the Federal Medical Council for Quality Assurance of Quantitative Analyses in Laboratory Medicine" [6] consequently requires the use of reference measurement procedures for setting target values for a number of analytes, to ensure the comparability of results in laboratory medicine.

The main characteristics of reference measurement procedures are described in the ISO Standard 15193: 2005 "In vitro diagnostic medical devices- Measurement of quantities in samples of biological origin- Presentation of reference measurement procedures" [7]. In the introduction of this ISO Standard a definition is given for the use of reference measurement procedures:

"*Reference measurement systems are needed for producing useful and reliable results of measurement, whether in science, technology, or routine service so as to be comparable and ultimately traceable to measurement standards of the highest metrological level. Analytical reference measurement procedures play a crucial role in this metrological system because they can be used*

- *in assessing performance characteristics of measuring systems – comprising measuring instruments, auxiliary equipment as well as reagents,*
- *in demonstrating the functional interchangeability of different routine measurement procedures purporting to measure the same quality*
- *in assigning values to reference materials that are then used to purposes of calibration or control of routine measurement procedures and*
- *in detecting analytical influence quantities in patient sample."*

Descriptions, definitions, and classifications of reference materials are given in the ISO Standard 15194- "In vitro diagnostics medical devices- Measurement of quantities in samples of biological origin- Description of reference materials" [8].
According to this ISO Standard reference materials are "*standards of highest metrological level*" and "*are used to obtain this traceability, both through time, distance, and different measurement procedures."*
The use of the reference materials can be used to establish the "*unbroken chain of comparisons"*, required in VIM [2] and ISO Standards [4]. The requirements for reference laboratories to fulfil the criteria of the reference measurement system are defined in the ISO

Standard 15195- "Laboratory Medicine- Requirements for reference measurement laboratories" [9].

Summarizing, the advantages of using reference measurement procedures for setting target values are:

1. There is only one target value for each analyte in an EQA sample.
2. The results between different methods and laboratories are directly comparable.
3. There will be uniform reference intervals in a population for the analytes in all laboratories.
4. An analytical basis of international epidemiological studies is given.
5. Methods with the highest accuracy are favoured on the market.
6. Obsolete methods can be identified and eliminated.

Reference measurement procedures should be used

1) for the determination of target values for internal and external quality control samples
2) for calibrating of kits and instruments
3) to get identical reference ranges for a defined population in all laboratories
4) for evaluation of the performance of in-vitro diagnostic medical devices
5) to establish a basis for the comparability of results of epidemiological studies

According to the ISO Standards 17025 [10] the development of reference measurement procedures is a dynamic process with continuous quality improvement. For reference measurement procedures it is desirable to use measurement systems of highest quality and performance. Therefore, established reference measurement procedures should be continously evaluated and optimized or replaced if necessary by a new method, if better analytical equipments, methods or materials are available. This continuous process of improvement in analytical quality is one basic principle of quality management in medical laboratories and also the aim of this study.

In the ISO Standard 15193 [7] a reference measurement procedure is defined as a "*thoroughly investigated measurement procedure shown to yield values having an uncertainty of measurement commensurate with its intended use, especially in assessing the trueness of other measurement procedures for the same quantity and characterizing reference materials"*.

Unfortunately, the ISO Standard does not define the main features of a candidate and an accepted reference measurement procedure. An essential requirement of an accepted reference measurement procedure is that the procedure is confirmed by international intercomparison studies (see JCTLM and IFCC Working Group on Standardization of HbA_{1c}).

In contrast to reference measurement procedures routine methods used in the clinical laboratories are mostly designed for a high throughput of analyses combined with a short turnaround time at acceptable costs and performance. The continuous process of improvement in analytical quality is also an important strategy for routine measurement procedures.

Examples for the progression in analytical quality are the optimization of the reference measurement procedure for triacylglycerols [IV] and the method development for determination of glibenclamide and theophylline in biological matrices [11,I,II,III].

In our reference laboratory 31 reference measurement procedures have been established and are in use. This analytical program covers the requirements of the actual "Guideline of the Federal Medical Council for Quality Assurance of Quantitative Analyses in Laboratory Medicine" [6].

The analytical principles of the reference measurement procedures are listed in table 1.

analytical principle	**analyte**
coulometry	chloride
flame emission photometry	Na^{+}, K^{+}
atomic absorption spectrometry	Ca^{++}, Mg^{++}, Li^{+}
photometry	total protein, albumin, phosphate, bilirubin
enzyme-kinetic measurement	ALT, ASAT, CK, γ-GT, LDH
GC-MS	glucose, cholesterol, creatinine, triglycerides, theophylline, steroid hormones (cortisol, 17β-oestradiol, progesterone, aldosterone, testosterone) thyroxine, uric acid, urea
LC-MS and LC-MS/MS	digoxin, digitoxin, HbA_{1c}

Table 1 **Analytical principles of reference measurement procedures**

These reference measurement procedures are used for the determination of target values in the EQAS in several countries. The fundamental difference of this evaluation procedure from the "consensus value principle" is that the same target values with the same acceptability range are set for all laboratories and by this way comparable results are collected in laboratory medicine. The reliability of the reference measurement procedure must be permanently controlled and continuously evaluated with the effect, that the reference measurement procedures are dynamic, because of this continuously evaluation and improvement process. Manufacturers are carefully observing the performance of the reference measurement procedures and the results of their customers in the EQAS.

The Federal Medical Council of Germany favours the development of reference measurement procedures. These are enclosed in the Guideline of the Federal Medical Council for Quality Assurance of Quantitative Analyses in Laboratory Medicine [6].

This strategy promoting the comparability of laboratory analyses at the highest level of accuracy supports the high quality of patient care and reduces costs in the health care system.

2. AIMS AND SCOPE

The aim of this study was to improve the quality of the medical laboratory performance by providing reference measurement procedures for several analytes for internal and external quality assessment. The study focused on the implementation, improvement and evaluation of existing reference measurement procedures and the development of new reference measurement procedures using GC-MS and LC-MS.

The Guideline of the Federal Medical Council for Quality Assurance of Quantitative Analyses in Laboratory Medicine of Germany requires reference measurement procedures for setting target values for selected analytes in internal and external quality control. Following the Guideline this study deals with the reference measurement procedures for HbA_{1c}, theophylline, digoxin, digitoxin, and additionally for glibenclamide, and immunosuppressive drugs.

A highly sensitive method for determination of glibenclamide has been developed in my diploma thesis [11] and was used for drug monitoring in the German Diabetes Research Institute and for preclinical studies [I]. On the basis of this method a reference measurement procedure is on the way to be established, which will then be used for setting target values in the new EQAS.

The reference measurement procedure for theophylline has been evaluated and established in our laboratory and is used in our EQA schemes for determination of the target values [II,III].

The reference measurement procedure for HbA_{1c} had been developed in an international cooperation [12]. This reference measurement procedure has been improved through extensive evaluation with different mass spectrometers. The use of this modified reference measurement procedure is demonstrated in international intercomparison studies and in EQAS.

For digoxin and digitoxin a new reference measurement procedure has been developed. With this method a new analytical principle in LC-MS/MS is introduced, by means of quantitative analysis of Cs^+ adducts, both in SIM and in MRM mode [V,VI].

The measurement of Cs^+ adducts in LC-MS/MS described here is a new principle in the determination of immunosuppressive drugs (sirolimus, everolimus, tacrolimus, and cyclosporin A). This new method has been established in this study and will be a solid basis for the development of an accepted reference measurement procedure [VII].

3. MATERIALS AND METHODS

3.1 HPLC measurements

3.1.1 Measurement conditions for determination of glibenclamide [I]

The HPLC system was from Merck Hitachi (Merck, Darmstadt, Germany), consisting of an L-6000 pump and an F-1050 fluorescence spectrophotometer. For sample injections a manual injection system from Rheodyne (Rheodyne Europe GmbH, Bensheim, Germany) was used.
Chromatographic separation was performed with a LiChrospher Si 60 column, 4 x 250 mm, 10 µm from Agilent Technologies Deutschland GmbH (Böblingen, Germany).
Isocratic elution was performed by using a mobile phase consisting of 1-octanol/2-methoxy-2-methylpropane/n-heptane (15:10:75; v/v/v). The flow rate was 2 ml/min. The fluorescence detector was set to the excitation wavelength of 450 nm and to the emission wavelength of 510 nm.

Sample preparation was performed as follows:
For extraction 1 ml serum was used and 200 ng of the 4-methylcyclohexyl analogue of glibenclamide (N-4-beta-(2-methoxy-5-chlorobenzamido) -ethylbenzosulfonyl-N'- (4-methylcyclohexyl)-urea) was added as internal standard. The pH of the sample was set to pH 3 by addition of 1 mol/l HCl. 5 ml chloroform was added to the acidified sample.
The sample was shaken for about 3 min and then centrifuged for 5 min at 3000 x g. The organic phase was evaporated to dryness under a stream of nitrogen. For derivatization the residue was dissolved in 450 µl 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl) in a concentration of 1 mg/ml 3-methyl-1-butanol. The solution was heated to 120 °C for 30 min. After the derivatization the solution was evaporated to dryness and dissolved in elution buffer/3-methylbutyl acetate (4:1, v/v) for HPLC injection.

For determination of glibenclamide in brain, the brain tissue of one rat was weighed, 100 ng internal standard (N-4-beta-(2-methoxy-5-chlorobenzamido) -ethylbenzosulfonyl-N'- (4-methylcyclohexyl)-urea) was added and the sample was homogenized in 2 ml perchloric acid (5 % v/v) using an Ultra Turrax® homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany). The sample was centrifuged (5 min, 3000 x *g*) and the supernatant was extracted with 5 ml chloroform. Derivatization was performed as described for serum samples.

3.1.2 Measurement conditions for determination of theophylline [II]

The HPLC system used was from Merck Hitachi (Merck, Darmstadt, Germany), consisting of an L-7100 pump, L-7200 autosampler and an L-7450 diode-array detector.
Chromatographic separation was performed with a Nautilus C18 column 4.6 x 250 mm, 5 µm from Macherey und Nagel (Düren, Germany).
Isocratic elution was performed by using a mobile phase consisting of 0.02 mol/l acetate-phospate buffer, pH 3.0 containing 9.6 % v/v acetonitrile. The run time was 10 min. The flow rate was 0.9 ml/min. The injection volume was 10 µl.
The diode-array detector was set to 275 nm.

Sample preparation was performed as described in [II]. 1,3-dimethyl-7-(2-hydroxyethyl)-xanthine was used as internal standard.

3.2 GC-MS measurements

3.2.1 Measurement conditions for determination of theophylline [III]

The following equipment was used for GC-MS measurements:
The gas-chromatograph was from Varian model 3400 (Darmstadt, Germany) coupled with a mass spectrometer TSQ 7000 from Thermo Electron Corporation (Dreieich, Germany). The injection system was a cold-start injector from Gerstel (Mülheim an der Ruhr, Germany).
Standard material of theophylline (declared purity 99 %) was purchased from Sigma-Aldrich (Taufkirchen, Germany). 2-^{13}C ,1,3-$^{15}N_2$-theophylline (declared purity 2-^{13}C,99%;1,3-$^{15}N_2$ 98%) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA)). Other chemicals were purchased as described in [III].

Chromatographic separation was performed using a FS- Supreme-5 GC column from Chromatography Service (Langerwehe, Germany) with a length of 30 m, an internal diameter of 0.25 mm and a film thickness of 0.25 µm. The temperature programmes of the GC and the cold-injection system are shown in table 2:

Event	**Setting** Gas chromatograph	**Setting** Cold injection system
initial temperature [°C]	170	60
initial isothermal period [min]	5	
programme rate [°C/min]	30	10
final temperature [°C]	300	280
final isothermal period [min]	3	1

Table 2 **Temperature programme of the gas chromatograph and the cold injection system**

The transfer line was set to 280 °C. The injection volume was 2 µl.

The settings of the TSQ 7000 mass spectrometer and the detection masses for theophylline and the internal standard 2-^{13}C ,1,3-$^{15}N_2$-theophylline are shown in tables 3 and 4.

Event	**Setting**
filament	200 µA
multiplier	1500 V
scan time	0.5 s
scan width	0.4 amu
gas pressure	10 psi

Table 3 **Settings of the TSQ 7000 mass spectrometer**

analyte	**m/z [amu]**
theophylline	237
2-^{13}C ,1,3-$^{15}N_2$-theophylline	240

Table 4 **Detection masses for theophylline and internal standard**

Sample preparation and derivatization is described in [III].

The volume of serum and standards was adjusted to an amount of 5 µg theophylline in the sample. By an iterative approach the internal standard 2-^{13}C ,1,3-$^{15}N_2$-theophylline was added giving the ratio of sample to internal standard of 1:1.

3.3 LC-MS and LC-MS/MS measurements

LC-MS and LC-MS/MS measurements were performed using the following equipment:
The HPLC system used was from Shimadzu (Duisburg, Germany) consisting of a SCL-10A system controller, three LC-10AD vp pumps (A, B, C), a DGU-14A degasser; a SIL-10AD autoinjector; a CTU-10AS column oven; and a FCU-12A Flow Switch (rotary valve)
The Mass Spectrometer was an API 4000 equipped with a TurboV^{TM} ESI source with TurboIon Spray^{TM} probe from Applied Biosystems (MDS-Sciex, Darmstadt, Germany).
A syringe pump was used for optimization of the ESI source parameters (DP, EP, CUR, GS1, GS2, TEM) during method development and for daily control of analytical sensitivity and for determination of the accurate masses.

3.3.1 Measurement conditions for determination of HbA_{1c}

The original reference measurement procedure for the determination of HbA_{1c} in human blood has been developed in collaboration with an international group of laboratories and has been described in detail in [17].
The principle of measurement of the IFCC reference measurement procedure is based on the determination of the ratio of glycated to non-glycated N-terminal hexapeptide of the β-chain of haemoglobin.
The method has three steps:
In the first step the whole blood sample is hemolysed. In the second step a proteolytic cleavage of haemoglobin is performed.
For proteolysis the haemolysed sample is treated with endoproteinase Glu-C to cleave the amino acid chain at the Glu-Glu positions. During the enzymatic cleavage of the β-chain of haemoglobin glycated and non-glycated N-terminal hexapeptides are generated.
In the third step the hexapeptides are separated chromatographically and measured by LC-ESI-MS.
Quantification is performed by external calibration. For calibration six mixtures of chromatographically purified HbA_{1c} and HbA_0 are analysed. For the calibration curve the ratio of the concentration of HbA_{1c} and HbA_0 is plotted against the ratio of their peak areas. The percentage of HbA_{1c} in the sample can be calculated from the regression curve.

For proteolysis endoproteinase Glu-C (EC: 2326424) was purchased from Roche Diagnostics (Penzberg, Germany). Calibrators and controls were delivered by the IFCC Working Group on HbA_{1c} Standardisation [18]. All reagents used for sample preparation and HPLC separation were of *supra pure* quality.

The hexapeptides were separated by HPLC with a ZORBAX SB-CN column (2.1 x 150 mm, 5µm, Axel Semrau GmbH, Sprockhövel, Germany).

The optimized conditions for determination of HbA_{1c} were as follows:

Elution was performed by using a binary gradient consisting of eluent A (pump A): 0.07 % v/v TFA in water and eluent B (pump B): 0.07 % v/v TFA in acetonitrile. The flow rate was 350 µl/min. The column temperature was 50 °C. In order to avoid contamination of the ESI source a switching valve system (rotary valve A) was introduced. At position 0 of rotary valve A the eluate was passed into the ESI source. At position 1 the eluate was discarded, during which the ESI source is supplied with 50 % acetonitrile, delivered by pump C. The injection volume was 2 µl.

The HPLC conditions for determination of HbA_{1c}, the settings of the API 4000 mass spectrometer and, the detection masses in the Q1 multiple ion mode for the ß-N-terminal hexapeptides of HbA_{1c} and HbA_0 are shown in tables 5, 6, and 7.

Time	**Module**	**Events**	**Setting**
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	10
16.40	subcontroller	rotary valve A	1
16.50	pumps	% B	10
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 5 **HPLC conditions for determination of HbA_{1c}**

Event (program abbreviation)	Setting
CUR	50.00
GS1	40.00
GS2	70.00
IS	5000.00
TEM	150.00
ihe	ON
DP	30.00
EP	10.00

Table 6 **Settings of the API 4000 mass spectrometer for Q1 multiple ion mode**

analyte	Q1 multiple ion m/z [amu]
ß-N-terminal hexapeptide of HbA_0	348.3
ß-N-terminal hexapeptide of HbA_{1c}	429.3

Table 7 **Detection masses in Q1 multiple ion mode for the ß-N-terminal hexapeptides of HbA_{1c} and HbA_0**

The MS detection of the hexapeptides was performed in the positive ion mode.

Lyophilised EQA samples were reconstituted according to the instruction of the sample manufacturers. Samples, calibrators and controls and the protocol of measurement were prepared according to the IFCC reference measurement procedure for HbA_{1c} [17,18].

According to this reference measurement procedure for HbA_{1c} the analyses were performed in the following sequence:

calibrator A → calibrator B → calibrator C → calibrator D → calibrator E → calibrator F
→ control A → control B → sample 1 to 10 → control C → control D
→ calibrator A → calibrator B → calibrator C → calibrator D → calibrator E → calibrator F

All samples are injected in duplicate.
Four sequences of measurement are performed for setting one target value.

3.3.2 Measurement conditions for determination of digoxin and digitoxin [V,VI]:

Separation of the drugs was performed on a LiChrospher RP-18 column (5 µm, 125 x 2 mm) equipped with a LiChrospher RP-18 pre-column (5 µm, 10 x 2 mm).
Standard material of digoxin (declared purity 99.8 %) and digitoxin (declared purity 99.0%) were delivered by LGC Promochem GmbH (Wesel, Germany). Other chemicals were purchased as described in [V,VI].

Elution was performed by using a binary gradient consisting of eluent A (pump A): 0.1 % v/v formic acid in water + 100 µmol/l caesium hydroxide (adjusted to pH 7 with formic acid) and eluent B (pump B): 0.1 % v/v formic acid in methanol + 100 µmol/l caesium hydroxide (adjusted to pH 7 with formic acid). The flow rate was 300 µl/min. The column temperature was 40 °C. In order to avoid contamination of the ESI source a switching valve system (rotary valve A) was introduced. At position 0 of rotary valve A the eluate was passed into the ESI source. At position 1 the eluate was discarded, during which the ESI source is supplied with 50 % CH_3OH, delivered by pump C. The injection volume was 10 µl.

The HPLC conditions for the separation of digoxin and digitoxin, the settings of the API 4000 mass spectrometer and, the detection masses in Q1 multiple ion mode and MRM mode of native and deuterated glycosides are shown in tables 8, 9, 10, and 11.

Time	**Module**	**Events**	**Setting**
0.01	pumps	%B	50
0.02	subcontroller	rotary valve A	1
4.50	subcontroller	rotary valve A	0
8.00	pumps	%B	77
8.50	pumps	%B	100
8.51	subcontroller	rotary valve A	1
9.50	pumps	%B	100
10.00	pumps	%B	50
14.50	subcontroller	rotary valve A	0
15.00	system controller	stop	

Table 8 **HPLC conditions for the separation of digoxin**

Time	Module	Events	Setting
0.01	pumps	%B	50
0.02	subcontroller	rotary valve A	1
4.50	subcontroller	rotary valve A	0
5.00	pumps	%B	80
9.00	pumps	%B	80
9.50	pumps	%B	100
9.51	subcontroller	rotary valve A	1
10.50	pumps	%B	100
11.00	pumps	%B	50
14.50	subcontroller	rotary valve A	0
15.00	system controller	stop	

Table 9 **HPLC conditions for the separation of digitoxin**

Event (program abbreviation)	Setting
CAD	5.00
CUR	20.00
GS1	30.00
GS2	60.00
IS	5500.00
TEM	350.00
ihe	ON
DP	111.00
EP	10.00
CE	49.00
CXP	26.00

Table 10 **Settings of the API 4000 mass spectrometer for Q1 multiple ion mode and MRM mode, respectively**

analyte	Q1 multiple ion	MRM	
	m/z [amu]	precursor ion m/z [amu]	product ion m/z [amu]
digitoxin	897.3	897.3	132.9
digitoxin-$^2H_3$	900.3	900.3	132.9
digoxin	913.3	913.3	132.9
digoxin-$^2H_3$	916.3	916.3	132.9

Table 11 **Detection masses in Q1 multiple ion mode and MRM mode of native and deuterated glycosides**

The MS detection was performed in the positive ion mode.

Preparation of the deuterated standards and the extraction procedure for serum and plasma samples is described in [V,VI]. The volume of serum and standards was adjusted to an absolute amount of 1ng digoxin or digitoxin present in the sample. By an iterative approach the deuterated internal standard was added giving the ratio of sample to internal standard of 1:1.

3.3.3 Measurement conditions for determination of sirolimus, everolimus, tacrolimus and cyclosporin A [VII]:

The chromatographic separation of the immunosuppressive drugs was performed on a phenyl-hexyl-RP column (Luna®, 2 x 150 mm, 5 µm, Phenomenex, Aschaffenburg, Germany).
Standard materials of sirolimus (declared purity > 99 %) and tacrolimus (declared purity > 99 %) and cyclosporin A (declared purity > 99 %) were delivered by LC Laboratories (Woburn, MA, USA). Everolimus was a gift from Recipe (München, Germany). 32-desmethoxy-rapamycin was a donation from Wyatt, St. Davids, PA, USA. Ascomycin was purchased from Sigma-Aldrich, (Taufkirchen, Germany). Other chemicals were obtained as described in [VII].
A ternary gradient was used for elution consisting of eluent A (pump A): 0.1 % v/v formic acid in water + 0.1 mmol/l caesium hydroxide (adjusted to pH 7 with formic acid); eluent B (pump B): 0.1 % v/v formic acid in methanol + 0.1 mmol/l caesium hydroxide (adjusted to pH 7 with formic acid) and eluent C (pump C): 0.1 % v/v formic acid in acetonitrile + 0.1 mmol/l caesium hydroxide (adjusted to pH 7 with formic acid). The flow rate was 300 µl/min. The column temperature was set at 50 °C. In order to avoid contamination of the ESI source a switching valve system (rotary valve A) was introduced. At position 0 of rotary valve A the eluate was passed into the ESI source. At position 1 the eluate was discarded. The injection volume was 10 µl.

The HPLC conditions for the separation of for the immunosuppressive drugs and the internal standards, the settings of the API 4000 mass spectrometer, and the detection masses in the MRM mode are shown in tables 12, 13, and 14.

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
31.05	pumps	% B	0
37.05	pumps	% C	100
37.10	pumps	% B	0
38.00	pumps	% C	0
38.05	pumps	% B	50
42.00	controller	Stop	

Table 12 **HPLC conditions for the separation of immunosuppressive drugs and the internal standards**

Event (program abbreviation)	Setting
CAD	12
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 13 **Settings of the API 4000 mass spectrometer for MRM mode**

analyte	MRM	
	precursor ion m/z [amu]	product ion m/z [amu]
tacrolimus	936.9	132.9
sirolimus	1046.8	132.9
everolimus	1090.8	132.9
cyclosporin A	1335.0	132.9
ascomycin	924.9	132.9
32-desmethoxy-rapamycin	1016.5	132.9
cyclosporin D	1349.2	132.9

Table 14 **Detection masses in the MRM mode for the immunosuppressive drugs and the internal standards**

The MS detection was performed in the positive ion mode.

Standard solutions were prepared by dissolving the pure standard material of sirolimus, everolimus, tacrolimus and cyclosporin A, respectively in methanol.

4. RESULTS AND DISCUSSION

According to the ISO Standard 15193 [7] "useful and reliable results of measurement" for reference measurement procedure are required which are "ultimately traceable to measurement standards of highest metrological order". Additionally, the uncertainty of measurement of the values has to be determined.

In this study the traceability of the results is given by reference measurement procedures using primary and secondary standards.

The trueness of measurements has been determined by using certified secondary reference materials. When no certified reference material was available, the trueness of measurements was determined by recovery studies.

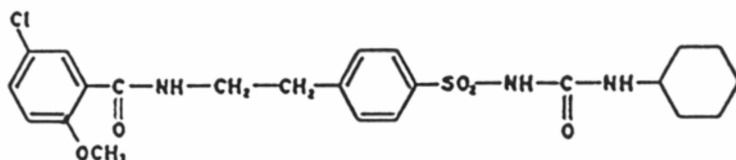
The uncertainty of measurement was determined by repeated measurements of a sample. The measurement uncertainty is generally caused by different factors. These factors have been well determined in our laboratory for the accreditation process. A main component of the total measurement uncertainty is the uncertainty of the primary or secondary reference material. Other components of the total uncertainty originate from several steps in sample preparation, such as pipetting and weighing, or influences due to fluctuations in room temperature, humidity during sample preparation, or from peak integration. The uncertainty of measurement in this study is represented by an estimated relative standard deviation (coefficient of variation).

When no international acceptability criteria for accuracy and precision are given, in this study the criteria for the evaluation of the reference measurement procedure were derived from the "German Guideline of the "Federal Medical Council for Quality Assurance of Quantitative Analysis in Laboratory Medicine" (RiliBÄK) [6]. The requirements for precision and accuracy for reference laboratories using reference measurement procedures were set at 1/3 of those for precision and accuracy of the routine laboratories (see column 5 and 6 of the current RiliBÄK).

The reference measurement procedures in this study have been established and evaluated with these analytical criteria.

4.1 Determination of glibenclamide by HPLC and fluorescence detection [I]

Glibenclamide is a member of sulfonylureas and is used as oral antidiabetic drug for the therapy of diabetes type 2.



chemical formula	$C_{23}H_{28}ClN_3O_5S$
molecular weight [g/mol]	494,0

Figure 2 **Structure of glibenclamide**

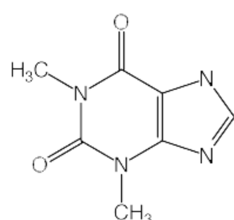
In the early 1990s mainly immunological methods were used for therapeutic drug monitoring of glibenclamide [13,14]. The immunological methods were sensitive for the detection and quantification of glibenclamide, but they had a poor analytical specificity. They were not able to distinguish the drug from its metabolites and from structurally related sulfonylureas.

To advance the analytical quality of routine measurement of glibenclamide in patient samples a reliable, sensitive and specific analytical method by the use of HPLC and fluorescence detection was developed at the German Diabetes Research Institute (see diploma thesis "Methoden-Entwicklung zur Bestimmung von Glibenclamid in biologischem Material mittels Hochdruckflüssigkeitschromatographie unter Verwendung eines Fluoreszenz-Detektors" [11]). This analytical principle of measurement was used for routine measurement in the clinical laboratory for many years. Moreover, the method could be adapted to determine glibenclamide for preclinical research. In a research study the rat brain tissue concentrations of glibenclamide after intravenous or local application have been determined, to investigate the effect of the drug on the ischemia-induced K^+ efflux changes [I].

Since the strategy of continuous improvement in analytical quality is still in process, in the meantime an LC-MS method for the determination of glibenclamide in serum has been developed on the basis of this diploma thesis [11] in the German Diabetes Research Institute [15,16]. As will be shown with digoxin, digitoxin (see 4.4) and immunosuppressive drugs (see 4.5) this method is on the way to be further improved by using the new analytical principle in LC-MS/MS (measurement of Cs^+ adducts) to a reference measurement procedure at INSTAND e.V. and will be used for setting target values in a new EQAS, thus promoting the comparability of results in drug monitoring of antidiabetic drugs.

4.2 Determination of theophylline by GC-IDMS and HPLC diode array detection [II, III]

Theophylline is a member of the xanthine family and is used in the therapy of bronchial asthma and chronic obstructive pulmonary diseases.



chemical formula	$C_7H_8N_4O_2$
molecular weight [g/mol]	180.17

Figure 3 **Structure of theophylline**

Several analytical measurement procedures for determination of theophylline in blood are available [19,20,21], but analytical problems may occur in routine methods by interferences of other xanthines and metabolites of theophylline. Theophylline is closely related in structure to naturally occurring xanthines in beverages such as coffee, tea and cocoa, which may either interfere with the immunoassay determination or – in the case of caffeine – be metabolised to theophylline. The main substance group which may interfere with the determination of the closely related theophylline (1,3-dimethylxanthine) are the dimethylxanthines theobromine (3,7-dimethylxanthine) from cocoa – and paraxanthine (1,7-dimethylxanthine) the main metabolite of caffeine. They differ in structure in the positions of the methyl groups on the xanthine moiety.

According to the Guideline of the German Federal Medical Council for Quality Assurance of Quantitative Analyses in Laboratory Medicine reference measurement procedure values are required for setting target values for theophylline in EQAS.

The existing routine method for the determination of theophylline in serum [20] was optimized and further developed to be established as reference measurement procedure. The principle of the method is a gas chromatography, isotope dilution-mass spectrometry (GC-IDMS) procedure. The internal standard used for this method was the purchasable 2-^{13}C,1,3-$^{15}N_2$-theophylline. The derivatization of all xanthines was made with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA). The extraction and derivatization procedures were examined in detail and optimized stepwise during the development of the method. By optimization of the chromatographic

conditions a separation of all dimethylxanthines was obtained. To obtain high accuracy and a good precision standard solutions and serum samples were quantified gravimetrically during sample preparation. The volume of serum and standards was adjusted giving an amount of 5 µg theophylline in the sample. Internal standard was then added so that the ratio of sample to internal standard was 1:1.

Since no certified reference material was available the evaluation of the trueness of the established GC-IDMS method was performed by recovery studies.

In serum samples, spiked with known amounts of theophylline, the mean recoveries in therapeutic ranges for theophylline was 99.78 % with a mean bias of 0.22 % (n = 20). The imprecision in EQAS samples (n=27 over a 4-day period) was between 0.527 % and 1.14 %, respectively.

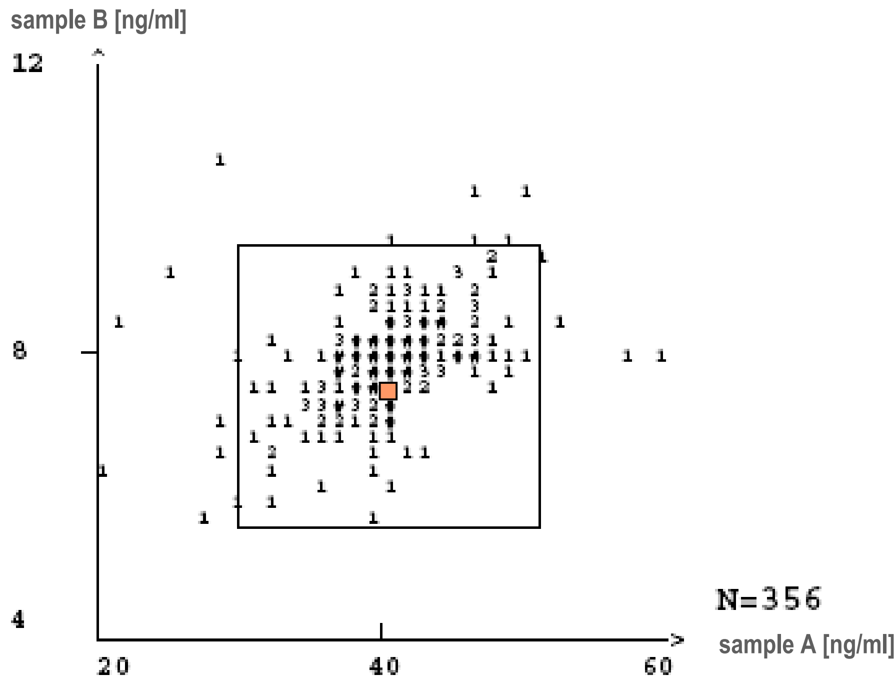
The analytical specificity and trueness of the GC-IDMS method was verified by comparison with a HPLC-diode array method, following the well accepted strategy that the reliability of the results is given, when agreement is achieved by two principally different methods. Therefore, a HPLC method has been developed, which allows the baseline separation of all dimethylxanthines and related compounds, which could lead to interferences: theophylline, theobromine, paraxanthine, paracetamol, caffeine, and uric acid.

The mean recoveries in spiked serum samples with the HPLC method were between 86 % and 101 % between 1.25 and 100 ng/ml for all compounds tested.The imprecision in EQAS samples was between 2.87 % (n=10) and 3.27 % (n=10), respectively.

A good correlation of the results between GC-IDMS and HPLC-diode array measurements was obtained, which indicates the good accuracy and specificity of the established GC-IDMS measurement procedure.

Unfortunately, an international network of reference laboratories for theophylline does not exist at the present time.

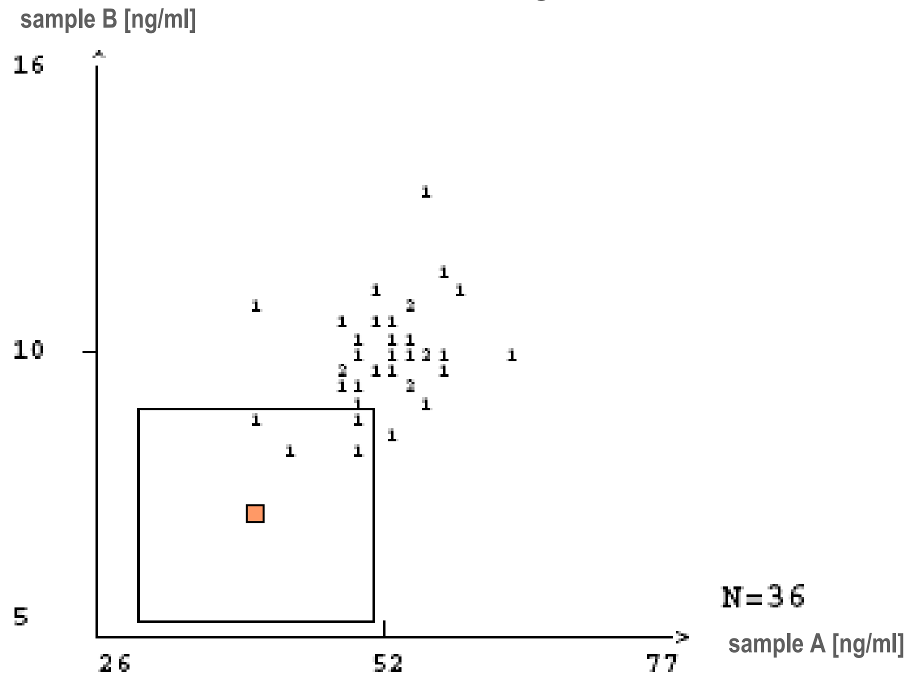
Our reference measurement procedure for theophylline is used for setting target value in the EQAS. An example for the evaluation of an EQA scheme for theophylline is shown in figure 4. The acceptability range for routine measurement of the participants of ± 30 % for theophylline is given by the Guideline of the Federal Medical Council for Quality Assurance of Quantitative Analysis in Laboratory Medicine. The central square dot indicates the target values for sample A and sample B. 96.3 % of the participants had successfully analysed the sample A and 98.6 % the sample B.



sample	*target value [ng/ml]*	*cv [%]*	*acceptability range [ng/ml]*	*mean value [ng/ml]*	*cv [%]*	*successful participations [%]*
A	40.1	0.488	29.6 – 50.6	39.7	9.02	96.3
B	7.45	0.539	5.51 – 9.39	7.86	7.98	98.6

Figure 4 **Youden-plot with target value, acceptability criteria and statistical data of the participants in an External Quality Assessment Scheme for theophylline run by INSTAND e. V. (2005)**

In the same survey for theophylline the results of the customers of one manufacturer (figure 5) had systematic deviation from the reference method value, indicating an analytical problem (calibration, specificity) of the routine measurement system used. The manufacturer of this analytical system had been informed, according to Article 11 of the Directive 98/79/EC of the European Parliament and of the Council [1].



sample	target value [ng/ml]	cv [%]	acceptability range [ng/ml]	mean value [ng/ml]	cv [%]	successful participations [%]
A	40.1	0.488	29.6 – 50.6	51.1	9.00	41.7
B	7.45	0.539	5.51 – 9.39	10.4	9.40	13.9

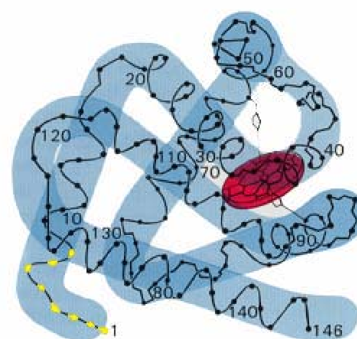
Figure 5 **Youden-plot with target value, acceptability criteria and statistical data of the participants in an External Quality Assessment Scheme for theophylline run by INSTAND e. V. (2005)**

Demonstration of an analytical problem of a routine method

A development of an isotope-dilution LC-MS/MS method for the determination of theophylline is on the way in our laboratory. On the basis of the publication of Beaudry et al. [22] the development of a method for setting target values in EQAS samples for theophylline will be further investigated.

4.3 Determination of HbA_{1c} by LC-MS

Glycated haemoglobin is an important parameter for the assessment of diabetes management in patient care [23,24]. The main analyte of glycated haemoglobins is HbA_{1c}. HbA_{1c} is a postsynthetic modification of haemoglobin A_0 by covalent attachment of glucose to the N-terminal valine of the β- chains. The reaction is thermodynamically controlled, i.e. the extent of formation of HbA1c is controlled by the concentration of glucose in blood.



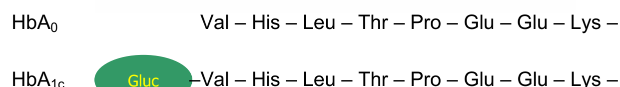


Figure 6

Model of the quarternary structure of haemoglobin

Figure 7

N-terminal aminoacids of the β-chain of haemoglobin

HbA_{1c} is a retrospective analyte reflecting the mean glucose concentration of the patient during the previous four to six weeks. The mean HbA_{1c} level reflects the compliance of the patient with the doctor in the management of the carbohydrate metabolism and is an indicator for the therapeutic success of the diabetes centres.

Several clinical studies, such as the DCCT (Diabetes Control and Complications Trial) study [23], have demonstrated, that an intensive metabolic control of diabetes reduces the onset and progression of diabetic complications in patients. According the DCCT study, a well managed diabetic patient should have an HbA_{1c} level less than 6.5 % of the haemoglobin. For the comparability of the analytical results is of great importance to have a reference measurement procedure and a standardized calibration of the routine methods. The organizers of the DCCT study agreed to standardize the measurement of HbA_{1c} according to the calibration used for

NGSP (National Glycohaemoglobin Standardisation Program), an HPLC method based on cation-exchange chromatography [25,26,27]. The agreement of a consensus value for calibration made it possible to compare the results of the different diabetes centres, however it did not improve the analytical accuracy. The calibration of the NGSP method is not traceable to a primary standard of HbA_{1c}. The so called "HbA_{1c}" peak in the HPLC chromatogram of cation-exchange chromatography is contaminated with about 2 % with other haemoglobin fractions. There was a need for a reference measurement procedure for the determination of HbA_{1c}. Such a reference measurement procedure has been developed and validated by the IFCC Working Group on HbA_{1c} Standardisation [17]. This IFCC reference method allows the international comparison of HbA_{1c} measurements at the highest level of accuracy.

In contrast to the NGSP calibration in the IFCC calibration the traceability of measurement results is achieved by chromatographically purified calibration standards of HbA_0 and HbA_{1c}.

Two different principles of measurement for HbA_{1c} are introduced by the IFCC, the LC-ESI-MS method and the HPLC/capillary electrophoresis method. Both methods have been established in our reference laboratory at INSTAND e.V.

The quantification of the IFCC reference method is based on calibration using external standards. For this kind of calibration an excellent long-term stability of the analytical measurement system is of utmost importance. According to the protocol of the IFCC reference measurement procedure for HbA_{1c} the calibrators must be measured before and after the controls and EQAS samples for which the target values have to be analysed. The total analytical time for measurement, including calibrators, controls and EQAS samples is about 24 h. The quality of the results is highly dependent on the long-term stability of the mass spectrometer used. In a stable analytical system, the repeated calibration curves should be congruent.

We tested the long-term stability (measuring period of about 24 h) of three different electrospray ionisation source- mass spectrometers:

- MSQ (Thermo Finnigan)
- Quantum ultra (Thermo Finnigan)
- API 4000 (Applied Biosystems).

All mass spectrometers were equipped with an HPLC-system from Shimadzu. The three LC-MS systems were tested under standardised conditions as requested in the IFCC reference measurement procedure over one measuring period of 24 h (see 3. MATERIALS AND METHODS].

Two of the mass spectrometers were not appropriate for this type of analysis. Both the MSQ and the Quantum ultra mass spectrometers revealed a long-term system instability (figure 8 and figure 9).

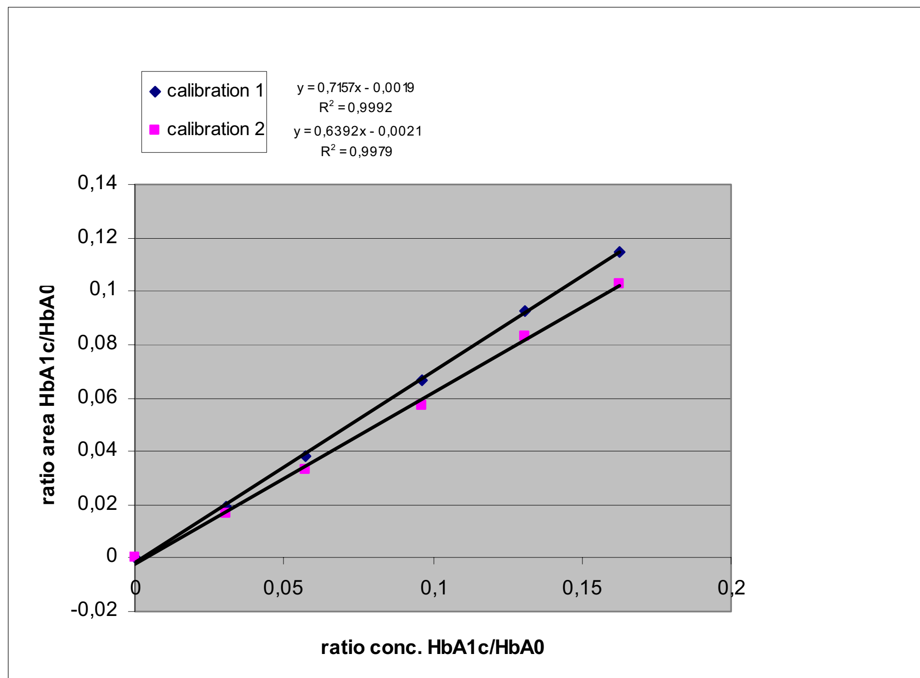


Figure 8 **Calibration curves at the beginning (calibration 1) and at the end (calibration 2) of a measuring sequence (24 h) for HbA_{1c} performed by using a MSQ mass spectrometer**

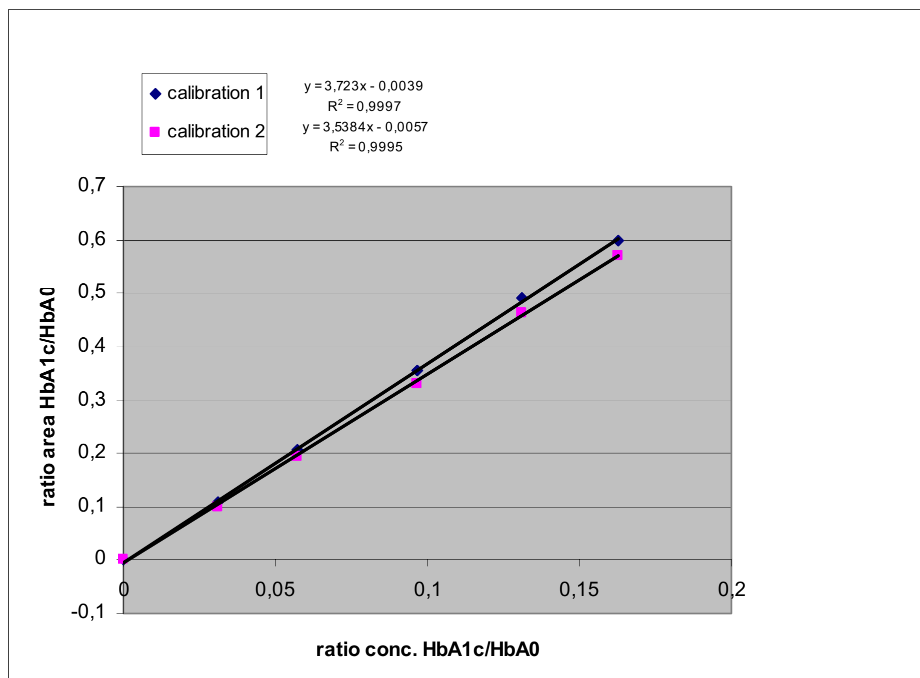
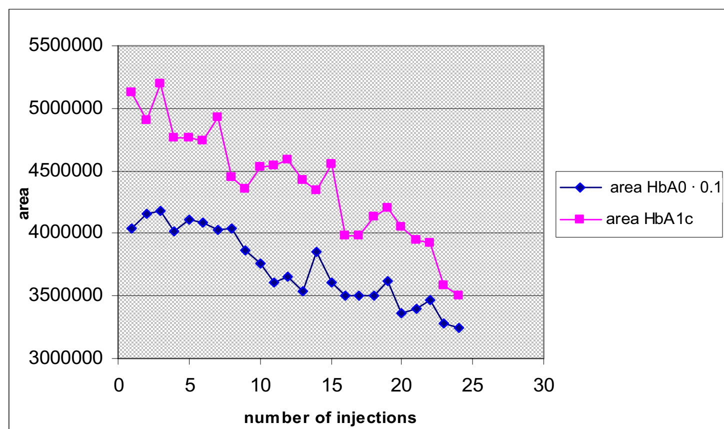


Figure 9 **Calibration curves at the beginning (calibration 1) and at the end (calibration 2) of a measuring sequence (24 h) for HbA_{1c} performed by using a Quantum ultra mass spectrometer**

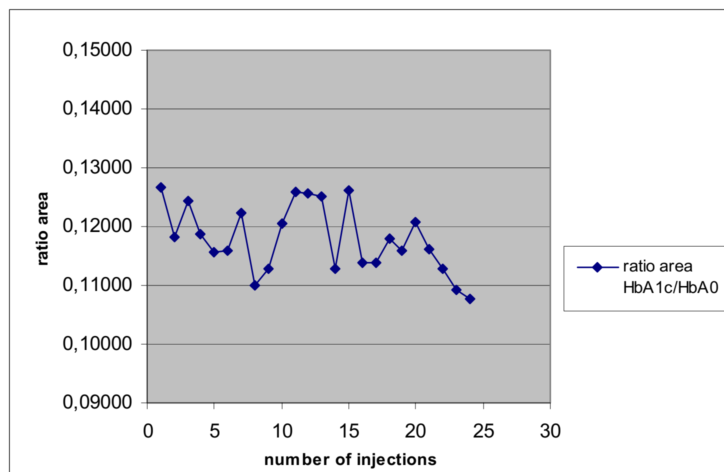
The calibration curve 1 (in the beginning of the sequence) and calibration 2 (in the end of the sequence) were calculated by plotting the ratio of the concentration of HbA_{1c} and HbA_0 versus the ratio of the peak area of the N-terminal hexapeptides of HbA_{1c} and HbA_0 and following linear regression.

Using the MSQ and Quantum ultra a decrease of the slope of the calibration curves 2 in relation to calibration 1 was observed.

The decreasing slope of the calibration curves is caused by different decrease of the absolute signal intensity of the glycated and native hexapeptides in the LC-MS systems. This was demonstrated by repeated injection of the same haemolysate sample (see figure 10).



<u>Figure 10</u> **Stability-test of absolute signal intensities for HbA_{1c} and HbA_0 by repeated measurements of a haemolysate performed by using a MSQ mass spectrometer**



<u>Figure 11</u> **Stability-test of the peak area ratio for HbA_{1c} and HbA_0 by repeated measurements of a haemolysate performed by using a MSQ mass spectrometer**

During the measuring period a continuous decay of signal intensity occurred, both for HbA_{1c} and HbA_0. Moreover, the rates of decay were different. This results in a continuous decay of the ratio of the peak areas of the β-N-terminal hexapeptides of HbA_{1c} and HbA_0 (see figure 11). Constant ratios are very critical for the accuracy and precision of the results. With the drifting systems we were not able to accomplish the acceptability criteria of the IFCC network for HbA_{1c} standardisation.

The best results were obtained using the mass spectrometer API 4000 from Applied Biosystems. The calibration curves were nearly congruent within one measuring period (figure 12).

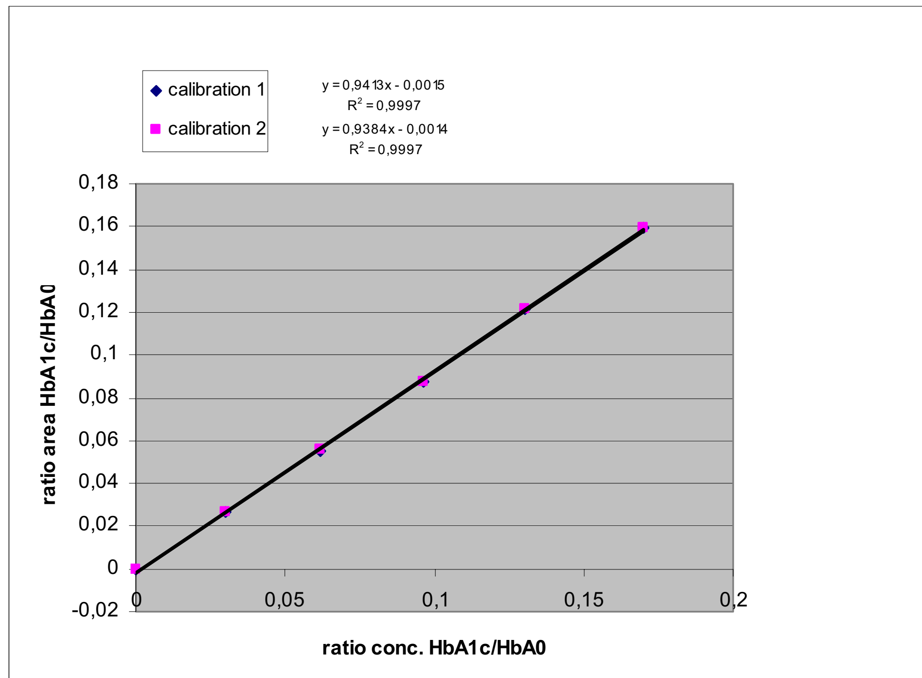


Figure 12 **Calibration curves at the beginning (calibration 1) and at the end (calibration 2) of a measuring sequence (24 h) for HbA_{1c} performed by using an API 4000 mass spectrometer**

Repeated injections of a haemolysate sample resulted in constant absolute signal intensities as (figures 13 and 14).

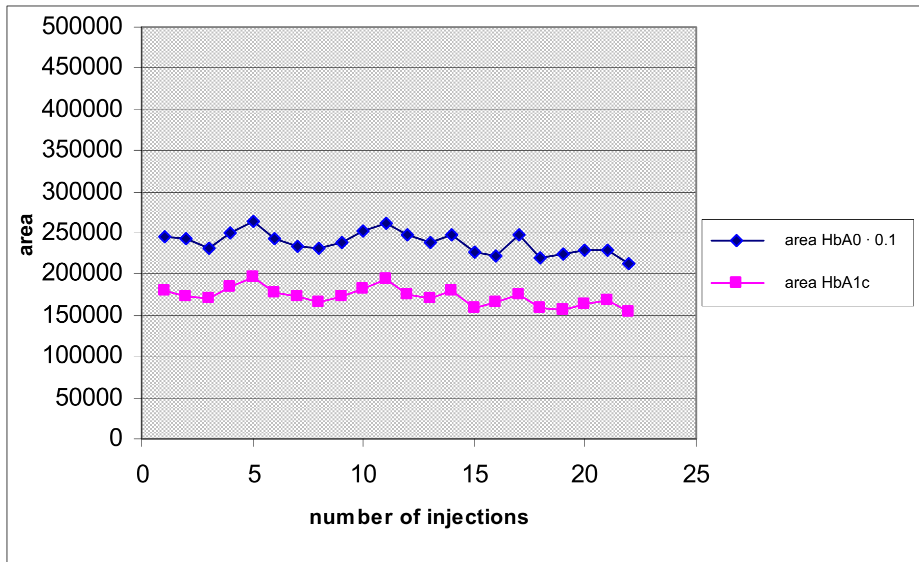


Figure 13 **Stability-test of absolute signal intensities for HbA_{1c} and HbA_0 by repeated measurements of a haemolysate performed by using an API 4000 mass spectrometer**

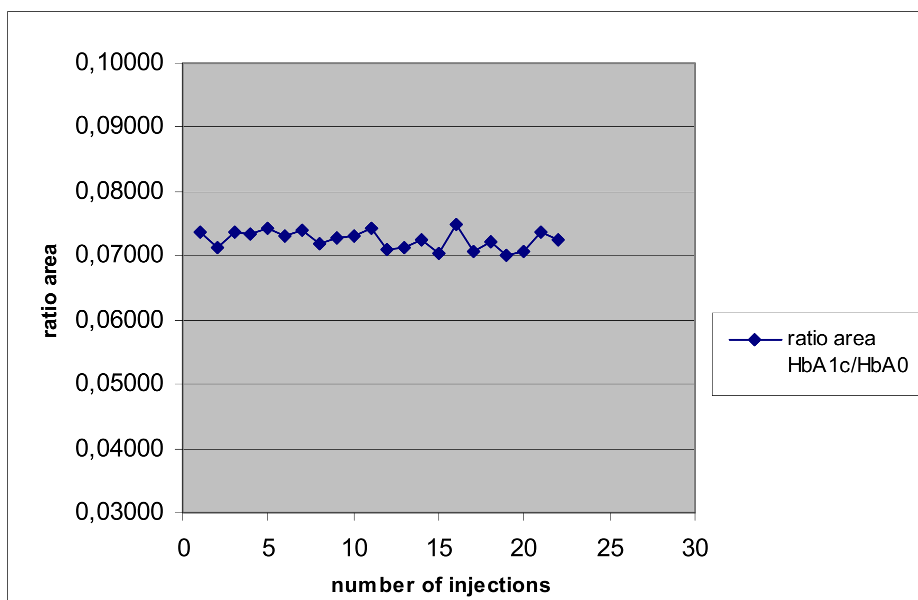


Figure 14 **Stability-test of the peak area ratio for HbA_{1c} and HbA_0 by repeated measurements of a haemolysate performed by using an API 4000 mass spectrometer**

The technical design of the electrospray ionisation interface of the LC-MS system is the critical element for the stability of calibration and analysis. The long-term instability of signal intensity could not be corrected, since no internal standards were available and calibration using external standards had to be used.
Therefore the API 4000 electrospray ionisation – mass spectrometer was used for the further measurements.

During optimization of the HPLC conditions for the determination of HbA_{1c} we modified the IFCC reference measurement procedure in several steps. In long-term evaluation, peak tailing, unexpected peaks and an instability of the retention time in the chromatographic separation was observed. A poor reproducibility of column selectivity and separation characteristics from batch-to-batch was detected.
The precision is highly dependent on the stability of the retention time. The stability of the retention times and the peak form was improved by increasing the concentration of TFA in the elution buffer, which was originally at 0.025 %. The best results were obtained by adding 0.07 % TFA to the eluents. By changing the gradient elution profile the β-N-terminal hexapeptides of HbA_{1c} and HbA_0 eluted in the plateau phase of 10 % of eluent B. The restitution of the equilibrium of the cyano-column after rinsing the column with acetonitrile at the end of each analytical cycle is critical to achieve highest precision of measurement. The run time was necessarily extended from 23 min to 28 min. Using this modified HPLC conditions column selectivity and separation characteristics were highly reproducible from batch-to-batch. These modifications in the HPLC conditions improved the imprecision of repeated measurements from more than 3 % of the original IFCC conditions (depending on the column batch) to a coefficient of variation of 1.35 %.

Another important aspect is the prevention of system overloading. We observed, that the concentration of HbA_{1c} in samples, which are prepared according the IFCC reference procedure, is too high for injection direct into the LC-MS system, when using the API 4000. Injecting 1 µl of the haemolysates the calibration curve was not linear over the required concentration range. Additionally, accuracy and precision was not satisfactory. The dilution of samples did not improve the results. Therefore, a post column flow splitting system was installed, leading to 1/10 of the elution flow into the ESI-MS system. Applying these modifications of the HPLC conditions, stable retention times, a symmetric peak form without any peak tailing, no unexpected peaks and linear regression curves were obtained. A typical HPLC-MS chromatogram of a haemolysate sample is shown in figure 15.

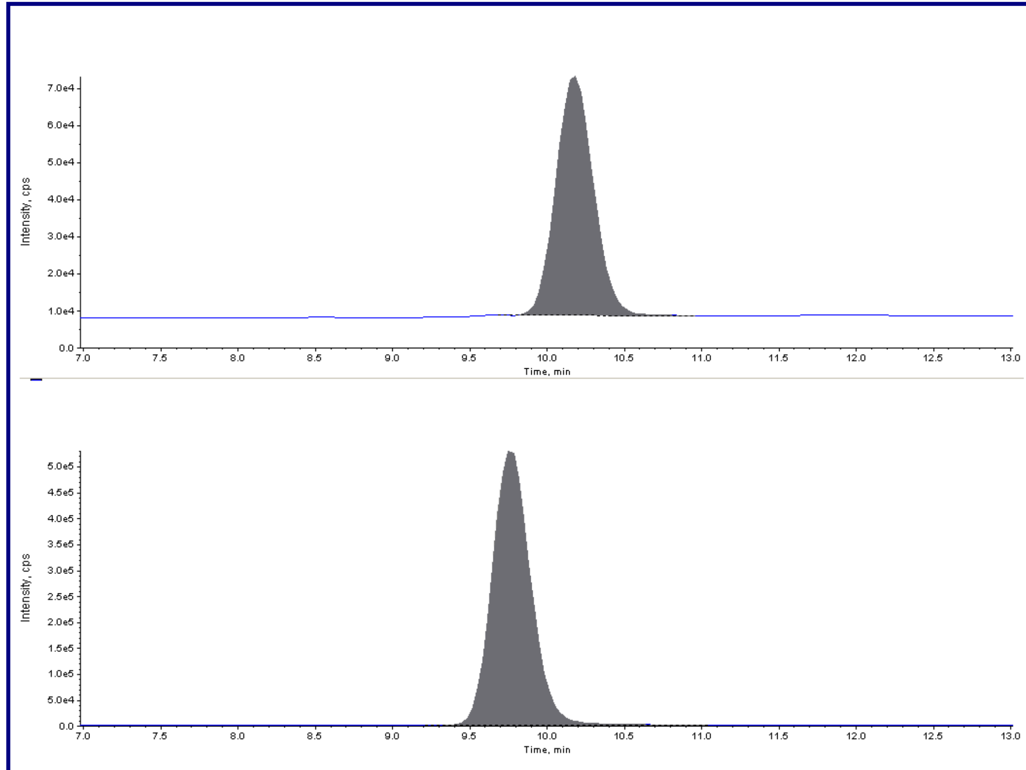


Figure 15 **HPLC-MS chromatogram of a haemolysate sample containing 8.22 % HbA_{1c}**
upper trace: **ß-N-terminal hexapeptide of HbA_{1c} (RT 10.2 min, m/z 429.3 amu)**
lower trace: **ß-N-terminal hexapeptide of HbA_0 (RT 9.8 min, m/z 348.3 amu)**

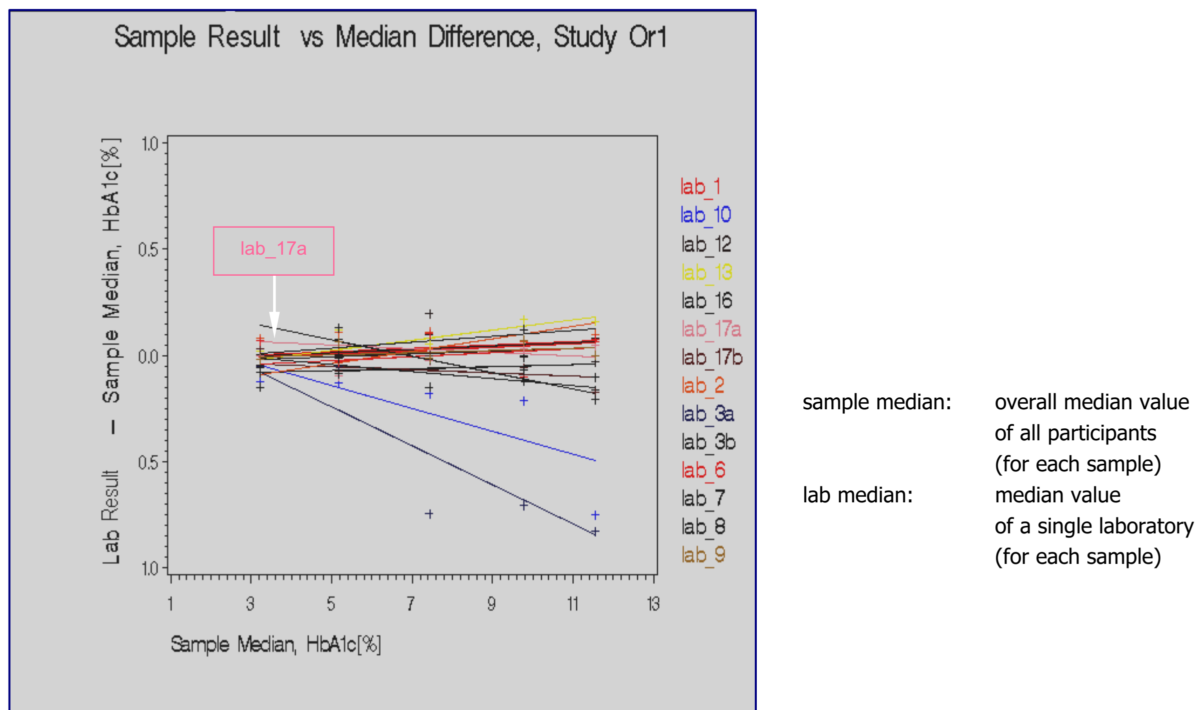
Using these modifications we have assessed the analytical trueness by measurements of a certified reference material supplied by the network for HbA_{1c} standardisation. The method had a bias between 0.1 % and 0.65 %. The coefficients of variation were between 0.92 % and 1.98 %. Additionally, we verified the analytical specificity and accuracy of the optimized LC-ESI-MS method by comparing the results with those obtained by the HPLC/capillary electrophoresis IFCC reference method.

Using this optimized measurement procedure we participated in several studies of the IFCC Working Group on Standardisation of HbA_{1c}.
This working group includes about 15 laboratories from various countries. Twice a year international intercomparison studies were organized.

In these studies five samples of unknown concentration were analysed according to the measurement protocol of the IFCC reference measurement procedure for HbA_{1c} (sequence see 3. MATERIAL AND METHODS).

For evaluation of the measurements of each sample the difference between the median value of each individual laboratory and the overall median value of all participants are plotted against the overall median value. A linear regression analysis of the results of each participant is made. The slope of the regression curve is a measure for the proportional bias, the intercept a measure for the systematic bias. Slope and intercept define the limit of acceptability.

A typical evaluation is shown in figure 16. The results of our laboratory are presented as laboratory number 17a.



(by kind permission of the IFCC Working Group on Standardization of HbA_{1c})

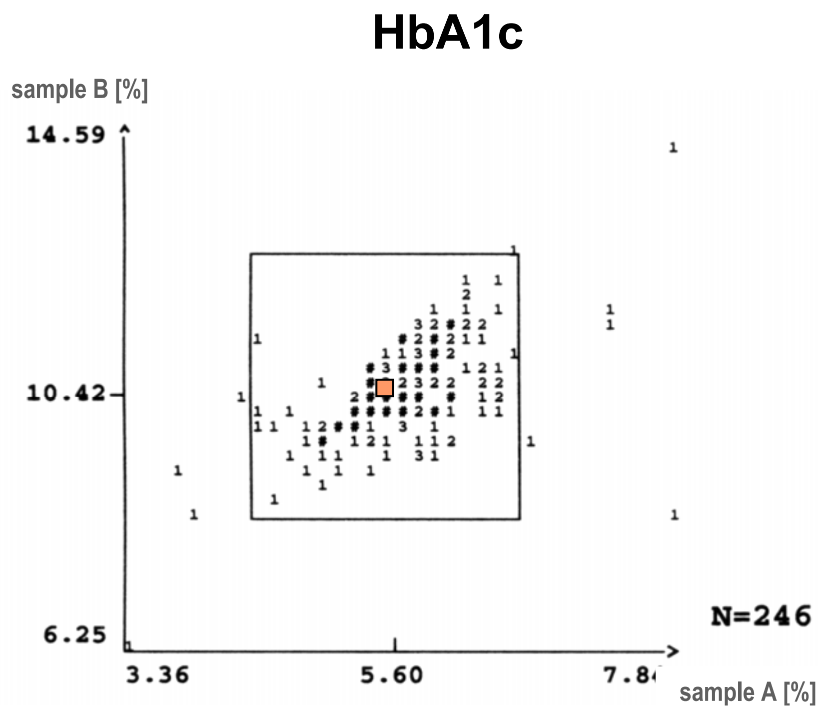
Figure 16 **Evaluation of an international intercomparison study of the IFCC Network Standardisation of HbA_{1c} (Results of INSTAND e.V.: lab_17a)**

The consensus mean of the approved data of each sample is the new assigned value. The intercomparison studies are used to renew the approval status of the network laboratories and for setting target values for new batches for controls and calibrators.

The established reference measurement procedure was used for setting target values in the external quality assessment schemes. The results of a typical survey are shown in figure 17. The acceptability criteria of ± 20 % for routine measurements of HbA_{1c} are shown as a frame. The central square dot indicates the two reference measurement values (target values) for

sample A and sample B. 97.2 % of the participants had successfully analysed the sample A and 98.8 % the sample B.

Unfortunately, for routine laboratories the reference measurement values for HbA_{1c} have to be transformed according the calculation formula of the IFCC Working Group into NGSP values, because the manufacturers decided to comply with DCCT-requirements and calibrate their commercial test systems with reference to the NGSP procedure for the next few years, before introducing the IFCC calibration.



sample	target * value [%]	cv [%]	acceptability range [%]	mean value [%]	cv [%]	successful participations [%]
A	5.52	2.07	4.41 – 6.63	5.59	8.20	97.2
B	10.50	2.19	8.39 – 12.70	10.50	6.50	98.8

Figure 17 **Youden-plot with target value, acceptability criteria and statistical data of the participants in an External Quality Assessment Scheme for HbA_{1c} run by INSTAND e. V. (2005)**

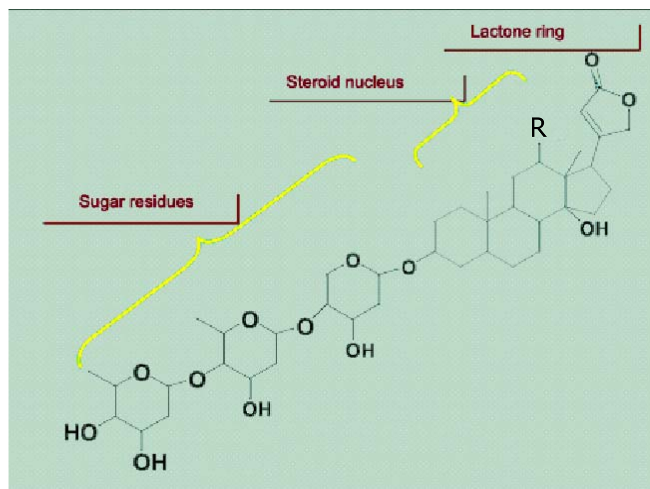
** converted from IFCC to NGSP values with the following formula:*

NGSP = (0.9148 x IFCC) + 2.152

Further development of the reference measurement procedure to variant haemoglobins (like HbF, HbC or HbS) and using labelled glycated and non glycated β-N-terminal hexapeptides as internal standards for a LC-IDMS reference measurement procedure is in progress.

4.4 Determination of digoxin and digitoxin by LC-MS and LC-MS/MS [V, VI]

Digoxin and digitoxin are cardiac glycosides, which are used for the therapy of cardiac diseases. The structure of digoxin is shown in figure 18. Digoxin is an analogue of digitoxin and differs only in the presence of a hydroxy-group at position 12 (see – R in figure 18) of the steroid residue.



	chemical formula	molecular weight [g/mol]
digoxin	$C_{41}H_{64}O_{14}$	780.94
digitoxin	$C_{41}H_{64}O_{13}$	764.94

Figure 18 **Structure of digoxin (R = OH) and digitoxin (R = H)**

The Guidelines of the Federal Medical Council for Quality Assurance of Quantitative Analyses in Laboratory Medicine made it mandatory to establish reference measurement procedures for determination of digoxin and digitoxin. The reference measurement procedure was published in 2003 [V]. The method describes a LC-MS method, using the SIM mode, combined with isotope dilution after a liquid-liquid extraction. The essential benefit of the method was the observation that digoxin and digitoxin form caesium (Cs^+) adducts. This new principle of measurement was adapted to LC-MS/MS [VI] to allow quantitative determination of the cardiac glycosides at high yield and improved specificity in the MRM mode.

In LC-MS many molecules form adducts with alkali ions such as Na^+ and K^+ [28,29]. Adduct formation with Na^+ or K^+, originating from the HPLC system (solvent, glasware, column), turned out as problem in analysis because the analytical sensitivity may be reduced [28] and tandem mass spectroscopic studies may be limited [30,31]. Several studies have been conducted, to control the problems of adduct formation during LC-ESI-MS analysis. One strategy is to avoid the formation of alkali metal adducts using ultra pure solvents, which are free of alkali metal impurities [31] or through competition by forming a stable complex with the alkali metal ions [30]. Another strategy enhances adduct formation by use of solvent additives, such as sodium

salts [32], primary amines [28], silver nitrate [49] or ammonium salts [33]. It must be admitted, that the analysis of the fragmentation pattern of these adducts is not convenient [33].

Analytical sensitivity [34] and specificity are highly affected by the composition of the mobile phase. Adding an excess of Cs^+ ions to the elution buffer, it is possible to shift the equilibrium of adduct formation to one main product and the Na^+ and K^+ adducts are reduced or even eliminated.

The mass spectrometry full-scan (Q1 scan) of a standard solution of digoxin (figure 19) shows, that without addition of Cs^+ ions digoxin is distributed into numerous fragments. The digoxin adduct with sodium ions $[M+Na]^+$ m/z 803.6 amu is dominant. Additionally the mass of protonated digoxin $[M+H]^+$ m/z 781.6 amu and the protonated and sodiated fragments of digoxin minus one, two and three digitoxose molecules can be detected. The mass of the K^+ adduct of digoxin $[M+K]^+$ m/z 819.7 amu is also detectable, but not labelled in this spectrum.

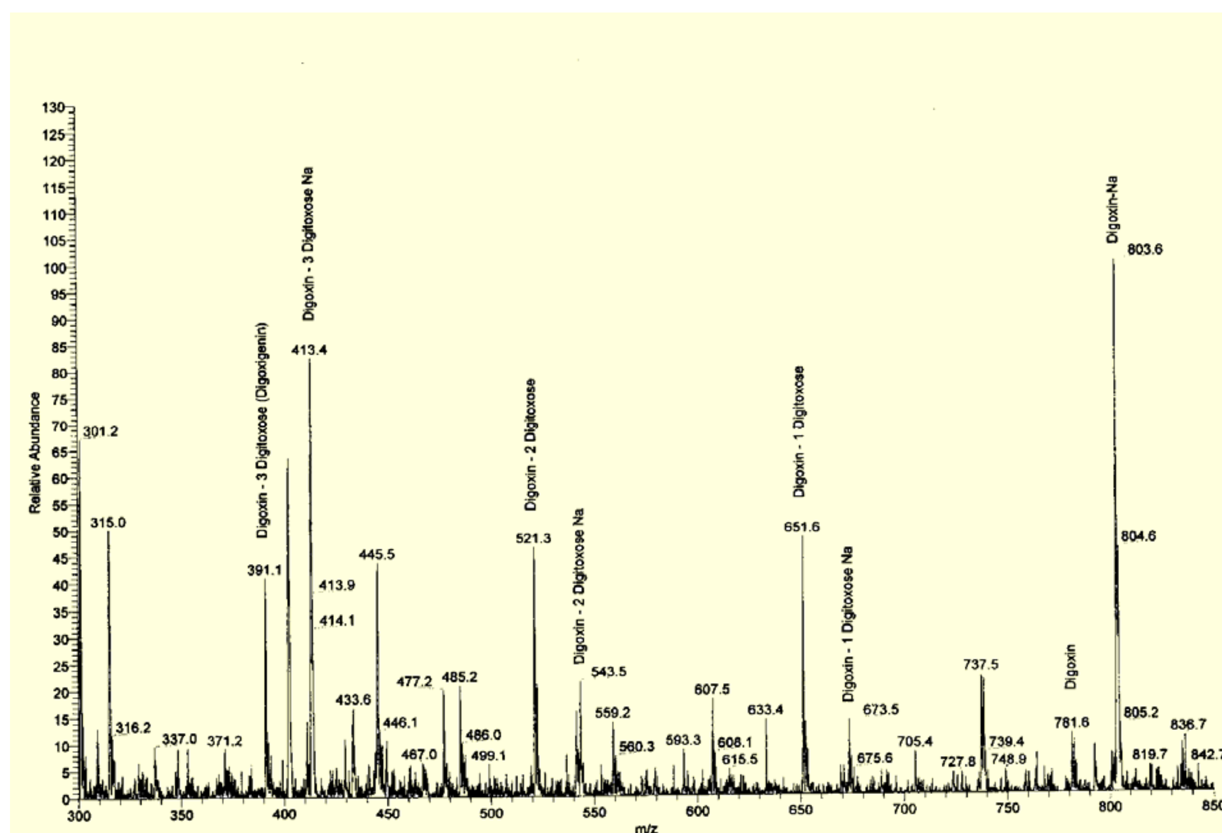


Figure 19 **Q1 scan of a digoxin standard solution (without Cs^+ ions)**

When Cs^+ ions are added to the elution buffer (figure 20), nearly the full signal intensity enters in the Cs^+ adduct of digoxin $[M+Cs]^+$ m/z 913.6 amu. The Cs^+ adduct formation has a stabilizing effect on the whole molecule, avoiding an undesirable fragmentation as seen without Cs^+ ions.

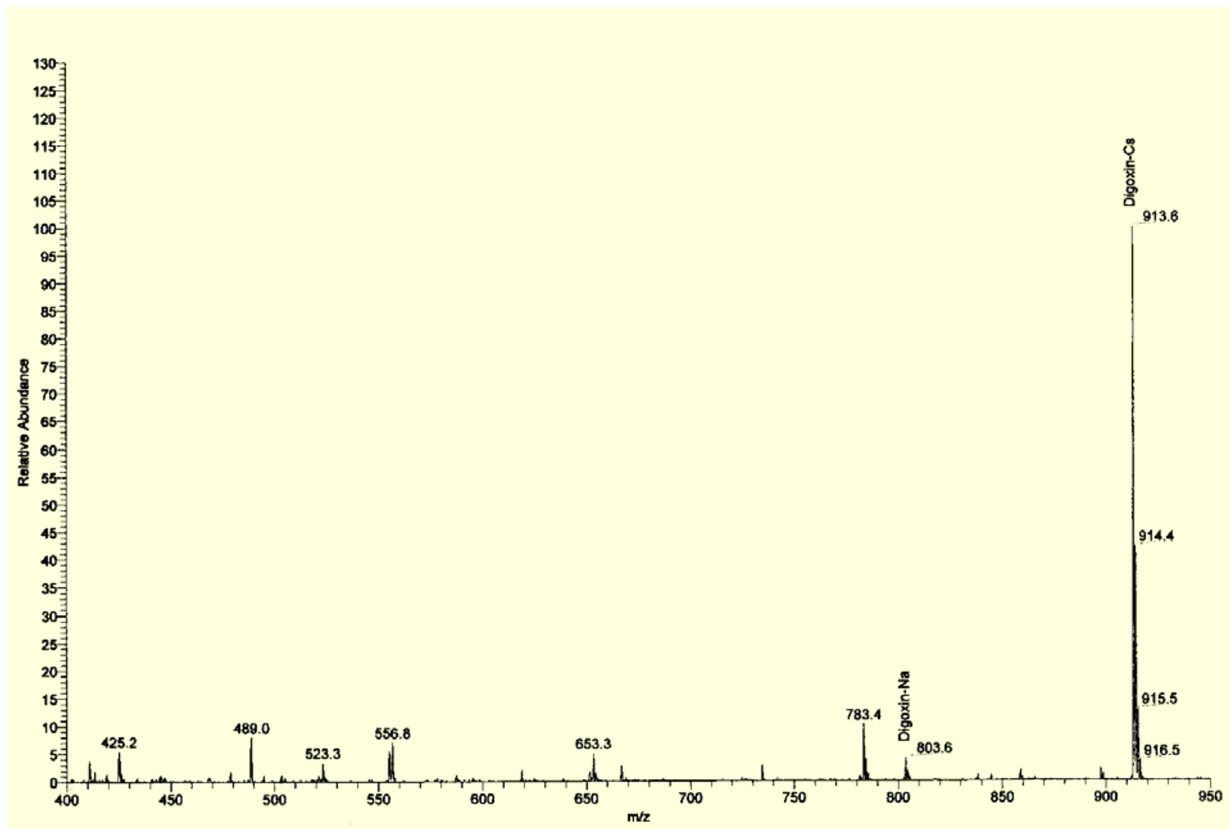


Figure 20 **Q1 scan of a digoxin standard solution (with Cs^+ ions)**

The same observation is made in the LC-MS spectrum of digitoxin.
At the same digitoxin concentration the Cs^+ adduct produces much higher peak values than the sodium adduct. The depression of ion signal intensity caused by high concentrations of electrolytes has been described previously [50]. Therefore, the influence of Cs^+ concentration in the elution buffer on the signal intensity of the Cs^+ adducts was examined. A reduction of Cs^+ concentration from 0.1 mmol/l to 0.01 mmol/l decreases the signal intensity. An increase of the Cs^+ concentration above 0.1 mmol/l in the elution buffer does not increase the signal intensity of the Cs^+ adducts. By the use of 0.1 mmol/l Cs^+ in the elution buffer we obtained stable and reproducible results.
The advantage of measuring Cs^+ adducts in the Q1 Multiple Ion mode is an increase in signal and an increase in analytical specificity. This effect results in higher precision and a lower limit of detection.

In the MRM mode the Cs^+ adduct decomposes in a way that the Cs^+ ion is detectable as product ion. The Cs^+ adduct is split into a neutral molecule and a positive charged caesium ion. By optimized selection of the ESI source parameters, in the product ion scan the signal of the

Cs^+ adduct can be completely reduced, resulting in a maximized intensity of the Cs^+ ion signal. An efficient dissociation of up to 80 % from the caesium-drug-complex to the caesium ion could be obtained. Under our conditions the Cs^+ ion is the only charged product from Cs^+ adducts. The Cs^+ adducts of digoxin and digitoxin become measurable as Cs^+ ion in the MRM mode in high signal intensity (figure 21).

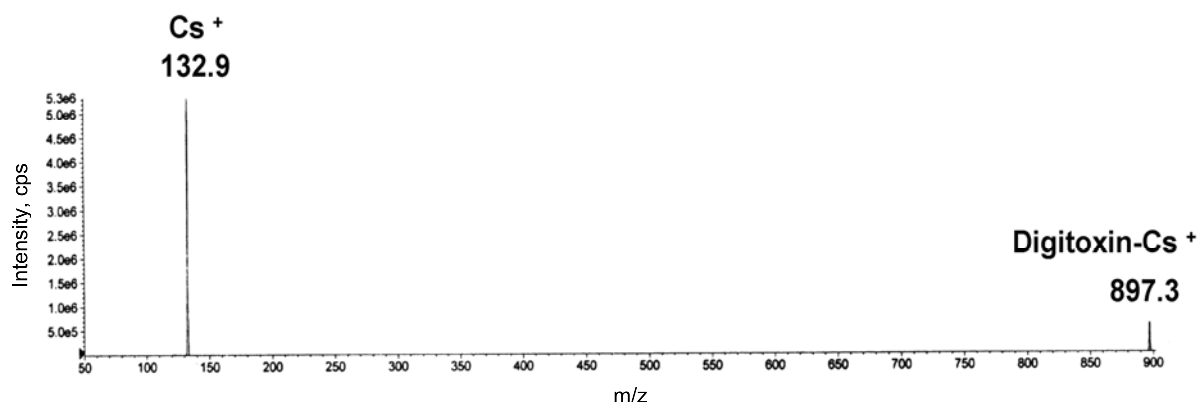


Figure 21 **Product Ion scan of the Cs^+ adduct of digitoxin (m/z 897.3 amu) in a digitoxin standard solution**

Correspondingly, we investigated the effect of the other alkali metals Na^+, K^+ and Rb^+ in MRM transmission. The Na^+ ions were not detectable in the same way like Cs^+ ions in the MRM mode. By addition of K^+ and Rb^+ ions the same kind of MRM transmission as seen by addition of Cs^+ ions was observed. In the Q1 multiple ion mode K^+ and Rb^+ adducts could be detected, which were measurable as K^+ and Rb^+ ion, respectively, as product ion in the MRM mode. In contrast to caesium, which is mono isotopic, potassium and rubidium have naturally occuring isotopes. Therefore, the signal of the product ion of the K^+ or Rb^+ adduct is split into two components, thus reducing the signal intensity of the target mass. Additionally, the higher molecular weight of caesium is more convenient for a specific detection.

The loss of a neutral molecule and the clear origin of the Cs^+ ion as product ion of the CS^+ adduct was clarified in [VII].

A scheme of the measuring principle of Cs^+ adducts in SIM (Q1 Multiple Ion) mode and MRM mode is shown in figure 22 [VI].

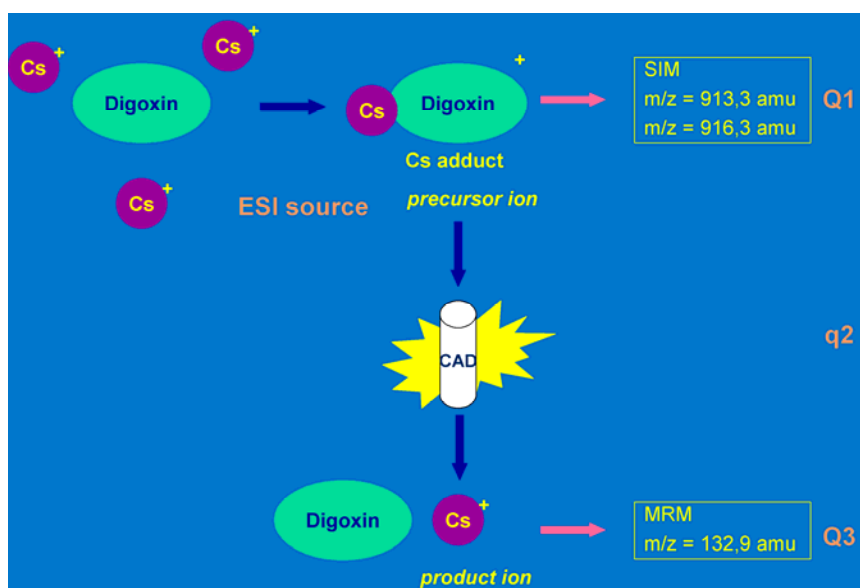
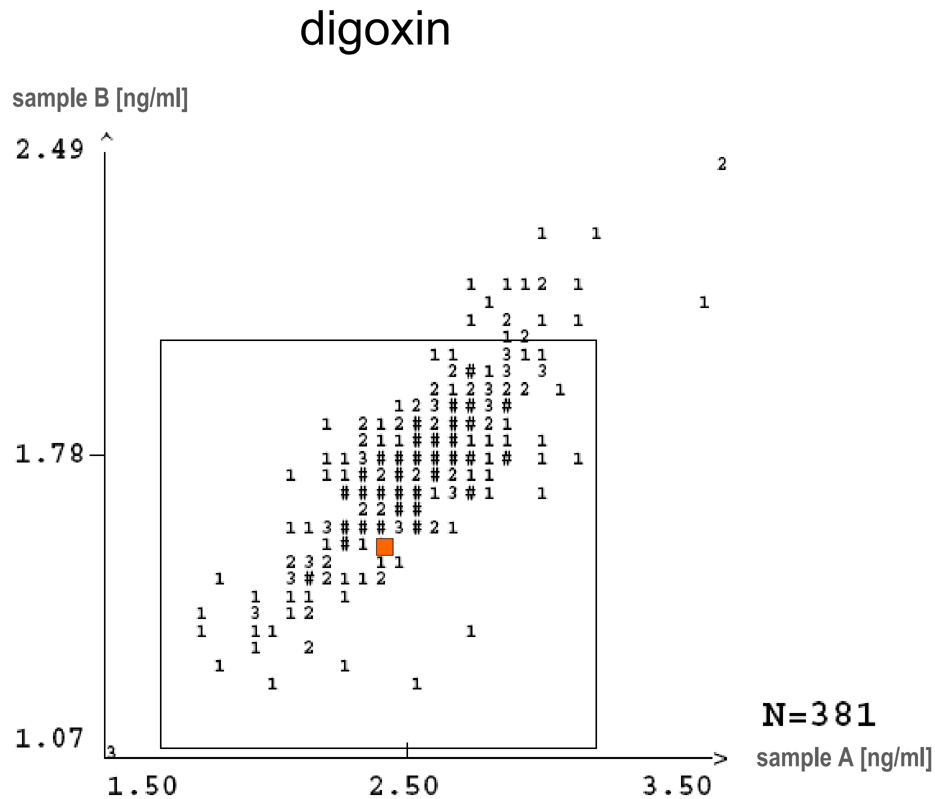


Figure 22 **Scheme of the principle of measurement of Cs^+ adducts SIM and MRM**
(by kind permission of Clin.Lab.)

For serum samples a special extraction procedure was developed as described in [V,VI].
As internal standards stable isotope-labelled compounds were synthesized in our laboratory with modification of the original procedure [48,V,VI]. To obtain high accuracy and a good precision standard solutions and serum samples were quantified gravimetrically during sample preparation. The volume of serum and standards was adjusted in such a way that an amount of 1 ng of the drug was present in the sample. Internal standard was then added giving the ratio of sample to internal standard of 1:1.
Since no certified reference material is available the trueness of the newly established method was investigated by recovery studies. In spiked serum samples the mean recoveries in therapeutic ranges for digoxin was 99.95 % (n=28) and for digitoxin 100.2 % (n=35), with a bias of 0.05 % and 0.2 % respectively. In the Q1 Multiple Ion mode the between-run imprecision was between 1.26 % and 2.91 % (n=7) for digoxin and 0.79 % and 2.00 % (n=7) for digitoxin. The corresponding coefficients of variation in EQAS samples were between 1.26 % and 3.18 % (n=6) and 1.21 % and 1.46 % (n=8), respectively. In the MRM mode the coefficients of variation were between 1.65 % and 2.28 % (n=6) for digoxin and 1.13 % and 1.44 % (n=6) for digitoxin.
These results are well within the requirements of the Guidelines of the German Federal Medical Council for Quality Assurance of Quantitative Analyses in Laboratory Medicine.
Unfortunately, an international network of reference laboratories for digoxin and digitoxin does not exist for interlaboratory comparison studies.

Our candidate reference measurement procedure is used for setting target values in the EQAS. A typical evaluation of an EQA survey for digoxin is shown in figure 23. The acceptability limits for routine measurement of ± 30 % for digoxin are shown as a frame. The central square dot indicates the two target values for sample A and sample B. 98.9 % of the participants had successfully analysed the sample A and 94.5 % the sample B.



sample	*target value [ng/ml]*	*cv [%]*	*acceptability range [ng/ml]*	*mean value [ng/ml]*	*cv [%]*	*successful participations [%]*
A	2.40	2.54	1.68 – 3.12	2.49	9.42	98.9
B	1.57	1.68	1.09 – 2.50	1.76	10.1	94.5

Figure 23 **Youden-plot with target value, acceptability criteria and statistical data of the participants in an External Quality Assessment Scheme for digoxin run by INSTAND e. V. (2005)**

Similar results have been collected for digitoxin in EQAS.

4.5 Determination of sirolimus, everolimus, tacrolimus, cyclosporin A by LC-MS/MS [VII]

The therapeutic drug monitoring (TDM) of immunosuppressive drugs in blood of organ-transplanted patients is of utmost importance to prevent intoxication or the rejection of the transplanted organ due to incorrect dosage. The most frequently used immunosuppressive drugs are the mTOR inhibitors sirolimus and everolimus and the calcineurin inhibitors tacrolimus and cyclosporin A.
The chemical structures of these compounds are shown in figures 24-27.

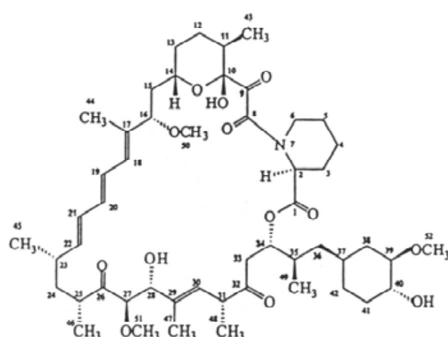


Figure 24 **Structure of sirolimus**

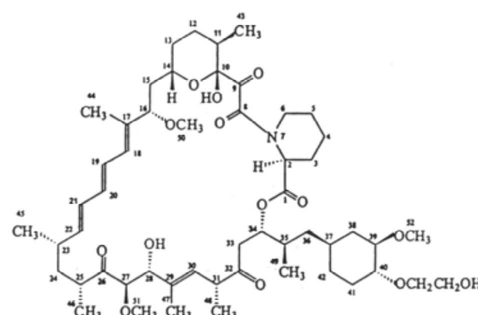


Figure 25 **Structure of everolimus**

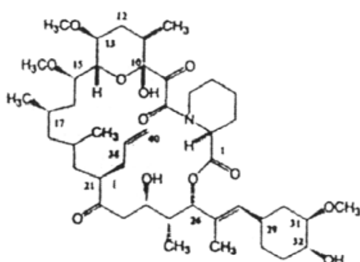


Figure 26 **Structure of tacrolimus**

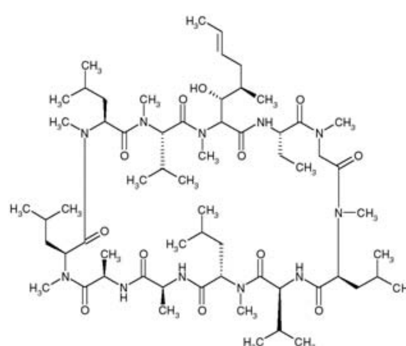


Figure 27 **Structure of cyclosporin A**

	chemical formula	molecular weight [g/mol]
sirolimus	$C_{51}H_{79}NO_{13}$	914.19
everolimus	$C_{53}H_{83}NO_{14}$	958.2
tacrolimus	$C_{44}H_{69}NO_{12}$	804.03
cyclosporin A	$C_{62}H_{111}N_{11}O_{12}$	1202.64

The routine methods for the determination of immunosuppressive drugs are immunoassays [35, 36,37], HPLC [38] and LC-MS [39,40,41] methods. In LC-MS routine measurement NH_4^+ adducts are measured in MRM mode without chromatographic separation of the drugs. This procedure has a low specificity [33] and is prone to mass interferences [42]. In patient samples problems may occur, due to the presence of metabolites, causing a positive bias of published LC-MS methods [43,33]. Until now no accepted reference measurement procedure for these drugs is available. Recent data from EQAS clearly show the poor comparability of the results obtained by routine methods for the same analyte. Large differences of results were observed using the kits of different manufacturers (figure 28).

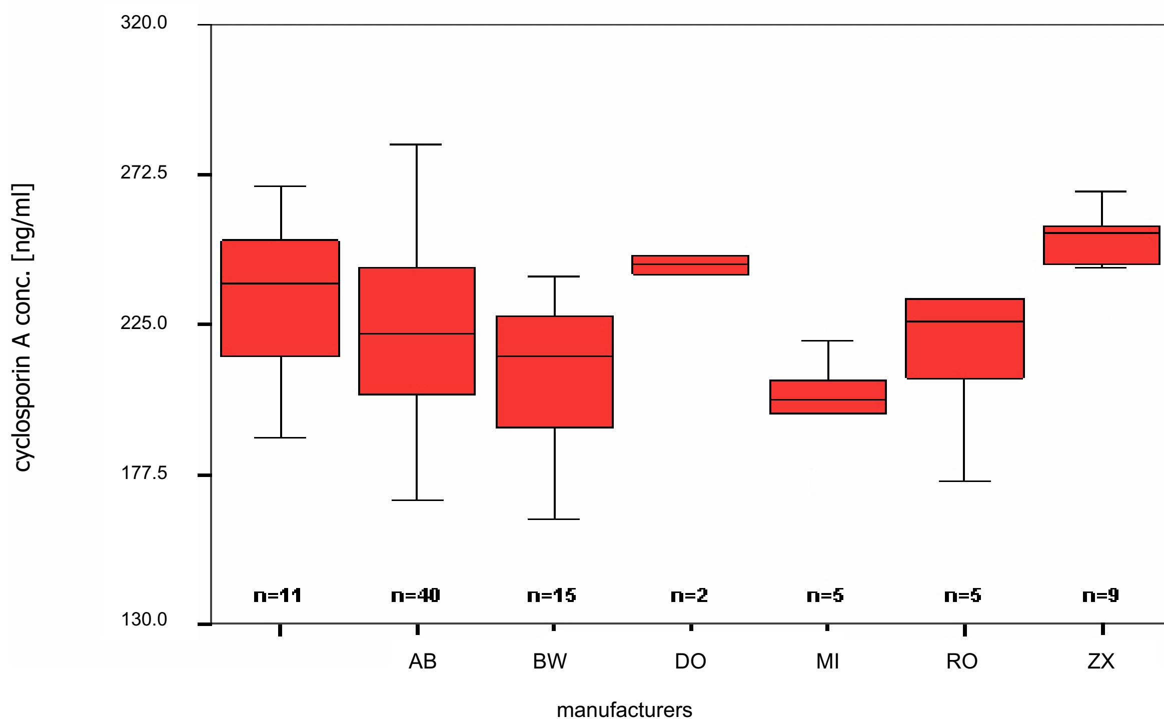


Figure 28 **External quality assessment scheme for cyclosporin A, run by INSTAND e.V (2005). Box-plots of the results of participating laboratories using different analytical systems**

There is a need for development of reference measurement procedures in order to achieve comparability of results. On the way to develop reference measurement procedures for these drugs we applied the experience of the Cs^+ adduct formation to the measurement of the immunosuppressive drugs.

All four immunosuppressive drugs form adducts with Cs^+ that are measurable in the MRM mode with Cs^+ ion as product ion.

The main charged product in the MRM transmission for the immunosuppressive drugs is the Cs^+ ion, like in digoxin and digitoxin, (figure 29).

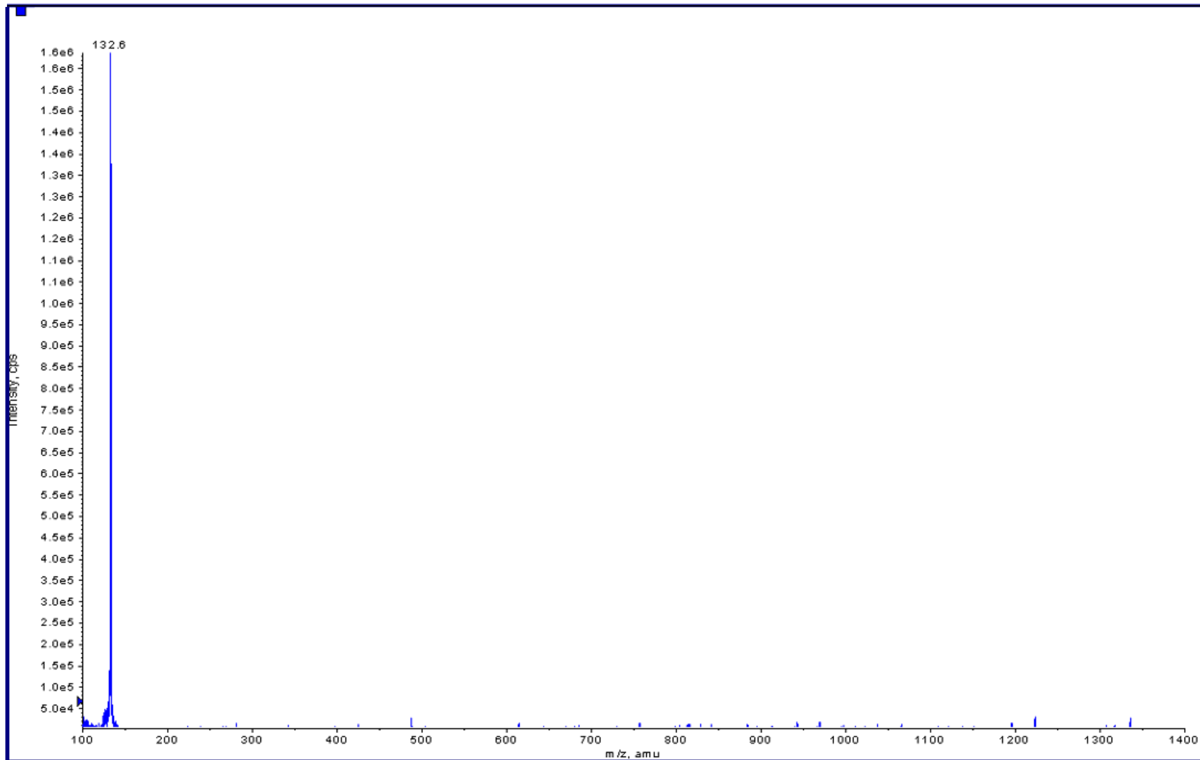


Figure 29 **Product Ion scan of a cyclosporin A standard solution (1 µg/ml in eluent B) infused at a flow rate of 10 µl/min**

For chromatographic separation various stationary phases were tested with different mobile phases, containing Cs^+, water, methanol and/or acetonitrile, with different elution profiles. Mobile aqueous phases with different pH, adjusted with formic acid, were also tested. Since the four immunosuppressive drugs are highly lipophilic, C18 reversed phase columns show a strong retention, especially for cyclosporin A [44]. We tested several C18 columns, consisting of different polymer and silica based materials and chemically modified reversed phase materials, with different kinds of end-capping, particle, and pore sizes. The best results were obtained using a phenyl-hexyl-phase. Using a ternary gradient elution profile we could separate all four drugs and cyclosporin D (figure 30).

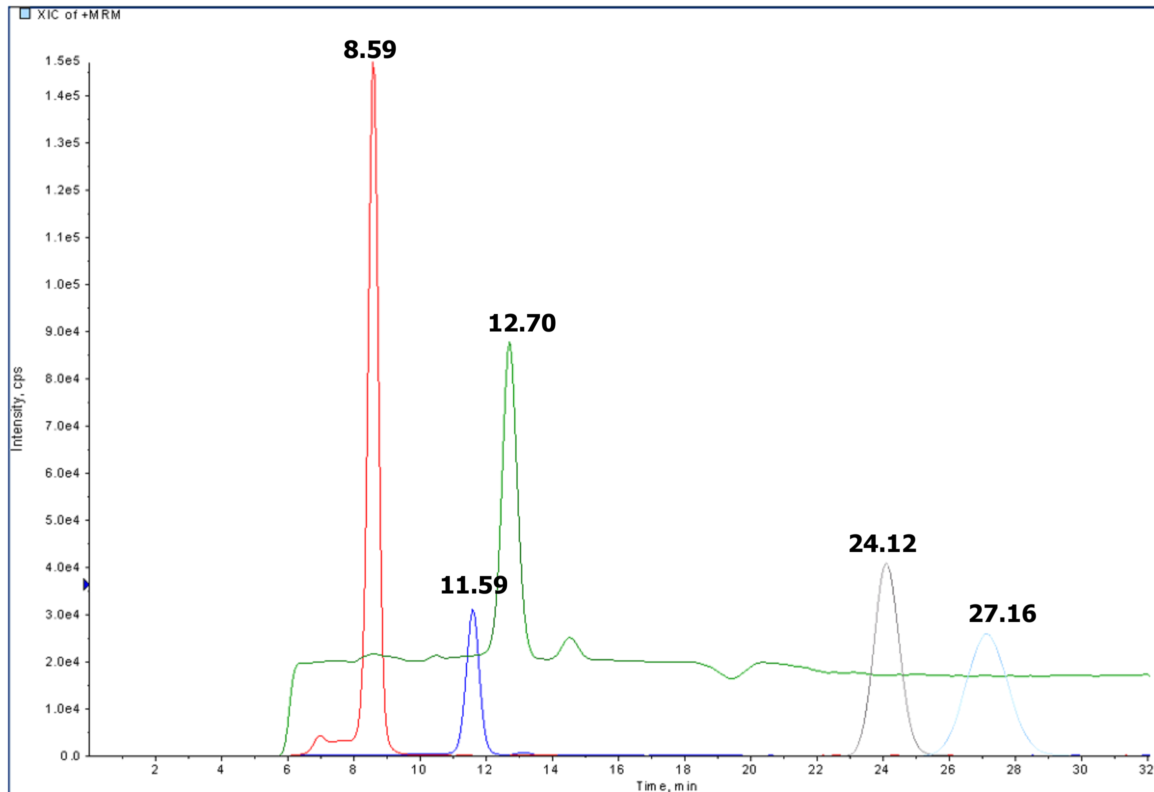


Figure 30 **HPLC-MS/MS chromatogram (overlaid graphs of single mass traces) of a standard mixture of tacrolimus (8.59 min), sirolimus (11.59 min), everolimus (12.70 min), cyclosporin A (24.12 min) and cyclosporin D (27.16 min), 10 µl (1 µg/ml) injected**

Since labelled internal standards are not available, analogue substances as internal standards were used for quantification. Ascomycin was used for the quantification of tacrolimus, 32-desmethoxy-rapamycin for sirolimus and everolimus and cyclosporin D for cyclosporin A.

area ratio	*sirolimus / 32-desmethoxy-rapamycin*	*everolimus / 32-desmethoxy-rapamycin*	*tacrolimus / ascomycin*	*cyclosporin A / cyclosporin D*
mean	2,52	2,89	1,20	1,09
n	25	25	25	25
SD	0,06	0,06	0,03	0,02
CV [%]	**2,57**	**2,11**	**2,31**	**2,11**

Table 15 **Imprecision of measurements of a standard mixture of tacrolimus, sirolimus, everolimus, cyclosporin A, cyclosporin D, ascomycin, and 32-desmethoxy-rapamycin**

Whereas the precision of measurement in a standard mixture of pure substances of tacrolimus, sirolimus, everolimus, cyclosporin A, cyclosporin D, ascomycin and 32-desmethoxy-rapamycin is excellent (table 15), the accuracy has to be determined using certified reference materials, which is unfortunately not available.

The HPLC separation of the immunosuppressive drugs and the use of Cs^+ ion for forming adducts is a good precondition to establish a reference measurement procedure. This new principle of measurement of these drugs may be improved by using stable-isotope-labelled drugs as internal standards, which will be produced in our laboratory.

New technologies using electrospray ionisation sources as interfaces, considerably improved the stability in LC-MS and LC-MS/MS analysis. Hence the accuracy and precision of results became comparable to those attained by GC-MS. In contrast to GC-MC methods LC-MS analysis does not require chemical derivatization of the analytes. Therefore GC-MS methods are more and more substituted by LC-MS, even in reference measurement procedures [45].

High throughput LC-MS methods may be used in routine analysis, sometimes called "quick-and-dirty" procedures rely exclusively on the specificity of the mass spectrometer by selecting one "specific" product ion mass. They are not appropriate for reference measurement procedures. The reference measurement procedure requires a higher specificity, accuracy and precision, which is only given by careful sample preparation, chromatographic separation of the analytes [38,47] and mass analysis, thus eliminating undesirable matrix effects and ion suppression [29,46]. Only by this way mass interferences can be avoided [37].

The most reliable quantification is performed using isotope-labelled internal standards. These internal standards may be purchased (as for 2-^{13}C ,1,3-$^{15}N_2$-theophylline for determination of theophylline) , produced by specialized institutes (labelled glycated and not glycated hexapeptides for determination of HbA_{1c}) or self prepared (for instance digoxin-$^2H_3$ and digitoxin-$^2H_3$ for determination of digoxin and digitoxin).

The isotope-labelling of analytes may be difficult as is the case with immunosuppressive drugs. In this field studies in our laboratory are on the way.

Development and optimization in the reference laboratory is a never completed process. This study demonstrates an actual status in a dynamic system.

A LC-MS/MS reference measurement procedure is on the way to be established for glibenclamide, measuring Cs^+ adducts of the drug for setting target values in a new EQAS. For theophylline an isotope-dilution LC-MS/MS method is actually developed. A further development of the reference measurement procedure for HbA_{1c} using labelled gycated and non glycated ß-

N-terminal hexapeptides as internal standard for a LC-IDMS reference measurement procedure is in process. The method for determination of the immunosuppressive agents sirolimus, everolimus, tacrolimus and cyclosporin A measuring Cs^+ adducts will be further developed to be proposed as a reference measurement procedure.

Reference measurement procedures improve calibration of routine methods, the comparison of the performance of routine methods and the objective monitoring of commercially available test kits. Reference measurement procedures lead to comparable reference intervals of analytes thus forming a reliable basis for epidemiological studies and better patient care.

In the future additional international networks should be established, to get reference measurement procedures confirmed and accepted at international level.

5. SUMMARY

Reference measurement procedures in laboratory medicine are necessary to get reliable and comparable results in laboratory analyses and thus improve patient care.
This study demonstrates that the development of reference measurement procedures is a highly dynamic process. Maintenance, evaluation, and progressive improvements to the highest technical level are a permanent objective of reference measurement laboratories.

Three reference measurement procedures and the basis for the development of two additional reference measurement procedures are presented in this study:

- reference measurement procedure for determination of theophylline [II, III]
- reference measurement procedure for determination of HbA_{1c}
- reference measurement procedure for determination of digoxin and digitoxin [V, VI]
- basis for a reference measurement procedure for determination of sirolimus, everolimus, tacrolimus, cyclosporin A [VII], and glibenclamide [I]

An isotope dilution GC-MS method for determination of theophylline was optimized and further developed to be proposed as a reference measurement procedure.
In serum samples spiked with theophylline in therapeutic range the mean recovery was 99.78 % with a bias 0.22 %. The imprecision in commercial control material was between 0.527 % and 1.14 %, respectively. To verify the analytical specificity and accuracy of the GC- IDMS method a HPLC-diode array method was developed for comparison and confirmation of results. The HPLC-diode array method allows the separation of all dimethylxanthines and related compounds, that may cause interferences: theophylline, theobromine, paraxanthine, paracetamol, caffeine, and uric acid. The good correlation between the results obtained from GC-IDMS and HPLC-diode array measurements confirms the specificity of GC-IDMS measurement procedures.
The GC-IDMS reference measurement procedure is actually used for setting target values in EQAS for theophylline.

The IFCC reference measurement procedure for HbA_{1c} is a LC-MS method with quantification by external standards. This method has been optimized and evaluated. The long-term stability of several LC-MS measuring instruments and their effect on the quality of the results has been evaluated. The HPLC conditions were optimized by changing the composition of the buffer, the

gradient elution profile and using a splitting system. By the use of the appropriate LC-MS equipment and the optimized HPLC conditions successful measurements were made in several international intercomparison studies of the IFCC Working Group on HbA_{1c} Standardisation. The method had a bias between 0.1 % and 0.65 %. The imprecision of the procedure was below 2.0 %, which was well within the acceptability criteria for the reference measurement laboratories of the IFCC network for HbA_{1c}. The optimized reference measurement procedure for determination of HbA_{1c} is actually used for setting target values in our external quality assessment schemes at INSTAND e.V. The reference laboratory at INSTAND e.V. is accepted as an IFCC-Reference Laboratory for HbA_{1c} (one of less than 20 in the world).

An important progress in the development of LC-MS methods as reference measurement procedures for the determination of digoxin, digitoxin and the immunosuppressive drugs sirolimus, everolimus, tacrolimus and cyclosporin A was the discovery, that these compounds are forming adducts with Cs^+ ions, which are detectable both in SIM and in MRM mode. By adding an excess of Cs^+ ions to the elution buffer Cs^+ adducts could be obtained. In MRM Cs^+ is measurable as product ion of the Cs^+ adducts of the drugs. This new principle in HPLC-mass spectrometry analysis results in a higher analytical specificity and better signal-to-noise ratio, by

- replacement of undesirable adduct formations with Na^+ and K^+
- a stabilizing effect of the Cs^+ adduct on undesirable in source fragmentation
- a clearly defined fragmentation pattern in MRM transmission

Our reference measurement procedure for the determination of digoxin and digitoxin is an isotope dilution LC-MS analysis with electrospray ionisation after a liquid-liquid extraction. The isotope-labelling of the pure analytes was performed in our laboratory.
In the SIM mode the Cs^+ adduct of deuterated and native digoxin and digitoxin has been measured. The LC-MS measurement procedure was further modified to a LC-MS/MS method. The mean recovery in spiked serum samples in the therapeutic range for digoxin was 99.95 % and for digitoxin 100.2 %, the bias was 0.05 % and 0.2 % respectively. In the SIM mode the imprecision was between 1.26 % and 2.91 % for digoxin and 0.79 % and 2.00 % for digitoxin. In EQAS samples the corresponding coefficients of variation were between 1.26 % and 3.18 % and 1.21 % and 1.46 %, respectively. In the MRM mode the coefficients of variation were between 1.65 % and 2.28 % for digoxin and 1.13 % and 1.44 % for digitoxin.

The newly developed reference measurement procedure for determination of digoxin and digitoxin is used for setting target values in External Quality Assessment Schemes.

A new basis was developed for a reference measurement procedure for the determination of immunosuppressive drugs. The immunosuppressive drugs sirolimus, everolimus, tacrolimus and cyclosporin A were measured as Cs^+ adducts in the MRM mode. Analogues of the drugs were used as internal standards. In contrast to existing routine methods the immunosuppressive drugs could be chromatographically separated using a ternary gradient profile. In standard solutions the coefficient of variation was between 2.11 % and 2.57 %. On the basis of this principle of measurement the further development of the method to a reference measurement procedure is on the way. For a better quantification we are producing stable isotope-labelled internal standards in our laboratory.

The analytical basis for a reference measurement procedure for glibenclamide and other sulfonylureas is established. The development of a reference measurement procedure for glibenclamide for setting target values in EQAS is an actual project at INSTAND e.V.

The study demonstrates how reference measurement procedures are developed, improved and evaluated and shows, that reference measurement procedures are thoroughly investigated measurement procedures enclosed in a dynamic process of optimization, leading to traceable and reliable results on highest metrological order. Providing and using reference measurement procedures improves the quality of the medicine laboratory performance.

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