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Therapeutic drug monitoring in the dried spotlight

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Ghent, 2019,

The promoter,

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Dankwoord

Trial and error, woorden die ik vaak liet vallen wanneer iemand mij vroeg 'Hoe gaat je doctoraat?'. In de afgelopen 4.5 jaar leerde ik dat niet alles steeds van een leien dakje loopt, maar dat alles uiteindelijk goed komt, zolang je er zelf maar in blijft geloven.

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List of abbreviations

%RE	% relative error
°C	Degrees Celsius
AEDs	Anti-epileptic drugs
ANOVA	Analysis of variance
CAD	Collision-activated gas
CBZ	Carbamazepine
CBZ-E	Carbamazepine-10,11-epoxide
CDC	Center for disease control and prevention
CDT	Carbohydrate-deficient transferrin
CE	Collision energy
CIs	Confidence interval(s)
CMIA	Chemiluminescent magnetic microparticle immunoassay
CMS	Capillary plasma microsampling
Conc	Concentration
COPD	Chronic obstructive pulmonary disease
CUR	Curtain gas
CV	Coefficient of variation
CXP	Collision cell exit potential
CYP	Cytochrome P
DBS	Dried blood spot(s)
DBSA	DBS Autosampler™
DP	Declustering potential
DPS	Dried plasma spot
DRC	Democratic Republic of the Congo
DRUID	Driving under the influence of drugs, alcohol and medicines
EBC	Exhaled breath condensate

EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalography
EMA	European Medicines Agency
EP	Collision cell entrance potential
ESI	Electrospray ionization
FDA	U.S. Food and Drug Administration
GC-MS	Gas chromatography-mass spectrometry
GH	Growth hormone
h	Hour
Hb	Hemoglobin
HbA1c	Hemoglobin A1c
Hct	Hematocrit
HRMS	High resolution mass spectrometry
IGF-1	Insulin-like growth factor-1
IS	Internal standard(s)
ISF	Interstitial fluid
LC-(MS/)MS	Liquid chromatography coupled to (tandem) mass spectrometry
LLOQ	Lower limit of quantification
LoA	Limits of agreement
m/z	Mass-to-charge-ratio
MeOH	Methanol
MF-DBS	Microfluidic dried blood spot(s)
min	Minutes
MRM	Multiple reaction monitoring

NA	Not applicable
NBS	Newborn screening
NIR	Near infrared
OXC	Oxcarbazepine
PB	Phenobarbital
PCI-IS	Post-column infused internal standard
PEth	Phosphatidylethanol
PHT	Phenytoin
PI-DBS	Partial-punch pipetted dried blood spot(s)
POC	Point-of-care
PT	Proficiency testing
QC(s)	Quality control (samples)
RBC	Red blood cell
RDE	Ring disk electrode
rpm	Revolutions per minute
RSD	Relative standard deviation
SCAP	Sample Card and Prep System
SLS-Hb	Sodium lauryl sulphate-Hemoglobin
SoHT	Society of hair testing
SPE	Solid phase extraction
T4	Thyroxine
TDM	Therapeutic drug monitoring
Tr	Retention time

U(H)PLC	Ultra-performance liquid chromatography
ULOQ	Upper limit of quantification
UV-VIS	Ultraviolet-visible
VAMS	Volumetric absorptive microsampling
VAPD	Volumetric absorptive paper disc
VAPDmini	Volumetric absorptive paper mini-disc
VPA	Valproic acid

AIM AND OUTLINE OF THE THESIS

Recently, different types of devices capable of generating dried blood samples, besides classical dried blood spots (DBS), have entered the market [1-9]. Dried blood samples have been used for highly diverse applications, starting from newborn screening, over therapeutic drug monitoring (TDM) throughout toxicology [10-17]. The growing interest in dried blood sampling during the last decade results from the many advantages coupled to this sampling technique. Since dried blood samples are mostly obtained by a finger prick, sampling can be performed by the patient himself, eliminating the need for a phlebotomist. Furthermore, the small sample volume is beneficial when envisaging use in a pediatric or anemic population. The non- or less contagious nature of dried blood samples and the fact that the dried matrix increases analyte stability poses fewer difficulties in terms of transport and storage. Besides, the sample preparation procedure is also straightforward and amenable to automation.

On the other hand, the use of dried blood samples for quantitative analysis also suffers from some challenges, with the hematocrit (Hct) effect certainly being the most discussed issue coupled to classical DBS analysis [18]. In essence, because of a different viscosity, blood with lower respectively higher Hct will spread more respectively less than control blood, typically leading to under- respectively overestimation of analyte concentrations when analyzing a DBS sub-punch. Furthermore, capillary concentrations can be different from venous concentrations and using blood/plasma ratios to calculate plasma concentrations based on capillary concentrations has proven to be challenging. Other limitations coupled to dried blood sampling include the dependence on adequate sampling, risk of contamination, influence of the spotted volume and spot inhomogeneity. This imposes a more complicated analytical and clinical validation procedure [11, 15, 18]. In addition, due the smaller sample size typically associated with dried blood samples, the amount of analytes and the number of analyses is limited. Therefore, sensitivity requirements may not always be met by available analytical instrumentation.

In recent years, many efforts have been made to facilitate correct dried blood sampling, to improve dried blood sample bioanalysis and to cope with challenges related to classical DBS analysis. New sampling devices are entering the market, automated analysis leads to a higher efficiency, sample throughput and reproducibility and different strategies to cope with the Hct effect have been put forward [18-20]. Innovations include whole-spot analysis of volumetrically applied DBS, development of alternative dried blood sampling devices, introduction of special

types of filter paper, as well as cards for generating dried plasma spots, and set-up of approaches for Hct prediction of classical DBS, allowing the correction of Hct-skewed results [18].

Alternative sampling strategies, especially dried blood samples, are the common thread throughout this work. The objective of this thesis is twofold; on the one hand we aim at evaluating the applicability of dried blood samples (**Part A**), on the other hand we address the most discussed issue coupled to classical DBS analysis, being the hematocrit effect (**Part B**).

Alternative sampling strategies can in fact be seen as the collection of 'traditional' samples in an alternative way (i.e. the collection of dried blood samples) or as the collection of 'alternative' samples in all kind of ways (i.e. oral fluid, hair, …). Either way, the implementation of the alternative sampling strategies within clinical routine requires sensitive analytical instrumentation due to the generally low amount of sample/analyte available. Therefore, **Chapter A.1.** provides an overview of the different kinds of alternative sampling strategies in clinical routine, measured with (liquid chromatography)-tandem mass spectrometry ((LC-)MS/MS). In addition, for several subdisciplines within the clinical lab the alternative sampling strategies readily implemented on a routine basis, as well as strategies with potential for future implementation are discussed.

Amongst those subdisciplines, TDM is a field with growing interest in the use of non- and minimally invasive alternative sampling strategies [11, 12, 15]. TDM is a multidisciplinary clinical practice in which the concentration of a drug is measured within a patient with the aim of minimizing the risk for toxicity, inadequate efficacy, or therapy resistance and of detecting compliance problems, pharmacokinetic abnormalities, and/or drug interactions [21]. Therefore, drugs with variable and complex pharmacokinetics, narrow therapeutic indices, a high risk of toxicity and multiple possible drug-drug interactions are considered good candidates for TDM. Definitely for non-hospitalized patients needing a long-term treatment and consequently a long-term follow-up, the relevance of dried blood samples becomes clear. The ease of sampling coupled to the latter, making home-sampling a possibility, in combination with the possibility of transport and storage at ambient temperature can be of great benefit in the field of TDM. Furthermore, as dried blood samples can be sent to a clinical laboratory via regular mail, laboratory results may already be available before a patient visits the clinician for a routine follow-up. The generally narrow therapeutic indices of first-

generation anti-epileptic drugs (AEDs) (amongst which carbamazepine (CBZ), phenobarbital (PB), phenytoin (PHT) and valproic acid (VPA)), making toxicity a common issue, together with their frequent use (i.e. for epilepsy, but also for pain and bipolar disorder) have led to the fact that TDM of these drugs has become an established application, in general as well as in special patient populations. Furthermore, the complex and variable pharmacokinetics and the frequently high degree of protein binding also make the above-mentioned AEDs good candidates for TDM [22]. Additionally, CBZ, PB, PHT and VPA are targets of many drug-drug pharmacokinetic interactions, since CBZ, PB and PHT are strong inducers and VPA is an inhibitor of multiple CYP enzymes. Therefore, **Chapter A.2.** describes the development and validation of a novel LC-MS/MS method for the determination and quantification of four AEDs and one active metabolite, including CBZ, PB, PHT, VPA and carbamazepine-10,11-epoxide (CBZ-E), making use of volumetric absorptive microsampling (VAMS) devices. The latter is one of the alternative sampling strategies which have been designed in an attempt to maintain the benefits of blood microsampling but eliminate the Hct issue coupled to classical DBS analysis. The VAMS devices consist of a hydrophilic polymeric tip connected to a plastic handler, which wicks up an accurate and precise volume (10, 20 or 30 µL) when contacting a blood surface, this across a broad Hct range [2, 23]. Furthermore, the validity and applicability of the developed method was demonstrated through a successful application on external quality control materials and on left-over patient samples.

Another advantage coupled to dried blood sample analysis is the compatibility with automated sample extraction procedures. For DBS in particular, to date, different set-ups exist, ranging from semi-automated platforms (e.g. automated DBS punchers) to fully automated extraction systems. Those fully automated procedures can be online coupled to standard LC-MS/MS methods, limiting the hands-on time and, hence, making it extremely interesting for high-throughput settings. Therefore, in **Chapter A.3.** a DBS-based LC-MS/MS method for the determination of the same set of AEDs as listed above was developed and validated, making use of the DBS-MS 500 autosampler. Method development revealed the need for adjustment of proposed, generic direct elution conditions, depending on the analytes of interest and the used set-up [24]. Furthermore, applicability of the method was demonstrated *via* a successful application on authentic patient samples.

The potential for remote or home sampling, combined with the non- or less contagious character of dried blood samples, makes it very attractive for TDM. Following sample collection at home, the patients can send the obtained samples via regular mail to the analyzing laboratory, allowing lab results to be available before a patient visits a clinician for routine follow-up or even rendering a routine follow-up consultation superfluous. In this context, **Chapter A.4.** describes the application of the developed LC-MS/MS methods on VAMS and DBS samples originating from patients with Nodding syndrome or another form of epilepsy living in developing countries, more specifically in Uganda and the Democratic Republic of the Congo. Nodding syndrome is a mysterious neurological disorder characterized by head nodding, mental retardation, malnutrition, stunted growth, etc., which typically has its onset in 5-15y old children in subregions of sub-Saharan Africa [25]. As the actual cause still remains unclear to date, Nodding syndrome patients are only treated symptomatically (seizure control). Especially older-generation AEDs are used, amongst which the 4 AEDs included within the validated LC-MS/MS methods [26, 27]. The nature of the VAMS and DBS samples is truly fit-for-purpose here, given the highly challenging context in which sampling may take place: in remote areas devoid of electricity, running water, etc. Furthermore, in contrast to classical liquid samples, the dried samples can be conveniently transported and stored at ambient temperature.

As described above, dried blood samples also suffer from some challenges. Here, the Hct-related issues certainly remain the major barrier for (regulatory) acceptance of the classical DBS analysis in the bioanalytical and clinical field. Seeing the major impact of this issue and the fact that tackling this issue is quite challenging, **Chapter B.1.** gives an overview on new strategies that try to cope with this Hct effect (going from avoiding to minimizing), on methods estimating the DBS volume, and on methods estimating or measuring the Hct of a classical DBS.

The Capitainer-B devices are one of the new strategies recently designed to avoid the Hct issue. The device consists of an inlet port to which a drop of blood (e.g. from a fingertip) can be applied, allowing a straightforward absorption of a fixed volume (±13.5 µL) of blood by a pre-perforated paper disc[7]. In **Chapter B.2.**, an evaluation of the potential of this new sampling strategy to effectively overcome the Hct bias is described together with an investigation whether the amount of blood applied at the inlet of the device has an influence on the device

performance. Using a fully validated LC-MS/MS method, model analytes caffeine and paraxanthine were determined in 133 Capitainer-B samples originating from hospital patients with varying Hct values. The results, and those of corresponding partial-punch DBS samples, were compared with corresponding whole blood concentrations. Furthermore, different volumes of whole blood originating from hospital patients with a low or high Hct were applied to the devices, to evaluate whether the amount of blood applied at the device inlet has an influence on the performance of the device. This set-up, being the first in-human Capitainer-B study in which the impact of the Hct and the applied volume was evaluated, allowed an in-depth evaluation of this new alternative sampling technique.

Finally, the broader international context, the relevance and the future perspectives are described, together with the general conclusions of this work.

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PART A

APPLICATION OF ALTERNATIVE SAMPLING STRATEGIES

CHAPTER A.1.

OPENING THE TOOLBOX OF ALTERNATIVE SAMPLING STRATEGIES IN CLINICAL ROUTINE: A KEY-ROLE FOR (LC-)MS/MS

Based on

Velghe S, Capiau S, Stove CP. Opening the toolbox of alternative sampling strategies in clinical routine: a key-role for (LC-)MS/MS. *Trends in Analytical Chemistry* 84, 61-73 (2016).

Abstract

Alternative sampling strategies such as dried blood sampling, liquid microsampling and the sampling of oral fluid, hair, meconium, interstitial fluid, sweat, exhaled breath condensate and sputum offer interesting opportunities for many applications in clinical routine. In this Chapter, we provide an overview of different applications, with special attention to the pivotal role of LC-MS/MS in facilitating analysis of the collected matrices. Covered clinical fields include newborn screening, endocrinology, therapeutic drug monitoring, phenotyping, toxicology, proteomics and metabolomics. Furthermore, specific advantages, challenges and limitations of each alternative sampling strategy are discussed, along with recent advances and future trends that may contribute to routine implementation of these sampling strategies. Given the development of many recent potentially valuable clinical applications, the possibility of home sampling and the opportunity to obtain information that is hard to procure using traditional sampling, a well-balanced role for alternative sampling strategies can be envisaged in patient healthcare in the (near) future.

A.1.1. Introduction

Alternative sampling strategies include the collection of 'traditional' samples (blood, plasma, serum or urine) in an alternative way, as well as the collection of 'alternative' samples in all kind of ways. A typical example of the former is the collection of dried blood spots (DBS) (i.e. the collection of blood in an unconventional manner), while examples of the latter include sampling of oral fluid, hair and a wide variety of other matrices. Both the 'alternative sampling' and the 'alternative samples' offer interesting opportunities for clinical applications, as they do not only imply easier sample collection (particularly in special patient populations such as small children and neonates), but can also provide information that is impossible or hard to obtain using traditional sampling strategies, such as venipuncture and urine collection. Moreover, the use of alternative sampling strategies is often coupled to matrix-specific advantages such as increased analyte stability and/or the possibility of home sampling. However, the implementation of alternative sampling strategies in clinical routine requires sensitive analytical techniques, since generally only minute amounts of sample are available and/or low analyte levels may be present. For the quantitative analysis of traditional samples in clinical routine, detection methods such as gas chromatography-mass spectrometry (GC-MS) and particularly immunoassays have been and are still being employed. Over the last decade, a clear trend towards implementing liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been observed [1, 2]. Especially in larger clinical laboratories, the added-value of LC-MS/MS has been advocated. For the analysis of samples obtained via alternative sampling strategies, LC-MS/MS is also the technique of choice when it comes to combining sufficient sensitivity with utmost selectivity.

A.1.2. Alternative sampling strategies

In this Chapter we will focus on the implementation of patient-friendly, minimally or non-invasive alternative sampling strategies which are promising for clinical routine, with special attention to the role of LC-MS/MS. Microsampling of blood to collect liquid microsamples or to generate dried blood or dried plasma spots (DPS), as well as sampling of oral fluid, hair, meconium, interstitial fluid (ISF), sweat, exhaled breath condensate (EBC) and sputum are the alternative sampling strategies covered in this Chapter. While there is a plethora of reports on

the use of alternative sampling strategies for research purposes, (pre)clinical and epidemiological studies, we focus here on those methods that are -in our opinion- most promising for application in clinical routine. The different areas in which alternative sampling strategies may have added-value will be highlighted and for each category some key examples will be discussed, without being comprehensive.

One of the best known alternative sampling strategies is DBS sampling. DBS are generally prepared by depositing a drop of capillary blood, obtained by a finger or heel prick, on a filter paper. The application field of DBS is highly diverse, going from newborn screening (NBS, the screening for inborn errors of metabolism), over therapeutic drug monitoring (TDM) to toxicology and pharmacokinetic studies in drug development. Other microsampling approaches closely related to DBS sampling are DPS and liquid microsampling, as well as volumetric absorptive microsampling (VAMS). The latter, applied for TDM of anti-epileptic drugs in Chapter A.2., is performed using a handheld device consisting of a plastic handle and a hydrophilic polymer tip which absorbs a fixed volume of blood (±10 µL). The advantages and challenges posed by (dried) blood microsampling have been subject to many reviews [3-6]. Briefly, common advantages of dried samples in general include the ease of sampling and the convenient transport and storage under ambient conditions. In addition, these samples pose a reduced risk of infection due to deactivation of pathogens upon drying. In DBS analysis, the hematocrit issue is undoubtedly the most widely discussed challenge. Variations in hematocrit influence the spreading of blood on filter paper, thereby impacting the spot size and, possibly, homogeneity. Furthermore, hematocrit may also influence recovery and matrix effect. As outlined further (see section A.1.6.1. Development of new formats), several approaches have been developed that allow to cope with the issues imposed by varying hematocrit [3, 7, 8]. Aside from the hematocrit effect, DBS analysis is also affected by the volume of blood deposited on the filter paper and the punch location [9]. Another issue, which applies to all microsampling strategies, is the possible difference in concentration between capillary and venous blood.

Liquid microsampling is the sampling of liquid capillary blood using a precision capillary. It is used in the pharmaceutical industry, primarily in the preclinical phase of drug development, to obtain pharmacokinetic and toxicokinetic information, e.g. from laboratory animals. Also in the clinical lab, liquid microsamples (typically taken from children) are already being used, e.g. for hemoglobin A1c (HbA1c) monitoring. As the precision capillaries are filled with liquid

blood, immediate analysis or processing (e.g. centrifugation, dilution in a stabilizing buffer and/or freezing) after sampling is generally required, making transport and storage less practical when compared to dried microsamples (e.g. DBS, DPS and VAMS).

Another widely used alternative matrix is oral fluid. Oral fluid is composed of saliva (an aqueous secretion produced by the major salivary glands), the secrete of the accessory glands, gingival fluid, enzymes, other proteins, electrolytes, bacteria, epithelial cells, ora-naso-pharyngeal secretions, and other debris [10]. Although oral fluid, as an alternative to plasma, has arisen as a potential alternative matrix for TDM, the best established oral fluid-based application to date is roadside drug testing. A major concern coupled to oral fluid analysis, is the risk of contamination. Indeed, contamination with food and/or beverages, other debris from the mouth or smoke are commonly seen [11]. In addition, oral fluid analysis is also prone to oral contamination. Therefore, sampling should be performed immediately before drug intake or after an adequate 'wash-out' period [12]. Furthermore, blood contamination of the oral fluid caused by leakage from the oral mucosa as a result of microinjuries, such as burns or abrasions and due to gingivitis and periodontitis or even following regular mouth hygiene, might compromise analyte quantitation in oral fluid. The latter can have a major impact on the analysis of compounds with a blood to oral fluid ratio which strongly deviates from 1, as was demonstrated for the measurement of e.g. salivary cortisol and testosterone [13-15]. Another important issue in oral fluid analysis is the fact that analyte levels may depend on a multitude of variables such as compound pKa, molecular weight, charge and lipid solubility, as well as oral fluid pH, flow rate and metabolism [10, 16]. Consequently, the measured oral fluid concentrations can be heavily influenced by the employed collection procedure [16, 17]. Oral fluid can either be collected without stimulation (e.g. via passive drooling) or with mechanical or chemical stimulation (e.g. by chewing on paraffin or by using citric acid, respectively) [16, 17]. Moreover, adsorption may occur to collection devices and -important when sample analysis is to be performed by LC-MS/MS-matrix effects may arise from incorporated buffers, preservatives or surfactants [16-18]. Due to the above-mentioned issues, careful selection and standardization of the oral fluid collection procedure is essential to obtain reliable and reproducible results.

The most important advantage of hair sampling is undoubtedly the wider window of detection, due to the fact that incorporated compounds are no longer subject to biotransformation.

However, substances of interest can gradually leach out of the hair or can be removed to an important extent by hair damage caused by cosmetic treatment, such as bleaching or dyeing, resulting in an underestimation of exposure or use [19]. Other advantages are the non-invasive nature of the sampling and the fact that the collection of a hair sample does not pose any privacy issues. However, it needs to be mentioned that hair sampling can be considered somewhat intrusive. One of the main challenges in hair analysis is external contamination or passive drug exposure. Therefore, a decontamination step is an essential factor in hair analysis, although also the decontamination itself poses challenges, as outlined in section A.1.4. Limiting factors [20-22].

Sweat, having a detection or collection window that may range from 30 minutes up to 1-2 weeks, is commonly collected using transdermal absorptive sweat patches, typically applied to the back, upper arm or lower chest and generally worn for several days. While the measurement of chloride in sweat for diagnosis of cystic fibrosis is likely the best studied application, LC-MS/MS-based applications -offering the required sensitivity- include the determination of prescription drugs or drugs of abuse. The main disadvantages of this sampling technique are the potential influence of external contamination of the skin or the sweat patches and the unknown sample volume (rendering interpretation of a quantitative result challenging), as sweat production may vary in function of physical activity or ambient temperature. Given the limited potential of sweat analysis in the routine clinical lab, the interested reader is referred to other recent reviews [23, 24].

EBC collection only requires quiet breathing in a specially designed collection device for several minutes. Various measures can be taken to avoid EBC contamination, as recently reviewed by Konstantinidi *et al*. [25]. Generally, it is recommended to analyze EBC samples immediately after collection, otherwise immediate freezing, including inconvenient storage, is necessary [26].

The collection of sputum, mucus coughed up from the lungs (after induction or not), is considered a semi-invasive sampling method. Sputum is typically -although not on a routine basis- used to investigate chronic lung diseases such as asthma, chronic obstructive pulmonary disease (COPD) and interstitial lung disease. LC-MS/MS has been used to measure a variety of analytes in sputum, amongst which leukotrienes, iso(desmosine), mucins, as well as therapeutic drugs [27-30].

Meconium, a neonate's first stool, is widely accepted as the matrix of choice for determining fetal drug exposure (other alternative matrices include umbilical cord, placenta, hair and nails). The wide detection window, covering approximately the last 2-3 months of pregnancy, is, just as the easy sampling, a major advantage [31, 32]. Although meconium is hitherto not used in general clinical routine, its use can be beneficial for example for the analysis of ethanol markers in centres specialized in the follow-up of neonates from mothers at risk of alcohol abuse [33].

Finally, although less applied in clinical practice up till now, ISF is also an interesting alternative matrix. The composition of ISF, the fluid which surrounds tissue cells, is determined by the continuous exchange of water and small, non-protein bound solutes such as therapeutic drugs, between whole blood and the ISF under the influence of hydrostatic and osmotic pressure. Since ISF concentrations often closely reflect free plasma concentrations of drugs and endogenous substances, this matrix can be particularly valuable in the field of TDM. Over the years, different technologies have been developed for ISF sampling, such as reverse iontophoresis and microneedles [34, 35]. The latter have the advantage of being more convenient for patient self-sampling. On the other hand, when sampling capillary blood via e.g. a fingerprick, the presence of ISF can be seen as a diluting factor. Therefore, clear instructions on how to sample correctly (e.g. wiping off the first drop of blood to avoid the collection of tissue fluid) is of key importance.

Despite the many advantages accompanying the various alternative sampling strategies, several challenges still remain. Table A.1.1 summarizes the main advantages and challenges of the different types of alternative sampling strategies. Alternative sampling strategies readily being implemented on a routine basis in clinical labs, as well as strategies with potential for future implementation, will be discussed in the next section, which covers several subdisciplines of the clinical lab.

Table A.1.1. The main advantages and challenges of the different types of alternative sampling strategies from the author's point of view.

	Different types of alternative sampling strategies										
	Dried blood spots	Dried plasma spots	Capillary liquid sampling	Volumetric absorptive microsampling (VAMS)	Oral fluid	Hair	Meconium	Interstitial fluid (micro-needles)	Sweat	Exhaled breath condensate	Sputum
Patient comfort (e.g. ease of sampling, invasiveness)	++	+	+	++	++	+	+++	++	++	+++	+
Small sample volume	++	++	++	+++	++	+	-	+++	NA	+++	++
Analyte stability	+++	+++	+++	+++	+/-	+/-	++	+	++	-	-
Convenient storage and transport	+++	+++	-	+++	+	+++	-	-	+	-	-
Reduced infection risk (compared to blood samples)	+	+/-	+	++	+++	+++	+	+	+++	+++	+
Resistance to contamination	-	-	+	-	-	-	++	++	-	+	++
Resistance to hematocrit effect	-*	+	++	++	NA	NA	NA	NA	NA	NA	NA

+++ / ++ / + / - : indication to what extent the statement on the left holds true; NA: not applicable

* The hematocrit effect is a major issue, but to date, several attempts to cope with the issue have been explored.

A.1.3. Clinical applications involving alternative sampling strategies

A.1.3.1. Newborn screening

The use of DBS sampling as an alternative for conventional blood sampling in neonates has become a widespread technique in NBS programs, ignited by the demonstration of Guthrie and Susi in 1963 to use newborn DBS to determine phenylketonuria [36]. DBS-based NBS by (LC-)MS/MS has exponentially increased since the 1990s and has become an established procedure in developed countries [37]. Moreover, the experience gained from NBS has undoubtedly facilitated the development of DBS-based applications in other fields as well. NBS can be divided into primary screening tests and second-tier tests. The primary tests are designed to identify as many inborn errors as possible. Since diagnostic sensitivity is favored over specificity for disorder detection here, the number of false-positive tests increases. Therefore, second-tier tests have been implemented, enabled by the introduction of MS/MS methods, to improve the specificity of disorder detection. A second-tier test is performed using the same DBS, is characterized by a lower sample throughput and is extremely suitable to confirm or refute an initial positive result, due to the measurement of additional metabolites [38]. In addition, a distinction has to be made between direct and indirect screening. Direct screening examines endogenous substances, while indirect screening focusses on the conversion of substrates by specific enzymes [39]. Current NBS programs screen for up to over 50 disorders [40, 41]. Of these, 20 to over 40 disorders can be screened for by LC-MS/MS [37]. However the exact number of disorders that is screened for varies strongly from country to country [37, 41]. A key advantage of the (LC-)MS/MS technology is that it is highly multiplexeable (e.g. a multiplex assay of lysosomal enzymes in DBS), making the procedure very attractive in routine NBS as a diagnostic platform for the early detection and confirmation of genetic disorders [42, 43]. As it is beyond the scope of this Chapter to provide a full overview of the metabolic diseases that can be screened for by tandem MS, we refer to a comprehensive review by Lehotay *et al*. on this topic [37]. Furthermore, DBS can also be used at a later age, for the follow up of (treatment) of inborn errors of metabolism, as is currently applied for patients with e.g. maple syrup urine disease, phenylketonuria or tyrosinemia type 1 [44].

A.1.3.2. Endocrinology

The measurement of sex steroids, testosterone and especially estradiol, serves as a key tool in the diagnosis or management of a wide range of disorders, such as hypogonadism, polycystic ovary syndrome, amenorrhea, disorders of puberty, male and female infertility and tumors of prostate, testes, breast and ovary [45]. Sex steroid testing has known a noticeable transition from colorimetric assays using urine, over manual radio-immunoassays and automated immunoassays using serum to LC-MS/MS methods [45]. Improved precision, sensitivity and selectivity compared to automated immunoassays and the capability of multiplexing methods has resulted in substitution of traditional sex steroid immunoassays by LC-MS/MS methods in large reference clinical laboratories.

Oral fluid serves, next to plasma and serum, as a matrix for sex steroid determination. Progress in LC-MS/MS has allowed to -at least partly- cope with the low hormone concentration, one of the challenges coupled to oral fluid analysis. However, still, sensitivity remains the limiting factor when considering e.g. the assessment of testosterone in children and women, and, to a lesser extent, in hypogonadal men via alternative sampling strategies. Furthermore, cortisol and progesterone are also detectable in oral fluid by LC-MS/MS [46]. Cortisol has been determined in oral fluid for the diagnosis of Cushing's syndrome and numerous stress-related disorders, as it is considered a 'stress' biomarker. Importantly, oral fluid can be used for home sampling, which can be of interest for example in the diagnosis of Cushing's syndrome, since late-night measuring of cortisol levels in oral fluid is recommended as a first-line screening test [47]. Furthermore, additional stress -which could affect cortisol test results- due to venous sampling and/or hospital visits is avoided in this way. Progesterone, a hormone that plays a pivotal role in the regulation of the menstrual cycle and in the maintenance of pregnancy, has also been measured in oral fluid, to determine luteal and placental functions in non-pregnant and pregnant women, respectively [48]. Besides oral fluid, DBS can also be used for the LC-MS/MS-based determination of steroid hormones, including corticosterone, deoxycorticosterone, progesterone, 17-hydroxyprogesterone, 11-deoxycortisol, 21-deoxycortisol, androstenedione, testosterone, dihydrotestosterone and cortisol, although not in all cases the required sensitivity will be achievable [49]. An upcoming tool for the assessment of long-term cortisol secretion, as a biomarker of chronic stress in various settings, is the analysis of hair cortisol concentrations via LC-MS/MS [50].

Anti-Müllerian hormone is a predictor of the ovarian response in women undergoing ovarian stimulation for *in vitro* fertilization. Since it can be quantified in DBS, this minimally invasive sampling strategy could be another possible future LC-MS/MS-based application in women undergoing fertility treatment [51]. Other hormones that have been measured in oral fluid and/or DBS via LC-MS/MS include 25-hydroxyvitamin D, melatonin and thyroxine (T4) [46, 52, 53]. T4 determination in oral fluid may serve as a simple and cost-effective alternative to free T4 measurement in serum, used in the diagnosis of thyroid disorders. Clinical application of the method could be an interesting future prospect, since T4 measurement in oral fluid turned out to be useful in the diagnosis of Graves disease [46]. Total T4 can also be measured in DBS via MS/MS, along with immunoassay-based determination of thyroid stimulating hormone and antithyroid antibodies [54-56].

Overall, oral fluid- and DBS-based hormone tests are an upcoming tool allowing patient friendly evaluation of endocrine functions. While immunoassays will undoubtedly continue to be used for routine measurement of hormones, LC-MS/MS methods are increasingly being integrated in clinical routine due to the disadvantages (e.g. sometimes poor specificity and accuracy) associated with immunoassays. Clearly, for the accurate determination of hormones in oral fluid and DBS, LC-MS/MS is the method of choice. For oral fluid it should be noted, though, that although the concentrations measured in this matrix may correlate with the serum/plasma free fraction, they are not necessarily equivalent. For example, Fiers *et al*. nicely demonstrated that salivary testosterone concentrations measured by LC-MS/MS are not identical to free testosterone concentrations in serum [57]. Moreover, as mentioned in section A.1.2. Alternative sampling strategies, pre-analytical issues, amongst which contamination of the oral fluid with blood, as well as the choice of the collection method, may have an impact on the result [58]. Hence, the decision whether oral fluid may truly serve as a more convenient and inexpensive alternative to serum/plasma for free active hormone testing may actually depend on the clinical question.

A.1.3.3. Toxicology

Alternative samples like hair and oral fluid have become an established part of toxicological investigations in many countries, with analyses being performed in both forensic and clinical labs. Although these samples can provide valuable information, their analysis is also

accompanied by some challenges, as discussed in section A.1.2. Alternative sampling strategies and A.1.4. Limiting factors. As already mentioned above (see section A.1.2), in several countries, oral fluid has become -or will soon become- the matrix of choice for immunoassay-based on-site drug screening. Whereas blood is the classical matrix for unequivocal MS-based confirmation of a positive on-site screening test, oral fluid can also serve this purpose. For this confirmation the toxicology section of clinical labs may play a role. Given the potentially high sample-throughput, focus has been put on the development of automated procedures, e.g. applying automated solid phase extraction (SPE) (on-line or off-line) and on-line sample clean-up procedures, typically followed by LC-MS/MS [59]. Another matrix often considered in the context of drugs and driving -albeit covering another time frame- is hair. Indeed, hair analysis is increasingly being used for demonstrating drug abuse or for the determination of (bio)markers, such as the alcohol markers ethyl glucuronide and fatty acid ethyl esters. This approach is currently readily being applied on a routine basis in several countries (e.g. in Sweden, Switzerland and Germany) in driving license regranting programs, providing some labs with a throughput of several thousands of samples per year [60-62]. Such throughputs, combined with the need of ultimate sensitivity, are offered by LC-MS/MS.

The use of DBS for toxicology purposes has also been advocated [6]. Samples can be obtained from adults or from newborns, e.g. to assess exposure to drugs, alcohol and other xenobiotics prior to birth. For an overview of analytes of particular forensic interest that have been measured in DBS the interested reader is referred to previous work of our group [6, 63]. The ease of (rapid) sampling and the stabilizing effect are two significant advantages coupled to DBS sampling in (forensic) toxicology. In newborn DBS, benzoylecgonine and cotinine, respectively metabolites of cocaine and nicotine, have been determined to evaluate the use of cocaine and tobacco products among childbearing woman [6]. For most applications, the limited amount of material, combined with the required sensitivity, imposes the need for a dedicated LC-MS/MS configuration. Recent progress in this field includes the set-up of hands-off on-line systems (see also section A.1.5.1. Automation) [64]. When considering the cut off for driving under the influence of drugs, alcohol and medicines (DRUID), for most analytes procedures have been described that are able to achieve the required sensitivity when starting from a ≤6.4-mm DBS punch or when starting from 10 µl or less dried blood [63]. Like oral fluid, DBS may offer more convenient sample collection in the context of driving under the influence of drugs, as the

usual urine sampling is coupled to privacy issues and the collection of a conventional blood sample by summoned medical staff is cumbersome and time-consuming [65]. In the context of driving license regranting programs, in which drivers with a history of alcohol abuse are followed up, DBS sampling may also be beneficial. We recently demonstrated this for phosphatidylethanol (PEth), a direct alcohol marker that is used to monitor alcohol consumption during the past few weeks: capillary concentrations of PEth were equivalent to those found in venous blood, demonstrating that capillary DBS are a valid alternative for venous blood for this purpose [66]. Since the sampling procedure does not require dedicated staff and PEth outperforms indirect markers like carbohydrate-deficient transferrin (CDT), capillary DBS sampling offers a promising avenue for routine follow-up of drivers with a history of alcohol abuse. As is the case for CDT now, PEth determination might become a routine procedure integrated in the clinical lab. Again, LC-MS/MS is the method of choice, combining both high-throughput and sensitivity. In the toxicology lab, DBS can also be used as a sampling preparation strategy. We routinely use DBS (as well as other dried matrix spots) for quantitative determination of gamma- and beta-hydroxybutyric acid. Although for these particular analyses we use “on-spot derivatization” and GC-MS [67-69], the use of dried (blood, urine, …) spots as an analytical tool (also allowing automation - see section A.1.5.1. Automation) prior to LC-MS/MS can be applied for other compounds as well [6, 70, 71]. When considering toxicology screening in an acute setting, liquid microsampling, coupled to e.g. on-line sample cleanup procedures like turbulent flow chromatography and MS/MS detection, are more likely to be used than DBS, since in most cases it would not make sense to wait for a sample to dry. Yet, it is conceivable that for screening purposes approaches like paper spray-MS/MS or -HRMS (see further in section A.1.6.4. MS(/MS)-based point-of-care testing) might be employed in future, to get an instant identification of an intoxicant. Again, a drop of blood might suffice.

Over the last few years, meconium has proven to be a valuable matrix in the assessment of prenatal exposure to drugs of abuse and has gained a lot of interest due to the higher sensitivity, the easier sample collection and the larger detection window than traditional matrices, such as neonatal hair and urine [32]. To date, effort has been put in the development of advanced broad-spectrum screening methods using LC-MS/MS, facilitating the use of meconium in clinical routine screening for drugs of abuse. Ristimaa *et al*. developed in this

context an LC-MS/MS-based targeted analysis method for a wide range of drugs of abuse, amongst which MDMA, MDA and THC-COOH [72]. Another application of meconium analysis is the quantification by LC-MS/MS of meconium fatty acid ethyl esters, ethyl glucuronide and ethyl sulfate, three alcohol markers used for the identification of *in utero* alcohol exposure [73]. Furthermore, the use of other non-invasive matrices in toxicology, such as nails, sweat and breast milk, has been enabled by the introduction of sensitive analytical techniques. Although these matrices can be useful in some instances (e.g. doping control, determination of exposure to environmental contaminants), their more widespread implementation in clinical routine is less likely, given the specialized nature of these samples.

A.1.3.4. TDM

TDM serves as an excellent tool in the optimization and individualization of drug therapy in both the general and special populations. Most often, TDM is performed on venous blood samples (whole blood, plasma or serum). Unfortunately, these samples are collected in an invasive way and the amounts of blood that are required are relatively large for e.g. neonates or anemic patients. In addition, samples need to be obtained by a phlebotomist, which obliges patients to visit a hospital or doctor's office for a blood draw. Therefore, there is a growing interest in the use of non- and minimally invasive alternative sampling strategies for TDM.

The most widely used alternative matrix in this regard is DBS [4, 5]. The use of DBS for TDM offers several benefits. As DBS are mostly obtained by a finger prick, the patient himself can perform the finger prick at home. In addition, as DBS are considered non- or less contagious, they can be sent via regular mail to the clinical laboratory [5]. Like that, laboratory results may already be available before a patient visits the clinician for routine follow-up. However, the small sample size (typically 3 - 12 µL) associated with DBS imposes the need for sensitive instrumentation [5]. This need can generally be met with LC-MS/MS. Whereas throughput can be considered a limitation of DBS analysis - at least when considering manual handling of DBS - the emergence of automated DBS analyzers could be of great benefit for clinical routine, as outlined further (see section A.1.5.1. Automation).

Table A.1.2 provides an overview of therapeutic drug classes, with selected examples, for which DBS-based TDM via LC-MS/MS has been reported [5, 74-86]. It needs to be mentioned,

though, that to date in clinical routine only few therapeutic drugs are determined in DBS via LC-MS/MS. A search throughout lab guides of different clinical laboratories only yielded 4 drug classes for which DBS are used for TDM in clinical routine: tricyclic antidepressants, antibiotics, anticonvulsants (see also Chapter A.3.) and immunosuppressants. Especially in the Netherlands, several hospitals have put major efforts to implement DBS for TDM (and other applications) in clinical routine [87-91]. As mentioned above for the use of DBS in an acute toxicology setting, also for TDM, liquid microsampling might be preferred over DBS sampling when feedback on the sample concentration is urgent. Still, in a hospital context, where staff is acquainted with traditional sampling and where patients are sampled anyway for evaluation of a variety of parameters, the implementation of alternative sampling strategies may not be a logical option in many cases. Microsampling may be a valuable option in those cases that require repeated measurement of drug levels e.g. for the abbreviated area under the curve estimation for the follow-up of tacrolimus treatment [92]. Outside the hospital, TDM may also play a crucial role in assessing patient adherence to prescription regimens of medication, as patient non-adherence is a worldwide problem and leads to serious consequences (e.g. additional use of scarce healthcare sources and higher costs of care, negative impact on the efficacy of treatments and patient's wellbeing) [93]. In this context, TDM via DBS also fits within the concept of "precision medicine", where a patient should not only get the right drug, but also at the right dosage to achieve the right concentration, eventually leading to optimized medication usage.

Although the small sample volume is one of the main advantages of DBS, it can be a limiting factor in certain cases as well, e.g. when a physician wants to evaluate various parameters in the same blood sample during treatment follow-up. The simultaneous determination of the kidney function, for example, can be of great importance given the fact that many drugs are excreted by the kidneys and/or may cause renal failure. In this case, creatinine (endogenous) or iohexol (administered) can be determined to assess the glomerular filtration rate. Koster *et al*. recently developed an LC-MS/MS method for the combined analysis of creatinine and several immunosuppressants in the same DBS extract, which is of great importance given the risk of renal failure associated with immunosuppressant use [94]. Since iohexol, a contrast agent, has already been analyzed in DBS, its determination in a DBS together with a drug of interest appears another feasible future perspective [95]. In summary, DBS can offer a lot of

advantages in the context of TDM. However, the choice to switch from traditional sampling to DBS sampling needs to be well-balanced, taking into account the clinical question and the context in which both sampling and analysis need to take place.

Another alternative matrix which has been extensively evaluated in the context of TDM is oral fluid. Since oral fluid is often regarded as a natural ultrafiltrate of whole blood, its use has been advocated as a convenient alternative to ultrafiltration or equilibrium dialysis to assess the free concentrations of therapeutic drugs [96]. In addition, oral fluid has the advantage of being obtained in a non-invasive manner, yielding the possibility of home sampling. Obviously, the latter is only feasible when a compound is sufficiently stable under ambient conditions, which needs to be evaluated during method development and validation. The stability of newer anti-epileptic drugs, for example, has proven to be adequate, allowing samples to be sent to the laboratory via postal service [97]. Furthermore, preservatives can be added to collection devices to enhance analyte stability [98].

Although oral fluid levels are suggested to correlate with the plasma free fraction of a drug, a correlation between both (or between oral fluid levels and total plasma levels) is often lacking. Moreover, even when a correlation is observed, the latter might be time- or concentration dependent and/or intra- and interpatient variability may be too large to allow reliable use in clinical practice [17, 99]. Therefore, oral fluid is probably not suitable for TDM of most therapeutic drugs [99]. Generally, non-ionizable drugs (at least within the pH range of oral fluid) are considered the best candidates [10, 16]. However, this always needs to be evaluated on a case-by-case basis. Antiepileptics are one of the drug classes for which oral fluid-based TDM can be performed successfully. Specific antiepileptic drugs for which oral fluid provides a good alternative include carbamazepine, clobazam, ethosuximide, gabapentin, lacosamide, lamotrigine, levetiracetam, oxcarbazepine, phenobarbital, phenytoin, primidone, topiramate, and zonisamide. For valproic acid on the other hand, this approach is not useful [12].

Additionally, other alternative matrices such as tears, hair, sweat, exhaled breath and ISF have also been evaluated for TDM purposes. Although some of these (e.g. ISF sampling via microneedles [34]) seem promising, currently, their use in routine TDM laboratories is non-existing or limited at best.

Table A.1.2. Overview of therapeutic drug classes, with selected examples of drugs, for which DBS-based TDM via LC-MS/MS has been reported.

Drug class	Medicines	
Anticonvulsant [75, 82]	Phenobarbital	Clobazam
	Topiramate	Clonazepam
	Rufinamide	Phenytoin
	Carbamazepine	Valproic acid
	Lamotrigine	
Antiviral [5, 74, 76, 85]	Atazanavir	Efavirenz
	Nevirapine	Darunavir
	Indinavir	Ribavarine
	Ritonavir	Tenofovir
	Saquinavir	Etravirine (TMC125)
	Nelfinavir	Raltegravir
	Lopinavir	(Val)Ganciclovir
Immunosuppressant [5, 76, 83]	Cyclosporine	Sirolimus
	Tacrolimus	Everolimus
	Mycophenolic acid	
Cytotoxic [5, 76, 78, 84]	Actinomycin-D	Imatinib
	Vincristine	Nilotinib
	Busulfan	Dasatinib
	Paclitaxel	Tamoxifen
	Docetaxel	
Analgetic [5, 86]	Acetaminophen	Oxycodone
Angiotensin II receptor antagonist [5]	Losartan	
Antibiotic [5, 74, 81]	Ertapenem	Moxifloxacin
	Linezolid	Rifaximin
	Claritromycin	Rifampicin
	Ramoplanin	
Antidepressant [5]	Venlafaxine	
Antimalarial [5]	Mefloquine	
Antimycotic [5, 74]	Voriconazole	Posaconazol
	Fluconazole	Metronidazole
Diuretic [5]	Canrenone	
Histamine H_2-receptor antagonist [5]	Ranitidine	
β-blocker [5]	Propranolol	
Antipsychotic [79]	Amisulpride	(Dehydro-)Aripiprazole
	Asenapine	Bromperidol
	(Nor)Clozapine	(OH-)iloperidone
	Haloperidol	Lurasidone
	(Levo)Sulpiride	Paliperidone
	Risperdone	Pipamperone
	Sertindole	Quetiapine
	Zuclopenthixol	
Antidiabetics [80]	Metformin	Sitagliptin
Enzyme-inhibitors [77]	Nitisinone	

A.1.3.5. Phenotyping

Phenotyping aims at determining the exact actual enzymatic activity of Cytochrome P (CYP) 450 enzymes. In general, phenotyping for a drug-metabolizing enzyme consists of administering a selective probe drug of the enzyme, followed by determining a specific pharmacokinetic metric (e.g. systemic clearance of the probe drug, single-point concentrations or metabolite/parent drug concentration ratios) [100]. CYP450 enzymes mainly catalyze phase I metabolism reactions, the first step in the enzymatic biotransformation which is chiefly responsible for the elimination of drugs and other xenobiotics. Interindividual variability is seen in CYP450 enzyme expression and function, which is determined by genetic, epigenetic and non-genetic host factors (e.g. sex, age, pathophysiological conditions) and by environmental influences, such as tobacco smoke, drug intake and diet [100]. Therefore, every person has his own CYP450 enzyme activity profile, resulting in variability in drug metabolism and -consequently- variability in drug response. This variability is the prime reason why CYP450 phenotyping can be implemented in clinical routine for a selection of drugs, including tricyclic antidepressants (imipramine, nortriptyline), antipsychotics (haloperidol, risperidone, clozapine, olanzapine), opioid analgesics (codeine, tramadol), proton-pump inhibitors (omeprazole, lansoprazole) and antithrombotic agents (clopidogrel, warfarine) [100]. The use of alternative sampling strategies for CYP450 phenotyping in combination with LC-MS/MS is currently confined to oral fluid and DBS. Although data obtained using EBC for CYP450 phenotyping are in some respect comparable with those obtained during oral fluid-based phenotyping, the use of EBC in this context is less obvious due to the highly specialized equipment necessary for the measurement of $^{13}CO_2/^{12}CO_2$ ratios in breath. To date, reliable phenotyping methods for various clinically relevant CYP450 enzymes, including CYP1A2, CYP2C19 and CYP2D6 are available using alternative sampling strategies. For CYP3A4 on the other hand, additional studies are necessary [100]. For more details, the interested reader is referred to the recent comprehensive review by De Kesel *et al*. [100].

A.1.3.6. Metabolomics, proteomics and protein analysis

The use of alternative sampling strategies in the 'omics' arena is to date limited to DBS, oral fluid and EBC. In a clinical setting, metabolomics and proteomics are typically targeted

approaches, following a discovery phase in which a selected set of biomarkers (proteins and small molecules, respectively) has been identified.

Metabolomics, the global study of metabolites in human body fluids, is an emerging 'omics' science which intends, just as proteomics, to discover specific disease biomarkers. Clinical metabolome studies by LC-MS/MS have been performed for transplantation, cancer, diabetes, lipid profiling and coronary heart disease. LC-MS/MS is an indispensable partner for performing quantitative metabolomics [101]. Alternative sampling strategies in the metabolomics field include EBC as well as oral fluid, with as an example the measurement of salivary biomarkers for the early diagnosis of various types of cancer [102-104].

EBC is an example of an alternative sampling strategy for which proteomic analysis has been performed for the detection of biomarkers related to asthma and COPD. Here, untargeted proteomics plays a distinct role in the search for the underlying pathobiology of these two most common chronic airway diseases. Analysis of EBC by an LC-MS/MS method revealed in this context the promising possibility of using a panel of proteins in the quest for the etiology of COPD [105].

Currently, clinical proteomics can be defined as the (large-scale) study of peptides/proteins in human biological matrices, aiming at validating and/or implementing biomarkers for the diagnosis, prognosis and/or therapeutic monitoring of diseases. Improvements in MS technology partly explain the increased focus on proteomics and protein analysis over the past decade, with applications in a variety of disciplines [106]. Recently, Chambers *et al*. published a multiplexed approach for the (semi-) quantification of a panel of 97 proteins in DBS [107]. However, the performance of proteomics and protein analysis in clinical routine, both by the use of conventional and alternative biological matrices (e.g. DBS), has remained rather limited to date and mainly focusses on studying one or several proteins. DBS-based protein analysis by MS includes quantitative and qualitative hemoglobin analysis, used in the diagnosis of sickle cell disease and other clinically relevant hemoglobinopathies [108]. Furthermore, Dewilde *et al*. developed a method for the determination of ceruloplasmin, a biomarker for Wilson's disease, in DBS using LC-MS/MS [109]. Proteins used in doping can also be determined in DBS via LC-MS/MS. Examples are insulin-like growth factor-1 (IGF-1), a biomarker of growth hormone (GH) abuse and Synacthen®, a synthetic human adrenocorticotropic hormone, causing increased plasma levels of cortisol [110, 111]. Further, the rapid emergence of protein

therapeutics will likely bring along the need in some clinical labs to measure these proteins in at least a subset of patients. This is typically done by targeted LC-MS/MS-based analysis of a representative set of peptides, generated by proteolytic digestion of a sample, such as a DBS.

Next to LC-MS/MS methods, several immunoassays were developed for the determination of relevant proteins in alternative samples, including DBS. Examples include thyroglobulin and prostate-specific antigen [112, 113]. Although more challenging to set up and implement, LC-MS/MS assays offer the advantage over immunoassays that they do not suffer from false positive results, caused by autoantibodies (e.g. against thyroglobulin) and rely on unequivocal identification rather than on antibody-based recognition. Another disadvantage coupled to many immunoassays is the lack of reliable reference methodologies, sometimes causing incomparable results. In this context, LC-MS/MS methods could be developed as reference measurement procedures [114]. An example of such a reference method is the determination of HbA1c, a fundamental biomarker in the long-term follow-up of the glycemic state of diabetic patients, in whole blood. Several publications readily unveiled the advantages of HbA1c determination in dried blood samples [115-117]. As blood lipids are also important risk determinants in patients with diabetes, a combined LC-MS/MS determination of HbA1c and lipids in DBS could be beneficial. Since LC-MS/MS-based quantification of cholesterol and related metabolites in DBS has already been performed in the screening of inborn errors, we believe that a combined method is certainly possible in the monitoring of diabetes patients [118].

A.1.4. Limiting factors

Although the use of alternative sampling strategies may be appealing for certain applications, their use in clinical routine is hampered by some practical hurdles, technical challenges and inherent (minor) disadvantages. First of all, the development of methods using alternative matrices generally takes longer, since more variables need to be evaluated. Examples include the evaluation of hematocrit, volume and chromatographic effect in DBS analysis and the influence of the collection method and collection device used in oral fluid analysis. In addition, method development may be further complicated due to interferences originating from e.g. DBS filter paper or oral fluid collection devices [18, 119]. Unfortunately, the above-mentioned

additional variables are often not included in standard validation guidelines and matrix-specific guidelines are not always available.

Furthermore, matrix-specific issues exist, which can lead to erroneous results or may complicate data interpretation. Hair analysis, for example, is subject to several issues, as already mentioned in section A.1.2. Alternative sampling strategies. First of all, it has been shown that external contamination can lead to false positive results, since contaminants can be introduced in the hair matrix of a non-user in various ways, including during washing steps carried out in the lab [21, 22, 120]. Secondly, for certain compounds a single dose may yield positive results not only in the hair segment corresponding to the moment of intake, but throughout the entire length of the hair, falsely indicating chronic use [121]. Thirdly, cut-offs employed for toxicological hair analysis are not available for every compound or may vary between different guidelines [122]. In DBS analysis on the other hand, the hematocrit effect is the most prominent issue affecting data accuracy and interpretation [3]. Another practical issue in interpreting DBS results is the fact that existing reference intervals and therapeutic ranges are generally established using serum or plasma. So either new reference intervals need to be set up for the specific matrix or bridging studies have to be conducted to correlate alternative matrix levels to systemic plasma or serum levels, whenever a correlation between both is assumed [123]. However, thorough clinical validation is often lacking, as generally only a limited number of samples are included in these studies or the included samples are not true patient samples. In addition, studies on the effect of the use of alternative matrix analysis on patient outcome have not been conducted to the best of our knowledge.

Moreover, as mentioned above, the analysis of alternative matrices requires sensitive equipment, since only a limited sample volume may be available and/or concentrations present may be very low. Hence, when obtaining sufficient sensitivity using traditional matrices (e.g. 100 µL of plasma) is already challenging, analysis of a DBS, for example, may not be feasible. Furthermore, the analysis of alternative matrices such as DBS and hair often includes a lot of manual steps and hands-on time, limiting sample throughput and increasing turn-around-time. The set-up of a quality control (QC) program is also particularly challenging for alternative matrices (as outlined in section A.1.5.2. Quality assurance and harmonization). Furthermore, it needs to be taken into account that in the case of home sampling of e.g. DBS or oral fluid there is no control of sample collection and storage conditions, as the collection

is carried out by the patient or his caregiver, instead of by trained personnel. Although the collection of these samples is not that hard, quality issues with patient samples may pose problems. Also important in a clinical setting is the fact that these alternative tests may not be included in nomenclature and hence may not be reimbursed by the public healthcare system.

A.1.5. Towards routine implementation

To overcome the above-mentioned hurdles a lot of work has been done and is still ongoing, including the development of new, sensitive and robust analysis techniques, new sampling formats, automated analyzers, and a surrounding support system comprising e.g. proficiency testing (PT) programs and matrix-specific best practice guidelines.

A.1.5.1. Automation

An important step in incorporating the analysis of alternative samples in routine laboratories is automation; not only to increase throughput and safety, but also to decrease hands-on time and to exclude human errors. Ideally, automation encompasses the pre-analytical, analytical and post-analytical phase. More specifically in the case of DBS or DPS, this means a lab technician would only have to introduce a patient's card into an analyzer after which the sample is automatically analyzed and the obtained result communicated into a laboratory management information system. Promising advancements have been made in this regard. The tedious punching step can be replaced by (semi-) automated punching devices, whilst sample preparation can be automated using e.g. readily available liquid handling systems [124]. Furthermore, completely automated DBS/DPS analyzers have become commercially available and can be directly coupled to standard LC-MS/MS configurations. These analyzers have accessories such as e.g. barcode readers that allow for sample registration and traceability. Every type of DBS/DPS automated analyzer uses solvents to elute a fixed area of matrix from a collection card, obviating the need for punching. The way the elution is performed depends on the type of analyzer: the DBS Autosampler™ and the Sample Card and Prep system™ both employ flow through desorption, whilst in the DBS-MS 500 system (see also Chapter A.3.) extraction solvent is guided horizontally through the DBS during a surface sealed extraction, after which the extract is guided into a sample loop [125-127]. After elution, the extract can be

subjected to on-line sample clean-up and/or separation on an LC column or even direct injection into the MS, depending on the chosen configuration [8, 64, 125-127]. Importantly, using automated DBS analyzers, the entire DBS extract is introduced into the (LC-)MS/MS system, thereby increasing method sensitivity, since in off-line approaches only part of the extract is injected into the analyzing system. The DBS-MS 500 system is currently the only analyzer in which internal standard can be sprayed onto the DBS before extraction [125]. In the two other types of analyzers the internal standard is automatically added to the elution solvent. To verify whether the correct portion of the card has been analyzed these analyzers can take a picture of the sample before and after analysis. In addition, it is also possible to use the DBS analyzers as automated sample preparation devices that are not coupled to an LC-MS/MS system. To reduce hands-on-time even further, also the preparation and spotting (in the case of DBS/DPS) of calibrators, QCs and blanks can be automated using a commercially available liquid handling system [59, 128]. This procedure showed similar accuracy and precision as manual preparation, but was safer, more efficient and yielded samples of predictable quality.

For the analysis of other alternative matrices, automation is also important to allow convenient implementation in a routine setting. Therefore, commercially available workstations can be employed to automate laborious sample pretreatment steps as much as possible. This was e.g. done for the analysis of drugs of abuse in preserved oral fluid samples collected using Quantisal™ devices [59]. For this application the workstation was not only used for IS addition, but also for automated SPE. The only remaining manual step during sample pretreatment was the evaporation of the SPE eluate which was automatically collected in LC vials. However, for some matrices automation may prove challenging. In the case of hair analysis for example, the inability to handle the solid hair sample limits the degree of potential automation [129].

Importantly, when using LC-MS/MS in a routine setting, not only the sample handling and the on-line sample preparation need to be automated, also the LC-MS/MS modules and software programs should become more user-friendly. More specifically, to make LC-MS/MS technology as convenient as possible for lab technicians, ideally a sort of “black box” LC-MS/MS unit should be integrated in existing chemistry analyzers. One suggestion that has been made in this regard is the development of an analyzing module with hybrid technology combining the characteristics of immunoassays and tandem MS [130], potentially omitting the need for LC.

Another, complementary way of increasing throughput to which LC-MS/MS lends itself perfectly, is to multiplex different analytes in a single run. The latter also aids in retrieving as much information as possible from a limited sample. However, in hair analysis the development of such multi-analyte procedures might prove challenging, since authentic hair samples are required for e.g. extraction optimization during method development [129]. Additionally, multiple LC systems can be multiplexed on one MS, further allowing a more economic use of the MS. This can be via staggered analysis (in which the chromatographic eluate only enters the MS/MS system during the time window where the compounds of interest elute) and/or via a more convenient switching between different methods, since no hardware changes need to be performed. Even sample multiplexing (i.e. the simultaneous introduction of two samples that were differentially derivatized) may be considered an option to increase throughput [131].

A.1.5.2. Quality assurance and harmonization

LC-MS/MS methods, including those for alternative matrix analysis, are typically developed in-house in clinical laboratories and are (at least up till now) generally not approved by the Food and Drug Administration [132]. Therefore, every laboratory is completely responsible for each test it implements. Unfortunately, QC materials for alternative matrices - necessary to help guarantee method quality - are generally not commercially available. QCs should be prepared in native matrix and whenever this is not feasible, should at least be commutable with true samples. Although a few standard kits exist for LC-MS/MS, these are not necessarily suitable for e.g. DBS methods, since in that case calibrator and QC materials should also have the same viscosity as true blood to have similar spreading properties. Therefore, calibrators and QCs are currently often prepared in-house from different (non-)certified starting materials. The development of more LC-MS/MS kits, encompassing calibrators and QCs in a suitable matrix, as well as e.g. internal standards, extraction solvents and mobile phases would hence be a tremendous step forward. Although such a kit has, for example, already been developed for the analysis of amino acids and acylcarnitines in DBS, it needs to be pointed out that it was developed for MS/MS analysis and not specifically for LC-MS/MS analysis [133]. Some alternative matrices also have special QC requirements. For DBS, for example, it is advisable to include different hematocrit levels [134], whilst for hair analysis it is important to include

different hair types [135]. In addition, to ensure appropriate accuracy, methods should ideally be traceable to a higher order reference method. However, these reference methods are often lacking.

Concerning external quality assurance, the NBS quality assurance program has played an important role for DBS analysis, since it offers both certified PT materials and external QCs for (MS/MS-based) NBS assays [136]. However, external QC and PT materials are not yet available for all performed NBS tests. Furthermore, an initiative has been launched by The Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology to set up a pilot PT program for TDM of immunosuppressive drugs in DBS [137]. For hair analysis the Society of Hair Testing (SoHT), HAIRVEQ and the German Society of Toxicological and Forensic Chemistry organize PT programs for some drugs of abuse and ethanol markers [122]. Also for oral fluid and sweat PT schemes exist for drug testing. An extra complicating factor in PT programs for alternative matrices are the different types of substrates that are used to collect samples (e.g. different types of filter paper cards in DBS sampling and different collection devices in oral fluid sampling) [137]. Therefore, to facilitate these types of programs, harmonization/ standardization will be important in the future.

Not only the analytical method itself, but also the patient samples (even when collected at home) have to be of sufficient quality. A first step towards achieving this is by guaranteeing the quality of the substrate on which the sample is collected. For DBS sampling this is achieved via the Filter Paper Evaluation Project, set up by the center for disease control and prevention (CDC) which also offers its services to filter paper manufacturers [136]. The quality criteria postulated by the CDC resulted in the laboratory standard, Clinical and Laboratory Standards Institute NBS01-A6, which has greatly contributed to the reduction of analytical imprecision due to batch-to-batch variability in filter paper. Furthermore, the quality of a DBS is also evaluated before analysis, either by experienced laboratory personnel or in an automated fashion using an optical scanning instrument [138]. Such instruments objectively evaluate spot area, circularity, convexity and consistency (i.e. DBS size, symmetry and uniformity). Thus, acceptable, marginally acceptable, and unacceptable DBS are distinguished and acceptable punch areas identified. To maximize the amount of acceptable spots in the event of home sampling, patient education via demonstration folders and movies has proven to be essential. To evaluate whether contamination occurred during sample collection, incurred sample

reanalysis or analysis of a blank filter paper area close to the DBS has been advocated [6, 139]. For other matrices which are usable for home monitoring, evaluation of sample quality is also essential. Therefore, it can for example be advised to perform oral fluid home sampling using collection devices with volume indicators.

To help harmonization and standardization of alternative matrix analysis, best practice guidelines have been proposed by several committees (e.g. SoHT or the European Bioanalysis Forum consortium) highlighting important pitfalls and validation requirements [122, 134]. Harmonization of data interpretation is also an essential goal of these committees. The SoHT and the DRUID project, for example, have played an important role in the determination of cut-offs for toxicological analysis of hair and oral fluid, respectively [122, 140].

A.1.6. Future trends

A.1.6.1. Development of new formats

New formats and approaches have been developed to improve the acceptance of dried blood (spot) analysis by tackling the crucial hematocrit issue (see also Chapter B.1. for a more detailed description of this hematocrit issue). Strikingly, most of these approaches have been designed to be compatible with automated analysis. First of all, volumetric approaches have been suggested which are able to produce fixed-volume DBS from a non-volumetric whole blood droplet. Whilst some of these approaches use formats which are compatible with existing DBS analyzers [141, 142], others such as VAMS employ a different type of collection device [143, 144]. However, also in this case, sample preparation can still be automated, more particularly via readily available liquid handling systems. Secondly, others have suggested the use of DPS instead of DBS. In such instance the former can be prepared via filtration of a blood drop over a size-exclusion membrane, which prevents blood cells from passing through [145, 146]. The DPS cards developed by Sturm *et al*. are compatible with commercially available automated analyzers [147]. Unfortunately, also DPS appear to be subject to a certain hematocrit effect [147]. A third possibility is to estimate the hematocrit of a DBS and to correct for the anticipated hematocrit effect [148]. Recently, we developed a non-contact hematocrit estimation method which could potentially be more easily automated than the potassium-based hematocrit estimation method we previously established [7, 149, 150].

For other alternative matrices innovation is also ongoing. An example is the development of a new exhaled breath collection device for the analysis of drugs of abuse, which allows to standardize the volume of matrix collected (at least to some degree) and facilitates remote collection by non-trained personnel [151]. The device is composed of a filter which is located in a filter holder and a mouth piece which is attached to a plastic bag. When a person breathes into the device, aerosol particles will be collected onto the filter and the plastic bag will inflate. When the plastic bag is full, sufficient matrix has been collected onto the filter. Subsequently, the filter holder and the mouthpiece can be detached from one another and the filter holder can be closed with plugs and sent to a laboratory via regular mail.

A.1.6.2. Microfluidics

Microfluidics have been employed for alternative matrix collection and sample preparation in conjunction with tandem MS. One example includes the use of a lab-on-a-chip for sample preparation of either a DBS punch or a directly applied capillary blood droplet [152]. Multiplexed extraction of DBS using this type of chips was demonstrated by Lafrenière *et al*. using automated droplet control [153]. Due to its relative simplicity this technique has even been suggested to be able to bring clinical analysis closer to the patient. In this context, a proof of principle was published by Kirby *et al*. who used digital microfluidics to extract dried urine spots and to transport the extract to a nanoelectrospray emitter to allow tandem MS-based detection of drugs of abuse using a portable mass spectrometer [154]. Evans *et al*. on the other hand, employed capillary-scale LC to analyze DBS extracts, since this increases assay sensitivity [155]. To avoid problems with column connections, a chip can be employed onto which column, connections and MS emitter and spray are co-located [156]. Although no published examples could be found by the authors, chip-based microfluidic extraction could potentially be made compatible with LC by integrating the required technology into a single chip format for a seamless workflow. Notwithstanding the promising nature of these new developments, latest technological advancements are often only steadily implemented in a routine setting, as robustness first has to be well established.

A.1.6.3. High resolution mass spectrometry (HRMS)

Aside from its use in e.g. biomarker discovery research (possibly in oral fluid or DBS), untargeted screening using HRMS is increasingly becoming a valuable tool for toxicological purposes, e.g. allowing detection of new psychoactive compounds and their metabolites [157]. In other cases where untargeted screening is advised, e.g. for the evaluation of the chemical and biological exposure of humans (which has been suggested to become more important in the future) [158], HRMS may also be an ideal screening tool. In addition, for the detection of inborn errors of metabolism, the use of HRMS has been advocated as an alternative for the traditionally used electrospray ionization-MS/MS, since multivariate pattern recognition analysis would lead to better specificity and the identification of comorbidities and interferences caused by medical treatment or damaged DBS [159]. Last, although LC-MS/MS is likely to remain the workhorse for quantitative bio-analysis during the next couple of years, LC-HRMS/MS is increasingly advocated as a suitable alternative, further facilitating quantitative analysis of complex mixtures.

A.1.6.4. MS(/MS)-based point-of-care testing (POC)

Another important trend in clinical analysis is the development of MS(/MS)-based POC testing and/or near-patient analysis. The latter may be performed at a professional healthcare center, at the emergency unit or even in operating theatres. However, development of these decentralized analyses is most often -and ideally- still under the supervision of the clinical laboratory, which is responsible for e.g. quality assurance. In general, these POC techniques do not require sample preparation steps nor a separation step and employ ambient ionization techniques (although a multitude of ambient ionization techniques have been employed, selected examples will be discussed). In this context as well, alternative sampling strategies have been employed. Examples include the use of paper spray MS for the analysis of blood or oral fluid collected on filter paper for e.g. TDM purposes and abstinence monitoring [160, 161]. In paper spray MS, a drop of the biological sample is deposited on a triangle-shaped filter paper which is part of a disposable collection cartridge. After the cartridge has been positioned in front of the MS, a solvent is applied to the filter paper, as well as a high voltage. This causes a spray to be formed at the tip of the triangle, which is then transmitted into the MS.

Subsequently, the resulting signal is recorded for a fixed period of time, which results in a signal vs. time plot (called a chronogram). To quantify the amount of the target compound present, the area under the curve of this chronogram is employed. To be workable in a POC-setting, the blood on the filter paper is either analyzed when it is still wet or after it has been quickly dried using either pre-spotted coagulants or heat application [160-162]. A first step towards automation has been accomplished by the development of a tray, which can hold multiple filter paper triangles which are consecutively analyzed [163]. In addition, to render POC-MS feasible, portable MS systems have been developed [164]. The mini 12, for example, has been employed for the analysis of the therapeutic drug amitriptyline using paper spray MS [165]. The goal of this instrument is to be able to offer a sample to a miniature MS after which analysis and data analysis are performed automatically and a result is directly generated on the screen. Similarly, the concept of touch spray MS has been developed. This refers to the direct analysis of oral fluid collected on a medical swab. In this case as well, a solvent and a voltage are applied to the collection device after which a spray is formed. Applications of the technique include the semi-quantification of various drugs and the detection of lipids which are specific to S. pyogenes to quickly diagnose strep throat [166, 167]. In addition, even surgical smoke has been sampled via a surgical knife to help differentiate between cancerous and non-cancerous tissue during surgery [168].

A.1.7. Conclusion

Although ample clinically valuable applications have been developed using alternative sampling strategies, their adoption in routine clinical laboratories is still limited. Aside from the vast use of DBS in NBS programs, only few examples can be found which employ alternative sampling strategies for routine analyses using LC-MS/MS. Examples include the determination of salivary cortisol levels, the determination of drugs of abuse in oral fluid, and the use of DBS for TDM purposes. The growing automation of alternative matrix analysis will definitely contribute to the acceptance and introduction in routine practice. In addition, matrix-dependent issues are (successfully) being tackled and new, more robust formats are being developed, bridging the gap between research and clinical laboratories. To guarantee the quality of alternative matrix-based assays, initiatives to set up guidelines for the development and validation of these assays (which take into account matrix-specific requirements) as well as matrix-specific PT programs are essential. Although alternative sampling strategies will never replace traditional sampling, they should definitely be regarded as a complementary approach, which may be particularly valuable to extend the window of detection and/or to allow specific applications such as home monitoring, sampling of special patient populations (such as neonates, children and elderly), and sample collection in remote or resource limited areas. In this context, alternative sampling strategies, combined with LC-MS/MS, can be looked at as an additional tool, with the potential to provide high quality results where adequate information cannot be (conveniently) obtained using traditional approaches.

A.1.8. References

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CHAPTER A.2.

VOLUMETRIC ABSORPTIVE MICROSAMPLING AS AN ALTERNATIVE TOOL FOR THERAPEUTIC DRUG MONITORING OF FIRST-GENERATION ANTI-EPILEPTIC DRUGS

Based on

Velghe S, Stove CP. Volumetric absorptive microsampling as an alternative tool for therapeutic drug monitoring of first-generation anti-epileptic drugs. *Analytical and Bioanalytical Chemistry* 410(9), 2331-2341 (2018).

Abstract

Dosage adjustment of anti-epileptic drugs by therapeutic drug monitoring (TDM) is very useful, especially for the first-generation anti-epileptic drugs (AEDs). Microsampling -the collection of small volumes of blood- is increasingly considered a valuable alternative to conventional venous sampling for TDM. Volumetric absorptive microsampling (VAMS) allows accurate and precise collection of a fixed volume of blood, eliminating the volumetric blood hematocrit bias coupled to conventional dried blood spot collection. The aim of this Chapter was to develop and validate an LC-MS/MS method for the determination and quantification of four anti-epileptic drugs (carbamazepine, valproic acid, phenobarbital and phenytoin) and one active metabolite (carbamazepine-10,11-epoxide) in samples collected by VAMS. The method was fully validated based on international guidelines. Precision (%RSD) was below 10%, while, with a single exception, accuracy (%bias) met the acceptance criteria. Neither carry-over nor unacceptable interferences were observed, the method being able to distinguish between the isomers oxcarbazepine and carbamazepine-10,11-epoxide. All compounds were stable in VAMS samples for at least 1 month when stored at room temperature, 4 °C and -20 °C and for at least 1 week when stored at 60 °C. Internal standard-corrected matrix effects were below 10%, with %RSDs below 4 %. High (>85%) recovery values were obtained and the effect of the hematocrit on the recovery was overall limited. Successful application on external quality control materials and on left-over patient samples demonstrated the validity and applicability of the developed procedure.

A.2.1. Introduction

Whether a patient with epilepsy is free from seizures and is able to live a normal life depends on the correct administration of appropriate anti-epileptic drugs (AEDs). AEDs can be divided into three subclasses: the 'classical' or 'first-generation' AEDs, the 'second-generation' AEDs and the 'third-generation' AEDs [1]. Carbamazepine, phenobarbital, phenytoin and valproic acid were introduced prior to 1990 and belong to the first-generation AEDs [1]. Oxcarbazepine, vigabatrin and topiramate, amongst others, are examples of the second-generation AEDs, while lacosamide, retigabine and eslicarbazepine are categorized as third-generation AEDs [1]. Significant interindividual variability in pharmacokinetics (absorption, distribution, metabolism and excretion) is one of the commonly known properties of first-generation AEDs, making optimization and individualization of the therapy quite challenging [2].

Therapeutic drug monitoring (TDM) serves as an excellent tool in the optimization and individualization of drug therapy. The generally narrow therapeutic indices of first-generation AEDs, causing toxicity to be a common issue, have led to the fact that TDM has become an established application, in general as well as in special populations (e.g. children, elderly and pregnant women with epilepsy).

TDM is most often performed on venous blood samples (whole blood, plasma or serum). However, given the invasive nature of the associated sampling and the relatively large amounts of blood that are typically taken, this sampling procedure becomes increasingly less attractive in clinical practice. In addition, as sampling requires a phlebotomist, patients are obliged to visit a hospital or doctor's office for a venous blood draw. Therefore, there is a growing interest in the use of non- and minimally invasive alternative sampling strategies for TDM [3, 4].

One of the most commonly used alternative sampling strategies is dried blood spot (DBS) sampling. Generally, DBS are prepared by depositing a drop of capillary blood, obtained by a finger or heel prick, on a dedicated filter paper. Over the past few years, several methods were published using DBS for the determination of both first- and second-generation AEDs [5-12]. DBS sampling -for TDM and in general- has several advantages over conventional venous blood sampling. As DBS are mostly obtained by a finger prick, the patients themselves can perform sampling at home. Furthermore, as the resulting dried matrix is considered non- or less contagious, sending DBS via regular mail to the clinical laboratory is allowed [13]. This way,

laboratory results may already be available before a patient visits the doctor's office for follow-up. Besides, sending the samples by airmail can also be advantageous in countries where patients have to cover a long distance to clinical practices. The small sample volume (typically 3 - 12 µL) associated with DBS sampling is another benefit, particularly for special populations, such as neonates and anemic patients. In addition, the sampling procedure is accompanied by increased analyte stability and by fewer difficulties with respect to sample handling, storage and transport [13]. Given all these benefits, DBS sampling can serve as an excellent alternative to conventional venous sampling for TDM of AEDs.

On the other hand, DBS sampling is also struggling with some challenges, with the hematocrit (Hct) issue undoubtedly being the most widely discussed one. In essence, because of Hct-dependent spreading of blood on filter paper (blood with higher Hct spreading less), partial punch analysis of a DBS (which is the approach mostly used) will most often yield a bias for DBS generated from blood with divergent (either low or high) Hct. However, several strategies have been developed that allow to cope with the issues coupled to a varying Hct [14-22]. One of the proposed approaches is the use of volumetric absorptive microsampling (VAMS) devices [23, 24]. The latter are handheld devices consisting of a hydrophilic polymer tip connected to a plastic handler, which are able to accurately and precisely wick up a fixed volume (approximately 10, 20 or 30 µL) when contacting a blood surface [24]. Using authentic samples with a wide Hct range (0.21-0.50), our lab readily demonstrated that VAMS effectively results in absorption of a fixed volume of blood, irrespective of the hematocrit [23]. Furthermore, VAMS still maintains the benefits associated with DBS sampling and was reported to be preferred over DBS sampling by patients in a home sampling context [25]. The associated cost, as well as current incompatibility with on-line analysis systems, as developed for DBS analysis, may be considered disadvantages. In addition, we -as well as others- found that, while VAMS effectively allows volumetric sampling (thereby not suffering from a Hct effect as observed in DBS), recovery may be impacted by Hct [23, 26].

The aim of this Chapter was to develop, validate and apply an ultra-performance liquid chromatography - tandem mass spectrometry (UPLC®-MS/MS) method for the determination and quantification of four AEDs and one active metabolite, including carbamazepine (CBZ), valproic acid (VPA), phenytoin (PHT), phenobarbital (PB) and carbamazepine-10,11-epoxide (CBZ-E), making use of VAMS devices. We thereby paid particular attention to the recovery

issue associated with analysis of VAMS devices. CBZ, VPA, PHT and PB were chosen since they belong to the first-generation AEDs class, for which the strongest evidence for TDM exists. Furthermore, this type of AEDs is still frequently used for seizure control in developing countries, where microsampling may offer the largest benefits [27, 28]. CBZ-E, an active metabolite of CBZ, was also incorporated in the multi-analyte method as it is equipotent to CBZ and hence contributes significantly to its therapeutic (or toxic) effects [29]. Furthermore, since in MS/MS the multiple reaction monitoring (MRM) transitions characteristic for CBZ-E are the same as those for its isomer oxcarbazepine (OXC), OXC was also incorporated to assess the capability of the method to distinguish between CBZ-E and OXC, rather than to quantitatively determine OXC.

A.2.2. Materials and methods

A.2.2.1. Chemicals and stock solutions

LC-MS grade acetonitrile was obtained from Biosolve (Valkenswaard, The Netherlands). A Synergy® Water Purification System (Merck Millipore, Overijse, Belgium) provided ultrapure water. Valproic acid, valproic acid-D6, phenytoin, phenytoin-D10, carbamazepine, carbamazepine-D10, oxcarbazepine, carbamazepine-10,11-epoxide and ammonium acetate were purchased from Sigma Aldrich (Diegem, Belgium). Phenobarbital and phenobarbital-D5 were derived from LGC standards (Molsheim Cedex, France). Oxcarbazepine-D4 was obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany) and carbamazepine-10,11-epoxide-d10 from J.H. Ritmeester B.V. (Nieuwegein, The Netherlands).

Taking into account the upper and lower limit of the therapeutic range of each compound, methanolic stock solutions were prepared at 40.00, 50.00, 10.00, 10.00 and 5.00 mg/mL for VPA, PB, PHT, CBZ and CBZ-E, respectively. For OXC, a 1.67 mg/ml stock solution was prepared in acetonitrile. For the preparation of the calibrators and quality control samples (QCs), independently prepared stock solutions were used. For the internal standards (IS) of PHT, CBZ and PB, methanolic stock solutions of 0.100 mg/mL were purchased, while for VPA the concentration of the IS stock solution was 1.00 mg/mL. IS stock solutions for CBZ-E and OXC (1.00 mg/mL) were prepared in methanol and acetonitrile, respectively. Working solutions of the standards and the IS were prepared the day of analysis by diluting the stock solutions with

water. All solutions -except for the stock solution of CBZ-E (4 °C)- were stored at -20 °C in 1.5 mL amber glass vials derived from VWR® (Leuven, Belgium).

A.2.2.2. Sample collection

Venous whole blood from an AED abstinent healthy, female volunteer was collected in EDTA tubes (BD Vacutainer® with BD Hemogard® closure 10 mL) for method development and validation purposes. VAMS devices (Mitra™) were obtained from Neoteryx (Torrance, CA, USA). Samples were prepared by dipping the tip into spiked whole blood in 2 mL eppendorf tubes. Overfilling of the devices was prevented by not completely immersing the tip into the blood. After completely filling the tips, the devices were dried in the accompanying clamshells for 2 hours at ambient temperature. Once dried, the VAMS devices were stored at room temperature in zip-closure plastic bags, containing two 5 g packages of desiccant (Minipax® absorbent packets, Sigma Aldrich) until UPLC®-MS/MS analysis.

A.2.2.3. Sample preparation

Sample preparation was performed by separating the VAMS tips from the plastic handlers and transferring these into 2 mL eppendorf cups. Extraction was carried out using a Thermo-shaker TS-100C (BioSan, Riga, Latvia). In order to optimize the extraction conditions, different combinations of water and acetonitrile were evaluated, as well as different extraction solvent volumes (varying from 70 to 140 µL), extraction times and temperatures. For each of the tested conditions, spiked VAMS devices were analyzed in triplicate and the final sample preparation method was selected based on a comparison of the peak areas obtained for each condition.

A.2.2.4. Preparation of calibrators and QCs

Calibrators were made at eight concentration levels in blank whole blood. For each compound -except for VPA- the lower limit of the therapeutic range in plasma divided by two was set as the lower limit of quantification (LLOQ) and the upper limit of the therapeutic range times four as the upper limit of quantification (ULOQ). The resulting calibrator concentrations were 1, 1.50, 2.00, 33.60, 65.20, 96.80, 128.40 and 160.00 µg/mL for PB; 4.00, 6.00, 8.00, 22.40, 36.80, 51.20, 65.60 and 80.00 µg/mL for PHT; 2.00, 3.00, 4.00, 12.80, 21.60, 30.40, 39.20 and 48.00

µg/mL for CBZ and 0.25, 0.38, 0.50, 5.20, 9.90, 14.60, 19.30 and 24.00 µg/mL for CBZ-E. For VPA, detector oversaturation occurred with concentrations at four times the upper limit of the therapeutic range, therefore the upper limit of the therapeutic range times 1.5 was used as ULOQ, yielding calibrators at 25.00, 37.50, 50.00, 70.00, 90.00, 110.00, 130.00 and 150.00 µg/mL. When taking into account blood/plasma ratios (see further), these calibration lines also cover the anticipated therapeutic ranges in blood. QC solutions (LLOQ, Low, Mid, High, respectively) were prepared in blank whole blood at 25.00, 55.00, 100.00, 112.50 µg/mL for VPA; 1.00, 3.00, 40.00, 120.00 µg/mL for PB; 4.00, 8.00, 20.00, 60.00 µg/mL for PHT; 2.00, 5.00, 12.00, 36.00 µg/mL for CBZ; 0.25 and 1.50, 6.00, 18.00 µg/mL for CBZ-E. Non-matrix solvents were never added in a proportion higher than 5 % of the total sample volume.

A.2.2.5. UPLC® -MS/MS method

A Waters Acquity UPLC® system (Waters, Milford, MA, USA) coupled to a SCIEX API™ 4000 mass spectrometer (SCIEX, Framingham, MA, USA) was used for all analyses. The hardware system was controlled by SCIEX Analyst® 1.6.2 and by the Waters Acquity console software.

The deviating characteristics of the 6 compounds, combined with the inability of the utilized configuration to switch between positive and negative ionization modes, necessitated development of two different UPLC®-MS/MS methods, one operating in negative ionization mode (method I, monitoring VPA, PB and PHT) and one in positive ionization mode (method II, monitoring CBZ, CBZ-E and OXC).

For both methods, a Chromolith® reversed phase (RP)-18 endcapped 100x4.60 mm column (Merck Millipore, Overijse, Belgium), equipped with the corresponding guard column, was chosen as it gave the best results in terms of compound separation. The column oven was set at 45 °C. A mobile phase consisting of 5 mM ammonium acetate (A) and 5 mM ammonium acetate in acetonitrile/water (95/5, v/v) (B) at a flow rate of 1.4 mL/min turned out to be the best option. The total run time was 10 min, including a 4-min run for method I and a 6-min run for method II. The mobile phase gradient program for method I started with 20 % solvent B, linearly increased to 60 % in 1 min, followed by an increase to 98 % in 0.5 min, maintained for 1 min and finally, reversal to starting conditions. For method II, the gradient started with 10 % solvent B, followed by a linear increase to 27 % in 0.22 min, isocratic conditions for 0.28 min,

an increase to 31 % in 1.72 min, followed by a rise to 98 % in 0.88 min, kept for 0.8 min and finally, returning to starting conditions.

The API™ 4000 mass spectrometer was equipped with an ESI source (TurboIonSpray®) and used an optimized multiple reaction monitoring (MRM) algorithm for detection. The source temperature was set at 600 °C, the ion spray voltage at -3000 V for method I and at 2000 V for method II. Nitrogen was used as nebulizer (gas 1), heater (gas 2), curtain (CUR) and collision-activated dissociation (CAD) gas, with following gas pressure settings: 90 psi for gas 1, 10 psi for gas 2, 20 and 40 psi (respectively for method I and II) for CUR and the CAD vacuum was set at 12 for both methods (arbitrary settings).

For PB, PHT, CBZ, CBZ-E and OXC, two characteristic precursor-to-product ion transitions were monitored, while for the corresponding internal standards one transition was analyzed. Since no stable ion fragments are created for VPA, a pseudo mass transition (143.1/143.1) was monitored. Table A.2.1 shows all MRM transitions, together with the compound-specific MS parameters (optimized following infusion).

Table A.2.1. Multiple reaction monitoring transitions and compound-specific MS parameters for valproic acid, phenobarbital, phenytoin, carbamazepine, carbamazepine-10,11-epoxide, oxcarbazepine and the corresponding internal standards.

	Precursor ion (m/z)	Product ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)	Tr (min)
Valproic acid	143.1	143.1	-59.2	-10.0	-6.43	-7.13	1.53
Phenobarbital	231.0	188.0	-70.0	-10.0	-18.0	-5.00	1.79
		84.80	-70.0	-10.0	-14.0	-9.00	
Phenytoin	251.0	207.9	-80.0	-10.0	-30.0	-5.00	2.02
		102.0	-80.0	-10.0	-24.0	-13.0	
Carbamazepine	237.2	194.1	41.0	10.0	10.0	6.00	3.65
		179.0	41.0	10.0	30.0	4.00	
Carbamazepine-10-11-epoxide	253.2	180.2	51.0	10.0	33.0	16.0	2.64
		210.2	51.0	10.0	21.0	16.0	
Oxcarbazepine	253.2	180.3	81.0	10.0	41.0	14.0	2.88
		208.0	81.0	10.0	27.0	14.0	
Valproic acid-D6	148.9	148.9	-59.2	-10.0	-6.43	-7.13	1.51
Phenobarbital-D5	236.0	193.0	-70.0	-10.0	-18.0	-5.00	1.78
Phenytoin-D10	261.0	218.1	-80.0	-10.0	-24.0	-13.0	1.99
Carbamazepine-D10	247.0	204.1	41.0	10.0	31.0	12.0	3.61
Carbamazepine-10,11-epoxide-D10	263.1	220.2	51.0	10.0	21.0	16.0	2.57
Oxcarbazepine-D4	257.3	184.0	81.0	10.0	41.0	14.0	2.84

A.2.2.6. Method validation

Method validation was based on U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation [30, 31] and covered accuracy, precision, carry-over, selectivity, homoscedasticity, calibration model, stability, matrix effect, recovery and Hct effect. Control blanks (i.e. VAMS samples prepared with blank blood and analyzed without IS in the extraction solvent) and zero samples (i.e. VAMS samples prepared with blank blood and analyzed with the regular extraction solvent) were assessed throughout each sequence.

Accuracy (%bias) and precision (% relative standard deviation, %RSD) were assessed by analyzing QCs (LLOQ, Low, Mid and High) in duplicate on 4 different days. The within day and total precision were determined using ANOVA, whereas the accuracy was calculated by dividing the difference between the obtained concentration and the nominal value by the nominal value, and multiplying by 100 [32]. The %bias and %RSD should be within ±15 % for the QC samples, except for the LLOQ, where they should be within ±20 % [31].

Carry-over was examined by analysis of 2 blank samples after measurement of the highest calibrator (ULOQ), on 4 different days (n=8). Carry-over for the analytes should not exceed 20 % of the peak area found for the LLOQ and 5 % for the IS [31]. For selectivity, identical criteria were applied. Selectivity was assessed by analyzing blank VAMS samples prepared with whole blood from 6 different individuals.

The LLOQ was defined for each of the AEDs as the lower limit of the therapeutic range divided by two. These concentrations gave a signal of at least 10 times the signal of a blank sample. A chromatogram of each compound at the LLOQ level is provided in Figure A.2.1.

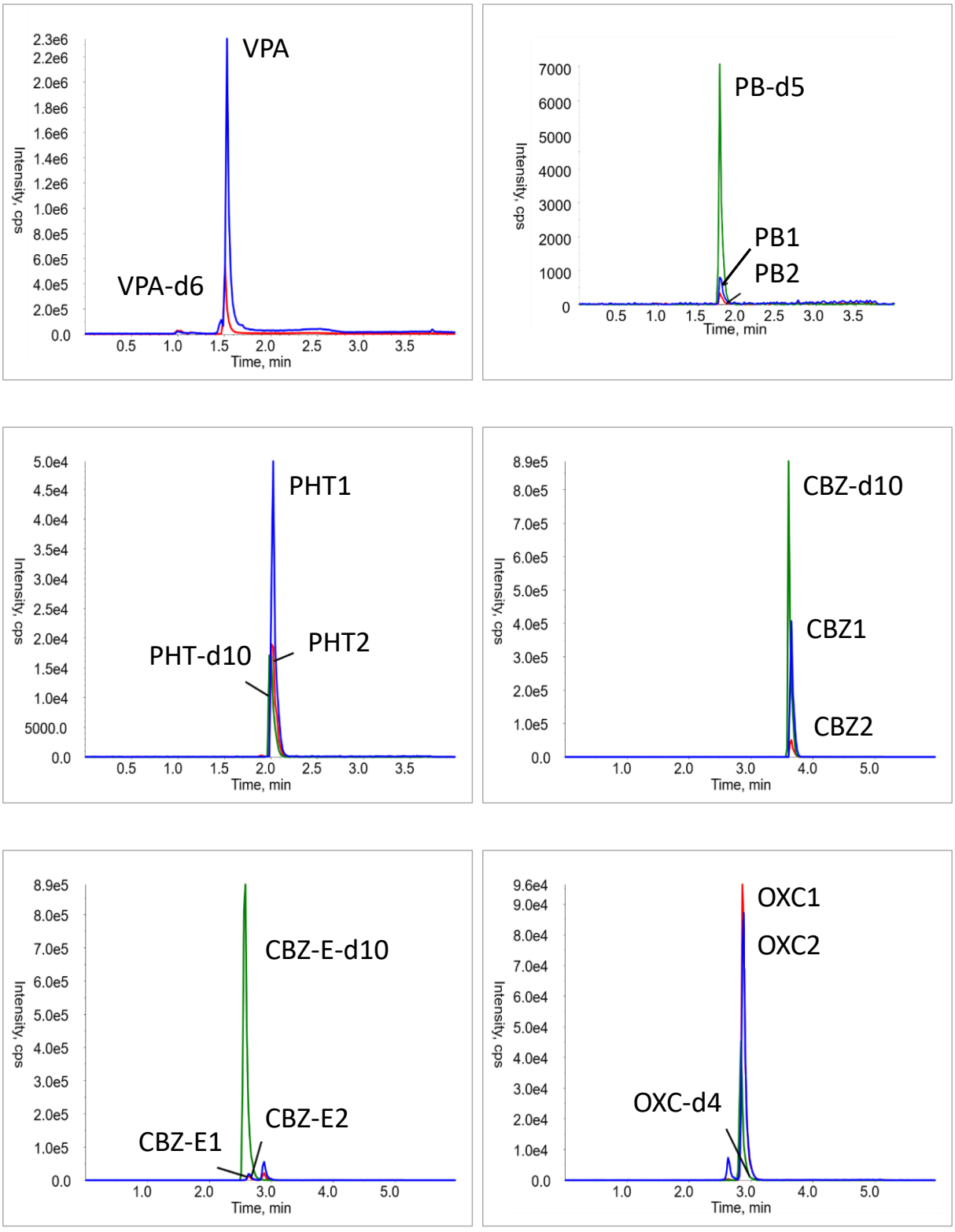

Figure A.2.1. Chromatograms at the LLOQ level for VPA (25 µg/mL), PB (1 µg/mL), PHT (4 µg/mL), CBZ (2 µg/mL), CBZ-E (0.25 µg/mL) and OXC (7.5 µg/mL).

Homoscedasticity and the calibration model were evaluated by generating eight eight-point calibration curves. Homoscedasticity was tested by performing an F-test (α=1 %) at the lowest and highest calibrator. Furthermore, for the calibration model, both weighted (1/x, 1/x2, 1/√x, 1/y, 1/y2 and 1/√y) and unweighted linear and quadratic regression were performed in order to find the best fitting model. The resulting models were compared by calculating the sum% relative error (%RE) and by plotting the %RE against nominal concentrations. Before accepting a selected model, a back-calculation was performed in which the mean concentrations of the calibrators should be within ±15 % of the nominal value or within ±20 % for the LLOQ [31].

Short- and long-term stability were assessed by analyzing Low and High QCs (n=3) in duplicate after storage for 4, 7 and 30 days at different temperatures (-20 °C, 4 °C, room temperature and 60 °C) in a zip-closure plastic bag containing two 5 g packages of desiccant. As reference, QCs, prepared at the same day of the QCs used for stability testing, were analyzed at time point zero. Autosampler stability (4 °C) was evaluated by storing the extracts of Low and High QCs for 24h in the autosampler before reinjection. At each day of analysis, an eight-point calibration curve was freshly prepared in order to calculate the concentration of the stored VAMS/extracts. Here again, the mean concentration of the QCs, at a particular time point should not deviate more than ±15 % from the nominal concentration [31].

Matrix effects were investigated by comparing the peak areas obtained at two concentration levels (Low or High QC), spiked to blank blood extract (from six different individuals, with a hematocrit ranging from 0.335 to 0.495) (A), with those obtained using a neat aqueous mixture containing the analytes and their IS at corresponding concentrations (B). The ratios of peak areas of (A) to those of (B), multiplied by 100 represent the IS-corrected matrix effect. Overall, the %RSD of the IS-corrected matrix effect should not exceed ±15 % [31].

The impact of the Hct on the recovery was evaluated for Low and High QC's (n=6) at four different Hct levels (target values at 0.21, 0.42, 0.52 and 0.62), prepared by centrifuging an aliquot of blood with a hematocrit of 0.40 in 2 mL eppendorf tubes in an Eppendorf 5804R centrifuge (Hamburg, Germany) for 5 min at 1000g and by removing or adding plasma. Here, two sets of VAMS samples were compared to one another, i.e. VAMS samples prepared by pipetting 10 µL of spiked blood (C) and VAMS samples prepared by pipetting 10 µL of blank blood and to which the analytes were only spiked post-extraction (D). The absolute recovery values (%) were calculated by multiplying the ratios of peak areas of (C) to those of (D) by 100.

To further evaluate the impact of the Hct, VAMS samples (n=6) were also prepared by dipping them into spiked blood at four different Hct levels (target values at 0.21, 0.42, 0.52 and 0.62). The latter better reflects the reality when compared to pipetting of a fixed volume onto the VAMS devices.

Where relevant, statistical analyses were performed using the Minitab® software.

A.2.2.7. Application

In order to objectively indicate the validity of the obtained results, 3 sets of external serum QC materials were used to generate QCs in blood and VAMS samples derived thereof. The QC materials were the ClinCal®-calibrator (Recipe®, Munich, Germany) containing PB 37.5 µg/mL, PHT 18.3 µg/mL, VPA 90.7 µg/mL, CBZ 11.2 µg/mL and CBZ-E 5.60 µg/mL in serum and the Liquichek™ Therapeutic Drug Monitoring Control (TDM) Levels 2 and 3 (Bio-Rad, California, USA) containing PB 34.7 µg/mL, PHT 15.2 µg/mL, VPA 85.6 µg/mL and CBZ 9.11 µg/mL in serum (level 2) and PB 65.0 µg/mL, PHT 31.8 µg/mL, VPA 134 µg/mL and CBZ 13.1 µg/mL in serum (level 3). In order to be comparable with a calibration curve prepared in whole blood, the external QC materials were diluted 1 on 4 with whole blood, by replacing 250 µL of plasma (obtained by centrifugation of 1 mL of whole blood) by 250 µL of the external QC materials. Due to this dilution, some concentration were no longer within the calibration range and, hence, could not be quantified. The resulting concentrations in blood of the used external QC materials were 33.5 µg/mL for VPA (Liquichek™ Level 3); 8.68, 9.38 and 16.3 µg/mL for PB (Liquichek™ Level 2, ClinCal® and Liquichek™ Level 3, respectively); 4.58 and 7.95 µg/mL for PHT (ClinCal® and Liquichek™ Level 3, respectively); 2.28, 2.80 and 3.28 µg/mL for CBZ (Liquichek™ Level 2, ClinCal® and Liquichek™ Level 3, respectively) and 1.40 µg/mL for CBZ-E (ClinCal®).

Furthermore, as a proof of concept, we analyzed 70 samples, collected at Ghent University Hospital from patients who visited the Hospital for evaluation of a variety of parameters, including follow-up of their AED treatment. VAMS samples were prepared by wicking up EDTA-anticoagulated blood from routine leftover whole blood samples that had been stored at room temperature for maximum 72 h. Approval for this study was provided by the Ethics Committee of Ghent University Hospital (EC2017/0572).

A.2.3. Results and discussion

A.2.3.1. Sample preparation

As the IS are in the extraction solvent, these do not compensate for recovery issues [33]. Therefore, optimization of sample extraction was comprehensively implemented. During this optimization, extraction at 22 °C and at 60 °C was compared, using freshly prepared VAMS samples, VAMS samples stored for 3 days at room temperature and VAMS samples stored for 3 days at 60 °C. The VAMS were prepared by using spiked whole blood (Low QC level) with a Hct of 0.41. Also a comparison was made with VAMS samples prepared from blood at a Hct of 0.62, stored for 3 days at 60 °C and extracted at 22 °C and at 60 °C.

As Figure A.2.2 depicts, extraction of the VAMS at elevated temperature (60 °C) provided overall the best results in terms of absolute recovery. Based on these findings, hundred µL of an acetonitrile/water (80/20, v/v) mixture, containing 5 mM ammonium acetate and the deuterated internal standards (0.5 µg/mL) was eventually used to extract VAMS devices by shaking for 10 min at 1000 rpm and 60 °C. Following centrifugation at ambient temperature for 10 min at 10 000 g, 70 µL of supernatant was diluted 1 on 1 with water containing 5 mM ammonium acetate. This mixture was transferred to an amber glass vial before injection of 10 µL onto the LC column.

A.2.3.2. Method validation

With the exception of VPA (18.2 %bias at Low QC), the acceptance criterion for accuracy (%bias below 20% at LLOQ and below 15% at the other QC levels) was met. With a within day and total precision (%RSD) below 10%, acceptance criteria were met for all compounds (Table A.2.2).

No carry-over was detected when injecting blank samples after the highest calibrator and, regarding selectivity, no unacceptable interferences were observed in VAMS samples prepared from blank blood originating from 6 different donors. Importantly, a considerable advantage is the possibility to distinguish between CBZ-E and OXC (retention times of 2.64 and 2.88 min, respectively), although they have the same MRM transitions (see Figure A.2.1 for

chromatograms at LLOQ levels). Therefore, the presence of OXC in a patient sample will not interfere with the calculated CBZ-E concentration.

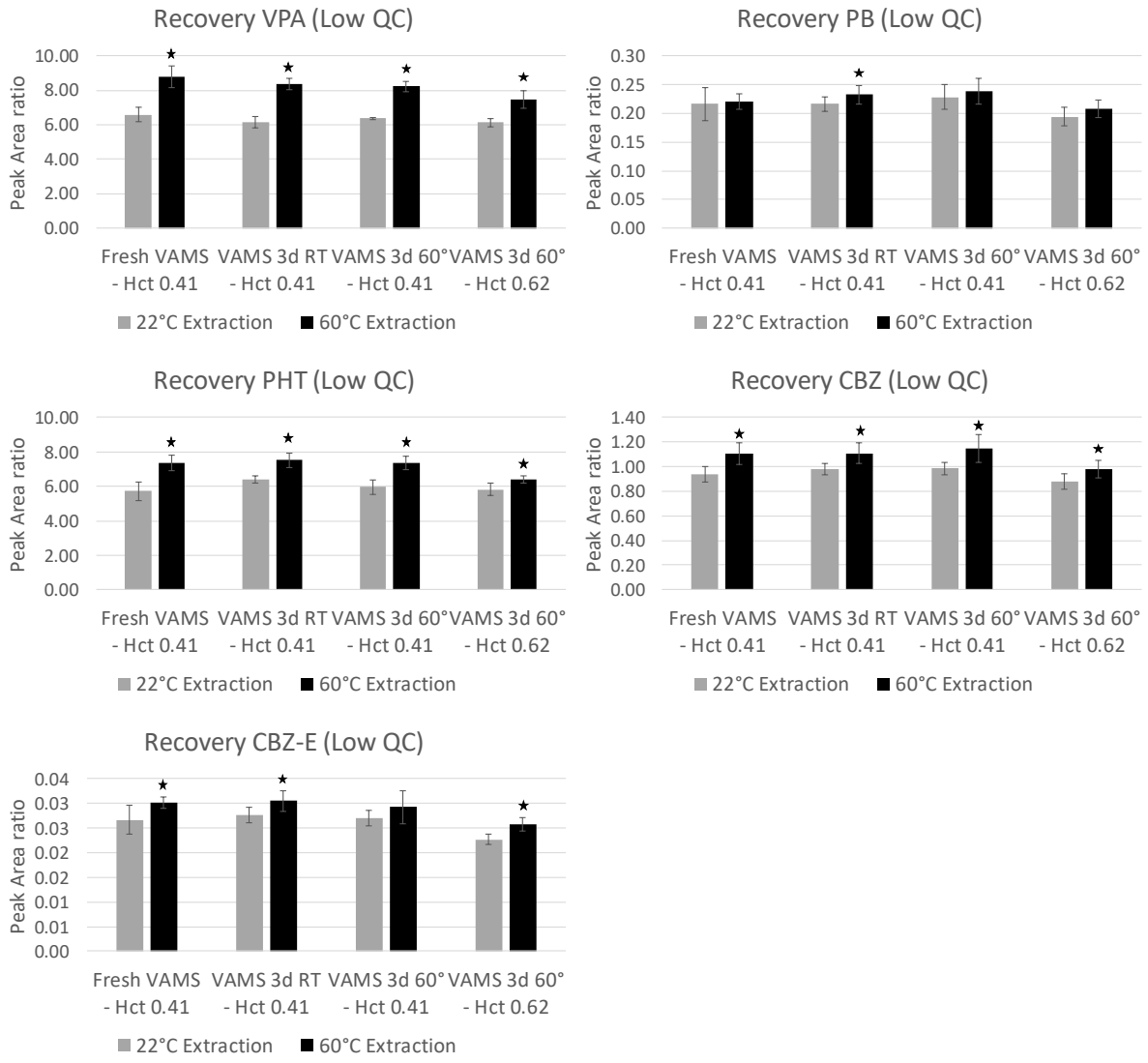

Figure A.2.2. Optimization of sample extraction: comparison of extraction at 22 °C and at 60 °C, using VAMS samples, spiked at Low QC, which were freshly prepared or stored for 3 days at RT (Hct 0.41) or were stored for 3 days at 60 °C (Hct 0.41 and Hct 0.62). Shown are the mean peak area ratios of the analyte and its corresponding IS and their standard deviation (n=6). Asterisks denote statistical differences ($p<0.05$, 2-sided t-test) between extraction at 22 °C and 60 °C.

Calibration data for all compounds were found to be heteroscedastic. Only for PHT, weighted regression did not improve the %RE, therefore the simplest model, i.e. unweighted linear regression, was chosen. The selected weighting factors for PB, CBZ and CBZ-E were $1/x$, $1/x^2$ and $1/x$, respectively. Based on %RE values, weighted quadratic regression was chosen for VPA, with a weighting factor $1/x$. Using these selected models, mean back-calculated

concentrations did not differ more than 7 % for all calibrators, which is in line with the acceptance criteria [31].

Table A.2.2. Within day and total precision (%RSD) and accuracy (%bias) for QCs of VPA, PB, PHT, CBZ and CBZ-E at four concentration levels in VAMS samples (n=4 x 2).

QC	Within day precision (%RSD) (n=4x2)				
	VPA	PB	PHT	CBZ	CBZ-E
LLOQ	7.47	9.76	8.60	8.76	7.67
Low	3.83	7.25	6.60	7.48	6.54
Mid	5.61	4.49	7.86	5.99	5.32
High	8.29	3.87	4.11	8.96	5.08
	Total precision (%RSD) (n=4x2)				
	VPA	PB	PHT	CBZ	CBZ-E
LLOQ	7.47	9.76	8.60	8.76	7.67
Low	8.15	7.83	6.60	7.48	6.63
Mid	5.61	4.49	7.86	7.34	5.32
High	8.29	6.16	7.68	8.96	5.08
	Accuracy (%Bias) (n=4x2)				
	VPA	PB	PHT	CBZ	CBZ-E
LLOQ	-15.3	-1.42	4.22	9.85	4.02
Low	18.2	-1.48	0.87	0.72	14.0
Mid	-1.14	-2.70	3.71	8.15	8.22
High	-1.32	1.51	4.84	2.01	4.97

As displayed in Table A.2.3, all compounds were stable in VAMS samples for at least 1 month when stored at room temperature, 4 °C and -20 °C and for at least 1 week when stored at 60 °C. The latter is important when envisaging e.g. sampling in and/or shipping from countries with high ambient temperatures. Re-analysis of Low and High QCs after storage for 24h in the cooled autosampler (4 °C) revealed autosampler stability for all compounds and their corresponding IS.

Based on the results provided in Table A.2.4, the values for the non-IS-corrected analyte matrix effects indicated relevant (> 15 %) suppression of ionization for PHT, while relevant (>15 %) enhancement of ionization was observed for CBZ and CBZ-E. However, the IS-corrected matrix effects were all within 90-103 %, indicating that the IS compensate for the differences in ionization. Importantly, the %RSD of the IS-corrected matrix effects was below 4 % in all instances, meeting the pre-set acceptance criterium of 15 % [31].

Table A.2.3. Stability data for VPA, PB, PHT, CBZ and CBZ-E in VAMS samples at Low and High QC (n=3), measured in duplicate. Data are presented as the percentage difference between the concentration measured at the specific time points and the nominal values.

Temp	Stability for 4 days (%difference) (n=3)									
	VPA		PB		PHT		CBZ		CBZ-E	
	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC
RT	-6.04	-9.32	-5.34	-6.15	-8.43	4.16	-9.22	-5.51	7.45	-4.31
4 °C	2.06	2.03	5.16	-0.40	-0.75	12.4	-0.31	4.69	15.9	5.29
-20 °C	12.1	0.25	8.29	-6.23	1.54	9.95	0.38	-3.61	17.5	0.02
60 °C	2.30	-2.33	0.03	-7.84	-4.85	3.03	-12.1	-5.12	2.87	-9.24
	Stability for 1 week (%difference) (n=3)									
RT	11.5	0.74	-6.01	-10.5	-8.33	9.60	-13.7	-8.83	5.94	-0.10
4 °C	11.8	-7.97	-7.22	-13.6	-8.37	3.75	-13.9	-13.5	6.89	-5.42
-20 °C	11.8	-4.42	-3.09	-11.8	-6.12	5.20	-13.4	-14.9	4.98	-0.76
60 °C	6.30	-7.97	0.56	-12.97	-10.3	2.31	-10.9	-12.3	-5.94	-15.3
	Stability for 1 month (%difference) (n=3)									
RT	17.6	10.4	5.07	0.27	11.9	9.18	-4.58	3.15	4.01	-4.12
4 °C	15.1	18.0	7.00	5.88	11.8	7.75	-3.64	-0.91	11.4	-1.66
-20 °C	15.5	17.6	13.2	7.36	9.46	7.38	-1.75	1.67	5.11	-0.22
60 °C	16.3	28.1	15.5	6.77	11.0	13.3	-10.8	-0.85	-23.6	-26.1

Table A.2.4. Analyte matrix effect and IS-corrected matrix effect (n=6) for VPA, PB, PHT, CBZ and CBZ-E.

	Analyte matrix effect (n=6)									
	VPA		PB		PHT		CBZ		CBZ-E	
	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC
Mean of 6 donors (%)	95.0	95.0	112	103	81.2	79.5	127	131	134	138
%RSD	3.64	3.36	5.11	4.82	2.29	2.12	10.7	8.08	12.4	11.6
	IS-corrected matrix effect (n=6)									
	VPA		PB		PHT		CBZ		CBZ-E	
	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC
Mean of 6 donors (%)	103	102	98.3	95.7	98.6	95.6	93.1	90.4	95.4	99.0
%RSD	1.21	1.83	1.92	1.58	3.91	3.01	1.67	0.93	1.70	3.97

The possibility of a Hct-dependent recovery, when using VAMS, is a well-known issue [23, 26]. Furthermore, as demonstrated by Abu-Rabie *et al*., a high recovery is important to minimize the risk of being confronted with a significant Hct-based recovery bias [33]. In a first stage, recovery was evaluated by pipetting a fixed volume (10 µL) of blood onto the VAMS. High recoveries were obtained for all compounds, at 85.2 ± 6.1 % for VPA, 93.7 ± 4.6 % for PB, 85.4 ± 5.9 % for PHT, 86.4 ± 5.9 % for CBZ and 91.4 ± 4.6 % for CBZ-E, these values corresponding to the averages calculated from all values obtained at all Hct levels and at Low and High QC level.

Furthermore, as shown in Figure A.2.3, overall, apart from VPA (High QC) at high Hct (0.62), the Hct did not significantly affect the recovery. In addition, when normalizing the 0.42 Hct level to 100 % (see Figure A.2.4), all recoveries -except for VPA- were within 15 % of the 0.42 Hct reference sample. For VPA, the low QC sample at the extreme Hct of 0.62 Hct differed 18 % from the 0.42 Hct sample, which can still be considered acceptable.

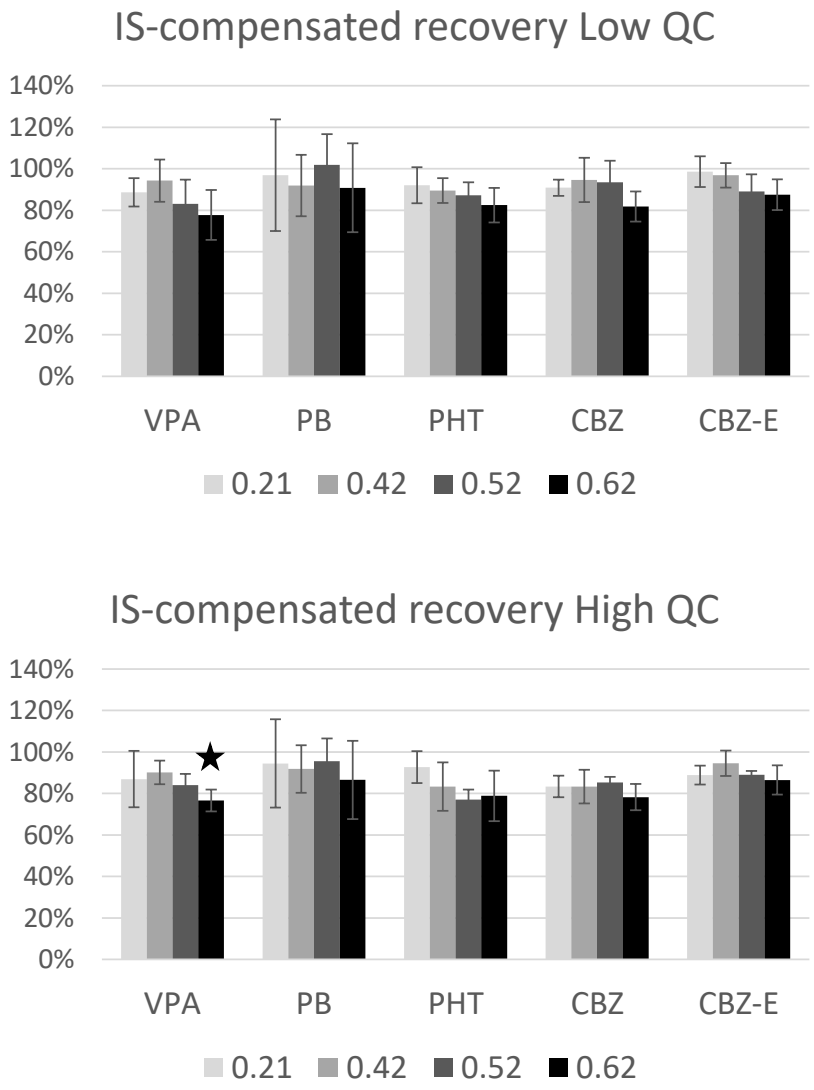

Figure A.2.3. IS-compensated recovery (%) at Low and High QC level (n=6) for VPA, PB, PHT, CBZ and CBZ-E measured in VAMS samples, prepared by pipetting 10 µl blood at 4 different Hct levels (target values at 0.21, 0.42, 0.52 and 0.62). Shown are the mean and standard deviation. Asterisk denotes statistical difference ($p<0.05$, one-way ANOVA test) from the 0.42 Hct reference sample.

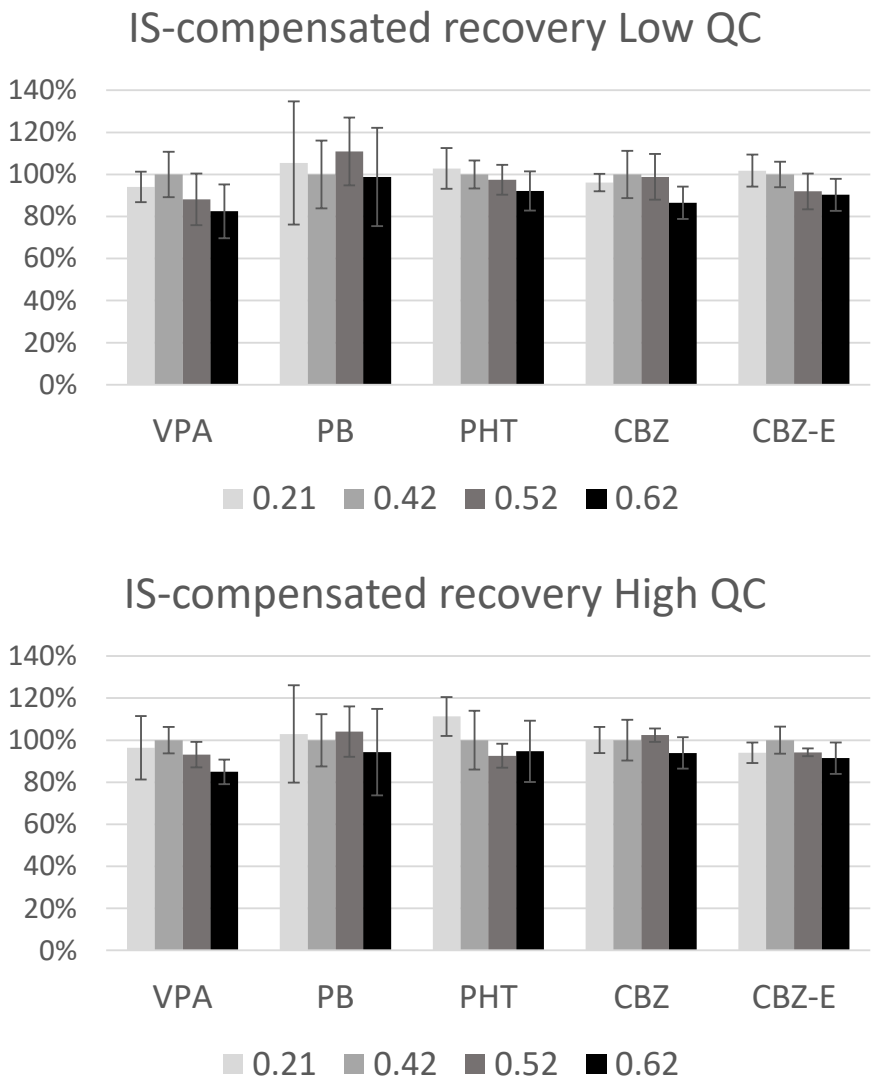

Figure A.2.4. IS-compensated recovery (%) at Low and High QC level (n=6) for VPA, PB, PHT, CBZ and CBZ-E measured in whole blood at 4 different Hct levels (0.21, 0.42, 0.52 and 0.62), with the 0.42 Hct sample being normalized to 100 %.

To further evaluate the impact of the Hct, VAMS samples were also prepared by dipping them into spiked blood (Low and High QC) at four different Hct levels (target values at 0.21, 0.42, 0.52 and 0.62). As Figure A.2.5 depicts, all were within 16 % of the 0.42 Hct sample, except for PB at 0.62 Hct (Low QC) and 0.52 Hct (High QC). However, one-way ANOVA analyses revealed that the observed differences for PB were not statistically significant (p=0.303 and 0.082, respectively).

Taking these findings into account, it can be concluded that if there is an effect of the Hct on the recovery, it is overall limited. As in some cases there is a trend towards somewhat lower

recoveries in samples with very high Hct values, it is recommended to be cautious when analyzing patient samples with a Hct above 0.60. Given the overall limited influence at low to normal Hct values and since we aim at applying the developed method on patient samples originating from children living in developing countries, making a high Hct rather exceptional, we do not foresee any Hct-related recovery problems.

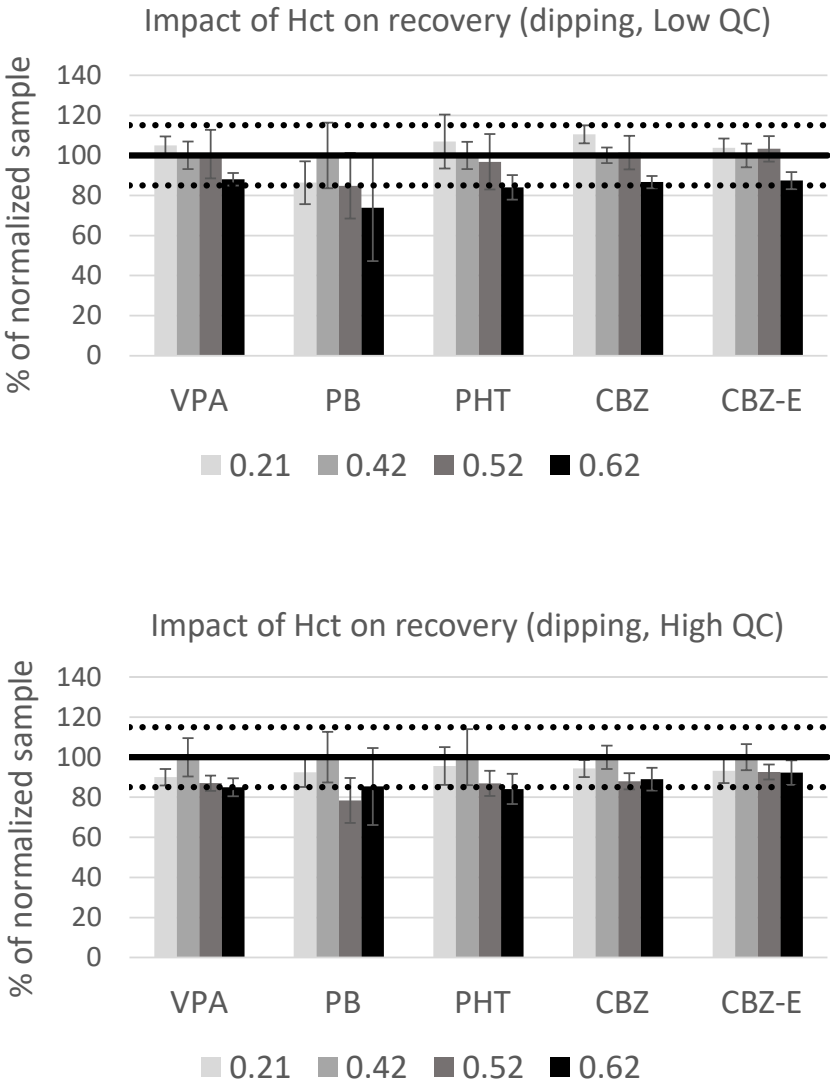

Figure A.2.5. Influence of the hematocrit on the recovery of VPA, PB, PHT, CBZ and CBZ-E, with the 0.42 Hct sample being normalized to 100 %. Here, VAMS samples were prepared by dipping into spiked blood (Low and High QC) at 4 different Hct levels (target values at 0.21, 0.42, 0.52 and 0.62). The full line indicates the 0.42 Hct sample normalized to 100% and the dotted lines indicate the ±15 % deviation limits.

A.2.3.3. Application

The developed method was applied in quadruplicate on 3 sets of VAMS QCs, generated from blood in which external serum QC materials had been diluted 1 on 4 by replacing 250 µL of plasma with 250 µL of the external QC. As outlined in Figure A.2.6, 35 out of the 40 measurements deviated less than 20 % from the target value and the mean concentrations were within ±20 % in all cases. No trend was evident from the distribution of the means, compared to the target concentrations and, with the exception of PB from set C (owing to one deviating value), the %RSD was below 15% for the quadruplicates. Overall, this objectively indicates the validity of the developed method.

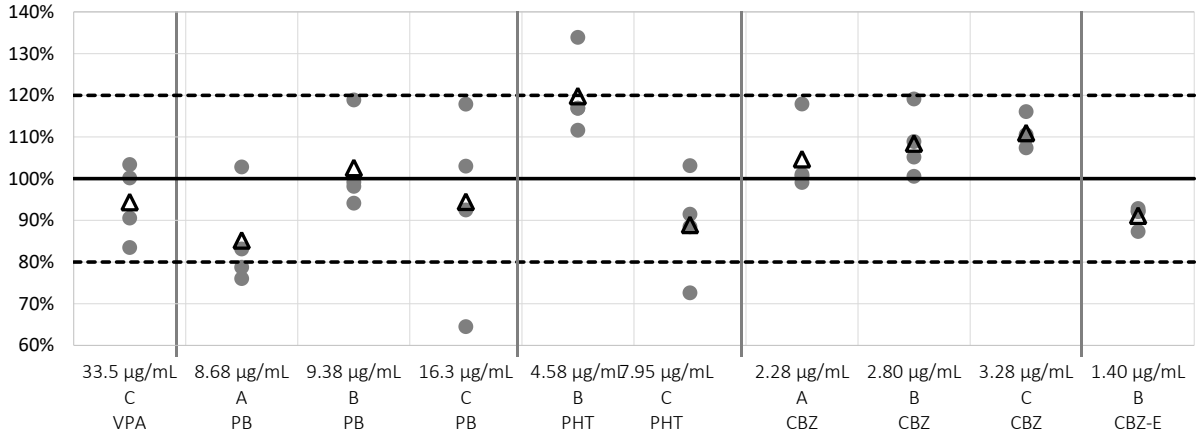

Figure A.2.6. Percentage of the obtained concentration in VAMS samples versus the target concentration present in 3 sets of external QC materials (n=4) (A: Liquicheck™ Level 2; B: ClinCal®; C: Liquicheck™ Level 3). The triangles depict the mean concentrations, the full line indicates the target concentration normalized to 100 % and the dotted lines represent a deviation of ±20 %.

Next, the method was applied on 70 real-life left-over whole blood samples (Table A.2.5). Analysis was performed within one month after collection (storage at -20 °C). Of the collected samples, 19 contained CBZ (and consequently also CBZ-E), 26 VPA, 13 PB and 14 PHT. Five of the VPA samples, 1 of the PHT samples and 1 of the CBZ samples had a concentration below the used LLOQ and hence were not quantified. As a reference, serum concentrations were obtained using chemiluminescent magnetic microparticle immunoassay technology (CMIA, Architect i2000SR). For VPA the mean of the VAMS concentrations were 68.2 ± 17.7 % of those measured in serum, for PB 95.5 ±12.4 %, for PHT 78.0 ± 15.4 % and for CBZ 112.9 ± 16.7%. These ratios are in line with published blood/plasma ratios of 0.70 for VPA, 0.90 for PB, 0.71 for PHT and 1.02 for CBZ [34-36]. In theory, blood/plasma ratios could be used to calculate the

serum concentrations, based on VAMS concentrations – with as a limitation that other techniques (i.e. immunoassay vs LC-MS/MS) were used for both assessments. However, as is readily clear from our limited dataset, there is a substantial variation in observed blood/plasma ratios between individuals. Linder *et al*. also observed substantial variations in blood/plasma ratios for CBZ and VPA, with a role played by Hct and concentration, albeit using spiked samples [37]. More specifically, in our dataset, the %RSD on the observed blood/plasma ratios was between 12 and 18 %, yielding a significant level of uncertainty when applying an 'average' conversion coefficient. Using the published blood/plasma ratios to calculate serum concentration from VAMS concentrations resulted in a mean bias of -2.57, 2.79 , 9.81, and 10.7 %, for VPA, PB, PHT and CBZ, respectively [34-36]. Given the small number of samples, no definitive conclusions can be drawn from this. Therefore, in conclusion, whereas this application on patient samples revealed applicability of the developed method on real-life patient samples, interpretation of the observed concentrations ideally involves the establishment of reference ranges in blood.

Table A.2.5. %Difference between patient serum concentrations and calculated serum concentrations (calculated by dividing the VAMS concentration by the corresponding blood/plasma ratio) and observed blood/serum ratios for VPA, PHT, PB and CBZ. Serum samples were analyzed using the chemiluminescent magnetic microparticle immunoassay technology and the VAMS samples were analyzed with the developed LC-MS/MS method.

	Conc VAMS (µg/mL)	**Calc serum Conc (µg/mL)**	**Serum conc (µg/mL)**	**%difference between calc serum conc and serum conc**	**vams conc/ serum conc (%)**
VPA[1]	29.3	41.9	49.5	-15.4	59.2
	56.2	80.3	79.0	1.63	71.1
	39.2	56.0	64.6	-13.3	60.7
	61.7	88.1	74.7	18.0	82.6
	30.0	42.9	50.6	-15.3	59.3
	42.7	61.0	66.8	-8.68	63.9
	44.9	64.2	61.7	3.98	72.8
	38.3	54.7	57.4	-4.78	66.7
	45.9	65.6	86.3	-24.0	53.2
	29.1	41.6	34.7	19.8	83.9
	73.5	105.0	112.3	-6.49	65.5
	42.0	60.0	63.5	-5.49	66.2
	35.1	50.1	59.7	-16.0	58.8
	28.0	39.9	30.8	29.6	90.7
	72.2	103.2	104.7	-1.46	69.0
	48.7	69.6	82.1	-15.2	59.4
	63.7	91.0	93.8	-2.99	67.9
	28.1	40.1	30.0	33.8	93.7
	51.0	72.8	100.8	-27.8	50.6
	36.5	52.2	45.1	15.7	81.0
	53.9	77.0	95.8	-19.7	56.2
				Mean ± SD	Mean ± %RSD
				-2.57 ± 17.2 %	68.2 ± 17.7 %
PB[2]	34.9	38.8	37.8	2.59	92.3
	7.35	8.17	8.70	-6.13	84.5
	41.6	46.2	43.6	6.01	95.4
	9.88	11.0	10.6	3.56	93.2
	8.82	9.80	8.20	19.5	107.6
	14.2	15.8	20.5	-23.0	69.3
	16.9	18.8	19.4	-3.25	87.1
	16.6	18.4	20.7	-11.1	80.1
	19.8	21.9	23.6	-7.00	83.7
	40.7	45.2	41.2	9.66	98.7
	36.9	41.0	33.6	22.1	109.9
	22.0	24.4	21.4	14.1	102.7
	27.3	30.4	27.8	9.30	98.4
				Mean ± SD	Mean ± %RSD
				2.79 ± 12.8 %	95.5 ± 12.4 %

Table A.2.5. (continued)

PHT[3]	14.3	20.1	15.8	27.5	90.5
	5.47	7.7	5.32	44.8	102.8
	8.31	11.7	12.4	-5.61	67.0
	7.26	10.2	10.0	2.25	72.6
	7.23	10.2	9.10	11.9	79.5
	8.13	11.5	13.4	-14.5	60.7
	10.3	14.6	13.6	7.01	76.0
	4.00	5.6	6.00	-6.14	66.6
	4.34	6.1	5.20	17.4	83.4
	8.24	11.6	9.50	22.2	86.8
	6.61	9.3	7.80	19.3	84.7
	10.6	14.9	13.3	12.4	79.8
	4.23	6.0	6.70	-11.0	63.2
				Mean ± SD	Mean ± %RSD
				9.81 ± 16.9 %	78.0 ± 15.4 %
CBZ[4]	12.2	12.0	8.60	39.1	141.9
	8.56	8.4	6.50	29.1	131.7
	5.91	5.8	5.20	11.4	113.7
	8.75	8.6	6.40	34.0	136.7
	2.71	2.7	2.30	15.5	117.8
	11.7	11.5	13.1	-12.4	89.3
	6.51	6.4	5.40	18.2	120.6
	9.36	9.2	10.6	-13.4	88.3
	6.97	6.8	7.70	-11.3	90.5
	7.17	7.0	6.30	11.6	113.8
	6.56	6.4	6.80	-5.40	96.5
	4.76	4.7	5.00	-6.61	95.3
	7.87	7.7	5.40	42.9	145.8
	4.11	4.0	3.20	26.1	128.6
	10.6	10.4	8.80	18.2	120.6
	14.4	14.1	11.7	20.3	122.7
	10.3	10.1	8.00	26.1	128.6
	7.79	7.6	6.10	25.2	127.7
				Mean ± SD	Mean ± %RSD
				10.7 ± 18.5 %	112.9 ± 16.7 %

1. Blood/plasma ratio 0.70[35]
2. Blood/plasma ratio 0.90[36]
3. Blood/plasma ratio 0.71[36]
4. Blood/plasma ratio 1.02[34]

A.2.4. Conclusion

In TDM there is a growing interest in the use of non- and minimally invasive alternative sampling strategies, VAMS being one of the recent developments. For anti-epileptic drugs, most evidence for TDM exists for the first-generation anti-epileptic drugs, due to their significant interindividual variability in pharmacokinetics and due to the narrow therapeutic indices related to those drugs.

In this Chapter, an LC-MS/MS method for the determination and quantification of 4 anti-epileptic drugs and one active metabolite, i.e. CBZ, VPA, PHT, PB and CBZ-E, making use of VAMS devices, was developed and validated. The final method was extensively validated, including both bioanalytical and VAMS-specific parameters and overall the pre-set acceptance criteria were met. Thorough optimization of the extraction procedure helped enabling a Hct-independent, consistent recovery.

Application of the method on external quality control materials and on real-life patient samples demonstrated the validity and applicability of the developed procedure. We successfully used external serum QCs to replace part of the plasma fraction of control blood, thereby yielding blood (and VAMS) samples with known concentrations. This represents a feasible approach to cope with the lack of external reference materials for dried blood matrices.

To date, the limited availability of clinical validation data still remains one of the constraints preventing the widespread implementation of dried matrix approaches in clinical practice [1]. Furthermore, divergent results have been reported on the ratio between blood and plasma or serum concentrations. Therefore, calculating serum concentrations based on blood concentrations is challenging [9, 37-39]. Having at hand reference ranges in blood could allow to cope with this.

As one of the advantages coupled to dried matrices is the extreme usefulness for sampling in remote or resource-limited settings (e.g. ease of collection and storage), in a next step, we aim at applying this newly developed method on patient samples originating from developing countries.

A.2.5. References

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CHAPTER A.3.

FULLY AUTOMATED THERAPEUTIC DRUG MONITORING OF ANTI-EPILEPTIC DRUGS MAKING USE OF DRIED BLOOD SPOTS

Based on

Velghe S, Deprez S, Stove CP. Fully automated therapeutic drug monitoring of anti-epileptic drugs making use of dried blood spots. *Journal of Chromatography A* 1601, 95-103 (2019).

Abstract

Fully automated dried blood spot (DBS) extraction systems, online coupled to standard liquid chromatography-tandem mass spectrometry (LC-MS/MS) configurations, decrease the hands-on time associated with conventional DBS analysis, resulting in a higher sample throughput, making the technique more compatible with a high-capacity bioanalytical workflow. The aim of this Chapter was to develop and validate an LC-MS/MS method, using a DBS-MS 500 autosampler, for the determination and quantification of four anti-epileptic drugs (carbamazepine, valproic acid, phenobarbital and phenytoin) and one active metabolite (carbamazepine-10,11-epoxide) in DBS samples. Method development included thorough optimization of the fully automated extraction procedure (i.e. extraction solvent, extraction (loop) volume, internal standard application, internal standard drying time, etc.). The method was fully validated based on international guidelines. Accuracy (%bias), as well as precision (%RSD) (with a single exception) were below 13%. Neither carry-over nor unacceptable interferences were observed. All compounds were stable in DBS for at least 1 month when stored at room temperature, 4 °C and -20 °C and for at least 4 days when stored at 60 °C. Internal standard-corrected matrix effects were below 8%, with %RSDs below 9.1%. Reproducible relative recovery values (around 60% for all analytes) were obtained and the effect of the hematocrit on the relative recovery was overall limited. Successful application on capillary patient samples originating from developing countries demonstrated the applicability of the developed procedure in a remote setting.

A.3.1. Introduction

During the past several years, DBS have increasingly been used, with applications in different fields, e.g. in toxicology [1, 2], therapeutic drug monitoring (TDM) [3-5] and phenotyping [6]. One of the advantages associated with DBS analysis is the possibility for automation [4, 7]. Benefits coupled to automation are increased sample throughput and safety, in combination with decreased hands-on time and risk for human errors. Furthermore, it has been demonstrated that a fully optimized, automated sample extraction can result in an increased assay sensitivity compared to a manual DBS extraction [8]. To date, for DBS, different possibilities exist regarding this automation, going from semi-automated set-ups (e.g. automated DBS punching instruments) to fully automated DBS analyzers, no longer needing the DBS punching step. The latter are also compatible with standard LC-MS/MS configurations. Furthermore, besides DBS, also dried plasma spots have shown to be compatible with the existing fully automated platforms [9]. The latter pose the advantage that no blood to serum or plasma conversion is required for many drugs (at least, if the DPS concentrations are equivalent with plasma concentrations). As a result, the only manual activity needed from the analyst in such a fully automated set-up is loading DBS cards into the sample racks, allowing an efficient workflow, certainly when processing large numbers of DBS samples. Different fully automated systems have been developed, differing from each other in the way sample elution is performed, in the possibility to perform an online sample clean-up and in the way of internal standard (IS) application [10]. The DBS Autosampler™ (DBSA, Spark Holland) and the Sample Card and Prep System (SCAP, Prolab) employ a flow-through desorption technology of a vertically placed card, whilst the DBS-MS 500 (CAMAG) utilizes a horizontal extraction [11-15]. The DBS-MS 500 system is currently the only system equipped with a built-in IS spray, whilst for the other two systems, the IS is added during sample extraction [11-16]. Spray addition of the IS onto DBS samples before sample extraction or spray addition of the IS onto DBS cards prior to blood application *via* the built-in IS spray of the DBS-MS 500 system have proven to be able to nullify the Hct-based recovery bias, this in contrast to when the IS is added *via* the extraction solvent [17]. Online sample clean-up via the use of a solid phase extraction (SPE) column, prior to analytical separation, is possible with the DBSA and SCAP system [12, 13, 15, 16, 18]. The DBS-MS 500, on the other hand, handles a direct elution technique, resulting in a simple and quick sample extraction method. However, care should be taken in the choice of the extraction

solvent, to avoid the introduction of extracts that are too 'dirty' into the LC-MS/MS system. The potential robustness of the technique was already demonstrated by Abu-Rabie et al. in a high-capacity setting, excluding the need for more complex and time-consuming clean-up steps [19].

Thorough optimization of different key conditions during the development of a fully automated direct DBS method has proven to be of utmost importance for both the magnitude of response and acceptable chromatography [8]. Optimization of sample extraction typically involves testing of different extraction solvents, different extraction volumes and different extraction solvent delivery speeds. Furthermore, the IS application needs to be optimized, including testing of different volumes of IS and different IS drying times. Extraction of prewetted DBS (i.e. by using the IS spray in combination with a short drying time) can result in increased analyte response, probably due to a more efficient extraction [8]. On the other hand, implementing an IS drying time of e.g. 10 min can enable the IS to become integrated in the sample, allowing co-extraction of both, resulting in nullification of the hematocrit(Hct)-based recovery bias [8].

In this Chapter the use of the DBS-MS 500, online coupled to an LC-MS/MS system, was evaluated for TDM of four first-generation anti-epileptic drugs (AEDs) and one active metabolite, including carbamazepine (CBZ), valproic acid (VPA), phenobarbital (PB), phenytoin (PHT) and carbamazepine-10,11-epoxide (CBZ-E). In a first stage, particular attention was paid to the different key parameters in the optimization procedure of an automated extraction method. Once optimized, the method was completely validated, before application on capillary patient samples originating from developing countries.

A.3.2. Materials and methods

A.3.2.1. Chemicals and stock solutions

Ultrapure water was provided by a Synergy® Water Purification System (Merck Millipore, Overijse, Belgium). LC-MS grade acetonitrile and methanol were obtained from Biosolve (Valkenswaard, The Netherlands). LC-MS grade 2-propanol was derived from Merck Milipore (Overijse, Belgium). Ammonium acetate, VPA, VPA-D6, PHT, PHT-D10, CBZ, CBZ-D10 and CBZ-E were purchased from Sigma Aldrich (Diegem, Belgium). CBZ-E-D10 was derived from J.H.

Ritmeester B.V. (Nieuwegein, The Netherlands) and PB and PB-D5 from LGC standards (Molsheim Cedex, France).

Based on the upper and lower limit of the therapeutic ranges, stock solutions for VPA, PB, PHT, CBZ and CBZ-E were prepared in methanol at 40.00, 50.00, 10.00, 10.00 and 5.00 mg/mL, respectively. Calibrators and quality control samples (QCs) were prepared using independently prepared stock solutions. Methanolic stock solutions of 0.10 mg/mL for the IS of PB, PHT and CBZ were purchased, while for VPA the concentration of the IS stock solution was 1.00 mg/mL. For CBZ-E-D10, a stock solution of 1.00 mg/mL was prepared in methanol. Working solutions of the standards and the IS were prepared the day of analysis, making a dilution of the stock solutions with water. All solutions, except for the stock solution of CBZ-E (4°C), were stored at -20°C in 1.5 mL amber glass vials derived from VWR ® (Leuven, Belgium).

A.3.2.2. Preparation of calibrators and QCs

Since the purpose of this study was to optimize and validate a fully automated DBS-based LC-MS/MS method for therapeutic drug monitoring of VPA, CBZ, PB, PHT and CBZ-E and since calibrators should be prepared in such a way that they cover the expected sample concentration range, relatively high concentration levels (micrograms/mL) were used in this study, which posed a challenge, as discussed below. Other studies have already shown the suitability of the DBS-MS 500 to accurately and precisely measure analytes at lower concentration levels [20-22].

Calibrators were prepared at eight concentration levels in blank whole blood with a concentration of 25.00, 37.50, 50.00, 70.00, 90.00, 110.00, 130.00 and 150.00 µg/mL for VPA; 1.00, 1.50, 2.00, 33.60, 65.20, 96.80, 128.40 and 160.00 µg/mL for PB; 4.00, 6.00, 8.00, 22.40, 36.80, 51.20, 65.60 and 80.00 µg/mL for PHT; 2.00, 3.00, 4.00, 12.80, 21.60, 30.40, 39.20 and 48.00 µg/mL for CBZ and 0.25, 0.38, 0.50, 5.20, 9.90, 14.60, 19.30 and 24.00 µg/mL for CBZ-E. In this way, the range between the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ) covers the therapeutic range set in serum, with the LLOQ corresponding to the lower end of the therapeutic interval, minimally divided by 2, and the ULOQ corresponding to the upper end of the therapeutic interval, minimally times 1.5 [23, 24]. QC solutions (LLOQ, Low, Mid and High, respectively) were prepared in blank whole blood at

25.00, 55.00, 100.00, 112.50 µg/mL for VPA; 1.00, 3.00, 40.00, 120.00 µg/mL for PB; 4.00, 8.00, 20.00, 60.00 µg/mL for PHT; 2.00, 5.00, 12.00, 36.00 µg/mL for CBZ; and 0.25, 1.50, 6.00, 18.00 µg/mL for CBZ-E. Non-matrix solvents were never added in a proportion higher than 5 % of the total sample volume.

A.3.2.3. Sample collection

Blank venous whole blood (Hct 0.42) from an AED abstinent healthy, female volunteer was collected in EDTA tubes (BD Vacutainer® with BD Hemogard® closure 10mL) and used for the preparation of calibrators and QC samples. PerkinElmer 226 Bioanalysis RUO cards were purchased from PerkinElmer (Greenville, SC, USA). DBS were generated by pipetting 25 µL of whole blood onto the DBS cards, using a calibrated pipette. After completing sampling, the cards were left to dry for approximately 2h at ambient temperature, before LC-MS/MS analysis.

For the generation of DBS with a wide Hct range, a specific amount of plasma from whole blood was added or removed, this by centrifuging an aliquot of whole blood with a Hct of 0.41 in an Eppendorf 5804R centrifuge (Hamburg, Germany) for 5 min at 1000 x g. A Sysmex XE-5000 hematology analyzer (Sysmex Corp., Kobe, Japan) was used to determine the (adapted) Hct [25].

A.3.2.4. Sample preparation

Sample preparation was performed by using the fully automated DBS-MS 500 system (CAMAG, Muttenz, Switzerland), operated using Chronos software. The system incorporates a robotic arm to move cards from one of the five racks (each with a capacity of 100 DBS cards) towards the different workstations, being an optical recognition system (locating the position of the spots on the cards and determining barcode information); an IS module (spraying of an IS solution onto the cards before extraction); and an extraction module containing a 4 mm clamp head for clamping of a DBS card and horizontally guiding the extraction solvent throughout a DBS into a sample loop [8, 11]. Furthermore, the autosampler is equipped with a wash station in order to avoid cross contamination between different samples [8, 11].

Method development included the evaluation of different extraction (different combinations of water and acetonitrile or methanol, in different compositions) and rinsing solvents, different ways of adding the IS (spraying versus adding the IS into the extraction solvent), different drying times after the IS application, different sample loop volumes, as well as different volumes of extraction solvent.

A.3.2.5. LC-MS/MS method

Chromatography was performed on a Shimadzu Prominence setup (Shimadzu, Brussels, Belgium) including a CBM-20A system controller, two LC-20AD pumps, a DGU-20A5R degasser, a SIL-20ACHT autosampler and a CTO-20AC column oven containing a Chromolith® reversed-phase (RP)-18 endcapped 100x4.60 mm column (Merck Millipore, Overijse, Belgium), equipped with the corresponding guard column, maintained at 45°C. A mobile phase consisting of 5 mM ammonium acetate (A) and 5 mM ammonium acetate in acetonitrile/water (95/5, v/v) (B) at a flow rate of 1 mL/min was used, with the following proportions of solvent B in the 8 minute gradient elution program: 20% for 0.34 min, linearly increased to 46% in 3.21min, followed by a short isocratic period of 46% for 0.55 min, a fast increase to 95% in 0.4 min, maintained for 1.0 min and finally, reversal to starting conditions, maintained for 2.5 min. The LC system was coupled to a QTRAP 5500 instrument (SCIEX, Nieuwerkerk aan den Ijsel, The Netherlands), controlled by Sciex Analyst® 1.6.2 software. The MS was equipped with an electrospray ionization (ESI) source and detected all compounds using an optimized multiple reaction monitoring (MRM) algorithm, operating in negative ionization mode for VPA, PHT and PB and in positive ionization mode for CBZ and CBZ-E, making use of the polarity switch option. The source temperature was set at 600°C, the ion spray voltage at -4500 V and at 4500 V. The gas settings were as follows: curtain gas: 30 psi, ion source gas 1: 50 psi and ion source gas 2: 50 psi. For PB, PHT, CBZ and CBZ-E, two characteristic precursor-to-product ion transitions were monitored, while for the corresponding IS one transition was analyzed. For VPA, a pseudo mass transition (143.1/143.1) was monitored, since no stable ion fragments are generated. Here, an optimization of source- and compound-dependent MS parameters was performed to get the best specificity with respect to any other possible matrix ion with a mass to charge ratio of 143. MRM transitions, as well as the compound-specific MS parameters, are listed in Table A.3.1.

Table A.3.1. MRM transitions and compound-specific MS parameters for valproic acid, phenobarbital, phenytoin, carbamazepine, carbamazepine-10,11-epoxide and the corresponding internal standards (DP: declustering potential, EP: entrance potential, CE: collision energy, CXP: collision cell exit potential).

	Precursor ion (m/z)	Product ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)	Tr (min)
Valproic acid	143.0	143.0	-5.00	-10.0	-25.0	-37.0	2.15
Phenobarbital	231.0	187.8	-40.0	-10.0	-18.0	-5.00	3.20
		84.80	-5.00	-10.0	-14.0	-10.0	
Phenytoin	251.1	102.2	-155	-10.0	-16.0	-49.0	4.50
		208.2	-150	-10.0	-24.0	-13.0	
Carbamazepine	237.0	194.1	230	10.0	50.0	52.0	4.64
		179.0	80.0	10.0	25.0	30.0	
Carbamazepine-10-	253.1	180.0	140	10.0	80.0	52.0	3.84
11-epoxide		210.1	140	10.0	45.0	15.0	
Valproic acid-D6	148.9	148.9	-5.00	-10.0	-20.0	-55.0	2.12
Phenobarbital-D5	236.0	193.0	-5.00	-10.0	-14.0	-11.0	3.18
Phenytoin-D10	261.0	218.1	-70.0	-10.0	-24.0	-13.0	4.45
Carbamazepine-D10	247.0	204.1	60.0	10.0	10.0	6.00	4.57
Carbamazepine-10,11-epoxide-D10	263.1	220.2	8.00	10.0	15.0	4.00	3.77

A.3.2.6. Method validation

Method validation was based on the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) guidelines for bioanalytical method validation [26, 27], as well as on the Official International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) guideline on the 'Development and Validation of Dried Blood Sample-based methods for Therapeutic Drug Monitoring' [28] and covered calibration model, homoscedasticity, accuracy, precision, carry-over, selectivity, stability, matrix effect, recovery and Hct effect. Throughout each sequence, control blanks (i.e., DBS prepared with blank blood and analyzed without IS) and zero samples (i.e., DBS prepared with blank blood and analyzed by adding IS) were assessed.

Six (n=3x2) 8-point calibration lines were analyzed to evaluate homoscedasticity and the calibration model. An F-test (α = 1%) at the lowest and highest calibrators was performed for testing the homoscedasticity. Furthermore, unweighted and weighted ($1/x$, $1/x^2$, $1/\sqrt{x}$, $1/y$, $1/y^2$ and $1/\sqrt{y}$) linear and quadratic regression were applied to find the best fitting calibration model. The sum% relative error (%RE) and plotting of the %RE against the nominal

concentrations was used to compare the resulting models. A back-calculation, in which the mean concentrations of the calibrators should be within ±15% of the nominal value or within ±20% for the LLOQ, was performed before accepting a selected model [27].

QCs (LLOQ, Low, Mid and High) were analyzed in duplicate on three different days to assess accuracy (%bias) and precision (%relative standard deviation, %RSD). The within day and total assay precision were determined using a single factor analysis of variance (ANOVA), while the accuracy was calculated by dividing the difference between the obtained concentration and the nominal value by the nominal value, this multiplied by 100 [29, 30]. The %bias and %RSD should be within ±15% for the QC samples, except for the LLOQ (within ±20%) [27].

For the evaluation of carry-over, two blank samples were analyzed after the ULOQ, on three different days (n=3x2). For the analytes, carry-over should not exceed 20% of the peak area found for the LLOQ and 5% for the IS [27]. Identical criteria were applied for the selectivity, which was evaluated by analyzing blank DBS, prepared with blank whole blood originating from 6 different volunteers.

Former experiments (see Chapter A.2.) did not reveal any problems with the stability of VPA, PB, PHT, CBZ and CBZ-E in dried blood samples. Based on literature, VPA and CBZ are stable for at least 1 year, PB for at least 6 weeks and PHT for at least 1 month at room temperature [31-33]. Moreover, we found all compounds to be stable for at least 6 months in volumetric absorptive microsampling samples when stored at -20°C (see Chapter A.4.). Therefore, only a limited stability study was carried out here. Stability was assessed by analyzing low and high QCs in triplicate after 4 days of storage at 60°C, after 1 week of storage at 60°C, room temperature, 4°C and -20°C and after 1 month of storage at room temperature, 4°C and -20°C in zip-closure plastic bags containing two 5 g packages of desiccant (Minipax® absorbent packets, Sigma Aldrich). Freshly prepared calibration curves were used at each day of analysis to calculate the concentration of the stored DBS. The mean concentration of the QCs at a particular point of time should not deviate more than ±15% from the nominal value to be acceptable [27].

Matrix effects were investigated by comparing the peak areas obtained at two concentration levels (Low and High QC), dissolved -together with the IS- in the extraction solvent, used for extracting blank DBS (originating from 6 different individuals and from blood with a Hct

ranging from 0.22 to 0.53) (A) and for extracting blank cards (B). The ratio of the peak areas of (A) to those of (B), multiplied by 100, was used to calculate the IS-corrected matrix effect. Overall, the %RSD of this IS-corrected matrix effect should be less than 15% [27].

Since the automated platform does not allow a conventional evaluation of the recovery (i.e., a comparison of pre- and post-extraction spiked DBS samples), the relative recovery was determined by using repeated sample extraction [16, 20, 34]. Here, three different concentration levels (Low, Mid and High QC) were prepared in blank whole blood at four different Hct levels (target values at 0.21, 0.42, 0.52 and 0.62). Despite programming a drying time of 15 minutes in between two extractions, a perforation of certain DBS cards was observed when extracting a spot four times or more. We therefore limited the number of extractions of each spot to three times. By using the built-in camera of the autosampler, the extraction module automatically locked the same area in the center of each DBS during the repeated extractions. The relative recovery was calculated by dividing the analyte peak area of the first extraction by the sum of the analyte peak areas of all three extractions [15, 16, 20]. Moreover, 20 DBS, originating from leftover whole blood patient samples, were analyzed to examine the relative recovery when starting from real-life patient samples. Here again, each DBS was extracted three times.

Furthermore, to evaluate the effect of a varying Hct on the spreading of blood on filter paper, DBS (n=3) were prepared from blank whole blood at four different Hct levels (target values at 0.21, 0.42, 0.52 and 0.62), spiked at three concentration levels (Low, Mid and High QC). The Hct effect was estimated by comparing the area ratios obtained at the 0.21, 0.52 and 0.62 Hct level with those at the 0.42 reference Hct level.

Where relevant, statistical analyses were performed using the Minitab® software.

A.3.2.7. Application

As a proof of concept, 5 DBS samples originating from Ugandan patients with Nodding syndrome (an unexplained, highly debilitating generalized seizure disorder, typically having its onset in 5-15-year-old children in subregions of sub-Saharan Africa), 5 DBS samples originating from epilepsy patients (Uganda) and 5 DBS samples originating from epilepsy patients with an *O. volvulus* co-infection (Democratic Republic of the Congo) were analyzed.

Upon signing an informed consent form, patients were asked to provide some blood via a fingerprick for the preparation of DBS samples. In a first step, the hands were disinfected, followed by the execution of a fingerprick with a BD Microtainer contact activated safety lancet (BD, Franklin Lakes, USA). The first drop of blood was wiped off with a clean tissue, to avoid collection of tissue fluid. Afterwards, the second drop of blood was applied to a DBS card. Patients were asked to -when possible- completely fill one DBS card (i.e. 4 spots). In this way, spare samples were available. Once collected, the samples were left to dry at room temperature for at least 2 h, before storage at -20°C in zip-closure plastic bags, containing two 5 g packages of desiccant, until shipping of the samples to Belgium.

Approval for this study (including the use of patient and volunteer blood samples) was provided by Ethics Committee of Ghent University Hospital (EC2017/0572 and EC2017/1165, for Uganda and the Democratic Republic of Congo, respectively), by the Ugandan Institutional Review Board (IRB; 2015 – 146), by the Ugandan National Council for Technology (UNCST; HS 1986) and by Kinshasa School of Public Health (ESP/CE/06/2017).

A.3.3. Results and discussion

A.3.3.1. Sample preparation

Figure A.3.1 depicts the complete workflow, including the DBS-MS 500. After sample collection, the DBS cards are put in the racks in the DBS-MS 500 system. Subsequently, a robotic gripper moves a card to the subsequent processes, and back into their original position after sample extraction. In a first step, the built-in camera was used to take a photo of each DBS card before each run to check for the presence of a DBS and to allow the extraction head to adjust the center of each spot. In a next step, the IS mixture (with all IS at 0.50 µg/mL) was added and the sample was extracted from the DBS card. A mixture of acetonitrile and water (80/20, v/v), containing 5 mM of ammonium acetate, was used as extraction (E) solvent. When using the built-in high precision IS sprayer (20 µL, fast spraying) during method development, a decrease in IS peak area was observed for CBZ-D10 and CBZ-E-D10 with an increasing analyte concentration (CV% IS >33%, see Table A.3.2), possibly indicating saturation at the ESI source. In an attempt to prevent this phenomenon, an extraction volume exceeding the loop volume was tested (i.e. an extraction volume of 60 µL in combination with a 20 µL loop). This way, the

excess extraction solvent (i.e. the first 40 µL) was directed to waste. When applying this strategy, the IS peak areas of PB-D5, PHT-D10, CBZ-D10 and CBZ-E-D10 remained stable (CV% IS <15%), however, VPA-D6 was not detectable anymore, even when a drying step (at 50°C overnight) between IS application and extraction was introduced, in an attempt to integrate the IS within the sample. Importantly, VPA itself was detectable, indicating a different extraction behavior of VPA and VPA-D6. As these are the first eluting compounds, VPA-D6 is likely first and more easily extracted from the DBS and, as a result, lost via the 40 µL that was directed to waste. Finally, when excluding the IS sprayer and adding the IS mixture via the extraction solvent, still using an extraction volume of 60 µL in combination with a 20 µL loop, all IS were detectable and no decrease in IS area was observed with increasing analyte concentrations (CV% IS <12.5%, see Table A.3.2). Afterwards, the extraction module was rinsed for 50 seconds, using a mixture of methanol, water, 2-propanol and acetonitrile (25/25/25/25, v/v/v/v) as rinsing (R) solvent. At the start of each day of analysis, an automated system prime method was performed, including priming of the extraction solvent for 5 cycles and flushing of the rinsing solvent for 5 minutes. Prior to a large set of analyses, the extraction head was cleaned with a mixture of water and methanol (50/50, v/v) in an ultrasound bath for 10 minutes.

Table A.3.2. differences in CV% of the IS peak area between adding the IS via the built-in IS sprayer versus via the extraction solvent (n=4)

Spraying of IS	**VPA-d6**	**PB-d5**	**PHT-d10**	**CBZ-d10**	**CBZ-E-d10**
QC1	1.67E+06	7.74E+05	1.16E+06	2.20E+06	1.30E+07
QC2	1.58E+06	7.79E+05	1.16E+06	1.80E+06	1.17E+07
QC3	1.39E+06	7.21E+05	1.08E+06	1.36E+06	7.90E+06
QC4	1.34E+06	6.64E+05	1.05E+06	8.51E+05	4.69E+06
CV%	9.61%	6.84%	4.99%	33.4%	36.2%
IS Added to extraction solvent	**VPA-d6**	**PB-d5**	**PHT-d10**	**CBZ-d10**	**CBZ-E-d10**
QC1	1.18E+06	3.16E+06	8.83E+06	1.71E+07	4.53E+07
QC2	1.20E+06	3.15E+06	8.87E+06	1.60E+07	4.25E+07
QC3	1.20E+06	3.21E+06	8.74E+06	1.49E+07	4.11E+07
QC4	1.20E+06	3.12E+06	8.70E+06	1.27E+07	3.82E+07
CV%	6.06%	6.27%	9.18%	12.3%	9.56%

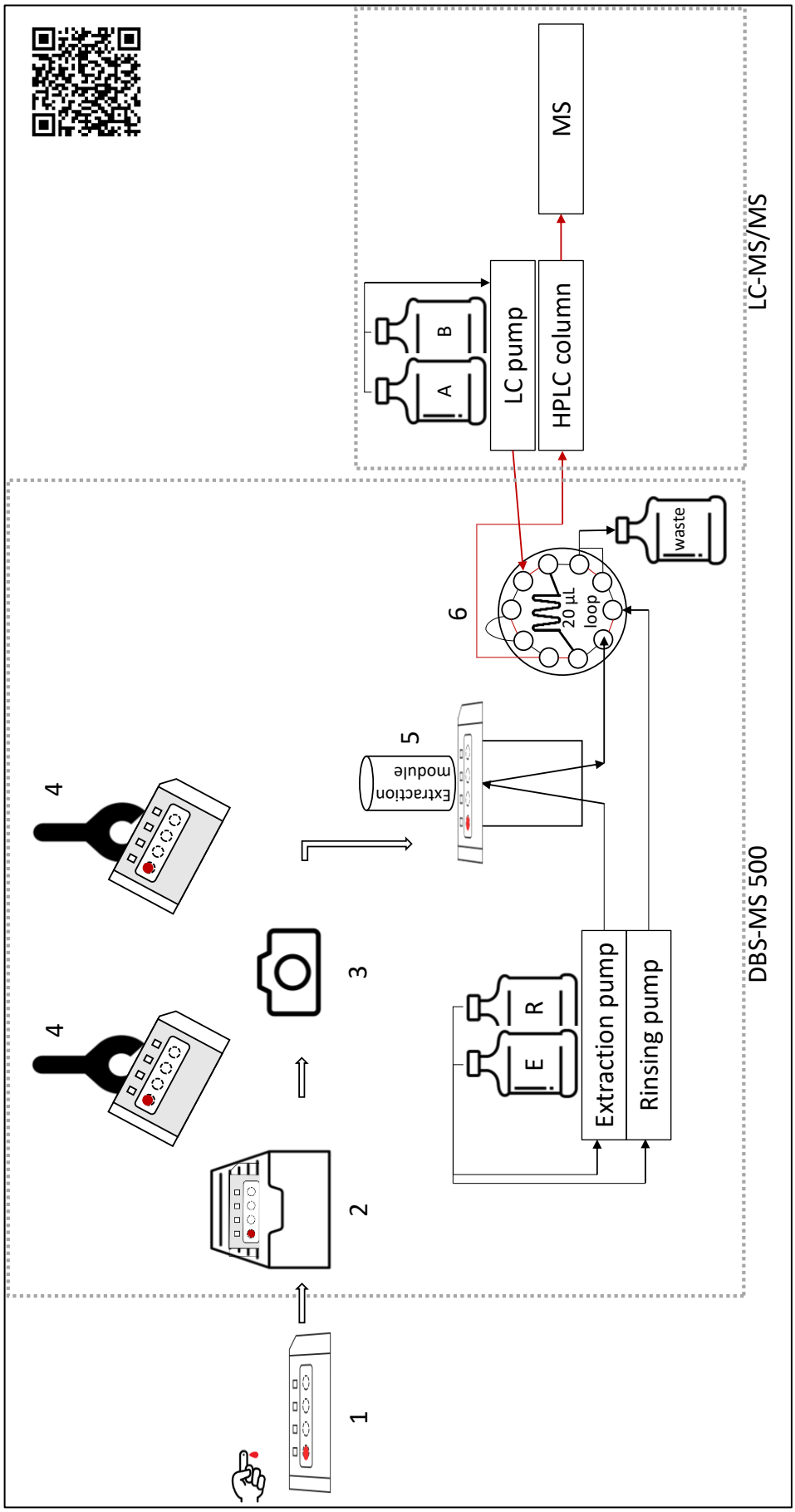

Figure A.3.1. Fully automated DBS-MS 500 workflow.
[1] Sample collection; [2] DBS card rack; [3] built-in camera; [4] robotic gripper; [5] extraction module containing a 4 mm clamp head; [6] 10-port valve equipped with a 20 µL sample loop; [E] extraction solvent: acetonitrile/water (80/20, v/v) containing 5 mM ammonium acetate and the IS mixture (with all IS at 0.50 µg/mL); [R] rinsing solvent: methanol/water/2-propanol/acetonitrile (25/25/25/25, v/v/v/v); [A] mobile phase A: 5 mM ammonium acetate; and [B] mobile phase B: acetonitrile/water (95/5, v/v) containing 5 mM ammonium acetate.

A.3.3.2. Method validation

Calibration data for all 5 compounds were found to be heteroscedastic. In all instances, weighted regression did improve the %RE and based on the %RE values, a weighted linear regression with a weighting factor of $1/x^2$ was chosen for all compounds. Using these selected models, the mean back-calculated concentrations did not differ more than ±15%, which is in line with the acceptance criteria [27].

As displayed in Table A.3.3, %bias was below 13%, meeting the acceptance criterion for accuracy (%bias within ±20% for LLOQ and within ±15% for other QCs). With the single exception of PB at Low QC level (with a total precision of 16.5 %RSD), the acceptance criteria (%RSD within ±20% for LLOQ and within ±15% for other QCs) for within day and total precision were met [27].

Table A.3.3. Within day and total precision (%RSD) and accuracy (%bias) for QCs of VPA, PB, PHT, CBZ and CBZ-E at four concentration levels in DBS samples (n=3x2).

	Within day precision (%RSD) (n=3x2)				
	VPA	PB	PHT	CBZ	CBZ-E
LLOQ	2.57	3.69	3.04	3.94	3.23
Low QC	7.41	8.94	10.0	8.97	11.5
Mid QC	7.25	2.50	2.83	5.64	2.92
High QC	8.70	5.97	6.15	5.79	5.44
	Total precision (%RSD) (n=3x2)				
	VPA	PB	PHT	CBZ	CBZ-E
LLOQ	11.9	9.42	9.42	9.42	9.42
Low QC	12.1	16.5	10.0	12.8	12.5
Mid QC	7.25	7.52	8.84	9.03	9.55
High QC	8.70	8.50	10.6	12.9	6.49
	Accuracy (%bias) (n=3x2)				
	VPA	PB	PHT	CBZ	CBZ-E
LLOQ	4.40	7.25	-5.46	-11.8	4.00
Low QC	1.45	3.67	-10.1	2.33	-12.8
Mid QC	-1.32	-0.17	2.67	3.06	12.2
High QC	-1.33	10.7	3.19	2.64	3.98

When blank samples were injected after analysis of the highest calibrator, no carry-over was detected for the compounds. Regarding selectivity, no unacceptable interferences were observed when analyzing blank DBS samples originating from six different volunteers.

As displayed in Table A.3.4, all compounds were stable in DBS samples for at least 1 month when stored at room temperature, 4 °C and -20 °C and for at least 4 days when stored at 60 °C (representing an accelerated stability experiment).

Table A.3.4. Stability data for VPA, PB, PHT, CBZ and CBZ-E in VAMS samples at Low and High QC (n=3). Data are presented as the percentage difference between the concentration measured at the specific time points and the nominal values.

Temp	**Stability for 4 days (%difference) (n=3)**									
	VPA		PB		PHT		CBZ		CBZ-E	
	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC
60 °C	-7.76	-2.66	13.53	14.93	0.59	-4.28	-11.2	-5.10	-3.42	-9.50
	Stability for 1 week (%difference) (n=3)									
RT	-7.45	-10.5	-4.76	1.67	-9.83	-0.17	17.2	-9.54	-6.89	-7.96
4 °C	-1.88	-11.4	-5.11	-1.11	-8.33	-13.2	11.8	-9.81	-6.89	-5.19
-20 °C	-1.45	-11.7	5.78	-1.67	-6.88	-11.7	13.3	-8.52	-7.33	-5.93
	Stability for 1 month (%difference) (n=3)									
RT	-10.4	-7.61	5.97	7.84	-7.83	-0.93	10.2	4.16	0.00	-0.97
4 °C	-4.77	-4.59	15.0	7.96	-3.85	-0.46	0.84	0.31	-2.51	-1.77
-20 °C	-0.47	-4.11	12.8	7.71	-4.06	2.94	8.86	-2.35	0.91	-4.51

As can be concluded from the non-IS-corrected analyte matrix effects provided in Table A.3.5, a relevant (>15%) ionization suppression is observed for CBZ-E. Remarkably, this suppression was even concentration dependent. However, the IS-corrected matrix effect was 99.2% and 96.0% for Low and High QC, respectively, indicating that the IS compensates for the differences in ionization, substantiating the need for the inclusion of a labeled IS. Furthermore, the %RSD of the IS-corrected matrix effects was overall below 10%, meeting the pre-set acceptance criterion of 15% [27].

Table A.3.5. Analyte matrix effect and IS-corrected matrix effect (n=9) for VPA, PB, PHT, CBZ and CBZ-E.

	Analyte matrix effect									
	VPA		PB		PHT		CBZ		CBZ-E	
	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC
Mean of 9 donors (%)	97.4	98.2	105.1	97.7	102.9	102.5	94.7	84.1	74.1	43.7
%RSD	1.45	1.94	1.36	0.81	2.11	0.89	1.63	1.68	8.77	5.79

	IS-corrected matrix effect									
	VPA		PB		PHT		CBZ		CBZ-E	
	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC
Mean of 9 donors (%)	92.4	97.7	100.3	98.4	101.6	101.2	97.7	92.9	99.2	96.0
%RSD	9.02	1.19	1.83	1.40	1.61	1.08	2.13	1.80	5.32	1.73

The relative recovery was evaluated by spiking three different concentration levels in blank whole blood with a Hct of 0.21, 0.41, 0.53 and 0.60. Upon extracting each spot three times, recovery values of 58.7 ± 8.33% for VPA, 61.2 ± 9.79% for PB, 59.5 ± 8.75% for PHT, 62.6 ± 9.36% for CBZ and 61.0 ± 9.99% for CBZ-E were obtained, these values corresponding to the averages calculated from all values obtained at all Hct levels and concentration levels. Compared to a previously reported method for TDM of AEDs, making use of volumetric absorptive microsampling devices [35], the relative recovery values obtained here were on the low side. A possible -partial- explanation for this observation is that the extraction module may not be able to lock the exact same area to the sub-millimeter, resulting in an underestimation of the relative recovery, due to a small amount of 'new' analyte extracted during the repeated extractions. However, since the relative recovery values turned out to be reproducible (CV% <10%, overall) and since sensitivity was not an issue, the lower relative recovery values were not problematic here. Furthermore, as the CV% values (shown in Table A.3.6) calculated over the different Hct levels are below 15% for all analytes, it can be concluded that the HCT did not affect the recovery. Importantly, analysis of real-life patient samples revealed similar relative recovery values, i.e. 62.4 ± 7.08% for VPA (average calculated from 12 samples with a Hct ranging from 0.29 to 0.47), 63.1 ± 8.56% for CBZ (average calculated from 3 samples with a Hct ranging from 0.34 to 0.44), 57.4 ± 7.84% for PHT (average

calculated from 5 samples with a Hct ranging from 0.38 to 0.44) and 69.7% for PB (1 patient sample with a Hct of 0.29).

Table A.3.6. Analyte recovery (%) at Low, Mid and High QC level for VPA, PB, PHT, CBZ and CBZ-E determined by using repeated (n=3) sample extracting of the DBS, this at four different Hct levels (i.e. 0.21, 0.42, 0.52 and 0.62, being the target values).

	VPA			PB			PHT			CBZ			CBZ-E		
	Low QC	**Mid QC**	**High QC**	**Low QC**	**Mid QC**	**High QC**	**Low QC**	**Mid QC**	**High QC**	**Low QC**	**Mid QC**	**High QC**	**Low QC**	**Mid QC**	**High QC**
0.21	58.4	65.7	65.7	63.9	71.9	70.7	59.8	68.3	66.3	67.2	71.8	71.7	65.7	71.0	70.4
0.42	54.6	50.7	53.2	58.1	53.0	54.9	55.3	50.9	53.6	58.9	53.9	55.8	58.1	52.2	53.9
0.52	63.3	55.7	56.9	62.7	55.8	59.1	62.9	55.2	59.1	64.8	57.0	61.4	63.4	55.1	58.8
0.62	57.5	63.0	59.8	58.3	65.6	59.6	59.0	63.7	60.3	60.4	65.7	62.1	59.4	64.3	59.4
CV%	6.15	11.7	8.95	4.92	14.2	11.0	5.26	13.3	8.73	6.14	13.2	10.6	5.74	14.2	11.5

Finally, DBS (n=3), prepared at three concentration levels (Low, Mid and High QC) in blank whole blood at four different Hct levels, were extracted to evaluate an influence of the Hct. When using partial-spot analysis of DBS, it is expected that a given surface of DBS, originating from blood with a higher Hct value, contains a larger volume of blood (due to the higher viscosity), when compared to DBS prepared from blood with a lower Hct [36]. Consequently, when using a calibration line established in blood with an intermediate Hct (here a Hct of 0.42), analysis of DBS with a higher Hct may result in an overestimation [36]. However, here, the opposite was observed for the 0.62 Hct level: for PB, PHT, CBZ and CBZ-E at the Low QC level and for CBZ and CBZ-E at the Mid QC level, results were more than 15% (but less than 20%) lower (see Figure A.3.2). This suggests a Hct-dependent recovery, albeit only at extremely high Hct values (0.62), which are only seldomly encountered [36]. As neither matrix effects, nor the 'relative' recovery (as determined by repeated extractions) provide an explanation for this observation, we conclude that at extremely high Hct values there may be a slight negative impact on 'extractability'. While this demonstrates that Hct remains an important parameter to be evaluated, also when using fully automated extraction of DBS, the overall relevance is limited, given the fact that Hct reference ranges for men and women lie at approximately 0.41-0.50 and 0.36-0.44, respectively [25]. Hence, it can be concluded that the impact of the Hct is limited overall and is very unlikely to skew the analytical result to an important extent.

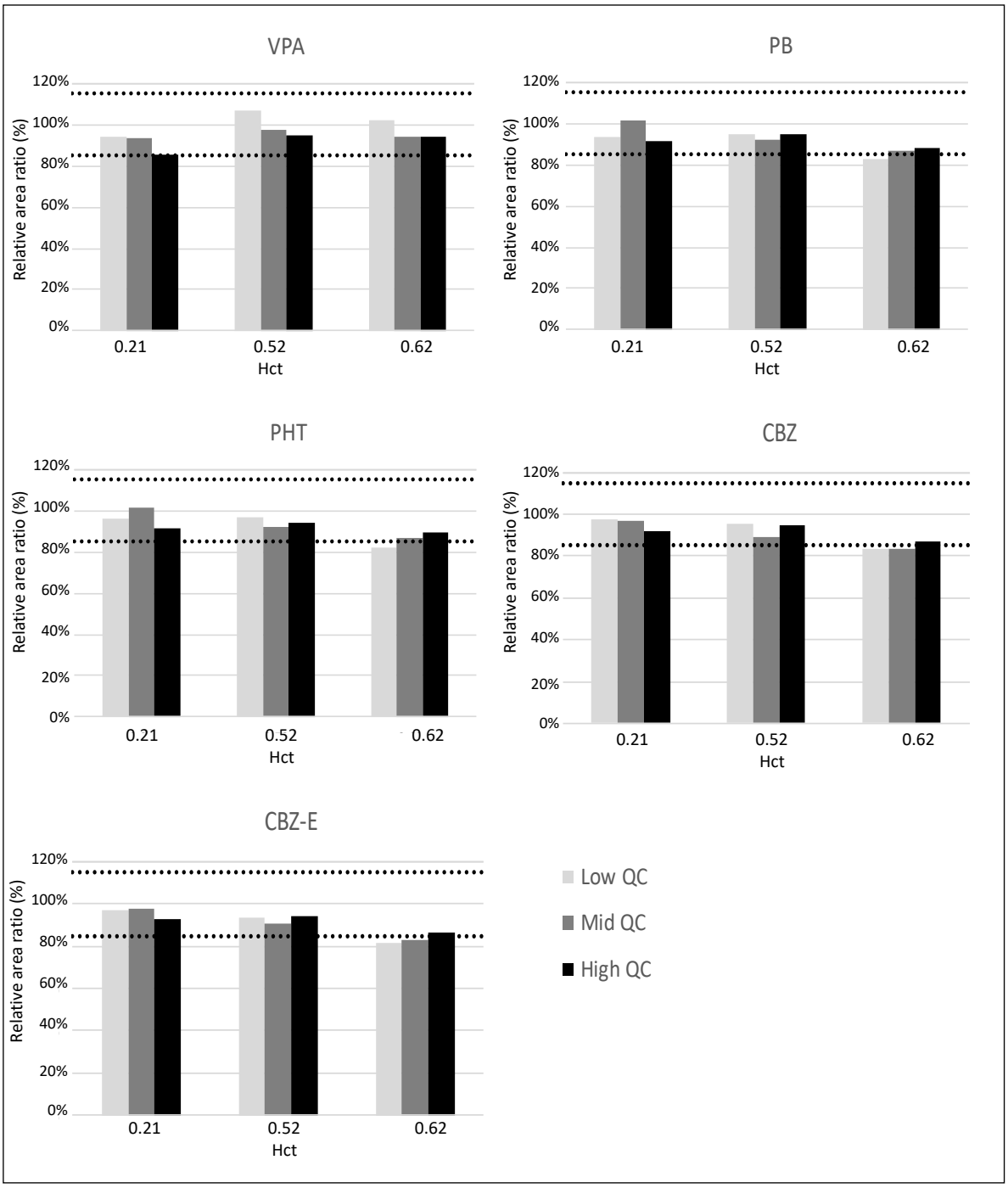

Figure A.3.2. Relative area ratio compared to the 0.42 reference Hct (target value) at three different concentration levels (Low, Mid and High QC) for VPA, PB, PHT, CBZ and CBZ-E.

A.3.3.3. Application

Fifteen capillary DBS samples, originating from patients receiving AED therapy in remote areas in sub-Saharan Africa, were analyzed. The DBS contained VPA (concentrations ranging from 33.9 to 47.1 µg/mL), CBZ (concentrations ranging from 3.05 to 7.84 µg/mL) and PB

(concentrations ranging from 4.54 to 14.2 µg/mL), originating from patients with Nodding syndrome, epilepsy patients and epilepsy patients with an *O. volvulus* co-infection, respectively. Incurred sample reanalysis revealed that for all of the analyzed samples the % difference between the initial concentration and the concentration measured during the repeat analysis was lower than 20% (see Figure A.3.3), which is in line with the acceptance criterion [27] and demonstrates the robustness of the procedure, from sampling to analysis [37]. To compare capillary DBS and venous blood results, ideally, bridging experiments are performed, to evaluate capillary-venous differences and to exclude a potential effect of (the absence of) an anticoagulant. While not within the scope of this study, various studies have already shown a good agreement between corrected (capillary) DBS concentrations and plasma concentrations for the included AEDs [38-40].

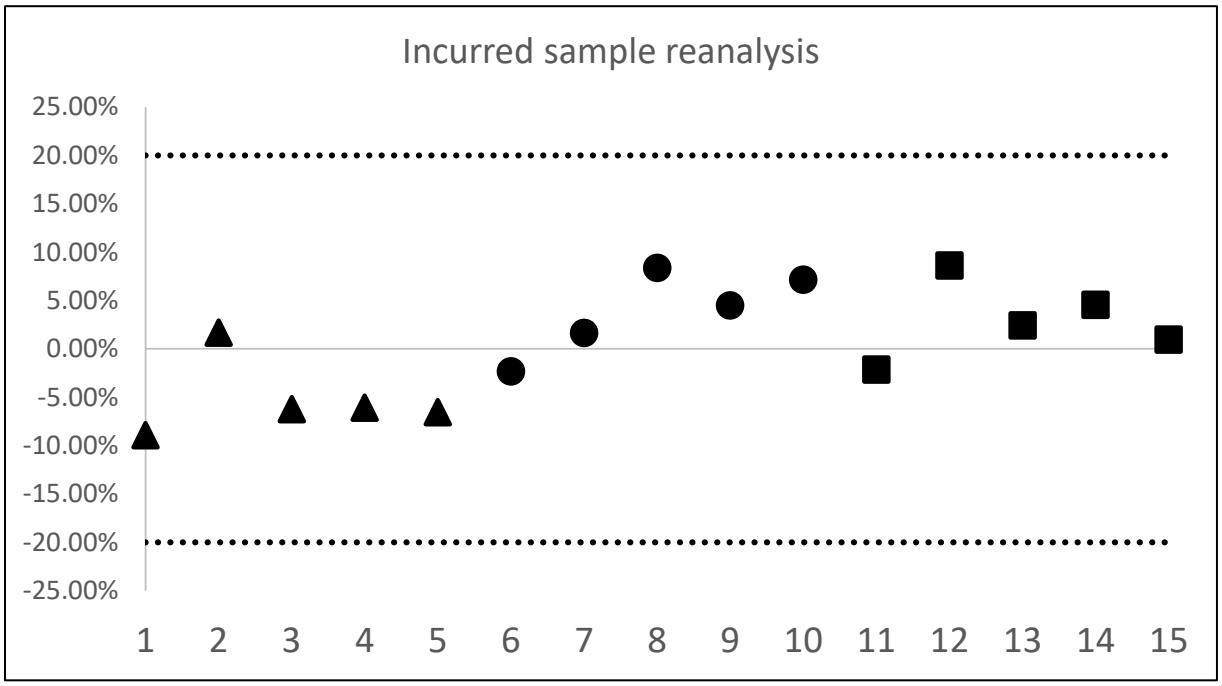

Figure A.3.3. Percent difference between the initial concentration and the concentration measured during the incurred sample reanalysis study. The triangles correspond to the VPA samples, the bulbs to the CBZ samples and the squares to the PB samples. The dotted lines represent the ± 20% deviation limits.

A.3.4. Conclusion

The DBS-MS 500 is one of the fully automated DBS autosamplers currently available on the market. With a total capacity of 500 DBS cards, the system is truly fit for high-capacity settings. Previously, the quantitative performance, the ability of nullifying the Hct-based recovery bias and the possibility of an increased assay sensitivity -compared to manual extraction procedures- have been demonstrated.

In this Chapter, an LC-MS/MS method for the determination and quantification of 4 anti-epileptic drugs and one active metabolite, i.e. CBZ, VPA, PHT, PB and CBZ-E, in DBS, making use of the DBS-MS 500 autosampler, was developed and validated. Several issues during method development had to be tackled, including the diverse character of the analytes and the high concentrations of the included AEDs (which, although only 4-mm areas were analyzed, posed a saturation issue for the highly sensitive instrumentation to which the autosampler was coupled). Therefore, thorough optimization of the fully automated extraction method was of utmost importance, finally resulting in the exclusion of the built-in IS spray. Our findings demonstrate that proposed, generic direct elution conditions, while of value for orientation, may require (substantial) adjustment, depending on the analytes of interest and the used instrumentation.

The final method was extensively validated, the validation including both bioanalytical and DBS-specific parameters and overall the pre-set acceptance criteria were met. Evaluation of matrix effects emphasized the relevance of the inclusion of a labeled IS, in order to compensate for matrix effects.

Finally, successful application of the method on real-life patient samples demonstrated the applicability of the developed procedure to analyze samples from remote areas.

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CHAPTER A.4.

DRIED BLOOD MICROSAMPLING-BASED THERAPEUTIC DRUG MONITORING OF ANTI-EPILEPTIC DRUGS IN CHILDREN WITH NODDING SYNDROME AND EPILEPSY IN UGANDA AND THE DEMOCRATIC REPUBLIC OF THE CONGO

Based on

Velghe S, Delahaye L, Ogwang R, Hotterbeekx A, Colebunder R, Mandro M, Idro R, Stove CP. Dried blood microsampling-based therapeutic drug monitoring of anti-epileptic drugs in children with Nodding syndrome and epilepsy in Uganda and the Democratic Republic of the Congo. Submitted for publication in *Therapeutic Drug Monitoring*.

Abstract

Nodding syndrome is a highly debilitating, generalized seizure disorder, affecting children in subregions of sub-Saharan Africa. Despite many efforts towards finding its etiology, the exact cause of the syndrome still remains obscure. Therefore, to date, patients only receive a symptomatic care, including the administration of first-generation anti-epileptic drugs (AEDs) for seizure control. Since information about medication effectiveness in this population is completely lacking, the aim of this Chapter was to perform therapeutic drug monitoring (TDM) to seek whether an answer could be provided to the question why for some patients the symptoms decrease, whilst in others the epileptic seizures remain poorly controlled. Seeing the challenging context in which sampling needed to take place (remote areas, devoid of electricity, running water, etc.), dried blood matrices (i.e. dried blood spots (DBS) and volumetric absorptive microsampling (VAMS) devices) were considered fit-for-purpose. Seeing the similarities between the syndrome and other forms of epilepsy, samples originating from patients suffering from (onchocerciasis-associated) epilepsy were also included. VAMS samples and DBS were analyzed using fully validated methods, involving manual extraction or fully automated extraction, respectively, prior to quantification using liquid chromatography coupled to tandem mass spectrometry. Analysis revealed that serum concentrations (calculated from DBS) within the respective reference ranges were attained for only 52.9% of the 68 Nodding syndrome patients treated with valproic acid, for 21.4% of the 56 Ugandan epilepsy patients treated with carbamazepine, and for 65.7% of the 137 onchocerciasis-associated epilepsy patients from the Democratic Republic of the Congo treated with phenobarbital. Furthermore, when comparing DBS to VAMS concentrations, an inexplicable overestimation was observed in the latter. Finally, no obvious link could be observed between the obtained drug concentrations and the number of seizures experienced during the last month before sampling, disclosing the fact that the level of improvement of some patients cannot simply linked to reaching therapeutic concentrations.

A.4.1. Introduction

Nodding syndrome, an unexplained, highly debilitating generalized seizure disorder, typically having its onset in 5-15-year-old children in subregions of sub-Saharan Africa, was first described in Tanzania by Dr. Louise Jilek-Aall in the 1960s [1, 2]. It is a distinct clinical entity, characterized by repetitive head nodding (an atonic seizure event triggered by e.g. cold and food), frequently progressing towards generalized convulsions and accompanied by malnutrition, behaviour problems, delayed physical development, lack of secondary sexual characteristics and cognitive defects [2-7].

Since 2009, various investigations into the possible causes were performed, resulting in several hypotheses with respect to its etiology, e.g. specific micronutrient malnutrition, ingestion of toxic substances, post-conflict stress, contaminated relief foods, infectious encephalitis, viral infection (e.g. measles), etc. [2, 7-9]. However, none of these hypotheses being confirmed. Another -currently leading- hypothesis for the cause is an infection with the parasitic worm *Onchocerca volvulus* (transmitted by the black fly) and that Nodding syndrome is one of the phenotypic presentations of onchocerciasis-associated epilepsy [1, 2, 8, 10-15]. Yet, the evidence for its contribution as a causal factor in the onset of Nodding syndrome has been inconclusive. Recently, Johnson *et al.* hypothesized that the syndrome may be an autoimmune-mediated disease, in which neurotoxic autoantibodies to leiomodin-1, a protein expressed in smooth muscle cells, the thyroid and in the central nervous system, play an important role [16]. The latter could be the result of a molecular mimicry between an *O. volvulus* protein and the human leiomodin-1 [16].

Since the pathophysiological mechanism causing Nodding syndrome is not known yet, there is still no known cure. Therefore, an action plan was developed to provide patients with symptomatic care [17]. This symptomatic treatment has a 3-fold intention: (1) relief of symptoms; (2) offering primary and secondary prevention for disability; and (3) rehabilitation to improve function. Furthermore, the action plan also includes vector control with larviciding of black fly breeding sides and mass drug treatment for Onchocerciasis twice a year with ivermectin [17].

Pharmaceutical treatment of Nodding syndrome patients mainly consists of seizure control [4]. Most used are the first-generation anti-epileptic drugs (AEDs) valproic acid (VPA),

carbamazepine (CBZ), phenytoin (PHT) and phenobarbital (PB). Idro *et al*. demonstrated that the multidisciplinary symptomatic treatment (i.e. anti-epileptic drugs, behavioural interventions, and nutritional and physical rehabilitation) leads to clinical and functional improvements in the majority of patients [18]. The pharmaceutical treatment resulted for 25% of the Nodding syndrome patients in seizure relief and furthermore, a reduction of 70% in head nodding and convulsive seizure frequency was reported [18]. However, information about medication effectiveness is completely lacking: whereas in some patients, symptoms decrease, in others, epileptic seizures remain poorly controlled. There is no information as to whether this may be simply linked to a failure of reaching therapeutic concentrations. Therapeutic drug monitoring (TDM) may provide an answer to this question. Other factors supporting the need for TDM are the poor nutritional status (impacting pharmacokinetics) and the difficulty of assessing toxicity or side effects in young and/or mentally disabled children.

Therefore, the aim of this Chapter was to perform TDM of AEDs in children suffering from Nodding syndrome treated in the treatment centre in Kitgum. Furthermore, seeing the clinical overlap, patients with other forms of (onchocerciasis-associated) epilepsy originating from Uganda and the Democratic Republic of the Congo (DRC) were also included in this study [2].

Dried blood microsamples were considered fit-for-purpose, given the potentially highly challenging context in which sampling was to take place: in remote areas devoid of electricity, running water, etc. In contrast to classical liquid samples, dried spot matrices can be conveniently transported and stored at ambient temperature [19-22]. Besides the advantage of increased analyte stability, these samples also offer the advantage that they are considered as non- or less contagious and can be sent via regular mail, without special precautions (which is highly relevant, given the still high prevalence of HIV in Northern Uganda [23]). The latter is also an important benefit in countries where patients still have to cover a long distance to clinical practices.

A.4.2. Materials and methods

A.4.2.1. Study sites

In Uganda, samples were collected in Kitgum, Pader and Lamwo districts, three of the districts in Northern Uganda which have been hit by Nodding syndrome. Within Kitgum General

Hospital, a Nodding syndrome ward was opened to treat Nodding syndrome patients with severe symptoms. The study was conducted on the same cohort of patients included in a case control study, evaluating the presence and levels of *O. volvulus* induced auto-antibodies against neuron surface proteins among Nodding syndrome patients, age matched children with other forms of generalized epilepsy and healthy community control patients. Ugandan participants in this study included children with Nodding syndrome and those other forms of epilepsy.

Furthermore, the study included participants with other forms of generalized epilepsy from an Onchocerciasis endemic region in the DRC, Ituri Province. The villages of Ituri Province are characterized by a high prevalence of epilepsy (3.6-6.2%), being 2-10 times higher compared to non-onchocerciasis endemic regions in Africa [24]. For the DRC, study participants were patients with onchocerciasis-associated epilepsy enrolled in a clinical trial investigating the effect of an ivermectin treatment on the frequency of epileptic seizures [25].

In order to obtain trustworthy samples, dried blood sampling was performed by a trained laboratory technician.

Approval for this study was provided by the Ethics Committee of Ghent University Hospital (EC2017/0572 and EC2017/1165, for Uganda and the DRC, respectively), by the Makerere University School of Medicine Research and Ethics Committee (IRB; 2015 – 146), by the Ugandan National Council for Technology (UNCST; HS 1986) and by Kinshasa School of Public Health (ESP/CE/06/2017).

A.4.2.2. Participants

In total, 68 patients with Nodding syndrome from Uganda, 58 Ugandan patients with epilepsy and 137 patients with onchocerciasis-associated epilepsy from the DRC were included in this study.

Patients from Uganda were identified as eligible Nodding syndrome patients based on the case definition for Nodding syndrome [17]. In this case definition, a distinction is made between a suspected case, a probable case and a confirmed case [17]. Only confirmed Nodding syndrome patients were included in the study, i.e. patients where a nodding episode was observed by a trained healthcare worker, or videotaped, or was observed on

electroencephalography (EEG). Upon inclusion, these patients were hospitalized for 1-2 weeks at Kitgum General Hospital, in order to conduct baseline tests, such as clinical, EEG, cognitive and laboratory assessments and to adapt the AED dose based on the clinical manifestation. Eligible Nodding syndrome patients received a VPA treatment. For patients already treated with (an)other AED(s), conversion to VPA was done after a withdrawal period. For VPA, it is suggested to start with 10 mg/kg/day in two divided doses. Dosage adjustments should be done by 5 mg/kg/day until seizure control is achieved or until the maximal dose is reached, being 40 mg/kg/day in children [17].

A person was considered to have epilepsy if he/she met the 2014 International League Against Epilepsy (ILAE) criteria: having experienced at least two seizures, unprovoked and without fever, with a minimal time difference of 24 hours between the two events. Samples from the DRC originated from patients with onchocerciasis-associated epilepsy [15]. An infection with the parasitic worm *O. volvulus* was confirmed by demonstrating the presence of microfilariae (produced by adult female worms) in a skin snip obtained from the patients or via the detection of *O. volvulus* Ov 16 antibody in blood [26]. Furthermore, for the DRC, patients were excluded when they already received an anti-epileptic or ivermectin treatment before the study.

A.4.2.3. Sample collection

Upon signing an informed consent form by the parents, patients were asked to provide some blood for the assisted preparation of dried blood samples, i.e. dried blood spots (DBS) and volumetric absorptive microsampling (VAMS) samples. DBS samples were collected on DBS cards, suitable for automated analysis. VAMS devices consist of a plastic handler, to which a polymeric, absorptive tip is connected, allowing the straightforward collection of a fixed volume of blood (in this case 10 µL) [27]. All dried blood samples were collected in the morning right before the first medication intake of the day. During sampling, the hands were disinfected, before executing a fingerprick with the help of a BD Microtainer contact activated safety lancet (BD, Franklin Lakes, USA). In a next step, the first drop of blood was wiped off with a clean tissue, to avoid the collection of tissue fluid. Afterwards, the second drop of blood was applied onto a DBS card or absorbed by the tip of a VAMS device. Subsequently, the samples were left to dry at room temperature. In total, 4 DBS samples and 2 VAMS samples

were collected per patient. For the Nodding syndrome patients (Uganda), sampling took place in a hospital environment, making it possible to immediately store the samples once dried (after approximately 2h) at -20°C in zipclosure plastic bags, containing a 10 g package of desiccant (Minipax® absorbent packets, Sigma Aldrich). For the epilepsy patients (Uganda and the DRC), sampling took place in the field and therefore samples could only be stored at -20°C upon arriving in the lab, this within 2-8 hours after sample collection. In order to be able to adequately interpret the obtained blood concentrations, some relevant information was collected: date of sample collection, time of sample collection, time of last medication intake, number of seizures experienced during the last month and type of medication.

A.4.2.4. Sample analysis

Once all samples were obtained, the dried samples were transported via regular mail to Belgium, where they were stored at -20°C prior to analysis. Concentrations of the AEDs were determined in DBS and VAMS samples using the fully validated methods described in Chapter A.2. and A.3. For the DBS, a fully automated DBS extraction system (DBS-MS 500, CAMAG), online coupled to a standard liquid chromatography-tandem mass spectrometry (LC-MS/MS) configuration was used, controlled by SCIEX Analyst 1.6.2. For the VAMS samples, the LC-MS/MS system was controlled by SCIEX Analyst 1.6.2 and by the Waters Acquity console software. The VAMS samples were extracted in 100 µL of an acetonitrile/water (80/20, v/v) mixture, containing 5 mM ammonium acetate and the deuterated internal standards. After gently shaking for 10 min at 60°C, VAMS samples were centrifuged and the resulting supernatants were diluted 1 on 1 with water, containing 5 mM ammonium acetate. Further details on the analytical methods can be found in Chapter A.2. and A.3.

A.4.3. Results

A.4.3.1. Valproic Acid

68 DBS samples originating from patients with Nodding syndrome were analyzed, VPA being detectable in all samples. In 57 samples the VPA concentration lay above the used lower limit of quantification (LLOQ) (i.e. 25 µg/mL). Furthermore, the analysis also revealed the presence of CBZ and its active metabolite CBZ-E in 2 patients, in contrast to what was expected, since

patients included in the clinical trial were supposed to only receive VPA.

Therapeutic reference ranges are usually set in serum/plasma and for the used AEDs it has been demonstrated that serum and VAMS (whole blood) concentrations may indeed differ [28]. Since therapeutic reference ranges in blood are still lacking to date, serum concentrations were calculated, starting from blood/plasma ratios, which were based on VAMS-assisted LC-MS/MS analysis of 21 left-over blood samples (Ghent University Hospital). For these blood samples, serum concentrations were available, albeit analyzed with another quantification method, i.e. chemiluminescent magnetic microparticle immunoassay technology (CMIA, Architect i2000SR). Based on the measured blood and obtained serum concentrations, a blood/plasma ratio of 0.66 was calculated for VPA (see Table A.4.1). This calculated blood/plasma ratio was used to calculate the serum concentrations for the 57 DBS samples with a VPA concentration above the used LLOQ. In total, this revealed that only 36 out of the 68 DBS samples (i.e. 52.9%) had a concentration within the therapeutic reference range (i.e. 50-100 µg/mL [29]) (Figure A.4.1, panel A, circles). There were no samples with a VPA concentration above the upper limit of the therapeutic reference range.

It is important to note that there is a substantial variation in blood/plasma ratios between individuals, as is readily clear from our limited data set (see Supplementary Table 1). This is in line with observations by Linder *et al.,* who also demonstrated substantial variations in blood/plasma ratios for CBZ and VPA [30]. Therefore, calculating serum concentrations based on blood concentrations remains a challenge, because this conversion introduces an additional factor of uncertainty. Furthermore, it is difficult to predict whether these calculated blood/plasma ratios, based on samples originating from patients suffering from epilepsy at Ghent University Hospital, reflect the blood/plasma ratios for these AEDs in children suffering from Nodding syndrome or epilepsy in sub-Saharan Africa. Ideally, blood reference intervals should be available.

To evaluate the accuracy of the entire procedure, from sampling to analysis, a reanalysis of DBS samples was performed in separate runs, with an interval of two weeks. Taking only the DBS samples with a VPA concentration above the used LLOQ in the first analysis into account and after visually inspecting the DBS cards (i.e. excluding spots with a size smaller than 4 mm and/or spots which were obviously obtained by the application of 2 separate blood drops), 43

samples were included in this incurred sample reanalysis. The results of this incurred reanalysis are depicted in Figure A.4.1 (panel A, triangles). The overall outcome of this incurred sample reanalysis was evaluated by dividing the difference between the repeat value and the initial value by the mean of both values. Since in 87.8% (36 out of 41, after exclusion of 2 outliers, detected *via* the Generalized Extreme Studentized Deviate test, with a %difference of -51.0 and 34.7%) of the samples the %difference between the initial VPA concentration and the concentration measured during the repeat DBS analysis was not higher than 20% of their mean, it can be concluded that the acceptance criterion for incurred sample reanalysis (i.e. the %difference between the concentration of the two repeats should be lower than 20% of their mean for at least 67% of the repeats) was met [31]. This indicates that, when also including visual inspection, for the vast majority of the samples, from sampling to analysis, acceptable data can be obtained. The median (range) %difference that was observed was -9.70 (-29.9 - 5.26)%. As the samples were processed on different days, this difference (overall negative bias in the repeat analysis) could be explained by a slight variation in calibration. However, this could not be explained by the analysis of quality control (QC) samples, since for all of these (LLOQ, Low, Mid and High QC) a %bias between 5 and 15% was observed on both analysis days.

Table A.4.1. Calculated blood/plasma ratios for VPA, CBZ and PB based on the analysis of 21, 18 and 13 left-over whole blood samples, respectively.

	VAMS concentration (µg/mL)	Serum concentration (µg/mL)	Calculated blood/plasma ratio
VPA	29.30	49.50	0.59
	56.20	79.00	0.71
	39.20	64.60	0.61
	61.70	74.70	0.83
	30.00	50.60	0.59
	42.70	66.80	0.64
	44.91	61.70	0.73
	38.26	57.40	0.67
	45.92	86.30	0.53
	29.11	34.70	0.84
	73.51	112.30	0.65
	42.01	63.50	0.66
	35.09	59.70	0.59
	27.95	30.80	0.91
	72.22	104.70	0.69
	48.74	82.10	0.59
	63.70	93.80	0.68
	28.10	30.00	0.94
	50.97	100.82	0.51
	36.53	45.10	0.81
	53.87	95.80	0.56
			Median ± SD
			0.66 ± 12.04%
CBZ	12.2	8.60	1.42
	8.56	6.50	1.32
	5.91	5.20	1.14
	8.75	6.40	1.37
	2.71	2.30	1.18
	11.7	13.1	0.89
	6.51	5.40	1.21
	9.36	10.6	0.88
	6.97	7.70	0.91
	7.17	6.30	1.14
	6.56	6.80	0.96
	4.76	5.00	0.95
	7.87	5.40	1.46
	4.11	3.20	1.29
	10.6	8.80	1.21
	14.4	11.7	1.23
	10.3	8.00	1.29
	7.79	6.10	1.28
			Mean ± SD
			1.21 ± 18.30%

Table A.4.1. Continued

PB	34.90	37.80	0.92
	7.35	8.70	0.84
	41.60	43.60	0.95
	9.88	10.60	0.93
	8.82	8.20	1.08
	14.20	20.50	0.69
	16.89	19.40	0.87
	16.57	20.70	0.80
	19.75	23.60	0.84
	40.66	41.20	0.99
	36.92	33.60	1.10
	21.97	21.40	1.03
	27.35	27.80	0.98
			Mean ± SD0.93
			0.93 ± 11.50%

Besides DBS, VAMS samples were also collected from the 68 children with Nodding syndrome. Analysis of the VAMS samples revealed that 58 had a VPA concentration above the used LLOQ. Applying the blood/plasma ratio to calculate the corresponding serum concentrations revealed that 46 samples (i.e. 67.6%) had a VPA concentration within the therapeutic reference range (Figure A.4.1, panel B, circles). Also here, incurred sample reanalysis was performed, with 90.2% of the repeats yielding a concentration within 20% of their mean, meeting the acceptance criterion for incurred sample reanalysis (Figure A.4.1, panel B, triangles) [31]. To compare the results obtained via the analysis of DBS and VAMS samples, we plotted the average DBS concentrations *vs.* the average VAMS concentrations. As can be deduced from Figure A.4.1, panel C, VPA concentrations were slightly overestimated in VAMS samples when compared to DBS concentrations, with a median %difference±SD of 13.6±11.2% (range -7.91 - 41.7%). In 69.8% of the VAMS - DBS comparisons, the difference in concentration did not exceed 20%. Although in Figure A.4.1, panel C, the 95% confidence intervals (C.I.) of the slope and intercept contained 1 and 0, respectively, it is clear that overall, the differences between DBS and VAMS are too large. Indeed, the 95% C.I. are too wide to consider the results of DBS and VAMS equivalent.

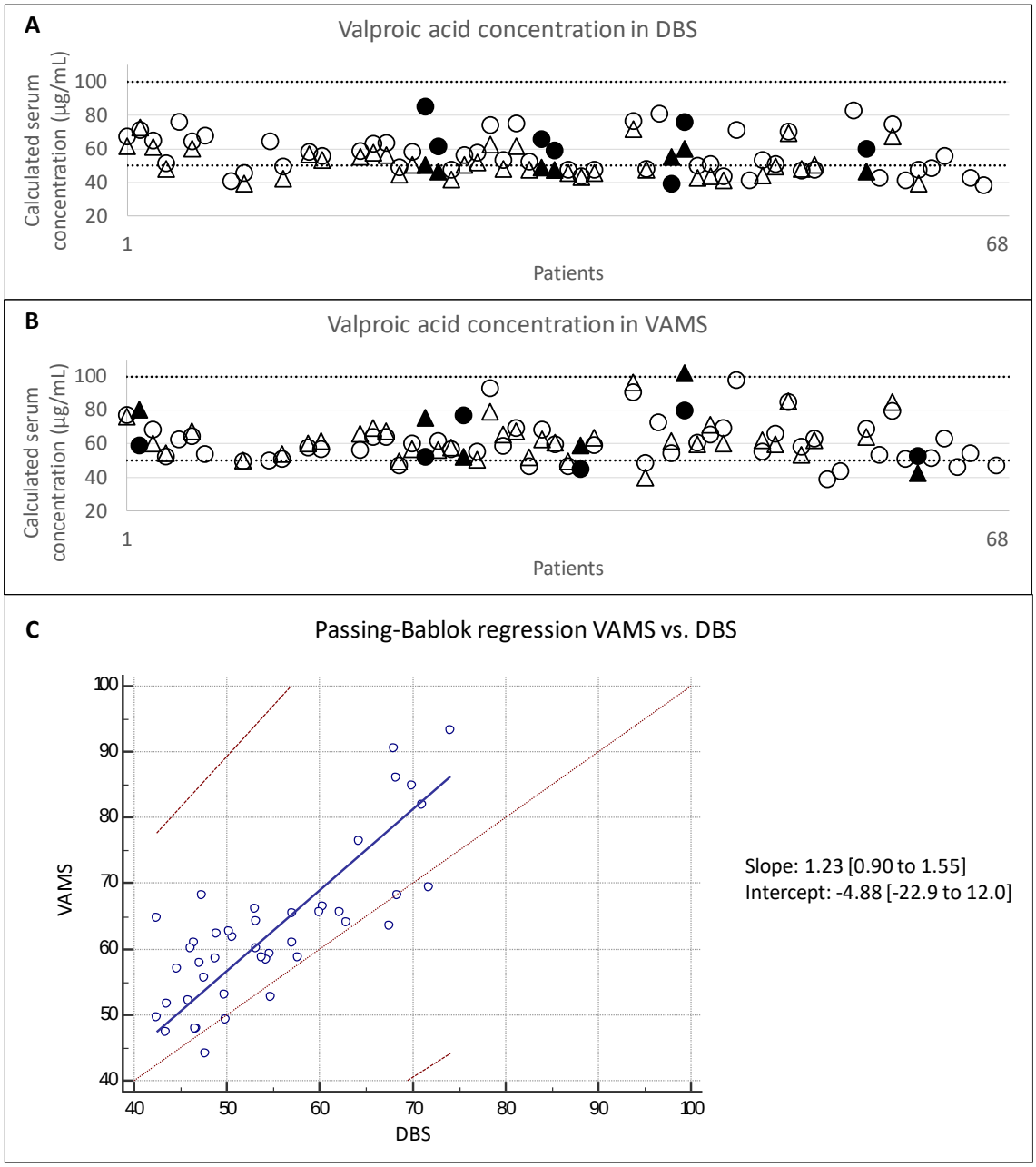

Figure A.4.1. A: incurred sample reanalysis for DBS samples. B: incurred sample reanalysis for VAMS samples. The circles correspond to the initially analyzed samples and the triangles to the repeated samples. The black colored symbols indicate the repeats with a %difference > 20%. The space between the dotted lines corresponds to the therapeutic reference range, set in serum. C: Passing-Bablok regression for the comparison of average calculated serum concentrations (µg/mL) obtained via the analysis of DBS samples and VAMS samples.

A.4.3.2. Carbamazepine

In all of the 56 DBS samples originating from Ugandan patients with epilepsy CBZ could be detected. While in 4 instances the use of VPA for several years and in 1 instance the use of PHT was claimed, these analytes were not found in the dried blood samples. Of the 56 analyzed samples, only 27 DBS samples had a CBZ concentration above the used LLOQ (i.e. 2 µg/mL).

As for VPA, also here, a blood/plasma ratio was calculated, based on a comparison between blood concentrations and available serum concentrations (see Table A.4.1), being 1.21 for CBZ. Using the latter for the calculation of serum concentrations, based on DBS concentrations, revealed that only 12 out of the 27 DBS samples had a CBZ concentration within the therapeutic reference range (i.e. 4-12 µg/mL [29]). So, in total only 21.4% (12 out of 56) of the included patients had a concentration within the therapeutic reference range (Figure A.4.2, panel A, circles).

For the Ugandan epilepsy patients, 23 DBS samples were suitable (i.e. a concentration above the LLOQ and visibly good spots) for incurred sample reanalysis (see Figure A.4.2, panel A, triangles). As can be concluded from Figure A.4.2, panel A, 100% of the repeats had a %difference lower than 20%, meeting the acceptance criterion for incurred sample reanalysis [31]. The median %difference was 5.01% (range -10.32 - 8.36%).

In a next step, also 56 VAMS samples originating from the same patients were analyzed and only 26 out of these had a CBZ concentration above the used LLOQ. When using the calculated blood/plasma ratio of 1.21 to calculate the serum concentrations, only 16 samples had a CBZ concentration within the therapeutic reference range (Figure A.4.2, panel B, circles). Furthermore, as for the DBS samples, also 23 VAMS samples were included in the incurred sample reanalysis experiment (Figure A.4.2, panel B, triangles). Although 78.3% of the samples did not differ more than 20%, meeting the acceptance criterion, the concordance was less good than that observed for DBS (median %difference 6.47%; range -9.39 - 42.1%) [31]. In Figure A.4.2, panel C, a comparison is displayed between the mean concentrations derived from VAMS and DBS samples. With a median %difference±SD between VAMS and DBS samples of 17.5±11.2%, it can be concluded that, when using VAMS samples, the concentrations are, as for VPA, overestimated, when compared to DBS concentrations. Only

52.1% of the concentrations differed less than 20% from one another. Here, the 95% C.I. of the slope did not include 1, pointing at a proportional difference between DBS and VAMS. Also here, the width of the 95% C.I. was too wide to be considered acceptable.

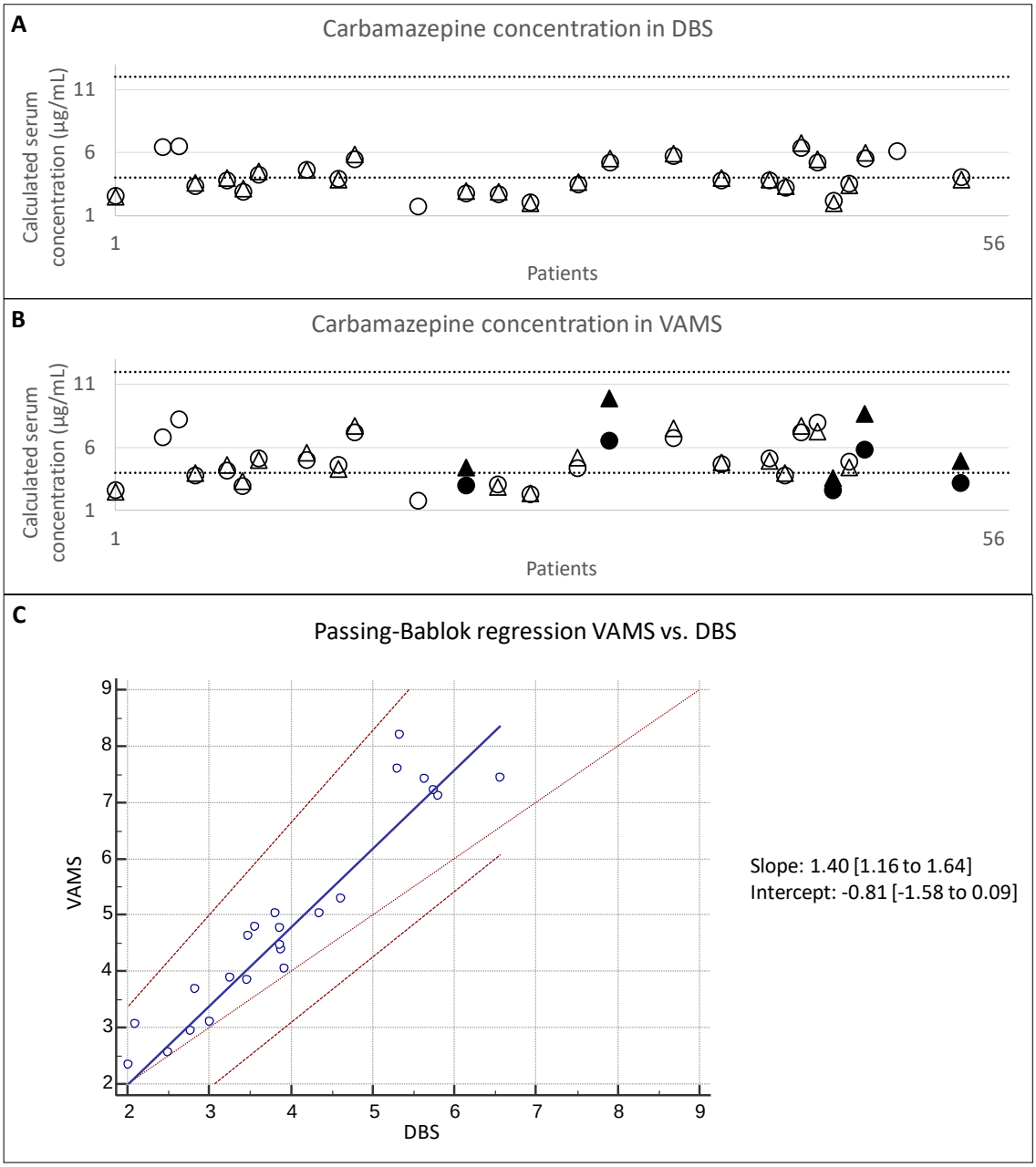

Figure A.4.2. A: incurred sample reanalysis for DBS samples. B: Incurred sample reanalysis for VAMS samples. The circles correspond to the initially analyzed samples and the triangles to the repeated samples. The black colored symbols indicate the repeats with a %difference > 20%. The space between the dotted lines corresponds to the therapeutic reference range, set in serum. C: Passing-Bablok regression for the comparison of average calculated serum concentrations (µg/mL) obtained via the analysis of DBS samples and VAMS samples.

A.4.3.3. Phenobarbital

Finally, from the DRC, 137 DBS samples originating from epilepsy patients were analyzed. Here, patients received a monotherapy with PB and for 133 patients a PB concentration above the used LLOQ (1 µg/mL) was observed. In the remaining 4 patients, no PB was detectable. When using the calculated blood/plasma ratio (see Table A.4.1) of 0.93, 90 DBS samples had a PB concentration within the therapeutic reference range (i.e. 10-40 µg/mL [29]), being 65.7% of all included DBS samples (Figure A.4.3, panel A, circles).

Here, taking the first 75 samples with a concentration above the LLOQ into account, 59 DBS samples were deemed suitable for incurred sample reanalysis upon visual inspection (see Figure A.4.3, panel A, triangles). After exclusion of 2 outliers (detected *via* the Generalized Extreme Studentized Deviate test) with a %difference of -93.4 and -27.9%, all of the remaining repeats had a %difference within 15.8% (see Figure A.4.3), meeting the acceptance criterion for incurred sample reanalysis [31]. The median %difference was -0.91% (range -11.6 - 15.8%).

Analysis of 137 VAMS samples revealed PB concentrations above the used LLOQ for the same 133 patients as observed with the DBS analysis. Calculating the serum concentration out of the VAMS concentration resulted in 105 VAMS samples with a concentration within the therapeutic reference range (Figure A.4.3, panel B, circles). Furthermore, the same cohort of patient samples as for DBS was included in an incurred sample reanalysis experiment (Figure A.4.3, panel B, triangles). Although with 69.0% of the samples not differing more than 20% of their mean, the acceptance criterion was, strictly taken, met, it was clear that there was a large spread between the concentrations of the initial and the incurred reanalysis [31]. In Figure A.4.3, panel C, a comparison is displayed between the VAMS and DBS samples. Here, a median %difference±SD of 46.6±13.3% between concentrations obtained from VAMS *vs.* from DBS was observed, indicating a serious underestimation of DBS concentrations compared to VAMS concentrations. Apart from a considerable proportional difference, the spread was less pronounced than that observed for VPA and CBZ, as evidenced by a less wide 95% C.I. of the slope. No systematic difference was seen here, as the 95% C.I. of the intercept included 0.

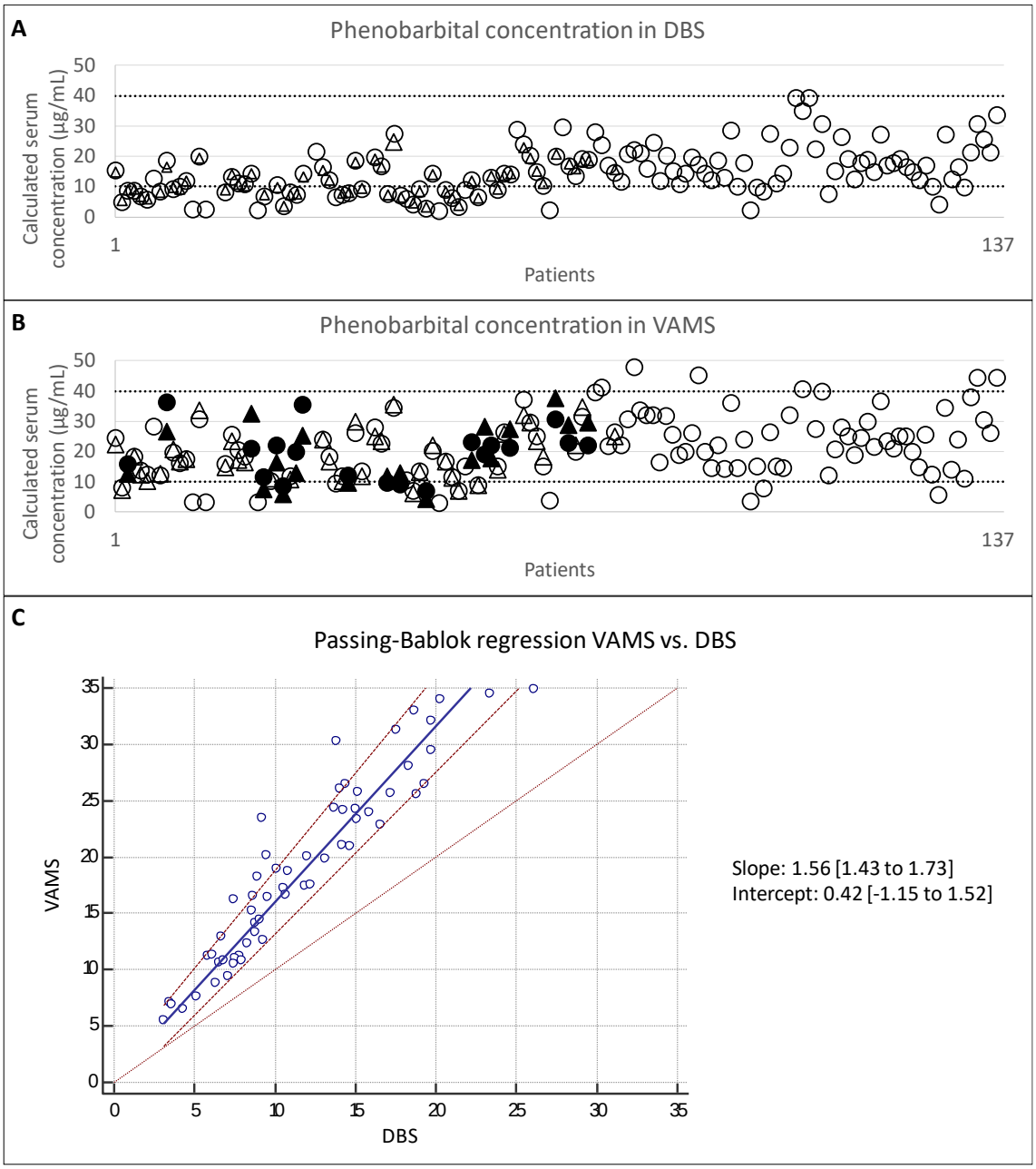

Figure A.4.3. A: incurred sample reanalysis for DBS samples. B: incurred samples reanalysis for VAMS samples. The circles correspond to the initially analyzed samples and the triangles to the repeated samples. The black colored symbols indicate the repeats with a %difference > 20%. The space between the dotted lines corresponds to the therapeutic reference range, set in serum. C: Passing-Bablok regression for the comparison of average calculated serum concentrations (µg/mL) obtained via the analysis of DBS samples and VAMS samples.

A.4.4. Discussion

Conclusively, as can also be deduced from Figure A.4.4, overall, the variability observed upon incurred reanalysis was (much) lower for DBS than for VAMS samples. In addition, a relevant overestimation of VAMS concentrations compared to DBS concentrations was observed, which was most pronounced for the samples containing PB.

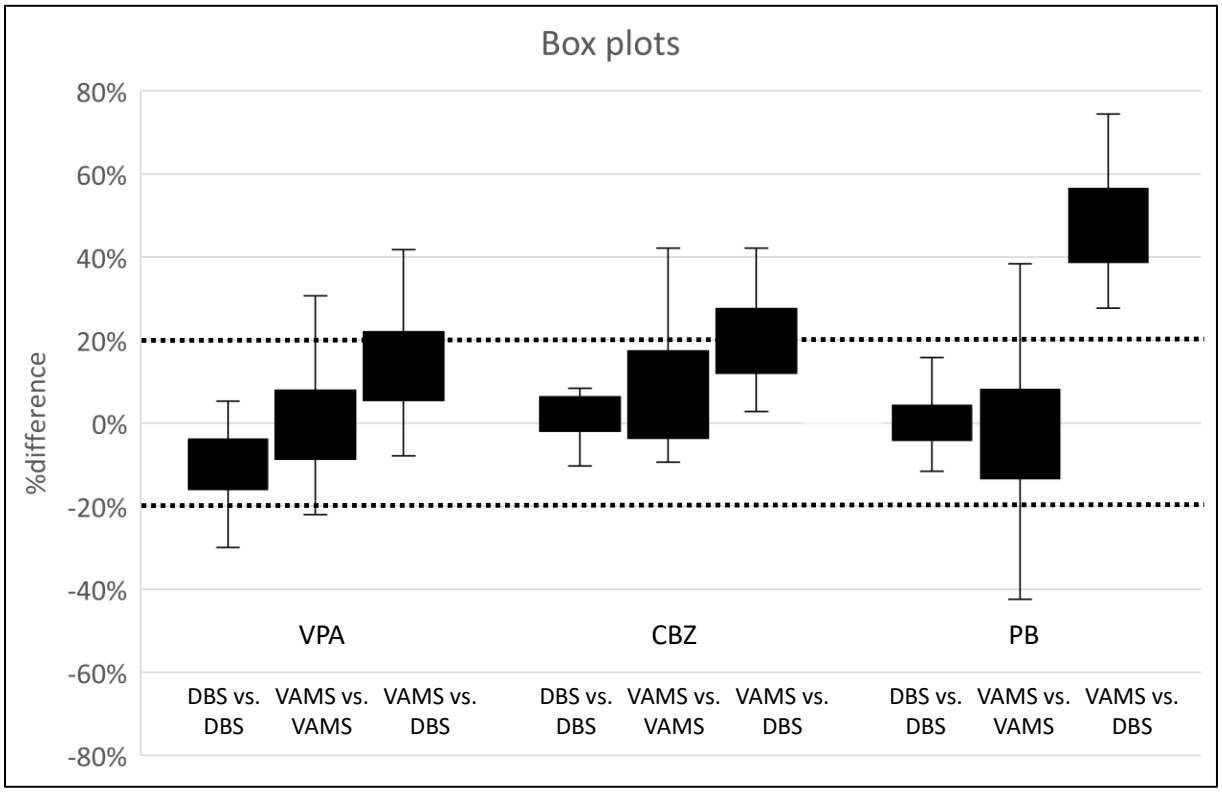

Figure A.4.4. Box plots for %difference of DBS vs. DBS, VAMS vs. VAMS and VAMS vs. DBS for VPA, CBZ and PB. The dotted lines indicate the ± 20% deviation limits.

Stability is an obvious parameter to look at when the results of incurred reanalysis are not entirely satisfactory. During method validation, stability of the VAMS samples at -20°C was evaluated by analyzing low and high QC samples (n=6) after 4, 7 and 31 days of storage. Afterwards, an extra stability study was conducted, in which samples (n=3) were assessed after 93 and 186 days of storage at -20°C in zip-closure plastic bags containing two 5 g packages of desiccant. Furthermore, also 9 left-over hospital patient samples were taken along during this extra stability experiment. As can be concluded from Table A.4.2, VPA, CBZ and PB were stable for at least 6 months in VAMS devices when stored at -20°C.

Table A.4.2. Stability data for VPA, CBZ and PB in VAMS samples at Low and High QC (n=3) and left-over patient blood samples (n=3). Data are presented as the percentage difference between the concentration measured in samples stored at -20°C and samples stored at -80°C.

Temp	**Stability for 3 months at -20°C (%difference) (n=3)**			**Stability for 6 months at -20°C (%difference) (n=3)**		
	VPA	CBZ	PB	VPA	CBZ	PB
Low QC	15.22	5.13	12.54	-1.42	-5.48	-6.90
High QC	10.32	1.54	4.05	-1.35	-4.88	-10.35
Patient 1	-5.90			4.09		
Patient 2	13.03			0.75		
Patient 3	-6.63			-2.93		
Patient 4	-3.44			-10.99		
Patient 5		-2.47			-5.05	
Patient 6		-5.06			7.93	
Patient 7		1.45			-0.40	
Patient 8		-1.75			4.25	
Patient 9		-10.63			-7.71	

However, the first set of VAMS samples was analyzed 9 months before the repeats and the DBS samples. As, owing to logistical reasons, it was not possible to cover the entire storage period of the samples in the validation stability experiments, the data of the stability study was used to make an extrapolation on the 9 months stability (see Figure A.4.5). Here, linear regression revealed that zero was included within the 95% confidence interval of the slopes. Furthermore, extrapolation of stability data predicted concentration changes within acceptable limits (±15%) compared to nominal values. These findings, in combination with literature data (VPA and CBZ are stable in dried blood samples for at least 1 year and PB for at least 6 weeks at room temperature [32, 33]), suggest that stability issues were unlikely to cause the observed differences in concentrations between VAMS and DBS samples and between the original and incurred analysis of VAMS samples.

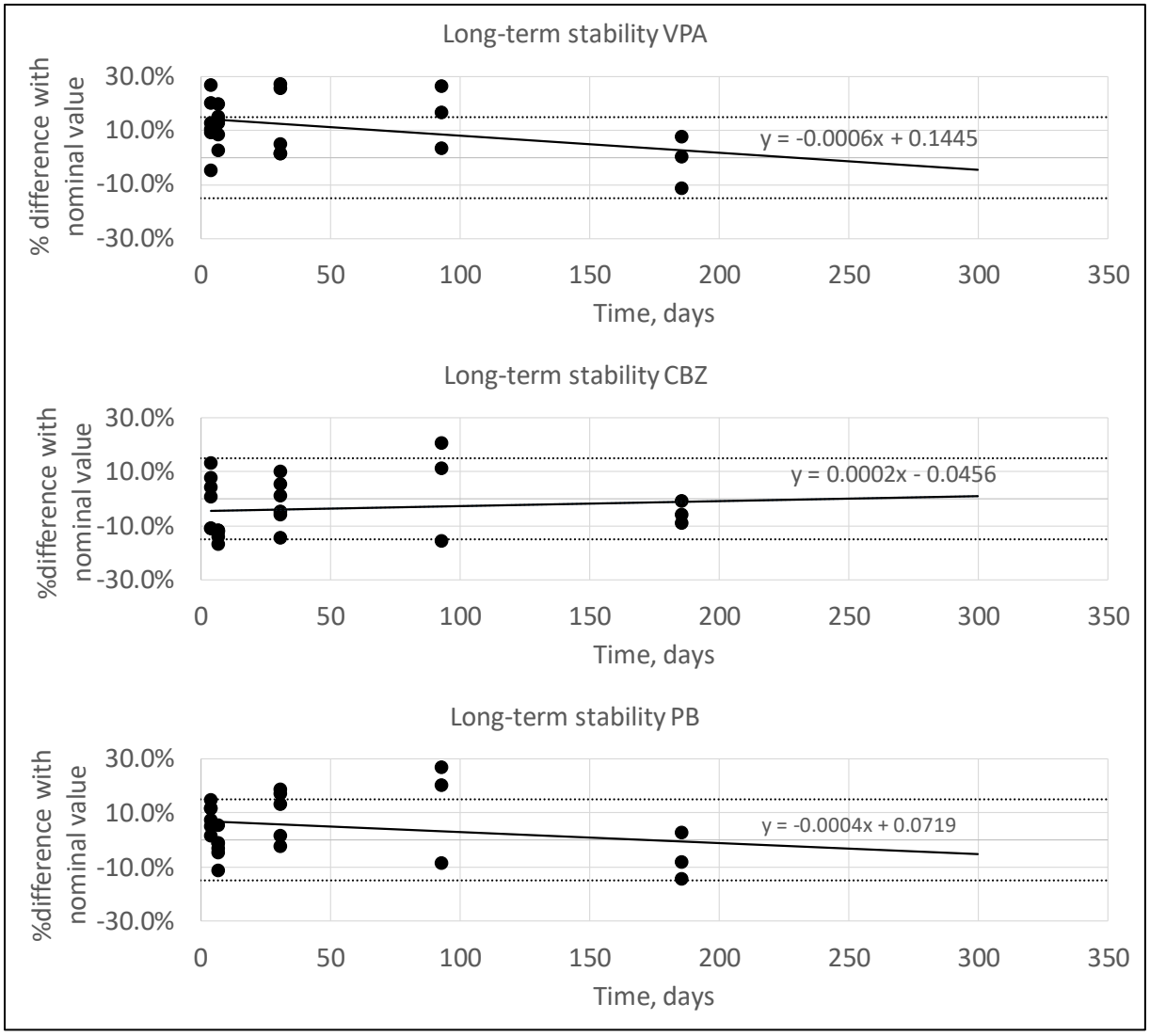

Figure A.4.5. Long-term stability prediction at -20°C for VPA, CBZ and PB in VAMS samples. Dotted lines indicate the ±15% acceptance limits.

Since stability (experiments) did not reveal an answer, another possible explanation for the overall lower concentrations in DBS vs. VAMS samples, is an analytical impact due to a hematocrit (Hct) effect, being the most widely discussed DBS-related problem when using a partial-punch DBS approach [34, 35]. The Hct is defined as the volume percentage of blood taken in by red blood cells and is determined by the amount and the size (volume) of these cells. It is influenced by different factors, e.g. age, sex, health and nutritional status. When preparing DBS, blood with a higher Hct (e.g. 50%) will spread less over cellulose-based DBS cards, compared to blood with a lower Hct (e.g. 30%), due to differences in the viscosity of the blood. When applying partial-spot analysis, this may impact the validity of the obtained results, since the analyzed area (e.g. a 3-mm punch or, as in our case, a 4-mm flow-through area) originating

from a DBS with a higher Hct will contain a larger volume of blood compared to DBS with a lower Hct [34]. For the Ugandan patients (treated with either VPA or CBZ), whole blood was also collected for a full blood count and therefore information on the Hct of the included patients was available. For the Nodding syndrome patients, a median Hct level of 38.1% (range 20.9 - 47.9%) was observed and for the epilepsy patients a median Hct of 38.8% (range 32.7 - 47.1%). These median Hct levels lay close to the Hct of the blood that was used to prepare the DBS and VAMS calibrators (approximately 39%). Moreover, as outlined in Chapter A.2. and A.3., the method validation for both DBS and VAMS samples included an extensive evaluation of the potential impact of the Hct, with no major Hct-related issues. Furthermore, in Figure A.4.6, a graphical representation of the %difference between VAMS and DBS samples in function of the Hct is provided. Since for both VPA and CBZ the slope was not significantly different from 0 (p = 0.75 and 0.36, respectively), it could be concluded that there is no relationship between the Hct and the %difference between VAMS and DBS. Based on this, we conclude that also the Hct does not provide an explanation for the observed underestimation in DBS relative to VAMS and consequently, no conclusive answer could be found for this underestimation. Furthermore, in every analysis batch, 4 QC samples (LLOQ, Mid, Low and High QC) were taken along to assure the validity and reliability of the obtained results. Seeing that the %bias was always within ±15% for all QCs, calibration issues could also be ruled out.

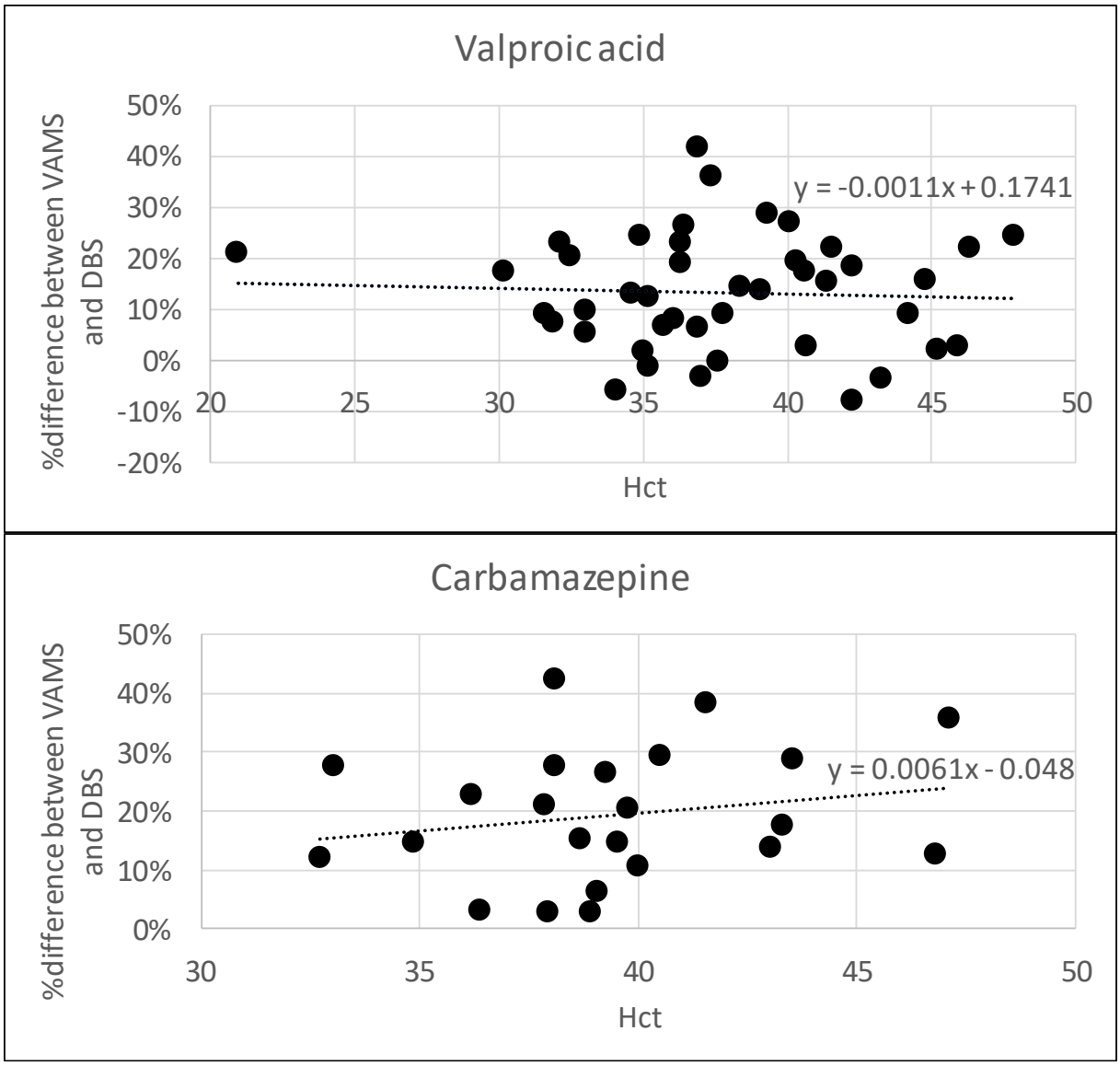

Figure A.4.6. %difference between concentrations obtained via the analysis of VAMS and DBS in function of a patient's hematocrit.

Finally, when comparing the measured blood concentrations with the number of seizures experienced during the last month before sampling, ambiguous results were obtained, e.g. for the Nodding syndrome patients treated with VPA, the average number of seizures experienced the month before sampling was 2.92 for the patients with a VPA concentration within the therapeutic reference range, compared to 2.97 for the patients with a concentration below the therapeutic range. For Ugandan epilepsy patients treated with CBZ, the average number of seizures was even higher for patients with a CBZ concentration within the therapeutic reference range compared to patients with a CBZ concentration below the therapeutic reference range (i.e. 5.42 seizures compared to 4.18). Therefore, it can be concluded that the

fact that in some patients symptoms decrease, whilst in others, epileptic seizures remain poorly controlled is not simply linked to a failure of reaching therapeutic concentrations. Hence, dosage adjustment should preferably be performed by combining the results of TDM with the clinical outcome. In other words, ideally, at the start of an AED treatment, a clinician aims at obtaining an AED blood concentration within a set reference range, followed by a titration upwards or downwards, depending on the clinical symptoms. In this context, the concept of the 'individual therapeutic concentration/range' arose, being the AED concentration or range of concentrations for which an individual patient experiences an optimum response [36].

A.4.5. Conclusion

Using DBS and VAMS samples, we monitored the concentration of 3 AEDs (VPA, CBZ and PB) in children suffering from Nodding syndrome, as well as in patients with epilepsy living in Northern Uganda and in an Onchocerciasis endemic region in the DRC.

The serum concentrations calculated from DBS lay within the respective reference ranges for 52.9% of the Nodding syndrome patients treated with VPA, for only 21.4% of the Ugandan epilepsy patients treated with CBZ and for 65.7% of the epilepsy patients from the DRC treated with PB. However, since divergent results have been reported on the ratio between blood and serum concentrations, calculating serum concentrations based on blood concentrations remains challenging, accentuating the need for reference ranges in blood. Furthermore, for all analytes, an inexplicable underestimation was observed for DBS concentrations in comparison with VAMS concentrations. A comparative study including VAMS, DBS and whole blood samples originating from epileptic patients with varying Hct levels, could help to address this phenomenon. Furthermore, in general, considering the incurred sample reanalysis results, the DBS-based method performed better in terms of variability. It is not clear whether this is related to the sampling or has to do with a possible added-value of a fully automated extraction procedure for processing the DBS.

Finally, when comparing the obtained concentrations with the number of seizures experienced during the last month before sampling, no obvious link between concentrations and (control of) seizures could be observed, since for some patients with a concentration below the therapeutic reference range epilepsy symptoms decreased, whilst for other patients, with a concentration within the therapeutic range the epileptic seizures remained poorly controlled. The latter emphasizes the need for a dosage adjustment based on the combination of TDM results and the clinical outcome.

A.4.6. References

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PART B

THE HEMATOCRIT ISSUE COUPLED TO DRIED BLOOD MICROSAMPLING

CHAPTER B.1.

IS THE HEMATOCRIT STILL AN ISSUE IN QUANTITATIVE DRIED BLOOD SPOT ANALYSIS?

Based on

Velghe S, Delahaye L, Stove CP. Is the Hematocrit Still an Issue in Quantitative Dried Blood Spot Analysis? *Journal of Pharmaceutical and Biomedical Analysis*. 163, 188-196 (2019)

Abstract

Hematocrit-related issues remain a major barrier for (regulatory) acceptance of the classical dried blood spot (DBS) analysis in the bioanalytical and clinical field. Lately, many attempts to cope with these issues have been made. Throughout this Chapter, an overview is provided on new strategies that try to cope with this hematocrit effect (going from avoiding to minimizing), on methods estimating a DBS volume, and on methods estimating or measuring the hematocrit of a DBS. Although many successful strategies have been put forward, a combination of different technologies still provides the most complete solution. Therefore, further efforts and the availability of a straightforward guideline for analytical and clinical method validation should help to overcome the hurdles still associated with DBS sampling.

B.1.1. Introduction

In the field of quantitative DBS analysis, many attempts to cope with the hematocrit (Hct) issue have been made over the past few years. The lack of a simple, universally applicable approach to overcome the Hct issue has been a main hurdle for the widespread implementation of this sampling technique in the clinical field. The potential applications are numerous and distributed over many different areas, amongst which newborn screening, therapeutic drug monitoring, toxicology, drug development [1-11]. As outlined below, the Hct issue is multifaceted, often also involving the need to convert(dried) blood-based results to reference values that are plasma-based [12, 13].

Tackling the Hct issue is quite challenging, since the impact of Hct is a compound-dependent matter which is affected by many factors. The latter was demonstrated by Abu-Rabie *et al*., who concluded that the overall Hct-based bias can be subdivided into a Hct-based area bias, a Hct-based recovery bias and a Hct-based matrix effect bias [14]. The Hct-based area bias is the best documented bias and is the physical consequence of a difference in spreading of whole blood with varying Hct over cellulose-based DBS cards. More specifically, blood with a high Hct (e.g. 50%) will spread less than blood with a lower Hct (e.g. 30%), this due to the differences in viscosity of the blood. This difference in spreading leads to a substantial difference in DBS area, which in turn leads to a difference in sample proportion when a fixed diameter sub-punch is taken from the DBS. The slower spreading of blood with high Hct was demonstrated by Chao *et al*., who investigated the effect of blood Hct on the kinetics of blood spreading on Whatman 903 filter paper, a commonly used filter paper for the generation of DBS [15].

As will be outlined further in this Chapter, a whole punch analysis after volumetric application of a fixed volume of blood can nullify this Hct-based area bias. The recovery bias on the other hand, is a result of the fact that for conventional DBS extraction, the internal standard (IS) is typically only added during the extraction step. Therefore it is unable to correct for variations in recovery from the dried blood. Furthermore, a DBS sample with a different Hct can be considered as being a different matrix, which can give rise to a Hct-dependent matrix effect bias in procedures using liquid chromatography-(tandem) mass spectrometry (LC-(MS/)MS). Since this matrix effect bias can have an impact on the accuracy of an analytical result, it can lead to an under- or over-estimation, depending on the samples' Hct. In addition, both the Hct-based recovery bias and the Hct-based matrix effect bias can also affect the precision of

an analytical result. Therefore, the inclusion of blood samples covering a broad Hct range is very valuable for the evaluation of recovery and matrix effects during method validation.

Many attempts have been made to develop new devices and strategies to collect samples for which the quantitative result is not (or less) influenced by the Hct (cfr. infra). However, the low cost and accessibility of a 'classical' DBS might cause some hesitation towards the use of these newer alternatives. Therefore, researchers have also explored ways to correct for the Hct-induced bias in quantitative DBS analysis. For this purpose, two strategies can be followed. The first one is to determine the volume of blood in a DBS (or sub-punch). Based on this volume, the result from a quantitative analysis can be adjusted. Hence, in this approach the Hct is not determined, but the Hct-based volume bias is dealt with. A second approach consists of determining or estimating the Hct of a DBS (or sub-punch). The determination of Hct can then be used to (1) apply a Hct dependent correction factor to correct the quantitative analysis; (2) determine if the Hct of the sample is within the validated Hct range of the method or (3) convert DBS concentrations into plasma concentrations.

An extensive description of the Hct, the Hct effect, and the possible impacts of deviating Hct values on quantitative DBS analysis can be found in a review by De Kesel *et al*. [13]. Therefore, these topics will not be extensively discussed throughout this Chapter, which aims at providing an overview of the different strategies that have recently been suggested in order to cope with the Hct issue present in quantitative DBS analysis. Here, a distinction will be made between attempts to avoid the Hct issue, strategies to minimize the Hct issue and approaches to measure or estimate the volume and/or Hct of a DBS.

B.1.2. New strategies to cope with the Hct issue

B.1.2.1. Avoiding the Hct issue

Recently, different alternative sampling strategies have been designed that allow to maintain the benefits of DBS but eliminate the Hct-based area bias coupled to DBS. A common theme here is that the dried blood sample is generated volumetrically, followed by analysis of the entire dried blood sample. Besides, Abu-Rabie *et al*. have demonstrated that the overall Hct-based bias can be avoided when this whole spot analysis is combined with a strategy that nullifies the Hct-based recovery bias [14]. As mentioned above, the recovery bias is inherent to

the addition of the IS in the extraction solvent. Therefore, different IS application techniques were investigated for their ability to allow quantitative coextraction of analyte and IS, to eliminate the recovery bias [14]. Three different IS addition strategies were actually able to eliminate the recovery bias: (1) spiking of the IS into blood before spotting of the blood, (2) spray addition of the IS onto DBS samples before sample extraction, using the IS spray module integrated within a commercially available DBS direct elution instrument (CAMAG DBS-MS500), and (3) spray addition of the IS onto the DBS card prior to blood application [14]. However, only the second nullifying-option is likely to be practical for most applications. Furthermore, Hempen *et al*. showed that the recovery can also be improved by using heated flow-through desorption [16]. Combining the latter with whole spot analysis also enables a Hct-independent automation (by using the commercially available flow-through desorption-solid phase extraction system provided by Spark Holland) of the entire DBS workflow [16].

As already mentioned by De Kesel *et al*., volumetric application by using anticoagulant-coated microcapillaries or an accurate pipetting technique will in practice only be possible in situations where dedicated staff (e.g. an experienced nurse or trained laboratory personnel) is available (e.g. patients in a hospital setting, postmortem sampling or preclinical studies) [13]. A recent, more user friendly application of precision capillaries, is the HemaPEN® technology (Trajan Scientific and Medical, Australia). This is a device that consists of 4 end-to-end capillaries, dispensing a fixed volume of 2.74 µL of capillary blood onto 4 pre-punched DBS paper spots (Figure B.1.1). Since patients only need to make contact between a blood drop (obtained by a fingerprick) and the tip of the device, the problem of accurate deposition on filter paper is overcome. Hence, this collection seems far better suited for patient self-sampling compared to conventional precision capillary-assisted preparation of DBS. However, still, as also noted by Neto *et al*., attention should be paid to a difference in collection and transfer velocity of the glass capillaries, depending on the Hct of the collected blood (collection took 1.2 s, 3 s or 6-8 s, for 25, 43 and 61% Hct samples, respectively). While a promising proof-of-concept has been published, making use of an experimental setup comparable to the working of the HemaPEN® [17], further research, including real life applications, are necessary to demonstrate the (Hct-independent) applicability and robustness of the proposed technology.

Figure B.1.1. HemaPEN device (with permission from Trajan Scientific and Medical, Australia.)

In 2014, Neoteryx introduced the Mitra® microsampling device based on volumetric absorptive microsampling (VAMS™) technology. These devices consist of an absorbent polymeric tip connected to a plastic handler (Figure B.1.2), which allows a straightforward collection of an accurate and precise amount (10, 20 or 30 µL) of a liquid sample across a broad Hct range [18, 19]. Careful handling of these devices is necessary in order to exclude a variation in the amount of blood retained on the absorbent tips. E.g., Denniff *et al*. demonstrated that misuse of the tips can lead to a higher or lower amount of blood retained, by touching the tips before sampling with greasy fingers or with poorly dried hands, respectively. Furthermore, incorrect handling of the tips after sampling resulted in a sample loss of up to 19%, depending upon the absorptivity of the materials to which the wet tips were touched [18]. In the context of the Hct effect, Spooner *et al*. demonstrated that the VAMS technique is able to minimize or eliminate the volumetric Hct effect coupled to DBS sampling. They did so by investigating the volume of blood with varying Hct values (20, 45 and 65%) and from multiple species (rat and human) that was absorbed by the tips. This Hct-independent uptake of blood was later confirmed using authentic patient samples [20]. However, even though the blood uptake is Hct-independent, a divergent Hct value can still have an impact on other parameters, e.g. analyte recovery or matrix effect. We -as well as others- found that VAMS samples, as compared to DBS, may even be somewhat more susceptible to an impact of the Hct on the analyte recovery [20-22]. In this context, Mano *et al*. found that the inclusion of a sonication step in the sample

extraction procedure can help to (partially) overcome the Hct-based recovery bias [23]. In a procedure for quantitative analysis of conventional anti-epileptics, extraction of the tips at elevated temperature (60 °C) provided the best results in terms of robust absolute recovery [21]. Others showed that combining a sonication step with an elevated extraction temperature and extended vortex mixing, may also be a good option to obtain a Hct-independent recovery [24]. Furthermore, various extraction solvents should be comprehensively evaluated in order to achieve a good and consistent recovery across a broad Hct range [25]. Importantly, as shown by Xie *et al*., improving the recovery (>80%) is not only necessary to avoid a significant Hct effect, but also to minimize apparent stability issues that are actually related to an unoptimized extractability, having a negative impact on aged VAMS samples [26]. Furthermore, we demonstrated that when using the same procedure for extracting paracetamol from blood-filled VAMS, no recovery issues were encountered, whereas this was the case when using water-filled VAMS, concluding that an extractability-mediated recovery bias can also be matrix-dependent [27].

In conclusion, VAMS helps to overcome the volumetric Hct effect coupled to DBS sampling whilst maintaining all the benefits coupled to regular DBS analysis. However, Hct-dependent recovery can be an issue. To (partially) resolve this issue, thorough optimization of the extraction procedure is necessary. This is particularly the case for compounds with a limited absolute recovery, since the Hct-based recovery bias has been demonstrated to be more prominent in such cases [14]. Furthermore, as the complete tip is used during sample extraction, analysis is simplified when compared to partial-spot DBS analysis. Moreover, as recently demonstrated by Verougstraete *et al*. analysis of 'wet' VAMS samples is a possibility as well, offering opportunities in a hospital as well as a home-based setting [28, 29].

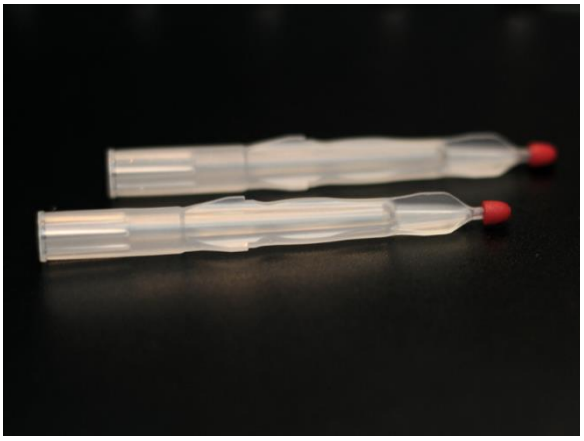

Figure B.1.2. The Mitra® microsampling devices.

Recently, Nakahara *et al*. reported on a volumetric absorptive paper disc (VAPD) and mini-disc (VAPDmini), which combine the principles of classical DBS and VAMS. These devices consist of a filter paper disc, which can hold an accurate volume of blood, fixed with adhesive tape within a filter paper sheet containing slightly larger holes than the disc [30]. It was demonstrated that, by using clozapine and its metabolites as model compounds, analyte recovery was Hct independent, since the percentage difference between a 30%, 40% and 60% Hct sample, compared to a 50% Hct sample, was within 15% [30]. Therefore, it was concluded that these devices could serve as an alternative for the VAMS tips or as a Hct independent alternative for classical DBS analysis. However, these findings should be confirmed using other compounds, since it is known that the recovery of compounds from VAMS sampler tips may be Hct-dependent and this could also be the case for these devices.

Another device, recently designed by KTH Royal Institute of Technology (KTH, Stockholm, Sweden) to overcome the volumetric Hct effect coupled to regular DBS analysis, is the Capitainer-B device (Figure B.1.3). This device consists of an inlet port to which a drop of blood (produced by a fingerprick) is added, which fills a capillary microchannel, with a defined volume of 13.5 µL. When the capillary channel is filled, a thin film at the inlet dissolves, ensuring that the excessive amount of blood is absorbed by a paper matrix, resulting in a separation of the excess blood and the filled channel. Finally, by complete dissolution of a thin film at the outlet, the capillary channel is emptied through capillary forces, leading to the absorption of 13.5 µL of blood by a pre-perforated paper disc [31]. Once dried, the pre-perforated paper discs can easily be removed using tweezers, for further sample processing. A proof-of-concept was provided by Spooner *et al*., who demonstrated that these devices are able to precisely dispense an average blood volume of 13.5 µL across a broad Hct range (25 to 65%) [32]. Furthermore, by evaluating the recovery of radiolabeled material they could also conclude that recovery from the derived DBS was Hct-independent. In addition, as described in Chapter B.2., we also demonstrated the Hct-independence of the devices, when measuring caffeine and its metabolite paraxanthine, using authentic patient samples with a broad Hct range (18-55%) [33]. The latter was also confirmed by Lenk *et al.* when making use of authentic capillary patient samples [34]. For the direct alcohol marker phosphatidylethanol 16:0/18:1 a good agreement was also found when comparing the results of the Capitainer-B devices with those of liquid whole blood [35]. Our data also suggest that there is no impact of the amount of

blood, pipetted to the inlet of the device on the accuracy and precision of the dispensed amount of blood at the device outlet [33]. Further research is required to demonstrate the user-friendliness and robustness of the device in real-life applications, including the direct application of unknown amounts of blood, obtained via fingerprick.

Figure B.1.3. The Capitainer-B device.
Picture 1 (left) showing the inside of the device containing user instructions; Picture 2 (middle) demonstrating the application of a drop of blood at the inlet port; Picture 3 (right) displaying 4 filled pre-perforated paper discs.

The HemaXis DB device (DBS System SA, Gland, Switzerland) is another example of a device for generating volumetric DBS (Figure B.1.4). This is a plastic foldable microfluidic-based device, comprising of a standard DBS paper card and a microfluidic chip containing 4 capillaries. The sampling procedure is straightforward and should easily lend itself to patient self-sampling [36]. Following contact between the capillary inlet and a drop of blood, the capillary channel fills itself with a fixed amount of blood (5.5 or 10 µL) [36, 37]. After repeating this step 4 times, the book-like plastic cover can be closed and needs to be pressed for 10 seconds to enable quantitative transfer of the fixed amount of blood from the outlet of the capillary channels to the opposing DBS card [36]. When followed by whole spot analysis, these devices are also able to nullify the volumetric Hct effect coupled to conventional DBS analysis. In contrast to the above-mentioned HemaPEN®, Capitainer-B and Mitra® devices, this sampling technique still maintains the format of a classic DBS card, which allows compatibility with the automated systems available for DBS analysis, as demonstrated by Verplaetse *et al*. [36]. Furthermore, DBS samples prepared by a volumetric pipet and samples prepared by using the HemaXis DB devices were shown to be comparable in providing acceptable data, independent of the Hct of a sample [36, 37].

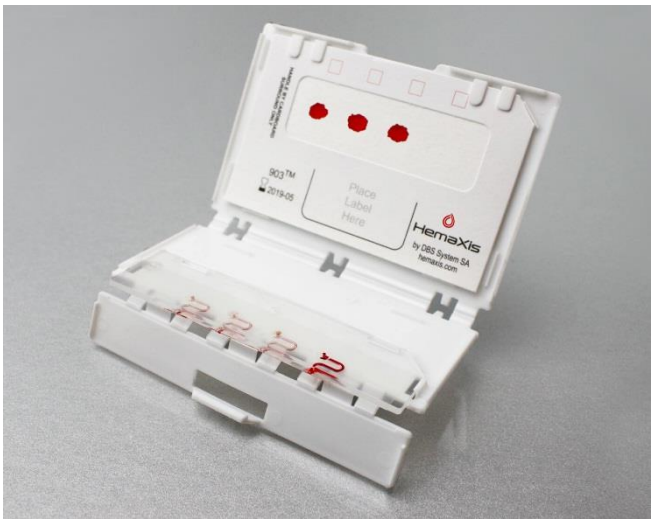

Figure B.1.4. HemaXis DB device (with permission from DBS System SA.)

Besides the volumetric application of DBS, the Hct bias can also be eliminated by using plasma instead of whole blood for the generation of dried matrix spots, resulting in dried plasma spots (DPS). The use of DPS requires a longer sample preparation time, since -normally- a centrifugation step is necessary. Hence, this also implies the impossibility of patient self-sampling. On the other hand, certainly for therapeutic drug monitoring, using plasma instead of blood definitely has benefits (e.g. reference ranges of several drugs are set in plasma) and a good correlation has been described between DPS results and results obtained from liquid plasma analysis [38-44]. In 2012, Astra Zeneca introduced capillary plasma microsampling (CMS), in which 8 µL of plasma was obtained by centrifugation of a Hct tube, containing 32 µL of whole blood, followed by a manual cutting step in which the plasma compartment in the tube was separated from the red blood cell (RBC) compartment [45]. Later, GlaxoSmithKline commercialized, in cooperation with Drummond Scientific, an application on the CMS introduced by Astra Zeneca. Here, a 75 µL glass capillary tube was developed containing a thixotropic gel, which is able to create a physical barrier between the plasma and RBC fraction after centrifugation, excluding the need for cutting the capillary [46]. However, since the workflows of both CMS methods include many manual steps and require an extensive training of lab technicians, the techniques were not widely accepted outside the preclinical area. Therefore, the interest arose in the development of a centrifugation-independent device for the collection of DPS. In 2015, Sturm *et al*. reported the autoDPS Card, which was designed as an improved version of the Yorktest plasma separator, used by Li *et al*. [47, 48]. The prototype autoDPS Card consists of a RBC filtration membrane connected to a plasma collection material,

consisting of four 2 or 4 mm diameter collection wells, separated from one another via hydrophobic wax barriers. Over 50 materials were tested during card development and asymmetric polymer membranes turned out to be the best option for RBC filtration, whilst cellulose-based materials were selected for plasma collection [47]. Evaluation of the analytical performance revealed that the card is able to produce accurate and precise results at a Hct level of 45% [48]. However, to date, the autoDPS Card is not able to produce acceptable bioanalytical data independently from a patients' Hct. A positive bias was observed when comparing the extraction of a whole plasma spot originating from 30% Hct blood with a spot from 45% Hct blood, whilst a negative bias was observed when using 60% Hct blood. The latter can be caused by a varying volume of plasma generated by blood with a different Hct [48]. Therefore Sturm *et al*. concluded that a redesign of the card is necessary in order to deliver a Hct independent device [47]. A Hct compatible alternative is the NoviPlex plasma extraction card (Novilytic LLC, IN, USA) [49]. In contrast to the above-mentioned autoDPS card, DPS are not collected on a card but on pre-punched discs, which can be considered a disadvantage when aiming at integration with systems for automated DBS analysis. To date, two different formats of the card exist: the original NoviPlex cards containing 1 plasma spot and the NoviPlex Duo cards which generate 2 plasma spots from a single blood deposition. The NoviPlex cards are able to generate a fixed amount of plasma (i.e. 2.5 or 3.8 µL, for the original and Duo card, respectively), starting from a variable amount of (capillary) blood [50]. The top layer of the NoviPlex card contains a 'test area' to which an unmeasured amount of (capillary) blood needs to be applied. When the volume of this drop is sufficient, a control spot should appear. In a next step, the blood rapidly dissipates across a spreading layer, before 2.5 or 3.8 µL of plasma is generated by a separation membrane. The generated plasma is collected on a reservoir, which can be removed after a drying time of 15 minutes. Kim *et al*. gravimetrically tested the impact of Hct (20, 41 and 71%) on the sample volume. The 20 and 41% Hct samples had a within card volume variation of less than 1%, whilst the 71% sample had a variation of 3.4%. Furthermore, a %RSD of less than 1% was reported for the 3 different Hct levels, suggesting the Hct compatibility of the NoviPlex card [49]. However, Sturm *et al*. detected an unacceptable Hct bias when applying 20 µL of whole blood onto the NoviPlex cards when using 30 or 60% Hct blood, compared to samples with a 45% Hct. On the other hand, application of 50 µL of whole blood resulted in an acceptable bias for 30, 45 and 60% Hct samples [47]. In conclusion, the NoviPlex card may serve as a user-friendly plasma collection technology, with as limitation

that an application of 50 µL of whole blood is a necessity. Another device is the HemaXis DX (DBS system SA, Gland, Switzerland), which should also be able to generate pure plasma or serum, starting from whole blood, without a centrifuge [51]. To date, the device is not yet available for research evaluation and therefore no data is available about the analytical performance of the device. Finally, there is the HemaSpot™-SE device (Spot on Sciences, Austin Texas), which consists of a spiral-like filter paper in a plastic holder [52]. The design should allow the separation of whole blood in serum and blood cells, resulting in a high concentration of red blood cells, platelets and leucocytes in the center of the filter paper, whilst serum and serum components are found at the end of the spiral [52]. However, to date, no data have been published concerning this device. It will be important e.g. to determine whether chromatography effects take place in the paper. A common benefit of the plasma generating systems is that they eliminate the need for a centrifugation step, making the technique accessible for anyone, anywhere, without the need of a phlebotomist. It needs to be demonstrated on a case-by-case basis that the DPS concentrations are effectively equivalent to those in plasma – also when starting from blood with atypical Hct values.

B.1.2.2. Minimizing the Hct issue

Besides different strategies to avoid the Hct issue, different approaches that try to minimize the Hct issue have been put forward by various stakeholders. One of the proposed approaches is the development of special filter material or special filter paper formats. In this context, HemaSpot™-HF (Spot on Sciences, Austin, Texas) was developed and claims to have a reduced Hct effect. This collection device consists of a cartridge, containing an 8-spoked filter paper disk and desiccant covered by an application surface with a small hole allowing blood to enter [53]. However, to date, there are only few reports available and no data concerning a possible reduction in Hct effect is available [54, 55].

Furthermore, Mengerink *et al*. presented the use of a hydrophilically coated woven polyester fiber as a Hct-independent substrate for DBS analysis, named Qyntest cards (Qynion, Geelen, The Netherlands). It was demonstrated that the cards generate spots with a Hct-independent size within a Hct range of 20 to 70%, allowing to use partial spot analysis without the Hct volume bias [56]. A clear drawback here is that this is a completely distinct material, which also

may be more expensive to produce, as compared to conventional cellulose-based cards. No follow-up reports have been published during the last few years.

Chitosan and alginate foams, two non-paper substrates that can be dissolved, may also offer the potential to overcome Hct-related recovery issues due to generally higher recovery values compared to classical commercial DBS cards [57].

As readily discussed by De Kesel *et al*., one of the easiest strategies may be to use calibrators prepared from blood with a Hct close to the expected median value of the target population [13]. Although one can never exclude that there may still be Hct extremes, this approach will make sure that the Hct impact will be minimized for the majority of the investigated population.

B.1.2.3. Measuring or estimating the volume of a DBS

As the Hct-based area bias (or volume bias) can be nullified by simply determining the volume in a DBS or DBS punch, strategies will be discussed below, where the volume of a DBS is determined or estimated, without determination of the Hct as such. The final aim of these strategies is to correct the analytical result based on the volume of the DBS (punch). The reasoning behind is that it shouldn't matter whether an analyte is measured in e.g. 10 µl dried or liquid blood – the result should be the same. A general remark concerning methods that aim at allowing a correction for the Hct-based area (or volume) bias, is that they do not take into account the other possible effects of Hct on a quantitative measurement (i.e. the Hct-based recovery bias and matrix effect bias). Therefore, during method development, special attention should be paid to the effect of the Hct on these parameters.

A first approach to estimate the volume of blood in a DBS was developed by Liao *et al*. Here, the extent of suppression of ionization in LC-MS/MS, caused by early eluting non-volatile salts in blood, is used to estimate the blood volume on a DBS card. The degree of suppression is measured by means of a post-column infused internal standard (PCI-IS). The reciprocal of the minimum response of the PCI-IS is calculated and correlated to the blood volume in a DBS via a calibration curve. To demonstrate the applicability of this method, the researchers compared the quantitative determination of voriconazole from DBS and plasma samples from 26 patients. A good correlation was found between the concentrations in DBS and plasma

samples, and furthermore, Hct variation did not have a significant effect on the estimation of the DBS volume. One of the main advantages, according to the authors, is the additional function of the PCI-IS for the correction of matrix effect-caused quantification errors [58]. However, other variations that may occur during sample analysis are not accounted for by this approach, and therefore an additional IS, which is included early in the sample preparation procedure, remains necessary. Moreover, as this approach measures the extent of ion suppression, it is only applicable when the analyte of interest is quantified via liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS(/MS)), as the phenomenon of ion suppression is specific for this technique. In addition, a more elaborate LC development is necessary and the selection of the IS and optimization of the PCI parameters are key for the success of this method. While in this report only whole spot analysis was performed it remains to be determined whether this approach can also be used with DBS sub-punches.

Kadjo *et al*. introduced a different technique, based on the electrical conductivity of a DBS extract, measured by a ring disk electrode (RDE). This technique to determine the blood volume of a DBS, is based on the strict regulation of electrolyte concentrations in human blood. As electrolyte concentrations are more or less constant between and within individuals, the electrical conductivity of a DBS extract could indicate the blood volume present in the DBS punch. The conductivity of the DBS extract is dependent on the composition of the extract, but also on the solution depth and the extraction solvent. Therefore, the authors also investigated the minimum required liquid depth necessary to obtain reproducible results with a RDE type electrode [59]. A custom made RDE was fabricated, since commercially available electrodes were too large for measurements in a typical 1.5 mL microcentrifuge tube. The extraction solvents tested in the experiments were 100 µL water, 100 µL 50:50 methanol-water, and 100 µL methanol. All extraction solvents performed well, provided that calibration of the electrode is done with standard solutions generated in the same solvent as the extraction solvent. The authors concluded that 100 µL, when put in a 1.5 mL microcentrifuge tube, provides sufficient immersion depth for reproducible measurements with this specific type of RDE. The developed method was also applied on a limited set of patient samples, to demonstrate the repeatability and reproducibility. However, no real comparison was made with a reference method for the estimation of blood volume in a DBS sub-punch from the

patient samples. The benefits of this method are its non-destructiveness (the extraction solvent can be used further for sample preparation) and the automatability of the conductivity measurement. However, this method cannot be used for patients with deviating electrolyte levels, which can occur in certain diseases.

B.1.2.4. Measuring or estimating the Hct of a DBS

A first spectrophotometric technique used to tackle the Hct issue was described by Miller IV *et al*. This technique uses the UV-VIS reflectance of a DBS to estimate the Hct of the blood. First, the researchers investigated if there was a correlation between the Hct of the DBS and the reflectance at 540 and/or 570 nm, two hemoglobin(Hb)-specific wavelengths. A correlation at these wavelengths was not found, but a relationship was seen between the background scattering at 980 nm and the Hct of a DBS. The researchers determined the correlation between the reflectance and the Hct, and between the Hct and the sample volume. Thereby, they were able to estimate the sample volume in a 3 mm DBS punch based on the reflectance at 980 nm. The authors pointed out that this technique should only be used for DBS of similar size, since spotted volume can also have an influence on the reflectance measured from spots with the same Hct. Therefore, they recommend it to be used for spots generated on pre-printed filter paper, so that an evaluation of spot size is possible [60]. Importantly, an evaluation of the effect of the age of a DBS on the measured reflectance was not included in the validation. Since the color of a DBS changes with age, this can have a major influence on the reflectance [13].

Capiau *et al*. also developed a spectroscopic technique, based on diffuse UV-VIS reflectance, for the prediction of Hct from a DBS [61]. In the approach that was developed, broadband light from a halogen source is guided to the surface (5.9 mm-diameter spot) of the DBS and a reflectance spectrum is recorded. Three Hb derivatives, oxyhemoglobin, methemoglobin, and hemichrome, are taken into account to determine the total Hb content. This is important, since over time -as the DBS dries and ages- the relative amounts of these derivatives change. Comparison of different anticoagulants during method development showed that the choice of the anticoagulant for the set-up of a calibration curve is important, since not all yielded the same reflectance spectrum as non-anticoagulated blood. The influence of spotted volume and DBS age was investigated, and no significant effect on the predicted Hct value was found.

Application on patient samples showed very good agreement (95% of the predicted Hct values were within ±20% of the true Hct) between the actual Hct (measured via routine whole blood analysis) and the predicted Hct via this method [61]. The downside of this method is that a specialized software program and complicated algorithm are necessary to estimate the amount of each of these three Hb derivatives. Therefore, Capiau and colleagues continued to improve this method and developed a method that can determine the Hb content via a single-wavelength (589 nm) reflectance measurement. The reflectance at this wavelength (1) remained constant for DBS with equal Hct but different age (2 h up to 5 months) and (2) could be used to accurately predict the Hct. Application on patient samples yielded even slightly better results than the more complicated full spectrum-based method in terms of Hct prediction (more than 98% of the predicted Hct values were within ±20% of the true Hct). The predicted Hct was used to correct caffeine concentrations from DBS analysis with a Hct-dependent correction factor, resulting in a drastic improvement of the accuracy of the caffeine determinations (i.e. 54.5% of the samples had a %difference within 20% before correction, whilst 95.5% after correction) in DBS samples, which had a broad Hct range (20 to 50%) [62]. The main benefits of this technique are that it is nondestructive, does not require any sample preparation and in principle should be easily automatable. Incorporation in a stand-alone instrument or even in an automated DBS analyzer may be possible. However, as for now, such dedicated equipment is not yet commercially available.

Not only UV-VIS spectroscopy can be used for the estimation of Hct, but also near infrared (NIR) spectroscopy can be employed for this purpose. Oostendorp *et al*. developed a NIR-spectrum based method for the determination of Hct from DBS samples. The Hct values obtained with this method were compared with Hct determinations via routine hemocytometry and both methods showed good correlation. The authors investigated the influence of drying time, albumin concentration, age and sex of patients, but none of these parameters were identified as significant covariates. For the development of this method actual patient samples were used to set up calibration curves[63]. As application on only a limited set of DBS samples was reported, statements based on these results should be interpreted with caution. Also here, the nondestructive nature and absence of sample preparation are the major advantages of this method.

A different approach to estimate the Hct of a DBS, also based on Hb quantification, was developed by Richardson *et al*. [64]. These authors used a method based on the formation of a sodium lauryl sulphate-Hb complex (SLS-Hb), which has an optimal absorbance range between 500 nm and 560 nm, and is therefore very well suited to be measured with simple UV-VIS spectrophotometry at 550 nm. For the formation of this complex, a commercially available SLS containing reagent (Sulfolyser) is added to 10 µL of an aqueous DBS extract. A 6 mm DBS-punch, extracted with a total volume of 100 µL water, was used to reach the optimal sensitivity. The influence of spotted volume, punch location, storage time, and storage condition was evaluated. None of these parameters had a significant influence on the predicted Hct. The good long term stability (up to 6 months when stored at 4 °C) is due to the ability of SLS to bind with all forms of Hb. Application of this method on a set of 59 patient samples, with Hct varying from 27 to 51%, demonstrated the ability of the method to predict the Hct of a DBS compared to a routine analysis in a clinical lab [64]. The basic instrumentation and chemicals that are required for this method allow it to be implemented in every laboratory. Although it is a destructive method, the major part of the extract remains available for further analysis. However, the extraction can only be done with water, or combinations of water-organic solvent via which proteins are extracted from the DBS. This can be a disadvantage, since a protein precipitation step can (often) be avoided by working with extraction solvents with high percentages of organic solvents. Furthermore, the effect of using organic solvents in the extraction solvent on the absorbance measurements and Hct prediction was not evaluated. In addition, a 30 min extraction was necessary to ensure complete desorption of the blood from the DBS. Furthermore, it might be challenging to implement this procedure in an automated DBS analyzer, as part of the extraction solvent needs to be accurately separated. On the other hand, this method is easily automatable with a liquid handler system, which is present in many clinical labs.

In addition, LC-MS/MS analysis -the gold standard technique for the quantification of small molecules- has also been used for quantification of Hb in DBS [65]. Although the method was not developed with the aim to cope with the Hct issue, the determination of Hb is a good surrogate for Hct, as demonstrated by the methods described above, and this method could in theory also serve that purpose. However, as Hb is a protein, the sample preparation is very labor intensive, requiring protein digestion with trypsin. Therefore, its practical use will

probably be rather limited, as the determination of the Hct will take much more time and effort, compared to the determination of the small molecule analyte. Furthermore, this technique is destructive, and part of the DBS has to be sacrificed for the Hct determination. Since more and more proteins are being quantified from DBS, this approach is not unlikely to be incorporated in such analysis, as simultaneous determination of Hb and the protein of interest might be possible. However, it remains to be demonstrated that determination of Hb with this method effectively allows to correct for the Hct-induced bias.

As discussed by De Kesel *et al*., potassium (K^+) is also a suitable candidate as a marker of Hct [13]. The first method using K^+ for the prediction of Hct from non-volumetrically applied DBS was developed by Capiau *et al*. [13]. In short, the K^+ content of a 3 mm DBS-punch extract, measured with a routine clinical chemistry analyzer, was shown to correlate with the Hct. A successful prediction of the Hct was demonstrated by application on patient samples. The influence of punch location and spotted volume was investigated, but neither one of these parameters had a relevant effect on the K^+ measurement. The Hct range covered by this method (19 to 63%) represents the majority of the patient population [66]. In a follow up study, De Kesel and colleagues evaluated the use of this method for a Hct-correction in the analysis of caffeine and its major metabolite paraxanthine from DBS [67]. These two analytes were chosen as model compounds since they are susceptible to a Hct-dependent bias [20]. First, a simple K^+-based algorithm was derived by plotting the whole blood/DBS caffeine concentration ratio against the K^+ concentration (as a surrogate for Hct) for a sub-set of samples. These samples were obtained from healthy volunteers, as well as from hospitalized patients. Application of this algorithm on DBS samples from an independent test set (n=50), for caffeine as well as paraxanthine, largely alleviated the clear Hct bias observed in [68]. The major benefit of this method is the use of standard instrumentation, available in any clinical laboratory and not requiring high-end instrumentation such as LC-MS/MS, which may not be present in resource limited settings. While a downside is the need for an additional 3-mm punch for the K^+ measurements, in most cases it is possible to obtain (at least) two 3-mm punches from 1 capillary DBS. However, some analytes require a larger punch or multiple 3-mm punches, which implies that an additional DBS is necessary for the K^+ measurement. This K^+-based strategy has been applied by several groups [69, 70].

Capiau *et al*. also investigated the use of this K^+-based Hct prediction starting from dried blood samples obtained via VAMS devices [Capiau *et al*., unpublished data]. Both an aqueous and organic extraction method were evaluated, and both were able to estimate the Hct within pre-defined acceptance criteria based on international guidelines. However, the organic extraction procedure was only performed with a limited set of patient samples and should still be further evaluated, Moreover, for the organic extraction procedure, evaporation of the organic extract is necessary, as the routine clinical analyzer is not compatible with organic solvents. On the other hand, for the analysis of most compounds, the organic extraction procedure decreases the workload. Hence, taking both analyses into account, the organic extraction procedure might be preferred. Again here, this process should be easily automatable. In addition, Bloem *et al*. also demonstrated the applicability of K^+ as a good marker for the estimation of the Hct when making use of VAMS devices [71].

Den Burger *et al*. used a modified method for the correction of creatinine measurements from DBS [72]. The adaptations of this method compared to that of Capiau *et al*. encompassed, amongst others, the measurement of the analyte and K^+ from one 8-mm DBS punch. A part of the DBS extract was used for the quantification of creatinine via LC-MS/MS and a part was used for the K^+ measurement. Also here, the influence of spotted volume and punch location were investigated, but the effects were not noteworthy. In this report, an improvement was also seen in the correspondence between DBS and whole blood analysis when DBS concentrations were corrected for the Hct [72], although the Hct range was more limited (18 to 44%) in comparison to that of Capiau *et al*. On the other hand, both measurements could be performed starting from one 8-mm DBS punch, which requires less total sample preparation.

In addition, Rufail *et al*. investigated whether a perimeter ring sample from a DBS can serve for the measurement of K^+ and subsequent Hct prediction, as an alternative for the regularly used center sub-punch. To this end, a fixed volume (40 µL) DBS spot was prepared, the area of the center punch and perimeter ring were determined via image analysis, and K^+ was measured from both samples. It is important to note that the very outer edge of the DBS was also included in the perimeter ring sample. Next, a value of K^+ per area (α) was calculated. Both the circular center punches and the perimeter ring samples showed a good correlation between α and Hct, but values for α were higher for perimeter ring samples compared to central sub-punches. This resulted in a higher resolution for α vs Hct, and hence a smaller

standard deviation when α was used for Hct prediction [73]. This result is not surprising, as it is known that some accumulation of RBCs occurs at the very outer edge of a DBS (known as the "volcano" effect). As these perimeter ring samples are typically not used for analyte determinations, their use for Hct prediction could eliminate the necessity of an additional DBS. A hurdle here is the requirement for volumetric DBS application and the requirement to measure the DBS area. This measurement is easily automatable, but adds a supplementary step, and complicates the implementation of DBS in clinical practice.

Finally, Liao *et al*. used a lipidomics profiling strategy to identify markers for Hct estimation [74]. Via LC-MS the most abundant RBC membrane lipids, including phosphatidylcholines and sphingomyelins, were investigated as potential Hct estimation markers. Three sphingomyelins were identified as potential Hct estimation markers. As a next step, the measurement of these markers was applied to estimate the Hct values of two sets of DBS, DBS generated from blood with artificially prepared Hct on the one hand, and DBS collected from patients on the other hand. All three sphingomyelin compounds were able to estimate the Hct from these patient samples, within ±20% limits. However, the Hct range of the patient samples was rather limited (20 to 40%). Furthermore, the performance of the method with DBS punches from non-volumetrically applied DBS was not demonstrated, which is essential for its implementation in clinical practice. As this method uses LC-MS, it may be possible to determine analyte concentration and the suggested Hct markers simultaneously. To achieve this, more elaborate optimization of the extraction conditions and chromatographic separation may be necessary, in particular for more polar compounds, which are very different from the lipophilic sphingomyelin markers.

B.1.3. Conclusion

While the advantages of DBS sampling over classical venous sampling have been generally recognized, the Hct-dependent assay bias (Hct-based area bias, Hct-based recovery bias, and Hct-based matrix effect bias) has been an important barrier for (regulatory) acceptance of DBS.

In summary, the Hct-based area bias can be overcome in two ways: either by whole sample analysis preceded by volumetric application of a fixed volume of whole blood or by correction for the bias introduced by varying Hct levels.

Many suggestions to overcome the Hct-based recovery bias have been proposed. As demonstrated by several researchers, this recovery bias can have an important impact on the total assay bias. In most cases, this issue can be tackled via thorough optimization of the sample extraction method, resulting in a maximum absolute recovery of the analyte(s) from the dried sample.

Compared to the area and recovery bias, the Hct-based matrix effect bias has generally no significant contribution to the total assay bias. However, its evaluation during method validation remains required.

Besides the attempts to overcome the total Hct bias coupled to the use of whole blood, efforts have been made to develop devices enabling the generation of DPS starting from capillary blood. In theory the use of DPS instead of DBS inherently offers the advantage of avoiding all Hct-related issues – at least, if DPS concentrations are equivalent to those in plasma.

Moreover, the Hct-based area bias can be coped with by determining the volume of blood present in a DBS via several analytical techniques, followed by full-spot analysis. Still, attention should be paid here to a potential Hct-based recovery bias.

Rather than trying to minimize or avoid the Hct bias, the Hct of the blood contained in a DBS can also be measured or estimated. Different strategies have been proposed, based on the measurement of endogenous compounds. As some of these techniques can potentially be incorporated in stand-alone instruments or even in commercially available automated DBS analyzers, we believe that these incorporations may become standard in the future, also given the non-destructive nature of some of the proposed technologies. Importantly, incorporation of some technologies might even be used to serve as a “quality control”, either by controlling whether the Hct of a given DBS lies within the validated range, or by providing an assessment

of DBS quality as a whole, which is relevant in all fields of DBS analysis, irrespective of whether it's e.g. newborn screening or therapeutic drug monitoring.

Although many successful efforts have led to the introduction of several new alternative sampling devices and/or methods to correct for the Hct, the implementation of these methods in clinical practice remains limited. A more widespread implementation of the presented alternatives, resulting in a cost reduction, will definitely help extensive spreading of the DBS technique across laboratories. Furthermore, novel capillary blood collection devices (e.g. from seventh sense biosystems, fluisense®, and BloodTrackR device) in combination with volumetric absorptive microsampling strategies can serve as a patient-friendly alternative in the future. The availability of a straightforward guideline for analytical and clinical method validation should also help to streamline DBS analysis and help to overcome the hurdles associated with DBS sampling and analysis [75].

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CHAPTER B.2.

EVALUATION OF THE CAPITAINER-B MICROFLUIDIC DEVICE AS A NEW HEMATOCRIT INDEPENDENT ALTERNATIVE FOR DRIED BLOOD SPOT COLLECTION

Based on

Velghe S, Stove CP. Evaluation of the Capitainer-B Microfluidic Device as a New Hematocrit Independent Alternative for Dried Blood Spot Collection. *Analytical Chemistry* (2018).

Abstract

The hematocrit-bias still remains one of the most discussed issues when it comes to dried blood spot (DBS) analysis. Therefore, many attempts to cope with this issue have been made, amongst which the development of novel sampling tools such as the Capitainer-B (further referred to as MF (microfluidic)- DBS) devices. These are designed to allow a straightforward absorption of a fixed volume (13.5 µL) of blood by a pre-perforated paper disc, which can be analyzed afterwards. The aim of this Chapter was to evaluate the potential of these devices to nullify the hematocrit-based area bias and to investigate whether the amount of blood applied has an influence on the device performance. An LC-MS/MS method for the quantification of caffeine and paraxanthine in MF-DBS was fully validated, meeting all pre-set acceptance criteria. In a next step, using a set of 133 authentic, venous patient samples with a hematocrit range of 18.8 to 55.0, concentrations of both compounds in MF-DBS were compared to those in corresponding partial-punch pipetted DBS (PI-DBS) and liquid blood samples. When compared to blood as a reference, the concentrations obtained in MF-DBS were not affected by a bias in function of the evaluated hematocrit, in contrast to those obtained from partial-punch PI-DBS. Furthermore, analysis of samples resulting from spiking different volumes of whole blood at different hematocrit levels, revealed that the amount of blood applied at the device inlet has no influence on the performance of the devices. Therefore, it can be concluded from this study, being the first in which the impact of the hematocrit and the applied volume is evaluated by analyzing authentic, venous patient samples, that MF-DBS devices effectively assist in eliminating the hematocrit-based area bias, independently from the applied blood volume.

B.2.1. Introduction

Lately, many efforts have been made in an attempt to overcome the well-discussed hematocrit (Hct)-issue coupled to classical dried blood spot (DBS) analysis. Theoretically, the overall Hct-based bias can be seen as a threefold problem, i.e. a distinction can be made between a Hct-based area bias, a Hct-based recovery bias and a Hct-based matrix bias [1]. The Hct-based recovery bias is resulting from the fact that the internal standard is typically only added during the extraction step for conventional DBS extraction, leading to the inability to correct for variations in recovery from the dried blood. Furthermore, a Hct-based matrix bias can be the result of the fact that a DBS sample with a different Hct can be considered as being a different matrix. Therefore, for the evaluation of recovery and matrix effects during method validation, the inclusion of blood samples with a broad Hct range is very valuable. The Hct-based area bias is best documented and is the consequence of the different spreading of whole blood -with a varying Hct- over classical DBS cards, due to differences in the viscosity of the blood: blood with a higher Hct (e.g. 50%) will spread less compared to the same amount of blood with a lower Hct (e.g. 30%). This phenomenon gives rise to DBS with a substantially different area, which in turn leads to a difference in the amount of analyte sampled when using a fixed diameter sub-punch. Logically, a whole spot analysis after volumetric application of a fixed amount of blood should nullify this area bias. However, volumetric application can only be achieved when using a dedicated device.

Volumetric application by using anticoagulant-coated microcapillaries or calibrated pipettes will in clinical practice only be possible in situations where dedicated staff (e.g. an experienced nurse or trained laboratory personnel) is available (e.g. patients in a hospital setting, postmortem sampling or preclinical studies) [2]. When this volumetric application needs to be performed by non-experienced people (e.g. patients at home) this non-direct application of a drop of blood constitutes a non-negligible disadvantage. Therefore, in order to render volumetric dried blood sampling possible at home, different strategies have been proposed to volumetrically generate the dried samples, without the necessity of using a calibrated pipette. The HemaPEN® technology (Trajan Scientific and Medical, Australia), the volumetric absorptive microsampling (VAMS™) technology (Neoteryx, USA), the volumetric absorptive paper disc (VAPD) and mini-disc (VAPDmini) and the HemaXis DB device (DBS System SA, Gland, Switzerland) are examples of techniques proposed for a user friendly generation of

volumetric dried blood samples [3-9]. However, although direct application of a blood drop from a fingertip onto the above mentioned devices may be relatively straightforward, proper instructions still need to be given to self-sampling patients in order to avoid incorrect sampling, leading to erroneous results. Here, we focus on the Capitainer-B (further referred to as MF (microfluidic)-DBS) device, recently designed by the KTH Royal Institute of Technology (KTH, Stockholm, Sweden) and commercialized by Capitainer AB (Stockholm, Sweden). The device is equipped with an inlet port to which a drop of blood (e.g. obtained via a fingerprick) is added, resulting in the filling of a capillary microchannel with a fixed volume of 13.5 µL. Upon completely filling this capillary channel, a thin film at the inlet dissolves, resulting in the absorption of the excessive amount of blood by a paper matrix, leading to the separation of the excess blood and the filled channel. Finally, upon dissolving of a thin film at the outlet, the capillary channel is emptied through capillary forces, resulting in the absorption of 13.5 µL of blood by a pre-perforated paper disc (Ahlstrom 222 filter paper) [10]. The QR code to the right leads to a short video showing the sampling mechanism. Spooner *et al.* readily provided a proof of concept in which it was demonstrated that the devices are able to precisely dispense an average blood volume of 13.5 µL across an artificially generated broad Hct range (25 – 65%) [11]. Furthermore, a good agreement was found when comparing the results of the MF-DBS devices with those of liquid whole blood, making use of the direct alcohol marker phosphatidylethanol 16:0/18:1 [12]. Based on these proof of concepts, the MF-DBS devices show promise to overcome the Hct-based area bias, while maintaining the benefits coupled to classical DBS analysis. However, although for phosphatidylethanol 16:0/18:1 determinations, authentic, venous patient samples were used, no information was provided on the Hct levels of the used patient samples, meaning that no conclusion could be made on the ability of the MF-DBS devices to overcome the Hct bias. Therefore, the impact of the Hct on the analysis of authentic patient samples remained to be established. Furthermore, it remained to be investigated whether the amount of blood dispensed at the device inlet has an impact on the accuracy and precision. Therefore, the aim of this Chapter was to evaluate the potential of MF-DBS devices to eliminate the Hct-based area bias by analyzing 133 left-over patient samples across a wide Hct range (18.8-55.0), with caffeine and paraxanthine as model compounds. To do so, concentrations measured in MF-DBS were compared to corresponding whole blood and partial-punch pipetted DBS (PI-DBS).

Moreover, different volumes of blood (25, 30, 35, 40 and 50 µL) were applied, originating from patient samples with a very low or very high Hct, in order to check whether the amount of blood added at the inlet port has an influence on the performance of the devices. Since the included samples originated from patients admitted to 17 hospital departments (including surgery, radiology, rheumatology, endocrinology and nephrology, amongst others), the samples used in this study include a wide variety of characteristics, which is highly relevant to assess the device's practical relevance and robustness.

B.2.2. Experimental section

B.2.2.1. Chemicals and stock solutions

Formic acid, caffeine, paraxanthine and the internal standards (IS) caffeine-$^{13}C_3$ and paraxanthine-$^{13}C_4$-$^{15}N_3$ were obtained from Sigma-Aldrich (Diegem, Belgium). LC-MS grade methanol was purchased from Biosolve (Valkenswaard, the Netherlands). A Synergy® Water Purification System (Merck Millipore, Overijse, Belgium) provided ultrapure water. For caffeine and paraxanthine, stock solutions of 1 mg/mL were prepared by dissolving 10 mg of the compound in 10 mL of water. For the IS, an appropriate dilution of a commercially available 1 mg/mL solution of caffeine-$^{13}C_3$ in methanol and a dilution of 2 mg of paraxanthine-$^{13}C_4$-$^{15}N_3$ in 20 mL of methanol provided a 100 µg/mL stock solution. Working solutions of the standards and the IS were prepared on the day of analysis by diluting the stored (-20°C) stock solutions with water. Independently prepared stock solutions were used to prepare the calibrators and quality control samples (QCs) as described before [13].

B.2.2.2. Sample collection

Blank venous whole blood from a caffeine abstinent healthy, female volunteer was collected in EDTA tubes (BD Vacutainer® with BD Hemogard® closure 10mL) and used for the preparation of calibrators and QC samples. Whole blood samples were generated by transferring 50 µL of blood into 2 mL Eppendorf tubes. MF-DBS devices were generously provided by Capitainer AB (Stockholm, Sweden). MF-DBS were generated by pipetting 35 µL of whole blood at the inlet port of the device using a calibrated pipette. After completing sampling, the devices were left to dry for approximately 3 hours at room temperature prior to storage at ambient temperature

in the presence of desiccant (two 5g Minipax®absorbent packets, Sigma Aldrich) in zip-closure plastic bags until analysis. PI-DBS were prepared by pipetting 25 µL of whole blood onto Whatman 903 filter paper (GE Healthcare, Dassel, Germany) using a calibrated pipette. The drying conditions were the same as described above for the MF-DBS.

When necessary, a Sysmex XE-5000 hematology analyzer (Sysmex Corp., Kobe, Japan) was used to determine the (adapted) Hct.

B.2.2.3. Sample preparation and UPLC® -MS/MS method

For the MF-DBS, sample preparation was performed by removing the pre-perforated paper discs using tweezers and transferring these into 2 mL Eppendorf tubes, before adding 225 µL of a methanol/water (80/20, v/v) mixture, containing 0.01% formic acid and both labeled IS, at 33 ng/mL and 16.5 ng/mL for caffeine-$^{13}C_3$ and paraxanthine-$^{13}C_4$-$^{15}N_3$, respectively. Subsequently, the samples were shaken for 10 min at 1000 rpm and 60°C on a Thermoshaker TS-100C (BioSan, Riga, Latvia), followed by a centrifugation step at ambient temperature for 10 min at 10 000 x g. Before injection of 10 µL onto the UPLC® column, 90 µL of the supernatant was diluted with 550 µL of water, containing 0.01% formic acid of which 250 µL was transferred to a vial with plastic insert. For quantification of caffeine and paraxanthine in PI-DBS (Whatman 903 filter paper) and whole blood samples, readily available fully validated methods were used, with a central 3-mm punch being used for PI-DBS [13].

A Waters Acquity UPLC® system (Waters, Milford, MA, USA) coupled to a SCIEX API 4000™ mass spectrometer (SCIEX, Framingham, MA, USA) was used to analyze all samples. The LC-MS/MS system was controlled by SCIEX analyst® 1.6.2 and by the Waters Acquity console software. Mobile phases A and B consisted of 0.01% formic acid in water and methanol, respectively. The same chromatography and mass spectrometry parameters as described elsewhere were used for the analysis of PI-DBS, whole blood and MF-DBS [13].

B.2.2.4. Validation of the MF-DBS method

Method validation was based on U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation [14, 15] and included the evaluation of accuracy, precision, carry-over, selectivity, homoscedasticity, calibration model, stability, matrix effect, recovery and Hct effect. At the start of each series, control blanks (i.e. MF-DBS prepared with blank blood and analyzed without IS in the extraction solvent) and zero samples (i.e. MF-DBS prepared with blank blood and extracted using the regular solvent) were analyzed.

Accuracy and precision were determined by analyzing QCs at four concentration levels (lower limit of quantification (LLOQ), low, mid and high) in duplicate on three different days. The used concentration levels for caffeine and paraxanthine, respectively, were 0.05 and 0.025 µg/mL (LLOQ), 0.12 and 0.06 µg/mL (low), 4.0 and 2.0 µg/mL (mid) and 8.0 and 4.0 µg/mL (high). The within day and total assay precision (%relative standard deviation, %RSD) were determined by using a single factor analysis of variance (ANOVA), while the accuracy (%bias) was evaluated by dividing the difference between the obtained concentration and the nominal value by the nominal value, this multiplied by 100 [16, 17]. Both, the %bias and the %RSD should be within ±15%, except for the LLOQ (within ±20%) [15].

Carry-over was assessed by injecting 2 blank samples after the highest calibrators, on four different days (n=8). Carry-over is considered acceptable when the obtained responses for the analytes were less than 20% of the LLOQ peak area and less than 5% for the IS [15]. For selectivity, identical acceptance criteria were applied. Selectivity was evaluated by analyzing blank MF-DBS, prepared with whole blood originating from 8 different volunteers.

Eight-point calibration lines were measured in duplicate on each of four days to evaluate homoscedasticity and the calibration model. The nominal concentrations of the calibrators were 0.050, 0.10, 0.25, 0.50, 1.0, 2.5, 5.0 and 10.0 µg/mL for caffeine and 0.025, 0.050, 0.10, 0.25, 0.50, 1.0, 2.5 and 5.0 µg/mL for paraxanthine. An F-test (α = 1%) at the lowest and highest calibrators was used to test the homoscedasticity. Furthermore, both, unweighted and weighted (1/x, 1/x2, 1/√x, 1/y, 1/y2 and 1/√y) linear and quadratic regression were applied to find the best fitting model. The sum% relative error (RE) and plotting of the %RE against the nominal concentrations was used to compare the obtained models. A back-calculation, in

which the mean concentrations of the calibrators should be within ±15% of the nominal value or within ±20% for the LLOQ, was performed before accepting a selected model [15].

Former experiments did not reveal any problems with the (processed sample) stability of caffeine and paraxanthine [5, 13]. However, since the DBS paper included in the MF-DBS device is not completely identical to the paper used in the previously validated PI-DBS method, a limited stability study was carried out here. Stability was assessed by analyzing low and high QCs in triplicate after 4 days of storage at 60°C and after 3 months of storage at -20°C and at room temperature in zip-closure plastic bags containing two 5 g packages of desiccant. A freshly prepared eight-point calibration line was used at each day of analysis in order to calculate the concentration of the stored MF-DBS. To be acceptable, the mean concentration of the QCs at a particular time point should not deviate more than ±15% from the nominal value [15].

Two concentration levels (low or high QC) together with the IS were spiked to a blank blood extract (originating from 7 different donors) (A) or to a neat MeOH/H_2O + 0.01% formic acid solution (B) to investigate the matrix effects. Furthermore, the Hct range was broadened by adding or removing a specific amount of plasma from whole blood, this by centrifuging an aliquot of blood with a Hct of 38.7 in an Eppendorf 5804R centrifuge (Hamburg, Germany) for 5 min at 1000 x g. In this way 10 different blank blood extracts could be obtained with a Hct ranging from 24.4 to 50.5. The IS-corrected matrix effect is calculated as the ratio of the peak areas of (A) to those of (B), multiplied by 100. Overall, the %RSD of this IS-corrected matrix effect should be less than 15% [15].

The recovery was evaluated for low and high QCs (n=6) at three different Hct levels (target values at 21.0, 42.0 and 62.0), prepared by adding or removing a certain amount of plasma from whole blood with a Hct of 40.3. Two sets of MF-DBS were compared: (C) MF-DBS obtained by pipetting 13.5 µL of spiked blood directly onto the pre-perforated paper discs and (D) MF-DBS generated by pipetting 13.5 µL of blank whole blood and to which the analytes were only spiked after the extraction. The average of the peak area of (C) divided by that of (D) multiplied by 100, revealed the absolute recovery values (%).

Where relevant, statistical analyses were performed using the Minitab® software.

B.2.2.5. Application

To get a complete view on the performance of the MF-DBS devices, a comparative study between MF-DBS, partial-punch PI-DBS and whole blood samples was carried out. Caffeine and paraxanthine concentrations were determined in 133 hospital patient blood samples (collected in EDTA tubes). Approval for this study (the use of left-over venous blood samples from patients and control blank blood samples from volunteers) was provided by the Ethics Committee of Ghent University Hospital (EC2018/0519). Blank blood, from a single donor (Hct 46.2), was used to prepare matrix-matched calibration curves. The impact of the Hct on the MF-DBS and PI-DBS results was evaluated by plotting the percentage difference between MF-DBS or PI-DBS and whole blood in function of the different Hct levels. The differences between MF-DBS or PI-DBS and whole blood concentrations, divided by the whole blood concentrations, multiplied by 100, represented these percentage differences. Furthermore, Medcalc statistical software version 14.12.0 (Medcalc Software bvba., Ostend, Belgium) was used to create Bland-Altman plots to compare MF-DBS and whole blood samples.

Additionally, to check whether the amount of blood added at the inlet port of the device has an influence on the measured caffeine and paraxanthine concentrations, different volumes (25, 30, 35, 40 and 50 µL) of blood, originating from patient samples with a very low or high Hct, were applied.

Furthermore, to better reflect realistic conditions, the use of capillary blood (collected via a fingerprick), obtained from 4 healthy volunteers, was tested. Here, a comparison was made between the caffeine and paraxanthine concentrations obtained via the use of MF-DBS devices with those in liquid capillary blood. A BD Microtainer® contact activated safety lancet (BD, Franklin Lakes, USA) was used to perform the fingerprick. Approval for this study was provided by the Ethics Committee of Ghent University Hospital (EC2018/0519) and a written informed consent was obtained from each volunteer.

B.2.3. Results and discussion

B.2.3.1. Method validation

As can be concluded from Table B.2.1, the overall %bias was below 6.15%, meeting the acceptance criterion for accuracy. Furthermore, with a within day and total assay precision (%RSD) below 10%, the acceptance criterion for precision was also met for both compounds.

No carry-over was found when injecting 2 blank samples after the highest calibrators. For selectivity, no unacceptable interferences were observed in blank MF-DBS, prepared with whole blood from 8 different volunteers (Hct range: 37.1-43.1).

Table B.2.1. Within day and total precision and accuracy (n=3x2) for QCs of caffeine and paraxanthine at four concentration levels (LLOQ, low, mid and high) in MF-DBS.

QC	Within day precision (%RSD) (n=3x2)		Total precision (%RSD) (n=3x2)		Accuracy (%bias) (n=3x2)	
	Caffeine	Paraxanthine	Caffeine	Paraxanthine	Caffeine	Paraxanthine
LLOQ	6.40	9.42	6.40	9.42	-1.03	3.13
Low	3.07	3.69	3.31	4.35	1.67	6.14
Mid	3.35	7.48	3.35	7.80	2.92	1.92
High	7.39	5.16	8.84	5.37	4.54	5.92

Calibration data for both caffeine and paraxanthine were found to be heteroscedastic. For both compounds, weighted regression did improve the %RE, therefore weighted linear regression was selected, using a weighting factor $1/x^2$. When using these models, the mean back-calculated concentrations did not differ more than ±10%. Hence, a linear calibration model could be accepted for both compounds.

As can be concluded from Table B.2.2, both caffeine and paraxanthine were stable for at least 4 days in MF-DBS when stored at 60°C (representing an accelerated stability experiment) and for at least 3 months when stored at room temperature or -20°C.

Table B.2.2. Stability data for caffeine and paraxanthine in MF-DBS at Low and High QC (n=3). Data are presented as the percentage difference between the concentration measured at the specific time points and the nominal values.

QC	4 days at 60°C (%difference) (n=3)		3 months at -20°C (%difference) (n=3)		3 months at RT (%difference) (n=3)	
	Caffeine	Paraxanthine	Caffeine	Paraxanthine	Caffeine	Paraxanthine
Low	3.33	8.00	7.78	12.39	15.28	14.94
High	7.17	13.5	7.54	0.33	5.04	-0.25

As displayed in Table B.2.3, the values for the analyte matrix effect indicate a relevant (>15%) ionization enhancement for paraxanthine. However, since the IS-corrected matrix effect was within 98.9-107.1% for both compounds, it can be concluded that the IS compensates for the differences in ionization. Furthermore, the pre-set acceptance criterion (<15% %RSD) for the IS-corrected matrix effects was met.

Table B.2.3. Analyte matrix effect and IS-corrected matrix effect for caffeine and paraxanthine.

	Caffeine		Paraxanthine	
	Low QC	High QC	Low QC	High QC
	Analyte matrix effect			
Mean of 10 donors (%)	98.9	84.9	128.7	115.8
%RSD	4.83	2.27	5.48	2.69
	IS-corrected matrix effect			
Mean of 10 donors (%)	107.1	104.6	105.8	98.9
%RSD	6.80	2.44	6.62	2.10

To evaluate the recovery, low and high QCs (n=6) were prepared in blank blood with three different Hct levels (21.5, 40.7 and 58.3), which was used to generate MF-DBS. High recovery values were obtained for both compounds, at 105.97 ± 8.96% for caffeine and 97.36 ± 10.1% for paraxanthine, these values corresponding to the averages calculated from all values obtained at all Hct levels and at both QC levels. Furthermore, when the 40.7 Hct level is normalized to 100%, all recoveries were within ±15% of this 40.7 Hct reference sample, demonstrating that the Hct has no statistical significant effect ($p > 0.1$, one-way ANOVA test) on the recovery (Figure B.2.1). This confirms the Hct-independence of the MF-DBS devices, as also observed by Spooner *et al*., who evaluated the recovery of radiolabeled material [11].

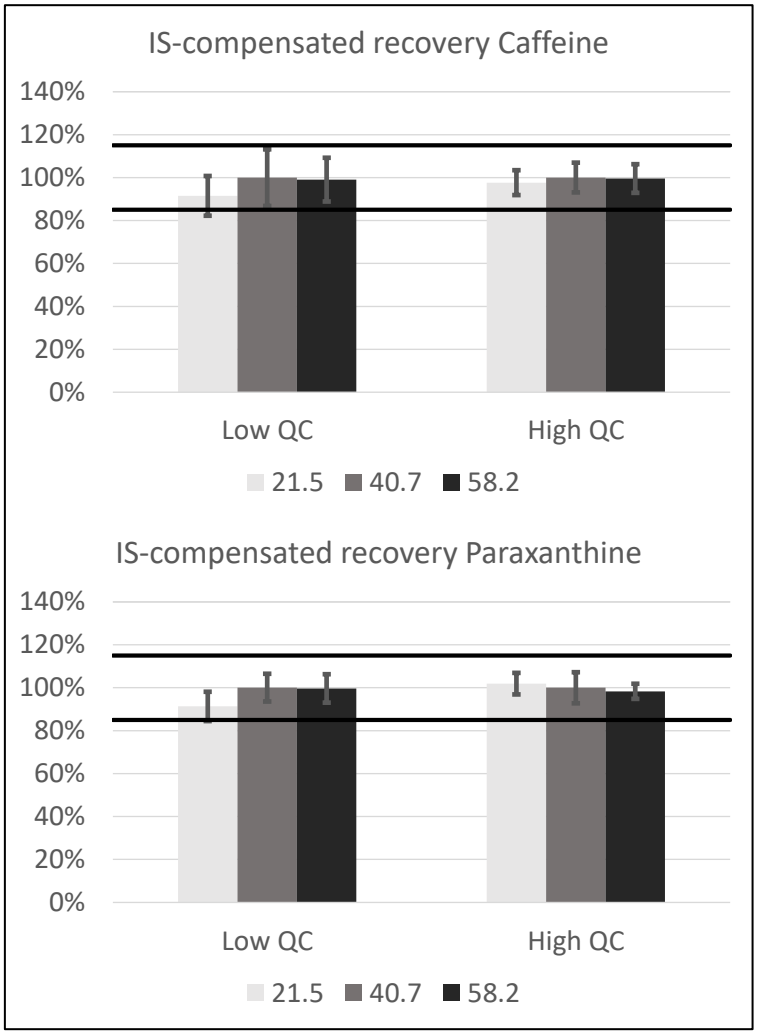

Figure B.2.1. IS-compensated recovery (%) at low and high QC level (n=6) for caffeine and paraxanthine measured in MF-DBS at 3 different Hct levels (21.5, 40.7 and 58.2), with the 40.7 Hct sample being normalized to 100%. The full lines indicate the ±15% deviation limits.

B.2.3.2. Application

The validated method was applied on 133 authentic, venous left-over patient samples with a Hct ranging from 18.8 to 55.0, with 35.95 being the median. In 105 respectively 110 of the 133 patient samples (corresponding MF-DBS, PI-DBS and whole blood samples), caffeine respectively paraxanthine concentrations were above the respective LLOQs (0.05 µg/mL and 0.025 µg/mL). All samples were analyzed against a calibration curve prepared using blood with a Hct of 46.2. This relatively high Hct was deliberately chosen, since in this way the Hct effect on PI-DBS concentrations can be clearly visualized. Furthermore, it enables to create a worst

case scenario for MF-DBS and PI-DBS, allowing an exhaustive assessment of (a possible) Hct effect. Figure B.2.2 depicts the percentage difference between MF-DBS or PI-DBS concentrations and whole blood concentrations in function of the Hct. Regression lines fitted to the differences between PI-DBS and whole blood concentrations had a slope of 0.851 (95% CI; [0.628 to 1.08]) and 0.716 (95% CI; [0.480 to 0.952]) and an intercept of -43.2 (95% CI; [-51.3 to -35.0]) and -44.3 (95% CI [-52.9 to -35.7]) for caffeine and paraxanthine, respectively, revealing for both compounds a similar Hct-induced bias, impacting PI-DBS concentrations. PI-DBS concentrations clearly decreased with a decreasing Hct, which is in line with previous findings for both compounds [5, 18]. In contrast, the concentrations obtained from the MF-DBS were not affected by a suchlike Hct-effect (Figure B.2.2, lower panels). The regression lines fitted to the differences between the MF-DBS and whole blood concentrations had a slope of -0.103 (95% CI; [-0.288 to 0.0819]) and -0.0707 (95% CI; [-0.256 to 0.114]) and an intercept of -1.07 (95% CI; [-7.77 to 5.63]) and -7.92 (95% CI; [-14.7 to 1.18]) for caffeine and paraxanthine, respectively. Incurred sample reanalysis resulted in regression lines with a slope of 0.00398 (95% CI; [-0.164 to 0.172]) and -0.00122 (95% CI; [-0.232 to 0.230]) and an intercept of -3.79 (95% CI; [-9.85 to 2.27]) and -2.59 (95% CI; [-11.0 to 5.81]) for caffeine and paraxanthine, respectively. Since the 95% CIs of the slopes for both compounds, for both analyses, included 0, it can be concluded that the differences between the MF-DBS and whole blood concentrations did not change in function of the Hct, this within the Hct range of 18.8 to 55.0. Therefore, the Hct-independence of the devices, as readily suggested by Spooner *et al*., who evaluated the recovery of radiolabeled material, is supported by these findings on authentic, venous patient samples [11].

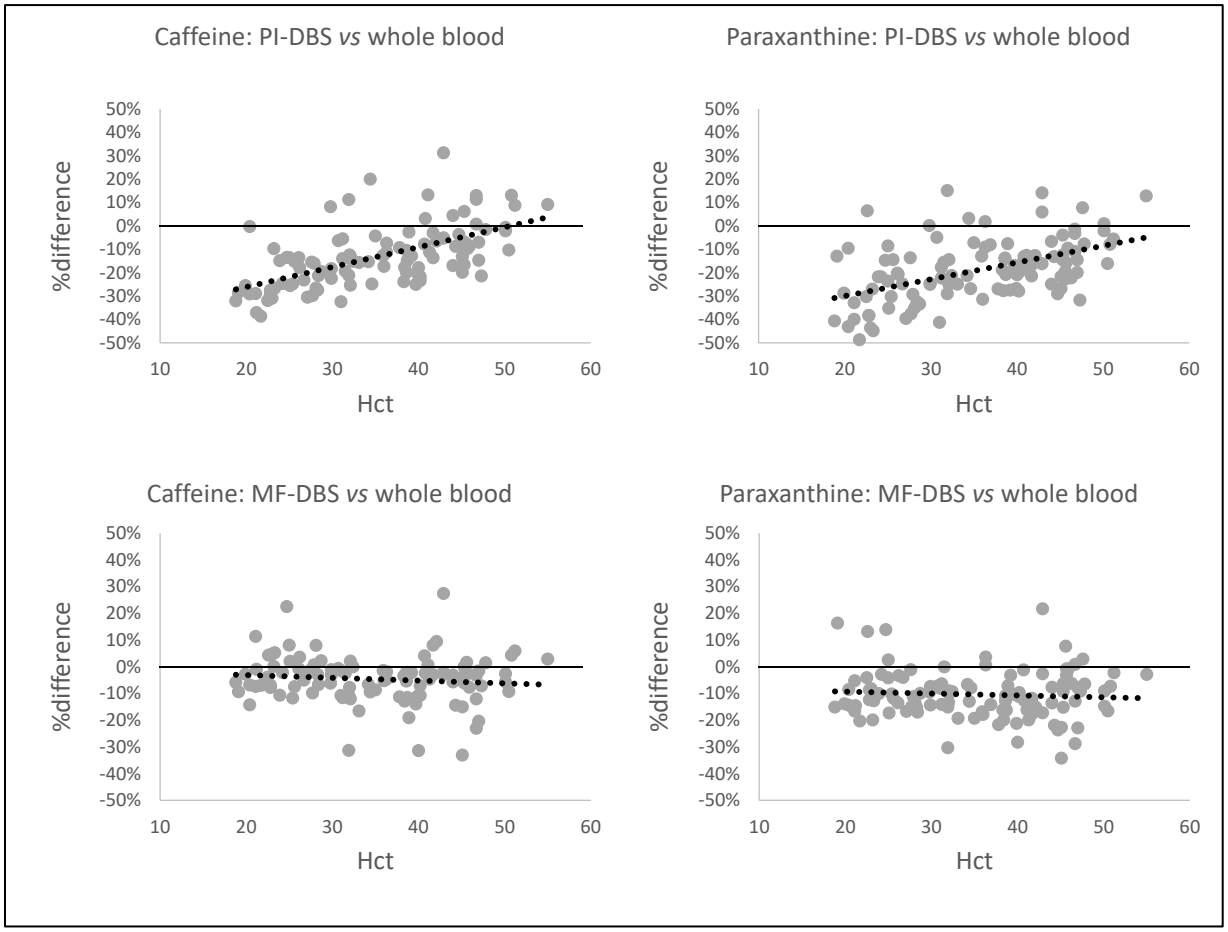

Figure B.2.2. Percentage difference between MF-DBS or PI-DBS concentrations and whole blood concentrations, plotted against hematocrit for caffeine (n=105) and paraxanthine (n=110). Broken lines represent linear regression lines.

Bland-Altman plots for the differences between MF-DBS and whole blood concentrations (Figure B.2.3) revealed a mean negative bias of -5.2% (95% CI; [-6.87 to -3.29%] and -11.5% (95% CI; [-13.23 to -9.39%]) for caffeine and paraxanthine, respectively. Incurred samples reanalysis revealed a mean negative bias of -4.0% (95% CI; [-5.47 to -2.42%] and -3.3% (95% CI; [-5.23 to -1.08%]) for caffeine and paraxanthine, respectively. From this it can be concluded that in our experiments the concentrations were slightly underestimated in MF-DBS versus whole blood. However, taking into account that results from different matrices (i.e. dried MF-DBS vs. liquid whole blood), obtained by different extraction methods and analyzed in separate analytical runs on different days, were compared, it can be concluded that these deviations were limited. For example, 93.3% and 89.1% of the caffeine and paraxanthine samples, respectively, did not differ more than 20% from the whole blood concentrations. For the incurred sample reanalysis, this was the case for 97.1% and 93.6% of the caffeine and paraxanthine samples, respectively, meeting the acceptance criterion for incurred sample

reanalysis [15]. Moreover, when comparing the incurred sample reanalysis to the original MF-DBS analysis, 97 out of the 105 caffeine samples and 90 out of the 110 paraxanthine samples were within ±20% of the mean, meeting the acceptance criterion for incurred samples reanalysis [14]. Furthermore, the different types of samples were analyzed against calibration curves prepared in the respective matrices, with accuracy values (%bias) for QCs of caffeine and paraxanthine within ±6% and ±5% (±14% at LLOQ level) for MF-DBS and whole blood samples, respectively. The storage conditions used during the actual study (1 day at 4°C for whole blood samples and 1 day at room temperature for MF-DBS) did definitely not exceed the storage conditions in which both analytes were stable in both whole blood (stable for at least 7 days at 4°C) and MF-DBS devices (stable for at least 3 months when stored at room temperature) [13]. Moreover, Hct had no effect on ion suppression or enhancement for MF-DBS. Therefore, we believe that a possible explanation for the positive bias observed in our study may be found in the fact that the patient samples were analyzed against a calibration curve prepared from blood with a Hct of 46.2, which was rather high, since only 15 out of the 133 included patient samples had a Hct value above 46.2. Although our recovery experiments had not revealed a Hct-dependent impact on recovery during method validation, this limited bias could still be induced by a small difference in recovery, taking into account that recovery experiments were performed using a small number (n=6) of spiked samples, whilst here a number of 133 authentic, venous patient samples were evaluated. As noted earlier, here, we wished to 'stress' the system, by using calibrators set up in a Hct at the higher end of the normal range, rather than using calibrators at about the median Hct of the anticipated range, as we recommend for dried blood analysis [2, 19].

In reality, samples will be collected by applying an undefined volume of blood, resulting from a finger prick, rather than by pipetting an amount of blood by the help of a calibrated pipette. We therefore evaluated whether the amount of blood added at the inlet port of the device influences the measured analyte concentrations. To this end, we applied different volumes (25, 30, 35, 40 and 50 µL) of blood, originating from authentic, venous patient samples with a Hct of 18.8, 22.8, 50.1 and 55. Results are depicted in Figure B.2.4.

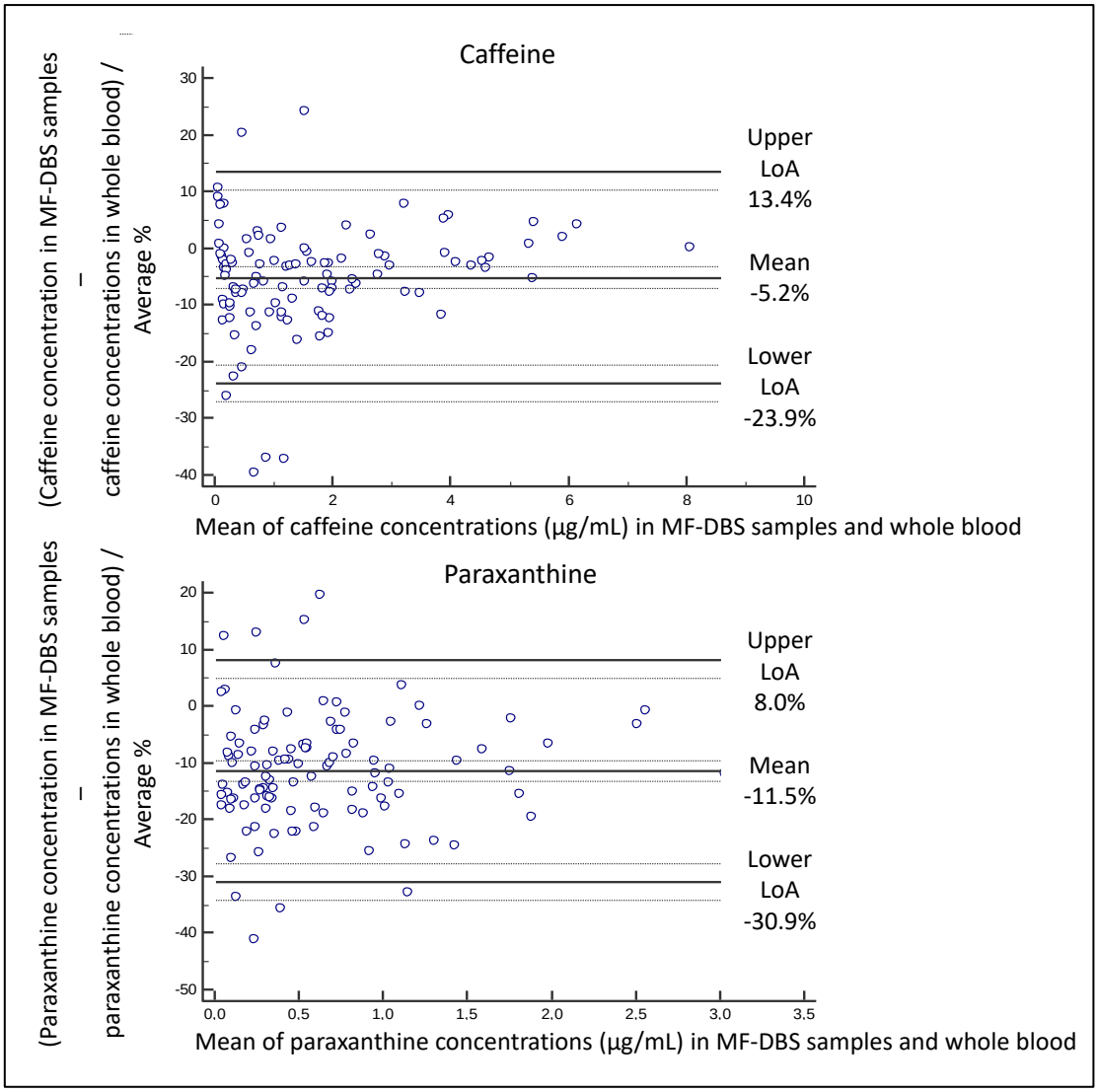

Figure B.2.3. Bland-Altman plots for the comparison between whole blood and MF-DBS concentrations for caffeine (n=105) and paraxanthine (n=110). Mean differences and limits of agreement (LoAs) are represented by full lines, 95% confidence limits by broken lines.

When comparing the concentrations obtained by pipetting different volumes, a CV% lower than 6.20% is obtained for both compounds. Therefore, it can be concluded that the amount of blood applied at the inlet of the device does not have an impact on the caffeine and paraxanthine concentration measured, this independent from a patient's Hct. Lastly, to exclude that coagulation may pose a problem, a small preliminary study was set up in which the use of non-anticoagulated capillary blood (obtained via a fingerprick) was evaluated. Capillary blood, obtained from 4 healthy volunteers, was pipetted onto MF-DBS devices and as a reference measurement, liquid capillary blood obtained from the same fingerprick was analyzed. In all cases, the difference between MF-DBS and capillary liquid blood

concentrations was below ±13.5% for both compounds, suggesting that also with non-anticoagulated blood the specifications are met. Further, large-scale, experiments in which the capillary blood is applied directly onto the devices are necessary to substantiate this conclusion.

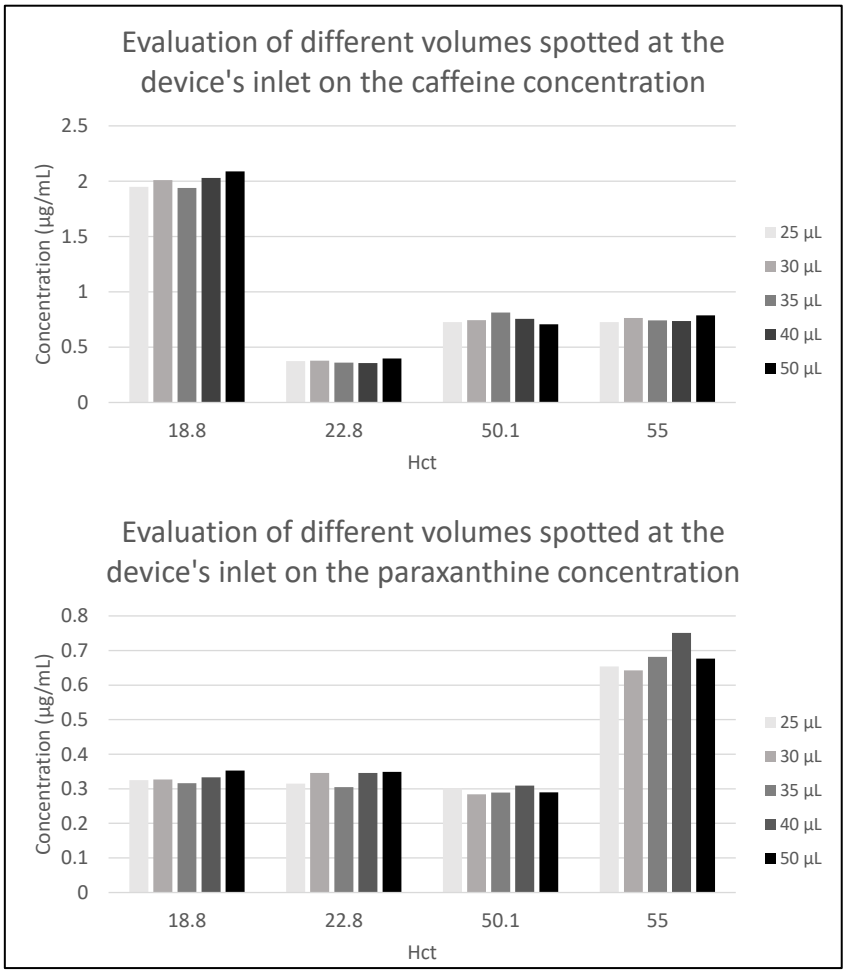

Figure B.2.4. Evaluation of the impact of applying different volumes of blood, originating from patient samples with a Hct of 18.8, 22.8, 50.1 and 55, at the device's inlet.

B.2.4. Conclusion

MF-DBS devices, one of the recently proposed strategies to help overcoming the Hct-based area bias, allow an accurate collection of a fixed volume (13.5 µL) of blood. In this Chapter, the potential of the devices to effectively nullify the Hct-based area bias was evaluated by analyzing 133 samples, prepared from whole blood covering a wide Hct range (18.8-55.0) originating from hospital patients. To this end, an LC-MS/MS method for the quantification of caffeine and paraxanthine, making use of MF-DBS devices, was completely validated, including the evaluation of both bioanalytical and dried blood sample specific parameters, with all pre-set acceptance criteria being met. Analyte concentrations measured in MF-DBS samples were compared to those measured in corresponding partial-punch PI-DBS and liquid whole blood samples. This comparison revealed that there was no Hct-dependent impact on the concentrations measured in MF-DBS, in contrast to parial-punch PI-DBS concentrations. However, we did observe a limited mean negative bias for both analytes in MF-DBS, when compared to whole blood. The use of blank blood with a rather high Hct (46.2) for the preparation of the calibrators might explain this, although no impact of Hct on recovery was observed during method validation. Given the results obtained and pending confirmation by others, using the same or other analytes, we conclude on the cautious side, that also for the MF-DBS devices it may be advisable to set up calibration lines at the anticipated median of the population-to-be-investigated. Evaluation of a potential volume impact by analyzing caffeine and paraxanthine concentrations in MF-DBS, derived from patient samples with a very low (18.8 and 20.8) or a very high (50.1 and 55) Hct, demonstrated that the amount of blood added at the inlet of the devices has no influence on the performance of the device, independently from the blood's Hct. Moreover, a preliminary study using capillary blood of 4 healthy volunteers indicated that the use of non-anticoagulated blood did not cause a problem.

Based on our findings, obtained by applying fully validated methods on authentic, venous patient samples, we conclude that the MF-DBS devices effectively assist in nullifying the Hct-based area basis for caffeine and paraxanthine and that the volume added at the inlet of the device does not have an impact on the measured analyte concentrations. Since these results were published, Lenk *et al.* also demonstrated for the MF-DBS devices, with caffeine as a model compound, a good volumetric performance, comparable to pipetting of blood with calibrated pipettes and independent from a patient's Hct [20].

It is important that these findings, which seem promising, should be corroborated by others, using other compounds with different features. In addition, further evaluation of the device by using non-assisted capillary microsamples, applied directly from a fingertip by the patient himself, is essential for evaluating its robustness in real practice. The latter is important to accept MF-DBS devices as a reliable alternative for whole blood analysis in existing and emerging applications.

B.2.5. References

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BROADER INTERNATIONAL CONTEXT, RELEVANCE AND FUTURE PERSPECTIVES

Worldwide, different working groups have been founded, focusing on the possibilities and the limitations coupled to dried blood sample analysis. The International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) has an Alternative Sampling Strategies Committee, the European Bioanalysis Forum (EBF) had/has a dried blood spot (DBS)/microsampling consortium and the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3R^s) established an international microsampling user group. Discussions within these groups resulted in recommendations for the validation of bioanalytical methods making use of dried blood samples, this in the framework of harmonization and standardization of alternative sample analysis [1], setting up guidelines for dried blood spot sampling for therapeutic drug monitoring [2] or for the implementation of microsampling techniques within toxicokinetic assessment studies [3] and for evaluation of the hematocrit issue [4].

Throughout this work, we mentioned some of the advantages and challenges coupled to the use of dried blood samples. A summary can be found in Table 1.

Table 1. Advantages and challenges coupled to dried blood sample analysis.

Advantages	**Challenges**
▪ Ease of sampling, rendering self/home-sampling possible	▪ Correlation between venous and capillary blood concentrations
▪ Minimally invasive sampling	▪ Only small sample volumes available: sensitive techniques required
▪ Suitable for automation	▪ Hematocrit issue
▪ Small sample volume	▪ Risk of contamination
▪ Increased analyte stability	▪ Extensive validation required (e.g. impact of Hct)
▪ Convenient transport and storage (ambient conditions)	▪ Need for adequate sampling
▪ Reduced risk of infection	

The small sample volume in combination with the minimally invasive character of dried blood samples, makes the technique extremely suitable for sample collection during preclinical and toxicokinetic studies involving animals and for special patient populations (e.g. anemic or pediatric patients). A drawback of this small sample volume is the need for highly sensitive

analytical instrumentation, a need which can generally be met with LC-MS/MS (see **Chapter A.1.**).

The ease of sampling makes home-sampling a possibility, which can be of great benefit in the field of TDM (see **Chapters A.2. and A.3.**). This advantage definitely resulted in a growing interest for the use of dried blood samples for TDM applications. The latter can be deduced from the increasing number of research papers covering dried blood-based methods for TDM (see Figure 1). For anti-epileptic drugs (AEDs) in particular, different indications for TDM have been put forward: (1) dose optimization after initialization of an AED treatment, after dose adjustments or after a change in AED formulation; (2) uncontrolled seizures despite an appropriate dosage of a convenient AED (e.g. due to poor compliance or to pharmacokinetic abnormalities); (3) special patient populations (e.g. children, elderly, pregnant women); (4) suspected toxicity; (5) presence of comorbidities which have a significant effect on the pharmacokinetics of AEDs (e.g. liver or kidney disorders, decreased cardiac function, …); and (6) suspected drug-drug interactions [5].

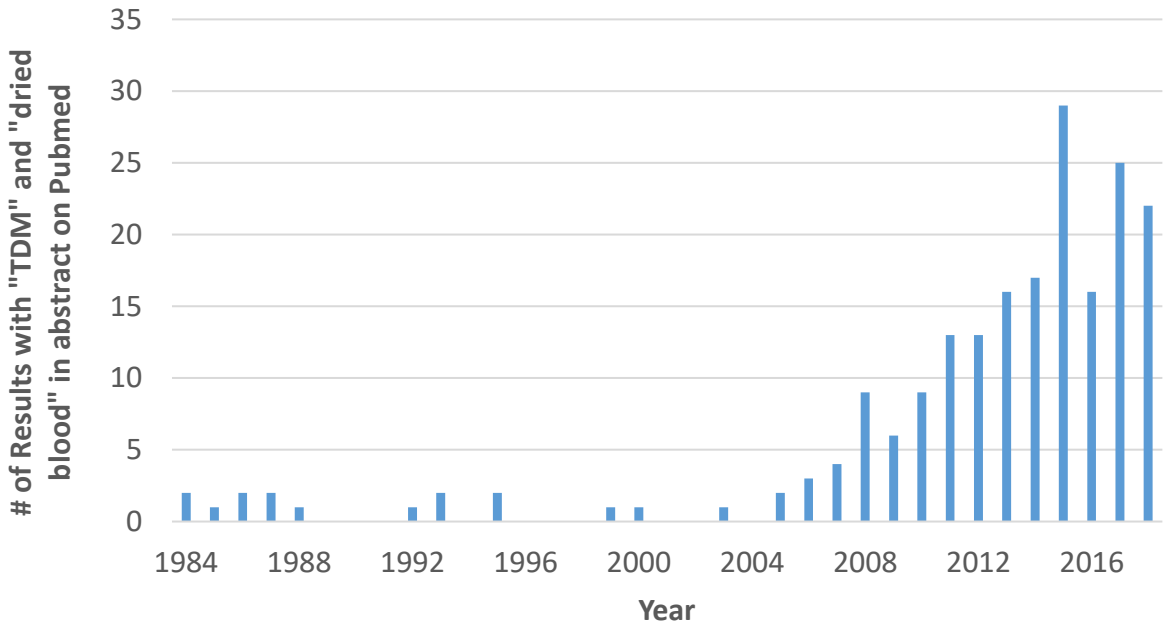

Figure 1. Overview of the number of research papers dealing with dried blood-based methods for TDM in Pubmed.

Due to the large inter-individual variety in types of epilepsy and in the severity of epileptic seizures, the same dosage of an AED causes in some patients a symptom decrease, whilst in others, epileptic seizures remain poorly controlled. Furthermore, some patients experience

complete seizure control with an AED blood concentration below or above a set reference range, making TDM of AEDs quite challenging. Therefore, dosage adjustment should preferably be performed by combining the results of TDM with the clinical outcome. In other words, at the start of an AED treatment, a clinician must aim at obtaining an AED blood concentration within a set reference range, followed by a titration upwards or downwards depending on the clinical symptoms. In this context, the concept of the 'individual therapeutic concentration/range' arose, being the AED concentration or range of concentrations for which an individual patient experiences an optimum clinical response [6]. Defining this 'individual therapeutic concentration/range' can also be seen as an indication for TDM of AEDs. Determining the latter concentration or range can be performed for every AED, also including the AEDs for which a reference range is currently still lacking. To do so, the steady-state AED(s) concentration(s) should preferably be measured twice (2-4 months apart) once a patient has reached his/her optimum AED regimen [5]. Furthermore, since TDM is most often performed on plasma or serum samples, reference ranges for AEDs are typically also set for these matrices, imposing the need for a case-by-case evaluation of the correlation between levels measured in samples different from plasma or serum (e.g. oral fluid or capillary blood) and plasma or serum levels. However, defining the 'individual therapeutic concentration/range' by using those alternative matrices renders this correlation assessment superfluous.

The possibility of convenient transport and storage in combination with the reduced risk of infection, makes dried blood sampling an attractive strategy for TDM in resource-limited areas (see **Chapter A.4.**). Poor knowledge about diseases and their therapy, low level of education, the presence of side effects, long distance to medication distribution centers, religious beliefs, etc. are all factors contributing to poor medication adherence in low- and middle-income countries. In the case of AEDs, especially first-generation AEDs, amongst which carbamazepine, phenobarbital, phenytoin and valproic acid, are prescribed in developing countries. The later generation AEDs are less available due to the high treatment cost for this class of drugs [7]. Furthermore, treatment cost and drug availability are, rather than the type of epilepsy and/or the patient's profile (e.g. co-morbidities), the decisive factors for an AED treatment choice [7]. Overall, availability, accessibility and AED quality poses a problem in these countries. Subsequently, most developing countries have a gigantic epilepsy treatment gap (i.e. the proportion of people with epileptic disorders who require treatment but do not

receive it) [7, 8]. Due to this treatment gap, 9 out of 10 people in Africa do not have access to treatment, this compared to only 1 out of 10 in industrialized countries [7, 9-13]. Furthermore, lack of sufficiently qualified staff, funds and transport causes a suboptimal follow-up of the outreach patients. An evaluation of different programs established to improve therapeutic management of epilepsy patients in low- and middle-income countries revealed the lack of TDM and adaptation/optimization of treatment dose [7]. So here, TDM can be of utmost benefit, definitely when considering the narrow therapeutic windows, the large number of drug-drug interactions and the severe side effects associated with the use of first-generation AEDs. To date, the need for technical facilities, trained personnel and the cost are limiting factors for the implementation of TDM in those countries, being limitations which can possibly be tackled by dried blood sample analysis.

Besides TDM of AEDs, the utility of alternative sampling strategies in resource-limited areas has also been demonstrated for TDM of other drugs, as well as for diagnostic purposes (e.g. hepatitis B and C [14] and HIV [15]). A literature search revealed the utility of dried blood-based methods, including mainly DBS, in hard-to-reach populations for TDM of e.g. antimalarials [16], antiretrovirals[17] and anti-tuberculosis drugs[18, 19]. In the case of human immunodeficiency virus (HIV), DBS have proven to be of utmost benefit. Here, adherence monitoring of antiretrovirals has evaluated from pill count and pharmacy refills, over HIV viral load monitoring to plasma/urine concentration measurements [20]. It should be noted though, that the latter has a limited utility due to the short half-lives of most antiretrovirals in these matrices and to the fact that concentrations derived from a single dose usually match steady-state concentrations, resulting in 'white coat' adherence [20]. Here, DBS offer the possibility to quantify both the recent and the cumulative antiretroviral adherence exposure, due to the trapping and accumulation of an active metabolite in the red blood cells [20]. This wider window of detection makes DBS preferable above urine and plasma [20]. On the other hand, having information on the recent exposure makes DBS also preferable above hair analysis. The information on both cumulative and recent exposure is possible for patients on a combination therapy of tenofovir and emtricitabine, by simultaneously quantifying their active metabolites in red blood cells [21, 22]. Besides the use of DBS for TDM of antiretrovirals and viral load testing, it has also been described for HIV drug resistance testing [23]. For tuberculosis, the Bill and Melinda Gates foundation is supporting the development of an on-site saliva test and a DBS-

based test for therapeutic drug monitoring of anti-tuberculosis drugs in patients originating from low-resource settings. Since low levels of anti-tuberculosis medication adherence contribute to the emergence of deadly multi-drug resistant tuberculosis, optimization of the drug dosage, based on concentration levels, is extremely important [24]. Furthermore, recently, the usefulness of DBS for pharmacokinetic studies in resource limited settings has been demonstrated for ivermectin, a broad-spectrum antiparasitic agent, being used in mass drug administration campaigns [25, 26].

Overall, in the context of TDM, we believe there is a bright future for dried blood samples, for several reasons. First, there is an increased demand from patients to allow drug monitoring at home and dried blood samples may perfectly serve this purpose. Second, there is an increased financial pressure on social security systems: dried blood-based monitoring may be a cost-effective approach, not only allowing patient-tailored therapy, but also serving as a tool to check patient compliance [27]. Indeed, with several medications costing thousands of dollars per month (e.g. oncology drugs), we have come beyond the point of having the right drug for the right patient: instead we wish to attain the right concentration of the right drug at the right time in the right patient. Third, as exemplified in **Chapter A.3.**, automated processing of dried blood samples can be made fully compatible with fully automated LC-MS/MS workstations that are about to enter clinical laboratories, allowing “from card to report, with no manual intervention”. These systems may for example be equipped with tools for automated recognition of correctly deposited DBS and for coping with variables like Hct, e.g. via the integration of reflectance spectroscopy for Hct prediction [28]. This tailoring to current (and future) workflows of routine clinical laboratories, focusing on high-throughput, is essential for a more widespread implementation.

To date, the proof of principle for dried blood samples has been convincingly established for many applications and it is now time for a more widespread implementation. Of course, strict quality criteria need to be taken into account if dried blood sampling and analysis is to be used for clinical or legal decision-making. Initiatives to set up proficiency testing programs, as well as widely accepted best practice guidelines on analytical and clinical validation for dried blood-based methodologies are essential for further integration of dried blood-based methods into routine practice [29, 30]. Besides a pilot proficiency testing program for the immunosuppressant tacrolimus [31], external QC programs specially designed for testing the performance of dried

blood-based methods are currently lacking, although being of urgent need for facilitating the uptake of dried blood samples within a routine workflow. Furthermore, existing external QC materials are mostly developed for the evaluation of plasma-based methods, making it difficult to use these samples to evaluate the performance of dried blood sample-based methods. However, as described in **Chapter A.2.**, a possibility here, is to remove a part of the plasma of a blank whole blood sample and replace it with this plasma-based, existing external QC material. The realization of a validation guideline has been one of the actions of IATDMCT's Alternative Sampling Strategies Committee, which resulted very recently in 'A development and validation guideline of dried blood spot-based methods for therapeutic drug monitoring' [2]. This guideline, being the first to describe a combination of existing guidelines for traditional matrix analysis (i.e. the EMA, FDA and CLSI guidelines) with DBS-specific guidelines, should enable a standardization of a dried blood-based method evaluation, which will definitely help the (regulatory) acceptance of dried blood-based analyses in the bioanalytical and clinical field.

Throughout this thesis, the enormous progress in the highly dynamic field of alternative sampling strategies was also put in the spotlight:

- The classical DBS sampling preparation evolved from a time-consuming handling, including a manual punching step, over a semi-automated procedure (i.e. the introduction of an automatic DBS puncher) to a fully-automated, high-throughput-capable approach (see **Chapter A.3.**).
- Besides the classical DBS cards, different new sampling devices, allowing to overcome the Hct issue related to classical DBS analysis, have entered the market (see **Chapter B.1.**), e.g. the Mitra® microsampling device (see **Chapter A.2.**), the hemaPEN® technology, Capitainer-B devices (see **Chapter B.2.**) and the HemaXis DB device, all able to volumetrically generate dried blood spots.
- Different kinds of devices capable of a centrifugation-independent generation of dried plasma spots have been introduced as another way to avoid the Hct effect (see **Chapter B.1.**).
- Different strategies for Hct estimation have entered the field, with non-destructive Hct prediction possibly being the most promising one for the future (see **Chapter B.1.**).

Conclusively, the work of various groups has resulted in the development of a more convenient sample preparation flow and of new strategies to tackle or to minimize the well-known Hct issue. However, the extra cost associated with the proposed approaches compared to classical DBS analysis may be a prohibiting factor for their widespread implementation. The latter is definitely important when considering sampling in resource-limited areas, but also holds true for Western clinical laboratories. To date, it has been demonstrated for DBS that home-sampling can result in an important decrease in costs on the patients' side compared to regular blood sampling, especially by a decrease in travel expenses and in costs related to loss of productivity [27]. Furthermore, both patients and social health care facilities may benefit from reduced healthcare costs, since e.g. no phlebotomist is necessary. On the other hand, the latter may lead to a loss of income for the healthcare facilities. Furthermore, one should bear in mind that the proportion of hospital visits has an enormous influence on the possible cost reduction and therefore, cost-savings should be evaluated for every type of disease. Seeing these possible savings associated with DBS sampling compared to regular blood sampling, the benefits of the newly proposed devices will need to be compared to the disadvantage of the extra cost before a more widespread routine implementation. Another important question here is, who will pay for the new devices? Probably the patient will still - as for DBS- benefit in terms of costs compared to regular blood sampling, however, on the other hand, for the laboratories, the time and costs associated with sample analysis may both increase (because existing workflows may not be adapted to microsamples) or decrease (e.g. when fully-automated high-throughput analysis "from card to result" is possible). Another major point of attention here is that while implementation of alternative sampling devices for home-sampling may allow a better patient follow-up, it can never fully replace a doctor's appointment. As is clear from the TDM examples in resource-limited areas outlined above, classical DBS are the dried blood samples of choice in these areas, mostly due to the lower cost of classical DBS cards (e.g. 3€ for 1 PerkinElmer 226 bioanalysis card, for applying 4 spots) when compared to some of the proposed alternatives (e.g. 7.7€ for a Mitra® microsampler clamshell, containing 4 Mitra® devices). Therefore, the Bill and Melinda Gates foundation is currently funding two projects focusing on the development of low-cost (1) new paper-based cards by the group of Mace [32]; and (2) novel dried plasma generating devices by the group of Anderson for the diagnosis of patients with Hepatitis B and C in low-resource areas [33, 34].

Last, a key for trustworthy conclusions are trustworthy data, obtained after analysis of trustworthy samples. Therefore, upon non-supervised sampling at home, providing patients with clear instructions on how to sample correctly is of key importance when it comes to valid results. Furthermore, it should be realized that dried blood sampling and analysis is not suitable for every analyte in every context and will never replace traditional sampling: as outlined in **Chapter A.1.**, dried blood sampling and analysis should rather be looked at as an additional tool in the analytical toolbox. If properly performed, it has the ability to provide high quality results where adequate information cannot be (conveniently) obtained using traditional procedures (e.g. sample collection in remote or resource-limited areas and at patients' homes).

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SUMMARY

In this work we focus on dried blood samples. Seeing the multiple advantages, an increased interest in the use of dried blood samples, with different kinds of applications in various fields, has arisen. On the other hand, dried blood sample analysis is still struggling with some issues. Therefore the objective of this thesis was twofold: first we focused on the possibilities of dried blood samples, with therapeutic drug monitoring (TDM) being the field of interest; second we discussed a major issue related to the use of classical dried blood spots (DBS), being the hematocrit (Hct) effect and applied a new strategy able to cope with this issue.

Alternative sampling strategies can be looked at from two sides: (1) traditional samples (i.e. blood, plasma, serum or urine) collected in an alternative way (e.g. DBS); and (2) alternative samples collected in all kind of ways (e.g. oral fluid or hair). In **Chapter A.1.**, a broader view is provided on distinct alternative sampling strategies used throughout different fields of application, with special attention to the use of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The latter is the technique of choice when considering sensitivity and selectivity. Specific benefits as well as challenges and limitations which have been associated with the different alternative sampling strategies were discussed, this together with recent advances and future trends, which are important for a more routine implementation of these sampling strategies.

One of the best known alternative sampling strategies is DBS sampling. DBS are generally collected as a spot, obtained by a finger or heel prick, on dedicated filter paper. TDM can be considered as one of the fields with an increased interest in the use of dried blood samples (amongst which DBS). The reason is multifaceted: (1) the ease of dried blood sample collection, making home sampling possible; (2) the dried matrix generally induces an improved analyte stability; (3) the compatibility with automated systems, making dried blood sample analysis high-throughput-capable; and (4) the ease of transport and storage [1]. On the other hand, classical DBS analysis also suffers from some issues, with the Hct effect definitely being the most discussed one [2]. Therefore, in an attempt to cope with this issue, different alternative types of devices capable of generating dried blood samples have entered the market, amongst which the volumetric absorptive miscrosampling (VAMS) devices [3, 4]. VAMS devices preserve all advantages coupled to classical DBS, with the profit of eliminating the Hct effect. Therefore, **Chapter A.2.** describes the development, validation and application of an ultra-performance liquid chromatography - tandem mass spectrometry (UPLC®-MS/MS)

method for the determination and quantification of four anti-epileptic drugs (AEDs) and one active metabolite, including carbamazepine (CBZ), valproic acid (VPA), phenytoin (PHT), phenobarbital (PB) and carbamazepine-10,11-epoxide (CBZ-E), making use of VAMS devices. These first-generation AEDs, characterized by narrow therapeutic windows, large numbers of drug-drug interactions and severe side-effects, are still frequently used for seizure control in developing countries, where dried blood sampling may be of utmost benefit [5]. Both bioanalytical and VAMS-specific parameters were included within the method validation and overall the pre-set acceptance criteria were met. In this Chapter we also describe how existing external serum quality control (QC) samples can be used as an alternative for -usually missing- whole blood QC samples to evaluate the performance of a dried blood-based method.

One of the advantages coupled to DBS analysis is the possibility for automation, making the sampling technique feasible for high-throughput settings. In **Chapter A.3.**, we successfully validated a fully automated DBS method, using a DBS-MS 500 autosampler, online coupled to an LC-MS/MS system, for the determination and quantification of the same set of AEDs as used in Chapter A.2. The DBS-MS 500 system consists of a robotic arm, able to transport cards from the card racks to the different workstations; an optical recognition system used for spot location and information collection; an internal standard (IS) module, which sprays the IS solution onto the DBS cards before extraction; an extraction module, holding a 4 mm clamp head; and a wash station. Method development revealed the importance of thorough optimization of the fully automated extraction procedure, finally resulting in the exclusion of the built-in IS spray. Here again, method validation included the evaluation of both bioanalytical and DBS-specific parameters.

The ease of sample collection and storage in combination with the reduced risk of infection, makes dried blood matrices extremely useful for sampling in remote or resource-limited settings. Therefore, in **Chapter A.4.** the VAMS- and DBS-based methods described above were used for the analysis of samples originating from children receiving AEDs in Uganda and the Democratic Republic of the Congo. Here, we observed that AED concentrations within the specific therapeutic reference ranges were only present in a relatively low number of patients. However, when comparing these results with the amount of seizures obtained during the last month before sampling, no obvious link could be observed: some patients with a concentration below the therapeutic reference ranges mentioned a seizure decrease, whilst

others with a concentration within the therapeutic range were poorly controlled. The latter illustrates the complexity of TDM of AEDs. Furthermore, an inexplicable underestimation was observed for all analytes when comparing DBS concentrations with VAMS concentrations. Here, a comparative study including VAMS, DBS and whole blood samples could help to address this finding.

The Hct is defined as the volume percentage of blood taken in by red blood cells. The Hct is determined by the amount and the size (volume) of these cells and influenced by different factors, e.g. age, sex, health and nutritional state. Overall, reference ranges lie at approximately 41-50% and 36-44%, for men and women, respectively. However, inter- as well as intra-individual differences exist. When preparing DBS, blood with a higher Hct (e.g. 50%) will spread less over cellulose-based DBS cards, compared to blood with a lower Hct (e.g. 30%), due to differences in the viscosity of the blood. When applying partial-punch analysis, this may impact the validity of the obtained results, since this partial-punch (e.g. 3 mm) originating from a DBS with a higher Hct will contain a larger volume of blood compared to DBS with a lower Hct [2]. Seeing the important impact of the Hct on DBS analysis, **Chapter B.1.** provides an overview on the recently suggested strategies that may help to cope with the Hct issue. More particularly, a distinction is made between attempts to avoid the Hct issue, strategies to minimize the issue and approaches able to measure or estimate the volume and/or Hct of a DBS.

One of the suggested strategies to avoid the Hct issue is volumetric collection of blood, followed by a whole spot analysis. Recently, different devices, utilizing this strategy, have entered the stage, allowing to maintain the benefits coupled to classical DBS, but eliminating the Hct issue. One example of such recently designed devices is the Capitainer-B device (referred to as microfluidic-DBS or MF-DBS device). In **Chapter B.2.** we evaluated the potential of this device to effectively eliminate the Hct effect by analyzing 133 left-over patient samples across a wide Hct range (18.8-55%). Furthermore, we investigated whether the amount of blood applied is influencing the performance of the device. To this end, an UPLC®-MS/MS method, making use of caffeine and its metabolite paraxanthine as model compounds, was fully validated, meeting all preset acceptance criteria. It could be concluded that, based on a comparison between analyte concentrations measured in MF-DBS and in corresponding partial punch DBS, the Hct had no impact on the measured concentrations in MF-DBS, this in

contrast to partial punch DBS. Furthermore, addition of different volumes of blood originating from patients with different Hct values at the inlet of the device, demonstrated that the amount of blood added has no influence on the device performance, this independently from a patient's Hct.

Conclusively, in the field of alternative sampling strategies an enormous progress has been made during the past few years. However, despite the numerous new developments, there are still some limitations which need to be tackled, e.g. the (relatively) high cost associated with most of the newly developed strategies. Furthermore, an evaluation of several of the new strategies by using capillary blood, applied directly from a fingertip under real-life circumstances, is still often missing, which is essential for the evaluation of their user-friendliness and robustness in real practice. The latter is very important for the acceptance of the different new developments as a reliable alternative for whole blood analysis. Finally, one should also bear in mind that there will always be some limitations coupled to dried blood based-methods, e.g. in some cases the analytical result is urgent, excluding the possibility to wait until the sample has dried. In these circumstances, the use of the classical, venous sampling technique may be necessary. However, an -already proven- alternative is the use of wet microsamples. On the other hand, the use of dried blood samples can definitely be encouraged when wet sampling is not possible, e.g. in remote areas or for home monitoring.

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SAMENVATTING

Gedroogde bloedstalen vormen de rode draad doorheen dit werk. Gezien de vele voordelen, is er een toegenomen belangstelling voor het gebruik van gedroogde bloedstalen, dit met een grote verscheidenheid aan toepassingen in verschillende vakgebieden. Anderzijds zijn er aan de analyse van gedroogde bloedstalen nog steeds een aantal nadelen verbonden. Het doel van dit proefschrift is daarom tweeledig: eerst hebben we ons gericht op de mogelijkheden van gedroogde bloedstalen, waarbij therapeutische geneesmiddelenbewaking (TDM) het interessegebied is; ten tweede gingen we dieper in op één van de grootste nadelen verbonden aan het gebruik van klassieke gedroogde bloedspots, zijnde het hematocriet (Hct)-effect, waarbij we een nieuwe strategie toepasten die dit probleem kan verhelpen.

Alternatieve bemonsteringsstrategieën kunnen van twee kanten worden bekeken: (1) traditionele stalen (bloed, plasma, serum of urine) kunnen worden verzameld op een alternatieve manier (bijvoorbeeld aan de hand van gedroogde bloedspots); en (2) alternatieve stalen kunnen worden verzameld op allerlei manieren (bijvoorbeeld speeksel of haar). In **hoofdstuk A.1.** wordt een bredere kijk gegeven op een waaier aan alternatieve bemonsteringsstrategieën, dit in verschillende toepassingsgebieden, met speciale aandacht voor het gebruik van vloeistofchromatografie gekoppeld aan tandem massaspectrometrie (LC-MS/MS). Deze laatste is de beste techniek wanneer we denken aan gevoeligheid en selectiviteit. Specifieke voordelen, evenals uitdagingen en beperkingen die geassocieerd zijn met de verschillende alternatieve bemonsteringsstrategieën werden besproken, dit in combinatie met recente ontwikkelingen en toekomstige trends, die belangrijk zijn voor een meer routinematige implementatie van deze bemonsteringsstrategieën.

Een van de bekendste alternatieve bemonsteringsstrategieën zijn de gedroogde bloedspots. Gedroogde bloedspots worden over het algemeen verzameld als een spot, verkregen door een vinger of hielprik, op een speciaal filterpapier. TDM kan worden beschouwd als een van de gebieden met een toegenomen belangstelling voor het gebruik van gedroogde bloedstalen (waaronder gedroogde bloedspots). De reden hiervoor is veelzijdig: (1) het gemak waarmee gedroogde bloedstalen verzameld worden, waardoor thuisafname mogelijk wordt; (2) de gedroogde matrix zorgt in het algemeen voor een verhoogde stabiliteit van analyten; (3) de compatibiliteit met geautomatiseerde systemen, waardoor analyse van gedroogde bloedstalen een hoge verwerkingscapaciteit mogelijk maakt; en (4) het gemak van transport en opslag [1]. Aan de andere kant kent de klassieke gedroogde bloedspot-analyse ook enkele

problemen, waarbij het Hct-effect absoluut het meest besproken is [2]. Daarom zijn, in een poging om dit probleem aan te pakken, verschillende alternatieven op de markt gekomen die in staat zijn om gedroogde bloedstalen te genereren onafhankelijk van het Hct. Een voorbeeld hiervan is de *volumetric absorptive microsampling* (VAMS) techniek [3, 4]. De VAMS techniek behoudt alle voordelen gekoppeld aan klassieke gedroogde bloedspots, met daarenboven de mogelijkheid tot het elimineren van het Hct-effect. Daarom beschrijft **hoofdstuk A.2.** de ontwikkeling, validatie en toepassing van een LC-MS/MS methode voor de bepaling en kwantificatie van vier anti-epileptica en één actieve metaboliet, waaronder carbamazepine (CBZ), valproïnezuur (VPA), fenytoïne (PHT), fenobarbital (PB) en carbamazepine-10,11-epoxide (CBZ-E), gebruikmakend van de VAMS techniek. Deze eerste generatie anti-epileptica, die worden gekenmerkt door nauwe therapeutische referentie intervallen, veel geneesmiddeleninteracties en ernstige bijwerkingen, worden nog steeds vaak gebruikt ter voorkoming van epileptische toevallen in ontwikkelingslanden, waar gedroogde bloedstalen een groot voordeel kunnen bieden [5]. Zowel bio-analytische als VAMS-specifieke parameters werden opgenomen in de methodevalidatie en over het algemeen werd aan de acceptatiecriteria voldaan. In dit hoofdstuk beschrijven we ook hoe bestaande externe serum kwaliteitscontrolestalen kunnen worden gebruikt als alternatief voor -meestal ontbrekende- volbloed kwaliteitscontrolestalen om de kwaliteit van een op gedroogde bloedstaal-gebaseerde methode te evalueren.

Een van de voordelen gekoppeld aan de analyse van gedroogde bloedspots is de mogelijkheid tot automatisering, wat de techniek aantrekkelijk maakt voor laboratoria met een hoge doorvoer. In **hoofdstuk A.3.** werd daarom een volledig geautomatiseerde gedroogde bloedspot-methode succesvol gevalideerd. De gebruikte opstelling omvatte een DBS-MS 500-autosampler, online gekoppeld aan een LC-MS/MS systeem en werd gebruikt voor de bepaling en kwantificering van dezelfde reeks anti-epileptica als gebruikt in hoofdstuk A.2. Het DBS-MS 500 systeem bestaat uit een robotarm die kaarten vanuit één van de 5 rekken naar de verschillende werkstations kan verplaatsen; een optisch herkenningssysteem dat wordt gebruikt voor het lokaliseren van de spotlocatie, alsook voor het verzamelen van informatie (bv. barcode gegevens); een interne standaard (IS) module, die de IS-oplossing vóór extractie op de gedroogde bloedspot-kaarten spuit; een extractiemodule met een klemkop van 4 mm; en een wasstation. Methodeontwikkeling benadrukte het belang van een grondige

optimalisatie van een volledig geautomatiseerde extractieprocedure en resulteerde hier uiteindelijk in het omzeilen van het gebruik van de ingebouwde IS module. Ook hier omvatte de methode validatie de evaluatie van zowel bioanalytische als gedroogde bloedspot-specifieke parameters.

Het gemak waarmee gedroogde bloedstalen verzameld en bewaard kunnen worden, in combinatie met het verminderde risico op infecties, maakt gedroogde bloedstalen uiterst geschikt voor staalname in afgelegen gebieden. Daarom werden in **hoofdstuk A.4.** de VAMS- en gedroogde bloedspot-gebaseerde methoden, die hierboven beschreven werden, gebruikt voor de analyse van stalen afkomstig van kinderen die een anti-epileptica behandeling verkrijgen in Uganda en de Democratische Republiek Congo. In deze studie werd vastgesteld dat slechts een klein percentage van deze patiënten anti-epileptica-concentraties had binnen de vooropgestelde therapeutische referentie intervallen. Het vergelijken van deze resultaten met het aantal epileptische toevallen in de maand voorafgaand aan de staalname leverde echter geen eenduidige link op: sommige patiënten met een concentratie onder een welbepaald therapeutisch referentie interval ervoeren een afname in het aantal toevallen, terwijl anderen met een concentratie binnen het therapeutische bereik een toename aanhaalden. Dit laatste illustreert de complexiteit van TDM van anti-epileptica. Bovendien werd voor alle componenten een onverklaarbare onderschatting waargenomen bij het vergelijken van DBS-concentraties met VAMS-concentraties. Een vergelijkende studie waarin zowel VAMS, DBS als volbloed stalen geanalyseerd worden, zou meer inzicht kunnen verschaffen met betrekking tot deze bevinding.

Het Hct wordt gedefinieerd als het volumepercentage van bloed dat wordt ingenomen door rode bloedcellen. Het Hct wordt bepaald door de hoeveelheid en de grootte (volume) van deze rode bloedcellen en wordt beïnvloed door verschillende factoren, waaronder o.a. leeftijd, geslacht, gezondheid en voedingstoestand. Over het algemeen liggen de referentiewaarden bij mannen tussen 41-50% en bij vrouwen tussen 36-44%, hoewel er zeker ook inter- en intra-individuele verschillen bestaan. Bij het bereiden van gedroogde bloedspots zal bloed met een hoger Hct (bijvoorbeeld 50%) zich minder verspreiden in vergelijking met bloed met een lager Hct (bijvoorbeeld 30%) over de op-cellulose-gebaseerde gedroogde bloedspot-kaarten, dit vanwege verschillen in de viscositeit van het bloed. Wanneer de analyse vervolgens gebeurt op een partieel gedeelte van de spot (bijvoorbeeld op een 3 mm

punch) kan dit een impact hebben op de betrouwbaarheid van de verkregen resultaten, aangezien een punch uit een gedroogde bloedspot afkomstig van bloed met een hoog Hct een groter volume aan bloed zal bevatten in vergelijking met een punch afkomstig van bloed met een laag Hct [2]. Gezien de belangrijke impact van het Hct op de analyse van gedroogde bloedspots werd in **hoofdstuk B.1.** een overzicht gegeven van recent voorgestelde technieken die werden ontwikkeld om het Hct-probleem het hoofd te bieden. In het bijzonder wordt in dit hoofdstuk een onderscheid gemaakt tussen pogingen om het Hct-probleem te vermijden, strategieën om het probleem te minimaliseren en benaderingen die in staat zijn om het volume en/of het Hct van een gedroogde bloedspot te meten of te schatten.

Een van de voorgestelde strategieën om het Hct-probleem te voorkomen is de bloedspots op een volumetrische manier te verzamelen, gevolgd door een analyse van de volledige spot. Onlangs werden verschillende technieken, die gebruik maken van deze strategie, geïntroduceerd. Op deze manier kunnen de voordelen gekoppeld aan klassieke gedroogde bloedspots behouden worden en wordt het Hct-probleem teniet gedaan. Een voorbeeld hiervan is de Capitainer-B-techniek (aangeduid als *microfluidic-DBS* of *MF-DBS-device*). In **hoofdstuk B.2.** werd het potentieel van deze techniek om het Hct-effect effectief te elimineren geëvalueerd. Hiervoor werden 133 patiënt-reststalen met een breed Hct-bereik (18,8-55%) geanalyseerd. Verder werd onderzocht of de hoeveelheid bloed toegevoegd aan de ingang van het apparaat de prestaties beïnvloedt. Daartoe werd een LC-MS/MS methode, gebruikmakend van cafeïne en diens metaboliet paraxanthine als modelcomponenten, succesvol gevalideerd. Een vergelijking tussen de analytconcentraties gemeten in de *MF-DBS-devices* en in overeenkomstige partiële-spot gedroogde bloedspots toonde aan dat het Hct geen invloed had op de gemeten concentraties in de *MF-DBS-devices,* dit in tegenstelling tot de analyse van gedroogde bloedspots op de klassieke manier. Bovendien toonde de toevoeging van verschillende volumina bloed (afkomstig van patiënten met uiteenlopende Hct-waarden) aan de ingang van het apparaat aan dat de hoeveelheid toegevoegd bloed geen invloed heeft op de werking van de *MF-DBS-devices.* Dit laatste was tevens onafhankelijk van het Hct van een patiënt.

Als besluit kunnen we stellen dat dat er de afgelopen jaren een enorme vooruitgang geboekt werd op het gebied van alternatieve bemonsteringsstrategieën. Ondanks de talrijke nieuwe ontwikkelingen zijn er echter nog steeds enkele beperkingen die moeten worden aangepakt,

b.v. de (relatief) hoge kosten verbonden aan de meeste nieuw-ontwikkelde strategieën. Bovendien ontbreekt tot op heden van de nieuwe strategieën vaak nog informatie met betrekking tot hun gebruiksvriendelijkheid en robuustheid in de praktijk onder reële omstandigheden, hetgeen geëvalueerd kan worden door gebruik te maken van capillair bloed, rechtstreeks verkregen vanaf de vingertop. Dergelijke evaluatie is zeer belangrijk met betrekking tot de acceptatie van de nieuwe strategieën als een betrouwbaar alternatief voor volbloedanalyse. Tenslotte dient men in gedachten te houden dat er altijd beperkingen gekoppeld zullen zijn aan methoden die gebruik maken van gedroogde bloedstalen, b.v. in sommige gevallen is het analyseresultaat dringend, waardoor het onmogelijk is om te wachten totdat het monster opgedroogd is. In deze omstandigheden kan het noodzakelijk zijn om naar de klassieke, veneuze bemonstering terug te grijpen. Anderzijds kan het gebruik van gedroogde bloedstalen zeker een voordeel bieden wanneer de klassieke, veneuze staalname onmogelijk is, b.v. in afgelegen gebieden in ontwikkelingslanden of in het geval van thuismonitoring.

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CURRICULUM VITAE

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Education

2014-2019	PhD in Pharmaceutical Sciences, Ghent University
2012-2014	Master in Pharmaceutical Care, Ghent university (magna cum laude) Master dissertation: Evaluation of pharmaceutical care in the treatment of Nodding syndrome in northern Uganda
2009-2012	Bachelor in Pharmaceutical Sciences, Ghent University (magna cum laude)
2003-2009	Science-mathematics, Bisschoppelijk College Veurne

Work experience

PhD student at the Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University (Prof. C. Stove)

Extra-scientific contributions

- Education
    - Practical courses Toxicology (2nd Master in Pharmaceutical Care and Drug Development)
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    - Support of demonstrations for courses Chemical Criminalistics (students Criminology)
    - Supervisor of Pharmaceutical Bachelor dissertations (3rd Bachelor in Pharmaceutical Sciences)
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        2017-2018: Lauren Debacker. Evaluatie van een nieuw volumetrisch systeem voor hematocriet onafhankelijke collectie van gedroogde bloedspots.
    - Supervisor of Honours Program in Life Sciences

        2018-2019: Anne-Sophie Bamelis. Detection and quantification of four anti-epileptic drugs in dried blood spots using LC-MS/MS.
- Laboratory
    - Support in forensic toxicological services

Scientific Curriculum

A1-publications

Velghe S*, Capiau S*, Stove CP. Opening the toolbox of alternative sampling strategies in clinical routine: a key-role for (LC-)MS/MS. *Trends in Analytical Chemistry* 84, 61-73 (2016).

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Other publications

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Velghe S, Stove CP. Dried blood sampling for TDM of anti-epileptics. *IATDMCT Compass* December 2018.

Membership of scientific organizations

Belgium Luxembourg Toxicology Society (BLT)

International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT)

Congress participation

Oral presentations:

1. Fully automated analysis of DBS for five anti-epileptic drugs and one active metabolite, European Bioanalysis Forum, 3rd Young Scientist Symposium (YSS), Barcelona, Spain, November 15th, 2016. (Velghe S, Stove CP)

2. Volumetric absorptive microsampling as an alternative tool for therapeutic drug monitoring of first-generation anti-epileptic drugs, European Bioanalysis Forum, 4th Young Scientist Symosium (YSS), Ghent, Belgium, March 15th-16th, 2018. (Velghe S, Stove CP)

3. Capitainer devices as a hematocrit-independent alternative for classical DBS analysis, 56th TIAFT (The International Association of Forensic Toxicologists) conference, Sciex workshop, Ghent, Belgium, August 26th-30th, 2018. (Velghe S, Stove CP)

Poster presentations:

1. Fully automated direct extraction and analysis of dried blood spots for the determination of five anti-epileptic drugs and one active metabolites, 2nd Annual European Congress of Mass Spectrometry: Applications to the Clinical Lab (MSACL), Salzburg, Austria, September 8th-11th, 2015. (Velghe S, Stove CP)

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4. Fully automated direct extraction and analysis of dried blood spots for the determination of five anti-epileptic drugs and one active metabolites, 9th International Society for Neonatal Screening (ISNS) International Meeting, The Hague, The Netherlands, September 11th-14th, 2016. (Velghe S, Stove CP)

5. Volumetric absorptive microsampling as an alternative tool for therapeutic drug monitoring of first-generation anti-epileptic drugs, 15th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology (IATDMCT), Kyoto, Japan, September 24th-27th, 2017. (Velghe S, Stove CP)

6. Evaluation of the Capitainer-B device as a new hematocrit independent alternative for dried blood spot collection, 16th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology (IATDMCT), Brisbane, Australia, September 16th-19th, 2018. (Velghe S, Stove CP)