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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

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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 within this population is completely lacking, the aim of this study 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, also samples originating from patients suffering from (onchocerciasis-associated) epilepsy were included. In total, 68 patients with Nodding syndrome from Uganda, 58 Ugandan patients with epilepsy and 137 patients with onchocerciasis-associated epilepsy from the Democratic Republic of the Congo (DRC) were included in this study. 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 DRC treated with phenobarbital. In all other instances, concentrations were subtherapeutic. 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 amount 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.

1. Introduction

Nodding syndrome is an unexplained, highly debilitating generalized seizure disorder, typically having its onset in 5-15-year-old children in subregions of sub-Saharan Africa[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 have been performed, resulting in several hypotheses with respect to its etiology, however, none of these hypotheses has been formally confirmed [1, 2, 7-15]. As there is still no known cure, an action plan was developed to provide Nodding syndrome patients with symptomatic care [16]. 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 [16].

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 [17]. 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 [17]. Information about medication effectiveness is completely lacking: whereas in some patients, symptoms decrease, in others, epileptic seizures remain poorly controlled. As there is no information as to whether this may be simply linked to a failure of reaching therapeutic concentrations, the aim of this study was to perform therapeutic drug monitoring (TDM) of AEDs in children suffering from Nodding syndrome, since 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. Furthermore, also patients with other forms of (onchocerciasis-

associated) epilepsy originating from Uganda and the Democratic Republic of the Congo (DRC) were included in this study as also in these patients older generation AEDs are used [2].

Dried blood microsamples (i.e. dried blood spots (DBS) and volumetric absorptive microsampling (VAMS) devices) 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 [18-21]. Besides the advantage of increased analyte stability, these samples also offer the advantage that they are considered as non-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 [22]). The latter is also an important benefit in countries where patients still have to cover a long distance to clinical practices. Fully validated methods applying liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) were used for the analysis of VAMS samples (manual extraction) and DBS (fully automated extraction) [23, 24].

2. Materials and methods

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 within 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 within 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 [25]. For the DRC, study participants were

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patients with onchocerciasis-associated epilepsy enrolled in a clinical trial investigating the effect of an ivermectin treatment on the frequency of epileptic seizures [26].

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).

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 [16]. Within this case definition, a distinction is made between a suspected case, a probable case and a confirmed case [16]. 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 [16].

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

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(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 [27]. Furthermore, for the DRC, patients were excluded when they already received an anti-epileptic or ivermectin treatment before the study.

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. DBS and 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) [28]. 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. 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 within 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 obtained during the last month and type of medication.

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 fully validated methods, described elsewhere [23, 24]. 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 elsewhere [23, 24]. Briefly, validation of the fully automated DBS method revealed an accuracy (%bias), as well as a precision (%RSD) (with a single exception of PB (total precision 16.5 %RSD) at Low QC level) below 13% and for the method making use of VAMS devices, precision was below 10%, while, with a single exception (i.e. VPA 18.2 %bias at Low QC), accuracy also met the acceptance criteria.

3. Results

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 within 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 [23]. 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 Supplementary Table 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 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].

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 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 83.7% (36 out of 43) of the samples the %difference between the initial VPA concentration and the concentration measured during the repeat DBS analysis was not higher than 20% from 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 (-51.0 - 34.7)%.

Besides DBS, also VAMS samples were 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 1, panel B, circles). Also here, incurred sample reanalysis was performed, with 86.1% of the repeats yielding a concentration within 20% of their mean, meeting the acceptance criterion for incurred sample reanalysis (Figure 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 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 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.

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 Supplementary Table 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]). The remainder of the samples had CBZ concentrations below the lower limit of the therapeutic reference range. So, in total only 21.4% (12 out of 56) of the included patients had a concentration within the therapeutic reference range (Figure 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 2, panel A, triangles). As can be concluded from Figure 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 2, panel B, circles). Furthermore, as for the DBS samples, also 23 VAMS samples were included within the incurred sample reanalysis experiment (Figure 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 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.

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 Supplementary Table 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 3, panel A, circles). As was also observed for VPA and CBZ, there were no DBS samples with a PB concentration above the upper limit of the therapeutic reference range.

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 3, panel A, triangles). After exclusion of 2 outliers (detected *via* the Grubbs test for outliers) 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 3, panel B, circles). Furthermore, the same cohort of patient samples as for DBS was included in an incurred sample reanalysis experiment (Figure 3, panel B, triangles). Although with 67.8% 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 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.

3.4. Comparison of the variability within and between DBS and VAMS

The variability which was observed in the two DBS analyses, the two VAMS analyses, and between the DBS and the VAMS analyses, was further investigated. As can be deduced from Figure 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.

3.5 Stability of AEDs in DBS and VAMS

Considering the outcome of the incurred sample reanalysis (section 3.4), the stability was examined more in detail, as this 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 Supplementary Table 2, VPA, CBZ and PB were stable for at least 6 months in VAMS devices

when stored at -20°C. 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 within the validation stability experiments, the data of the stability study was used to make an extrapolation on the 9 months stability (see Supplementary Figure 1). 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.

3.6 Evaluation of the impact of hematocrit

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 [36]. 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, 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 [23, 24]. Furthermore, in Supplementary Figure 2, 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.

4. Discussion

In this study, we applied both DBS- and VAMS-based sampling for the determination of the concentrations of three anti-epileptics, VPA, CBZ and PB in children with epilepsy from Uganda or the Democratic Republic of the Congo (DRC). 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. VAMS samples and DBS were analyzed using fully validated methods, involving manual extraction or fully automated extraction, respectively, prior to quantification using LC-MS/MS. To assess whether the measured concentrations were therapeutic, a conversion from blood (DBS) concentrations to serum concentrations was necessary, as therapeutic reference intervals are only available in the latter. This calculation of serum concentrations based on blood concentrations remains a challenge, as this conversion introduces an additional factor of uncertainty. Furthermore, it is difficult to predict whether the 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. Hence, in an ideal scenario, blood reference intervals should be available. With this limitation in mind, we applied the blood/plasma ratios to calculate serum concentrations from the DBS concentrations. This revealed that 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 DRC treated with phenobarbital. In all other instances, concentrations were subtherapeutic.

Furthermore, when comparing DBS to VAMS concentrations, an inexplicable overestimation was observed in the latter. When considering the results of the incurred sample reanalysis, a larger spreading was observed for VAMS than for DBS. Several possibilities were explored to find an explanation for this observation. Our stability data, 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. On the other hand, since it is known that humidity can have an impact on sample stability and since -for the samples obtained in the field- the local

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storage conditions (combination of temperature with humidity) were not included in the validation experiments of the used methods, we cannot fully exclude a stability issue. Ideally, an expanded stability study, covering the entire sampling process (at local temperature and humidity conditions of sampling and transport prior to storage at -20°C), with reference samples that are instantly frozen, could be performed. However, this was logistically not possible within our study.

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]. Based on our results, however, we could 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, within 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, also calibration issues could be ruled out. 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, 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 measured blood concentrations with the number of seizures experienced during the last month before sampling, ambiguous results were obtained, e.g. some patients with a VPA concentration below the therapeutic reference range experienced

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no seizures, whilst others with a concentration within the therapeutic reference range still had for example 10 seizures. 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 [37].

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. For all other DBS samples, either the analyte was not detected, the signal was below the LLOQ, or the calculated serum concentration fell below the lower limit of the therapeutic reference range. 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.

Finally, when comparing the obtained concentrations with the amount of seizures obtained 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.

6. Acknowledgements

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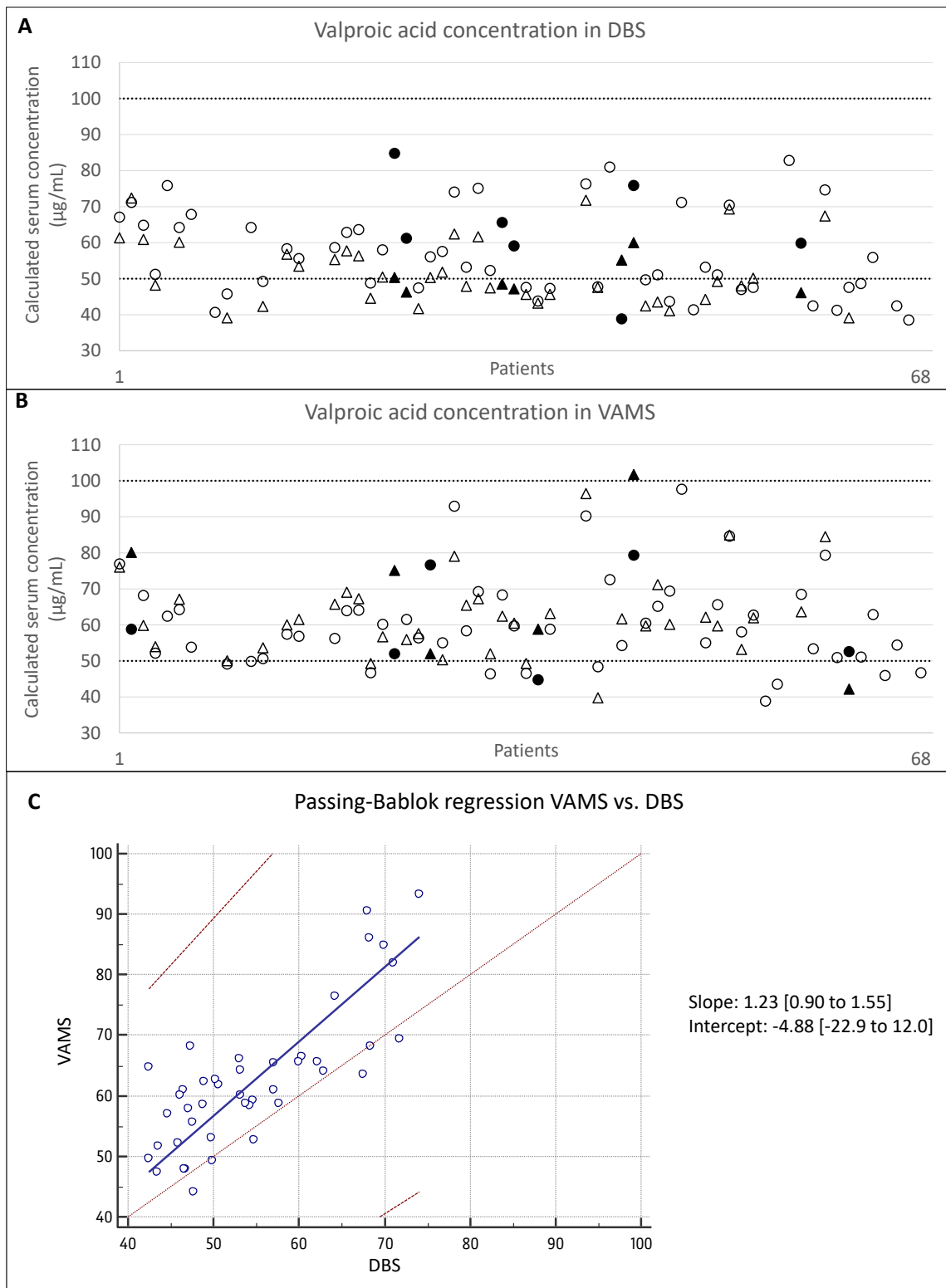
Figure 1. A: incurred sample reanalysis for DBS samples. B: incurred sample reanalysis for VAMS samples. The circles correspond to the calculated serum concentrations in the initially analyzed samples and the triangles to the calculated serum concentrations in the repeated samples. The black colored 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.
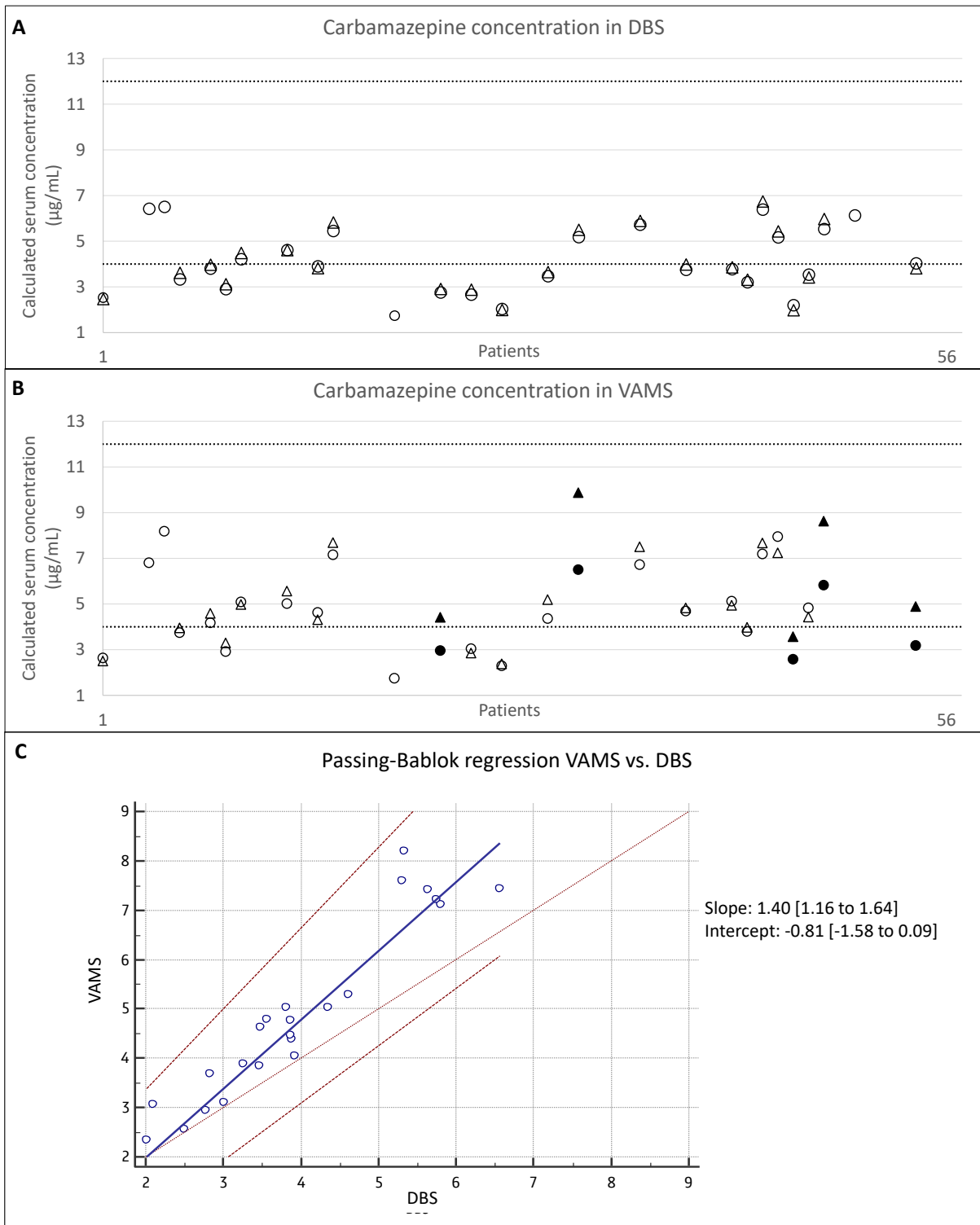
Figure 2. A: incurred sample reanalysis for DBS samples. B: Incurred sample reanalysis for VAMS samples. The circles correspond to the calculated serum concentrations in the initially analyzed samples and the triangles to the calculated serum concentrations in the repeated samples. The black colored 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.
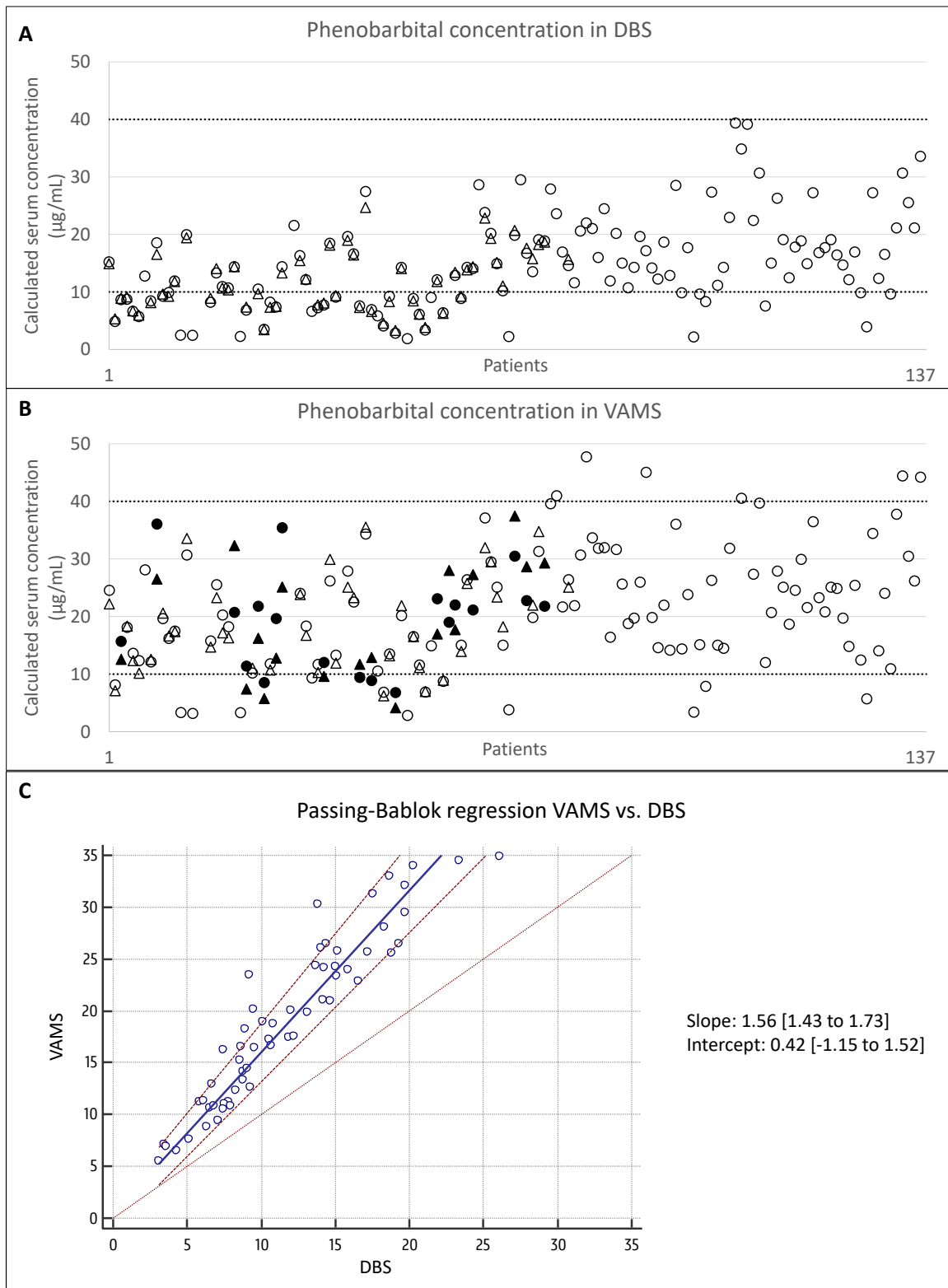
Figure 3. A: incurred sample reanalysis for DBS samples. B: incurred samples reanalysis for VAMS samples. The circles correspond to the calculated serum concentrations in the initially analyzed samples and the triangles to the calculated serum concentrations in the repeated samples. The black colored 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.
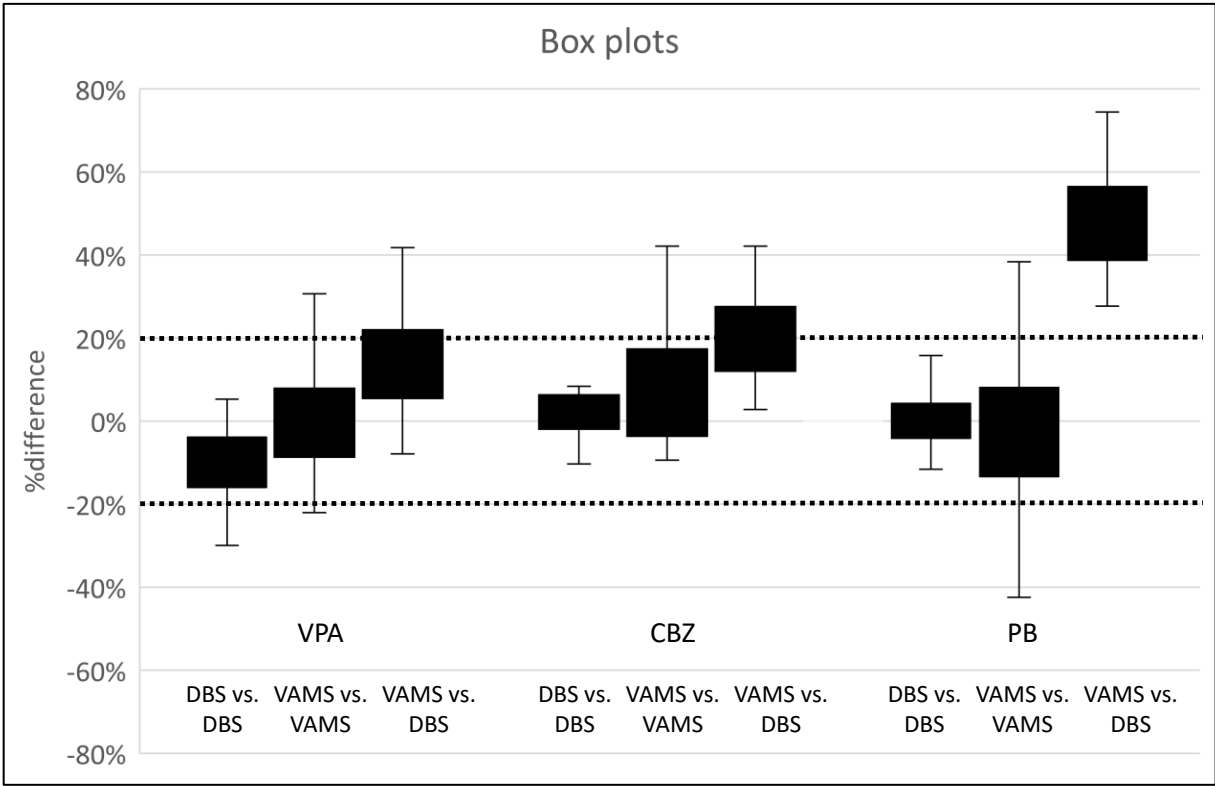
Figure 4. *Box plots for %difference of DBS vs. DBS, VAMS vs. VAMS and VAMS vs. DBS for VPA, CBZ and PB. The dotted lines indicate the ± 20% deviation limits.*

Supplementary data

Calculated blood/plasma ratio based on the analysis of 21 (VPA), 18 (CBZ) and 13 (PB) left-over whole blood samples.

	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%

Supplementary Table 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%

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	

Long-term stability prediction at -20°C for VPA, CBZ and PB in VAMS samples. Dotted lines indicate the ±15% acceptance limits.
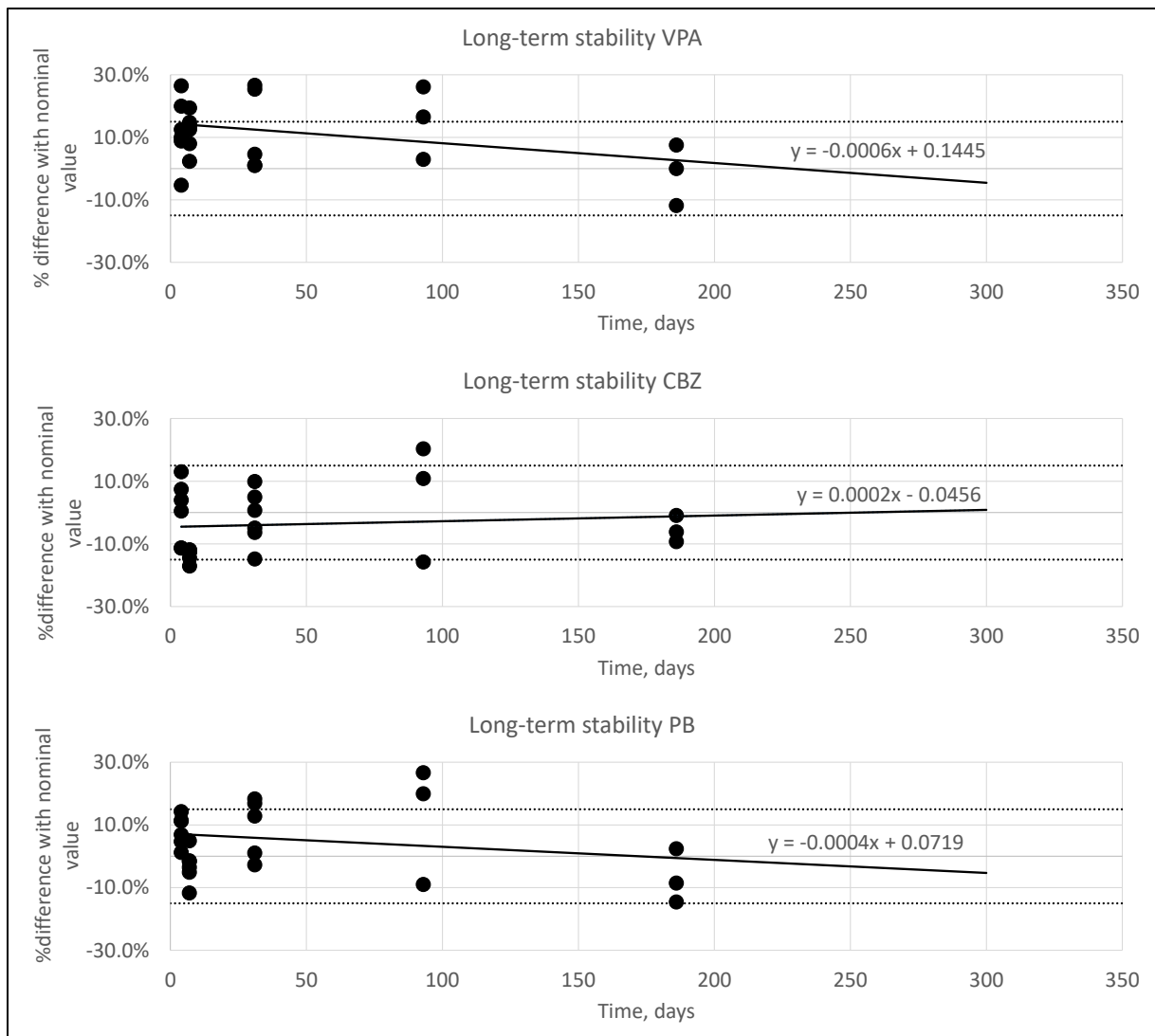
%difference between concentrations obtained via the analysis of VAMS and DBS in function of a patient's hematocrit.