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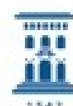

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Tesis Doctoral

MASS SPECTROMETRY APPLICATION STRATEGIES OF DRIED BLOOD SPOTS ANALYSIS

Autor

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UNIVERSIDAD DE ZARAGOZA
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INSTITUTO DE CARBOQUÍMICA

MASS SPECTROMETRY APPLICATION STRATEGIES OF DRIED BLOOD SPOTS ANALYSIS

TESIS DOCTORAL

Stefan Gaugler

- UNIVERSIDAD DE ZARAGOZA -

FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA ANALÍTICA

GRUPO DE NANOSENSORES Y SISTEMAS BIOANALÍTICOS

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D. Vicente Luis Cebolla Burillo, Investigador Científico del Instituto de Carboquímica (CSIC) de Zaragoza,

CERTIFICA,

que la presente Memoria titulada:

“**MASS SPECTROMETRY APPLICATION STRATEGIES OF DRIED BLOOD SPOT ANALYSIS”**,

correspondiente al Proyecto de Tesis aprobado por el Departamento de Química Analítica de la Universidad de Zaragoza, ha sido realizada por **Stefan Christian Gaugler** bajo mi dirección para optar al grado de Doctor.

Y, asimismo,

AUTORIZA

la presentación de la citada Memoria de Tesis Doctoral para su defensa y calificación ante el Tribunal correspondiente.

Zaragoza, 30.04.2019,

Fdo: Vicente Luis Cebolla Burillo

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For my daughter Malou Joline

For my wife Jarah Maria

This Doctoral Thesis is a compendium of the following papers:

Stefan Gaugler *et al.*, "Extended and fully automated newborn screening panel for mass spectrometry detection", Int. Journal of Neonatal Screening, **2018**, 4, 2; doi:10.3390/ijns4010002

Stefan Gaugler, Jana Rykl, Vicente L. Cebolla, "Validation of an Automated Extraction Procedure for Amino Acids and Acylcarnitines for Use with Tandem Mass Spectrometry for Newborn Screening", Endocrinology, Diabetes and Metabolism Journal, **2019**, 3 (1): 1–9

Stefan Gaugler *et al.*, "Fully automated drug screening of dried blood spots using online LC-MS analysis", Journal of Applied Bioanalysis, **2018**, Vol. 4, No. 1, p. 7-15; doi: 10.17145/jab.18.003.

Stefan Gaugler *et al.*, "Fully automated forensic routine dried blood spot screening for workplace testing", Journal of Analytical Toxicology, **2018**;1–9, doi: 10.1093/jat/bky074

M. Luginbühl, S. Gaugler, W. Weinmann, "Fully automated determination of phosphatidylethanol 16:0/18:1 and 16:0/18:2 in dried blood spots", Journal of Analytical Toxicology, **2019**;1–8, doi: 10.1093/ jat/bkz035

Urs Duthaler, Claudia Suenderhauf, Stefan Gaugler, Beatrice Vetter, Stephan Krähenbühl, Felix Hammann, "Development and validation of a LC-MS/MS method for the analysis of ivermectin in plasma, whole blood, and dried blood spots using a fully automatic extraction system", Journal of Pharmaceutical and Biomedical Analysis, **2019**, 172, 18-25, JPBA_2019_744, doi: 10.1016/j.jpba.2019.04.007

Although these papers belong to the research carried out, they were not included in this compendium due to the involvement of other Doctoral students:

M. Haschke, U. Duthaler, B. Berger, S. Erb, M. Battegay, E. Letang, S. Gaugler, S. Krähenbühl, "Automated high throughput analysis of antiretroviral drugs in dried blood spots.," Journal of Mass Spectrometry, ***2017****, vol. 52, no. 8, pp. 534–542; doi: 10.1002/jms.3952.*

Urs Duthaler, Benjamin Berger, Stefan Erb, Manuel Battegay, Emili Letang, Stefan Gaugler, Alex Natamatungiro, Dorcas Mnzava, Massimiliano Donazelli, Stephan Krähenbühl, Manuel Haschke, "Using dried blood spots to facilitate therapeutic drug monitoring of antiretroviral drugs in resource-poor regions", Journal of Antimicrobial Chemotherapy, ***2018****; 73 (10), 2729-2737, doi: 10.1093/jac/dky254*

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Abstract

Dried blood spot analysis (DBS) is a well-known analysis technology, which first routine applications date back to the 1960s. Advancements in mass spectrometry instruments during the last century, enabled to reach the required sensitivity to open up new markets. Some uncertainties and missing method understanding remain and this is holding back the technology from wide spread market-acceptance.

In this doctoral thesis, various DBS analysis techniques, conditions and workflows have been developed and validated, demonstrating the feasibility and application of the DBS-LC-MS technology.

Advanced methods for the field of newborn screening were developed, where the analysis panel has been extended and standardized.

Also new fields of application, such as therapeutic drug monitoring and forensic toxicology were investigated. Drug monitoring of antiretroviral drugs in remote and resource poor regions are described and new approaches for alcohol abuse monitoring are presented. Finally, a breakthrough method, where over 1200 illicit drugs can be detected from one single blood droplet, was introduced within this thesis.

The scientific research carried out is presented in the form of a compendium of publications (6), which are included in this report. Two other works of that do not appear officially in this compendium, are attached as an appendix. All the works constitute a coherent thematic unit on the technique of the DBS and its coupling to the mass spectrometry.

Abstract in Spanish

El análisis de manchas de sangre seca (DBS) es una tecnología de análisis bien conocida, cuyas primeras aplicaciones de rutina se remontan a la década de 1960. Los avances en los instrumentos de espectrometría de masas durante el siglo pasado, permitieron alcanzar la sensibilidad necesaria para abrir nuevos mercados. Sin embargo, algunas incertidumbres y la falta de comprensión de los métodos han impedido hasta el momento que la tecnología tenga una amplia aceptación en el mercado.

En esta tesis doctoral se han desarrollado y validado diversas técnicas, condiciones y flujos de trabajo de análisis DBS, que demuestran la viabilidad y el potencial de aplicación de la tecnología DBS-LC-MS.

Se han desarrollado métodos avanzados para su aplicación en el campo de la pediatría neonatal, en particular la prueba del talón en recién nacidos, donde se ha ampliado y estandarizado el panel de análisis.

También se han investigado nuevos campos de aplicación, como la vigilancia de medicamentos terapéuticos y la toxicología forense. Se describe la aplicación de la farmacovigilancia remota de antirretrovirales en regiones de escasos recursos y se presentan nuevos enfoques analíticos para la vigilancia del abuso de alcohol. Finalmente, en esta tesis se ha introducido un método innovador con el que se pueden detectar más de 1.200 drogas ilícitas a partir de una sola gota de sangre.

La investigación científica realizada se presenta en forma de compendio de publicaciones (6), que son incluidas en esta Memoria. Se adjuntan a modo de apéndice otros dos trabajos del candidato que no constan oficialmente en dicho compendio. Todos los trabajos constituyen una unidad temática coherente sobre la técnica de la DBS y su acoplamiento a la espectrometría de masas.

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Objectives of the thesis

Dried blood spot (DBS) analysis is a well-known technology in the field of newborn screening. Recent advancements like increasing sensitivity of mass spectrometry instrumentation opened up the scope and possibilities for DBS in other fields. Goal of this doctoral thesis is to investigate emerging application fields and to find and develop new potential applications. The applications should align with current micro sampling trends and contribute to the implementation of DBS in new fields. Validation and regulation hurdles should be addressed. The methods will be established on a fully automated DBS sample preparation platform and an according laboratory will be set-up for method development.

Introduction dried blood spot analysis

History of dried blood spot analysis

The first documented concept of applying biological fluids to filter paper, drying, and transporting to a laboratory date back more than 100 years [1], [2]. Filter paper sampling simplified matrix (such as blood) collection, handling and storage over alternative methods at the time. Over the years, this methodology has emerged to a variety of fields of study application such as newborn screening (NBS), forensic toxicology, therapeutic drug monitoring, toxico- and pharmacokinetic studies of drugs, the field of omics, infectious diseases, epidemiology and environmental research [2], [3]. The handling of filter paper seems conceptually simple, however there are some points which add additional complexity to the process such as matrix effects. Blood of different hematocrit (the proportion of red blood cells) and certain drying effects have resulted in unexpected analytical challenges. However, the utility and acceptance of DBS has increased over the last decades due to concerted efforts to control, minimize, and eliminate these analytical variations [2], [3].

The first routine application of DBS dates back to 1961, where Dr Robert Guthrie published a unique bacterial inhibition assay for measuring phenylalanine in newborns to detect phenylketonuria (PKU) [4]. This breakthrough was based on previous investigations of a urine approach from 1958, where a method for the collection of urine from infants onto squares of Whatman filter paper was described as part of a program for early detection of PKU [5]. Here, a square absorbent filter paper was either dipped in urine or placed into a baby's diaper, to let it soak the urine and then allowing it to dry. If the urine appeared too light, the square was dipped once again and dried a second time. Up to this point, dipping the filter paper into the matrix was the standard procedure. Only after 1961, identification circles were printed on the filter paper as target for blood collection [6]. For user friendliness, four circles were printed onto the filter paper. One circle requires approximately 65 µL to fill up completely. The dried specimens' samples were shipped by mail to a centralized laboratory for analysis. It was more cost effective to analyze batch wise with standardized reagents [3], [5].

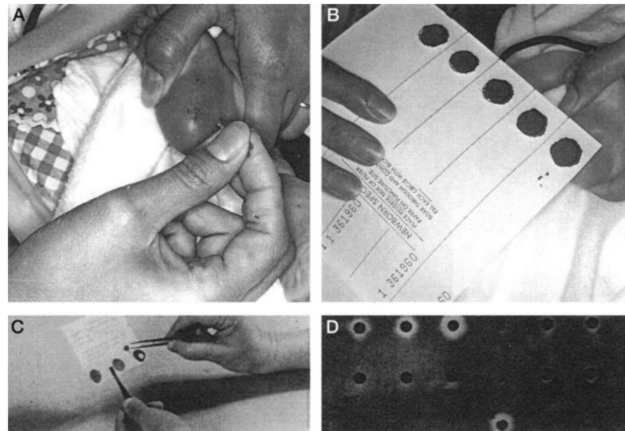

Figure 1, Picture from the Discovery of Phenylketonuria showing a DBS punch process [7]

The approach of screening PKU from filter paper, which is easily transportable and inexpensive, made the application on large scale possible (Figure 1). Due to this new methodology, public health programs adapted and PKU analysis from DBS became a standard for NBS. Due to a large number of false-positive results found in another research team, Guthrie's article from 1961 was initially rejected [8]. Only later on, Guthrie found that different filter paper sources in that study might explain their high false-positive rate [9]. Guthrie found that the filter paper from that Schleicher and Schuell Grade 903 suited best for his test and suggested some minor adaptions for further improving the paper. Such as the DBS should be between 9.5 and 12.5mm in diameter, the paper needs to look uniform on both sides and the area of collection should be close to the edge to allow easy punching with a paper-hole puncher [3], [9].

The Grade 903 filter paper has been produced since 1960 and is still used tofday in many NBS laboratory. This filter paper contains no additives or hardeners and it is highly regulated and controlled to produce consistent quality. Over the years, new regulation had to be fulfilled. Today, blood collection papers need to fulfill the ASTM International standards [10] and are certified to meet the performance standards for sample absorption and lot-to-lot consistency as set by the approved standard.

For the past 50 years, the novel and low-cost approach of blood collection introduced by Dr. Robert Guthrie has led to worldwide population screening of inherited metabolic diseases in newborns and the application has been expanded to many other fields. The initial protocol was also limited to qualitative analysis, because analytical instruments did not have the required sensitivity. During the past years, a revival of the DBS technique can be observed, indicated by the number of newly reported applications [3, 4]. Key driver of this trend was the development and application of tandem mass spectrometry, which enabled far more sensitive analysis [3], [13].

The advantages of DBS compared to conventional blood analysis techniques are numerous (summarized in Table 1). The DBS technique requires much less biological material for the analysis, which is connected to less invasive sampling and hence more comfort for the patients. Additionally, the storage, shipping and handling costs are lower. Also, the working risks for the technicians are significantly reduced by using DBS. DBS seems to be a powerful alternative to conventional blood sampling, since trends in biomedical analysis are going into direction of reduction of biological material, time and analysis costs.

Table 1, Advantages of DBS to conventional blood analysis techniques

Parameter	Conventional Analysis	DBS Analysis	Advantage of DBS
Blood volume	> 500 µL	10 -30 µL	Less biological material needed Suitable for newborn and elderly people Ethical reasons
Blood collection technique	Venipuncture with a sample and stabilization system	Finger or heel pricks using a capillary	Less invasive sampling No trained personal needed Less patient discomfort
Sample processing	Precipitation, centrifugation and further preparation steps depending on the assay	Blood spotting and drying (2 h). The extraction is either performed manually or automated.	Less steps needed Significant reduction of technician work load No laboratory environment required Less risk of handling the bio-fluid
Storage and transport	Plasma and serum must be stored and shipped under frozen conditions	Stable after drying at room temperature. Can be shipped without any biohazard labeling.	Cards require less space to store or ship No need for dry ice Patient can prepare the card at home and sent it via post mail
Cost	Samples must be frozen and treated as hazard bio fluids	Reduced storage, shipping and labour costs	Overall costs are significantly reduced

State of the art and methodology

Manual DBS procedure

For DBS sample preparation and analysis, there are manual punchers, semi-automated punchers and complete automation devices available. Punching is still used in many laboratories (especially in the field of newborn screening) nowadays. Here, the DBS are punched out and extracted in a micro centrifuge tube. The extraction process can be varied in terms of solvent, time and treatment. The extracts are centrifuged and either analysed directly, or dried and reconstituted in the mobile phase. The punching of blood spots and the offline extraction are time consuming and labour intensive (Figure 2). Additionally, there is a high waste of consumables and it is not suitable for high throughput applications. However, additional extraction or derivatisation steps of the analytes can be introduced, and the sample can be reconstituted before measurement.

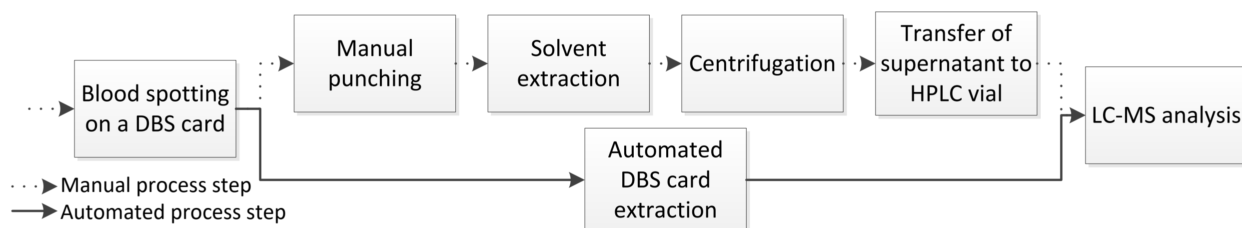

Figure 2, Comparison between working steps in the manual and in the automated DBS approach

Automated and semi-automated DBS instrumentation

In recent years there have been several approaches to automate the filter card extraction procedure. Spark Holland developed the first on-line extraction system in 2007 [14]. The group of Neil Spooner at GalaxoSmithKline (GSK) tested this technology in comparison with the new system from CAMAG for on-line DBS-LC-MS/MS (LC-MS/MS: liquid chromatography tandem mass spectrometry) in 2009 [15]. Later on, in 2010, ProLab launched the first commercial available on-line DBS instrument. The first ProLab system (SCAP) was equipped with a robotic gripper, which places the DBS cards into a clamp system, an online trapping column for desorption and a coupling to a LC-MS/MS system. Spark Holland launched another fully automated DBS Autosampler (DBS-X) in 2012 and the DBS-MS 500 system from CAMAG was introduced in 2013.

Nowadays, there are two high end technology systems which show good results concerning sensitivity, quantitative performance, automation and throughput and which are appropriate for LC-MS coupling. On the one hand the DBS-MS 500 from CAMAG (Figure 3, left), which was initially developed to couple cost-effective thin layer chromatography (TLC) separation with selective and sensitive mass spectrometry (MS) detection. Here a nonporous material can be extracted with the sealing surface sampling probe procedure. On the other hand there is the Spark Holland DBS X system (Figure 3, right).

Figure 3, The automated extraction systems DBS-500 [16] **(left) and DBS X** [17] **(right)**

The flow scheme of the DBS X system from Spark Holland (Figure 4) consists of a combination of three 6-port valves. The internal standard is first loaded into a defined loop. After clamping the DBS card in the extraction cell, the elution pump brings solvent through the internal standard (ISTD) loop and trough the DBS onto a trapping column. The standard–solvent mix is forced horizontally through the filter paper, which functions as a form of membrane. Different clamp sizes for desorption and an option to heat the clamp are available. The analytes elute off the trap by switching it into the LC-MS cycle and by increasing the organic content of the mobile phase, where the extract is guided via a column into the MS. The system can handle up to 96 DBS cards and is equipped with a camera, which takes a picture from each DBS card before and after the extraction. The centre of each spot is determined by image analysis plus providing several advantages:

- Read barcode information / identify card
- Check card position
- Determine offset to the centre, to preform several extractions of one DBS
- This allows multiple measurements with several internal standards [17], [18]

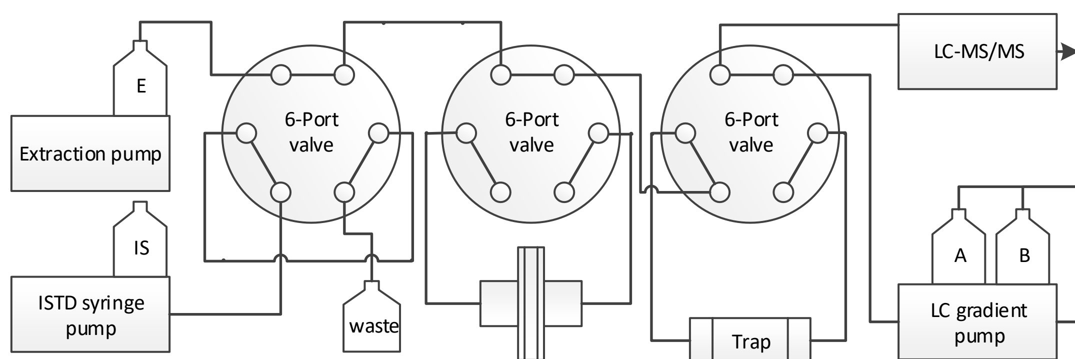

Figure 4, Flow scheme of the DBS X system from Spark Holland

This system is also distributed by Gerstel and Axel Semrau as OEM (Original equipment manufacturer) product.

Likewise, the CAMAG DBS-MS 500 is an automated extraction module with an integrated camera and internal standard application. However, the basic principle is different to the DBS X system. The main differences are the configuration of the extraction setup and the application of the internal standard.

The extraction head of the CAMAG device consists of a cone with two small holes connected to a “dual capillary” inlet and outlet (Figure 5; Extraction plunger). A circular plunger seals a hole of 4 mm inner diameter in the card. Afterwards the extraction solvent passes through the card from the inlet to the outlet and is then collected in a sample loop. This sample loop is independent from the LC-MS system and thus allows a high flexibility for different extraction conditions. The loop size can vary from 20 to 200 µL or a trapping column can be installed. After the extraction procedure, a start signal is sent to the LC-MS/MS and the loop volume is injected into the column.

The second difference is the application of the ISTD which is sprayed onto the filter card. This allows the possibility to add the standard as early as possible to the sample. The spraying unit sprays the ISTD solutions in volumes of 5 to 40 µL directly on one spot (Figure 5; ISTD sprayer). Four different ISTD bottles can be selected individually and the needle is washed before each run to avoid carry-over. The application of the internal standard just before the extraction of the DBS card is much easier from a logistic point of view than adding the internal standard before the blood sample is spotted onto the DBS card.

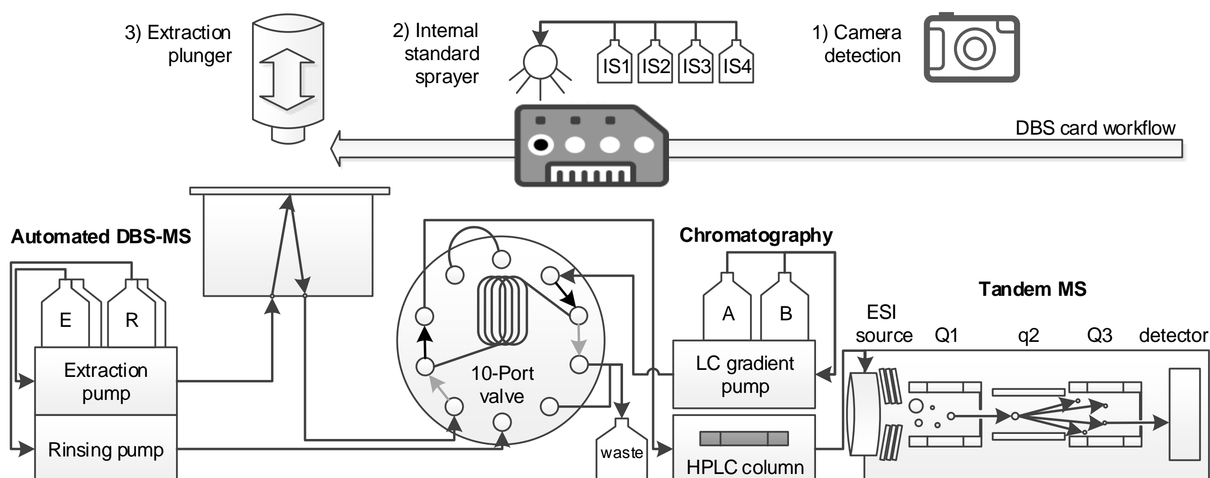

Figure 5, DBS-MS 500 flow scheme

The flow scheme (Figure 5) of the DBS-MS 500 setup can be divided into two basic process steps. First, the card extraction flow path and second the rinsing of the extraction unit when the ten-port valve is switched. The card is trapped on the cone of the extraction head and extracted with solvent coming from the extraction pump. The flow rate and the volume can be adjusted to reach optimum extraction conditions. This extraction unit is cleaned after each run. The extraction head and its outlet to the 10 port valve are flushed backwards by a rinsing unit. The rinsing program can be set manually making use of up to four different solvents to avoid carry-over effects. The DBS-MS 500 is also equipped with a camera, which recognizes the blood spots like in the DBS X system. This optical card recognition unit determines the card properties like spot number and position, checkbox status and barcode information. The DBS card is only accepted for analysis if all parameters comply. The rinsing unit was successfully proven to prevent carry-over effects. Furthermore, the function of the spraying unit was positively investigated in terms of reliability and consistency and became the gold standard in DBS [19],[20].

There is a third system on the market, the SCAP from Prolab or also referred as Agilent automated card extraction (AACE). This instrument was bought by Agilent Technologies in 2012, however not continued after 2015 and therefore back at Prolab. The flow scheme of the AACE device (Figure 6) consists of two high pressure binary pumps and one isocratic pump for extraction, dilution and chromatographic separation. At first the clamped DBS card is extracted with a mixture of a solvent (flask A and B) and an internal standard (ISTD), which is loaded into an injection loop and guided to the first trap. The standard–solvent mix is forced through the filter paper. The analytes elute off the first trap by increasing the organic content of the solvent. Afterwards, the dilution pump adds the aqueous mobile phase (flask A) to allow retention of the analytes to the second trapping column. The second valve is then switched to start the chromatographic separation by eluting the analytes backwards of the second trap column with the gradient pump (flask A and C) [10, 11].

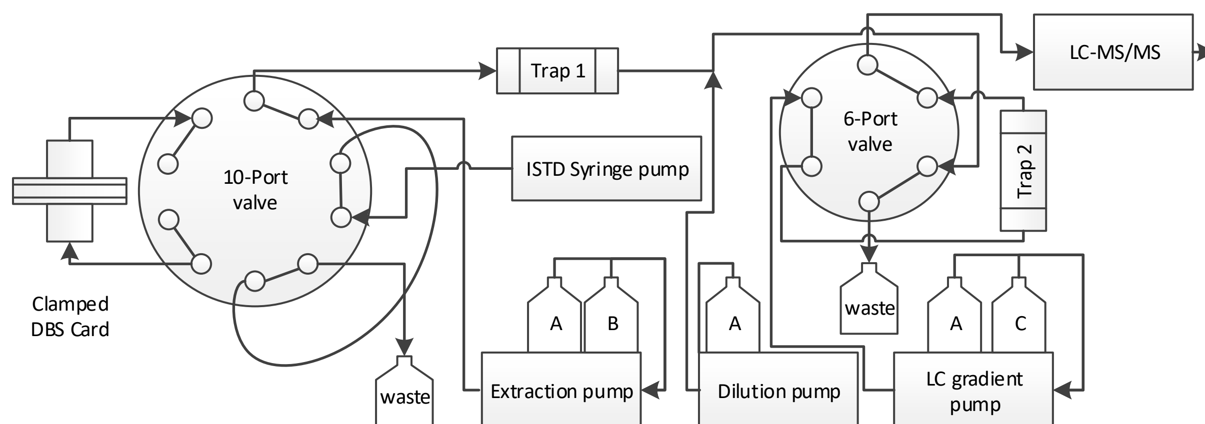

Figure 6, AACE flow scheme

In general, all systems have advantages and disadvantages depending on the field of application. Hence, it has to be evaluated individually which system fits best for the actual purpose. The core parameters are summarized in the table below (Table 2).

Table 2, Hardware comparison of the DBS-MS 500, the DBS X and SCAP system

	CAMAG DBS-MS 500	Spark Holland DBS X	Prolab SCAP
Extraction	Horizontally from the bottom back to the bottom	Flow through	Flow through
Internal standard application	Sprayed on the card prior extraction	Added to the extraction solvent	Added to the extraction solvent
Optical card recognition	OCR-module	Optical camera	Optical camera
Card capacity	500 cards	96 cards	40 cards
Samples per spot	1	Up to 4	Up to 4
Consumables	20-50 µL extraction solvent	>50 µL extraction solvent plus SPE cartridge	>50 µL extraction solvent plus SPE cartridge
MS integration	Full online integration	Full online integration	Agilent interface

Indirect alternatives to the fully automated DBS platforms are automated card punchers, which allow semi-automated analysis workflows. There are several of those punchers from PerkinElmer (Panthera), Hamilton (STAR-led), and BSD robotics (600plus) on the market and very wide spread in newborn screening laboratories. Another recent development is the CLAM-2000 system from Shimadzu, which enables automated solid extraction and LC-MS injection of the extract.

The CAMAG DBS-MS 500 instrument was used for the sample preparation within this work, therefore the other technologies will not be further discussed.

DBS filter paper

The DMS filer paper is the most critical consumable in the workflow. As Guthrie described in 1963 [9], the quality and thickness of the paper has significant impact on the analysis result. Therefore, paper manufacturers established together with the Center for Disease Control and Prevention (CDC) the CLSI NBS-01-A6 guideline. The main pints can be summarized [10]:

- Filter paper should be made of 100% pure cotton fiber, with no wet-strength additives.
- Basis weight should be 110 lb ± 5% per ream (179 g/m2 ± 5%). A ream is defined as 500 sheets 2411 x 36" (ASTM D646-96).
- The pH should be 5.7-7.5 (Test method ISO 6588:1981).
- Ash %: 0.1 % maximum (Test method A of ASTM D586-97a).

The first filter paper from Schleicher and Schuell called 903 was chosen by Guthrie and became the market standard. The English company Whatman entered the filter paper market in the early 1990 and provided a paper called BFC 180, this paper was then accepted by the Europe Food and Drug Administration (FDA). Lateron, Whatman purchased Schleicher and Schuell in 2005 and replaced their BFC 180 with the acquired 903. In 2007, Ahlstrom entered the market with the FDA approved 226 filter paper. PerkinElmer purchased this paper type exclusively [3] and Ahlstom registered another type called TFN.

In the early 2000, pharmaceutical companies began to evaluate filter paper to collect and transport blood samples for drug discovery and development. This opportunity motivated the filter paper manufacturer to take a closer look at their process in order to supply a product that meets the pharma requirements and is also suited for quantitative assays [2]. Since Ahlstrom produces the 226 filter paper exclusively for PerkinElmer, they introduced a new brand called TFN in 2013. These three papers, 903, 226 and TFN are recognized medical class II devices. Further filter papers in the market are 818 from Machery Nagel, 2992 from Kühne Nagel and further Ahlstrom types such as 222.
Besides the pure cotton filter paper types, there was a filter card from Agilent Technologies, which was composed of a nylon membrane. Whatman also offers coated filter paper such as the DMKA-A and DMPK-B cards.

DBS related additional analysis complexity

The handling of filter paper seems conceptually simple, however there are some points which add additional complexity to the process [23]. Due to the growing interest in DBS analysis, the American FDA added a new section to their bioanalytical method validation guideline [24] for DBS. These points and further are addressed in the following sections:

Hematocrit effects

The hematocrit (HCT) effect is probably the most discussed topic in the DBS community. The blood volume within a given area may increase significantly when HCT levels fluctuate from 30% to 70% [2]. Since usually the volume and the HCT are unknown, exact quantification represent significant analytical challenges in method validation. A 6.0 mm punch from the center of a 100 µL DBS made from 30% HCT blood contained 47% more serum volume than a similar punch from 70% HCT blood [25]. Although, this is not a realistic scenario, as blood droplets are usually around 15-25 µL. Blood samples with low haemoglobin concentrations (hence having a low HCT) spread more widely due to lower viscosity than those with normal and high haemoglobin concentrations (Figure 7).

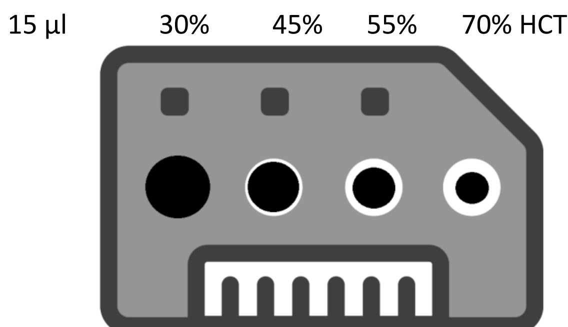

Figure 7, Visualized impact of varying hematocrit on DBS size

During the past years, different approaches have been suggested to overcome this issue. There are basically three ideas; modifying the filter paper card, using an optical tool to detect the actual HCT or using a known value from the HCT portion as reference (Figure 8).

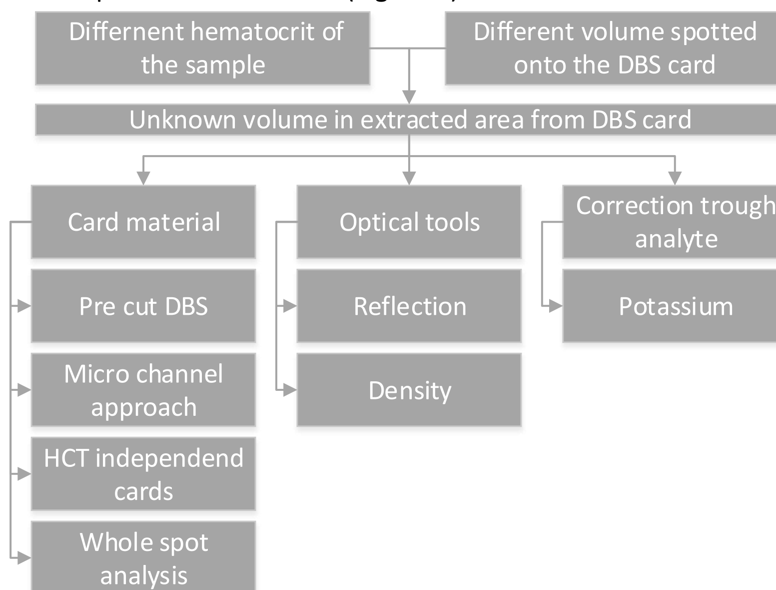

Figure 8, Strategies to handle the HCT effects

Most efforts went into the development of alternative card materials and devices where a fixed volume of blood is applied on the filter paper card. Two commercial systems are on the market which apply a fixed volume through micro channel devices [26], [27]. Also, cards were designed with a lateral plasma separation layer. There is no significant difference in plasma viscosity and the existing plasma methods doesn't need to be adapted to whole blood [28] (see section DBS devices and alternatives).

Another idea was to use optical devices to scan the DBS prior analysis to detect its HCT. The density measurement didn't deliver reliable results. However, the reflection measurement of haemoglobin content and its metabolites seems to be a very good option which is investigated more deeply at the moment. The key advantage of this method is the fact that it is non-destructive and it could be integrated into an automated DBS sample preparation system [29].

The third approach was to derive the approximate HCT from a non-volumetrically applied DBS based on its potassium content. Although this method yielded good results and was straightforward to perform, it was destructive, required sample preparation and additional analysis equipment [30].

Depending on market, this effect has different consequences. The variation of HCT is highest in newborns, however newborn screening is a semi quantitative method and analyte concentrations of metabolic diseases differ in most cases very significantly from the normal range. Therefore the HCT based variance is often neglected. In a healthy adult population, the variance in blood HCT for men is between 38 to 50 percent and for women between 35 to 45 percent [31]. The variance in this range is much less as in the range of 30 to 70 % as requested for method validation. In other cases, such as clinical studies, the patient HCT is often known.

Sample volume

As described in the section hematocrit effects, the applied volume is the second unknown in the sampling process. Imprecision can (and should) be minimized by using standardized collection procedures. Using the pre-printed target rings on the filter paper can help control the volume of blood collected. Here, the circle should be filled to its edge during the collection. Capillary tubes with a predefined fill volume were used in some studies to avoid volume-associated effects. However, this might not be a preferred workflow for the collection of NBS samples. Automated DBS sample preparation instruments, such as the DBS-MS 500 can detect the area of the DBS. If the HCT is known, a correction algorithm for volume correction could be applied.

Stability and humidity effects

Although most analytes are more stable in a dried state as in the liquid blood sample, the FDA guideline [24] requires extended shelf life study of the DBS sample investigating temperature and humidity.
Humidity affects the blood spreading, the sample drying process and the stability of the target analytes within the DBS. Each of these factors has been addressed and examined [10], [32], [33]. The stability has to be validated for each analyte transferred to DBS. In general, studies showed that humidity has the biggest influence, rather than elevated temperature. In a study of 34 compounds, 27 compounds showed accelerated degradation caused by humidity and only four by temperature above 37 °C [2], [32]. By shipping the DBS in sealed bags with desiccant, this could be avoided easily.

Drying effects

Drying or evaporation effects of the blood droplet on the filter paper card could lead to a ring-like solid stain. The volatile species first evaporate at the contact line, whereas a capillary force draws the non-volatile solutes to the outer ring [31]. This phenomenon is often called the coffee-ring or volcano effect. This variance can be compromised by using positioning the extraction to the middle of each DBS as this is automatically done by the DBS-MS 500.

Chromatographic effects

Similar to a thin layer chromatography plate, certain analytes can show chromatographic effects on the filter paper fibres. The analyte concentration then may vary from the center to the edges depending on the extent of this effect [25]. Center punches vary only 1-2% from punches near the edge of the DBS. This effect is also neglected by the automated center positioning of the DBS-MS 500.

Anticoagulants

Some collection techniques, mainly the use of capillary tubes, may involve anticoagulants. Ion suppression effects to the target analytes of these reagents need to be checked during validation. Common anticoagulants are acid citrate dextrose, ethylene diamine tetra acetic acid (EDTA), potassium oxalate, and sodium heparin. Pre-coated cards, such as the DMPK-A and DMPK-B card from Whatman are not recommended for mass spectrometry analysis, since it may contain sodium dodecyl sulphate.

Analyte Extraction

The nature of extraction solvent significantly affects the analysis. The extraction solvent depends on the target analytes and on the chosen analysis technology. Samples are usually diluted during extraction processes depending on protocol. Using automation, the dilution process can be minimized. In a conventional protocol, a 3.2 mm disc is extracted in 100 µL extraction solvent. A comparison study of the DBS-MS 500 showed that the same efficiency can be achieved with 20 µL [34]. On one hand, the automated extraction is performed with increased pressure and on the other hand the continuous extraction process always delivers fresh solvent maintaining optimal extraction efficiency.

Blood Source

As most data of the last decades was gathered from liquid serum or plasma, whole blood analysis in the form of DBS is usually not considered as standard. The results from DBS might not be directly comparable with the existing reference levels and cut-off concentrations from serum and plasma. Therefore, new analytical decision levels need to be established for the DBS matrix [34]. Correlative studies with traditional sampling should be conducted and compared during DBS method development and implementation [24].

DBS devices and alternatives

Beside the card-based systems, micro sampling in the form of DMS is also being transferred to several devices and alternatives. Most effort goes into the direction of sampling a defined volume of blood onto a filter paper or cartridge and into collecting plasma from the blood droplet.

Defined volume absorption

By using a capillary system, the companies HemaXis and Capitainer developed two-card based devices, which apply a defined volume of 5 to 15 µL blood onto a filter paper card. The HemaXis micro blood sampling platform uses microfluidics to collect and prepare accurate volume-controlled samples of whole blood and transfer it to a standardized DBS card with no bias due to HCT. The repeatability in volume control is comparable to a volumetric micropipette so there is no need for a sub punch as the entire spot can be used for extraction [35]. Also the Capitainer system uses a microfluidic platform which draws 10 µL of whole blood, through dissolution of an interim layer the volume is spotted on a filter paper disc [36]. The card is not in the standardized format of 84.67 mm × 53.2 mm (generally agreed card dimension for automation) and therefore not feasible direct for automation with the commercial instruments available in the market.

The second approach is the usage of a micro sampling device which has got a capacity of absorbing 10 µL of whole blood. A commercial solution composed of a circular holder and a porous absorption tip called Mitra [36] is available and is being investigated within the potential micro sampling fields.

Lateral flow plasma separation

Separating whole blood into plasma within the collection devices has two advantages, first existing plasma assays can be used without the need for large whole blood versus plasma comparison studies, and second there is no significant viscosity difference in plasma and therefore the HCT effect can be neglected.

HemaXis developed a modification of their initial product, where a passive plasma separation was implemented within the micro capillary which is drawing the blood. The device called HemaXis DX was launched in Q3 2018 [35]. Lateral plasma separation was also implemented into the card format by several researchers and vendors. There are the following products in the market; AdvanceDx100, Noviplex and book-type DPS card [37]. Since those are very new products, solution for automation is currently being investigated.

Other devices

Next to the devices described above, there have been many other approaches published, such as absorbing papers to tear off as the HemaSpot-HF from Spot on sciences, MS-W^2 from Shimadzu or the HemaPen from Trajan. Also, capillary micro sampling devices for storing 5-15 µL liquid blood are suggested as alternative, whereas the sample handling gets more tedious and the workflow loses the general simplicity of DBS. Further approaches for the automation of the DBS card extraction are liquid micro-junction surface sampling probe (LMJ-SSP) or ambient ionisation MS techniques like desorption electrospray ionisation (DESI) and paper spray (PS) [4]. However, these techniques showed some disadvantages concerning sensitivity and are therefore not further discussed here.

Liquid chromatography and mass spectrometry for DBS

The principle of the analysis technology liquid chromatography (LC) and mass spectrometry (MS) can be found in literature [38], [39]. LC coupled to MS (LC-MS) using electrospray ionization (ESI) is a powerful analytical tool for the analysis of different compounds such as polar, semi-volatile and thermally labile compounds of large molecular weight range. Different mass spectrometry instruments are available, including systems based upon quadrupole, time-of-flight (TOF), ion trap, and orbitrap technology, triple-quadrupole–based mass analyzers (MS/MS) are the most popular for quantitation. The MS/MS setup allow for different experiments such as full scan, neutral loss, single or multiple reaction monitoring (MRM), where the MRM mode is by far the most used setting. Here the system works like a double mass filter with a fragmentation in-between, resulting in highest selectivity and sensitivity [39],[40]. All work done within this doctoral thesis was conducted on triple quadrupole mass spectrometer using ESI with or without an analytical column prior mass analysis.

There are targeted and un-targeted LC-MS/MS methods, whereas targeted means that the molecules of interest are known. Typical methods consist of a certain number of target compounds, where the mass spectra and retention times are set. Both sensitivity and selectivity for those compounds are optimized, however the amount of measured date is limited. Other chemicals, which may be present in the sample, are not monitored. Where the un-targeted approach detects any chemicals present in the sample. A typical mass spectrometer for this approach would be a TOF or orbitrap instrument, since they monitor the exact mass with high resolution [41], [42]. In a second step the MS/MS spectra are acquired for identification. This method results in a huge amount of data which needs to be processed accordingly [43], [44].
In this thesis, the targeted approach was chosen for all projects. The parameters which need special consideration when working with DBS, will be addressed in the following sections.

Analytical column

In general, it is always recommended to use a short analytical column. It makes sense from an analytical point of view to separate the target compounds from ions and other small molecules which may affect the ionization process. Also, in any case it is recommended to use a LC inline filter prior the analytical column or mass spectrometer entrance. When working with complex matrices, such as blood, the LC system is prone to clog. Hence, the system backpressure should always be monitored very carefully. The ripple of the pressure curve must be smaller than 1%, otherwise there might be some air inclusions. The connection tubes between the LC and the DBS-MS 500 should be as short as possible to have a minimal system backpressure. Alternatively, also columns with large particles or poroshell materials work well for DBS applications.
In certain cases, for example newborn screening, a column is not required due to speed requirements and semi-quantification. Here it is crucial to use an LC inline filter prior the ESI source.

Extraction solvent and mobile phase

The standard loop setting for collecting the DBS extract is 20 µl. This relatively large volume must not corrupt the chromatographic separation. The extraction solvent should be as similar as possible to the initial mobile phase of the LC method. To extract small organic molecules, the maximum water content should not exceed 30 % and the extraction solvent should not contain any buffer, since high aqueous content or acidic buffers might lead to unwanted dissolution of matrix components. Higher aquatic content of the extraction solvent might dissolve haemoglobin and proteins which may lead to matrix effects. Otherwise, another stationary phase such as HILIC can be used.

Matrix effects and ion suppression

When working with complex matrices such as blood, matrix effects and ion suppression should be closely monitored. The fastest approach to assess such effects is to infuse an appropriate concentration of target analytes into the mobile phase flow. By using a syringe pump and a T-piece connected after the analytical column, this effect can be monitored very easily. A typical DBS sample should then be injected through the DBS-MS 500 as per the usual analytical parameters. Another approach would be to compare the sample with standards prepared in pure solvents, which gives the best-case scenario. However, the extraction behavior might be dependent on the matrix applied to the filter paper. Best strategy is to always use internal standards and to use matrix matched calibration standards.
The background signal of the tandem MS should be checked on a regular basis. This can be done by either observing the online spectrum plot directly on the screen in scan mode or by measuring the background noise as a sample run without an injection (always using the same mobile phase). The advantage of a sample run is that it can be compared with former system checks. This long-term system performance can be tracked and simplifies a troubleshooting if any error occurs. The tune settings of a tandem MS should also be monitored and adapted to assure the quality of the analysis. If the system sensitivity decreases significantly, the ESI source should be cleaned manually.

Method validation

Additional validation steps need to be examined when setting up a new DBS method. Storage and handling temperatures, homogeneity of sample spotting, hematocrit, stability, carryover and reproducibility should be addressed [24]. For clinical applications, correlative studies with traditional sampling should be conducted.

DBS markets and applications

DBS shows significant advantage in the fields of omics (genomics, proteomics and metabolomics), newborn screening, forensic toxicology, pre-clinical studies and therapeutic drug monitoring [45] (Figure 9).

Omics

Whole blood omics
Stabilized sample
Reduced volume

NBS
Less invasive
Minimal pain
Sterility
Low volume

TDM
Home-sampling
Easy training
Easy logistics
Stabilized sample
Non-contagious

Preclin and Clin
Three R's rule
Reduced pain/stress
Low volume collected
Reduced number of animals

Forensic toxicology
Easy logistics
Remote sampling
Untrained personal
Large scale if required
Non-contagious

Figure 9, Fields of interest and summarized DBS advantages

Newborn Screening (NBS)

Newborn screening is a public health program provided by most of the countries around the world aimed at screening newborns for a list of serious genetic and metabolic disorders [23], [46], [47]. Early diagnosis of those conditions can help prevent their further development which untreated often results in brain damage, organ damage and even death.

Nowadays, a routine neonatal screening procedure requires that a health professional takes a few drops of blood from the baby's heel, applies them onto a filter paper and sends such prepared samples to a laboratory for a number of analytical tests. Sample preparation taking place before analysis may be labor-intensive, time-consuming and not very precise due to carry-over when processed with traditional "punch-and-elute" methodology. Most laboratories perform the MS analysis from one DBS disc and the biological assays from a separate disc.

USA and Canada have an expanded neonatal screening. The majority of states have centralized screening laboratories, equipped with tandem MS and biological assays. In Europe, especially in UK, Ireland, Germany, Switzerland, Sweden and France the newborn screening programs are implemented and very advanced. There are NBS programs in Asia [48], [49], however they do not routinely use LC-MS analysis for their screening, since the instruments are too expensive. Arabic countries have been implementing new NBS programs during the last years. In general, the newborn screening market is not very regulated and analysis procedures vary regionally.

Application of DBS-MS 500 would on one hand standardize the sample preparation and analysis process and on the other hand simplify the workflow and eliminate any manual working steps. Trends in newborn screening are that the screening panel (list of all screened compounds) is growing and there is a unification trend going on. It is discussed that in the future, NBS will be performed by next generation sequencing of the babies' DNA. However, it will take several years to make this method affordable and to find the specific DNA mutations which are linked to certain diseases.

Forensic toxicology

The field of forensic toxicology in regard of dried matrix spot analysis is distinguished into the fields doping analysis and testing for illicit drugs.

Doping control

Urine is the preferred biological fluid, where most commonly used performance enhancing compounds are analyzed. The collection of this biological fluid is non-invasive and cheap. However, blood test are becoming more and more common within the anti-doping and sport organizations and thus the detection of substances in blood was included in the World Anti-Doping Agency (WADA) prohibited list [50]. Blood analysis was not convenient for large scale screening in the past, but with new DMS methods it might replace the urine analysis to some extent, since for some compounds it is more accurate and un-metabolized drugs can be found. Blood analysis provides a snap shot of the compounds in the blood stream in the very moment of sampling, whereas urine often reflects an average over several days. An

official implementation of DBS in doping control is the method from *Laura Tretzel et al.* from 2016 [50] where nicotine and its major metabolites are measured in whole blood. Nicotine-containing products for performance-enhancing purposes have been monitored by the WADA for several years.

Dried matrix spot analysis is also a topic for race animal doping control. Especially for small animals, for example race falcons, only a minor blood amount can be drawn for analysis [51]. These animals have a very low total blood volume, for which DBS is a very suitable and trending technology. There is a tendency that more and more DMS methods will be implemented within the next years, DMS will certainly gain popularity in this market.

Illicit drug control

Illicit drug control is routinely performed for workplace testing, roadside testing, rehabilitation programs, screening for suspects or post-mortem investigations. Often, a drug wipe test is done, where surfaces are analyzed for traces of drug residues. This technology is non-invasive but has a high error rate. Immune assay strips containing antibodies bind to components of the different drugs [45], [52]–[57]. If those tests are positive, blood drawing at hospitals or external sites follows. There, usually plasma analysis is performed to assure or reject the first result. For post-mortem investigations, the sample goes directly to the analysis site.

DMS applications in literature are increasing during the last year, especially after the work of the group of Sebastian Dresen et al. [58] in 2014, where a method for the detection of 64 psychoactive substances from one single blood droplet was introduced. Further, it has been found that enzyme activity in collected liquid blood samples can manipulate analysis results, for example in blood alcohol marker measurements. Drying a sample on a DBS card immediately stops this activity and could lead to more reliable results.

Screening of other prohibited compounds could be performed with little equipment by police officers anywhere. Nowadays in Europe, a police officer has to bring the suspect to a facility where blood is drawn by trained staff or the emergency doctor has to drive to the road side testing site. With DBS sampling, such procedures could be significantly accelerated and designed more cost effective. Also screening for illicit drugs in larger organizations, for example the military, gets affordable.

Therapeutic drug monitoring (TDM)

Therapeutic drug monitoring (TDM) aims at personalized patient care where drug dosage is individually adjusted to optimize the treatment outcome.

There has been a great dynamic around TDM related to DBS for the last 5 years. Personalized health care is a big topic in pharma industry and DBS is a well-suited technique to monitor a patient's drug uptake (ADME studies). Drugs, which have to be taken long term, are especially suited. Enabling an "at home" sampling for patients would bring significant cost reductions and comfort for the patients [59]–[62].

The implementation of DBS would avoid that the patients need to visit the hospital several times to draw up to 5 mL of blood for the plasma testing. With DBS, patients could draw samples by them self at home and send it with standard mail to a centralized laboratory. Also, the correct administration of drugs can be monitored very easily by DBS [63]. Therefore, there is a clear trend of using DBS for monitoring anti-cancer drugs, anti-retroviral drugs, antiepileptic drugs, immunosuppressive drugs, drugs for mass administration campaigns, etc.

Preclinical and clinical trials

The preclinical and clinical market summarizes the testing of new pharmaceuticals on animal (preclinical) and human (clinical) studies. The DBS technology offers huge advantages here, however has not been fully accepted by regulatory bodies so far [64]–[67]. The market is strongly controlled by the FDA, EMA and CFDA, who are not accepting the technology as long as the described hematocrit effects is not fully solved and large-scale comparison studies on plasma and DBS samples have been conducted. To fulfill all requirements, an extensive investment is needed, and the certification process would be for specific drugs only and not for DBS in general.

Main advantage in preclinical studies is the reduced number of test animals (due to the need for lower blood volumes) which is in accordance with the 3R requirement of animal studies (replacement, reduction, refinement) [68]. Drugs could be studied on a single mouse over several time points, whereas several mice are required with conventional methods. In clinical studies, there is an ease in sampling and more patient comfort. As soon as first companies push the certification process of DBS and the DBS technology is gaining in acceptance, there might be a boom of DBS analysis in this market.

Omics

The term omics summarizes the fields of genomics, proteomics and metabolomics. For DMS analysis, all fields are of interest, where genomics and metabolomics get most of the attention.

There are many methods for recovering DNA from DBS and doing PCR analysis [69]. Also, a general trend shows the study of DNA for health care markers, discovering a person's ancestry composition and DNA family.

However, focus of this work is the field of metabolomics, since most metabolites can be detected by LC-MS/MS [23], [45], [66], [70]. Here, health care biomarkers are of major interest to deduce a person's (or animal's) health status regarding specific markers such as Vitamin D as example, which directly effects bone mineralization and calcium regulation in the body.

The approach of general medicine is changing from broad-spectrum drugs towards personalized healthcare. The new trend is fuelled by an increasing level of information flow, transparency, customisation and patient choice, all together leading to new services. Patients can gather an enormous amount of medicinal data from the internet or even order blood and biomarker tests themselves. Direct analysis blood tests are available online, for example from DirectLabs or Life Extension Foundation, where several healthcare test kits are offered [71]. The growing awareness of healthcare issues and recent developments of minimal invasive blood biomarker tests show the huge future potential of this life science branch. In future, personalized drug dosage could be achieved by almost online monitoring the particular biomarker, thus providing more safety for the patient based on smart dosages and dynamically updated dosage profiles. Biomarker analysis cannot only be used to monitor a drug dosage, they can be applied much earlier – before the disease even appears.

Final conclusions

The developed methods within this doctoral thesis contribute to the field of:

i. New born screening
ii. Forensic toxicology
iii. Therapeutic drug monitoring

i. Extended and standardized NBS screening

In newborn screening, two applications were developed on the fully automated DBS-MS 500 platform. First, the existing methodology was transferred to the automated platform and extended by an additional steroid panel. Nowadays, most newborn screening programs measure amino acids and acyl carnitines by mass spectrometry (derivatized and non-derivatized methods) and thyroid-stimulating hormone (TSH), immunoreactive trypsinogen (IRT), galactose, galactose-1-phosphate uridyl transferase, biotinidase, Thyroxine (T4) und 17α-hydroxyprogesterone (17OHP) by enzyme and immune assay.

Goal of the first publication was to transfer the amino acids and acyl carnitines analysis onto the automated DBS-MS 500 platform. Also, a steroid panel of 17OHP, cortisol and androstenedione was defined to exclude the 17OHP from the immune assay panel and to transfer this as well onto the fully automated DBS-LC-MS/MS. The conventional 17OHP enzyme-linked immunosorbent assay (ELISA) based on a manually punched DBS disc leads to a high percentage of false positive. First, cortisol increases when the babies are stressed, which also leads to elevated 17OHP concentrations and secondly, the ELISA has significant cross reaction potential with steroid sulfate which can be monitored with androstenedione. Both, the amino acid and acyl carnitine detection plus the integration of a steroid panel into the DBS-LC-MS/MS workflow was successfully achieved [34]. Unlike most protocols used nowadays in NBS, a short analytical column was used. The steroids had to be separated in time, since some compounds share similar multiple reaction monitoring (MRM) transitions within the mass spectrometer. The method requires 4 minutes per sample, however it is significantly improved in robustness and sensitivity. Due to the chromatographic separation (Figure 10), the analytes enter the mass spectrometer much more focused which results in better signal to noise and therefore also in robustness. Disadvantage of this method is the lack of certification for now, the method describes a protocol how to manually prepare the mobile phase, the rinsing and extraction solvent and the internal standard mixture. Quality control cards are prepared by the laboratory themselves. This is the most cost-effective workflow and allows full flexibility for the laboratory. However, this workflow is manly applicable in countries with low regulation as the self-made consumables are not certified. If a kit manufacturer produces the here described consumables in a certified process and provide it to the laboratories, all centers would be able to switch to the automated and extended MS screening.

Nonetheless, the existing methodology was significantly improved by the degree of automation and process standardization. Many manual steps are no longer needed, and all DBS cards are treated in the same way by removing the human factor. The abstract of the this study has already been downloaded for more than 1000 times according to the statistic tool provided by the journal of neonatal screening and has a world-wide impact [34].

Newborn screening panels are not unified across borders and sometimes even differ within countries and laboratories. Since more than a decade, there is a trend to unify the screening method to allow cross-checking of results. The implementation of the fully automated DBS platform could be a good starting point of standardization and unification of those programs. Since the first method required a chromatographic column and was not based on certified reference material, a second method based on an official newborn screening kit was introduced in a follow up publication [72]. The analysis takes only 2 minutes per sample; however it is limited to amino acids and acyl carnitines only. The DBS extract is directly guided to the mass spectrometer without any column. This is a well-known procedure in newborn screening and allows speeding up the process to its optimum. The drawback of the direct injection is that all compounds elute at the same time and are only distinguished by their MRM transitions (Figure 10). Since this method is considered as semi quantitative, the partially bad peak shape and fast switching time of the mass spectrometer between all MRM is accepted. The sample signals are referred to the certified high and low control samples from the kit and the signal is reported relative to those samples. Since the difference in concentration from a normal NBS sample (healthy) to a positive sample (metabolic disorder) varies significantly for most diseases, no higher sensitivity and precision of the amino acids and acyl carnitines is required.

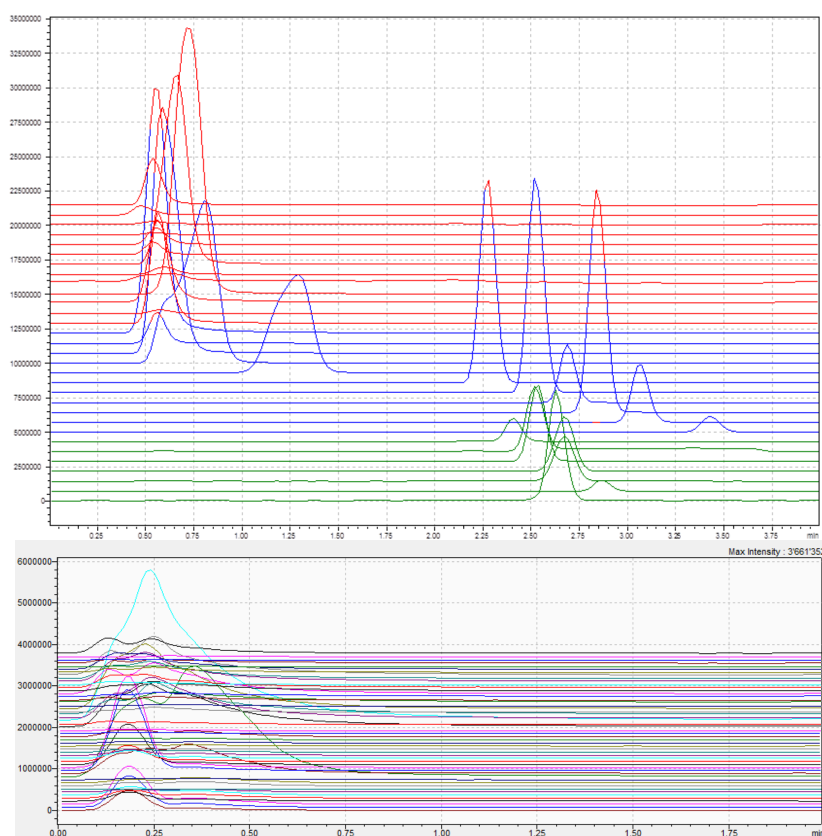

Figure 10, spectra comparison method one with column (above) and method two below (below)

ii. Screening of over 1200 drugs from a single blood droplet

In forensic toxicology, it was shown that DMS is a suitable technology for large scale DBS applications. The first DBS methods in the field of forensic toxicology with high impact are from 2014, where Sebastian Dresen et al. [58] describes a screening of 64 psychoactive substances from one blood spot. The whole blood spot was cut out manually and extracted in two step process including evaporation of the extract and reconstitution. This approach was further investigated and advanced within this thesis. The outcome is a fully automated method, where either 1200 drugs can be screened from a DBS or a quantitative follow up method focusing on the 28 most abundant drugs of abuse can be performed. This was documented within two publications, where the first publication was a prove of concept study to determine if the detection limits can be reached using the DBS-LC-MS/MS methodology [73]. The compounds fentanyl and LSD are among the most active drugs in the chosen panel and appear in very low blood concentrations. One blood droplet (15 µL) of the fentanyl sample at level 1 contains only 7.5 pg of standard. The droplet spreads with an average hematocrit to an area of approximately 40 mm^2. An extraction circle of 4 mm diameter equals 30 percent of the droplet, which means that with a theoretical extraction efficiency of 100 %, only 2.5 pg of standard reaches the LC-MS/MS system (visualized in Figure 11) [73].

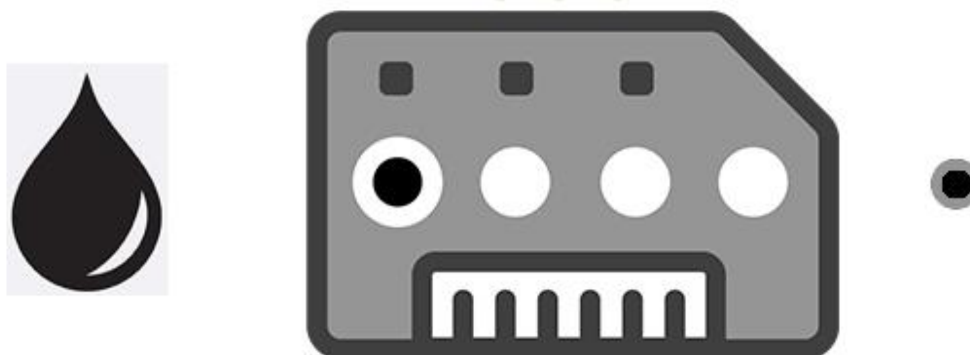

Figure 11, 15 µL droplet blood results in approx. 40 mm^2 DBS, 12.6 mm^2 are extracted

The according cut-off values were reached and the stability of the drugs in the DBS showed promising results.

The second, follow up publication was a specific method development and implementation. The introduced DMS workflow eases sampling, sample transport and analysis through full automation. More than 1200 illicit drugs can be screened from a single DBS or DUS within 20 minutes per sample using a Forensic Toxicology Database. A quantitative follow up method on positive screening results was developed for 28 target compounds and their according deuterated internal standards. The quantitative method gives confident positive/negative results for all tested drugs at their individual cut-off concentration [73]. The criteria of bio-analytical method validation guidelines were fulfilled, and the method was transferred successfully. It is already used on a daily basis at a routine laboratory in Dammam, Saudi Arabia, and has raised world-wide interest for workplace testing and rehabilitation programs. It was proved that DBS stops all enzyme activity and delivers reliable analysis results.

The second forensic DBS investigation within this thesis was alcohol monitoring. Alcohol measurement is usually performed by direct gas chromatography (GC)-MS measurement of breath or blood alcohol [74]. Long term use is monitored by ethyl glucuronide (EtG) and ethyl sulfate (EtS) LC-MS detection in urine. Due to the short window of detection (3-5 days), these two markers require frequent sampling. This results in a large number of liquid urine samples which need to be transported and stored prior to analysis. Furthermore, to prevent post-sampling formation and degradation of EtG, compliance with the cold chain, the use of sodium azide sampling tubes [75], or sampling as dried urine spots [76] is required. Sampling as dried urine spot (DUS) insures post-sampling stability, allows shipping without biohazard labeling in a standard envelope, and does not require any sample cooling. The detection of the biomarkers EtG and EtS from a DUS was transferred to the automated DBS-LC-MS/MS platform (not published yet).

Furthermore, the discovery of phosphatidylethanol (PEth) as direct alcohol marker prolonged the window of detection for alcohol consumption to several weeks. PEth allows to distinguish heavy from occasional drinkers and to monitor alcohol abstinence [77]. PEth proved to be instable during storage of liquid blood samples. By using DBS sampling, this biomarker can be stabilized due to the inactivation of enzymatic activity. Also, for PEth, a fully automated DBS-LC-MS/MS was established for the determination of the two most abundant PEth homologs in a range from 20–1500 ng/mL. Automated DBS card handling and online solid phase extraction LC-MS/MS permits baseline separation and detection of PEth 16:0/18:1 and PEth 16:0/18:2 within 7 minutes per sample [78].

Long term alcohol monitoring is important for the investigation of chronical alcohol misuse of e.g. new drivers, patients after liver transplantation and pilots or other sensitive jobs.

DBS is heavily discussed within the forensic toxicology lobby, however there are still a few hurdles to overcome before the technology will find wide spread implementation. The analytical result must be bullet prove against a court, since this will be questioned by a lawyer. The bias caused by the hematocrit remains an issue which might affect the results and drawing a blood sample by a non-medical professional is in most countries considered invasive. Also, the analysis sensitivity for certain analytes such as THC, LSD or fentanyl is at its limit using a micro sampling approach. However, mass spectrometry instruments are gaining impressively in sensitivity with each new model and several solutions to overcome the hematocrit effect are becoming available (see section: Outlook).

On the other hand, the advantages of transferring the well-known DBS technology from the field of newborn screening into the forensic toxicology offers some bold advantages. It is very easy to pack and ship the samples anonymized and protected against misuse to the laboratory. After analysis, the samples can be easily stored long term using minimal storage space. Therefore, next to the above described markets, also roadside testing, sport medicine, doping control for human and animal are investigated.

iii. Monitoring HIV drug dosages in remote areas

For therapeutic drug monitoring in remote settings, DBS are particularly advantageous, as blood sample collection and handling is uncomplicated. During this thesis, a university laboratory in Basel (Switzerland) was supported in terms of method development of TDM in rural areas. The first method focuses of the three antiretroviral drugs nevirapine, efavirenz and lopinavir [63]. The study highlights the transportation advantages of DBS, without any biohazard labeling neither requiring cooling chains (Figure 12). DBS were drawn in Tanzania, Africa, and sent to Switzerland where the samples went through different climate zones. Nonetheless, the samples showed very good results and stability of the monitored drugs. We ran a follow up study on some of the samples approximately one year after the publication, where still all analytes could be recovered in the same concentration as published. Also the DBS sampling itself was investigated in a rural area of Tanzania [79] and the technique proved to be suitable.

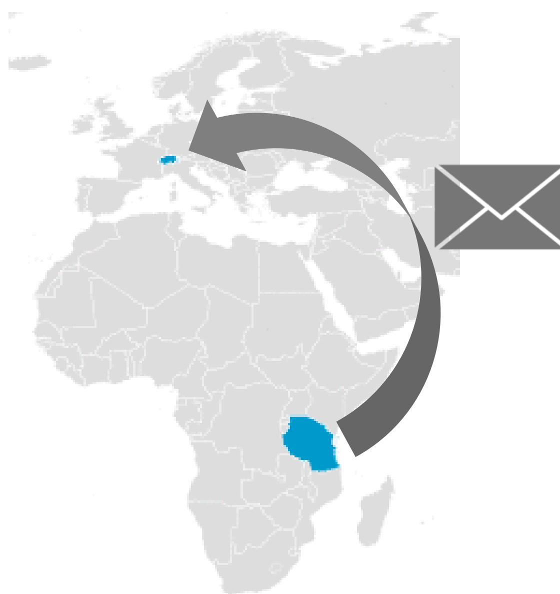

Figure 12, Transportation of DBS samples via standard mail

Another therapeutic drug, where a more efficient sampling process is required for mass drug administration (MDA) campaigns, is Ivermectin. This drug lowers the incidence of river blindness and lymphatic filariasis infections. Further, recent studies demonstrated that Ivermectin is also active against several other parasites and even against certain mosquitoes. As one of these mosquitoes is Anopheles gambiae, the major vector of malaria in Africa, Ivermectin could be mass administered to fight malaria [80]–[82]. In other words, the drug makes the human blood lethal for the Anopheles gambiae and therefore reduces the number of vectors in an area. Still, more safety data is required from a larger population. Here, an according fully automated DBS method has been developed and validated. In addition, a comparison study to plasma samples, stability and hematocrit impact has been studied [82].

Outlook

More and more LC-MS methods will be transferred to micro sampling approaches due to the simplified sample drawing, shipping and analysis. The micro sampling technology gaining the highest market percentage is to date not decided, however the tendency is a card-based approach with the option of automation. Basically, the detection of the sample hematocrit is still the highest hurdle, whereas several solution concepts are documented and are being validated at the moment. The reflection measurement of haemoglobin content shows a good solution, since it can be integrated into an automated DBS platform. As soon as the hematocrit bias is fully resolved, DBS will experience its revival and will grow rapidly. The qualitative or semi-quantitative methods will then be possible to enable confident quantitation.

As soon as pharma companies start to invest into micro sampling and finance large scale plasma comparison studies, the technology will gain in general acceptance. Mass spectrometer instruments are becoming more sensitive and robust, enabling the transfer of even more compounds to micro sample workflows with very low volume and low concentration. Also, the introduction of multiple charge ionization technologies for MS enables the detection of larger molecules such as proteins. This will then soon replace many biological assays. A limitation for mass spectrometry is the detection of enzyme activity, which cannot directly be deduced from the molecule mass. Limitations of the DBS technology itself are compounds which are prone to oxidation. Here, a DBS card coating with an anti-oxidant might enhance the stability [23].

The publication number in the field of DBS is increasing recently and the interest can be directly monitored. The journal of neonatal screening provides open statistics for authors regarding the abstract views and pdf downloads of the published articles. For the first NBS publication [34], highest interest comes from US and China markets, followed by Europe, Russia and India (Figure 13).

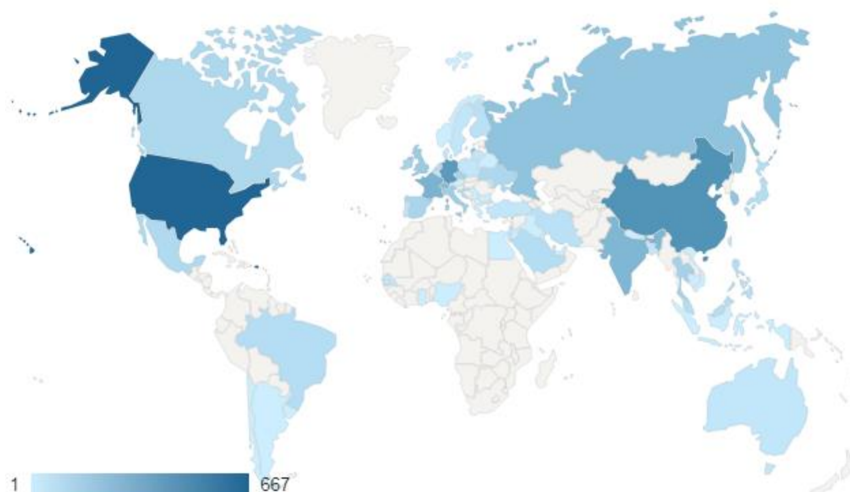

Figure 13, Article access statistic of the first NBS publication of this doctoral thesis (status: 08.01.2019)

Publications (copy of each)

9 Pages 41-50 Stefan Gaugler, Jana Rykl, Irene Wegner, Tamara von Däniken, Ralph Fingerhut, Götz Schlotterbeck, “Extended and fully automated newborn screening panel for mass spectrometry detection”, Int. J. Neonatal Screen. **2018**, 4, 2

7 Pages 51-58 Stefan Gaugler, Jana Rykl, Vicente L. Cebolla, “Validation of an Automated Extraction Procedure for Amino Acids and Acylcarnitines for Use with Tandem Mass Spectrometry for Newborn Screening”, Endocrinology, Diabetes and Metabolism Journal, **2019**, 3 (1): 1–9

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Extended and Fully Automated Newborn Screening Method for Mass Spectrometry Detection

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Abstract: A new and fully automated newborn screening method for mass spectrometry was introduced in this paper. Pathological relevant amino acids, acylcarnitines, and certain steroids are detected within 4 min per sample. Each sample is treated in an automated and standardized workflow, where a mixture of deuterated internal standards is sprayed onto the sample before extraction. All compounds showed good linearity, and intra- and inter-day variation lies within the acceptance criteria (except for aspartic acid). The described workflow decreases analysis cost and labor while improving the sample traceability towards good laboratory practice.

Keywords: newborn screening (NBS); dried blood spots (DBS); automation

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1. Introduction

Newborn screening (NBS) is a public health program provided by most of the countries around the world aimed at screening newborns for a list of serious genetic and metabolic disorders [1–6]. Early diagnosis of these conditions can help prevent their further development, which untreated often results in brain damage, organ damage, and even death. A routine neonatal screening procedure requires that a health professional takes a few drops of blood from the baby's heel, applies them onto special filter paper and sends such prepared samples to a laboratory for a number of analytical tests [7]. The sample preparation before analysis may be labor-intensive and time-consuming when processed with traditional "punch-and-elute" methodology. To date probably all NBS programs use punchers (manual, semi-automated, or totally automated) which transfer a 3.2 mm punch of the dried blood spot (DBS) into a microtiter plate. Although most puncher software programs register which sample has been punched into which well of the respective microtiter plate, there is no control as to whether a DBS has flipped out of the respective well due to static electricity.

Application of automated DBS card handling systems, which are connected to mass spectrometry analyzers, offers a modern and fast approach where a circular area of the DBS is directly eluted from the filter paper card without any punching. A panel of relevant biomarkers for the mass spectrometry newborn screening, including amino acids and acylcarnitines, was chosen for this study. Additionally, a steroid panel has been added to integrate new biomarkers which may lead to clinical relevant data. Automation for this study was implemented by the CAMAG DBS-MS 500 equipment.

Today, with the advancement of suitable instrumentation, more and more analytes are transferred from being detected by biological assays into the mass spectrometry screening panel with the advancement of those instruments [6,8–11].

2. Materials and Methods

2.1. Chemicals

Gradient grade water and LC-MS grade methanol for liquid chromatography plus the non LC-MS grade rinsing solvents 2-propanol and acetonitrile as well as formic acid (puriss.) and ammonia fluoride (puriss.) were purchased from Carl Roth (Carl Roth, Rothenfels, Germany). A collection of all L-amino acids and carnitine, acetylcarnitine, propionylcarnitine, butyrylcarnitine, valerylcarnitine, hexanoylcarnitine, octanoylcarnitine, decanoylcarnitine, lauroylcarnitine, myristoylcarnitine, palmitoylcarnitine and stearoylcarnitine, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The deuterated internal standards (IS) Cortisol $^{2}H_4$, Progesterone $^{2}H_9$, 17-Hydroxyprogesterone $^{2}H_8$, 11-Deoxycortisol $^{2}H_5$, 21-Deoxycortisol $^{2}H_8$, Androstenedione $^{13}C_3$, Corticosterone $^{2}H_4$, 11-Deoxycorticosterone $^{13}C_3$ were also products of Sigma-Aldrich (St. Louis, MO, USA). The deuterated amino acids and acylcarnitines were obtained from the MassChrom® Amino Acids and Acylcarnitines from Dried Blood kit from Chromsystems (Munich, Germany). Dried blood spot cards (TFN filter paper) were provided by CAMAG (Muttenz, Switzerland). Fresh whole blood was obtained from the local blood donation center (Basel, Switzerland).

2.2. Analytical Methods

Analytical Materials and Methods

Chromatography was performed on a modular HPLC system from Shimadzu (Kyoto, Japan); it contained a system controller (CBM-20A), two Nexera X2 pumps, a degasser (DGU-20ASR), and a column oven (CTO-20AC). Automated extractions were carried out with a DBS-MS 500 (CAMAG, Muttenz, Switzerland). Analytes were separated on a Shim-pack GIST (4.6 × 50 mm, 5 µm STEAROYL, 227-30017-3) analytical column (Shimadzu, Kyoto, Japan). A filter frit (KrudKatcher Ultra, Phenomenex, Torrance, CA, USA) was connected upstream to the analytical column. Mobile phase A consisted of water plus 0.1% formic acid and 2 mM ammonia fluoride, while methanol supplemented with 0.1% formic acid and 2 mM ammonia fluoride was used as mobile phase B. The following stepwise gradient was applied: 40% A (0–1.0 min), 40–90% A (1.0–2.0 min), 90% A (2.0–3.0 min), and 40% A (3.01–4.0 min). The flow rate was set at 1.0 mL/min at 40 °C. The HPLC liquid stream was connected to an 8060 tandem mass spectrometer (Shimadzu, Kyoto, Japan). The mass transitions and compound specific settings were included in Table S1.

2.3. DBS-MS 500 Settings

The extraction solvent on the DBS-MS 500 (CAMAG, Switzerland) was a mixture of methanol and water (70:30 *v/v*) and was connected to extraction port E1/R3. The wash solution, consisting of methanol, acetonitrile, 2-propanol and water (25:25:25:25, *v/v/v/v*), with 0.1% formic acid, was connected to the rinsing bottle R1. The internal standard mix was connected to IS2, and IS4. IS wash was filled with methanol. The system was prepared by priming methanol through the internal standard port (10 cycles) followed by 2 cycles of IS2. The extraction head was cleaned in an ultra sound bath at 40 °C for 10 min prior to a large set of analyses. The extraction solvent was primed for 5 cycles and the rinsing solvents were flushed for 1 min (this process is an automated system prime method). The DBS cards were photographed with the built-in camera of the DBS-MS 500 before and after each extraction to check for the presence of a blood spot and to adjust the extraction head to the center of each spot. The Chronos for CAMAG software automatically recognized inadequate dried blood spots based on their roundness, diameter, and area. Inadequate DBS were excluded from

analysis. Twenty microliters of internal standard were sprayed in a homogenous layer onto each spot. After a 20 s drying time, the samples were extracted with a volume of 20 µL and a 200 µL/min flow rate. To complete the automated DBS extraction cycle, the system was rinsed for 20 s with R1 [12].

2.4. DBS-MS 500 and LC-MS/MS Interface

The flow scheme of the fully automated card extraction system and the coupled LC-MS/MS is shown in Figure 1. The DBS cards are moved to the extraction unit, where a plunger seals a circular region of the card. The extraction solvent is pumped through this sealed region of the card and loaded into a loop (Figure 1: red arrows). By switching the 10-port valve, the loop volume is connected to the LC-MS/MS flow path (Figure 1: green arrows) and guided to the column and after separation to the tandem MS. Meanwhile, the extraction head is cleaned by a rinsing cycle to avoid carry over.

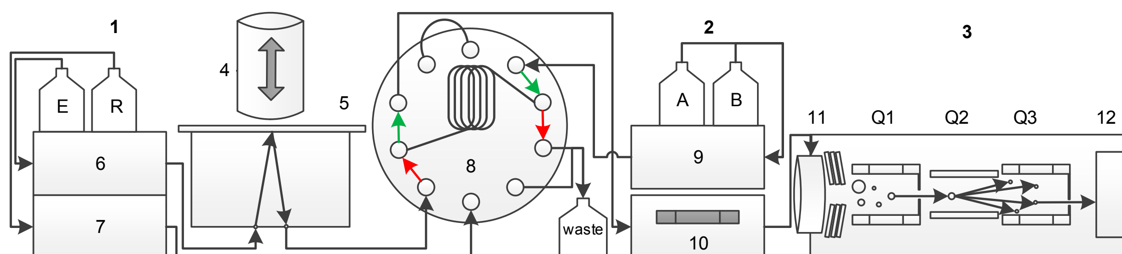

Figure 1. Flow scheme of the automated DBS-LC-MS/MS approach: 1, Automated dried blood spot (DBS) system; 2, HPLC; 3, MS/MS; 4, Extraction head; 5, DBS card; 6, Extraction pump; 7, Rinsing pump; 8, 10-port valve interface; 9, HPLC pump; 10, HPLC oven and analytical column; 11, ESI source; and 12, detector.

2.5. Sample and Standard Preparation

According to reference values, stock solutions were prepared and spiked to fresh blood in three different amounts to generate four levels in total, including the endogenous level from the male donor. The amino acids, carnitine, and acetylcarnitine were dissolved in water to prepare spike solution A (Table S2). The remaining carnitines were dissolved in methanol (spike B). The steroids were diluted with methanol to 100 µg/mL and mixed (spike C). Spike solutions A, B and C were stored at 4 °C.

2.5.1. Working Standards for LC-MS/MS Tuning

The spike solutions were used for LC-MS/MS method development and tuning of the MRM transitions. Spike A was diluted 100-fold and spike B and C 10-fold prior to injection.

2.5.2. Internal Standards

The deuterated steroids were dissolved in methanol to prepare 100 µg/mL standard solutions. Then, a mix was prepared with a final concentration of 10 µg/mL for all deuterated steroids. A MassChrom® internal standard mix (intended for 50 mL extraction buffer) was dissolved in 4.95 mL methanol and 50 µL of the deuterated steroid mix was added. This solution was used as an internal standard mix on the DBS-MS 500.

2.5.3. Dried Blood Spot Samples

The method should be applicable for using a calibration curve and for the detection via a reference signal, which improves the accuracy of the method. Therefore, 4 calibration levels were prepared. The calibration curve can be drawn using the 4 points and the zero concentration can be derived through the standard addition method. Alternatively, level 2 and 4 can be used as low and high controls when comparing the analyte peak and IS ratio. Freshly collected human blood was obtained from the local blood donation center (Basel, Switzerland). EDTA was used as an anticoagulation

agent (vacutainer tubes, BD, Allschwil, Switzerland). Spiked blood samples (prepared according to (Table S3) were gently mixed, after which 50 μL aliquots were spotted by an Eppendorf pipette onto CAMAG DBS cards (CAMAG, Muttenz, Switzerland). DBS cards were dried at room temperature for at least 3 h and were subsequently stored at 4 °C in sealed plastic bags containing desiccants. All DBS were prepared from the same blood source, prepared with the same procedure to neutralize potential hematocrit effect [12,13], which were not investigated in this study.

2.6. Quantification Method

All compounds were spiked in three different concentrations, resulting in levels 2, 3, and 4; level 1 reflects the endogenous concentration of the donor blood. Quantification can be performed by the standard addition method using the three calibration points or by comparing the analyte to internal standard peak ratio using level 2 as low and level 4 as high control. Those calibration cards with level 1–4 can directly be used as quality control standards in the developed method. Quantification by comparing the peak ratio is often used in newborn screening labs, whereas the three point calibration is a more widely used method in instrumental analytics [14,15], is accurate [16] and which is applicable on any commercial mass spectrometry software.

2.7. Documentation

The documentation process is given by the DBS-MS 500 instrument which takes a picture of the DBS card before and after each extraction to assure sample traceability. All pressures on the DBS card sampler as well as on the LC-MS/MS unit are monitored and documented.

The camera documentation system checks for preset values, where quality control parameters can be integrated. If the quality criteria are not met, the system automatically checks the next spot and continues the preset program. In addition, the camera detects already extracted spots and blocks those to prevent reanalysis. The results and preset criteria from the DBS card shown in Figure S1 are listed in Table S4. The system checks the xy shift of the circle on the card and the blood spot to center the internal standard spray and the extraction spot to the middle of each DBS (to avoid inhomogeneous distribution effects). Further, the inbuilt pressure sensors are monitoring the extraction and rinsing pressure (in this study, the extraction pressure was 0.9 bar and the rinsing pressure 41.5 bar). The maximum extraction pressure is 1.5 bar, depending on the age of the blood sample, and the system can be rinsed with pressure up to 100 bar to prevent carry over.

3. Results

3.1. LC-MS/MS Method

A C18 column was used to have a quick separation of the target compounds. It is important to separate the acylcarnitines and some of the steroid, since they fragment into the same daughter ions. Figure 2 shows the total ion chromatogram of all target compounds without internal standards. The amino acids and the short-chain acylcarnitines (C0–C4) elute in the first section before 0.75 min, the longer chained acylcarnitines (C5–C18) between 0.75 and 4 min, and the steroids between 2.25 and 3 min. The main goal of the column integration was to separate acylcarnitines from steroids, since some of them have similar multiple reaction monitoring (MRM) transitions. Therefore, the chromatographic separation was not further optimized.

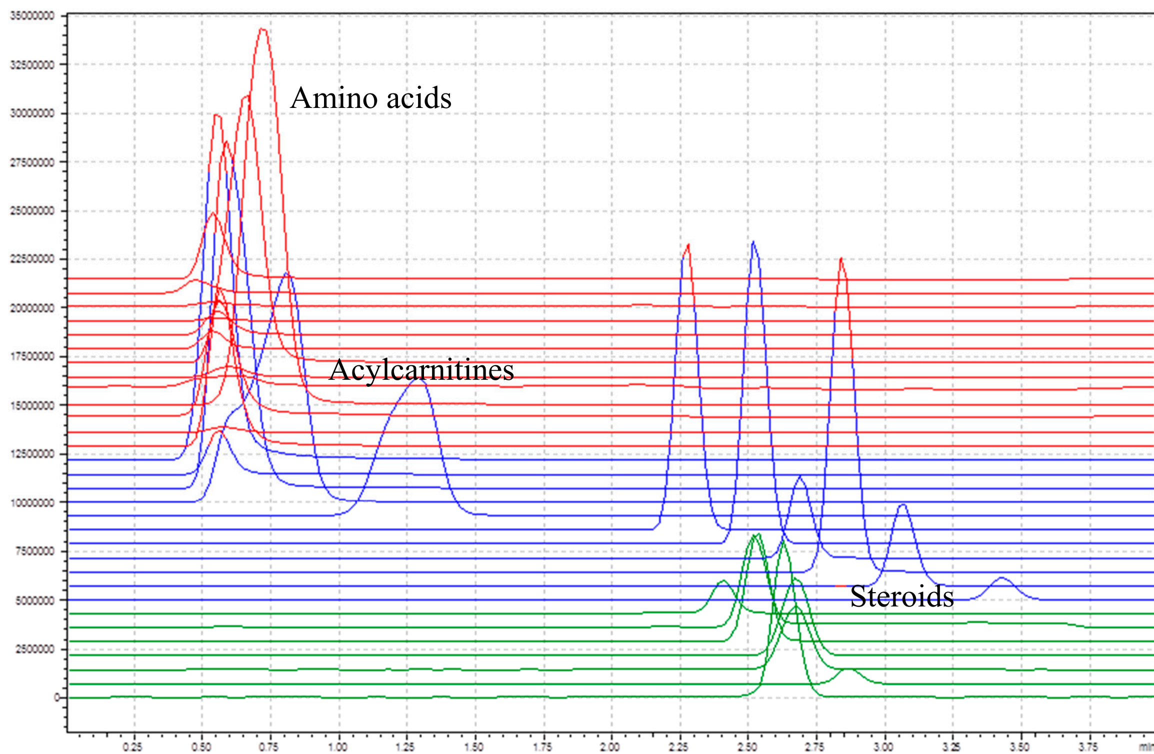

Figure 2. Total ion chromatogram (TIC) of all unlabeled target compounds (amino acids in red, acylcarnitines in blue and steroids in green).

3.2. Linearity and Storage Conditions

All DBS samples were measured 10-fold to determine the method robustness and intra-day variations. The samples were then stored for seven days at three different storage conditions: 4 °C in the fridge, at room temperature, and at 40 °C in an oven. Of each substance class, a compound was chosen for visualization (Figures 3–5).

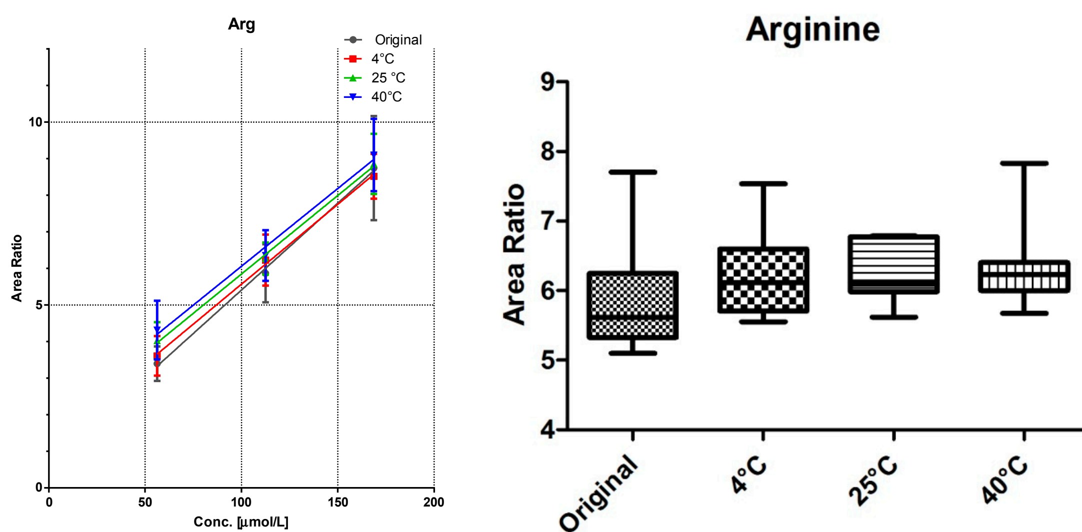

Figure 3. LC-MS/MS analysis of arginine from DBS cards stored at different storage temperatures: (**left**) linearity (mean ± sd of n = 7); and (**right**) box and whiskers graph of level 2 with 5–95 percentiles of the same data.

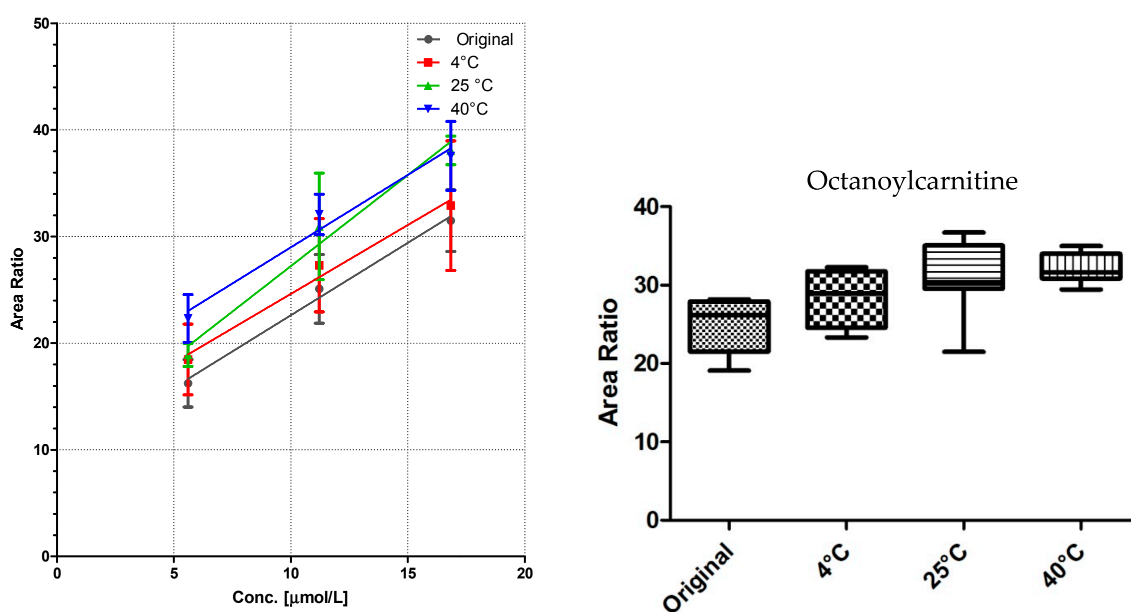

Figure 4. LC-MS/MS analysis of octanoylcarnitine from DBS cards stored at different storage temperatures: (**left**) linearity (mean ± sd of $n = 7$); and (**right**) box and whiskers graph of level 2 with 5–95 percentiles of the same data.

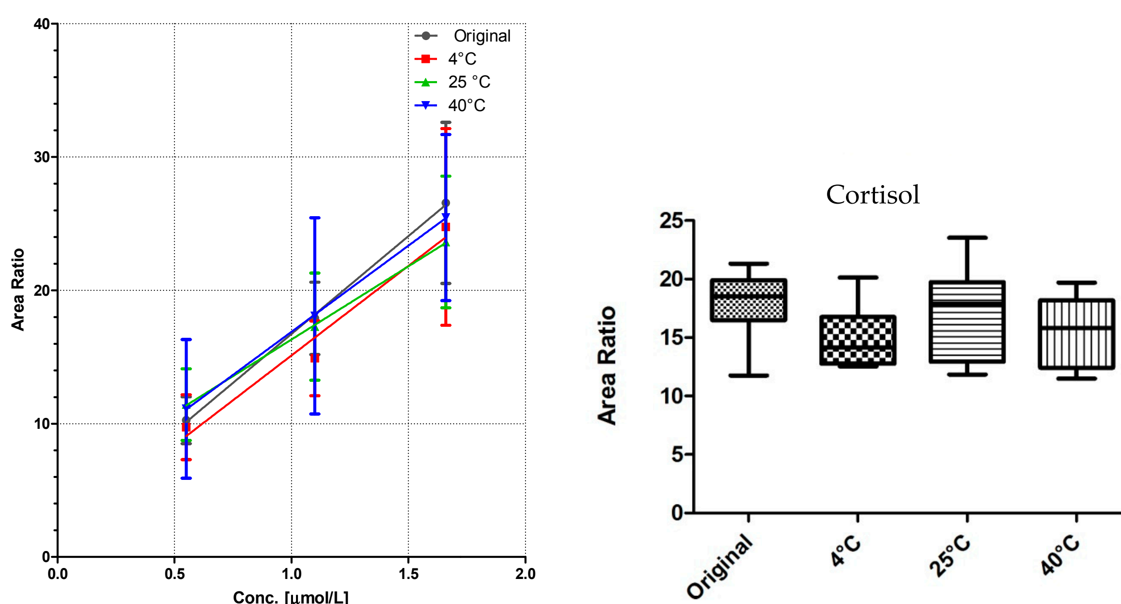

Figure 5. LC-MS/MS analysis of cortisol from DBS cards stored at different storage temperatures: (**left**) linearity (mean ± sd of $n = 7$); and (**right**) box and whiskers graph of level 2 with 5–95 percentiles of the same data.

Comparison of the data shows no significant differences (calculated by GraphPad Prism software) between the first time point ("original") and the results from differently stored cards. As seen with octanoylcarnitine (Figure 4), the mean signal intensity may even be higher if stored at elevated temperatures. However, this effect is not significant from the gathered data and has to be monitored more closely in a follow up study.

A sufficient linearity was reached for all compounds (Table 1), with the exception of myristoylcarnitine. The storage temperature within one week has no influence on the calibration function, as shown in Figures 3–5.

3.3. Intra-Day and Inter-Day Variations

The intra- and inter-day variations as well as the coefficients of determination (R^2) of the three calibration standards are summarized in Table 1. The experiments performed with the DBS-LC-MS/MS system showed excellent linearity. Performances of accuracy and precision were acceptable according to the objectives of these explorations with all CV and biases <20% with two exceptions for the inter-day variation: aspartic acid and glycine. This is due to a shift in peak height between the two compared time points. In a routine setup, this could be avoided by measuring a quality control card as reference each day of use. In addition, carry over was monitored by injecting blank samples after measuring the high QC level number 4, where the blank signal was less than 1% of the high signal.

Table 1. Intra-day and inter-day precision and R^2 of calibrators.

Compound	Intra-Day *	Inter-Day **	R^2	Compound	Intra-Day *	Inter-Day **	R^2
	[%]	[%]			[%]	[%]	
Alanine	8.6	19.0	0.993	Valeryl-carnitine	3.9	12.8	0.999
Arginine	13.6	11.4	0.998	C5DC-carnitine	14.0	17.2	0.616
Aspartic acid	9.3	41.4	0.994	Hexanoyl-carnitine	4.3	11.6	0.999
Citrulline	15.6	18.5	0.999	Octanoyl-carnitine	14.0	18.7	0.996
Glutamic acid	8.2	8.5	0.999	Decanoyl-carnitine	11.8	17.4	0.971
Glycine	8.0	20.3	0.991	Lauroyl-carnitine	3.3	7.3	0.999
Leucine	3.4	11.4	0.999	Myristoyl-carnitine	8.4	19.3	0.875
Methionine	7.0	10.9	0.998	Palmitoyl-carnitine	3.6	5.8	0.997
Ornithine	10.0	10.6	0.999	Stearoyl-carnitine	2.7	5.6	0.999
Phenylalanine	5.5	13.8	0.997	Cortisol	15.2	18.2	0.999
Proline	4.3	11.7	0.995	21-Deoxy-cortisol	10.0	9.7	0.994
Tyrosine	4.5	6.3	0.997	11-Deoxy-cortisol	8.9	13.2	0.999
Valine	5.5	10.1	0.985	17-Hydroxy-progesterone	19.9	16.2	0.972
Carnitine	6.2	11.3	0.999	11-Deoxy-corticosterone	7.6	10.9	0.996
Acetyl-carnitine	5.7	6.8	0.998	Progesterone	11.5	13.5	0.992
Propionyl-carnitine	10.7	12.4	0.998	Androstene-dione	3.4	8.8	0.997
Butyryl- carnitine	5.7	9.0	0.983	Corticosterone	13.2	17.9	0.996

* Of first time point, level 2, ** Storage at 25 °C, level 2, R^2 from day 1, level 1–3.

4. Discussion

The described method follows the trend of the industry towards fully automated processes and also presents a new expanded LC-MS/MS method for newborn screening. In addition to the complete panel of all clinically relevant amino acids and acylcarnitines, a steroid panel was integrated for the first time in a single, fast and fully automated LC-MS/MS method.

The three point calibration curve allows accurate quantification with independent analysis software. The different storage conditions examined in this work had no significant influence on the results and the inter- and intra-day variations are in an acceptable range for a screening method.

The method takes 4 min per sample using the Shim-pack GIST column. Due to the chromatographic separation, the extract is purified, which increases the system robustness. There is no maximum sample amount per column given by the manufacturer, however it is approximated to be plus 1000 samples per column. The total system backpressure was less than 100 bar and the method runs each sample subsequently. Coversely, the traditional punch-and-elute is a batch approach via the 96 well plate format. In contrast to the conventional method, the DBS-MS 500 extracts a centered 4 mm circular area. This does not allow extracting the same DBS twice, however it neutralizes inhomogeneous distribution effects [17] through the software centration of the punching location. In most DBS protocols [3], at least 100 µL extraction solution with internal standard plus sample preparation consumables such as vials and pipette tips are used. The DBS-MS 500 system only uses 20 µL extraction solution and the internal standard is added prior the extraction. To monitor the extraction efficiency and to follow method development guidelines, the internal standard should be added as early as possible into the workflow [18].

The DBS-LC-MS/MS system is capable of handling up to 1200 bar in the analytical circuit and preliminary studies have shown that the process can easily be accelerated to 2.5 min per samples by using denser columns and higher pressure on the system. Bypassing the column is not an option, since the steroids and some acylcarnitines have to be separated for a reliable detection through MRM, since they fragment into the same product ions.

This preliminary study shows the method feasibility and potential of using such automated equipment in the sector of new born screening. To implement such a method, a follow-up study with an extensive validation is planned. In addition, there should be a direct comparison between the punch-and-elute methodology and the direct elution approach described in this study.

Further, the preparation of the calibrators and the quality control cards should be investigated more closely. Basically, the blood can be spiked directly (as shown here), the red blood cells (RBC) can be separated from the plasma, whereas the plasma is then spiked or exchanged with a spiked saline or bovine serum albumin solution, prior remixing with the RBC. The direct addition of spiked solvents may cause hemolysis or protein denaturation above a certain percentage.

5. Conclusions

In addition to the complete coverage of all amino acids and acylcarnitines of interest, an additional steroid panel was integrated to allow screening for congenital adrenal hyperplasia currently determined by separate biological assays. This screening method decreases the analysis cost through full automation and an economical use of solvents. Only 20 µL HPLC grade extraction solvent are used per extraction and the volume of internal standard is reduced through the integrated sprayer. Since the rinsing solution flows through separate channels, non LC-MS grade solvents can be used here. The extended newborn screening panel for MS detection therefore not only reduces the amount of solvents and labor, it also provides the foundation of good laboratory practice (GLP) by an integrated documentation system. The dried blood spots are directly eluted from the filter cards without any punching and are therefore traceable at all times during the analysis process. Each card is documented before and after analysis to ensure the highest standards of quality and documentation.

Supplementary Materials: The following are available online at www.mdpi.com/2409-515X/4/1/2/s1.

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Author Contributions: Ralph Fingerhut, Stefan Gaugler, and Götz Schlotterbeck participated in the concept and design of the study. Jana Rykl contributed to the optimization of the MS methods. Tamara von Däniken Stefan Gaugler, Jana Rykl, and Irene Wegner did the practical work. Stefan Gaugler was responsible for drafting of the manuscript. Ralph Fingerhut and Götz Schlotterbeck were responsible for revising of the manuscript. All authors approved the manuscript as submitted.

Conflicts of Interest: Jana Rykl is an employee of Shimadzu Schweiz GmbH (Reinach, Switzerland) and Stefan Gaugler is an employee of CAMAG (Muttenz, Switzerland). None of the other authors report any conflict of interest regarding this study. One DBS-MS 500 was setup at the Children´s Hospital as part of this KTI project. All other instrumentation is property of the respective companies and Children´s Hospital.

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Research Article

Validation of an Automated Extraction Procedure for Amino Acids and Acylcarnitines for Use with Tandem Mass Spectrometry for Newborn Screening

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Abstract

A certified reagent kit for newborn screening was transferred on a fully automated dried blood spot platform. The dried blood spot cards are directly eluted and the extract is online guided to tandem mass spectrometry instrument, where the amino acid and acyl carnitine panel is detected. The method takes 2 minutes per sample and requires no human interaction for up to 500 samples. The method is fully standardized through the automation and the usage of only certified consumables and reference material. The manual reagent kit was first modified to fit the automated platform, secondly validated and third, successfully transferred into a routine newborn screening laboratory.

Keywords: *Dried blood spot, Newborn, Screening, Amino acid, Carnitine, Automation*

Introduction

Newborn screening (NBS) is a public health program provided by most of the countries around the world aimed at screening newborns for a list of serious genetic and metabolic disorders. Early diagnosis of these conditions can help prevent their further development, which if untreated often results in brain damage, organ damage, and even death [1–4]. A routine neonatal screening procedure requires that a health professional takes a few drops of blood from the baby's heel, applies them onto special filter paper and sends such prepared samples to a laboratory for a number of analytical tests [5].

The amino acids and acyl carnitines are detected in modern methods with tandem mass spectrometry (MS/MS). MS/MS is a fundamentally different technology than systems previously used by most newborn screening laboratories, such as bacterial inhibition assays. It is a versatile and modular system that can be easily adapted to the preferred testing approach by the user. This has led to numerous variations of newborn screening by MS/MS, and it became a challenge to compare results between laboratories. There is a recognized need to develop consensus solutions to provide more consistency between MS/MS screening programs [6–9].

This validation addresses a certified screening method using fully automated analysis equipment from sample recognition towards extraction and analysis. The method was based on the commercial MassChrom® Reagent Kit from Chromsystems to allow full standardization of the complete process. The method development was performed at the Shimadzu laboratory (Reinach, Switzerland) and the application has been transferred to a routine NBS laboratory in Switzerland (Childrens Hospital Zurich). After the method development, a validation was performed, followed by a transfer into a routine laboratory focusing on inter-, intra-day variations, correlation and robustness. To standardize the sample preparation, handling and extraction, a fully automated DBS system from CAMAG (DBS- MS 500) was integrated into the workflow. The main goal of this study was to validate this change in analytical procedure.

Application of automated DBS card handling systems, which are connected to mass spectrometry analyzers, offers a modern and fast approach where a circular area of the DBS is directly eluted from the filter paper card without any punching [9–12].

Materials and Methods

Chemicals

The following MassChrom Reagents (Chromsystems Munich, Germany) were used; Mobile Phase (No. 57001), Internal Standard (No. 57004), Extraction Buffer (No. 57008) and Mass Check Controls propionylcarnitine, decanoylcarnitine, lauroylcarnitine, myristoylcarnitine, palmitoylcarnitine and stearoylcarnitine, were purchased from Sigma-Aldrich (St. Louis, USA). MassChrom rinsing solution (No. 55007) was used for the rinsing process and also purchased from Chromsystems (Munich, Germany). Dried blood spot cards (903, TFN, MN818 and 2992) were provided by CAMAG

(Muttenz, Switzerland). Fresh whole blood was obtained from the local blood donation center (Basel, Switzerland). The blood was previously tested for infectious diseases.

Analytical Methods

A DBS-MS 500 unit (CAMAG, Muttenz, Switzerland) was attached as front end to a modular HPLC system from Shimadzu (Kyoto, Japan), containing a system controller (CBM-20A), a Nexera X2 pump and a degasser (DGU-20ASR). The loop outlet of the DBS-MS 500 system was connected with a 1.8 m PEEK tubing (yellow 1/16» OD x .007» ID) using a KrudKatcher Ultra (KrudKatcher Ultra, Phenomenex, Torrance, CA, USA) inline filter at the mass spectrometer inlet. Analysis was performed in positive multiple reaction monitoring (MRM) mode on an electrospray ionization tandem mass spectrometry system 8060 in Reinach and 8050 in Zurich (Shimadzu, Kyoto, Japan). The extract was directly injected into the mass spectrometer without an analytical column.

The elution was performed isocratic with the mobile phase from the kit using a flow gradient starting at 0.2 mL/min to 0.6 mL/min in 0.3 min to 1.9 min, 1.91 min back to 0.2 mL/min, 2.0 min controller stop. The following m/z transitions were programmed for the mass spectrometry detection (Table 1);

Table 1. m/z transitions

Name	Precursor m/z	Product m/z	Dwell (msec)	Q1 Pre Bias (V)	CE	Q3 Pre Bias (V)
Alanine	90.2	44.2	10	-10	-12	-19
Alanine-$^{2}H_{4}$	94.2	48.2	10	-10	-12	-19
Arginine	175.2	70.2	10	-10	-24	-16
Arginine-$^{2}H_{7}$	182.2	77.2	10	-10	-24	-16
Aspartic acid	134.2	134.2	10	-15	-12	-13
Aspartic acid-$^{2}H_{3}$	137.2	75	10	-16	-16	-27
Citrulline	176.1	113.1	10	-10	-16	-25
Citrulline-$^{2}H_{2}$	178.1	115.1	10	-10	-16	-25
Glutamic acid	148.15	84.1	10	-17	-17	-14
Glutamic acid-$^{2}H_{5}$	153.1	88.2	10	-17	-18	-19
Glycine	76	30	10	-11	-12	-30
Glycine-$^{13}C_{3}$/ $^{15}N_{1}$	79	32	10	-10	-16	-28
Leucine	132	86.2	10	-16	-12	-19
Leucine-$^{2}H_{3}$	135	89.2	10	-16	-12	-19
Methionine	150.1	104.1	10	-18	-14	-22
Methionine-$^{2}H_{3}$	153.1	107	10	-18	-13	-18
Ornithine	133.2	133.2	10	-16	-12	-27
Omithine-$^{2}H_{6}$	139.2	76	10	-15	-19	-15
Phenylalanine	166.2	120.2	10	-18	-14	-28
Phenylalanine-$^{2}H_{5}$	171.2	125.2	10	-18	-14	-28
Proline	116.2	70.1	10	-14	-18	-23
Proline-$^{2}H_{7}$	123.2	77.1	10	-13	-18	-16
Tyrosine	182.1	123.1	10	-10	-18	-24
Tyrosine-$^{2}H_{4}$	186.1	127.1	10	-10	-18	-24
Valine	118.2	72.1	10	-14	-13	-17
Valine-$^{2}H_{8}$	126.2	80.2	10	-14	-13	-17
Carnitine	162	85	10	-21	-23	-20
Carnitine-$^{2}H_{9}$	171	85.1	10	-10	-23	-17

Name	Precursor m/z	Product m/z	Dwell (msec)	Q1 Pre Bias (V)	CE	Q3 Pre Bias (V)
Acetylcarnitine	204	84.9	10	-20	-25	-20
Acetylcarnitine-$^{2}H_{3}$	207	85.1	10	-11	-19	-18
Propionylcarnitine	217.9	85	10	-20	-25	-20
Propionylcarnitine-$^{2}H_{3}$	221	85.1	10	-12	-23	-18
Butyrylcarnitine	231.9	85	10	-20	-25	-20
Butyrylcarnitine-$^{2}H_{3}$	235	85.1	10	-13	-23	-17
Valerylcarnitine	246	85.1	10	-20	-14	-21
Valerylcarnitine-$^{2}H_{9}$	255	85.1	10	-14	-24	-17
C5DC-carnitine	276.2	85	10		-46	
C5DC-carnitine-$^{2}H_{6}$	282.6	85	10	-15	-26	-19
Hexanoylcarnitine	260	85	10	-20	-25	-20
Hexanoylcarnitine-$^{2}H_{3}$	263	85.1	10	-13	-22	-14
Octanoylcarnitine	288	85	10	-20	-30	-20
Octanoylcarnitine-$^{2}H_{3}$	291	85.1	10	-15	-23	-14
Decanoylcarnitine	316	85.1	10	-10	-24	-17
Decanoylcarnitine-$^{2}H_{3}$	319	85.1	10	-10	-24	-17
Lauroylcarnitine	344	85	10	-20	-28	-20
Lauroylcarnitine-$^{2}H_{3}$	347	85.1	10	-11	-25	-19
Myristoylcarnitine	372.2	85	10	-19	-30	-20
Myristoylcarnitine-$^{2}H_{3}$	375	85.1	10	-11	-27	-17
Palmitovlcarnitine	399.9	85.1	10	-12	-28	-18
Palmitoylcarnitine-$^{2}H_{3}$	402.9	85.1	10	-12	-28	-18
Stearoylcarnitine	427.9	85.1	10	-13	-29	-17
Stearoylcarnitine-$^{2}H_{3}$	430.9	85.1	10	-13	-29	-17

ESI mode, Nebulizing gas; 1.5L/min, Heating gas Flow; 10 L/min, Interface Temperature; 300 °C, DL Temperature 300°C, Heat Block Temperature 400°C, Drying gas Flow; 10 L/min

The data analysis uses a linear curve type using the internal standard for the area calculation. The two Chromsystems MassCheck levels are used according to the specified concentrations. All peaks are as default integrated from 0.05 to 1.5 min with a width of 25 sec.

DBS-MS 500 Instrumentation and Settings

The MassChrom internal standard 57004 was dissolved in 25 mL extraction buffer 57008 according to the Chromsystems procedure and connected to elution bottle 1, this solution was used for extracting the DBS. The MassChrom rinsing solution was connected to rinsing bottle 1 (R1). The extraction head was cleaned in an ultra sound bath at 40 °C for 10 min prior to a large set of analyses. The extraction solvent was primed for 5 cycles and the rinsing solvents were flushed for 1 min (this process is an automated system prime method). The DBS cards were photographed with the built-in camera of the DBS-MS 500 before and after each extraction to check for the presence of a blood spot and to adjust the extraction head to the center of each spot. The Chronos for CAMAG software automatically recognized inadequate dried blood spots based on their roundness, diameter, and area. Inadequate DBS were excluded from analysis. The samples were extracted with a 4 mm diameter clamp and a volume of 60 µL and a 200 µL/min flow rate into a 20 µl loop (the 40 µl upfront volume is directed to the waste). The extraction solvent passes the sealed area on the DBS card horizontally from the bottom back to the bottom into a sample loop, which is online guided to the mass spectrometer after the elution step. The area next to the 4 mm extraction ring is not affected by the solvent and could be reused if needed. To complete the automated DBS extraction cycle, the system was rinsed for 20 s with R1 [11].

Standard and DBS Sample Preparation

DBS calibration samples for method development and implementation were prepared manually in the laboratory (described as calibrators). Later on for method validation, external quality control material from Chromsystems (Masscheck) was integrated into the analysis workflow and used for reference (described as controls).

Five calibration points were prepared for the validation process. Here, 10 mg of each analyte was dissolved in 10 ml of water for the amino acids and in 10 ml methanol for the acyl carnitines. For each level, a mix was prepared according to reference values provided by the MassCheck Controls from Chromsystems. EDTA stabilized blood was pooled and centrifuged at 1300 rcf for 5 minutes. The plasma and buffy coat layer was removed and replaced with saline (0.9% NaCl) [13]. After gently mixing, this washing procedure was repeated twice. After removing the wash solution, a spiked saline with amino acids and acyl carnitines in five different levels (A-E, Supplementary data) was added and gently mixed [14].

Aliquots of the five calibrator blood levels were spotted in 15, 30 and 50 µl (standard 30 µl) droplets by an Eppendorf pipette onto TFN, 226, 903, 818 and 2992 filter paper cards from different vendors and dried in a horizontal position for a minimum of three hours. After drying, the calibrators were placed in a plastic bag with desiccant and stored at -20 °C. Calibrators B and E were used to derive data for intra- and interday imprecision. Those levels were used to compare to the high and low control levels from the MassChrom kit which will be used for the routine afterwards.

To measure the external MassCheck control card, a DBS frame in the standardized format of 84.67 mm × 53.2 mm (w × h) was fastened to the previously cut out reference DBS spots (Figure 1). By using the X-offset of the DBS-MS 500, several extractions per reference spot can be performed.

Figure 1. Manually prepared QC card.

All DBS were prepared from the same blood source, prepared with the same procedure to neutralize potential hematocrit effects [11], [15], [16] , which were not investigated in this study. All samples were prepared at one site and shipped to the other laboratory under controlled environment, to also eliminate the inaccuracy of different sample preparation.

The five calibration points were used for method validation and transfer, afterwards the routine measurement will be referred to the two Chromsystems MassCheck control levels high and low.

Validation Procedure

The inter- and intra-assay precision was obtained by measuring calibrator levels B and E at 3 consecutive days six fold. Precision was evaluated within a single validation run (intra-day) as well as between three runs recorded on different days (inter-day). The precision was calculated as the percentage relative standard deviation (CV, %) within an analytical run (intra-day precision, n = 6) and over all three runs (inter-day precision, n = 18). Depending on the analyte, CVs for within-run precision can range from 15% to 25% and for inter-run precision can range from 20% to 35% for newborn screening MS/MS assays [14]. The accuracy was assessed from the overall mean of each MassCheck control concentration divided by its nominal value (bias, %). Since all signals are related to official and certified reference material from Chromsystems [17], no other accuracy assays were conducted.

The extraction recovery of the DBS-MS 500 autosampler was investigated for DBS samples at level D. The five compounds methionine, proline, valine, carnitine and lauroyl-carnitine were chosen for this experiment. Each spot was extracted five times in triplicates. Between two extractions, a drying time of approximately 15 min was programmed. Using the built-in camera of the DBS-MS 500, the extraction head automatically locked onto the same area in the center of the blood spot. The recovery was finally estimated as the percentage ratio of the analyte peak area of the first extraction to the sum of the peak areas of all subsequently conducted extractions [11, 14].

Correlation and robustness was performed on each three days in two different laboratories. The correlation is determined using the 5 calibrator levels on 903 type filter paper, measured four fold. Each day, the run included a double measurement of each MassCheck level high and low as reference.

The carryover was assessed to study the possible effects from specimens with a high concentration of an analyte on the result of the subsequent specimen or specimens. Although there are several potential sources for carryover within the MS/MS system, the most common source is the autosampler injection port and the tubing leading to the MS/MS electrospray unit. To determine the carry over in the automated DBS-MS 500 system, an extraction with a high concentration at level E is performed, followed by three extractions of blank DBS cards. For no carryover, the analyte concentrations of the blank sample should be below the LOD concentration previously determined. This should be repeated a minimum of five times [14].

Although no interferences are known or have been documented [17], the blank filter papers used for specimen collection were checked for possible interferences of common m/z for any of the analytes measured, especially since new filter paper sources and new lot numbers are used. Here, we investigated the most commonly used filter paper types from different vendors used for manufacturing the DBS cards. Paper types are TFN and 226 from Ahlstrom, 903 from EBF, MN 818 from Macherey Nagel and 2992 from Hahnemühle. Calibrator level B, D and F were measured as triplicates on each paper type to detect precision and any significant off-set.

The robustness is determined during the interference and repeatability study by varying the following parameters; technician, card type, eluent preparation (using solvent and buffer from different lots), elapsed times (with or without breaks between the measurements) and laboratory environment (location, temperature

and humidity). All cards used for the interference study were spotted with level a calibrator.

Matrix effects, an extended interference study and stability tests were not performed, since this data is given by the MassChrom kit and its process remain unchanged [17].

Documentation

The documentation process is given by the DBS-MS 500 instrument which takes a picture of the DBS card before and after each extraction to assure sample traceability. All pressures on the DBS card sampler as well as on the LC-MS/MS unit are monitored and documented.

The camera documentation system checks for preset values, where quality control parameters can be integrated. If the quality criteria are not met, the system automatically checks the next spot and continues the preset program. In addition, the camera detects already extracted spots and blocks those to prevent reanalysis. The system checks the xy shift of the circle on the card and the blood spot to center the extraction spot to the middle of each DBS (to avoid inhomogeneous distribution effects). Further, the inbuilt pressure sensors are monitoring the extraction and rinsing pressure (in this study, the extraction pressure was 0.5 bar and the rinsing pressure 45 bar). The maximum extraction pressure is 1.2 bar, depending on the age of the blood sample, and the system can be rinsed with pressure up to 100 bar to prevent carry over [9].

Results

MS/MS Method

The method from the MassChrom kit was transferred to the automated DBS-MS 500 platform allowing full automation of this process. The method was successfully installed at a routine NBS laboratory. Five calibrator levels of blood spotted on DBS cards were established for the method validation and installation. For routine, the two levels high and low from the MassCheck controls were used. Since the extract is guided online to the tandem mass spectrometry system, it only takes 2 minutes per sample. All processes are overlapping, where the DBS-MS 500 runs a wash program and extracts the consecutive sample while the MS/MS is detecting and reporting the target analytes of the previous sample.

Method validation

First, validation including additionally carry-over, interference, volume effects and recovery was performed after setting up the method on the automated platform. Evaluation of accuracy was not part of this method validation, since control material from the MassChrom kit was used as reference in all experiments. The intra- and inter-day variations as well as the coefficients of determination (R2) of the calibration standards are summarized in Table 2. All factors were within the general acceptance criteria for NBS screening methods [17]. Intra-day variations are all below 15% with the exception of aspartic acid and glutamic acid which are slightly above but still acceptable since the criteria sets a value of < 25%. Also, glutamic acid gave a relative high variation for the inter-day variation (still within the criteria), compared to the other values which are all below 25%. Therefore the optimization of the MRM transition of glutamic acid was carefully monitored for the method installation at the routine site.

Table 2. Selected recovery comparison to MassCheck controls

MassCheck	Methionine	Proline	Valine	Carnitine	Laurovl-carnitine
Extraction 1	0.6159	1.421	1.597	15.499	56.966
Extraction 2	0.1284	0.460	0.527	2.416	9.854
Extraction 3	0.0557	0.154	0.184	0.653	4.461
Extraction 4	0.0276	0.063	0.083	0.293	3.981
Extraction 5	0.0154	0.031	0.035	0.152	3.368
	[%]	[%]	[%]	[%]	[%]
MassCheck recovery	73.1	66.7	65.8	81.5	72.4
Sample recovery	79.9	70.3	61.5	80.0	76.4

DBS spots using 15, 30 and 50 µl blood were prepared at calibrator level A, C and E and compared as triplicates. The deviations from 50 µl to 30 µl spots were between 90.8 – 102.2 % and the deviations from 30 µl to 15 µl were between 78.2 – 88.8 %. There is a trend of smaller signals towards the 15 µl spots, however there is no obvious trend between 30 µl and 50 µl spots.

Carry-over from the high concentration to a blank DBS card passed the ICH guideline criteria and all investigated filter paper types were feasible for being used with this method. The best results were accomplished with the TFN filter paper (2.6% variation), paper types 226, 903, 229 were below 5% variation and the 818 paper moved with 6.0% on the last rank (supplementary data). The standard deviation of the complete panel from the triplicate measurement was taken into account.

The recovery of the compounds from the DBS card lies within 40 to 80%. This recovery remains constant for each analyte with the chosen extraction parameters. Since the results are always considered relative to the used MassChrom quality control card values with the same consistent recovery, this has no effect to the screening method, (see Table 2 and supplementary data).

Also the extraction behavior remains constant. This was investigated by comparison of freshly spotted blood and blood spots with 4 days of age stored at -20 °C (see supplementary data).

DBS spots using 15, 30 and 50 µl blood were prepared at calibrator level A, C and E and compared as triplicates. The deviations from 50 µl to 30 µl spots were between 90.8 – 102.2 % and the deviations from 30 µl to 15 µl were between 78.2 – 88.8 %. There is a trend of smaller signals towards the 15 µl spots, however there is no obvious trend between 30 µl and 50 µl spots.

Method Transfer into a Routine Environment

The previously developed and validated method was transferred into a routine environment. A short validation focusing on correlation and robustness was performed, where all five calibration levels were measured four-fold on 903 filter paper on three consecutive days. Each day was referred to high and low Mass Check control measured before the sample run.

Table 3. Intra-day, inter-day precision and R2 of calibrators level B (L1) and E (L2) from the spiked DBS samples (*Xle refers leucine/ Isoleucine)

	Intraday day 1		Correlation	Intraday day 2		Correlation	Intraday day 3		Correlation	Inter day	
	LI [%]	L2 [%]	R^2	LI [%]	L2 [%]	R^2	LI [%]	L2 [%]	R^2	LI [%]	L2 [%]
Alanine	6.8	7.6	0.994	3.7	7.1	0.981	8.6	5.4	0.998	24.5	13.0
Arginine	6.1	4.1	0.998	8.1	4.6	0.998	7.8	1.9	0.997	8.5	5.7
Aspartic acid	15.7	6.8	0.964	13.1	6.8	0.987	5.3	10.9	0.925	13.9	10.4
Citrulline	4.8	5.9	0.999	6.5	5.3	0.998	6.5	4.3	0.999	7.9	8.4
Glutamic acid	15.5	7.2	0.998	1.4	4.3	0.997	3.5	3.4	0.995	32.4	11.7
Glycine	9.2	9.9	0.998	9.2	9.9	0.998	7.7	12.3	0.987	8.7	18.9
Leucine (Xle*)	6.5	4.2	0.999	2.8	5.0	0.999	5.0	3.5	0.901	12.3	9.5
Methionine	10.3	4.8	0.999	9.6	6.1	0.985	11.9	4.8	0.999	24.9	5.8
Ornithine	6.9	4.0	0.999	6.5	5.7	0.998	11.1	4.6	0.996	13.3	14.8
Phenylalanine	5.7	3.9	0.999	4.3	4.9	0.999	8.6	8.3	0.998	11.7	6.0
Proline	4.0	4.1	0.999	4.6	5.0	0.999	11.0	8.8	0.999	9.4	6.9
Tyrosine	7.8	6.8	0.999	14.5	5.4	0.991	14.1	5.3	0.997	16.2	11.1
Valine	6.0	4.3	0.999	4.7	4.2	0.999	4.8	2.9	0.999	6.5	6.3
Carnitine	6.9	8.9	0.999	1.7	9.4	0.999	6.1	8.1	0.999	6.6	15.5
Acetvlcarnitine	9.3	2.8	0.978	9.7	6.3	0.986	8.0	6.2	0.983	9.4	7.4
Propionylcarnitine	2.3	2.9	0.998	1.5	3.3	0.999	3.8	3.6	0.996	12.9	3.3
Butyrylcarnitine	8.8	2.9	0.999	6.3	4.0	0.998	10.2	8.1	0.999	11.1	7.8
Valerylcarnitine	6.3	3.5	0.999	6.3	4.4	0.999	7.6	4.3	0.996	8.0	6.2
Hexanoylcarnitine	9.2	3.1	0.999	5.0	5.6	0.998	6.5	7.7	0.998	15.0	7.0
Octanoylcarnitine	5.7	3.5	0.999	6.6	3.6	0.999	5.4	7.4	0.997	10.0	6.7
Decanoylcarnitine	5.0	2.4	0.978	3.7	12.5	0.998	7.0	4.5	0.997	15.4	9.4
Laurovlcarnitine	14.0	4.2	0.999	11.5	4.2	0.999	7.8	3.5	0.999	12.8	4.2
Myristovlcarnitine	3.2	5.0	0.997	3.4	3.3	0.998	5.9	6.6	0.999	10.8	5.6
Palmitoylcarnitine	1.6	2.7	0.996	1.3	1.8	0.997	1.3	2.5	0.996	14.8	10.6
Stearoylcarnitine	2.3	2.8	0.995	1.8	2.4	0.999	2.1	3.2	0.995	18.1	14.1

At the Children's hospital in Zurich, the Shimadzu MS/MS 8050 was coupled to a DBS-MS 500 unit as used for the method development. The criteria were met for intra- and inter-day variation following the validation procedure (supplementary data).

Discussion

The described method is fully automated and uses exclusively certified consumables and reference material. Through this degree of standardization, the application can be directly transferred in-between newborn screening laboratories and therefore following the trend towards the development of standardized programs. The method represents a high throughput application with only 2 minutes per sample. All processes are well documented by the reporting system of the DBS- MS 500, where a picture is taken of each spot before and after the extraction and all pressures for extraction, rinsing and LC pump are monitored. The DBS card picture is analyzed with image recognition software, providing results for the spot diameter, area and roundness, which can be used as quality control criteria. Also, the system detects a spot which has been previously extracted, to avoid multiple extractions of the same area. In a standard setting, the extraction is performed automatically from the center of each spot to overcome inhomogeneous distribution effects within the DBS sample [18].

We encountered a relative high endogenous concentration of the target analytes in the available donor blood. To bring this into the range of interest, the blood was washed with saline following a protocol. This procedure allowed gently spiking the washed red blood cells with the target analytes in the according concentrations. The five calibration points were used for method development, validation

and installation at the routine site. After implementing the method, all results were referred to externally prepared and certified reference cards from the MassChrom kit.

The protocol of the MassChrom kit describes an extraction of a 3.2 mm disc in 100 µl extraction buffer (including the internal standard). Here, a 4 mm area was sealed on the DBS card and extracted with 60 µl into a 20 µl loop, whereas the first 40 µl are guided into waste. This was experimentally optimized using different loop volumes, extraction volumes and flow rates. This outcome could be due to an initially high portion of certain analytes, such as salts and phospholipids, causing matrix effects and ion suppression in the ESI source. However, the basic principle of this result needs to be further investigated. The method uses 60 µl of extraction buffer per sample instead of 100 µl from the protocol, which reduces the amount of solvent and analytical standard. In addition, consumables for sample preparation according to the protocol such as Eppendorf vials and pipette tips are no longer required.

Leucine, isoleucine, hydroxy l proline and allo-isoleucine are detected as sum (Xle) and are not separated within this method. An abnormal result for this parameter will have the automatic consequence of a second tier method using an analytical column prior the mass spectrometer to properly distinguish between all isobars. This is needed for the detection of maple syrup urine disease (MSUD).

A volume of 30 µl blood was chosen for the preparation of the calibrator spots. The 50 µl standard volume from literature resulted in relatively large spots on the standard filter paper card for automation with a spot-to-spot distance of 13.7 mm. The 50 µl spots were overlapping if all four positions on the DBS card were spotted. A comparison of 30 and 50 µl droplets showed no difference in the results.

Conclusion

A validated reagent kit for the extraction and analysis of dried blood spots in the field of newborn screening was transferred and validated on an automated DBS card extraction platform. First, a validation was performed and the method was then successfully transferred into a routine environment. Each process step is well documented and all analysis steps follow Good Laboratory Practice (GLP) [19]. The method can be easily modified or extended and transferred to other routine laboratories.

Author Contributions

Stefan Gaugler and Vincente Luis Cebolla Burillo participated in the concept and design of the study. Jana Rykl contributed to the optimization of the MS methods. Stefan Gaugler and Jana Rykl did the method development work, Stefan Gaugler did the practical work at the Children's Hospital Zurich. Stefan Gaugler was responsible for drafting the manuscript. Vincente Luis Cebolla Burillo was responsible for revising the manuscript. All authors approved the manuscript as submitted.

Conflict of Interest

Jana Rykl is an employee of Shimadzu Schweiz GmbH (Reinach, Switzerland) and Stefan Gaugler is an employee of CAMAG (Muttenz, Switzerland). None of the other authors report any conflict of interest regarding this study. All instrumentations are property of the respective companies and laboratories.

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RESEARCH ARTICLE

Fully automated drug screening of dried blood spots using online LC-MS/MS analysis

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A new and fully automated workflow for the cost effective drug screening of large populations based on the dried blood spot (DBS) technology was introduced in this study. DBS were prepared by spotting 15 µL of whole blood, previously spiked with alprazolam, amphetamine, cocaine, codeine, diazepam, fentanyl, lysergic acid diethylamide (LSD), 3,4-methylenedioxymethamphet-amine (MDMA), methadone, methamphetamine, morphine and oxycodone onto filter paper cards. The dried spots were scanned, spiked with deuterated standards and directly extracted. The extract was transferred online to an analytical LC column and then to the electrospray ionization tandem mass spectrometry system. All drugs were quantified at their cut-off level and good precision and correlation within the calibration range was obtained. The method was finally applied to DBS samples from two patients with back pain and codeine and oxycodone could be identified and quantified accurately below the level of misuse of 89.6 ng/mL and 39.6 ng/mL respectively.

Keywords: drugs of abuse, dried blood spot, DBS, automation, TDM.

Introduction

The dried blood spot (DBS) technology was introduced in 1963 by Guthrie and Susi for the detection of phenylketonuria in newborns [1]. The technology evolved to be the method of choice in newborn screening laboratories around the world [2]. One major drawback was the sensitivity since very small sample volumes are applied on the DBS cards. However, with modern mass spectrometry instruments, this issue is no longer a hurdle. The DBS technology emerged to further applications and markets such as therapeutic drug monitoring [3–5], pharmaco-toxicokinetic studies [6] and forensics [7–13]. Advantages of using DBS are simplified blood collection, reduced shipping, and storage costs and reduced analysis time and labor costs due to full automation [4,14]. Automation of the DBS workflow was achieved by automated punching equipment [15], where a manual transfer step of transporting the discs remains, and by direct elution technologies [16,17].

Forensic applications are of major interest since the DBS technology allows screening of a large population with minimum equipment in a cost-effective way. Samples can be drawn easily and shipped to a centralized lab for analysis. The samples are non-hazardous after drying and

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can be shipped by standard mail to the fully automated laboratory. Each sample is anonymized after drawing using a barcode, which is later connected to the analysis results in a database. In this study, a panel of different psychoactive drugs was chosen for introducing the automated drug screening concept. Psychoactive drugs act on normal brain functions and may alter an individual's consciousness, mood or thinking processes. There are legal drugs used for medication such as benzodiazepines, antidepressants and sedatives and illicit drugs such as opiates, cannabis, hallucinogens, and cocaine [18,19]. The detection of these drugs is of major interest in workplace drug testing programs, roadside testing, therapeutic drug monitoring, rehabilitation programs and post-mortem investigations.

We here present the development of an automated DBS process, including card recognition, sample preparation and extraction, and online analysis by ultra-high performance liquid chromatography-tandem mass spectrometry (DBS-LC-MS/MS) for the simultaneous determination of a panel of psychoactive drugs in dried blood spots. Automation for this study was implemented by the CAMAG DBS-MS 500 equipment and a Shimadzu LC-MS/MS-8060 triple quadrupole coupled to a Nexera X2 UHPLC System. The method has been applied to two real cases to illustrate the process and to show the potential of this approach. The acquired MRM data from MS was used for quantitation and additionally for compound verification by screening against a forensic toxicology spectral library from Shimadzu.

Materials and methods

Chemicals and consumables

Gradient grade water and methanol for liquid chromatography (rinsing solvents 2-propanol and acetonitrile), and formic acid were purchased from Carl Roth (Carl Roth, Germany). Ammonium formate (LCMS Grade) was purchased from Sigma Aldrich (Sigma Aldrich, USA). All analytical standards were purchased from Lipomed (Lipomed, Switzerland): alprazolam, amphetamine, cocaine, codeine, diazepam, fentanyl, lysergic acid diethylamide (LSD), 3,4-methylenedioxymethamphetamine (MDMA), methadone, methamphetamine, morphine and oxycodone, and their deuterated standards: alprazolam-D5, amphetamine-D3, cocaine-D3, codeine-D3, diazepam-D3, fentanyl-D5, lysergic acid diethylamide-D3, 3,4-methylenedioxymethamphetamine-D5, methadone-D3, methamphetamine-D5, morphine-D3 and oxycodone-D3. Dried blood spot cards (Ahlstrom TFN filter paper) were provided by CAMAG (Muttenz, Switzerland). Fresh whole blood was obtained from the local blood donation center (Basel, Switzerland). For the DBS drawing BD Microtainer contact-activated lancets (Becton, USA) and soft-zellin alcohol prep-pads (Paul Hartmann AG, Germany) were used.

Analytical material and methods

LC-MS/MS instrumentation and settings

Chromatography was performed on a modular HPLC system from Shimadzu (Kyoto, Japan), which contained a system controller (CBM-20A), two Nexera X2 pumps, a degasser (DGU-20ASR), and a column oven (CTO-20AC). Automated extractions were carried out with a DBS-MS 500 (CAMAG, Muttenz, Switzerland). Analytes were separated on a Shim-pack GIST (2.3 x 50 mm, 5 µm C18, PN 227-30017-3) analytical column (GL Science, Japan). An inline filter (KrudKatcher Ultra, Phenomenex, USA) was connected upstream to the analytical column. Mobile phase A consisted of water with 10 mM ammonia formate, and mobile phase B of methanol with 10 mM ammonia formate. The following stepwise gradient was applied: 5%-95% (0.0-6.0 min), 95% (6.0-8.0 min), 5% (8.1-10.0 min). The flow rate was set to 1.0 mL/min at 40°C. The HPLC liquid stream was connected to a 8060 tandem mass spectrometer (Shimadzu Kyoto, Japan). At least 5 MRM transitions were recorded for each analytical compound. The most abundant three mass transitions were used for quantitation. The detailed MS settings are listed in **Table 1**.

DBS-MS 500 instrumentation and settings

The extraction solvent on the DBS-MS 500 (CAMAG, Switzerland) was a mixture of methanol and water (70:30 v/v) and was connected to the extraction port. The wash solution, consisting out of methanol, acetonitrile, 2-propanol and water with 0.1% formic acid (25:25:25:25, v/v/v/v), was connected to the rinsing bottle. The internal standard mix was connected to internal standard port 2. Internal standard port 4 and the wash port were also filled with methanol. The system was prepared by priming methanol through the internal standard port 4 (10 cycles) followed by 2 cycles through port 2. The extraction head was cleaned in an ultra sound bath at 40°C for 10 min prior a large set of analyses. The extraction solvent was primed for 5 cycles and the rinsing solvents were flushed for 1 minute (this process is an automated system prime method). The DBS cards were photographed with the built-in camera of the DBS-MS 500 before and after each run to check for the presence of a blood spot and to adjust the extraction head to the center of each spot. The Chronos for CAMAG software automatically recognized inadequate dried blood spots based on their roundness,

Table 1. m/z transitions of all compounds.

Name	Pre.	Quant.	Qual.	Qual 2.	Qual 3.	Q1	Q2	Q3
	m/z	*m/z*	*m/z*	*m/z*	*m/z*	V	V	V
Alprazolam	309.1	281.05	205.1	274.1	151.1	-21	-25	-29
Alprazolam-D5	314.1	269.35	286.1	105.05	-	-30	-15	-28
Amphetamine	136.1	91.1	119.15	65.1	39.1	-16	-22	-16
Amphetamine-D3	139.0	92.1	122.15	93.1	-	-15	-18	-16
Cocaine	304.15	182.15	82.05	77.05	105.05	-20	-19	-21
Cocaine-D3	307.15	185.3	85.1	105.1	-	-22	-18	-30
Codeine	300.15	152.1	215.15	165.15	128.1	-15	-63	-28
Codeine-D3	303.05	215.1	226.05	199.1	-	-15	-27	-22
Diazepam	285.1	193.05	154.0	222.1	257.0	-19	-31	-20
Diazepam-D3	288.0	157.05	225.1	260.1	-	-30	-28	-30
Fentanyl	337.25	188.15	105.1	132.1	77.05	-20	-23	-22
Fentanyl-D5	342.25	105.25	188.15	137.2	-	-25	-31	-19
LSD	324.2	223.15	207.15	208.15	281.15	-22	-24	-23
LSD-D3	327.2	226.3	208.1	211.1	-	-24	-30	-23
MDMA	194.1	163.1	105.1	135.05	77.05	-23	-13	-28
MDMA-D3	197.0	163.25	105.1	135.1	-	-22	-14	-29
Methadone	310.2	265.15	105.05	77.05	223.15	-21	-15	-23
Methadone-D3	313.2	268.35	105.0	60.1	-	-23	-13	-29
Methamphetamine	150.15	91.1	119.15	65.1	39.1	-26	-22	-21
Methamphetamine-D5	155.15	92.2	121.1	-	-	-30	-20	-28
Morphine	286.15	152.1	201.1	165.1	128.05	-14	-59	-15
Morphine-D3	289.15	152.1	201.15	-	-	-14	-59	-26
Oxycodone	316.15	241.15	256.15	212.1	187.1	-16	-29	-25
Oxycodone-D3	319.15	301.1	259.1	-	-	-23	-19	-21

The general settings of the mass spectrometer were: nebulizing gas 2 L/min (N_2), heating gas 9.7 L/min (N_2), drying gas 10 L/min, positive and negative mode, and source temperature 300°C. Labsolutions software (Shimadzu Kyoto, Japan) was used to operate the LC-MS/MS system. Pre. = precursor ion; Quant. = daughter ion for quantification; Qual. = qualifier ion; Qual. 2,3 = qualifier ion 2 and 3; Q1 = first quadrupole massfilter ; Q2 = quadrupole collision cell ; Q3 = second quadrupole massfilter

diameter, and area. Inadequate DBS were excluded from analysis. 20 µL of internal standard was sprayed in a homogenous layer onto each spot. After a 20 second drying time the samples were extracted with a volume of 20 µL and a 200 µL/min flow rate. To complete the automated DBS extraction cycle, the system was rinsed for 20 seconds [14].

Sample preparation

Working standards for LC-MS/MS tuning

The preset MRM transition coming from the forensic and toxicology LC-MS/MS method package of Shimadzu were confirmed using standard solutions (1 µg/mL) and were used afterwards for the LC- MS/MS method development. The deuterated drugs were dissolved in methanol to prepare 100 µg/mL standard solutions. Then, a mix was prepared by two dilution steps to generate a final concentration of 100 ng/mL for all deuterated standards. This solution was used as internal standard mix on the DBS-MS 500, each sample was sprayed with 20 µL in "fast" mode. The internal standard module of the DBS-MS 500 was purged with methanol prior mounting and priming the internal standard.

Dried blood spot samples

According to documented cut-off levels in whole blood

Table 2. Analyte concentration and levels [20].

Name	Cutt-off level ng/mL	Toxic level ng/mL	L1 ng/mL	L2 ng/mL	L3 ng/mL	L4 ng/mL
Alprazolam	100	> 350	10	100	500	1000
Amphetamine	100	> 1000	10	100	500	1000
Cocaine	150	> 1000	15	150	750	1500
Codeine	300	> 1100	30	300	1500	3000
Diazepam	100	> 3000	10	100	500	1000
Fentanyl	5	> 34	0.5	5	25	50
LSD	0.5	> 2	0.05	0.5	2.5	5
MDMA	5	> 1000	0.5	5	25	50
Methadone	200	> 1000	20	200	1000	2000
Methamphetamine	100	> 1000	10	100	500	1000
Morphine	300	> 200	30	300	1500	3000
Oxycodone	100	> 200	10	100	500	1000

[20], a calibration was set-up with four levels: level 1 was 10-fold below the cut-off, level 2 was the cut-off concentration, level 3 was 5-fold the cut- off concentration and level 4 10-fold the cut-off concentration (**Table 2**). Except for diazepam and MDMA, level 4 is above the toxic concentration and therefore covers the whole range. Freshly collected human blood was obtained from the local blood donation centre (Basel, Switzerland). EDTA was used as an anticoagulation agent (vacutainer tubes, BD, Allschwil, Switzerland). Endogenous blood of a healthy male donor was chosen as zero control. A stock solution of the standards was prepared in methanol and gently mixed with the donor blood in four different concentrations to prepare levels 1-4. 15 µL aliquots were spotted onto CAMAG DBS cards (CAMAG, Muttenz, Switzerland) and dried at room temperature for at least 2 h. The cards were subsequently stored at 4°C in sealed plastic bags containing desiccants. Stock solutions were stable for two weeks.

Results

Correlation and precision

The calibration levels were measured 7-fold to determine the method robustness and validity. The relative standard deviations of the internal standards, which were applied by spraying, are below 10% for all target compounds by comparing the data through all four levels **(Table 3)**.

The correlation and intra-day variations of all target compounds are listed in **Table 4**. The target to internal standard ratio was used to compare the results and to develop the calibration line. The calibration line is shown for codeine and oxycodone in **Figure 1**, since those compounds were measured in the patient samples using the developed method (section: Patient samples).

The intra-day variations as well as the coefficients of determination (R^2) of the four calibration standards are summarized in **Table 4**. Fentanyl, LSD, morphine and MDMA can be quantified at their cut-off level, however level 1 represents the limit of detection with a signal to noise ratio of 10.3 for fentanyl, 8.0 for LSD (shown as an example in **Figure 2**), 9.0 for morphine and 3.3 for MDMA, which are therefore not represented in **Table 4.** Excellent correlation was obtained for all target compounds in the panel (**Table 4**). All points of the calibration functions were sufficiently precise with relative standard deviations below 15%.

Table 3. Internal standard imprecision.

Name	Relative standard deviation %
Alprazolam-D5	6.0
Amphetamine-D3	6.7
Cocaine-D3	5.9
Codeine-D3	6.1
Diazepam-D3	9.6
Fentanyl-D5	6.0
LSD-D3	6.1
MDMA-D3	7.0
Methadone-D3	3.8
Methamphetamine-D5	9.4
Morphine-D3	5.4
Oxycodone-D3	5.9

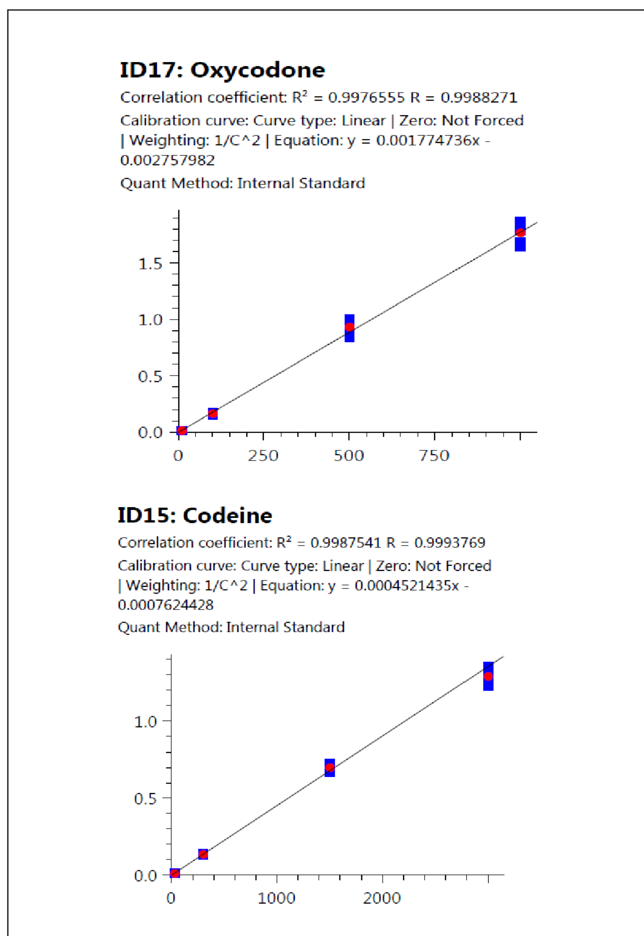

Figure 1. Calibration of top: oxycodone (n=7 each level) and bottom: codeine (n=7 each level).

Patient samples

To check the feasibility of the method and the applicability in a real setting, two anonymized DBS samples were acquired from healthy donors using medication for back pain. The samples were drawn by the patients themselves according to an introduction sheet. They were provided with a DBS card, a 1.8 mm lancet, an alcohol prep pad and packaging material with desiccant. The returned samples were analyzed using the newly developed DBS-LC-MS/MS method. 89.6 ng/mL codeine were detected in sample 1 and 39.6 ng/mL oxycodone in sample 2. The MRM data quality was sufficient enough to be used for screening against a spectral library search option to confirm the identity of the quantified compounds. The software displays the chromatographic peak, the calculated concentration according to the calibration function and the results from the library search (**Figure 3**).

Automation

The flow scheme of the fully automated card extraction system and the coupled LC-MS/MS is shown in **Figure 4**. The DBS cards are moved to the extraction unit, where a plunger seals a 4 mm circular hole in the card. The extraction solvent is pumped through the card and loaded into a loop (**Figure 4**: red arrows). By switching the 10-port valve, the loop volume is connected to the LC-MS/MS flow path (**Figure 4**: green arrows), guided to the column and to the MS/MS. Meanwhile, the extraction head is cleaned by a rinsing cycle to avoid carry over [21].

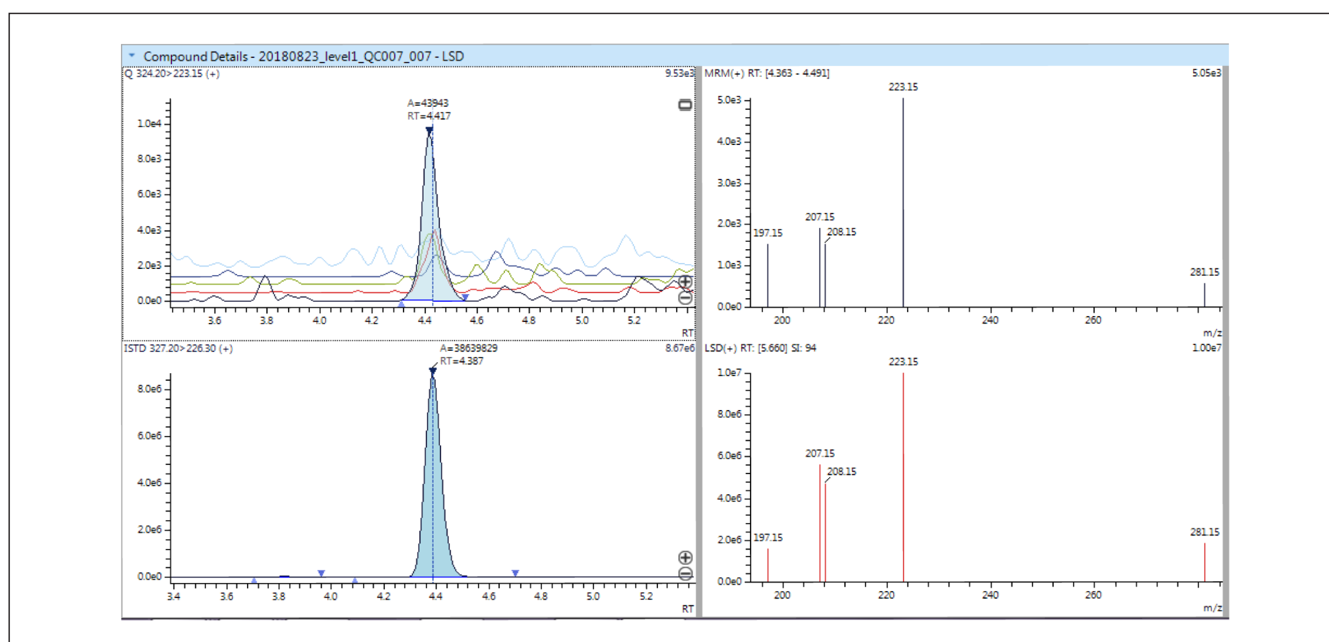

Figure 2. LSD peak at level 1 (0.05 ng/mL) (top) and its deuterated standard to represent the LOD (bottom).

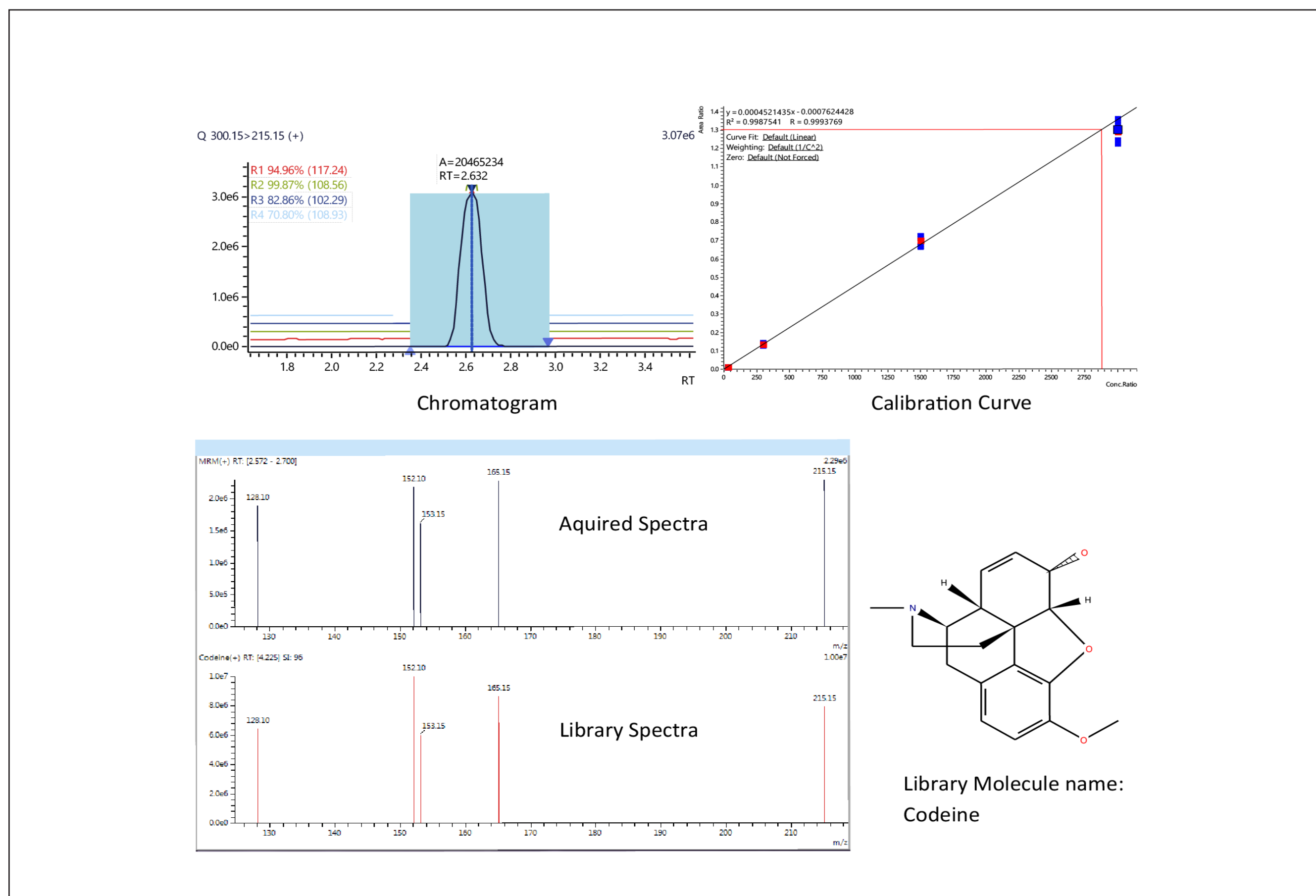

Figure 3. Sample report from the Insight® software with chromatographic peak, overlay of acquired and library spectra, calibration function and molecular structure.

The DBS card contains a barcode which is linked to a data administration sheet. The patient information is sent separately to an administration office and the DBS card containing the blood sample is sent to the centralized laboratory. The system reports the barcode, a picture of the card before and after extraction, blood spot details such as roundness, area, diameter and the location on the card, all fluidic pressures and the applied method. This report is then matched with the patient information. The advantage of this workflow is a completely anonymous and standardized sample handling process, which is suitable for anti-doping or police laboratories.

Carry-over

Carry over was monitored by measuring blank blood after injecting a mix of all standards at level 4 (**Table 2**). The criteria of bio-analytical method validation guidelines were fulfilled [22,23]. No carry over was observed for the chosen analyte panel. **Figure 5** shows the codeine peak for level 4 and the subsequent blank sample. This was optimized by programming a wash sequence by the DBS-MS 500. Here, the outlet capillary (between extraction and 10-port valve, **Figure 4**) was rinsed with methanol, acetonitrile, 2-propanol and water with 0.1% formic acid (25:25:25:25, v/v/v/v) for 20 seconds at 45 bar after which the extraction chamber inlet was flushed with extraction solvent for 10 seconds.

The investigation of the feasibility and routine applicability of the method using real patient samples was successful. By spotting micro volumes, such as DBS, a very good sensitivity is obtained. The compounds fentanyl and LSD are among the most active drugs in the chosen panel and appear in very low blood concentrations. One blood droplet (15 µL) of the fentanyl sample at level 1 contains only 7.5 pg of standard. The droplet spreads with an average hematocrit to an area of approximately 40 mm². An extraction circle of 4 mm diameter equals 30 percent of the droplet, which means that with a theoretical extraction efficiency of 100%, only 2.5 pg of standard reaches the LC-MS/MS system. Nonetheless, this low amount is detectable and at slightly higher concentrations quantification is possible. The 8060 MS/MS system

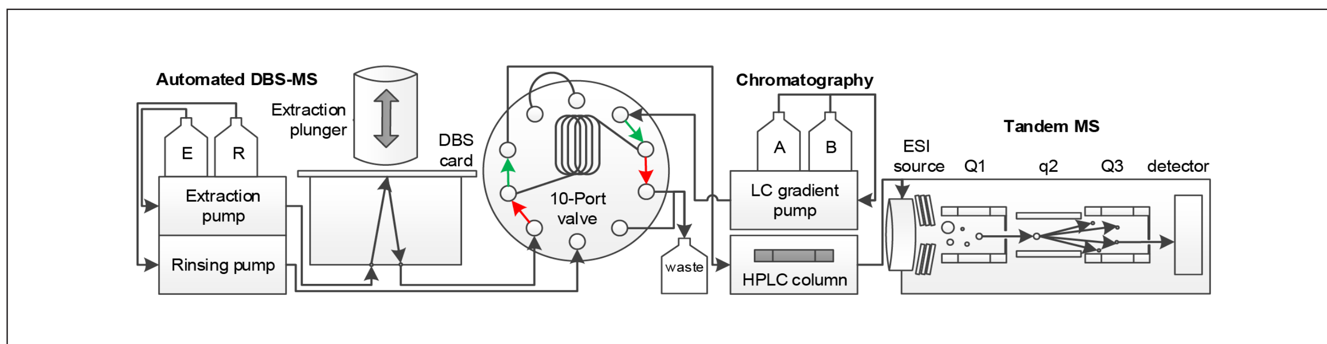

Figure 4. Flow scheme of the automated DBS-LC-MS/MS approach.

is a high-end mass spectrometer and those instruments are gaining in sensitivity with each new generation. This DBS-LC-MS/MS workflow allows screening of hundreds of drugs in parallel.

When analyzing smallest drug amounts in a complex matrix such as blood, it is useful to include a short separation on an analytical column prior the MS detection. The ion source of the MS can be protected from suppressing ions when the first portion from the column is guided directly to waste. Ions for example are not retained on the C18 column and can be eliminated by washing before opening the path into the ion source. In this method, a C18 column was used to isolate the target compounds from the blood extract. Also, 20 µL injection volume is a relatively high amount for the LC system. The extraction solvent should be as similar as possible to the mobile phase (at the time point of extraction) for a good LC separation. Here, it was found that the extraction volume does not corrupt the analytical separation, although the organic contend at the start conditions differs from the extraction solvent.

Both patient samples contained a drug concentration below the cut-off level of abuse (89.6 ng/mL codeine, cut-off 300 ng/mL and 39.6 ng/mL oxycodone, cut-off 100 ng/mL), which shows in both cases a good therapeutic dosage. Additionally a morphine signal was detectable in the patient taking codeine, since typically approximately 10 % of the codeine is metabolized via CYP2D6 to morphine [24].

The internal standards are used to monitor the extraction efficiency and to ensure that the system is working properly [14,25]. 20 µL of the internal standard mix was sprayed on the DBS spot prior extraction. This process of integrating the internal standard by spraying is the method of choice for quantification. Abu-Rabie et al. stated that the hematocrit level can have an influence on the extraction efficiency, so that the internal standard should better be applied prior to the extraction [25]. All deuterated standards were detected easily and could be used in much lower concentrations to decrease costs. Also, for a routine setup, internal standards can be used for substance classes to further reduce the analysis cost.

The extraction setup of the DBS-MS 500 features a horizontal extraction, where the solvent passes from the bottom through the sealed area on the DBS back to the bottom of the card. The extraction is performed under increased pressure to fully dissolve the target molecules producing 20 µL of highly concentrated extract (volume can be adjusted), which is online coupled to the analytical system. The full automation of the analysis workflow is important to exclude error sources. Each process is monitored and documented automatically and large batches of samples can be measured over night without any human interaction. Each dried blood spot card is prepared and handled the same way in a standardized process. Analytes are less stable in solution and this method minimizes this time period where those remain in solution.

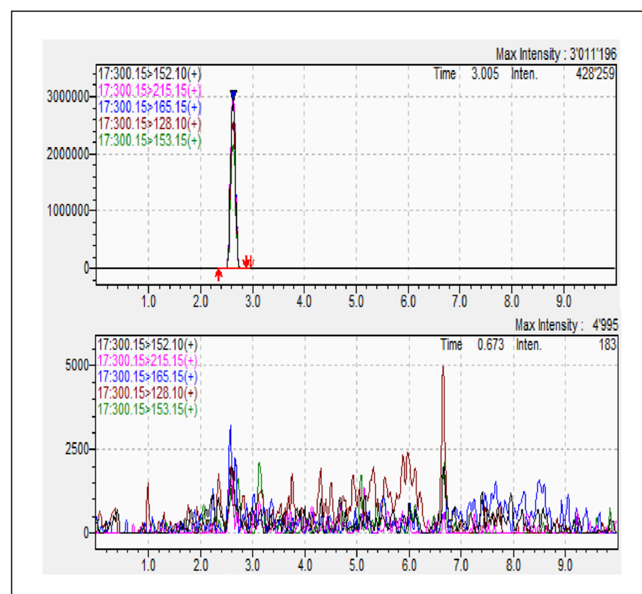

Figure 5. Codeine peak at level 4 (3000 ng/mL) (top) and the following blank blood injection (bottom).

With the presented method, the analysis of one sample takes 10 minutes. The analysis time could be decreased by using denser packed columns and a higher LC flow rate. The method needs to be validated as a next step prior to application for routine testing. Also the transportation and storage conditions have to be further examined to develop analysis guidelines.

Conclusion

A new workflow for drug screening in large populations was introduced for a panel of 12 drugs of abuse from a wide variety of structural categories. The analysis process is fully automated by using an online DBS-LC-MS/MS analysis system. The feasibility for such an approach was shown and the method has been successfully applied to real cases showing the potential. The panel was chosen with a variety of different illicit and legal drugs from different substance classes to cover a broad field of substances. Highly active drugs, such as LSD and fentanyl, which appear in very small blood concentrations, were included in the panel and quantified at their cut-off concentration. The process from sampling to generating the report was linked with a barcode system to enable forensic applications. Each process step is well documented and all analysis steps follow Good Laboratory Practice (GLP) [22]. The method can be easily extended and validated according to individual laboratory guidelines.

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Fully Automated Forensic Routine Dried Blood Spot Screening for Workplace Testing

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Abstract

In this study, we describe the transfer of a new and fully automated workflow for the cost-effective drug screening of large populations based on the dried blood spot (DBS) technology. The method was installed at a routine poison control center and applied for DBS and dried urine spot (DUS) samples. A fast method focusing on the high-interest drugs and an extended screening method were developed on the automated platform. The dried cards were integrated into the automated workflow, in which the cards were checked in a camera recognition system, spiked with deuterated standards via an in-built spraying module and directly extracted. The extract was transferred online to an analytical LC column and then to the electrospray ionization tandem mass spectrometry system. The target compounds were analyzed in positive multiple-reaction monitoring mode. Before each sample batch or analysis day, calibration samples were measured to balance interday variations and to avoid false negative samples. An internal standard was integrated prior the sample extraction to allow in process control. A total of 28 target compounds were analyzed and directly extracted within 5 min per sample. This fast screening method was then extended to 20 min, enabling the usage of a Forensic Toxicology Database to screen over 1,200 drugs. The method gives confident positive/negative results for all tested drugs at their individual cut-off concentration. Good precision (±15%, respectively ±20% at limit of quantification) and correlation within the calibration range from 5 to 1,000 ng/mL was obtained. The method was finally applied to real cases from the lab and cross-checked with the existing methodologies.

Introduction

The fast and cost-effective detection of prescription and illicit drugs is of major interest in workplace drug testing programs, roadside testing, rehabilitation programs and post-mortem investigations (1). Psychoactive drugs act on normal brain functions and may alter an individual's consciousness, mood or thinking processes. There are legal drugs used for medication such as benzodiazepines, antidepressants and sedatives and illicit drugs such as opiates, cannabis, hallucinogens and cocaine (2, 3). The integration of the dried blood spot (DBS) technology in drug testing offers various advantages such as simplified blood collection, reduced shipping, storage costs, reduced analysis time and labor (4–7). After sample collection, each sample is anonymized using a barcode, which is later connected to the analysis results in a database. The advantages of DBS technology are not restricted to blood samples but can also be applied to other matrices such as dried urine spots (DUS).

The DBS technology was introduced in 1963 by Guthrie and Susi for the detection of phenylketonuria in newborns (8, 9). The technology evolved to be the method of choice in newborn screening laboratories around the world (10). One major drawback was the

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sensitivity, since very small sample volumes are applied on the DBS cards. However, with modern mass spectrometry instruments this issue is no longer a hurdle. The DBS technology emerged to further applications and markets such as therapeutic drug monitoring (11–13), pharmacotoxicokinetic studies (14, 15) and forensics. Although the DBS technology has been applied to individual drug tests, no fully automated system that includes a screening of a wide range of drugs is available (16–20). When it comes to high throughput of tests or to drug testing where no professional laboratory environment or expert knowledge is available, automation is crucial.

In this study, two fully automated methods for DBS and DUS were established, including card recognition, sample preparation and extraction, and online analysis by ultrahigh-performance liquid chromatography-tandem mass spectrometry (DBS–LC–MS-MS). First, a fast 5-min screening method for the 28 most abundant illicit drugs at the Regional Poison Control Center Dammam was developed and second, the DBS samples were analyzed using a Forensic Toxicology Database method (21). Automation for this study was implemented by the CAMAG DBS-MS 500 equipment and a triple quadrupole coupled to a UHPLC system. The method has been applied to real cases from the laboratory to illustrate the process and to show the potential of this approach. The focus of this study was to develop an automated method that gives a confident positive/negative result at the cut-off concentration of the individual drug.

Experimental

Apparatus

Gradient-grade water and methanol for liquid chromatography (rinsing solvents 2-propanol and acetonitrile) and formic acid were purchased from Carl Roth (Carl Roth, Germany). Ammonium formate (LC–MS Grade) was purchased from Sigma Aldrich (Sigma Aldrich, USA).

All analytical standards were purchased from Lipomed (Lipomed, Switzerland): Codeine, 6-acetylmorphine, 3,4-methylenedioxymethamphetamine, amphetamine, Methamphetamine, *N*-methyldiethanolamine, 4,4′-methylene dianiline, heroin, cocaine, morphine, 7-aminoclonazepam, chlorphenamine, clozapine, bromazepam, midazolam, clonazepam, clobazam, oxazepam, alprazolam, chlorpromazine, nordiazepam, diazepam, 11-nor-delta9-tetrahydrocannabinol carboxylic acid, 4′-methyl-AM-2201, 5″-fluoro-JWH-122, THCA-A, delta-9-tetrahydrocannabinol and Cannabidiol. Also the deuterated internal standards alprazolam-D5 and cocaine-D3 were purchased from Lipomed (Lipomed, Switzerland).

DBS cards (Ahlstrom TFN filter paper) were provided by CAMAG (Muttenz, Switzerland). Fresh whole blood was obtained from the local blood donation center (Basel, Switzerland) and from the regional poison control center (Dammam, Saudi Arabia). EDTA tubes and blood drawing equipment were supplied by the University Hospital of Basel (Basel, Switzerland).

Table I. Mass-to-charge ratio transitions of all compounds

Name	Dwell (ms)	Pre.	Quant.	Qual. 1	Qual. 2	Qual. 3	Qual. 4	CE [V]
Codeine	20	300.15	152.10	215.15	165.15	128.10	153.15	−15
6-Acetylmorphine	5	328.15	165.10	211.15	152.10	193.10	181.15	−16
3,4-Methylenedioxymethamphetamine	5	194.10	105.10	163.10	135.05	77.05	133.05	−23
Amphetamine	5	136.10	91.10	119.15	65.10	39.10	51.10	−16
Methamphetamine	5	150.15	91.10	119.15	65.10	39.10	51.10	−26
3,4-Methylenedioxyethylamphetamine	5	208.15	163.10	105.10	135.05	133.05	77.05	−14
3,4-Methylenedioxyamphetamine	5	180.00	77.10	105.10	135.00	133.10		−21
Heroin	20	370.15	165.05	268.10	328.10	211.05	152.10	−19
Cocaine-D3	5	307.15	85.05					−20
Cocaine	2.5	304.15	182.15	82.05	77.05	105.05	150.15	−20
Morphine	20	286.15	152.10	201.10	165.10	128.05	153.10	−14
7-Aminoclonazepam	5	286.05	121.10	222.10	250.05	195.10	77.00	−20
Chlorphenamine	5	275.15	230.10	167.10	166.15	139.10	201.10	−20
Clozapine	5	327.15	270.10	192.10	227.05	164.10	296.10	−20
Bromazepam	5	316.00	182.15	209.10	181.10	208.15	288.00	−21
Midazolam	5	326.10	291.10	223.10	244.00	249.05	209.05	−22
Clonazepam	5	316.05	270.05	214.05	241.15	151.10	207.10	−22
Clobazam	5	301.05	259.05	224.10	223.10	153.05	105.10	−21
Oxazepam	5	287.05	241.00	104.05	77.00	231.00	269.00	−19
Alprazolam-D5 (internal standard)	5	314.10	286.00					−30
Alprazolam	2.5	309.10	281.05	205.10	274.10	151.10	241.05	−21
Chlorpromazine	5	319.10	86.10	58.05	246.00	214.05	239.05	−22
Nordiazepam	5	271.05	140.05	208.10	165.05	243.05	104.10	−18
Diazepam	5	285.10	154.00	193.05	222.10	257.00	228.00	−19
11-nor-delta9-tetrahydrocannabinol carboxylic acid	20	345.10	327.05	299.20	118.90	193.00	229.15	−19
4′-Methyl-AM-2201, 5″-fluoro-JWH-122 (MAM-2201)	2.5	374.10	168.95	141.10	115.00	232.05	143.85	−30
THCA-A	20	345.20	299.20	193.15	119.20	187.15	327.20	−17
Delta-9- tetrahydrocannabinol	20	315.25	193.15	259.15	123.15	135.15	235.20	−11
Cannabidiol	20	314.95	192.90	93.05	135.10	123.00	259.15	−26

The general settings of the mass spectrometer were nebulizing gas 2 L/min (N_2), heating gas 10 L/min (N_2), drying gas 10 L/min, positive mode only and source temperature 300°C. Labsolutions software (Shimadzu Kyoto, Japan) was used to operate the LC–MS-MS system. Dwell = duration of collecting each *m*/*z* ion signal; Pre. = precursor ion; Quant. = daughter ion for quantification; Qual. = qualifier ion; Qual. 2,3 = qualifier ion 2 and 3; CE = collision energy of quantifier ion. 6-MAM, 6-acetylmorphine; MDMA, 3,4-methylene-dioxymethamphetamine; MDEA, *N*-methyldiethanolamine; MDA, 4,4′-methylene dianiline; 11-nor-delta9-THC COOH, 11-nor-delta9-tetrahydrocannabinol carboxylic acid.

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Methods

The DBS-MS 500 system (CAMAG, Switzerland) was attached as front end to a modular HPLC system from Shimadzu (Kyoto, Japan); it contains a system controller (CBM-20A), a Nexera X2 pump and a degasser (DGU-20ASR). Analysis was performed in positive multiple-reaction monitoring (MRM) mode on an electrospray ionization tandem mass spectrometry system 8040 and 8060 (Shimadzu, Kyoto). Data interpretation was performed by Labsolution, all samples are referred to quality control (QC) samples.

LC–MS-MS instrumentation and settings

Chromatography was performed on Shim-pack GIST (2.1 × 50 mm, 2 μm C18) (GL Science, Japan) and Kinetex (2.1 × 100 mm, 2.6μm, XB-C18) (Phenomenex, USA) analytical columns. An inline filter (KrudKatcher Ultra, Phenomenex, USA) was connected upstream to the analytical column. Mobile phase A consisted of water with 10 mmol/L ammonium formate + 0.1% formic acid and mobile phase B methanol with 10 mmol/L ammonium formate + 0.1% formic acid. For the 5-min method on the Shim-pack GIST column, the following stepwise gradient was applied: 45%B (0.0–2.0 min), 45–90%B (2.0–2.5 min), 90%B (2.5–3.5 min), 45%B (3.51–5.0 min). The flow rate was set to 0.5 mL/min at 50°C. The screening method on the Kinetex column was set according to the Shimadzu protocol (21). The HPLC liquid stream was connected via the DBS-MS 500 autosampler to the LC–MS system. At least five MRM transitions were recorded for each analytical compound. The most abundant mass transition was used for quantitation. The signals of all acquired mass transitions were used for compound identification. The detailed MS settings are listed in Table I.

DBS-MS 500 instrumentation and settings

The extraction solvent on the DBS-MS 500 (CAMAG, Switzerland) was a mixture of methanol and water (70:30 *v*/*v*) and was connected to the extraction port (Figure 1). The wash solution, consisting out of methanol, acetonitrile, 2-propanol and water with 0.1% formic acid (25:25:25:25, *v*/*v*/*v*/*v*), was connected to the rinsing bottle. The internal standards D5-alprazolam or D3-cocaine were connected to an internal standard port 1. The calibration mix 1 (bases) and calibration mix 2 (acids) were connected to ports 2 and 3 (explained in the section Results, QC cards and internal standard) and internal standard port 4 and the wash port were filled with methanol. The system was prepared by priming methanol through the internal standard port 4 (10 cycles) followed by two cycles through port 1. The extraction head was cleaned in an ultrasound bath at 40°C for 10 min prior a large set of analyses. The extraction solvent was primed for five cycles and the rinsing solvents were flushed for 1 min (this process is an automated system prime method). The DBS cards were photographed with the built-in camera of the DBS-MS 500 before and after each run to check for the presence of a blood spot and to adjust the extraction head to the center of each spot. The Chronos for CAMAG software automatically recognized inadequate DBSs based on their roundness, diameter and area. Inadequate DBSs were excluded from the analysis. A total of 20 μL of internal standard were sprayed in a homogenous layer onto each spot. After a 20-s drying time, the samples were extracted with a volume of 22 μL and 50 μL/min flow rate. To complete the automated DBS extraction cycle, the extraction outlet was rinsed for 20 s with the wash solution and for 20 s with the extraction solution followed by 5 s of inlet rinsing with the extraction solution (15).

Sample preparation

Working standards for LC–MS-MS tuning and QC

To balance inter-day variations of this screening method, each day high- and low-QC samples are measured before starting the sample analysis. The sample amount of those QC's is known and the later results are referred to these values. The QC cards were prepared in the according laboratory by using the DBS-MS 500 spraying module. Here, two calibration mixes were prepared in methanol, first all alkali target compounds are mixed to prepare a 100 ng/mL standard solution (drug panel except for THCA-A and 11-nor-delta9-THC COOH) and second the same is done for the two acidic target compounds. Both solutions are connected to the DBS-MS 500 internal standard ports (2 and 3). DBS cards with negative blood were prepared by pipetting 20 μL of whole blood into the dashed rings and allowed to dry for 2 h. The dried cards were inserted the DBS-MS 500 instrument and the cards were coated with the two 100 ng/mL standard solutions by using the spray option only. Low controls are coated with each standard mix with 10 μL and high control with 40 μL (both in fast mode), the acidic and alkali standard solutions were sprayed on the top of each other. Separate cards for low and high controls were used. The DBS-MS 500 sprays a quadratic area of 1 × 1 cm. If 10 μL of a 100 ng/mL solutions are sprayed on the blank DBS, this equals a concentration of 0.126 ng under the plunger area which is 4 mm in diameter. The stock solutions are stable for 1 month at −20°C. All target analytes from the panel of the

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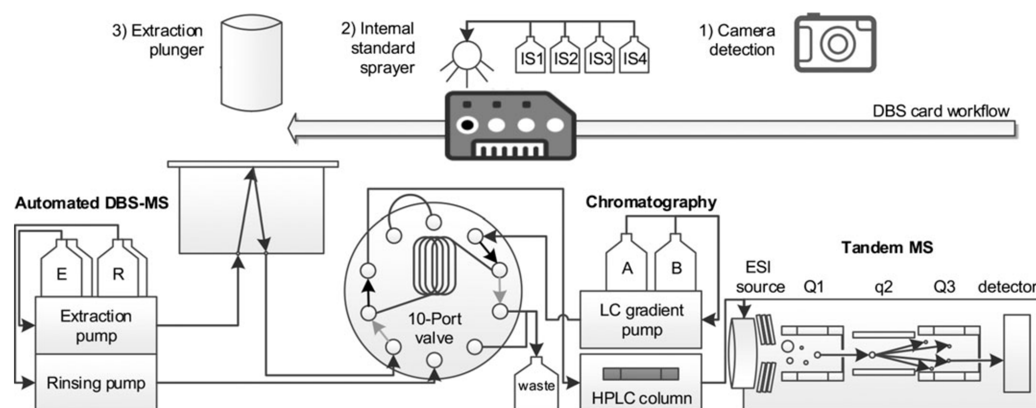

Figure 1. Fully automated workflow of the DBS-MS 500.

Table II. Analysis workflow

	Step	Comment
1	Solvent	Blank DBS card extraction without internal standard (ISTD) spray
2	Negative matrix	Negative blood sample with ISTD spray, signals must be below LOD
3	2 × Low control	QC low sample with ISTD
4	2 × High control	QC high sample with ISTD
5	2 × Negative matrix	Negative must be clean, signals must be below LOD
6	Patient 1	Negative: Only ISTD signal is detected Positive: Drug signal above cut-off detected, measure sample again with alternative technology
7	Negative matrix	Negative must be clean, signals must be below LOD
8	Patient 2	Repeat for further patients
9	Negative matrix	
n	Low control	Low control after 15 patients and at the end of each analysis batch
$n + 1$	Negative matrix	

5-min method, except for heroin, are stable on the dried card for 2 days at room temperature.

Internal standard integration

To get an in-process control, at least one deuterated standard must be integrated into the analysis workflow. Depending on which laboratory either D5-alprazolam or D3-cocaine was used, those analytes were chosen since they elute in the middle of the chromatographic separation and they show good ionization properties. The internal standard is dissolved in methanol to generate a 0.1 µg/mL solution, which is connected to the spray port 1 of the DBS-MS 500. If the method will be used for absolute quantitative analysis, each target compound must be compared to the according deuterated analog.

DBS samples

According to documented cut-off levels in whole blood (22) and according to the reference values of the Regional Poison Control Center Dammam, the target concentration range for the fast screening method was chosen between 5 and 1,000 ng/mL. Six calibration points were chosen to simplify the sample preparation, since it was intended to acquire a five-point calibration from 5 to 500 ng/mL for certain target compounds and also a five-point calibration from 25 to 1,000 ng/mL for the remaining analytes. Freshly collected human blood was obtained from the local blood donation center. EDTA was used as an anticoagulation agent (vacutainer tubes, BD, Allschwil, Switzerland). Volunteer blood of a healthy male donor was chosen as a zero control. A stock solution of the standards was prepared in methanol and gently mixed with the donor blood in six different concentrations to prepare the levels. The maximum organic spike was 2.5% to the donor blood. A total of 20 µL aliquots were spotted onto CAMAG DBS cards (CAMAG, Muttenz, Switzerland) and dried at room temperature for at least 2 h. The cards were subsequently stored at 4°C in sealed plastic bags containing desiccants. Stock solutions were stable for 2 weeks.

Analysis workflow

To avoid false positive and false negative, the following analysis workflow was defined (Table II):

Blank DBS cards, cards with negative blood and the QC cards are filled in separate racks. Negative blood is measured between patients to get physical prove that there is no carryover. If the samples are drawn from different sites, an optional process step can be integrated to exclude contamination of the whole filter paper card. Here, spot 1 is left empty while sampling and negative blood is applied in the laboratory prior the analysis.

Results

DBS and DUS cards were successfully automated for the intended analysis workflow. The cards are checked with image recognition software, sprayed with internal standard and extracted in a fully automated process. Up to 500 such cards with four samples each can be handled without any human interaction. The total time-to-result is 5 min per sample using the method with 28 target analytes and 20 min for the database method screening for over 1,200 target compounds. Correlation was measured from below the according cut-off concentration to the toxic blood level of each drug (Table III). Each compound is detected by five MRM transitions to enhance total signal intensity of a compound and to acquire enough data to confirm the target drugs. To further improve the signal of certain drugs and to lower their limit of quantification (LOQ), the dwell time of the mass spectrometer was adjusted for certain analytes to either increase or decrease their signal intensity (Table I). To speed up the overall cycle time of the MRM data acquisition, specific retention time windows were defined for each compound (Supplementary Data).

Correlation and precision

The calibration levels were measured 6-fold on two different days to determine the method robustness and validity. The relative standard deviation of the internal standard was below 15% for all target compounds by comparing the data through all levels (5–1,000 ng/mL). The correlation and inter-day variations of all target compounds are listed in Table III. The target-to-internal standard ratio was used to compare the results and to develop the calibration line. Excellent correlation was obtained for all target compounds. All points of the calibration functions were sufficiently precise with relative standard deviations below 15%, respectively, below 20% at LOQ with the exception of THCA-A, 6-MAM and diazepam which have to be further investigated and validated. Table III also includes the cut-off and toxicological levels from literature for blood samples (7, 22). The same cut-off concentrations were used for the urine samples in this phase, however this will be adjusted with growing data from routine analysis. The goal of the analysis method was to detect the cut-off concentration with confidence; everything above this concentration will be reported and retested with alternative technologies.

The calibration line is shown for amphetamine (25 ng/mL) in Figure 2, since this compound was found in one of the toxicological routine samples from the laboratory after method implementation (section Method Transfer to Routine Laboratory, Real samples from a routine environment). Also illustrated in Figure 2 is the butterfly spectrum from the MRM spectrum mode and the according molecule structure generated by the Shimadzu Forensic Toxicology Database. There was a 100% hit for the amphetamine spike and the MS fragment spectra are overlapping nicely. The spike in the measurement below was alparzolam-D5.

Carryover

Carryover was monitored by measuring blank blood right after extracting a mix of all standards at 1,000 ng/mL. The criteria of

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Table III. Results from six-point calibration on two separate days

	Relative inter-day standard deviation				Correlation R^2			
	5 ng/mL (%)	25 ng/mL (%)	250 ng/mL (%)	500 ng/mL (%)	5–500 (ng/mL)	25–1,000 (ng/mL)	Cut-off (22) (ng/mL)	Tox. Level (ng/mL)
Codeine	10.4	10.5	9.7	11.6	0.992	0.997	50	1,100
6-MAM	22.3	9	13.4	15.6	0.992	n/a	10	n/a
MDMA	14.3	8.5	6.8	6.3	0.995	0.999	100	1,000
Amphetamine	12.6	9.6	4.9	7.1	0.996	0.999	100	1,000
Methamphetamine	13.2	11.3	8	9	0.995	0.998	100	n/a
MDEA	11.8	8.7	6.9	6.6	0.994	0.997	100	n/a
MDA	9.4	5.1	5.3	5.8	0.995	0.998	100	n/a
Heroin (n/a, not stable on card)	*4.8*	*7.4*	*4.6*	*5.6*	*0.995*	*0.997*	*50*	*200*
Cocaine	14.8	13.3	14.7	7.7	0.999	n/a	20	1,000
Morphine	18.6	9	8.2	8.6	0.993	0.998	50	200
7-Aminoclonazepam	12.6	7.7	7.4	6.6	0.995	n/a	25	n/a
Chlorphenamine	11.6	3.4	1.9	7.1	0.999	n/a	25	n/a
Clozapine	11.2	5.1	6.5	3.9	0.994	n/a	25	n/a
Bromazepam	15.2	7.6	6.5	5.7	0.998	n/a	25	n/a
Midazolam	10	4.5	3.1	4	0.995	n/a	25	n/a
Clonazepam	14.7	6.9	5.7	8.2	0.995	n/a	25	80
Clobazam	13.3	6.6	5.8	8.1	0.997	n/a	25	n/a
Oxazepam	13.8	13.3	11.1	12.6	0.999	n/a	25	1,400
Alprazolam	11.9	3.9	4.5	7.1	0.991	n/a	25	350
Chlorpromazine	16.6	13.9	13.1	5.6	0.999	n/a	25	n/a
Nordiazepam	13	11.1	6.9	7	0.999	n/a	25	n/a
Diazepam	*19.4*	*29.9*	*24.8*	*31.8*	*0.995*	n/a	25	3,000
11-nor-delta9-THC-COOH	15.4	11.8	11.9	7.4	0.998	n/a	10	n/a
MAM-2201	16.8	11.1	10	5.6	0.996	n/a	10	n/a
THCA-A	13.6	11	15.4	8.7	0.998	n/a	10	200
Delta-9- tetrahydrocannabinol	13.2	12.3	6.8	8.7	0.995	n/a	10	n/a
Cannabidiol	10.5	8.4	12.1	9	0.996	n/a	10	n/a

bioanalytical method validation guidelines were fulfilled (18, 19). No carryover was observed for the chosen analyte panel. Figure 3 shows the amphetamine peak for 1,000 ng/mL and the subsequent blank sample. This was optimized by programming a wash sequence within the DBS-MS 500 workflow. Here, the outlet capillary from the DBS-MS 500 system was rinsed with methanol, acetonitrile, 2-propanol and water with 0.1% formic acid (25:25:25:25, *v*/*v*/*v*/*v*) for 20 s at 45 bar after which the extraction chamber inlet was flushed with extraction solvent for 10 s.

QC cards and internal standard

Prior the analysis, high and low control samples were measured. The low control reflects a concentration close to the cut-off of the THC derivates and the high control gives a value significantly higher to allow controlling of the analysis performance. The control cards were prepared by the laboratory using the DBS-MS 500 spraying module.

A total of 10 µL of the deuterated alprazolam or cocaine standard are sprayed as 0.1 µg/mL solution in methanol directly on the DBS prior extraction. The internal standard solution can be used for up to 2 weeks if stored in the fridge overnight. Alprazolam-D5 was used during method development phase and cocaine-d3 for the method implementation due to standard availability.

Method Transfer to Routine Laboratory

DBS method transfer

The method described in the section Results was developed in a research laboratory using a Shimadzu 8060 LCMS system. In the next step, this method was transferred to a routine laboratory equipped with an 8040 LCMS. After transferring the method and adjusting the MRM voltages of the target compounds by the Labsolution optimization tool, a five-point calibration each with three repetitions was measured in the range of 10–500 ng/mL target drug in blood, since this was the region of interest for the routine laboratory. Presprayed QC samples were measured before and after the calibration samples according to the described workflow. Excellent correlation >0.995 and standard deviation below 15% were obtained for all compounds except for the THC derivates, where the correlation was above 0.985 and standard deviation below 25% (except for LOD, shown in Supplementary Data). The sensitivity of the THC derivates is at its limit on the 8040 LCMS system, however, when the cut-off is put at 25 ng/mL rather than at 10 ng/mL the result can be detected reliably.

Method adaption for urine samples

Since sometimes samples are also acquired as urine by the laboratory, the method was adapted to measure the same analytes also from a DUS. Negative urine was spiked with the same standard mix used for the blood samples and a three-point calibration was measured in the range of 10–400 ng/mL. Here, 10 µL of urine was spotted on a urine card. The urine cards are coated to make the colorless liquid visible (picture in supplementary data) and detectable by the DBS-MS 500 in-built camera. Excellent correlation >0.995 and standard deviation below 15% (below 25% at LOQ) were obtained for all compounds except for the THC derivates, where the standard deviation was below 25% (except for LOQ, shown in

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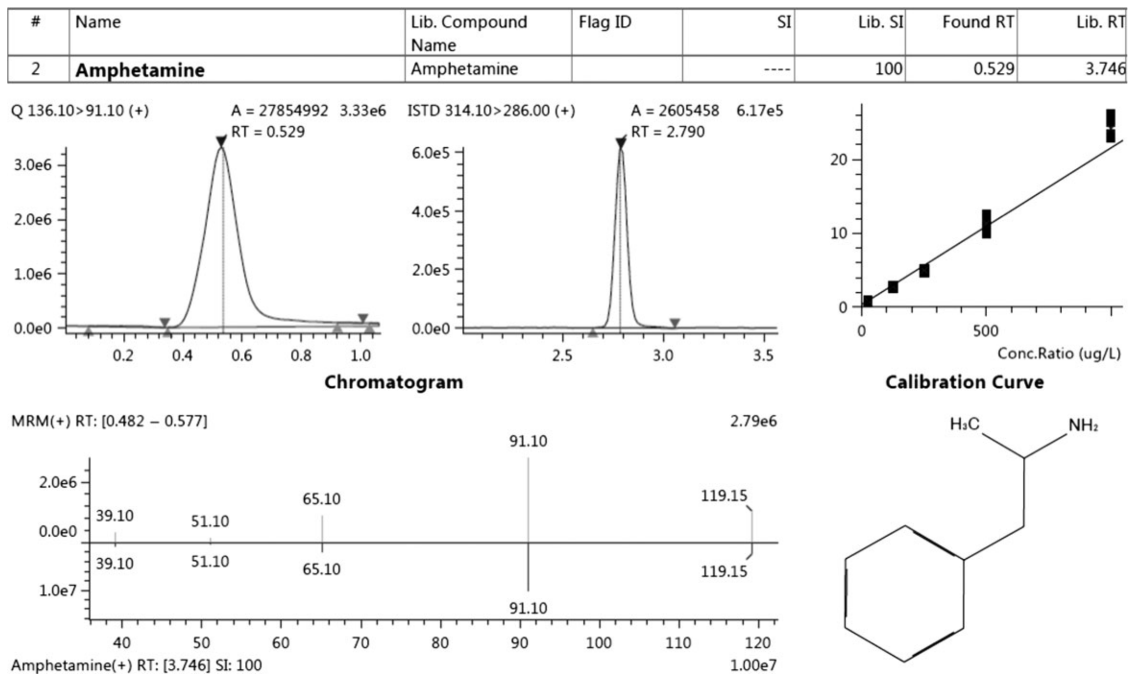

Figure 2. Database report of amphetamine sample showing the peak, the internal standard peak, the calibration curve, the MRM spectrum mode with database spectra and the structure.

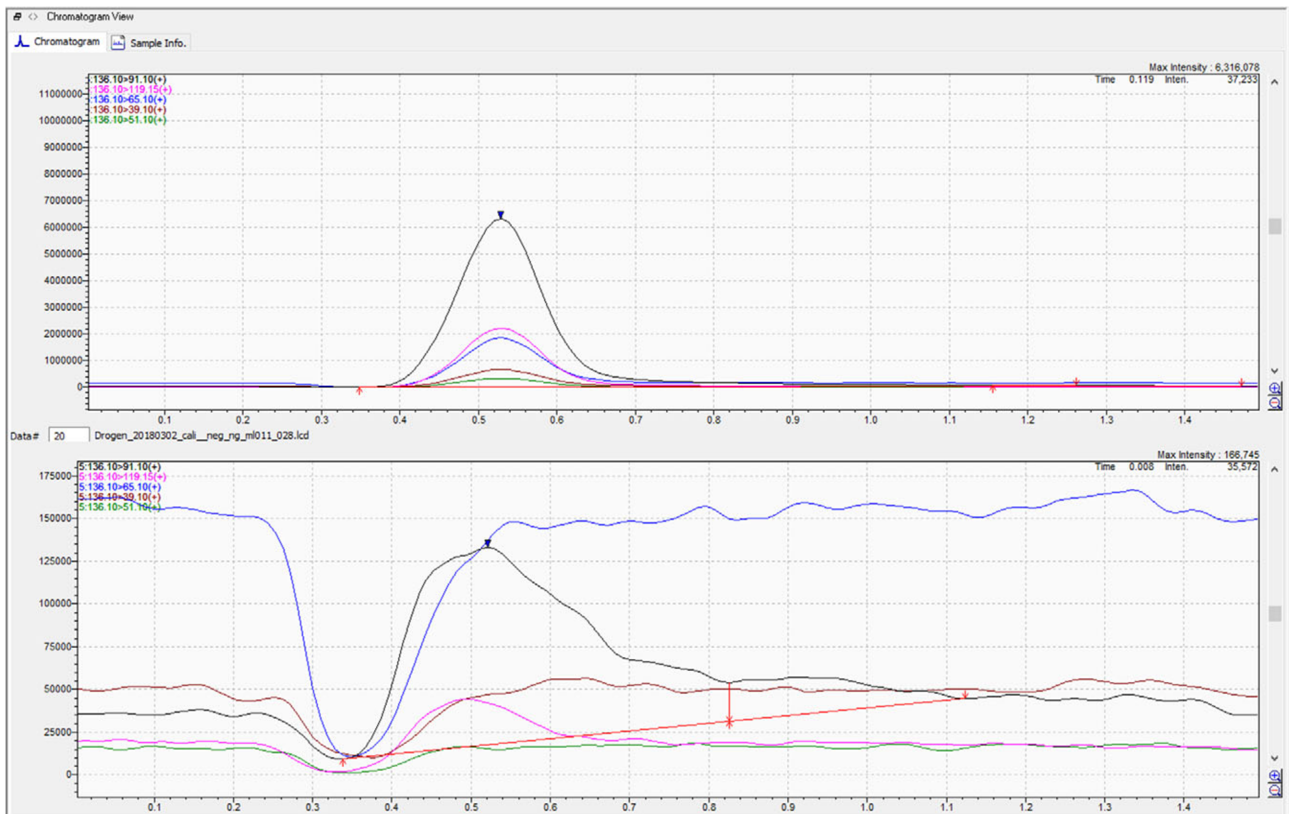

Figure 3. 1,000 ng/mL amphetamine peak (above) and subsequent blank sample (below) showing no carryover.

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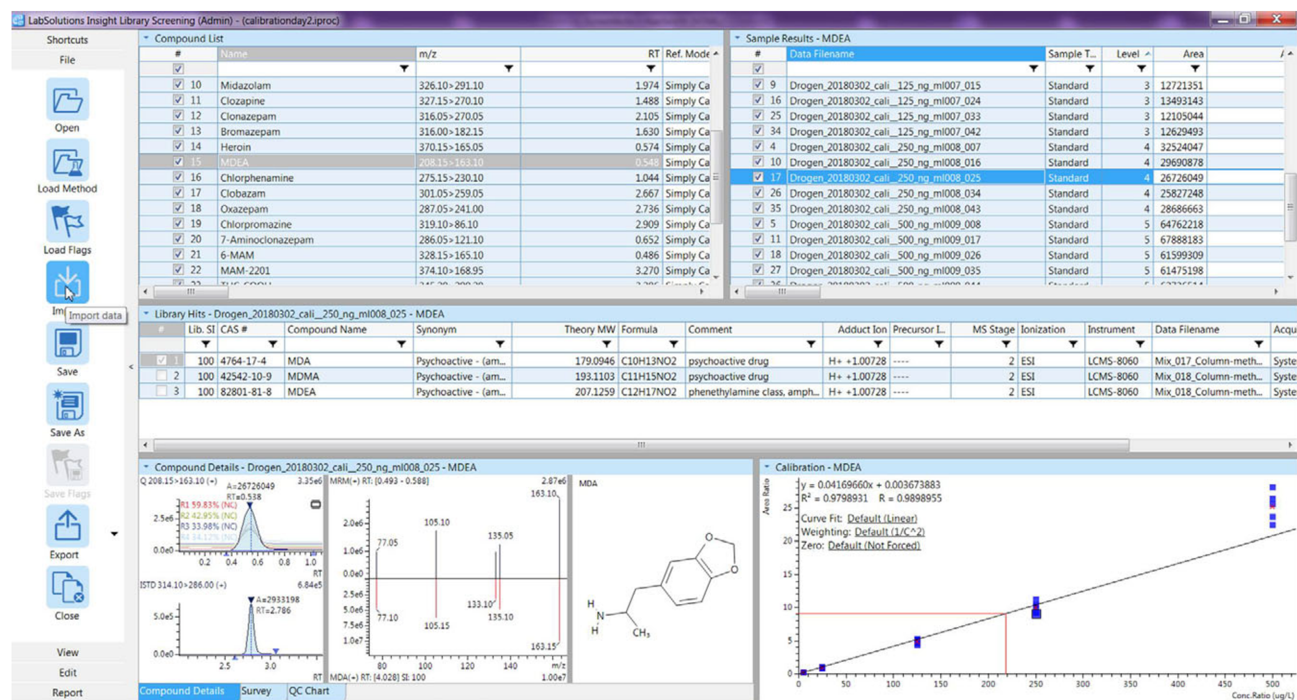

Figure 4. Insight report for MDA showing also a positive library hit for MDMA and MDEA.

Supplementary Data). Also, morphine and MDA could not be detected due to interferences.

Real samples from a routine environment

To further investigate the method, a positive sample from the Dammam laboratory was measured with the newly installed DBS–LC–MS-MS method. Blood and urine from the same suspect were analyzed. The sample was positive for amphetamine and 11-nor-delta9-tetrahydrocannabinol carboxylic acid in both, urine and blood, where the same cut-off concentration was considered for both matrices. Delta-9-tetrahydrocannabinol and cannabidiol was positive in blood and THCA-A was positive in urine. This reflects the metabolism of THC, where the native THC is found in blood and the carboxylated THC is found in urine. All data were in correlation with the results from alternative analysis by the Dammam laboratory. This is a proof of concept for the function of the new dried matrix spot method.

Discussion

The method was successfully transferred from a research into a routine environment. The sensitivity of the 8040 LCMS instrument was sufficient for the described application, however the cut-off level of the THC derivates is at its limit of sensitivity on this instrument. On the other hand was the MRM signal saturated for e.g., midazolam and clozapine at the level 1,000 ng/mL and therefore only the correlation up to 500 ng/mL is displayed in the results. Very small amount of drugs can be measured on these instruments allowing the trend toward microsampling approaches. When reducing the volume to one droplet of blood with a realistic drug concentration, only a few picograms reach the analytical system. Nonetheless, this low amount is detectable and quantification is possible. Using the automated instrumentation, up to 500 sample cards can be processed without any human interaction enabling the fast and cost-effective screening of large populations.

When using tandem mass spectrometry, not all compounds have to be baseline-separated by the chromatography, since they can be distinguished by the MRM information. Attention must be paid if certain compound shares the same MRM transitions and are not separated. Here, morphine and 7-aminoclonazepam both elute at 0.62 min and share a similar parent ion mass of 286 *m/z*. However, the fragmentation patterns are different and therefore both compounds can be identified.

To enhance the signal of certain drugs and to lower their LOQ, the dwell time was adjusted. Increasing the dwell time from 5 to 20 ms increases the signal-to-noise ratio (SNR) significantly. For example, codeine has SNR of 11.67 at 5 ms, 13.81 at 10 ms and 20.35 at 20 ms. The absolute signal area does not increase significantly, but the background noise is lowered and therefore increase the SNR, which then decreases the detection limit.

The internal standards are used to monitor and normalize the extraction efficiency, to ensure that the system is working properly and to avoid false negatives (15, 24). A total of 20 µL of the internal standard solution were sprayed on the DBS spot prior to extraction. This process of integrating the internal standard by spraying is the method of choice for quantification. Abu-Rabie *et al.* stated that the hematocrit level can have an influence on the extraction efficiency, so that the internal standard should better be applied prior to the extraction (24). It is not possible to spike the internal standard to the blood sample prior spotting, since the spots are usually drawn at remote areas.

Low- and high-QC cards were introduced for the screening workflow. For the initial phase, the QC cards were prepared directly in the laboratory using the DBS-MS 500 spraying module. This process step is evaluated to outsource into a certified reference

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laboratory to better control the workflow at the routine site. The goal would be that the low control reflects the individual cut-off concentration of each target drug and the high control should be around the toxic concentration. This would allow a direct comparison of the routine samples to the low control to generate a either positive or negative screening result with more confidence and to cover the complete analytical relevant range. Also the analyte on card stability needs to be further investigated. The DBS-MS 500 reference cards are stored at deep freeze and long-term stability studies are running. Deep freeze and room temperature stability studies are running investigation at the Dammam Poison control center, since the DBS-MS 500 is not cooled and the cards remain at room temperature during sample queue. The cut-off and toxicological ranges were defined according to literature (22) and internal reports of the Dammam Poison control center.

The extraction setup of the DBS-MS 500 features a horizontal extraction, where a small volume of solvent is flushed under increased pressure from the bottom through the sealed area on the DBS back to the bottom of the card. No other HPLC-grade solvents or consumables are required for the extraction process, which makes this application more cost-effective and therefore well suited for high-throughput applications such as the described method. Since the DBS card, which is the main consumable, is a natural product from cellulose, the analysis workflow is eco-friendly compared to alternative sample preparation processes.

The analysis method was transferred successfully to urine samples, except for MDA and morphine. Both compounds are rapidly metabolized to sulfates and glucuronide before urine excretion (25). Therefore, the urine method should be adapted for their according metabolites. Those analytes have to be further investigated.

Using the Shimadzu Forensic Toxicology Database method allows to detect next to the list of integrated drugs also new drugs, since the search tool compares homologous fragment ions and does use the mass of the precursor ion as a prefilter of the candidates during the database search. With this new option, for example, new synthetic cannabinoids can be detected by the identical fragmentation pattern of the molecule ground structure independent of their intact precursor mass. On the other hand, this can also lead to more than just one database hits for certain structurally related drugs. For example, an MDA sample gives also a 100% database hit for MDMA and MDEA (Figure 4), since they share the same fragmentation pattern. They need to be distinguished by the precursor mass in this case.

The whole analysis workflow was fully automated. Each process is monitored and documented without any human interaction required. Each sample preparation step is standardized through the automation. Using the 28 target drug methods, each sample takes 5 min. This workflow was extended by using the Shimadzu Forensic Toxicology Database to open up the analysis possibility to identify and quantify more than 1,200 drugs within 20 min of sample analysis time.

Conclusion

A new workflow for high-throughput drug screening was developed and transferred into a routine environment. The method is already used on a daily basis to monitor workplace safety and for suspects from roadside controls. Blood and urine samples are analyzed via the DBS–LC–MS-MS platform and depending on the context, either the drug panel or the database method is acquired. The process from sampling to generating the report was linked with a barcode system. QC cards can be prepared by the laboratory using the spraying module of the DBS-MS 500. Each process step is well documented and all analysis steps follow Good Laboratory Practice (23). The method can be easily modified or extended and transferred to other routine laboratories.

Supplementary Data

Supplementary material is available at *Journal of Analytical Toxicology* online.

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Fully Automated Determination of Phosphatidylethanol 16:0/18:1 and 16:0/18:2 in Dried Blood Spots

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Abstract

Purpose: Direct alcohol markers are widely applied during abstinence monitoring, driving aptitude assessments and workplace drug testing. The most promising direct alcohol marker was found to be phosphatidylethanol (PEth). Compared to other markers it shows a long window of detection due to accumulation in blood. To facilitate and accelerate the determination of PEth in DBS, we developed a fully automated analysis approach.

Methods: The validated and novel online-SPE-LC-MS/MS method with automated sample preparation using a CAMAG DBS-MS 500 system reduces manual sample preparation to an absolute minimum, only requiring calibration and quality control DBS.

Results: During the validation process, the method showed a high extraction efficiency (>88%), linearity (correlation coefficient >0.9953), accuracy and precision (within ±15%) for the determination of PEth 16:0/18:1 and PEth 16:0/18:2. Within a run time of about 7 min, the two monitored analogs could be baseline separated. A method comparison in liquid whole blood of 28 authentic samples from alcohol use disorder patients showed a mean deviation of less than 2% and a correlation coefficient of >0.9759. The comparison with manual DBS extraction showed a mean deviation of less than 8% and a correlation coefficient of >0.9666.

Conclusions: The automated analysis of PEth in DBS can provide a fast and accurate solution for abstinence monitoring. In contrast to the manual extraction of PEth in DBS, no laborious sample preparation is required with this automated approach. Furthermore, the application of the internal standard by a spray module can compensate for extraction bias and matrix effects.

Introduction

A simple method to monitor alcohol abstinence is the determination of ethyl glucuronide (EtG) and ethyl sulfate (EtS) in urine. Due to the short window of detection, these two markers require frequent sampling. This results in a large number of liquid urine samples which need to be transported and stored before analysis (1). The discovery of phosphatidylethanol (PEth) as a direct alcohol marker extended the window of detection for alcohol consumption to several weeks. Due to the fast formation rate and slow degradation, it accumulates in cell membranes at about 1–2% of the total cellular phospholipid pool (2, 3). PEth allows one to distinguish heavy from occasional drinkers and to monitor alcohol abstinence (4–6).

PEth is present as a group of abnormal phospholipids with a polar phosphoethanol head. Fatty acid chains are attached at positions sn-1 and sn-2. Up to 48 different PEth analogs have been detected with various combinations of fatty acyl chain lengths and numbers of double bonds. The predominant species in blood after alcohol consumption are PEth 16:0/18:1 (30–46%) and PEth 16:0/18:2 (16–28%) (7–12). PEth in whole blood proved to be unsuitable for routine analysis, as it is unstable during storage and transportation above −80°C. Furthermore, PEth may be generated *in vitro*, post-sampling, if ethanol is present. This can lead to false positive results (10, 13).

By using dried blood spot (DBS) sampling, PEth can be stabilized due to the inactivation of enzymatic activity. Sampling as DBS

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prevents post-sampling formation of PEth, allows shipping without biohazard labeling in a standard envelope and does not require any sample cooling. Several DBS methods for monitoring PEth have been developed, showing the feasibility of PEth determination in the dried format. The protocols involve manual punching of DBS cards and long sample preparation steps, including incubation, centrifugation and sonification (14–16).

The aim of this study was to develop a method for the fully automated sample preparation, extraction and online LC-MS/MS quantification for the two most abundant PEth species: PEth 16:0/18:1 and PEth 16:0/18:2. The main focus was thereby put on a system offering high-throughput and minimal turnaround time. Automation for this study was implemented by the CAMAG DBS-MS 500 extraction system and a triple quadrupole mass spectrometer coupled to an HPLC system.

Experimental

Chemicals and reagents

The internal standards for the PEth determination (16:0/18:1-D_5 and PEth 16:0/18:2-D_5) were synthesized as described elsewhere (17). The internal standard solution was prepared by mixing both analogs with 2-propanol.

Ammonium acetate, fractopur and methanol, with a purity of Reag. Ph Eur (European Pharmacopoeia), were obtained from Merck (Darmstadt, Germany). 2-Propanol, HPLC grade, was purchased from Fisher Chemical (Reinach, Switzerland). Acetonitrile, p. a., was ordered from Acros Organics (Geel, Belgium). Certified spiking solution for phosphatidylethanol 16:0/18:1 and 16:0/18:2 were purchased from Cerilliant (Round Rock, USA) and Avanti Polar Lipids, Inc. (Alabaster, USA). Formic acid, puriss p.a. 98%, was purchased from Sigma-Aldrich (Buchs, Switzerland). De-ionized water was produced with a Milli-Q water system from Millipore (Billerica, USA). BioSample TFN filter paper DBS cards from Ahlstrom were used to produce the volumetric DBS (supplied by CAMAG, Switzerland).

Calibrator and quality control samples

For the quantification of PEth, a six-point calibration was prepared at 20, 75, 188, 375, 750 and 1,500 ng/mL (K1–K6). For the verification of the calibration curve, three quality control samples were prepared at 20, 45 and 1,180 ng/mL (QC1–QC3). To prepare the samples, 240 µL of blank blood from a nondrinker (containing no PEth) was spiked with 10 µL of stock solution containing PEth 16:0/18:1 and PEth 16:0/18:2 in 2-propanol. Subsequently, the spiked samples were mixed for 4 h. Afterwards, DBS spots containing 20 µL of blood were prepared on DBS cards. Before the extraction, the cards were dried for three hours. Afterwards, they were stored in a mingrip bag together with a silica gel pack and stored at −20°C.

Batch design and QC strategy

To provide a reliable measurement and to monitor the robustness of the automated system, each batch of analysis contained at least one set of six calibrator samples (K1–K6), a quality control sample at each concentration (QC1–QC3), negative controls (with and without internal standard added) and a blank (card without any blood added).

Fully automated sample extraction

The DBS-MS 500 system (CAMAG, Switzerland) was attached as front end to the online-SPE-LC-MS/MS system. The extraction solvent for the DBS elution consisted of water, acetonitrile, 2-propanol and formic acid (34.5:15:50:0.05, v/v/v/v). Each DBS was photographed with the built-in camera prior and after each run to check for the presence of a spot, to adjust the extraction head and to verify that an extraction took place. Internal standard solution (20 µL) containing the deuterated PEth analogs was applied by a spray head in a homogenous layer onto each spot. In contrast to spiking the internal standard into the sample tube, this procedure enables to compensate for extraction differences (recovery bias) (18). Afterwards, the card was dried for 30 s, before the extraction of a 4.2 mm sub-punch with a volume of 40 µL at a flow rate of 40 µL/min took place. After the extraction process, the extraction head and outlet were rinsed with the inbuilt wash station three times, each step taking 20 s. Rinsing solution 1 (85% 2-Propanol, 15% water, v/v, containing 13 mM ammonium acetate), rinsing solution 2 (50% water, 50% acetonitrile, v/v) and rinsing solution 3 (2-propanol).

Manual DBS extraction

Spots were generated by adding 20 µL of blood on DBS cards. To compensate for a potential extraction bias, the internal standard was sprayed with the CAMAG DBS-MS 500 autosampler. Manual extraction of the complete DBS spot (20 µL) was performed by using 1 mL of methanol and subsequently shaking the sample for 4 h on a Vortex-Genie 2 (Scientific Industries Inc., New York, USA). Afterwards, centrifugation for 10 min at 16,000 g took place (Mikro 220 R, Hettich, Switzerland). The supernatant was transferred into champagne-cup glass vials, evaporated to dryness in a heating block set at 50°C, with a gentle stream of nitrogen, and reconstituted in 100 µL of elution solvent (water, acetonitrile, 2-propanol and formic acid (34.5:15:50:0.05, v/v/v/v)).

Manual whole blood extraction

To determine PEth in whole blood, 250 µL of blood was mixed with 100 µL 2-propanol and vortexed. Afterwards, 150 µL 2-propanol and 750 µL acetonitrile were added. The sample was vortexed again, and shaken for 45 min, before centrifugation for 10 min at 16,000 g was performed. The supernatant was transferred into glass vials, evaporated to dryness in a heating block set at 50°C, with a gentle stream of nitrogen, and reconstituted in 800 µL of elution solvent (water, acetonitrile, 2-propanol and formic acid (34.5:15:50:0.05, v/v/v/v)).

Online SPE-LC-MS/MS analysis

The online SPE-LC-MS/MS system consisted of an UltiMate® 3000 UHPLC system (Dionex, Thermo Scientific Instruments, Reinach, Switzerland) coupled to a Sciex 5500 QTRAP (Toronto, Canada) operated in negative SRM mode at −4,500 V, see Figure 1 and Table I. At first, the analytes were trapped on a polar-RP column, 20 mm × 2 mm, 5 µm particle size (Phenomenex, Brechbühler, Schlieren, Switzerland) which was preconditioned with A1 (water containing 0.1% formic acid v/v). Substances not interacting with the column were directed to the waste container. After 0.25 min elution with 100% B1 (70% acetonitrile, 30% water, v/v, containing 2 mM ammonium acetate) started during 2.25 min, whereby the ten-port valve at the UHPLC system was switched from the waste to the analytical column at 0.8 min. As analytical column, a Luna RP-C5 column, 50 mm × 2 mm, 5 µm particle size (Phenomenex, Brechbühler, Schlieren, Switzerland) preheated to 60°C was used.

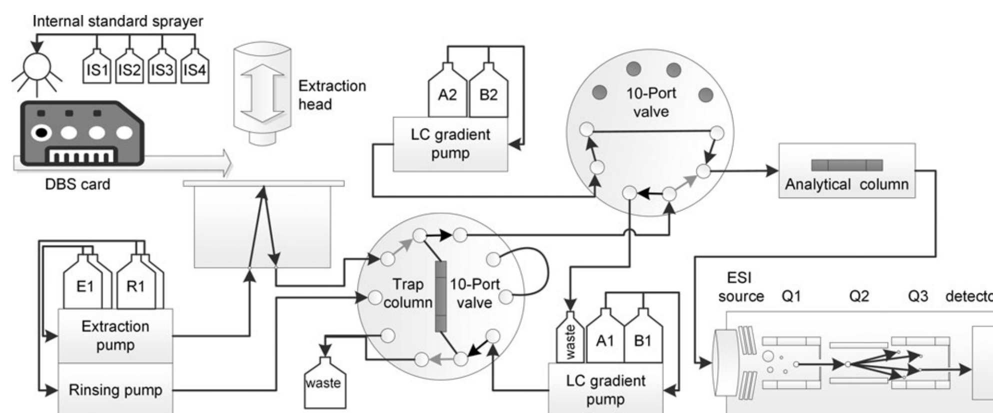

Figure 1. Instrumental setup for the fully automated analysis of DBS samples. The internal standard is sprayed onto the DBS, followed by extraction and concentration on a trapping column by the DBS-MS-500 system. Chromatographic separation is achieved by liquid chromatography, before the detection by tandem mass spectrometry is performed. Black arrows indicate the loading phase, gray arrows indicate the elution phase.

Table I. Parameters used for the selected reaction monitoring (SRM) of PEth 16:0/18:1 and PEth 16:0/18:2

Compound	Q1 [*m/z*]	Q3 [*m/z*]	Dwell time [ms]	DP [V]	EP [V]	CE [V]	CXP [V]	RT [min]
PEth 16:0/18:1 SRM 1	701.3	255.2	20	−32	−10	−40	−14	3.53
PEth 16:0/18:1 SRM 2	701.3	281.3	20	−20	−10	−40	−14	
PEth 16:0/18:1–D_5 SRM 1	706.3	255.3	20	−20	−10	−40	−14	
PEth 16:0/18:1–D_5 SRM 2	706.3	281.1	20	−32	−10	−40	−14	
PEth 16:0/18:2 SRM 1	699.5	279.4	20	−5	−10	−40	−14	3.29
PEth 16:0/18:2 SRM 2	699.5	255.3	20	−5	−10	−40	−14	
PEth 16:0/18:2–D_5 SRM 1	704.5	279.5	20	−5	−10	−40	−14	
PEth 16:0/18:2–D_5 SRM 2	704.5	255.3	20	−5	−10	−40	−14	

An inline filter (KrudKatcher Ultra, Phenomenex) was connected upstream to the analytical column. The analytical column was preconditioned with A2 (30% water, 70% acetonitrile, v/v, containing 0.6 mM ammonium acetate). The valve was switched back to the initial position at 1.7 min. Afterwards, the gradient elution with B2 (100% 2-propanol) started: The gradient for the analytical column was as follows: Start at 0% B2, followed by an increase to 25% B2 from 2.0 to 4.0 min. Afterwards, 100% B2 from 4.1 to 5.0 min. The flow rate for all pumps was set to 0.5 mL/min. The total run time was 5 min. For a chromatogram, see Figure 2.

Results

Linearity

Linearity was established during three validation series, whereby the calibrators K1–K6 were measured in duplicate. A linear calibration model with weighting $1/x^2$ was chosen. The correlation coefficient for SRM 1 and SRM 2 was 0.9980 ± 0.0016 (range: 0.9954–0.9996) for PEth 16:0/18:1. The correlation coefficient for SRM 1 and SRM 2 was 0.9974 ± 0.0005 (range: 0.9953–0.9982) for PEth 16:0/18:2. Extended calibration ranges were tested, with additional calibration samples at 1,750, 2,000, 2,250, 2,500 and 3,000 ng/mL. An extended linear range up to 2,500 ng/mL was investigated for all four monitored transitions with a correlation coefficient of at least 0.9975. The LOQ was set at 20 ng/mL, as samples below this concentration are associated with light or no alcohol consumption. Inaccuracy and imprecision at this concentration were below 15%. The LOD was observed at 10 ng/mL based on the repetitive analysis of samples at this concentration with an observed signal-to-noise ratio of at least 3.

Accuracy and precision

Accuracy and precision were investigated during the validation process by the analysis of spiked samples at the quality control concentrations. Six individual blood spots at each quality control concentration were analyzed on three different days, see Table II. Accuracy and precision remained within the required ±15% during intra-assay and inter-assay evaluation for all three measurements.

Selectivity

The chromatography has been optimized for both analogs, PEth 16:0/18:1 and PEth 16:0/18:2. Base peak separation successfully prevented cross-talk between PEth 16:0/18:2-2 × ^{13}C and PEth 16:0/18:1 (relative abundance of 8.7%). Samples from six alcohol abstinent subjects were examined for chromatographic interferences. There were no interfering peaks observed with the same retention time as PEth 16:0/18:1 or PEth 16:0/18:2 in all of the investigated samples.

Carry-over

Carry-over was tested in each run by the injection of a blank sample directly after the highest calibrator (K6, 1,500 ng/mL), and was found to be up to 0.5% (7.5 ng/mL).

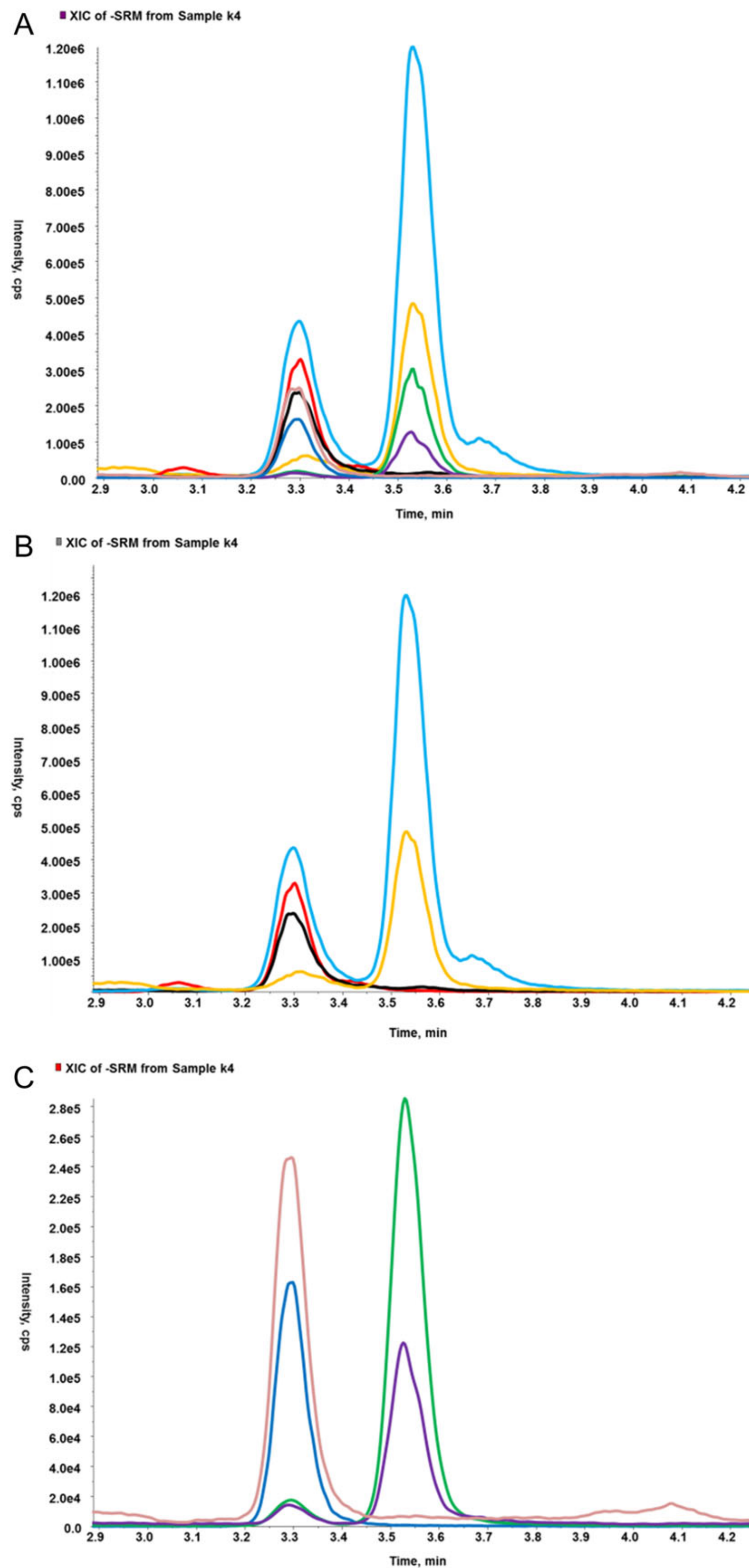

Figure 2. Chromatogram for a calibrator sample spiked at K4 (375 ng/mL). Part (A) represents all monitored SRM transitions. Part (B) represents the monitored analyte SRM transitions. Part (C) represents the monitored internal standard SRM transitions. Orange: PEth 16:0/18:1 SRM 1, Red: PEth 16:0/18:2 SRM 1, Cyan: PEth 16:0/18:1 SRM 2, Black: PEth 16:0/18:2 SRM 2, Green: PEth 16:0/18:1–D_5 SRM 1, Blue: PEth 16:0/18:2–D_5 SRM 1, Purple: PEth 16:0/18:1–D_5 SRM 2, Wine: PEth 16:0/18:2–D_5 SRM 2.

Table II. Intra-assay and inter-assay accuracy and imprecision determined during three validation runs with six quality control samples at each concentration

Analyte	Concentration (ng/mL)	Intra-assay accuracy (%, $n = 6$)	Inter-assay accuracy (%, $n = 3$)	Intra-assay imprecision (%, $n = 6$)	Inter-assay imprecision (%, $n = 3$)
PEth 16:0/18:1 SRM 1	20	95–104	100.0	3.7–4.3	4.57
	45	102–106	103.3	2.7–4.6	1.99
	1,180	87–97	91.2	2.5–6	5.69
PEth 16:0/18:1 SRM 2	20	97–101	98.9	2.5–4.3	2.11
	45	97–99	98.5	2.8–6.6	1.16
	1,180	93–104	98.0	2.3–5.1	5.82
PEth 16:0/18:2 SRM 1	20	97–103	99.3	2.8–7.4	3.12
	45	94–105	98.3	3.3–6.7	5.84
	1,180	100–107	103.7	2.9–5.1	2.89
PEth 16:0/18:2 SRM 2	20	97–106	100.8	3.4–8.3	4.52
	45	97–100	99.2	3.2–4.9	1.91
	1,180	93–104	95.1	3.3–6.5	8.14

Matrix effects

Matrix effects were tested by the injection of matrix extracts from six different nondrinkers spiked with PEth concentrations at K3 and K6. Each sample was prepared in duplicate. The internal standard corrected concentration was compared to samples containing no matrix, spiked at concentration K3 and K6. The obtained results are listed in the Supplementary Material S1. The investigated matrix effects can be considered as negligible. Ion enhancement and ion suppression were investigated by comparing the analyte area without any internal standard correction: For PEth 16:0/18:1 a response of 97–129% and for PEth 16:0/18:2 a response of 123–198% was observed.

Extraction efficiency

Extraction efficiency was investigated by the repetitive extraction of calibrator samples. Thereby calibrator samples at K4–K6 (375-–1,500 ng/mL) were extracted six times. The monitored analyte peak area after each extraction was thereby compared to the total analyte peak area obtained after six extractions. For PEth 16:0/18:1, a mean extraction efficiency of 89% after the first extraction, and 7% after the second extraction was observed. For PEth 16:0/18:2 a mean extraction efficiency of 88% after the first extraction, and 8% after the second extraction was observed.

Application of internal standard

To investigate if the time point of the internal standard application affects the outcome of the measurement, two different approaches were compared: on the one hand direct application of the internal standard before the extraction, on the other hand, application of the internal standard 8 h before the extraction.

Considering the quantification of ~~real 28 case~~ samples from alcohol use disorder (AUD) patients, no differences were observed: The mean agreement was 105 ± 10% (R = 0.9821) for PEth 16:0/18:1 SRM 1 and 101%±10% (R = 0.9832) for PEth 16:0/18:1 SRM 2. For PEth 16:0/18:2 SRM 1, a mean agreement of 93 ± 10% (R = 0.9897), and for PEth 16:0/18:2 SRM 2 of 95%±16% (R = 0.9877) was observed.

Comparison with PEth in liquid whole blood

To compare the fully automated extraction with the determination of PEth in liquid whole blood, 28 samples from AUD patients were compared.

A comparison between ~~the manually extracted DBS and fully automated extraction~~ revealed that PEth results obtained with both methods are comparable: For PEth 16:0/18:1 SRM 1 a mean difference of 100 ± 9% (range: 84–128%), and for PEth 16:0/18:1 SRM 2 a mean difference of 101 ± 9% (range: 81–122%) was observed. For PEth 16:0/18:2 SRM 1 a mean difference of 98 ± 8% (range: 87–118%), and for PEth 16:0/18:2 SRM 2 a mean difference of 99 ± 9% (range: 82–116%) was observed. The linearity of the two methods of PEth determination was high, see Figure 3.

Comparison between manual and automated DBS extraction

To compare the fully automated extraction with manual DBS extraction, 28 DBS samples from AUD patients were compared.

A comparison between the manually extracted DBS and fully automated extraction revealed that PEth results obtained with both methods are comparable: For PEth 16:0/18:1 SRM 1 a mean difference of 108 ± 11% (range: 87–125%), and for PEth 16:0/18:1 SRM 2 a mean difference of 107 ± 10% (range: 87–126%) was observed. For PEth 16:0/18:2 SRM 1 a mean difference of 96 ± 10% (range: 75–117%), and for PEth 16:0/18:2 SRM 2 a mean difference of 95 ± 10% (range: 79–118%) was observed. The linearity of the two methods of PEth determination was high, see Figure 4.

Discussion

The aim of this study was the development of a method for the fully automated sample preparation, extraction and online LC-MS/MS quantification of PEth 16:0/18:1 and PEth 16:0/18:2. In contrast to only analyzing the most abundant PEth species, PEth 16:0/18:1, the here presented method has a distinct advantage: the quantification of the two most abundant PEth homologs allows confirmation of the result within the run. In abstinent subjects, both of the analogs are present at low concentrations; in AUD patients, on the other hand, both analogs are present at elevated concentrations.

During the validation process, the automated extraction method proved to be a fast and reliable method for the determination of PEth samples. The method proved to be linear, accurate and precise. Considering the linearity, the observed correlation coefficient was at least 0.9953. Accuracy and precision remained below the required ±15% for all of the monitored transitions, independent of intra-assay or inter-assay comparison. The LOQ of 20 ng/mL permits the

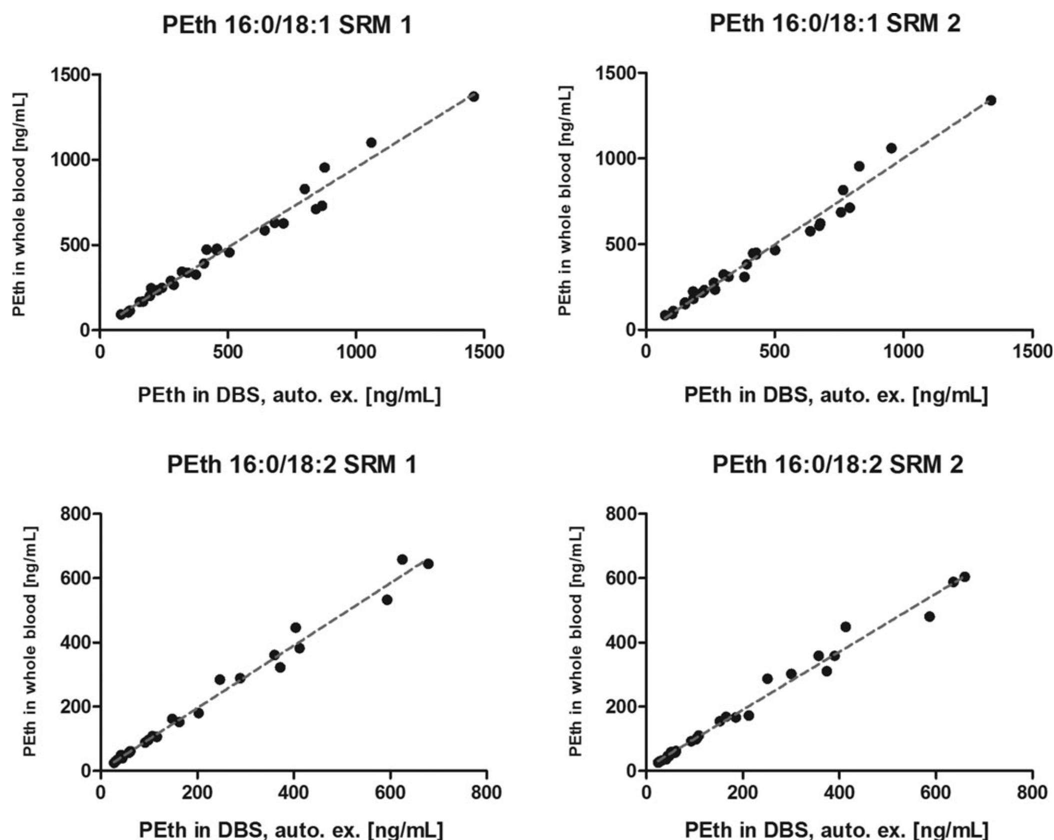

Figure 3. Comparison of PEth concentrations determined in liquid whole blood and automated DBS extraction for 28 samples from AUD patients (data points above LOQ). PEth 16:0/18:1 SRM 1: Slope: 0.9349 ± 0.02824 ($R = 0.9768$), $P < 0.0001$. PEth 16:0/18:1 SRM 2: Slope: 1.007 ± 0.03102 ($R = 0.9759$), $P < 0.0001$. PEth 16:0/18:2 SRM 1: Slope: 0.9706 ± 0.02339 ($R = 0.9863$), $P < 0.0001$. PEth 16:0/18:2 SRM 2: Slope: 0.9029 ± 0.02489 ($R = 0.9821$), $P < 0.0001$.

identification of samples at common PEth reference interval concentrations such as 35 ng/mL (19).

Furthermore, no interferences were observed for blood samples from nondrinkers. By the use of two internal standards for PEth 16:0/18:1 and 16:0/18:2 it was possible to compensate for potential matrix effects occurring throughout the measurement. In addition, the application of internal standard directly to the DBS by the spray module allows compensating for potential extraction bias. In comparison to manual DBS extraction using passive elution from the spot using shaking in solvent, the active extraction under pressure showed high extraction efficiencies of about 90%. In contrast, Kummer *et al.* reported PEth extraction efficiencies for manual DBS extraction >55% (RSD < 18%) (20).

Concerning the investigated carry over of up to 0.5%, we recommend the reanalysis of samples close to the lower limit of reporting, if preceded by a sample with a high concentration. Another option is the extraction of a blank after each real case sample. For the manual injection of liquid extracts, comparable carry over was investigated, leading to the conclusion, that the carry over may be related to the column material. In general, PEth appears to have properties which lead to minimal carry over: Isaksson *et al.* reported carry over of about 0.2% for their high-throughput method for whole blood (19).

As the sample preparation is performed within the autosampler unit, laboratory work is reduced to an absolute minimum: only the calibrator and quality control samples have to be prepared manually. However, this can be performed in advance of the analysis, as PEth was found to be relatively stable on DBS (21). The potential of the automated system for high-throughput analysis of PEth provides a short turnaround time for large quantities of samples. Based on low-key calculation with a run time of about 8 min, 180 samples can be analyzed each day. This simplified and fast acquisition method may lead to a broader acceptance of PEth for routine analysis.

By comparing samples from AUD patients which contain endogenous PEth, it could be shown that the PEth concentrations determined by the fully automated system are comparable to the measurement in liquid whole blood or manual DBS extraction. This allows results to be comparable independent of the applied extraction method. To be comparable, the protocol for the manual calibration and quality control sample preparation had to be prolonged, as differences concerning the results between authentic PEth samples from AUD patients were observed between the manual PEth DBS extraction and the automated PEth DBS extraction. The manually prepared quality control samples with spiked PEth did not require the prolonged incubation protocol, as no differences between the extraction methods were observed for these samples. This implies that there is a difference between PEth that is integrated into the blood cells and exogenous PEth which is spiked into blood from a nondrinker. However, prolonged incubation of spiked PEth and blank blood appears to successfully incorporate PEth, leading to comparable results. Therefore, careful cross-validation between different extraction methods with authentic PEth samples is highly recommended for the validation of PEth methods.

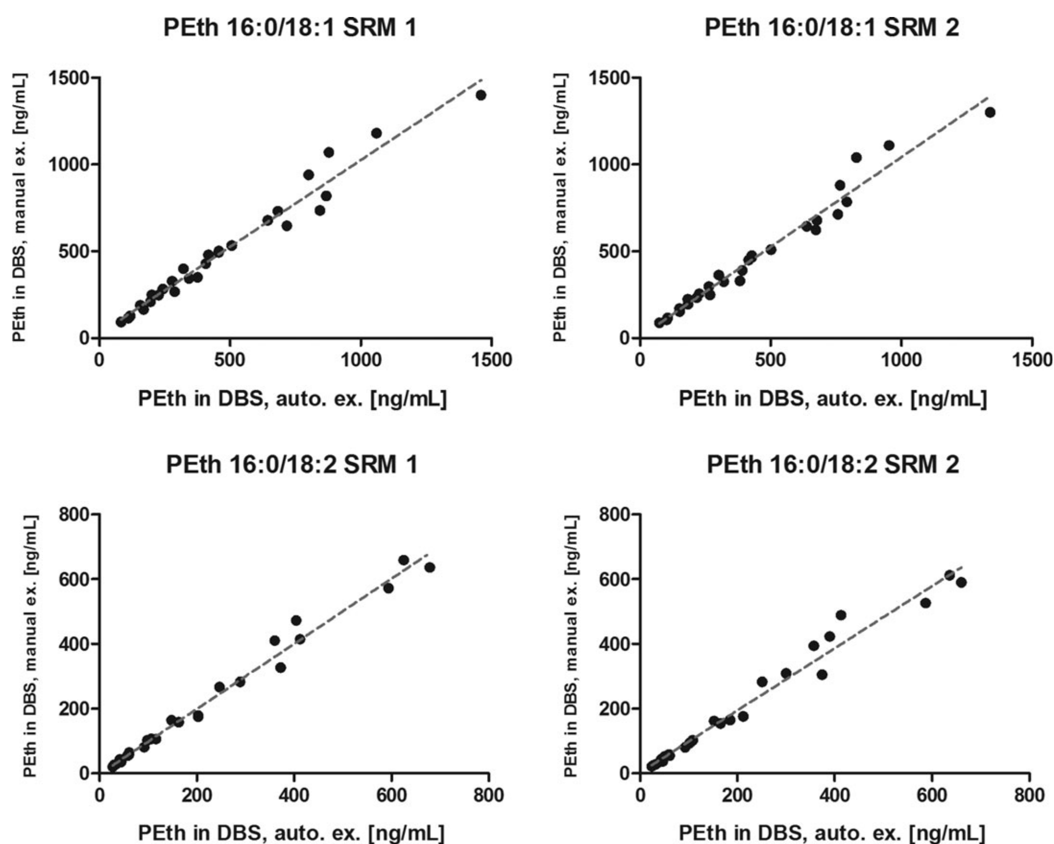

Figure 4. Comparison of manual DBS extraction and automated DBS extraction for 28 samples from AUD patients (data points above LOQ). PEth 16:0/18:1 SRM 1: Slope: 0.9995 ± 0.03644 ($R = 0.9666$), $P < 0.0001$. PEth 16:0/18:1 SRM 2: Slope: 1.033 ± 0.03613 ($R = 0.9692$), $P < 0.0001$. PEth 16:0/18:2 SRM 1: Slope: 1.006 ± 0.02542 ($R = 0.9849$), $P < 0.0001$. PEth 16:0/18:2 SRM 2: Slope: 0.9602 ± 0.03170 ($R = 0.9745$), $P < 0.0001$.

Conclusion

A new workflow for high-throughput determination of PEth in DBS was developed by automated sample work-up with a DBS-MS 500 coupled to online-SPE-LC-MS/MS.

The analytical process is fully automated by using an online DBS-SPE-LC-MS/MS analysis system. The feasibility of the automated analysis was successfully validated and has been applied to real case samples. The obtained results are comparable to the widely applied manual extraction of PEth in DBS or the analysis of PEth in liquid blood samples. Automation with the DBMS-500 includes the possibility of sample identification by a barcode label on the DBS card—with a link to a laboratory information system (LIMS).

Supplementary data

Supplementary material is available at *Journal of Analytical Toxicology* online.

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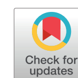
Development and validation of an LC-MS/MS method for the analysis of ivermectin in plasma, whole blood, and dried blood spots using a fully automatic extraction system



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Microsampling
Dried blood spots
Automated extraction

ABSTRACT

Ivermectin is deployed in mass drug administration (MDA) campaigns to control parasitic diseases in the tropics, with billions of treatments having been administered in the last three decades. Simple blood sampling tools, like the dried blood spots (DBS) technique, are needed to monitor treatments in such challenging settings. Thus, we developed a fully automated method for the analysis of ivermectin in DBS microsamples, including a bioanalytical and clinical validation.

Automated extraction was carried out using a DBS-MS 500 autosampler which was coupled to a LC–MS/MS system. DBS were extracted with 20 μL solvent and eluted on a C8 analytical column. Analysis was performed by multiple reaction monitoring in the positive mode.

Automated DBS extraction resulted in consistent recoveries (62.8 ± 4.3%) and matrix effects (68.0 ± 8.1%) between different donors and concentration levels. Intra- and inter-day accuracy and precision deviations were ≤15%, while samples with hematocrits from 20 to 60% could be quantified reliably. The achieved sensitivity of 1 ng/mL in DBS samples is sufficient to analyze ivermectin at the dose given (single oral administration of 12 mg) over a period of at least 72 h post treatment. Importantly, DBS samples are stable after one-month storage at room temperature (accuracy: 88.8–96.2%), thus samples collected in the field must not be shipped on dry ice. Ivermectin concentrations in venous and capillary blood agreed strongly, with a mean difference of −4.8%. Moreover, the drying process of DBS did not alter the analysis and importantly plasma concentrations can be estimated from DBS data using the hematocrit and red blood cell partitioning as correction factor.

Our method enables uncomplicated sample collection and shipment as well as automated analysis of large amounts of samples, which is key to surveying MDA campaigns in remote settings.

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1. Introduction

William C. Campbell and Satoshi Ōmura received the Nobel price in Medicine in 2015 for the discovery and development of ivermectin, a fermentation product of *Streptomyces avermitilis*. Ivermectin initially has been used for the treatment of roundworm infections in farm animals. In 1987, it has been registered for human use and it drastically lowered the incidence of river blindness and lymphatic filariasis infections as a result of mass drug administration (MDA) campaigns. Recent studies demonstrated that ivermectin is also active against several other parasites and even against arthropods like mosquitoes, which further underscores its clinical significance and potential. Ivermectin for instance kills *Anopheles gambiae* mosquitoes, the major vector of malaria in Africa, after feeding on blood of treated humans [1,2]. Thus, it might play a role in vector control of malaria, by decreasing the mosquito's survival, fitness, and fertility [3,4]. Importantly, ivermectin was given to 260 million adults alone in 2014 in order to control river blindness and lymphatic filariasis, which indicates that the drug is generally safe [5]. In addition, doses 10 times higher than approved were well tolerated by healthy volunteers [6]. Therefore, ivermectin is potentially eligible for vector control programs.

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Still, safety data in children (<15 kg) and during pregnancy are not yet available [5].

To evaluate the safety of ivermectin in children, a blood sampling technique is required which is minimally invasive and uses only little amounts of blood. Moreover, the technique must be applicable in field settings to support mass drug administrations with pharmacokinetic (PK) data collection. The dried blood spot (DBS) technique fulfills these requirements because blood is collected by a simple finger prick, and only few microliters are dropped onto filter cards [7,8]. Drugs in dried matrices are usually more stable and can thus be stored and shipped at room temperature, which additionally simplifies the whole sampling procedure. Moreover, sample processing and analysis can be automated, giving the possibility to screen a large population [9]. However, a sensitive method is required to quantify ivermectin levels in such low sample volumes.

Numerous bioanalytical methods have already been developed for quantification of ivermectin in different matrices [10–12]. Samples were analysed by high-performance liquid chromatography (HPLC), which was in most cases coupled to a UV or fluorescence detector. Analysis by tandem mass spectrometry, which facilitates a selective, sensitive, and fast measurement, was less frequently used. Moreover, only one method showed that ivermectin can be analysed in DBS but this method was not fully-automated, which is essential if a large number of samples have to be determined efficiently [12].

Recently, we investigated the population pharmacokinetics of ivermectin in human plasma and DBS samples using the bioanalytical method which we present here in full detail [13]. The aim of this study was to show the entire development process and the thorough validation of our LC–MS/MS method for the quantification of ivermectin in plasma and blood as well as in DBS using a fully automated extraction system. The DBS validation comprised parameters like extraction recovery and matrix effect, which are more complex for an automated system and therefore requires modification of the standard procedures. Moreover, the blood hematocrit and spot homogeneity were investigated as they may impact the reliability of DBS analyses.

Finally, we treated three healthy volunteers with 12 mg ivermectin to clinically validate if the DBS technique can be used as an alternative to conventional venous blood sampling. We compared the ivermectin levels in plasma and whole blood using different anticoagulants as well as in capillary blood which was withdrawn from the fingertip and pipetted accurately into a vial and onto a DBS card. With this setup, we aimed to investigate the effect of the anticoagulation, as well as the difference between venous plasma, venous blood, and capillary blood. In addition, we assessed the influence of the drying process and compare manual whole-spot and automated sub-spot extractions.

2. Materials and methods

2.1. Chemicals, reagents and reference compounds

Ivermectin and ivermectin-d2 were purchased from Toronto Research Chemicals (Toronto, Canada). Gradient grade methanol, water and formic acid were products from Merck (Darmstadt, Germany). Ammonium formate and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, USA). DBS cards (grade 903 filter paper) were purchased from Whatman (Sanford, ME, USA) and lithium heparin coated Minivette® POCT capillaries (20 μL) from Sarstedt (Nümbrecht, Germany). Calibrators and quality control (QC) samples were prepared in drug free plasma or whole blood from the local blood donation center (Basel, Switzerland). Ethylendiaminetetraacetic acid (EDTA) was used as anticoagulant.

2.2. LC–MS/MS instrumentation and settings

Ivermectin was analysed on a modular UPLC system from Shimadzu (Kyoto, Japan) containing a system controller (CBM-20A), four pumps (LC-30AD), a degasser (DGU-20A5), a column oven (CTO-20AC), and an autosampler (SIl-30ACMP), which was coupled to an API 5500 QTRAP tandem mass spectrometer (AB Sciex, Framingham, MA, USA).

A Kinetex C8 analytical column (2.6 μm, 100 A, 50 × 2.1 mm) was installed in the 55 °C tempered column oven. A filter frit (SS 0.5 μm 0.62 × 0.65, Ercatech AG, Bern, Switzerland) was used to prolong the lifetime of the analytical column. Mobile phase A was an aqueous solution of 20 mM ammonium formate supplemented with 0.1% formic acid (pH 3.5). Mobile phase B was methanol plus 0.1% formic acid. The column was conditioned at 2% mobile B during the first 0.25 min of each analytical run. Afterwards, the mobile B concentration was linearly increased to 75% over 0.5 min and to 95% within 1.75 min. 95% mobile phase B was kept constant for 0.75 min. The run was terminated by reconditioning the column for 0.5 min at 2% mobile B. The flow rate was set to 0.6 ml/min and the injection volume was 10 μL. Ivermectin eluted after 1.9 min, therefore the UPLC system was only connected during minute 1.5 and 2.5 with the mass spectrometer to avoid superfluous contamination of the system.

Ivermectin and ivermectin-d2 were analysed by electrospray ionization in the positive mode. Multiple reaction monitoring (MRM) of 892.4 → 569.1 m/z and 895.4 → 571.8 m/z was applied for ivermectin and ivermectin-d2, respectively, in plasma and whole blood. To note, the second most abundant mass was used for ivermectin-d2 (877 + 18 (NH_4^+) m/z: 51.9%). For DBS samples the sum of two transitions, 892.4 → 569.1/307.2 m/z (ivermectin) and 895.4 → 571.8/309.3 m/z (ivermectin-d2), was used to increase the sensitivity of the method. The compound specific settings are given in supplementary Table S1. Nitrogen was used as ion source (gas 1 30 L/min, gas 2 60 L/min), curtain (20 L/min) and collision gas (medium). The ion spray voltage was set at 5500 V (positive mode) and the source temperature was 300 °C. The LC–MS/MS system was operated using Analyst software 1.6.2 (AB Sciex, Framingham, MA, USA).

2.3. Preparation of calibration and quality control samples

Ivermectin and ivermectin-d2 were dissolved in DMSO at a final concentration of 1 mg/mL. Calibration and quality control (QC) stock solutions of ivermectin originated from different weighing.

For plasma and whole blood analyses, the calibration stock solution was serially diluted with DMSO to cover a range from 25 – 0.05 μg/mL and 10 – 0.05 μg/mL, respectively. The QC stock was diluted with DMSO to receive final concentrations of 10, 1, 0.1, and 0.05 μg/mL for plasma and of 5, 1, 0.1, and 0.05 μg/mL for whole blood. Calibrators and QC samples were prepared by spiking blank plasma or blood with the analyte dilution series in a ratio of 1:100 (v/v). Plasma calibration samples encompassed a range of 0.5–250 ng/mL and blood calibrators of 0.5–100 ng/mL. Plasma and blood QC samples were prepared at the lower limit of quantification (LLOQ: 0.5 ng/mL), low (QC_{LOW}: 1 ng/mL), mid (QC_{MID}: 10 ng/mL) and high (plasma $QC_{HIGH:}$ 100 ng/mL, blood $QC_{HIGH:}$ 50 ng/mL) concentration levels.

In the case of DBS analyses, a calibrator and QC working solution of 100 μg/mL were prepared in DMSO. Calibrators and QC samples were prepared by spiking blood with the analyte so that the DMSO content was less than 0.1% in order to avoid hemolysis. Calibrations were prepared from 100 to 1 ng/mL using blank blood for the dilutions. QC samples were made in the same way at 50 ng/mL (QC_{High}), 10 ng/mL (QC_{MID}), 2.5 ng/mL (QC_{LOW}), and 1 ng/mL (LLOQ). The samples were gently mixed and kept agitated on a roll-agitator

(RM 5, CAT, Staufen, Switzerland) until 15 μL aliquots were spotted onto DBS filter cards. DBS were dried at room temperature for at least 2 h, packed together with desiccants, and stored at −20 °C until analysis.

A calibration set contained one double blank sample, which was processed without internal standard (IS), one blank sample which was extracted with IS, and more than seven calibrators. Calibration lines were fitted by a linear regression of the ivermectin concentration (x) against the peak area ratio (y) of ivermectin to ivermectin-d2 (IS). The regressions were weighted by $1/x^2$.

2.4. Sample extraction

2.4.1. Plasma and blood analysis

Aliquots of 50 μL plasma or blood samples were extracted with 150 μL methanol containing 50 ng/mL ivermectin-d2. The samples were vortex-mixed for about 1 min and afterwards centrifuged at 10 °C and 3220 g for 30 min. The samples were kept at 10 °C within the autosampler and 10 μL supernatant were subjected to LC–MS/MS analysis.

2.4.2. Manual DBS extraction

The entire blood spots (20 μL blood) were punched out and transferred to a 0.75 mL autosampler matrix tube (Thermo Scientific, Reinach, Switzerland). The DBS were extracted with 350 μL of a 70:30 (v/v) mixture of IS solution (50 ng/mL ivermectin-d2) and water. The samples were mixed for 10 min, sonicated for 20 min, and centrifuged as described above. An aliquot of 10 μL supernatant was injected into the LC–MS/MS system.

2.4.3. Automated DBS extraction

The DBS-MS 500 autosampler was conditioned as described previously [9]. Before and after the extraction, the cards were photographed in order to check for the presence of an adequate blood spot, to center the extraction head to the middle of each spot, and to verify a reasonable extraction. Too small or deformed DBS were recognized by the software (Chronos, AS, Germany) and were excluded from the analysis. Internal standard, 10 μL of 1 μg/mL ivermectin-d2 dissolved in methanol, was sprayed onto each spot. The DBS were dried for 20 s and afterwards extracted with a 20 μL methanol and mobile phase A (7:3 v/v) at a flow rate of 40 μL/min. After the extraction, the autosampler was cleaned for 20 s with a mixture of methanol:acetonitrile:isopropanol:water (1:1:1:1 v/v/v/v) and for 20 s with water containing 0.1% formic acid.

2.5. Method validation

The LC–MS/MS method was validated according to the guideline on bioanalytical method validation of the European Medicines Agency (EMA) [14]. Selectivity, sensitivity, accuracy, precision, linearity, extraction recovery, matrix effect, and analyte stability were taken into account. Moreover, the impact of the hematocrit and the homogeneity of DBS samples as well as the influence of the anticoagulation were assessed during the validation.

2.5.1. Selectivity and sensitivity

Blank samples from seven different subjects were processed with and without internal standard and examined for interfering matrix components. Moreover, the same blank samples were spiked at the designated LLOQ. An imprecision of less than 20% and a mean accuracy of 80–120% were accepted in this study, while at least 67% of the LLOQ samples had to be within this range.

2.5.2. Linearity

The linear regression between the analyte concentration and the analyte:IS peak area ratio had to result in a correlation coefficient (R) of at least 0.99. In addition, 75% of the calibration samples must be within ±15% (LLOQ: ±20%) of the nominal value including at least six calibrators, a LLOQ and upper limit of quantification (ULOQ) sample.

2.5.3. Intra- and inter-day accuracy and precision experiments

Six QC sample replicates at four concentration levels (LLOQ, QC_{LOW}, QC_{MID}, and QC_{HIGH}) were analysed to determine the accuracy and precision of the method. Precision and accuracy were assessed within a single validation run (intra-day) as well as between three runs recorded on different days (inter-day). The QC samples were placed between two calibration lines for each validation run to calculate their concentrations. The calculated concentration was divided by the nominal value to determine the accuracy (bias, %). A mean accuracy of 85–115% (LLOQ: 80–120%) was accepted, while at least 67% of the QC samples of each concentration level had to be within these limits. The coefficient of variation (CV, %) was used to calculate the precision for each QC concentration within an analytical run (intra-day precision, n = 6) and over all three runs (inter-day precision, n = 18). The precision had to be <15% and <20% at the LLOQ.

In addition, the effect of the blood hematocrit on the reliability of the DBS analysis was evaluated. Therefore, blood with hematocrit values of 20, 40, and 60% was prepared by adding plasma to or removing from blood with a known hematocrit. Modified hematocrit values were verified for quality control reasons using a hematology analyzer (Sysmex KX-21 N, Kobe, Japan). The blood samples were spiked at QC_{LOW}, QC_{MID}, and QC_{HIGH} levels. The accuracy of the samples (n = 4 per QC level) was determined at three different days using a calibration line with a hematocrit value of 40%. Blood spots were photographed using the built-in camera of the CAMAG autosampler, and the areas were digitally measured with GIMP software (GNU Image Manipulation Program 2.8.10).

The influence of the spotted blood volume and homogeneity was investigated. QC samples were prepared at low, medium and high concentration level using 30 μL and 15 μL blood. A mean deviation of less than 15% was considered acceptable.

2.5.4. Recovery and matrix effect

The extraction recovery and matrix effect was investigated in samples of six different subjects at QC_{LOW}, QC_{MID}, and QC_{HIGH} concentration levels. The recovery of ivermectin was calculated by comparing the peak areas of blank matrix samples spiked before and after extraction. The ivermectin peak areas were normalized by the ivermectin-d2 peak area. Matrix effects were assessed as the ratio of the peak areas of blank matrix samples spiked after extraction to the peak areas of the analytes prepared in extraction solvent. The recovery and matrix effect of the automated extracted DBS samples was assessed as described previously [9]. Overall, the recovery and matrix effect should be consistent, precise (<15%) and reproducible.

2.5.5. Stability

The stability of ivermectin was evaluated under different storage conditions at QC_{LOW}, QC_{MID}, and QC_{HIGH} concentration levels. In the case of plasma and blood samples, the stability was assessed following three freeze-and thaw cycles (24 h), 8 h at room-temperature (bench-top stability), and twelve months at −20 °C (long term stability). Ivermectin stability in DBS samples was evaluated following 1-month storage at room temperature, in the fridge, and at −20 °C, respectively. Three replicates were analysed per condition and compared to a calibration which was freshly prepared on the day of analysis.

2.6. Method application

Three healthy volunteers were treated with an oral dose of 12 mg Ivermectin (4 tablets of 3 mg ivermectin, Stromectol®, MSD, USA) in a fasted state. The procedure was brought to the attention of the Ethics Committee Northwest/Central Switzerland (EKNZ, notification No. EKNZ UBE-15/17).

Whole blood samples were drawn before treatment (T = 0) and 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h post treatment by peripheral venipuncture into 2.7 mL EDTA as well as Li-heparin coated tubes. An aliquot of 500 μL was placed in a cryotube and the rest was centrifuged to produce plasma. The fingertip was punctured with a sterile one-way finger pricker (BD Microtainer, Becton Dickinson and Company, NJ, USA) to obtain capillary blood directly after the withdrawal of the venous whole blood samples. Aliquots of 20 μL blood were collected in heparin coated capillaries (Minivette POCT 20 μL LH, Sarstedt, Germany) and either directly transferred into autosampler tubes or spotted onto DBS cards (903 grade, Whatman, ME, USA). The cards were allowed to dry for at least 2 h and stored in sealed plastic bags with desiccants. All samples were stored at −80 °C until analysis and processed as outlined above.

Pharmacokinetic parameters were calculated using non-compartmental analysis in Gnu R (Version 3.3.3; R Foundation for Statistical Computing, http://www.R-project.org, Vienna, Austria) and the ncappc package [15].

Bland-Altman plots were generated with GraphPad Prism 6.04 (La Jolla, CA, USA) to compare the concentrations measured in the different matrices. The %difference ($= \frac{(Concentration\ of\ matrix\ 1 - Concentration\ of\ matrix\ 2)}{mean\ concentration\ of\ matrix\ 1\ and\ 2} \times 100$) was plotted against the mean concentration of two matrices. Moreover, the mean %difference was illustrated in the plots as well as the 95% limits (±2 standard deviations). For a good agreement, at least 67% of the samples had to be within ±20% limits according to cross-validation guidelines [14]. Plasma concentrations were extrapolated from blood data using the equation $[Analyte]_{Plasma} = \frac{[Analyte]_{Blood}}{(1-HCT)+K_{RBC:Plasma} \times HCT}$ as proposed by Jager et al [16]. The hematocrit (HCT) was determined for each patient and the mean partitioning of the drug between plasma and red blood cells ($K_{RBC:Plasma}$) was calculated by rearranging the equation. $K_{RBC:Plasma}$ was determined at each time-point using the measured plasma and blood concentrations, whereas the mean $K_{RBC:Plasma}$ was used to extrapolate plasma concentrations from blood values.

3. Results and discussion

3.1. Method development

Mass drug administration campaigns using ivermectin are frequently carried out in order to control onchocerciasis and lymphatic filariasis infections in Africa. Moreover, ivermectin shows promise to be used as a malaria vector control agent [1]. However, blood sampling techniques are needed which enable uncomplicated sample collection in the field and transportation to bioanalytical laboratories for the purpose of surveying treatments within the campaigns. Furthermore, automatisation of sample processing should be possible. Here, we present a fully automated LC–MS/MS method for the quantification of ivermectin in DBS, which fulfills the demanding requirements of studies executed in remote environments (see graphical abstract).

Method development focused mainly on the optimization of the automated DBS analysis since a lot of information about the chromatography and mass detection of ivermectin has already been described in literature. In brief, ivermectin binds strongly to reverse phase columns. A C8 instead of a C18 column was used to retain ivermectin, given that ivermectin eluted earlier and at a higher percentage of mobile phase A. The ammonium formate in mobile A was important for the ionization of ivermectin as the ammonia adducts are the most intense and reliable ions. 569.1 and 307.2 m/z were the most abundant fragments of ivermectin, whereas 569.1 was selected for quantification in plasma and blood. The sum of both fragments was used for DBS analyses to improve method sensitivity.

The extraction of plasma and whole blood samples was not optimized since the sensitivity was sufficient using a simple protein precipitation. In the case of the automated DBS extraction the extraction-flow, -volume, and -solvent composition were improved. Different methanol:water mixtures were tested (60:40, 70:30, 80:20, and 90:10 v/v). All extraction mixtures yielded a recovery of approximately 60–70%. The methanol:mobile A 70:30 v/v mixture was chosen, because it appeared to be a good compromise between risking to clog the extraction head using high amounts of water and to be more compatible with the aqueous starting condition of the mobile phase gradient. A sample loop of 20 μL was installed on the DBS-MS 500, thus an extraction-volume of 20 μL was used to avoid superfluous loss of sample. The extraction-flow was set to the standard setting of 40 μL/min as it had no significant impact on method sensitivity. The same wash cycle as described previously was used in order to reduce analyte carry-over [9]. This wash program resulted in an ivermectin carry-over of less than 20% of the LLOQ peak area in the first blank sample measured after an ULOQ sample.

3.2. Method validation

3.2.1. Selectivity and sensitivity

The selectivity and sensitivity of ivermectin was determined in blank plasma, blood, and DBS from seven different donors (Fig. 1). No interference could be observed in double blank samples. Ivermectin-d2 caused a small but constant interference, which was approximately 30% of the LLOQ peak area. To lower the IS concentration would reduce the interference, however it would also alter method linearity as the ivermectin-d2 signal is increased by high ivermectin concentrations. Thus, the chosen IS concentration is a compromise between method linearity and selectivity. Overall, the interference did not affect the accuracy and precision at the LLOQ. A mean accuracy of 94.3% ± 12.4%, 105.0% ± 7.1%, and 107.4 ± 13.3% was calculated for the LLOQ samples of seven different donors in plasma, blood, and DBS, respectively. Moreover, the achieved sensitivity was sufficient to also monitor low ivermectin levels as would be expected at 72 h post-treatment [13]. Therefore, the developed method is selective and sensitive enough for the analysis of ivermectin in various matrices.

3.2.2. Linearity

The correlation coefficient (R) of the linear regression between the analyte to IS peak area ratio and the nominal concentration was >0.997 for all validation runs. Hence, ivermectin can be quantified by linear regression within the chosen calibration range of 0.5–250 ng/mL for plasma, 0.5–100 ng/mL for blood, and 1–100 ng/mL for DBS samples. Based on published data, we expect this range to be sufficient to analyse PK studies of ivermectin [13]. A smaller ULOQ sample was chosen for blood and DBS calibrations, because ivermectin is highly bound to plasma proteins and thus only marginally distributed into the red blood cells, which results in lower blood than plasma concentrations [17].

3.2.3. Accuracy and precision

Intra- and inter-day accuracy and precision data are presented in Table 1. Intra-day precisions were less than 10%, 10% and 14% determined in plasma, blood, and DBS, respectively. The inter-day precisions were <12%. Mean intra-day accuracies were between

Table 1
Intra- and inter-day accuracy and precision of ivermectin determined in plasma, blood, and dried blood spots (DBS).

Matrix	Nominal concentration [ng/mL]	Day 1		Day 2		Day 3		Day 1–3	
		Calculated Concentration [ng/mL]	Accuracy ± CV [%]	Calculated Concentration [ng/mL]	Accuracy ± CV [%]	Calculated Concentration [ng/mL]	Accuracy ± CV [%]	Calculated Concentration [ng/mL]	Accuracy ± CV [%]
Plasma	0.5	0.50	99.0 ± 9.4	0.46	91.0 ± 7.2	0.48	96.3 ± 5.6	0.48	95.4 ± 8.0
	1	1.00	99.8 ± 8.9	0.97	96.5 ± 2.7	1.04	103.8 ± 4.5	1.00	100.1 ± 6.4
	10	10.4	104.4 ± 1.8	10.2	102.3 ± 1.5	10.3	102.6 ± 2.0	10.3	103.1 ± 1.9
	100	100	100.7 ± 1.4	99.7	99.7 ± 0.8	94.5	94.5 ± 2.1	98.3	98.3 ± 3.2
Blood	0.5	0.53	106.3 ± 9.2	0.49	97.3 ± 7.4	0.52	104.7 ± 5.5	0.51	102.8 ± 8.1
	1	1.07	107.2 ± 9.0	0.96	96.0 ± 7.8	1.00	100.0 ± 3.3	1.01	100.4 ± 8.2
	10	10.7	107.0 ± 4.4	10.8	108.4 ± 4.7	10.5	105.2 ± 3.7	10.7	106.9 ± 4.2
	50	49.4	98.7 ± 1.8	50.6	101.1 ± 3.4	49.5	99.0 ± 1.5	49.8	99.6 ± 2.5
DBS	1	0.98	97.7 ± 10.5	1.03	103.3 ± 13.3	1.03	103.2 ± 12.2	1.01	101.4 ± 11.7
	2.5	2.53	101.2 ± 6.3	2.50	100.1 ± 8.6	2.53	101.1 ± 1.7	2.52	100.8 ± 5.9
	10	9.75	97.5 ± 4.3	9.64	96.4 ± 4.9	10.1	100.5 ± 6.3	9.81	98.1 ± 5.2
	50	49.3	98.7 ± 2.0	46.7	93.3 ± 1.9	45.4	90.7 ± 3.4	47.1	94.2 ± 4.3

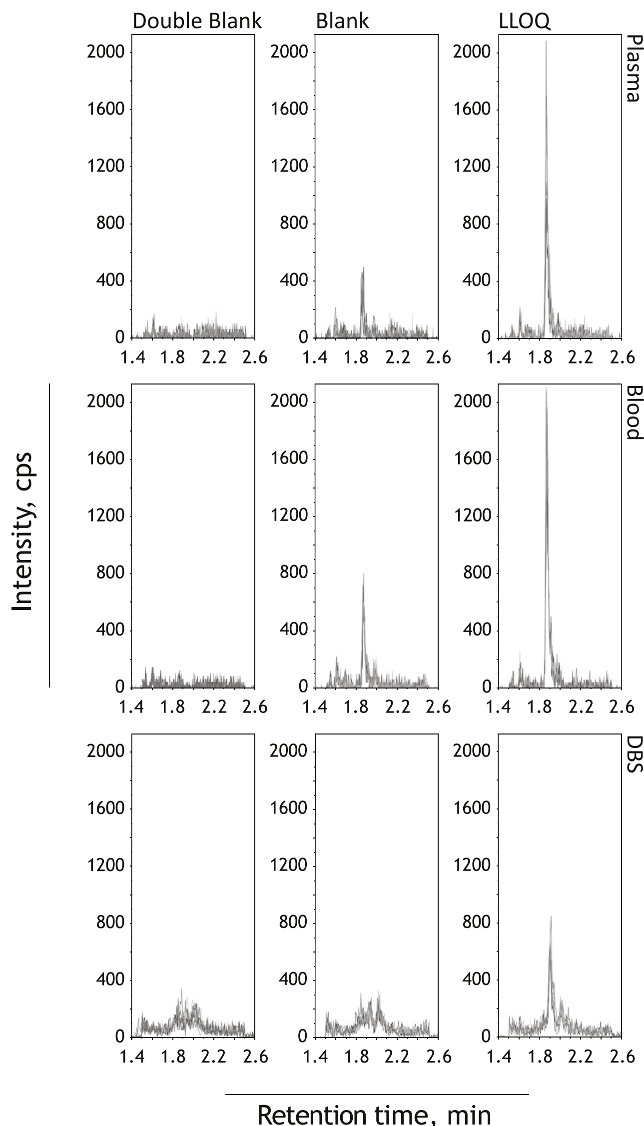

Fig. 1. Selectivity and sensitivity of ivermectin monitored in plasma, blood, and dried blood spots (DBS). Overlay of seven blank chromatograms, which were extracted without (double blank: 1st column) and with internal standard (blank: 2nd column). Seven chromatograms of LLOQ samples from different subjects were overlaid (3rd column). No interference could be observed in double blank samples. Ivermectin-d2 (IS) caused a small but constant interference of approximately 30% compared to the LLOQ peak area.

91–105% for plasma, 96–109% for blood, and 90–104% for DBS samples. In any case, not more than one QC samples per concentration level was outside the 85–115% (LLOQ: 80–120%) limits. Overall, the results were in line with conditions specified by regulatory guidelines [14].

The hematocrit influences the extent of blood diffusion on the filter paper. The larger the hematocrit, the smaller is the resulting blood spot area [18]. Hence, DBS samples at QC_{LOW}, QC_{MID}, and QC_{HIGH} were prepared using blood with a hematocrit of 20, 40 and 60%. The accuracy of the QC samples was calculated based on a calibration line exhibiting a hematocrit of 40% (Fig. 2A). Not more than 6 out of 108 QC samples were beyond 85–115% accuracy margins. The median deviation between 20 and 60% hematocrit samples was 16%, which is in line with the corresponding deviation of the blood spot area (Fig. 2B). Therefore, the hematocrit has an effect on the accuracy of the DBS analysis, but this effect can be reduced to an

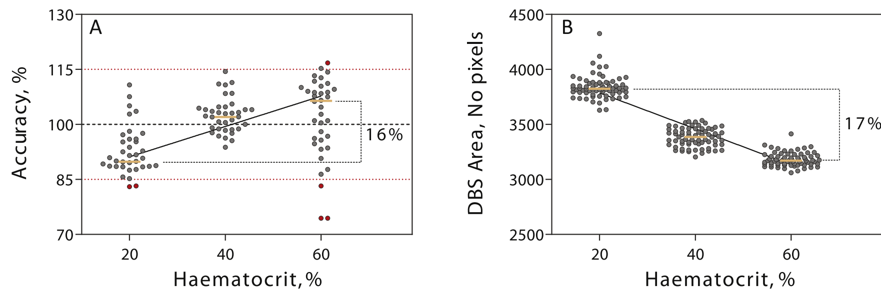

Fig. 2. The effect of the hematocrit on the accuracy of dried blood spots analysis (DBS). **2 A.** Accuracy measurements of QC_{LOW}, QC_{MID}, and QC_{HIGH} samples in blood exhibiting a hematocrit of 20, 40, and 60%. Accuracies were calculated on three different days based on a calibration line with a hematocrit of 40%. Red colored circles (5 out of 108 samples) were beyond 85–115% accuracy limits. Median difference between 20 and 40% hematocrit measurements was 16% **2B.** Spot area determined for DBS with a hematocrit of 20, 40, 60%. Median change in area was 17% between a hematocrit of 20 and 60%.

Table 2
Recovery and matrix effect of ivermectin determined in plasma, blood and dried blood spot (DBS) samples.

Matrix	QC Level	Nominal Concentration [ng/mL]	Recovery ± CV [%]	Mean ± CV [%]	Nominal Concentration [ng/mL]	Matrix effect ± CV [%]	Mean ± CV [%]
Plasma	Qc_{LOW}	1	111.2 ± 4.6	108.8 ± 1.9	1	73.0 ± 10.9	70.6 ± 3.8
	Qc_{MID}	10	107.8 ± 3.9		10	71.2 ± 10.0	
	Qc_{HIGH}	100	107.4 ± 5.5		100	67.7 ± 13.4	
Blood	Qc_{LOW}	1	98.1 ± 2.9	97.9 ± 0.6	1	112.8 ± 5.7	119.9 ± 6.2
	Qc_{MID}	10	98.4 ± 2.0		10	119.2 ± 7.6	
	Qc_{HIGH}	50	97.3 ± 2.3		50	127.7 ± 5.9	
DBS	Qc_{LOW}	2.5	64.1 ± 2.8	62.8 ± 4.3	0.2	73.2 ± 7.0	68.0 ± 8.1
	Qc_{MID}	10	63.2 ± 5.8		1	62.3 ± 5.7	
	Qc_{HIGH}	50	61.1 ± 2.6		5	68.5 ± 3.7	

acceptable degree when the calibration line is made in blood with an average hematocrit of e.g. 40%.

Importantly, the applied blood volume did not alter the distribution of ivermectin within the DBS, because concentrations of QC samples differed by less than 7.6% when 30 μL instead of 15 μL blood was spotted onto cards (Table S2). In addition, accuracies were always between 87–98% with an imprecision of less than 15%. Thus, the applied blood volume did not interfere with the sub-spot analysis.

3.2.4. Recovery and matrix effect

The extraction recovery and matrix effect were tested in plasma, blood, and DBS from six different donors at QC_{LOW}, QC_{MID}, and QC_{HIGH} concentration levels (Table 2). In plasma, a mean recovery of 108.8% was observed, which was consistent between subjects (CV: <5.5%) and over two orders of magnitude (CV: 1.9%). The extraction recovery of ivermectin in blood was almost complete (97.9%) and very consistent with deviations lower than 2.9%. Remarkably, a mean recovery of 62.8% with an inter-subject variation of less than 5.8% was obtained for DBS samples using only 20 μL extraction solvent.

The plasma matrix suppressed the ivermectin signal uniformly by about 30%, since the bias between different donors and over different concentration levels was less than 13.4%. In contrast, blood matrix increased the ivermectin signal slightly about 20% with minor variations between donors and concentration levels. DBS decreased the ivermectin ionization as observed for plasma of about 30% (CV < 8.1%). Overall, the recovery and matrix effect was very consistent between different subjects and concentration levels.

Table 3
Stability of ivermectin in plasma, blood, and dried blood spots (DBS) under several conditions.

Matrix	Condition	Nominal concentration [ng/mL]	Calculated Concentration [ng/mL]	Accuracy ± CV [%]
Plasma	Freeze/thaw	1	1.04	100.4 ± 4.0
		10	10.4	104.1 ± 2.5
		100	95.2	95.2 ± 1.9
	8 h at RT	1	0.96	96.1 ± 4.3
		10	10.4	103.8 ± 0.5
		100	95.6	95.6 ± 0.8
	12 month at −20 °C	1	0.98	98.0 ± 2.0
		10	9.74	97.4 ± 1.2
		100	93.1	93.1 ± 2.0
Blood	Freeze/thaw	1	0.97	97.5 ± 3.0
		10	9.50	95.0 ± 3.3
		50	45.1	90.2 ± 2.1
	8 h at RT	1	0.98	98.2 ± 7.3
		10	9.65	96.5 ± 0.5
		50	47.6	95.2 ± 4.4
	12 month at −20 °C	1	0.99	99.0 ± 4.6
		10	9.93	99.3 ± 4.0
		50	48.4	96.8 ± 1.3
DBS	1 month at RT	2.5	2.30	90.8 ± 1.8
		10	9.60	96.2 ± 3.0
		50	44.4	88.8 ± 3.0
	1 month at 5 °C	2.5	2.40	94.3 ± 4.0
		10	9.80	98.4 ± 5.0
		50	48.3	96.7 ± 0.6
	1 month at −20 °C	2.5	2.60	105.0 ± 4.1
		10	9.90	99.5 ± 1.0
		50	42.7	85.4 ± 1.3

3.2.5. Stability

Stability data are summarized in Table 3. Plasma and blood samples are stable after three freeze and thaw cycles, can be stored for 8 h at ambient temperature or for at least 12 months at −20 °C. The calculated accuracies of the different conditions varied between 90.2–104.1% with an imprecision of not more than 7.3%. These results are in line with data from other studies [11,12]. Schulz and colleagues found a tendency of ivermectin instability in blood samples following 2–3 months of storage and repetitive freezing and thawing. However, the instability could not be observed consistently over all QC levels [12]. Nevertheless, our data indicate

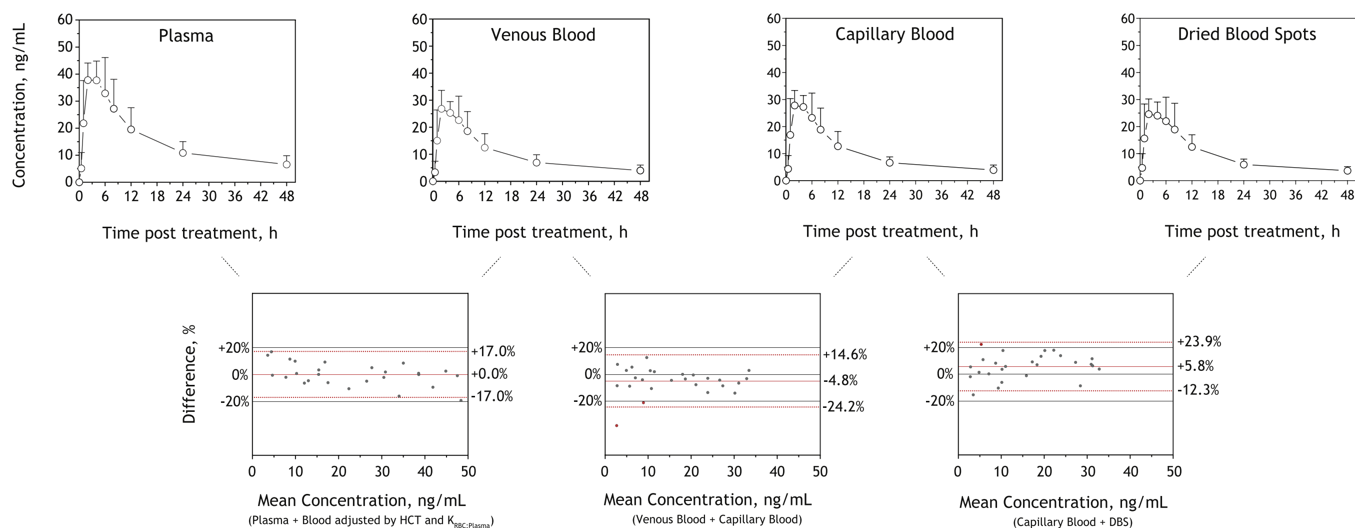

Fig. 3. 1st row: Pharmacokinetic profiles of three healthy volunteers treated with an oral dose of 12 mg ivermectin. Ivermectin concentrations were determined in plasma, venous blood, capillary blood, and dried blood spots. Error bars correspond to standard deviations. 2nd row: Bland-Altman plots of plasma and venous blood (adjusted by the hematocrit and red blood cell partitioning, $K_{RBC:Plasma}$ = 0.22), venous and capillary blood, and capillary blood and dried blood spots (DBS). Sub-spot areas were extracted in the case of DBS samples using the DBS-MS 500 autosampler. Values with a difference of more than +20% difference (grey line) between the two matrices are shown using red circles. The Red line corresponds to the mean difference and the dashed lines to the 95% limits of agreement.

that ivermectin is stable under the investigated conditions. The stability of DBS was evaluated after one-month storage at room temperature (25 °C), in the fridge (5 °C), and in the freezer (−20 °C). Importantly, DBS samples are stable under all conditions (Accuracy: 85.4–105.0%, Precision: <5.0%), observations which are in line with data from literature [12]. Thus, samples collected in the field must not immediately be frozen which simplifies sample collection and shipment as there are no cold chain requirements.

3.3. Method application

Plasma, whole blood and DBS samples were collected from three healthy volunteers who received a single oral dose of 12 mg ivermectin. EDTA and heparin were used as anticoagulants. The difference and 95% limits of agreement calculated between the different matrices are summarized in Table S3. No difference was observed between the EDTA and heparin anticoagulation. Ivermectin plasma concentrations were approximately 40% higher than in whole blood, as ivermectin is highly bound to albumin and consequently only marginally diffuses into red blood cells [17]. Blood concentrations have to be corrected by the hematocrit and $K_{RBC:Plasma}$ in order to achieve a good agreement between blood and plasma (Fig. 3). There is no difference between capillary and venous blood (%difference: -4.8%, 95% limits: 24.2% to 14.6%). Thus, plasma concentrations can be extrapolated from DBS measurements using the hematocrit and $K_{RBC:Plasma}$ of ivermectin. Importantly, the drying process did not affect the analysis, because a good agreement could be found between capillary blood which was directly pipetted into autosampler tubes and capillary blood which was spotted and dried onto filter cards (DBS). Moreover, manually extracted whole DBS spots and automated sub-spot extractions agreed strongly with a minor bias of 5.8% (-16.1% to 27.7%).

PK parameters were calculated based on a non-compartmental analysis (supplementary Table S4). Maximal ivermectin concentrations of about 30 ng/mL in blood and DBS and 42 ng/mL in plasma were reached 4 h post-treatment, which is in line with PK data retrieved from literature [6,19,20]. Therefore, sample dilution was not necessary as the calibration line reached up to at least 100 ng/mL. The mean (standard deviation) half-live, peak concentrations, and area under the curve in automated extracted DBS were 17.9 (2.4) h, 28.2 (5.3) ng/mL, and 525 (171) ng×h/mL, respectively. The sensitivity of the method was sufficient to monitor ivermectin over 72 h and importantly at least 5 times lower than the LC_{50} against anopheles' mosquitoes. Overall, the method is suitable for investigating PK and pharmacodynamic interactions in various matrices.

4. Conclusions

We developed and validated a LC–MS/MS method for the analysis of ivermectin in plasma, blood, and DBS. We demonstrate for the first time that ivermectin can be measured fully automated in DBS, which is on the one hand essential for PK studies carried out in remote settings and on the other hand to process large amounts of study samples. Importantly, one DBS can be extracted and analysed in approximately 5 min and the method uses only 20 μL solvent to extract reproducible about 60% ivermectin out of the dried matrix. The highly concentrated extract suppressed the ionization (∼30%) only negligibly. The method was linear, accurate, and precise over a reasonable concentration and hematocrit range. Overall, all validation parameters were in accordance with regulatory guidelines. The clinical validation showed that DBS can be used to estimate ivermectin concentrations in plasma using the hematocrit and partitioning of ivermectin between plasma and red blood cells. Moreover, the anticoagulants used (EDTA and heparin), the drying of the blood, as well as sub-spot analysis did not question the reliability of the measurements. In conclusion, the developed method enables the analysis of ivermectin in micro-samples which renders the possibility to monitor the drug in various settings.

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Conflict of interest

Stefan Gaugler is an employee of CAMAG AG (Muttenz, Switzerland). None of the other authors reports any conflict of interest regarding this study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2019.04.007.

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Abbreviations

CAMAG	Chemie-Erzeugnisse und Adsorptionstechnik AG
CDC	Center for Disease Control and Prevention
DBS	Dried blood spot
DMS	Dried matrix spot
DUS	Dried urine Spot
EDTA	Ethylenediarninetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
GC	Gas chromatography
HCT	Hematocrit
IRT	Immunoreactive trypsinogen
ISTD	Internal standard
LC	Liquid chromatography
MDA	Mass drug administration
MRM	Multiple reaction monitoring
MS	Mass spectrometry/ spectrometer
MS/MS	Tandem mass spectrometry/ spectrometer
NBS	Newborn screening
17OHP	17α-hydroxyprogesterone
PhD	Doctor of Philosophy
PKU	Phenylketonuria
TDM	Therapeutic drug monitoring
TSH	Thyroid-stimulating hormone
T4	Thyroxine

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Declaration

I hereby declare under oath that the present dissertation attached to this declaration was prepared independently and without any unauthorized help, that it has not yet been submitted to any other body for examination and that it has not been published either in full or in excerpt. The passages of the work ¬ including tables, illustrations etc., ¬ which are taken from other works and sources (including Internet sources) in terms of wording or meaning, I have identified in each individual case as borrowings with exact reference to the source.

Place:	Date:	Signature:
Muttenz	30.04.2019	S. Gangler

Appendix

Co-authored publications excluded from this compendium

Research article

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Automated high throughput analysis of antiretroviral drugs in dried blood spots

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For therapeutic drug monitoring in remote settings, dried blood spots (DBS) are particularly advantageous, as blood sample collection and handling is uncomplicated. The aim of this study was to develop and validate an automated extraction method for the analysis of nevirapine, efavirenz and lopinavir in DBS samples. Automated extraction was performed with methanol : water (70 : 30 *v/v*), using a DBS-MS 500 autosampler coupled to a liquid chromatography tandem mass spectrometry system. The autosampler used digital images of each DBS to position the extraction head, sprayed 10 μl of internal standard onto each DBS and extracted a 4-mm disc (Ø) from the centre of each spot by unilateral flow using 25-μl extraction solvent. The analytes were baseline separated on a pentafluorophenyl column and analysed by using electrospray ionization with multiple reaction monitoring in positive polarity mode for nevirapine and lopinavir and in negative mode for efavirenz. The method was linear between 10 and 10 000 ng/ml for all analytes. Automated sample extraction resulted in consistent recoveries (nevirapine: 70 ± 6%, efavirenz: 63 ± 11% and lopinavir: 60 ± 10%) and matrix effects between different donors and concentration levels. Intra-day and inter-day accuracy and precision deviations were ≤15%. Manual and automated extractions of DBS samples collected within the framework of an adherence assessment study in rural Tanzania showed good agreements with deviations of less than 10%. Our study highlights that therapeutic drug monitoring samples obtained in the resource-constrained setting of rural Africa can be reliably determined by automated extraction of DBS. Overall, automatization improved method sensitivity and facilitates analysis of large sample numbers. **Copyright © 2017 John Wiley & Sons, Ltd.**

Keywords: nevirapine; efavirenz; lopinavir; liquid chromatography tandem mass spectrometry; dried blood spots; automated extraction

Introduction

The dried blood spot (DBS) technique facilitates minimally invasive blood sampling, whereby capillary blood is spotted onto a filter paper, ideally leading to a homogenous blood spot. After complete drying, a fixed blood spot area can be punched out for drug analysis, making exact pipetting unnecessary.[1] In contrast to conventional plasma sampling, the DBS technique does not require a trained phlebotomist. Only a few drops of blood are withdrawn after a simple and minimally invasive finger prick: a process that can even be performed by adequately instructed patients.[2] Moreover, DBS samples minimize biohazard risk during further sample handling and are generally stable at room temperature.[3] Hence, the collection of DBS samples is especially attractive for field studies in remote or resource-constrained settings, where uninterrupted cold chains cannot be guaranteed.[4]

Worldwide, an estimated 37 million people are HIV positive, of which the majority reside in Sub-Saharan Africa.[5] Antiretroviral therapy leading to viral suppression has been strongly correlated with increase in survival and improved quality of life.[6,7] Good adherence to antiretroviral therapy is paramount, and patients with suboptimal adherence are at risk of HIV progression and the development of drug resistance, which consequently narrows options for future treatment.[6,8] Circulating antiretroviral drug concentrations are characterized by a high degree of between-patient variability, due to genetic and nongenetic heterogeneity in drug disposition. Therapeutic drug monitoring (TDM) is an approach to standardize drug exposure through dosage individualization and to prevent both the toxicity associated with high exposure and the inefficacy associated with insufficient exposure.[9–12] However, particularly in resource-limited settings,

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TDM is infrequently performed due to technical challenges.[6,8,13] In such settings, the previously mentioned advantages of DBS sampling could facilitate implementation of regular TDM.

On the other hand, the development of bioanalytical methods for DBS samples is more complex than for conventional liquid matrices, as variable haematocrit values alter not only analyte recovery but also the extent of blood diffusion within the filter paper, thereby impacting the reliability of the DBS analysis.[14] Moreover, the small amount of blood collected on a filter card requires highly sensitive bioanalytical methods.[14] Finally, concentrations in DBS samples must be compared with conventional plasma samples to allow correct interpretation of DBS measurements.[15–18]

The aim of the present work was to develop and validate a fully automated DBS extraction method for the analysis of nevirapine, efavirenz and lopinavir, with sufficient robustness to handle large sample numbers. Certain steps of the validation procedure such as assessment of extraction recovery and matrix effects are more complex when automated extraction systems are used and require modifications of standard validation procedures. Feasibility of the automated extraction method was demonstrated by comparing concentrations obtained after automated and manual DBS extraction of samples from a combination antiretroviral therapy adherence assessment study conducted at the Chronic Diseases Clinic of Ifakara, Tanzania. The DBS samples of patients treated with the non-nucleoside reverse transcriptase inhibitors efavirenz and nevirapine and the protease inhibitor lopinavir were collected.[19] In contrast to previous studies that used manual DBS extraction for the measurement and validation of combination antiretroviral therapy compounds,[20–23] we extracted DBS by using a fully automated DBS autosampler (CAMAG, DBS-MS 500).[24] This autosampler exhibits a TLC-based extraction head, with a circular plunger that seals a vent of 4-mm inner diameter on the blood spot. The extraction solvent passes horizontally from the inlet capillary through the blood spot to the outlet capillary and into a sample loop (unilateral extraction). Thus, in contrast to other online DBS extraction systems,[25–28] the extraction solvent is not forced vertically through the filter paper (flow-through extraction). The autosampler is connected to a liquid chromatography tandem mass spectrometry (LC–MS/MS) system, features 500 DBS card slots, takes an image of the blood spot before and after the extraction process, sprays the internal standard solution onto each blood spot and works with a low volume (~25 μl) of extraction solvent.

Materials and methods

Chemicals, reagents, and reference compounds

Gradient grade water and methanol for liquid chromatography as well as formic acid (98–100%) were purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, USA). The reference compounds, nevirapine, efavirenz and lopinavir and its deuterated internal standards (IS) nevirapine-d3, efavirenz-d5 and lopinavir-d8 were products of Toronto Research Chemicals (Toronto, Canada). DBS cards (grade 226 filter paper) were kindly provided by CAMAG (Muttenz, Switzerland). Fresh whole blood was obtained from the local blood donation centre (Basel, Switzerland).

LC–MS/MS instrumentation and settings

Chromatography was performed on a modular high-performance liquid chromatography system from Shimadzu (Kyoto, Japan); it contained a system controller (CBM-20A), four pumps (2× LC-20AD and 2× LC-20AD XR), a degasser (DGU-20A5) and a column oven (CTO-20A). A CTC HTS PAL autosampler (CTC analytics, Zwingen, Switzerland) was used in the case of manual extraction of DBS samples. Automated extractions were carried out with a DBS-MS 500 autosampler (CAMAG, Muttenz, Switzerland). Analytes were separated on a Kinetex 2.6 μ F5 100 Å (50 × 2.1 mm) analytical column (Phenomenex, Torrance, USA). A filter frit (SS 0.5 μm 0.62 × 0.65, Ercatech AG, Bern, Switzerland) was connected upstream to the analytical column. Mobile phase A consisted of water plus 0.1% formic acid, while methanol supplemented with 0.1% formic acid was used as mobile phase B. The following stepwise gradient was applied: 5% (0–0.25 min), 5%–60% (0.25–0.4 min), 60%–80% (0.4–2.0 min), 80%–95% (2.0–2.2 min), 95% (2.2–3.0 min) and 5% (3.0–3.3 min). The flow rate was set at 0.5 ml/min at 45 °C. The high-performance liquid chromatography liquid stream was connected to an API 4000 Q-trap tandem mass spectrometer (AB Sciex, Framingham, MA, USA) only between minute 0.6 and 2.5 of each run to reduce system contamination. The analytical run was divided into three multiple reaction monitoring periods, whereas electrospray ionization was switched from positive to negative mode between minute 1.3 and 1.7 (period 2) of each run. The following mass transitions and compound specific settings were used: 267 → 226 *m/z* for nevirapine [declustering potential (DP): 76 V, collision energy (CE): 20 V, entrance potential (EP): 10 V, collision cell exit potential (CXP): 16 V], 270 → 229 *m/z* for nevirapine-d3 (DP): 121 V, CE: 37 V, EP: 10 V, CXP: 16 V), 314 → 244 *m/z* for efavirenz (DP: −95 V, CE: −26 V, EP: −10 V, CXP: −13 V), 319 → 248 *m/z* for efavirenz-d5 (DP: −75 V, CE: −28 V, EP: −1 V, CXP: −15 V), 629 → 155 *m/z* for lopinavir (DP: 111 V, CE: 35 V, EP: 10 V, CXP: 10 V) and 637 → 163 *m/z* for lopinavir-d8 (DP: 66 V, CE: 75 V, EP: 10 V, CXP: 10 V). The general settings of the mass spectrometer were as follows: ion source gas-1 60 l/min (N_2), ion source gas-2 50 l/min (N_2), curtain gas 10 l/min, collision gas 4 l/min, ion spray voltage 5500 V (positive mode) and −4200 V (negative mode) and source temperature 350 °C. ANALYST software 1.6.2 (AB Sciex, Framingham, MA, USA) was used to operate the LC–MS/MS system.

Preparation of standards and extraction solvents

Nevirapine, efavirenz and lopinavir stock solutions were prepared in DMSO (10 mg/ml) and stored at −20 °C. Stock solutions were pooled and serially diluted with DMSO to cover a range from 1000 to 1 μg/ml. The dilution series for calibrators and QC samples originated from different weightings. IS stock solutions were likewise prepared in DMSO at a final concentration of 1 mg/ml. The extraction solvent was a mixture of methanol and water (70 : 30 *v/v*). The IS was dissolved in methanol at a concentration of 1 μg/ml for nevirapine-d3 and at 2 μg/ml for efavirenz-d5 and lopinavir-d8.

Preparation of calibration and quality control samples

Freshly collected human blood was obtained from the local blood donation centre (Basel, Switzerland). Ethylenediaminetetraacetic acid was used as an anticoagulation agent (vacutainer tubes, BD, Allschwil, Switzerland). Calibrators and Quality control (QC) samples were prepared by spiking blank blood with the analyte dilution series in a ratio of 1 : 100 (*v/v*). Calibration samples for automated extractions encompassed a range from 10 to 10 000 ng/ml and 50 to 10 000 ng/m for manual extractions. QC samples were prepared at the lower limit of quantification (LLOQ, 10 ng/ml), as well as at

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low (50 ng/ml), medium (500 ng/ml) and high (5000 ng/ml) concentration levels. In the case of manual extractions, the LLOQ was set to 50 ng/ml. Spiked blood samples were gently mixed and agitated on a roll-agitator (CAT RM 5 Staufen, Switzerland), after which 15-μL aliquots were spotted onto CAMAG DBS cards (CAMAG, Muttenz, Switzerland). DBS cards were dried at room temperature for at least 2 h and were subsequently stored at −20 °C in sealed plastic bags containing desiccants. Each calibration set consisted of 1 blank sample (DBS sample processed without IS), 1 zero sample (DBS sample processed with IS) and 11 calibrators (9 calibrators in the case of manual extractions). Calibration lines were established by linear regression of the nominal analyte concentration against the analyte : IS peak area ratio by using a weighting factor of $1/x^2$.

Dried blood spot sample extraction

Automated extraction

The extraction head was cleaned in an ultra sound bath at 40 °C for 10 min prior each set of analyses because filter paper debris can clog the port after several extractions. Moreover, the internal standard module was primed for at least 3 cycles to clear air bubbles from the syringe. Each extraction solvent was primed for more than 3 cycles, and the rinsing solvents were flushed for 4 min. Twisted DBS cards were pressed overnight under a heavy weight before use in the autosampler.

The DBS cards were photographed with the built-in camera of the DBS-MS 500 autosampler before and after each run to check for the presence of a blood spot and to adjust the extraction head to the centre of each spot. The software of the autosampler automatically recognized inadequate DBS based on their roundness, diameter and area. Inadequate DBS were excluded from analysis. Ten μl of internal standard was sprayed in a homogenous layer onto each spot. After a 20-s drying time, the samples were extracted with a volume of 25 μl and a 40-μl/min flow rate. As a 20-μl loop was installed, the first 5 μl of each extraction was discarded. To complete the automated DBS extraction cycle, the system was first rinsed for 20 s with a methanol : acetonitrile : isopropanol : water (1 : 1 : 1 : 1 *v/v*) mixture, after which it was cleaned for a further 20 s with water containing 0.1% formic acid.

Manual extraction

Ten μl of internal standard was sprayed by using the internal spraying device of the DBS-MS 500 autosampler onto each spot. The card was left to dry at room temperature, and then a disc of 3 mm in diameter was manually punched out from the centre of each spot by using a manual hole puncher (Whatman, Sanford, ME, USA) and transferred to a 0.75-ml autosampler matrix tube (Thermo Scientific, Reinach, Switzerland). Afterwards, 200-μl extraction solvent, methanol : water (70 : 30 *v/v*), was added to each disc. The samples were mixed for 3 min, centrifuged (30 min; 3220 g; 10 °C, Eppendorf, Hamburg, Germany) and kept at 10 °C in the autosampler. To perform the analysis, an aliquot of 20-μl supernatant was injected into the LC–MS/MS system. Subsequently, the system was washed with methanol and a methanol : water mixture (1 : 1 *v/v*).

Method validation

The automated DBS extraction LC–MS/MS method was validated following the FDA guidance for bioanalytical method validation for industry.[29] The method was validated in terms of selectivity, sensitivity, accuracy, precision, linearity, extraction recovery, matrix effect and analyte stability. In addition, the impact of the applied blood volume was evaluated during the validation process.

Selectivity and sensitivity

Blank DBS samples from seven different subjects were examined for interfering endogenous matrix components. The signal at the designated LLOQ was set to be at least five times higher than the noise signal, with a bias in precision of less than 20% and accuracy between 80 and 120%.

Linearity

The coefficients of variation (R^2) of the linear regression, between the analyte peak area, normalized by the internal standard peak area and the nominal concentration, had to be ≥0.99. At least 75% of the calibration samples had to be within ±15% (LLOQ: ±20%) of the nominal value.

Intra-day and inter-day accuracy and precision experiments

The accuracy and precision of the method were determined by analysing QC samples from seven different subjects at four concentration levels (LLOQ, low-level, medium-level and high-level QC). Placing the QC samples between two calibration lines, one spot was analysed per condition (n = 28 QC samples, n = 20 calibrators). Precision and accuracy were evaluated within a single validation run (intra-day) as well as between three runs recorded on different days (inter-day). The precision was calculated as the percentage relative standard deviation (CV, %) for each QC concentration within an analytical run (intra-day precision, n = 7) and over all three runs (inter-day precision, n = 21). A precision of <15% (<20% at the LLOQ) was accepted in our study. The accuracy was assessed from the overall mean of each QC concentration divided by its nominal value (bias, %). A mean accuracy of 85–115% (LLOQ: 80–120%) was acceptable; however, at least 67% of the QC samples of each concentration level had to be within the acceptance range.

In addition, DBS spots using 15 and 30-μl blood were prepared at LLOQ, low, medium and high concentration levels. The change in concentration of 15 to 30 μl spots was calculated. A deviation of ≤15% (LLOQ ≤20%) implied that the method does not depend on the applied volume of blood.

Recovery and matrix effect

The extraction recovery of the DBS-MS 500 autosampler was investigated for DBS samples of seven different subjects. DBS spots at 50, 500 and 5000 ng/ml were prepared for the recovery experiments. Each spot was extracted six times for medium and high concentration samples (500 and 5000 ng/ml), while low concentration samples (50 ng/ml) were extracted three times. Between two extractions, a drying time of approximately 15 min was programmed. Using the built-in camera of the autosampler, the extraction head automatically locked onto the same area in the centre of the blood spot. The recovery was finally estimated as the percentage ratio of the analyte peak area of the first extraction to the sum of the peak areas of all subsequently conducted extractions.

Blank DBS samples from seven different subjects were prepared to quantify the effect of the blood matrix on the analyte signal intensity. The extraction solvent was spiked with 10, 100 or 1000-ng/ml nevirapine, efavirenz and lopinavir. Each blank DBS sample

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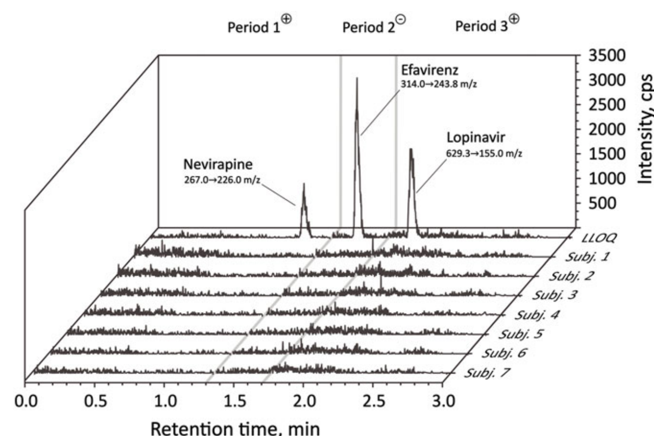

Figure 1. Chromatograms of blank DBS samples (n = 7 donors) were placed next to an LLOQ (10 ng/ml) sample. The method is selective for the quantification of nevirapine, efavirenz and lopinavir in DBS samples. [Colour figure can be viewed at wileyonlinelibrary.com]

and a corresponding card without a blood spot were processed at each concentration level. The matrix effect was calculated as the ratio of the analyte peak areas measured, following extraction of filter cards containing a blank DBS, to the peak areas of filter cards without matrix.

Stability

Stability tests of nevirapine, efavirenz and lopinavir were performed under different conditions at a medium concentration level of 500 ng/ml. Stability was evaluated in the fridge (4 °C) and the freezer (−20 °C) after 4 weeks of storage. Five replicates were analysed per condition and compared with a set of QC samples, prepared on the day of analysis.

Method application

Clinical application of the LC–MS/MS method was demonstrated by analysing a series of randomly selected DBS samples. The DBS samples contained nevirapine, efavirenz and lopinavir and were collected during an adherence assessment study conducted in Tanzania.[19] Ethical approval was obtained from the Institutional Review Board of the Ifakara Health Institute (reference no IHI 28-2013), the Tanzanian National Institute of Medical Research, Dar es Salaam, Tanzania (reference no NIMR/HQ/R.8a/V01. IX/I762) and the Tanzanian Commission for Science and Technology (no 2014-276-NA-2014-195). For each analyte, 30 DBS samples were

Figure 2. Optimization of the extraction parameters for the automated analysis of nevirapine, efavirenz and lopinavir from DBS samples. High concentration QC samples (n = 4) were extracted for each condition. Effect of the methanol : water mixture, extraction volume and extraction flow on the extraction yield was evaluated. Conditions with grey-coloured box plots were selected for the final extraction method. The percent figures above the boxes are CV%.

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processed by automated and manual extraction. In both cases, the IS was sprayed onto the DBS. Bland–Altman plots were created with GRAPHPAD PRISM 6.04 (La Jolla, CA, USA) to compare the two extraction methods.[30] Mean-difference plots were generated by using the mean %difference bias [%difference = (concentration automated extraction − concentration manual extraction/mean concentration) • 100] and the 95% limits of agreement (±2 standard deviations). At least 67% of the samples had to be within ±20% limits according to cross-validation guidelines.[31,32]

Results and discussion

Method development

Assessment of adherence to antiretroviral therapy is essential to assure sufficient viral suppression and improve survival and quality of life in HIV-infected patients. While adherence in industrialized nations where TDM belongs to the standard of care generally is high, less is known about adherence to antiretroviral treatment in resource-limited countries. In such settings, the advantages of DBS samples could facilitate monitoring of antiretroviral therapy. Importantly, it has been demonstrated that antiretroviral drugs can be analysed in DBS samples, and moreover, a good correlation between plasma and DBS concentrations has been found for efavirenz and nevirapine.[20–23,33,34] However, compared with conventional plasma or serum samples, working with DBS samples entails several method-specific drawbacks. The preparation of calibrator and QC samples, as well as the extraction procedure, is more laborious. Moreover, the small amount of blood available in the DBS sample is a challenge and requires development of particularly sensitive methods. Here, we demonstrate that these challenges can, at least, in part, be overcome for the analysis of nevirapine, efavirenz and lopinavir by using a DBS autosampler system.

In the first stage of method development, mass spectrometer voltages were adjusted to maximize the peak response of the parent mass and the product ions for each compound. The best results were obtained by using the transitions 267 → 226 *m/z*, 314 → 244 *m/z* and 629 → 155 *m/z* for nevirapine, efavirenz and lopinavir, respectively. Similar transitions were used for the internal standards, with the addition of the respective amount of deuterium atoms (nevirapine-d3: 270 → 229 *m/z*, efavirenz-d5: 319 → 248 *m/z*, lopinavir-d8: 637 → 163 *m/z*). The same MS/MS transitions were also used in other published LC–MS/MS methods.[20,21,23,33] Nevirapine and lopinavir were optimized in positive ionization mode, while efavirenz was optimized in negative ionization mode. Therefore, efavirenz had to be chromatographically separated from nevirapine and lopinavir, as negative and positive ionization modes cannot be run in parallel with the employed mass spectrometer. Figure 1 illustrates that baseline separation of all analytes was

Table 1. Intra-day and inter-day accuracy and precision of nevirapine, efavirenz and lopinavir (10–10 000 ng/ml)

Analyte		Intra-day						Inter-day	
		Day 1		Day 2		Day 3		Day 1–3	
	QC level [ng/ml]	Conc. found at [ng/ml]	Accuracy ± CV [%]	Conc. found at [ng/ml]	Accuracy ± CV [%]	Conc. found at [ng/ml]	Accuracy ± CV [%]	Conc. found at [ng/ml]	Accuracy ± CV [%]
Nevirapine	10	9.4	94.1 ± 7.6	10.8	108.4 ± 6.8	10.8	107.9 ± 8.6	10.3	103.4 ± 9.8
	50	44.5	88.9 ± 8.1	47.5	95 ± 13.9	47.9	95.7 ± 6.7	46.6	93.2 ± 10.2
	500	470	93.9 ± 5.2	466	93.3 ± 4.7	481	96.1 ± 6.8	472	94.4 ± 5.5
	5000	4750	95 ± 7.4	4680	93.6 ± 5.4	4923	98.5 ± 9.9	4784	95.7 ± 7.8
Efavirenz	10	9.2	92.4 ± 8.1	9.5	95.2 ± 14.5	10.6	105.7 ± 10.4	9.8	97.8 ± 12.3
	50	46.7	93.5 ± 8.6	47.1	94.2 ± 11.5	49.6	99.2 ± 9.2	47.8	95.6 ± 9.7
	500	479	95.9 ± 5.2	475	94.9 ± 4.6	489	97.7 ± 6.6	481	96.2 ± 5.4
	5000	4767	95.3 ± 8.4	4593	91.9 ± 8	4861	97.2 ± 9	4741	94.8 ± 8.4
Lopinavir	10	9.7	96.8 ± 6.2	10.0	99.7 ± 12	10.9	109.3 ± 8.5	10.2	101.9 ± 10.3
	50	46.2	92.4 ± 10.3	45.8	91.5 ± 13.3	47.0	94.1 ± 10.1	46.3	92.7 ± 10.8
	500	486	97.2 ± 6.9	453	90.6 ± 6.3	461	92.2 ± 7.2	467	93.3 ± 7.2
	5000	4757	95.1 ± 9.4	4474	89.5 ± 8.9	4583	91.7 ± 12.2	4605	92.1 ± 10.1

Table 2. Deviation between DBS samples using 15 or 30-µl blood spot volume

Qc level [ng/ml]	Nevirapine			Efavirenz			Lopinavir		
	Concentration found at [ng/ml]			Concentration found at [ng/ml]			Concentration found at [ng/ml]		
—	15 µl	30 µl	Change [%]	15 µl	30 µl	Change [%]	15 µl	30 µl	Change [%]
10	9.4	9.5	0.7	9.2	9.7	5.1	9.7	9.5	−1.7
50	44.5	44.8	0.6	46.7	45.1	−3.4	46.2	43.2	−6.5
500	470	479	2.0	479	478	−0.2	486	455	−6.4
5000	4750	4986	5.0	4767	4790	0.5	4757	4710	−1.0

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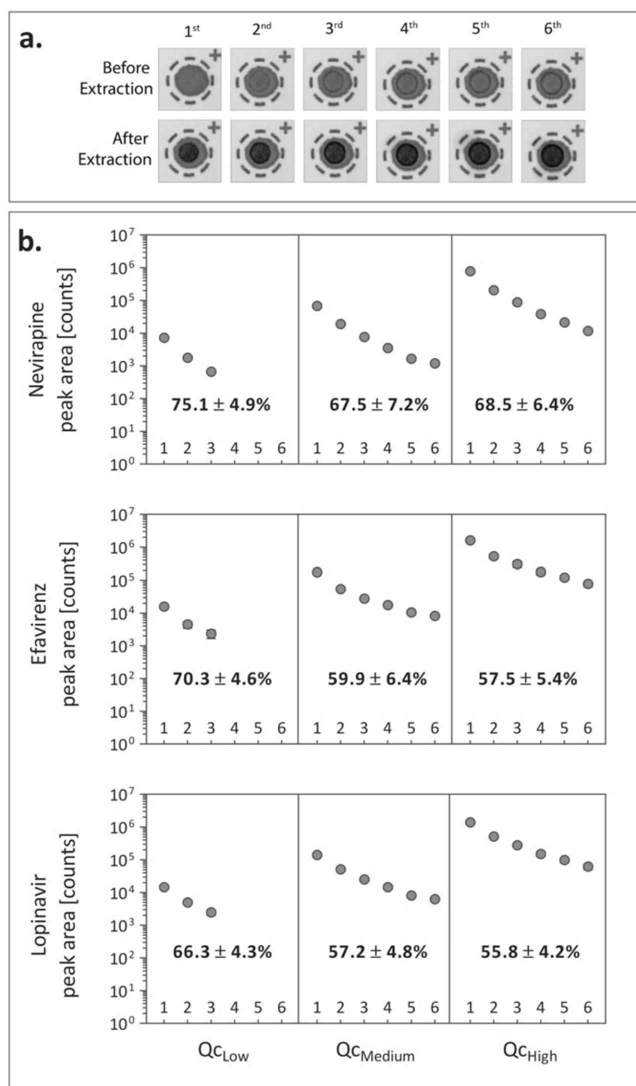

Figure 3. (a) Pictures of DBS before and after six consecutive extractions. Using the built-in camera of the autosampler, the extraction head automatically locked on the same position in the centre of the blood spot. (b) Extraction recovery of nevirapine, efavirenz and lopinavir determined at low, medium and high concentrations of four different donors. Decrease in peak area after six (medium and high QC) or three (low QC) repetitive extractions is shown. Recoveries were consistent between different subjects and over different concentrations. Bold numbers are recoveries +/− CV%.

efficiently achieved within a 3-min run time, using a core-shell pentafluorophenyl phase column. Peak symmetry was satisfactory using methanol and water supplemented with formic acid (0.1%) as mobile phase.

Further method development focused on improving the automated extraction process where the main adjustable instrument parameters are the amount of IS sprayed onto the DBS, as well as the extraction flow, extraction volume and extraction solvent composition. Parameters giving the highest signal intensities, best precisions and optimal peak shapes were selected (Fig. 2). First, different methanol : water mixtures (50 : 50, 60 : 40, 70 : 30, 80 : 20 and 90 : 10 *v/v*) were tested. A high water amount increased the risk of clogging the extraction head, as presumably more biomolecules and cellular components are removed from the DBS. Robustness of the extraction was thereby limited. Overall, a mixture of 70 : 30 methanol : water resulted in the highest signal intensities and best precision. Peak symmetry was disturbed by methanol concentrations exceeding 80% in the extraction solvent. Replacing methanol with acetonitrile did not improve extraction yield and worsened peak shapes. The extraction volume was increased stepwise from 20 to 70 μl (20, 25, 30, 40, 50, 60 and 70 μl), while the last 20 μl of each extraction were trapped in the sample loop. Signal intensities decreased with larger extraction volumes to about 80–90% of the initial value. Almost 50% is extracted within the first 10 μl of the extraction fraction. An extraction volume of 25 μl was selected because the precision was enhanced compared with 20 μl, and the signal intensity was only marginally lower. As extraction flow did not seem to have a significant impact, the standard setting of 40-μl/min flow was used to perform automated extraction. The wash cycle was optimized in order to reduce analyte carry-over. The signal in a blank sample after injection of the highest calibrator was ten times lower than the signal detected at LLOQ. Hence, carry-over of the automated extraction of the analytes is negligible.

Method validation

Selectivity and sensitivity

Selectivity of the method was tested for interfering matrix components in seven blank human DBS samples (Fig. 1). Noise level baselines of the blank samples did not show coeluting peaks at the retention time of nevirapine (1.1 min), efavirenz (1.5 min) or lopinavir (1.9 min). Moreover, internal standards that were sprayed onto blank DBS spots did not cause interfering signals. Hence, the developed method is selective for the analysis of the investigated antiretrovirals.

A sensitivity of 10 ng/ml was achieved for all analytes. Based on published data, we expect this quantification limit to be sufficient to perform therapeutic monitoring of nevirapine, efavirenz and lopinavir.[35] Figure 1 illustrates that the signal intensity at LLOQ is at least five times higher than the background noise level.

Linearity

Linearity was attained over a calibration range of 10 to 10 000 ng/ml for all analytes. Taking all validation experiments into account, the coefficient of variation (R^2) was always >0.99 for each

Table 3. Matrix effect (ME) of nevirapine, efavirenz and lopinavir in DBS samples

Concentration	Nevirapine		Efavirenz		Lopinavir	
	ME ± CV [%]	Mean ± CV [%]	ME ± CV [%]	Mean ± CV [%]	ME ± CV [%]	Mean ± CV [%]
10 ng/ml	92.2 ± 1.0	92.0 ± 0.4	52.7 ± 4.9	54.8 ± 10.4	100.6 ± 2.0	94 ± 6.2
100 ng/ml	91.5 ± 1.8	—	50.4 ± 5.4	—	91.5 ± 2.0	—
1000 ng/ml	92.1 ± 3.4	—	61.2 ± 11.0	—	89.7 ± 2.0	—

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Table 4. Stability of nevirapine, efavirenz and lopinavir evaluated under different conditions. DBS samples at a concentration of 500 ng/ml were used

Condition	Nevirapine			Efavirenz			Lopinavir		
	Concentration [ng/ml]	Accuracy ± CV [%]	Change in concentration [%]	Concentration [ng/ml]	Accuracy ± CV [%]	Change in concentration [%]	Concentration [ng/ml]	Accuracy ± CV [%]	Change in concentration [%]
RT	519.5	103.9 ± 3.6	—	481.6	96.3 ± 6.1	—	532.7	106.5 ± 3.3	—
4 weeks, 4 °C	532.4	106.5 ± 7.3	2.5	511.1	102.2 ± 8.8	6.1	537.2	107.4 ± 8.2	0.8
4 weeks, −20 °C	517.4	103.5 ± 7.2	−0.4	477.7	95.5 ± 8.2	−0.8	559.2	111.8 ± 6.7	5.0

analyte. The upper limit of quantification also encompasses high therapeutic through concentrations.[11,12,36,37] This is especially important for the automated extraction of DBS, as dilution of the DBS samples cannot be easily performed. Thus, clinically occurring concentrations can be quantified by linear regression within the chosen calibration range.

Accuracy and precision

Intra-day and inter-day accuracy and precision data are summarized in Table 1. QC samples were derived from seven donors. Intra-day precisions of less than 13.9%, 14.5% and 13.3% were found for nevirapine, efavirenz and lopinavir respectively. Mean intra-day accuracies were between 88.9 and 108.4% for nevirapine, 91.9 and 105.7% for efavirenz and 90.6 and 109.3% for lopinavir. Inter-day precisions were ≤12.3% for all three analytes. On all occasions, not more than two out of the seven QC samples per concentration were outside the 85–115% (LLOQ: 80–120%) limits.

In addition, concentrations of QC samples differed by less than 6.5% when 30 μl instead of 15 μl of blood was spotted onto cards (Table 2). Hence, the applied blood volume produced reproducible, volume-dependent spot areas and therefore did not affect accuracy and precision of the analysis. Overall, the results were consistent with conditions specified by regulatory guidelines.[29]

Recovery and matrix effect

The relative recovery and matrix effect was determined in four different donors at low, medium and high QC levels (Fig. 3). In addition, the recovery of the analytes was assessed by using aqueous analyte solutions spotted onto the filter paper ('dried water spots'). The highest mean recovery was achieved for nevirapine with 70%, followed by efavirenz (63%) and lopinavir (60%). A bias in recovery of less than 7.2% was determined between subjects, indicating high consistency in the sample extraction process. Recovery at low concentration was overestimated by approximately 10% compared with medium and high concentration samples, as the limit of quantification was already reached after three instead of six consecutive extractions. The recovery of dried water spots was >97%, indicating that blood acts as an extraction barrier.

The signal intensity of nevirapine and lopinavir was only suppressed by the DBS matrix by about 8% and 6% respectively (Table 3). However, a significant suppressive matrix effect was observed for efavirenz, where the signal was reduced to approximately 50% due to the biological matrix. Importantly, the matrix effect was independent of concentration, as its deviation was ≤10.4% over the tested concentration range and it did not vary between subjects (CV ≤ 11.0%). Hence, the matrix of different donors did not alter the reliability of the method. Online solid phase

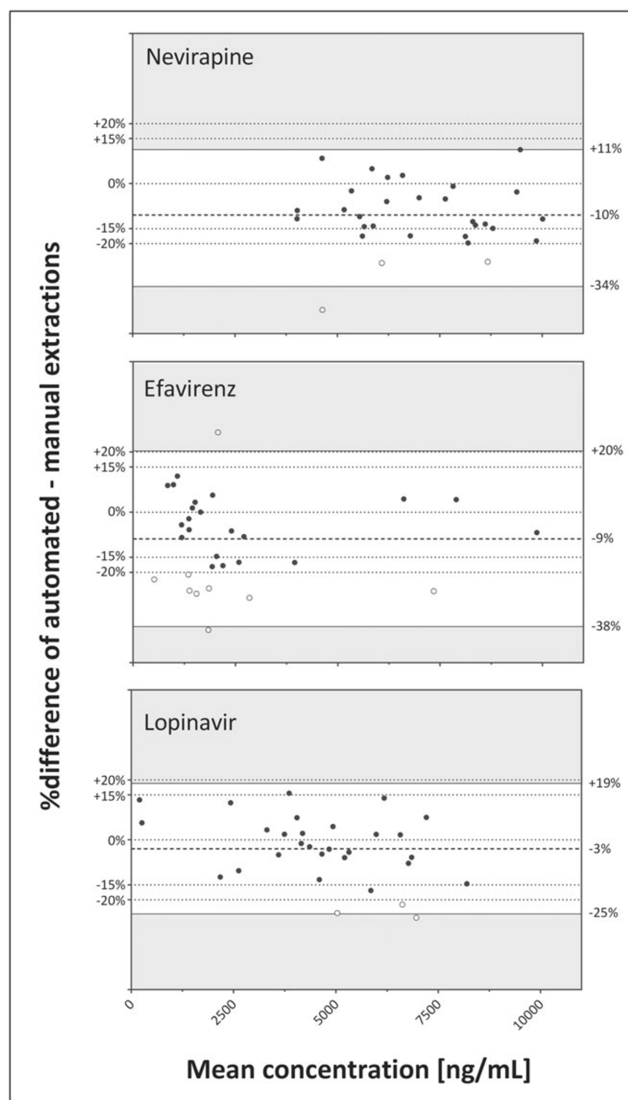

Figure 4. Bland–Altman plots of nevirapine, efavirenz and lopinavir were generated for automated and manual DBS extractions. Values were coloured grey if the %difference of the two extraction methods was less than ±20%; otherwise, values were coloured white. The white plot area illustrates the 95% limits of agreement. The black dashed line describes the mean percentage difference between automated and manual extraction.

purification of DBS extracts, via column switching or by more thorough separation of efavirenz and blood ingredients, may eliminate suppressive matrix effects.[38,39] In summary, the automated extraction was reproducible and delivered consistent matrix effects.

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Stability

Stability data are summarized in Table 4. The data implied that analytes were stable in DBS for 4 weeks at −20 °C or 4 °C, as the change in concentration compared with a freshly prepared DBS sample was less than 6.1%. These results are in line with stability data published in previous studies.[23,33,40,41] Therefore, DBS samples containing nevirapine, efavirenz and lopinavir are not likely to be degraded by storage in the fridge for 1 month.

Method application

Ninety DBS samples (30 DBS per analyte) collected in the framework of a drug adherence study in Ifakara (Tanzania) were analysed after manual extraction as well as after automated extraction using the DBS-MS 500 autosampler. Nevirapine concentrations in DBS were between 3360 and 10 800 ng/ml, efavirenz concentrations were between 262 and 10 200 ng/ml, and lopinavir concentrations were between 41.8 and 8 800 ng/ml; thus, the selected calibration range was suitable for the quantification of the collected TDM samples. Sample dilution was therefore not necessary, which is relevant because it cannot be easily performed through automated extraction.

Overall, the results obtained after automated and manual extractions were in good agreement (Fig. 4). The mean bias of automated to manual extractions was −10.5% for nevirapine, −8.9% for efavirenz and −3.0% for lopinavir. Ninety-five per cent limits of agreement were narrow with a range of about ±25% (nevirapine: −32.3 to +11.3%; efavirenz: −38.1% to +20.4%; lopinavir: −24.7% to +18.8%). Consequently, when comparing automated and manually extracted samples, 70% or more of the respective samples showed a deviation of less than 20%, according to cross-validation guidelines.[31,32] No concentration-dependent trend was observed between the ratios of the two extraction methods across the entire concentration range. Overall, automated extraction was five times more sensitive than manual extraction, which is mainly due to DBS being extracted with less solvent through the automated sample workup (25 *vs* 150 μl) resulting in more concentrated samples. Both extraction procedures achieved similar results, and the data obtained were in agreement with afore mentioned criteria.[31] A thorough clinical validation including the comparison of plasma and DBS samples will be published elsewhere.

Conclusions

We developed and validated an innovative LC–MS/MS method by using on-line DBS extraction for the quantification of three frequently used antiretroviral drugs. The short time of approximately 3 min to extract and analyse one DBS sample makes the method suitable for handling larger sample loads. Automated DBS extraction entails specific challenges regarding method validation and requires novel approaches to assess, e.g. extraction recovery and matrix effects. The calculated interindividual recoveries and matrix effects were very consistent, and validation parameters were in accordance with regulatory guidelines. The method was successfully applied to analyse TDM samples from a field study. The results obtained by automated and manual extraction were comparable; however, sample processing time and method sensitivity were improved by the automated extraction procedure. Nevertheless, a comprehensive clinical validation comparing DBS with plasma samples is required before the developed method can be implemented for TDM in daily practice.

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Conflict of interest

Stefan Gaugler is an employee of CAMAG (Muttenz, Switzerland). None of the other authors reports any conflict of interest regarding this study.

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Using dried blood spots to facilitate therapeutic drug monitoring of antiretroviral drugs in resource-poor regions

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Objectives: We evaluated whether dried blood spots (DBS) are suitable to monitor combined ART when samples are collected in rural Tanzania and transported over a long distance to a specialized bioanalytical laboratory.

Methods: Plasma and DBS samples were collected in Tanzania from study patients treated with nevirapine, efavirenz or lopinavir. In addition, plasma, whole blood and DBS samples were obtained from a cohort of HIV patients at the site of the bioanalytical laboratory in Switzerland. DBS samples were analysed using a fully automated LC-MS/MS method.

Results: Comparison of DBS versus plasma concentrations of samples obtained from the bridging study in Switzerland indicated an acceptable bias only for nevirapine (18.4%), whereas for efavirenz and lopinavir a pronounced difference of −47.4% and −48.1% was found, respectively. Adjusting the DBS concentrations by the haematocrit and the fraction of drug bound to plasma proteins removed this bias [efavirenz +9.4% (−6.9% to +25.7%), lopinavir +2.2% (−20.0% to +24.2%)]. Storage and transportation of samples from Tanzania to Switzerland did not affect the good agreement between plasma and DBS for nevirapine [−2.9% (−34.7% to +29.0%)] and efavirenz [−9.6% (−42.9% to +23.8%)]. For lopinavir, however, adjusted DBS concentrations remained considerably below [−32.8% (−70.4% to +4.8%)] corresponding plasma concentrations due to decay of lopinavir in DBS obtained under field conditions.

Conclusions: Our field study shows that the DBS technique is a suitable tool for therapeutic drug monitoring in resource-poor regions; however, sample stability remains an issue for certain analytes and therefore needs special consideration.

Introduction

Therapeutic drug monitoring (TDM) is an inherent part of medical care in industrialized countries.[1] It is an important tool for physicians not only to guide dose adaptation but also to assess patients' adherence. However, well-equipped and specialized laboratories are required, which often are not available in resource-poor regions.[2–4] To promote further the TDM in developing countries, blood sampling techniques are needed that allow collection of patient samples easily and at low-cost under field conditions.[5] Moreover, the samples should be stable under local climate conditions, as uninterrupted cold-chains are often not available.[6] Using dried blood spots (DBS) offers many advantages in this context. Collecting a few capillary blood drops from the fingertip is minimally invasive and does not require a trained phlebotomist.[7] Moreover, the drying of blood drops on filter paper in many cases improves sample stability compared with the use of wet matrices, which often allows storage at room temperature and reduces the biohazard risk.[8]

The DBS technique was introduced in the 1960s and is nowadays still routinely used in neonatal screening, but less frequently in highly regulated areas, e.g. in drug development or for routine TDM of small molecules.[9] Literature data are usually based on plasma and not on capillary blood concentrations, so that the

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relationship between the two matrices has to be understood for the correct interpretation of DBS measurements. In addition, bioanalytical aspects, e.g. lower assay sensitivity, analytical bias caused by variable haematocrit values and the complexity of extraction, limit the widespread acceptance of the DBS technique.[10] Nevertheless, recent technical advances in the automation of DBS extraction and analysis have led to more time-efficient, robust and sensitive measurements.[10,11]

TDM is a valuable tool to improve efficacy and safety in HIV therapy by identifying patients with toxic, subtherapeutic or appropriate drug concentrations.[12] To maintain sufficient viral suppression, which is linked to increased survival and reduced morbidity, patients must adhere to $\geq$95% of the prescribed dosages.[13–16] In addition, insufficient drug exposure increases the risk of HIV progression and the development of drug resistance. Worldwide an estimated 37 million people are infected with HIV; the highest prevalence can be found in Sub-Saharan Africa.[17] Considering the previously mentioned advantages of DBS, the technique could help to establish TDM as an important tool in the management of patients with HIV living in rural areas with high HIV prevalence.

The aim of this study was to evaluate whether DBS are suitable to monitor combined ART, when samples are collected in rural Tanzania and transported over a long distance to a specialized bioanalytical laboratory. DBS, whole blood and plasma TDM samples were acquired at the site of the bioanalytical laboratory (the bridging study) from patients treated with either the NNRTIs efavirenz or nevirapine, or the PI lopinavir. In addition, DBS and plasma samples were collected in Tanzania (the field study[18]) and transported to Switzerland. DBS samples were extracted using a fully automated system to facilitate the analysis of the large number of study samples.

Patients and methods

Ethics

Ethics approval for the field study was received from the Institutional Review Board of the Ifakara Health Institute (no. IHI 28-2013), the Tanzanian National Institute of Medical Research, Dar es Salaam, Tanzania (no. NIMR/HQ/R.8a/V01. IX/I762) and the Tanzanian Commission for Science & Technology (no. 2014-276-NA-2014-195). For the bridging study, anonymized samples from patients enrolled in the Swiss HIV Cohort Study were used. Ethics approval was received from the ethics committee of Northwest and Central Switzerland (no. EK 76/13). The bridging study was conducted from April until June 2013, whereas the field study samples were collected from October 2013 to September 2014. Informed consent was obtained from all participants.[18]

Study design

Bridging study

Sixty HIV-positive patients followed at the University Hospital Basel, Switzerland, who received either nevirapine ($n = 20$), efavirenz ($n = 20$) or lopinavir ($n = 20$) were enrolled in the bridging study. Patients were treated with a median oral dose of 400 mg nevirapine (range 200–400 mg), 600 mg efavirenz (range 200–800 mg) or 800 mg lopinavir (range 400–800 mg). Plasma, whole blood and DBS samples were collected simultaneously during a routine clinical visit. Whole blood was sampled by venepuncture from the antecubital vein into EDTA-containing tubes. Plasma was obtained by centrifugation using standard settings. DBS samples were collected via capillary puncture from the tip of the middle or ring finger. The first blood drop was discarded and subsequent drops of blood were transferred onto DBS filter paper cards (226 grade; PerkinElmer, Waltham, MA, USA). DBS samples were dried for 2 h at room temperature and placed in small plastic bags containing a desiccant. All samples were stored at −20°C until analysis to ensure sample stability.

Field study

DBS and the matching plasma samples (nevirapine $n = 182$, efavirenz $n = 481$ or lopinavir $n = 64$) were collected within the framework of an adherence assessment study in Tanzania, including 299 patients.[18] Between one and three samples were collected per patient on different occasions. Patients were treated with a single daily dose of 600 mg efavirenz, two daily doses of 200 mg nevirapine, or lopinavir/ritonavir 400/100 mg twice daily. Plasma and DBS samples were collected as described above. Plasma samples were stored at −20°C and shipped on dry ice to the bioanalytical laboratory (Basel, Switzerland). DBS samples were stored in the fridge and shipped at room temperature. At the bioanalytical laboratory, plasma and DBS samples were stored at −80°C and analysed after all samples had been received to minimize inter-batch variability.

Bioanalytical analyses

The antiretrovirals (ARVs) were analysed in DBS using a recently developed fully automated LC-MS/MS method.[11] Plasma and DBS samples were analysed using methods validated according to the FDA guideline on bioanalytical method validation.[19] A qualified method was used for the analysis of whole blood samples (see Table S1, available as Supplementary data at *JAC* Online). More details about the method and the performance of the analyses are given in the Supplementary data. A precision of 15% and a mean accuracy of 85%–115% were accepted in this study.

Stability of the ARV was assessed in DBS under accelerated storage conditions mimicking the climate in Tanzania. Stability was evaluated after 1 day and after 1, 2, 3 and 4 weeks of storage at 40°C and 75% relative humidity (rH). Five replicates (500 ng/mL) were analysed and compared with a set of freshly prepared samples. Samples were considered stable if the change in concentration was <15%.

Statistical analyses

The level of agreement of the ARV concentrations measured in DBS and plasma was assessed using Bland–Altman analysis. The measured concentrations in the different biofluids were compared pairwise by plotting the percentage differences ($\%difference = \frac{concentration\ in\ fluid\ 1 - concentration\ in\ fluid\ 2}{mean\ concentration} \times 100$) against their mean values. The mean deviation and the 95% limits of agreement were calculated. DBS concentrations were adjusted with the patient's haematocrit and with the fraction of drug bound to plasma protein (f_{bpp}) as proposed by Kromdijk *et al.*[20] ($[Analyte]_{Plasma} = \frac{[Analyte]_{DBS}}{(1-HCT)} \times f_{bpp}$). The haematocrit was determined for each patient while the f_{bpp} used for nevirapine, efavirenz and lopinavir was 0.6, 0.995 and 0.98, respectively.[20,21] Another algorithm for plasma and DBS concentrations was evaluated ($[Analyte]_{Plasma} = \frac{[Analyte]_{DBS}}{(1-HCT)+K_{RBC/plasma} \times HCT}$), which considers the haematocrit as well as the partitioning of the drug between plasma and red blood cells ($K_{RBC/plasma}$).[22] $K_{RBC/plasma}$ was calculated based on the measured haematocrit, and the whole blood and plasma concentrations from the participants of the bridging study. The mean $K_{RBC/plasma}$ ($n = 20$) was then determined for each analyte by rearranging the equation published by Jager *et al.*[22]

A cross-validation of the concentrations obtained in the different study sample matrices was performed according to bioanalytical method validation guidelines, whereby at least 67% of the samples were required to be

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Table 1. Nevirapine, efavirenz and lopinavir concentrations [median (IQR)] determined in plasma, DBS and whole blood

	Nevirapine		Efavirenz		Lopinavir	
Matrix	bridging study (Switzerland)	field study (Tanzania)	bridging study (Switzerland)	field study (Tanzania)	bridging study (Switzerland)	field study (Tanzania)
Plasma	5305 (3495–6708)	7240 (5050–10 125)	2705 (1963–3468)	2630 (1895–4225)	9245 (6720–14 125)	8870 (6645–11 350)
DBS	6800 (4255–7598)	7325 (5070–10 625)	1575 (1295–2250)	1530 (1105–2505)	6120 (4563–8063)	4005 (3103–5395)
Whole blood	5650 (3713–7455)	ND	1580 (1253–2230)	ND	5840 (4108–7318)	ND

ND, not determined. All concentrations are in ng/mL.

within the ±20% limits.[23,24] Statistical analyses were carried out with GraphPad Prism 7 (La Jolla, CA, USA).

A subtherapeutic drug concentration was defined as any concentration below the 2.5th percentile of published population-based pharmacokinetic models for the single daily dose of 600 mg efavirenz,[25] two daily doses of 200 mg nevirapine[26] and lopinavir/ritonavir 400/100 mg twice daily.[27]

Results

Participant characteristics

Sixty participants were enrolled in the bridging study, with an equal number of patients receiving treatment either with nevirapine, efavirenz or lopinavir. Of the participants, 65% were male and the median age was 50 years (range 27–73 years). The median haematocrit was 42% and ranged between 30% and 52%. Samples were withdrawn at a median of 11 h (range 1–36 h) post treatment.

The characteristics of the patients enrolled in the field study were summarized by Erb *et al.*[18] In brief, samples were collected within 2–24 h post-dosing, which was comparable to the bridging study. Haematocrits were generally lower than in the bridging study with a median of 35.9% and a range from 10.9% to 50.8%. Concentrations determined in plasma, whole blood and DBS are summarized in Table 1.

RBC partitioning

$K_{RBC/plasma}$ of the ARV was calculated based on plasma and blood data obtained within the bridging study. Concentrations measured in blood and plasma following nevirapine treatment resulted in a mean $K_{RBC/plasma}$ of 1.15 (95% CI 1.07–1.24). In comparison, the $K_{RBC/plasma}$ for efavirenz and lopinavir indicated that only a small amount of efavirenz [0.13 (95% CI 0.08–0.18)] and almost no lopinavir [–0.039 (95% CI −0.10–0.02)] distributed into the red blood cell (RBC).

Agreement between plasma and blood samples

The percentage differences between blood and plasma concentrations for nevirapine, efavirenz and lopinavir were consistent without a trend across the monitored concentration range. Blood versus plasma concentrations indicated only a small bias of +5.7% for nevirapine (Table 2). In contrast, comparison of efavirenz and lopinavir blood and plasma concentrations displayed a pronounced difference of −47.8% and −55.2%, respectively. The 95% limits of agreement for all three drugs were narrow with a range of less than ±25%. For nevirapine, no correction between plasma and blood was necessary, because the difference of all samples is already within the ±20% limits (Table 2). Efavirenz and lopinavir blood concentrations had to be adjusted by the haematocrit and the $K_{RBC/plasma}$ or f_{bpp} to estimate correctly the concentrations in plasma, due to the low amount of drug distributing into the RBCs. Both correction modalities significantly improved the agreement between plasma and blood concentrations so that the mean percentage difference was <9% and at least 95% of the samples were within the ±20% limits (Table 2).

Agreement between plasma and DBS samples

Overall, the percentage differences between plasma and DBS samples did not display a trend across the observed concentrations (Figures 1 and 2).

The mean percentage difference of nevirapine plasma and DBS concentrations was higher in the bridging study (+18.4%) compared with the field study (+3.6%). Thus, only 60% of the nevirapine samples were within the ±20% limits in the bridging study, whereas 87% of the field study samples were within these boundaries (Figures 1 and 2). Correction of the DBS samples by the haematocrit and $K_{RBC/plasma}$ improved the agreement between plasma and DBS samples of the bridging study samples so that 90% of the samples were within ±20%. However, the agreement was not improved when adjusting the DBS concentrations by the haematocrit and the protein binding.

Efavirenz concentrations found in DBS were very similar to levels measured in whole blood, but amounted to only about half of the concentration found in plasma. The mean percentage differences of the DBS and plasma samples collected within the bridging (−47.4%) and the field study (−52.0%) were similar, but samples from Tanzania were more scattered, leading to wider 95% limits of agreement. Almost none of the plasma and DBS samples exhibited a difference of <20% if no correction was applied to DBS samples. Correction by the haematocrit and the protein binding or the $K_{RBC/plasma}$ significantly improved the agreement between plasma and DBS measurements. Both correction functions nullified the bias between plasma and DBS samples in case of the bridging study so that no samples had a >20% deviation and the mean percentage difference was <10%. The agreement between efavirenz plasma and DBS samples collected in Tanzania was improved by both correction modalities as well, resulting in a mean percentage

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Table 2. Percentage differences (95% limits of agreement) determined for nevirapine, efavirenz and lopinavir between different matrices

	Bridging study (Switzerland)				Field study (Tanzania)	
Analyte/correction	plasma and blood	no. within ±20% limits (%)	plasma and DBS	no. within ±20% limits (%)	plasma and DBS	no. within ±20% limits (%)
Nevirapine						
no correction	+5.7 (−6.1 to +17.7)	20 (100)	+18.4 (+2.1 to +34.8)	12 (60)	+3.6 (−25.0 to +32.1)	158 (87)
corrected by HCT and f_{bpp}	+7.7 (−12.3 to +27.8)	18 (90)	+20.3 (−6.3 to +46.9)	9 (45)	−2.9 (−34.7 to +29.0)	142 (78)
corrected by HCT and $K_{RBC/plasma}$[a]	−0.4 (−12.6 to +11.8)	20 (100)	+12.4 (−3.7 to +28.5)	18 (90)	−1.7 (−30.3 to +26.8)	160 (88)
Efavirenz						
no correction	−47.8 (−65.2 to −30.4)	0 (0)	−47.4 (−63.9 to −30.9)	0 (0)	−52.0 (−84.8 to −19.3)	2 (0.4)
corrected by HCT and f_{bpp}	+9.0 (−6.6 to +24.4)	19 (95)	+9.4 (−6.9 to +25.7)	20 (100)	−9.6 (−42.9 to +23.8)	364 (76)
corrected by HCT and $K_{RBC/plasma}$[a]	−0.3 (−16.0 to +15.3)	20 (100)	+0.1 (−15.7 to +16.0)	20 (100)	−16.1 (−48.9 to +16.8)	379 (58)
Lopinavir						
no correction	−55.2 (−77.6 to −33.0)	0 (0)	−48.1 (−69.0 to −27.2)	0 (0)	−72.3 (−107.5 to −37.2)	0 (0)
corrected by HCT and f_{bpp}	−5.6 (−23.1 to +11.9)	19 (95)	+2.2 (−20.0 to 24.2)	19 (95)	−32.8 (−70.4 to +4.8)	16 (25)
corrected by HCT and $K_{RBC/plasma}$[a]	−3.6 (−21.1 to 13.9)	20 (100)	+4.2 (−17.8 to + 26.2)	18 (90)	−31.0 (−68.6 to +6.7)	17 (27)

f_{bpp}, fraction of drug bound to plasma protein; $K_{RBC/plasma}$, partition of the drug between plasma and RBCs; HCT, haematocrit.
[a]$K_{RBC/plasma}$ of 1.15, 0.13 and 0 was used for nevirapine, efavirenz and lopinavir, respectively. Values were determined from *in vivo* blood and plasma drug distribution data.

difference of less than −16.1%. Seventy-six percent of the samples were within the specified limits of ±20% after adjusting DBS concentrations by the haematocrit and the protein binding, and thus passed the criterion of 67% for cross-validation.

Lopinavir concentrations measured in DBS were more than 50% lower than in plasma, similar to the observation accounted for in efavirenz samples. Furthermore, the 95% limits of agreement between plasma and DBS were similar compared with plasma and blood samples. Adjusting the lopinavir concentrations in DBS samples from the bridging study based on their haematocrit and the protein binding or $K_{RBC/plasma}$ resulted in a substantial improvement of the agreement. More than 90% of the samples were within 20% limits and the mean percentage difference was less than +5%. However, lopinavir concentrations in DBS samples from Tanzania were still ∼30% lower than in plasma after correction, and only a quarter of the samples were within ±20%.

Overall, 4.2% ($n = 20$), 2.2% ($n = 4$) and 6.3% ($n = 4$) of the measured efavirenz, nevirapine and lopinavir plasma samples, respectively, were in a subtherapeutic range (Figure S1). The fractions of subtherapeutic samples increased to 14.0% ($n = 67$) for efavirenz and to 10.9% ($n = 7$) for lopinavir if calculations were based on uncorrected DBS measurements. For nevirapine, DBS analysis revealed the same percentage of subtherapeutic samples as for plasma analysis. Interpretation of the TDM samples using DBS instead of plasma was comparable if the DBS concentrations were corrected by the haematocrit and the protein binding, so that 5.2% (plasma 4.2%) of the efavirenz, 2.7% (plasma 2.2%) of the nevirapine and 6.3% (plasma 6.3%) of the lopinavir concentrations were judged as subtherapeutic.

Stability of DBS samples

The stability of the ARV drugs was tested under accelerated storage conditions (40°C, 75% rH) because the percentage differences of lopinavir DBS and plasma samples from Tanzania were unexpectedly large in comparison with the data obtained within the bridging study (Figure 3).

Nevirapine and efavirenz concentrations were stable during 4 weeks of incubation at 40°C and 75% rH. Maximal deviation from freshly prepared samples was <5%, which is smaller than the accepted analytical error of ±15%. In contrast, lopinavir DBS concentrations decreased time-dependently by −14% after 2 weeks and by −25% after 4 weeks of incubation. Hence, the observed difference between DBS and plasma concentrations in the field study can be explained by degradation of lopinavir in DBS samples under the climatic conditions encountered in Tanzania.

Discussion

TDM of ARV has been shown to improve treatment outcome by preventing toxicity and insufficient viral suppression due to inadequate drug levels. However, TDM is frequently not implemented in resource-poor regions due to the shortage of infrastructure. Our field study shows that automated DBS analysis alone (e.g. for nevirapine) or in combination with an algorithm correcting for blood/plasma partitioning (e.g. for efavirenz) can successfully be applied in such a setting.

Automation of the extraction process is central to establish DBS-based TDM in medical laboratories, as it improves robustness, sensitivity, efficiency and thus overall cost-effectiveness of

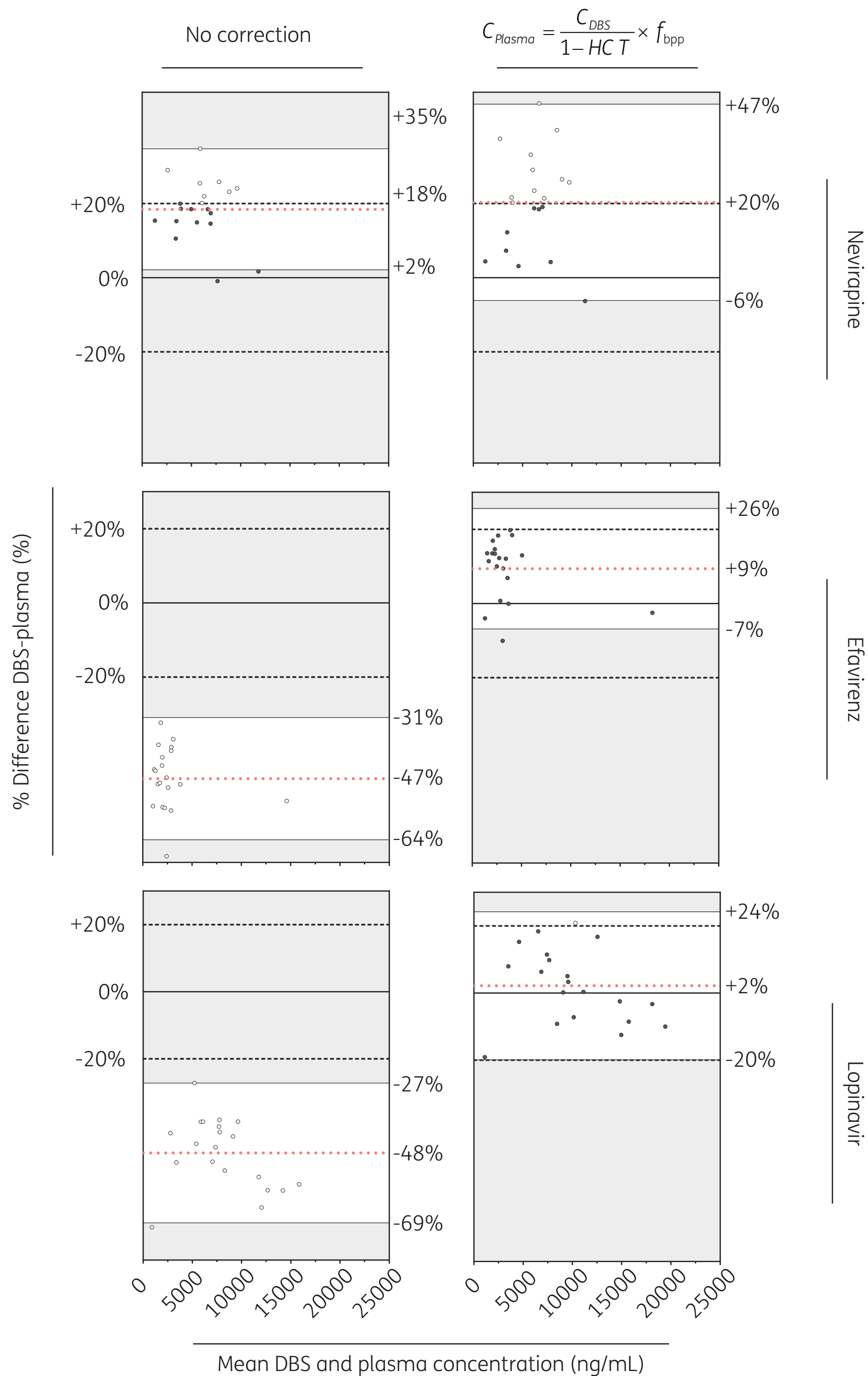

Figure 1. Bland–Altman plots of nevirapine, efavirenz and lopinavir concentrations measured in plasma and DBS of samples collected within the bridging study in Switzerland. Values with a difference of less than ±20% between the two matrices are shown using filled symbols; values with larger differences are shown using open symbols. Dotted line (red) shows the mean difference between plasma and DBS samples, the dashed lines the ±20% limits. White plot area illustrates the 95% limits of agreement. DBS concentrations are uncorrected (left column) or adjusted by haematocrit (HCT) value and protein binding (f_{bpp}, right column). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

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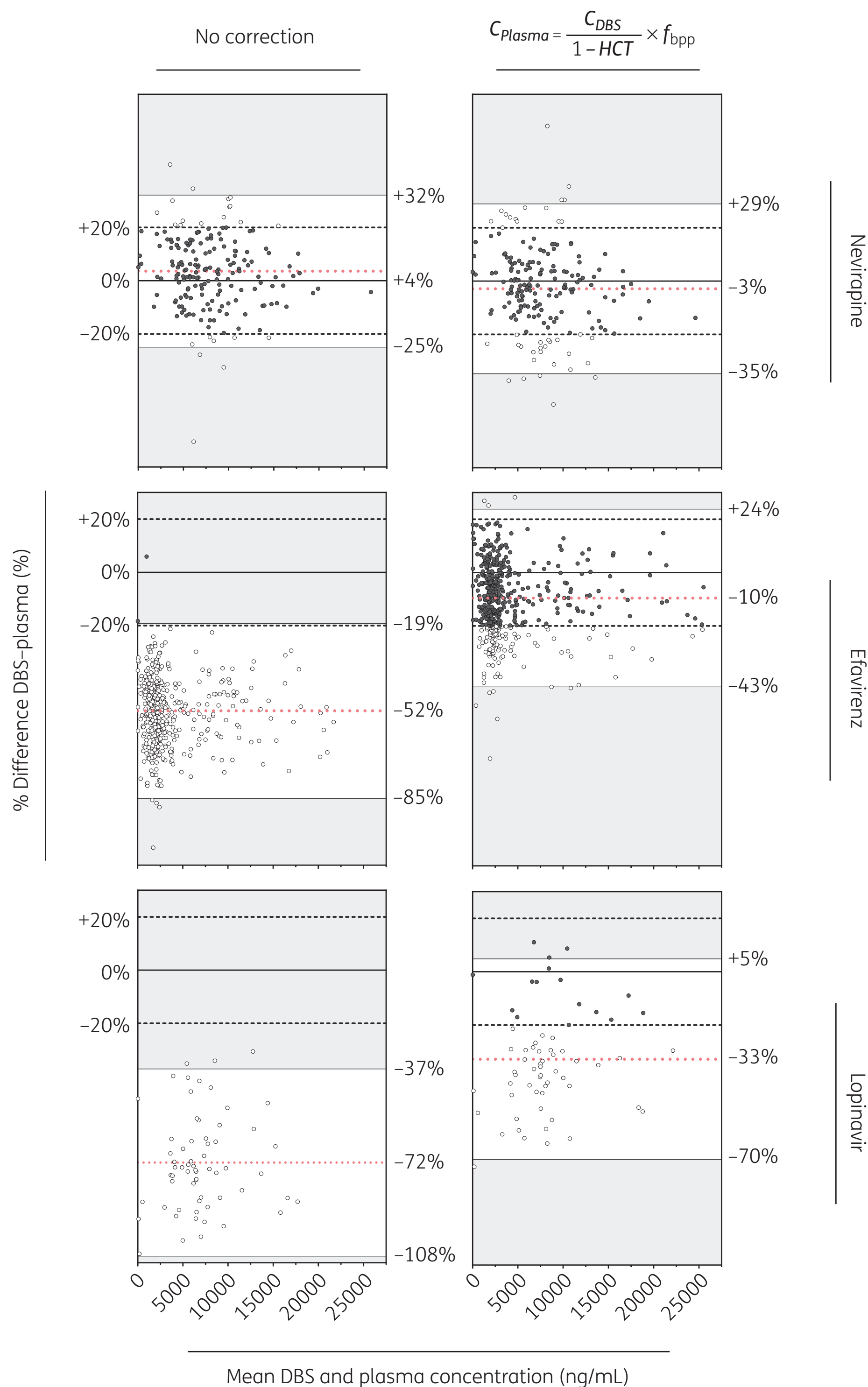

Figure 2. Bland–Altman plots of nevirapine, efavirenz and lopinavir concentrations measured in plasma and DBS of samples collected within the field study in Tanzania. Samples were collected at one to three occasions for each patient. Values with a difference of less than ±20% between the two matrices are shown using filled symbols; values with larger differences are shown using open symbols. Dotted line (red) shows the mean difference between plasma and DBS samples, the dashed lines the ±20% limits. White plot area illustrates the 95% limits of agreement. DBS concentrations were not corrected (left column) or adjusted by haematocrit (HCT) value and protein binding (f_{bpp}, right column). *Y*-axes were kept small for better visualization of the data; thus, a few data points were outside the axis limits (left column: nevirapine $n = 1$ and efavirenz $n = 2$; right column: nevirapine $n = 1$ and efavirenz $n = 3$). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

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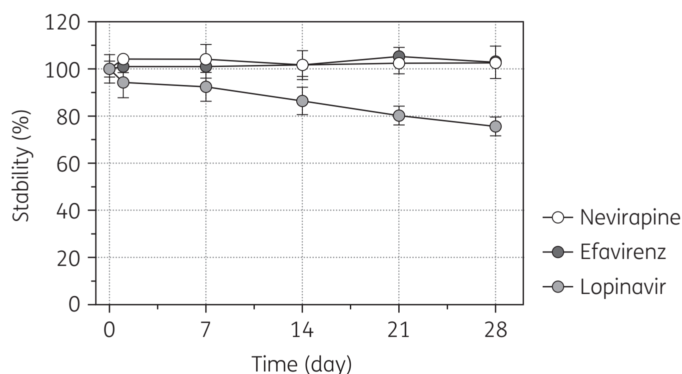

Figure 3. Stability of nevirapine, efavirenz and lopinavir under accelerated storage conditions at 40°C and 75% rH over 4 weeks. Five replicates were analysed after 1 day and after 1, 2, 3 and 4 weeks. Freshly prepared samples were used as comparators to assess the stability of the ARV drugs. Circles indicate the calculated mean percentage stability and the error bars are standard deviations.

measurements.[28] To date, several techniques for the automation of DBS samples have been introduced,[10,29–32] but only a few have so far been used to quantify concentrations in clinical patient samples.[28,33] With our setup we demonstrated that a large number of DBS study samples can be analysed in a fully automated way. Accuracy and precision data show that the method was reliable (Table S1) and time-efficient, considering the analysis time of ~4 min per sample. Hence, this type of automated DBS analysis holds promise to further DBS sampling for TDM.

However, as serum or plasma and not venous or capillary blood is typically used for TDM analysis, a comparison of the two matrices is necessary. Factors affecting suitability of a matrix for TDM are the unbound drug fraction in plasma and blood, the haematocrit and the distribution of the analyte into RBCs. According to Emmons and Rowland[34,35] the blood/plasma concentration ratio is a useful metric to select the appropriate matrix. Blood and plasma concentrations of nevirapine agreed strongly (ratio of 1.06), indicating that nevirapine distributes almost equally into the cellular and plasma compartments of the blood ($K_{RBC/plasma}$ 1.15). An *in vivo* study using radioactive-labelled nevirapine also showed an even distribution of nevirapine between human plasma and whole blood.[36] Therefore, either plasma or whole blood could be used for TDM. In contrast, efavirenz and lopinavir are highly bound to plasma proteins;[37–39] thus, distributing only partially into the cellular compartment resulting in low blood/plasma ratios of 0.62 and 0.57, respectively. To our knowledge, no data are available in the literature about the blood/plasma ratios of efavirenz and lopinavir. However, observed DBS/plasma ratios (efavirenz 0.6; lopinavir 0.51) were similar to blood/plasma ratios and importantly these data were comparable with published DBS/plasma ratios for efavirenz.[20,40,41] At this low ratio, blood cells basically act as diluent and hence (under the assumption of constant protein binding), the haematocrit value primarily alters the proportion between blood and plasma concentrations. Thus, according to the decision tree proposed by Emmons and Rowland,[35] blood and plasma can equally be used for all three ARV drugs assuming constancy of the parameters, e.g. haematocrit, the unbound drug fraction and the blood cell partitioning. However, the haematocrit values in our study population ranged between 10% and 50%. Consequently, concentrations measured in blood or DBS have to be adjusted by the haematocrit to allow reasonable prediction of the plasma concentration. We applied two different correction algorithms; both incorporated the haematocrit, as well as either the RBC partitioning or the plasma protein binding of the drugs.[20,22,42]

As expected, the agreement between plasma and blood concentrations was good for nevirapine but insufficient for efavirenz and lopinavir. Both methods of correction considerably improved agreements with only few samples exhibiting a bias >20%. Blood and DBS concentrations were largely similar, except that nevirapine concentrations in DBS samples tend to be ~10% higher than in whole blood samples. Therefore, the agreement between blood and plasma was closer than for DBS and plasma. However, interpretation is difficult as the bias still lies within the generally accepted accuracy of 85%–115% for bioanalytical methods.[23] Moreover, in the field study, which included a larger number of samples, almost no bias was detected between nevirapine concentrations determined in plasma and DBS samples. Similar results have been observed by Kromdijk and colleagues.[20] Our data also showed that neither the haematocrit nor the protein binding altered the agreement between DBS and plasma, as the applied corrections had no impact. For nevirapine, the outcome of bridging and the field study was similar; thus, prolonged storage and shipment did not affect nevirapine analysis in DBS samples. This result is in line with our stability recorded under accelerated storage conditions and data published in the literature.[43,44]

The DBS and plasma comparison of efavirenz and lopinavir samples collected within the bridging study was very promising. The mean bias was <10% after correction by the haematocrit and protein binding or $K_{RBC/plasma}$ while almost none of the samples was beyond ±20%. The agreement between plasma and DBS samples collected within the field study was acceptable for efavirenz but not for lopinavir. The efavirenz concentration in DBS was on average 52% lower than in plasma, which is in line with studies from Kromdijk *et al.*[20] and Amara *et al.*,[40] who found a 39.8% and 41.9% decrease, respectively. Only a small negative bias of −10% was observed after correcting the DBS concentration by the haematocrit and protein binding and three-quarters of all samples exhibited a bias of <20%. This result further strengthens the usefulness of this algorithm to correlate DBS with plasma data as proposed by Li and Tse[42] and Kromdijk *et al.*[20] The small negative bias might be due to some decay of efavirenz; however, neither our stability experiments under accelerated storage conditions nor published data indicate relevant instability.[20,40,41,43]

In the case of lopinavir, the agreement between plasma and DBS samples collected in the field study was surprisingly poor, considering the promising data obtained in the bridging study. Overall, despite the correction, concentrations measured in DBS samples were ~30% lower than in plasma, whereas the bias observed in the bridging study was negligible. Only a few studies about lopinavir analysis in DBS have been published so far,[45–48] but, to the best of our knowledge, no data are available comparing concentrations in DBS and plasma samples. Our data indicate that fresh DBS samples can be used for TDM of lopinavir; however, prolonged storage and sample shipment affect the quality of DBS samples and lead to unreliable results. This is most likely due to degradation of lopinavir in DBS samples and fits our observations of lopinavir degradation under accelerated storage conditions. Lopinavir is generally considered to be stable at room temperature and after heat

exposure at 58°C for 35 min.[43,46,49–51] However, one study shows that the lopinavir concentration decreases by ~13% in DBS kept at 20°C in desiccators for 2 years, which supports our assumption of lopinavir decaying in DBS over time.[45]

Our study shows that concentrations determined in whole blood are highly correlated with plasma concentrations. In the case of efavirenz and lopinavir, blood samples need to be corrected by the haematocrit and the cellular fraction of the drugs. Using the same correction algorithms, a good correlation between DBS and plasma concentrations of samples collected within the bridging study was reached. Agreement between plasma and DBS concentrations was also convincing for nevirapine and efavirenz for samples collected under field conditions in Tanzania. However, lopinavir was not stable in DBS under the conditions of this study, an aspect that should be considered when planning future studies. Importantly, the analysis of adjusted DBS and plasma concentrations lead to the same clinical interpretation of most TDM samples. Overall, automation facilitated DBS measurements by increasing method sensitivity and by decreasing the overall workload; thus, enhancing attractiveness of the DBS technique for TDM, pharmacokinetic studies and assessment of treatment adherence. However, before DBS can be implemented in low- and middle-income countries, obstacles, e.g. cost of sampling and analysis, have to be overcome.

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Transparency declarations

S. G. is an employee of CAMAG (Muttenz, Switzerland). The remaining authors have none to declare.

Author contributions

U. D. and B. B. conducted analyses, wrote the first draft of the manuscript and were supervised by M. H. and S. K. M. D. was involved in the sample analyses within the bridging study. S. G. provided technical support for the automated bioanalyses. S. E. with oversight from M. B. and M. H. established the study design. S. E., A. N., D. M. and E. L. were involved with leading field data collection and ensuring the quality of data. All authors contributed to the writing of the paper and have read and approved the final manuscript.

Supplementary data

Additional Methods information, Table S1 and Figure S1 are available as Supplementary data at *JAC* Online.

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