DEVELOPMENT AND OPTIMIZATION OF REFERENCE MEASUREMENT PROCEDURES

PhD thesis

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2006

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)

[VII] **Kaiser, P.**, Akerboom, T., Wood, W.G., Reinauer, H. A new approach for the determination of immunosuppressive drugs using HPLC-MS/MS and Cs^+ adducts. Ger.Med.Sci.4: Doc01 (2006)

ACKNOWLEDGEMENTS

I would like to thank my supervisors Prof. Dr. Dux and Prof. Dr. Reinauer for giving me the topic and opportunity to perform this study and for all their help during these years.

I am also grateful to Prof. Dr. Wood for all his useful advice and support during the preparation of this thesis.

My thanks are due to my colleagues in the reference laboratory at INSTAND e.V. Udo Kramer, Michael Kress, Dieane Meißner, Ulrike Speith, and Dr. Akerboom for their valuable suggestions and support, and I express my thanks to Karin Schumann for help in preparation of documents and presentation for publications and lectures.

Finally I like to thank my husband Dipl.-Ing. Christian Kaiser for all his support and understanding patience.

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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.

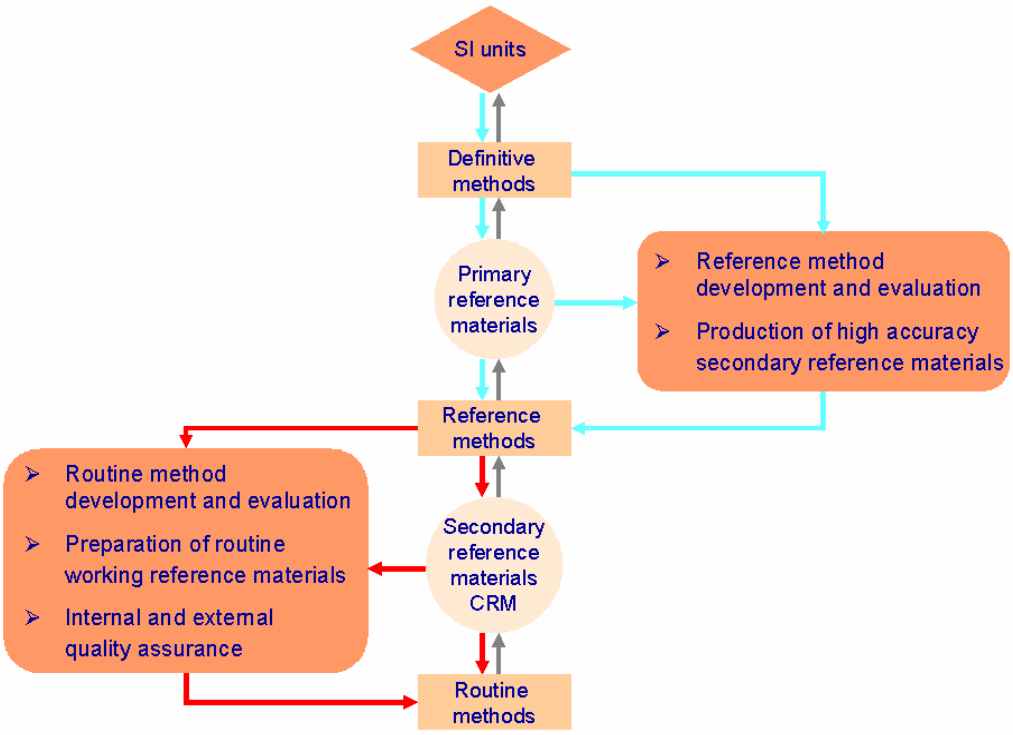


Figure 1 **Flowchart of the traceability of reference methods and routine methods to the highest metrological order that is the SI units**

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-methylcyclo-hexyl)-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-methyl-cyclohexyl)-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™ ESI source with TurboIon Spray™ 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-$^{2}H_3$	900.3	900.3	132.9
digoxin	913.3	913.3	132.9
digoxin-$^{2}H_3$	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.

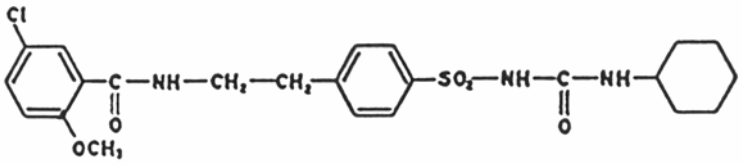
The uncertainty of measurement was determined by repeated measurements of a sample. The measurement uncertainty is generally caused by different factors. These factors have been well determined in our laboratory for the accreditation process. A main component of the total measurement uncertainty is the 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**

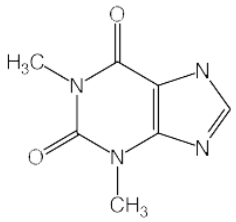
In the early 1990s mainly immunological methods were used for therapeutic drug monitoring of glibenclamide [13,14]. The immunological methods were sensitive for the detection and quantification of glibenclamide, but they had a poor analytical specificity. They were not able to distinguish the drug from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

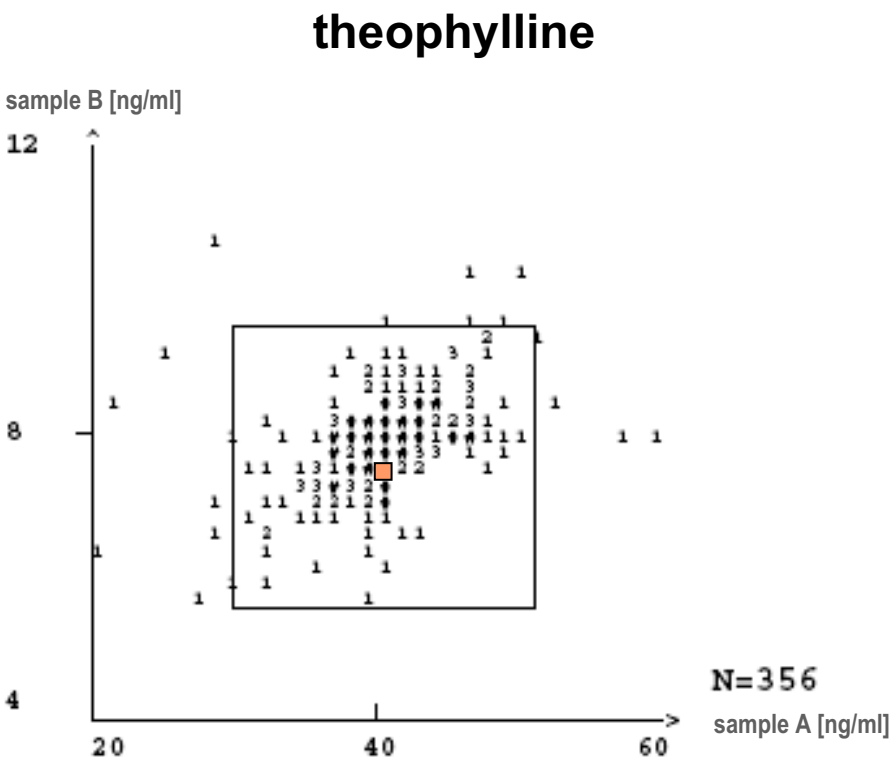
The existing routine method for the determination of theophylline in serum [20] was optimized and further developed to be established as reference measurement procedure. The principle of the method is a gas chromatography, isotope dilution-mass spectrometry (GC-IDMS) procedure. The internal standard 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.

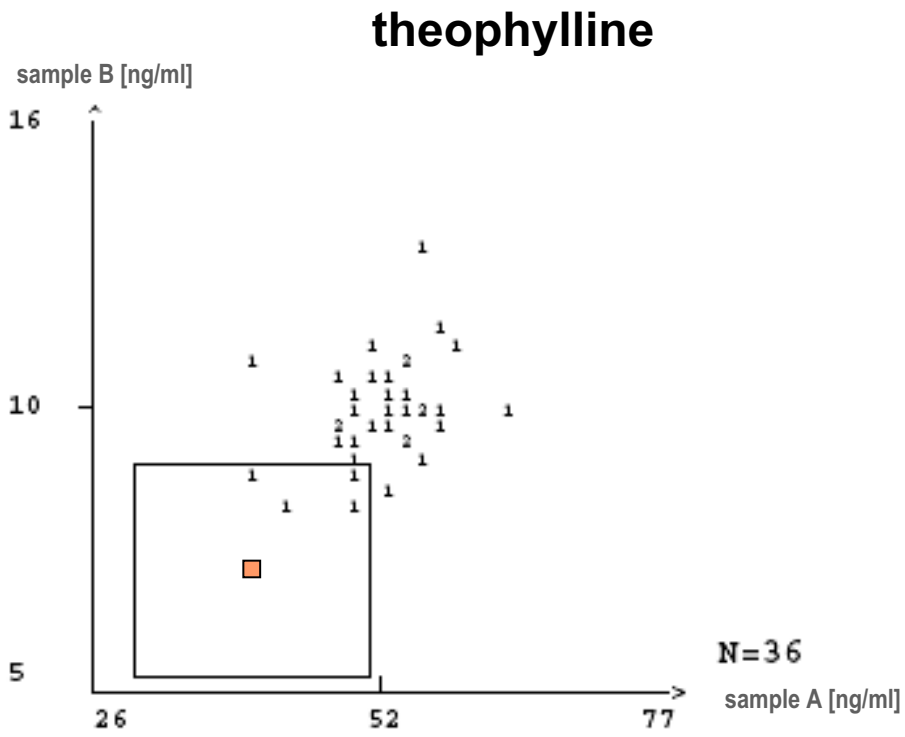
Our reference measurement procedure for theophylline is used for setting target value in the EQAS. An example for the evaluation of an EQA scheme for theophylline is shown in figure 4. The acceptability range for routine measurement of the participants of ± 30 % for theophylline is given by the Guideline 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.

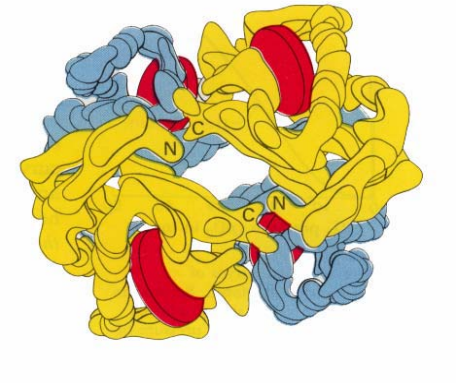


Figure 6

Model of the quarternary structure of haemoglobin

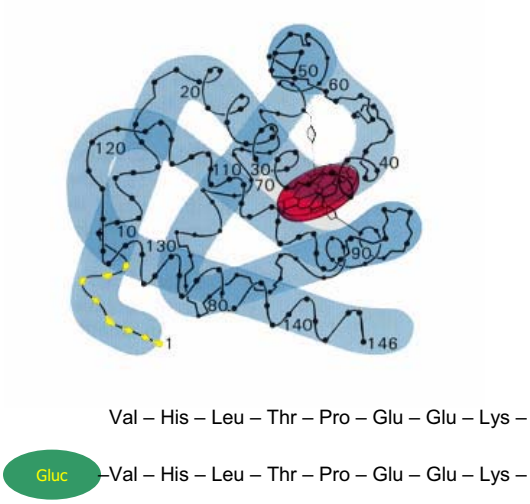


Figure 7

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).

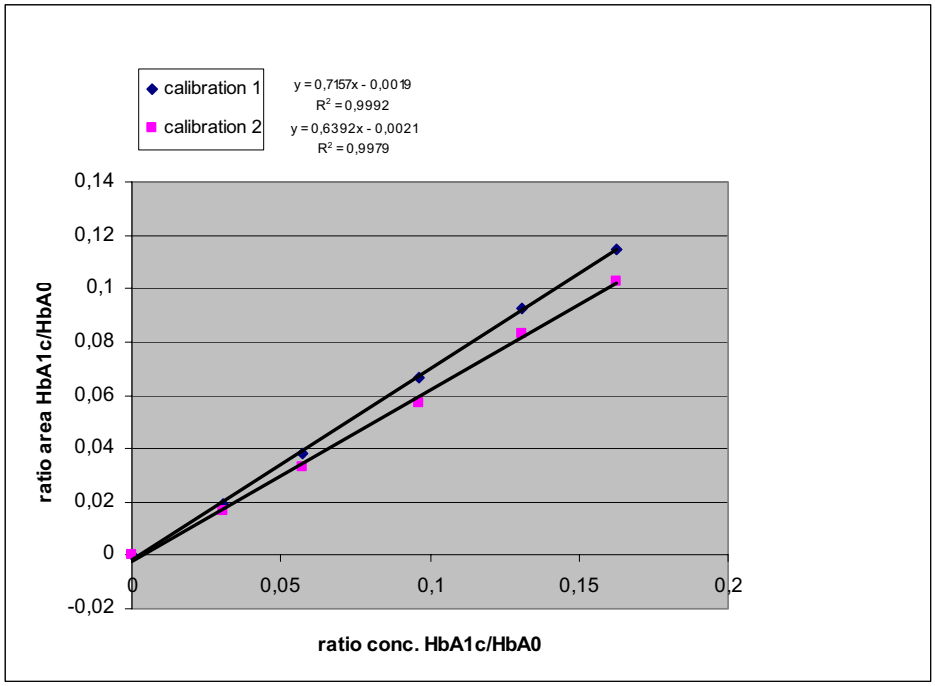


Figure 8 **Calibration curves at the beginning (calibration 1) and at the end (calibration 2) of a measuring sequence (24 h) for HbA_{1c} performed by using a MSQ mass spectrometer**

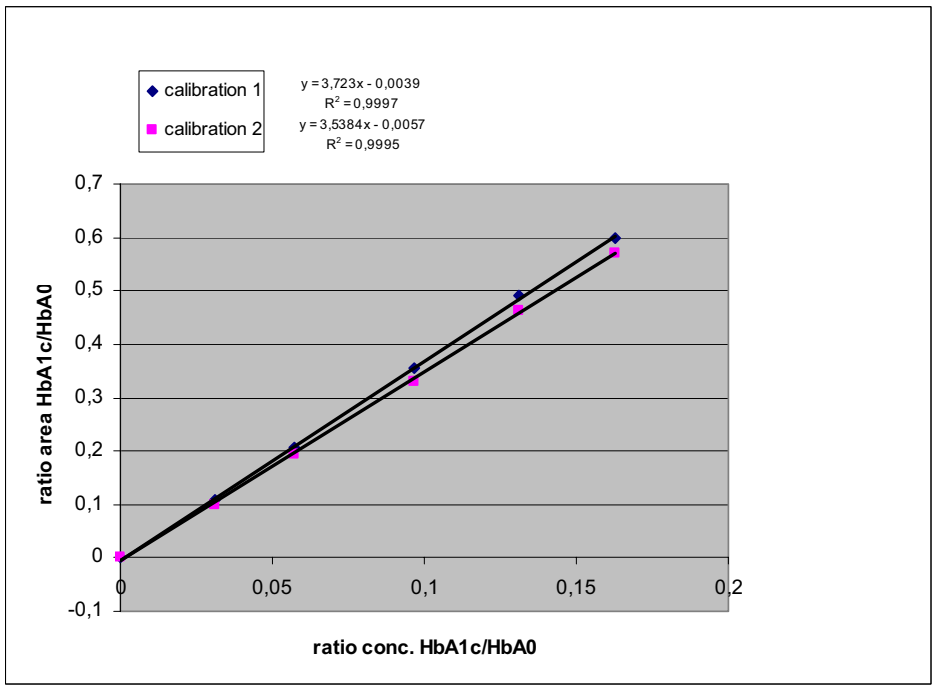


Figure 9 **Calibration curves at the beginning (calibration 1) and at the end (calibration 2) of a measuring sequence (24 h) for HbA_{1c} performed by using a Quantum ultra mass spectrometer**

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).

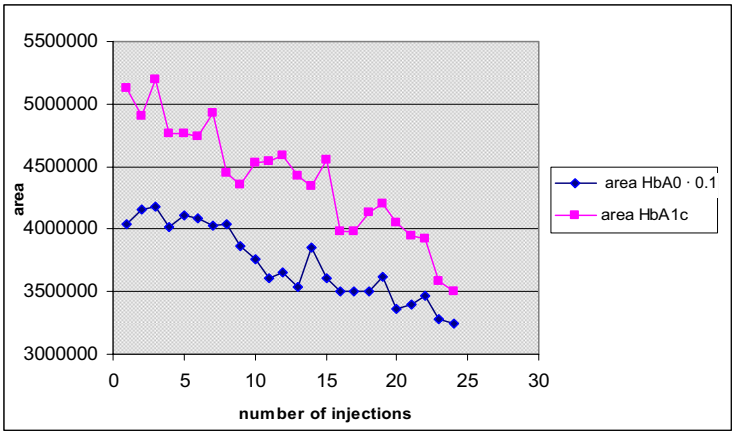


Figure 10 **Stability-test of absolute signal intensities for HbA_{1c} and HbA_0 by repeated measurements of a haemolysate performed by using a MSQ mass spectrometer**

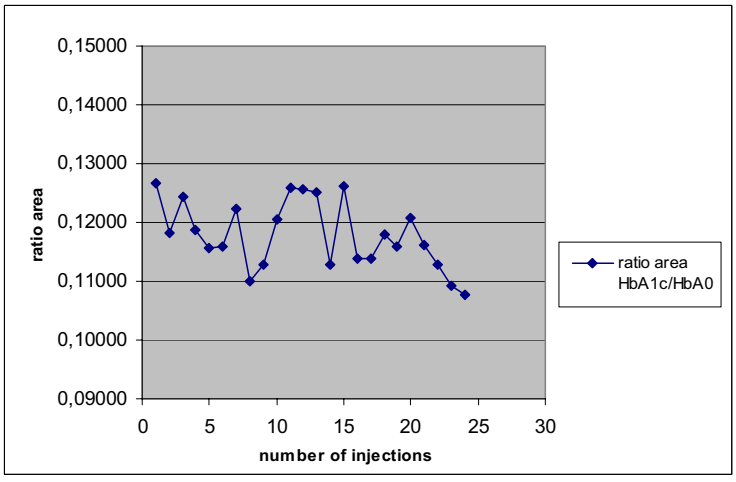


Figure 11 **Stability-test of the peak area ratio for HbA_{1c} and HbA_0 by repeated measurements of a haemolysate performed by using a MSQ mass spectrometer**

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).

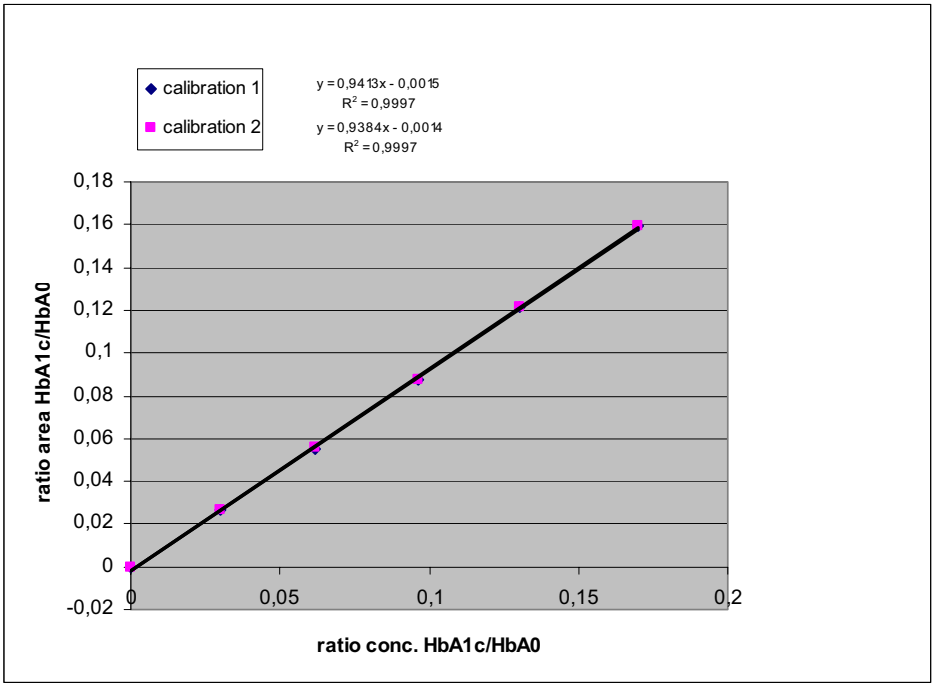


Figure 12 **Calibration curves at the beginning (calibration 1) and at the end (calibration 2) of a measuring sequence (24 h) for HbA_{1c} performed by using an API 4000 mass spectrometer**

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

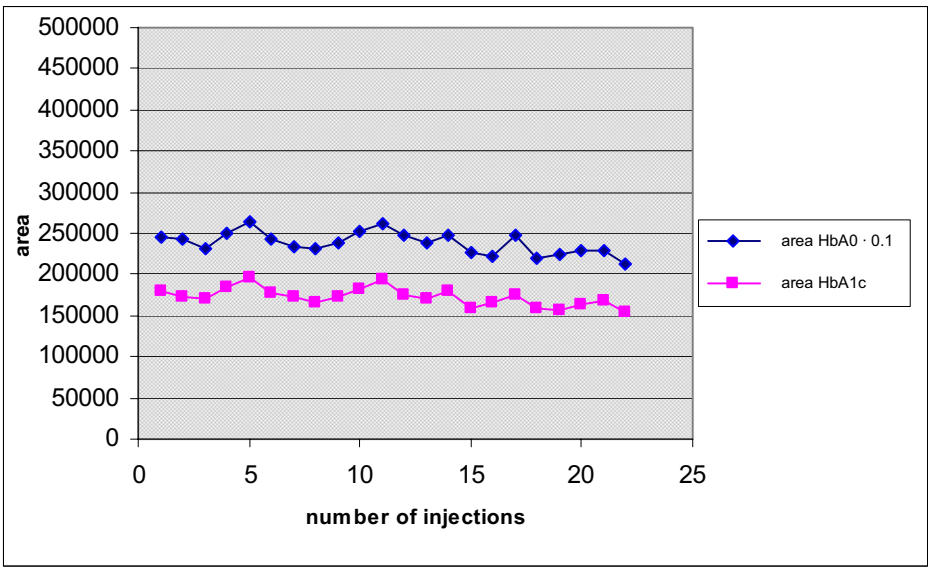


Figure 13 **Stability-test of absolute signal intensities for HbA_{1c} and HbA_0 by repeated measurements of a haemolysate performed by using an API 4000 mass spectrometer**

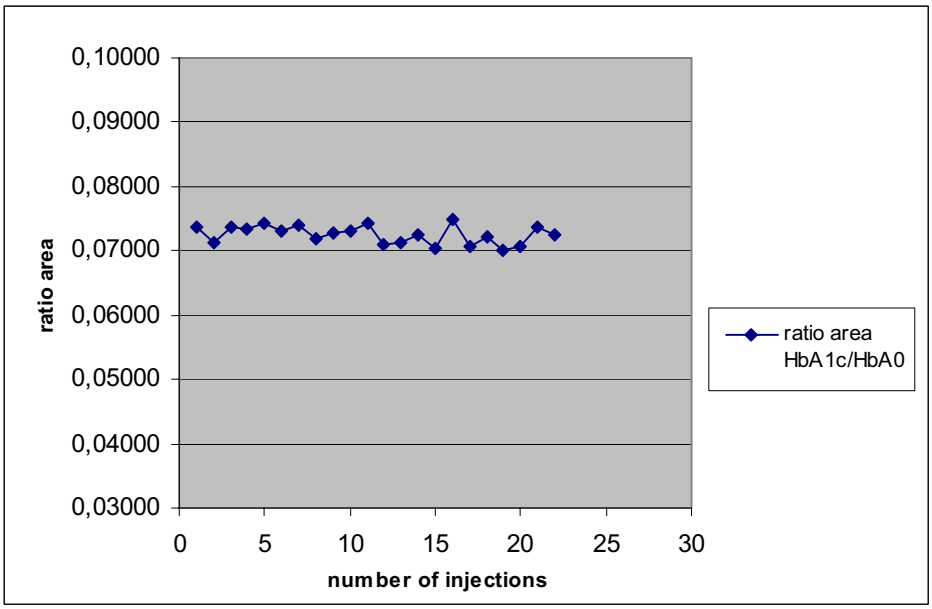


Figure 14 **Stability-test of the peak area ratio for HbA_{1c} and HbA_0 by repeated measurements of a haemolysate performed by using an API 4000 mass spectrometer**

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.

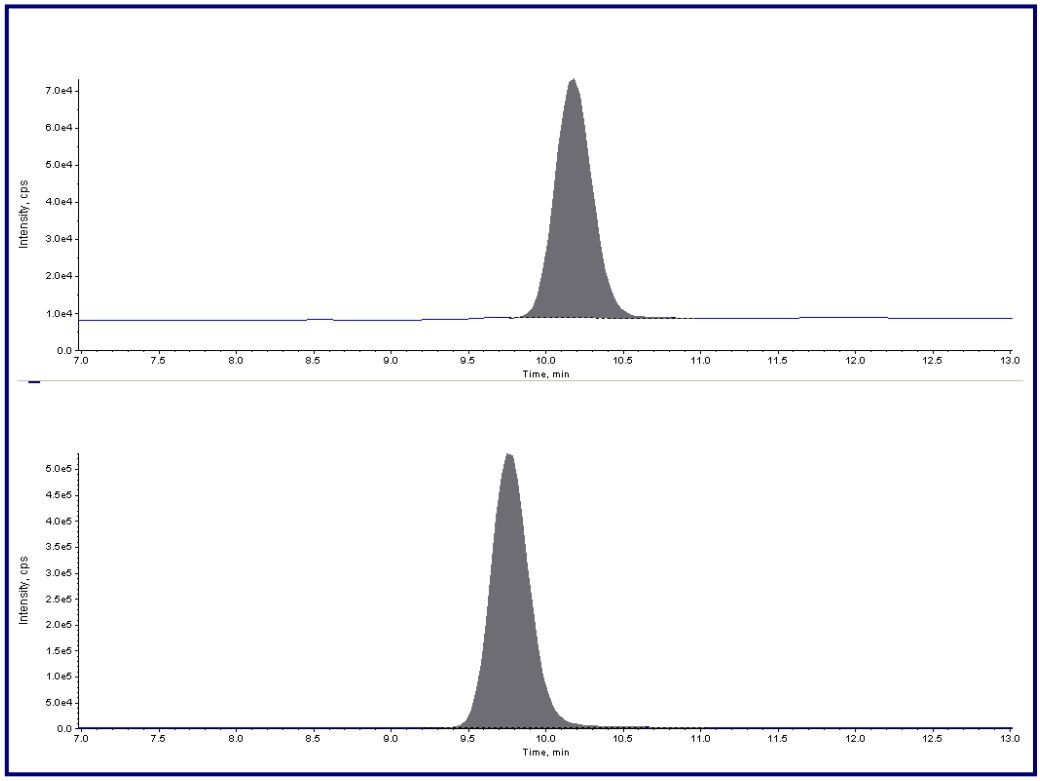


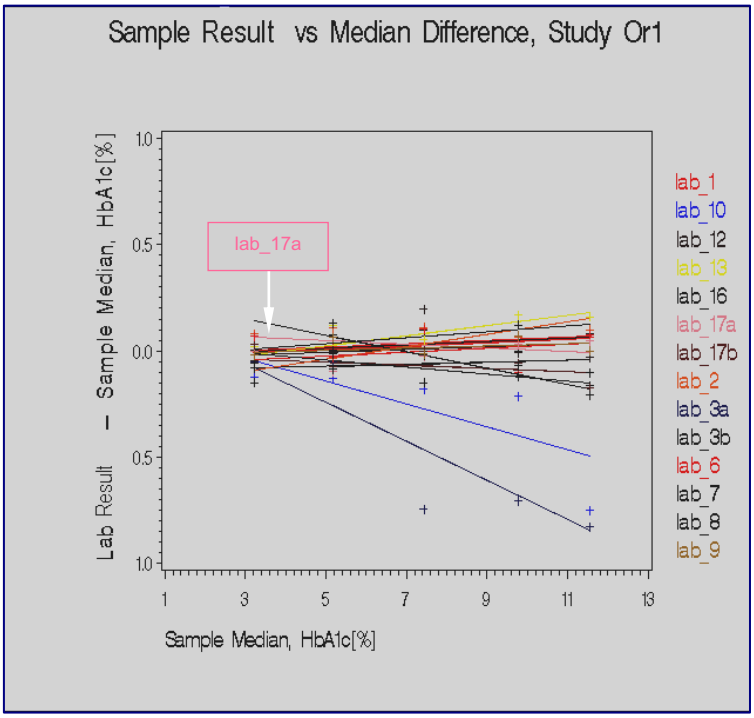
Figure 15 **HPLC-MS chromatogram of a haemolysate sample containing 8.22 % HbA_{1c}**
upper trace: ß-N-terminal hexapeptide of HbA_{1c} (RT 10.2 min, m/z 429.3 amu)
lower trace: ß-N-terminal hexapeptide of HbA_0 (RT 9.8 min, m/z 348.3 amu)

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.



sample median: overall median value of all participants (for each sample)

lab median: median value of a single laboratory (for each sample)

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

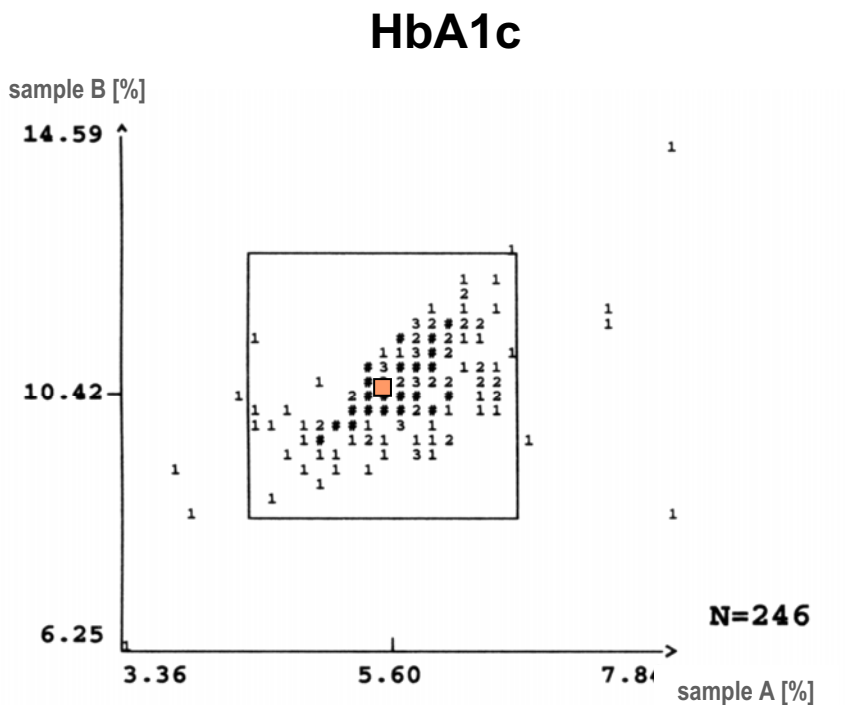
Figure 16 **Evaluation of an international intercomparison study of the IFCC Network Standardisation of HbA_{1c} (Results of INSTAND e.V.: lab_17a)**

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)**

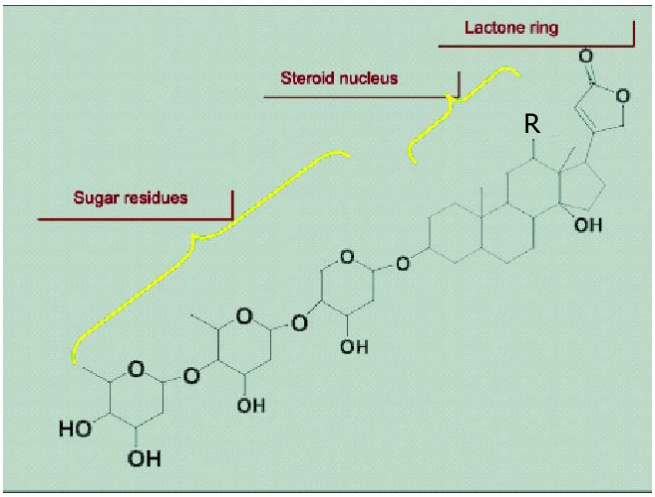
** converted from IFCC to NGSP values with the following formula:*

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.

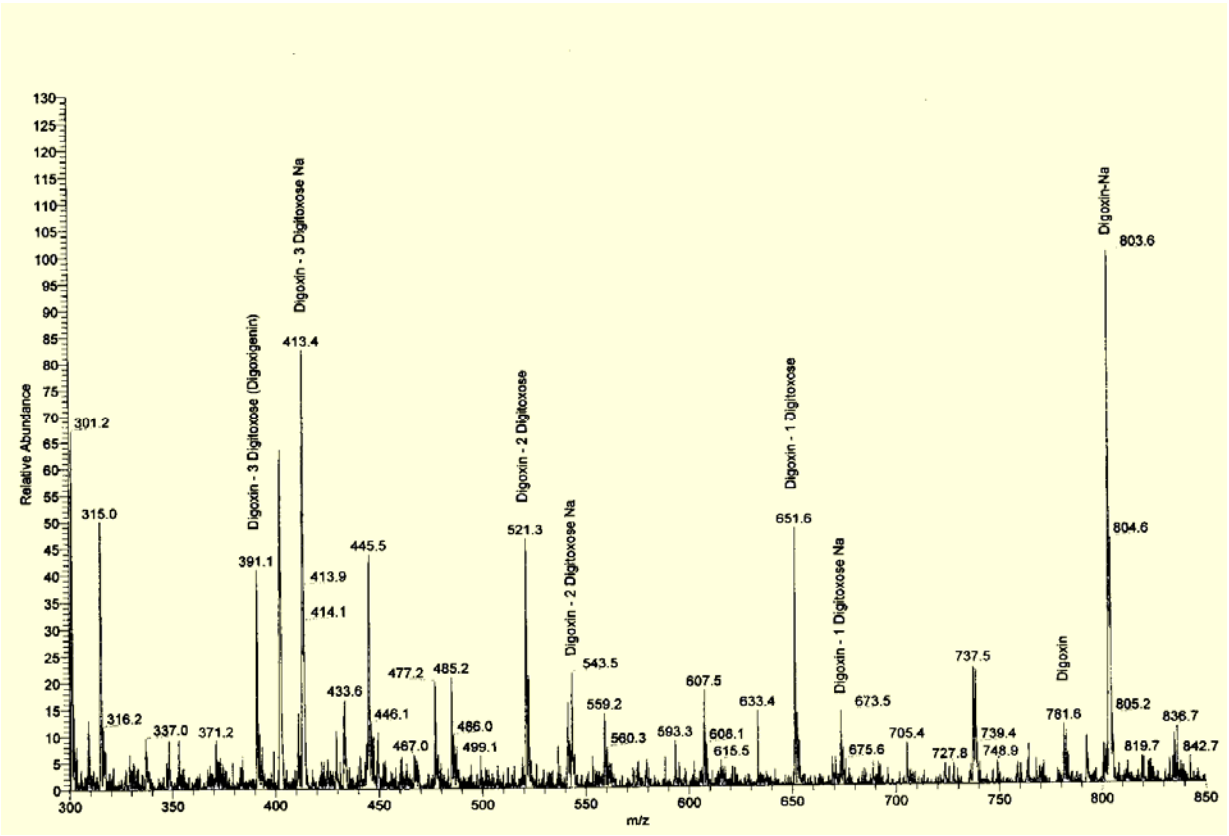


Figure 19 **Q1 scan of a digoxin standard solution (without Cs^+ ions)**

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.

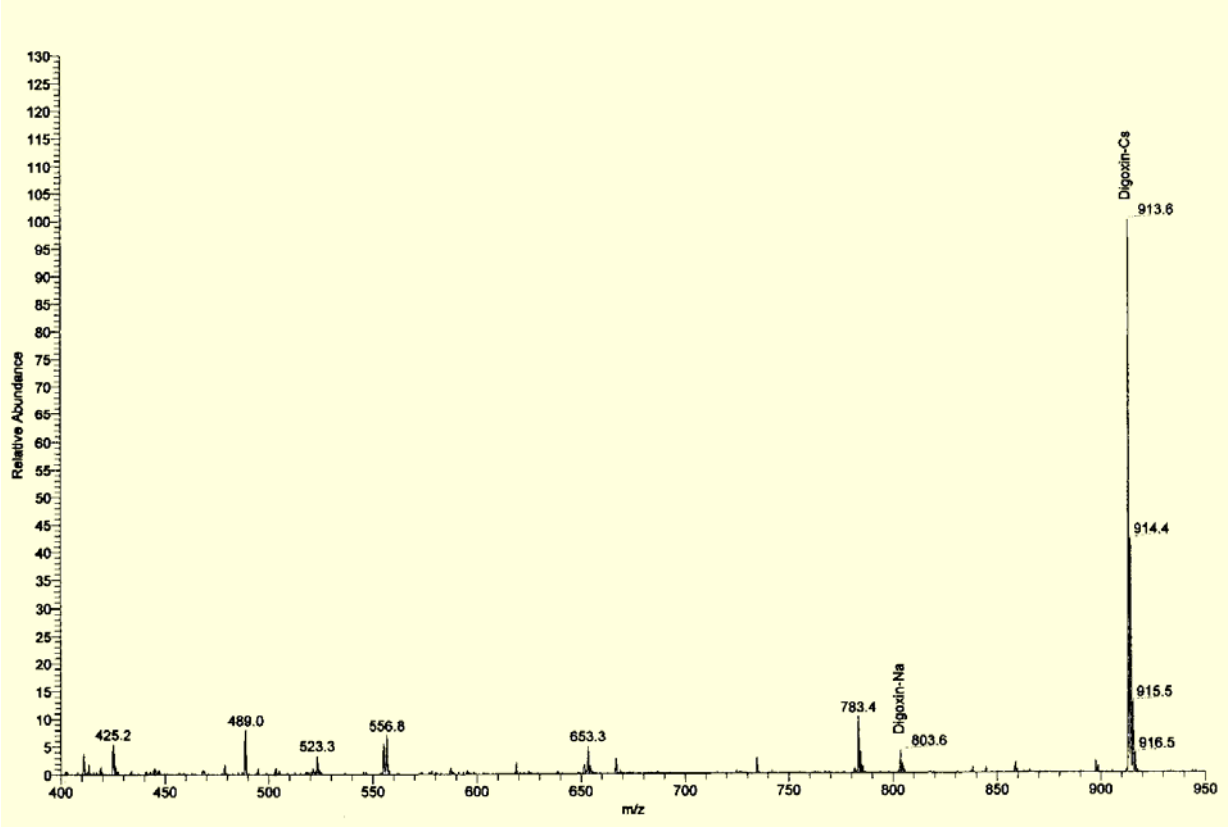


Figure 20 **Q1 scan of a digoxin standard solution (with Cs^+ ions)**

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).

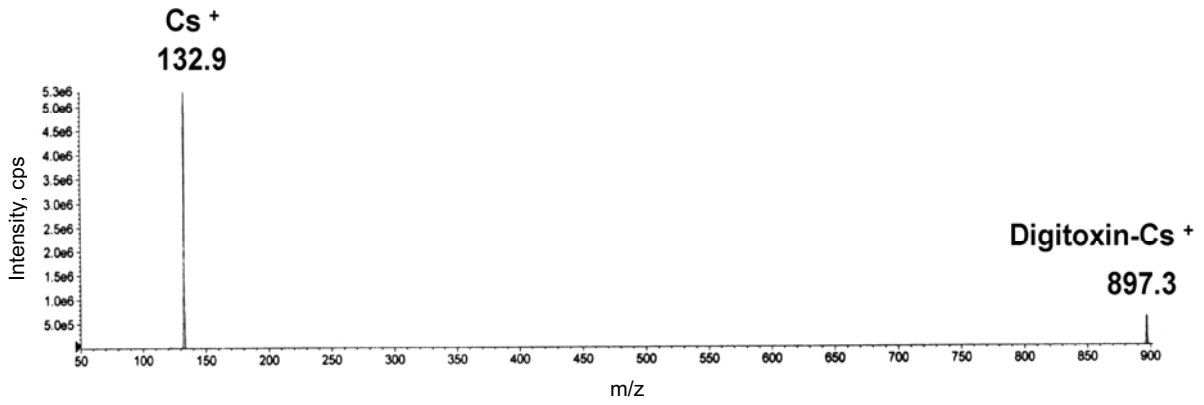


Figure 21 **Product Ion scan of the Cs^+ adduct of digitoxin (m/z 897.3 amu) in a digitoxin standard solution**

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].

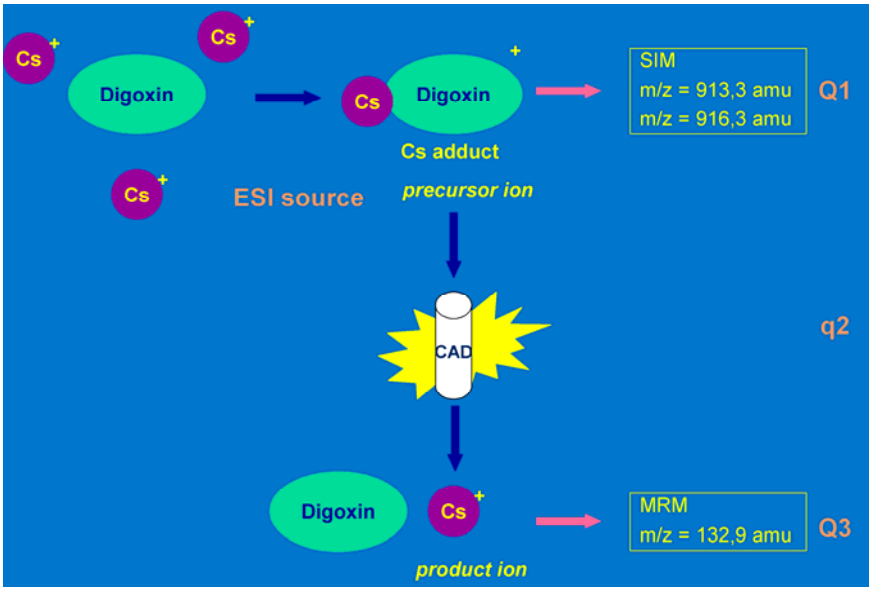


Figure 22 **Scheme of the principle of measurement of Cs^+ adducts SIM and MRM**
(by kind permission of Clin.Lab.)

For serum samples a special extraction procedure was developed as described in [V,VI].

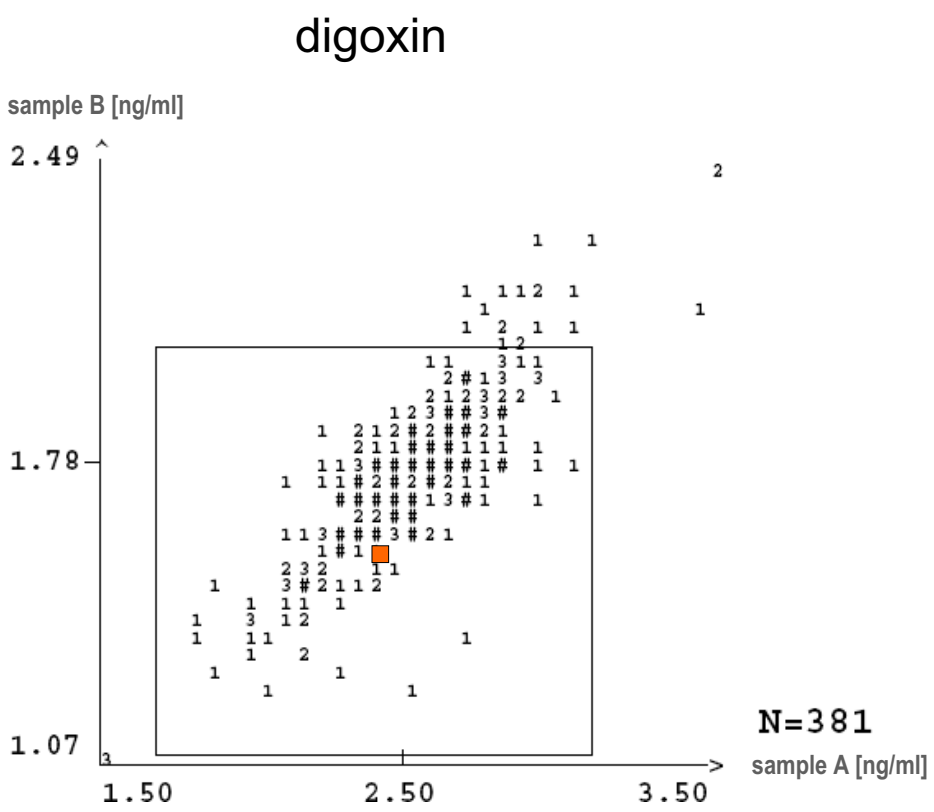
As internal standards stable isotope-labelled compounds were synthesized in our laboratory with modification of the original procedure [48,V,VI]. To obtain high accuracy and a good precision standard solutions and serum samples were quantified gravimetrically during sample preparation. The volume of 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.

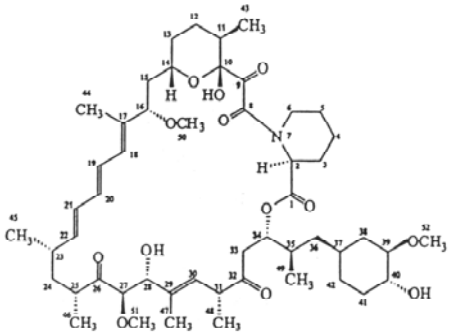


Figure 24 **Structure of sirolimus**

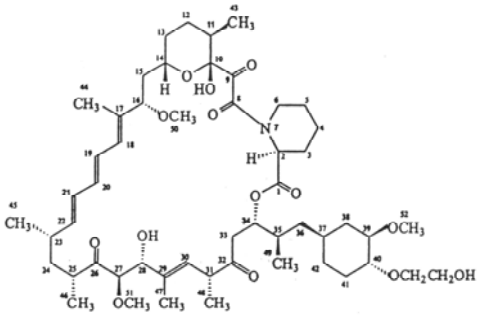


Figure 25 **Structure of everolimus**

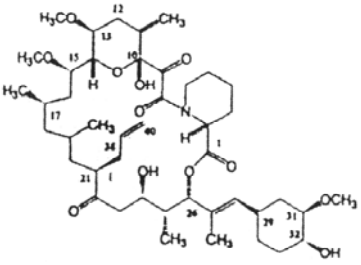


Figure 26 **Structure of tacrolimus**

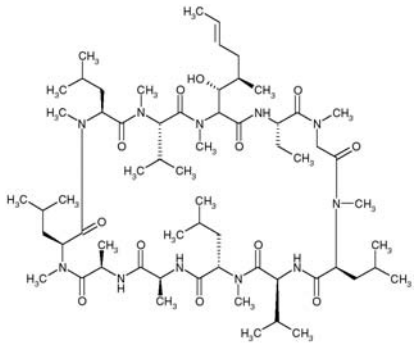


Figure 27 **Structure of cyclosporin A**

	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).

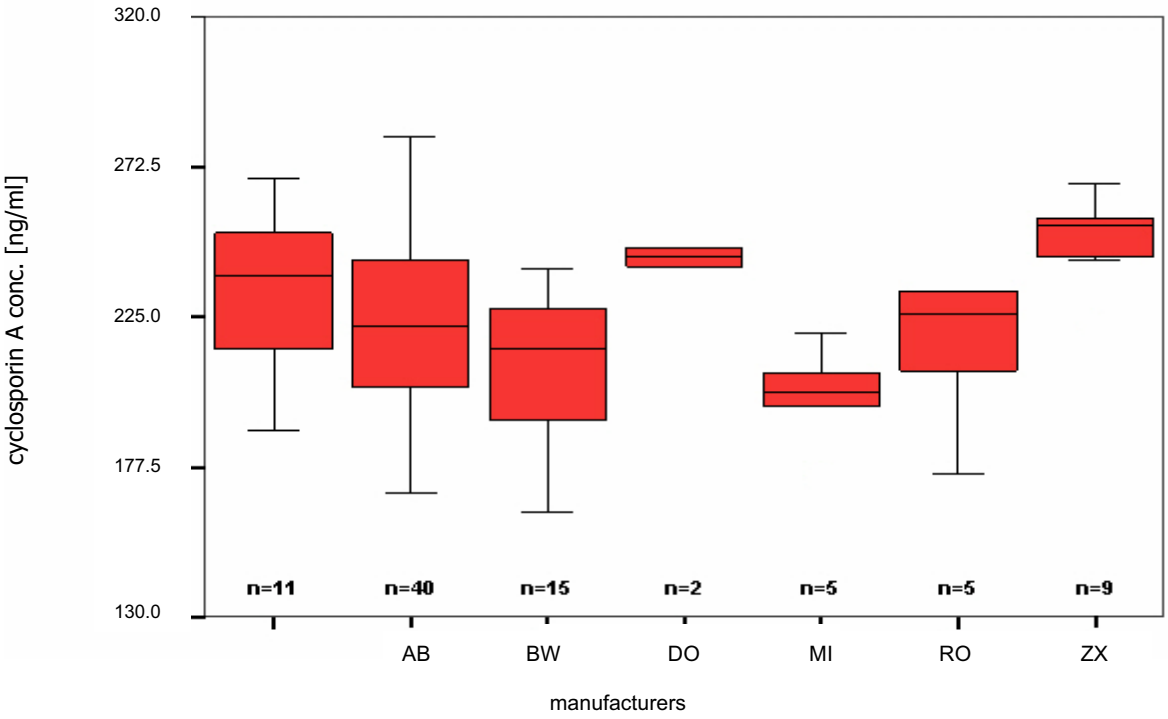


Figure 28 **External quality assessment scheme for cyclosporin A, run by INSTAND e.V (2005). Box-plots of the results of participating laboratories using different analytical systems**

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).

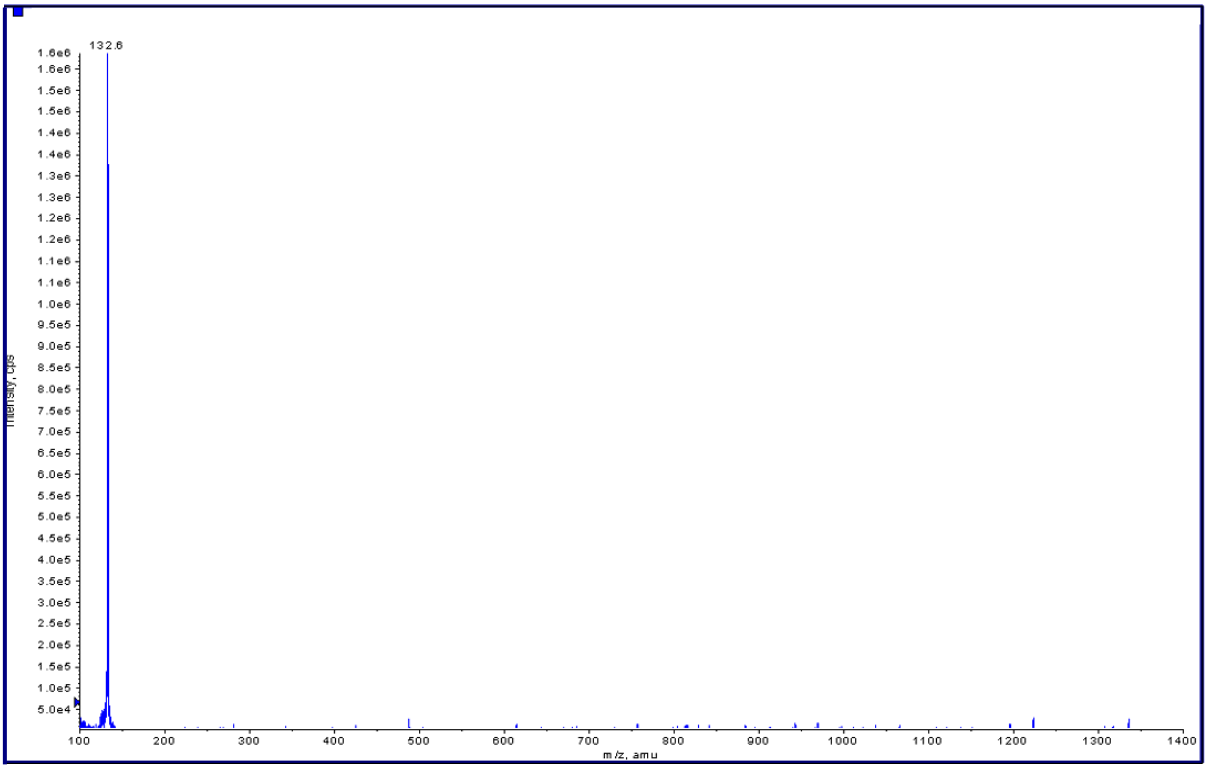


Figure 29 **Product Ion scan of a cyclosporin A standard solution (1 µg/ml in eluent B) infused at a flow rate of 10 µl/min**

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).

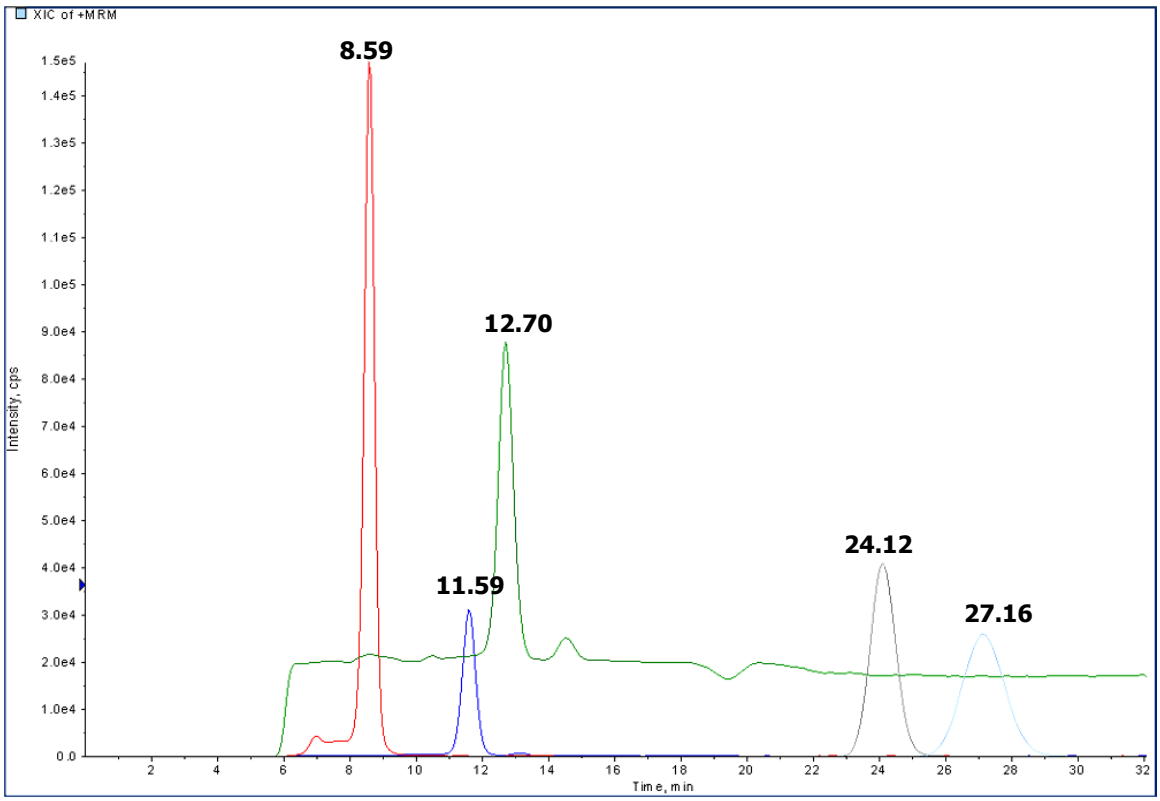


Figure 30 **HPLC-MS/MS chromatogram (overlaid graphs of single mass traces) of a standard mixture of tacrolimus (8.59 min), sirolimus (11.59 min), everolimus (12.70 min), cyclosporin A (24.12 min) and cyclosporin D (27.16 min), 10 µl (1 µg/ml) injected**

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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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)

Journal of Cerebral Blood Flow and Metabolism
15:587–594 © 1995 The International Society of Cerebral Blood Flow and Metabolism
Published by Raven Press, Ltd., New York

Ion Channel Involvement in Anoxic Depolarization Induced by Cardiac Arrest in Rat Brain

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Summary: Anoxic depolarization (AD) and failure of ion homeostasis play an important role in ischemia-induced neuronal injury. In the present study, different drugs with known ion-channel-modulating properties were examined for their ability to interfere with cardiac-arrest-elicited AD and with the changes in the extracellular ion activity in rat brain. Our results indicate that only drugs primarily blocking membrane Na^+ permeability (NBQX, R56865, and flunarizine) delayed the occurrence of AD, while compounds affecting cellular Ca^{2+} load (MK-801 and nimodipine) did not influence the latency time. The ischemia-induced $[Na^+]_e$ reduction was attenuated by R56865. Blockade of the ATP-sensitive K^+ channels with glibenclamide reduced the $[K^+]_e$ increase upon ischemia, indicating an involvement of the K_{ATP} channels in ischemia-induced K^+ efflux. The K_{ATP} channel opener cromakalim did not affect the AD or the $[K^+]_e$ concentration. The ischemia-induced rapid decline of extracellular calcium was attenuated by receptor-operated Ca^{2+} channel blockers MK-801 and NBQX, but not by the voltage-operated Ca^{2+} channel blocker nimodipine, R56865, and flunarizine. **Key Words:** Anoxic depolarization—Calcium—Cerebral ischemia—Cerebral pharmacology—Ion homeostasis.

Anoxic depolarization (AD) is a well known phenomenon occurring if tissue energy supply ceases for a certain time. The normal compartmentation of ions between the intra- and extracellular space breaks down and the cerebral direct current (DC) potential exhibits a sudden negative shift (Hansen, 1985). Previous studies have shown that the severity of the ischemic neuronal injury was significantly correlated with the time period during which the brain tissue was in a depolarized state (Balestrino et al., 1989; Somjen et al., 1990; Gill et al., 1992). Some drugs beneficial in the treatment of cerebral ischemia are able to delay the occurrence of AD (Balestrino and Somjen, 1986; Höller et al., 1986). Therefore, the latency from onset of ischemia until occurrence of AD has been considered an indicator for the tissue resistance against ischemic insults (Bures and Buresova, 1957).

However, for an effective search of antiischemic drugs and to get a plausible explanation of the drug properties needed to delay the occurrence of AD, better understanding of this phenomenon is required. A primary event upon cerebral ischemia preceding and coinciding with AD is the change in membrane ion permeability. The present study is an attempt to investigate which types of ion channels are involved before, during, and after the negative DC shift and whether the ischemia-induced ion movements influence the latency time from onset of ischemia until the occurrence of AD. For this purpose, we examined the effects of selected compounds with known ion-channel-modulating properties in a rat model of global cerebral ischemia induced by cardiac arrest.

Received July 8, 1994; final revision received November 9, 1994; accepted November 11, 1994.

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Abbreviations used: AD, anoxic depolarization; AMPA; DC, direct current; NMDA, *N*-methyl-D-aspartate; TTX, tetrodotoxin.

MATERIALS AND METHODS

Animal preparation

Male Wistar rats (230–280 g body wt) were anesthetized by intraperitoneal injection of urethane (1.2–2.0 g/kg). Femoral artery and vein were cannulated to monitor arterial blood pressure, collect blood samples, and apply drugs. The rectal temperature was kept at 37.5°C by means of a warm water blanket surrounding the animal body. The animals were immobilized by intravenous injection of succinylcholine and artificially ventilated with O_2/N_2 (30:70) gas mixture. A hole of 3-mm diameter was drilled in the parietal skull. A double- or triple-barrel glass

electrode was inserted into the cortex at 1-mm depth. In the majority of the experiments, the animals received a 10-min lasting intravenous infusion of drugs or the same volume (≈0.5 ml) of solvents. Glibenclamide and cromakalim were applied locally for 60 min on the cortical surface by using a superfusion technique. In this case, the drugs were dissolved in artificial CSF at a concentration of 50 μ*M* and the brain surface exposed by the craniectomy was covered with this solution. A pump delivered continuously 1 μl/min fresh drug solution to the brain surface to compensate for evaporation. The control animals received CSF superfusion without drug. In Table 1 the examined compounds, the dosage used, and the corresponding solvents are presented. Cardiac arrest was induced by intravenous injection of saturated $MgCl_2$ 30 min after the beginning of the intravenous drug treatment or 60 min after the beginning of the superfusion.

Chemicals

NBQX was a gift from Novo Nordisk (Bagsvaerd, Denmark). MK-801 and glibenclamide were purchased from Research Biochemicals (Natick, MA, U.S.A.). Nimodipine was obtained from Bayer AG (Leverkusen, Germany) and cromakalim from Beecham Pharmaceuticals (Surrey, U.K.). R56865 and flunarizine were delivered by the Janssen Research Foundation (Beerse, Belgium).

The organic ionophores for the measurement of ion activities with ion-sensitive microelectrodes were purchased from Fluka (Neu-Ulm, Germany). Other chemicals and reagents, if not otherwise stated, were obtained from Merck (Darmstadt, Germany).

Monitoring techniques

The arterial Po_2, Pco_2, and blood pH values were monitored by means of a blood gas analyzer (Eschweiler, Kiel, Germany). Glibenclamide concentration in the cortical tissues was determined by a modified HPLC method described by Wahlin-Boll and Melander (1979). The ion-sensitive barrels of the microelectrode were filled alternatively with different organic ionophores. The cortical DC potential was recorded as the potential difference between the reference barrel of the microelectrode and an extracranially located Ag/AgCl electrode. The potential difference between the ion-sensitive and the reference barrels reflected the extracellular ion activity. The microelectrodes were calibrated in artificial CSFs containing different concentrations of the corresponding ions before and after each individual experiment.

The latency (s) to AD was assessed by measuring the time period from induction of the cardiac arrest to the sudden negative deflection of the extracellular DC potential. The measurement of the amplitude (mV) of AD is illustrated in Fig. 1. The extracellular K^+ concentration (m*M*) from which $[K^+]_e$ starts to rise rapidly during ischemia was defined as K^+ threshold (arrow in Fig. 1). The maximal $[K^+]_e$ concentration and the final (lowest) $[Ca^{2+}]_e$ and $[Na^+]_e$ concentrations were determined ~5 min after occurrence of AD.

Statistics

The values measured in the drug-treated groups were compared with the control values by using one-way factorial analysis of variance and sequential Dunnett *t* test for multiple comparison of independent samples (two tailed). $p < 0.05$ was considered significant.

RESULTS

Control animals

Control animals treated individually with different solvents did not show any significant differences in all measured parameters. Therefore, the results from these animals were pooled into one control group for further analysis.

General physiological variables

The arterial blood Po_2, Pco_2, and pH were kept within the normal range during the experiments (Table 2). At the dosages used, nimodipine, flunarizine, and R56865 reduced the arterial blood pressure, whereas NBQX and systemic application of glibenclamide increased the blood pressure slightly (Table 2). Heart rate was significantly accelerated by NBQX and slowed down by R56865 and flunarizine (Table 2). The ECG morphology was normal and unchanged.

Responses of cortical DC potential to cerebral ischemia

After an initial small positive shift, the cortical DC potential exhibited a sudden negative deflection (AD) upon cerebral ischemia (Fig. 1). In the animals receiving solvents, the latency from the induction of

TABLE 1. *Overview of drugs used*

Compound	Dosage	Solvent
Voltage-dependent cation channel modulators		
Nimodipine (L-type Ca^{2+} blocker)	50 μg/kg i.v.	0.6% polyethylene glycol 400
Flunarizine (Na^+/Ca^{2+} modulator)	5 mg/kg i.v.	10% cyclodextrin
R56865 (Na^+/Ca^{2+} modulator)	5 mg/kg i.v.	10% cyclodextrin
Glutamate antagonists		
MK-801 (NMDA type)	2 mg/kg i.v.	0.9% NaCl
NBQX (AMPA type)	30 mg or 60 mg/kg i.v.	Aqua dest
ATP-sensitive potassium channel modulators		
Glibenclamide (blocker)	5 mg/kg i.v.	10% cyclodextrin
Glibenclamide (blocker)	50 μ*M* superfusion	Artificial CSF
Cromakalim (opener)	50 μ*M* superfusion	Artificial CSF

For abbreviations see the text.

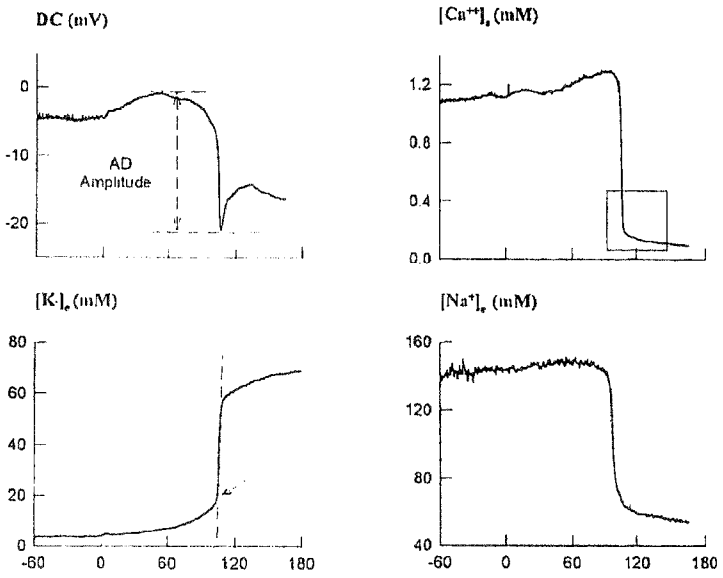

FIG. 1. Representative traces of the direct current (DC) signal **(top left)**, the extracellular cortical calcium activity **(top right)**, the extracellular cortical potassium activity **(bottom left)**, and the extracellular cortical sodium activity **(bottom right)** before (−60 to 0 s; *x*-axis) and after (0 to 180 s; *x*-axis) induction of cardiac arrest. The arrow in the $[K^+]_e$ trace indicates K^+ threshold. The $[Ca^{2+}]_e$ recordings marked by the box are displayed in the Fig. 2 with high magnification.

cardiac arrest until the DC negative shift ranged from 80 to 150 s (mean 130.2 s). Table 3 summarizes the effects of different drugs on ischemia-induced AD. Three compounds significantly prolonged the latency to AD: NBQX by 34%, flunarizine by 24%, and R56865 by 26%. Combined treatment with NBQX plus MK-801 showed a similar result (30% increase) as when NBQX was used alone. Systemic application of 5 mg/kg glibenclamide shortened the latency to AD by 25%. This effect was not observed when glibenclamide was applied locally, although both kinds of treatment resulted in a similar drug tissue concentration (15.6 ng/g in intravenous and 12.5 ng/g in superfusion group, respectively). The amplitude of the DC negative shift was not influenced by any of the drugs used (Table 3).

Ischemia-induced changes of extracellular ion activities

Immediately after the onset of cerebral ischemia, there was a transient $[K^+]_e$ elevation to 4 m*M*. The $[K^+]_e$ usually declined again and was followed by a secondary slow elevation to the so-called K^+ threshold from which the extracellular potassium concentration rose steeply (Fig. 1). The $[K^+]_e$ threshold measured in the control animals was 16.7 ± 3.3 m*M*, and the maximal $[K^+]_e$ 5 min after occurrence of AD was 67.4 ± 6.5 m*M* (steady state). Only one of the examined compounds, glibenclamide, significantly influenced the ischemia-induced extracellular $[K^+]$ changes (Table 4). Given either intravenously or locally, it reduced the $[K^+]_e$ threshold by −21 and −13%, respectively.

TABLE 2. *General physiological variables*

Drug before	MABP (mm Hg) after		Heart rate (beats/min) before	after	P_aO_2 (mm Hg)	P_aCO_2 (mm Hg)	pH
Solvents	106 ± 22	103 ± 19	417 ± 37	410 ± 37	132 ± 15	39.6 ± 5.1	7.35 ± 0.04
Nimodipine	109 ± 14	88 ± 12[a]	415 ± 34	413 ± 46	129 ± 22	41.1 ± 6.1	7.37 ± 0.09
Flunarizine	101 ± 25	67 ± 15[a]	407 ± 41	303 ± 45[a]	100 ± 9.0	38.3 ± 5.7	7.37 ± 0.04
R56865	114 ± 16	80 ± 15[a]	410 ± 43	291 ± 27[a]	139 ± 11	34.3 ± 5.0	7.38 ± 0.04
MK-801	119 ± 17	111 ± 19	436 ± 27	422 ± 36	140 ± 12	39.3 ± 3.4	7.36 ± 0.03
NBQX	80 ± 29	111 ± 12[a]	392 ± 62	463 ± 23[a]	129 ± 8.6	40.9 ± 3.3	7.34 ± 0.04
MK-801 + NBQX	118 ± 12	117 ± 10	430 ± 36	415 ± 32	130 ± 8.4	34.0 ± 3.9	7.40 ± 0.02
Glibenclamide							
i.v.	101 ± 16	117 ± 12[a]	410 ± 36	404 ± 32	114 ± 13	41.9 ± 10.4	7.34 ± 0.04
Superfusion	108 ± 16	90 ± 21	385 ± 47	359 ± 40	131 ± 9.4	41.3 ± 6.2	7.33 ± 0.04
Cromakalim	100 ± 17	97 ± 14	392 ± 23	413 ± 29	123 ± 7.9	40.9 ± 4.5	7.36 ± 0.04

Values are means ± SD. The MABP and heart rate were measured 10 min prior to and 10 min after the drug treatment. The blood gas values were obtained just before induction of cardiac arrest.

[a] $p < 0.05$ (difference before and after treatment, paired *t* test).

TABLE 3. *Cardiac-arrest-induced changes of cortical direct current potential*

Drug	Latency (s) to AD	AD amplitude (mV)
Control	130.2 ± 24.6 (50)	19.5 ± 3.0 (50)
Nimodipine	130.9 ± 20.1 (11)	20.2 ± 3.1 (11)
Flunarizine	161.4 ± 21.1[a] (7)	20.1 ± 4.1 (7)
R56865	164.5 ± 40.4[a] (13)	18.3 ± 2.6 (13)
MK-801	144.6 ± 18.9 (13)	19.8 ± 2.6 (13)
NBQX (30 mg/kg)	174.3 ± 62.1[a] (6)	20.9 ± 2.2 (6)
MK-801 + NBQX	168.8 ± 11.7[a] (6)	20.1 ± 4.1 (6)
Glibenclamide		
i.v.	97.8 ± 21.6[a] (10)	19.6 ± 2.7 (10)
Superfusion	141.1 ± 18.1 (7)	18.8 ± 4.0 (7)
Cromakalim	143.3 ± 24.9 (6)	16.0 ± 2.8 (6)

Numbers in parentheses indicate numbers of measurements. Values are means ± SD. AD, anonic depolarization.

[a] $p < 0.05$ (drugs versus solvent); one-way factorial analysis-of-variance test followed by Dunnett *t* test for multiple comparison of independent samples.

The maximal $[K^+]_e$ during ischemia was also reduced after both glibenclamide treatments (Table 4). All other compounds slightly increased $[K^+]_e$ threshold. However, this effect failed to reach statistical significance when one-way factorial analysis of variance and the multiple comparison test were used (Table 4).

The change of $[Ca^{2+}]_e$ upon cerebral ischemia was triphasic (Fig. 1). It exhibited at first an initial elevation from 1.09 ± 0.1 to 1.28 ± 0.1 m*M*. At the beginning of the negative DC deflection, the $[Ca^{2+}]_e$ dropped rapidly to ~0.25 m*M* and subsequently declined slowly to the final level of 0.07 ± 0.03 m*M* measured at 5 min after AD (Table 4). Neither the final $[Ca^{2+}]_e$ concentration during ischemia nor the speed of the rapid $[Ca^{2+}]_e$ reduction was affected by any of the tested compounds. However, the ischemia-induced $[Ca^{2+}]_e$ reduction was slowed down by both glutamate antagonists MK-801 and NBQX, which was revealed by the measurement of time to the 90% reduction of $[Ca^{2+}]_e$ (Fig. 2; Table 4).

The $[Na^+]_e$ activity was recorded only in control animals and animals treated with NBQX and R56865. The ischemia-induced $[Na^+]_e$ changes resembled the $[Ca^{2+}]_e$ profile. It exhibited an initial minor elevation and a rapid fall associated with AD (Fig. 1). The final minimal $[Na^+]_e$ concentration measured at 5 min after AD was 53.3 ± 5.3 m*M* in the control group (Table 4). R56865 (5 mg/kg) significantly increased the final $[Na^+]_e$ to 65.9 ± 4.9 m*M*, whereas the NBQX treatment (30 mg/kg) did not show a clear effect (Table 4). We also treated two animals with 60 mg/kg NBQX. The minimal $[Na^+]_e$ concentrations during ischemia in these two animals were both >70 m*M* and markedly higher than the level of 53 m*M* measured in the control animals (Table 4).

DISCUSSION

Resistance to ischemic AD

In the present study, three of the seven compounds tested were able to delay the occurrence of AD upon cerebral ischemia. These compounds, NBQX, flunarizine, and R56865, have all been proven to block membrane Na^+ fluxes. NBQX is a selective antagonist of the glutamate receptor of the AMPA subtype (Watkins et al., 1990). The AMPA receptor-regulated ion channels have been shown to be predominantly permeable to sodium ions (Young and Fagg, 1990). Flunarizine and R56865 have been characterized as Na^+/Ca^{2+} overload blockers (Pauwels et al., 1991). They block not only the voltage-dependent Ca^{2+} channels, especially the T and N type (Takahashi et al., 1989; Panchenko et al., 1993), but also the voltage-dependent Na^+ channel (Kiskin et al., 1993). In contrast, the drugs that principally interfere only with transmembrane Ca^{2+} movement like MK-801 [*N*-methyl-D-aspartate (NMDA) antagonist] and nimodipine (L-type Ca^{2+} channel blocker) did not influence the latency time to AD. At the dosage used, the ATP-sensitive K^+ channel modulators (glibenclamide and cromakalim) did not affect the latency time to AD. Thus, it seems reasonable to assume that the ischemia-induced Na^+ influx is the major factor in determining the latency time from onset of ischemia to the occurrence of AD.

AD and Na^+ fluxes

In previous studies it was shown that ischemia caused an early Na^+ influx preceding the complete breakdown of ion homeostasis (Jiang et al., 1992*a*). This early Na^+ influx was strongly inhibited by tetrodotoxin (TTX), and the latency time to the occurrence of AD was enormously prolonged (Xie et al., 1994). The prolonged onset time to AD observed in the present study suggested that the ischemia-induced early Na^+ influx could also be inhibited by R56865, flunarizine, and NBQX. In other words, both voltage- and receptor-operated Na^+ channels may be involved in the ischemia-induced early Na^+ influx. The linkage between the Na^+ blocking effect and the prolongation of the latency time to AD is probably an energy-saving mechanism. The transmembranal Na^+ gradient is maintained by the Na^+/K^+ pump at the expense of energy. Previous studies showed that the DC negative deflection occurred in the rat brain when the tissue ATP content was reduced to 30% of the normal

TABLE 4. *Cardiac-arrest-induced changes of extracellular ion activity in rat brain*

Drug	Min. $[Na^+]_e$ (m*M*)	$[K^+]_e$ threshold (m*M*)	Max. $[K^+]_e$ (m*M*)	Min. $[Ca^{2+}]_e$ (m*M*)	Time (s) until 90% reduction of $[Ca^{2+}]_e$
Solvent	53.3 ± 5.3 (7)	16.7 ± 3.3 (35)	67.4 ± 6.5 (38)	0.07 ± 0.03 (13)	11.6 ± 6.6 (15)
Nimodipine	ND	18.2 ± 1.3 (5)	72.9 ± 12.1 (5)	0.05 ± 0.01 (5)	15.6 ± 10.4 (5)
Flunarizine	ND	18.5 ± 1.5 (6)	65.1 ± 4.2 (7)	0.08 ± 0.03 (6)	14.4 ± 8.1 (6)
R56865	65.9 ± 4.9[a] (7)	18.9 ± 2.9 (7)	67.7 ± 4.7 (6)	0.07 ± 0.01 (5)	19.8 ± 6.7 (5)
MK-801	ND	17.8 ± 0.9 (7)	65.1 ± 4.8 (7)	0.06 ± 0.01 (6)	69.6 ± 23.9[a] (6)
NBQX	58.7 ± 7.1 (5)	18.1 (1)	62.0 (1)	0.09 ± 0.01 (6)	34.0 ± 22.1[a] (6)
MK-801 + NBQX	ND	ND	ND	0.08 ± 0.02 (6)	69.0 ± 29.6[a] (6)
Glibenclamide					
i.v.	ND	13.2 ± 3.5[a] (8)	57.1 ± 10.1[a] (10)	ND	ND
Superfusion	ND	14.6 ± 4.4[a] (7)	53.5 ± 17.1[a] (7)	ND	ND
Cromakalim	ND	17.5 ± 0.7 (6)	65.6 ± 7.0 (6)	ND	ND

Numbers in parentheses indicate numbers of measurements. Values are means ± SD.
[a] $p < 0.05$ (drugs versus solvent); one-way factorial analysis-of-variance test followed by Dunnett *t* test for multiple comparison of independent samples.

level (Obrenovitch et al., 1990). Comparative studies demonstrated that the brain ischemia tolerance (maintenance of the cellular ion homeostasis) was clearly dependent on the preservation of energy (Doll et al., 1991; Xia et al., 1992). A limited Na^+ influx will reduce the mismatch between cellular metabolic demand and energy supply and thereby delay the occurrence of AD. In fact, it has been shown that TTX was able to slow down the ischemia-induced ATP fall (Boening et al., 1989). Our results are also in line with this hypothesis. The massive Na^+ movement coinciding with the AD, evidenced by the rapid $[Na^+]_e$ fall from 135 to 53 m*M*, was only marginally affected by the drugs. Even at a very high dose (10 μ*M*), TTX did not influence the ischemia-induced $[Na^+]_e$ fall, indicating that the voltage-dependent Na^+ channels may not be essential in this process (Xie et al., 1994).

The AMPA receptor antagonist NBQX seemed to attenuate the $[Na^+]_e$ decline at a high dose of 60 mg/kg (see Results), implying a possible involvement of the receptor-operated Na^+ channels in the rapid redistribution of Na^+ ions coinciding with the AD.

An intriguing finding is that the rapid $[Na^+]_e$ decrease associated with AD was slightly, but nevertheless significantly, attenuated by R56865, while the strong Na^+ channel blocker TTX was ineffective (Xie et al., 1994). Which channels did R56865 block? Recent studies using patch clamp and fluorescence techniques showed that the Na^+ blockade effect of R56865 differed principally from that of TTX (Verdonck et al., 1990; Kiskin et al., 1993). R56865 preferably affected the open Na^+ channels and enhanced the inactivation process irreversibly. R56865 also increased the cell membrane resistance

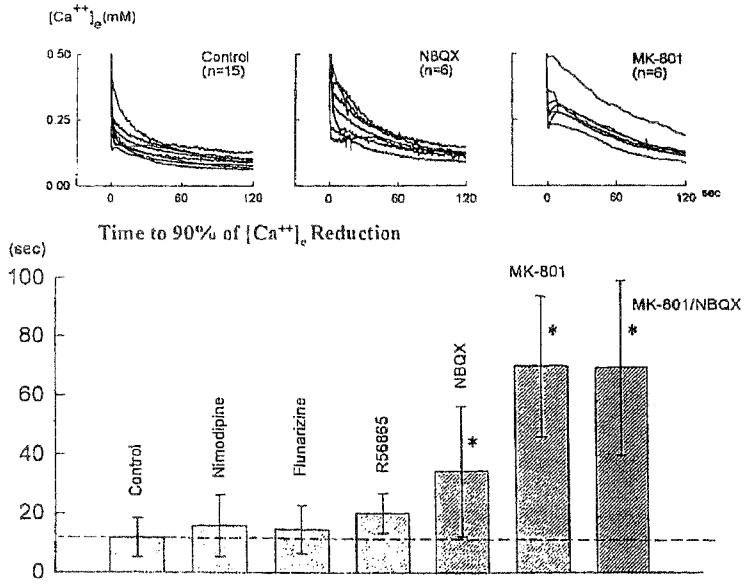

FIG. 2. Top: Extracellular Ca^{2+} activities after anoxic depolarization in control animals and animals that were treated with MK-801 and NBQX. Note that the time scale is different from that in Fig. 1. The time "zero" indicates the onset of anoxic depolarization but not the induction of cardiac arrest. Upon anoxic depolarization, extracellular calcium drops rapidly to a level of ~0.25 m*M* followed by a slow, exponential further decline that is significantly slowed down by MK-801 and NBQX. **Bottom:** Time needed for the extracellular calcium signal to reach 90% of the decline. The error bars represent SD. Only MK-801, NBQX, and the combination of NBQX with MK-801 delayed the time to 90% $[CA^{2+}]_e$ reductions significantly ($p < 0.05$).

and reduced the leakage current (Kiskin et al., 1993). These effects can be enhanced by ischemic conditions. Accordingly, the R56865 effect observed in the present study might suggest that the nonspecific cation pathway (carrier of the leakage current?) becomes dominant for Na^+ movement after massive cell depolarization and/or the voltage-operated Na^+ channels undergo certain modifications upon ischemia that makes them more sensitive to R56865 than to TTX.

AD and ATP-sensitive K^+ (K_{ATP}) channels

Another important factor regulating cell membrane potential is the K^+ permeability. Previous studies have shown that a blockade of the ischemia-induced K^+ efflux facilitated the AD (Ben-Ari, 1990; Jiang et al., 1992*b*). Several in vitro studies using cultured neurons or brain slices suggested that the K_{ATP} channel is intimately involved in mediating K^+ efflux under ischemic/anoxic conditions (Ben-Ari et al., 1990; Jiang and Haddad, 1991). The present data confirm these observations in in vivo experiments. Glibenclamide, a rather specific ligand and blocker for K_{ATP} channels, was able to curb the ischemia-induced $[K^+]_e$ elevation in the cortex. The K^+ threshold was significantly lower in the glibenclamide-treated than in the control animals, while the AD latency remained unaffected. This indicates that the ischemia-induced potassium efflux is lowered when ATP-dependent potassium channels are blocked. In addition to this, the potassium level measured after AD was lower than in controls (Table 4). Thus, the K_{ATP} channels may mediate both the early slow K^+ efflux and the rapid transmembrane K^+ redistribution coinciding with the AD. A facilitation of AD (shortening of the latency time to AD) was not observed in our experiments.

There are conflicting data concerning the effects of K^+ channel blockers on cellular hyperpolarization. This may be due to the different preparations used. It is well known that cellular potassium efflux is clearly affected by the size of the extracellular space, which is larger in in vitro preparations than in vivo.

Since it is well known that glibenclamide affects the blood glucose at the intravenous dosage used in the present study, we administered the compound also locally via the cup technique. After either systemic and local application, the glibenclamide concentration in the brain tissue and its effects on the ischemia-induced $[K^+]_e$ changes were comparable (Table 4). Therefore, it could be assumed that the effects on the extracellular K^+ levels observed in the present study may rather be mediated via its parenchymal effect in the brain than via possible systemic changes in the blood glucose concentration. However, the observed AD latency shortage in the animals receiving glibenclamide intravenously (Table 3) can be at least partly explained by the reduced plasma glucose content (Hansen, 1978), which did not occur if the drug was applied locally. Since only a single dose of glibenclamide was used, we cannot exclude that this dosage might have been too low to affect the AD latency directly.

Opening K_{ATP} channels hyperpolarizes the membrane potential and limits the neuronal excitability. Consequently, it should prevent or delay the AD and the subsequent deterioration (Duty and Weston, 1990; Miller, 1990; Murphy and Greenfield, 1991). In animal experiments, the K_{ATP} channel opener aprikalim (RP52891) was used successfully to reduce myocardial infarct size (Grover et al., 1990; Auchampach et al., 1991). In in vitro experiments, the K_{ATP} channel openers have also been proven to antagonize anoxia-induced neuronal depolarization (Ben-Ari, 1990; Ben-Ari et al., 1990). In the present study, however, cromakalim treatment did not delay the occurrence of the AD or influence the ischemia-induced K^+ efflux. In a pilot experiment, 5 mg/kg cromakalim given intravenously was also ineffective (data not shown). The reason for this negative result may possibly be an inadequate dosage or application route. Cromakalim has been shown to be effective at doses ranging from 20 to 100 μM in brain slices (Alzheimer et al., 1989), but the tissue drug concentration in our experiments could not be determined. Another possible explanation is that the neuronal K_{ATP} channels are already maximally activated in cardiac-arrest-induced cerebral ischemia. Thereafter, no further activation could be achieved by the drug. This consideration may be supported by the observation of Ripoll and colleagues (1990). In their experiments, the K_{ATP}-channel-activating effect of 40 μM cromakalim was lost if ATP was absent in the tissue bath.

AD and Ca^{2+} fluxes

Our results showed that the ischemia-induced cortical $[Ca^{2+}]_e$ decrease was slowed down by the glutamate receptor antagonists MK-801 and NBQX, while the voltage-operated Ca^{2+} channel blockers nimodipine (L type), flunarizine, and R56865 (T and N type) were ineffective. This is in agreement with observations of other authors (Pluta et al., 1989; Silver and Erecinska, 1990; Salinska et al., 1991) who also observed that the ischemia-induced Ca^{2+} influx cannot be blocked by voltage-operated Ca^{2+} channel blockers. The ineffective-

ness of nimodipine, R56865, and flunarizine can probably be explained by the intrinsic electrophysiological properties of the voltage-operated calcium channels that undergo rapid inactivation upon cell depolarization. The receptor-operated ion channels, therefore, become the predominant pathway for Ca^{2+} influx. Channels linked to glutamate receptors of NMDA subtype are well known to be more permeable to calcium ions than to sodium ions (Mayer and Miller, 1990). Under ischemic conditions, a persistent and pronounced activation of the NMDA receptors is favored by the increased extracellular glutamate concentration (Beneveniste et al., 1984) and the relief of the magnesium blockade by membrane depolarization (Mayer et al., 1984). The effect of AMPA receptor antagonist NBQX on ischemia-induced $[Ca^{2+}]_e$ decline could be explained in several ways. First, AMPA receptors promote the NMDA receptor activation by modulating membrane potentials (Murphy and Miller, 1989; Collingride and Singer, 1990). Second, the AMPA receptor-mediated Na^+ influx may reverse the Na^+/Ca^{2+} exchanger and lead to a secondary Ca^{2+} influx (Waxman et al., 1991). Finally, there is increasing evidence indicating that the AMPA receptors per se can be permeable to calcium ions (Hollmann et al., 1991). Such calcium permeability of the AMPA receptor was already reported in cultured hippocampal neurons (Lino et al., 1990) and cerebellar glial cells (Burnashev et al., 1992). The effects of NBQX on ischemia-induced decrease of $[Ca^{2+}]_e$ observed in the present study may imply the existence of calcium-permeable AMPA receptors in the cortical neurons and glial cells.

In summary, we demonstrated that the blockade of ischemia-induced Na^+ influx showed the most pronounced effect on the latency time to occurrence of AD. This effect may be the consequence of an energy-saving process. The K_{ATP} channels are present in the rat cortical nervous tissue. They are involved in the ischemia-induced K^+ efflux and may contribute to the development of AD. The ischemia-evoked Ca^{2+} influx, mainly mediated by the receptor-operated but not the voltage-dependent Ca^{2+} channels, might not be the cause but rather the consequence of cell depolarization. Hence, attenuating cellular sodium overload might be the primary route to increase tissue resistance against ischemia/anoxia.

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II

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"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)

Clin. Lab. 2002;48:541-551

ORIGINAL ARTICLE

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

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SUMMARY

The aim of this study was to develop and compare high-performance liquid chromatography (HPLC) and gas chromatography coupled with isotope dilution-mass spectrometry (GC-IDMS) methods with a common extraction procedure for the determination of substituted xanthines in biological matrices such as serum and urine. For HPLC both isocratic and gradient methods were evaluated. Difficulties occurred in separation of all 6 xanthines of interest – uric acid, theobromine, theophylline, paraxanthine, caffeine and 1,3-dimethyl-7-(2-hydroxyethyl) xanthine as internal standard. In addition, paracetamol was seen to interfere at higher concentrations, which meant that a system had to be developed to separate all 7 components of interest. The final solution chosen consisted of precipitation of serum samples with 6 mol/l trichloroacetic acid followed by neutralisation with 3 mol/l KOH and chromatography on a 150 x 4.6 mm Nautilus C-18 column (Macherey & Nagel) using an isocratic elution consisting of 0.02 mol/l acetate-phosphate buffer, pH 3.0 containing 9.6% v/v acetonitrile and monitoring at 273 ± 7 nm. Comparisons with GC-IDMS and FPIA were acceptable. Run times of 10 minutes were possible. An additional "safe time" of 5 minutes was allowed to elute any substances with similar absorption maxima which were sometimes present in commercial control sera. Precision of the method was 1.64% (intra-assay) and 2.87% (inter-assay) at 4.1 mg/l and 1.51% respectively 2.15% at 25 mg/l including extraction and measurement steps. Recovery was between 86 and 101% between 1.25 and 100 mg/l and peak time deviations for all 7 components between 0.07% and 0.34% (coefficient of variation) in 7 consecutive measurements. (Clin. Lab. 2002;48:541-551)

KEY WORDS

HPLC, GC-IDMS, theophylline, caffeine, 1,7-dimethyl-xanthine, theobromine, 1,3-dimethyl-7-(2-hydroxy-ethyl)-xanthine, acetaminophen, paracetamol, human plasma, extraction, internal standard, derivatisation, FPIA

INTRODUCTION

Although several methods for the determination of theophylline using high-performance liquid chromatography (HPLC) (1-5) and gas chromatography coupled with isotope dilution-mass spectrometry (GC-IDMS) (1,6-9) have been published, the effects of the closely related xanthine derivatives caffeine, theobromine, 1,7-dimethylxanthine and 1,3-dimethyl-7-(2-hydroxyethyl)-xanthine (internal standard for HPLC), while having been closely examined in the HPLC determination, have been less extensively studied using GC-IDMS. Recently, combinations of liquid chromatography and isotope dilution-mass spectrometry (LC-IDMS) have been used to determine theophylline, theobromine and caffeine in human plasma and urine (10).

The aim of this study was to use a common sample extraction for both HPLC and GC-IDMS and to study the effects of the above mentioned xanthine derivatives during the development of both methods, including the effects of the derivatisation process prior to GC-IDMS.

The need for close examination is because the metabolism of caffeine (from coffee, tea and mate drinkers), (11-13) which gives rise to theophylline (less than 10%)

Manuscript accepted June 3, 2002

Table 1: Gradient Elution Profiles

a. Waters – Spherical C-18 Resolve – 30 x 0.46 cm.

Elution Time - min	Buffer A - %	Buffer B - %	Flow Rate - ml/min
0.0	0	100	0.700
4.0	0	100	0.700
12.0	100	0	0.700
15.0	100	0	0.700
15.1	0	100	0.700
20.0	0	100	0.700

Buffer A – 0.02 mol/l acetate-phosphate/ acetonitrile – 88:12 v/v – pH 3.0
Buffer B – 0.02 mol/l acetate-phosphate / acetonitrile – 94:6 v/v – pH 3.0

Column temperature – ambient (20 – 25 °C)

(11) and paraxanthine (1,7-dimethylxanthine – above 80%) (11,14) as well as theobromine, (3,7-dimethylxanthine), a metabolite of caffeine (around 10%) (11) and the main xanthine component of cocoa may interfere with the determination of the closely related theophylline (1,3-dimethylxanthine).

MATERIALS AND METHODS

HPLC

Eluant, Standards and Column

Isocratic Procedure - The columns used were (a) a 30 x 0.39 cm 5 µm Spherical C-18 Resolve (Waters, Milford, MA, USA) using 0.02 mol/l sodium acetate adjusted to pH 3 with 85% orthophosphoric acid and acetonitrile 90:10 as the mobile phase; and (b) a 25 x 0.46 cm Nautilus C-18 (Macherey & Nagel, Düren, Germany) first using a 70:30 water:methanol mixture and then, because of unacceptable precision, 70:30 0.1 mol/l acetic acid: methanol, finally with 0.02 mol/l acetate-phosphate buffer, pH 3.0, containing 9.6% v/v acetonitrile to exclude cross-reactivity due to acetaminophen (paracetamol). The columns were run at ambient temperature. The run time was set initially at 20 minutes for both systems. The Nautilus C-18 column was then run for 10 minutes using the acetate-phosphate-acetonitrile combination.

Elution Gradient Procedure – A gradient was incorporated into the run for the Waters column. Buffer A was as above for the isocratic procedure, but with 12% acetonitrile. Buffer B was the same, but with 6% acetonitrile. The run set-up is shown in Table 1.
Attempts at running the Nautilus C-18 column using an elution gradient gave rise to separation problems with paraxanthine and/or paracetamol.

Standards and Related Xanthines
The internal standard used was 1,3-dimethyl-7-(2-hydroxyethyl)-xanthine dissolved in 0.025 mol/l potassium hydroxide to give a final concentration of around 10 mg/l and a pH between 10 and 11.
Caffeine, theobromine and 1,7-dimethylxanthine were also dissolved in 0.025 mol/l KOH to give final concentrations between 1 and 4 g/l. Stock solutions were appropriately diluted to give final concentrations of around 10 mg/l.
A solution of acetaminophen (paracetamol) was made up by dissolving an effervescent paracetamol-seltzer tablet (500 mg paracetamol – Superdrug Stores, Croydon, Great Britain) in 50 ml distilled water to give a concentration of 10 g/l.
Control sera from BioRad, Munich, Germany, (Lyphochek Immunoassay Control; Lots 40091, 40092 and 40093 and Assayed Clinical Chemistry Control; Lot 14081) were used. Internal standard was added to each sample or control before trichloroacetic acid precipitation (100 µl of a 6 mol/l trichloroacetic acid (TCA) solution in distilled water per ml sample or serum standard. The supernatant was then transferred to a clean screw-top glass vial and neutralised with ca. 250 µl 1 mol/l KOH per ml starting material to give a final pH between 7 and 9. This solution was used for injection into the HPLC and as starting material for the derivatisation for GC-IDMS. Recovery and linearity experiments were made by addition of stock solutions of theophylline to drug-free human sera before TCA-precipitation.
Virtual interfering substances (see above) were added to a solution of theophylline and internal standard, so that the end concentration of all three substituted xanthines was around 10 mg/l. Uric acid was added to the standard mixture to give a final concentration of 32 mg/l, a physiologically relevant concentration; acetaminophen (4-acetamidophenol, paracetamol) was added to give a

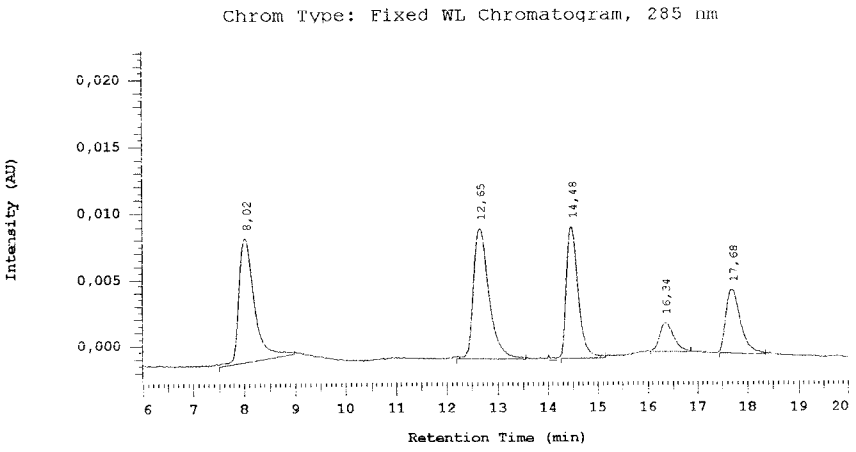

Figure 1a:
Isocratic elution on the Waters C-18 column as described in table 2a using the Bio-Rad control serum 40092. The elution times were: Paracetamol at 8.02 min; theophylline at 12.65 min; internal standard at 14.48 min; unknown component at 16.34 min and caffeine at 17.68 min. In the diode array spectrum (not shown), paracetamol exhibited a typical "Bunsen-burner" profile.

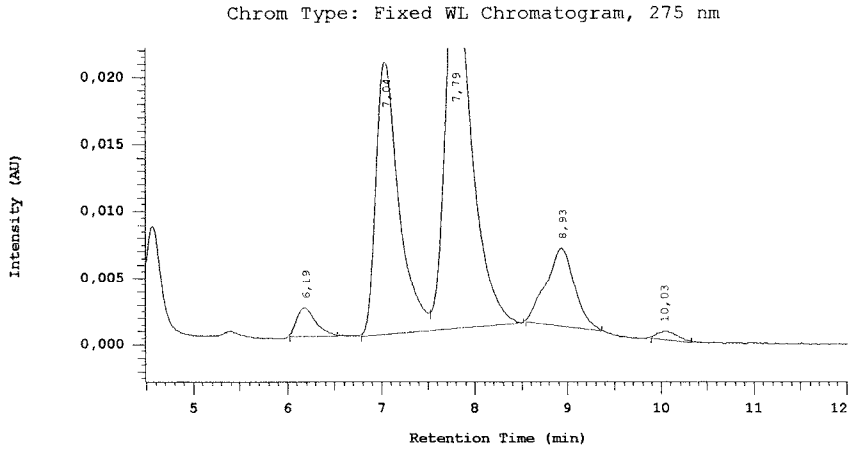

Figure 1b:
Chromatogram of Bio-Rad control serum 40092 on the Macherey and Nagel Nautilus C-18 column. Theophylline eluted at 7.04 min, internal standard and paracetamol co-eluted at 7.79 min and caffeine at 8.93 min. The eluent was acetic acid methanol 70:30 v/v (see table 2d).

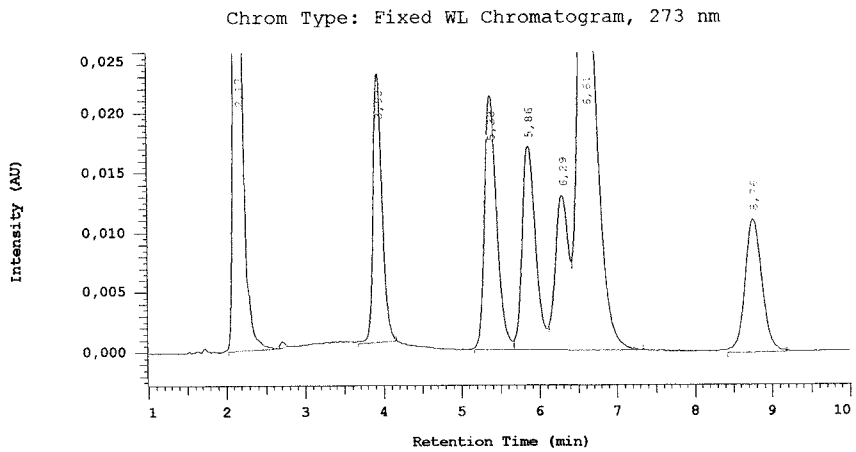

Figure 1c:
Chromatogram of all xanthines under optimised conditions on the Nautilus C-18 column. Uric acid eluted at 2.17 min, theobromine at 3.93 min, paraxanthine at 5.38 min; theophylline at 5.86 min; internal standard at 6.29 min, paracetamol (500 mg/l) at 6.61 min and caffeine at 8.76 min. The eluent was acetate-phosphate buffer containing 9.6% acetonitrile v/v, pH 3 (see table 2e).

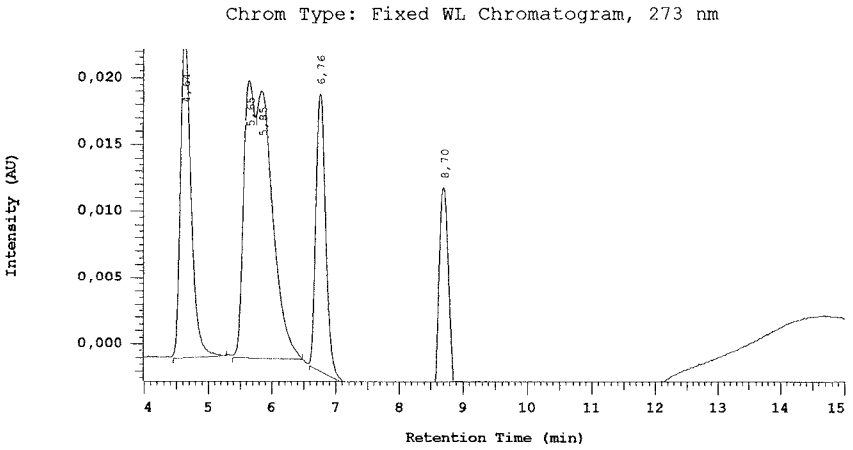

Figure 2a:
Chromatogram of the 5 component calibration-mixture on the Waters C-18 Spherosil column. Theobromine elutes at 4.64 min; theophylline at 5.65 min; paraxanthine at 5.85 min; internal standard at 6.76 min and caffeine at 8.76 min. The eluent was 6 – 12% acetonitrile gradient in acetate-phosphate buffer (see table 2c.). The diode array spectrum for reference was simultaneously recorded (not shown).

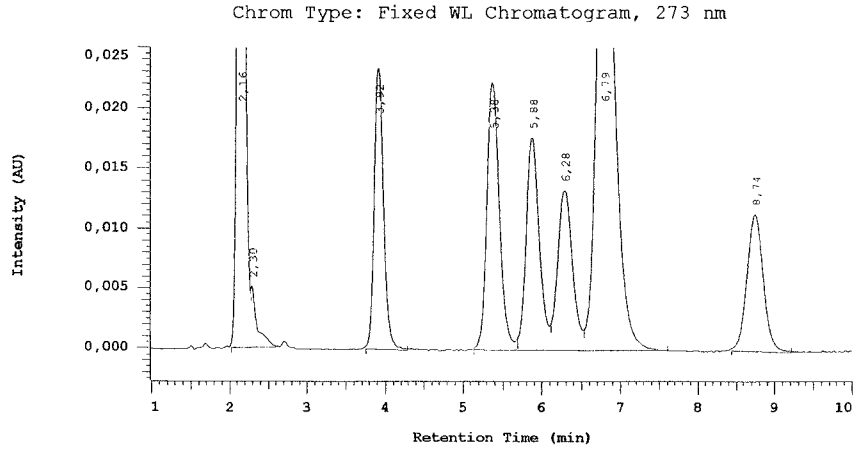

Figure 2b:
Isocratic elution of the 7-component mix on the Nautilus C-18 column with conditions as in figure 1c. The elution times were: Uric acid at 2.16 min; theobromine at 3.92 min; paraxanthine at 5.38 min; theophylline at 5.88 min; internal standard at 6.28 min; paracetamol at 6.79 min and caffeine at 8.74 min. Paracetamol was indicated by the simultaneously recorded diode array spectrum (not shown).

final concentration of 100 mg/l – around 5-10 times higher than the expected serum levels under therapy and paraxanthine – the main metabolite of caffeine – to give a final concentration of 10 mg/l. The complete mixture with potential interfering compounds was referred to as "Calmix-7". The basic calibration mixture containing about 10 mg/l each of theobromine, theophylline, internal standard and caffeine was termed "Calmix-4"

Serum samples from patients undergoing theophylline therapy as well as from employees who had had their yearly routine examination were analysed, and the results were compared with routine fluorescence polarisation immunoassay (FPIA) from Abbott Diagnostics (Wiesbaden-Delkenheim, Germany) run automatically on the AxSYM (Abbott).

RESULTS

a) HPLC – Isocratic Methods

Quality Control Sera

The quality control materials from Bio-Rad contained not only theophylline and caffeine (declared values) but also acetaminophen in measurable quantities. A typical chromatogram of a control serum is shown in Figure 1a for the Waters column and Figures 1b and 1c for the Nautilus C-18 column.

Elution Profiles of Mixtures of Known Xanthines

Figures 2a and 2b show a mixture of pure synthetic xanthine derivatives – 1,3 dimethyl-xanthine (theophylline), 3,7-dimethylxanthine (theobromine), 1,3,7-trimethylxanthine (caffeine), 1,7-dimethylxanthine (paraxan-

Table 2: Retention Times of Related Substituted Xanthines

a. Waters C-18 Column – Isocratic – 10% Acetonitrile – Flow Rate 0.45 ml/min

Compound	Elution Time – min	Ratio – E_T/E_X
Theophylline – 1,3-dimethylxanthine	7.58	1.00
Theobromine – 3,7-dimethylxanthine	6.80	0.897
Paraxanthine -1,7-dimethylxanthine	7.68	1.01
1,3-dimethyl-7-(2-hydroxyethyl)-xanthine*	8.22	1.08
Caffeine – 1,3,7-trimethylxanthine	12.3	1.62

b. Waters C-18 Column – 6-12% Acetonitrile Gradient – Flow Rate 0.70 ml/min

Compound	Elution Time – min	Ratio – E_T/E_X
Theophylline – 1,3-dimethylxanthine	9.90	1.00
Theobromine – 3,7-dimethylxanthine	7.67	0.775
Paraxanthine -1,7-dimethylxanthine	10.42	1.05
1,3-dimethyl-7-(2-hydroxyethyl)-xanthine*	12.35	1.25
Caffeine – 1,3,7-trimethylxanthine	16.65	1.68
Paracetamol	5.90	0.596
Uric acid	3.81	0.385

c. Macherey-Nagel Nautilus C-18 Column – Isocratic – 70% Water, 30% Methanol – Flow rate 0.9 ml/min

Compound	Elution Time – min	Ratio – E_T/E_X
Theophylline – 1,3-dimethylxanthine	8,84	1.00
Theobromine – 3,7-dimethylxanthine	5.96	0.674
Paraxanthine -1,7-dimethylxanthine	7.91	0.895
1,3-dimethyl-7-(2-hydroxyethyl)-xanthine*	8.24	0.932
Caffeine – 1,3,7-trimethylxanthine	10.1	1.14
Paracetamol	8.84	1.00

d. Macherey-Nagel Nautilus C-18 Column – Isocratic – 70% 0.1 mol/l CH_3COOH, 30% Methanol – Flow rate 0.9 ml/min

Compound	Elution Time – min	Ratio – E_T/E_X
Theophylline – 1,3-dimethylxanthine	7.67	1.00
Theobromine – 3,7-dimethylxanthine	5.35	0.698
Paraxanthine -1,7-dimethylxanthine	6.67	0.870
1,3-dimethyl-7-(2-hydroxyethyl)-xanthine*	6.94	0.905
Caffeine – 1,3,7-trimethylxanthine	8.82	1.15
Paracetamol	7.66	0.999
Uric acid	2.10	0.274

Note that the elution sequence has changed, the internal standard elutes before theophylline on the Nautilus column using water or acetic acid / methanol as eluant

e. Macherey-Nagel Nautilus C-18 Column – Isocratic – 0.02 mol/l acetate-phosphate buffer, pH 3, containing 9.6 % (v/v) acetonitrile – Flow rate 1.6 ml/min

Compound	Elution Time – min	Ratio – E_T/E_X
Theophylline – 1,3-dimethylxanthine	5.61	1.00
Theobromine – 3,7-dimethylxanthine	3.78	0.674
Paraxanthine -1,7-dimethylxanthine	5.13	0.914
1,3-dimethyl-7-(2-hydroxyethyl)-xanthine*	5.96	1.06
Caffeine – 1,3,7-trimethylxanthine	8.26	1.47
Paracetamol	6.60	1.18
Uric acid	2.12	0.378

*Internal Standard; E_T – Elution Time for Theophylline; E_X – Elution Time for analyte in question

Note that the elution sequence has changed, the internal standard elutes before theophylline on the Nautilus column using the acetic acid / methanol eluant.

Table 3: Effects of Paracetamol on the measured values from commercial control sera containing theophylline and paracetamol

	40091 – mg/l	40092 – mg/l	40093 – mg/l
Theophylline-eluant 1*	9.16	28.6	68.9
Theophylline-eluant 2**	5.31	16.5	25.6
Quotient	1.73	1.73	2.69
Caffeine-eluant 1	2.60	6.52	14.0
Caffeine-eluant 2	2.30	7.00	13.5
Quotient	1.13	0.93	1.04

- Eluant 1 – 0.1 mol/l acetic acid / methanol 70:30
- Eluant 2 – 0.02 mol/l sodium acetate, 2 ml/l 85% orthophosphoric acid, 9.6% acetonitrile.

Caffeine values are included to show that the interference was only seen with theophylline.
The control sera were Lyphochek (Bio-Rad), the lot numbers being shown in the column headers.

thine) and 1,3 dimethyl-7-(2-hydroxyethyl)-xanthine (internal standard) in 0.025 mol/l KOH .
It is clear that 1,7-dimethylxanthine interferes with the determination of theophylline when using the Waters column with isocratic elution, whereas the other substances which could potentially interfere, are well separated from theophylline. The Nautilus C-18 column with the same buffer but with 9.6% acetonitrile did not show this effect. The elution times of the xanthines tested in the system and which constitute the major source of potential error are given in Table 2.
The main thing to note when using the Nautilus C-18 column with water/methanol or acetic acid/methanol as eluent was the change in position of the theophylline and internal standard peaks as well as the shortening of the retention time for caffeine (Table 2.)

Unexpected Interference in Commercial Control Sera when Eluting with Acetic Acid-Methanol
The use of the acetic acid –methanol eluant for the Nautilus C-18 Column seemed to be acceptable until the control sera were measured. The theophylline concentrations found were approximately double those expected (Table 3). On examining the diode-array (DAD) spectra it could be seen that the maximum absorption did not occur at 273 nm but at 250 nm for the theophylline peak (Figure 1b). The peak form was symmetrical and there was no evidence of a double or superimposed peak. In patient sera this absorption maximum for the theophylline peak was seen at irregular intervals which pointed to a superimposed peak of an unknown compound present in some but not all patient samples.
On examining the list of drugs in the control sera and their corresponding molar extinction coefficient and maximum absorption wavelength, a candidate was found for the interfering peak, namely acetaminophen or paracetamol. To check whether acetaminophen was in fact the culprit, a 500 mg paracetamol-seltzer tablet was dissolved in 50 ml distilled water. 10 µl of this solution was added to 990 µl calibration mixture to give an end concentration of 100 mg/l. 10 µl of the mixture was injected and the DAD-spectrum between 240 and 320 nm compared with the peaks generated using a specific wavelength of 273 nm (band width 7 nm). The paracetamol peak co-eluted exactly with theophylline and the spectrum was identical, proving paracetamol to be the interfering compound.
As paracetamol is a commonly used analgesic, it was decided to change the acetic acid-methanol for the sodium acetate-phosphoric acid eluent (without acetonitrile) used for the Waters column. Paracetamol continued to co-elute with theophylline so that the Waters column eluent was tried with varying concentrations of acetonitrile. An optimum separation was achieved with a final concentration of 9.6% acetonitrile. This concentration was able to separate both paracetamol as well as 1,7-dimethylxanthine from theophylline with a run time of 10 minutes as shown in Figure 1c. Acetonitrile concentrations below 9.2% resulted in incomplete separation of paracetamol, whereas concentrations above 10% resulted in incomplete separation of 1,7-dimethylxanthine.

b) HPLC – Gradient Elution Method – Waters C-18 Column

Elution Profiles of the Different Xanthine Derivatives
Although separation of 1,7-dimethylxanthine was not complete (Figure 2a), it was better than using an isocratic elution (Figure 1b). The separation between theophylline and 1,7-dimethylxanthine was 5% in retention time using the gradient elution, compared with only 1% using an isocratic elution (Table 2).

Table 4: Intra- and Inter-assay Precision

a) Gradient Elution – Waters Column

Control Serum	Intra-assay⁺ mean	Intra-assay CV	Inter-assay mean	Inter-assay CV
Lyphochek-1*	4.06 mg/l	1.64 %	4.16 mg/l	2.87 %
Lyphochek-2	16.3 mg/l	2.29 %	14.4 mg/l	2.27 %
Lyphochek-3	25.1 mg/l	1.51 %	24.6 mg/l	2.15 %

* Lot Number 40091/92/93; ⁺ Intra-assay: n = 6; Inter-assay: n = 6.

Retention Time Precision for Theophylline and the Internal Standard
Theophyllin mean 9.90 min (Range: 9.84 – 9.96 min) – n = 6
1,3-dimethyl-7-(2-hydroxyethyl)-xanthine – mean 12.34 min (Range 12.29-12.38 min) – n = 6

b) Isocratic Elution
i) Macherey-Nagel Nautilus C-18 Column – Acetic Acid:Water – 30:70

Control Serum	Intra-assay⁺ mean	Intra-assay CV
Lyphochek-1*	8.52 mg/l	2.59 %
Lyphochek-2	28.1 mg/l	1.46 %
Lyphochek-3	67.3 mg/l	2.22 %

* Lot Number 40091/40092/40093; ⁺ Intra-assay: n = 6; Inter-assay: n = 6.

Retention Time Precision for Theophylline and the Internal Standard
Theophyllin – mean 7.89 min (Range: 7.75 – 7.89 min) – n = 6
1,3-dimethyl-7-(2-hydroxyethyl)-xanthine – mean 7,04 min (Range 7.00 - 7.13 min) – n = 6

ii) Macherey-Nagel Nautilus C-18 Column – Acetate/Phosphate/ 9.6% Acetonitrile, pH 3#

Control Serum	Intra-assay⁺ mean	Intra-assay CV	Inter-assay mean	Inter-assay CV
Lyphochek-1*	5.32 mg/l	1.59 %	5.26 mg/l	2.87 %
Lyphochek-2	16.8 mg/l	2.17 %	16.4 mg/l	3.27 %
Lyphochek-3	26.2 mg/l	2.21 %	26.6 mg/l	2.85 %

* Lot Number 40091/40092/40093; ⁺ Intra-assay: n = 6; Inter-assay: n = 10.
# This was the final combination with the Nautilus column, which was then used for routine use. The buffer composition was – per litre: 902 ml HPLC-grade water: 1.64 g anhydrous sodium acetate, 2 ml 85% orthophosphoric acid, 96 ml acetonitrile. The pH was approximately 3.

Retention Time Precision for all 7 components*

	Mean - min	Median -min	Std Dev. - min	CV - %
Uric acid	2.15	2.15	0.007	0.34
Theobromine	3.91	3.91	0.005	0.07
Paraxanthine	5.37	5.37	0.006	0.12
Theophylline	5.88	5.88	0.005	0.08
Int. Standard	6.28	6.28	0.006	0.10
Paracetamol	6.79	6.79	0.005	0.07
Caffeine	8.73	8.74	0.010	0.12

* Results from 7 consecutive measurements

Intra-Assay Precision – Standards*

	Mean – mg/l	Median –mg/l	Std Dev. – mg/l	CV - %
Theobromine	10.6	10.5	0.076	0.727
Theophylline	10.5	10.6	0.075	0.706
Caffeine	10.1	10.2	0.255	2.52

Results from 6 consecutive measurements of the 7-component mixture – standard concentrations: caffeine and theobromine 10 mg/l; theophylline 10.5 mg/l.

Table 5: Recovery of Theophylline (TP) using a single point calibration with internal standard and comparison of peak heights and areas with concentration.

TP mg/l	Found mg/l	Recovery %	P-Height	PHx:PH1.25	P-Area	PAx:PA1.25
1.25	1.21	96.8	908	1.00	17245	1.00
2.5	2.51	100	1901	2.09	37325	2.16
5	5.06	101	3785	4.16	70038	4.06
10	10.0	100	8058	8.87	167646	9.72
25	24.1	96.4	17504	19.3	336939	19.5
50	43.0	86.0	35013	38.6	670711	38.9
100	98.8	98.8	74290	81.9	1428690	82.8

Key:
TP – Theophylline weighed-in standard. Internal standard was added to each standard to give a final concentration of 10 mg/l. Sample concentrations determined from the ratio peak area internal standard : peak area unknown.
Found – Recovery of theophylline using the single point calibration – here 11.2 mg/l of theophylline containing 10 mg/l internal standard.
P-Height / P-Area – Peak height, Peak area – arbitrary units.
PHx:PH1.25 – Ratio between the peak height of standard x and peak height of standard 1.25 mg/l
PAx:PA1.25 – Ratio between the peak area of standard x and peak area of standard 1.25 mg/l

Table 6: Comparison of Theophylline concentrations measured by HPLC, GC-IDMS and FPIA

Sample No.	HPLC mg/l	GCMS mg/l	FPIA mg/l
1	n.d.	n.d.	n.d.
2	n.d	n.d.	n.d.
3	8.37	9.33	9.28
4	19.9	16.0	15.9
5	23.7	22.7	22.9
6	0.66	n.d	n.d
7	9.70	10.7	10.4
8	18.5	18.3	18.7
9	13.9	13.5	13.1
10	15.2	16,2	16.6
11	5.72	5.62	5.73
12	10.8	10.8	10.4
13	5.28	n.m.	4.86
14	5.54	n.m.	5.79
15	17.0	n.m.	16.0
16	n.d.	n.m.	n.d
17	n.d	n.m.	n.d
18	n.d	n.m.	n.d

n.d. – below detection limit n.m. – sample not measured

c) Method Precision

The intra-assay precision was measured on at least six consecutive injections of extracted control sera at three therapeutically important concentrations. The time cycle of 25 minutes between injections prevented the conventional 10 or 20 samples usually used for the intra-assay precision for the Waters column. Inter-assay precision was determined from measurements made on at least 6 consecutive days. Table 4 shows the results for both the intra- and inter-assay precision for all combinations of column and elution buffers/configurations.

d) Recovery of Theophylline Using a Single Point Calibration at around 10 mg/l

Concentrations of theophylline were made between 1.25 and 100 mg/l in drug-free human serum (Theophylline not detectable by HPLC and GC-IDMS). Each concentration had a concentration of 10 mg/l internal standard as used in the routine method. Protein removal was performed as for routine serum samples. The ratios between peak height and peak area and concentration were also calculated, the results being shown in Table 5.

e) Comparison of GC-IDMS, HPLC and Fluorescence Polarisation Immunoassay (FPIA) Measurements of Theophylline in Human Serum

18 sera – 12 from patients under theophylline therapy and 6 from young healthy subjects not under medication – were measured using GC-IDMS, HPLC (Nautilus column – isocratic elution with 0.1 mol/l acetic acid-methanol 70:30) and FPIA (Abbott TDx) and the measured concentrations compared. 5/6 healthy subjects had no detectable theophylline in both HPLC and GC-IDMS, although measurable concentrations of theobromine (< 1.5 mg/l) and caffeine (< 2 mg/l) were present in the majority of samples measured by HPLC. The remaining healthy control had minimal levels of theophylline (0.66 mg/l), when measured by HPLC. The results are shown in Table 6.

DISCUSSION

The determination of theophylline in human serum or plasma is a part of routine therapy control of patients under treatment for reversible airway obstruction and acute left ventricular failure (15). The usual method routinely used for determination is the automated fluorescence polarisation immunoassay (FPIA). Advantages are the short assay times and the ease of determination without prior extraction of plasma (15).

Theophylline is closely related in structure to naturally occurring xanthines in beverages such as coffee, tea and cocoa, which can either potentially interfere with the immunoassay determination or – in the case of caffeine from coffee – be metabolised to theophylline. The main substances which can cause problems are the dimethyl xanthines. Theophylline (1,3 dimethylxanthine), theobromine (3,7-dimethylxanthine) from cocoa – and paraxanthine, (1,7-dimethylxanthine) the main metabolite of caffeine, – only differ in structure in the positions of the methyl groups on the xanthine moiety.

Over 80% of caffeine is metabolised to 1,7-dimethylxanthine (6-8) in the adult. The serum concentrations of caffeine have been reported to reach levels around 4 – 5 mg/l, dependent on coffee consumption (13,16). The half-life of caffeine in serum is usually between 1 and 2 hours (16), whereas that of 1,7-dimethylxanthine lies between 3 and 6 hours (16). The latter results in serum 1,7-dimethylxanthine levels in normal coffee drinkers lie between 0.5 and 0.7 mg/l (13,16). Under normal conditions, the concentrations of 1,7-dimethylxanthine can be ignored; however, cases of caffeine intoxication in children have been reported in which serum caffeine levels were above 100 mg/l (17) and in such cases the serum levels of 1,7-dimethylxanthine cannot be ignored.

The starting point for the HPLC determination of theophylline was based on the publication of Susanto et al (1). This isocratic method was unable to separate theophylline from paraxanthine (1,7-dimethylxanthine). Other routine HPLC-methods also have difficulties in separating these two components (15). The use of an acetonitrile gradient made it possible to partially separate these components on the Waters C-18 column, as can be seen in Table 2 above. The run time of 20 minutes and the waiting time of 5 minutes before the next injection does not make this method suitable for routine use, where more than a few samples have to be determined. It does, however, have the advantage of being able to measure the different dimethylxanthines present in the serum of persons consuming beverages containing such compounds as well as persons with excessive caffeine ingestion. The need for separation of 1,7-dimethylxanthine and theophylline is important when using HPLC as the routine method of choice for the determination of theophylline in biological samples and control materials such as human serum or plasma or in cases of caffeine overdose (17).

The use of the Nautilus C-18 column with its stabilised C-18 groups allows the use of acetonitrile-free eluents according to the manufacturer's data. Water-methanol, suggested in the company's catalogue, was not optimal for neutralised extracted serum as the retention times and peak-separation were not stable during a run. Changing water for 0.1 mol/l acetic acid appeared at first to resolve this problem when aqueous mixtures of the xanthines were injected onto this column. The discrepancies seen with the commercial control sera, where the concentrations of "theophylline" found were approximately twice as high as those given by the manufacturer, led to the adoption of an acetate-phosphate-acetonitrile eluant after it was seen that without acetonitrile, paracetamol always co-eluted with theophylline on the Nautilus C-18 column.

This experience showed that unexpected and chemically unrelated substances can cause interference in HPLC, which is not always easy to correct. The broad absorption spectrum of paracetamol led to sufficient UV-absorption at 273 nm to substantially elevate the measured "theophylline" concentrations. The Waters Spherosil C-18 column was not susceptible to this interference, as can be seen in Figure 1a. The advantages of the Nautilus C-18 column were the possibility of isocratic elution and a reduction in total run time between injections of 60% compared with the Waters column.

It was possible to determine the purine derivatives uric acid, theophylline, theobromine, paraxanthine and caffeine as well as paracetamol in a single isocratic HPLC run within 10 minutes of injecting the sample with the Nautilus C-18 column using the Calmix-7 calibrator.
The GC-IDMS method allows separation of all dimethylxanthines, including 1,7-dimethylxanthine. This fact makes GC-IDMS the method of choice as a reference as well as a routine method for determining theophylline in control materials, the latter only if the presence of 1,7-dimethylxanthine in the sample to be measured cannot be excluded. Caffeine is not derivatised as all three NH-groups are occupied by methyl groups.
The aim of using a single extraction procedure for GC-IDMS and HPLC was only possible when using the same internal standard. In practice it was shown that although a single precipitation reagent was used, namely 6 mol/l trichloroacetic acid (TCA), the use of $^{13}C,^{15}N$-theophylline as internal standard in GC-IDMS prevented the use of this extract in HPLC which used 1,3-dimethyl-7-(2-hydroxyethyl)-xanthine as internal standard. The use of TCA-precipitation for HPLC was more practical and economical than the ultrafiltration method described by Susanto et al. (1). The neutralisation of the extract before injection onto the HPLC-column was performed in order to prolong the life of the column and increase the stability of the extract. Extracted samples could be kept at 4 – 7 °C for at least 7 days without demonstrable loss of theophylline.

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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)

Clin. Lab. 2002;48:535-540

ORIGINAL ARTICLE

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

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SUMMARY

A method is described which uses a combination of gas chromatography and isotope dilution-mass spectrometry (GC-IDMS) to determine the concentration of theophylline (1,3-dimethyl xanthine) in human plasma or serum samples. The effects of similar substituted xanthines – namely theobromine (3,7-dimethyl xanthine), paraxanthine (1,7-dimethyl xanthine) 1,3-dimethyl-7-(2-hydroxyethyl) xanthine (internal standard HPLC) and caffeine (1,3,7-trimethyl xanthine) were tested to confirm the specificity of the method. The derivatisation of all xanthines was performed with N-methyl-N-trimethylsilyl trifluroacetamide (MSTFA). The internal standard used was 2-^{13}C ,1,3-$^{15}N_2$-theophylline. The extraction and derivatisation procedures were examined in detail and optimised stepwise during the development of the method. High-performance liquid chromatography (HPLC) was used for comparison. (Clin. Lab. 2002;48:535-540)

KEY WORDS

xanthines, theophylline, caffeine, theobromine, paraxanthine, gas chromatography, mass spectrometry, isotope dilution, human serum

INTRODUCTION

The aim of the study was to develop a potential reference method for the specific determination of theophylline in serum or plasma, as this was required for setting the concentration of this analyte in external quality control materials according to the new guidelines of the German Medical Council (Richtlinie der Bundesärztekammer – Rili-BÄK) (1,2).
High-performance liquid chromatography (HPLC) was used to control the development of the GC-IDMS method and has also been published in this journal (3).
As other biologically active substituted xanthines are either used pharmacologically or occur naturally in foodstuffs, the specificity of the method was an important criterion during development.

Manuscript accepted June 3, 2002

MATERIALS, METHODS AND RESULTS

Materials

Theophylline, theobromine, caffeine and β-hydroxytheophylline (7-(2-hydroxyethyl)-theophylline) were purchased from Sigma-Aldrich, Deisenhofen, Germany.
Isotope labelled theophylline (2-^{13}C, 1,3-$^{15}N_2$ theophylline) was obtained from Cambridge Isotope Laboratories, USA.
N-methyl-N-trimethylsilyl trifluroacetamide (MSTFA) was bought from CS, Langerehe, Germany.
All other chemicals and solvents (HPLC-grade) were obtained from Merck, Darmstadt, Germany.
Control sera were purchased from Bio-Rad, Munich, Germany. These were Lyphochek Immunoassay Control Sera 1,2 and 3 (Lots 40091, 40092 and 40093).
The internal standard for the HPLC method was β-hydroxytheophylline (1,3-dimethyl-7-(2-hydroxyethyl)

Table 1: Experiments on two quality-control samples containing "theophylline".

Sample 1: Measured Concentration by GC-IDMS 14.0 mg/l

C_{12}-Theophylline added	C_{12}-Theophylline found	Recovery
0 mg/l	14.0 mg/l	100.0%
5	19.0	100.0
10	23.8	99.17
25	39.1	100.3
50	64.3	100.5

Sample 2: Measured Concentration in GC-IDMS <0.1 mg/l; in one test kit 8.9 mg/l

C_{12}-Theophylline added	C_{12}-Theophylline found	Recovery
0 mg/l	Not detectable - <0.1 mg/l	100.0%
5	4.81	96.20
10	9.84	98.40
25	25.3	101.2
50	51.0	102.0

Admixture test. Sample 1 + Sample 2 in different proportions

Proportion Sample 1 : Sample 2	Theoretical Concentration	Concentration Found
100:0	14.0 mg/l	13.9 mg/l
80:20	11.2	11.0
50:50	7.00	6.87
20:80	2.80	2.72
0:100	0.00	<0.10

xanthine). This could not be used as an internal standard in the GC-IDMS method (Figure 1, see below).

Apparatus and Adjustments

The mass spectrometer used was the TSQ 7000 from Finnegan-MAT, which was coupled to a gas-chromatograph from Varian (Model 3400). The injection system was a cold-start injector from Gerstel. The GC-capillary column was an FS-Supreme-5 from CS with a length of 30 m; internal diameter 0.25 mm and film thickness 0.25µm. The system gave optimal results with the following settings:

GC-Programme – 170 °C – 5 min; stepwise 30 °C/min to 300 °C; gas pressure 10 psi; injection volume 2 µl.

Cold injection system: 60:10:280 15 s split, then splitless

MS-settings: m/z 237, 240; sw 0.5; st 0.5; F 200; Mp 1500; 70 eV

Experimental Procedures

Preparation of Working Solutions

All working solutions of the substituted xanthines were prepared so that an end concentration of 0.2 g/l was attained. Solutions were made up in HPLC-grade water (SG: 0.9982 at 20 °C)

Extraction Media

Extraction of theophylline and the other xanthines was performed using the following solutes: ethyl acetate, butan-1-ol, chloroform, propan-2-ol, dichloromethane and varying proportions of mixtures of chloroform:propan-2-ol.

The extraction volumes were 1 ml and 2 x 500 µl. The best result was obtained with a mixture of chloroform:propan-2-ol 9:1.

Protein Precipitation

Different classical precipitation methods were tested, which included 6 mol/l trichloroacetic acid; acetone-HCl 200:1 (v/v); methanol and acetone. The best results

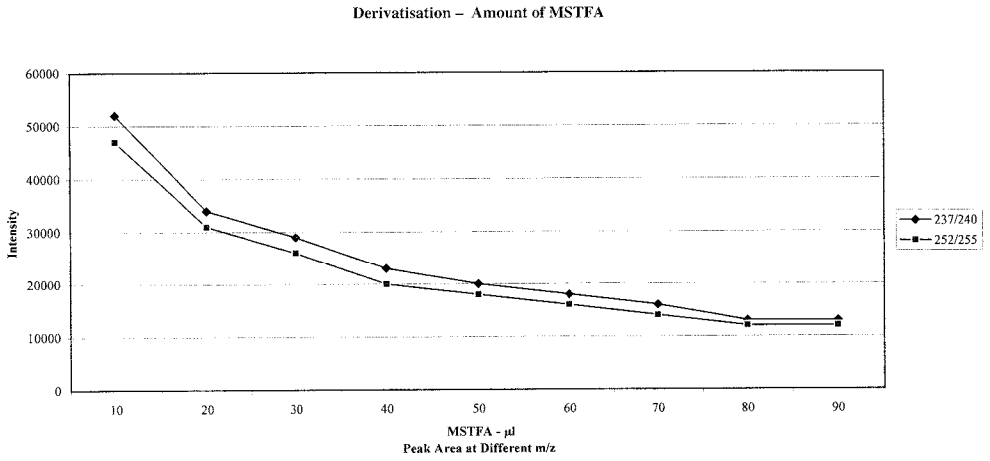

Figure 1: Different amounts of MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide) (abscissa) used for derivatisation of theophylline. Derivatisation time – 25 min; temperature 90 °C.
♦ - m/z 237/240; ■ – m/z 252/255

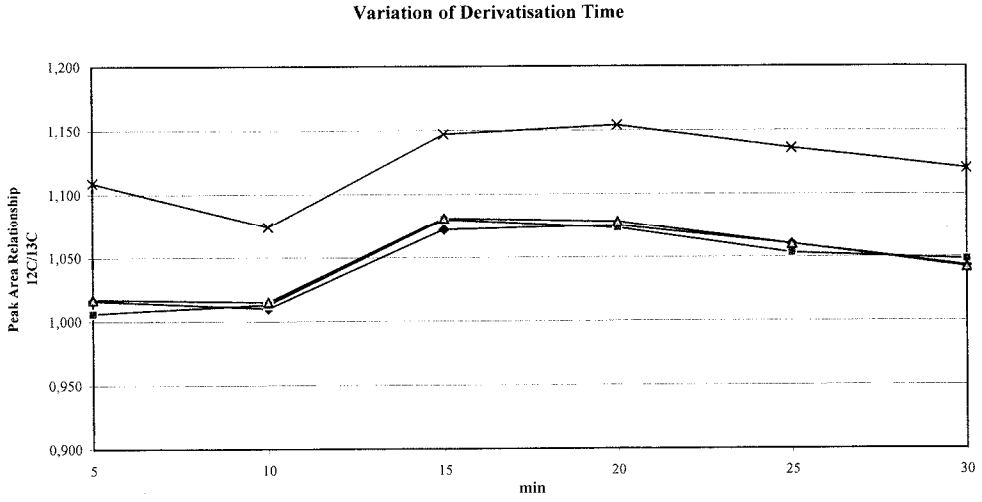

Figure 2: Variation of derivatisation time. Time of derivatisation shown on the abscissa. Storage of the derivatives over a period of 0 minutes to 3 days at room temperature shown as graphs. (25 µl of MSTFA was used for derivatisation).
♦ - derivative injected immediately after derivatisation
■ – derivative injected 120 minutes after derivatisation
▲ - derivative injected 24 hours after derivatisation
× - derivative injected 3 days after derivatisation.
The peak area relationship $^{12}C/^{13}C$ is shown on the ordinate.

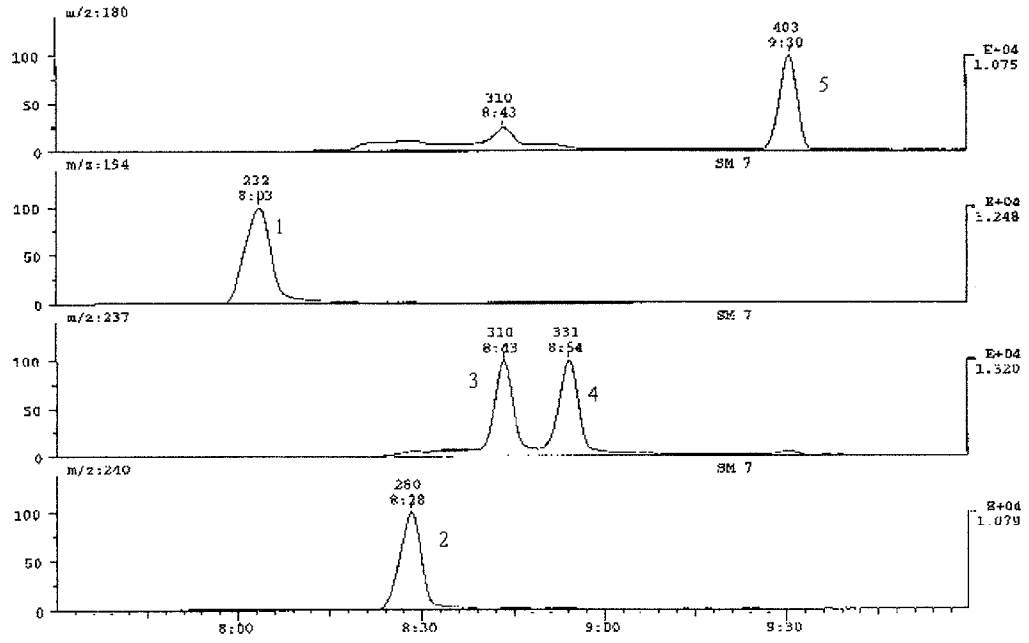

Figure 3: GC-separation of xanthines shown as full-scans on the MS.
The left ordinate shows the relative intensities of the mass-ion, the right ordinate the relative signal intensity. The abscissa shows the scan time in minutes. Above each peak is the scan number and scan time.

Peak identity: 1 – Caffeine, 2 – ^{13}C $^{15}N_2$ theophylline, 3 – 1,7-dimethyl xanthine (paraxanthine), 4 – Theobromine, 5 – 1,3-dimethyl-7(2-hydroxyethyl) xanthine (=7(2-hydroxyethyl)-theophylline

were obtained with 6 mol/l trichloroacetic acid (TCA), whereby 100 µl TCA was added to 500 µl serum or plasma, vortexed and centrifuged (10 min at 2000 g).
If the supernatant remained cloudy after centrifugation, 100 µl TCA was added, the tube vortexed and then centrifuged a second time.
TCA was made up fresh every 14 days as it was seen that the efficiency of precipitation declined continually after 14-21 days standing at room temperature (22-25 °C).

Derivatisation

Volume: As the derivatisation mixture was injected directly into the GC, the intensity was proportional to the final volume of the derivatisation mixture. The best results were obtained using a derivatisation volume of 25 µl MSTFA. With volumes of MSTFA less than 25 µl, the peak form was typical for overloading the column (tailing-effects). With derivatisation volumes above 30 µl, loss of signal intensity was seen. The result of the derivatisation-volume test can be seen in Figure 1.

Time: An optimal derivatisation was obtained after 25 min at 90 °C. (Figure 2). Storage of the derivatives at room temperature over a period of 7 days did not lead to a loss in signal at the measured masses, which showed that the derivatives could be stored before injection into the GC.

Temperature: The time needed to complete derivatisation was reduced by increasing the temperature. Temperatures of 50 – 100 °C were tested using steps of 10 °C. The time chosen was 25 min at 90 °C (Figure 2).

Retention times of different xanthines on the GC-column

The retention times of the different derivatised xanthines on the GC column operated as described above were as follows:

caffeine	– m/z 154; elution time 8.02 min
theophylline	– m/z 237/240; elution time 8.28 min
theobromine	– m/z 237; elution time 8.54 min
paraxanthine	– m/z 237; elution time 8.43 min
7-(2-hydroxyethyl)-theophylline	– m/z 180; elution time 9.30 min

The results are shown in Figure 3.

Caffeine (1,3,7-trimethyl xanthine) was not derivatised due to the absence of a free hydroxyl group. The internal standard used for HPLC (7-(2-hydroxyethyl)-theophylline) could not be used in GC-IDMS as it had different properties after derivatisation and did not give rise to reproducible results.

Quantitative Determination of Theophylline in Serum or Plasma

The determination is carried out in glass vials. The volume of serum and standards is adjusted so that an absolute amount of 5 µg theophylline is present in the sample. Internal standard is then added so that the ratio of sample to internal standard is 1:1.

The mixture is vortexed and allowed to equilibrate at room temperature for 15 min. Serum or plasma samples are then mixed with 100 µl TCA for each 500 µl sample, vortexed and centrifuged at 2000 g for 10 min.

The supernatant is pipetted into new vials and extracted with 1 ml chloroform-propan-2-ol, vortexed and centrifuged as above. The lower organic phase is then transferred to new screw-top vials and evaporated to dryness under nitrogen at 90 °C.

Derivatisation is then performed on the dry extract by adding 25 µl MSTFA, sealing the vials with screw tops and heating at 90 °C for 25 min. 2 µl of this mixture is then injected onto the GC column and the ions with m/z 237 / 240 are measured in the MS.

Precision of measurements

A total of 27 measurements of theophylline were made over a 4-day period using the BioRad Lyphochek 40093 control serum. The results were as follows:

Day 1: mean: 14.1 mg/l; cv 0.885 %; n = 12
Day 2: mean: 14.0 mg/l; cv 1.14 % ; n = 5
Day 3: mean: 14.1 mg/l; cv 0.827 %; n = 5
Day 4: mean: 14.2 mg/l; cv 0.527 %; n = 5

The measurements were made throughout the day at regular intervals (day 1 every 4th sample; days 2-4 every 8th sample.)

Comparison of measurements with GC-IDMS and HPLC performed on the same materials on the same day

The results are shown in the accompanying publication on the HPLC-determination of xanthines in serum or plasma.

DISCUSSION

The use of GC-IDMS as a reference method is widespread in the field of clinical laboratory analysis. In the routine determination of theophylline in biological fluids, mainly serum or plasma, however, GC-IDMS does not play a large role because of its time-consuming nature.

GC-IDMS for theophylline extracted from plasma or urine was first published almost two decades ago (4), although its use as a candidate reference method for use in clinical samples is much more recent (5,6). The latter use is of importance in setting reliable and specific and method-independent target values for theophylline in internal and external quality survey materials. The comparison between GC-IDMS and HPLC was shown to be excellent (3) when identical samples were assayed. The cross-reactivity of antibodies used in commercial assays can be controlled using either GC-IDMS or HPLC, especially with respect to caffeine.

HPLC is used much more often for routine method comparisons, as the necessary apparatus is present in most analytical laboratories (7,8).

For most purposes, semi- or fully automated immunoassays are routinely used in emergency and/or pharmacological laboratories to determine this analyte.

The advantages of GC-IDMS – and to an extent HPLC – are that the analyte can be measured specifically as well as simultaneously in a mixture of metabolites and chemically related substances, here such as caffeine and theobromine, both of which have similar biological activity, and which are present in natural beverages such as coffee and cocoa. The latter situation may be of interest when the patient presents with unexpected symptoms or signs of overdose – or in forensic cases.

In conclusion, we describe a method which uses GC-IDMS to determine the concentration of theophylline (1,3-dimethyl xanthine) in human plasma or serum. The effects of similar substituted xanthines – namely theobromine (3,7-dimethyl xanthine), paraxanthine (1,7-dimethyl xanthine) 1,3-dimethyl-7-(2-hydroxyethyl) xanthine (internal standard HPLC) and caffeine (1,3,7-trimethyl xanthine) were tested to confirm the specificity of the method. The derivatisation of all xanthines was performed with MSTFA. The internal standard used was 2-^{13}C ,1,3-$^{15}N_2$-theophylline. The extraction and derivatisation procedures were examined in detail and optimised stepwise during the development of the method. High precision and accuracy have been confirmed. HPLC was used for comparison.

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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)

Clin. Lab. 2002;48:635-646

PERSONAL EXPERIENCE

How to Make Things Work Again – Troubleshooting Using the GC-IDMS Determination of Triacylglycerols as an example

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SUMMARY

This article describes the process of "repairing" a method which has gone out of control, using the gas-chromatographic isotope-dilution mass spectrometric (GC-IDMS) determination of total glycerides in serum, measured as glycerol. The original method used $^{13}C_2$-glycerol as aqueous internal standard and ^{12}C-tripalmitin dissolved in toluene as external standard. The modified method used tripalmitin as internal and external standard, the former being labelled uniformly on the glycerol moiety of tripalmitin ($^{13}C_3$-tripalmitin). In addition, glycerol-free human serum albumin was added to the external standards to "trap" the glycerol physically during evaporation of the extraction solvent after alkaline hydrolysis. The modified method was more stable than the original one and the intensity of the MS-signal in the modified method was at least 100 times stronger in the external standards than in the original method. The precision of the modified method in measuring total glycerides in serum samples (as total glycerol) was better than in the original method, the coefficients of variation being under 1.5% at concentrations between 0.8 and 3.5 mmol/l. (Clin. Lab. 2002;48:635-646)

KEY WORDS

Extraction, recovery, precision, accuracy, troubleshooting. gas chromatography, mass spectrometry, isotope dilution, GC-IDMS, internal standard, derivatisation, glassware, tripalmitin, glycerol

INTRODUCTION

Laboratory methods are frequently published with details as to how a method (should) function(s). However, very little is seen in the literature which describes how a method which has ceased to function can be resuscitated. Even though the symptoms of system-failure are different, the ways to examine and correct such failures are similar enough to allow the use of a specific example to describe such corrective actions.

Many newcomers to scientific research are taught how to perform an established method or use a commercial kit. Very few are shown how to develop and/or repair a method, which has ceased to function properly. Worse still, many researchers are unable to detect that a method is failing, so that results are produced which may give rise to "false significances". The use of statistics alone is insufficient to produce meaningful results!

This short article describes the resurrection of the determination of triacylglycerols by gas chromatography isotope dilution mass spectrometry (GC-IDMS) as an example of successful troubleshooting.

Reagents used

$^{13}C_2$-glycerol (Cambridge Isotope Laboratories, Andover, MA, USA) and $^{13}C_3$-tripalmitin (declared purity > 99%) were used as internal standards. Tripalmitin (NIST / US Department of Commerce, Washington, DC, USA, purity 99.5 ± 0.2%) was used as the external standard. Tripalmitin was dissolved in toluene, glycerol diluted with HPLC-grade water. $^{13}C_3$-tripalmitin was custom-synthesised, all glycerol carbon-atoms being labelled.

Certified control materials were serum-based and obtained from the National Institute for Standards and Technology, (NIST), Gaithersberg, MD, USA – the standard reference material 909b, levels I and II were used for the determination of total glycerides in serum.

Manuscript accepted July 5, 2002

Human-based control serum was obtained from Rolf Greiner Biochemicals, Flacht, Germany (Serodos and Serodos plus).
Solvents and extraction reagents were HPLC-grade and were purchased from Merck, Darmstadt, Germany, or Sigma-Aldrich, Taufkirchen, Germany. Potassium hydroxide pellets (pro analysi grade) were ordered from Merck. Heptafluorobutyric anhydride (HFBA) was purchased from Macherey-Nagel, Düren, Germany.
The GC-IDMS measurements were performed on a Trace GC-MS (Thermo-Finnegan, Egersbach, Germany.)

Basics of the Original Reference Method
The determination of triacylglycerides is dependent upon the hydrolysis of the ester linkage between the glycerol moiety and fatty acid residues, the derivatisation of the glycerol and its determination with GC-IDMS. The following table shows the steps in performing the assay.

1. Weigh the components into clean 4 ml screw-top vials. (^{12}C-tripalmitin as standard, ^{13}C-glycerol as internal standard, control materials – for example NIST-909b and samples)
2. Hydrolyse the triacylglycerols with methanolic KOH
3. Neutralise with HCl and precipitate proteins with acetone
4. Centrifuge and transfer supernatant to clean 4 ml screw-top vials
5. Evaporate to dryness under nitrogen
6. Derivatise with heptafluorobutyric anhydride (HFBA)
7. Add n-heptane, vortex and centrifuge
8. Transfer supernatant to clean 1 ml screw-top vials
9. Evaporate to dryness and take up in a small volume of n-heptane
10. Inject into GC and measure ion-pairs with m/z 253/255 and 467/469 (for dually labelled glycerol).
11. Calculate results of unknowns from the standards and internal standards.

Concentrations and volumes have been omitted on purpose at this stage, as these are assay-specific and have to be optimised during the method-development and validation stages.

Potential Problems, Signs and Symptoms
The method was always fraught with problems, even though it was defined as a reference method.
The first potential problem was that the internal ^{13}C standard (glycerol) was not identical with the ^{12}C-standard (tripalmitin) used.
The next problem point was the hydrolysis with methanolic KOH.
Other trouble areas included the evaporation to dryness, derivatisation with HFBA and removal of the latter before injection onto the GC column.
The volumes of standards and samples are adjusted to give a theoretical peak area ^{13}C:^{12}C glycerol of unity. This allows a 1-point calibration to be used.
Signs that the assay is beginning to malfunction include the following:

a. Precision begins to worsen – shown by increasing values of the coefficient of variation.
b. The peak-area coefficient (^{13}C:^{12}C) begins to deviate from 1.00
c. The peak-area changes – increases or decreases are possible
d. The change of sample appearance during hydrolysis or derivatisation becomes apparent.

A further problem was that reproducibility worsened over a very short period of time. After four weeks trouble-shooting it was found that the new – more economical – glass vials used for extraction and derivatisation were contaminated with an invisible lipid film, which varied from vial to vial. This was verified by running different blanks and carrying out the derivatisation and extraction steps in vials without sample. As soon as the lipid-contaminated vials were replaced with the original ones, which included changing the supplier, the non-reproducibility vanished. This shows that saving money on disposables can be expensive – here around 400 man-hours of enlightenment (and frustration)!

Curing the Patient
The steps used are identical to the numeration in "Basics of the Method" – see above.

Step 1
The glass vials are checked by running a derivatisation without samples. If ^{12}C-peaks corresponding to glycerol appear, then the vials are contaminated with fat. This was the case with one supplier. By changing the vial supplier, this problem was solved.
The use of 1,2-^{13}C-glycerol as internal standard was taken from the first publication of a GC-IDMS method for triglycerides (determined as total glycerol) (1). A better comparison is obtained by replacing the 1,2-^{13}C-glycerol by 1,2,3-^{13}C-glyceryl tripalmitate. Standard and internal standard are then identical and correction factors for ^{13}C-glycerol are no longer needed. Finally the separation of 3 mass units allows for better discrimination between ^{12}C and ^{13}C labelled glycerol-HFBA derivatives (m/z 252/255; m/z 467/470). Any loss during the initial hydrolysis is compensated for.

Step 2
If glycerol is evaporated to dryness, like water, nothing remains! The original method did not add any carrier to the standards. The method only "worked" with freshly prepared methanolic KOH. Even then, the absolute intensity of the derivatised standards was much lower than for derivatised serum samples. By addition of 50 µl glycerol-free human serum albumin solution (ca. 45 g/l) a matrix was provided, which physically prevented the glycerol from evaporating. The signal intensity of the derivatised standards was equivalent to that of the derivatised serum samples. The methanolic KOH could be stored for at least 4 weeks, compared with 4-7 days without the use of carrier albumin. The testing of suitable albumin was made using a "dummy" derivatisation of the albumin solution (albumin blank). The albumin is suitable if no peak occurs at m/z 255 or 467 after HFBA-derivatisation.

Step 3
The amount of HCl was adjusted to allow neutralisation of the KOH (25 µl 30% HCl for 500 µl 1 mol/l KOH)

Step 4
After centrifugation around 2/3 of the supernatant was transferred to a fat-free clean glass vial (see step 1 above!)

Step 5
Evaporation to dryness was carried out under nitrogen at 70 °C. This took 15-20 minutes. It is important that the samples are dry, otherwise the derivatisation with HFBA does not function.

Step 6
The vials are briefly vortexed every 5 minutes during derivatisation. The sediment dissolves during hydrolysis and the colour of the derivatised extract changes to yellow or orange-brown. A bordeaux-red coloration usually (but not always) denotes that the reaction has not taken place. This is often due to moisture carried over from step 5 above.

Step 7
500 µl of n-hexane are added to the derivative (in the fume cupboard!), the cap is screwed firmly onto the vial and the vial vortexed several times over a period of 1-2 minutes. The vials are then centrifuged and 400 µl of the supernatant transferred to a clean 1 ml vial.

Step 8
The hexane is evaporated off at 70 °C under nitrogen. This takes ca. 2 minutes.

Step 9
The vials are allowed to cool and 50 µl n-hexane are added and the caps screwed tightly onto the vials which are vortexed to redissolve the residue.

Step 10
1 µl of derivative is injected into the GC and the mass ion pairs m/z 253/256; 467/470 are detected in the MS. The derivative can be stored for at least 10 days at 4-7 °C without loss in signal or change in numerical results.

Examples of GC-IDMS Results
The effects of the changes made to the system during the process of optimising the method are best demonstrated by showing the visual differences in the observed signals. The different effects are shown in the following tables and figures.
Figures 1a-1d show variations on the original procedure for GC-IDMS (1) in which the internal standard (^{13}C-glycerol) was not identical with the external standard (^{12}C-tripalmitin).
This method only functioned – and then unreliably – with fresh methanolic KOH. When considered physically, glycerol vapourises when heated, analogous to water. The glycerol which is used for derivatisation must be retained physically on a matrix. In the original procedure, this is purely due to chance and seems to be partly dependent upon the presence of a residue from the fresh methanolic KOH. The addition of a glycerol-free protein-matrix – here human serum albumin – seems to bind the released glycerol, so that loss during evaporation under nitrogen is minimised.
Figures 2a-2d show the results from the modified method, which used $^{13}C_3$-tripalmitin as internal standard instead of $^{13}C_2$-glycerol together with the addition of a glycerol-free protein-matrix for the external standards. Advantages of this system are the use of identical internal and external standards and a human protein-matrix for the standards.
Figures 2a-2c show the excellent discrimination between ^{12}C and ^{13}C for the tripalmitin standards. The intensity of the signal (see diagram right side – NL: a,b,c,d) for the modified method was on average more than 100 times higher than in the original method. The retention times on the GC-column (Rtx-5MS [15 meters] Thames-Restek, Windsor, GB) were constant.
The NIST sera showed a good separation of hydrolysed glycerol (not shown). The stability of the retention times were tested over a five-week period using the same column and GC-MS programme. The retention times on day 1 were between 5.90 and 5.92 min; on day 35 between 6.10 and 6.11 minutes.
Figure 2d shows the advantage of measuring the m/z pair at 467/470 amu. The interfering peak at m/z 253 amu is much reduced at m/z 467 amu. It is advisable to measure the higher mass pair to avoid potential interference even though the absolute intensity of this ion-pair (see Figure 1a, trace 1) is much lower.

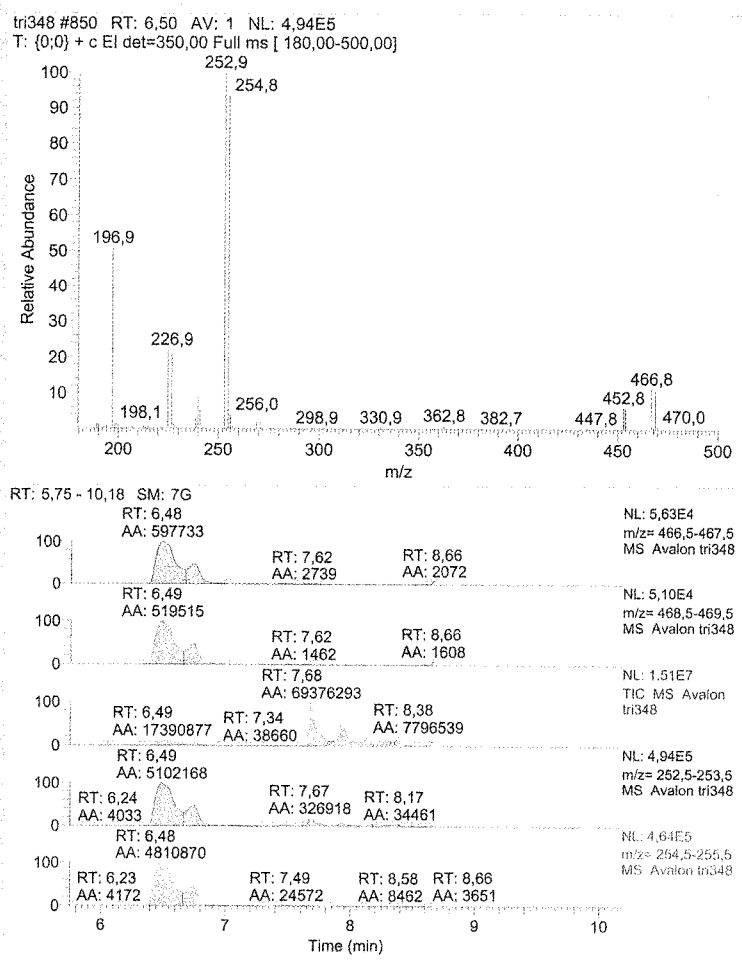

Figure 1a:

Please note that in all figures the decimal point (.) is represented by a comma (,). The software is adapted to the German convention, in which the use of decimal point and comma for the separation of thousands is reversed when compared with the Anglo-American convention.

Abbreviations used in all figures: RT – retention time (min); NL – Intensity of the highest peak in arbitrary units; MA – area under the peak in arbitrary units; trixxx – sample number (lab-internal).

a.) Full scan for the NIST-Reference Material 909 b II (serum) – from above:
Trace 1: The m/z peaks between 180 and 500 amu. The ion-pairs of interest are: m/z 253/255 and 467/469 (shown here as 252.9/254.8 and 466.8/468.7).
Traces 2 & 3: The GC-MS chromatogram for m/z 467/469. The specific peak has a retention time of 6.48 min.
Trace 4: Full scan – all detected peaks with m/z between 180 and 500 amu.
Traces 5 & 6: As traces 2 & 3 but for the ion pair m/z 253/255.

Internal standard $^{13}C_2$-glycerol, external standard ^{12}C-tripalmitin.
Vials used – from "economic" supplier with lipid-film resulting in double peak form. The peak with the highest intensity is at 7.68 min and is non-specific.
Hydrolysis was carried out with fresh KOH/methanol.
The abscissa is the time scale in minutes (GC-column); the ordinate represents the relative ion-intensity expressed as % of the highest peak.

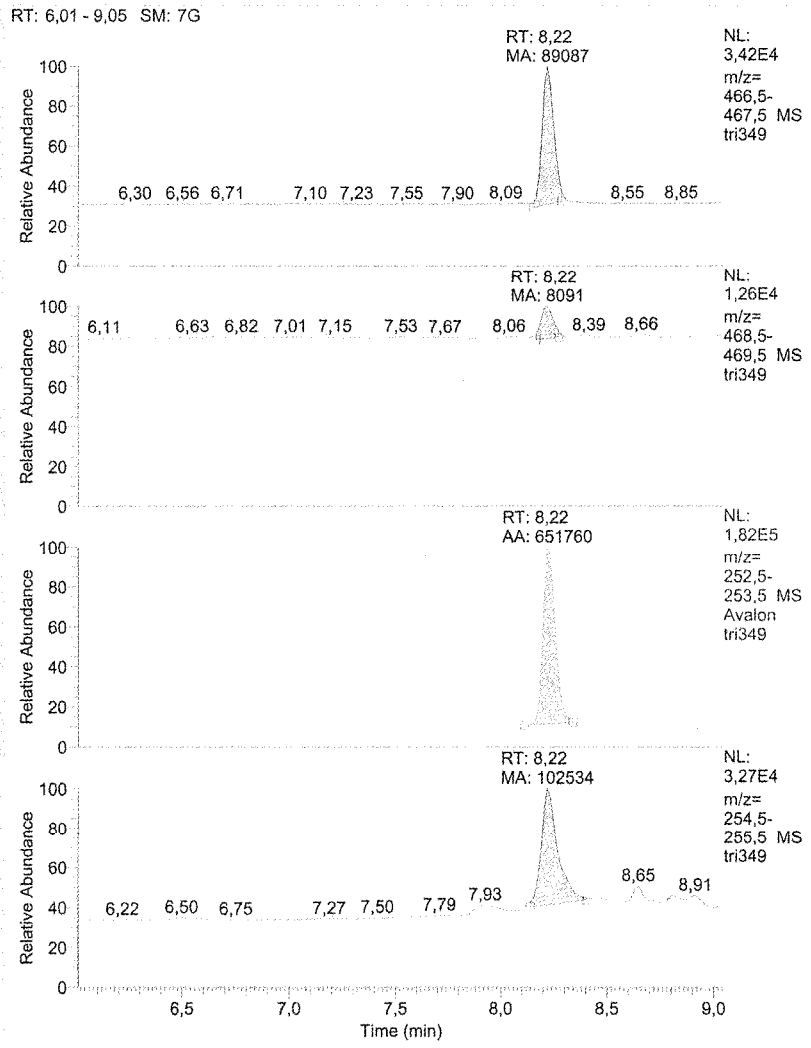

Figure 1b:

Please note that in all figures the decimal point (.) is represented by a comma (,). The software is adapted to the German convention, in which the use of decimal point and comma for the separation of thousands is reversed when compared with the Anglo-American convention.

Abbreviations used in all figures: RT – retention time (min); NL – Intensity of the highest peak in arbitrary units; MA – area under the peak in arbitrary units; trixxx – sample number (lab-internal).

b.) Water blank taken through the full extraction and derivatisation procedure. Vials as in Figure 1a above. Abscissa and ordinate as in Figure 1a. From above:
traces 1 & 2 m/z pair 467/469; traces 3 & 4 m/z pair 253/255 amu.

The single peak at 8.22 min shows "^{12}C-glycerol" contamination. The elution time differed from the normal ^{12}C-glycerol derived from the hydrolysis of the tripalmitin standard and from the ^{13}C-glycerol internal standard.

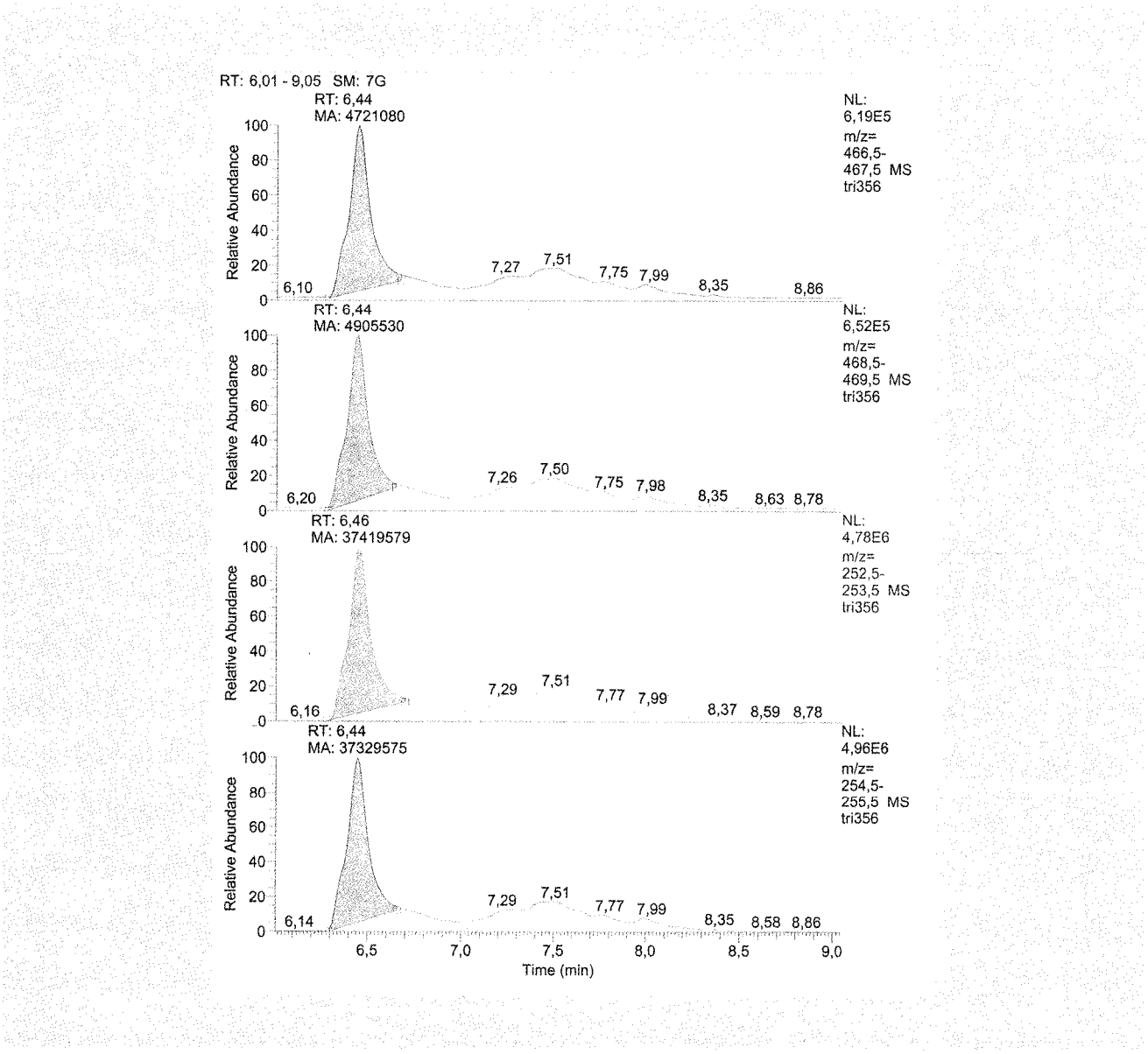

Figure 1c:

Please note that in all figures the decimal point (.) is represented by a comma (,). The software is adapted to the German convention, in which the use of decimal point and comma for the separation of thousands is reversed when compared with the Anglo-American convention.

Abbreviations used in all figures: RT – retention time (min); NL – Intensity of the highest peak in arbitrary units; MA – area under the peak in arbitrary units; trixxx – sample number (lab-internal).

c.) ^{12}C-tripalmitin external standard with ^{13}C-glycerol internal standard after extraction and derivatisation, no additional albumin, fresh methanolic-KOH, vials, abscissa and ordinate as above. Specific peak with shoulder at 6.44 min. Additional contamination peaks between 7.26 and 8.86 min. The latter were only present when the "economy" vials were used.

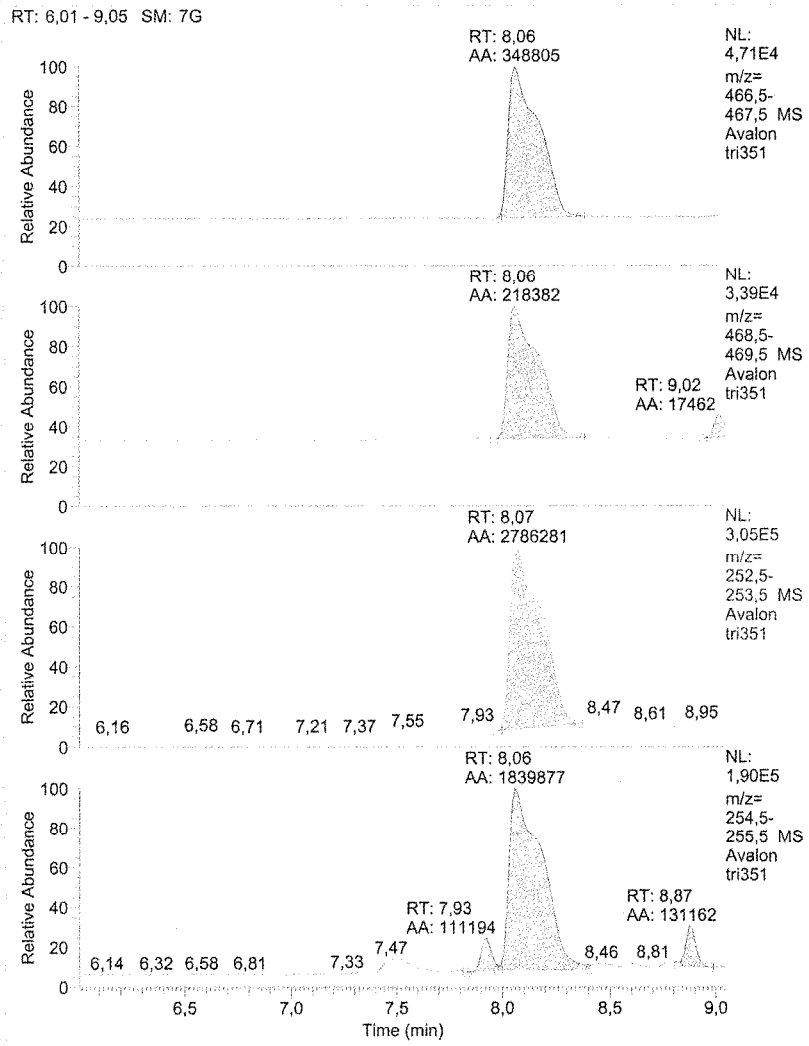

Figure 1d:

Please note that in all figures the decimal point (.) is represented by a comma (,). The software is adapted to the German convention, in which the use of decimal point and comma for the separation of thousands is reversed when compared with the Anglo-American convention.

Abbreviations used in all figures: RT – retention time (min); NL – Intensity of the highest peak in arbitrary units; MA – area under the peak in arbitrary units; trixxx – sample number (lab-internal).

d.) Human serum albumin blank (50 µl of a 45 g/l solution in physiological saline). Conditions as in Figures 1a-1c above.

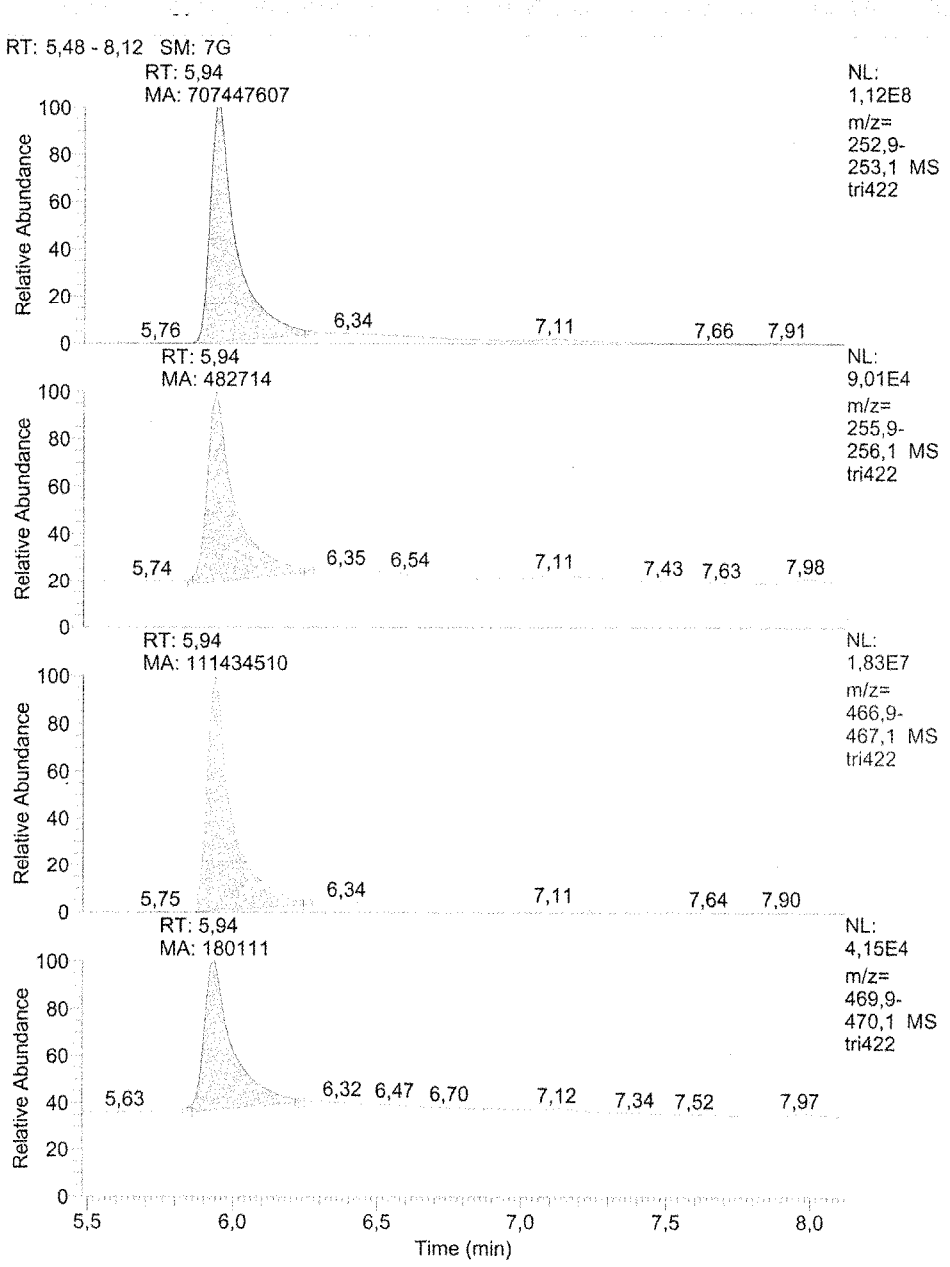

Figure 2a:

The layout of all the figures is similar to that in Figure 1. Note the m/z pairs are 3 amu apart, namely 253/256 and 467/470 due to the triple labelling of the tripalmitin internal standard. The abbreviations are as in Figure 1 above.

a.) ^{13}C-blank containing ^{12}C-tripalmitin and human serum albumin carrier taken through the full procedure.

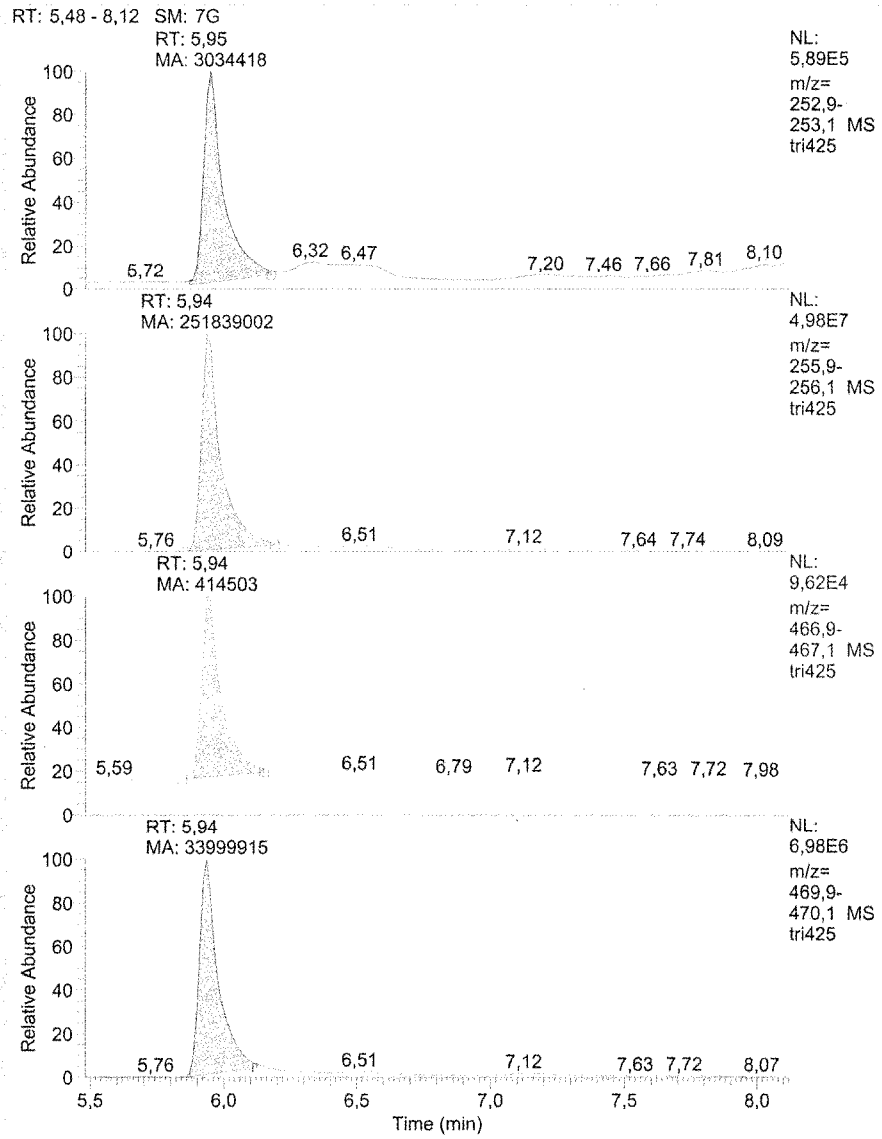

Figure 2b:

The layout of all the figures is similar to that in Figure 1. Note the m/z pairs are 3 amu apart, namely 253/256 and 467/470 due to the triple labelling of the tripalmitin internal standard. The abbreviations are as in Figure 1 above.

b.) ^{12}C-blank containing ^{13}C-tripalmitin and human serum albumin carrier taken through the full procedure of extraction and derivatisation with HFBA.

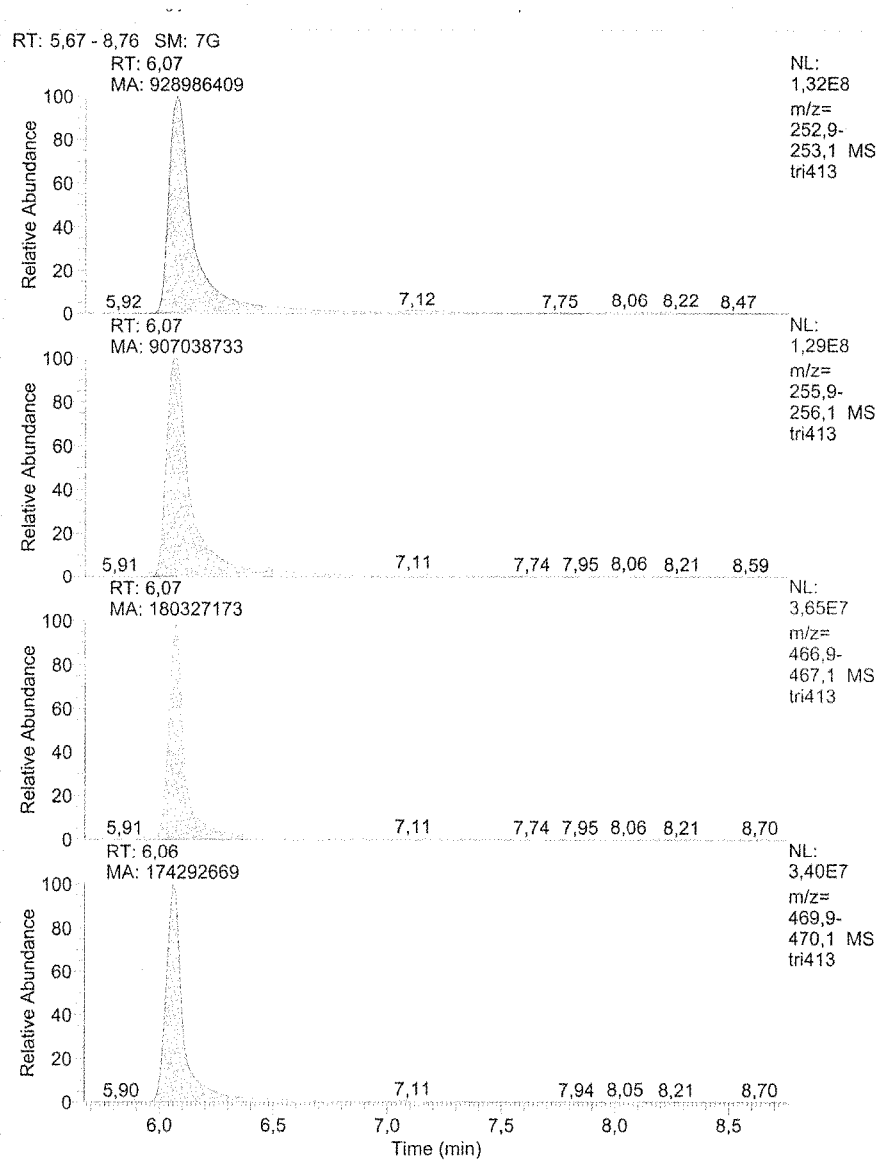

Figure 2c:

The layout of all the figures is similar to that in Figure 1. Note the m/z pairs are 3 amu apart, namely 253/256 and 467/470 due to the triple labelling of the tripalmitin internal standard. The abbreviations are as in Figure 1 above.

c.) As Figures 2a and 2b but with labelled and unlabelled tripalmitin

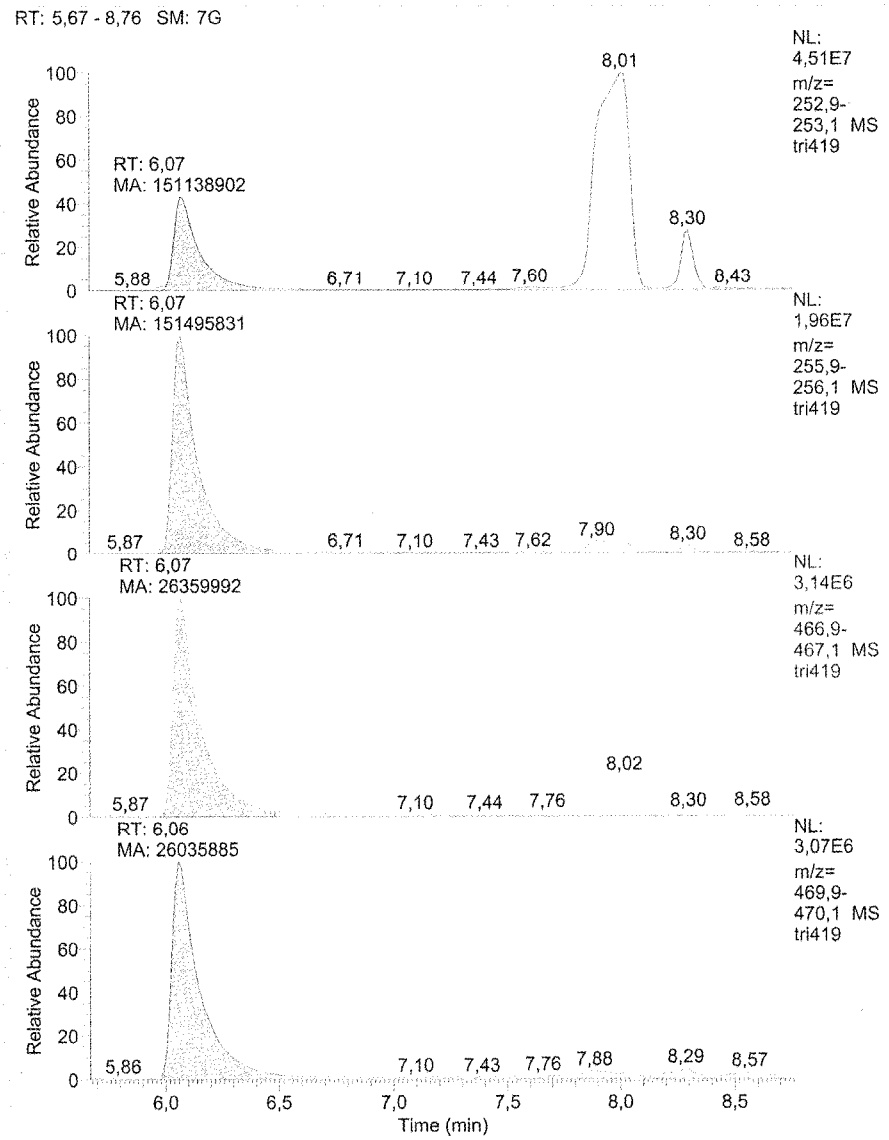

Figure 2d:

The layout of all the figures is similar to that in Figure 1. Note the m/z pairs are 3 amu apart, namely 253/256 and 467/470 due to the triple labelling of the tripalmitin internal standard. The abbreviations are as in Figure 1 above.

d.) Control serum Serodos plus – additional peaks (8.02 and 8.30 min) do not interfere, and are hardly present in the higher m/z trace no. 3.

General Performance Data

The declared levels of total glycerides – expressed as triolein – for the NIST materials were 0.949 ± 0.061 mmol/l for level I and 1.529 ± 0.035 mmol/l for level II. The modified method gave values of 1.014 ± 0.013 for level I and 1.554 ± 0.018 mmol/l for level II. These results were obtained from 10 measurements each made from separate extraction and derivatisation of the original material.

The precision of measurements using the modified method and commercial human-based control sera lay between 1.25 and 1.47 % when expressed as the coefficient of variation. These values were for samples with total glycerides lying between 0.8 and 3.5 mmol/l and were derived from at least 16 independent measurements.

The corresponding data for the original method were: 1.102 ± 0.019 for NIST level I and 1.566 ± 0.027 mmol/l for NIST level II ($n \geq 24$ independent measurements).

DISCUSSION

This exercise shows an example of optimising a method, taking into account various physical properties of the substance to be measured as well as the necessity of using the same materials for internal and external standards.

In a method which has been declared as a reference method, it is more important to avoid unneccesary compromises, especially in the choice of standards. The use of tripalmitin for internal and external standard alleviates the use of a conversion factor needed when glycerol is used as the internal standard. As both the ^{12}C and ^{13}C-tripalmitin have to be hydrolysed to glycerol, a further potential source of error has been eliminated, namely that of the non-hydrolysis of the internal standard if ^{13}C-glycerol is used.

The simple addition of a protein-trap to retain the glycerol during the evaporation of the extraction solvents after the hydrolysis of the tripalmitin not only increases the intensity of the final signal by a factor of at least 100-fold in the mass spectrometer, but improves the precision of the measured signal by over 30%. In addition, the methanolic KOH can be used over a longer period of time – up to at least 4 weeks – in the modified procedure, most probably due to the addition of human serum albumin to the otherwise protein-free external standards.

This short presentation shows how one can "repair" methods which no longer perform to expected standards, taking into consideration both physical and chemical properties of the substances to be quantitated.

Other aspects – such as the importance of evaporation to dryness before derivatisation with HFBA – are also discussed.

Reference.

1. Siekmann L, Schönfelder A, Siekmann Anita. Isotope dilution-mass spectrometry of total glycerol in human serum – a reference method in clinical chemistry. Z Anal Chem 1986: 324; 280-1

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V

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"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)

Clin. Lab. 2003;49:329-343

ORIGINAL ARTICLE

Determination of the Cardiac Glycosides Digoxin and Digitoxin by Liquid Chromatography Combined with Isotope-Dilution Mass Spectrometry (LC-IDMS) – a Candidate Reference Measurement Procedure

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SUMMARY

This article describes a method of high analytical sensitivity, reproducibility and trueness for the determination of digoxin and digitoxin in serum or plasma at therapeutic levels using a combination of high-pressure liquid chromatography (HPLC), isotope-dilution mass spectrometry (IDMS) and caesium-adduct formation. A method for threefold deuterium substitution in the glycosides was developed, which could be performed within 24 hours without distillation giving yields >98% of the theoretical value. Extraction from a serum or plasma matrix was performed using a liquid-phase extraction with ammonium acetate buffer/tertiary butylmethyl ether/ethyl acetate at pH 9.5. The HPLC-separation used a 10 x 2 mm LiChrospher RP-18 5 µm guard column in combination with a 125 x 2 mm main column of the same material and a gradient containing methanol, caesium ions and formic acid. Quantification of digoxin and digitoxin was made with IDMS using deuterated internal standards and the system run in single ion monitoring (SIM) mode. The methods had a lower limit of determination of 0.25 µg/l for digoxin and digitoxin, a trueness between 97.5 and 104% for digoxin and between 98 and 101% for digitoxin, respectively and had a coefficient of variation of less than 3% in the therapeutic range for both glycosides. Maximally 1 ml serum or plasma was needed for the procedure. The method is used to set target values for materials used in external quality assessment surveys (EQAS) run by INSTAND as part of a national EQAS-programme.
(Clin. Lab. 2003;49:329-343)

KEY WORDS

digoxin, digitoxin, digoxigenin, digitoxigenin, LC-IDMS, liquid-phase extraction, reference measurement procedure, caesium adducts, quality-control.

INTRODUCTION

The development of methods of higher metrological order is an essential strategy for improving EQAS and is therefore an integral part of the work of a laboratory involved in developing and validating reference measurement procedures. This article describes the development, optimisation and validation of a method for measuring digoxin and digitoxin in body fluids using a combination of high-pressure liquid chromatography (HPLC) and isotope-dilution mass spectrometry (IDMS).

One aim of an EQAS is to compare the results of participating laboratories with target values obtained from the method nearest to the true value, thus evaluating both the performance of a single laboratory and the analytical systems used. In the case of small organic molecules found in blood, reference measurement procedures can be used for the determination of target values using gas-chromatography (1,2) or high-pressure liquid chromatography (3-6) coupled with mass spectrometry.

Manuscript accepted March 26, 2003

Table 1a – HPLC conditions for the separation of digoxin

Run time (min)	Eluent (%B)
0.01	50
6.50	72
6.60	100
9.60	100
13.0	Stop

Table 1b – HPLC conditions for the separation of digitoxin

Run time (min)	Eluent (%B)
0.01	50
10	83
14	100
16	50
20	Stop

By choosing an internal standard with (almost) identical properties as the substance to be measured, problems in extraction and chromatography can be overcome. The use of stable isotopic labelling can be used to produce such an internal standard. The physical separation of the native and isotope-labelled analyte by mass-spectrometry is easier the greater the difference in molecular mass.

The choice of LC-IDMS as method was made as the alternative, GC-IDMS did not give any reproducible results. Moreover, methods have been published for the determination of digoxin and digitoxin using LC-IDMS (5,6). One of these methods formed the basis for the present communication (5).

MATERIALS AND INSTRUMENTS

Digoxin (purity 96.7%, certificate of analysis by Sigma-Aldrich), digitoxin (purity 100%, certificate of analysis by Sigma-Aldrich), triethylamine (purity 99%), 1,4-dioxane (purity > 99.9%) ammonia (25%, p.a.), formic acid (puriss.p.a. 98%), deuterium oxide (purity 99.9%) and caesium hydroxide (purity 99.97%) were purchased from Sigma-Aldrich, Taufkirchen, Germany. Methanol (LiChrosolv), water (Lichrosolv), ethyl acetate (for organic trace analysis), ammonium acetate (p.a.) and tetrahydrofurane (p.a.) were obtained from Merck, Darmstadt, Germany. Other buffer substances were either obtained from Merck or Sigma-Aldrich.

The HPLC-system used was from Shimadzu and consisted of two LC-10 ADVP pumps, a system controller (SCL-10AVP CE [LV]), an on-line degasser (DGU-14A) and a semi-micro mixer.

Separation of components was obtained by using a 125 x 2 mm LiChrospher 100 RP-18 5 μm (Merck), together with a guard column (10 x 2 mm) of the same material. Manual injections were made via a Rheodyne injection valve.

The MS-system used was a Finnigan-MAT TSQ 7000 triple-stage quadrupole tandem mass spectrometer equipped with an electrospray ionisation interface (ESI) (Finnigan-MAT, Egersbach, Germany). Data processing was done with the Finnigan-MAT software packet ICIS and Xcalibur.

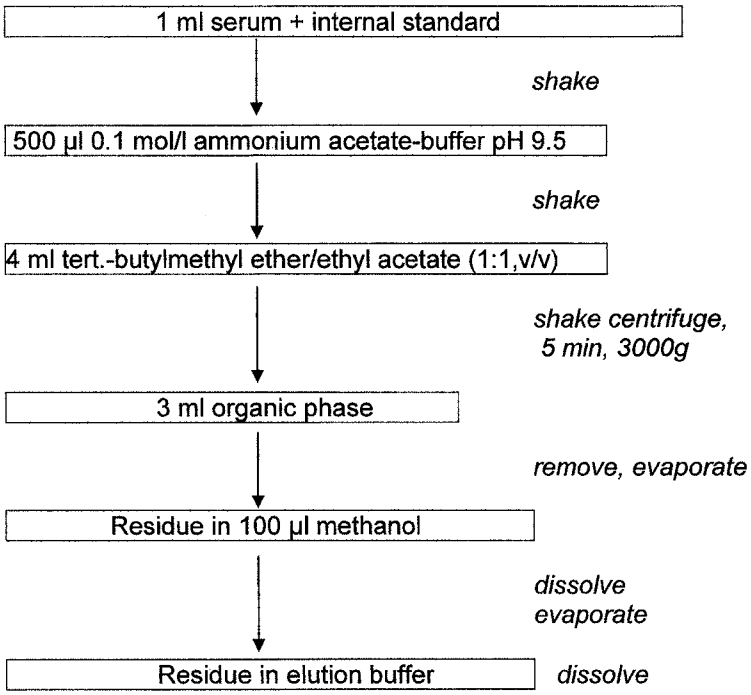
METHODS

Synthesis of labelled glycosides

Basic catalysis for deuterium substitution of steroids has been known for many years (7). The original synthesis of deuterated digoxin and digitoxin was taken directly from the method described by Kessler (5,8) with slight modification. This method consisted of mixing the components and letting them stand in a dark cupboard for at least 21 days at room temperature (22-26 °C). The product formed was almost exclusively triply labelled. The yield was around 70% of the theoretical value, 30% of the glycoside being degraded. Whereas digoxin was exclusively triply labelled, around 6% of the digitoxin remained unlabelled in this method. Stability of the deuterated products could be improved by addition of deuterium oxide before storage. Solutions were used over a period of 4 weeks, after which they were discarded. During this period, no loss of deuterium was observed.

An improved synthesis of deuterated glycosides was performed as follows:
1 ml 1-4 dioxane, 1 ml tetrahydrofurane, 1 ml deuterium oxide and 0.5 ml triethylamine were mixed in a Wheaton vial and 1 mg of the glycoside added. The Wheaton vial was then closed with a screw-cap, shaken until the glycoside had dissolved and allowed to react at 70 °C for 24 h in a heating block. The solutions were then allowed to cool, evaporated to dryness under nitrogen, and redissolved in HPLC-eluant (50% methanol + 50% 4.8 mmol/l formic acid) and tested by direct injection into the mass spectrometer using a full-scan mode. All glycoside solutions showed a 100% conversion into the d_3 derivatives. The spectra were "cleaner" than by the 3-week method and the degradation was much lower. By this method, labelled glycosides could

Table 2: Extraction of digoxin and digitoxin from serum or plasma



be prepared overnight, i.e. directly before use. The yields for digoxin and digitoxin were >98% of the theoretical value, respectively. For storage, the reaction mixture was evaporated to dryness at 70 °C under nitrogen and stored at 4 °C until required. The residues were dissolved in CD_3OD before use to prevent any possible hydrogen/deuterium exchange.

HPLC-conditions

The HPLC conditions were optimised using unlabelled and labelled cardiac glycosides. The extraction of the biological matrices is described below. The chromatographic separation was performed using a binary gradient – see Table 1a for digoxin and Table 1b for digitoxin.

Eluent A was 26 mmol/l formic acid containing 100 µmol/l caesium ions adjusted to pH 3.5. Eluent B was made up of the same concentrations of Cs ions and HCOOH but in methanol. The flow rate was 300 µl/min. CsOH was first titrated with HCOOH to pH 7 to give a stock solution of Cs^+ ions in a neutral solution of caesium formate.

Mass spectrometry

The measurements were made under the following conditions: Spray voltage 4.5 kV; emult 1800 kV; capillary temp 250 °C; sheath gas 60 psi; aux gas 17 ml/min.

Measurements were made in the positive-ion mode using single-ion monitoring in a scan-range from 912.8 - 913.8 and 915.8 - 916.8 for digoxin and digoxin-d_3, respectively and 896.8 - 897.8 and 899.8 – 900.8 for digitoxin and digitoxin-d_3. These masses define the window for the caesium-ion adducts of digoxin and digitoxin.

Sample preparation

The procedure for the extraction of digoxin and/or digitoxin from serum was as shown in Table 2.

Figure 1a

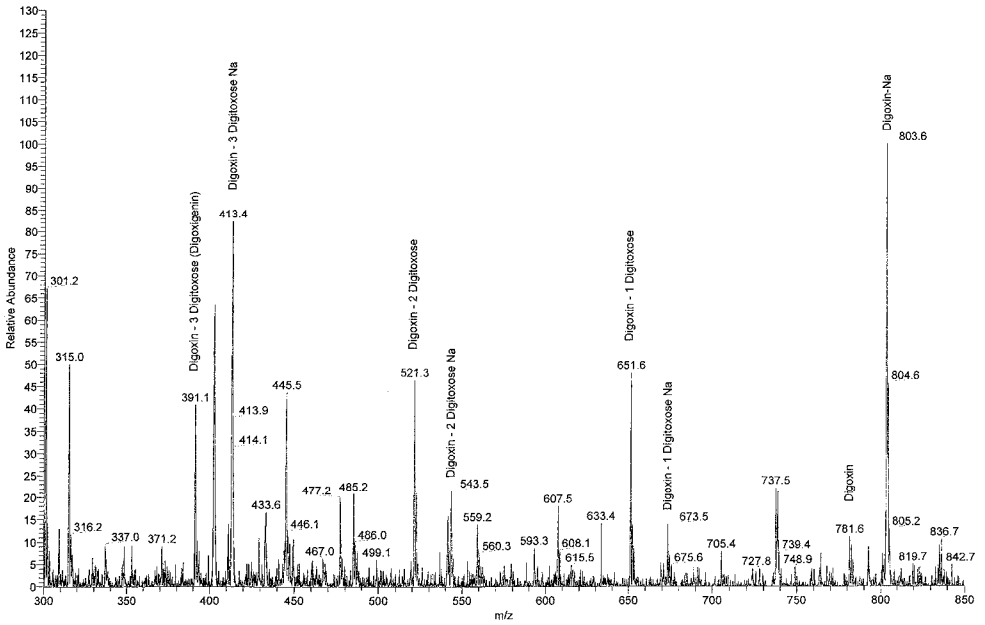


Figure 1b

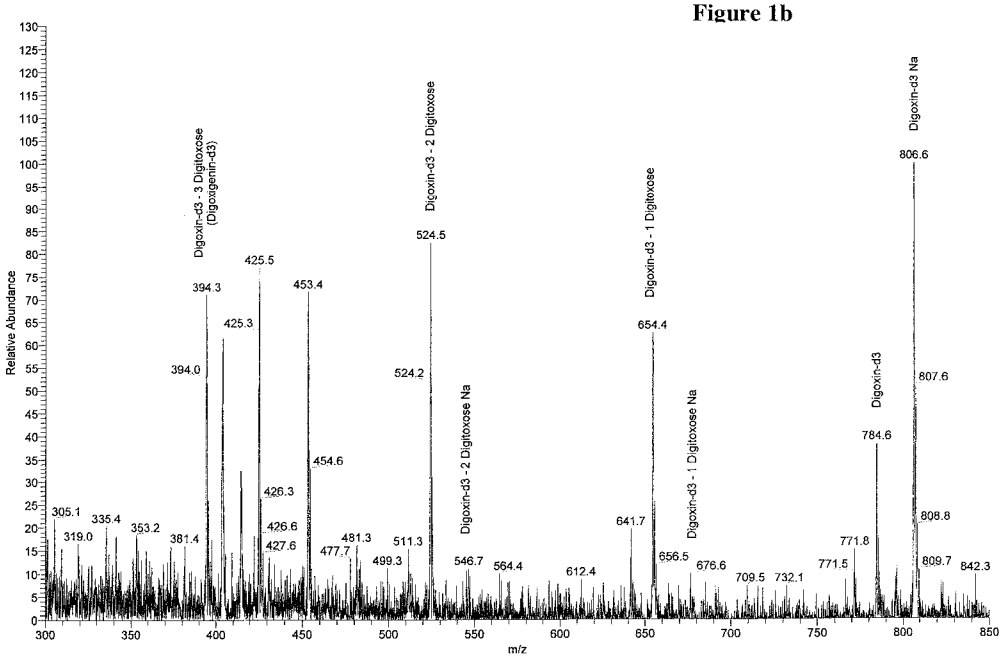


Figure 1c

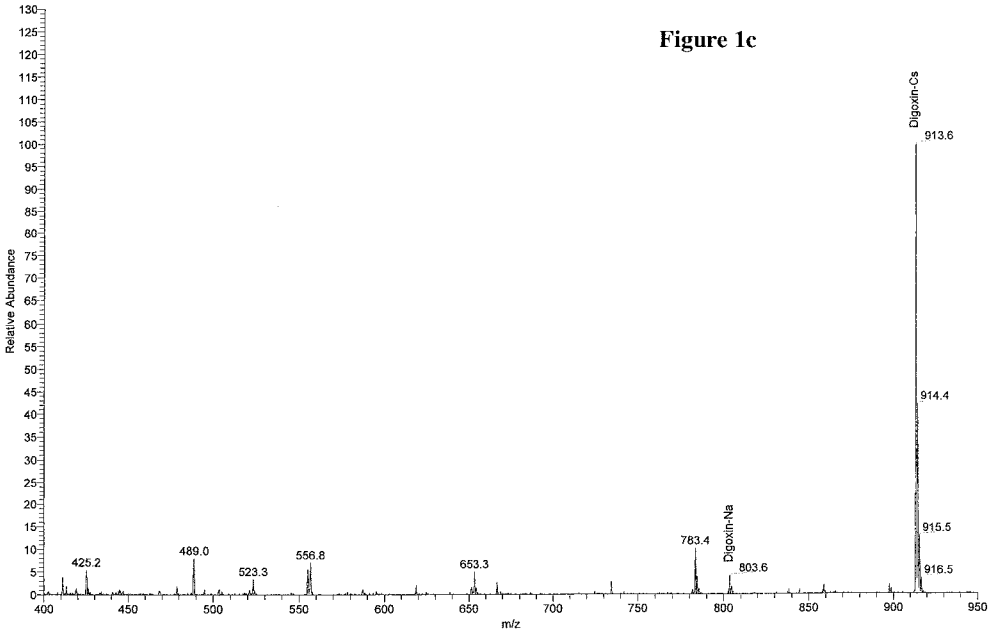
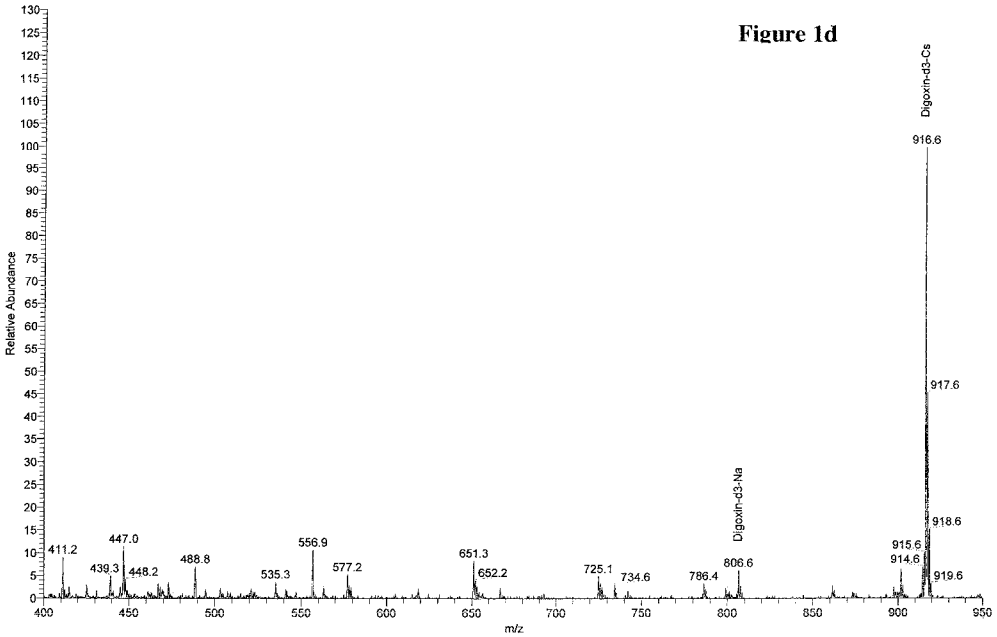


Figure 1d



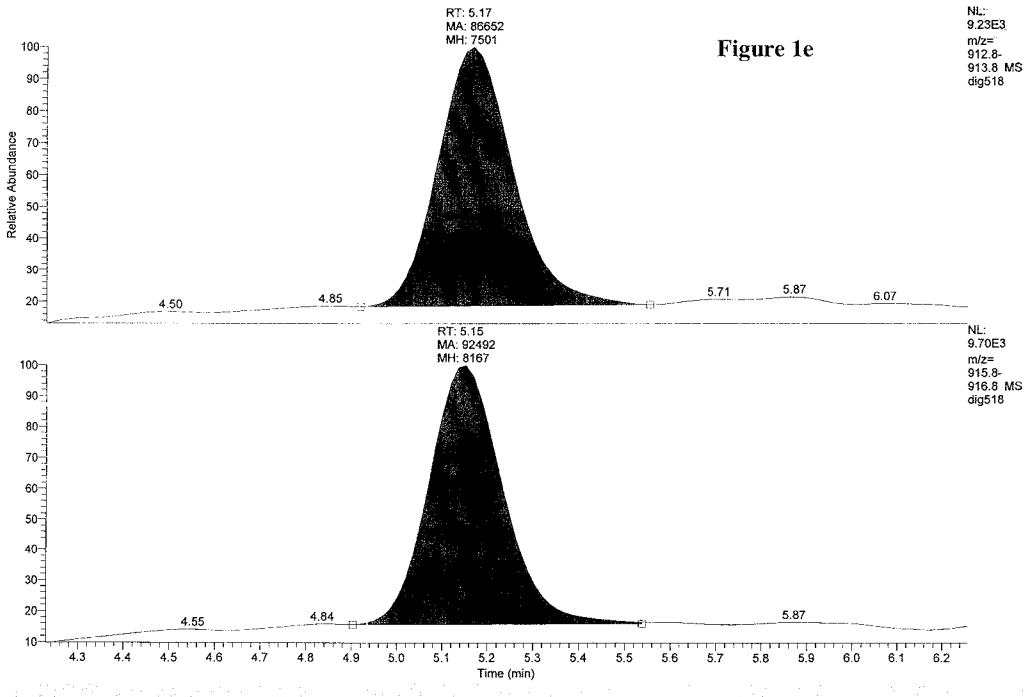


Figure 1:

a. Mass spectrometry full-scan for native digoxin.
Digoxin was dissolved in HPLC-medium (see text) and was injected directly into the mass spectrometer. The pH was adjusted so that the different peaks of interest were visible. The flow-rate was 10μl/min. The following peaks are of interest:
Digoxin Na-adduct – 803.6 amu: Digoxin – 781.6 amu
Digoxin – 1 digitoxose: Na-adduct – 673.5 amu; native – 651.6 amu
Digoxin – 2 digitoxose: Na-adduct – 543.5 amu; native – 521.3 amu
Digoxigenin: Na-adduct – 413.4 amu; native – 391.1 amu

b. Mass spectrometry full-scan for deuterated digoxin (digoxin-d_3).
The procedure was identical with Figure 1a above. Peaks of interest are:
Digoxin-d_3 Na-adduct – 806 amu; Digoxin-d_3 – 784.6 amu
Digoxin-d_3 – 1 digitoxose: Na-adduct – 676.6 amu; native – 654.4 amu
Digoxin-d_3 – 2 digitoxose: Na-adduct – 546.7 amu; native – 524.5 amu
Digoxigenin-d_3: Na-adduct – 416.4 amu; native – 394.3 amu

c. Mass spectrometry full-scan for digoxin after injection of the sample in the presence of 100 μmol/l Cs^+ ions using the full-scan mode. Conditions as in Figure 1a above. Only the digoxin Cs adduct (Digoxin-Cs); 913.6 amu and Digoxin Na-adduct (Digoxin-Na) were visible.

d. Mass spectrometry full-scan for digoxin-d_3 after injection of the sample in the presence of 100 μmol/l Cs^+ ions using the full-scan mode. Conditions as in Figure 1a above. Only the digoxin-d_3 Cs adduct (Digoxin-d_3 Cs); 916.6 amu and digoxin-d_3 Na-adduct (Digoxin-d_3 Na) were visible.

e. Mass spectrometry in single-ion mode (SIM). The upper trace represents the native (unlabelled) digoxin, the lower trace the deuterated digoxin. The signal intensity is on the ordinate; the time scale on the abscissa. The time scale for the chromatogram is the peak ± 1 min. This figure is derived from an actual quantification of a serum sample in which the complete extraction and measurement procedures were carried out. The digoxin concentration is 2.0 μg/l, i.e within the therapeutic range.
Key: RT – retention time; MA – peak area in arbitrary units; MH – peak height in arbitrary units.

Figure 2a

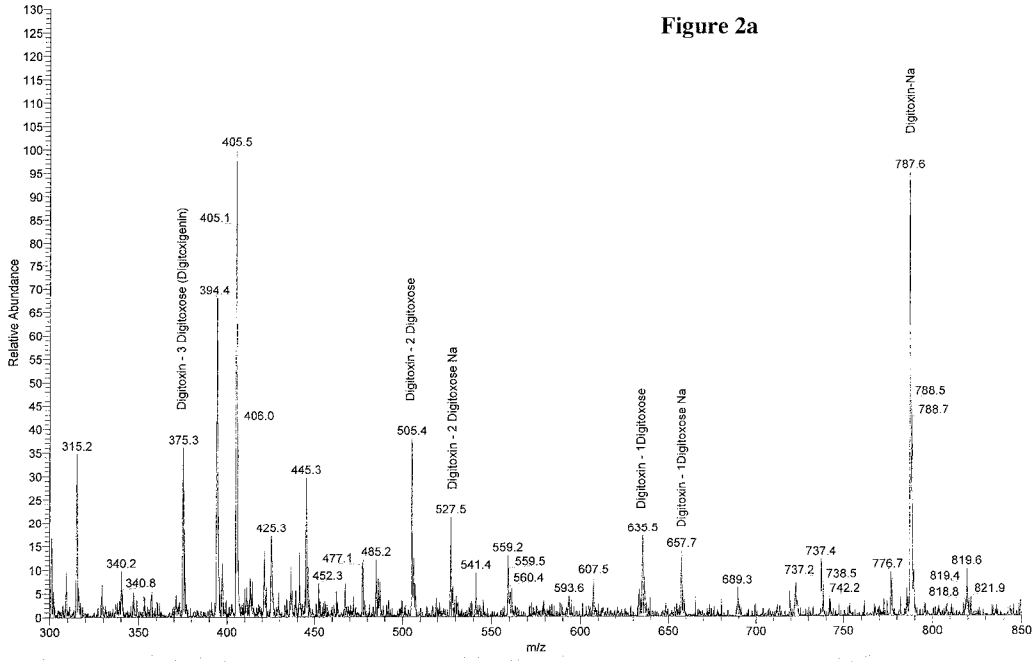
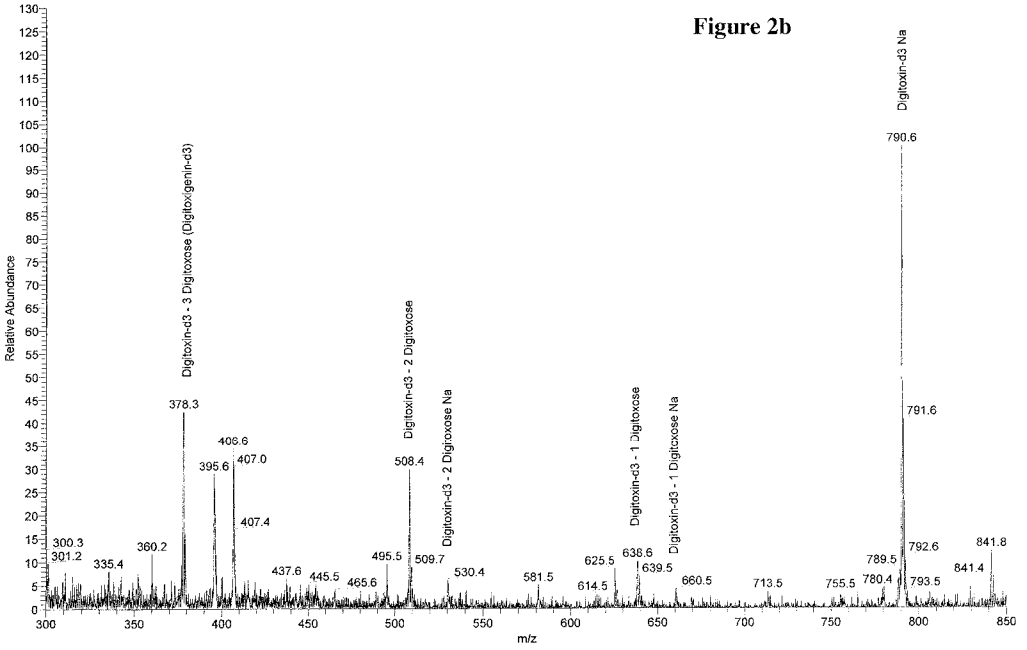


Figure 2b



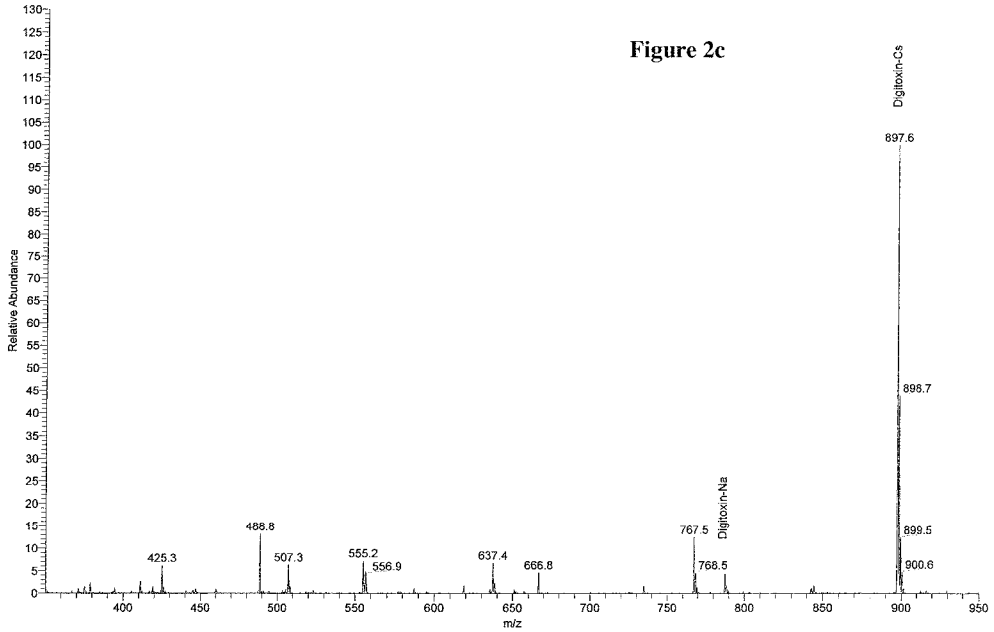


Figure 2c

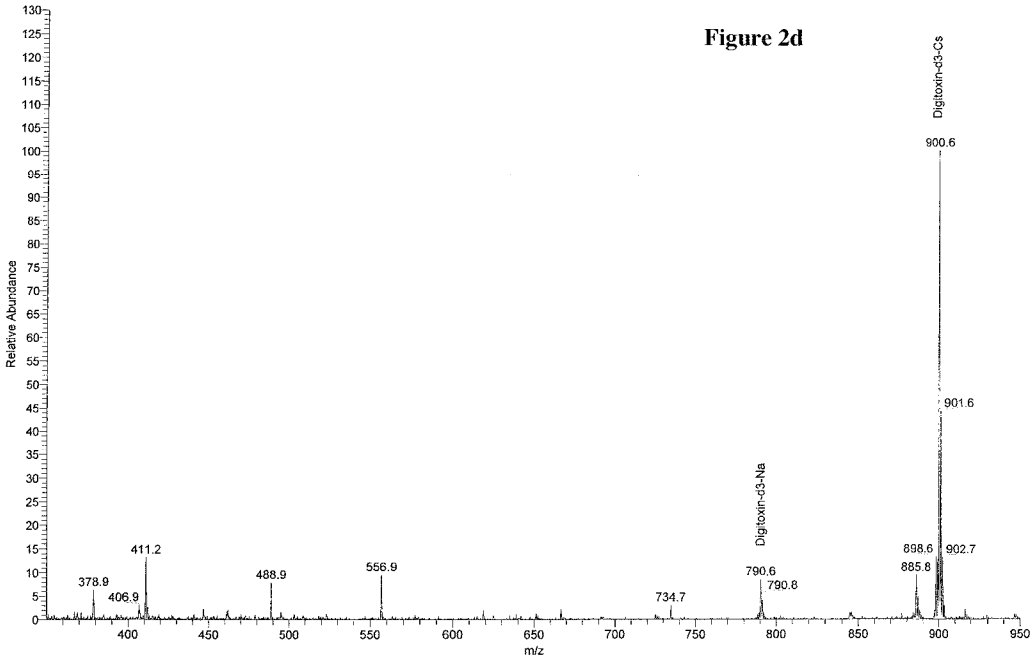


Figure 2d

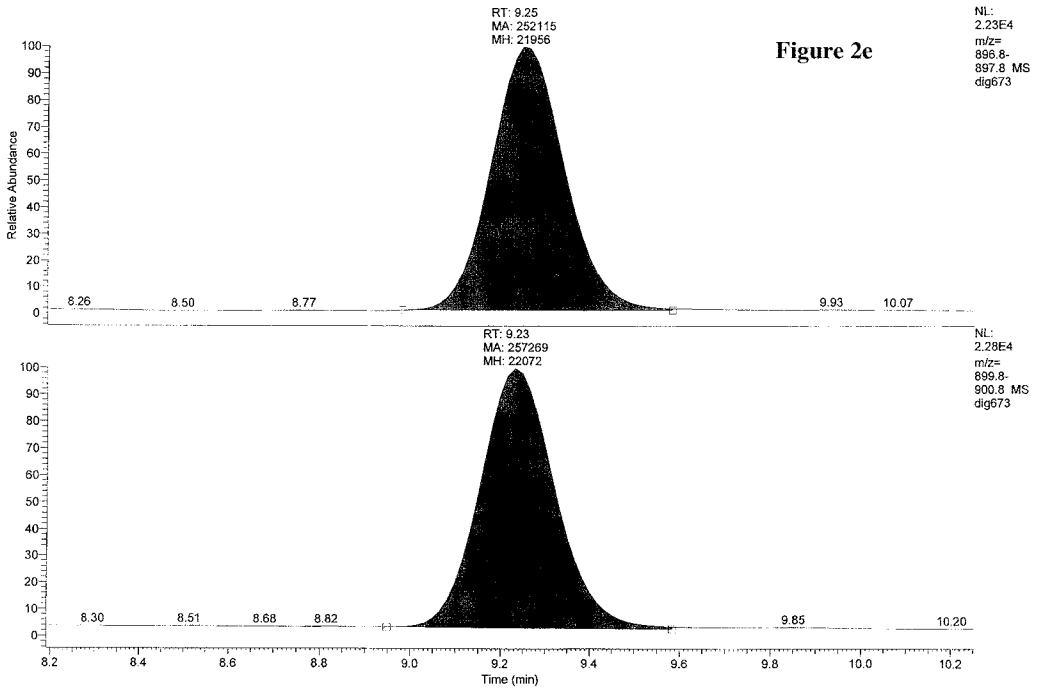


Figure 2:

a. Mass spectrometry full-scan for native digitoxin.
The procedure was as in Figure 1a above. The peaks of interest are:
Digitoxin Na-adduct – 787.6 amu; the native form was not seen.
Digitoxin – 1 digitoxose – Na-adduct 657.7 amu; native – 635.5 amu
Digitoxin – 2 digitoxose – Na-adduct 527.5 amu; native – 505.4 amu
Digitoxigenin – not observed

b. Mass spectrometer full-scan for deuterated digitoxin (digitoxin-d_3)
The procedure was as in Figure 1a above. The peaks of interest are:
Digitoxin-d_3 Na-adduct – 790.6 amu; the native form was not seen.
Digitoxin – 1 digitoxose - Na-adduct 660.5 amu; native – 638.6 amu
Digitoxin – 2 digitoxose – Na-adduct 530.4 amu; native – 508.4 amu
Digitoxigenin – Na-adduct not observed; native – 378.3 amu

c. Mass spectrometry full-scan for digitoxin after injection of the sample in the presence of 100 μmol/l Cs^+ ions using the full-scan mode. Conditions as in Figure 1a above. Only the digitoxin Cs adduct (Digitoxin-Cs); 897.6 amu and Digitoxin Na-adduct (Digoxin-Na) were visible.

d. Mass spectrometry full-scan for digitoxin-d_3 after injection of the sample in the presence of 100 μmol/l Cs^+ ions using the full-scan mode. Conditions as in Figure 1a above. Only the digitoxin-d_3 Cs adduct (Digitoxin-d_3 Cs); 900.6 amu and Digitoxin-d_3 Na-adduct (Digitoxin-d_3 Na) were visible.

e. Mass spectrometry in single-ion mode (SIM). The upper trace represents the native (unlabelled) digitoxin, the lower trace the deuterated digitoxin. The signal intensity is on the ordinate; the time scale on the abscissa. The time scale for the chromatogram is the peak ± 1 min. This figure is derived from an actual quantification in which all the extraction and measurement steps were carried out. The digitoxin concentration is 20 μg/l, i.e within the therapeutic range.
Key: RT – retention time; MA – peak area in arbitrary units; MH – peak height in arbitrary units.

RESULTS

Mass spectrum data

Figures 1a-1b and 2a-2b show the mass spectra for unlabelled and deuterated (d_3) digoxin and digitoxin from solutions directly injected into the mass-spectrometer after limited hydrolysis with 26 mmol/l formic acid to visualise these fractions.The molecular-ion (M^+) from each glycoside was accompanied by the fragments with zero, one and two digitoxose molecules, resulting from cleavage. The M+23 ions, formed from traces of sodium in the system, are also detectable.

To remove the random effects of the sodium adduct, caesium-ions were added in excess to give a stable, more intensive and constant signal (M+133). The caesium adducts measured were 913.6 and 916.6 for digoxin / digoxin-d_3 and 897.6 and 900.6 for digitoxin / digitoxin-d_3. (Figures 1c, 1d and 2c, 2d). Figures 1e and 2e show MS-traces of digoxin and digitoxin taken through the extraction and measurement steps.

Method validation

Figure 3 and Table 3 show the validation data for digoxin; Figure 4 and Table 4 the same for digitoxin. The spiked amounts of digoxin and digitoxin used were determined by the therapeutical and clinically relevant concentration ranges.

As there are no certified reference materials available for digoxin and digitoxin, commercially available preparations with purity declarations were used (see Materials above). HPLC-analysis of both glycosides using a diode array detector showed no measurable impurities. Calibration, trueness and precision data were obtained from digoxin and digitoxin weighed into glycoside-free serum. The latter was defined as serum giving no signal at the examined molecular-ion caesium-adduct mass.

Lower limits of determination

The limit of determination was determined practically as the lowest measurable concentration with a CV < 4%. This was determined by serial dilutions of each glycoside in serum, taken through the extraction steps and injected onto the HPLC-column. The lower limit of determination was 0.25 μg/l for both digoxin and digitoxin.

The upper limit of determination was not determined. The dilution/recovery curve was linear over the range 0.25 – 100 μg/l for digitoxin and 0.25 and 10 μg/l for digoxin. Higher concentrations of glycoside were not tested due to the clinical irrelevance of such concentrations.

Positions of the deuterium atoms in the internal standard

From full scans of the labelled and unlabelled glycosides, which were directly injected into the MS without addition of caesium ions to the mixture, it was not clear whether the labelling had taken place in the steroid moiety or on the sugar molecules as proposed by Kessler (5,8).

Digoxigenin was labelled by the same method and was also present as the d_3 derivative with mass-ions 413.2 and 416.2 for the sodium adduct of the native and deuterated products, respectively, thus arguing against a labelling of the digitoxose residues. Further evidence of the labelled steroid part of the molecule in the intact molecule was given in as much as the digoxigenin peak in the deuterated digoxin MS-scan was also labelled with 3 atoms of deuterium. The digitoxose in the same mixture, however, had a molecule-ion of 171 m/z, which pointed to the sodium adduct of the unlabelled digitoxose molecule.

The position of the deuterium atoms was not further determined, although double-substitution at position 16 in the D-ring and a single substitution at position 6 in the B-ring is a possibility (7).

Stability of the internal standard.

The stability of the deuterium-labelled glycosides in solution (methanol:D_2O) was studied by measurement in the full-scan mode over a time period of several weeks. The quality of digitoxin and digitoxigenin, as seen by additional MS-peaks, was significantly poorer than for digoxin and digoxigenin in the 3-week synthesis.

Solutions of the glycosides from this synthesis were used over a maximum period of 4 weeks when stored in CH_3OH/D_2O mixture. During this time exchange of hydrogen for deuterium was not observed.

The internal standard produced by the 24-hour synthesis method was stored dry under nitrogen until use. It was then solubilised with a mixture of CD_3OD to prevent isotope exchange over longer periods of time.

Uncertainty of measurement.

The uncertainty of measurement is reflected by the overall coefficient of variation of the method. There are many components which make up the uncertainty in a measurement, some of which are so small that they can be ignored, others contributing a measurable amount. The main components of the uncertainty of measurement are to be found in the addition of components to the system (pipetting or weighing-in of standards and

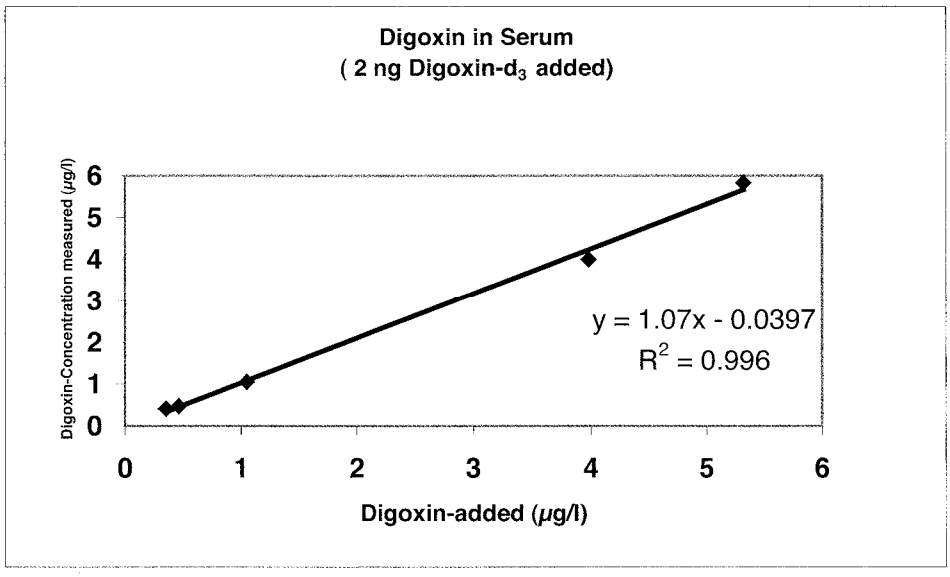


Figure 3: Recovery and linearity data for digoxin (mean of 7 measurements per data point)

Table 3: Inter-run precision and trueness at four different concentrations of digoxin in serum

Added [ng/ml]	Found [ng/ml]	Recovery [%]	CV [%]
0.50	0.50	100	1.97
1.16	1.14	98.3	2.91
2.01	1.96	97.5	2.62
4.50	4.65	104	1.26

Determined for 7 runs at each concentration.
* Recovery reflects the trueness of the measurement

unknowns), the preparation (extraction/possible derivatisation steps) and the measurement procedure itself (injection, peak-integration method, system variability).

By substituting a pipetting (volume-addition) step with a weighing-in of the components, a considerable amount of uncertainty can be avoided. As an example, the coefficient of variation of pipetting 10 μl of water or serum was around 3%, 50 μl of water by 0.6% and 50μl of serum by 1.2%. The coefficients of variation for weighing-in 10 mg and 50 mg of a substance were 0.1 and 0.015%, respectively, representing an potential improvement of up to almost 3% at 10 mg and 1.1 % at 50 mg.

By use of an internal standard, the variation in the efficiency of extraction and derivatisation and injection volume at the measurement stage can be eliminated, thus further reducing the uncertainty of measurement.

In this method, no derivatisation of the glycosides was necessary prior to injection.

The largest component in the uncertainty of measurement, which accounts for at least 90% of this, is the measurement step itself, which is composed of variations in the injection of sample, integration of the signal and instrument variation. To investigate the influence of each component, the following scheme was used: (a) tenfold integration of the same m/z peak (for operator-precision); (b) tenfold integration of the same standard

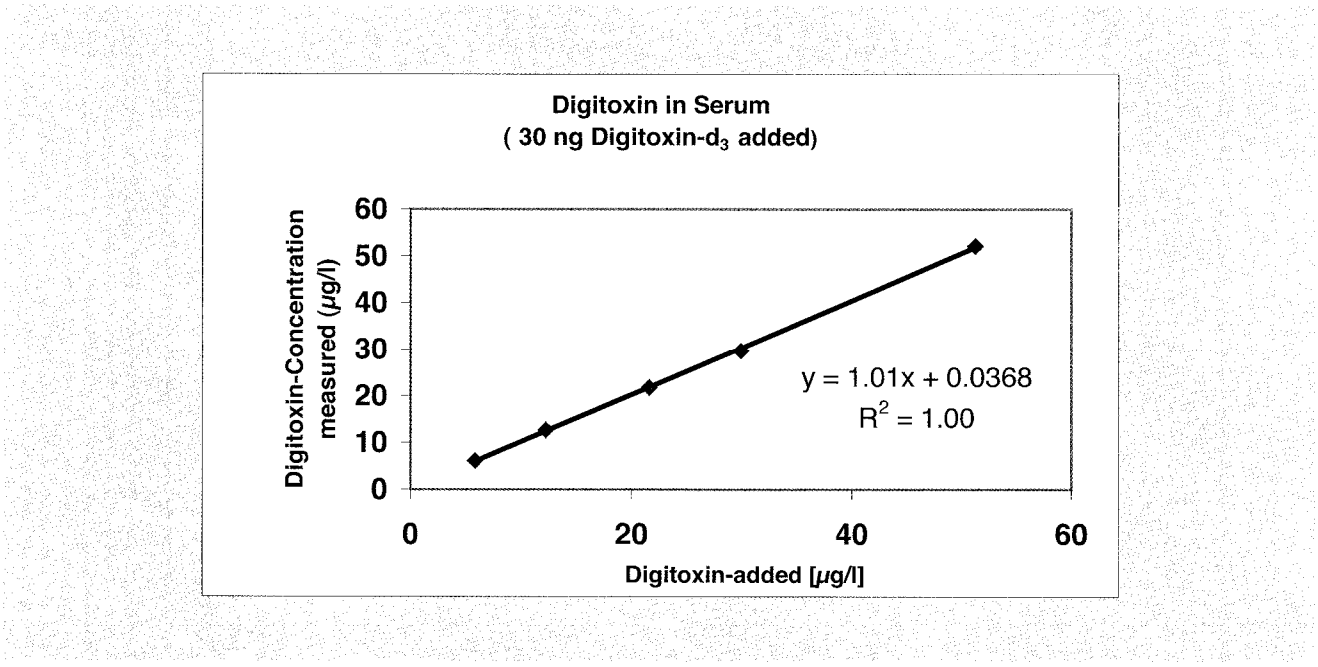


Figure 4: Recovery and linearity data for digitoxin(mean of 7 measurements per data point)

Table 4: Inter-run precision and trueness at five different concentrations of digitoxin in serum.

Added [ng/ml]	Found [ng/ml]	Recovery* [%]	CV [%]
4.77	4.82	101	1.27
10.3	10.6	103	2.00
20.0	19.6	98.0	1.38
30.5	30.2	99.0	1.11
45.0	44.9	99.8	0.79

Determined for 7 runs at each concentration.
* Recovery reflects the trueness of the measurement

m/z peak and the same sample m/z peak (for accuracy of the quantitation-step); (c) repeat of (a) but using 10 different extractions of the same sample (for precision of extraction); (d) repeat of (b) using 10-fold extraction of standard/sample pairs (for precision of the quantitation step).

The following results were obtained using serum samples to which digoxin had been added to give a final concentration around 2 µg/l (schemes (a) and (b)) and 3 µg/l (schemes (c) and (d)), i.e. within the clinically relevant range. The results from scheme (a) were 0.72 and 1.07% CV for the peak area and 0.26 and 0.28% CV for the peak height for 10 measurements made by two observers.

The results from scheme (b) were 3.08 and 4,34% CV for peak area and 2.55 and 2.88% CV for the peak height, again from two observers well acquainted with LC- and GC-MS procedures.

Scheme (c) was performed by a single observer and gave the following results for peak height (CV 22.8%) and peak area (CV 20.6%). After correction using the internal standard as in scheme (d), the results for a mean digoxin concentration of 3.01 µg/l gave a coefficient of variation of 2.22%.

Components which do not influence the measurement of uncertainty in a statistically significant way include atmospheric pressure and humidity.

The practical effect of temperature on the uncertainty of measurement depends to a large extent on whether the laboratory has a stable temperature-contolled air conditioning or not. This laboratory is situated on the north side of the building and has a temperature constancy (air conditioning) of ± 0.2 °C. The normal laboratory temperature was between 20 and 24 °C. All solutions were brought to ambient temperature before use.

Those components which influence the uncertainty of measurement in a "borderline" way include the specific density measurement of the serum/plasma samples to be determined.

From the data collected in the laboratory, the uncertainty of measurement in the worst case for the use of a small volume (10 µl) and peak area (personal and device components) was estimated to be 5.28%. Using weighed-in components (10 mg) and the peak-height (personal and device components) the uncertainty of measurement was calculated to be 1.70%. The actual uncertainty of measurement found was 2.20%, as expressed by the coefficient of variation of the final analyte concentrations.

DISCUSSION

It could be shown that the LC-IDMS method described here was suitable for the specific detection of digoxin and digitoxin in small volumes of serum and plasma and EQA samples, although other methods have a lower detection limit – for example immunoassays. These, however, are normally specific for the steroid moiety digoxigenin or digitoxigenin, so that not only digoxin and digitoxin are detected but also some of their metabolites in which the steroid structure is not changed (9) as well as drugs with closely related structures (10,11). Immunoassays are apt to measure "digoxin-like substances" (12), a term often used for non-defined cross-reacting compounds which may interfere with immunological-based systems. Other combinations of HPLC separation coupled to a specific detection system have been described for HPLC-radioimmunoassay (13), HPLC-fluorescence-polarisation immunoassay (14) and HPLC-immunoassay-fluorescence-energy-transfer (15).

Several LC-MS methods have been described for cardiac glycosides, but none has attained the necessary precision (6, 16) for a candidate reference method procedure.
The basis for this method development was the thesis of Dr. A. Kessler (5). This method was improved to achieve a better sample extraction which led to results within a working day (maximum 8 hours) using smaller volumes of sample and extraction solutions. The analytical recovery of both glycosides was around 70%.

The overnight method developed for deuterium substitution in the glycosides made the method more practicable, so that the time between starting the synthesis and the appearance of the first results was less than two working days, an improvement of three weeks when compared with the original method (5,8).

The LC-IDMS method is specific for digoxin and digitoxin due to the detection of specific masses, here the caesium adducts. The caesium adduct was chosen for the increase in mass and that it was added in defined quantities, rather than to allow for chance, as is often the case in the measurement of the sodium adducts, the sodium ions more likely originating from the HPLC-column rather than the glassware as has been suggested (5). In this way, the specificity of the method can be improved. Improvement in precision is obtained by extraction of the glycosides in the presence of a fixed amount of labelled internal standard (see Figures 3 & 4).

The identification of the components of the uncertainty of measurement drastically show the influence of the use of an internal standard in reducing the overall imprecision of the method. Similarly, the practice of weighing in components, when compared with volume addition, also led to a significant increase in precision, reflected by the reduction of the coefficient of variation.

The LC-IDMS method, although not suitable for a routine laboratory with many samples daily, may be used to determine digoxin and digitoxin in materials suitable for EQAS-programmes, as well as for examining abnormal metabolism in patients under therapy with cardiac glycosides, who do not respond to treatment as expected, or in intoxication cases treated with Fab antibody fragments (17) where immunoassays often give wrong results.

Definitions used in this publication – (see also reference 18)
As many similar terms are used to express or define a quantity, parameter or measurement, the definitions - as used in this publication - are listed below.

Accuracy of measurement: closeness of the agreement between the result of a measurement and a true value of the measurand [VIM:1993, 3.5] (18)

Higher limit of quantification (= *higher limit of determination* (19)): highest result of a measurement that can be obtained by a stated measurement procedure, and that can be given with a statement of uncertainty of measurement.

Inaccuracy of measurement/inaccuracy: discrepancy between the result of a measurement and a true value of a measurement. (20).

Lower limit of determination/minimal quantifiable value (18,19): lowest result of a measurement, that can be obtained by a stated measurement procedure, and that can be given with a statement of uncertainty of measurement.

Precision of measurement/precision: closeness of agreement between independent results of measurements obtained under stipulated conditions [ISO 3534-1: 1993, 3.14] (21).

Recovery measurement: indication of a measuring system obtained by measurement of an analytical sample containing an added known amount of analyte minus the indication of another analytical sample of the same laboratory sample without the added analyte, as related to the amount added (18).

Reference method procedure/reference procedure: thoroughly investigated measurement procedure, shown to have an uncertainty of measurement commensurate with its intended use, especially in assessing the trueness of other measurement procedures for the same quantity/ quantities, and in characterising reference materials (22).

Standard deviation: positive square root of the variance [ISO 3534-1:1993, 2.34]

True value: value perfectly consistent with the definition of a given measurable quantity [VIM:1993, 1.19]

Trueness of measurement/trueness: closeness of agreement between the average value obtained from a large series of results of measurements and a true value (20)

Uncertainty of measurement/uncertainty: parameter associated with the result of a measurement that characterises the dispersion of the values that could reasonably be attributed to the measurand [VIM:1993, 3.9]

The term bias has not been used as such. It has been replaced by inaccuracy, which expresses the same thing. Where several terms are used for the same definition, these have been included.

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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)

Clin. Lab. 2006;52:37-42

ORIGINAL ARTICLE

A Novel LC-IDMS/MS Method for the Determination of the Cardiac Glycosides Digoxin and Digitoxin using Caesium Adducts

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SUMMARY

This article describes an essential improvement of the published candidate reference measurement procedure for digoxin and digitoxin and compares it with the original method. The novelty of the method lies in the measurement of the caesium (Cs^+) ion as product ion in the multiple reaction monitoring mode (MRM) with potentially improved analytical specificity whilst retaining a comparable accuracy and precision at therapeutic levels. The original measurement procedure used the single-ion mode (SIM). The dissociation of the Cs^+ adducts in MRM leads to the formation of Cs^+ ions as main charged product in high yield. The present method results in a product ion signal intensity in MRM for digoxin and digitoxin of up to 80% of the precursor ion signal intensity in SIM. The precision, expressed as the coefficient of variation of the new method for digoxin was 3.18% (SIM) and 2.28% (MRM) at a concentration of 0.66 µg/l and 1.26% (SIM) or 1.65% (MRM) at 2.0 µg/l. The corresponding data for digitoxin were 1.21% (SIM) and 1.62% (MRM) at 24 µg/l and 1.46% (SIM) and 1.13% (MRM) at 42 µg/l.
(Clin. Lab. 2006;52:37-42)

INTRODUCTION

The inclusion of digoxin and digitoxin in Annex 1 of the current Guidelines of the Federal Medical Chamber for Quality Assurance of Quantitative Analyses in Laboratory Medicine has made it necessary to develop matrix independent methods of analysis of high metrological order (reference measurement procedures) (1,2). In EQA-surveys the values from participants are compared with the reference method value as the unique target value.

A candidate reference measurement procedure using HPLC-MS has already been developed and published (3,4). A further improvement of the method was obtained using HPLC-MS/MS. The novelty of this method was the measurement of the caesium ion as target mass. After specific detection of the caesium complex of the glycoside in question on quadrupole Q1, this was fragmented in the collision cell (q2) before allowing the caesium ion (mass 132.9 amu) to be measured in MRM on quadrupole Q3.– (see Figure 1)

It is well known that many molecules form adducts with alkali metals such as Na^+ and K^+ and ammonium ions. Adducts with Na^+ or K^+ ions are often seen as problems in analysis as the potential analytical sensitivity may be worsened [production and detection of several mass ion products, or no fragmentation]. By shifting the equilibrium of adduct formation by adding an excess of caesium ions, two positive effects were seen, namely the formation of a stable Cs^+ adduct with the reduction of non-specific Na^+ and K^+ adducts and an increased mass ion signal of the analyte. The atomic mass of caesium (132.9 amu) also shifts the signal of the Cs^+ adduct to a region of the mass spectrum which is well away from that of the original compound.

One reason for developing the new method was the process of continuous improvement according to the ISO-Standards 17025 and 15189.

Manuscript submitted July 13, 2005 and accepted October 8, 2005

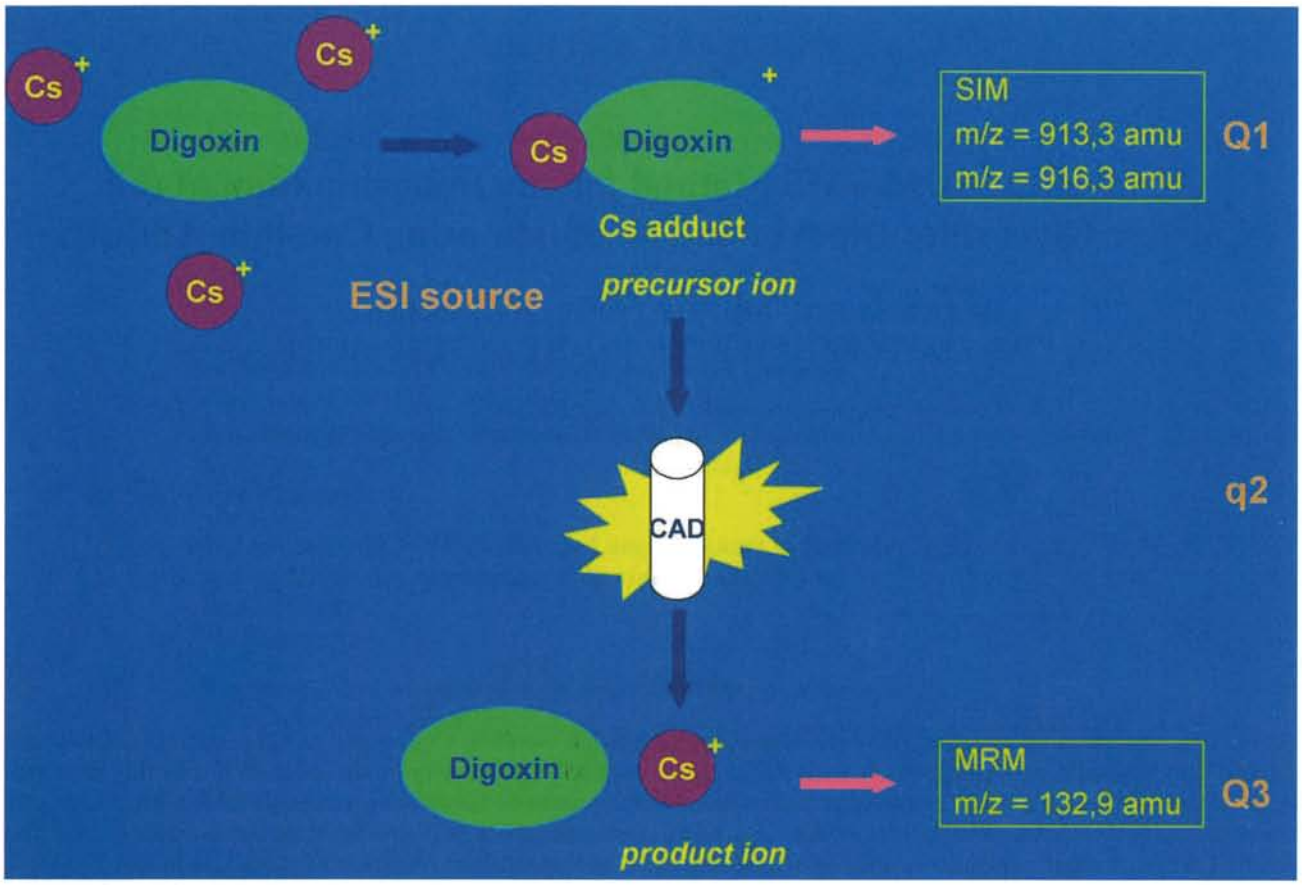

Figure 1: Measurement principle of Cs^+ adducts in SIM and MRM using digoxin as example.
Schematic representation of the detection of the native [913.3 amu] and deuterated [916.3 amu] caesium adducts of digoxin using electrospray ionisation (ESI). The principle for digitoxin is identical, the masses being 897.3 amu (native) and 900.3 amu (deuterated) respectively.
The caesium adducts are measured at quadrupole Q1, the fragmentation takes place at q2 (CAD – collision activated dissociation), the measurement of the Cs^+ ion at Q3.

MATERIALS AND METHODS

Chemicals

Digoxin (purity 99,8%, certificate of analysis by Promochem, UN 2811), digitoxin (purity 99,0%, certificate of analysis by Promochem, UN 3249), triethylamine (purity 99 %), 1,4-dioxane (purity > 99.9 %), ammonia (25%, p.a.), formic acid (98%, puriss. p.a.), deuterium oxide (purity 99.9%), tert.-butylmethyl ether (purity 99.8%) and caesium hydroxide (purity 99.97%) were purchased from Sigma-Aldrich, Taufkirchen, Germany. Methanol (LiChrosolv), ethyl acetate (for organic trace analysis), ammonium acetate (p.a.) and tetrahydrofuran (p.a.) were obtained from Merck, Darmstadt, Germany. Albumin (bovine, Cohn fraction V) and Sigmacote® were purchased from from Sigma-Aldrich, Taufkirchen, Germany. Water was prepared using the water purification system Direct-Q™ 5 (Millipore GmbH, Eschborn, Germany). The caesium hydroxide was adjusted with hydrochloric acid to pH 7 before use.

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™ ESI source with Turboion Spray™ probe from Applied Biosystems (MDS-Sciex, Darmstadt, Germany)

Preparation of Labelled Digoxin and Digitoxin.

Digoxin and digitoxin were labelled with a modification of the method described in (3). The resulting substitution was 3 deuterium atoms in the steroid moiety of both glycosides. The deuteration procedure was as follows:
1 ml 1,4 dioxane, 1 ml tetrahydrofuran, 1 ml deuterium oxide and 0.5 ml triethylamine were mixed in a 5 ml Wheaton vial and 1 mg of the glycoside (either digoxin or digitoxin) added. The Wheaton vial was shaken until the glycoside had dissolved. The mixture was allowed to react overnight (15 – 18 h) at 70 ± 2 °C in a thermostatically controlled heating block.
The resulting deuterated glycoside was stable in the above mixture for at least 7 days at 4 – 7 °C. Portions of the deuteration mixture could be removed at 60 °C under a stream of nitrogen or argon, the residue being dissolved in 50% CH_3OH in water for immediate use in the LC-IDMS method

Table 1a. Conditions for measurement of digoxin

Time	Module	Events	Parameter
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 1b: Conditions for measurement of digitoxin

Time	Module	Events	Parameter
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	

described here. If the solution was to be stored for a longer period of time, CD_3OD and D_2O could be used to prevent any back-exchange of deuterium.

Sample Preparation

Sample preparation was as described in (3). Briefly, the internal standard (glycoside-d_3) was added to 1 ml sample (serum or standard) in a 10 ml glass vial with screw top and PTFE seal and equilibrated on a roller mixer for 20 min at ambient temperature. 500 µl of ammonium acetate buffer (0.1 mol/l, pH 9.5) were added, the vial resealed and the mixture briefly mixed on a vortex mixer before 4 ml tert. butylmethyl ether/ethyl acetate (1:1 v/v) were added. The resulting mixture was shaken vigorously for 3 minutes on a horizontal shaker at 300 min^{-1}. The vial was then centrifuged for 5 min at 3000 g and 3 ml of the upper (organic) phase removed before being evaporated to dryness under nitrogen at 60 °C. The residue was redissolved in 100 µl methanol and the methanol removed under nitrogen as above. Directly before assay, the residue was dissolved in 50 µl elution buffer B. A volume of 5-10 µl was injected into the HPLC module.

HPLC-MS/MS measurement conditions

Separation 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).

Table 2: Detection masses in SIM and MRM of native and deuterated glycosides

analyte	SIM [amu]	MRM [amu]
digitoxin	897.3	897.3 → 132.9
digitoxin-d_3	900.3	900.3 → 132.9
digoxin	913.3	913.3 → 132.9
digoxin-d_3	916.3	916.3 → 132.9

Key: SIM – Single Ion Monitoring; MRM – Multiple Reaction Monitoring; amu – atomic mass unit (1 amu = 1.660538 x 10^{-27} kg)

Table 3: Settings for MRM mode

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

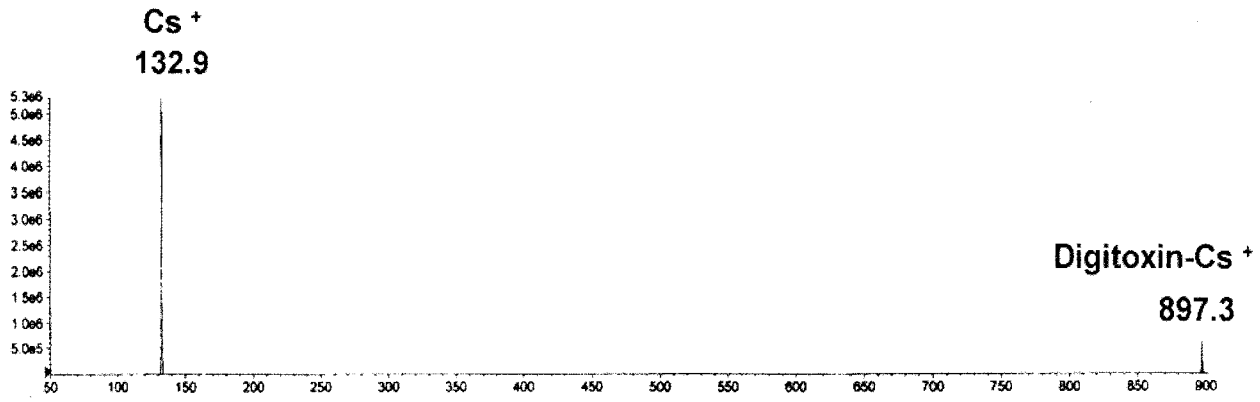

Figure 2: MS/MS Spectrum of a Standard Solution of Digitoxin (5 µg/l) in CH_3OH/H_2O (50:50 v/v) with 100 µmol/l Cs^+ and 0.1% HCOOH.
This figure shows a full scan of a standard solution of digitoxin in caesium formate / methanol (Eluant B). The abscissa shows the atomic mass units (amu), the ordinate the relative signal strength (peak height in arbitrary units).

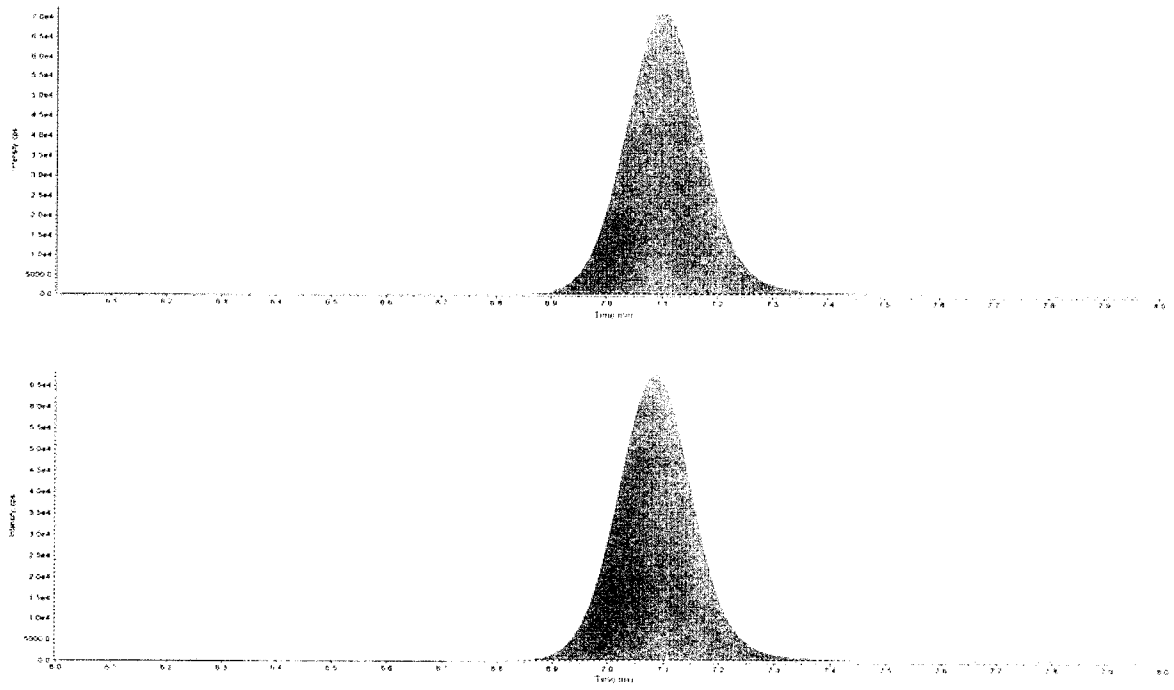

Figure 3: HPLC-MS/MS chromatogram for digitoxin in a serum sample
The upper trace shows the native digitoxin in a serum sample, the lower trace the added internal standard (deuterated digitoxin) after fragmentation and detection of the caesium ion (132.9 amu). The abscissa shows the retention time in minutes, the ordinate the peak height in arbitrary units (counts per second; cps).

Table 4: Comparison of peak properties in SIM (LC-IDMS) and MRM (LC-IDMS/MS) modes using an albumin-based d_0-d_3 standard mixture of digitoxin (5µg/L)

	SIM	MRM
area d_0 (counts / 10 µl)	9.3 x 10^4	7.8 x 10^4
ratio area (d_0/d_3)	1.0533	0.9929
std. deviation	0.0113	0.0096
coeff. of variation (%)	1.08	0.97
number of injections	15	15

Table 5a: Concentrations of digitoxin in samples analysed in external quality control surveys

	SIM			MRM		
Sample	INSTAND #248	JCTLM A	JCTLM B	INSTAND #248	JCTLM A	JCTLM B
Mean [µg/l]	24.0	41.4	24.7	23.9	42.2	24.2
n	8	8	8	8	6	6
SD [µg/l]	0.291	0.805	0.334	0.388	0.478	0.349
CV [%]	1.21	1.46	1.36	1.62	1.13	1.44
RMV [µg/l]	23.8			23.8		

Table 5b: Concentrations of digoxin in samples analysed in external quality control surveys

	SIM			MRM		
Sample	INSTAND #246	JCTLM A	JCTLM B	INSTAND #246	JCTLM A	JCTLM B
Mean [µg/l]	1.620	0.659	2.01	1.60	0.658	2.0
n	9	6	6	7	6	6
SD [µg/l]	0.033	0.021	0.025	0.033	0.015	0.033
CV [%]	2.07	3.18	1.26	2.04	2.28	1.65
RMV [µg/l]	1.57			1.57		

Key: RMV – Reference Method Value; JCTLM – Joint Commission on Traceability in Laboratory Medicine

Elution was performed by using a binary gradient consisting of eluent A (pump A): 0.1% v/v HCOOH in water + 100 µmol/l CsCl (see above); and eluent B (pump B): 0.1% v/v HCOOH in CH_3OH + 100 µmol/l CsCl. 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 is passed into the ESI source. At position 1 the eluate is directed into waste, during which the ESI source is supplied with 50% methanol, delivered by pump C (Table 1). The MS detection was performed in the positive ion mode. The masses and mass transitions (m/z) for MS detection are given in Table 2.
The settings of the mass spectrometer are given in Table 3. (The abbreviations for settings are specific to the API 4000 software used to control the system).

RESULTS

In previous work (3) we showed that the introduction of Cs^+ ions in the elution buffer of the chromatographic system lead to a formation of caesium adducts of digoxin and digitoxin with high signal intensity in the SIM mode in the ESI-MS system.
Here we observed that in the MRM mode the Cs^+ adduct as parent ion decomposes in a way that the Cs^+ ion becomes detectable as product ion. (Figure 2)
Figure 3 shows a chromatogram of a digitoxin serum sample spiked with threefold deuterated digitoxin (internal standard) measured as Cs^+ ion in MRM. The upper panel shows the peak of the native digitoxin (d_0) and the lower panel the peak of the deuterated digitoxin (d_3). As demonstrated in Table 4 a high signal intensity was obtained in MRM. If the absolute signal intensity was compared under these conditions, up to 80% of the corresponding signal in SIM was observed.
The validity of the LC-IDMS/MS method is shown by the results of the measurement of three serum samples (Tables 5a, b). The samples were processed on separate days and each analysed in duplicate. Unfortunately, no certified reference material is available. The reference method values (RMV) given here are derived from our method (3) previously used for EQA-surveys run by INSTAND e.V.

Tables 5a,b show the data obtained for the quantitation of digitoxin and digoxin from different extractions of the same serum sample and demonstrate that the accuracy and precision of both methods are comparable.

DISCUSSION

The use of caesium as adduct-forming ion introduces new aspects into the field of HPLC-mass-spectrometry. This new principle has already been published for digoxin and digitoxin in the SIM mode (3). The further development of this idea led to the use of the caesium ion as detector-ion in a LC-IDMS/MS procedure as described here. (Figure 1)

The results of the new MRM procedure are comparable with those from the original SIM procedure with regard to accuracy and precision. The advantage of the new method is an improvement in analytical specificity which may be of importance when analysing biological matrices often known to give rise to high unspecific signals (background noise) when using conventional LC-MS procedures. This fact has been observed by other groups working with reference method procedures with analyte concentrations near to the limit of quantification (5), in which elimination of non-specific signals is of utmost importance. Moreover this mechanism can be transferred to the measurement of other compounds which tend to form adducts and are therefore prone to problems in fragmentation when using conventional measurement techniques.

The described effect may be of use in improving the routine determination of pharmaceutical agents in whole blood, plasma or serum, where a short measurement cycle must be achieved – often at the cost of the quality of the analysis – in order to be able to run several hundred samples per day, as is the case in large diagnostic laboratories. The prerequisite is, that the analyte in question forms adducts with alkali metals. This is the case for immunosuppressive agents such as ciclosporin-A, tacrolimus, everolimus and sirolimus, which must be measured regularly after organ transplantation (6). An improvement in the quality of the analysis of immunosuppressive agents can be expected in using LC-MS/MS determinations as described in this article in terms of improved precision coupled with increased signal to noise ratio and thus a lowering of the limit of quantification, which is necessary for routine use in this group of analytes.

Fragmentation usually leads to different products in which the signal charge intensities are distributed between the fragments. The only charged product from Cs^+ adducts under our conditions is the Cs^+ ion (Figure 1) which is obtained in high yield. Normally the absolute signals in MRM are much lower (often less than 10%) than in SIM. The present method results in a single product ion signal intensity for digoxin and digitoxin of up to 80% of the precursor ion signal intensity in SIM mode.

Acknowledgements.
The authors wish to thank Frau Karin Schumann for help in constructing the figures in this manuscript.

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Kaiser, P., Akerboom, T., Wood, W.G., Reinauer, H.

"A new approach for the determination of immunosuppressive drugs using HPLC-MS/MS and Cs^+ adducts"

Ger.Med.Sci.4: Doc01 (2006)

Research Article

A new approach for the determination of immunosuppressive drugs using HPLC-MS/MS and Cs^+ adducts

Neuer Ansatz zur Bestimmung von Immunsuppressiva unter Einsatz von HPLC-MS/MS und Cs^+-Addukten

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GMS Ger Med Sci 2006;4:Doc01 **provisional PDF**

Abstract

In this study a new principle of measurement in LC-MS/MS (liquid chromatography mass spectrometry) for determination of the immunosuppressive drugs sirolimus, everolimus, tacrolimus, and cyclosporin A has been introduced by using the Cs^+ ion as the product ion in the multiple reaction monitoring mode (MRM).

Separation of the immunosuppressive agents was achieved using a phenyl-hexyl-RP column together with a ternary gradient elution profile, consisting of water, methanol and acetonitrile combined with 0.1% v/v formic acid and 0.1 mmol/l Cs^+.

Quantification was performed using cyclosporin D, ascomycin and 32-desmethoxy-rapamycin as internal standards.

The inter-run precision of this new method, expressed as the coefficient of variation, was 2.57% for sirolimus, 2.11% for everolimus, 2.31% for tacrolimus and 2.11% for cyclosporin A.

Keywords: immunosuppressive drugs, LC-MS/MS, Cs^+ adducts

Zusammenfassung

Ein neues Messprinzip in der LC-MS/MS (Flüssigchromatographie mit Massenspektrometrie) wird zur Bestimmung der Immunsuppressiva Sirolimus, Everolimus, Tacrolimus und Cyclosporin A vorgestellt. Hierbei wird das Cs^+-Ion als Produkt-Ion im MRM (multiple reaction monitoring) Modus gemessen. Eine chromatographische Trennung der Immunsuppressiva wurde mit einer Phenyl-Hexyl-RP Säule erzielt. Die Elution erfolgte unter Verwendung eines ternären Gradienten aus Wasser, Methanol und Acetonitril mit 0,1% v/v Ameisensäure und 0,1 mmol/l Cs^+. Für die Quantifizierung wurden Cyclosporin D, Ascomycin und 32-Desmethoxy-Rapamycin eingesetzt. Die Wiederholpräzision der neuen Methode

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hat einen Varianzkoeffizienten (VK) von 2,57% für Sirolimus, 2,11% für Everolimus, 2,31% für Tacrolimus und 2,11% für Cyclosporin A.

Schlüsselwörter: Immunosuppressiva, LC-MS/MS, Cs^+-Addukte

Introduction

The therapeutic drug monitoring (TDM) of immunosuppressive drugs in blood of organ-transplanted patients is of utmost importance to prevent intoxication or rejection due to incorrect dosage. Commonly used immunosuppressive agents are calcineurin inhibitors such as cyclosporin A and tacrolimus or the mTOR inhibitors sirolimus and everolimus. Recently a new therapeutic strategy has been to combine immunosuppressive drugs with different mechanisms of action in order to reduce undesirable side effects. This often leads to a lowering of the therapeutic concentrations of these drugs. Analytical problems occur in the detection of clinically relevant low levels of immunosuppressive agents, which in turn requires a more sensitive analytical measurement procedure at low analyte concentrations for all the drugs used.

Several LC-MS/MS methods for routine measurement of immunosuppressive drugs in whole blood have been described [1], [2], [3]. These routine methods have been designed for high throughput combined with a short turnaround time. Methods without chromatographic separation of the analytes require cycles of only a few minutes per sample. Unfortunately, co-elution of the compounds usually leads to a bias in the analytical results, due to a lack of specificity of the mass transition in the MRM mode of the LC-MS/MS measurements [4], [5]. Avoidance of this phenomenon requires separation and detection of the parent substances from the different metabolites with biological activity.

LC-MS/MS procedures for determination of serum levels of digoxin and digitoxin have been recently published from this laboratory [6], [7] using Cs^+ adduct formation, which allows potential improvement of both analytical sensitivity and specificity.

The aim of the present study was to develop a method for determination of immunosuppressive agents, by utilising the principle of measurement of Cs^+ adducts, thus forming the basis of a reliable method for defining target values for immunosuppressive drugs in external quality assessment schemes (EQAS).

This article describes a LC-MS/MS method with complete chromatographic separation of the immunosuppressive drugs cyclosporin A (cyclosporin A, Sandimmune®), tacrolimus (FK-506, Prograf®), sirolimus (rapamycin, Rapamune®) and everolimus (Certican®), with cyclosporin D, ascomycin and 32-desmethoxy-rapamycin as internal standards.

Materials and methods

Chemicals

Rapamycin (>99%), tacrolimus (FK-506, >99%) and cyclosporin A (>99%) were obtained from LC Laboratories, Woburn, MA, USA. Cyclosporin D and everolimus were a kind gift from Recipe, München, Germany. 32-desmethoxy-rapamycin was a kind donation from Wyatt, St. Davids, PA, USA. Ascomycin, formic acid (98%, puriss. p.a.) and cesium hydroxide (purity 99.97%), were purchased from Sigma-Aldrich, Taufkirchen, Germany. Methanol (LiChrosolv) was obtained from Merck, Darmstadt, Germany.

Water was prepared using the purification system Direct-Q™ 5 (Millipore GmbH, Eschborn, Germany).

Apparatus

The HPLC system used was from Shimadzu (Duisburg, Germany) consisting of a SCL-10A system controller, three LC-10ADvp 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 a 4000 QTrap equipped with a TurboV™ ESI source with Turboion Spray™ probe from Applied Biosystems (MDS-Sciex, Darmstadt, Germany).

HPLC-MS/MS measurement conditions

The chromatographic separation was performed on a phenyl-hexyl-RP column (Luna®, 2 x 150 mm, 5 µm, Phenomenex, Aschaffenburg, Germany).

A ternary gradient was used for elution consisting of eluent A (pump A): 0.1% v/v HCOOH in water + 0.1 mmol/l CsOOCH; eluent B (pump B): 0.1% v/v HCOOH in methanol + 0.1 mmol/l CsOOCH and eluent C (pump C): 0.1% v/v HCOOH in CH_3CN + 0.1 mmol/l CsOOCH. CsOOCH was produced by neutralising CsOH with HCOOH to pH 7. 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 is passed into the ESI source. At position 1 the eluate is directed into waste (Figure 1, Table 1).

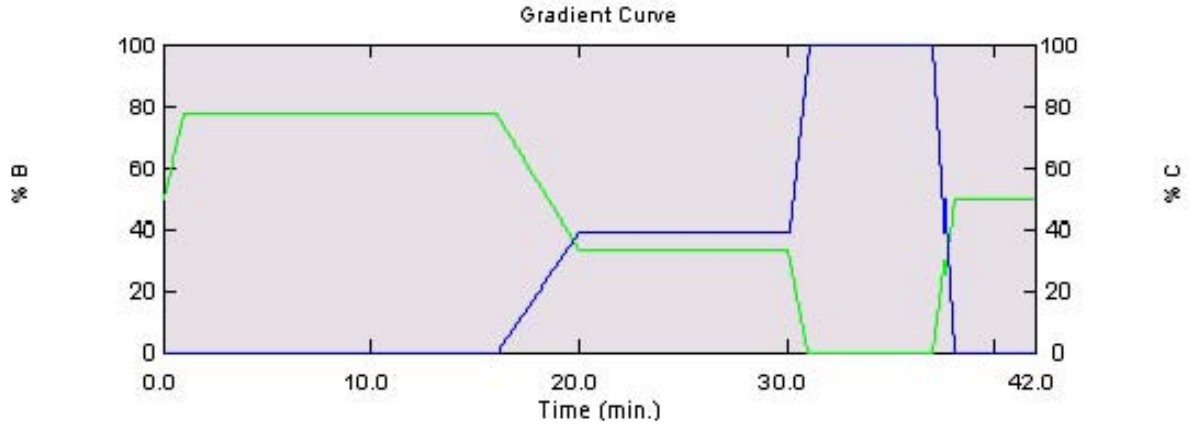

Figure 1: Gradient profile for HPLC separation (% of eluent B in green, % of eluent C in blue)

Table 1: Gradient profile for HPLC separation in detail

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

The MS detection was performed in the positive ion mode. The mass transitions (m/z) for MS detection are given in Table 2. The m/z of 132.9 amu is the mass of the Cs^+ ion.

Table 2: Mass transitions of the four immunosuppressive agents tacrolimus, sirolimus, everolimus and cyclosporin A and the internal standards ascomycin, 32-desmethoxy-rapamycin and cyclosporin D

analyte	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

The settings of the mass spectrometer are given in Table 3. (The abbreviations for settings are specific to the 4000 Q TRAP software used to control the system.)

Table 3: Instrument settings in the 4000 Q TRAP for MRM mode

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

Results

As shown in previous publications [6], [7] the generation of Cs^+ ion adducts combined with LC-MS/MS offered great advantages in analysis of digoxin and digitoxin, both in SIM and in MRM mode. It was observed, that in the MRM mode, the Cs^+ adduct as parent ion decomposes in a way that the Cs^+ ion becomes detectable as product ion.

This principle of measurement was investigated for the immunosuppressive agents tacrolimus, sirolimus, everolimus, cyclosporin A together with the analogues ascomycin, 32-desmethoxy-rapamycin and cyclosporin D used as internal standards.

The generation of Cs^+ adducts is examplified for cyclosporin A in Figure 2. The Q1 scan was performed by using a standard solution, dissolved in eluent B, which contained 0.1 mM Cs^+. Under these conditions the singly charged Cs^+ adduct generates the major signal (1335 amu). Only small amounds of the protonated form (1203 amu), the Na^+ adduct (1225 amu) and the K^+ adduct (1241 amu) are visible. Na^+ and K^+ probably originate from the manufacturing procedure and can be separated by HPLC.

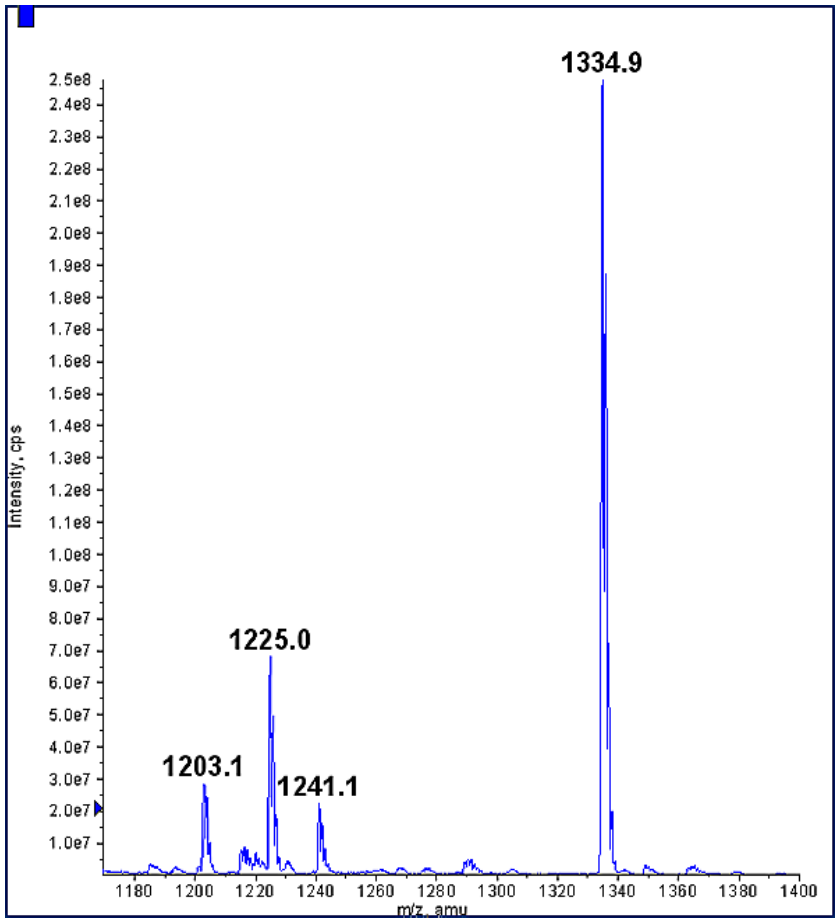

Figure 2: Q1 scan of a cyclosporin A standard solution (1 µg/ml in eluent B) infused at a flow rate of 10 µl/min

Fragmentation of the cyclosporin A Cs^+ adduct was studied using an 4000 Q TRAP in the enhanced product ion scan mode (Figure 3). The ion trap option offers a high mass resolution. In the product ion scan of Cs^+ adduct of cyclosporin A with a parent mass of 1335 amu, the mass of 132.9 amu is the main signal. The isotope pattern of this signal and the defined mass indicates that this signal originates solely from Cs^+.

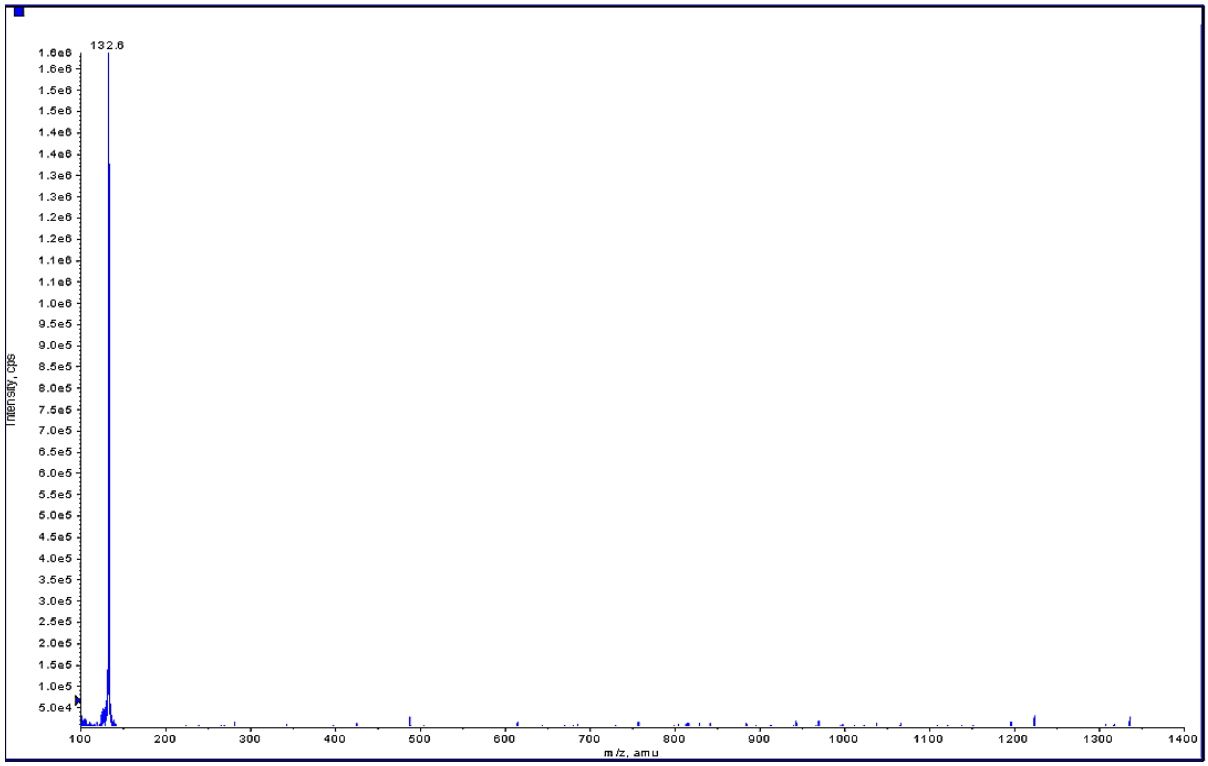

Figure 3: Enhanced product ion scan of a cyclosporin A standard solution (1 µg/ml in eluent B) infused at a flow rate of 10 µl/min

During the mass transition the mass of 1202 amu is lost (Figure 4). This value corresponds to the mass of the unprotonated cyclosporin A molecule. Thus, it can be considered, that cyclosporin A is split off in form of a neutral molecule in the fragmentation process. This concept is supported by the results of the neutral loss scan. This scan demonstrates, that the only source of the neutral loss of the unprotonated cyclosporin A molecule is the Cs^+ adduct of cyclosporin A with the mass of 1235 amu.

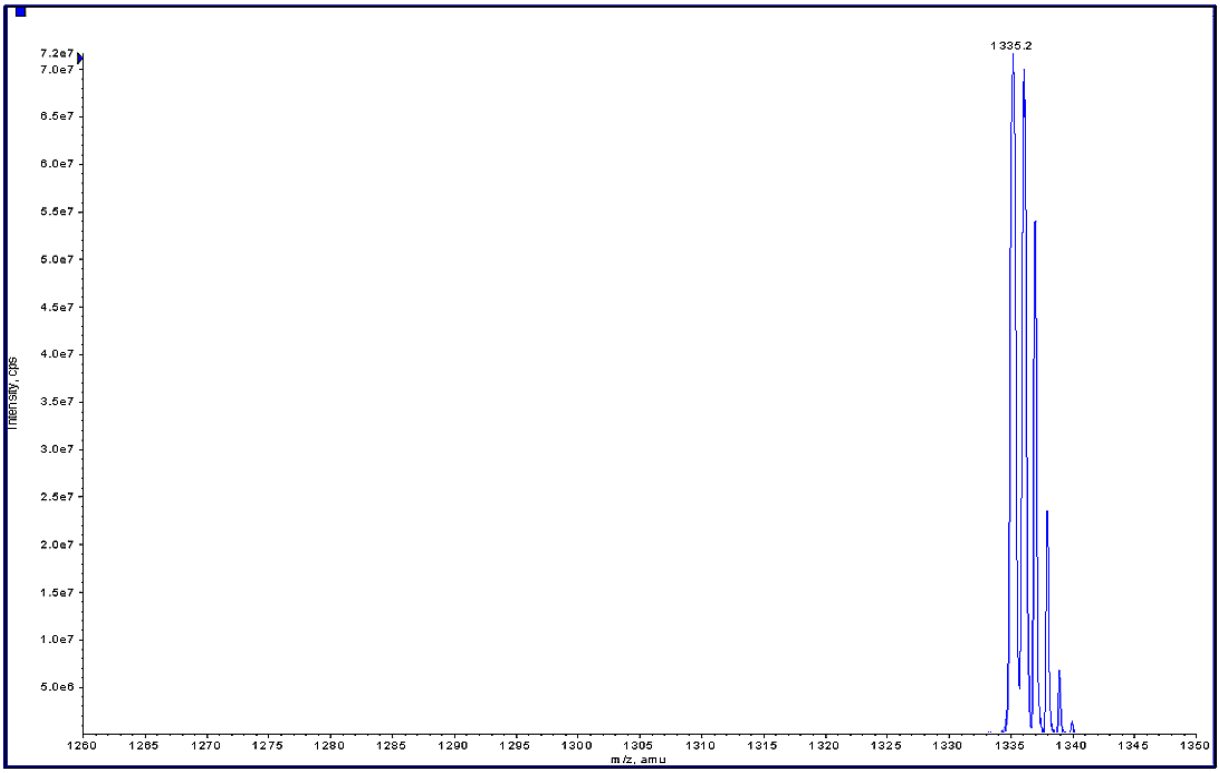

Figure 4: Neutral loss scan of mass 1202 amu (1 µg/ml in eluent B) infused at a flow rate of 10 µl/min

The adduct formation with Cs^+ and the subsequent collision induced dissociation with Cs^+ as the product ion was observed for all seven analytes. This principle of detection was used for the development of the chromatographic separation of all immunosuppressive drugs. A baseline separation was realized by using a ternary gradient (Figure 5).

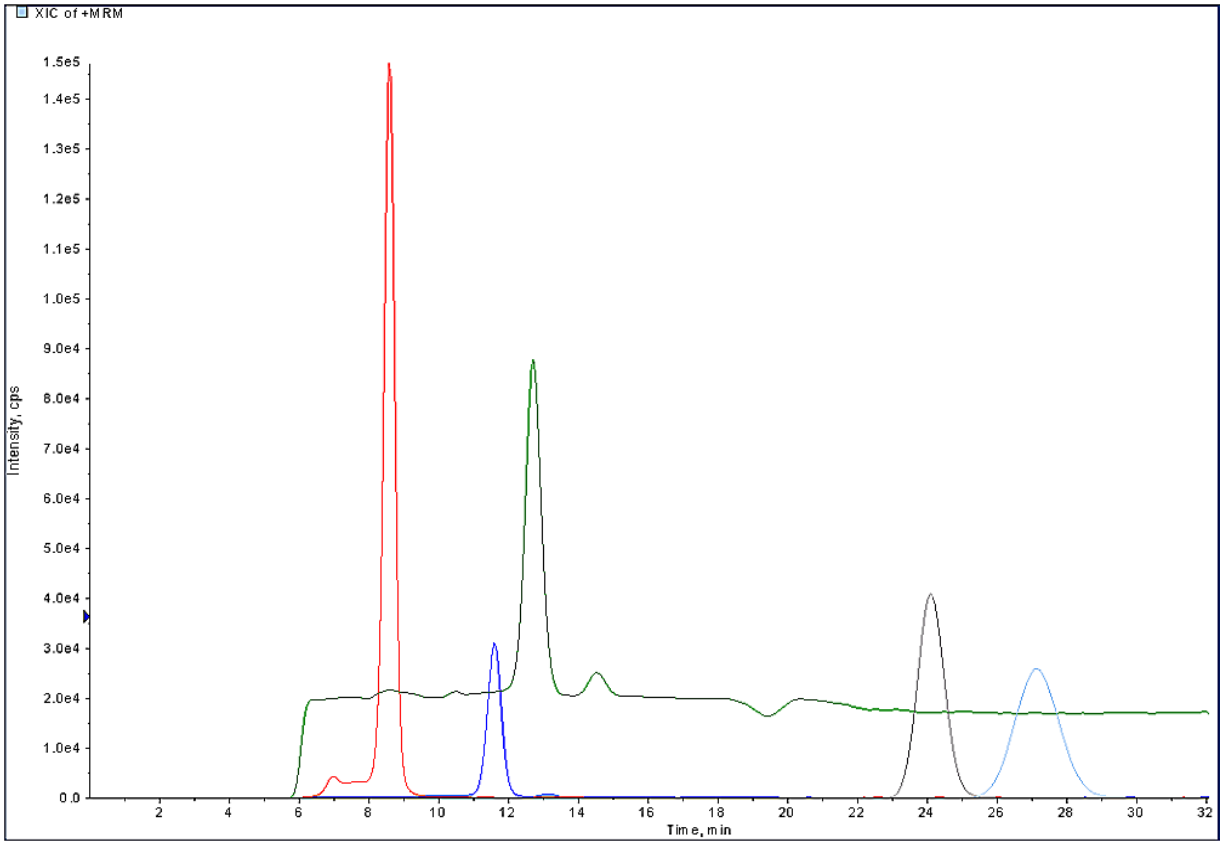

Figure 5: HPLC-MS/MS chromatogram (overlaid graphs of single mass traces) of a standard mixture of tacrolimus (8.59 min), sirolimus (11.59 min), everolimus (12.70 min), cyclosporin A (24.12 min) and cyclosporin D (27.16 min), 10 µl (1 µg/ml) injected (for details see Table 2, Figure1)

In Figures 6-12 chromatograms of standard solutions of tacrolimus (Figure 6), sirolimus (Figure 7), everolimus (Figure 8), cyclosporin A (Figure 9) and the analogues cyclosporin D (Figure 10), ascomycin (Figure 11) and 32-desmethoxy-rapamycin (Figure 12) are shown in separate runs. In addition to the declared components, these standard solutions contain additional substances (Figures 6-10), which are separated during the chromatographic procedure. Tacrolimus, sirolimus, everolimus, ascomycin and 32-desmethoxy-rapamycin contain isobaric components, which probably represent isomers, as has been described elsewhere [8]. For the cyclosporins A and D cross contamination is observed (Figures 11-12). Cyclosporin A contains about 5% cyclosporin D and cyclosporin D contains about 1% cyclosporin A. This is of importance, as cyclosporin D is used as internal standard for quantification of cyclosporin A.

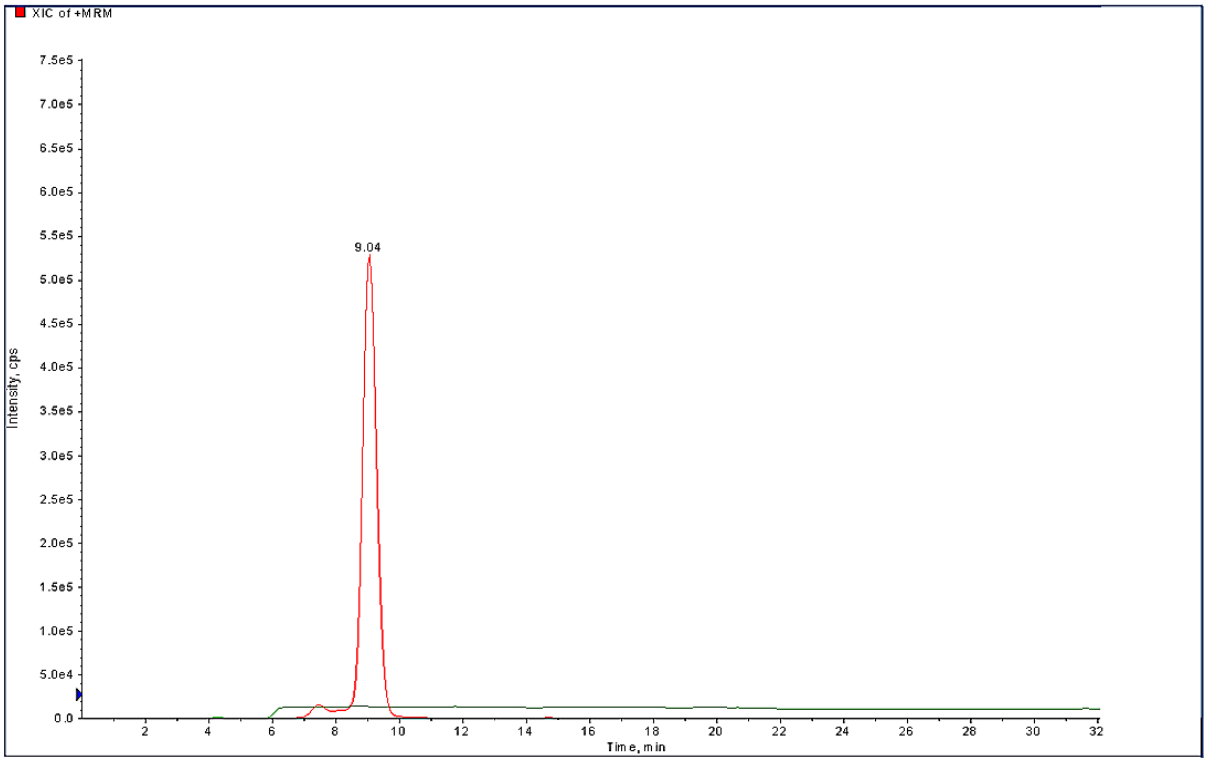

Figure 6: HPLC-MS/MS chromatogram (overlaid graphs of mass traces defined in Table 2) of a tacrolimus standard solution, 10 µl (1 µg/ml)

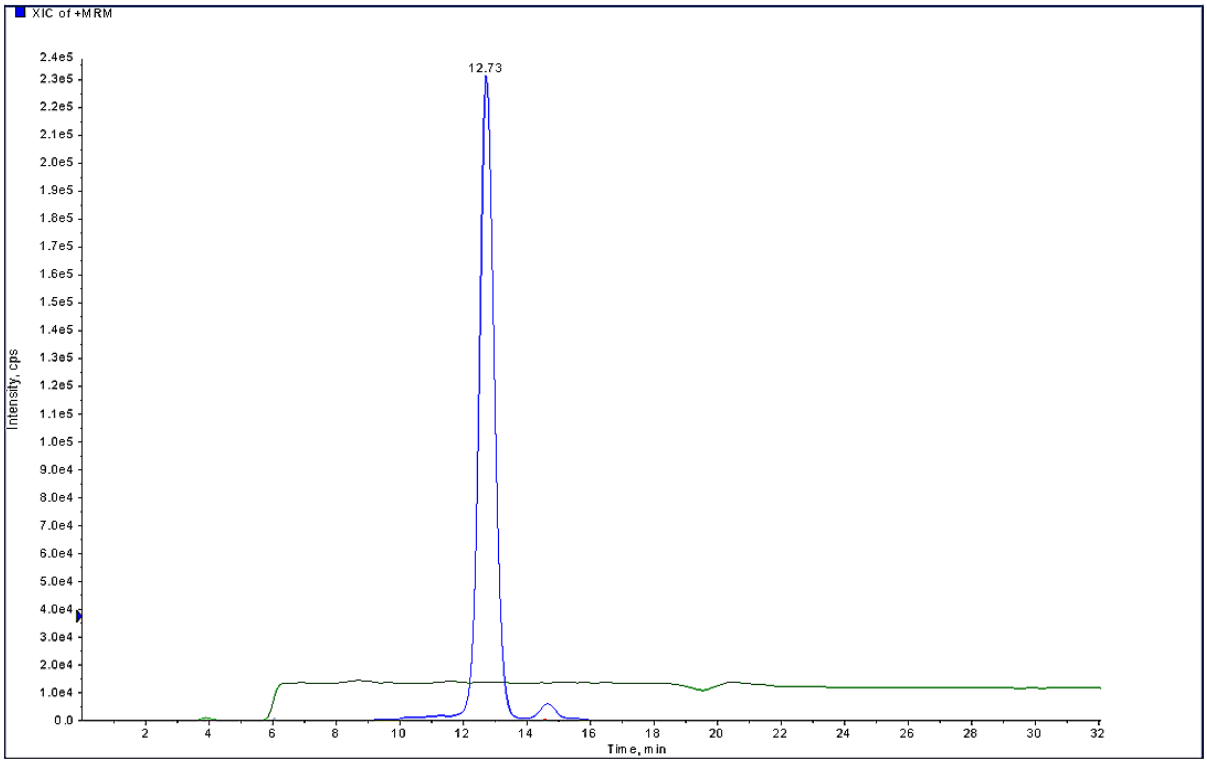

Figure 7: HPLC-MS/MS chromatogram (overlaid graphs of mass traces defined in Table 2) of a sirolimus standard solution, 10 µl (1 µg/ml)

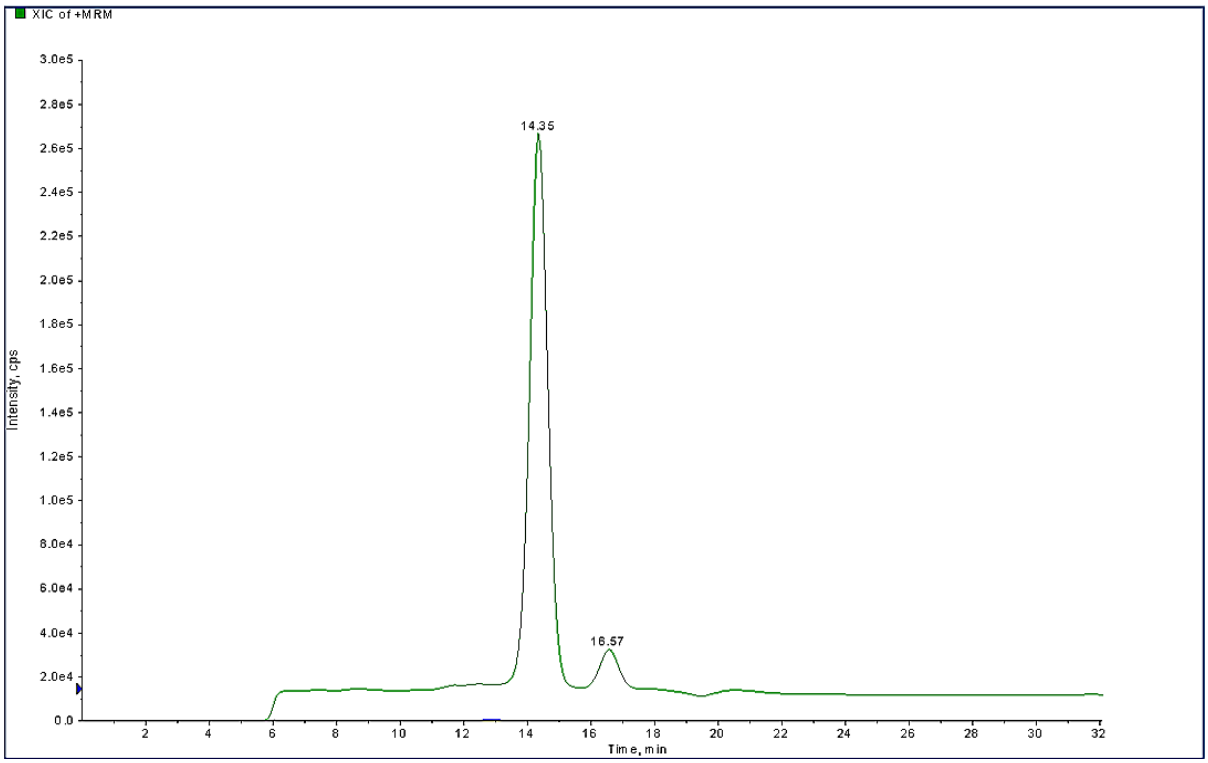

Figure 8: HPLC-MS/MS chromatogram (overlaid graphs of mass traces defined in Table 2) of an everolimus standard solution, 10 µl (1 µg/ml)

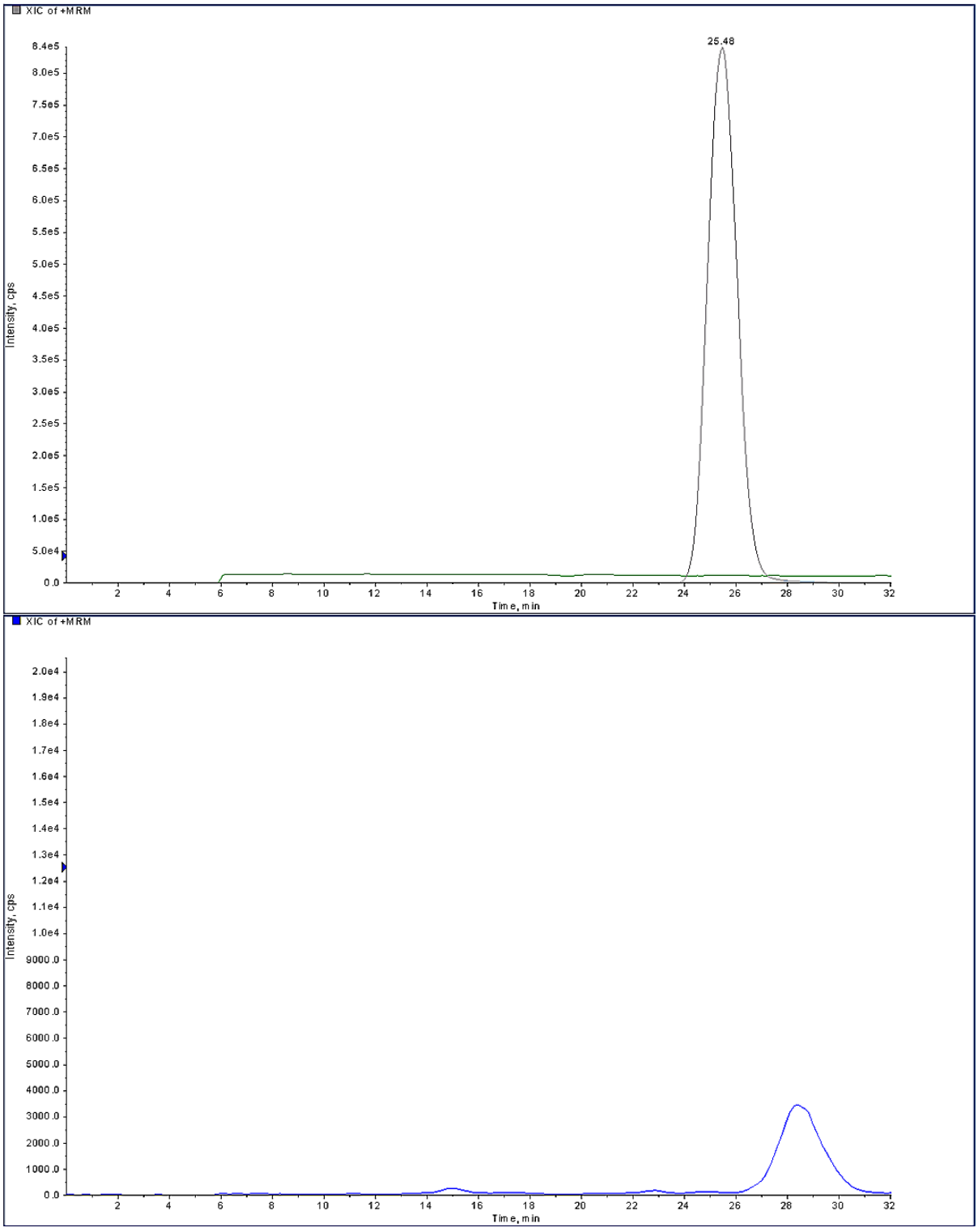

Figure 9: HPLC-MS/MS chromatogram of a cyclosporin A standard solution, 10 µl (1 µg/ml)
upper panel: overlaid graphs of mass traces defined in Table 2
lower panel: extracted mass trace of cyclosporin D

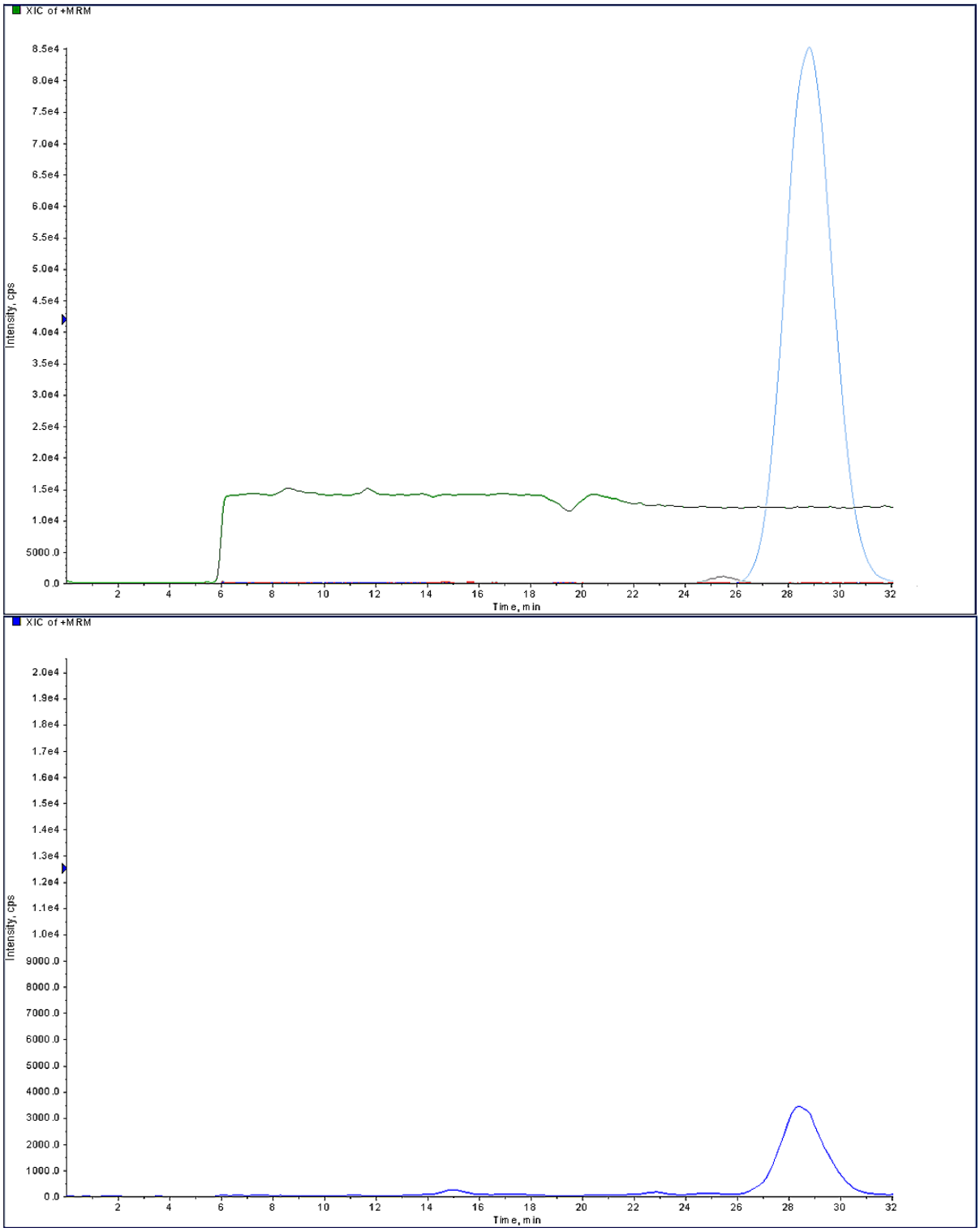

Figure 10: HPLC-MS/MS chromatogram of a cyclosporin D standard solution, 10 µl (1 µg/ml)
upper panel: overlaid graphs of mass traces defined in Table 2
lower panel: extracted mass trace of cyclosporin A

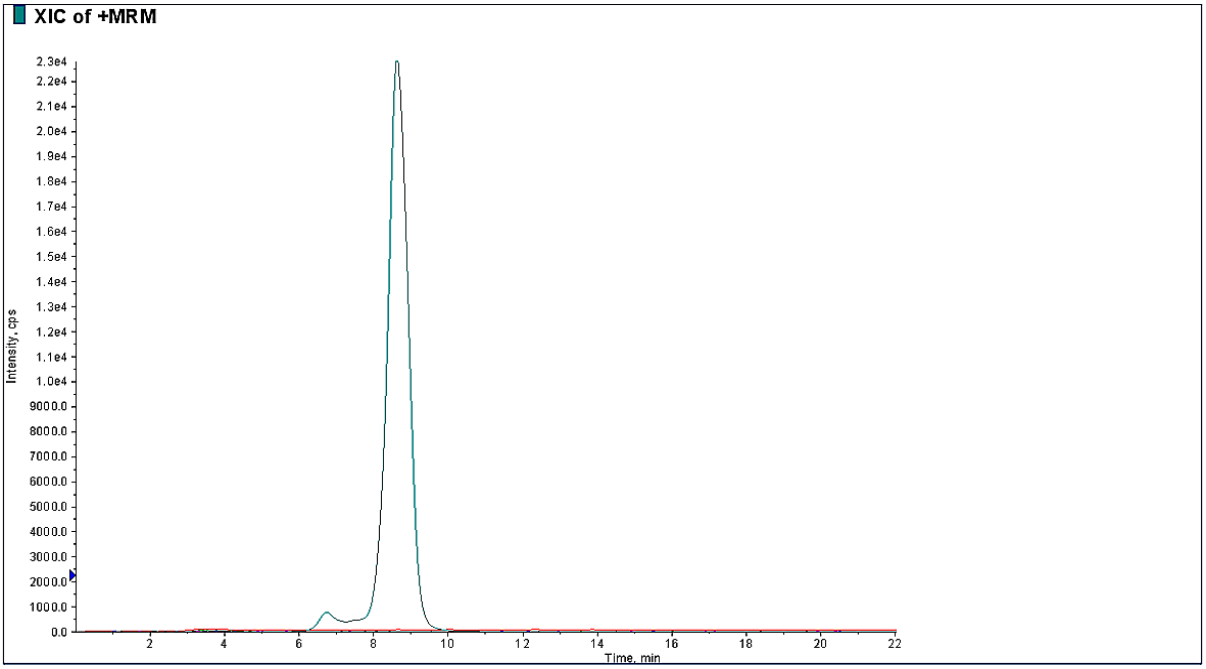

Figure 11: HPLC-MS/MS chromatogram (overlaid graphs of mass traces defined in Table 2) of a ascomycin standard solution, 10 µl (1 µg/ml)

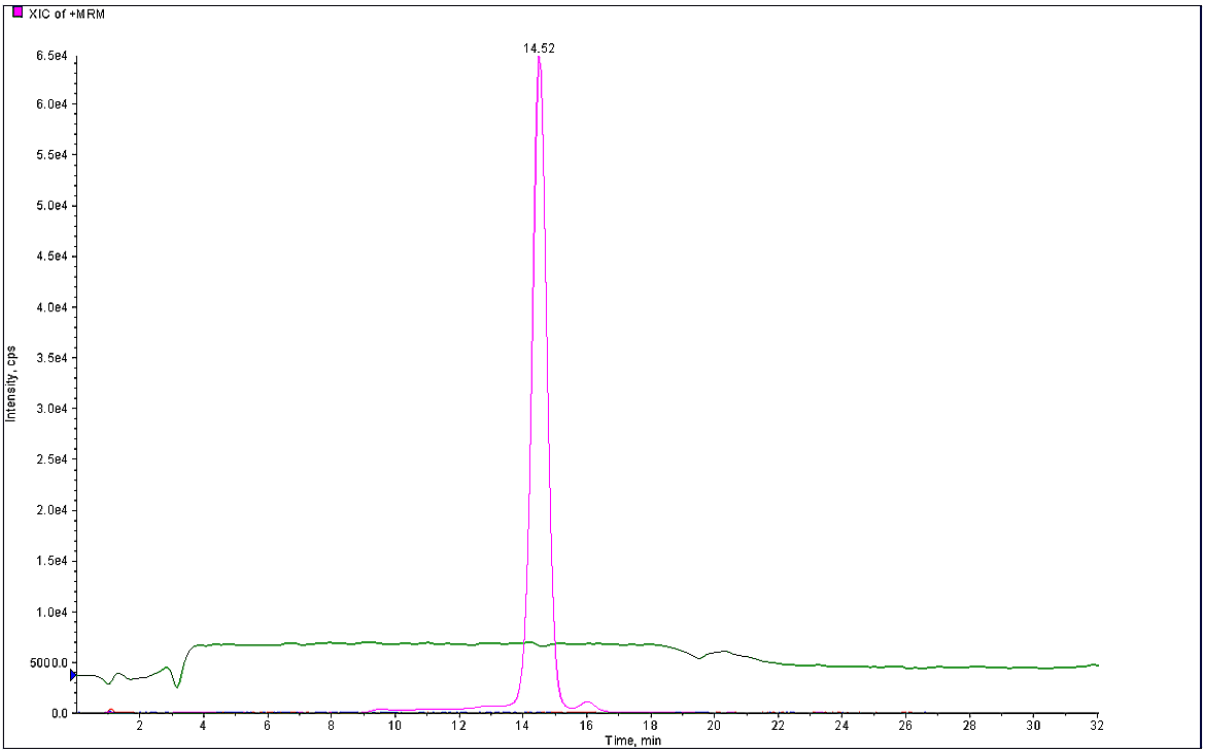

Figure 12: HPLC-MS/MS chromatogram (overlaid graphs of mass traces defined in Table 2) of a 32-desmethoxy-rapamycin standard solution, 10 µl (1 µg/ml)

For quantification of tacrolimus, ascomycin was used as internal standard, for sirolimus and everolimus the internal standard used was 32-desmethoxy-rapamycin.

The validity of this method was tested during the course of this study, in which the inter-run precision was measured using standard solutions. The precision measured – expressed as the coefficient of variation (CV) - under these conditions was 2.57% for sirolimus, 2.11% for everolimus, 2.31% for tacrolimus and 2.11% for cyclosporin A (Table 4).

Table 4: Inter run precision of a standard mixture of tacrolimus, sirolimus, everolimus, cyclosporin A, cyclosporin D, ascomycin and 32-desmethoxy-rapamycin

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**

Discussion

Recent LC-MS/MS measurements of immunosuppressive drugs have often used NH_4^+ adducts, which give rise to several different product ions [4]. Addition of Cs^+ ions reduced the number of product ions to one single product ion, namely the Cs^+ ion, which is measurable with high specificity and high signal intensity. It is of utmost importance to have a highly specific analytical method with high accuracy and precision for use as an intended reference measurement procedure. The specificity of current LC-MS/MS methods should be improved by chromatographic separation [9]. In the method presented here, all four immunosuppressive drugs are fully separated. Different internal standards listed in the text have been used for the quantification of the four immunosuppressive drugs. The best results were obtained using the corresponding analogues, namely ascomycin for tacrolimus, 32-desmethoxy-rapamycin for sirolimus and everolimus and cyclosporin D for cyclosporin A. This has been discussed elsewhere by Vogeser et al [4].

A baseline separation of cyclosporin D from cyclosporin A has been realized, as well as the separation of sirolimus from 32-desmethoxy-rapamycin. If needed, separation of everolimus from the internal standard 32-desmethoxy-rapamycin or tacrolimus from the internal standard ascomycin can be made by slight modifications of the ternary gradient profile.

Until now, there are no pure reference materials available for the immunosuppressive agents. All analytes used have been extracted from biological sources and contain sodium and potassium ions (see Figure 2) which can be removed by chromatographic separation so that the presence of Na^+ and K^+ adducts was less than 2%.

The high throughput routine methods which have been published for determination of immunosuppressive drugs do not separate the different immunosuppressive agents chromatographically. As a result, the routine methods currently in use are not suitable for setting target values in EQAS. They have limited specificity [4] and are prone to mass interferences [5]. In patient samples problems may occur during quantification, due to the presence of metabolites. At least 13 metabolites have been described for cyclosporin A [10]. The metabolites are usually more hydrophilic than the parent substance, due to hydroxylation, and can be separated by modifying the ternary gradient system used here.

For optimal quantification it is desirable to have stable isotope labelled analytes as internal standards [11]. Unfortunately, at present there are no commercial sources for isotopically labelled immunosuppressive agents. When stable-labelled compounds become available the method presented here can be developed into a reference measurement procedure for immunosuppressive drugs.

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