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Short Communication

The use of freeze-dried blood samples affects the results of a dried blood spot analysis

Marith I. Francke [a,b,c,d,*], Bart van Domburg [c], Daan van de Velde [c], Dennis A. Hesselink [a,b], Brenda C.M. de Winter [c,d]

[a] Department of Internal Medicine, Division of Nephrology and Transplantation, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[b] Erasmus MC Transplant Institute, Erasmus MC, University Medical Center Rotterdam, the Netherland
[c] Department of Hospital Pharmacy, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands
[d] Rotterdam Clinical Pharmacometrics Group, the Netherlands

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ABSTRACT

Dried blood spot (DBS) microsampling has several advantages over venous blood sampling. In a clinical validation study of tacrolimus microsampling it was noted that tacrolimus DBS concentrations ($[Tac]_{DBS}$) were systematically higher than tacrolimus whole-blood concentrations ($[Tac]_{WB}$). This observation was explored by investigating the effect of using freeze-dried standards (ST_{FD}) for $[Tac]_{DBS}$ measurement.

For all experiments, both non-frozen whole-blood samples and whole-blood samples that were frozen and thawed (to simulate freeze-drying) of 10 patients were analyzed. Multiple tacrolimus concentrations were measured: 1) $[Tac]_{WB,}$ 2) $[Tac]_{DBS}$, where 15 μL was volumetrically applied to a pre-punched DBS disk, and 3) $[Tac]_{DBS}$, where 50 μL was applied before a 6 mm DBS disk was punched from the card. All tacrolimus concentrations were determined independently using ST_{FD} and standards made of non-frozen blood spiked with tacrolimus (ST_{SP}).

In both non-frozen and frozen and thawed whole-blood samples, $[Tac]_{WB}$ measured with ST_{FD} appeared similar to $[Tac]_{WB}$ measured with ST_{SP} (Ratios 1.061 and 1.077, respectively). In non-frozen samples, the median ratio between the $[Tac]_{DBS}$ measured with ST_{FD}, and $[Tac]_{WB}$ measured with ST_{FD} (the reference method), was 1.396. When blood was volumetrically applied to the DBS card (to eliminate the effect of the spreading over the filter paper), this ratio was 1.009.

In conclusion, when using DBS microsampling to quantify concentrations of analytes, one should be aware that using the commercially available freeze-dried blood samples for the preparation of standards may affect the spreading of blood on the filter-paper, leading to a systematic error in the results.

1. Introduction

Dried blood spot (DBS) microsampling is a blood sampling method that is used for several purposes in the medical field. With DBS microsampling, a drop of capillary blood is collected on a filter-paper, from which different analytes can be measured. DBS has several advantages over venous blood sampling [1]. The method is less invasive and only a small volume of blood is required. Moreover, DBS sampling can be performed at home, which can limit the frequency of hospital visits, and allows blood sampling at specific time points.

Before implementing DBS microsampling in clinical practice, the method should be validated analytically and clinically [2]. In the Erasmus MC, University Medical Center Rotterdam, the results of the clinical validation of DBS for the measurement of tacrolimus concentrations in kidney transplant recipients showed a high proportional systematic deviation of the tacrolimus DBS concentrations ($[Tac]_{DBS}$) from the standard whole-blood concentrations ($[Tac]_{WB}$) collected by venipuncture, for which a correction factor was needed (Supplementary Data S1; Supplementary Fig. S1; [3]). As this deviation was not present when comparing $[Tac]_{WB}$ drawn from the finger with samples drawn by venipuncture, the hypothesis was that the cause of this deviation lies in the preparation of the standards. Here we describe the effect of using

* Corresponding author at: Dept. of Internal Medicine, Division of Nephrology & Renal Transplantation, Erasmus MC, University Medical Center Rotterdam, Room Rg-527, P.O. Box 2040, 3000 CA Rotterdam, the Netherlands.
E-mail address: M.FRANCKE@ERASMUSMC.NL (M.I. Francke).

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freeze-dried standards for the measurement of $[Tac]_{DBS}$.

2. Material and methods

2.1. Tacrolimus concentration measurement

In this study, two different standards were used for the determination of tacrolimus concentrations: 1) freeze-dried whole-blood standards (Chromsystems, cat.no. 0081 MassCheck®; ST_{FD}), and 2) blank whole-blood spiked with a tacrolimus (Sigma-Aldrich Chemie BV, Zwijndrecht, Netherlands) stock solution with a concentration of 0.39 mg/L (*i.e.* standards of fresh, non-frozen blood spiked with tacrolimus; ST_{SP}). Both the ST_{FD}, and the ST_{SP} were spotted on a DBS card to use as standards for DBS measurement ($ST_{FD\text{-}DBS}$ and $ST_{SP\text{-}DBS}$ respectively). All calibration curves were independently used to determine tacrolimus concentrations.

Details on the sample preparation and analysis can be found in Supplementary Data S2. Briefly, for DBS sampling, Whatman 903 Protein Saver DBS-Cards (art.no. 10531018, GE Healthcare) were used. For DBS samples, 50 μL of whole blood was pipetted on a DBS card. The DBS cards were dried for 24 h in a desiccator. For the sample preparation, a 6 mm hole was punched from the middle of the blood spot. Then, 200 μL internal standard solution was added to the 6 mm punch, which was then sonicated for 15 min at 40 °C. Next, 10 μL of the supernatant was injected into the LC-MS/MS system. Sample preparation for whole blood samples was done by mixing 50 μL of whole blood with 700 μL internal standard (5 μg/L, $^{13}C,^{2}H_4$-Tacrolimus, Alsachim, Illkirch Graffenstaden, France) in a 2:1 methanol and 0.1 M $ZnSO_4$ solution, which was then centrifuged for 10 min. Then, 10 μL of the supernatant was injected into the LC-MS/MS system.

All tacrolimus concentrations were measured using a validated LC-MS/MS method in an ISO15189 certified laboratory. The imprecision of the whole-blood method is < 15% with a bias < 15% over the validated range 1.0–35.0 ng/mL. The imprecision of the DBS microsampling method is < 20% with a bias < 20% over the validated range of 2.0–35.0 ng/mL.

2.2. Study design

Multiple experiments (Supplementary Fig. S2) were performed to evaluate whether the systematic differences between $[Tac]_{WB}$ and $[Tac]_{DBS}$ that were observed in the clinical validation study (Supplementary Data S1; Supplementary Fig. S1; [3]) could be explained by the use of ST_{FD}. For all experiments, left-over material of venous whole-blood EDTA samples of 10 patients that received tacrolimus as part of their immunosuppressive treatment was used. Half of each patient's sample was prepared following standard sample preparation (i.e. fresh non-frozen whole-blood samples prepared following the procedure described in Supplementary Data S2). The other half of the same sample was frozen at −20 °C for 20 h and thawed before further work-up to simulate the freeze-drying effect.

To compare the results of the different methods described below, ratios between the concentrations measured according to these methods were calculated. A deviation of < 10% was described as "similar" (ratios of 0.9–1.1), a deviation of 10–15% was described as "slightly different" (ratios of 0.85–0.9 and 1.1–1.15), and a deviation of > 15% was described as "different" (ratios < 0.85 and > 1.15).

2.2.1. Using freeze-dried versus fresh, non-frozen standards for $[Tac]_{WB}$

First, it was evaluated whether there was no inherent difference between frozen and non-frozen blood and standards. The $[Tac]_{WB}$ in both non-frozen whole-blood samples and frozen and thawed whole-blood samples was determined by using the calibration curves of ST_{FD} (as used in clinical practice) and ST_{SP}.

Table 1
Tacrolimus concentrations.

Sample	Tacrolimus concentration Median (range) (n = 10)	Ratio Sample: $[Tac]_{WB}$ ST_{FD}* Median (range) (n = 10)	Ratio ST_{FD}:ST_{SP} Median (range) (n = 10)
$[Tac]_{WB}$			
Standard work-up			
ST_{FD} (reference*)	7.6 (4.5–13.6)	1.00	1.061 (0.915–1.308)
ST_{SP}	7.0 (3.5–14.0)	0.941 (0.769–1.089)	
Frozen & thawed			
ST_{FD}	8.1 (2.4–14.3)	1.069 (0.452–1.406)	1.077 (1.051–1.200)
ST_{SP}	7.5 (2.0–13.6)	1.002 (0.368–1.335)	
$[Tac]_{DBS}$ 15 μL			
Standard work-up			
$ST_{FD\text{-}DBS}$	8.1 (4.4 – 16.0)	1.009 (0.848 – 1.320)	1.133 (1.111 – 1.173)
$ST_{SP\text{-}DBS}$	7.1 (3.7 – 14.4)	0.898 (0.751 – 1.159)	
Frozen & thawed			
$ST_{FD\text{-}DBS}$	7.2 (1.9 – 14.4)	1.023 (0.360 – 1.165)	1.139 (1.113 – 1.299)
$ST_{SP\text{-}DBS}$	6.3 (1.5 – 13.0)	0.898 (0.277 – 1.047)	
$[Tac]_{DBS}$ 50 μL			
Standard work-up			
$ST_{FD\text{-}DBS}$	10.8 (6.0 – 24.0)	1.396 (1.111 – 1.863)	1.771 (1.734 – 1.831)
$ST_{SP\text{-}DBS}$	6.1 (3.3 – 13.8)	0.794 (0.628 – 1.048)	
Frozen & thawed			
$ST_{FD\text{-}DBS}$	10.6 (4.6 – 20.8)	1.543 (0.854 – 1.953)	1.773 (1.738 – 1.872)
$ST_{SP\text{-}DBS}$	6.0 (2.5 – 12.0)	0.874 (0.456 – 1.122)	

*Prepared and measured according to standard practice.
$[Tac]_{WB}$, whole-blood tacrolimus concentration; $[Tac]_{DBS}$, Dried blood spot tacrolimus concentration; ST_{FD}, freeze-dried standard; $ST_{FD\text{-}DBS}$, freeze-dried standard spotted on a dried blood spot card; ST_{SP}, standards of fresh non-frozen blood spiked with tacrolimus; $ST_{SP\text{-}DBS}$, standards of fresh non-frozen blood spiked with tacrolimus spotted on a dried blood spot card.

2.2.2. $[Tac]_{DBS}$ in fixed volume samples using freeze-dried versus fresh, non-frozen standards

Second, the effect of ST_{FD} on $[Tac]_{DBS}$ measurement was investigated. A fixed volume (15 μL) of the patients' non-frozen whole-blood samples and the frozen and thawed whole-blood samples were applied volumetrically on pre-punched 6 mm DBS disks. Both $[Tac]_{DBS}$ from non-frozen blood samples and from blood that was frozen and thawed before application on a DBS card, were determined using the calibration curves of both $ST_{FD\text{-}DBS}$, and $ST_{SP\text{-}DBS}$.

Finally, to detect extractability issues, we measured $[Tac]_{WB}$ in 15 μL using the calibration lines of $ST_{FD\text{-}DBS}$ and $ST_{SP\text{-}DBS}$.

2.2.3. Spreading of freeze-dried blood over the DBS filter paper

Third, the effect of ST_{FD} on the spreading of the blood on the filter paper was evaluated. Fifty μL of the patients' non-frozen whole-blood samples, and the frozen and thawed whole-blood samples were spotted on DBS cards. There after a disk with a diameter of 6 mm was punched from the middle of the DBS spot. Both $[Tac]_{DBS}$ from non-frozen whole-blood samples and from blood samples that were frozen and thawed before application on a DBS card were determined using the calibration

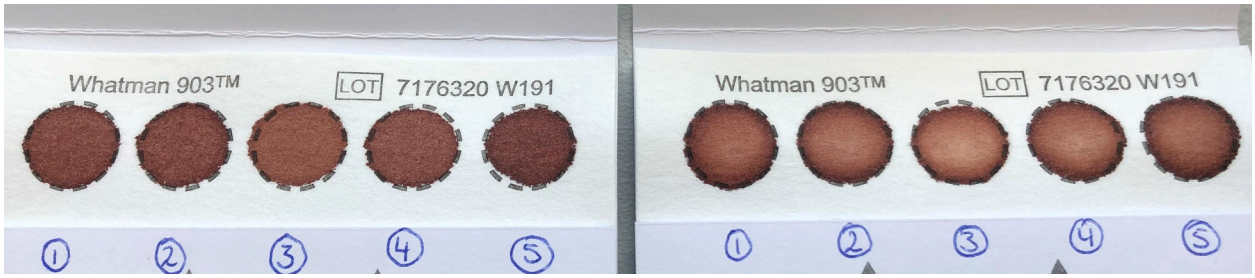

Fig. 1. Dried blood spot samples of fresh, non-frozen blood (left DBS-card) and dried blood spot samples of blood that was frozen and thawed before application to the DBS-card (right DBS-card).

curves of both $ST_{FD\text{-}DBS}$, and $ST_{SP\text{-}DBS}$.

3. Results

3.1. Using freeze-dried versus fresh non-frozen standards for $[Tac]_{WB}$

First, potential inherent differences between freeze-dried and non-frozen blood and standards were evaluated. The calibration curves of ST_{FD} and ST_{SP} for tacrolimus were comparable with intercepts of 0.021 and −0.001, and slopes of 0.0539 and 0.0552, respectively. In both non-frozen samples and frozen and thawed samples, the $[Tac]_{WB}$ measured with ST_{FD} were similar to $[Tac]_{WB}$ measured with ST_{SP} (Median ST_{FD}:ST_{SP} ratios 1.061 (range 0.915–1.308) and 1.077 (range 1.051–1.200), respectively; Table 1; Supplementary Table S1).

3.2. Using freeze-dried versus fresh non-frozen standards for $[Tac]_{DBS}$

3.2.1. Methodological effects of freeze-dried standards for $[Tac]_{DBS}$

Second, methodological and extraction effects of ST_{FD} on $[Tac]_{DBS}$ measurement were investigated by volumetrically applying 15 μL of blood on pre-punched disks (Table 1; Supplementary Table S1). In non-frozen blood samples, the $[Tac]_{DBS}$ measured with $ST_{FD\text{-}DBS}$ (Median 8.1 ng/mL (range 4.4–16.0)) were slightly higher compared to $[Tac]_{DBS}$ measured with $ST_{SP\text{-}DBS}$ (Median 7.1 ng/mL (range 3.7– 14.4); Median ST_{FD}:ST_{SP} ratio 1.133 (range 1.111–1.173). Similar results were observed with blood that was frozen and thawed before application on the DBS disk (Median ST_{FD}:ST_{SP} ratio 1.139 (range 1.113–1.229; Table 1). Finally, in 15 μL samples, $[Tac]_{WB}$ was similar to $[Tac]_{DBS}$ measured with $ST_{FD\text{-}DBS}$ (Median $[Tac]_{WB}$:$[Tac]_{DBS}$ ratio 1.018 (range 0.935–1.164) and with $ST_{SP\text{-}DBS}$ (Median $[Tac]_{WB}$:$[Tac]_{DBS}$ ratio 1.019 (range 0.933 – 1.168)).

3.2.2. Spreading of freeze-dried blood over the DBS filter paper

Third, the effect of ST_{FD} on the spreading of the blood over the filter paper and its effect on $[Tac]_{DBS}$ measurement was evaluated (Table 1; Supplementary Table S1).

In non-frozen samples, the median ratio between the $[Tac]_{DBS}$ measured with $ST_{FD\text{-}DBS}$, where blood was (non-volumetrically) applied prior to punching the DBS disk, and $[Tac]_{WB}$ measured with ST_{FD} (the reference method), was 1.396 (range 1.111–1.863). Moreover, using non-frozen blood samples, $[Tac]_{DBS}$ were higher with $ST_{FD\text{-}DBS}$ (Median 10.8 ng/mL (range 6.0–24.0)), than with $ST_{SP\text{-}DBS}$ (Median 6.1 ng/mL (range 3.3–13.8); Median ST_{FD}:ST_{SP} ratio 1.771 (range 1.734–1.831)). Also using frozen and thawed blood samples, the $[Tac]_{DBS}$ were higher with $ST_{FD\text{-}DBS}$ (Median 10.6 ng/mL (range 4.6–20.8)), than with $ST_{SP\text{-}DBS}$ (Median 6.0 ng/mL (range 2.5–12.0); Median ST_{FD}:ST_{SP} ratio 1.773).

Finally, the color of the blood spots was different comparing spots from fresh, non-frozen blood with spots from blood that was frozen and thawed before application to the DBS card (Fig. 1). The color of the fresh, non-frozen blood spots was uniform throughout the spot, while the frozen and thawed blood spots showed a light center and a much darker outer ring.

4. Discussion

The results of this study indicate that the spreading of blood over a DBS filter paper is different between freeze-dried and fresh, non-frozen blood samples. This explains the large deviation of $[Tac]_{DBS}$ from the $[Tac]_{WB}$ when using $ST_{FD\text{-}DBS}$ and ST_{FD}, observed previously in our clinical validation study (3).

Different factors that could affect $[Tac]_{DBS}$ measurement with the use of ST_{FD} were evaluated, among which methodological and extractability issues, and differential spreading over the filter paper. In the present study, both $[Tac]_{WB}$ and $[Tac]_{DBS}$ (when blood was volumetrically applied to a pre-punched DBS disk), were similar when determined with either ST_{FD} or ST_{SP} (the median ST_{FD}:ST_{SP} ratios ranged between 1.061 and 1.139). Moreover, in a fixed volume of blood, $[Tac]_{WB}$ and $[Tac]_{DBS}$ were similar using $ST_{FD\text{-}DBS}$ and $ST_{SP\text{-}DBS}$. These results indicate that the large deviation between $[Tac]_{DBS}$ and $[Tac]_{WB}$ cannot be (fully) explained by methodological or extraction effects of freeze-drying on $[Tac]_{DBS}$ measurement.

However, when blood was applied before punching the DBS disk from the DBS spot, like is done in clinical practice, the differences between $[Tac]_{DBS}$ determined with $ST_{FD\text{-}DBS}$ and $ST_{SP\text{-}DBS}$ were larger (median ST_{FD}:ST_{SP} ratios of 1.771 and 1.773, for non-frozen and frozen and thawed blood samples, respectively). As the punched DBS disk has a standard size (6 mm), the spreading of the blood over the filter-paper can affect the blood volume that is measured, when punching the DBS spot after the blood is applied. Therefore, these results indicate an effect of freeze-drying on the spreading of the blood over the filter paper. This is further supported by the high deviation that was observed between $[Tac]_{WB}$ measured with ST_{FD} (the reference method) and $[Tac]_{DBS}$ measured with $ST_{FD\text{-}DBS}$, where blood was applied before punching the DBS disk from the DBS spot (median $[Tac]_{WB}$:$[Tac]_{DBS}$ ratio of 1.396). This factor almost normalized to a ratio of 1 when blood was volumetrically applied to the DBS card (to exclude the effect of the spreading of the blood over the DBS filter paper): in non-frozen samples that were volumetrically applied to a pre-punched DBS card, the median ratio between $[Tac]_{DBS}$ determined with $ST_{FD\text{-}DBS}$ and $[Tac]_{WB}$ determined with ST_{FD} (reference) was 1.009. Finally, the middle of the DBS spot had a lighter color after the application of blood that was frozen and thawed before application to the DBS card, compared to non-frozen blood. As the DBS spot is punched from the middle of the blood spot, this can affect the measurement of the analyte.

Based on these results the methodological and extraction effects of freeze-drying appear limited, whereas the freeze-drying process appears to affect the spreading of the blood on the filter paper. Together, these results explain the high systematic deviation in our clinical validation study of DBS microsampling for the measurement of tacrolimus concentrations. In conclusion, when using DBS microsampling to quantify concentrations of analytes, one should be aware that using the commercially available freeze-dried blood samples for the preparation of standards may affect the spreading of blood on the filter-paper, leading to a systematic error in the results. Therefore we recommend to use fresh, non-frozen blood spiked with the component(s) of interest

in DBS microsampling method development.

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Declaration of Competing Interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2022.03.007.

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