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RESEARCH ARTICLE

The influence of the dried blood spot drying time on the recoveries of six immunosuppressants

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Investigation of the drying time of dried blood spots (DBS) is currently not included in DBS validations. The influence of the DBS drying time on the recovery of tacrolimus, ascomycin, sirolimus, everolimus, cyclosporin A and temsirolimus was evaluated by measuring DBS with a fixed blood volume at a hematocrit range between 0.1 and 0.6 L/L at 3, 24 and 48 hours of drying time. Results showed that the recovery of sirolimus, everolimus, temsirolimus and cyclosporin A was influenced by the DBS drying time, while the recovery of tacrolimus and ascomycin was not. A drying time of at least 24 hours is advised in order to stabilize hematocrit and concentration related recovery effects of sirolimus, everolimus, temsirolimus and cyclosporin A.

Keywords: dried blood spot, drying time, hematocrit, Whatman FTA DMPK-C cards, recovery, immunosuppressants

Introduction

One of the main advantages of DBS sampling is that it allows the patient to sample at home and send the DBS sample to the laboratory by mail [1-4]. This sampling is considered to be patient friendly because it is less invasive and saves patients transportation costs and time. DBS sampling also has a lower biohazard risk and requires a smaller amount of blood than venous sampling [2,4]. Solid organ transplant recipients are required to use a lifetime of immunosuppressant medications like tacrolimus (TaC), sirolimus (SiR), everolimus (EvE) and cyclosporin A (CyA) to prevent allograft rejection. Bioanalysis and Therapeutic Drug Monitoring of these drugs are necessary because efficacy and toxicity is associated with blood concentrations and/or pharmacokinetic parameters. Therefore, these patients could greatly benefit from immunosuppressant DBS analysis. Since the use of dried blood spot (DBS) analysis for therapeutic drug monitoring (TDM), more extensive validation procedures have been proposed in order to improve the quality of the analysis results. Variations of the hematocrit value, spot volume and DBS stability are among the parameters that should be investigated during method validation [1,5].

A perhaps unappreciated source of variability may be the drying time of a dried blood spot sample. After collection of the blood on the DBS card it should be dried dried at ambient temperature. It is already suggested by the European Bioanalysis Forum (EBF) that the required drying time may be influenced by the hematocrit (HT) and that this may affect the robustness and reproducibility of the assay [5]. Consequently it is recommended to investigate these parameters as part of the validation [5]. Although the DBS may appear dry after 3 hours, the extraction recoveries of the substances within the DBS

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could still suffer from further drying effects after those 3 hours. This could especially be the case when substance binding to the DBS card matrix by the hydrogen bond acceptors of the substance with the hydrogen groups in the cellulose of the DBS cards is suspected [6-8]. Despite this recommendation, many publications describe a drying time of 2 to 3 hours without presenting data to support this period. The most recent review of DBS validation procedures also noted that none of 68 reports included in the review described the effect of aging on the recovery [9].

The objective of this study was to investigate the influence of the drying time of the DBS on the recoveries for the immunosuppressants TaC, SiR, EvE, CyA and their structural analogues ascomycin (AsC) and temsirolimus (TeM).

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 2500 ng/mL in methanol and CyA at 50.000 ng/mL.

The following isotopically labelled internal standards (IS) were purchased from Alsachim (Illkirch Graffenstaden, France): TaC [$^{13}C,^{2}H_{2}$], EvE [$^{13}C_{2},^{2}H_{4}$] and CyA [$^{2}H_{12}$]. The extraction solution consisted of methanol:water (80:20 v/v%) and contained the isotopically labelled IS TaC [$^{13}C,^{2}H_{2}$], EvE [$^{13}C_{2},^{2}H_{4}$] and CyA [$^{2}H_{12}$] at concentrations of 2.5 ng/mL, 1.0 ng/mL and 10 ng/mL respectively. TaC [$^{13}C,^{2}H_{2}$] was used as IS for TaC and AsC. EvE [$^{13}C_{2},^{2}H_{4}$] was used as IS for EvE, SiR and TeM. And CyA [$^{2}H_{12}$] was used as IS for CyA. Previous research showed that some isotopically labelled IS were contaminated with one of the immunosuppressants [6]. TaC [$^{13}C,^{2}H_{2}$] was 1.1% contaminated with TaC, SiR [$^{13}C,^{2}H_{3}$] was 2.9% contaminated with SiR, EvE [$^{13}C_{2},^{2}H_{4}$] was 0.5% contaminated with EvE and was 0.7% contaminated with SiR. For this reason it was decided to use EvE [$^{13}C_{2},^{2}H_{4}$] as the internal standard for SiR and TeM. Low concentrations of the IS were used to minimize the effect of the contaminants, while still providing high and reliable peak areas.

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. This was performed by carefully tumble mixing the stored blood tube, followed by 2 minutes centrifugation at 2000 x *g* and visual inspection of possible hemolyis of the plasma.

Whatman FTA DMPK-C cards (Kent, UK), which are not impregnated with chemicals, were used for the DBS analysis. 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 1290 series combined LC system. All technical parameters were used as described by Koster et al [6]. 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.

Sample preparation

The preparation of the different target hematocrit values was by centrifuging tubes of citrate whole blood with a known HT (measured by a Sysmex XN-9000 analyzer) for 5 minutes at 1972 x *g*. The necessary volumes of plasma were omitted or added to achieve the target HT values [10]. The prepared HT values were always measured with the Sysmex XN-9000 analyzer in order to confirm the correct HT preparation. The sample preparation was performed according to a previously published method, which makes use of partial spot analysis [6]. For the preparation of the DBS samples an 8 mm disk 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 spiked or blank blood onto the DBS card punch in five-fold for each HT value and concentration. In this way, full spot punches were obtained for this research. The spots were air dried at ambient temperature. 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 10000 x *g* for 5 min, 20 µL of the extract was injected to the LC-MS/MS system.

Influence of the DBS drying time on the recovery (full spot punch)

Since recoveries could be negatively affected by lower HT values, combined with high concentrations [6-8],

Table 1. Mass spectrometer settings for all substances.

Substance	Precusor ion *(m/z)*	Product ion *(m/z)*	Fragmentor Voltage (Volts)	Collision Energy (Volts)
Tacrolimus	821.5	768.4	190	11
Tacrolimus [$^{13}C,^{2}H_2$]	824.5	771.4	140	15
Sirolimus	931.5	864.4	140	6
Everolimus	975.6	908.5	121	10
Everolimus [$^{13}C_2,^{2}H_4$]	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 [$^{2}H_{12}$]	1231.8	1414.8	170	16

blood with HT values of 0.10, 0.20, 0.30, 0.40 0.50 and 0.60 L/L were spiked at 100 ng/mL for TaC, SiR, EvE, AsC and TeM and at 2000 ng/mL for CyA.

After the DBS preparation, the spots were dried for 3, 24 and 48 hours at ambient temperature, directly followed by processing and analysis (solutions A). For the extraction recovery, extracts of blank DBS 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: recovery = A/B x 100%.

Results and discussion

The drying time results were evaluated at the HT values of 0.1 L/L and 0.4 L/L, as shown in **table 2** and **figure 1**. The HT value of 0.4 L/L represented a common patient HT value, while the HT of 0.1 L/L was expected to show lower recoveries compared to the HT value of 0.4 L/L, thereby confirming earlier data [6-8]. **Figure 1** showed no significant trends in declining recoveries of TaC and AsC due to the drying time at both HT values of 0.10 and 0.40 L/L. SiR, EvE and TeM showed considerable declining recoveries between 3 and 24 hours of drying, followed by a stabilization of the recoveries. At the low HT of 0.1 L/L, combined with the high concentration, the recoveries of SiR, EvE and TeM declined with 24%, 26% and 27% between 3 and 24 hours of drying time. At the HT of 0.40 L/L the decrease in recoveries between 3 and 24 hours of SiR, EvE and TeM was still observed but was less extreme with 13%, 17% and 21% respectively. With the drying time of 3 hours, the recoveries of SiR, EvE and TeM between 0.1 L/L and 0.40 L/L differed only 4%, 1% and 8% respectively. The short drying time of 3 hours apparently showed no HT related recovery effects. While a drying time of 24 hours or more showed

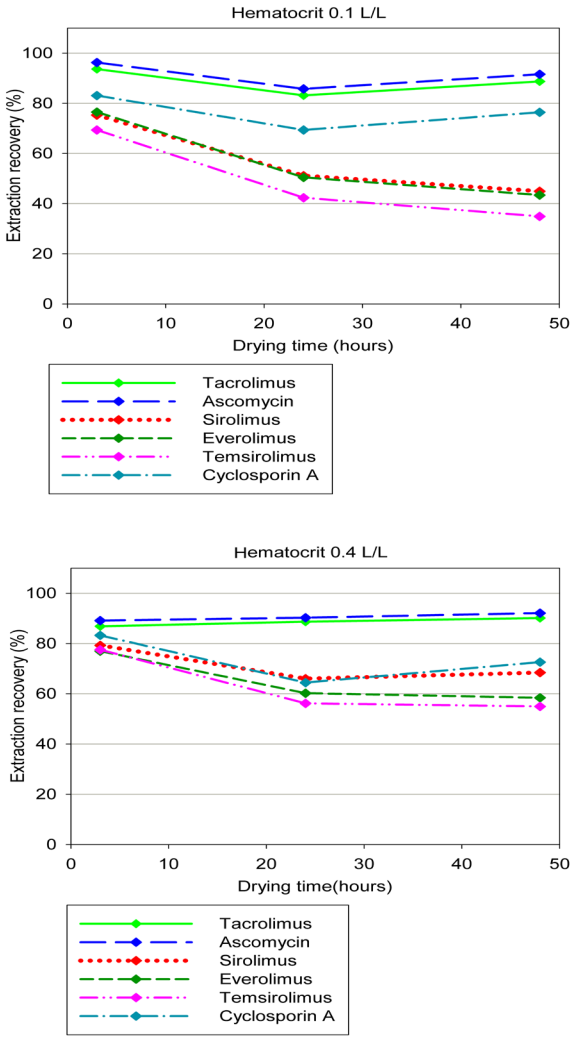

Figure 1. Influence of the DBS drying time on the substance recoveries using DBS full spot analysis at the hematocrit values of 0.1 and 0.4 L/L. The concentrations for tacrolimus, ascomycin, sirolimus, everolimus and temsirolimus were 100 ng/mL. And for cyclosporin A 2000 ng/mL. For every data point the mean of n=5 was reported.

Table 2. Mean recoveries and variation coefficients (CV) (data for figure 1) (n=5).

Substance	Hematocrit L/L	3 hours drying Mean recovery %	3 hours drying CV %	24 hours drying Mean recovery %	24 hours drying CV %	48 hours drying Mean recovery %	48 hours drying CV %
Tacrolimus	10	93.6	6.6	83.2	7.8	88.7	3.1
Ascomycin	10	96.3	7.2	85.8	9.4	91.6	2.3
Sirolimus	10	75.3	6.5	51.1	9.3	44.9	4.7
Everolimus	10	76.5	6.0	50.5	8.9	43.4	4.4
Temsirolimus	10	69.4	5.6	42.4	10.5	34.9	5.8
Cyclosporine A	10	83.1	6.0	69.3	9.6	76.4	3.8
Tacrolimus	40	86.9	0.7	88.7	1.9	90.1	1.0
Ascomycin	40	89.1	1.3	90.2	2.4	92.1	1.5
Sirolimus	40	79.3	3.6	66.0	3.7	68.4	1.7
Everolimus	40	77.0	2.8	60.6	4.3	58.4	2.0
Temsirolimus	40	77.5	3.0	56.2	3.8	54.9	2.2
Cyclosporine A	40	83.3	2.6	64.4	2.7	72.6	3.0

Table 3. Mean recoveries and variation coefficients (CV) for temsirolimus (data for figure 2) (n=5).

	3 hours drying		24 hours drying		48 hours drying	
Hematocrit L/L	Mean recovery %	CV %	Mean recovery %	CV %	Mean recovery %	CV %
0.1	69.4	5.6	42.4	10.5	34.9	5.8
0.2	72.7	6.7	51.2	6.3	45.7	4.6
0.3	75.9	3.9	54.8	3.4	55.7	3.2
0.4	77.5	3.0	56.2	3.8	54.9	2.2
0.5	75.9	7.4	51.4	11.4	55.1	6.1
0.6	71.0	8.1	43.4	3.6	50.0	5.4

declining recoveries for SiR, EvE and TeM at a normal HT of 0.40 L/L, while the low HT of 0.10 L/L showed additional HT related recovery effects with even lower recoveries. In line with a previous study, Cya showed no HT related drying time effects [6]. However, for both HT values of 0.10 L/L and 0.40 L/L CyA showed a decrease in recoveries of 14% and 19% respectively between 3 and 24 hours of drying. This showed that a drying time of 3 hours may also be insufficient when the substance recoveries are not influenced by binding to the cellulose of the DBS card. Since TeM recoveries were most affected by HT, **figure 2** illustrated the additional effects of the drying time combined with the HT effects at the HT range from 0.10 L/L to 0.60 L/L (see also **table 3**). It was observed that the whole HT range showed reduced recoveries after drying for 24 and 48 hours compared to those at 3 hours. A standardized drying period of 24 hours would result in lower, but more importantly, stable recoveries for SiR, EvE and TeM. **Table 3** also showed that the difference in recoveries for TeM between the drying times of 3 and 24 hours at the HT of 0.4 L/L was 21%. At the drying time of 3 hours, the difference in recoveries between a HT of 0.4 L/L and 0.1 L/L was 8%. At the drying time of 24 hours, the difference in recoveries between a HT of 0.4 L/L and 0.1 L/L was already 14%. And at the drying time of 48 hours, the difference in recoveries between a HT of 0.4 L/L and 0.1 L/L was 20%. This shows that both the drying time and the HT had significant influence on the recovery, and that the recovery is affected by the combination of these parameters. When the validation would be performed with a 3 hours drying time, the (recovery) results would be too optimistic, since patient DBS samples would probably dry for a longer time.

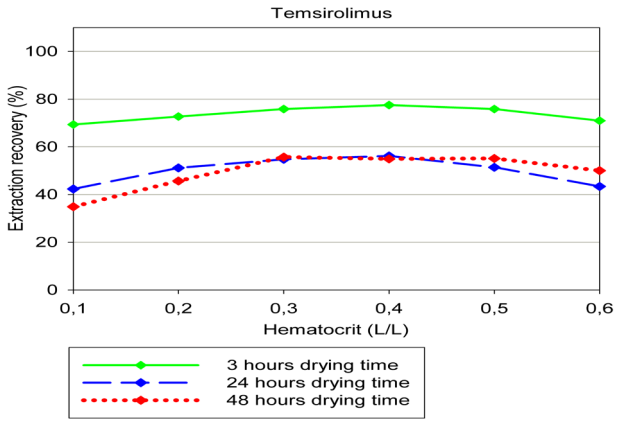

Figure 2. Recovery testing of temsirolimus (100 ng/mL) for 3, 24 and 48 hours at the hematocrit values of 0.10, 0.20, 0.30, 0.40, 0.50 and 0.60 L/L using DBS full spot analysis. For every data point the mean of n=5 was reported.

It should be noticed that the observed effects are only related to the (full spot punch) recoveries and that the distribution of the blood on the DBS cards due to HT would create an additional error.

The applied DBS extraction procedure made use of 15 minutes of sonication. In order to improve the recoveries, extended sonication times of 30 and 60 minutes were tested, without the desired results. Therefore, the current extraction procedure was considered optimal.

The low recoveries of less than 60% for SiR, EvE and TeM is however a worst case scenario that is negatively influenced by high concentrations and low HT values, in combination with the increasing number of hydrogen bond acceptors of SiR, EvE and TeM. At patient trough levels and normal HT values, the recoveries of SiR, EvE and TeM are higher than approximately 75%, which is considered suitable for therapeutic drug monitoring [6-8].

For this research, isotopically labeled structural analogues were used for AsC, SiR and TeM, instead of isotopically labeled IS. However, these IS showed to perform well, and identical conclusions were drawn based on the assessment on peak areas. The influence of the DBS dry-

ing time may overlap with substance stability effects at a certain point in time. This could imply that instability of the substances in the liquid or dried blood interfered with the conclusion of this study. However, the stability of TaC, SiR, EvE and CyA was previously validated in whole blood for three days at ambient temperature and were all found stable [11]. And the stability of TaC, SiR, EvE and CyA was previously validated in DBS for at least 7 days at ambient temperature [6]. Both validations support the conclusion that the lowered recoveries were due to the observed drying time effects and not due to stability issues.

Published DBS analysis methods for immunosuppressants mentioned drying times of 3 hours [12], at least 3 hours [13-15] and overnight drying [16, 17]. The lack of data supporting a 3 hour DBS drying time or more suggests that the effect of the drying time was underestimated. When a DBS drying time of 3 hours is applied during the validation, this will provide misleading results for SiR, EvE, TeM and CyA with less extreme HT related recovery effects and relatively high recoveries. Therefore, discrepancies in drying times between prepared standards and patient DBS samples may introduce significant bias during analysis of patient samples.

Conclusions

This study showed that the DBS drying time could have a significant influence on analyte recovery of SiR, EvE, TeM and CyA. For SiR, EvE and TeM, the drying time effect is also influenced by the HT of the blood, where lower HT values cause lower recoveries. It is clear that the DBS drying time is not yet acknowledged as a possible factor of influence in DBS analysis, since a recent review did not include the DBS drying time in their recommended validation practices [9]. A 3 hour DBS drying time is not recommended for SiR, EvE, TeM and CyA and a drying time of at least 24 hours is advised in order to minimize the risk on drying time related recovery effects for the compounds tested in this study. As a future perspective, DBS drying time effects should be investigated for a period of at least 48 hours during future method development and validation. Furthermore, the method validation should include a framework for the HT and substance concentration in which the results are within acceptable limits. Patient samples that are transported by mail will be allowed to dry sufficiently in a sealed bag with a silica sachet. However, the use of prepared standard and control samples that have not been dried sufficiently may introduce a significant bias during analysis of patient samples.

References

1. Wilhelm AJ, den Burger JC, Swart EL. Therapeutic Drug Monitoring by Dried Blood Spot: Progress to Date and Future Directions. Clin. Pharmacokinet. (2014).
2. Edelbroek PM, van der Heijden J, Stolk LM. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. Ther. Drug Monit. 31(3), 327-336 (2009).
3. Vu DH, Alffenaar JW, Edelbroek PM, Brouwers JR, Uges DR. Dried blood spots: a new tool for tuberculosis treatment optimization. Curr. Pharm. Des. 17(27), 2931-2939 (2011).
4. Li W, Tse FL. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. Biomed. Chromatogr. 24(1), 49-65 (2010).
5. Timmerman P, White S, Globig S, Ludtke S, Brunet L, Smeraglia J. EBF recommendation on the validation of bioanalytical methods for dried blood spots. Bioanalysis. 3(14), 1567-1575 (2011).
6. Koster RA, Alffenaar JW, Greijdanus B, Uges DR. Fast LC-MS/MS analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in dried blood spots and the influence of the hematocrit and immunosuppressant concentration on recovery. Talanta. 115, 47-54 (2013).
7. Koster RA, Alffenaar JW, Botma R et al. The relation of the number of hydrogen-bond acceptors with recoveries of immunosuppressants in DBS analysis. Bioanalysis. 7(14), 1717-1722 (2015).
8. Koster RA, Botma R, Greijdanus B et al. The performance of five different dried blood spot cards for the analysis of six immunosuppressants. Bioanalysis. 7(10), 1225-1235 (2015).
9. Jager NG, Rosing H, Schellens JH, Beijnen JH. Procedures and practices for the validation of bioanalytical methods using dried blood spots: a review. Bioanalysis. 6(18), 2481-2514 (2014).
10. Koster RA, Alffenaar JW, Botma R et al. What is the right blood hematocrit preparation procedure for standards and quality control samples for dried blood spot analysis?. Bioanalysis. 7(3), 345-351 (2015).
11. Koster RA, Dijkers EC, Uges DR. Robust, high-throughput LC-MS/MS method for therapeutic drug monitoring of cyclosporine, tacrolimus, everolimus, and sirolimus in whole blood. Ther. Drug Monit. 31(1), 116-125 (2009).
12. Sadilkova K, Busby B, Dickerson JA, Rutledge JC, Jack RM. Clinical validation and implementation

of a multiplexed immunosuppressant assay in dried blood spots by LC-MS/MS. Clin. Chim. Acta. 421, 152-156 (2013).
13. Hinchliffe E, Adaway JE, Keevil BG. Simultaneous measurement of cyclosporin A and tacrolimus from dried blood spots by ultra high performance liquid chromatography tandem mass spectrometry. J. Chromatogr. B. Analyt Technol. Biomed. Life. Sci. 883-884, 102-107 (2012).
14. van der Heijden J, de Beer Y, Hoogtanders K et al. Therapeutic drug monitoring of everolimus using the dried blood spot method in combination with liquid chromatography-mass spectrometry. J. Pharm. Biomed. Anal. 50(4), 664-670 (2009).
15. Rao RN, Maurya PK, Ramesh M, Srinivas R, Agwane SB. Development of a validated high-throughput LC-ESI-MS method for determination of sirolimus on dried blood spots. Biomed. Chromatogr. 24(12), 1356-1364 (2010).
16. Wilhelm AJ, den Burger JC, Vos RM, Chahbouni A, Sinjewel A. Analysis of cyclosporin A in dried blood spots using liquid chromatography tandem mass spectrometry. J. Chromatogr. B. Analyt Technol. Biomed. Life. Sci. 877(14-15), 1595-1598 (2009).
17. den Burger JC, Wilhelm AJ, Chahbouni A, Vos RM, Sinjewel A, Swart EL. Analysis of cyclosporin A, tacrolimus, sirolimus, and everolimus in dried blood spot samples using liquid chromatography tandem mass spectrometry. Anal. Bioanal Chem. 404(6-7), 1803-1811 (2012).

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