Introduction
Over the last decade, DBS analysis has gained popularity for TDM because it's a patient friendly sampling procedure [1-4]. Additional advantages are prolonged sample stability, lower risk of infections and transportation at ambient temperature [1-3,5]. These advantages may facilitate implementation of TDM in different clinical settings including resource limited areas. Patients will benefit from DBS analysis but the analytical development and validation of DBS methods is more complex compared to plasma or serum analysis.

Additional validation parameters, like the effect of the hematocrit (HT) and blood spot volume need to be evaluated. Drug substances may also interact with the blood matrix or with the DBS card, resulting in matrix related recovery effects. Unfortunately, official guidelines for validation of DBS are not available yet. However, in recent literature, several interesting issues related to analytical DBS research have been discussed [1,6-12]. Ongoing research and improved understanding of the factors that influence DBS analysis results will ultimately result in well-founded guidelines for DBS analytical method validation. This would be very helpful for daily practice but would also benefit patient safety because uniformity in method validation prevents potential pitfalls during validation or method development and increase credibility of assay results.

Our aim is to discuss some relevant topics related to DBS development prior to development of future guidelines on DBS development and validation.

The influence of blood hematocrit value on analytical results.
It is well known that the HT may affect analytical results. The blood HT affects the blood viscosity and that in turn influences the formation of a blood spot, which affects the analytical results. The preparation of the target HT values for standards and quality control samples is of great importance and a recent study has shown that the best result was obtained by centrifuging blood, followed by removal or addition of a calculated volume of plasma to adjust the HT [7]. However, it is advised to let the prepared HT be measured in order to confirm the correct preparation. In addition, extraction efficiency may also be influenced by the HT and concentration of the substance. It is important to investigate these influences before the analytical procedure can be implemented in routine patient care [13]. In daily practice, correction of analytical results for the HT value using a linear HT correction method is simple but may not always correct for all HT effects. In that case, a point-to-point relationship between recovery, HT and concentration may be more feasible. Recently, a DBS method for the determination of the HT by potassium measurement was published [14]. However, most likely a second spot is required for the potassium measurement using an immunoanalyzer. This implies a significant amount of work for the development and measurement of the HT value and subsequently for setting up the correction formula. When no HT correction is applied, the method validation should include a range that covers substance concentration and a HT of the target population with the analytical re-
results within acceptable limits. The approach of whole spot analysis or pre-cut disks will show no effects of the HT on the spot formation [15-18]. However, if extraction efficiency is affected by HT and concentration of the substance, this effect cannot be compensated. In addition, the main advantage of a procedure for partial spot DBS analysis is the ease of self-sampling for the patient, which is more complicated and more prone to sampling errors when exact blood volumes have to be applied. However, if a health care professional is performing the dried blood spot procedure and extraction efficiencies are stable, a whole spot analysis or pre-cut disks can be of advantage to avoid the HT effect.

**The influence of the matrix on DBS recovery**

Some substances may be difficult to extract from the spotting card matrix or they may form complexes with the endogenous components present in the DBS matrix [8, 9]. The extraction efficiency may be influenced by different aspects like nature of the DBS material, extraction conditions, HT and concentration of the substance. Although most analytical DBS procedures may (apparently) show no interaction of the analyte with the blood in the DBS card or with the DBS card matrix itself, some studies showed an altered analytical response. The researcher should be aware of these possible interactions in order to acknowledge them in an early stage of the analytical method development. For example, iron from the blood interacts with rifampicin to form a complex [8]. Addition of both deferoxamine and ethylenediaminetetraacetic acid (EDTA) to the extraction solution was applied to form a complex with the iron and recovery of rifampicin in the DBS extraction increased to approximately 100% [8]. Another study showed that the combination of low HT and high drug concentration does not only affect the spot size but also negatively affects the extraction recoveries [9]. An increased number of hydrogen bond acceptors was associated with more affinity to form hydrogen bonds with the cellulose of the DBS card, making the extraction less efficient.

**Sample dilution in DBS analysis**

When DBS analysis is performed for the measurement of trough levels, a sufficient linear range can easily be validated. For the analysis of pharmacokinetic curves, the linear range should be large enough or a dilution protocol should be validated. Various types of dilution protocols can be validated. The DBS extract can be diluted with extraction solvent or blank extract. With this approach, the dilution can be performed after the concentration is measured above the linear range. The DBS can be extracted with a larger extraction volume, or a smaller diameter can be punched and extracted with the original extraction volume. However, for both approaches, the concentration should be expected to be above the linear range beforehand. Including a smaller punch diameter in addition to the standard punch size during validation could also be an option for spots that would otherwise be too small for analysis and would be rejected.

**Measuring endogenous substances with DBS**

In order to make DBS analysis even more patient friendly, the analysis of multiple substances in a single DBS extract would be preferred. This could include exogenous and endogenous substances, like for example the additional analysis of creatinine. For the measurement of endogenous substances in DBS, the preparation of calibration standards and quality control samples pose a challenge, because it is impossible to obtain an analyte free matrix without changing the matrix itself. Changing the matrix by washing the red blood cells will have significant effects on the formation of the blood spot and gives possible other interactions with the blood matrix and DBS card.

A recent publication describing the validation of creatinine in DBS addressed this issue by using three different validation strategies; a 7-point calibration curve using the intercept of the calibration to correct for the natural presence of the creatinine in reference samples; a one point calibration curve at an extremely high concentration in order to diminish the contribution of the natural presence of creatinine and the use of creatinine-[H] with an eight-point calibration curve [19].

**Analytical vs clinical validation**

In order to implement DBS analysis in daily practice, clinical validation studies need to be performed capturing the variability that was not included during analytical validation. During clinical validation the overall results from the DBS procedure should be compared with conventional sampling. Till today no consensus has been reached for the number of patients to include in a clinical validation. Numbers of 10 to 50 have been mentioned in literature. Appropriate statistical tests should be used to compare results from DBS analysis and conventional blood sampling. To date, the most applied tests are Bland Altman analysis, Deming regression and Passing and Bablok regression [20-22]. Foreseeing daily routine, it seems important to have strict criteria considering the punching procedure (full and partial spot analysis) and type of DBS card, because these differences require a full validation during analytical de-
development. In addition, assessment of sample stability during ‘real life’ storage and shipment of samples, could be evaluated by sending QC samples to the sampling environment for execution of the routine procedures followed by re-sending the QC samples to the reference lab. For best implementation of the procedures, personal DBS sampling instructions should be performed and they should be available in a flyer and a video.

To summarize The DBS analytical procedures are influenced by parameters like concentration range, HT value, matrix interactions and patient population. Consequently, the validation parameters should be adjusted to the specific patient population. The HT can be set at the mean HT of the patient population and prepared appropriately as discussed before. QC samples can be set at therapeutic concentrations for trough levels and are used to assess the HT effect and recovery. The more restricted framework of parameters may show far less effects of the HT and the combination of HT and concentration dependent recovery. The approach of the restricted framework may make the analysis of an extra DBS for HT assessment unnecessary and will create a more efficient workflow in the laboratory. The use of DBS for TDM is very promising, but there are several additional parameters compared to plasma analysis that should not to be underestimated and need to be considered before performing patient analyses. To conclude, the gained knowledge for analytical DBS development and validation has certainly evolved to a higher level but has not yet reached its final stage. An official guideline developed by scientific societies which is periodically updated would be useful for the field of DBS analysis.

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