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Chapter 3.8

The performance of five different dried blood spot cards for the analysis of six immunosuppressants

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ABSTRACT

Background: The relation between hematocrit, substance concentration, extraction recovery and spot formation of tacrolimus, sirolimus, everolimus, ascomycin, temsirolimus and cyclosporin A was investigated for Whatman 31 ET CHR, Whatman FTA DMPK-C, Whatman 903, Perkin Elmer 226 and Agilent Bond Elut DMS DBS cards.

Results: We found that all DBS cards showed the same hematocrit and concentration dependent recovery patterns for sirolimus, everolimus and temsirolimus. At high concentrations, the total hematocrit effects were much more pronounced than at low concentrations for tacrolimus, sirolimus, everolimus, ascomycin and temsirolimus.

Conclusion: The tested card types showed differences in performance, especially at extreme concentrations and hematocrit values. It may be useful to investigate the performance of different types of DBS cards prior to analytical method validation.

INTRODUCTION

In the past years, several dried blood spot (DBS) methods have been published regarding the analysis of immunosuppressants [1-3]. These DBS methods used different DBS card types. However, the performance of different card types for these substances were never compared in one research. There are several factors that need to be taken into account when a DBS method is to be developed and validated. The hematocrit (HT) value can be considered as one of the most important parameters that can influence DBS analysis results. Since the HT represents the relative volume of the red blood cells in the blood it also affects the viscosity of the blood. Therefore the spreading of the blood drop on the DBS card is influenced by the HT of the blood where a high HT forms smaller spots and vice versa. The intended fixed volume of a set diameter punch would then contain a deviating blood volume, which causes analytical biases. The preparation of a range of hematocrit (HT) reference samples during method validation has been evaluated earlier. The effect caused by the HT on the measured concentration was linear correlated with the HT value and it was suggested to correct for this effect [4-6].

The influence of the combination of the HT and the concentration of the analyte on the recovery could also affect the analytical results [4]. This means that the spot size is not the only parameter that is influenced by the HT and that a simple linear HT correction may not correct for all HT related analytical biases. At low HT in combination with a high concentration resulted in decreased recoveries of sirolimus (SiR), everolimus (EvE) and especially temsirolimus (TeM). This was explained by the increasing number of hydrogen (H)-bond acceptors of 13 for SiR, 14 for EvE and 16 for TeM compared to those of tacrolimus (TaC) and ascomycin (AsC) with 12 H-bond acceptors [7]. Where a high number of H-bond acceptors explained the level of affinity to form H-bonds with the cellulose of the DBS card.

Because the spreading of the blood drop is not the only parameter that will affect the analytical results, the fixed punch size of a partial DBS will be affected by the combination of the spreading of the blood drop and possible variation of recoveries.

Different types of card matrices may, in combination with varying HT values, have different effects on the spreading of the blood spot and the extraction recovery of the substance. Whatman 903 and Perkin Elmer 226 (previously Ahlstrom) are the only types of DBS cards that are approved by the FDA as clinical collection devices. These types of cards are extensively monitored for consistent performance between batches [8, 9]. Several studies described the

effect of the HT and spot volume using different DBS card types. O'Mara et al. investigated the effect of the HT on the distribution on Perkin Elmer (Ahlstrom) 226, Whatman FTA, Whatman FTA Elute and DMPK C cards [10]. Vu et al. tested Whatman grade No3, 31 ET CHR, and 903 cards for the analysis of moxifloxacin [5]. A DBS card with an alternative material, the Agilent Bond Elut DMS cards, are made of non-cellulose material that should reduce non-specific binding of analytes to the card matrix [11]. This type of card could help to overcome the observed reduced recoveries due to binding to the cellulose matrix of other cards [4]. In this research, the performance of the DBS cards were assessed by testing a wide range of HT levels in combination with various substance concentrations. This was tested in two different ways. In the first, the influence of the changing HT values and concentrations were assessed by full spot analysis. This test included the influence on the substance recovery but ruled out the effect of the spot formation. In the second, the influence of the changing HT values and concentrations were assessed by partial spot analysis. This test included both the influence on the substance recovery and the effect of the spot formation.

For clinical use it is relevant to get more insight in the differences in performance of various DBS cards. Therefore, the objective of this study was to evaluate five types of DBS cards by measuring recoveries and spot formation effects at various concentrations and HT values for a range of immunosuppressants.

EXPERIMENTAL SECTION

Chemicals and materials

TaC was purchased from USP (Rockville, MA, USA). EvE was purchased from Sigma-Aldrich Inc. (St. Louis, USA). SiR was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and CyA was purchased from EDQM (Strasbourg, France). AsC was purchased from LC Laboratories (Woburn, USA). TeM was purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Combined stock solutions containing TaC, EvE, SiR, AsC and TeM were prepared at 2,500 ng/mL and CyA at 50,000 ng/mL in methanol. This combined stock solution was five times diluted with methanol to obtain concentrations of 500 and 10,000 ng/mL respectively. These combined stock solutions were used for the experiments. The following deuterated internal standards (IS) were purchased from Alsachim (Illkirch Graffenstaden, France): TaC [$^{13}\text{C}_2, ^2\text{H}_2$], EvE [$^{13}\text{C}_2, ^2\text{H}_4$] and CyA [$^2\text{H}_{12}$]. The extraction solution consisted of methanol:water (80:20 v/v%) and contained the deuterated internal standards TaC [$^{13}\text{C}_2, ^2\text{H}_2$], EvE [$^{13}\text{C}_2, ^2\text{H}_4$]

and CyA [$^2\text{H}_{12}$] at concentrations of 2.5 ng/mL, 1.0 ng/mL and 10 ng/mL respectively. TaC [$^{13}\text{C}_2,^2\text{H}_2$] was used as IS for TaC and AsC. EvE [$^{13}\text{C}_2,^2\text{H}_4$] was used as IS for EvE, SiR and TeM. And CyA [$^2\text{H}_{12}$] was used as IS for CyA. Citrate whole blood was purchased from Sanquin (Amsterdam, The Netherlands). The whole blood was stored at 4°C and was used within two weeks after blood donation. To assure the quality of the blood, it was checked for hemolysis prior to use. The following types of DBS cards were tested: Whatman 31 ET CHR, Whatman FTA DMPK-C, Whatman 903 (Kent, UK), Perkin Elmer 226 (Ahlstrom) (Helsinki, Finland) and Agilent Bond Elut DMS (Santa Clara, USA). A Hettich centrifuge model 460R was used to centrifuge the whole blood for HT preparation. A XN9000 hematology analyzer from Sysmex (Hyogo, Japan) was used for all hematocrit analyses. All experiments were performed on an Agilent 6460A (Santa Clara, Ca, USA) triple quadrupole LC-MS/MS system, with an Agilent 1200 series combined LC system. All technical parameters were used as described by Koster et al. [4]. All precursor ions, product ions, optimum fragmentor voltages and collision energy values were tuned and optimized in the authors' laboratory and are shown in table 1. Agilent Masshunter software for quantitative analysis (version B.04.00) was used for quantification of the analysis results.

Table 1 Mass spectrometer settings for all substances

Substance	Precursor ion (m/z)	Product ion (m/z)	Fragmentor voltage (V)	Collision energy (V)
Tacrolimus	821.5	768.4	190	11
Tacrolimus [$^{13}\text{C}_2,^2\text{H}_2$]	824.5	771.4	140	15
Sirolimus	931.5	864.4	140	6
Everolimus	975.6	908.5	121	10
Everolimus [$^{13}\text{C}_2,^2\text{H}_4$]	981.6	914.5	165	13
Ascomycin	809.5	756.5	160	16
Temsirolimus	1047.6	980.5	130	16
Cyclosporin A	1219.8	1202.8	200	30
Cyclosporin A [$^2\text{H}_{12}$]	1231.8	1214.8	170	16

Sample preparation

The preparation of the different target hematocrit values was by centrifuging tubes with citrate whole blood with a known HT (measured by a Sysmex XN-9000 analyzer) for 5 minutes at 1,972g. The necessary volumes of plasma were removed or added to achieve

the different target HT values [12]. The prepared HT values were always confirmed with the Sysmex XN-9000 analyzer.

The sample preparation was performed according to a previously published method [4]. For the preparation of the DBS samples an 8 mm disk from the central part of the blood spot was punched into an eppendorf tube. For recovery testing, the DBS card was first punched into an eppendorf tube, followed by the addition of 15 μL blood onto the DBS card punch. The spots were dried at ambient temperature for 24 hours. After addition of 200 μL extraction solution the samples were vortexed for 60 sec, sonicated for 15 min and then vortexed again for 60 sec. The extract was transferred into a 200 μL glass insert and placed at -20°C for 10 min to improve protein precipitation. After centrifugation at 10,000g for 5 min, 20 μL of the extract was injected to the LC-MS/MS system.

Influence of the HT and concentration on the recovery (full spot punch)

Blood samples with HT values of 0.10, 0.20, 0.30, 0.40, 0.50 and 0.60 L/L were spiked to produce concentrations of 3.0 and 100 ng/mL for TaC, SiR, EvE and AsC and of 60 and 2,000 ng/mL for CyA. TeM was also spiked at 10 and 50 ng/mL at the HT values of 0.10 and 0.40 L/L. Blank DBS card spots were punched from all 5 types of DBS cards, transferred to eppendorf cups and 15 μL blood was spiked on the punched spots for each HT and concentration, dried at room temperature for 24 hours and analyzed in fivefold (solutions A). For the extraction recovery, extracts of blank DBS of each card type were spiked at the tested concentrations (solutions B). The average peak area ratios of the substance with its internal standard were used to calculate the recovery. The calculation of the percentage recovery was as followed: $\text{recovery} = A/B \times 100$. In order to report the acquired data, the coefficient of variation (CV) of the 5 replicate analyses was required to be within 15%.

Influence of the HT on the formation of the spot area and recovery (partial spot punch)

Blood samples with HT values of 0.10, 0.20, 0.30, 0.40, 0.50 and 0.60 L/L were spiked at 3.0 and 100 ng/mL for TaC, SiR, EvE, AsC and TeM and at 60 and 2,000 ng/mL for CyA and 50 μL spots were made on all 5 types of DBS cards. The cards were dried at room temperature for 24 hours and analyzed in fivefold.

The biases that were caused by the HT were calculated with respect to the HT of 0.40 L/L, which was set as the standard HT value. Peak area ratios of the substance with its internal standard were used for the calculations. In order to report the acquired data, the CV was required to be within 15%.

RESULTS AND DISCUSSION

Influence of the HT and concentration on the recovery (full spot punch)

All tested DBS card types showed stable recoveries for TaC, CyA and AsC at HT values from 0.1 to 0.4 L/L, while higher HT values showed decreasing recoveries (figure 1). At 3.0 ng/mL for SiR, EvE and TeM, the extraction recoveries were stable at HT values from 0.1 to 0.4 L/L, while higher HT values also showed decreasing recoveries (figure 2). Compared to 3.0 ng/mL, the extraction recoveries at 100 ng/mL were lower for all HT values. The Agilent Bond Elut DMS cards also showed deteriorated extraction recoveries for SiR, EvE and TeM at the HT of 0.1 L/L and 100 ng/mL, which was unexpected because it was claimed to be of non-cellulose material. Although the Agilent Bond Elut DMS cards claimed to be of non-cellulose material, it may still contain H-bond donor groups, which are susceptible to binding to the investigated substances. In addition, the Agilent Bond Elut DMS cards claim to reduce non-specific binding of substances, which is normally of more impact at low concentrations than at high concentrations. This is in contrast to our findings, where the observed recoveries deteriorated when concentrations increased. This may support the suggested presence of H-bond donor groups in the Agilent Bond Elut DMS cards.

Figure 2 also showed the trends of declining recoveries of SiR, EvE and TeM influenced by the HT values at 3.0 and 100 ng/mL. Here it can be seen that the extraction recoveries at 3.0 ng/mL did not show declining recoveries at lower HT values for all card types. Compared to the HT of 0.40 L/L, the recoveries start to decline when the HT further increases. This effect is most prominent with the Whatman 31 ET CHR cards and at the HT of 0.60 L/L. The extraction recoveries of SiR, EvE and TeM at 100 ng/mL declined with 11% to 38% when the HT decreased from 0.4 L/L to 0.1 L/L. The HT value of 0.60 L/L also showed decreased extraction recoveries for all substances and all DBS card types compared to the HT of 0.50 L/L (figure 1 and 2). The decreased extraction recoveries with the Whatman 31 ET CHR cards seemed to be most affected by the changing HT values. The H-bonding theory for SiR, EvE and TeM has most effect at low HT values and high concentrations, because this leads to a

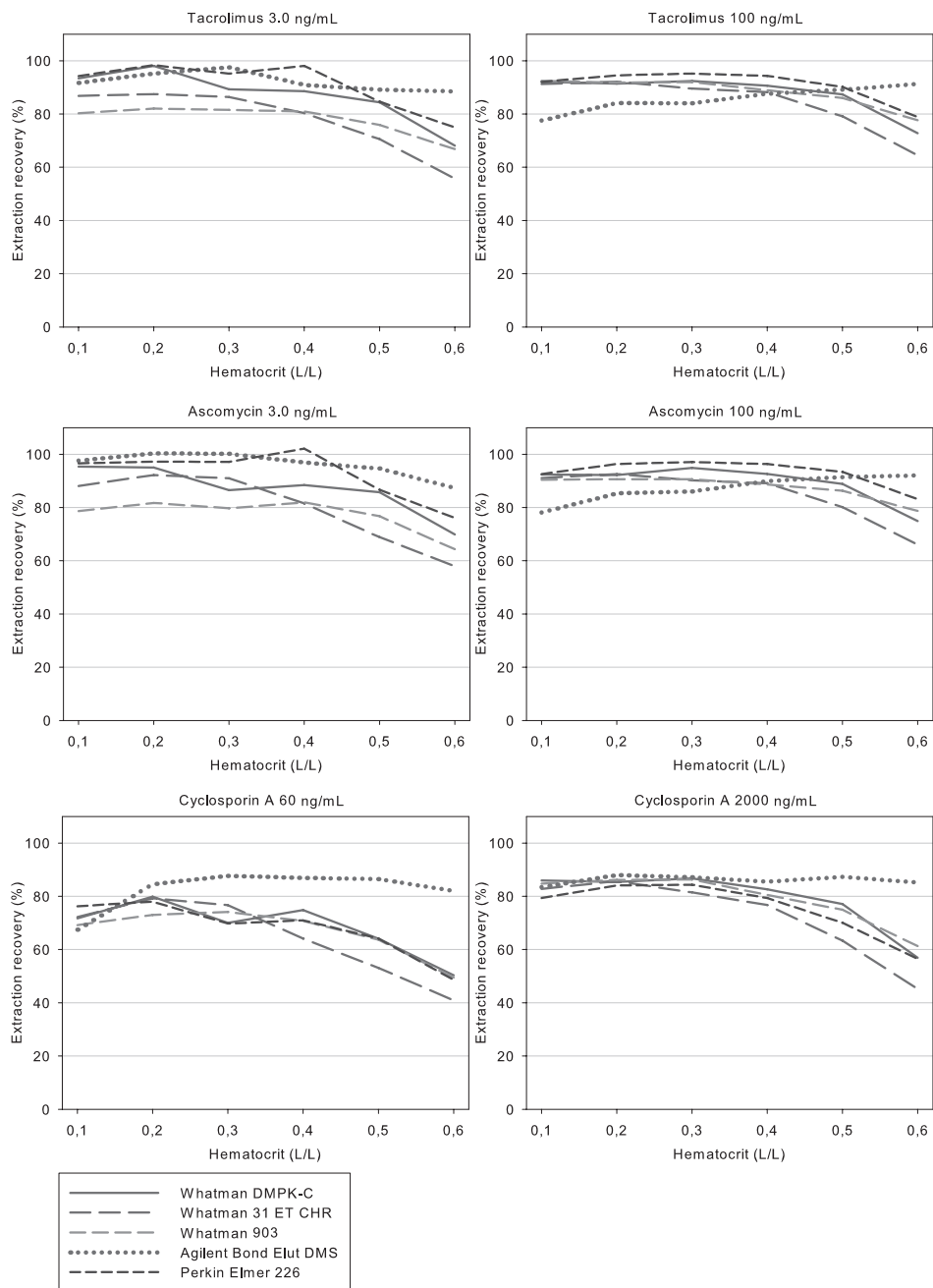


Figure 1 Recovery testing of tacrolimus, ascomycin and cyclosporin A with varying hematocrit values using DBS full spot analysis.

The card types were evaluated per substance. Tacrolimus and ascomycin were assessed at 3.0 and 100 ng/mL and cyclosporin A at 60 and 2,000 ng/mL. For every data point the mean of n=5 was reported.

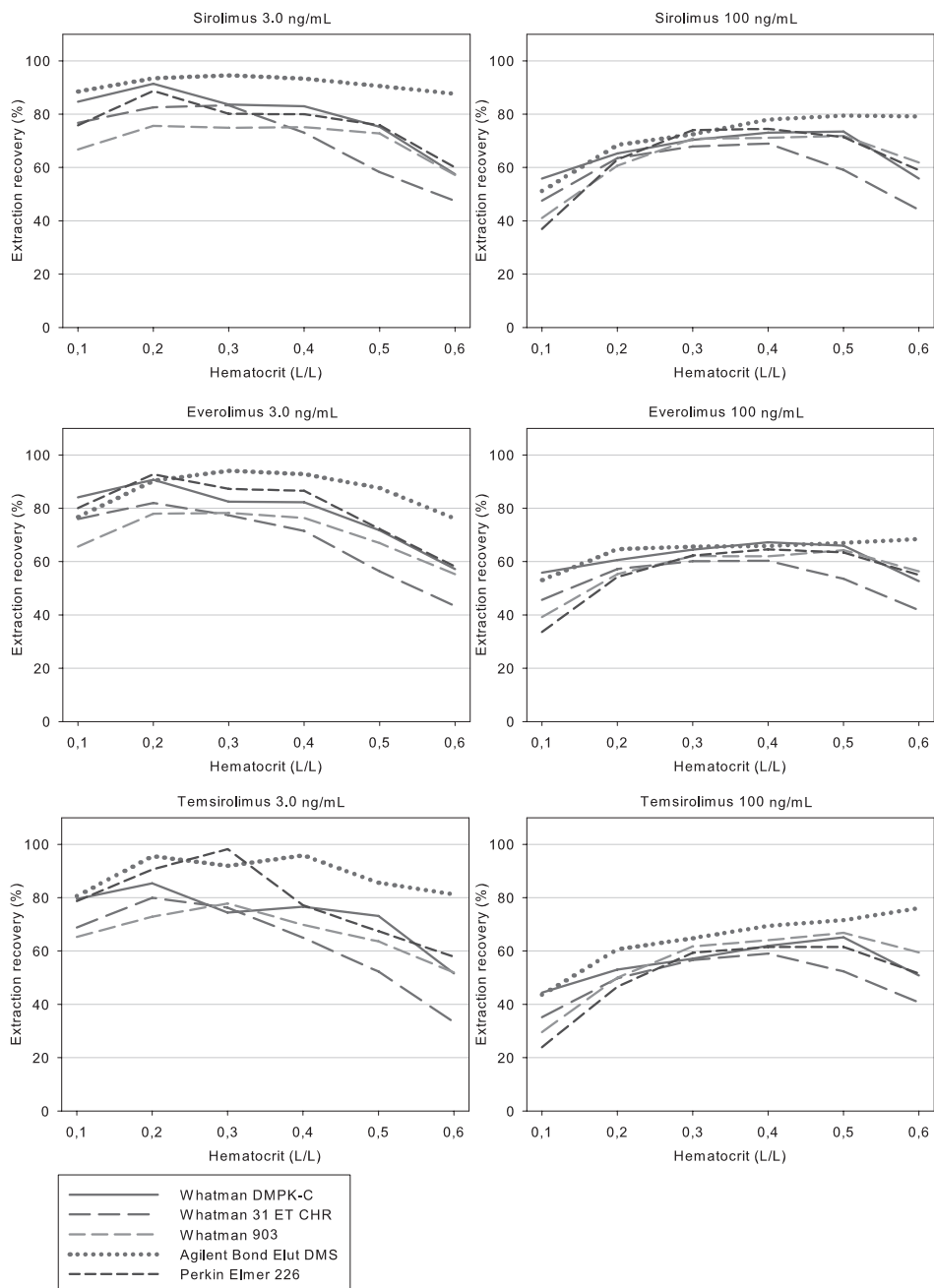


Figure 2 Recovery testing of sirolimus, everolimus and tamsirolimus with varying hematocrit values using DBS full spot analysis.

The card types were evaluated per substance. Sirolimus, everolimus and tamsirolimus were assessed at 3.0 and 100 ng/mL. For every data point the mean of $n=5$ was reported.

higher fraction of unbound substance, which can bind to the H-bond donor groups in the card matrix. This means that at the high HT value of 0.60 L/L the H-bonding theory is of much less effect. In addition, TaC, CyA and AsC also showed declined extraction recoveries, meaning that another factor like extraction efficiency may be responsible for this phenomenon. The lowered extraction efficiency may be caused by substance inclusion in the DBS caused by the high percentage of red blood cells. Therefore the current DBS extraction procedure with 15 minutes sonification was tested for extended sonication times of 30 and 60 minutes in order to improve the recoveries, without the desired results. Therefore, the current extraction procedure was considered optimal.

Since the extraction recoveries of TeM showed to be most affected by high concentrations and low HT values, the performance of the DBS cards was shown for TeM in figure 3. Here it can be seen that at low HT the extraction recoveries decreased when concentrations increased. The 5 tested DBS cards all showed the same concentration dependent recovery pattern. However, the performance varied between the DBS cards. The Whatman DMPK-C cards showed the highest extraction recoveries while the Perkin Elmer 226 cards showed 20% lower extraction recoveries than the Whatman DMPK-C cards at 100 ng/mL. At the HT of 0.4 L/L, the trend in extraction recoveries showed a much less decrease for all DBS cards when concentration increased. The performance of the Perkin Elmer 226 cards now showed to be as good as the Whatman DMPK-C cards. However, this showed that the extraction recovery of TeM was most affected by changes in HT with the Perkin Elmer 226 cards.

Influence of the HT on the formation of the spot area and recovery (partial spot punch)

The varying HT values will affect the DBS results by the different spreading of the blood drop due to the blood viscosity. For SiR, EvE and TeM, the HT and drug concentration will also influence the recovery.

For the calculation of the biases that were caused by the HT, the HT of 0.40 L/L was set as the standard HT value. At the low concentration of 3.0 ng/mL for TaC, AsC, SiR, EvE and TeM the HT effects exceeded the -15% bias at a HT of approximately 0.20 L/L, figure 4 and 5. At 60 ng/mL CyA, a HT of 0.30 L/L already showed -14% to -23% biases for all DBS cards, figure 4. Furthermore, HT effects didn't seem to vary a lot between the tested card types in the low concentration ranges of all substances.

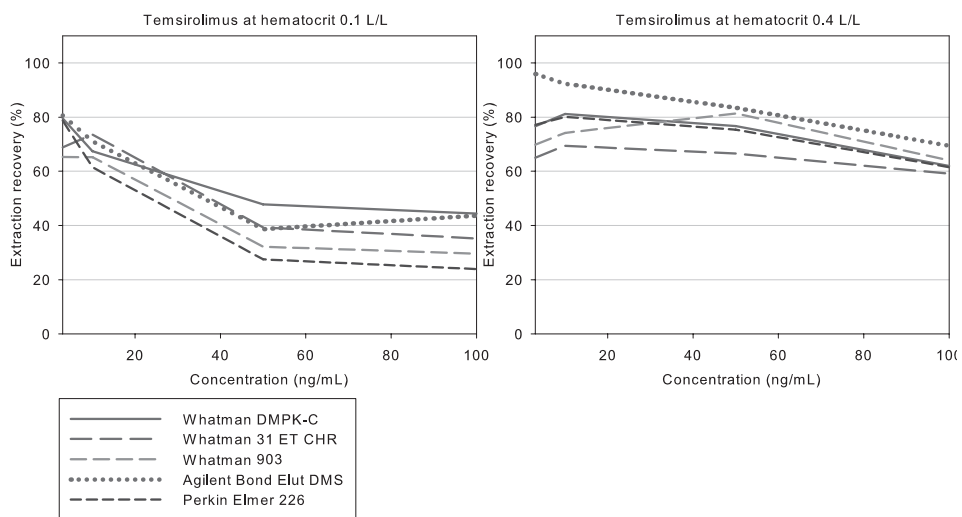


Figure 3 Recovery testing of tamsulosin with varying concentrations at hematocrit values of 0.1 and 0.4 L/L using DBS full spot analysis.

Each card type is evaluated at 3.0, 10, 50 and 100 ng/mL tamsulosin. For every data point the mean of $n=5$ was reported.

At the high concentration of 100 ng/mL for TaC, AsC, SiR, EvE and TeM and 2,000 ng/mL for CyA, the biases caused by the HT effects already exceeded -15% at HT values ranging from approximately 0.25 to 0.30 L/L. All DBS card types showed the downward trends in biases when HT decreased. Strangely, at the concentration of 100 ng/mL TaC and AsC also showed far more extreme HT effects than at 3.0 ng/mL. The previous tests showed that the extraction recoveries of TaC and AsC were not influenced by the concentration and HT, but the HT effect on the spot formation appeared to influence the results more at high concentrations than at low concentrations. The HT effects of CyA were not influenced by its concentration or changing recoveries, but extreme HT effects were observed at low and high concentrations concluding that the spot formation effect was the most critical parameter for the HT effects of CyA. There appeared to be another HT related parameter that was of influence on the DBS results. This parameter could have been the distribution of AsC, TaC and CyA between red blood cells and plasma, which depended on the concentration and HT.

The Agilent Bond Elut DMS cards showed the same downward trend in biases as the other DBS cards, especially at high concentrations. The overall results of the Agilent Bond Elut DMS cards sometimes seemed better than the other cards. However, the spots created on the Agilent Bond Elut DMS cards were very small and inconsistent in shape and the spot

material was very fragile. This has led to apparently full spot punches, while strangely still displaying HT effects. The overall conclusion of the Agilent Bond Elut DMS cards was that they also suffered from HT effects and were not suitable for its intended purpose.

The HT effects which were influenced by the HT and concentration showed to be of minor impact at low concentrations for TaC, AsC, SiR, EvE and TeM. This indicated the usefulness of DBS analysis for trough levels in patients with HT levels between 0.25 and 0.60 L/L for TaC, AsC, SiR, EvE and TeM.

Figures 4 and 5 showed that the biases between DBS card types were similar. It should be noted that these figures only showed the HT dependent trend in biases for all tested card types. Since the response ratio was set at 100% at a HT of 0.40 L/L for each card type, the biases in recoveries between the card types were not accounted for in these figures.

Based on figure 4 and 5, the total HT effects appeared not to have a linear correlation for all substances. However, most of the current HT correction methods make use of a linear correlation [4-6]. Especially CyA showed different HT effects above 0.40 L/L compared to lower HT values (figure 4). This would further complicate a correction of the clinical analysis results for the HT value. Therefore, a HT correction method should include the effect of the substance concentration on the recovery and the non linear relation of the HT with the measured substance concentration for each DBS card type. Since it would be complicated to set up such a correction method it may be more feasible to set up a (linear) point-to-point relation between recovery, HT and concentration.

When no HT correction is applied, the method validation should include a framework for the HT and substance concentration in which the results are within acceptable limits. In order to perform clinical DBS analysis for the investigated substances on either DBS card type without HT correction, it is suggested that DBS analysis of TaC, SiR, EvE and TeM is suitable for trough levels in patients with a HT range between 0.20 and 0.60 L/L. For CyA, the acceptable HT range is smaller and should be between 0.30 and 0.60 L/L. The DBS analysis and interpretation of pharmacokinetic curves requires caution and could be possible when the HT value of the patient is within the acceptable HT range.

The HT values of the calibration curve and control samples could be prepared at a HT of 0.35 instead of 0.40 L/L. This would increase the acceptable HT range were the biases remain within 15%. In addition, the development of alternative DBS card matrices may help to overcome the current HT effects.

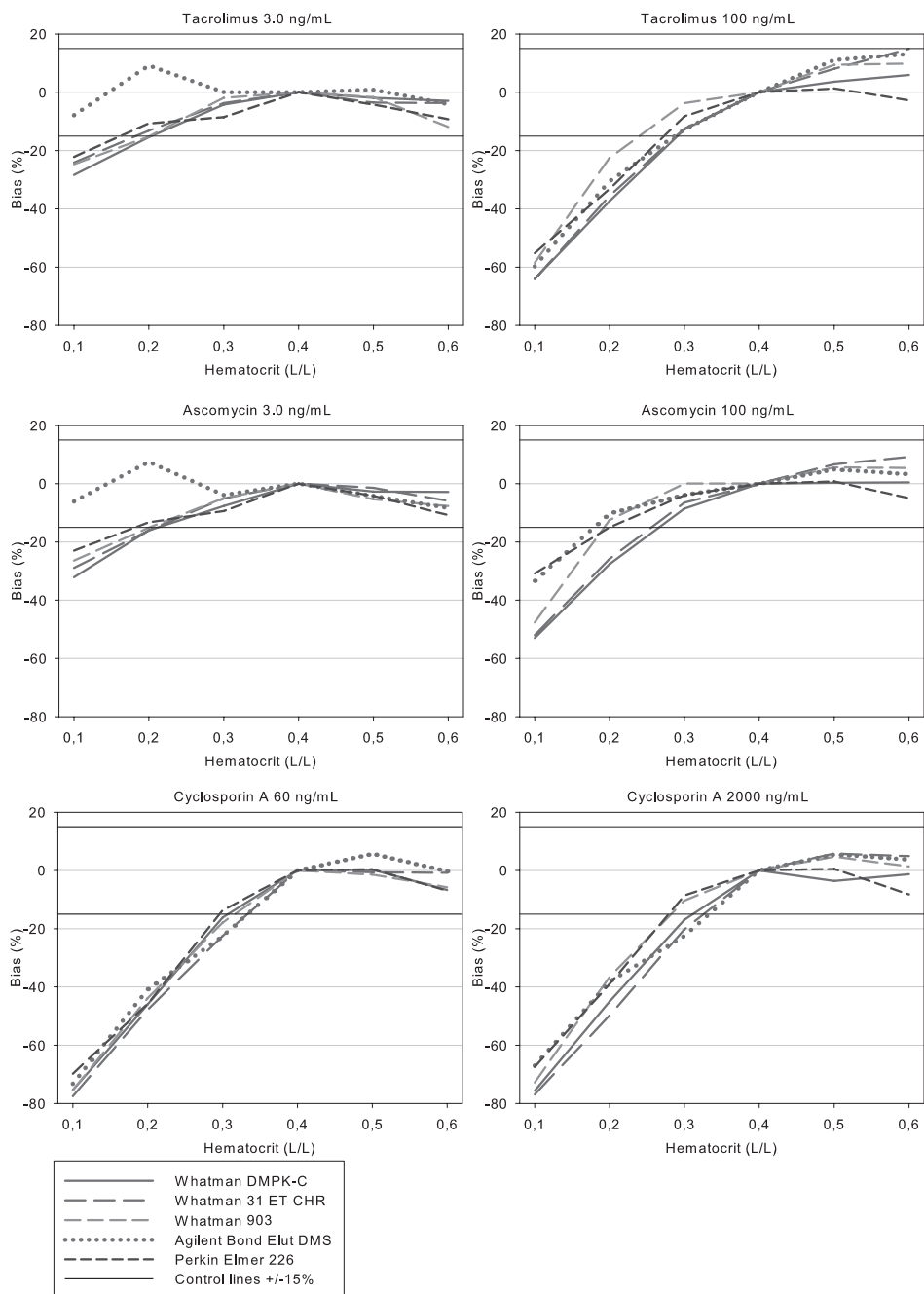


Figure 4 Influence of the HT on the formation of the spot area and recovery of tacrolimus, ascomycin and cyclosporin A using a partial spot punch.

The card types were evaluated per substance. Tacrolimus and ascomycin were assessed at 3.0 and 100 ng/mL and cyclosporin A at 60 and 2,000 ng/mL. For every data point the mean of n=5 was reported.

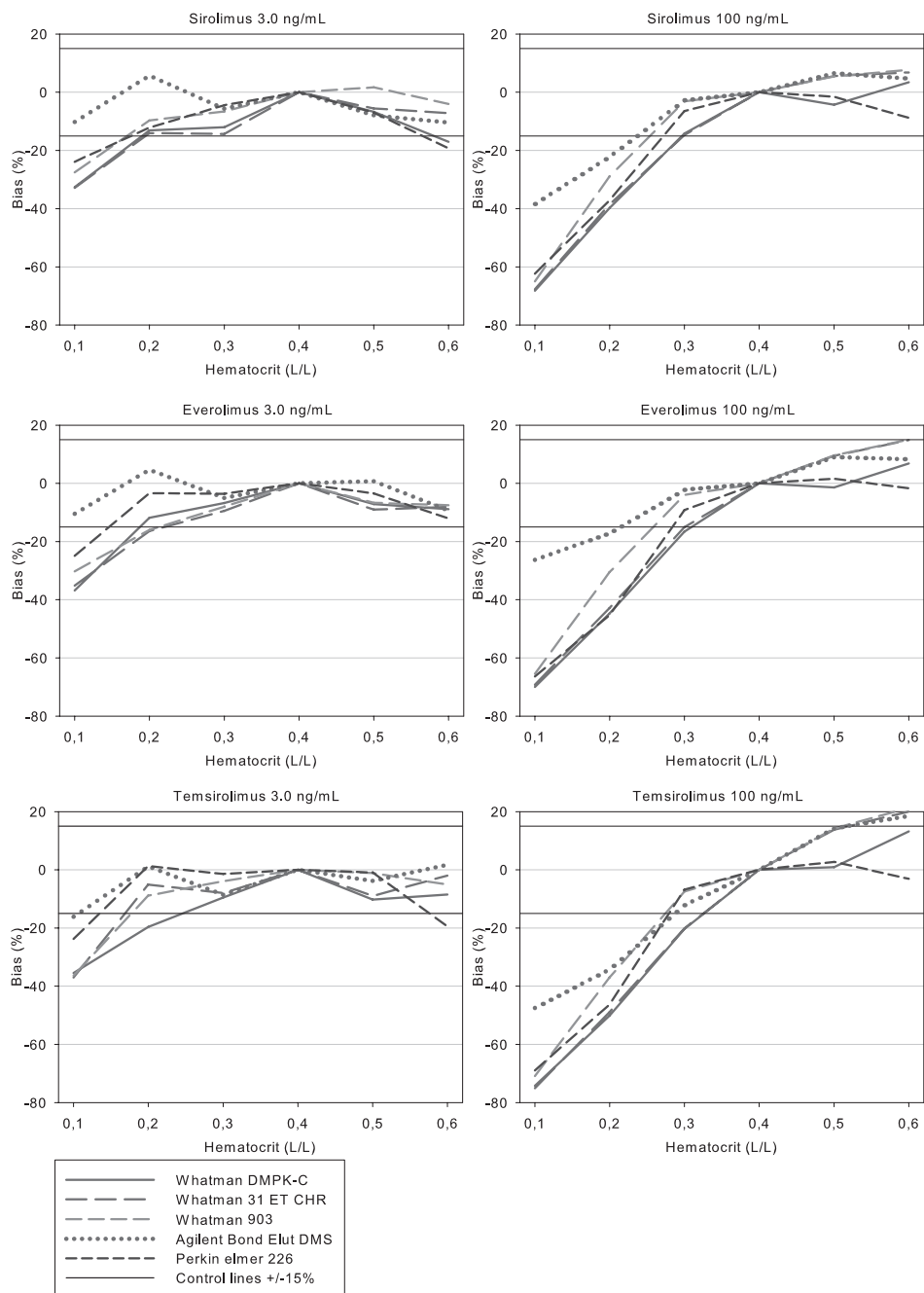


Figure 5 Influence of the HT on the formation of the spot area and recovery of sirolimus, everolimus and temsirolimus using a partial spot punch.

The card types were evaluated per substance. Sirolimus, everolimus and temsirolimus were assessed at 3.0 and 100 ng/mL. For every data point the mean of n=5 was reported.

CONCLUSIONS

A previous research investigated the performance properties of Whatman 903 and Perkin Elmer 226 (Ahlstrom) for 26 newborn screening analytes [9]. Their data demonstrated similarities of analyte recovery between the DBS cards and suggested the comparability for newborn screening and other applications. Our results indicated that there were differences in performance between the 2 tested DBS cards for the tested substances, which depended on concentration and HT value.

It can be concluded that HT and concentration related effects were observed for all DBS cards. The recovery performance with regard to the substance binding to the cellulose of the card matrix showed to vary between the tested DBS cards. The overall performance of the Whatman DMPK-C cards seemed to have the most constant and best performance. The tested DBS cards showed little differences in performance regarding the formation of the DBS during partial spot analysis. Based on this research, it is advised not to use different DBS card types in clinical routine analysis and to fully (re)validate the analytical method when the used DBS card matrix is changed. In addition, it may be useful to investigate the performance of different types of DBS cards and to validate the analytical method with the best performing DBS card.

FUTURE PERSPECTIVE

The current cellulose based DBS cards showed to have disadvantages that are inherent to the DBS cards. Better understanding of the performance of the DBS cards and the analytical methods may be used to provide a framework in which the analytical DBS performance is within the validation requirements. This can be done by a set HT and concentration range. In addition, new DBS card matrices may provide improved performance and can be tested according to the here described method.

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