Application of MALDI-triple quadrupole mass spectrometry for the quantification of small molecules in biomedical research

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Application of MALDI-triple quadrupole mass spectrometry for the quantification of small molecules in biomedical research

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voor Maria Victoria en Manuela

"Luck is when knowledge is coupled with the opportunity"
Lucius Annaeus Seneca (4 BC – 65 AD).

List of abbreviations

α-CHCA	α-cyano-4-hydroxycinnamic acid
AIDS	Acquired immunodeficiency syndrome
ALL	Acute lymphoblastic leukemia
ART	Antiretroviral therapy
AZT	Azidothymidine
2,5-DHB	2,5-dihydroxybenzoic acid
CID	Collision induced dissociation
CYP	Cytochrome P450 enzyme
DBS	Dried blood spot
FDA	US Food and Drug Administration
FPIA	Fluorescence polarization immunoassay
HIV	Human immunodeficiency virus
HPA	3-hydroxypicolinic acid
IDMS	Isotope dilution mass spectrometry
LLOQ	Lower limit of quantification
LOD	Limit of detection
LPV	Lopinavir
MALDI	Matrix-assisted laser desorption/ionization
MRM	Multiple reaction monitoring
NFV	Nelfinavir
NNRTI	Non-nucleotide reverse transcriptase inhibitor
NRTI	Nucleotide reverse transcriptase inhibitor
NSCLC	Non-small cell lung cancer
NVP	Nevirapine
OS	Oseltamivir
OSC	Oseltamivircarboxylate
QqQ	Triple quadrupole mass spectrometry
RA	Rheumatoid arthritis
RT	Reverse transcriptase
RTV	Ritonavir
SA	Sinapic acid
SCLC	Small cell lung cancer
SPE	Solid phase extraction
TCA	Trichloroacetic acid
TDF	Tenofovir disoproxil fumarate
TDM	Therapeutic drug monitoring
THAP	Trihydroxyacetophenone
TNV	Tenofovir
ULOQ	Upper limit of quantification

Contents

Chapter 1

General introduction

Mass spectrometry in biomedical research

A century after its introduction, mass spectrometry is still an innovative technology, which, due to continuous instrumental developments and improvements, has provided important scientific insights in biochemistry, molecular biology and medicine. The development of new ionization techniques in the 1990s brought a real breakthrough for mass-spectrometry-related biomedical research: suddenly, electrospray ionization (ESI) [1] and matrix-assisted laser desorption/ionization (MALDI) made it possible to ionize biomolecules that were polar, thermally instable, and of high molecular weight [2]. Now, in 2011, mass spectrometry is used to analyze various types of molecule, such as proteins, peptides, carbohydrates, oligonucleotides, estrogens, natural products, drugs, and drug metabolites.

Because modern mass spectrometers have high sensitivity-ranges (picomole (10^{-12} g)-to attomole (10^{-18} g)) [3], they have became a very popular analytical research tool in several areas of biomedical research. In pediatrics, for example, mass spectrometry is used with other analytical techniques to investigate the presence of congenital metabolic errors such as fatty-acid oxidation disorders, acidurias and organic acidurias in newborns. The first gas-chromatography mass-spectrometric (GC-MS) clinical application used to detect acidurias dates back to the 1960s [4]. Some twenty years later, later mass spectrometry technology had become suitable for routine measurements, and clinical laboratories started to use far more innovative and selective electrospray tandem-mass spectrometry (MS/MS) to detect inborn metabolic errors [5,6].

In oncology, mass spectrometry is used not only in the diagnosis of cancer [7, 8], but to a far greater extent in approaches to fundamental cancer research that use proteomics – the study of proteins, their functions and structure (the proteome being the entire complement of proteins, including post-translational modifications [9]). Proteomics can detect the changes to the human proteome that may occur in a diseased state, and which may alter protein-expression levels. These can eventually be used in clinical diagnostics or the development of fundamental new therapies and treatments [10]. Proteomics is one few of the biomedical research techniques to depend completely on sophisticated mass-spectrometric technology.

Like proteins, other distinct groups of small molecules and metabolites play important roles in the healthy and diseased states, participating in energy production and the synthesis (anabolism) and degradation (catabolism) of molecules and macro-molecules. Metabolomics, like proteomics, depends heavily on mass-spectrometric technologies [11,12]. The metabolome is the complete set of small-molecules and metabolites – such as metabolic intermediates, hormones and signaling molecules, and secondary metabolites – that are present in a single organism, cell or tissue [13]. To date, some two thousand biologically active molecules, metabolites and alterations [14] are known. As with the altered protein concentrations that are detected by proteomics, it is possible to use altered small-molecule concentrations in clinical diagnostics, therapy, and fundamental and biomedical research [15].

In clinical research, mass spectrometry is used mainly to determine drug concentrations in a patient's body fluids. In certain therapies, it is extremely important to determine drug concentrations, as the efficacy and outcome of therapy is determined by the inter-patient variability caused by pharmacokinetic differences. Greater knowledge of inter-patient variability will increase the consistency of clinical outcomes: this is because the equal-dosing principle – "one patient, one dose" (on the basis of the patient's body weight) – is therapeutically less effective, especially in extremely young or extremely old patients, or in patient populations with low drug tolerance or impaired or abnormal organ function [16].

In disease treatment, so-called personalized medicine – the proper drug dosing of individual patients – is becoming increasingly desirable, and also increasingly important. For the purpose of research into personalized medicine, techniques such as therapeutic drug monitoring (TDM) are now being developed and used. The clinical value of TDM was first shown in the treatment of epileptic patients, when it was proved that if adverse drug effects could be reduced and seizure control could be significantly improved. This improvement actually came when individual drug doses could be adjusted by using TDM to measure blood concentrations [17].

Although the use of mass-spectrometric technologies in biomedical research has many advantages, mass spectrometry has also limitations. For example, while its use in biomedical research is widespread, its use within routine clinical diagnostics is very limited; it is yet to be accepted as an analytical technique that is just as valuable as routine standard classical analytical techniques. Because the latter – photometry,

immunoassays and other techniques – have been much used over time, they have become highly automated high-throughput techniques. In many clinical diagnostic assays they have therefore become the “golden standard”, despite being limited by low analyte specificity and by cross-reactivity with other drugs or endogenous molecules.

Another limitation of mass spectrometry is the expense of its instrumentation. As a result, sample analysis costs are higher than those of routine analysis using immunoassays, but we can soon expect to see enormous analytical potential for routine clinical diagnostics [18]. Rather than quantifying just a few measured biomarkers as at present, mass-spectrometry applications may soon allow us to characterize the metabolic complicity of diseases in an individual patient.

After the introduction of mass spectrometry into routine clinical laboratories, we can expect future diagnostics to be driven by new developments in for example mass spectrometry. The overall diagnosis and treatment of diseases will be improved not only by the introduction of new clinical diagnostic parameters or better quality-standard regulations for biomarker discovery and analyses, but also by routine *in-vitro* diagnostics.

Although scientific research provides the foundation of our knowledge, a major future challenge in biomedical research is how new knowledge can be translated into clinical practice. Expectations are high that new clinical diagnostic parameters, more sensitive instrumentation, improved bioanalytical quality standards could influence modern medicine, and therefore especially highly efficient and individually orientated therapies will result from such research and the knowledge it generates, not only on the burden of certain diseases, but also on disabilities. Hopefully, biomedical mass spectrometry will help fulfill these expectations.

This thesis demonstrates the diagnostic potential and advantages of innovative mass-spectrometry technology in biomedical research. It covers three medical research fields –infectious diseases, oncology and auto-immune diseases – for which we developed new mass-spectrometric bioanalytical assays to support biomedical research on several specific drugs used to treat patients.

Aim of the thesis

Our aim was to develop, implement and test new bioanalytical assays using MALDI-QqQ-MS/MS technology for its clinical feasibility for ultrafast, high-throughput therapeutic drug monitoring of six different drugs used to treat diseases such as human immunodeficiency virus infection (HIV), acute lymphoblastic leukemia (ALL), non-small cell lung cancer (NSCLC), and rheumatoid arthritis (RA).

The statistical criteria applied to new bioanalytical assays

Some of the criteria applied in method development were that analysis of the drug concentrations could be based on small sample volumes ($\leq$ 100 µL), and that drugs could be analyzed in various body fluids, such as plasma, serum or whole blood. At the very least, the new to develop bioanalytical assays also had to be sensitive enough to measure pharmaceutically relevant drug concentrations. Next to the application of small sample volumes and the applicability of the new developed assays to different body fluids, bioanalytical assays were validated according to the most recent FDA guidelines on method development and the validation of bio-analytical assays [19].

The important statistical-validation parameters were accuracy and precision on the same day (within-run) or over three consecutive days (between-run). Here it should be noted that precision refers to the variation (%CV) in the multiple drug-concentration measurement of a quality-control sample (QC) whose concentration is known. Accuracy is a statistical validation parameter expressed as percentage error; it shows whether the drug concentrations observed correspond with nominal drug concentrations in QC samples whose drug concentrations are known. According to the most recent FDA guidelines, the precision and accuracy of bioanalytical assays should be $\leq$ 20% at concentrations at the lowest limit of quantification (LLOQ), and < 15% for concentrations between LLOQ and the upper limit of quantification (ULOQ).

Bland-Altman analysis was used to statistically compare the newly developed bioanalytical assays with routine clinical diagnostic assays [20, 21]. Bland-Altman analysis is not a statistical test using a *p*-value between two sets of data, but a statistical approach to determining the degree of agreement between two independent bioanalytical assays. Consisting of a scatter plot of variable means

plotted on the horizontal axis, with the differences plotted on the vertical axis, it shows the degree of disagreement between two observations (via the differences), and indicates how this disagreement relates to the magnitude of the measurements. A Bland-Altmann plot includes the mean and limits of agreement calculated at bias ± 1.96 STD (average difference ± 1.96 standard deviation of the difference). When the differences between two observations by both assays are not deemed to be clinically important, it is concluded from a Bland-Altmann plot that agreement between both methods is statistically confirmed.

Rationale behind the use of MALDI-QqQ-MS/MS technology

Since many patient's samples are collected in clinical studies, it was desirable for the newly developed assays to have ultrafast and high-throughput properties. We therefore decided to apply a relatively new mass-spectrometric technology that had been launched commercially in 2008 by Applied Biosystems/MDS Sciex (now known as AB Sciex), but had not yet been used extensively for the quantitative determination of small molecules.

This technology merges a high-repetition matrix-assisted laser desorption/ionization (MALDI) source (1000 Hz, 349 nm) with a triple quadrupole mass analyzer (QqQ) platform (FlashQuant workstation). Using this technology, small-molecule quantization is at least twenty-five times faster than it is using the fastest liquid chromatography-coupled (triple quadrupole) mass spectrometers (LC-MS) currently available [22]. Together with electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI) is one of the most used and most important ionization techniques for ionizing non-volatile, polar and high-molecular-weight molecules. It can also be used to ionize low-weight molecules [23-25].

Because the FlashQuant workstation omits liquid chromatographic separation, analysis times are considerably reduced to approximately 10 seconds per sample [26, 27] or even less. These very short analysis times mean that MALDI-QqQ-MS/MS is very suitable for ultrafast and high-throughput analysis of clinical samples. The analytical performance properties of this technology have been shown to be equal to that of (HP)LC-ESI-MS [28].

Three of the six newly developed bioanalytical assays presented in this thesis applied isotope dilution mass spectrometry (IDMS), an analytical procedure for applying stable-isotope-labeled substances. A known, exact quantity of stable-isotope-labeled substance is added to the sample with an analyte-unknown concentration, and the ratio of the two signal intensities between stable-isotope-labeled and non-labeled analyte is used to determine the concentration of the non-labeled analyte [29, 30].

MALDI-QqQ-MS/MS bioanalytical assays applied in different biomedical research fields

Infectious diseases

Infectious diseases are also known as communicable or transmissible diseases. Resulting from an infection (i.e., the presence or growth of a pathogenic microorganism in a host organism), they are caused by pathogens such as viruses, bacteria, fungi, protozoa, parasites and aberrant proteins (prions). The pathogens are transmitted by parasites, or through physical contact, body fluids, food, or inhalation [31].

Human immunodeficiency virus infection

This year (2011), it is exactly 30 years since the American Centers for Disease Control and prevention (CDC) reported the discovery of a new disease among homosexual men which was apparently causing death through a severe T-cell immune-deficiency disorder [32]. In 1983, the cause of the acquired immunodeficiency syndrome (AIDS) was discovered; in the same year, human immunodeficiency virus (HIV), the primary etiological agent of AIDS and HIV, was isolated [33, 34].

HIV belongs to the genus of lentiviridae (family of the retroviruses). It attacks and destroys specific activated $CD4^+$ T-cells and macrophages; after infection, viral enzyme RNA reverse transcriptase (RT) catalyses the synthesis of a double-stranded viral DNA. The viral DNA becomes integrated into the DNA of the host, and serves as

a template for viral RNA transcription, which is then packaged into new virions that leave (budding) the host cell through its cell surface. In the chronic phase of HIV infection, the HIV viral replication is relatively stable, but the $CD4^+$-T cell count gradually declines, eventually leading to other infections or malignancies.

Human influenza-virus infection

Influenza, commonly known as flu, is an infectious disease caused by RNA viruses from the family of Orthomyxoviridae. Its commonest symptoms are chills, fever, sore throat, muscle pains, severe headache, coughing, weakness/fatigue, and general discomfort. Influenza may also produce nausea and vomiting, particularly in children. Typically, it is transmitted through the air by coughs or sneezes, which create aerosols containing the virus. While influenza viruses can be inactivated by sunlight, disinfectants and detergents [35], vaccinations are usually used, the commonest human vaccine being trivalent influenza vaccine (TIV), which contains purified and inactivated antigens against three viral strains. Typically, this vaccine includes material from two influenza A virus subtypes and one influenza B virus strain [36]. TIV carries no transmission risk and has very low reactivity. Since the influenza virus evolves rapidly and new strains quickly replace the older ones, vaccines which are formulated in one year may be ineffective in the following year. Antiviral drugs are used to treat influenza, the neuraminidase inhibitors such as Oseltamivir (Tamiflu®) and Zanamivir (Relenza®) being particularly effective.

Oncology

Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths (approx. 13% of all deaths) in 2008 [37]. The major types are lung cancer (1.4 million deaths in 2008), stomach cancer (740,000), liver cancer (700,000), colorectal cancer (610,000), and breast cancer (460,000) [37]. Tobacco use is a major risk factor for the development of cancer, together with other risk factors such as excessive alcohol use, poor diet, and physical inactivity. Cancer can also be caused by viral infections such as hepatitis B and C. Similarly, some types of human papilloma virus (HPV) can cause cervical cancer (with up to 29% of cancer-related deaths in low and middle-income countries, and 9% in high-income countries). Although over 30% of all cancer

deaths can be prevented, it is estimated that deaths from cancer will rise to over 11 million by 2030.

Lung cancer

Defined as uncontrolled cell growth of the lung tissue, lung cancer can lead to metastasis, spreading of the cancer, and invasion into other organs. Most primary lung cancers are so-called carcinomas, i.e. cancer of the epithelial cells. As one of the most common causes of cancer-related deaths in men and women, lung cancer was responsible for 1.4 million deaths worldwide in 2008 [37]. The main lung-cancer types are small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NCCLC), which are treated very differently, surgery often being better for NSCLC, and chemotherapy and radiation usually achieving better responses in SCLC [31].

Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is a malignant proliferation of lymphoid cells [38] which are blocked at an early stage of development. Although ALL is primarily a disease of the bone marrow and peripheral blood, any organ or tissue can be infiltrated by abnormal cells. Most ALL cases feature chromosomal and genetic abnormalities that occur spontaneously in important regulatory genes in the lymphoid cell populations. ALL is a biologically heterogeneous disorder, meaning that morphologic, immunologic, cytogenetic, biochemical and molecular genetic characterizations of lymphoblasts are needed to establish the final diagnosis, and thus to exclude eventually other causes of bone marrow failure [38].

Auto-immune diseases

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease [31] of unknown cause that primarily affects peripheral joints in a symmetrical pattern. Symptoms include fatigue, morning stiffness and malaise. RA also involves joint destruction, which often leads to considerable morbidity and mortality. Thanks to the introduction of new and innovative therapies, RA treatment is now expanding rapidly, with the drug methotrexate (MTX) being used in low doses (5-25 mg/week) as a first-line drug [39].

Due to this low-dose therapy and the relatively short half life (8-15 hrs) of MTX, TDM of plasma MTX concentrations in low dose MTX therapy is useless [40]. Neither do MTX plasma levels correlate with the disease activity [41, 42]. In RA patients, however, intracellular MTX metabolite (MTX-polyglutamates) concentrations are used to predict MTX response. While the higher MTX-polyglutamates (i.e. MTXPG3-MTXPG5) have three or more glutamic acid residues, and are particularly associated with therapeutic responses, MTX and MTXPG2 are poorly associated with therapeutic efficacy [41].

Outline of the thesis

Chapter 2 describes the development and application of newly developed bio-analytical assays to support biomedical research in HIV and influenza infection. Bioanalytical assays for the quantitative measurement of protease-inhibitor drug concentrations in plasma and dried blood-spot specimens collected from pediatric HIV-1 infection and a quantitative bio-analytical assay in combination with an uses isotope-dilution MALDI triple quadrupole method to measure plasma concentrations of Tenofovir in adult HIV-1 infection are presented. Furthermore, a bioanalytical assay for the measurement of the antiviral drug Tamiflu® used to treat influenza infections, and a study how the addition of an internal standard in dried blood-spot analysis influences bioanalytical method development, especially in research on HIV-1 infections are presented.

Chapter 3 presents newly developed bio-analytical assays that use MALDI-QqQ-MS/MS technology to support oncology research. The chapter describes the application of this technology for the measurement of plasma concentrations of the recently introduced antifolate drug Pemetrexed in NSCLC patients. The second part of the chapter demonstrates the application of the MALDI-QqQ-MS/MS technology combined in combination with an isotope-dilution method approach for the determination of methotrexate and 7-hydroxymethotraxate metabolite concentrations in plasma samples from patients with acute lymphoblastic leukemia (ALL).

Chapter 4 describes a newly developed bioanalytical assay to measure therapeutically relevant intracellular erythrocyte concentrations of methotrexate and

methotrexate polyglutamate metabolites in packed erythrocyte pellet samples collected from Rheumatoid Arthritis (RA) patients.

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Chapter 2

Infectious diseases

Ultrafast and high-throughput mass spectrometric assay for therapeutic drug monitoring of antiretroviral drugs in pediatric HIV-1 infection applying dried blood spots

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Abstract

Kaletra® (Abott Laboratories) is a co-formulated medication used in the treatment of HIV-1 infected children and it contains the two antiretroviral protease inhibitor drugs lopinavir and ritonavir. We validated two new ultrafast and high-throughput mass spectrometric assays to be used for therapeutic drug monitoring of lopinavir and ritonavir concentrations in whole blood and in plasma from HIV-1 infected children. Whole blood was blotted onto dried blood spot collecting cards (DBS) and plasma was collected simultaneously. DBS collecting cards were extracted by an acetonitrile/water mixture while plasma samples were deproteinized with acetone.

Drug concentrations were determined by Matrix-Assisted Laser Desorption/Ionization-triple quadrupole tandem mass spectrometry (MALDI-QqQ-MS/MS). The application of DBS made it possible to measure lopinavir and ritonavir in whole blood in therapeutically relevant concentrations. The MALDI-QqQ-MS/MS plasma assay was successfully cross-validated with a commonly used HPLC-UV assay for the TDM of HIV-1 infected patients, and it showed comparable performance characteristics. Observed DBS concentrations showed as well a good correlation between plasma concentrations obtained by MALDI-QqQ-MS/MS as also obtained by the HPLC-UV assay. Application of DBS for TDM proved to be a good alternative to the normally used plasma screening.

Moreover, collection of DBS requires small amounts of whole blood which can be easily performed especially in (very) young children where collection of large whole blood amounts is often not possible. DBS is perfectly suited for TDM of HIV-1 infected children, but nevertheless DBS can also easily be applied for TDM of patients in areas with limited or no laboratory facilities.

Introduction

Protease inhibitors (PI) belong to a class of pharmaceuticals used in the treatment of the human immunodeficiency virus infection (HIV) [1]. This class of protease inhibitor drugs inhibits the viral HIV-1 protease enzyme, which processes viral polyproteins which are essential for the completion of the HIV viral life cycle. PI drugs are used within the combined antiretroviral therapy (CART), a therapy which has significantly improved the decrease in morbidity and mortality for HIV infected patients [2]. Kaletra® (Abbott) is a co-formulation, containing the two protease inhibitors lopinavir (LPV, 80 mg/mL) and ritonavir (RTV, 20 mg/mL). This co-formulation is used in pediatric HIV-1 pediatric treatment. RTV acts to boost up the Lopinavir plasma concentration by inhibition of the cytochrome P450 CYP3A isozymes [3] and the active transport by the P-glycoprotein [4].

Patient's drug intake compliance is very important in HIV treatment and the patient's compliance with the drug regimen can be monitored by determination of the LPV and RTV plasma concentration levels. More importantly, plasma concentrations of HIV protease inhibitors are used to optimize dosing with these drugs for each individual patient (therapeutic drug monitoring; TDM), which is in particular important for HIV infected children because of the profound changes in drug pharmacokinetics during development and maturation of the child. Patient compliance to the HIV drugs regimen and TDM is in general monitored by analyses of plasma or serum, sometimes also another body fluid such as saliva [5] is used. Heine et al. [6] and Koal et al. [7] used both DBS for the quantitative determination of PI concentrations by LC-MS/MS. The DBS assay was first described by Guthrie [8] in the 1960s and was primarily used to screen neonates for metabolic diseases. DBS assays are not only applied for screening of metabolic diseases in neonates but in general can also be used to determine drug concentrations in whole blood. Especially, DBS assays have been applied for the determination of concentrations of antimalarials [9], antiepileptics [10] and antiretrovirals [7]. Whole blood is collected from either heel or finger prick or by collection by vena-puncture. The whole blood is then blotted onto a DBS collection card. After the whole blood spots have been dried, a disk is punched out from the center of the DBS and PI are extracted from the disk with appropriate

solvents and extracts are analyzed. Over the last decades, several DBS assays have been applied for screening of metabolic disorders.

Analytical assays used for the determination of LPV, RTV and other PI in general are High Performance Liquid Chromatography (HPLC) with ultraviolet (UV) [11] detection, electrospray ionization-mass spectrometry (ESI-MS) [12,13] and tandem mass spectrometry (ESI-MS/MS) [7,14-17] and MALDI-QqQ-MS/MS [18]. MALDI-QqQ-MS/MS (Matrix-Assisted Laser Desorption/Ionization-triple quadrupole tandem Mass Spectrometry) is a relative new technique that combines two known aspects of mass spectrometry, MALDI and triple quadrupole mass spectrometry (QqQ), into a novel merged mass spectrometric technique. Biological samples are mixed with a solution containing the MALDI matrix, which is typically a small molecule that absorbs the energy of the laser and assists with desorption and gentle ionization of the analyte. A small aliquot (0.25-1.5 µL) of the sample/matrix solution is then spotted onto a MALDI (stainless steel) target plate. The spots are dried at ambient temperature and analyzed by ionization by a high repetition rate solid state UV-laser (1 kHz, 349 nm). The produced ions are then analyzed using multiple reaction monitoring (MRM). The MALDI-QqQ-MS/MS technique does not necessarily require liquid chromatographic separation of samples prior to mass MRM analyses. Therefore, an analysis time of MALDI-QqQ-MS/MS is primarily determined by the amount of laser shots required to obtain a reproducible MRM, the raster speed of the laser, and the repetition rate of the laser. Using our settings the analysis takes 5 seconds per sample or even less (depending on laser speed). The MALDI-QqQ-MS/MS assay has been previously used to determine the concentrations of different drug types [19]; antiretroviral drugs [20,21], benzodiazepines [22], beta-blockers and antibiotics [23] but was also successfully used as a screening tool in enzyme kinetic studies [24]. In this study, we present two newly developed MALDI-QqQ-MS/MS assays for ultrafast and high-throughput monitoring of lopinavir and ritonavir drug concentrations in plasma and in whole blood (DBS) from HIV-1 infected children. The new assays were validated according to the FDA guidelines [25] and tested and applied for its clinical use by analyses of DBS and plasma samples from a cohort study of HIV-1 infected children. Observed plasma concentration levels of LPV and RTV by the MALDI-QqQ-MS/MS plasma assay were also cross-validated with a validated HPLC-UV assay.

Materials and methods

Chemicals and reagents

Investigated PI drugs were a kindly donation from pharmaceutical companies; LPV and RTV by Abbott Laboratories (USA, Illinois, IL) and the internal standard nelfinavir (NFV) by Pfizer (USA, Groton, CT). A primary stock solution of LPV and RTV was prepared in methanol and contained 200 µmol/L LPV and RTV while a second primary stock solution also prepared in methanol contained 200 µmol/L of the internal standard NFV, respectively. All solvents used were obtained from commercial sources and were of LC-MS grade (Biosolve, Valkenswaard, the Netherlands) and other chemicals were of ACS grade from Sigma Aldrich (Zwijndrecht, the Netherlands). Chemical structures of lopinavir, ritonavir and used internal standard nelfinavir are illustrated in Fig.1.

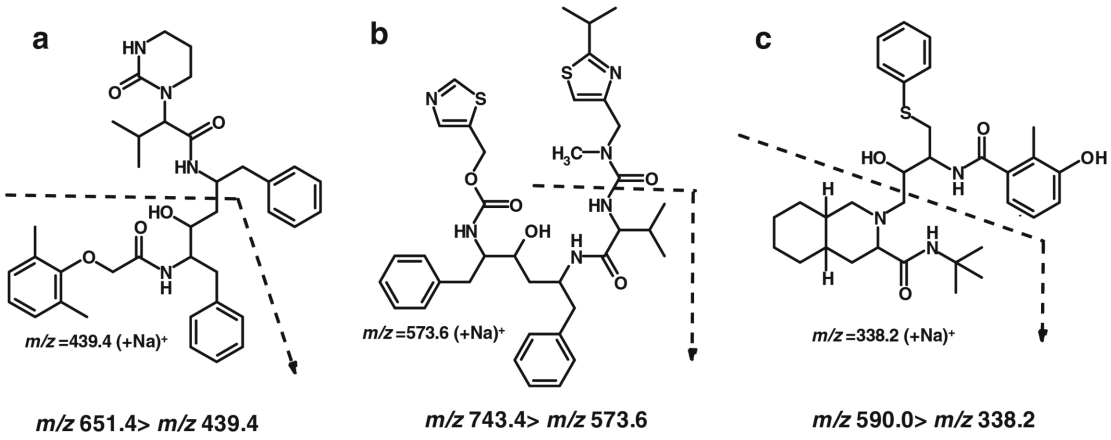

Figure 1. Molecular structures of (a) LPV, (b) RTV and (c) internal standard NFV and respective fragmentations of the sodium adducts of LPV, RTV and NFV and MRM transition.

Preparation of calibrators and calibration curves

Whole blood based calibrators for DBS analyses

Calibrators for the DBS assay were prepared by dilution of the primary stock solution with drug free whole blood (EDTA) from healthy donors that were obtained from the local blood bank (Sanquin Blood Supply Foundation, Rotterdam, the Netherlands). Whole blood based calibrators yielded following concentrations: 50, 25, 10, 5.0, 2.5, 1.0, 0.5, 0.25 µmol/L LPV and RTV. From the calibrators an aliquot of 25

µL were spotted (n=4) onto FTA PK DBS collecting cards from GE Healthcare (Piscataway, NJ, USA). DBS collecting cards were dried overnight at room temperature. Two disks (7 mm diameter) were punched out from two different DBS and punches were transferred into a safe-lock micro tube (1.5 mL, Eppendorf) and extracted with 250 µL of a acetonitrile/water mixture 80:20 (v/v) containing 5 µmol/L Nelfinavir as internal standard (IS) for 30 minutes in an ultrasonic bath. DBS extracts were centrifuged for 5 minutes at room temperature (2000 x *g*) to remove precipitated proteins. From the supernatants 20 µL were mixed with 20 µL of MALDI matrix α-CHCA (α-cyano-4-hydroxycinnamic acid, Agilent Technologies;6.2 mg/mL in methanol/acetonitrile/water 36:56:8 (v/v/v), pH=2.5 supplemented with 1 mmol/L sodium iodide (NaI) and 1.5 µL of the supernatant/MALDI matrix mixtures were spotted (n=5) on an Opti-TOF 96-well stainless steel target plate (123 x 81 mm; MDS Analytical Technologies, Concord, Canada) and sample spots were dried at room temperature for 5 min.

Plasma based calibrators

Plasma based calibrators were prepared from drug free plasma harvested from the drug free whole blood collected of healthy donors which were also used for development of the DBS assay. The plasma was prepared by centrifugation of whole blood for 10 min at a temperature of 4°C (400 x *g*). Plasma based calibrators were prepared by spiking with LPV and RTV and yielded the following calibrators: 4, 1, 0.5, 0.2, 0.1, 0.05 µmol/L and a blank. From each calibrator two aliquots of 10 µL were spiked with 5 µL of an IS solution and were deproteinized by the addition of 85 µL of acetone. Precipitated proteins were removed by centrifugation for 5 minutes at room temperature (2000 x *g*). The supernatants (20 µL) were mixed with α-CHCA solution (20 µL), 1.5 µL of the supernatant/matrix mixture was spotted (n=5) and the resulting sample spots were dried at room temperature for 5 min.

MALDI-triple quadrupole tandem mass spectrometry

MALDI-QqQ-MS/MS analyses were conducted on a Flashquant Workstation combined with a 4000 API mass analyzer (MDS Analytical Technologies, Concord,

Canada) operating in the positive ionization mode. MS/MS analyses were carried out by multiple reaction monitoring (MRM) at unit resolution. MRM parameters of the sodium adducts of analyte molecules $(M+Na)^+$ used in this study were: LPV (*m/z* 651.4>439.4), RTV (*m/z* 743.4>573.6) and NFV (*m/z* 590.0>338.2) and collision energies were 52, 50 and 50 V for LPV, RTV and NFV, respectively. Optimized MALDI-QqQ-MS/MS instrument parameters were as follows: laser power 60%, target plate voltage 60 V, skimmer voltage 0 V, CAD gas 6 arbitrary units (collision gas), collision cell energy exit potential (CXP) 10 V, source gas 10 arbitrary units, dwell time 20 ms and laser speed was 1 mm/sec. MALDI-MS instrument control and data analyses were performed using Flashquant 1.0 software and Analyst 1.4.2 application software (MDS Analytical Technologies, Concord, Canada).

Assay validation

The newly developed assays were validated for the following assay specific properties; limit of detection (LOD), lower limit of quantification (LLOQ), linearity, accuracy, precision and stability of LPV and RTV in DBS/plasma matrix using the FDA's Guidance for Industry on Bio-analytical method validation procedure [26]. Assay precision, accuracy and the stability of the analytes were determined from quality control samples (QC) prepared in drug free whole blood and drug free plasma by spiking LPV and RTV at three different concentrations of 50, 10 and 1.0 µmol/L for DBS analysis and 4.0, 2.0 and 1.0 µmol/L for plasma analysis, respectively.

Children cohort study and sample preparation

Nineteen HIV-1 infected children (n=19) were included into the cohort patient study during a two month period. Average age of the participants was 10.2±4.0 years. Whole blood samples from participants were collected at the outpatient clinic of the Pediatrics department at the Erasmus MC-Sophia Children Hospital (Rotterdam, the Netherlands). Patient blood samples were collected in compliance with the Helsinki. The whole blood samples from sixteen of the nineteen participants were collected at approximately the same time after oral intake of Kaletra® (mean collection time of 15.6 ± 0.7 h). From the three remaining patients whole blood samples were collected at 4.0, 18.25, and 20.50 h, respectively. Whole blood samples were drawn by vena-puncture and collected into BD Vacutainer EDTA tubes

(Becton Dickinson, Breda, the Netherlands). For the DBS assay 4 x 25 µL of collected whole blood was blotted onto a DBS collection card followed by overnight drying of the DBS collection card at ambient temperature. After the DBS collection cards were dried, they were stored in a plastic sealable bag (Minigrip, Lelystad, the Netherlands) at a temperature of 4°C. Plasma was prepared from an aliquot of collected EDTA whole blood by centrifugation for 6 min (400 x *g*, 4°C). Plasma was separated from the red blood cell pellet transferred into a separate vial and directly stored in a freezer at a temperature of -80°C.

Results

Assay development

The DBS assay was developed by spiking whole blood collected from healthy donors with LPV, RTV and internal standard NFV. First, extraction of the PI from the DBS matrix was optimized by blotting aliquots of 25 µL of spiked whole blood (20 µmol/L LPV and RTV) on DBS collection cards. After drying, the DBS were punched out and extracted with different organic solvent/water mixtures. Combinations of methanol/water (MeOH/H_2O) mixtures ranged from MeOH/H_2O 10:90 (v/v) to MeOH/H_2O 90:10 (v/v) and the same procedure was used for acetonitrile/water (ACN/H_2O) solvent mixtures to determine optimal conditions for PI measurement in organic/water solutions. The best extraction results for LPV, RTV and NFV were obtained with ACN/H_2O 80:20 (v/v) solvent mixtures. It was observed that extracts with higher water content (>20% v/v) were colored slightly too deep red/brown. These extracts showed severe ion suppression during MALDI ionization which is probably due to extracted (oxidized) hemoglobin. Moreover, more profound ion suppression was observed with MeOH/H_2O mixtures and less with ACN/H_2O mixtures. Ion suppression effects decreased significantly for ACN/H_2O with higher ACN content (> 40% v/v). Subsequently, we studied the maximum amount of whole blood that could be blotted onto the DBS collection cards without overloading the spot and causing it to spread. Volumes of 5, 15, 25, and 40 uL of spiked whole blood were blotted onto DBS collection cards. The 40 uL volume resulted in a spot that had a larger diameter than the maximum allowed by the DBS (marked by a printed circle), thus adjacent spots ran into each other. The optimal whole blood amount that could be blotted was

25 µL, covering approx. 90 % of the allowed surface of printed circles. Smaller whole blood amounts were also tested but these resulted in significantly higher detection limits. After determination of the optimal amount of whole blood, extraction volume and extraction time of DBS were studied by the application of 50, 100, 250 and 500 µL of ACN/H_2O 80:20 (v/v) solvent mixture and extraction times of 15, 30, 45 and 60 min using sonication. The optimal DBS extraction volume and extraction time were observed for 250 µL of ACN/H_2O 80:20 (v/v) mixture and extraction time of 30 min. The plasma assay was developed by the application of spiked plasma prepared from the drug free whole blood that was also used for the DBS assay development. To this end, plasma was spiked with LPV, RTV and NFV at a concentration level of 1 µmol/L.

Deproteinization of plasma was studied by the application of different deproteinization agents. Studied deproteinization agents were ACN, acetone (ACT) and MeOH. Aliquots of 10 µL of spiked plasma were deproteinized by the addition of 90 µL of ACN, ACT or MeOH and mixed on a vortex mixer for 1 min. Precipitated proteins were removed by centrifugation for 10 min (2000 x *g*) at room temperature and supernatants were mixed with MALDI matrix and spotted. The highest recoveries were observed for ACT deproteinization and were 93.4%, 93.2% and 98.5% for LPV, RTV and NFV, respectively. Recovery rates for LPV, RTV and NFV using acetonitrile and methanol were approx. 80% and 13%, respectively.Furthermore, the amount of ACT used for deproteinization was optimized and following amounts of ACT were tested on their deproteinization capacities; 40, 60, 80, 90 and 100 µL for the deproteinization of 10 µL plasma. Observed recovery rates for LPV were 36.8, 59.7, 114.0, 93.8 and 85.4%, for RTV 29.6, 64.6, 111.3, 92.3 and 77.6% and for NFV 28.4, 69.9, 108.5, 98.5 and 86.7%, respectively. Further deproteinization of plasma samples was performed by deproteinization of 10 µL of plasma sample with 90 µL of acetone.

Assay validation

Linearity of DBS and plasma assays

Selected range of whole blood and plasma based calibrators indicated the existence of linear relationships between the ratio of the peak areas of LPV/NFV ratio and RTV/NFV ratio (y) and concentration (x) of LPV and RTV in DBS and plasma,

respectively. All calibration curves showed linear relationships between the analyte peak areas and analyte concentration. For the plasma assay the calibration curves were linear between 0.05 and 4.0 µmol/L and between 0.25 and 50 µmol/L for the DBS assay, respectively. The linear regression analyses were calculated using GraphPad Prism software version 5.00 for windows (GraphPad Software, San Diego, USA). The regression equations of the DBS assay were y= 0.022x-0.0069 (LPV) and y= 0.0165x-0.0096 (RTV) and regression coefficients were 0.9941 and 0.9948 for LPV and RTV, respectively. The calibration curve regression equation and regression coefficients of the plasma assay were y= 4.5131x-0.0208 (LPV) and y= 30.0478x-0.1904 (RTV) and regression coefficients were 0.9932 and 0.9965 for LPV and RTV, respectively.

LLOQ and LOD of DBS and plasma assay

The lower limit of quantification (LLOQ) was defined as the lowest matrix-based calibrator of the calibration curve that could be measured with acceptable accuracy and precision (<20% error) in compliance with FDA regulations. The LLOQ of the DBS assay was 0.25 µmol/L for LPV as well as RTV and 0.05 µmol/L for both analytes for the plasma assay. The upper limits of quantification (ULOQ) were defined as the highest matrix based calibrator measured and was 200 µmol/L for LPV and RTV for the DBS assay and 4.0 µmol/L for the plasma assay, respectively.

The limits of detection (LOD), defined as three times the signal-to-noise ratio of drug free whole blood/plasma were 0.039 µmol/L (LPV) and 0.058 µmol/L (RTV) for the DBS assay and 0.0017 µmol/L (LPV) and 0.0093 µmol/L (RTV) for the plasma assay, respectively.

Within-and between run accuracy and precision

The accuracy and precision of the DBS and plasma assay for LPV and RTV were in good compliance with the FDA regulations (<15/20 %error and %CV). Observed accuracy and precision results at three studied concentration levels are listed in Table 1.

Stability of LPV and RTV

LPV and RTV were considered stable in the DBS and plasma matrix if during conservation of the quality control samples the decrease in concentration was <15% (expressed as %error). The observed stability of LPV and RTV in both biological matrices was <15% error for studied concentration levels for storage in a refrigerator (24h, 4°C), 20 days in a freezer (-20°C) and during three freeze-thaw cycles (Table 2 and 3).

Cross validation of plasma assay with HPLC-UV assay

Plasma LPV and RTV concentrations observed by the newly developed MALDI-QqQ-MS/MS plasma assay were cross validated with an HPLC-UV method as described previously [27]. HPLC-UV assay determinations of plasma LPV and RTV concentrations of participants from the cohort study were performed at the Dept. Clinical Pharmacy (Radboud University Nijmegen Medical Centre, the Netherlands). For cross-validation purposes, plasma samples were separately prepared for analysis at both laboratories and standard solutions were not exchanged between the laboratories.

Discussion

Currently, several papers describe the determination of PI concentrations and other antiretroviral drugs in plasma from HIV-1 infected patients. All papers applied HPLC or LC assays in combination with UV or mass spectrometric detection for the determination of drug concentrations. Moreover, the average analysis time of applied assays ranged between 5 minutes to approx. 20 minutes per sample depending on instrumentation and separation column used. Previously, we demonstrated that the measurement of intracellular concentrations of LPV and RTV in lysates of peripheral blood mononuclear cells (PBMC) was not only very sensitive (femto-mol amounts) but as ultrafast and high-throughput [18] compared with the LC-MS/MS assay published by Pelerine et al. [11] where an analysis time of 20 min was reported. The MALDI-QqQ-MS/MS assay analysis time for one sample was <15 sec which means that a full 96-well MALDI target plate could be analyzed within 16 to 17 minutes analysis time.

For the measurement of LPV and RTV concentrations in plasma and DBS two new bio-analytical assays were developed and validated. In the past we already applied the MALDI-QqQ-MS/MS for the analysis of LPV and RTV in plasma samples but this method was not validated in compliance with the FDA regulations [18]. A cross validation of the DBS assay was not possible because no HPLC-UV assay applying DBS samples was available but for the validation of the plasma assay an accredited HPLC-UV was used. The DBS and plasma assays were developed using quality control samples and both assays were validated in full compliance with the FDA regulations [25]. NFV was selected as internal standard because it was in the past successfully used in the assay for the intracellular PBMC concentrations for LPV and RTV [18]. Observed LOD of the DBS assay for LPV and RTV were 0.039 and 0.058 µmol/L respectively. Observed LODs were higher (2.8 times (LPV) and 3.6 times higher (RTV)) than reported LODs by Koal and coworkers [7]. Reported work by Koal et al. [7] injected 3.125 µL whole blood (calculated from extracted amount whole blood) from extracted 5 µL whole blood into the LC-MS instrumentation this in contrast to the MALDI-QqQ-MS/MS assay where 0.05 µL of the 25 µL DBS were used for analysis.

Moreover, the LODs of the MALDI-QqQ-MS/MS plasma assay were 0.0017 and 0.0093 µmol/L for LPV and RTV respectively, comparable with LODs published by Koal et al. [7] but they applied a total sample amount of 1.67 µL of plasma for the LC-MS/MS analysis and the MALDI-QqQ-MS/MS assay just 0.05 µL. By the calculation of total injected sample amounts of 0.05 µL of sample for as well DBS as plasma assay compared to the amounts used in the reported LC-MS/MS assay [7] it could be concluded that both MALDI-QqQ-MS/MS assays were approximately 30 times more sensitive. Besides the higher sensitivity, less sample amount is needed. Even the more significant advantage of the application of the MALDI-QqQ-MS/MS technology are the ultrafast and high-throughput characteristics of this technology.
Although LODs of the MALDI-QqQ-MS/MS assays are higher than for example LC-MS/MS, MALDI-QqQ-MS/MS is sensitive enough to be applied in the clinical practice.

In very short time TDM of DBS and plasma concentrations from HIV-1 infected children could be applied to verify if clinical relevant systemic concentrations were present. Almost all patients had therapeutically relevant concentrations of LPV in collected plasma and via DBS collected whole blood samples. Expected

therapeutically relevant concentrations had to range between 12 and 20 µmol/L. This relative broad range relates to if the HIV-1 child was native to Kaletra® or already previously treated with Kaletra® [28]. The assays described here were validated by the determination of linearity, recovery rates, within- and between run accuracy and precision and stability of LPV and RTV in both DBS and plasma matrix. The within- and between-run accuracy and precision as well as stability were all in compliance with FDA regulation for validation of (new) bio-analytical assays (Table 1 until Table 3).

After full validation of both assays, real patient samples from a cohort study were measured. Patients DBS samples were extracted and analyzed by the MALDI-QqQ-MS/MS DBS assay and corresponding plasma samples were prepared and analyzed in duplicate. Patients plasma samples (n=19) were prepared according to the optimal conditions determined. Samples (1 µL) were spotted in fivefold onto the MALDI target plate. Patient's DBS were punched out in duplicate, extracted and spotted in fivefold onto the MALDI target plate. Comparison of observed plasma concentrations and DBS concentrations of LPV and RTV showed a significant correlation. The correlation between patient's plasma concentrations and DBS concentrations of LPV and RTV are illustrated in Fig. 2.

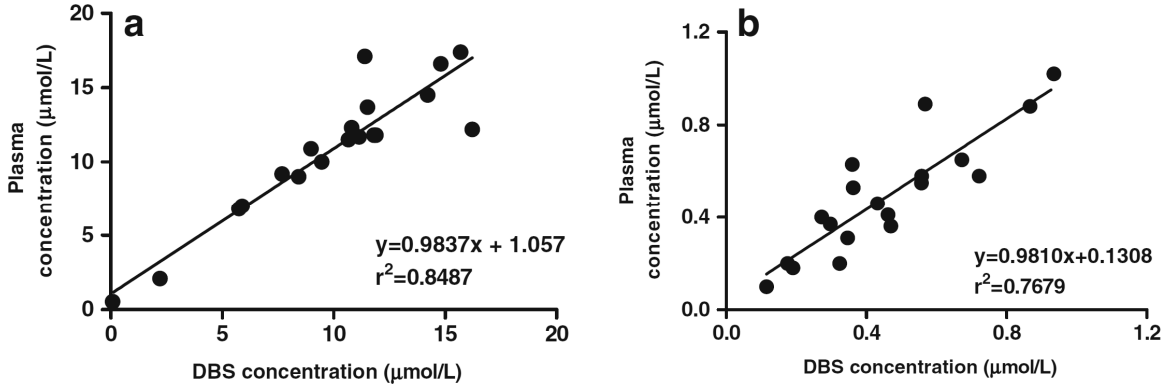

Figure 2. Comparison of drug levels in plasma and DBS samples from HIV-1 infected children; (a) LPV and (b) RTV

Observed plasma concentrations of LPV and RTV by the MALDI-QqQ-MS/MS plasma assay were also cross-validated with measured plasma concentrations by a validated HPLC-UV [27] at an accredited laboratory of the dept. Clinical Pharmacy

(Radboud University Nijmegen Medical Centre, the Netherlands) by calculating the bias between both methods according to Bland-Altman method comparison statistics [29, 30]. The differences for LPV and RTV concentrations observed by both assays are expressed as a Bland-Altman plot and are illustrated in Figure 3 and the linear regression between observed LPV and RTV concentrations patient's plasma samples of the HPLC-UV- and MALDI-QqQ-MS/MS assay are also presented.

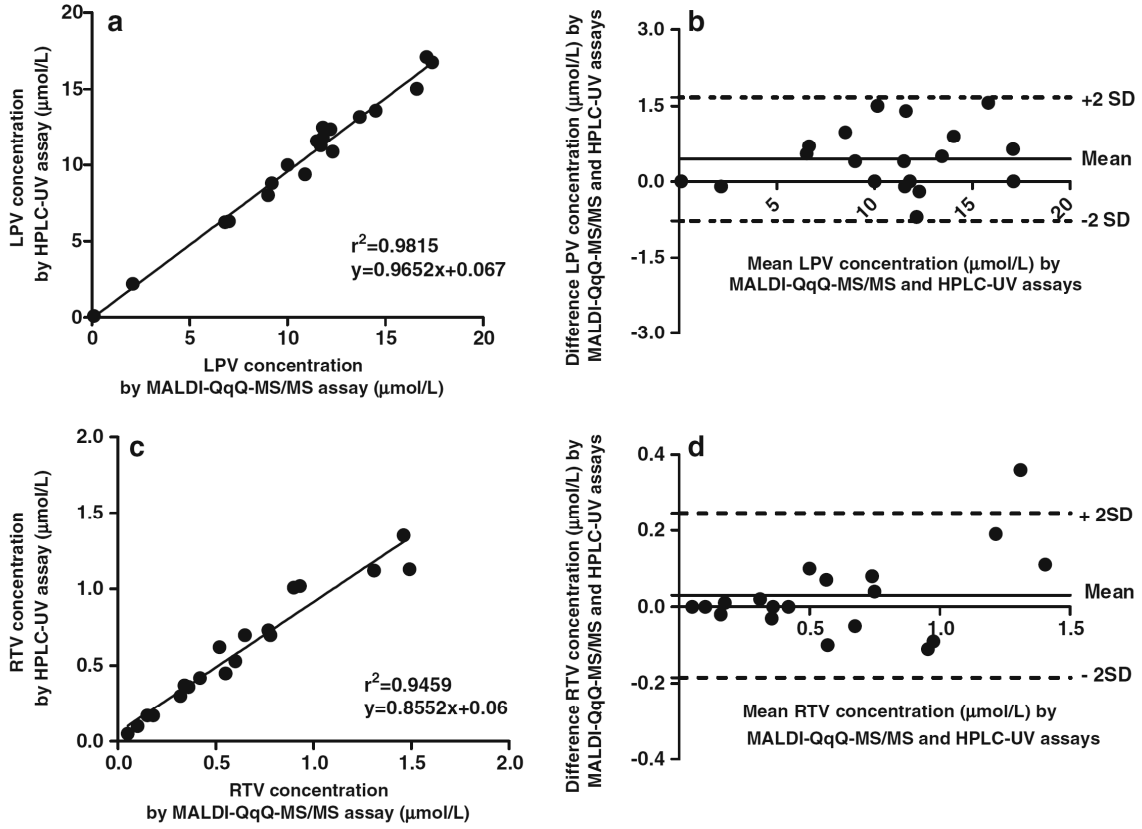

Figure 3. (a) Comparison of observed LPV concentrations by HPLC-UV assay and new developed MALDI-QqQ-MS/MS assay, (b) Bland-Altman plot of differences between observed LPV concentrations by MALDI-QqQ-MS/MS and HPLC-UV assay, (c) Comparison of observed RTV concentrations by HPLC-UV assay and the new MALDI-QqQ-MS/MS assay, (d) Bland-Altman plot of differences between observed RTV concentrations by MALDI-QqQ-MS/MS and HPLC-UV assay. Patients (n=19) LPV and RTV plasma concentrations were measured by the newly developed MALDI-QqQ-MS/MS plasma assay and cross validated with an accredited HPLC-UV assay. Solid line (—) represents mean and dotted lines represent mean ± 2 SD (standard deviation of mean).

Table 1. Precision and accuracy of the MALDI-QqQ-MS/MS assay at three DBS and plasma concentration levels

	Dried blood spots (DBS)						Plasma					
Analyte	Within-run validation[a]						Within-run validation[a]					
Nominal concentration (μM)	50.0		10.0		1.0		4.0		2.0		1.0	
	LPV	RTV	LPV	RTV	LPV	RTV	LPV	RTV	LPV	RTV	LPV	RTV
Mean observed concentration (μM)	49.9	50.7	10.7	10.6	0.9	0.9	3.7	4.0	1.9	2.1	1.1	1.2
Accuracy (%error[c])	-0.3	1.4	7.2	5.6	-8.1	-10.8	-8.6	0.5	-3.7	5.2	8.8	12.4
Precision (%CV)	4.7	6.4	11.7	11.9	16.5	12.8	7.5	12.5	10.3	13.4	11.2	8.8
	Between-run validation[b]						Between-run validation[b]					
Nominal concentration (μM)	50.0		10.0		1.0		4.0		2.0		1.0	
	LPV	RTV	LPV	RTV	LPV	RTV	LPV	RTV	LPV	RTV	LPV	RTV
Mean observed concentration (μM)	51.2	49.4	10.9	9.6	1.2	1.0	3.7	4.2	2.0	2.2	1.1	1.1
Accuracy (%error[c])	2.4	-1.2	9.0	-4.3	10.1	1.0	-7.1	3.8	1.5	10.8	8.4	13.7
Precision (%CV)	11.4	9.8	12.2	11.3	18.2	18.3	12.0	14.6	13.2	12.4	12.1	11.0

[a]within-run results summarize 10 spots per QC sample at each concentration level in one experiment,
[b]between-run results summarize three different experiments from 3 consecutive days with 10 spots per QC sample at each concentration level,
[c]% error = (mean observed concentration-nominal concentration)/(nominal concentration)* 100%

Table 2. Stability experiments with DBS-QC samples containing LPV and RTV under different storage conditions [a] results summarize 10 spots per QC

Nominal conc. (µM)	Storage Condition				Storage Time			
	Refrigerator (4°C, 24h)[a]				Dessicator (20°C, 20 days)[a]			
	LPV		RTV		LPV		RTV	
	Mean (SD)	%error[b]	Mean (SD)	%error[b]	Mean (SD)	%error[b]	Mean (SD)	%error[b]
50.0	49.9 (6.1)	-0.2	48.5 (4.0)	-3.0	49.7 (3.3)	-0.6	49.2 (3.9)	-1.6
10.0	10.5 (0.9)	5.0	10.4 (1.4)	4.0	9.4 (1.1)	-6.0	10.1 (0.7)	1.0
1.0	1.1 (0.1)	10.0	0.9 (0.1)	-10.0	0.93 (0.15)	-7.0	1.1 (0.2)	10.0

[a] results summarize 10 spots per QC sample at each concentration level in one experiment,
[b]% error = (mean observed concentration-nominal concentration)/(nominal concentration)* 100%

Table 3. Stability experiments with plasma QC samples containing LPV and RTV under different storage conditions

Nominal conc.(μM)	Storage Conditions/Time											
	Refrigerator (4°C, 24 h)[a]				Freezer (-20°C, 20 days)[a]				Freezer (-20°C, 3 freeze-thaw cycles)[a]			
	LPV		RTV		LPV		RTV		LPV		RTV	
	Mean (SD)	% error[b]	Mean (SD)	% error[b]	Mean (SD)	% error[b]	Mean (SD)	% error[b]	Mean (SD)	% error[b]	Mean (SD)	% error[b]
4.0	3.81 (0.05)	-4.8	3.92 (0.07)	-2.0	3.90 (0.36)	-2.5	3.79 (0.27)	-5.3	3.78 (0.53)	-5.5	3.76 (0.51)	-6.0
2.0	1.88 (0.12)	-6.0	1.86 (0.09)	-7.0	1.86 (0.38)	-7.0	1.75 (0.12)	-12.5	1.94 (0.35)	-3.0	1.97 (0.24)	-1.5
1.0	0.94 (0.24)	-6.0	1.07 (0.20)	7.0	0.95 (0.11)	-5.0	0.87 (0.05)	12.0	0.93 (0.16)	-7.0	0.94 (0.14)	-6.0

[a] results summarize 10 spots per QC sample at each concentration level in one experiment,
[b]% error = (mean observed concentration-nominal concentration)/(nominal concentration)* 100 %

All measured differences in concentrations for LPV and RTV for both analytical assays were between the mean of both assays ± 2 SD (95% confidence), except for one patient's sample (RTV) (Fig 3D). Both LPV and RTV concentrations were statistically compared, however from clinical point of view, the protease inhibitor LPV is more important than RTV because LPV is the active drug responsible for inhibition of the viral HIV-1 protease. RTV, as used in the concentration in the co-formulated Kaletra medication acts as a kind of pharmacokinetic booster of LPV preventing the metabolic degradation by drug-metabolizing CYP3A isozymes. In the clinical practice, the LPV concentration is important for TDM and for determination of the patient's compliance with the drug regimen. The MALDI-QqQ-MS/MS plasma assay shows a slightly positive bias compared to the HPLC-UV assay (Fig. 3B) while almost no bias is observed with RTV (Fig. 3D) analysis. For the observed positive bias of the LPV determination no explanation could be given.

In conclusion, the newly developed MALDI-QqQ-MS/MS assays described here can be used for the sensitive and ultrafast determination of the antiretroviral drugs LPV and RTV in plasma and in DBS samples from HIV-1 infected children. The assays showed significant correlations between observed LPV and RTV concentrations in whole blood (DBS) and in plasma. The MALDI-QqQ-MS/MS plasma assay was successfully cross-validated with an accredited HPLC-UV assay. The newly developed MALDI-QqQ-MS/MS DBS assay as well the MALDI-QqQ-MS/MS plasma assay can therefore be used for TDM and for monitoring of the patient's compliance to the prescribed drug regimen. The easy handling to obtain DBS and the possibility to store the DBS collection cards for prolonged periods should make this sampling technique suitable for obtaining whole blood samples from HIV-1 infected children in remote areas in developing countries. We expect that DBS can be used in general for quantification of metabolites, drugs and nutrients by MALDI-QqQ mass spectrometry.

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Determination of the antiretroviral drug tenofovir in plasma from HIV-infected adults by ultrafast isotope dilution MALDI-triple quadrupole tandem mass spectrometry

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Abstract

A new and reliable mass spectrometric method using an isotope dilution method in combination with Matrix-Assisted Laser Desorption/Ionization-triple quadrupole tandem mass spectrometry (ID-MALDI-QqQ-MS/MS) has been developed and validated for the determination of concentrations of the antiretroviral drug tenofovir (TNV) in plasma from HIV infected adults. The advantage of this new method is that (1) the method is ultrafast and (2) can be applied for high-throughput measurement of TNV in plasma. The method is based on a simple plasma deproteinization step in combination with the use of [adenine-$^{13}C_5$]-TNV as internal standard. TNV and [adenine-$^{13}C_5$]-TNV were monitored by multiple reaction monitoring using the transition *m/z* 288.0 → *m/z* 176.2 and *m/z* 293.2 → *m/z* 181.2 for TNV and [adenine-$^{13}C_5$]-TNV, respectively. The method was validated according to the most recent FDA guidelines for the development and validation of (new) bio-analytical assays. Validated method parameters were: linearity, accuracy, precision and stability of the method. The lowest limit of quantification (LLOQ) was 0.10 µmol/L while the limit of detection (LOD) determined at a signal-to-noise ratio (S/N=3:1) in pooled drug free human control plasma was 0.04 µmol/L. The validated method was successfully applied and tested for its clinical feasibility by the analysis of plasma samples from selected HIV infected adults receiving the pro-drug tenofovir disoproxil fumarate (TDF). Observed plasma TNV concentrations ranged between 0.11 and 0.76 µmol/L and measured plasma TNV concentrations were within the therapeutically relevant concentration range.

Introduction

TNV is an antiretroviral drug from the class of nucleotide reverse transcriptase inhibitors (NRTI). TNV can be applied together with other antiretroviral drugs (e.g. protease inhibitors) in the combined antiretroviral therapy (cART). Since1995–1996, cARTis successfully used in the treatment of the HIV infection [1]. TNV is administered orally as the prodrug tenofovir disoproxil fumarate (TDF; 9-(R)-(2-phosphonomethoxypropyl)-adenine) and TDF is, after absorption, rapidly converted into TNV by enzymatic hydrolysis followed by further intracellular metabolic conversion by kinase enzymes into TNV-diphosphate (TNV-DP) [2]. TNV-DP is actually the active antiretroviral drug; it acts as a competitive inhibitor of the viral HIV reverse transcriptase enzyme (HIV-RT). TNV-DP is inserted into the viral DNA chain and then blocks the HIV-RT enzyme in the elongation of the viral DNA chain leading to the termination of HIV viral DNA growth [3]. Patient's adherence to cART is very important to avoid the development of HIV resistance and consequent therapy failure.

One way to monitor the adherence of the patient is using therapeutic drug monitoring (TDM) by frequent determination of plasma concentrations of prescribed antiretroviral drugs on defined moments during treatment. More importantly, TDM can be used to optimize drug dosing for each individual and can serve to improve therapeutic efficacy. Currently, plasma TDM of TNV concentrations from HIV-infected patients are determined by pre-column derivatization of TNV with chloroacetaldehyde and measurement of the derivative by high-performance liquid chromatography (HPLC) with UV [4,5] or fluorescence detection [6,7] although liquid chromatography–tandem mass spectrometry (LC–MS/MS) [8,9] of the free TNV is also applied. Quantitative mass spectrometric analyses using MALDI sources in combination with mass spectrometers are not frequently applied within the mass spectrometry community. MALDI sources are general combined with time-of-flight (TOF) mass spectrometers and in the past, it has been shown that MALDI-TOF instrumentation has very limited use for quantitative purposes because coefficient of variance (CV) of multiple measurements was relative high (>15% CV). This high CV hampered a good quantitative determination of analytes especially when analyte concentrations were low and had to be measured in biological matrices (e.g. plasma, serum, urine and

cerebrospinal fluid) [10]. Since the introduction of a mass spectrometry platform which consists of MALDI source with a high repletion solid-state UV laser (1 kHz, 349 nm) in combination with a triple quadrupole mass spectrometer (MALDI-QqQ-MS) few articles have been reported demonstrating that this new technology can be applied for high accurate and precise determinations of concentrations of small molecules [11] with CVs <15/20%, according to the most recent FDA guidelines [12]. Therefore, we decided to use this new technology and to study its feasibility for high-throughput and ultrafast measurement of plasma concentrations of the antiretroviral drug TNV in plasma from HIV adults using an isotope dilution method. MALDI-QqQ-MS is a relative new mass spectrometric technology which does not necessarily require liquid chromatographic separation of samples prior to mass spectrometric analysis. Omitting liquid chromatographic separation reduces analysis time considerable and analysis time of approximately 10 s per sample [13] or less is possible. Due to these short analysis times, this new technology is (perfectly) suitable for ultrafast and high-throughput mass spectrometric analysis. We demonstrated previously that MALDI-QqQ-MS technology has a great usability and is a versatile tool in the determination of concentrations (extra- and intracellular) of small molecules such as antiretroviral drugs (protease inhibitors) [14,15], anticancer drugs [13] but also other types of drugs [16–18] and as screening tool in enzyme kinetic studies [19]. In a study by Volmer et al. [20] the MALDI-QqQ-MS technology demonstrated equal performances compared with LC–QqQ-MS/MS instrumentation applying electrospray ionization (ESI) as ionization source when applied in pharmacokinetic studies.

Previously, Notari et al. [21,22] demonstrated that MALDI-TOF could be applied for the determination of low concentrations of antiretroviral drugs in plasma samples although concentrations of the antiretroviral drugs were not determined by isotope dilution mass spectrometry but by means of internal standard quantification. We report to our best knowledge the first application of the MALDI-QqQ-MS technology in combination with an isotope dilution method used for TDM of TNV concentrations in plasma from HIV-infected adults with good therapeutically responses on cART with TDF. The new developed ID-MALDI-QqQ-MS method was validated according to recent FDA guidelines for bio-analytical method development

[12] and applied to determine TNV plasma concentrations in samples from HIV-infected adults.

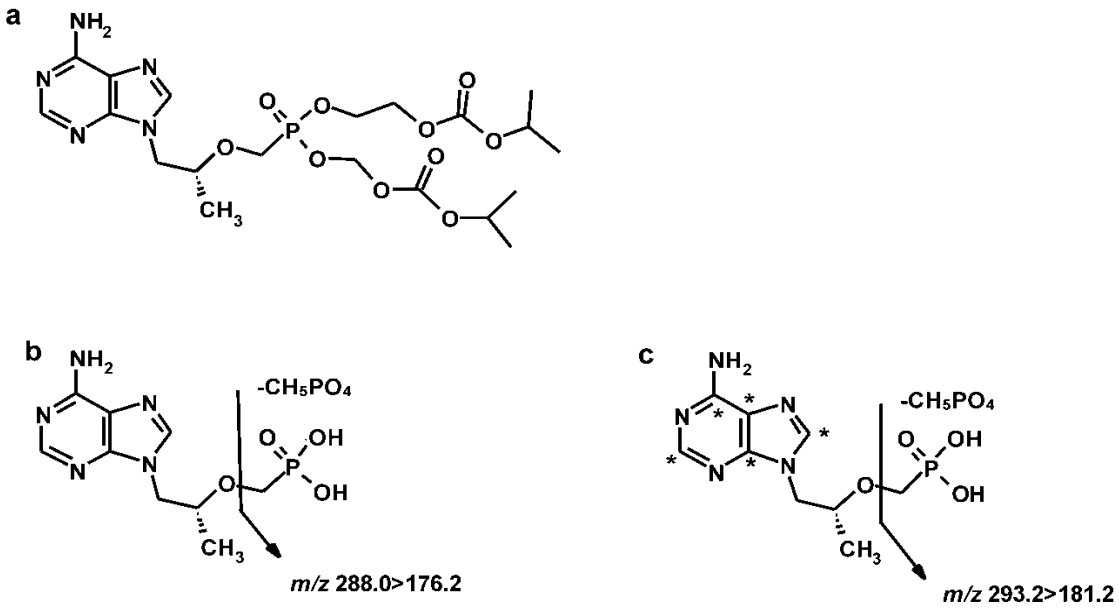

Figure 1. Structural formulas of (a) tenofovir disoproxil fumarate (b) tenofovir and (c) [adenine-$^{13}C_5$]-tenofovir and MRM fragmentation reactions, (*) depicts the position of the ^{13}C-labeling.

Experimental

Chemicals

TNV and the internal standard [adenine-$^{13}C_5$]-TNV (chemical purity 99.8%) were both purchased from Moravek (Brea, CA, USA). Primary stock solutions of TNV and [adenine-$^{13}C_5$]-TNV were prepared in water (500 μmol/l). All used solvents were obtained from commercial sources and were of LC–MS grade (Biosolve, Valkenswaard, The Netherlands), other chemicals were of ACS grade purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Chemical structures of the prodrug TDF, TNV and the [adenine-$^{13}C_5$]-TNV internal standard are presented in Fig. 1.

Determination of MALDI ionization efficacy

The influence of the MALDI matrix on the ionization efficacy of TNV was determined for the few frequently used MALDI matrices in MALDI mass

spectrometry. Tested were: 2,5-dihydroxy benzoic acid (2,5-DHB), the novel matrix compound 7-hydroxy-4-(trifluoromethyl)-coumarin (HFMC) [23], super-DHB (SDHB; mixture of 2,5-DHB and 5-methoxysalicylic acid), 9-amino acridine (9-AA) and α-cyano-hydroxy-cinnamic acid solution (α-CHCA). The MALDI matrices 2,5-DHB and SDHB were used at a concentration of 30 mg/ml, HFMC and 9-AA at 10 mg/ml and α-CHCA at 6.2 mg/ml, respectively. Ionization efficacy was determined by measurement of the total counts per second (CPS) signal for TNV in full-scan mode. For that purpose, 20 µl of a TNV stock solution (10 µmol/l TNV in 50% (v/v) methanol/water) were mixed at a ratio of (1:1) with the various MALDI matrix solutions and subsequently spots of 1 µl were measured. The positively charged ion of TNV (*m/z* 288.0) was detected using full scan mode (*m/z* = 250–350) at a scan time of 1s and step size of 0.2 Da (MS settings, see MALDI-QqQ-MS/MS Conditions Section).

MALDI-QqQ-MS/MS conditions

The MALDI-QqQ-MS/MS instrumentation used was a FlashQuant Workstation containing a FlashLaser source (349 nm, 1kHz) combined with a 4000 API mass analyzer (AB Sciex, Concord, Canada) operating in the positive ionization mode. Analyses were carried out by multiple reaction monitoring (MRM) at unit resolution. In MRM mode, monitored ion transitions were: *m/z* 288.0→176.2 for TNV and *m/z* 293.2→181.2 for [adenine-$^{13}C_5$]-TNV, respectively. Selected MRM corresponds to the $[M+H]^+ \rightarrow [M+H-CH_5PO_4]^+$-ion transitions and these fragment ions had the highest intensities of all ions observed in the product scan of the $[M+H]^+$ ions (Fig. 1). Optimized MALDI-QqQ-MS/MS instrument parameters used were: laser power 60%, target plate voltage 100 V, skimmer voltage 0 V, CAD gas 12 arbitrary units (collision gas), collision cell energy exit potential (CXP) 3V, source gas 20 arbitrary units, dwell time 10 ms and laser raster speed was set to 1 mm/s. Instrument control and data acquisition were performed by Flashquant 1.0 software and Analyst 1.4.2 application software (AB Sciex).

Calibration curve of TNV

Plasma-based calibrators at different TNV concentrations were used to determine the linear concentration range of the method. The calibrators were prepared in pooled drug-free control plasma from healthy controls (Sanquin Blood Supply Foundation, Rotterdam, The Netherlands) containing potassium-EDTA as anti-coagulant by serial dilution of a 100 µmol/l TNV plasma stock standard solution with drug-free control plasma to yield the following calibrators: 10, 5, 2.5, 1, 0.5, 0.25, 0.1 and 0.0 µmol/l (control plasma). Three aliquots (25µl) from each calibrator were spiked with 2.5 µl of an [adenine-$^{13}C_5$]-TNV solution (2.5 µmol/l) and deproteinized by the addition of 2.5µl of a trichloroacetic acid solution (TCA, 50% (w/v)). Precipitated proteins were removed by centrifugation (5 min at 2000 x *g*) and 10µl of the supernatant was diluted (1:10) with a methanol/water mixture (50:50% (v/v)) and was mixed (1:1) with MALDI matrix solution (α-cyano-4-hydroxycinnamic acid; 6.2 mg/ml in methanol/acetonitrile/water 36:56:8 (v/v/v), pH 2.5; α-CHCA) and 1 µl of each aliquot was spotted (n=3) onto a stainless steel MALDI target plate (123 mm × 81 mm; AB Sciex). Sample spots were let to crystallize for 5 min at ambient temperature. The regression equation of the calibration curve and regression coefficients were calculated by GraphPad Prism software version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Assay validation

The developed ID-MALDI-QqQ-MS/MS assay was validated according to recent FDA guidance for industry-bioanalytical method validation on following assay-specific parameters [12]; linearity, limit of detection (LOD), lower limit of quantification (LLOQ), within-run and between run accuracy and precision and stability of TNV in plasma. Within- and between-day precision and accuracy and the stability of TNV were determined using plasma-based quality control (QC) samples prepared in pooled drug-free plasma from healthy controls at concentration validation levels of 10, 2.5 and 0.25 µmol/l and spiked with [adenine-$^{13}C_5$]-TNV. Three replicates were analyzed at each QC concentration level for three different runs in order to access the within-run and between-run accuracy and precision. The stability of TNV in the plasma matrix was determined at different storage conditions also at three QC

concentration levels. Recovery for TNV was evaluated by comparing the peak areas of QC samples prepared through proposed method and untreated calibrators at the same QC concentration validation levels spiked with [adenine-$^{13}C_5$]-TNV. The influence of background signals on the TNV measurement by isotope dilution were studied by the measurement of 20 individual control plasma samples collected from 20 different healthy controls. For this purpose, the plasma samples were first measured on their background signals for TNV and [adenine-$^{13}C_5$]-TNV and then subsequently spiked with TNV and [adenine-$^{13}C_5$]-TNV both at a concentration of 0.25 μmol/l and reanalyzed for the determination of the precision.

Sample analysis protocol

Whole blood samples (anticoagulant potassium-EDTA) from HIV-infected participants receiving TDF were collected at the outpatient clinic of the Internal Medicine Department at the Erasmus University Medical Center Rotterdam. Patient blood samples were collected in compliance with the Helsinki regulations and patients gave written consent (MEC protocol 2007-274). In the time period between September 2009 and February 2010, whole blood samples from 20 selected patients were included in the present study. The average age (SD) of the patients was 47.3 years (8.5). Twenty-five-microliter aliquots (n=3) of patient plasma were spiked with 2.5 μl of an [adenine-$^{13}C_5$]-TNV solution (2.5 μmol/L) and prepared for ID-MALDI-QqQ-MS/MS analysis as described above in the previous section.

Determination of the accuracy of the isotopic ratio measurement

For the determination of TNV plasma concentrations by the measurement of the isotopic ratios between spiked [adenine-$^{13}C_5$]-TNV and the actual TNV concentration present in the patient plasma, it is from utmost importance that the isotopic ratio is measured with a high accuracy. To determine this accuracy in compliance with FDA guidelines accepted levels (<15% error at higher isotopic ratios; <20 %error at lower isotopic ratios), isotopic ratio calibrators with defined isotopic ratios at different atom percent excess ratio's (APE) were used. Calibrators were prepared by the addition of different amounts of [adenine-$^{13}C_5$]-TNV (10 μmol/l in pooled drug-free control plasma) to a 10 μmol/l TNV solution also in drug-free control

plasma. The procedure for the preparation of the isotopic ratio calibrators was as follows; 100 µl aliquots of TNV spiked control plasma were mixed with 100, 50, 25, 10, 5, 2.5 and 1 µl of [adenine-$^{13}C_5$]-TNV. The natural (m+5) isotopic ratio of TNV was determined from a 10-µmol/l TNV in control plasma without the addition of [adenine-$^{13}C_5$]-TNV. From prepared isotopic ratio calibrators, three aliquots of 25 µl were deproteinized by 2.5 µl of TCA, deproteinized calibrators were centrifuged and supernatants were diluted (1:10) with methanol/water (50:50%, v/v) mixture and mixed (1:1) with MALDI solution and isotopic ratios were measured by ID-MALDI-QqQ-MS/MS. Applied APE calibrators (calculation by Formula 1) for the accuracy measurements were 99.01%, 98.04%, 96.15%, 90.91%, 83.33%, 71.43%, 50.0% and 0% APE. For the calculation of the accuracy of the isotopic ratio measurement, observed APE ratios of calibrators were compared with calculated APE ratios. For calculation of the accuracy of the isotopic ratio measurement observed APE ratios of calibrators were compared with calculated APE ratios.

$$APE = \frac{AR_{corrected}}{1 + AR_{corrected}} \times 100\% \quad \text{(formula 1)}$$

Where $AR_{corrected}$ is the measured isotopic ratio between [adenine-$^{13}C_5$]-TNV and TNV (corrected for the (m+5) natural isotopic ratio of TNV).

Determination of TNV concentration in patient plasma by isotopic ratio measurement

By the measurement of the isotopic ratios between [adenine-$^{13}C_5$]-TNV and TNV to determine unknown TNV plasma concentration, the TNV concentrations of patient plasma samples were calculated from observed isotopic ratios using following formula:

$$C_{TNV} = \frac{V_{IS} \times C_{IS}}{AR_{std} \times V_{plasma}} \quad \text{(formula 2)}$$

where V_{IS} is the volume of [adenine-$^{13}C_5$]-TNV (µL), C_{IS} is the concentration of [adenine-$^{13}C_5$]-TNV (pmol/µL), AR_{std} is the isotopic ratio between [adenine-$^{13}C_5$]-TNV

and TNV (observed isotopic ratio is corrected for the natural (m+5) isotopic ratio of TNV) measured in the patient plasma sample and V_{plasma} the volume of plasma (µL) used for analyses.

Effect of triglycerides concentration on TNV measurements

HIV-infected patients receiving TNV or other antiretroviral drugs in combination with protease inhibitor drugs may develop hyperlipedemia during treatment [24]. In the present study, five patients received a combination of TNV in combination with protease inhibitors; lopinavir/ritonavir (n=2) or ritonavir/atazanavir (n=2) or atazanavir (n=1). The effect of the triglycerides concentration on the accuracy of the TNV measurement was studied by spiking ten plasma (potassium-EDTA) samples from HIV-negative controls in triplicate at a TNV concentration of 0.25 µmol/l. The triglyceride concentrations of the ten control plasma samples ranged between 3.06 and 5.33 mmol/l.

Results and Discussion

Method development

MALDI ionization efficacy

From tested MALDI matrix solutions, the α-CHCA matrix solution resulted in the highest total CPS; the ionization efficacy for α-CHCA was compared with other MALDI matrix solutions 20 times more efficiently than SDHB and even 500 times more effective than 9-AA (used in negative ionization mode). In the α-CHCA matrix, both analytes were protonated what resulted in the formation of $(M+H)^+$ ions; *m/z* = 288.0 (TNV) and *m/z* = 293.2 for [adenine-$^{13}C_5$]-TNV, respectively. These protonated molecular ions were subsequently used for the optimization of MALDI-QqQ- MS/MS instrument parameters such as: laser power, plate voltage, collision gas (CAD), source gas and collision energy (CE). Optimized MALDI-QqQ-MS/MS instrument parameters were CE 35V and collision cell energy exit potential (CXP) 10V for TNV and [adenine-$^{13}C_5$]-TNV, respectively. Collision-induced dissociation (CID) was applied for the determination of the MRM transitions of both protonated molecular

ions resulting in the formation of highly abundant protonated fragment ions. The fragment ion with the highest abundance was selected for MRM analyses, and the selected fragment ion was the result of ether bond cleavage within the TNV and [adenine-$^{13}C_5$]-TNV ion which resulted in the formation and loss of the neutral fragment CH_5PO_4 (Fig. 1(b and c)). The cleavage of the ether bond and selection of the corresponding fragment ion resulted for [adenine-$^{13}C_5$]-TNV in the conservation of the (m+5)-stabile isotope label within the fragment ion for MRM analyses. Selected MRM transitions for further optimization and analysis of patient samples were: TNV (*m/z* 288.0 → 176.2) and [adenine-$^{13}C_5$]-TNV (*m/z* 293.2→ 181.2).

Plasma assay development and FDA validation

Prior to analysis plasma samples had to be deproteinized to avoid ion suppression resulting in otherwise sensitivity loss. We decided to use TCA for deproteinization of the plasma samples because the use of organic solvent such as acetonitrile or methanol would dilute the plasma samples to much. However, deproteinization by TCA had one disadvantage; the highly concentrated TCA solution used for deproteinization caused ion suppression of the TNV signal and negatively influenced the crystallization quality of the sample spots. Both problems were solved by the dilution of the TCA deproteinized patient plasma with a water/methanol mixture (50:50%, v/v). The sensitivity and linearity range of the ID-MALDI-QqQ-MS/MS method was determined by the measurement of plasma-based calibrators and for the determination of LOD and LLOQ. The expected therapeutically relevant plasma concentrations were within selected range of plasma-based calibrators (0.1–10 μmol/l) [2]. The plasma-based calibration curve showed a linear relationship between the isotopic ratio [adenine-$^{13}C_5$]-TNV/TNV (y) and the TNV concentration (x) (Fig. 2(a); r^2= 0.9996, y = 0.8986x + 0.06478). The TNV calibration curve was applied to determine whether if collected patient samples could be analyzed directly or had to be diluted prior to sample preparation because the samples were outside of the linear range of the ratio measurement. By pre-analyses of few patient samples, it was concluded that TNV plasma concentrations were to be expected within the selected calibrated concentration range and that the dilution of the samples prior to sample preparation was not necessary. Patient plasma concentrations were determined from

measured isotopic ratio by calculation (Formula 2). The LLOQ and LOD were determined according to FDA guidelines; the LLOQ was defined as the lowest plasma-based calibrator from the calibration curve which could be measured with acceptable precision and accuracy (CV<20%). The observed LLOQ for TNV was 0.1 μmol/l. The upper limit of quantification (ULOQ) was defined as the highest plasma-based calibrator that could be measured with CV<15% which was 10 μmol/l. The LOD determined at a signal-to-noise ratio (3:1) of drug-free human control plasma was 0.04 μmol/L.

Accuracy of isotopic ratio measurements

The accuracy of isotopic ratio measurement was determined by the application of isotopic ratio calibrators prepared in pooled drug-free control plasma spiked at a concentration of 10 μmol/l TNV by the addition of different amounts of [adenine-$^{13}C_5$]-TNV whereby isotopic ratios were expressed as atomic percent excess. Accuracy of the observed isotopic ratio in the plasma matrix was determined by the calculation of % error values of each measured calibrator by comparison with prepared APE ratio calibrators with defined APE ratios (Formula 1).

Table 1. Precision and accuracy of the ID-MALDI-QqQ-MS/MS method using isotopic ratio measurements

APE theoretical	APE measured (mean)	Precision CV (%)	Accuracy (%error)[a]
99.0	99.1	0.03	0.10
98.0	98.3	0.02	0.26
96.2	96.7	0.06	0.55
90.9	92.3	0.22	1.49
83.3	85.6	0.27	2.77
71.4	75.5	0.72	5.66
50.0	55.9	1.61	11.90

[a] % error = (mean $APE_{observed}$-$APE_{theoretical}$)/($APE_{theoretical}$)* 100 %

The observed accuracy of the APE measurement at the different artificial prepared APE (99.01%, 98.04%, 96.15%, 90.91%, 83.33%, 71.43% and 50.0% above natural APE of TNV) is presented in Fig. 2(b), average accuracy and precision of the isotopic ratio determination were determined at 3.25 %error (calculated over the complete APE calibration line, Table 1) and 0.42 %CV, respectively.

The observed mean accuracy of the developed method was in good compliance with the FDA criteria (%error<15/20%) and it was concluded that accurate isotopic measurements were possible at high APE by the addition of high amounts of [adenine-$^{13}C_5$]-TNV but also at very low APE by the addition of low amounts of [adenine-$^{13}C_5$]-TNV. We selected the addition of a low amount of [adenine-$^{13}C_5$]-TNV (2.5 μl of a 2.5 μmol/l solution), the error in isotopic ratio measurement was 5.66% (Fig. 2(b)), which was according to FDA guidelines an acceptable accuracy (% error) because the accuracy was <15% for higher isotopic ratios.

Accuracy, precision and stability

Within-run and between-run accuracy and precision of the method were determined at three plasma-based QC concentration levels (0.25, 2.5 and 10 μmol/l TNV). Observed within-run and between run accuracy and precision were in good compliance with the FDA criteria for bio-analytical assays (Table 2).

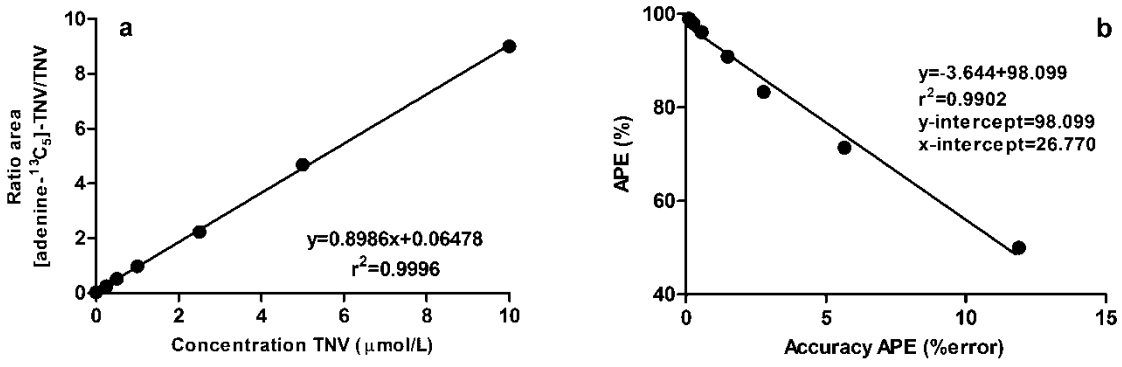

Figure 2. (a) isotopic dilution MRM calibration curve of TNV in plasma, (b) observed accuracy of the isotopic ratio measurement of TNV by addition of [adenine-$^{13}C_5$]-TNV at different APE ratios.

The precision of the assay expressed as %CV was between 8.1% and 9.7% for within-run measurements and between 9.5% and 11.1% for between-run measurements. The accuracy expressed as %error ranged between 4.0% and 10.0% for within-run measurements and between 4.0% and 11.1% for between-run measurements. We found that TNV was chemically stable in the plasma matrix since the decrease in TNV concentration for all three storage conditions was <15% (%error) as defined by the FDA guidelines [12] and it could be shown that the TNV stability in plasma matrix was good (Table 2).

Table 2. Precision and accuracy of the ID-MALDI-QqQ-MS/MS method at three TNV plasma concentration levels

	Within-run validation[a]			Between-run validation[b]		
Nominal conc. (µM)	0.25	2.5	10.0	0.25	2.5	10.0
Observed conc. (µM)	0.26	2.6	11.0	0.25	2.6	10.9
Accuracy (%error[c])	4.0	4.0	10.0	8.9	4.0	9.0
Precision (% CV)	9.7	9.3	8.1	11.1	9.5	9.6

	Storage Conditions/Time[a]					
Nominal conc. (µM)	Refrigerator (8ºC)		Freezer (-20ºC)		Freezer (-20ºC)	
	24 h		20 days		3 freeze/thaw cycles	
	Mean (SD)	%error[c]	Mean (SD)	%error[c]	Mean (SD)	%error[c]
10.0	9.0 (0.9)	-9.6	10.9 (0.8)	9.0	9.9 (1.3)	10.0
2.5	2.2 (0.2)	-12.1	2.7 (0.1)	8.0	2.6 (0.4)	4.0
0.25	0.23 (0.03)	-9.5	0.28 (0.05)	12.0	0.26 (0.05)	4.0

[a]results summarize 10 spots per QC sample (n=3) at each concentration level in three experiment, [b]results summarize three different, experiments from 3 consecutive days with 10 spots per QC sample (n=3) at each concentration level, [c]%error=(mean observed concentration-nominal concentration)/(nominal concentration)*100%

The influence of background signals on the precision of the TNV concentration determination by the addition of [adenine-$^{13}C_5$]-TNV was also studied. Areas of background signals of TNV and [adenine-$^{13}C_5$]-TNV observed in 20 analyzed plasma

samples from 20 different healthy controls prior to spiking with TNV and [adenine-$^{13}C_5$]-TNV showed to have no significant influence on the TNV determination.

Table 3. Determination of the precision of the ID-MALDI-QqQ-MS/MS method in plasma samples collected from twenty different healthy controls

Plasma	TNV[a]	IS[b]	TNV spiked at 0.25 µM	IS spiked at 0.25 µM	Observed TNV conc.	SD	Precision
#	CPS[a]	CPS[a]	CPS[a]	CPS[a]	µM	µM	%CV
1	55.5	83.5	1043.1	1041.1	0.26	0.04	17.0
2	29.4	57.5	1004.4	913.4	0.28	0.02	7.5
3	63.5	94.3	1100.1	1263.2	0.22	0.03	14.7
4	39.2	60.8	1028.5	1129.4	0.23	0.01	4.2
5	88.5	51.7	1062.5	1214.8	0.22	0.02	7.1
6	105.9	79.2	767.1	881.3	0.22	0.03	12.5
7	135.4	66.7	699.5	766.0	0.22	0.04	16.7
8	82.6	59.2	767.8	895.3	0.22	0.03	14.3
9	57.6	66.8	1167.2	1215.0	0.25	0.04	17.2
10	83.5	92.6	1071.8	1113.5	0.24	0.03	14.2
11	41.7	30.8	1463.6	1465.2	0.26	0.03	11.4
12	45.1	35.8	941.2	932.2	0.26	0.04	15.0
13	25.9	40.1	1279.3	1192.3	0.27	0.03	11.1
14	44.3	51.8	1157.3	1088.0	0.27	0.04	15.5
15	25.8	68.4	1222.5	1134.8	0.28	0.03	11.5
16	34.2	34.2	1067.5	1028.8	0.27	0.04	15.1
17	40.8	48.4	1191.6	1300.3	0.23	0.03	13.8
18	33.4	50.0	1244.6	1284.0	0.25	0.04	15.3
19	30.0	39.2	1153.8	1107.0	0.26	0.04	14.7
20	41.7	64.3	1529.7	1484.3	0.26	0.04	14.9
Mean	55.2	58.8	1098.2	1122.5	0.25	0.03	13.2

[a]TNV background signal (blank plasma),
[b][adenine-$^{13}C_5$]-TNV background signal (blank plasma), CPS=counts per seconds (area)

Effect of triglycerides concentration on TNV measurements

After spiking the 20 different control plasma samples with in triplicate with TNV and [adenine-$^{13}C_5$]-TNV at a concentration of 0.25 μmol/l, the mean TNV concentration determined was 0.25 (0.03) μmol/l (SD) and the observed mean precision was 13.2% which was lower than the maximum allowed precision (20% CV) reported by the FDA guidelines for bio-analytical method development and validation [12] (Table 3).

Plasma samples (potassium-EDTA coagulant) with high triglycerides concentrations (>1.9 mmol/l) spiked with TNV at a nominal concentration of 0.25 μmol/l showed no significant influence on the accuracy of the developed assay. Observed accuracies (Table 4) of the ID-MALDI-QqQ-MS/MS assay at different triglycerides concentrations were all in compliance with the FDA guidelines for bio-analytical assays (<20 %error). Average observed TNV concentration was 0.26 μmol/l with a mean accuracy of 3.7 %error.

Table 4. Accuracy of the new developed assay for the measurement of hyperlipidemic plasma.

Patient	Triglyceride conc. (mmol/L)	Measured TNV conc. μmol/L	Accuracy[a] (%error)
1	3.38	0.26	2.8
2	3.77	0.26	3.2
3	4.83	0.27	7.6
4	4.39	0.27	7.2
5	3.57	0.28	10.4
6	3.41	0.27	5.6
7	3.92	0.24	-6.8
8	5.33	0.25	-1.6
9	3.06	0.27	5.2
10	3.20	0.26	3.2

[a]%error=(mean obs.TNV conc.-nominal TNV conc.)/(nominal TNV conc.)*100%

The following cART regimens were used: nevirapine (NVP)+TDF+emtricitabine (FTC) (n=10), NVP+TDF+lamivudine (3TC) (n=1), efavirenz (EFV)+TDF+FTC (n=3), EFV+ TDF+3TC (n=1), ritonavir-boosted lopinavir (LPV/r)+TDF+FTC (n=2), ritonavir-boosted atazanavir+TDF+FTC (n=2) and atazanavir+TDF+3TC (n=1). Patient plasma samples were measured in duplicate and the mean results were within the therapeutically relevant steady-state concentration range ($C_{average}$) of about 100 µg/l (0.35 µmol/l) as reported previously in adult patients receiving 300 mg/day TDF [2] during 48 weeks, while maximum and minimum plasma concentrations were reported between 1.05 and 0.21 µmol/l, respectively [2].

Observed patients TNV plasma concentrations were also comparable with previously reported TNV plasma concentrations measured in HIV-infected adults. Delahunty et al. [8] reported a median TNV plasma concentration measured in 228 HIV-infected adults of 92.6 ng/ml (0.32 µmol/l) and a paper by Sentenac et al. [4] reported a mean TNV plasma concentration of 98.5 ng/ml (0.34 µmol/l).

Application of the assay to patient samples

After FDA validation of the new developed ID-MALDI-QqQ-MS/MS method, the method was tested for its clinical use by measurement of real patient samples. From the measurement of different isotopic ratios in collected patient samples and calculation of the TNV plasma concentrations (Formula 2), the relationship to the time after drug intake of TDF is illustrated in Fig. 3. Twenty plasma samples were used from 19 HIV-infected patients. One patient was infected with HIV type 2, whereas the remaining patients were infected with HIV type 1. All patients were treated with a once-daily cART regimen containing 300 mg TDF which corresponds to 245 mg TNV.

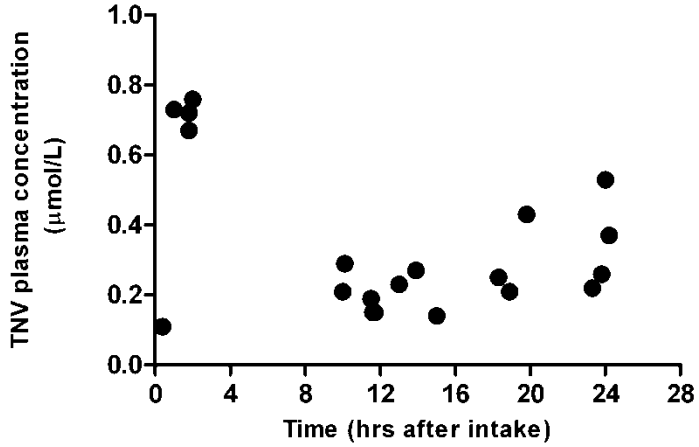

Figure 3. Observed TNV plasma concentrations from nineteen selected HIV-1 infected patients in time receiving 300 mg/day TDF.

Conclusions

The newly developed ID-MALDI-QqQ-MS/MS assay is suitable for accurate measurement of therapeutically relevant plasma concentrations of TNV in plasma from HIV infected adults. The new assay is ultrafast and high-throughput and can be applied for the measurement of large numbers of patient samples in short analysis times. The method was validated conform the most recent FDA guidelines for the development and validation of new bio-analytical methods [12]

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Dried blood spots UHPLC/MS-MS analysis of Oseltamivir and Oseltamivircarboxylate-a validated assay for the clinic

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Abstract

The neuraminidase inhibitor oseltamivir (Tamiflu®) is currently the first-line therapy for patients with influenza virus infection. Common analysis of the prodrug and its active metabolite oseltamivircarboxylate is determined via extraction from plasma. Compared with these assays, dried blood spot (DBS) analysis provides several advantages, including a minimum sample volume required for the measurement of drugs in whole blood. Samples can easily be obtained via a simple, noninvasive finger or heel prick. Mainly, these characteristics make DBS an ideal tool for pediatrics and to measure multiple time points such as those needed in therapeutic drug monitoring or pharmacokinetic studies. Additionally, DBS sample preparation, stability, and storage are usually most convenient. In the present work, we developed and fully validated a DBS assay for the simultaneous determination of oseltamivir and oseltamivircarboxylate concentrations in human whole blood. We demonstrate the simplicity of DBS sample preparation, and a fast, accurate and reproducible analysis using ultra high-performance liquid chromatography coupled to a triple quadrupole mass spectrometer.

A thorough validation on the basis of the most recent FDA guidelines for bioanalytical method validation showed that the method is selective, precise, and accurate ($\leq$15 %RSD), and sensitive over the relevant clinical range of 5–1500 ng/mL for oseltamivir and 20–1500 ng/mL for the oseltamivircarboxylate metabolite. As a proof of concept, oseltamivir and oseltamivircarboxylate levels were determined in DBS obtained from healthy volunteers who received a single oral dose of Tamiflu®.

Introduction

The neuraminidase (NA) are a family of enzymes, which cleave the sialic acid (derivative of neuraminic acids) residue from cellular glycoproteins and lipids [1]. These enzymes are essential for influenza viruses in order to spread within the host. The process allows the virus to detach from the host cell after replication and consequently infects further host cells. Oseltamivircarboxylate (OSC), the active metabolite of oseltamivir (OS) (Tamiflu®, Hoffmann-La Roche, Nutley, NJ) provides a pharmacophore, which resembles sialic acid and selectively inhibits the activity of the enzyme, thus preventing the virus from spreading in the host. Treatment of influenza virus infection is particularly important for patients who are at risk for a complicated course of influenza. These patient groups include neonates, infants, young children, pregnant women, elderly, and immune compromised patients [2–4]. Over the last couple of years, Tamiflu® resistance has emerged in seasonal influenza A (H1N1) viruses. However, it seems that this influenza strain has been replaced by the pandemic influenza A H1N1/09 virus, which is still sensitive to Tamiflu®. As with most of the drugs, a precise dosing regimen for infants and young children is based on calculations according to their body weight and due to empiric data on efficacy [5].

Determinations of a therapeutic window in this patient subgroup are often hampered by an inadequate blood/plasma sampling and sensitive analytical methods. Dried blood spot (DBS) analysis provides an ideal tool to monitor circulating drug levels and is also feasible to obtain drug pharmacokinetics (PK studies) [6,7]. From a finger or heel prick minimum amounts of blood, usually 10–30 μL are spotted directly on specifically designed filter cards, which are subsequently extracted in an on- or off-line procedure [8] to be further analyzed in a high-throughput manner by gas chromatography–mass spectrometry (GC-MS) [9] or ultra high-performance liquid chromatography (UHPLC) coupled to ultra violet (UV), fluorescence, or mass spectrometer (MS) detectors [10]. The use of MS detectors applying multiple reaction monitoring (MRM) mode has the advantage of being usually very sensitive and more selective than other common LC detectors.

Moreover, they provide the possibility to monitor multiple analytes simultaneously and do not depend on fluorescence or UV active chemical structures. The current work describes a novel DBS UHPLC-MS/MS method which allows to

simultaneously monitoring OS and OSC concentrations in human whole blood samples. This work expands on a just recently published DBS method for measurement of OS and OSC in rodent blood [8]. Here, we describe for the first time a method to measure OS and OSC concentrations in human whole blood via DBS. The assay was validated on the basis of the latest Food and Drug Administration (FDA) guideline for bioanalytical method validation [11], and was applied to a small set of samples obtained from healthy subjects, as a proof of concept. The assay requires minimum blood volumes, which can easily be obtained by finger or heel prick, and it obviates plasma centrifugation to simplify sample preparation. Previously reported ester hydrolysis [12] was not observed on DBS during method development and validation, also shown by the various stability tests of OS and OSC on DBS cards.

Materials and methods

Chemicals and reagents

All solvents were of ULC/MS grade and were purchased from Biosolve (Valkenswaard, the Netherlands). The standards for OS and OSC (see Fig. 1) and their respective stable isotope-labeled internal standards *d3*-OS and *d3*-OSC were kindly provided by the Hoffmann-La Roche Ltd. (Basel, Switzerland). Filter paper from Schleicher & Schuell 2992 (Schleicher & Schuell BioScience GmbH, Dassel, Germany) was used for the validation and the patient samples.

Sample preparation

For the preparation of the DBS during the validation, blank whole blood (WB) was collected from two healthy donors (Sanquin, Rotterdam, the Netherlands). Blood was sampled in single-use evacuated fluoride/oxalate blood collection tubes containing 10 mg sodium fluoride and 8 mg potassium oxalate (BD VACUTAINER®) supplied by BD (Franklin Lakes, NJ, USA). Aliquots were kept in the freezer and were prepared freshly every day. Twenty microliters of blood were spotted with an Eppendorf pipette (Wesseling-Berzdorf, Germany) onto the DBS cards and dried for a minimum of 2h at room temperature (RT). For the measurement of the clinical

samples, WB was collected by finger prick and spotted directly on DBS cards. The blood from the arm vein was collected in fluoride/ oxalate tubes and spotted directly from the tubes onto the DBS paper as described above. After drying, 4 μL of a mixture of the two internal standards (*d3*-OS 50 ng/mL and *d3*-OSC 250 ng/mL) were spotted onto the DBS and the cards were dried for another 30 min at RT. The extraction of the DBS was conducted by manually punching out a disk (5 mm Ø) into an Eppendorf tube. Extraction was achieved by the addition of 75 μL 5% methanol (MeOH) in water, followed by 10 min sonication. Forty-five microliters of the extract were transferred into a fresh Eppendorf tube and combined with 30 μL of acetonitrile (kept at −20°C) for protein precipitation. The mixture was centrifuged for 10 min (14000 rpm, 4°C), and the supernatant was diluted 1:4 with water prior to analysis.

Chromatographic conditions

An Ascentis® Express C18 reversed-phase column (100 × 2.1 mm, 2.7 μm) from Supelco (Munich, Germany) and an Ascentis® Express guard column (C_{18}, 2.1 mm I.D.) from Supelco (Munich, Germany) were used for LC separations. The chromatographic separation was carried out on an Ultimate 3000 Rapid Separation LC system from Dionex Benelux B.V. (Amsterdam, the Netherlands).

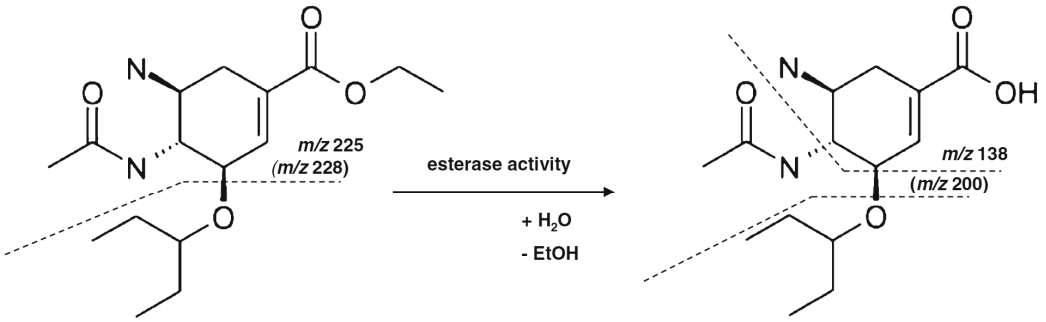

Figure 1. Enzymatic activation of the prodrug oseltamivir (OS) to oseltamivircarboxylate (OSC). The dotted lines represent the fragmentation of the compounds during MRM measurements, including the *m/z* value of the selected fragment ions of the analytes and the respective stable isotope internal standards (values in brackets). For the stable isotope-labeled internal standards, the methyl group of the amid function is replaced by $-CD_3$ group, respectively.

Chromatographic conditions were as follows: gradient elution was performed with two solvents: solvent A, 0.1% formic acid (FA) in water and solvent B, 0.1% FA in MeOH. The program was initiated with a linear gradient from 25% to 100% B at 0.25 min and kept constant for 2.75 min. At 3 min, the column was equilibrated again with 25% solvent B for 3 min. Total run time was 6 min with a constant flow rate of 350 μL/min and a constant column oven temperature of 30°C. The retention time of OSC was 1.88 min and for OS 3.20 min. The system was coupled to an API 4000 QTRAP linear ion trap mass spectrometer (AB Sciex, Concord, ON, Canada). The mass spectrometer was run in positive MRM mode with nitrogen as nebulizer, drying and collision gas. Following parameters were used: curtain gas, 25 (arbitrary units); collision gas pressure, medium; ion-spray voltage (IS), 5000 V; temperature, 650°C; nebulizer gas, 20 (arbitrary units); declustering potential, 20 V; entrance potential, 10 V; collision energy, 15eV for *d3*-OS, OS, and OSC and 30 eV for *d3*-OSC; collision cell exit potential, 10 V for all analytes, except OSC, for which it was set at 20 eV. The MRM transitions were (OS) *m/z* 313→225, (*d3*-OS) *m/z* 316→ 228, (OSC) *m/z* 285→138, and (*d3*-OSC) *m/z* 288→200, respectively (see also Fig. 1). Selection of each MRM transition was based on optimal intensities obtained for the fragment ion during method development (data not shown).

Post-column infusion experiments

A mixture of the two analytes OS (50 ng/mL) and OSC (200 ng/mL) was prepared in water and the solution was directly infused from a syringe pump (Harvard Instruments, Holliston, MA) into the ESI source via a T-piece, where the mobile phase was introduced from the UHPLC system at assay flow rate, column and gradient conditions. Once a steady selected ion current (XIC) for the MRM transitions of OS and OSC was achieved, 60 μL extract from blank blood spot sample were injected onto the column (Fig. 2).

Method validation

Validation samples for the validation samples, fresh WB was collected in BD Vacutainer® fluoride/oxalate tubes from two healthy donors and aliquots were stored at −20°C. Blank blood samples (spiked with 3 μL of water instead of analyte solution)

did not show any interfering signals under assay conditions. Furthermore, quality control (QC) samples prepared (see text below) in blood of donor 1 were calculated with a calibration curve of standards prepared in blood from donor 2, and vice versa to demonstrate the independency of the assay results from sample matrix. Stock solutions and dilutions thereof were prepared in water for all analytes. Calibration standards were prepared daily by adding 3 μL of the respective OS–OSC mixture to 27 μL of blood to obtain one sample. Calibration standards were chosen at the following concentrations for OS: 5, 10, 20, 30, 50, 100, 250, 500, 1,000, and 1500 ng/mL and for OSC: 20, 30, 50, 100, 250, 500, 1,000, and 1500 ng/mL. Calibration curves included a blank sample and two zero samples, spiked with each internal standard separately. QC samples were prepared in the following concentrations (OS/OSC): 0/750, 75/200, and 150/75 ng/mL and measured with n=4 for each QC sample.

Validation protocol

The method validation was performed in respect to the latest FDA guideline for method validation of bioanalytical assays [11]. The selectivity of the method was determined by the comparison of blood blank and two zero samples from two donors, respectively. Linearity of the DBS assay was verified by five calibration curves measured on five separate days. Each one consisted of ten non zero calibration standards for OS and eight for OSC, covering the concentration range from 5–1500 ng/mL for OS and 20–1500 ng/mL for OSC measured in the identical calibration standards. The calibration curves were calculated by linear regression using weighting for both analytes. The back-calculated values are required to be within 15% relative standard deviation (RSD) and 20% RSD at the lower limit of quantification (LLOQ), from the nominal concentration. The acceptance criteria for the correlation coefficients (r^2) of the calculated regression curves were 0.99 or higher.

The determination of the LLOQ was conducted by spiking six WB samples with OS and OSC at the lower end of the calibration curves (5 and 20 ng/mL, respectively). Results were recalculated with a freshly prepared calibration curve. Intra- and interday accuracy and precision were determined by analyzing three times

four replicates of each QC sample together with a freshly prepared calibration curve in one analytical run within 1 day (intraday) and by analyzing three times four replicates of each QC sample together with a freshly prepared calibration curve in one analytical run on three consecutive days (interday).

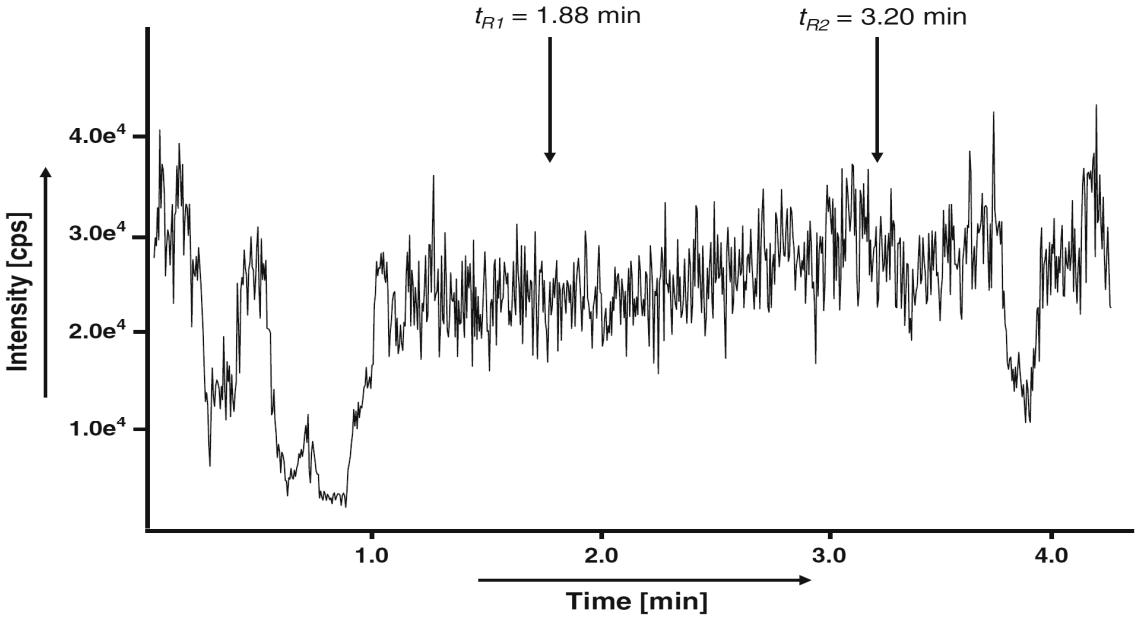

Figure 2. Post-column infusion chromatogram. Indicated by the arrows are the retention times of OSC tR1=1.88 min and OS tR2=3.20 min. The major drops in the XIC signal (for OS *m/z* 313→225 and OSC *m/z* 285→138) are due to ion suppression of eluting matrix compounds at the beginning of the gradient and due to a sudden change in solvent composition after 3.4 min.

The mean, the standard deviation, the RSD, and bias were calculated. The recovery of the method was determined by spiking three WB samples with OS and OSC (75 and 200 ng/mL, respectively), spotting and cutting out the whole blood spot. As a comparison, three blank blood spots, completely extracted and spiked in the extraction solvent with the same concentration were used. Samples were measured and results calculated by setting the samples spiked after extraction as 100%. Stability of OS and OSC was determined for all QC samples with n=4, for 0, 1, 3, and 7 days at RT and in the fridge at 4 °C; at –20°C and +40°C for 24 h. All samples were kept in air-tight plastic containers for time of storage. Additionally, 12 h auto sampler stability was tested for the extracted solution.

Clinical samples

As a proof of concept study, three healthy volunteers (three male adults) took a single Tamiflu® capsule (75 mg), after signing written informed consent. Blood obtained via finger prick and from the median cubital vein was collected 2.5 (±15 min) and 4.25 h (±15 min) after administration of the capsule by trained personnel.

Results and discussion

The method described in the current work shows the development of a Tamiflu® DBS assay in combination with a fast, accurate, precise, and reproducible UHPLCMS/MS application. The assay was validated based on criteria described by the most recent FDA guideline for bioanalytical method validation [11]. The feasibility of the analytical procedure was demonstrated in a clinical proof of concept study. To the best of the author's knowledge, this study is the first report in the field of DBS analysis which assesses the effect of the type of whole blood, i.e., venous WB in blood collection tubes containing additives or capillary WB obtained by finger prick, on the measured concentrations of OS and OSC. This comparison demonstrated that results obtained with the newly developed assay were not influenced by additives (fluoride/oxalate) in the blood collection tubes. Fluoride/oxalate tubes are the tubes of choice for OS blood sampling [13]. Further proof of the accurate and reproducible analytical method was obtained via post-column infusion chromatograms, demonstrating no interference (ion suppression or enhancement) at the respective retention times of the analytes (see Fig. 2), despite an extraction protocol for the DBS using 5% methanol in water. Watery extraction solvents are technically more prone to show matrix effects, as endogenous, hydrophilic compounds get easily extracted together with the drug of interest [14]. This interference can mainly be observed in the infusion chromatogram (see Fig. 2) during the first minute.

Method validation

Selectivity No interfering signals were observed at the respective retention times of t=1.88 min (OSC) and t= 3.20 min (OS) when blank or zero sample (spiked with IS) blood spots were analyzed.

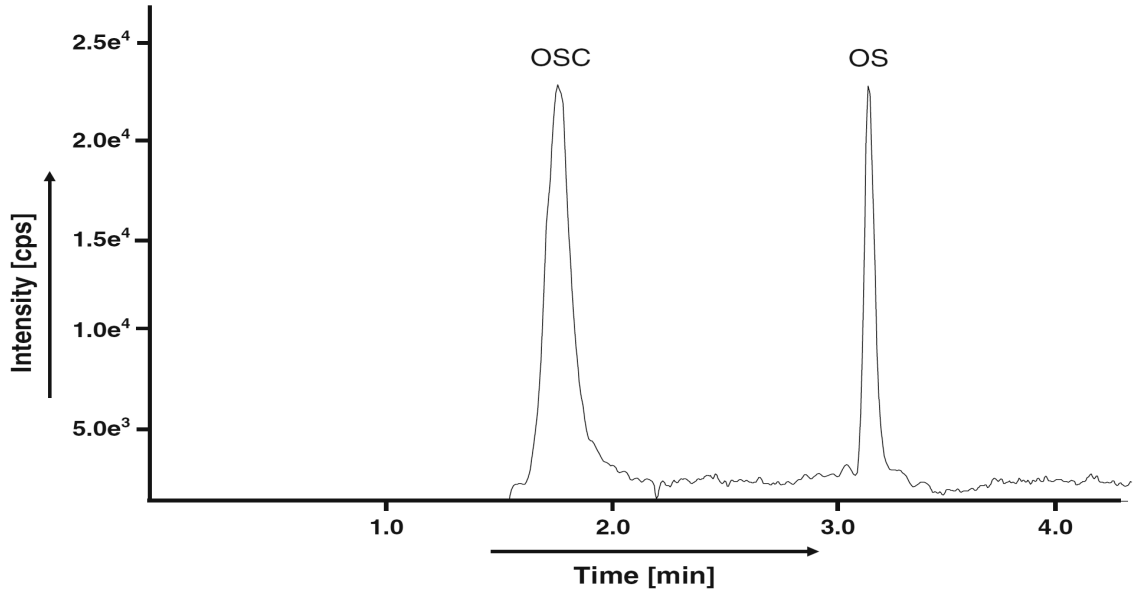

Figure 3. Exemplified chromatogram of OS (Rt=3.20 min) and OSC (Rt=1.88 min) at the LLOQ, monitored with scheduled MRMs for the following mass transitions OS m/z 313 →225 and OSC *m/z* 285 →138

Linearity Five measurements of the calibration standards, freshly prepared on five separate days, showed good linearity over the entire calibration range from 5–1500 ng/mL for OS and 20–1500 ng/mL for OSC.To obtain the best fit of the calibration standards a weighting factor of $1/x^2$ was used for each analyte. This factor was determined through a comparison of the calculated correlation coefficients (r^2 values) and the sums of the absolute values of the relative errors for the calibration curves when different weighting factors were applied in the calculation (data not shown). The r^2 values were consistently greater than 0.991, with a mean of 0.9938 for OS and 0.9987 for OSC, respectively. The RSD values of the calculated standard concentrations from their nominal values were constantly $\leq$15% for all values, including the respective LLOQs. These results were well in line with the FDA specifications [11].

Lower limit of quantification

The lower limit of quantification, determined in six spiked DBS samples was 5 ng/mL for OS and 20 ng/mL for OSC. Measurements for OS showed an average of 5.7 ng/mL ± 3.3% (bias=5.1%) and for OSC an average of 21.3 ng/mL ± 8.5% (bias=6.7%). These values are well below the general FDA acceptance criterion of

20% RSD for the LLOQ [11]. A representative chromatogram at the LLOQ is shown in Fig. 3.

Accuracy and precision

Accuracy and precision were determined for three independent measurements within 1 day and on three consecutive days. Results can be found in Table 1, which demonstrates that the acceptance criteria of a maximum RSD of 15% are fulfilled for all QC samples. The relative recoveries for OS (75 ng/mL) and OSC (200 ng/mL) were determined with 92.8% and 100.2%, respectively. These results demonstrate that there was almost no sample loss during sample preparation.

Table 1. Intra- and interday accuracy and precision

OS	nominal conc. (ng/mL)	mean calc. conc. (ng/mL)	Accuracy (bias %)	Precision (%CV)
intraday (n=3)	75	70.6	-5.9	4.7
	150	159.6	6.4	1.8
interday (n=3)	75	72.2	-3.7	6.5
	150	151.0	0.7	8.7
OSC				
intraday (n=3)	75	78.5	4.6	4.5
	200	219.3	9.7	1.0
	750	686.3	-8.5	1.4
interday (n=3)	75	74.7	-0.5	7.3
	200	221.1	10.5	0.8
	750	708.5	-5.5	4.4

Intra- and interday accuracy and precision measurements of three independent measurements of the respective QC samples for OS and OSC, n=4 respectively. The accuracy is expressed as the bias of the measurement and the precision as % CV.

Table 2. Results of various stability test conditions.

	nominal conc. (ng/mL)		accuracy (bias %)		precision (% CV)	
Condition	OS	OSC	OS	OSC	OS	OSC
RT at day 7		75		4.9		6.0
	75	200	- 11.2	3.8	3.8	3.1
	150	750	-8.9	-3.6	6.8	4.7
4°C at day 7		75		0.9		6.7
	75	200	-6.8	1.4	8.3	4.3
	150	750	-9.8	-12.2	4.1	1.7
24 h at -20°C		75		-0.1		5.5
	75	200	-10.6	-5.0	5.5	8.9
	150	750	-7.9	-7.6	10.0	4.0
24 h at +40°C		75		1.5		3.4
	75	200	-13.1	0.7	7.2	7.7
	150	750	-8.6	-5.4	5.6	6.1
Auto sampler at 4°C (12h, dark)		75		4.1		3.7
	75	200	-7.8	10.8	7.6	1.6
	150	750	8.7	-9.3	3.4	4.5

Table 3. Blood concentratios obtained form healthy volunteers

		OS concentration (ng/mL)			OSC concentration (ng/mL)		
donor	t (h)	finger prick	arm vein	Δ (%)	finger prick	arm vein	Δ (%)
1	2.5	27	25	6.4	125	149	-19.0
2		27	25	7.2	148	143	3.1
3		31	29	5.8	90	83	7.9
1	4.25	n.d.	n.d.	-	242	189	22.0
2		n.d.	n.d.	-	175	151	14.0
3		5	6	-19.9	140	129	7.5

Blood concentrations from healthy volunteers were measured using blood from the finger prick, compared to blood taken from the cubital vein via blood collection tubes.
All blood samples were spotted on DBS cards. Volunteers took one tablet of Tamiflu® at time point t=0 and samples were taken at t=2.5 and t=4.25h.
The delta values (Δ) show the difference between the value of the finger prick and the cubital vein, expressed in % of the finger prick value; n.d. = not determined, as values were below the LLOQ.

Stability

All stability tests showed sufficient stability of both analytes, OS and OSC under various test conditions. As shown in Table 2, on card stability of both analytes showed no significant sample loss over 7 days at RT and at 4°C, simulating collection, shipment, and storage condition. Values for 1 and 3 days stability were well below 15% accuracy (bias) and precision (data not shown). Moreover, stability could be demonstrated for 24 h at −20°C and +40°C, simulating shipment conditions. Auto sampler stability was shown for the extracted QC samples at 4°C in the dark to guarantee stability during higher sample throughput. No significant sample loss was shown under any of the test conditions.

The listed delta values describe the relative difference between the values obtained from the finger pricks and via the blood collected in fluoride/oxalate blood collection tubes from the cubital veins, prior spotting on DBS paper. The OS/OSC levels were comparable in DBS and venous WB, as judged by the delta values.

Analysis of clinical samples

Three healthy volunteers were treated once daily with 75 mg Tamiflu® and blood samples were collected at two different time points, 2.5 and 4.25 h after oral administration. These time points were chosen in a time window in which the t_{max} of the prodrug OS and the active metabolite OSC were expected, as previously described [15]. Concentrations were determined in DBS obtained from finger pricks (capillary WB) and in DBS from in BD Vacutainer® fluoride/oxalate tubes collected blood (venous WB). These tubes were also used for the collection of the blank blood used during the validation. Results are shown in Table 3. The values are well within the plasma concentrations described in literature [16].

Conclusions

The current work describes for the first time a complete method validation for the simultaneous measurement of OS and OSC in WB using DBS. In the fast growing field of pharmaceutical DBS analysis, we demonstrated with the current work that DBS are feasible for Tamiflu® concentration measurements in humans. The importance of this novel approach lies in the greatly facilitated approach for blood

collection, especially in neonates, infants, and small children (finger or heel prick), where collection of larger blood volumes may be problematic. The work described herein will also open possibilities for therapeutic drug monitoring (TDM), as again only small sample volumes (20 μL/spot) are needed and a more invasive venous puncture can be avoided. Furthermore, OS potentially undergoes further ester hydrolysis (esterase activity) in collected WB samples [12] and could therefore influence accurate determinations of plasma OS and OSC (OSC is the hydrolysis product of OS) concentrations. A recently published, elaborate study by Heinig et al. of Tamiflu DBS analysis in rat blood specifically investigated stability of OS during drying time of different DBS cards and found treated (DMPK-A and DMPK-B) in contrast to chemically untreated cards to stabilize the drug [8]. This stands in contrast to a report by D'Arienzo et al. who could not observe ester hydrolysis on untreated cards (Whatman 903 Protein Saver) [17]. In order to investigate this stability issue, we compared DBS spotted from fluoride/oxalate tubes to directly spotted blood from the finger prick. The fluoride/oxalate blood collection tubes were shown to be necessary to prevent ester hydrolysis of OS after blood sampling [13, 18]. The small pilot study presented in the current work did not show any significant differences in OS or OSC concentrations in DBS collected via these two ways. Furthermore, we did not observe any significant degradation during method development or in a separate calibration curve for OS. However, since these calibration samples were prepared in previously frozen blood a loss of esterase activity during as a consequence of freeze–thaw cycles cannot be ruled out. Further research comparing a validated plasma assay and the validated DBS assay, described in the current work will be necessary to specifically address this issue in a larger patient collective. In the future, studies will also be performed to determine ideal dosing regimes in infants and small children [19], where dosing is currently calculated according to the bodyweight or according to empiric amounts, but not based on actual blood levels in influenza-infected children. Besides pediatrics, this assay will also provide the possibility for future PK and TDM studies in clinical and nonclinical environments.

Taken together, further research in the context of DBS analysis of Tamiflu® will have to address more technical issues like, e.g., the influence of different hematocrit (Ht) values, paper quality, sampling instructions, etc. [14]. These

investigations will also depend on the study of interest and the setting of the sample collection.

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Impact of internal standard addition on dried blood spot analysis in bio-analytical method development

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Abstract

Background

Addition of internal standards to DBS specimen can be complicated. Therefore, we studied the feasibility of different internal standard addition procedures. Nevirapine and its stabile isotope analogue were used as a model compounds and concentrations in DBS specimen were determined by MALDI-triple quadrupole tandem mass spectrometry using selected reaction monitoring.

Results

The addition procedure of the stable isotope labeled internal standard had significant impact on observed Nevirapine concentrations. Relative recovery rates depending on the internal standrad addition procedure ranged between 11.4 and 107.9%. Experiments with different punch sizes (5 and 7 mm diameter) showed no significant influence on observed Nevirapine concentrations.

Conclusion

Application of internal standard prior to blood spotting provided good Nevirapine recoveries and this procedure is well suitable for applying DBS in infectious diseases, especially in HIV infection treatment.

Keywords

Dried blood spots, Nevirapine, antitretroviral drugs, matrix-assisted laser desorption/ionization mass spectrometry, isotope dilution, HIV-1 infection, internal standard addition, DBS punch size.

Introduction

The use of dried blood spot specimen (DBS) on cellulose paper reaches back more than 40 years, when it was introduced by Dr. Robert Guthrie in the 1960s. He used DBS specimen as a simple assay to determine relative blood concentrations of the amino acid phenylalanine in newborns, which were suspected of having phenylketonuria (PKU) [1]. Since then, DBS specimens became of popular use in pharmaceutical drug development and pharmacokinetic (PK)-studies [2,3] but also in many clinical assays for determination of patient's adherence to the drug regime by therapeutic drug monitoring (TDM) [4-7] like in infectious diseases such as the human immunodeficiency virus infection (HIV) [8-11] or influenza (H1N1) [12]. Compared to conventional sampling techniques (i.e. plasma or serum collection), DBS specimens provide distinct advantages. Sample collection is greatly facilitated as blood can be obtained from a simple finger-or heel prick (capillary blood) and medical supervision, as needed for the more invasive venepuncture (venous blood) can be omitted. Furthermore, storage and transport of dry DBS specimen is possible at ambient temperature while shipment and storage of frozen plasma and serum samples at -20°C or -80°C is necessary and is very costly. However, DBS specimens have also certain disadvantages especially with respect to analysis [4]. Due to the complexity of whole blood, DBS specimens are more prone to show matrix effects as compared to measurement of serum or plasma. Furthermore, chemical treatment of the cards (i.e. DMPK-B cards, GE Healthcare, USA) potentially adds to these effects and needs to be investigated during method validation.

The addition of internal standards (IS) prior to sample preparation and analysis is a common practice to compensate for sample matrix effects and differences in extraction efficiency, losses due to sample handling and variations in instrument sensitivity. In our study, to compensate for these effects in the best manner was only possible by applying a stable isotope labeled analogue of Nevirapine (NVP), since a surrogate IS would behave differently during DBS sample preparation and analysis.
When analyzing liquid samples like serum or plasma, IS addition is simply done by spiking it into the sample prior to sample preparation and analysis. However, the addition of an IS for analysis of DBS specimens can be complicated. Especially, DBS specimen sampling in infectious diseases like HIV infection the addition of the IS to

blood samples prior to spotting requires extra safety measurements in resource limited conditions. Because of the considerable value of an IS application for method reproducibility current study aims to investigate the impact of different IS addition procedures on the quality of DBS analysis. The experiments were carried out using the antiretroviral drug Nevirapine (NVP, Fig. 1) used in HIV-1 infection treatment [13], as a model compound. NVP concentrations were determined by an isotope dilution method. In isotope dilution analysis, known exact quantity of stable isotope labeled internal standard (i.e. NVP-*d3*) is added to the sample with an unknown concentration of the drug (i.e. NVP). The ratio of the two signal intensities of NVP-d3 and NVP (isotopic ratio of NVP-*d3*/NVP) is then used to determine their relative proportion and consequently from this isotopic ratio the NVP concentration can be calculated. For the measurement of the isotopic ratios a Matrix-Assisted Laser Desorption/Ionization mass spectrometer (MALDI-QqQ-MS) was used. This mass spectrometric technology omits a time consuming chromatographic separation what resulted in a reduced analysis time of approx. 10 seconds per sample or even less. This relatively new technology (launched in 2008) applies a MALDI source in combination with a triple quadrupole mass spectrometer (MALDI-QqQ-MS).This technology was previously successfully applied for the measurements of antiretroviral drug concentrations in DBS and plasma [8,11,14] but also for other types of drugs [15-18]. Due to these extremely short analysis times and the high specificity, given by the MS, MALDI-QqQ-MS/MS is perfectly suitable for ultrafast and high-throughput analysis and it has been demonstrated to have equal analytical performances as HPLC in combination with electrospray ionization (ESI) [19] coupled to MS. The aim of this study was to determine the impact of different IS addition procedures on the determination of the NVP concentrations applying DBS specimens during bio-analytical development. The results of this study will be implemented into a new to develop bio-analytical assay for the measurement of Nevirapine concentrations which will be used for TDM purposes of HIV-1 infected persons.

Experimental

Chemicals & materials

The paper used for DBS specimen collection was the Whatman protein saver 903® paper (GE Healthcare, Diegem, Belgium). Blood used for DBS specimen, needed in all experiments was from a single healthy donor and was obtained from a local blood product supplier company (Sanquin Blood supply Foundation, Rotterdam, the Netherlands) containing potassium-EDTA as anti-coagulant. NVP and NVP-*d3* were purchased from Campro Scientific (Veenendaal, the Netherlands). Methanol and high purity quality water (LC-MS) were obtained from Biosolve (Valkenswaard, the Netherlands).

MALDI-QqQ-MS/MS analysis of DBS specimen

The MALDI-QqQ-MS/MS instrumentation used for analysis of the DBS specimen was the FlashQuant™ Workstation containing a FlashLaser source (349 nm, 1000 Hz) combined with a API 4000 mass analyzer (AB Sciex, Concord, Canada) operating in the positive ionization mode. Analyses were carried out using selected reaction monitoring (SRM) at unit resolution. The following mass transitions of the protonated molecular ions were monitored: *m/z* 267.2→ *m/z* 226.0, *m/z* 270.2 →229.0, for NVP and NVP-*d3*, respectively (Fig. 1).

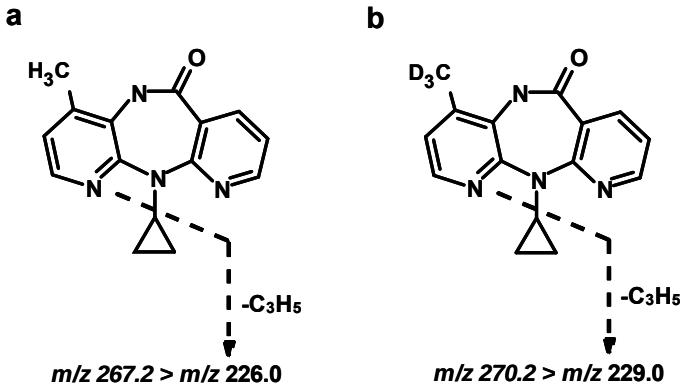

Figure 1. The non-nucleoside reverse transcriptase inhibitor (a) Nevirapine (NVP) and the (b) deuterated internal standard (NVP-*d3*) and their selected reaction monitoring (SRM) transitions

Optimized MALDI-QqQ-MS/MS instrument parameters used for analysis were: laser power 50%, MALDI target plate voltage 25 V, skimmer voltage 0 V, CAD gas 12 arbitrary units (collision gas), collision cell energy exit potential (CXP) 10V, source gas 20 arbitrary units, dwell time 10 ms and laser raster speed was set to 1 mm/ sec while the collision energy (CE) was 35 V for both analytes. Instrument control and data acquisition were performed by the Flashquant 1.0 software in combination with the Analyst 1.4.2 application software (AB Sciex, Concord, Canada).

Preparation of NVP calibration curve by an isotopic dilution protocol

DBS specimen calibrators applied for isotopic ratio calibration purposes were prepared by dilution of a primary NVP stock solution (291 µmol/L in 50:50% MeOH/water) with drug free control blood from the healthy control. Blood calibrators yielded following NVP concentrations: 50, 25, 10, 5.0, 2.5, 1.0, 0.5 and 0 (blank) µmol/L NVP. From all calibrators, a 100 µL-aliquot was spiked with 10 µL of IS (NVP-*d3*; 100 µmol/L in 50:50% MeOH/water) for the preparation of the isotopic ratio calibrators. From these calibrators three 25-µL aliquots were spotted onto the DBS paper and DBS specimen were dried for 90 min at ambient laboratory temperature (approx. 23°C, 40% relative humidity) prior to disk punching. From each DBS specimen a disk (7 mm diameter, centered) was punched out and transferred into a safe-lock micro tube (1.5 mL). The disk was extracted with 200 µL of an acetonitrile/water mixture (80:20% (v/v)) for 30 min using ultrasound. From the obtained DBS specimen extracts, 20 µL were mixed with 20 µL of the MALDI matrix solution α-CHCA (α-cyano-4-hydroxycinnamic acid, Agilent Technologies; 6.2 mg/mL in methanol/acetonitrile/water 36:56:8 (v/v/v), pH=2.5) and 0.5 µL were spotted in fivefold (*n=5*) on an Opti-TOF 96-well stainless steel target plate (123 x 81 mm; MDS Analytical Technologies, Concord, Canada) and dried at room temperature for 5 min prior to analysis by MALDI-QqQ-MS/MS. The NVP concentrations of the DBS specimens were calculated from using the isotopic ratio calibration curve.

Impact of NVP-*d3* addition procedure on NVP recoveries

The impact of the NVP-*d3* addition procedure on the NVP concentration determination by isotopic ratio measurement applying different NVP-*d3* addition

procedures in DBS specimen analysis was determined as follows: 100 µL of control blood was spiked with NVP at a concentration of 29.1 µmol/L (dissolved in 50:50% MeOH/water) and subsequently spiked with 10 µL of the NVP-*d3* stock solution (100 µmol/L, 50:50% MeOH/water). Three 25 µL-aliquots were spotted onto the DBS paper and DBS specimens were dried for 90 min at ambient temperature.

1. Three 2.5 µL-aliquots of the NVP-*d3* solution (100 µmol/L) were spotted onto the DBS paper prior to blood spotting and the DBS paper was dried for 30 min at ambient temperature. On top and in the center of the internal standard spots 25 µL-aliquots of spiked blood (29.1 µmol/L NVP) were added and DBS specimen were dried for an additional 60 min at ambient temperature.
2. Three 25 µL-aliquots of spiked blood (29.1µmol/L NVP) were spotted onto DBS paper and DBS specimen were dried for 60 min at ambient temperature. On top and in the center of the DBS specimen a 2.5 µL-aliquot of the NVP-*d3* (100 µmol/L) were added and DBS specimens were dried for an additional 30 min at ambient temperature.
3. Three-25 µL-aliquots of spiked blood (29.1 µmol/L NVP) were spotted on DBS paper and DBS specimens were dried for 90 min at ambient temperature.

All paper strips were manually punched producing disks with a diameter of 7 mm. DBS specimen disks obtained from the experiments 1, 2 and 3 were extracted by 200 µL of a mixture of acetonitrile/water (80/20% v/v) using ultrasonic sound (30 min) while disks from experiment 4 were extracted with 200 µL of a mixture of acetonitrile/water (80/20% (v/v)) containing 2.5 µL/mL NVP-*d3* (100 µmol/L) by ultrasonic sound. From the all obtained DBS specimen extracts, 20 µL were mixed with 20 µL of the MALDI matrix solution and 0.5 µL were spotted in fivefold onto the MALDI target plate and dried at room temperature for 5 min prior to analysis by MALDI-QqQ-MS/MS.

NVP-*d3* displacement and influence of disk punch size on the NVP concentrations

Since displacement of NVP-*d3* (previous section, experiment 2) within the dried NVP-*d3* spot by spotting blood onto the spot could occur [20], the influence of the spotted volume of blood on IS displacement was studied by addition of three

different NVP spiked blood amounts (10, 15 and 25 µL). Studied were NVP spiked blood volumes of 10, 15 and 25 µL (29.1 µmol/L, in six-fold), and the blood was spotted in the center of NVP-*d3* pretreated DBS paper (2.5 µL of 100 µmol/L NVP-*d3*), which was then dried for 90 min at ambient temperature. From each DBS specimens 7 mm and 5 mm disks (*n=3*, resp.) were punched out and extracted with 200 µL of the acetonitrile/water (80:20% (v/v)) extraction solution, mixed 1:1 with MALDI matrix solution and then 0.5 µL aliquots (*n=5*) were pipetted onto the MALDI target plate. NVP concentrations were determined by isotopic ratio measurement by MALDI-QqQ-MS/MS.

Results and discussion

NVP isotopic ratio calibration curve and LLOQ/LOD determination

The selected range of DBS specimen based calibrators showed a linear relationship between the isotopic ratio of NVP-*d3* and NVP (y-value) and the NVP concentration (x-value). The NVP-*d3*/NVP isotopic ration calibration line (y=0.0773x-0.0562) was linear between 0.5 and 50 µmol/L (r^2=0.993). The lower limit of quantification (LLOQ) was defined as the lowest calibrator that could be measured with an acceptable accuracy and precision of < 20% error according to the most recent FDA [21] guidelines for bio-analytical method development and validation.

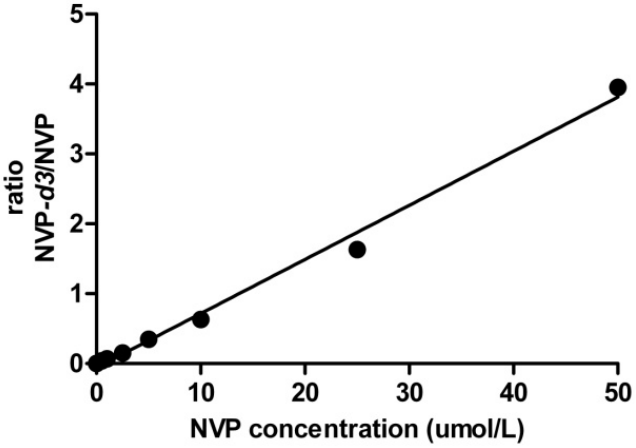

Figure 2. Isotopic calibration curve (NVP-*d3*/NVP) applying DBS based calibrators of 25 µL blood volume

The LLOQ was 0.5 µmol/L while the upper limit of quantification (ULOQ) defined as the highest DBS specimen calibrator that could be measured with CV<15%, was 50 µmol/L. The limit of detection (LOD), defined as three times the signal-to-noise ratio of the blank DBS specimen calibrator was 0.1 µmol/L.

As observed from preliminary DBS specimen experiments [11,12], we concluded that the addition of an IS was necessary to be able to correct for the blood matrix effects and analyte losses when analyzing NVP from DBS specimens. Based on these preliminary experiments we first investigated the impact of different addition procedures of the NVP-*d3* on the NVP measurements in DBS.

Procedures for the addition of NVP-*d3* applying DBS specimen for analysis

In total four different potential NVP-*d3* addition procedures were studied; (1) addition of NVP-*d3* into NVP spiked blood prior to spotting of the DBS specimens, (2) prior to spotting of the NVP spiked blood addition of NVP-*d3* onto DBS paper, (3) addition of NVP-*d3* onto a DBS specimen after drying and (4) addition of the NVP-*d3* into the solvent mixture used for extraction of the DBS specimen disks. The latter procedure is also for example used for online extraction of DBS specimens [20] or manual extraction [11,12]. Results from the NVP-*d3* addition experiments showed significant differences between observed NVP concentrations (Table 1) from each experiment. The addition of NVP-*d3* into the NVP spiked blood prior to DBS spotting provided the best recovery rates for NVP (mean conc. 31.4 µmol/L; recovery 107.9%) with good accuracy and precision (both<15%). Obtained recovery results were within expectancy because the addition of NVP-*d3* into NVP spiked blood prior to spotting will assure identical physical-chemical behavior of both compounds and matrix effects like ion-suppression during measurement were identical for analyte and IS and expected recovery rates of 100 % were obtained. By spotting NVP spiked blood onto NVP-*d3* treated DBS paper (experiment 2), the observed DBS specimen NVP concentration decreased by 18.8% (mean conc. 25.5 µmol/L; recovery 87.5%). The observed lower recovery could be explained by a better extraction of NVP-*d3* from the DBS specimen than the extraction of NVP distributed in the blood matrix, but also

a displacement of the NVP-d3 could influence the NVP concentration determination. A higher extraction rate for NVP-*d3* leads to a higher NVP-*d3*/NVP isotopic ratio and thus to a lower NVP concentration and/or recovery. By changing the addition procedure of NVP-*d3* from prior to blood spotting (experiment 2) to addition onto the dried DBS specimen (experiment 3) the observed NVP concentrations decreased even further, when compared to NVP concentrations in experiment 1 and 2. Mean observed NVP concentration was 16.0 µmol/L (mean recovery of 54.9%). This experiment showed that the extraction of NVP-*d3* from the surface of the DBS specimen was much higher than the NVP-*d3* extraction from underneath the dried blood matrix (experiment 2). A possible explanation for this could be that the extraction of the IS was hindered by the dried blood matrix covering the treated paper area but also the existence of strong polar-polar interactions of NVP-*d3* with the cellulose structures of the DBS paper. The influence of polar-polar interactions between DBS paper and analytes on their recovery rates was previously demonstrated [22]. The lowest NVP recovery was observed when DBS specimens were extracted with a solvent mixture containing NVP-*d3*. This low recovery can be explained by the fact that NVP-*d3* was already for 100% in solution, as no extraction of the IS was necessary. The extraction of NVP from the DBS specimen was comparable to the previous tests. From this experiment it was observed that the extraction efficiency of NVP from the blood matrix was 11.4%.

Determination of optimal spotted blood volume and DBS specimen punch size

The results from the IS addition experiments showed that the best recovery was obtained by adding the IS to the NVP spiked blood prior to spotting, a procedure which is impractical for DBS specimen sampling in HIV-1 infection due to safety risks for medical personnel. Therefore, we had to decide to use the IS-treated paper approach (experiment 2). However, spotting of spiked whole blood onto IS-treated paper might cause displacement of the IS from the center of the spot as previously [20, 23] due to possible chromatographic effects and/or re-dissolving of the IS during drying of the blood. To determine a possible displacement of the IS from the center of the IS spot towards the outsides of the IS spots, different amounts of NVP spiked

blood were spotted onto the IS-treated paper and from the DBS specimen disks with a diameter of 7 mm were punched and analyzed as described previously. It was observed that the amount of spiked whole blood onto the IS-treated paper had an explicit impact on the recovery of NVP. Spotting 25 and 15 µL of blood resulted in good recovery rates of almost 100%. Spotting of 10 µL of blood resulted in a significant increase of the recovery rates compared to the DBS specimen prepared by spotting 15 and 25 µL of NVP spiked blood (Table 2). Addition of 2.5 µL of NVP-*d3* solution (dissolved in 50:50% MeOH/water) onto the DBS paper resulted in NVP-*d3* spots with a mean area of 33.3 ± 4.6 mm^2 (data not shown). NVP-*d3* spot areas were determined by an independent experiment with a colored NVP-*d3* solution (bromothymol blue indicator was used). Measured NVP-*d3* spot areas were smaller than the 25 µL-DBS specimen areas (area 60.2 ± 3.8 mm^2, mean spot diameter 8.7 mm Table 2) but by applying 15µL- blood the areas of NVP-*d3* and of the DBS specimen were almost identical (37.6 ± 2.2 mm^2, Table 2). However, in case of 10 µL DBS specimens the NVP-*d3* spot areas were significantly bigger (25.3 ± 2.3 mm^2, Table 2). The observed mean NVP concentration of 15 µL DBS specimen was 28.9 µmol/L (recovery of 99.4%). Since the NVP-*d3* spot area and DBS specimen area were almost identical (33.3 mm^2 vs. 37.6 mm^2) and the IS/DBS specimen areas were (almost) completely punched out by 7 mm disks (area 38.5 mm^2 and mean spot diameter of 7.4 and 5.8) and recovery rates of NVP were as expected close to 100%. The obtained result of 99.4% was well in line with the expected recovery.When 10 µL DBS specimen were analyzed the NVP recovery was even higher (mean recovery 129.4%). One could expect that the extraction of NVP-*d3* which is not covered by the dried blood would be more efficient than the NVP extraction from the blood matrix. But, the mean observed recovery of 128.6% implicates that despite punching and extracting the complete IS/DBS specimen spot the extraction of free NVP-*d3* is less efficient than NVP from the blood matrix what can only be explained by the polar-polar interactions between NVP-*d3* and the DBS paper. Partial punching of DBS specimen as done with the 25 µL-DBS specimen resulted in lower recoveries of NVP (83.8%) than observed with other spotted blood volumes. We think that due to partial punching of the DBS specimen (although NVP-d3 spot is punched out completely!) the real NVP concentration of the DBS specimen disk was lower than the spiked 29.1 µmol/L, what could be a plausible explanation for the observed lower NVP recovery

rate. All above described experiments were also carried out with punching out 5 mm disks from the DBS specimen, but no significant differences (unpaired t-test, 95% confidence interval) were observed when compared to the results obtained from 7 mm punches (Fig. 3 and Table 3). No, clear evidence for IS displacement were obtained but more experiments are needed to be sure that no displacement of the IS occurs when the IS was spotted to the DBS paper prior to blood spotting. To sum all results up, extraction of all DBS specimen spotted with different volumes of NVP spiked blood resulted in significant different NVP recoveries.

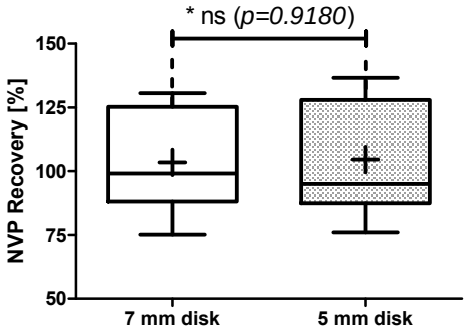

Figure 3. Comparison of observed NVP recovery rates from DBS specimen applying 5 and 7 mm disks (Box-whisker plot illustrates the median (solid line), min to max (wiskers) and 25-and 75 % percentiles (box) and mean (+); ns=not significant)

Table 3. Statistics of observed results from different DBS specimen disk punch sizes

Statistics	7 mm disk punch	5 mm disk punch
Minimum recovery rate (%)	75.10	76.00
25% Percentile	88.15	87.45
Median recovery rate (%)	99.10	95.00
75% Percentile	125.3	128.0
Maximum recovery rate (%)	130.6	136.6
Mean recovery rate (%)	103.5	104.5
SD (%)	19.68	22.18

Table 1. Different internal standard addition procedures (NVP-*d3*) and the sequential impact on the NVP recovery

	Experiment 1 Disk 1-3	Experiment 2 Disk 4-6	Experiment 3 Disk 7-9	Experiment 4 Disk 10-12
Nominal NVP concentration (µmol/L)	29.1	29.1	29.1	29.1
Mean observed concentration (µmol/L)	31.1	24.8	15.6	3.2
Recovery (%)	106.9 ± 4.4	85.3 ± 7.0	53.6 ± 7.6	11.1 ± 0.2
Accuracy (%error)	6.9	-14.8	-46.4	-89.0
Precision (%CV)	8.9	15.9	7.8	7.6

Table 2. Influence of spotted blood volume on the recovery of NVP

DBS spot (µL)	DBS spot diameter (mm ± SD[b])	DBS spot area (mm^2 ± SD)	Disk size diameter (mm)	Observed NVP concentration (µmol/L ± SD)	Accuracy (%error)	Precision (%)	Recovery (% ± SD)
25	8.7 ± 0.2	60.2 ± 3.8	7	25.4 ± 2.2	-12.7	9.1	83.8 ± 7.6
15	7.4 ± 0.3[a]	37.6 ± 2.2	7	28.9 ± 1.3	-0.7	4.6	99.4 ± 4.6
10	5.8 ± 0.4[a]	25.3 ± 2.3	7	37.4 ± 0.8	28.5	2.2	128.6 ± 5.4

[a] completely punched by a 7 mm disk punch

[b] SD ; standard deviation

Conclusion

From the observed results with NVP and NVP-*d3* as model compounds we conclude that the optimal procedure for the addition of the IS to DBS specimen was spotting the IS solution onto the DBS paper prior to blood spotting if DBS specimen were used for NVP analysis in HIV-1 infected patients. This addition procedure is a compromise between an acceptable recovery rate for NVP and the feasibility for the clinical practice when working with infectious blood samples and when collecting samples in areas with limited resources, especially in developing countries.

Further experiments will be necessary to tests the influence of various drugs (different logP values), extraction solvents and DBS card materials (impregnated paper). These and further tests will help to find a consensus for validation procedures for routine DBS drug analysis.

Future perspective

Since DBS specimen can offer advantages but also analytical chemical challenges more experiments will be crucial to get a better knowledge on physical and chemical behavior of various drugs in DBS specimen analysis.

Executive summary

- The addition of internal standards for DBS analysis in infectious disease requires extra safety measurements to avoid infection of laboratory-and medical personnel. Addition of the internal standard to the DBS paper prior to blood spotting was selected for DBS analysis in infectious diseases due to safety issues.
- The procedure of internal standard addition in DBS analysis has a great impact on the observed Nevirapine concentration, especially when stable isotopes labeled internal standards are applied.
- The size of the punched disks from DBS specimen had no significant impact on the Nevirapine recovery rate, precision and accuracy.

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Chapter 3

Oncology

A new ultrafast and high-throughput mass spectrometric approach for the therapeutic drug monitoring of the multi-targeted anti-folate Pemetrexed in plasma from lung cancer patients

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Abstract

An analytical assay has been developed and validated for ultrafast and high-throughput mass spectrometric determination of pemetrexed concentrations in plasma using Matrix Assisted Laser Desorption/Ionization-triple quadrupole-tandem mass spectrometry (MALDI-QqQ-MS/MS). Patient plasma samples spiked with the internal standard methotrexate (MTX) were measured by multiple reaction monitoring (MRM). The detection limit (LOD) was 0.4 fmol/µL, lower limit of quantification (LLOQ) was 0.9 fmol/µL and upper limit of quantification (ULOQ) was 60 fmol/µL, respectively. Overall observed pemetrexed concentrations in patient samples ranged between 8.7 (1.4) and 142.7 (20.3) pmol/µL (SD). The new developed mass spectrometric assay is applicable for (routine) therapeutic drug monitoring of pemetrexed concentrations in plasma from NSCLC patients

Introduction

Pemetrexed (PTX, Alimta®; N-[4-[2-[2-Amino-4,7-dihydro-4-oxy-3H-pyrrolo[2,3-d]-pyrimidine-5-ylethyl]-benzoyl]-L-glutamic acid; Fig.1a) is a multi-targeted anti-folate drug applied in the treatment of malignant pleural mesothelioma (MPM) and non-small cell lung cancer (NSCLC) [1]. Therapeutic drug plasma concentrations are defined for various types of drugs, offering a useful tool for monitoring drug dosage and avoiding drug toxicity and obtain therapeutic efficacy.

PTX plasma concentrations can be determined by (High Performance) Liquid Chromatography with ultraviolet (HPLC-UV) [2-4] or mass spectrometric detection (LC-MS) [5]. HPLC-UV and LC-MS methods are time-consuming and less suitable for routine measurement of many samples. To avoid long analysis time, we applied Matrix Assisted Laser Desorption/Ionization- triple quadrupole-tandem mass spectrometry (MALDI-QqQ-MS/MS) for the development of a new assay. MALDI-QqQ-MS/MS does not require liquid chromatographic separation prior to analyses and thus can result in analysis times of approx. 10 seconds per sample and is therefore very suitable for the analysis of many samples in short time. The MALDI-QqQ-MS/MS technology has been used previously for the determination of drug concentrations [6-9] and for enzyme inhibition studies [10]. Here, we report a new ultrafast and high-throughput MALDI-QqQ-MS/MS application for therapeutic drug monitoring of pemetrexed concentrations in plasma from NSCLC patients.

Methods and materials

Chemicals

Pemetrexed(Alimta®;N-[4-[2-[2-Amino-4,7-dihydro-4-oxy-3H-pyrrolo[2,3-d]-pyrimidine-5-yl)ethyl]-benzoyl]-L-glutamic acid disodium salt heptahydrate) was obtained from the Department of Pharmacy, Erasmus MC and methotrexate (4-amino-10-methylpteroylglutamic acid, MTX) used as internal standard was from Schircks Laboratories (Jona, Switzerland). Solvents were of LC-MS grade (Biosolve,Valkenswaard, the Netherlands) and other chemicals were from ACS grade (Sigma Aldrich, Zwijndrecht, the Netherlands).

Preparation of plasma matrix-based calibrators and calibration curve

Standard stock solution (PTX: 600 nmol/L and MTX: 550 nmol/L) were prepared in LC-MS quality water. PTX plasma matrix-based calibrators were prepared by dilution with drug free human control plasma (Sanquin Blood Supply Foundation, Rotterdam, the Netherlands) to yield following calibrators: 60, 30, 15, 7.5, 3.75, 1.88 and 0.94 pmol/µL. Ten microliter of the calibrators were pipetted into 10 mL volumetric flasks and simultaneously deproteinized/diluted (1:1000) with an acetonitrile/water (60/40% v/v) mixture. Calibrators were centrifuged (5 min at 2000 x *g*) and 20 µL of supernatant were mixed with α-CHCA (10µL, 6.2 mg/mL in methanol/acetonitrile/water 36/56/8% (v/v/v), pH=2.5) matrix solution and 1.5 µL were spotted (n=3, 10 spots per QC sample) on a Opti-TOF 96-well stainless steel target plate (123 x 81 mm; MDS Analytical Technologies, Concord, Canada) and spots were dried at room temperature (5 min).

MALDI-QqQ-MS/MS instrumentation

Analyses were conducted by a Flashquant Workstation with a 4000 API mass analyzer (MDS Analytical Technologies, Concord, Canada) operating in the positive ionization mode. MS settings were: skimmer voltage 0 V, CAD gas 8 arbitrary units, source gas 10 arbitrary units, dwell time 20 ms, plate voltage 45 V, laser power 45% and laser raster speed 1 mm/sec. Instrument control/data analyses were performed using Flashquant 1.0 and Analyst 1.4.2 application software. Different MALDI matrices such as 2,5-dihydroxybenzoic acid (2,5-DHB), 7-hydroxy-4-(trifluoromethyl)-coumarin (HFMC) [18] and α-cyano-hydroxy-cinnamic acid (α-CHCA) were used to determine the highest possible sensitivity. Therefore, 10 µL of a PTX/MTX solution (approx. 100 nmol/L) were mixed with all MALDI matrices (10 µL) and analyzed in positive full scan mode (*m/z*=150 to *m/z*=350).

Assay validation

Validated assay parameters according to FDA guidelines [26] were: limit of detection (LOD), lower limit of quantification (LLOQ), linearity, accuracy, precision,

recovery and stability of PTX using quality control (QC) samples prepared in drug free human control plasma at 6.0, 30.0 and 60.0 pmol/µL.

Preparation of patient samples

EDTA-blood was collected (one week after admission) from patients who had received one dose of PTX (500 mg/m^2) and who have written consent for using their blood samples. Plasma was prepared by centrifugation for 10 min (4°C, 1400 x *g*).Ten µL of plasma were transferred into a 10.00 mL volumetric flask and spiked with 10 µL MTX (27.5 µmol/L) and deproteinized/diluted with an acetonitrile/water mixture (60/40% v/v). Precipitated proteins were removed by centrifugation (5 min at 2000 x g) at ambient temperature and 20 µL supernatant were mixed with MALDI matrix (10 µL) and 1.5 µL were spotted (n=10).

Results

MALDI-QqQ-MS/MS analysis

From all matrices, α-CHCA matrix gave the highest signal intensity for PTX and MTX. protonated ions of PTX and MTX ($(MH)^+$) were *m/z*= 428.2 for PTX and *m/z*= 455.2 for MTX. These protonated ions were used for further assay optimization. Optimized instrument parameters were: collision energy (CE) 38 and 25 V and collision cell energy exit potential (CXP) 13 and 10 V for PTX and MTX, respectively. Collision induced dissociation (CID) resulted in the formation of protonated fragment ions (Fig.1). Selected MRM transitions were for PTX *m/z*=428.2>281.2 and for MTX *m/z*=455.2>308.2.

Assay validation

Analyte recovery

To avoid laborious sample preparations by SPE or extraction, we tried to isolate PTX from plasma by deproteinization with trichloroacetic acid (TCA). We determined recoveries using QC samples (30 pmol/µL) deproteinized with different TCA concentrations; 5, 10, 20 and 30% TCA (% w/v). Recoveries ranged between 13 and 30% for PTX and between 20 and 62% for MTX. To obtain probably higher

recoveries, we transferred 100 µL aliquots of the supernatant of QC sample (10% TCA) onto a 96-well SPE method development plate (25 mg/well) (Sigma Aldrich, Zwijndrecht, the Netherlands). Recovery rates on following SPE adsorbents; PS/PVB (polysterene divinyl benzene), octadecyl (C18), octyl (C8), cyanopropyl (CN), MCAX (mixed- mode strong cation exchange) and WCX (weak cation exchange) were determined. Conditioning of the wells was done by acetone (2x1 mL), methanol (2x1 mL) and water (2x1 mL) except for WCX/SAX the last conditioning step used was 2 x 1 mL H_2O/10% TCA (% w/v). Extraction was done by 100 µL of methanol, 20 µL of the extract was mixed with α-CHCA (10 µL) and 1.5 µL were spotted (n=10). Recoveries were still low (<60%). Because of the low recoveries we decided to just deproteinize the QC samples by addition of acetonitrile/water mixtures. Ten µL were deproteinized/diluted (1:1000) with different acetonitrile/water mixtures; 40/60, 50/50 and 60/40% (v/v). The highest recoveries were obtained for 60:40 %(v/v) acetonitrile/water mixture; PTX (91%) and MTX (80%), while recovery rates dropped to 61 and 66% and 58 and 69% for PTX and MTX if we used 50/50% (v/v) and 40/60% (v/v) mixtures, respectively.

Linearity

The selected matrix-based calibrators (0.94-60 pmol/µL) displayed a linear relationship between MRM peak area and PTX concentration (r^2= 0.9973, y= 101.96x+84.245). Linear regression of the results was done using GraphPad Prism version 5.00 (GraphPad Software, San Diego California USA).

LLOQ and LOD

The lower limit of quantification (LLOQ) was defined as the lowest calibrator of the calibration curve that could be analyzed with an accuracy and precision of CV < 20% [26]. The LLOQ of PTX was 0.94 pmol/µL and upper limit of quantification (ULOQ) was 60 fmol/µL (CV<15%). The limit of detection (LOD) of PTX, defined at a signal-to-noise ratio of (3:1) in drug free human control plasma was 0.4 fmol/µL.

Accuracy and precision

The accuracy and precision were within FDA criteria (CV <15%) at all three validation levels (Table 1), precision expressed as % CV was between 11.9 and 31.8 % for within run and between 8.5 and 14.5% for between-run experiments. Accuracy expressed as % RSD ranged between 2.5 and 5.7% for within run and between 1.7 and 6.0% for between-run experiments.

PTX stability

PTX is according to FDA criteria defined as stable when the concentration decrease in plasma matrix was <15% (expressed as %Error), this was the case at all three QC validation levels (Table 1).

Application of assay to patient samples

Observed PTX plasma concentrations in analyzed patient samples are presented in Table 2.

Table 2. Measured pemetrexed concentrations in plasma from NSCLC patients (n=3)

Patient code	Pemetrexed concentration (pmol/µL) Mean (SD)	Gender[b]	Age
A	80.0 (9.3)	f	51
A	142.7 (20.3)*	f	51
B	8.7 (1.4)	f	56
C	63.0 (7.0)	m	66
C	60.7 (8.3)*	m	66
D	33.3 (4.9)	m	75
E	42.7 (9.7)	m	48
F	49.1 (10.8)	f	56
G	121.0 (20.2)	m	61

[a] results summarize 10 spots per patient sample,
[b] female (f) and male (m),
[c] Second administration of 500 mg/m^2, three weeks after first administration

Table 1. Precision and accuracy and stability experiments of developed assay at three different plasma concentration levels (n=3)

Analyte	Pemetrexed					
	Within-run validation[a]			Between-run validation[b]		
Nominal concentration (pmol/µL)	6.0	30.0	60.0	6.0	30.0	60.0
Mean observed concentration (pmol/µL)	6.2	31.7	58.5	5.9	31.8	62.2
Accuracy (% RSD[c])	3.3	5.7	2.5	1.7	6.0	3.7
Precision (% CV)	12.9	13.8	11.9	13.7	14.5	8.5

	Storage Conditions/Time[a]					
Nominal concentration (pmol/µL)	Refrigerator (8°C) 24 h		Freezer (-20°C) 20 days		Freezer (-20°C) 3 freeze/thaw cycles	
	Mean (SD)	%Error[d]	Mean (SD)	%Error[d]	Mean (SD)	%Error[d]
60.0	62.8 (6.0)	4.7	59.3 (5.8)	-1.2	58.7 (4.9)	-2.2
30.0	29.5 (3.8)	-1.7	31.5 (3.9)	5.0	29.2 (2.7)	-2.7
6.0	6.5 (1.2)	8.3	6.2 (0.8)	3.3	5.9 (0.5)	-1.7

[a] results summarize 10 spots per QC sample at each concentration level in one experiment.,
[b] results summarize three different experiments from 3 consecutive days with 10 spots per QC sample at each concentration level,
[c] RSD, relative standard deviation of the mean observed concentration,
[d] %Error=(mean observed concentration-nominal concentration)/(nominal concentration)*100%

Results show that patient A has an increased plasma concentration of PTX after the second administration compared to first administration, patient C though showed no increased plasma concentration level after the second administration. Other patients (B,D,E,F and G) only had received their first administration of PTX at the moment the samples were collected.

Discussion

Recently, it was demonstrated that MALDI-QqQ-MS/MS is a ultrafast, high-throughput and sensitive mass spectrometric technique [7,8,13-16] for small molecules. Analysis times for pemetrexed on HPLC -or LC-MS instrumentations are between 7 and 20 min [3,5,17] per sample measured by HPLC and up to 30 min per sample for LC-MS [18] while the MALDI-QqQ-MS/MS assay has an analysis time of approx. 10 s per sample (Fig.1). Fig.1 illustrates that multiple measurement of a plasma sample (n=24) can be accomplished within 4 minutes with acceptable accuracy (CV 13.1%).

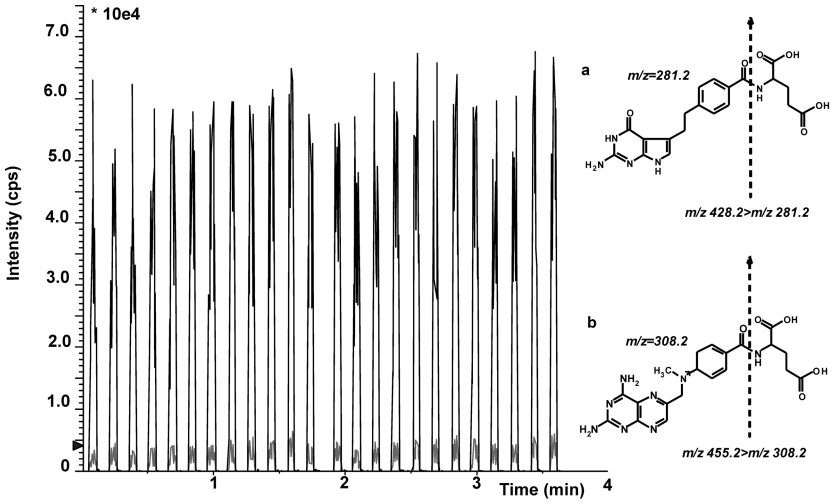

Figure 1. Example of ultrafast and high-throughput MALDI-QqQ-MS/MS analyses. Illustrated are reconstructed MRM traces of pemetrexed (black) and internal standard methotrexate (gray) from a QC sample containing pemetrexed at a concentration level of 30 fmol/µL. Chemical structure of (a) pemetrexed and (b) methotrexate and respective fragmentation and MRM transitions.

Some papers [2, 3, 5, 18] report the measurement of PTX in plasma and many papers use SPE. Hamilton et al. [3] reported recoveries for PTX depending on the PTX concentration, at a concentration of 15 ng/mL (35 fmol/µL), recoveries were 40% and at 1200 ng/mL (2760 fmol/µL) the recovery was 83%. Higher recoveries were reported by [2] (76.9%) while [5] reported recoveries of 99 and 124%. We studied also SPE but obtained only low recoveries for PTX and MTX. Instead, we tested different acetonitrile/water mixtures for PTX and MTX isolation from proteins in plasma. Due to expected PTX patient plasma concentrations in the micro molar range, simultaneous deproteinization and dilution (1:1000) of patient plasma samples were studied. The highest analyte recoveries were obtained with an acetonitrile/water (60:40% v/v) mixture, recoveries were 91% for PTX and 80% for MTX, respectively. The LOD of the assay is 0.4 fmol/µL, significantly lower than previously reported LODs of 23 fmol/µL and 6 fmol/µL [3-5], respectively.

The assay had been validated by the determination of linearity, recovery rates, within- and between run accuracy and precision and stability of PTX. The within-and between-run accuracy and precision as well as stability of PTX were all <15% CV and are in good compliance with the FDA regulation [12] (Table 1).

The total plasma amount spotted is 0.001 µL while others [35] injected in total 3.5 µL of plasma into the LC-MS system, demonstrating the very high sensitivity of the assay. After the validation the assay was applied to NSCLC patient samples. PTX concentrations ranged between 8.7 (1.4) and 142.7 (20.3) pmol/µL (SD). Two patients had already received a second dose (Table 2). One of the two patients showed a significant increase (142.7 (20.3) pmol/µL) compared to the first dose (80.0 (9.3) pmol/µL). This significant increase of the PTX plasma concentration cannot be explained by an increasing steady-state plasma concentration level between two administrations, since the half-life time of PTX is 3.5 hours [19]. More experiments/studies will be necessary to determine pharmacokinetic properties for extra-and intracellular concentrations of PTX to be able to understand significant increased plasma concentration levels in patients.

Conclusion

We have developed an ultrafast, sensitive and high-throughput assay for the determination of PTX concentrations in plasma from NSCLC patients. The assay can be used for therapeutic drug monitoring of PTX plasma concentration levels and the assay is so sensitive that it can support pharmacokinetic studies even with plasma amounts of few microliters.

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Ultra-fast selective quantification of methotrexate in human plasma by high-throughput MALDI-isotope dilution tandem mass spectrometry

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Abstract

Background

A new analytical mass spectrometric method using isotope dilution combined with MALDI-triple quadrupole MS/MS has been developed and validated for the determination of methotrexate and 7-hydroxymethotrexate in plasma. Methotrexate, methotrexate-*d3*, 7-hydroxymethotrexate and 7-hydroxymethotrexate-*d3* were monitored by selected reaction monitoring using the transitions *m/z* 455.2→308.2, *m/z* 458.2→311.2, *m/z* 471.2→324.2 and *m/z* 474.2→327.2 for methotrexate, methotrexate-*d3*, 7-hydroxymethotrexate and 7-hydroxymethotrexate-*d3*, respectively.

Results

The LLOQ was 1 nmol/l for methotrexate and 7-hydroxymethotrexate while the limit of detection was 0.3 nmol/l for both analytes. The new developed method was cross-validated by a fluorescence polarization immunoassay and tested for its clinical feasibility by measuring plasma samples from patients suffering from acute lymphoblastic leukemia. Plasma methotrexate concentrations ranged between 66.0 and 954 nmol/l and observed 7-hydroxymethotrexate/methotrexate ratios ranged between 0.1 and 32.4, respectively.

Conclusion

The new method showed comparable analytical performances as fluorescence polarization immunoassay, but analyte specificity and sensitivity of the new developed method were significantly better.

Introduction

Methotrexate (N-[4[[(2,4-diamino-6-pteridinyl) methyl] methyl-amino] benzoyl]-l-glutamic acid; MTX) is a prototype folate antagonist cytotoxic drug used in the treatment of a number of malignancies or non-malignant disorders such as acute lymphoblastic acute lymphoblastic leukemia (ALL) [1], severe psoriasis [2] and (adult) rheumatoid arthritis (RA) [3,4]. MTX is a cytotoxic compound acting in active replicating cells but not only in neoplastic cells but also to other cells and tissues. Moreover, patients receiving MTX sometimes develop adverse events due to increased patient susceptibility, excessive administration and therapeutic errors by patients due to wrong intake schedules (e.g. taking the drug daily instead of weekly). Toxic adverse effects may occur hours to weeks after the last recent MTX administration. MTX is administrated in a (high) toxic dose and after 24 h the patient starts with the administration of folic acid as a kind of 'antitoxic rescue' to reduce the MTX toxicity and plasma concentration. Therapeutic drug monitoring (TDM) of cytotoxic lack of established therapeutic plasma concentration levels [5]. One exception is MTX; (too) high plasma concentration levels are considered to be a reasonable risk for developing drug toxicity. Therefore, TDM of MTX plasma concentrations applied for observation of patient adherence is often mandatory, especially when used to determine patients with impaired MTX clearance. TDM is not only very suitable to reduce and prevent toxicity during treatment but also acute toxic adverse events of MTX might be observed especially during prolonged MTX therapy. While intrinsic factors determine an individual patient's response to MTX, adverse events are not yet fully understood, but a better understanding of individual differences in pharmacokinetics might help to improve the patient's treatment response and minimize MTX toxic adverse events.

Determination of MTX concentrations in biological fluids is mainly performed by HPLC in combination with fluorescence detection [6–8], UV detection [9] or MS detection [10–12], but CE [13] and the application of immunoassays are also possible. Fluorescence polarization immunoassay (FPIA) [14] and radioimmunoassay [15,16] are frequently used in clinical laboratories alongside the mentioned LC separation methods. Although these commercial immune assay based methods are relatively fast and easy to perform, they have the disadvantage of having a low

analyte specificity because of cross reactivity with MTX metabolites such as 7-OH-MTX and DAMPA (2,4-diamino-N10-methylpteroic acid) and MTX-polyglutamates.

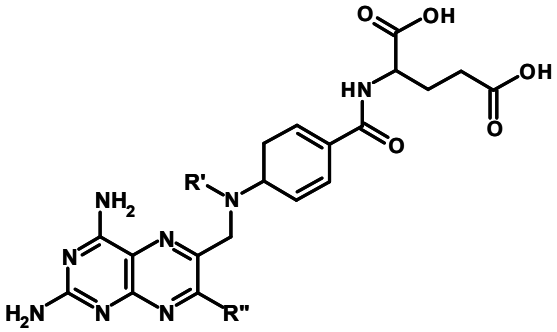
Sample treatment for MTX concentration determination is mandatory in several analytical methods, but sample treatment can sometimes be laborious and time consuming. Many methods applied in clinical laboratories for the determination of MTX concentrations in plasma, a simple removal of plasma proteins [17] is applied or MTX is isolated by liquid–liquid extraction [18,19] or SPE [7,12,20–22]. We choose to apply MALDI-triple quadrupole (QqQ)-MS/MS for TDM of MTX and 7-OH-MTX plasma concentrations in ALL patients on MTX therapy. MALDI-QqQ-MS/ MS is a relatively new MS technology (commercially launched in 2008) that does not necessarily require LC separation of samples prior to MS analysis. By omitting LC separation, analysis times are reduced considerable and analysis times of approximately 10 s per sample [23,24] or less is possible. Due to these very short analysis times and the high analyte specify due to MS detection, MALDI-QqQ-MS/MS is perfectly suitable for ultrafast and high-throughput analysis and it has been demonstrated to have equal analytical performances as HPLC in combination with ESI [25] and MS detection. We previously demonstrated that the new MALDI-QqQ-MS/MS technology has a great usability and is a versatile tool in the determination of concentrations (extra- and intra-cellular) of small molecules such as antiretroviral drugs [26–28], anticancer drugs [23]; others also reported good feasibility [24,29–31]. MALDI-QqQ-MS/MS could also be applied as a screening tool in enzyme kinetic studies [32] and it was very recently successfully used in combination with an isotope-dilution method for the quantitative determination of the antiretroviral drug tenofovir [28]. For the measurement of MTX and 7-OH-MTX concentrations we also selected isotopic-dilution analysis because in this way we could increase the precision and accuracy of the MS analysis. Therefore, to the plasma samples a known amount of the isotopic-labeled analytes (e.g., MTX-*d3* and 7-OH-MTX-*d3*) was added and then, by measuring the isotopic ratio between isotopic-labeled analytes and non-labeled analytes, plasma concentrations (MTX and 7-OH-MTX) could be calculated [33]. The aim of the present study was to develop a new MS assay applying isotope dilution in combination with MALDI-QqQ-MS/MS followed by statistical comparison of the new developed method with the FPIA assay frequently used in the clinical laboratory for MTX plasma analyses. We also applied the new

developed method for measurement of MTX concentrations over time (pharmacokinetics) and compared observed MTX concentrations with MTX concentrations determined by FPIA.

Experimental

Chemicals and reference materials

Methotrexate (MTX), 7-hydroxymethotrexate (7-OH-MTX) and the internal standards methotrexate-*d3* (MTX-*d3*) and 7-OH-methotrexate-*d3* (7-OH-MTX-*d3*) were purchased from Schircks Laboratories (Jona, Switzerland). LC–MS-grade methanol, acetonitrile and water were obtained from Biosolve (Valkenswaard, The Netherlands). The FPIA was purchased from Abbott Laboratories (Abbot Park, IL, USA). Chemical structures of MTX and the metabolite 7-OH-MTX and deuterated analogues applied are illustrated in Figure 1.



	R’	R”	SRM (*m/z*)
MTX	CH_3	H	455.2→308.2
MTX-*d3*	CD_3	H	458.2→311.2
7-OH-MTX	CH_3	OH	471.2→324.2
7-OH-MTX-*d3*	CD_3	OH	474.2→327.2

Figure 1. Chemical structures of analytes and deuterated analogues and its SRM transitions

MALDI-QqQ-MS/MS conditions

Prior to method development and US FDA validation of the method, the MALDI ionization efficacies of MTX and the 7-OH-MTX were studied by applying

different MALDI matrix ionization compounds. MALDI matrix compounds (10 mg/ml) such as 2,5-dihydroxy benzoic acid (2,5-DHB), 7-hydroxy- 4-(trif luoromethyl)-coumarin (HFMC) [34] and α-cyano-hydroxy-cinnamic acid solution (α-CHCA) were tested. For this purpose, a mixture containing 100 nmol/l MTX and 7-OH-MTX were mixed (1:1) with the different MALDI matrix solutions and analyte signal intensities were determined and compared. The highest signal intensities for both analytes were obtained by the α-CHCA MALDI matrix compound. Furthermore, the optimal mixing ratio between analyte amount and MALDI matrix solution was also determined. The MALDI-QqQ-MS/MS instrumentation used was a FlashQuant™ Workstation containing a FlashLaser source (349 nm; 1000 Hz) combined with a 4000 API mass analyzer (AB Sciex, Concord, Canada) operating in the positive ionization mode. Analyses were carried out by selected reaction monitoring (SRM) at unit resolution. In SRM mode, monitored ion transitions were: *m/z* 455.2→308.2, *m/z* 458.2→311.2, *m/z* 471.2→324.3 and *m/z* 474.2→327.2 for MTX, MTX-*d3*, 7-OH-MTX and 7-OH-MTX-*d3*, respectively. Optimized MALDI-QqQ-MS/MS instrument parameters used were: laser power 50%, target plate voltage 45 V, skimmer voltage 0 V, collision gas (CAD) 11 arbitrary units, collision cell energy exit potential (CXP) 12 V, source gas 15 arbitrary units, dwell time 10 ms and laser raster speed was set to 1 mm/s while collision energies were 25, 28, 30 and 30 V for MTX, MTX-*d3*, 7-OH-MTX and 7-OH-MTX-*d3*, respectively. Instrument control and data acquisition were performed by Flashquant 1.0 software and Analyst 1.4.2 application software (AB Sciex).

Calibration curves MTX and 7-OH-MTX

Calibration curves for both analytes were determined by the use of plasma-based calibrators. MTX and 7-OH-MTX plasma matrix-based calibrators were prepared by spiking drug free human control plasma (Sanquin Blood Supply Foundation, Rotterdam, The Netherlands) with MTX and 7-OH-MTX to yield following calibrators: 60, 40, 20, 10, 5, 4, 2, 1 and 0 (blank) μmol/l for MTX and 7-OH-MTX, respectively. From each calibrator 10 μl was transferred into 10-ml volumetric flasks and simultaneously deproteinized/diluted (1:1000) with methanol/water (60/40% v/v) , resulting in plasma-based calibrators in the concentration range 1.0 to 60 nmol/l. Subsequently, 20 μl of the diluted plasma-based calibrators were mixed with a-CHCA

(20 μl, 6.2 mg/ml methanol/acetonitrile/water 36/56/8% v/v/v; pH= 2.5) matrix solution and 0.5 μl were spotted (ten spots per calibrator) on an Opti- TOF 96-well stainless steel target plate (123 × 81 mm; AB Sciex, Concord, Canada) and spots were left to dry and crystallize for 5 min at ambient temperature prior to MALDI-QqQ- MS/MS analyses.

Method validation

The new developed MALDI-QqQ-MS/MS method was validated according to most recent FDA Guidelines for Industry – Bioanalytical Method Validation by validation of the following assay specific parameters [101]: linearity, limit of detection (LOD), LLOQ, intra- and inter-run accuracy and precision and stability of MTX in the plasma matrix. Intra-day and between-day precision and accuracy and the stability of MTX were determined using plasma-based quality control (QC) samples prepared in drug free plasma from healthy controls at MTX concentration validation levels of 55.0, 27.5 and 5.5 μmol/l and spiked with MTX-*d3* and diluted/deproteinized (1:1000) with a methanol/water (60/40% v/v) mixture to obtain QC samples at 5.5, 27.5 and 55.0 nmol/l. Only MTX was validated according to FDA guidelines because of its therapeutic relevance in plasma. The 7-OH-MTX metabolite has a 200-fold less potent inhibition capacity than MTX of the dihydrofolic acid reductase [35] and has no or limited relevance in a therapeutic and pharmacological perspective. Three replicates from each QC were measured by three separate runs in order to assess the intra-and inter-run accuracy and precision. The chemical and biological stability of MTX in the plasma matrix was determined at three different storage conditions at all three QC concentration levels.

Furthermore, the accuracy of the MTX-*d3*/MTX and 7-OH-MTX-*d3*/7-OH-MTX isotopic ratio measurements were determined by analyses of plasma matrix based isotopic ratio calibrators at different atom percent excess ratios (APE) prepared by mixing defined amounts of the MTX-*d3* or 7-OH-MTX-*d3* solution (40 μmol/l) with a MTX or 7-OH-MTX solution (40 μmol/l) spiked in drug-free control plasma from healthy controls, followed simultaneously deproteinized/diluted (1:1000) with a methanol/water (60/40% v/v) mixture. Applied APE calibrators for the accuracy determination were 99.01, 98.04, 96.15, 90.91, 83.33, 71.43, 50.0, 33.3 and 0%.

Preparation of defined APE ratio calibrators was using the following calculation [28,33]:

$$APE = \frac{AR_{corrected}}{1 + AR_{corrected}} \times 100\% \quad (1)$$

Where $AR_{corrected}$ is the measured isotopic ratio between MTX-*d3*/MTX and/or 7-OH-MTX-*d3*/7-OH-MTX (both ratios corrected for the (m+3) natural isotopic ratio of MTX and/or 7-OH-MTX.

Analyte specificity of the new developed method for MTX and 7-OH-MTX was determined by the measurement of twenty plasma samples collected from healthy controls. The plasma samples were prepared for MALDI-QqQ-MS/MS analysis by the protocol described in the patient sample preparation protocol section.

Patient sample preparation protocol

Whole blood samples from ALL patients receiving for during 24 hrs intravenous 2-5 g/m^2 MTX were collected 24 hrs after the last admission at the outpatient clinic of the Pharmacy Department at the Erasmus University Medical Center Rotterdam. Because we used anonymized and left-over patient material from routine diagnosis, no informed consent was necessary. In the time period between October, 2009 and May, 2010 whole blood from 50 selected patients were included in present study. Included patients were monitored by drawing blood at different time points after admission of the medication. Whole blood from patients were collected in potassium-EDTA blood collection tubes and after collection kept on ice until centrifugation for 10 min (1400 x *g*). Plasma was separated from the erythrocyte pellet and stored immediately at a temperature of -80°C. An aliquot of 10 µL of the patient plasma samples were spiked with 10 µL of a solution containing MTX-*d3* and 7-OH-MTX-*d3* solution (10 µmol/L) and diluted to 1.00 mL by addition of a methanol/water mixture (60/40% v/v). An aliquot of 20 µL of diluted plasma samples were mixed (1:1) with α-CHCA MALDI matrix solution, 0.5 µL of each aliquot was spotted (n=5) onto the MALDI target plate and sample spots were analyzed after drying and crystallization for 5 min at ambient temperature. The plasma MTX and 7-

OH-MTX concentrations (nmol/L) of patient plasma samples were calculated using the following formula (1) [28, 33]:

$$(2) \qquad C_{MTX/7OHMTX} = \frac{V_{IS} \times C_{IS}}{AR_{std} \times V_{plasma}} \times 1000$$

where V_{IS} is the volume of MTX-*d3*/7-OH-MTX-*d3* internal standard (µL), C_{IS} is the concentration of MTX-*d3*/7-OH-MTX-*d3* standard (pmol/µL), AR_{std} is the isotopic ratio between MTX-*d3*/MTX and/or 7-OH-MTX-*d3*/7-OH-MTX (both ratios corrected for the natural (m+3) isotopic ratio) measured in the patient plasma sample and V_{plasma} the volume of plasma (µL) used for measurements.

Cross-validation by FPIA

Plasma samples prepared from whole blood collected from ALL patients were divided into two aliquots at the laboratory of the Department Clinical Chemistry at the Erasmus University Medical Center in Rotterdam, The Netherlands.One aliquot was directly analyzed by FPIA for MTX concentration and the second aliquot was frozen directly at -80°C for MALDI-QqQ-MS/MS analyses. For FPIA analyses, the MTX II reagent Pack was purchased from Abbott Laboratories (Abbot Park, IL, USA). The reagent pack was applied according to the protocol described by the manufacturer and FPIA analyses were performed using a TDx analyzer (Abbott Laboratories). The MTX II assay has the capability of determining MTX concentrations up to 1000 µmol/l without manual dilution and the detection limit is 0.02 µmol/l. Observed MTX plasma concentrations by FPIA analyses and MALDI-QqQ-MS/MS analyses were statistically cross validated with Bland–Altman method comparison [36,37]. This comparison calculates the bias between both analytical methods and the 95% confidence interval.

Results and discussion

Method development

We applied the relative new MALDI-QqQ-MS/MS technology (launched in the year 2008) for the measurements of MTX plasma concentrations in plasma samples

from ALL patients. This technology omits a LC separation, but due to the application of SRM in combination with stable isotope-labeled analogues, has very high analyte specificity and sensitivity. MALDI-QqQ-MS/MS analyses are therefore ultrafast due to no chromatographic separation, and this technology is thus applied for high-throughput analyses of large numbers of (patient) samples. The analysis time of one sample with this technology is approximately 10 s, which provides the possibility of measuring a MALDI 96-well target plate within 20 min. We applied this technology for the development of a new bioanalytical method for the determination of MTX concentrations in plasma. The new method was cross validated against FPIA, a technique frequently used in the clinical laboratory for MTX concentration measurements. The new MALDI-QqQ-MS/MS method appeared to be ultrafast, high-throughput and very sensitive, the obtained sensitivity was 60-fold higher (LOD: 0.3 nmol/l) than the sensitivity of the FPIA (LOD: 20 nmol/l). Next to the high sensitivity obtained by the MALDI-QqQ-MS/MS technology, the significantly higher selectivity of MS for selected analytes was an important property of the method. Due to very limited cross-reactions possible with MS, this technology improved the reproducibility of the MTX concentration measurement in plasma, especially in the low-concentration range were cross-reaction problems with FPIA are observed and specificity becomes limited. The most frequently applied methods in the clinical laboratory for the determination of plasma MTX concentrations are LC in combination with UV, fluorescence or MS detection. Mean analysis times of these separation methods range between 2 and 40 min per sample [38] depending on the instrumentation and separation method used.

In contrast, immunoassay- based methods such as FPIA are mainly based on the change in fluorescence polarization induced by the drug (antigen) and antibody (monoclonal drug antibody). Immunoassays, in general, are analyte-specific although cross-reactions [14] with MTX-polyglutamate metabolites are frequently observed, especially in complex biological samples such as plasma from diseased patients or samples with low-analyte concentrations. MS methods can overcome these problems due to higher specificity and sensitivity in combination with, for example, stable isotope labeled internal standard

MALDI ionization efficacy

An analyte-MALDI matrix solution ratio of 1:1 resulted in the maximum signal intensity possible for MTX and 7-OH-MTX. Analytes in α-CHCA matrix resulted in the formation of protonated analyte ions $(MH)^+$; *m/z*= 455.2 and *m/z*= 471.2 for MTX and 7-OH-MTX, respectively. These protonated ions were used for further optimization of method parameters such as; laser power, plate voltage, collision gas (CAD), source gas and collision energy (CE). Collision induced dissociation (CID) was applied for determination of the SRM transitions of both protonated analyte ions resulting in the formation of several (protonated) fragment ions. The fragment ion with the highest abundance and selectivity was selected for SRM analysis. Selected fragment ions of MTX and 7-OH-MTX for SRM analysis were the result of the cleavage of the intermolecular peptide bond what resulted in the loss of a neutral glutamate moiety (loss of 147 Da) in combination with the rearrangement of hydrogen. Cleavage of the peptide bond resulted for 7-OH-MTX-*d3* and MTX-*d3* in the conservation of the d3-isotopic label within chosen SRM transition, selected transitions were: MTX (*m/z* 455.2 →308.2), MTX-*d3* (*m/z* 458.2→ 311.2), 7-OH-MTX (*m/z* 471.2→ 324.4) and 7-OH-MTX-*d3* (*m/z* 474.2→327.2), respectively.

Plasma method development & US FDA validation

The new developed method was validated by the determination of linearity, recovery rates, intra and inter-run accuracy and precision, and stability of MTX in plasma matrix. The intra- and inter run accuracy and precision as well as stability were all in compliance with recent FDA regulation for the validation of (new) bioanalytical assays (Table 1). After full validation, ALL patient samples were measured on MTX plasma concentrations. The method was validated for assay parameters according to the FDA's Guidance for Industry–Bioanalytical Method Validation procedure [39]: LOD, LLOQ, linearity, accuracy, precision, recovery and stability of the analyte in the plasma matrix. The sensitivity and linearity range of the MALDI-QqQ-MS/MS method were determined by measurement of plasma-based calibrators. The plasma-based calibration curve showed linear relationships between the SRM peak area (y) and concentration (x) (MTX: r^2= 0.9965, y= 1.891x-146.1 and 7-OH-MTX: r^2= 0.9987, y= 2.579x+16.15) for both analytes. The LLOQ and LOD were determined according to FDA guidelines; the LLOQ was defined as the lowest

plasma-based calibrator from the calibration curve that could be measured with acceptable precision and accuracy (CV<20%). The observed LLOQ for MTX and 7-OH-MTX were 1.0 nmol/l, respectively. The ULOQ was defined as the highest plasma-based calibrator that could be measured with CV<15% which was 60 nmol/l for MTX and 7-OH-MTX, respectively.The LOD determined at a signal-to-noise ratio (3:1) of drug-free human control plasma was 0.3 nmol/l for MTX and 7-OH-MTX. The accuracy of isotopic ratio measurement of MTX was determined by application of isotopic ratio calibrators prepared in drug-free control plasma spiked at a concentration of 40 nmol/l MTX by addition of different amounts of MTX-*d3*. Isotopic ratios were expressed as APE calibrators and accuracy (expressed as %error) of the isotopic ratio determination in the plasma matrix was determined by regression of calculated APEs (APE calibrators) and measured APEs. Measured APE indicated a good correlation between measured APE (y) and calculated APE (x), the linear regression line equation was y= 0.9731x with a regression coefficient of r^2= 0.9997 and average accuracy of the (m+3) isotopic ratio determination was in average 2.15% (determined over the complete APE calibration line).The specificity of the developed method for MTX and 7-OH-MTX by analysis of plasmasamples (in triplicate) from 20 healthy controls was good. For both analytes, MTX and 7-OH-MTX, using the SRM table (section MALDI-QqQ-MS conditions) observed concentrations were <LOD (0.3 nmol/l).

Accuracy, precision and stability

Intra- and inter-run accuracy and precision of the method were determined at three plasma based QC concentration levels (5.5, 27.5 and 55 nmol/l MTX). Observed intra- and inter-run accuracy and precision were in good compliance with the FDA criteria for bioanalytical assays.Precision of assay expressed as %CV was between 6.5 and 10.0% for intra-run measurements and between 8.1 and 11.1% for inter-run measurements. The accuracy expressed as a percentage ranged between 7.2 and 10.5% for intra-run measurements and between 5.5 and 9.1% for inter-run measurements. We found that MTX was chemically and biologically stable in the plasma matrix because the observed decrease in MTX concentration for all three storage conditions was <15% (expressed as %error) as defined by the FDA guidelines (Table 1) [101].

MALDI-QqQ-MS/MS assay application to patient samples

After FDA validation of the new developed MALDI-QqQ-MS/MS method, the method was tested for its clinical use by measurement of patient samples from ALL patients receiving intravenous MTX. Observed MTX and 7-OH-MTX plasma concentrations determined in 50 ALL patients are illustrated in Figure 2A, and calculated 7-OH-MTX/MTX ratios are illustrated in Figure 2B. Observed MTX plasma concentrations in the plasma of ALL patients showed a distribution of the MTX concentration between 66 and 954 nmol/l. This broad concentration range could be explained by variation in the time of blood collection after the intake of the MTX dose, which had to be at least 24 h or more. Cancellation of the admission of folic acid is possible if MTX plasma concentration level are <400 nmol/l at 48 h and/or <250 nmol/l at 72 h after MTX admission. Observed MTX plasma concentration levels of studied patients were all <250 nmol/l at 72 h after MTX admission. When plasma concentration levels at these two time points after oral intake rise above these levels, they are considered a high risk for developing MTX toxicity [7]. Moreover, it could be concluded from observed patients' MTX plasma concentrations at 72 h that each patient was in good adherence with the prescribed MTX drug regime. The 7-OH-MTX/OH ratios observed by the MALDI-QqQ-MS/MS method ranged between 0.1 and 32.4, which were in good correlation with previously reported ratios [19] observed at 24, 48 and 72 h after MTX admission.

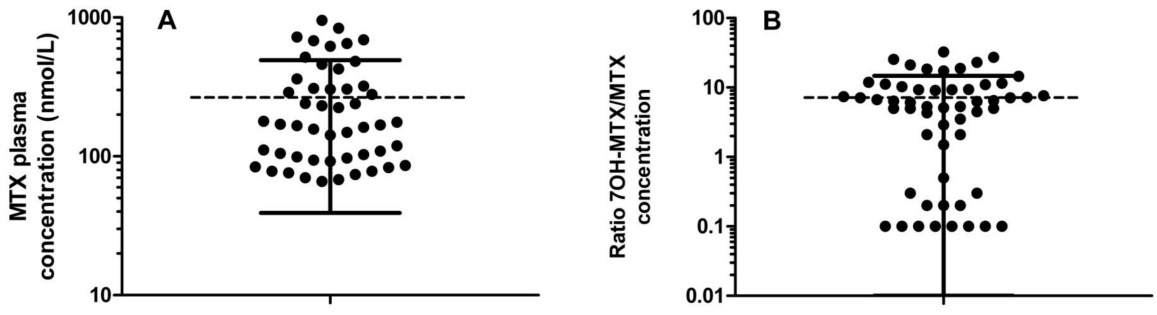


Figure 2. Observed concentrations of (A) MTX in 50 plasma samples from acute lymphoblastic acute lymphoblastic leukemia patients and (B) ratio between the 7-OH-MTX metabolite concentration and MTX concentration in patient plasma (7-OH-MTX/MTX). Dotted line represents mean, and solid lines represent mean ± 2SD (standard deviation of mean.

Table 1. Precision, accuracy and stability experiments of methotrexate at three different plasma concentration levels

Analyte	Methotrexate (MTX)					
	Within-run validation[a]			Between-run validation[b]		
Nominal concentration (nmol L^{-1})	5.5	27.5	55.0	5.5	27.5	55.0
Mean observed concentration (nmol L^{-1})	5.9	30.1	60.8	6.0	29.0	58.7
Accuracy (% error[c])	7.2	9.5	10.5	9.1	5.5	6.7
Precision (% CV)	10.0	7.9	6.5	10.6	11.1	8.1

	Storage Conditions/Time[a]					
Nominal concentration (nmol L^{-1})	Refrigerator (4°C)		Freezer (-20°C)		Freezer (-20°C)	
	24 h		20 days		3 freeze/thaw cycles	
	Mean (SD)	%Error[d]	Mean (SD)	%Error[d]	Mean (SD)	%Error[d]
55.0	58.7 (4.7)	6.7	54.4 (5.8)	-1.1	56.1 (3.9)	2.0
27.5	28.9 (3.2)	5.1	27.1 (5.4)	-1.5	27.1 (1.8)	-1.5
5.5	5.9 (0.6)	7.3	5.8 (4.7)	5.5	5.7 (0.6)	3.6

[a] results summarize 10 spots per QC sample (n=3) at each concentration level in one experiment,
[b] results summarize three different experiments from 3 consecutive days with 10 spots per QC sample (n=3) at each concentration level,
[c] %error=(mean observed concentration-nominal concentration)/(nominal concentration)*100%
[d] %error=(mean observed concentration-nominal concentration)/(nominal concentration)*100%

The therapeutically relevance of the 7-OH-MTX/MTX ratio has to be studied in more detail because a previously published paper reported the use of the 7-OH-MTX/MTX ratio as a kind of 'endogenous rescue' in high dose MTX admissions, this in contrast to the rescue with folic acid and patients were categorized as having a lower susceptibility to develop MTX toxicity with a high ratio [40].

Cross-validation by Fluorescence Polarization Immunoassay (FPIA)

Cross validation of the MTX plasma concentrations determined by the MALDI-QqQ-MS/MS method and FPIA (Pharmacy Department, Erasmus, MC) was carried out by calculating the variance in MTX concentrations observed between both methods using Bland–Altman method comparison statistics [37,38]. The cross validation showed a good correlation between both methods (Figure 3). For cross validation purposes, plasma samples were separately prepared prior to analysis at both laboratories (Neurology and Pharmacy), and MTX stock and calibrator solutions were not exchanged between both laboratories. The linear regression of observed MTX concentrations in patients' plasma samples by FPIA and MALDI-QqQ-MS/MS methods are illustrated in Figure 3A.

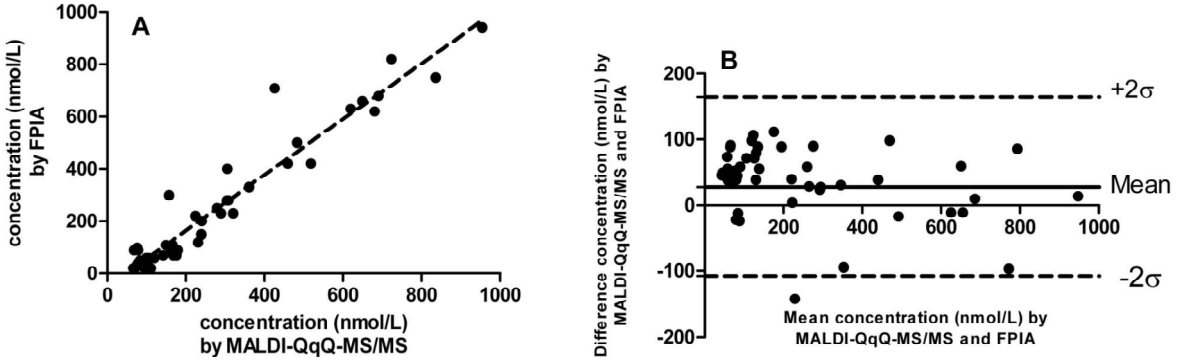


Figure 3. (**A**) Comparison of observed MTX plasma concentrations. Comparison by (A) FPIA and MALDI-QqQ-MS/MS method and

(**B**) Bland-Altman plot of differences between observed MTX concentrations by FPIA and MALDI-QqQ-MS/MS methods. Solid line represents mean, and dotted lines represent mean ± 2SD (standard deviation of mean)

Both methods illustrate a relative good correlation (r^2 = 0.9821, y = 1.065x - 48.58) between MTX plasma concentrations observed by both analytical methods but a better presentation of differences in MTX concentrations observed with both methods are better expressed by calculation and presentation of a Bland–Altman plot which is presented in Figure 3B. All observed differences in MTX concentrations by both analytical methods were between the mean of both assays ±2 SD (95% confidence from -103.5 to 166.2, mean bias 31.36), except for one patient (Figure 3B). It was concluded that there was a good agreement between the MALDI-QqQ-MS/MS method and FPIA assay. Nevertheless, the new developed MALDI-QqQ-MS/MS method had a higher sensitivity (LOD 0.3 nmol/l) compared with the FPIA method, which had a LOD of 20 nmol/l. The new developed MALDI-QqQ-MS/MS showed also comparable results with FPIA when the MTX plasma concentrations were measured over time. Figure 4 illustrates the MTX plasma concentration– time profile of MTX in one selected ALL patient after an intravenous dose of 2–5 g/m2 MTX. It can be observed that at lower MTX plasma concentrations, higher MTX concentrations by the MALDI-QqQ-MS/MS method are measured than by the FPIA method. No clear explanation for these phenomena could be given; in the future it will need more in-depth research. Measured MTX plasma concentration measured were above the LLOQ of both analytical methods, so a MTX concentration quantification problem was excluded.

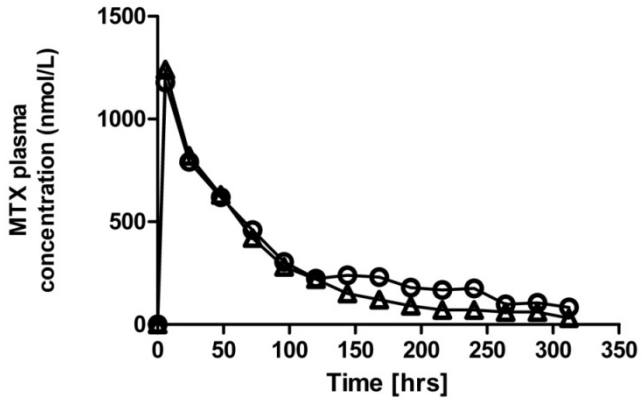


Figure 4. MTX plasma concentration-time profile from an acute lymphoblastic acute lymphoblastic leukemia patient after a 24 hrs intravenous admission of 2-5 g/m^2 MTX. Symbols illustrate analyses of the MTX plasma concentrations **(**∆**)** MALDI-QqQ-MS/MS and **(**○**)** FPIA

Overall, both methods perform statistically equal and the new developed method can therefore, from an analytical point of view, be applied for determination of MTX plasma concentration levels in plasma samples for TDM purposes or even for determination of patient related pharmacokinetics (Figures 3 and 4).

Conclusions

The newly developed MALDI-QqQ-MS/MS assay is suitable for accurate measurement of therapeutically relevant MTX plasma concentrations in ALL patients and also performed satisfactorily in MTX pharmacokinetic studies. The new developed MALDI-QqQ-MS/MS method performed statistically equally to the current clinical laboratory used FPIA assay, but the sensitivity and analyte specificity of the MALDI-QqQ-MS/MS method were higher. The new developed MS method is ultrafast and high-throughput and can thus be applied for the measurement of large numbers of patient plasma samples in short time.

Future perspective

Ultrafast determination of methotrexate plasma concentration levels offers the possibility of better and faster TDM of patients and will prevent the development of methotrexate toxicity especially in patients with impaired methotrexate clearance or a low adherence.

Executive summary

- An accurate, ultrafast, and high-throughput isotope dilution-MALDI-QqQ-MS/MS method has been developed and validated for the determination of methotrexate and its metabolite, 7-hydroxymethotrexate.
- Methotrexate and 7-hydroxymethoterxate were measured using a ABI Sciex 4000 API mass spectrometer combined with a FlashQuant MALDI source containing a 1000 Hz solid state UV laser and α-cyano-hydroxy-cinnamic acid as MALDI matrix compound
- Positive ionization was used in selected reaction monitoring mode applying following transitions: *m/z* 455.2→ 308.2, *m/z* 458.2→311.2, *m/z* 471.2→324.2 and

m/z 474.2→327.2 for methotrexate, methotrexate-*d3*, 7-hydroxymethotrexate and 7-hydroxymethotrexate-*d3*, respectively.

- The MALDI-QqQ-MS/MS method was so sensitive that plasma based calibrators and patient plasma samples subsequently had to be diluted, this in contrast to the FPIA assay were plasma without dilution can be analyzed. The linear concentration range of the MALDI-QqQ-MS/MS method was 1.0 to 60 nmol/L while the linear range of the FPIA assay was between 20 nmol/L up to 1000 µmol/L.
- Linear calibration curves of methotrexate and 7-hydroxymethotrexate were generated over the concentration range of 1.0-60 nmol/L and exhibited consistent linearity and reproducibility with correlation coefficients (r^2) greater than 0.9960.
- The LLOQ of methotrexate and 7-hydroxymethotrexate were 1 nmol/L and limit of detection (LOD) were 0.3 nmol/L, respectively. The LOD of the new developed MALDI-QqQ-MS/MS method was 60 times lower than the applied FPIA assay (20 nmol/L).
- Patient plasma samples were deproteinized and diluted with a mixture methanol/water (60/40% v/v)
- Methotrexate concentrations in patients suffering from acute lymphoblastic leukemia (ALL) ranged between 66.0 and 954 nmol/L while observed ratio's between the 7-hydroxymethotrexate metabolite and methotrexate ranged between 0.1 and 32.4, respectively.
- Cross-validation of the new developed method with a fluorescence polarization immune assay (FPIA) which is applied as the "golden standard" in this institution for methotrexate analysis showed comparable analytical performances with the difference that the new developed method was more sensitive and analyte specificity was better.Although the linear range of the MALDI-QqQ-MS/MS methods is significantly smaller, the advantages of this method are the application of stabile isotope labeled internal standards to correct for plasma matrix interferences, the possibility to measure next to MTX also the 7-OH-MTX metabolite and the high-throughput (360 samples/hr) and ultrafast properties (1 analysis in 10 sec) of the method and the increase of the sensitivity of the MTX measurement by MALDI-QqQ-MS/MS of a factor 60 compared to FPIA. The new

developed method is a desirable solution for the clinical laboratory, especially with big samples numbers and big patient studies

Future perspective

Ultrafast determination of methotrexate plasma concentration levels offers the possibility of better and faster therapeutic drug monitoring (TDM) of patients and will prevent the development of methotrexate toxicity especially in patients with impaired methotrexate clearance or a low adherence. A faster analytical method is costs effective which a desirable option for the clinical laboratory is.

Acknowledgement

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Chapter 4

Auto-immune diseases

Assessment of intracellular methotrexate and methotrexate-polyglutamate concentrations in erythrocytes by ultrafast MALDI-triple quadrupole tandem mass spectrometry

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Rapid Communications in Mass Spectrometry 2011; 25(20): 3063-3070

Abstract

A new ultrafast quantitative and high-throughput mass spectrometric method using Matrix-Assisted Laser Desorption/Ionization-triple quadrupole-tandem mass spectrometry has been developed and validated for determination of intracellular erythrocyte concentrations of the antifolate drug methotrexate (MTX) and its polyglutamate metabolites. The method consist a solid phase extraction of MTX and MTX-polyglutamate metabolites from deproteinized erythrocyte lysates spiked with aminopterin as internal standard. The new developed method was validated according to the most recent FDA guidelines on linearity, recovery, within-run and between run accuracy and precision and stability of the analytes. The low limit of quantification (LLOQ) was 10 nmol/L for all analytes while the limit of detection (LOD) determined at a signal-to-noise ratio (S/N=3:1) in drug free erythrocyte lysate was 0.3 nmol/L.

After validation, the new method was used in the measurement of intracellular erythrocyte concentrations of MTX and MTX-polyglutamate metabolites (MTXPG2 to MTXPG7) in packed human erythrocyte samples collected from patients with rheumatoid arthritis receiving low-dose oral methotrexate therapy. Mean (SD) intracellular erythrocyte concentrations observed in patient samples were 12.8 (12.6), 12.4 (9.4), 44.4 (30.0), 33.6 (35.9) and 9.4 (8.2) nmol/L for MTX to MTXPG5, respectively in 10^6 erythrocytes. The highest observed glutamylation degree of MTX was MTXPG5, the very long chain MTX-polyglutamate metabolites MTXPG6 and MTXPG7 were not detected in the packed erythrocyte pellets from rheumatoid arthritis patients.

Introduction

Methotrexate (N-[4[[(2,4-diamino-6-pteridinyl) methyl] methyl-amino] benzoyl]-L-glutamic acid; MTX) is a cytotoxic drug (folate antagonist) which is used to treat diseases such as leukemia, severe psoriasis and rheumatoid arthritis (RA). MTX inhibits competitively and reversibly the enzyme dihydrofolic acid reductase (DHFR) resulting in the inhibition of nucleic acid synthesis and in cell death[1]. MTX is applied in different doses depending on the disease; high dosage (up to 5000 mg/week) for treatment of different cancer types (e.g. leukemia) and in much lower doses for psoriasis [2] and rheumatoid arthritis (RA) [3] (5-25 mg/week). In RA, MTX is used as first-line drug. After admission of MTX, its plasma concentration decrease rapidly. MTX is transported into cells by the reduced folate carrier where it is retained, long after MTX has been eliminated from the plasma. MTX is metabolized intracellular by enzymatic polyglutamylation (folate-γ-glutamyl-tranferase) into MTX-polyglutamate metabolites by the addition of glutamate residues (max. 6 residues are added). Intracellular glutamylation increases the polarity of MTX resulting in intracellular retention prolonging drug action. MTX-polyglutamate metabolites can cause severe adverse events that can be counteracted by supplementation with folic acid (vitamin B9 or B11). Due to the low dose and the relatively short half life (8-15 hrs), it is no use measuring plasma MTX concentrations in low dose MTX therapy [4] and hence, MTX plasma levels do no correlate with disease activity [5]. In contrast, intracellular MTX-polyglutamates predict MTX response in RA patients especially the MTX-polyglutamates with three or more glutamic acid residues (MTXPG3-MTXPG5) are associated with this therapeutic response while MTX and MTXPG2 are poorly associated with therapeutic efficacy [6].

MTX and MTX-polyglutamate concentrations can be determined by analytical techniques such as high performance liquid chromatography (HPLC) with post column photo oxidation [4,6,7]. Total intracellular MTX can also be assessed after enzymatic hydrolysis of the polyglutamates followed by photometric measurement [8]. MTX-polyglutamate concentrations can also be determined by fluorescence polarization immunoassay (FPIA) [9] and capillary zone electrophoresis [10]. These techniques are laborious and influenced by interference from natural folates or other MTX related compounds. Recently, an ion-pairing liquid chromatography-tandem

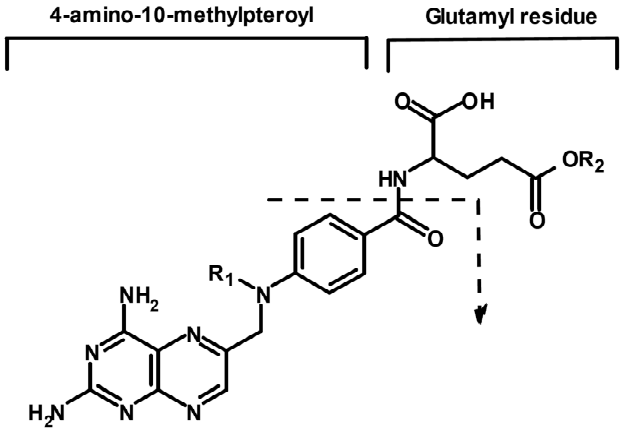
mass spectrometry (LC-MS/MS) technique was described with increased specificity [11]. We decided to select a different analytical technology to determine intracellular polyglutamate concentrations. We applied a relative new mass spectrometric technology. This technology combines Matrix-Assisted Laser Desorption/Ionization (MALDI) and triple quadrupole (tandem) mass spectrometry (MALDI-QqQ-MS(/MS)) and which was launched in 2008. It proved to be a robust and sensitive technology which can be applied for ultrafast and high-throughput analyses of small molecules because it does not necessarily require liquid chromatographic separation of samples prior to mass spectrometric analysis [12-14]. Omitting liquid chromatographic separation in combination with MALDI reduces analysis time considerably to approx. 10 seconds per sample [13] or less. MALDI-QqQ-MS technology has been proven to be a versatile quantitative tool in the ultrafast and high-throughput determination of concentrations of drugs (extra-and intracellular) such as antiretroviral drugs (protease inhibitors) [12,14,15], anticancer drugs [13,16] but also other types of drugs [17-19] and as screening tool in enzyme kinetic studies [20]. Moreover, Volmer et al. [21] demonstrated that MALDI-QqQ-MS technology has equal analytical performances in comparison to conventional LC-MS instrumentation using electrospray ionization (ESI). The aim of this study was to develop a new mass spectrometric method for measurement of intracellular MTX and MTX-polyglutamate concentrations in packed erythrocyte pellets from patients on low-dose MTX therapy.

Experimental

Materials and methods

4-amino-10-methylpteroylglutamic acid (MTX), 4-amino-10-methylpteroyldiglutamic acid (MTXPG2), 4-amino-10-methylpteroyltriglutamic acid (MTXPG3), 4-amino-10-methylpteroyltetraglutamic acid (MTXPG4), 4-amino-10-methylpteroylpentaglutamic acid (MTXPG5), 4-amino-10-methylpteroylhexaglutamic acid (MTXPG6),4-amino-10-methylpteroylheptaglutamic acid (MTXPG7) and internal standard 4-aminopteroylglutamic acid (Aminopterin; AO) were purchased from Schircks Laboratories (Jona, Switzerland). LC-MS grade methanol and water were obtained from Biosolve (Valkenswaard, the Netherlands) and all MALDI matrices and tricloroacetic acid (TCA) were purchased from Sigma Aldrich (Zwijndrecht, the

Netherlands) and were Matrix-Assisted Laser Desorption/Ionization time-of-flight mass spectrometric (MALDI-TOF) quality. The erythrocyte lysis buffer was purchased from Roche (Almere, the Netherlands. The 96-well SPE plates were purchased from Sigma Aldrich (DSC-C8 and DSC-C18; 25 mg) and from Waters (Oasis HLB-30 mg, Breda, the Netherlands). Primary stock solutions of MTX and MTX-polyglutamate metabolites (MTXPG2, MTXPG3, MTXPG4, MTXPG5, MTXPG6 and MTXPG7) were prepared in potassium hydroxide solution (0.02 mol/L) at 44, 44, 44, 44, 58, 22, 22 µmol/L for MTX, MTXPG2, MTXPG3, MTXPG4, MTXPG5, MTXPG6 and MTXPG7, respectively. Chemical structures of MTX, MTX-polyglutamates and the internal standard aminopterin are illustrated in Fig.1.



Name	R_1	R_2
MTX	CH_3	H
MTXPG2	CH_3	-[NHCHCOOH$(CH_2)_2$COO]-H
MTXPG3	CH_3	-[NHCHCOOH$(CH_2)_2$COO]$_2$-H
MTXPG4	CH_3	-[NHCHCOOH$(CH_2)_2$COO]$_3$-H
MTXPG5	CH_3	-[NHCHCOOH$(CH_2)_2$COO]$_4$-H
MTXPG6	CH_3	-[NHCHCOOH$(CH_2)_2$COO]$_5$-H
MTXPG7	CH_3	-[NHCHCOOH$(CH_2)_2$COO]$_6$-H
Aminopterin	H	H

Figure 1. Chemical structures of Methotrexate (MTX), MTX-polyglutamate metabolites and internal standard Aminopterin and respective SRM transitions of protonated molecular ions [11].

MALDI-QqQ-MS/MS conditions

The MALDI-QqQ-MS/MS instrumentation used was a FlashQuant™ workstation containing a high repetition rate solid state UV-laser (FlashLaser; 349 nm, 1000 Hz) combined with a 4000 API mass analyzer (AB Sciex, Concord, Canada) operating in positive ionization mode with selected reaction monitoring (SRM) of the selected analytes at unit resolution. SRM for MTX and MTX-polyglutamate metabolites corresponded to following transitions: $[M+H]^+ \rightarrow [M-((C_5H_9NO_4)_n-(H_2O)_m)+H]^+$ with *n=1 to 7* and *m=(n-1)* for MTX to MTXPG7, respectively illustrating the loss of the polyglutamate chain. Optimized MALDI-QqQ-MS instrument parameters used were: laser power 55%, skimmer voltage 0V; CAD gas 8 arbitrary units (3.0-3.33*10-5 torr, N_2); source gas 10 arbitrary units (3.33-4.17*10-5 torr, N_2), dwell time 10 ms and laser raster speed of 1 mm/sec. Instrument control and data analyses were performed using Flashquant 1.0 software and Analyst 1.4.2 application software (AB Sciex, Concord, Canada).

Determination of MALDI ionization efficacy

The type of MALDI ionization matrix compound used can have a significant impact on sensitivity of the MALDI-QqQ-MS/MS measurements since the ionization efficacy of the analyte by selected MALDI matrix compound can be significant different. Therefore, we determined the influence of the MALDI matrix compounds on the ionization efficacy of MTX and all MTX-polyglutamate metabolites. We applied different MALDI matrix compounds which are frequently applied in MALDI-TOF. Tested were: 2,5-dihydroxy benzoic acid (2,5-DHB),7-hydroxy-4-(trifluoromethyl)-coumarin (HFMC) [22], super-DHB (SDHB; mixture of 2,5-DHB and 5-methoxysalicylic acid), 9-amino acridine (9-AA) and α-cyano-hydroxy-cinnamic acid (α-CHCA). MALDI matrices such as 2,5-DHB and SDHB were used at a concentration of 30 mg/mL, HFMC and 9-AA at 10 mg/mL and α-CHCA at 6.2 mg/mL, respectively.

The ionization efficacy of each individual MALDI matrix compound was determined by the measurement of the total counts per second (CPS) signal for all analytes at one concentration using each MALDI ionization compound, respectively. The CPS signal was measured using following protocol: 20 µL of individual MTX and

MTX-polyglutamate metabolite stock solutions (100 nmol/L in 50% (v/v) methanol/water) were mixed at a ratio of (1:1) with the different MALDI matrix solutions and subsequently five spots of 0.5 µL were spotted onto the MALDI target plate. Detection of the positive charged (protonated) ions were done in full scan mode (*m/z*= 450 to 1250*)* using a scan time of 1s. After the experiments, the MALDI matrix compound with the highest ionization efficacy was used for further fine-tuning of MALDI-QqQ-MS/MS instrument settings and the optimal sensitivity by determination the best mixing ratio between analyte solution and MALDI matrix solution and the maximum sample amount (µL) to be spotted onto the stainless steel MALDI target plate.

Development of solid phase extraction procedure

A solid phase extraction (SPE) procedure was developed because MTX and MTX-polyglutamate metabolite concentrations are very low (fmol/10^6 erythrocytes) [4, 6, 9, 23]. At first, we determined the best SPE adsorbent material by determination of recovery rates for MTX and all MTX-polyglutamate metabolites applying 96-well SPE plates containing following adsorbents: octyl (C_8), octadecyl (C_{18}) and hydrophilic modified styrene polymer (HLB). The C_8 and C_{18} well plates contained 25 mg adsorbent while the 96-well SPE-HLB plate contained 30 mg of adsorbent. The recovery rates of MTX and all other individual MTX-polyglutamate metabolites on the C_8, C_{18} and HLB adsorbents were determined by spiking four different amounts of erythrocyte pellets (from healthy controls) with MTX and MTX-polyglutamate metabolites at one concentration (MTX-MTXPG2-MTXPG4; 44 nmol/L, MTXPG3; 58 nmol/L and MTXPG5-MTXPG6-MTXPG7 at 22 nmol/L,respectively). Erythrocyte pellet volumes of 25, 50, 75 and 100 µL (in triplicate) were homogenized and lyzed with 65 µL water, 10 µL of internal standard (500 nmol/L) and 150 µL of the erythrocyte lysis buffer solution followed by deproteinized of the lysate by 50 µL TCA (50% w/v). Collected deproteinized supernatants were diluted with 1000 µL of water and further processed by SPE using the three different types of adsorbents and recovery rates for MTX and all MTX-polyglutamate metabolites were determined.

Calibration curve of MTX and MTX-polyglutamate metabolites

The linear concentration ranges of the method for MTX and all MTX-polyglutamate metabolites were determined by applying calibrators prepared in whole blood by spiking MTX and MTX-polyglutamate metabolites at different concentrations. The whole blood applied for the preparation of calibrators was obtained from a healthy control (Sanquin Blood Supply Foundation, Rotterdam, the Netherlands) and calibrators were made by dilution of primarily stock solution containing MTX and MTX-polyglutamate metabolites (1000 nmol/L per analyte) with drug free whole blood yielding following calibrator concentrations: 1000, 500, 250, 100, 50, 25, 10 and 0 nmol/L (blank). The calibration curves were prepared by spiking each calibrator (25µL in triplicate) with 10 µL of the internal standard aminopterin (AO; 500 nmol/L) followed by lysis of the erythrocytes with 65 µL of water, 150 µL of erythrocyte lysis buffer (Roche, Almere, the Netherlands) and deproteinization of the erythrocyte lysates by 50 µL of TCA. Precipitated proteins were removed by centrifugation for 5 min at 400 x *g* at ambient temperature.After collection of the supernatants, 1000 µL of water were added and analytes were extracted from the supernatants by solid phase extraction (SPE) using an Oasis HLB 96-SPE well plate (Waters, Etten-Leur, the Netherlands) containing 30 mg adsorbent. The SPE adsorbent was conditioned by washing the adsorbent with 1 mL of methanol followed by 2 x1 mL of water. After adsorption of the analytes and washing of the SPE adsorbent by 1 mL of water, elution of the analytes from the SPE adsorbent was achieved by 200 µL of methanol.Aliquots of 20 µL from collected SPE extracts were mixed with 40 µL of α-HCA-MALDI matrix solution and 0.5 µL were spotted in fivefold onto a 96-well stainless steel MALDI target plate (123 x 81 mm).Pipetted spots were let to dry and crystallize for 5 min at ambient temperature prior to MALDI-QqQ-MS/MS analysis. Calibration curves from all analytes were fit by linear regression of the ratio between the SRM peak areas of analyte and internal standard versus analyte concentrations by using GraphPad Prism software version 5.00 for Windows (GraphPad software, San Diego, USA).

Accuracy and precision of the method

Within-run and between run accuracy and precision were determined by analyzing erythrocyte lysates prepared from 25 µL of whole blood in threefold spiked at three different concentration levels with all analytes at low, middle and high concentrations [24]. Within-run accuracy and precision were assessed with 3 replicate erythrocyte lysates spiked at 500, 100 and 20 nmol/L for each analyte, whereas between run accuracy and precision were assessed with 3 replicates of each concentration level analyzed on 3 subsequently different days. Accuracy was determined from the difference between measured concentrations and spiked nominal concentrations and was expressed as %error. Precision was determined by calculation of the coefficient of variation (%CV) of the replicate measurements (%CV).

Application of method to RA patient samples

During MTX administration, 8 mL CPT cell preparation tubes (Becton Dickinson, Breda, the Netherlands) were collected on t=3 months (patients 1-7) and 9 months (patients 8-10) during therapy by venapuncture from RA patients receiving low dose MTX therapy (15-25 mg MTX/week). The included patients had inflammatory joint complaints for less than 1 year. Patient blood samples were collected in compliance with the Helsinki regulations and patients gave written consent (MEC-2006-252).

Hematocrit, erythrocyte cell count, (differential) white blood cell count, hemoglobin concentration and platelet counts were determined from EDTA whole-blood using a Sysmex XE-5000 hemocytometer (Sysmex, Etten-Leur, The Netherlands). CPT tubes were centrifuged at room temperature for 20 minutes at 1500-1800 x *g* to separate blood cells (erythrocytes and monocytes) and obtained plasma was immediately stored at -80°C. From all RA patients hematological parameters such as hematocrit, erythrocyte count (10^{12}/L), hemoglobin concentration and platelet count were known, erythrocyte count and hematocrit values were used to determine the number of erythrocytes/µL in the packed erythrocyte pellet. Mean hematocrit (SD) was 0.43 (0.03) and and mean erythrocyte count (SD) was 4.56 x 10^{12}/L (0.37). Collected erythrocyte pellets contained approx. 10 million

erythrocytes/µL and for the measurement of the intracellular erythrocyte MTX and MTX-polyglutamate metabolite concentrations in average 250 million erythrocytes were used.

Results and discussion

Method development

MALDI ionization efficacy and sensitivity

The highest ionization efficacy for all MTX-polyglutamate metabolites was obtained using α-CHCA as MALDI matrix in combination with a sample/MALDI matrix solution ratio of 1:2 and sample spots of 0.5 µL. Application of α-CHCA as MALDI matrix compound resulted in significant higher total ion counts (CPS) for all protonated molecular ions of MTX polyglutamates (Fig. 2).

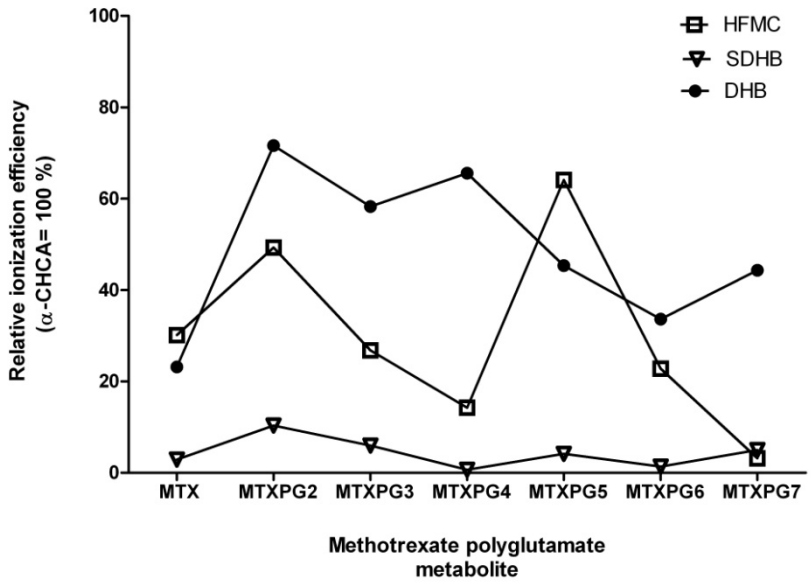


Figure 2. Ionization efficiencies of studied MALDI ionization compounds on methotrexate and metothrexate polyglutamate metabolites. Applied MALDI-QqQ-MS instrument parameters (full scan *m/z* 450 to 1250) laser power 55%, skimmer voltage 0V; source gas 10 arbitrary units (3.33-4.17*10-5 torr, N_2) and laser raster speed of 1 mm/sec

Protonated molecular ions of MTX and MTX-polyglutamate metabolites using α-CHCA were used for further optimization of MALDI and MS instrument parameters

such as plate voltage (V), collision energy (CE), collision cell exit potential (CXP), collision gas (CAD), source gas, and skimmer voltage setting. Optimized MALDI-QqQ-MS/MS instrument settings for MTX and MTX-polyglutamate metabolites and the internal surrogate standard aminopterin are presented in Table 1.

Mass spectrometric analysis of MTX and MTX-polyglutamate metabolites

Collision induced fragmentation (CID) of the protonated molecular ions from MTX and MTX-polyglutamate metabolites resulted in MS/MS spectra where one main high abundant fragment ion at a mass-to-charge ratio of *m/z* 308.2 was observed, although also few smaller fragment ions with lower abundance were observed; for the internal standard aminopterin, identical fragmentation behavior was observed with one difference that a high abundant fragment ion was observed at *m/z* 294.2. The high abundant fragment ions observed at *m/z* 308.2 for all analytes were selected for SRM analyses. The formation of this high abundant fragment ion originate from an intermolecular cleavage of the amide bond in MTX and MTX-polyglutamate metabolites situated between the first glutamyl-moiety and the 4-amino-10-methylpteroyl-backbone of MTX-polyglutamate molecules (Fig.1). The cleavage of the amide bond resulted after hydrogen rearrangement in the loss of a neutral glutamate molecule (M-$C_5H_9NO_4$; M-147) for MTX as well as the loss of neutral charged polyglutamyl-peptide containing between two and seven glutamate moieties for MTXPG2 until MTXPG7, respectively as also reported by van Haandel et al. [11] using LC-ESI-MS/MS.

Method development and validation

Significant different recovery rates for MTX and MTX-polyglutamate metabolites were observed for the three tested different SPE adsorbent materials. The lowest recoveries for all analytes were observed using the SPE-C8 adsorbent material, recovery ranges obtained applying this SPE adsorbent material ranged between 62.4% for MTX and 17.6% for MTXPG6. Higher recovery rates were obtained for the SPE-C18 material, recovery ranged here between 85.6% and 57.9 % for MTX and MTXPG7, respectively. The highest recoveries were observed for the HLB-SPE material, recoveries observed were 71.3, 75.1, 96.7, 86.3, 88.5, 90.3 and 97.7% for MTX to MTXPG7, respectively.

Table 1. MALDI-QqQ-MS/MS instrument settings for the quantitative measurement of MTXPGs[a,b]

Analyte	Abbr.	SRM (*m/z*)	Plate Voltage (V)	CE (V)	CXP (V)
4-amino-10-methylpteroylglutamic acid	MTX	455.2 → 308.2	45	25	10
4-amino-10-methylpteroyldiglutamic acid	MTXPG2	584.4→308.2	30	43	10
4-amino-10-methylpteroyltriglutamic acid	MTXPG3	713.4→308.2	35	43	10
4-amino-10-methylpteroyltetraglutamic acid	MTXPG4	842.4→308.2	45	60	10
4-amino-10-methylpteroylpentaglutamic acid	MTXPG5	971.6→308.2	60	70	15
4-amino-10-methylpteroylhexaglutamic acid	MTXPG6	1100.3→308.2	80	80	10
4-amino-10-methylpteroylheptaglutamic acid	MTXPG7	1229.4→308.2	75	90	25
4-amino-pteroyldiglutamic acid	AO	441.2→294.2	50	28	15

[a] Skimmer voltage (0 Volts), [b]As collision gas (CAD) N_2 was applied

The recovery of the internal standard aminopterin was in the range of MTX and the MTX-polyglutamate metabolites, the recovery was 81.2%. After determination of the SPE adsorbent material with the highest recovery rates for all analytes, the maximum sample loading of this adsorbent was determined by applying different amounts of packed erythrocyte pellet, tested were 25, 50, 75 and 100 µL of packed erythrocyte pellet and recoveries of MTX and MTX-polyglutamate metabolites were determined. Highest recovery rates for all analytes were observed when 25 µL of lyzed erythrocyte pellets were applied. Higher loading amounts resulted in significant lower recovery rates for all analytes (Fig.3). Obviously, higher amounts of erythrocyte cell pellets overloaded the SPE adsorbent and caused desorption (breakthrough) of the analytes.

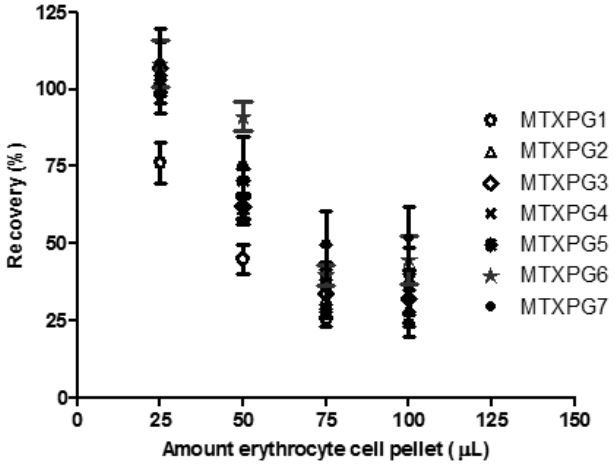


Figure 3. Recovery rates for individual MTX-polyglutamates in spkied erythrocyte pellets in relation to the erythrocyte pellet volume used for solid-phase extraction using Oasis HLB 96-well plates containing 30 mg adsorbent.

The selected concentration range using lysates produced from erythrocyte pellets based calibration standards indicated a linear relationship between the analyte peak area/IS ratio of each individual MTX-polyglutamate metabolites and their concentration (MTX: y=1.3224x+0.3546, r^2=0.9981; MTXPG2: y=0.4042x-0.0443, r^2=0.9936; MTXPG3: y=0.1649x-0.0317, r^2=0.9905; MTXPG4: y=0.0709x-0.0078, r^2=0.9945; MTXPG5: y=0.1137x-0.0189, r^2=0.9912; MTXPG6: y=0.2063-0.004, r^2=0.9928 and MTXPG7: y=0.01113x-0.0011, r^2=0.9961). The LLOQ for each

individual MTX-polyglutamate metabolite was defined as the calibrator with the lowest concentration used to calculate the calibration curve which could be measured with an accuracy and precision of <20 %CV [24]; the LLOQ for the MTX-polyglutamate metabolites was 10 nmol/L for MTX to MTXPG7, respectively. The upper limit of quantification (ULOQ) was defined as the highest concentration calibrator which could by measured with acceptable accuracy and precision of <15 %CV; the ULOQ was 1000 nmol/L for all MTX-polyglutamates.

The limit of detection (LOD) defined as three times the signal-to-noise ratio (3:1) of drug free control whole blood was 1 nmol/L for MTX and MTXPG2 and 3 nmol/L for MTXPG3 to MTXPG7, respectively. Validation of the method's accuracy and precision by determination of with-run and between run accuracy and precision using quality control samples prepared at three different concentration levels (500, 100 and 20 nmol/l for each analyte) were in compliance with FDA criteria (CV and %error < 20%) (Table 2). Precision of the new method was expressed as %CV from within-run validation and ranged between 5.5 and 14.4%, while within-run precision ranged between 4.9 and 14.6%, respectively.

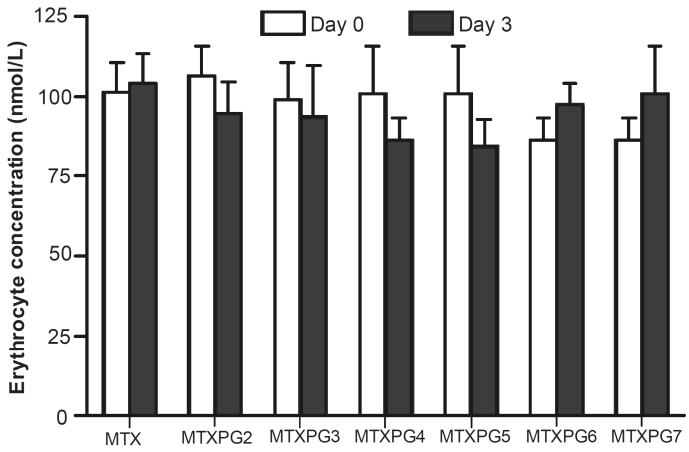


Figure 4. Stability of MTX and MTX-polyglutamate metabolites in a packed erythrocyte pellet spiked at a concentration of 100 nmol/L per analyte, illustrated are measured concentration of all analytes at day 0 and day 3 (mean ± SD).

Accuracy of the new method expressed as %error ranged for within-run validation between -8.5% and 9.8%, while the %error for the between run measurements were between -7.5% and 4.5%, respectively. The stability of MTX and

its MTX-polyglutamate metabolites in erythrocyte matrix were tested by an erythrocyte pellet (25 µL) spiked with MTX and MTX-polyglutamate metabolites at a concentration of 100 nmol/L by measuring at day 0 and day 3. The stability of all analytes in the erythrocyte matrix was stable (expressed as %error) and were found to be <20 %error (Figure 4.).Dervieux et al. [6] reported a mean sum of individual MTX- polyglutamate metabolite in seventy RA patients of 120 nmol/L (MTX to MTXPG5). The intracellular concentrations were determined applying HPLC in combination with fluorescence detection of the MTX-polyglutamate metabolites. Van Haandel et al. [11] used a LC-MS/MS assay for the determination of erythrocyte intracellular MTX and MTX-polyglutamate metabolite concentrations and reported a concentration of 153 nmol/L (sum of MTX to MTXPG6) for a JIA patient (Juvenile Idiopatic Arthritis) on MTX therapy.

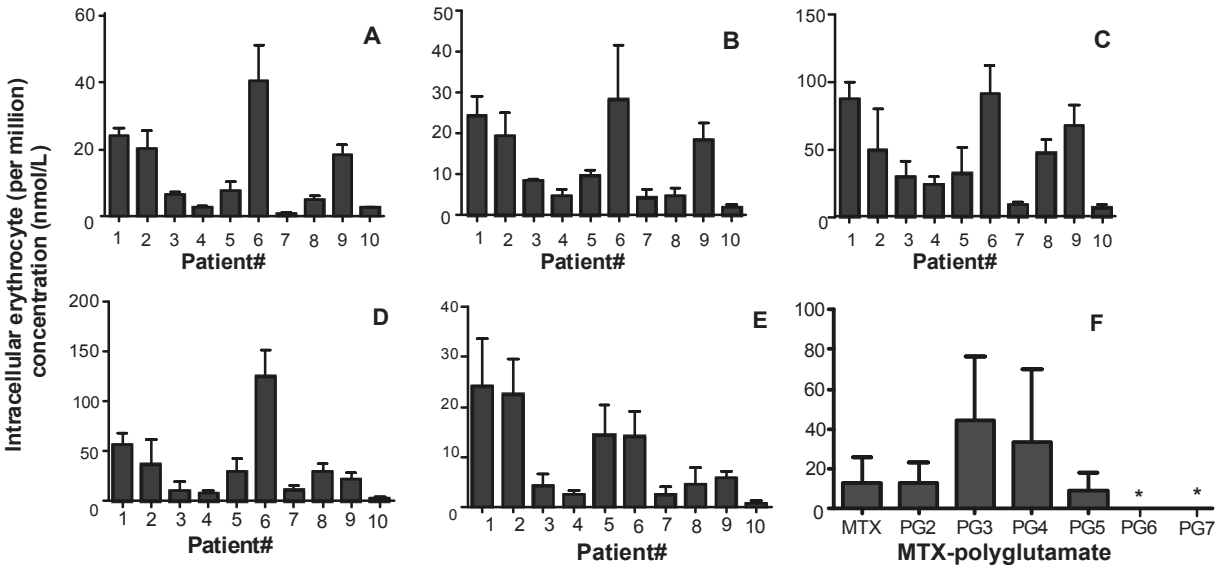


Figure 5. Individual intracellular erythrocyte MTX and MTX-polyglutamate metabolite concentrations in ten RA patients; (a) MTX, (b) MTXPG2, (c) MTXPG3, (d) MTXPG4 and (e) MTXPG5, (f) observed concentrations of individual MTX-polyglutamates. The concentrations of the very long chain MTXPG6 and MTXPG6 were < LOD. Illustrated are mean concentrations and SD obtained from an analysis of 25 µL of a packed erythrocytes pellet in triplicate by MALDI-QqQ-MS/MS. (*) depicts concentration of this MTX-polyglutamate metabolite is <LOD.

Table 2. Precision and accuracy of the MALDI-QqQ-MS/MS assay at three different MTX polyglutamate concentration levels

	Within-run validation[a]																				
Analyte	MTX			MTXPG2			MTXPG3			MTXPG4			MTXPG5			MTXPG6			MTXPG7		
Nominal conc. (nmol/L)	500	100	20	500	100	20	500	100	20	500	100	20	500	100	20	500	100	20	500	100	20
Observed conc. (nmol/L)	507	105	18	486	99	21	486	94	20	497	97	22	494	92	20	495	92	20	502	98	21
Accuracy (% error[c])	1.4	4.9	-7.9	-2.7	-1.3	4.4	-2.8	-6.1	0.26	-0.7	-2.7	9.8	-1.1	-8.3	0.9	1.0	-8.5	1.1	0.3	-2.3	5.5
Precision (% CV)	5.5	8.0	14.1	7.5	11.2	13.6	9.2	13.4	10.7	12.6	14.4	12.7	6.0	12.7	5.5	12.1	11.3	13.3	1.8	13.6	5.7
	Between-run validation[b]																				
Observed conc. (nmol/L)	502	103	19	494	100	20	490	96	20	498	102	20	484	93	20	503	99	20	491	102	21
Accuracy (% error[c])	0.3	3.2	-5.2	-1.1	0.3	-1.3	-2.1	-3.8	0.8	-0.5	1.9	-2.3	-3.1	-7.5	0.1	0.6	-1.3	-2.5	-1.7	1.8	4.5
Precision (% CV)	5.00	9.2	13.2	9.8	11.0	7.9	9.1	14.5	6.8	11.4	13.0	11.9	7.9	14.6	13.4	7.4	8.1	7.6	5.9	4.9	6.8

[a]within-run results summarize 3 spots per QC sample at each concentration level in one experiment, [b]between-run results summarize three different experiments from 3 consecutive days with 3 spots per QC sample at each concentration level, [c]% error = (mean observed concentration-nominal concentration)/(nominal concentration)* 100%

In comparison to measured sum of individual MTX and MTX-polyglutamate metabolite concentrations (139 nmol/L; MTX to MTXPG5) in our study of RA patients both publications are consistent with both publications. In contrast to reported concentrations we observed a concentration (SD) of the MTXPG4 metabolite of 33.6 nmol/L (35.9) was almost equal to the MTXPG3 metabolite concentration level which was 43.6 nmol/L (30.0). An unambiguous explanation is difficult to give since many parameters could cause this significant difference. These include differences in pharmacokinetics between patients, time point of sample collection, and storage of samples [7]. The time reaching steady state concentrations for MTX and MTX-polyglutamate metabolites can be significant different, especially for MTXPG4 time ranges between 16 and 832 weeks (mean 146 weeks) were observed with patients [7] while for MTX-MTXPG3 metabolite 6, 11 and 41 weeks and for MTXPG5 140 weeks were reported [7]. Depending on the point of the sample collection higher or lower concentrations can be expected [7]. Higher MTXPG4 concentrations were reported as being associated with improved MTX efficacy in RA patients [23]. In general, our findings are consistent with previously reported intracellular concentrations [6,26,27] although also a large variation in individual MTX-polyglutamate metabolite concentrations between our ten included RA patients per MTX-polyglutamate metabolite was observed (Fig.5). This large variation in individual MTX-polyglutamate metabolite concentrations was also observed by Dalrymple et al. [7] who reported a large interpatient variability of erythrocyte MTX and MTX-polyglutamate metabolite accumulation and elimination in adults with RA.

Conclusion

The newly developed MALDI-QqQ-MS/MS method allows a sensitive and accurate measurement of therapeutically relevant concentrations of MTX and MTX-polyglutamate metabolites in erythrocytes from patients receiving low dose MTX therapy. This new technology is in comparison to other analytical methods such as fluorescence polarization immunoassay (FPIA) and HPLC in combination with fluorescence detection after photo-oxidation or LC-MS/(MS) is that it is ultrafast and therefore can be applied for high-throughput measurements of large number of samples of large patient population studies. A major advantage of the MALDI-QqQ-

MS instrumentation is that the instrumentation is directly available for analysis of other substances without downtime of the instrumentation compared with common LC-MS instrumentation due to exchange of separation columns and equilibration of the applied instrumentation.

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Chapter 5

General discussion

In this PhD thesis we have explored the feasibility of using the new mass-spectrometric MALDI-QqQ-MS/MS technology for purposes of biomedical research. We determined the feasibility of this relatively new technology in three different biomedical research fields: infectious diseases, oncology and auto-immune diseases. In these fields, drug concentrations are routinely determined in biofluids such as plasma, serum and urine, not only to determine the patient's compliance, but also to assess the therapeutic efficacy of a particular treatment.

Although routine clinical diagnostic assays are used for some drugs, they have several disadvantages: not only are they laborious and expensive, their sensitivity and analyte specificity are sometimes low. To tackle these disadvantages, we therefore developed new bio-analytical assays using MALDI-QqQ-MS/MS technology. In the treatment of certain diseases, the frequent measurement of drug concentrations in patient's biofluids (therapeutic drug monitoring (TDM)) can play a very important role in increasing survival, quality of life, and treatment efficacy.

The rationale behind our application of the new MALD-QqQ-MS/MS technology was that it allows for ultrafast and high-throughput bio-analytical assays with better analyte sensitivity and selectivity than the classical analytical techniques used for routine clinical analysis.

Major findings

As reported in **Chapter 2,** two new bio-analytical assays were developed and validated for analytical support to HIV research: one to measure the protease inhibitors Lopinavir and Ritonavir (Kaletra®) in pediatric HIV-1 infection, and the other to measure the nucleotide reverse transcriptase inhibitor drug Tenofovir® in plasma from HIV-1 infected adult patients.

Pediatric HIV-1 infected patients received the protease-inhibitor co-formulation medication Kaletra®, plasma and dried blood-spot concentrations of which were measured using MALDI-QqQ-MS/MS. The bioanalytical assay developed for the support of pediatric HIV-1 infection research was validated according to the most recent FDA guidelines, and also cross-validated with a high-performance liquid chromatography-UV assay [1], which was used to routinely measure the plasma concentration levels of both antiretroviral drugs. Plasma concentrations are used not

only to assess a patient's compliance with the drug regime, but in therapeutic and medical interventions whenever severe adverse effects, such as drug toxicity, hyperlipidimia, or other drug-related adverse effects are observed.

A small cohort study which included nineteen HIV-1 infected pediatric patients was used to determine the extents to which the HPLC-UV assay and MALDI-QqQ-MS bioanalytical assay could establish the plasma concentrations of these antiretroviral drugs. A Bland-Altman comparison plot [2] was used to determine the degree of agreement between both bioanalytical methods. Both analytical assays were in a high degree of agreement, and provided statically equal results.

As well as using the MALDI-QqQ-MS/MS bioanalytical assay to analyze the antiretroviral drugs in plasma, we used it to measure Kaletra® whole-blood concentrations in dried blood-spot specimens (DBS). We found that DBS concentrations were in a high degree of agreement with the concentrations we obtained and cross-validated.

At some point in the future, the combination of MALDI-QqQ-MS/MS technology with DBS specimen sampling may be used not only for the TDM of local HIV-1 infected pediatric patients, but also for HIV-1 infected patients (pediatric and adult alike) in developing countries, where routine clinical diagnostics and measurements of antiretroviral plasma concentration levels are hampered by limited local resources, particularly in rural areas. In such environments, it would therefore be very advantageous if DBS specimens could be used for TDM purposes in HIV-1 infections, since the collection of DBS specimen can be faster, easier and less expensive than that of plasma and blood samples.

To gain experience and to determine the options for using DBS specimens in biomedical research and clinical diagnostics, we plan to improve the collection and use of DBS specimen-sampling (in HIV-1 infection). Although DBS specimens have many advantages over plasma sampling, they have one major drawback: the risk of infection for the medical and laboratory staff who collect them. DBS specimens can be prepared by spotting blood directly from the patient's finger after a finger prick, or by pipetting it from a blood-collection tube. The longer exposure time to HIV-1 infected blood makes both procedures inherently more risky.

Generally speaking, bio-analytical assays use internal standards to correct for interpatient blood matrix variations, loss of analyte and sample preparation losses.

To determine the most efficient way to include an internal standard in DBS specimen collection and analysis, we conducted a study in which various experiments were used to identify the most efficient internal standard inclusion. We determined the best inclusion procedure in a set of experiments using the antiretroviral drug Nevirapine as model compound. Used in combination with its stable-isotope-labeled analogue (IS, Nevirapine-d3), Nevirapine is a very common antiretroviral drug used to initiate treatment for the HIV-1 infection [3].

We obtained the best recovery results for Nevirapine when the IS was added to whole blood before spotting onto the DBS substrate material. But as blood-collection tubes have to be opened before IS is added, its direct addition to the whole blood might create extra safety risks for the medical and laboratory staff personnel who prepare DBS specimens. It is also the case that the blood droplets cannot be transferred directly from the finger onto the DBS specimen substrate material in this way. Neither is it possible to spike the IS into the blood droplet before it is transferred to the DBS specimen substrate material.

Thus, due to this higher infection risk for medical and laboratory staff, we automatically selected the second best IS-addition procedure, which consisted of adding the IS to the DBS substrate material before the whole blood was spotted. The recovery rate for Nevirapine was 15% lower than that it was when the IS was added directly into the blood before spotting. According to the most recent FDA guidelines, a 15% decrease in recovery decrease was still acceptable [4].

Even lower recovery rates for Nevirapine were achieved in two other experiments using different IS procedures: addition of the internal standard on top of dried DBS specimens (53%) and addition into the extraction solvent of the DBS specimen (11%). However, because these recovery rates were not acceptable under the FDA guidelines, the two procedures in question were not continued in further experiments.

Both papers on the use of DBS specimens in biomedical research described and demonstrated the advantages for bioanalytical measurements of drugs over the conventional handling of biofluids. DBS specimens are very interesting for sample collection, especially when only small amounts of biofluids are available, or when they can only be collected in neonates or, in animal tests, with small rodents. DBS specimens are also advantageous not just from a bioanalytical perspective, but also

from a financial one. There are three main reasons they can significantly reduce the cost per sample: expensive cooled transport of frozen plasma and blood is reduced, storage volumes are significantly smaller, and sample collection can be much easier, especially when resources are limited (as in remote rural areas and in developing countries with few laboratory or hospital facilities).

Chapter 2 presents a newly developed bio-analytical assay for the determination of Tenofovir plasma concentrations. Tenofovir is a nucleotide reverse transcriptase inhibitor (NRTI) pro-drug [5] used in HIV-1 infection. By combining the MALDI-QqQ-MS/MS technology with an isotope dilution method using stable-isotope-labeled [adenine-$^{13}C_5$]-TNV, the new assay made it possible to measure these plasma concentrations in adult HIV-1 infected patients in less than 10 seconds per sample. Precision and accuracy were in high agreement with the most recent FDA regulations (<15/20%) [4].

During long-term treatment, HIV-1 infected patients treated with antiretroviral drugs sometimes develop hyperlipidemia [6]. Because it was not known whether high (or higher) triglyceride plasma concentrations influenced the measurement of TNV concentrations, we studied their influence in the range from 3.20 to 5.33 mmol/L. Our MALDI-QqQ-MS/MS bioanalytical assay showed that they did not.

As well as our work to support research on HIV-1 infection, we also developed a new bio-analytical assay to support research on influenza infection. Because such infection can be life threatening, especially in specific patient populations such as neonates and the elderly, influenza-infected patients are sometimes treated with the antiviral drug Oseltamivir (Tamiflu®) [7-9]. While the Oseltamivir doses used in pediatric influenza infection are still based on bodyweight and empiric efficacy data obtained from adult patients, the dramatic changes in drug pharmacokinetic during a child's development and maturation do not provide the most effective basis for calculating individual doses. Because Oseltamivir is a pro-drug, with the actual active drug being formed after enzymatic transformation by an endogenous esterase enzyme, two factors are therefore important: the determination of Oseltamivir concentrations, and also the concentrations of the enzymatic product Oseltamivircarboxylate.

Due to low ionization efficacies for Oseltamivir and Oseltamivircarboxylate, which resulted in detection limits for both analytes that lay far above the actual therapeutically relevant plasma concentrations, MALDI-QqQ-MS/MS failed to simultaneously measure the concentrations of Oseltamivir and its carboxylate metabolite. We solved the problem of the low sensitivity of the MALDI-QqQ-MS/MS assay for Oseltamivir and Oseltamivircarboxylate by developing a new bioanalytical assay that used ultra-performance liquid chromatography with mass-spectrometric detection (UHPLC-MS). This had sufficiently high sensitivity for both analytes, and analysis time (6 min/sample) was satisfyingly fast.

We also used the UHPLC separation method to analyze DBS specimens on Oseltamivir and Oseltamivircarboxylate concentrations; this showed that DBS specimen concentrations were in a high degree of agreement with plasma concentrations measured for both analytes in the selected patient population. We concluded that the use of DBS specimens would be especially valuable in pediatric influenza infections, providing a perfect whole-blood sampling tool that could apply the small whole-blood quantities needed when sampling pediatric patients. In future clinical studies, we expect the combination of UHPLC bio-analytical assay and DBS-specimen collection to increase our knowledge not only of optimal dosing regimens for individual paediatric patients, but also of the determination of pharmacokinetic properties (PK) and TDM studies in clinical studies.

In **Chapter 3**, we demonstrate the bioanalytical feasibility of the new MALDI-QqQ-MS/MS technology in oncology research, for which we developed two new bioanalytical assays. After validation, we used these assays to measure drug concentrations in biofluids collected from patients. We show that measurement of anti-folate concentrations in plasma from lung-cancer patients (NSCLC) patients was possible only after the plasma samples had been deproteinized and diluted. The minimum amount of plasma needed to measure pemetrexed concentration in plasma samples was only 0.001 µL. The high sensitivity of the assay for the analyte thus makes it a perfect tool for future pharmacokinetic studies.

We were also able to demonstrate the ultrafast properties of the technology and the significantly higher sensitivity (factor 25-60) than possible with routine HPLC and LC-MS assays [11,13]. While the analysis time in routine diagnostics using

HPLC and LC-MS lies between approximately 7 and 30 min [10,11,12], analysis time of the MALDI-QqQ-MS/MS bioanalytical assay was approximately 10 seconds/sample.

Nevertheless, if more knowledge is to be obtained on interpatient variability in pharmacokinetic properties for pemetrexed, extracellular and intracellular concentrations, new and larger patient studies should be planned. Greater understanding of interpatient variability will help increase therapeutic efficacy, and probably answer why we observed significantly increased plasma concentration levels in some patients.

We present also a new bio-analytical assay for determining plasma concentrations for the antifolate drug methotrexate in patients suffering from acute lymphoblastic leukemia (ALL). For routine measurements of methotrexate plasma concentration levels, the hospital pharmacy at Erasmus University Medical Center uses polarization fluorescence immunoassay (FPIA), which, compared to our MALDI-QqQ-MS/MS assay, has a larger linear concentration range (up to 1000 µmol/L), against a range of up to 60 µmol/L for the MALDI-QqQ-MS/MS. The clinical feasibility and performance of the new assay was determined by cross-validation with the FPIA assay. While the methotrexate concentrations measured by MALDI-QqQ-MS/MS assay had a statistically high degree of agreement with those measured by the FPIA assay, the MALDI-QqQ-MS/MS assay had some advantages: selectivity and sensitivity were both significantly better, and it was also possible to simultaneously measure the 7-hydroxymethotrexate metabolite concentration, which was very difficult or even impossible with the FPIA assay.

Due to its better sensitivity and selectivity, the new MALDI-QqQ-MS/MS assay thus offers the possibility of fast and more precise therapeutic drug monitoring (TDM) of methotrexate and its 7-hydroxymethotrexate metabolite. More precise TDM can help prevent the development of adverse drug effects such as methotrexate toxicity, which is sometimes seen in patients with impaired methotrexate clearance or a low adherence to the drug.

In **Chapter 4,** we used the MALDI-QqQ-MS/MS technology to measure intracellular erythrocyte concentrations for methotrexate polyglutamate metabolites in packed

erythrocyte pellets collected from rheumatoid arthritis patients on low-dose methotrexate (25 mg/week) therapy.

Methotrexate and individual methotrexate polyglutamate concentrations (MTXPG2-MTXPG7) are routinely determined by analytical techniques such as high-performance liquid chromatography (HPLC) with post-column photo oxidation [14,15,16]. Total intracellular methotrexate (methotrexate and methotrexate polyglutamates) are assessed after enzymatic hydrolysis of the polyglutamates followed by photometric measurement [17], or by FPIA [18], capillary zone electrophoresis [19] and ion-pairing LC-MS/MS [20]. When used for routine diagnostics, these analytical techniques are very laborious, and also subject to interferences from natural folates and other methotrexate metabolites such as DAMPA or 7-hydroxymethotrexate.

The new MALDI-QqQ-MS/MS assay used fast, simple and straightforward solid-phase extraction (SPE) for the isolation of methotrexate polyglutamates from packed erythrocyte pellet lysate spiked with an internal standard (Aminopterin). After isolation of the methotrexate polyglutamates by the SPE material, methotrexate polyglutamates were eluted from the SPE adsorbent material, and individual MTX polyglutamate concentrations could be determined. At <20%, the accuracy and precision of the bioanalytical assay were in high agreement with the most recent FDA guidelines. The intracellular MTX-polyglutamate erythrocyte concentrations obtained were in good agreement with concentrations published previously [1, 14-17]. The most important advantage of the MALDI-QqQ-MS/MS assay was the ultrafast and high-throughput determination of methotrexate and methotrexate polyglutamate concentrations in 10 seconds per analysis – very favorable when compared with the analysis times of up to 30 minutes per sample using routine conventional assays.

Further research using the new MALDI-QqQ-MS/MS assay could provide data for the better and more effective treatment of rheumatoid arthritis patients. It will also prevent the development of methotrexate toxicity, especially in patients whose methotrexate clearance is impaired.

Conclusions

The application of ultrafast and high-throughput MALDI-QqQ-MS/MS technology in biomedical research can have many advantages. Quickly receiving answers to medical and clinical questions will not only result in a highly efficient therapy intervention and treatment, it will also provide knowledge on interpatient variances in pharmacokinetic and drug metabolism properties, for example in epidemiological studies with large patient populations. Biomedical research may be particularly improved by the fact that the bioanalytical performance of MALDI-QqQ-MS/MS technology is equal to that of the conventional analytical techniques such as the HPLC, LC-MS and immunoassays used in routine clinical practice. This improvement will naturally be obtained not only in the medical fields described in this thesis, but also in other medical disciplines supported by this new technology. The inclusion of stable-isotope-labeled internal standards in bioanalytical assays, which is not possible in immunoassays or HPLC-UV assays, will improve the reliability of qualitative data. In conclusion, the ultrafast and high-throughput properties of MALDI-QqQ-MS/MS mass spectrometry could have a major impact on future biomedical research.

Future perspectives

In this study, we used MALDI-QqQ-MS/MS technology to develop new bio-analytical assays for infectious diseases, oncology and auto-immune diseases. The strength of this mass-spectrometric technology lies not only in its ultrafast and high-throughput properties, but also in the fact that its sensitivity and selectivity are potentially higher than those of the more routine analytical techniques such as HPLC-UV, immunoassays and LC-MS.

Although one welcome improvement of this mass spectrometric technology would be automated spotting of the MALDI plates by a spotting device that could further improve the sample throughput, sample preparation, and reproducibility of this technology, the sensitivity and robustness of the MALDI-QqQ-MS technology has opened future possibilities for studying other molecules beyond drugs – such as peptides, hormones, and small proteins– by multi-reaction monitoring (MRM). MRM is a mass-spectrometry technique that uses knowledge of chemical structures of

proteins, peptides, small molecules, and their specific transitions during fragmentation, and makes it possible to quantitatively determine their concentrations.

MALDI-QqQ-MS/MS technology clearly holds potential for biomedical research, especially in studies with many subjects (patients and healthy controls) – for example, when new potential biomarkers in a disease are to be validated in relative large numbers of samples. However, unlike most current routine diagnostic assays in biomarker research, including Western blotting, ELISA and bead-bound antibody assays, MALDI-QqQ-MS/MS technology is not necessarily antibody-based. Since immunoassays are analyte-specific, the use is hampered by the fact that an immunoassay is not available for every potential analyte, and that an individual antibody would have to be produced, and its specificity demonstrated for the particular protein, peptide or small molecule.

The production and validation of individual antibodies for immunoassay testing is a costly and time-consuming process that has to be repeated for each candidate marker (for which no assay is available yet). To save time and sample volume, and to make it possible to measure many different potential biomarkers in a single run, multiplex immunoassays [21] have therefore been developed in which approximately 20-100 potential proteins or peptides can be analyzed in one single run and in one sample. The sensitivity (approx. 1 ng protein/mL) and specificity of these multiplex immunoassays differs per protein-antibody combination, and has to be carefully determined for each protein, peptide or small molecule. The best approach to measuring multiple compounds in minute sample amounts is probably by using combinations of well-designed immunoassays and MRM-based assays (i.e. assays based on multi-reaction monitoring) [22,-24].

In this thesis, we have demonstrated the applicability MALDI-MRM, which allows sample analysis in several seconds per sample. The ultrafast, high-throughput properties of this mass-spectrometric technology enable it to offer protein, peptide and metabolite analysis at a low cost. A major advantage of this technology over immunoassays is the fact that no antibody is required, and that proteins, peptides and small molecules can be measured directly in combination with or without appropriate isotope-labeled analogues. This could significantly improve validation of potential biomarkers and other interesting analytes, such as drugs and endogenous compounds in larger cohort studies.

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Chapter 6

Summary

Chapter 1

In the introduction chapter of this dissertation, an overview has been be presented about the application of MALDI mass spectrometry in selected biomedical research fields. Mass spectrometry has proven to be an important technology in research fields like proteomics and metabolomics. Due to the complexity of the samples sophisticated and high-throughput mass spectrometric instrumentation is needed, especially when low anlyte concentrations are expected and large sample sets of patient samples must be analyzed. Many samples demands also instrumental technology which is ultrafast (preferably analysis in seconds). The new mass spectrometric MALDI-QqQ-MS/MS technology combines high-throughput and ultrafast analyses. This thesis tested this new technology on its clinical-and biomedical research feasibility by analysis of six different drugs used for the treatment of HIV-1 and influenza infection, lung cancer and leukemia and the auto-immune disease rheumatoid arthritis. Finally, the aims and outline of this dissertation are presented.

Chapter 2

In **chapter 2**, the development and the application of three new bio-analytical assays is described. The bio-analytical assays were developed for the support of biomedical research in the infectious diseases field, especially for HIV-1 and influenza infections. The first assay was developed for the measurement of the antiretroviral drugs Lopinavir and Ritonavir (Kaletra®) in plasma and dried blood spots from pediatric patients. Observed DBS concentrations showed as well, a good correlation between plasma concentrations obtained by MALDI-QqQ-MS/MS and the HPLC-UV assay. Application of DBS for TDM applications in pediatric infectious diseases research was proven to be a suitable and diagnostic good alternative to the normally used plasma screening. Collection of DBS specimen requires small amounts of whole blood and is easily performed especially in (very) young children where collection of relative large whole blood amounts by venepunctre is often limited. DBS collection is perfectly suited for TDM of HIV-1-infected children and DBS specimen can also easily be applied for TDM of patients in areas with limited or no laboratory facilities. A bio-analytical assay developed to support HIV research was

used for the measurement of the nucleotide reverse transcriptase inhibitor Tenofovir in plasma from HIV-1 infected adults. The paper described to our knowledge the first combined application of MALDI-QqQ-MS/MS technology and an isotope dilution method. The assay was successfully applied and tested for its clinical feasibility by the analysis of plasma samples from selected HIV-infected adults receiving the prodrug tenofovir disoproxil fumarate. Observed Tenofovir plasma concentrations were within the therapeutically relevant concentration range and showed that compliance of the patients to the therapy was good.

The development of a bio-analytical assay for the measurement of the Oseltamivir (Tamiflu®), an antiviral drug used for the treatment of influenza infection is described. Since the application of DBS for the measurement of whole blood concentrations of Kaletra® provided us with results that well cross validated with the golden standard, we developed and fully validated a DBS assay for the simultaneous determination of oseltamivir and oseltamivircarboxylate concentrations in human whole blood. The application of MALDI-QqQ-MS/MS technology failed because the sensitivity for the oseltamivircarboxylate was not high enough and problems with the reproducibility were observed. We therefore decided to use ultra high-performance liquid chromatography- triple quadrupole mass spectrometry to apply for the determination of oseltamivir and oseltamivircarboxylate concentrations. We demonstrate the simplicity of DBS sample preparation and as a proof of concept, oseltamivir and oseltamivircarboxylate levels were determined in DBS obtained from healthy volunteers who received a single oral dose of Tamiflu®.

We also studied the addition of internal standards in DBS analysis from patients who are treated with the antiviral drug Nevirapine due to an HIV-1 infection. We observed that the addition procedure of the stable isotope labeled internal standard had significant impact on observed Nevirapine concentrations. The direct addition of the internal standard to the blood prior to spotting onto the DBS paper provided us with Nevirapine recoveries of approximately 100%. However, this procedure is not practical due to safety issue concerns for medical and laboratory personnel. Therefore, we selected the second best procedure where the internal standard is added to the paper prior to the blood spotting (NVP recovery 85.3%). Moreover, disks sizes of the punches (5 and 7 mm diameter) prior to analysis had no

significant influence on measured Nevirapine concentrations. Application of internal standard prior to blood spotting provided good Nevirapine recoveries and this procedure is well suitable for TDM applying DBS in infectious diseases, especially in HIV treatment.

Chapter 3

In **chapter 3**, the measurement of pemetrexed concentrations in plasma from lung cancer (NSCLC) patients is shown. In oncology plasma concentration levels of drugs are sporadic determined. Pemetrexed is a relative new anti-folate drug and we present here one of the few bio-analytical assays published on the determination of plasma pemetrexed concentrations. The new assay was ultrafast and demonstrated a sixty times lower detection limit than a previously described LC-MS assay but. The pemetrexed concentrention could be determined from 0.001 µL of patient plasma, a significant lower sample amount than necessary for LC-MS measurement where sample amounts of few microliters are regularly used.

The measurement of the plasma concentrations of the anti-folate methotrexate and its 7-hydroxy metabolite using MALDI-QqQ-MS/MS in combination with isotope dilution is also described. Methotrexate and 7-hydroxymethotrexate concentrations in plasma samples from acute lymphoblastic leukemia (ALL) patients were determined. Observed methotrexate concentrations were cross-validated with concentration determined by the Erasmus MC pharmacy laboratory using a standardized polarization fluorescence immunoassay (FPIA). The results from the cross-validation showed that the MALDI-QqQ-MS/MS assay provided equal analytical performance as the FPIA assay but the MALDI-QqQ-MS/MS assay had a better sensitivity (60 times more sensitive) and a better selectivity especially in the lower concentration range compared to the FPIA assay. Furthermore, it was possible with the MALDI-QqQ-MS/MS assay to quantify the 7-hydroxymethotrexate metabolite which could have a therapeutic relevance as a new biomarker for having a lower susceptibility to the development of MTX toxicity.

Chapter 4

In **chapter 4**, the application of a new developed MALDI-QqQ-MS/MS assay for the ultrafast quantitative determination of intracellular erythrocyte methotrexate

and ethotrexatepolyglutamate metabolite concentrations is described. The assay was used to measure these analytes in packed erythrocyte pellets from rheumatoid arthritis patients receiving low dose (25 mg/week) methotrexate therapy. The highest observed MTX glutamylation degree was MTXPG5, the very long chain MTX-polyglutamate metabolites MTXPG6 and MTXPG7 were not detected in anyone of the packed erythrocyte pellets and observed analyte concentrations were in good agreement with published data on intracellular MTX and MTX-polyglutamate metabolite concentrations.

Chapter 5

This chapter provides a general discussion of obtained results and results of are critically discussed and analyzed. Furthermore, a separate section with some considerations on future research applying the MALDI-QqQ-MS/MS technology is presented.

Addendum

Nederlandse samenvatting

In dit proefschrift beschrijven wij het gebruik van een nieuwe massa spectrometrische technologie die gebruik maakt van matrix-assisted laser desorption/ionization triple quadrupole massaspectrometrie (MALDI-QqQ-MS) voor de meting van geneesmiddelconcentraties in plasma en “dried blood spots”. De bepaling van geneesmiddelen in bloed (of plasma) van patiënten kan een moeizame en langdurige procedure zijn. In het bloed van de patient bevinden zich niet alleen buiten de gebruikte geneesmiddelen en afbraakproducten hiervan ook voedingsstoffen, hormonen, zouten, vetten, vitaminen en nog veel meer stoffen. In het klinisch laboratorium word daarom voor de bepaling van geneesmiddelen veelvuldig gebruik gemaakt van meetmethoden die veelal een chromatografische scheiding gebruiken om de te meten stoffen van andere stoffen aanwezig in het monster en die niet interessant zijn voor de behandeling van de ziekte te scheiden. Dit scheidingsproces kan veel tijd in beslag nemen en is in de klinische praktijk meestal de snelheidbepalende factor. In het jaar 2008 bracht het bedrijf Applied Biosystems/MDS Sciex (tegenwoordig AB Sciex genaamd) de Flashquant op de markt, deze massaspectrometer maakt niet gebruikt maakt van de veelvuldig gebruikte electrospray (ESI) bron voor de ionisatie van moleculen maar had een matrix-assisted laser desorption/ionization (MALDI) bron voor de ionisatie geïnstalleerd. Door de koppeling van de massaspectrometer met MALDI i.p.v. ESI kunnen moleculen geioniseerd worden in complexe biologische monsters zoals plasma, bloed en urine. Voor de MALDI koppeling met de massa spectrometer is het gebruik van een vloeistofchromatografische scheiding (HPLC) niet noodzakelijk nodig en hierdoor wordt de analysetijd van monsters korter maar de meting is in veel gevallen ook gevoeliger. De combinatie van MALDI en massaspectrometrie voor de kwantitatieve bepaling van geneesmiddelconcentraties wordt in het algemeen nu nog niet niet veel toegepast omdat er in het verleden slechte reproduceerbare resultaten werden verkregen. De reproduceerbaarheid werd echter significant verbeterd door gebruikmaking van een 1000 Hz laser in de Flashquant bron in plaats van een laser met lagere frequentie in klassieke MALDI bronnen. Hierdoor is de Flashquant uiterst geschikt is om geneesmiddelen te kwantificeren (triple quadrupole massaspectrometer)met hoge reproduceerbaarheid en een zeer aantrekkelijke

analysetijd (enkele seconden per monster). Wij hebben de toepasbaarheid van deze nieuwe massaspectrometrische technologie bestudeerd voor klinische diagnostieke doeleinden door het meten van verschillende geneesmiddelconcentraties gebruikt in verschillende ziektebeelden.

In **Hoofdstuk 2,** beschrijven we verschillende methoden om concentraties te meten van antivirale medicatie. Zo meten we protease inhibitoren, geneesmiddelen die gebruikt worden bij de behandeling van kinderen die met HIV-1 geïnfecteerd zijn. Bovendien wordt een methode beschreven om de concentratie te meten van Tenofovir, een ander type geneesmiddel die veelvuldig gebruikt word om volwassenen die met HIV-1 geïnfecteerd zijn te behandelen. Tenslotte, beschrijven we een methode om Tamiflu in plasma van patiënten te meten die een influenza (griep) infectie bezitten. Omdat er gebruikt gemaakt word van “dried blood spots” bij deze patiënten presenteren we in het laatse gedeelte van hoofdstuk 2 een studie waarbij onderzoek is gedaan naar de best mogelijke manier om interne standaarden te gebruiken in “dried blood spots” metingen in het algemeen.

In **Hoofdstuk 3,** presenteren we een methode die het mogelijk maakt om concentraties van het nieuwe geneesmiddel Pemetrexed te meten in plasma van longkanker patiënten en bovendien een methode om Methotrexaat en zijn afbraak product (7-hydroxymethotrexaat) te meten in plasma van patiënten met leukemie.

In **Hoofdstuk 4**, tenslotte beschrijven wij een method om methotrexate en methotrexaat polyglutamaat metaboliet concentraties in rode bloedcellen te meten van patiënten met rheumatoide arthritis.

In **Hoofdstuk 5**, word een algemene discussie gegeven waarin alle resultaten beschreven in dit proefschrift nogmaals kritisch worden bekeken en er worden verder nog enkele aanbevelingen gedaan voor toekomstig wetenschappelijk onderzoek.

In **Hoofdstuk 6**, worden tenslotte alle in dit proefschrift verkregen resulaten samengevat.

Dankwoord

Zo, eindelijk is mijn proefschrift klaar. Promoveren doe je echter niet alleen, hoewel dat door velen gedacht wordt. Daarom wil ik hier nogmaals iedereen uit het diepste van mijn hart bedanken. Ik wil graag de laboratoria voor Neuro-oncologie en Clinical & Cancer proteomics, de afdeling Virologie, Klinische Chemie en Longziekten bedanken voor de prettige en inspirerende werkomgeving waarbinnen dit promotieonderzoek heeft plaatsgevonden.

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PhD portfolio

Name:	Roland Jacob Willem Meesters
ErasmusMC department:	Neurology
PhD period:	2009- 2011
Research school:	Erasmus Postgraduate school Molecular Medicine
Promotor:	Prof.dr. P.A.E. Sillevis Smitt
Co-promotor:	Dr. T.M. Luider

In depth courses/workshops

- Assesment of DBS Technology for the detection of Therapeutic antibodies, FIP Pharmaceutical Sciences 2010 World Congress, American Association of Pharmaceutical Scientists, New Orleans, USA, 2010.
- Successful DBS in a rapid Drug Discovery Environment, FIP Pharmaceutical Sciences 2010 World Congress, American Association of Pharmaceutical Scientists, New Orleans, USA, 2010.
- Training network UEPHA, Marie Curie fellowship, Rotterdam, the Netherlands, 2010.
- MolMed course Biomedical Research Techniques, Rotterdam, the Netherlands, 2010.

Oral presentations

- **Roland J W Meesters** and Gero P Hooff, Dried blood spots: the past, present and future. Invited lecture DSM Resolve, Geleen, the Netherlands, 2011
- **Roland J W Meesters** and Gero P Hooff, Dried blood spots: the past, present and future. Invited lecture Unilever, Vlaardingen, the Netherlands, 2011

- **Roland J W Meesters**, Jeroen J A van Kampen, Mariska L Reedijk, Rachel D Scheuer, Lennard J M Dekker, David M Burger, Nico G Hartwig, Albert D M E Osterhaus, Theo M Luider, Rob A Gruters. MALDI-MS/MS analyses of antiretroviral drugs used in HIV-1 treatment HTC-11 congress, Bruges, Belgium 2010

- R A Gruters (presenting author), **R J W Meesters**, J J A van Kampen, D M Burger, N G Hartwig, T M Luider et al. Ultrafast and high-throughput therapeutic drug monitoring of antiretroviral drugs in pediatric HIV-1 infection applying dried blood spots. Oral presentation. Antiviral Congress, Amsterdam, the Netherlands, 2010

- **R J W Meesters**, J J A van Kampen, M L Reedijk, R D Scheuer, L J M Dekker, D M Burger, R de Groot, N G Hartwig, ADME Osterhaus, R A Gruters, T M Luider. Oral presentation. Pediatric therapeutic drug monitoring of the protease inhibitor Lopinavir and two metabolites in HIV-1 infected children treated with Kaletra®. FIGON 2010 Dutch Medicine Days, Lunteren, the Netherlands, 2010

- **Roland Meesters**, Jeroen van Kampen, Lennard Dekker, Marleen Reedijk, Rachel Schreuer, David Burger, Ab Osterhaus, Theo Luider, Rob Gruters. Oral presentation. Analyses of antiviral drugs with MALDI-MS/MS. FIGON 2009 Dutch Medicine Days, Lunteren, the Netherlands, 2009

- **Roland J W Meesters.** Toepassing van massa spectrometrie in de klinische chemie “diagnostiek m.b.v. MALDI-MS/MS bij HIV-geinfecteerde patienten”. OLVG Hospital, Amsterdam, the Netherlands, September 10, 2009

Poster presentations

- **Roland J W Meesters**, Jeroen J A van Kampen, Rachel D Scheuer, David M Burger, Albert D M E Osterhaus, Theo M Luider, Rob A Gruters. Rapid assessment of antiretroviral therapy efficacy by dried blood spot sampling in

HIV infection. Poster presentation, TiPharma Springmeeting 2011, Utrecht, the Netherlands, 2011

- **Roland J W Meesters**, Jeroen J A van Kampen, Mariska L Reedijk, Rachel D Scheuer, Lennard J M Dekker, David M Burger, Nico G Hartwig, Albert D M E Osterhaus, Theo M Luider, Rob A Gruters. Ultrafast and high-throughput therapeutic drug monitoring of antiretroviral drugs in pediatric HIV-1 infection applying dried blood spots. Poster presentation, TiPharma Spring Meeting 2010, Utrecht,the Netherlands, 2010

- **Roland J W Meesters**, Jeroen J A van Kampen, Mariska L Reedijk, Rachel D Scheuer, Lennard J M Dekker, David M Burger, Nico G Hartwig, Albert D M E Osterhaus, Theo M Luider, Rob A Gruters. Ultrafast and high-throughput therapeutic drug monitoring of antiretroviral drugs in pediatric HIV-1 infection applying dried blood spots. Poster presentation. FIGON Dutch Medicine Days, Lunteren, the Netherlands, 2010

- **Roland J W Meesters**, Jeroen J A van Kampen, Mariska L Reedijk, Rachel D Scheuer, Lennard J M Dekker, David M Burger, Nico G Hartwig, Albert D M E Osterhaus, Theo M Luider, Rob A Gruters. Ultrafast and high-throughput therapeutic drug monitoring of antiretroviral drugs in pediatric HIV-1 infection applying dried blood spots. Poster presentation. 1st AB SCIEX European Conference on MS/MS (ECMSMS), Noordwijkerhout, the Netherlands, 2010

- **Roland J W Meesters**, Jeroen J A van Kampen, Mariska L Reedijk, Rachel D Scheuer, Lennard J M Dekker, David M Burger, Nico G Hartwig, Albert D M E Osterhaus, Theo M Luider and Rob A Gruters. Ultrafast, high-throughput therapeutic drug monitoring of antiretroviral drugs in pediatric HIV-1 infection applying dried blood spots. Poster presentation. FIP Pharmaceutical Sciences 2010 World Congress, New Orleans, USA, 2010

- **Roland J W Meesters**, Yvonne M Bastiaansen-Jenniskens, Joris Harlaar, Johan F Lange, Gerjo J V M van Osch, Theo M Luider. A new ultrafast and high-throughput assay for disease-specific diagnostics of collagen and

collagen cross-links. Poster presentation. Biomedica 2010, Aachen, Germany, 2010

International conferences

- 11th congress International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analyzers, Bruges, Belgium, 2009.
- 4th Biomedica Life Summit congress, Aachen, Germany, 2010.
- FIP Pharmaceutical Sciences 2010 World Congress, New Orleans, USA, 2010

Awards

- Top Institute Pharma Spring Meeting 2010 best Poster price, Utrecht, the Netherlands
- Nominated for the best Poster price at the NVMS (Dutch Association for Mass spectrometry) autumn meeting 2010, Amsterdam, the Netherlands, 2010.

Extra-curricular activities

- Invited lecturer Mass Spectrometry, Zuyd University, Heerlen, the Netherlands, 2010.

Curriculum vitae

Roland Jacob Willem Meesters werd geboren op 19 maart 1967 te Bocholtz (Zuid-Limburg). Van 1979 tot 1985 doorliep hij de HAVO op het Sancta Maria college te Kerkrade-West. Na het behalen van het HAVO diploma heeft hij twee jaar aan de Zuid Limburgse Laboratorium (ZLS) school in Sittard voor chemisch analist gestudeerd maar moest die studie onderbreken vanwege de in die tijd nog verplichte militaire dienstplicht van 14 maanden. Gedurende zijn militaire dienstplicht heeft hij 's avonds een verkorte Middelbare Laboratorium Opleiding (MLO) op afstand gevolgd. Na afronding van de militaire dienstplicht is hij in 1990 aan een studie Hogere Laboratorium Opleiding (HLO) begonnen (avondopleiding). In 1994 behaalde hij zijn diploma terwijl hij overdag werkte als chemisch analist op een milieuchemisch laboratorium aan de Universiteit van Aken (RWTH Aachen, Duitsland). Op 25 april 2000 is hij getrouwd met Maria Victoria en in 2001 besloot weer gaan te studeren. Hij schreef zich in voor de Bacheloropleiding en Masteropleiding Milieu-en Natuurwetenschappen aan de OU, die met het doctoraal examen met goed gevolg werd afgesloten. Gedurende deze studie heeft hij nog als onderzoeks assistent bij de RWTH en als onderzoeks assistent aan de Universteit van Maastricht gewerkt. In 2007 is hij met zijn gezin naar de VS geëmigreerd waar hij twee jaar als onderzoeks assistent aan de University of Arkansas for Medical Sciences (UAMS) heeft gewerkt. In 2009 is hij met gezin terug gekeerd naar Nederland en is sindsdien werkzaam als wetenschappelijk medewerker en onderzoeker in opleiding (OIO) bij het Laboratory of Neuro-Oncology and Clinical & Cancer Proteomics van de afdeling Neurologie van het ErasmusMC te Rotterdam.