Review

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Preanalytical considerations in therapeutic drug monitoring of immunosuppressants with dried blood spots

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Abstract

Background: Dried blood spots (DBSs) could allow patients to prepare their own samples at home and send them to the laboratory for therapeutic drug monitoring (TDM) of immunosuppressants. The purpose of this review is to provide an overview of the current knowledge about the impact of DBS-related preanalytical factors on TDM of tacrolimus, sirolimus and everolimus.

Content: Blood spot volume, blood spot inhomogeneity, stability of analytes in DBS and hematocrit (Hct) effects are considered important DBS-related preanalytical factors. In addition, the influence of drying time has recently been identified as a noteworthy preanalytical factor. Tacrolimus is not significantly influenced by these factors. Sirolimus and everolimus are more prone to heat degradation and exhibited variations in recovery which were dependent on Hct and drying time.

Summary and outlook: DBS-related preanalytical factors can have a significant impact on TDM for immunosuppressants. Tacrolimus is not significantly influenced by the studied preanalytical factors and is a viable candidate for DBS sampling. For sirolimus and everolimus more validation of preanalytical factors is needed. In particular, drying conditions need to be examined further, as current protocols may mask Hct-dependent effects on recovery. Further validation is also necessary for home-based self-sampling of immunosuppressants as the sampling quality is variable.

Keywords: dried blood spot; dried blood spot testing; drying time; filter paper; hematocrit; immunosuppressants; microsampling; preanalytical phase; stability; therapeutic drug monitoring.

Introduction

The use of immunosuppressants after solid-organ transplantation contributes to the quality of life of organ recipients and offers a significant advantage in terms of survival [1]. However, the narrow therapeutic range of these drugs necessitates therapeutic drug monitoring (TDM) in order to avoid toxicity and transplant rejection [2]. TDM is often associated with a significant patient burden, as regular trips for blood sampling by a medical professional are necessary.

Dried blood spot (DBS) sampling consists of the deposition of a drop of capillary blood (preferably from the finger in adults) on filter paper by either the patient or a medical professional. The spot is then left to dry (drying times typically range from a couple of hours to overnight drying) and is shipped to the laboratory. After arrival at the laboratory, a sample is punched from the DBS, which is then followed by an extraction procedure and analysis of the sample [3]. DBS sampling has long been used for qualitative or semi-quantitative analysis such as the heel-prick for the detection of inborn errors of metabolism popularized by Guthrie [4].

Recent developments, in particular the use of triple quadrupole liquid chromatography-tandem mass spectrometry (LC-MS/MS), allows quantitative analysis of a wide range of analytes due to increased sensitivity. In the past, the small amount of analyte in a punch taken from a DBS posed a problem for less sensitive assays. Consequently, interest has been rising in DBS sampling as a minimally invasive sampling method that could provide a benefit in terms of cost, ease of use and transportation, patient comfort and sample stability [3, 5, 6]. A wide variety of analytes has been studied in DBSs, including therapeutic drugs for TDM, drugs

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of abuse, serological testing, microbiology, endocrinology, clinical chemistry and genetic testing [7].

In particular, TDM of immunosuppressive agents in DBSs may be of interest because of the possibility of self-sampling. This would allow patients to prepare their own DBS at home and send them to the laboratory through regular postal services as a primary means of TDM, or to perform additional self-sampling in order to better characterize the pharmacokinetic profile of their immunosuppressive drug [2, 8–10].

Multiple studies have been published regarding the TDM of immunosuppressive agents in DBSs (most notably tacrolimus, sirolimus and everolimus), all of which concluded that DBS sampling is a valid alternative for venous sampling. However, most studies have been performed in controlled environments and were focused on analytical performance, often using venous sampling i.e. the application of a drop of ethylenediamine tetraacetic acid (EDTA) blood by a laboratory technician] instead of true capillary sampling. The impact of preanalytical factors specific to DBSs may be underestimated. In as many as 40% of published assays using DBSs for various analytes, not

Figure 1: A summary of the important preanalytical factors for analysis of immunosuppressants with capillary DBS sampling.

Figure 2: DBSs at different Hct levels (panel A) and using different blood volumes (panel B).

The experiment was performed using K_3 -EDTA anticoagulated blood from a single volunteer. For panel A, each circle contains 75 µL K_3 -EDTA anticoagulated blood. For panel B, the Hct was 45%.

one DBS-specific validation parameter has been studied [11]. As preanalytical errors are the main cause of errors in laboratory testing [12], more attention for DBS-specific preanalytical factors is needed, especially if self-sampling by patients is to become a reality.

Possible sources of errors in the preanalytical phase described in the literature include, among others, the quality of the self-sampled blood spot, chromatographic effects (i.e. the inhomogeneity of the spot), the influence of blood spot volume and hematocrit (Hct) and the stability of the analyte in the DBS [3, 11, 13, 14]. Recently, more attention has also been given to the influence of variations in drying time [15]. Figure 1 gives an overview of the important preanalytical factors for TDM of immunosuppressants with DBS and Figure 2 illustrates the effect of volume and hematocrit.

We performed a thorough literature search to identify the current knowledge about the impact of DBS-related preanalytical factors on TDM of tacrolimus, sirolimus and everolimus, and to examine DBSs to what extent DBS-related preanalytical factors are taken into account in published methods. The following preanalytical factors were studied: the influence of blood spot volume, Hct and drying time, blood spot homogeneity and influence of punch location, stability of the analyte, the influence of the DBS card type and the quality of (self-sampled) DBSs.

Literature search

The MEDLINE database has been searched with the use of PubMed for the following MeSH terms: “Dried Blood

Spot Testing” [Mesh] combined with “Tacrolimus” [Mesh], “Sirolimus” [Mesh], “Everolimus” [Mesh]. In order to compensate for the relatively new MeSH term “Dried blood spot testing”, which was only introduced in 2012, a free-text search using the phrase “Dried blood” combined with the aforementioned immunosuppressants was also performed. The last search was performed on 10/05/2018. Studies were included if they described method development and/or validation, method comparison, clinical validation or correlation study in humans for TDM of tacrolimus, sirolimus and/or everolimus in DBSs using LC-MS/MS. As ciclosporin A use is diminishing in our center and the use of this drug is projected to further decrease, we decided not to include it in our analysis.

Twenty-one methods and clinical validations were included [8, 10, 16–34]. In addition, three studies evaluating a single DBS-specific parameter in the context of immunosuppressants [15, 35] and three studies using TDM of tacrolimus in DBSs as a validated method for pharmacokinetic studies were included [9, 36, 37]. Two studies concerning TDM of immunosuppressants in DBSs were rejected due to the use of a method other than LC-MS/MS for analysis.

Impact of DBS-related preanalytical factors

Differences between filter cards

Results of the literature search

The most commonly used card in the included validation studies is the Whatman 903, followed by the Whatman nr. 10535097 and various Whatman FTA cards.

One method validation performed a cross-validation between Whatman 31 ET CHR paper and Whatman FTA DMPK-C cards and found no significant differences in analyte concentration [32]. In a separate study by Koster et al., five DBS cards were compared for the analysis of immunosuppressants (Whatman 31 ET CHR, Whatman FTA DMPK-C, Whatman 903, Perkin Elmer 226 and Agilent Bond Elut DMS DBS cards) [35]. All cards displayed a concentration- and Hct- dependent recovery pattern. In the low concentration ranges of all tested immunosuppressants, the effects of Hct on the formation of the blood spot did not vary substantially between the tested filter cards and the cards showed little difference in performance. In terms of recovery, Whatman 31 ET CHR cards were most

affected by increasing Hct concentrations, which lead to decreasing recoveries. Whatman DMPK-C cards showed to be most resistant to the effect of increasing concentration at low Hct [35].

Discussion

A range of different filter cards is available with different physical characteristics. Typical properties of filter cards are particle retention, pore size, thickness and weight, which are properties that determine the spreading of whole blood on the paper and loading capacity [5]. While the most commonly used cards are untreated cellulose filter cards, some cards are impregnated with chemicals designed to lyse cells, inactivate pathogens or denature enzymes and other proteins [3]. Two specific cards are preferred in the literature due to experience that has been built up through the use of these cards for neonatal sampling. These cards are the Whatman 903 and Ahlstrom 226, both untreated cellulose filter cards, which are recommended by the Clinical and Laboratory Standards Institute (CLSI) guideline for neonatal screening, and are approved by the Food and Drug Administration, the Newborn Screening Quality Assurance Program (NSQAP) and the Centers for Disease Control [38, 39]. Antunes et al. reported that the Whatman 903 was used in 60% of all reviewed publications regarding TDM in DBS, which is consistent with our findings [40]. Other untreated (e.g. Whatman FTA DMPK-C) and chemically treated (e.g. Whatman FTA Elute, FTA DMPK-A and FTA DMPK-B) cards exists, however, these are not CLSI LA4-A5 compliant.

Due to their different physical characteristics, different types of filter card are likely to exhibit differences in terms of extraction recovery, matrix effects, analyte stability, chromatographic effects, Hct-related effects and volume effects [3]. Although similar results have been found in a limited cross-validation of two cards by Koster et al., the general consensus is that filter cards are not freely interchangeable and the European Bioanalysis forum (EBF) recommends that a method needs to be fully validated per filter card type and any change of card type or manufacturer requires at least a partial validation [14, 32].

Quality of capillary (self-)sampling

Results of the literature search

In 12 publications capillary sampling was performed in order to create the DBS, either as the primary sampling method or alongside the creation of DBSs from venous

blood by a medical professional. The quality of the DBS was assessed by visual inspection. The most common criteria used to judge the acceptability of a DBS sample included (almost) complete and homogenous filling of the predrawn circle and a dark red color on both sides of the paper [10, 19, 41, 42].

In five publications, capillary DBS sampling was performed by a trained professional. Three of these studies reported the percentage of rejected samples due too poor blood spot quality, which consisted of 4.8% ($n=210$), 11.1% ($n=18$), and 4.4% ($n=91$) rejected samples [26, 29, 41].

Capillary sampling was performed by the patients themselves (self-sampled DBS) in seven publications. Of these studies, four took place in a hospital or clinic setting and three studies evaluated or used home-based self-sampling. In publications that reported the percentage of unsuitable samples (three studies), the percentage of unsuitable samples consisted of 1.8% ($n=108$) for self-sampling in a hospital setting [10] and consisted of 3.8% ($n=26$) and 11.6% ($n=216$) in a home setting [23, 34]. Regarding the three pharmacokinetic trials using home-based self-sampled DBSs, one study reported that 3% ($n=400$) of pharmacokinetic profiles had to be repeated due to low quality or missing samples [36].

Discussion

When DBSs are prepared by capillary sampling, the quality of the blood spot becomes an important pre-analytical factor, especially in the context of home-based self-sampling. A crucial parameter for self-sampled DBSs is the amount of usable samples that patients can generate themselves. It has been found that adequate training is not a guarantee for successful sampling [5, 13]. Peck et al. showed a concerning lack of quality in blood spots for lead screening, even when sampling happened by trained professionals. Sixty-two percent of blood volumes were lower than 25 μL , well below the recommended 50 μL per spot for Whatman 903 paper [43]. In accordance with this, Martial et al. noted that the blood samples they had obtained from children for the analysis of immunosuppressants were much smaller than they had initially anticipated [22]. In addition, Peck et al. reported that the application of multiple drops (instead of one continuous drop, as recommended by existing guidelines) was prevalent. Application of multiple drops can lead to falsely elevated values due to a more concentrated blood spot [43]. A feasibility trial for home-based self-sampling for various analyses in a motivated population of women above 50 years of age reported that only 74.1%

of the received samples were taken according to instructions (although 92.9% were suitable for at least one biochemical analysis) [44]. Recently, a feasibility trial was conducted by Al-Uzri et al. to evaluate the possibility of home-based monitoring of tacrolimus and creatinine by DBSs in children. They reported that, although 88.4% of the DBS cards were deemed suitable at first, only 80% of the blood spots on these cards were properly saturated and suitable for analysis [23].

A visual inspection of the sample is the most commonly used method to judge the acceptability of a DBS. The most important visual criteria are: (almost) complete filling of the predrawn circle, a symmetrical spread around the center and an even, dark red color on both sides of the DBS card [5]. As for the size of the blood spot, Edelbroek et al. require a blood spot that is at least larger than the punch which will be taken [5], whereas most publications require complete filling of the pre-drawn spot [3, 8]. Furthermore, DBSs that exhibit clotting, layering, super-saturation, serum rings, visible traces of hemolysis or contamination should be rejected [3].

All studies concerning self-sampled DBSs for TDM of immunosuppressants in a hospital or clinic setting showed a sample quality comparable to that of trained personnel. In addition, the results from blood spots made by patient and trained personnel did not differ significantly [32], which was also true for blood spots made with and without nurse assistance [8]. In the case of home-based self-sampling, results were more variable. While rejection rates as low as 3% are reported, larger feasibility trials mimicking real-life situations report 11.6% rejected DBS cards, and 20% of the accepted blood spots being ultimately unsuitable for analysis. Several other studies concerning different analytes have reported similar percentages of rejected samples (17–19% rejected samples) [45]. This may be a realistic outcome for a patient population that has not been extensively pre-selected. A clinical trial in progress has been halted because of inadequate sample quality, suggesting that preanalytical sample quality in (self-sampled) DBSs is important [46]. Additionally, timing and adherence may be a problem, as evidenced by Al-Uzri et al., who noted that only 38% of the samples were drawn at the correct time [23].

Although self-sampled DBSs are often mentioned as a potential advantage of DBS sampling, further research is needed and a validation in a true home-based self-sampling scenario needs to be performed. Problems regarding sample quality, timing and adherence to self-sampling may occur. Self-sampled DBS at home may primarily be feasible in a selected population of motivated and capable patients.

Blood spot volume

Results of the literature search

Seven publications included in this review have examined the effect of blood spot volume on analyte concentration in their validation protocols (Table 1). The examined volumes ranged from 15 to 100 µL. All seven studies noted an effect of blood volume on immunosuppressant concentration, but this effect was not deemed to be significant, with the exception of den Burger et al., who reported biases of >15% for everolimus at low concentration and sirolimus at high concentration in 20 µL spots [25]. Knapen et al. reported that the slope of the calibration curve for everolimus differed significantly between different blood volumes at a low Hct level (20%), an effect not found at a Hct level of 40% [20]. Additionally, Hoogtanders et al. examined the effect of blood spot volume by weighing punches from blood spots made with different volumes of blood. They found that punches weighed less and thus contained less blood when 10 µL of blood was used to make the blood spot versus 20 or 30 µL, but no difference between the two latter options was found [41].

Discussion

The volume of the blood spot has been described as an important parameter which should be included in all validation studies [40]. The EBF calls the investigation of the effects of blood spot volume an integral part of the

validation of a DBS method [14]. The volume of blood that is deposited on the filter card through the use of a capillary prick is not controlled and may differ between spots on the same filter card and between patients. On cellulose-based cards (e.g. Whatman 903) an increase in blood volume will bring forth a linear increase in blood spot area. Theoretically, this should not affect the volume in a single punch, DBSs have been compared to very flat cylinders of blood which has spread continuously and homogenously through the paper. As such, a punch from a filter card should contain the same amount of blood regardless of blood spot volume [3]. However, with large differences in blood spot volume (>20 µL), differences in blood volume contained in the punch and consequently concentration differences in analytes such as phenylalanine have been described [3].

In addition to the volume of the blood drop used to make the spot, the saturation properties of the paper may influence the effect of blood spot volume. For Whatman 903 paper, concentration differences in various analytes were observed with volumes up to 50 µL, and a stabilization between approximately 50 µL and 100 µL was reported, presumably due to full saturation of the paper [3]. Den Burger et al. confirmed this effect for tacrolimus, sirolimus and everolimus [25]. As a consequence, the effects of blood spot volume are dependent on paper type and should be validated together [3].

No significant interference from blood spot volume on the concentration of immunosuppressants was reported, except for sirolimus and everolimus in 20 µL spots [25]. Den Burger et al. pointed out, however, that these spots would have been rejected in practice due to incomplete

Table 1: Influence of blood spot volume in method and clinical validations.

Study	Card type	Range, µL	Crit.	Drug	Effect of variation in blood spot volume
Koster et al. [32]	Whatman nr. 10535097	30–90	15%	TaC SiR EvE	No significant effect on analyte concentration
Sadilkova et al. [28]	Whatman 903	25–100	n/a	TaC SiR	No significant effect on analyte concentration
Den Burger et al. [25]	Whatman 903	20–100	15%	TaC SiR EvE	No significant effect on analyte concentration 20 µL spot and HC: approx. –17.5% bias 20 µL spot and LC: approx. –19% bias
Li et al. [30]	Whatman 903	15–50	15%	TaC	No significant effect on analyte concentration
Martial et al. [22]	Whatman 903	20–60	15%	TaC	No significant effect on analyte concentration
Knapen et al. [20]	Whatman 903	20–50	15%	EvE	At 20% Hct: difference of slope of calibration curve >15%
Koster et al. [21]	Whatman FTA DMPK-C	30–70	15%	TaC SiR EvE	No significant effect on analyte concentration

TaC, tacrolimus; SiR, sirolimus; EvE, everolimus; n/a, not available; crit., acceptability criterion; HC, high concentration; LC, low concentration.

filling of the predrawn circle [25]. The volume of a blood drop obtained by capillary sampling has been estimated to be approximately 30 μL [10, 42]. Knapen et al. also reported a combined effect of low Hct and blood spot volume on the slope of the calibration curve for everolimus. This effect was deemed to be primarily explained by the Hct effect [20].

The lack of clinically significant interference from blood spot volume is in accordance with a recent comprehensive review by Wagner et al., which concluded that the effect of blood spot volume appears to be negligible in a volume range of approximately 20 μL around the calibration standard and accurate control of blood spot volume is not necessary [3]. A volume of 30 μL is often used as a calibration standard, as it approximates the volume of a drop of blood and assures filling of the DBS spot [19].

Jager et al. recommend that a volume range of at least 15–40 μL should be investigated during validation at a minimum of two concentration levels (low and high) [11]. All included studies that performed a validation for the effect of blood spot volume examined the effect at different concentrations, but only one study used a range starting from 15 μL . In general, more attention was given to relatively high volumes. This does not reflect clinical practice, where low volumes are more prevalent and may be more likely to cause clinically significant bias [43]. The current overrepresentation of

higher volumes may mask clinically relevant effects, however, it should be noted that low blood spot volumes often lead to samples that would be rejected due to incomplete filling of the blood spot and thus would not contribute to false results.

Hematocrit

Results of the literature search

Eight publications studies examined the effect of Hct on the concentration of immunosuppressants (Table 2). The majority of method validations and all clinical validations and correlation studies reported measurable, but not necessarily clinically significant effects. In four studies, a correction was applied for the expected Hct value of the target population. Koster et al. found significant biases for sirolimus and everolimus (–20% and –28%, respectively) after correction at a Hct of 20% and high analyte concentration (40 $\mu\text{g/L}$) but found that these biases were much less pronounced when using concentrations that were more clinically relevant, and a different DBS card [21, 32]. Sirolimus showed a bias of –15.1% at a Hct of 28% and a concentration of 3 ng/mL . Knapen et al. found that bias exceeded 15% for everolimus when Hct was 20% and the concentration was 20 or 40 $\mu\text{g/L}$ [20]. Martial et al. reported

Table 2: Influence of Hct in method and clinical validations.

Study	Card type	Hct range or mean, %	Crit	Drug	Hct effect on bias
Koster et al. [32]	Whatman nr. 10535097	20–50%	15%	TaC SiR EvE	No significant effect after correction for target Hct Hct 20% and HC: –20% bias Hct 20% and HC: –28% bias
Koster et al. [32]	Whatman nr. 10535097	Mean: Outpatient: 38.4% Inpatient: 29.5%	15%	TaC SiR EvE	No significant effect (no correction)
Sadilkova et al. [28]	Whatman 903	20–45%	n/a	TaC SiR	No significant effect (no correction)
Den Burger et al. [25]	Whatman 903	22–41%	15%	TaC SiR EvE	No significant effect after correction for target Hct
Li et al. [30]	Whatman 903	23.2–48.6%	15%	TaC	No significant effect (no correction)
van Boekel et al. [9]	Whatman nr. 10535097	39%	–	TaC	No significant effect on correlation with venous blood (primary outcome)
Koster et al. [21]	Whatman FTA DMPK-C	23–53%	15%	Tac EvE SiR	No significant effect Hct 28% and LC: –15.1% bias Hct 20% and HC: –18 to –21.9% bias
Knapen et al. [20]	Whatman 903	20–50%	15%	EvE	Hct 15% and MC: –24% bias
Martial et al. [22]	Whatman 903	15–50%	15%	TaC	Hct 20% and MC: –16% bias

TaC, tacrolimus; SiR, sirolimus; EvE, everolimus; n/a, not available; Hct, hematocrit; crit., acceptability criterion; HC, high concentration; MC, medium concentration; LC, low concentration.

significant biases for tacrolimus at Hct levels of 15 and 20% at a medium concentration [22]. An evaluation of the performance of five DBS cards concluded that Hct effects were of minor impact at low concentrations of tacrolimus, sirolimus and everolimus [35].

One method validation examined the effect of Hct on the recovery of immunosuppressants. The recovery of tacrolimus was not affected by Hct. However, sirolimus and everolimus showed a decreased extraction recovery which was most pronounced at low Hct values (25%) and high concentration (50 µg/L) [32]. In an evaluation of five different DBS cards, decreasing recoveries were primarily found at higher Hct values, while recovery remained constant in a Hct range of 10–40% for tacrolimus and low concentrations (3 µg/L) of sirolimus and everolimus. For the latter two drugs, recoveries at high concentration (100 µg/L) were lower than at low concentration for all Hct levels [35].

The Hct levels in blood samples of 1508 patients that underwent TDM for immunosuppressants at our center between ranged from 10.5 to 57.2%. Of all patients, 95% percent had a Hct between 21.0% and 49.4%.

Discussion

The influence of Hct on the results obtained through DBS sampling has been called the single most important parameter defining compound behavior and DBS assay performance and may play a significant role in the slow uptake of DBSs in clinical practice [47]. The EBF recommends that every validation of a DBS method should evaluate the effect of Hct [14, 47]. In accordance with this, an increasing amount of studies have started including the effect of Hct in validation studies [11].

The potential effect of Hct is two-fold and may be divided into an analytical and a physiological aspect [48]. In regard to the analytical effect, Hct influences several factors such as the spread of blood on the filter card, the homogeneity of the blood spot and the drying time needed. In the classic Hct-associated interference, a higher Hct will lead to a smaller, denser blood spot on cellulose-based paper [48]. The blood spot area decreased by 15% on Whatman 903 paper when Hct was increased from 30 to 70%. A punched-out sample from a blood spot from a patient with a high Hct will therefore contain more blood, leading to an overestimation of the true concentration. In Whatman 903 paper, a blood volume difference of 35% was reported in punches taken from blood spots created with blood with a Hct of 20–80% [49].

The analytical effects of Hct are, however, not limited to the increased blood volume per spot. Changes in Hct

influence chromatographic effects and they may interfere in analyte extraction from the blood spot. A high Hct may lead to the formation of a barrier after drying, which interferes in extraction of the analyte [48]. In addition, a low Hct has been found to decrease recovery of certain analytes such as immunosuppressants as well [32].

The physiological effect of Hct consists of its effect on the blood-to-plasma ratio of certain analytes. As TDM is often based on measurements in serum or plasma, results obtained from whole blood need to be converted. This conversion relies on the blood-to-plasma ratio, which is dependent on Hct, especially for analytes that primarily reside in the plasma fraction and do not bind or enter erythrocytes [48]. However, the immunosuppressive drugs included in this review, i.e. tacrolimus, sirolimus and everolimus, are already measured and reported in whole blood and trough levels are measured before the administration of the next dose, minimizing the effects of the speed of drug distribution between capillary and venous blood. Consequently, no significant difference between venous samples and DBS samples is expected in this regard [5].

In general, the effect of Hct on the concentration of immunosuppressants was not deemed to be significant in clinical practice or could be corrected relatively easily. Tacrolimus was least affected by Hct. Only one of the included studies reported a significant bias at the very low end of clinically relevant Hct levels. The lack of Hct effect in regard to tacrolimus has been noted to be sufficiently proven by Veenhof et al., such that no additional correction for Hct was deemed to be necessary in their method validation [29].

Sirolimus and everolimus are more susceptible to interference from Hct. A bias that persisted after the correction has been noted at low Hct levels (20%) and high concentrations (40 µg/L) [25]. Additionally, a Hct-dependent recovery has been found, which may be related to the formation of hydrogen bridges between the analytes and the cellulose paper. A low Hct combined with a high concentration may increase the non-erythrocyte bound drug fraction, which is then free to form hydrogen bonds with the cellulose paper. As Koster et al. noted, this effect was found at Hct levels and drug concentrations which are very rare in clinical practice [15]. For tacrolimus, sirolimus and everolimus, it has been suggested that DBS analysis of trough levels is possible without any Hct correction in a range of 20–60% Hct, which corresponds well to the distribution of Hct in patients undergoing TDM for immunosuppressants at our center [35].

The effect of Hct was most pronounced in the setting of method validation, where extremes for both Hct and

analyte concentration can be produced. The lack of a significant Hct effect in a clinical setting can partly be explained by the infrequent occurrence of these extremes in clinical practice.

Several methods have been proposed to either avoid or correct for any Hct-related effects that may be present. The simplest method to avoid the effect is to use whole blood spot analysis, coupled with volumetric application of the blood spot [3]. This, however, adds complexity to the sampling process and lessens the applicability of self-sampling in a home-based setting. Recently, single-use devices have been developed which simplify the volumetric sampling process and could enable volumetric self-sampling. De Kesel et al. reported that volumetric absorptive microsampling (VAM) allows for a straightforward volumetric sampling of a drop of blood and assists in eliminating the effect of Hct [50].

For the correction of any Hct effects that may occur, several options exist. A correction for the estimated Hct of the target population is a simple but an effective approach to minimize the Hct effect for a large part of the study population [48]. This approach was used to good effect in several studies included in this review. In these studies, additional measurements were performed in order to measure the Hct of the target population. In clinical practice, available historical data could be used, but this approach needs to be validated. Estimation of the Hct of the patient is also possible by measurement of potassium in the DBS [51] or a photometric measurement of hemoglobin content [52]. A correction can then be applied, which enables an accurate measurement, even for Hct-sensitive analytes [53]. A novel extraction method which uses heated flow-through desorption has been studied for immunosuppressants in DBS and has been found to eliminate Hct effects [33]. However, only one publication currently exists using this method for immunosuppressants [33].

It is important to note that the impact of Hct cannot be evaluated alone. Factors such as blood spot volume and punch location interact with each other and with the Hct effect. Furthermore, the impact of the Hct depends on the analyte and the paper material [3]. The combined effect of Hct, blood spot volume and punch location should be evaluated.

Drying time

Results of the literature search

Drying time in the studies ranged from “at least 10 min” [34] or (at least) 3 h [28, 31] to overnight drying [25, 30, 32,

41, 54] and a drying time of one to several days [29]. None of the method or clinical validation studies included the effect of drying time in their protocol.

In a separate study, Koster et al. examined the influence of drying time on the recovery of immunosuppressive drugs on Whatman FTA DMPK-C filter cards [15]. Variation in drying time (3–48 h) had no effect on tacrolimus recovery, but significantly impacted the recovery of sirolimus and everolimus. The recovery of these immunosuppressants decreased significantly between 3 and 24 h of drying time, after which it stabilized. This effect was Hct-dependent and was more pronounced at low Hct levels (10%) combined with a high concentration (100 µg/L) where the decline in recovery between 3 and 24 h was 24% for sirolimus and 26% for everolimus [15].

Discussion

Standard protocols recommended by the CLSI for newborn screening with DBSs, the area in which most clinical expertise with DBSs has been gathered, recommend a drying time of at least 3 h at room temperature, shielded from direct sunlight [38]. Residual humidity may cause bacterial or fungal growth and can alter the elution time of the specimen [55]. Several factors, such as Hct and blood spot volume influence the drying time a blood spot may need [14]. The EBF recommends that every full and partial DBS validation include an evaluation of drying conditions (i.e. drying time and temperature) [14].

The drying time varied significantly between the studies included for review and none of the included method and clinical validations included an evaluation of the effect of drying time. In a separate study, Koster et al. examined this effect in detail and concluded that sirolimus and everolimus are susceptible to Hct-dependent changes in recovery when drying times exceed 3 h [15]. This effect may be explained by the formation of hydrogen bonds between sirolimus and everolimus and the cellulose paper [56]. Koster et al. noted that when drying time is only 3 h, this may mask the effects of Hct and may provide misleading results for sirolimus and everolimus, which underestimate the effects of Hct. A drying time of 24 h for sirolimus and everolimus was recommended to achieve a stable, albeit lower recovery [15]. Although the concentrations used in these studies were far above the expected concentrations in a real-life setting, the effect of drying time for sirolimus and everolimus needs further attention in method- and clinical validation to establish whether clinically relevant interference can occur.

Stability

Results of the literature search

Fourteen publications have examined the stability of immunosuppressants in DBSs. No studies reported significant degradation of tacrolimus at room temperature within the measured timeframes, which ranged from 5 days to 8 months [18, 22, 32]. When refrigerated or frozen, long-term stability for at least 1 week to 168 days has been demonstrated [16, 25]. At higher temperatures, tacrolimus was found to be stable at 37°C for at least 4–28 days [18, 30, 32, 41], at least 5 days at 60°C [28] and at least 1 day at 50°C and 70°C [30, 41]. Sirolimus and everolimus were reported to share the same stability at low and room temperature as tacrolimus but were found to be more susceptible to long-term degradation in warm temperature conditions, as they showed degradation at 37°C over a 4-week period [32]. Additionally, sirolimus showed degradation within 24 h at 60°C [28] and everolimus was deemed to be unstable in DBSs at 70°C [19].

Two studies examined the stability of DBS samples for the analysis of tacrolimus in long-distance shipping conditions. Hoogtanders et al. shipped patient DBS samples from Japan to the Netherlands and found no degradation of the samples [8]. Cheung et al. sent samples from Hong Kong to the Netherlands and found the samples to be stable during long-distance transport [10].

Discussion

DBSs have been described as a stable medium for a variety of analytes, even without the need for refrigeration or special precautions [3, 5, 13]. DBSs improve the stability of most analytes in comparison to plasma, serum or whole blood samples, possibly due to removal of esterase activity upon drying [40]. Omeprazole and nifedipine are examples of drugs that are stabilized in the DBSs and consequently are far more stable than in plasma [3]. Biomarkers such as acylcarnitines and several amino acids are stable for several weeks at room temperature [3] and nucleic acids may be stable for several months or even more than 1 year [7]. However, stability needs to be investigated on a case-by-case basis, as notable exceptions exist. Several analytes, such as ornithine, sarcosine and serine show a significant decrease after 1 day at room temperature [3].

It is important to measure analyte stability in several temperature conditions, as the possibility of self-sampling at home includes transport and handling. Temperature in

post boxes can be 20°C above outside temperature and may reach 60°C [57].

DBSs have proven to be a stable medium for immunosuppressants for both long- and short-term storage in a variety of temperature conditions. Tacrolimus showed an excellent stability, even at high temperatures. Sirolimus and everolimus are stable at room temperature but were reported to be more susceptible to higher temperatures, which may present problems during transport in warm months. Although the stability of immunosuppressants in DBS has been well documented, more validation on interfering factors such as drying time is needed.

Spot homogeneity and punch location

Results of the literature search

Two studies examined the effect of punch location. Den Burger et al. found no significant concentration difference at different punch locations (central versus peripheral) for tacrolimus, sirolimus and everolimus [25]. Li et al. found no significant chromatographic effects for tacrolimus on DBSs when comparing central and peripheral punches [30].

Sadilkova et al. unevenly soaked filter paper with 4 mL of patient blood and punched 21 8-mm punches spread throughout the card in order to study the effect of punch location. The coefficient of variation for tacrolimus and sirolimus concentrations between the spots was <15% [28].

Discussion

Due to chromatographic effects, i.e. the inhomogeneous spread of blood through the filter paper, the location of a punch may have an effect on the concentration of the analyte [3]. Chromatographic effects can be influenced by the Hct, filter paper, humidity, drying conditions and filter card position while drying [40]. A “volcano pattern” has been described for a number of analytes (a higher concentration between the center and the edge [58]) which is more pronounced on pre-treated cards (e.g. FTA Elute) than on untreated cellulose cards [3]. In addition, a “coffee-stain” effect is seen as well, which consists of the accretion of solid particles, e.g. red blood cells, at the edge of the blood spot [58]. An effect of the coffee-stain effect on analytes which are primarily located in erythrocytes has been described. The concentration of lead was increased 1.5 times when the punch was located within 1 mm of the blood spot edge when compared to the center [43].

None of the studies that investigated the effect of punch location found a significant effect on the concentration of tacrolimus, sirolimus and everolimus. The lack of significant effects of punch location is in accordance with guidelines by the EBF, which mention that spot homogeneity usually does not seem to be an issue and with Jager et al., who reviewed the validation of DBS methods and found no reports of significant interference because of spot inhomogeneity [11, 14].

The impact of any chromatographic effects that may be present is influenced by the size of the punch and the DBS. A large punch will punch out overlapping areas and will compensate for possible differences [3]. The lack of a chromatographic effect in the reviewed publications may partly be explained by the large punch sizes. For example, den Burger et al. used an 8-mm punch which punched out approximately 67% of the entire spot [25]. The EBF recommends taking a punch that is big enough to be a representative sample and/or to always punch from the same location on the blood spots. The punch size in the reviewed publications ranged from 6 to 10 mm and, when specified, all punches were made from the center of the blood spot. For analytes which are primarily located in erythrocytes, such as immunosuppressants, it may be important to avoid the edge of the blood spot, as red blood cell accretion at the perimeter has been noted. In this regard, it is crucial to reject samples with insufficient volume. Keeping the EBF recommendations in mind, no significant impact from spot inhomogeneity is to be expected.

Summary

DBS sampling is a promising sampling method with benefits in terms of ease of use, patient comfort, stability and cost. The possibility of self-sampling by the patient is interesting, especially in the context of immunosuppressant TDM. However, DBS-related preanalytical factors are not always part of the method validation in published methods: out of 21 method validations, seven publications (33%) studied the influence of blood spot volume, 14 (66%) the stability of immunosuppressants in DBSs, nine (42%) the influence of Hct, 3 (14%) the effect of punch location and no method validations included the influence of drying time.

Tacrolimus was not significantly influenced by blood spot volume, Hct effects, blood spot inhomogeneity and drying times. Furthermore, tacrolimus was found to be sufficiently stable at low and high temperatures, as no

significant degradation was noted after 5 days at 60°C. This lack of interference makes tacrolimus a viable candidate for DBS sampling, even in a home-based setting. Several pharmacokinetic trials have already used home-based self-sampled DBS of tacrolimus as an established method, demonstrating the feasibility of this method.

Sirolimus and everolimus were more prone to interference from the studied preanalytical factors. They exhibited significant bias in blood spots with low volume, although the clinical impact is minimal, as these spots would have been rejected due to insufficient filling. High temperatures significantly impacted the stability of these compounds at 60°C and 70°C, temperatures which can be reached in postal boxes during summer months.

Furthermore, sirolimus and everolimus were more susceptible to Hct-related effects than tacrolimus, as they exhibited variations in recovery (up to -26%) which were dependent on Hct, concentration and drying time. A significant Hct-dependent bias on concentration was also found at low Hct and high concentration. Although the impact of Hct seems to be minimal in clinical practice, where extremes of Hct and concentration are rare, further validation of these preanalytical factors is needed before their use in DBSs can become widespread. In particular, drying conditions for these compounds need to be examined further, as current protocols for drying time may mask Hct-dependent effects on recovery.

Finally, although the possibility of home-based self-sampling of DBSs is often reported, it has not been extensively studied. Several publications show promising results, but the quality of blood spot made by patients at home is variable (the amount of rejected samples ranges from 3% up to 11.6%) and problems with timing and adherence to self-sampling have been noted. Self-sampling at home may primarily be reserved for a selected population of patients and further validation in a home setting and development of protocols for patient training and selection are needed.

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