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Chapter 3.6

The relation of the number of hydrogen bond acceptors with recoveries of immunosuppressants in dried blood spot analysis

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ABSTRACT

Background: We investigated the influence of the number of hydrogen bond acceptors on the recovery of immunosuppressant drugs and their structural analogues. This hypothesis was tested by evaluation of the extraction recoveries of tacrolimus, ascomycin, sirolimus, everolimus and temsirolimus, with 12, 12, 13, 14 and 16 hydrogen bond acceptors respectively.

Results: With an increasing number of hydrogen bond acceptors of sirolimus, everolimus and temsirolimus a decrease in recoveries was found, while ascomycin showed recoveries corresponding to those of tacrolimus.

Conclusion: This study showed that the number of hydrogen bond acceptors of the analyte of interest may influence the recoveries in DBS analysis and is a relevant factor to be investigated during method development and validation.

INTRODUCTION

Tacrolimus (TaC), sirolimus (SiR) and everolimus (EvE) are used to prevent allograft rejection in solid organ transplantation [1]. Their narrow therapeutic ranges require individualized dosing using therapeutic drug monitoring (TDM) [2, 3]. To facilitate TDM, dried blood spot (DBS) sampling has been introduced to sample at home. This procedure is considered to be patient friendly because it saves patient's travel costs and time and requires only a small amount of blood [4-6].

In DBS analysis, the hematocrit (HT) effect can be considered as one of the most critical parameters during method development and validation [7]. The HT represents the relative volume of the red blood cells (RBC) in the blood and has a direct effect on the viscosity of the blood. The HT of the blood influences the permeability through the DBS card. Blood with a high HT has a low permeability through the DBS card and thus forms a smaller spot. The fixed diameter punch would then contain a higher blood volume causing a positive bias. Earlier publications have proven the viscosity effect caused by the HT on the measured concentration. It has been suggested to correct for this effect using a linear relation between HT and measured concentration [8-10]. Indirect measurement of the HT in the DBS by potassium analysis has also been proposed [11]. In order to avoid HT effects, several procedures used fixed volume coupled to a full spot analysis. However, the DBS sampling through a blood drop performed by patients produces a DBS with an unknown volume of blood, which can not be analyzed by means of a full spot analysis.

Despite being the most relevant clinical source of variability, not all observed variation can be explained solely by the effect of the HT on the blood spot formation. A recent study showed a significant reduction in recoveries at low HT in combination with high concentrations of SiR and EvE [8]. The decline in recoveries could potentially be related to differences in molecular properties of the compounds. The molecular properties of TaC, SiR and EvE showed an increasing number of hydrogen (H)-bond acceptors of 12, 13 and 14 respectively [12]. It was hypothesized that a higher number of H-bond acceptors induced a higher cellulose bound fraction, which was more difficult to extract than the non-cellulose bound fraction. This could explain the lower recoveries of the compounds with more H-bond acceptors.

The objective of this study was therefore to test the hypothesis that recovery was related to the number of H-Bond acceptors of the analyte by using a range of structural analogues with increasing number of H-bond acceptors; TaC and ascomycin (AsC) (n=12), SiR (n=13), EvE (n=14) and temsirolimus (TeM) (n=16).

MATERIALS AND METHODS

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). AsC was purchased from LC Laboratories (Woburn, USA). TeM was purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland).

The chemical and physical properties of the investigated substances are shown in table 1.

Table 1 Chemical and physical properties of the investigated substances

Substance	Molecular formula	Molecular weight g/mol	Hydrogen bond acceptor count	Hydrogen bond donor count	Hydrophobicity LogP	Water solubility *10 ⁻³ g/L	Protein binding %
Tacrolimus	C ₄₄ H ₆₉ NO ₁₂	804	12	3	5.6	4.0	99
Ascomycin	C ₄₃ H ₆₉ NO ₁₂	792	12	3	5.5	N.A.	N.A.
Sirolimus	C ₅₁ H ₇₉ NO ₁₃	914	13	3	7.5	1.7	92
Everolimus	C ₅₃ H ₈₃ NO ₁₄	958	14	3	7.4	1.6	74
Temsirolimus	C ₅₆ H ₈₇ NO ₁₆	1030	16	4	7.1	2.4	87

N.A. Not Available.

Data derived from <http://www.drugbank.ca/> and <http://www.chemicalize.org/>.

Combined stock solutions containing TaC, EvE, SiR, AsC and TeM were prepared at 2,500 ng/mL in methanol. This combined stock solution was five times diluted with methanol to obtain a concentration of 500 ng/mL. These combined stock solutions were used for the experiments. The deuterated internal standards (IS) TaC [¹³C₂,²H₄] and EvE [¹³C₂,²H₄] were purchased from Alsachim (Illkirch Graffenstaden, France). The extraction solution consisted of methanol:water (80:20 v/v%) and contained the deuterated internal standards TaC [¹³C₂,²H₄] and EvE [¹³C₂,²H₄] at concentrations of 2.5 ng/mL and 1.0 ng/mL respectively. TaC [¹³C₂,²H₄] was used as IS for TaC and AsC. EvE [¹³C₂,²H₄] was used as IS for EvE, SiR and TeM. 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. Whatman FTA DMPK-C cards (Kent, UK) 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. [8]. 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 2. Agilent Masshunter software for quantitative analysis (version B.04.00) was used for quantification of the analysis results, using peak area ratios of the substance and its internal standard.

Table 2 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 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

Sample preparation

The preparation of the target hematocrit values was by centrifuging tubes with 8 mL of 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 omitted or added to achieve the target HT values [13]. 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 [8]. 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 blood onto the DBS card punch. The spots were air 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 at 3.0, 10, 50 and 100 ng/mL for TaC, SiR, EvE, AsC and TeM. Blank DBS card spots were punched, transferred to eppendorf cups and 15 µL blood was spiked on the punched spots in fivefold for each HT and concentration, dried for 24 hours and analyzed (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: $\text{recovery} = A/B \times 100$. In order to report the acquired data, the coefficient of variation (CV) of the 5 replicate analyses was required to be within 15%.

RESULTS AND DISCUSSION

At low HT of 0.1 L/L stable extraction recoveries for TaC and AsC were observed and reduced extraction recoveries for SiR, EvE and TeM when concentrations were increased (table 3 and figure 1). The recoveries at the lowest concentration of 3.0 ng/mL showed that TeM has the lowest recovery of 80%. This was still close to the recoveries of SiR and EvE that showed recoveries of 85% and 84% respectively. When the concentrations of SiR, EvE and TeM increased, the recoveries reduced. This deterioration in recoveries was the worst for TeM, followed by EvE and SiR respectively. The recovery of TeM decreased with 32% with increasing concentration from 3.0 to 50 ng/mL, while the recoveries for EvE and SiR decreased with 25% and 21% respectively.

Table 3 Mean recoveries and variation coefficients (CV) at the hematocrit of 0.1 L/L and varying concentrations (data for figure 1) (n=5)

Substance	3 ng/mL		10 ng/mL		50 ng/mL		100 ng/mL	
	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)
Tacrolimus	93.4	6.1	94.9	6.3	92.8	2.4	92.1	1.9
Ascomycin	95.4	4.3	93.8	7.1	92.9	1.8	92.4	1.0
Sirolimus	84.7	8.4	79.4	9.1	63.3	3.8	55.9	4.3
Everolimus	84.1	5.4	75.5	4.0	58.9	3.5	55.8	3.7
Temsirolimus	79.5	10.7	67.4	6.0	47.8	4.3	44.4	7.0

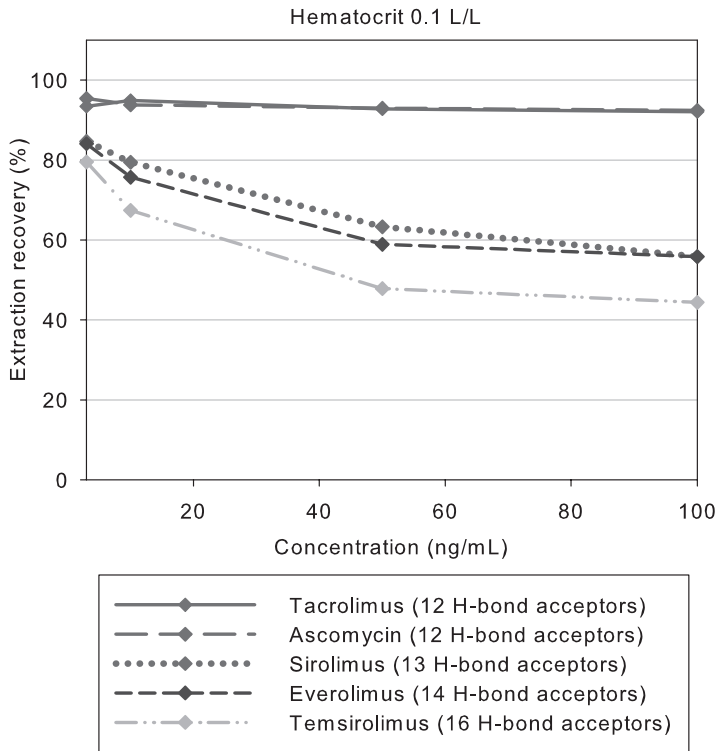


Figure 1 Recovery testing of tacrolimus, ascomycin, sirolimus, everolimus and temsirolimus at a hematocrit value of 0.1 L/L and at concentrations of 3.0, 10, 50 and 100 ng/mL using DBS full spot analysis. For every data point the mean of n=5 was reported.

In figure 2 (and table 4) it is demonstrated that at the HT values of 0.1 to 0.5 L/L and a fixed concentration of 3.0 ng/mL the extraction recoveries of all substances showed biases of no more than 10% compared to the extraction recoveries at a HT of 0.40 L/L, which is considered the mean HT of the patient population. The extraction recoveries at 100 ng/mL were also stable for TaC and AsC, while for SiR, EvE and TeM the recovery patterns showed decreasing extraction recoveries when the HT value decreased. At lower HT values, the recoveries of TeM were the lowest, followed by EvE and SiR.

The observations regarding the recoveries of TeM, EvE and SiR were in accordance with their number of H-Bond acceptors of 16, 14 and 13 respectively (table 1). TaC and AsC showed stable recoveries which was expected due to their 12 H-Bond acceptors. The inclusion of AsC and TeM seemed to confirm the theory that the number of H-bond acceptors was of

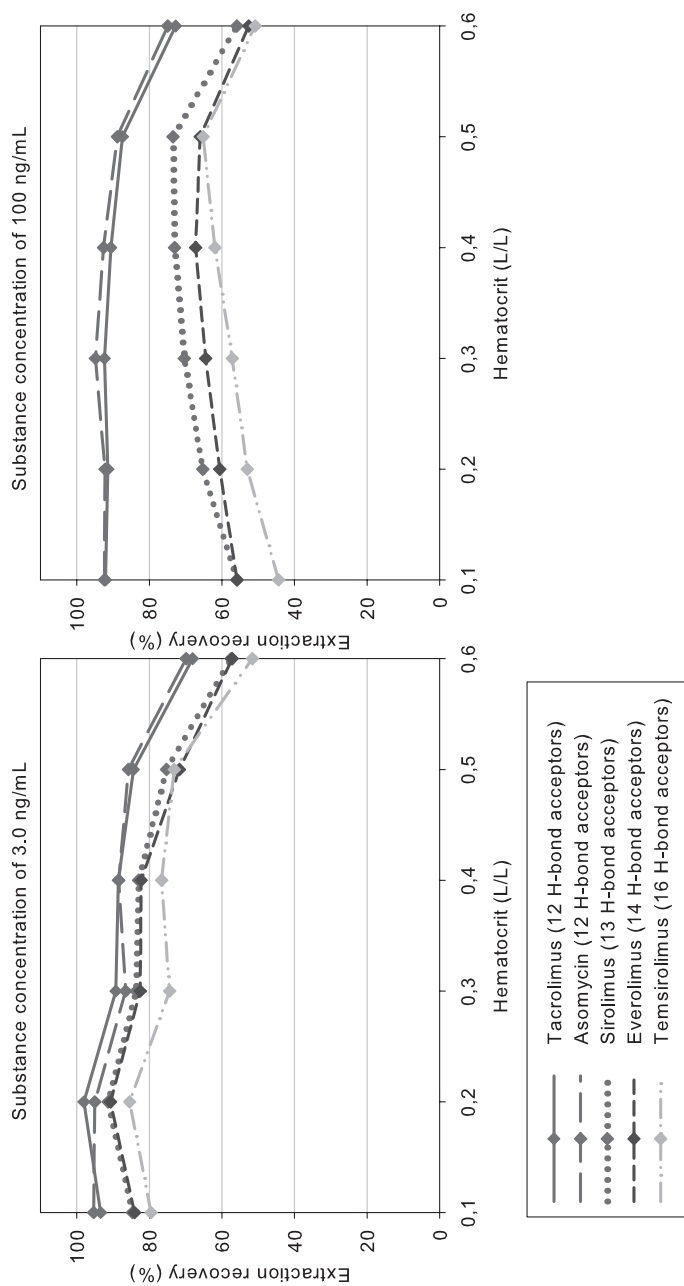


Figure 2 Recovery testing of tacrolimus, ascomycin, sirolimus, everolimus and temsirolimus at 3.0 and 100 ng/mL, at varying hematocrit values using DBS full spot analysis. For every data point the mean of n=5 was reported.

Table 4 Mean recoveries and variation coefficients (CV) at the concentrations of 3.0 ng/mL and 100 ng/mL and varying hematocrit values (data for figure 2) (n=5)

Substance	Concentration ng/mL	Hematocrit 0.1 L/L		Hematocrit 0.2 L/L		Hematocrit 0.3 L/L		Hematocrit 0.4 L/L		Hematocrit 0.5 L/L		Hematocrit 0.6 L/L	
		Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)
Tacrolimus	3.0	93.4	6.1	98.0	3.7	89.2	2.3	88.6	5.6	84.4	4.1	68.1	8.5
	100	92.1	1.9	91.4	2.4	92.4	2.5	90.6	1.0	87.4	1.3	72.7	3.4
Ascomycin	3.0	95.4	4.3	95.0	4.1	86.5	5.9	88.4	3.8	85.8	3.1	69.9	4.4
	100	92.4	1.0	92.2	2.2	94.8	5.8	92.6	0.7	88.8	1.4	74.9	3.6
Sirolimus	3.0	84.7	8.4	91.4	2.8	83.6	11.8	83.0	7.0	75.3	8.2	57.5	9.8
	100	55.9	4.3	65.3	2.5	70.4	6.2	73.0	4.7	73.5	4.8	55.8	8.3
Everolimus	3.0	84.1	5.4	90.7	3.8	82.5	9.6	82.3	4.0	71.7	10.2	57.2	10.8
	100	55.8	3.7	60.5	2.6	64.5	3.8	67.2	2.9	65.9	4.4	52.6	6.9
Temsirolimus	3.0	79.5	10.7	85.4	5.6	74.4	13.5	76.6	7.4	73.1	11.3	51.6	13.1
	100	44.4	7.0	53.0	1.1	57.1	11.6	61.9	8.1	65.2	5.5	50.8	6.8

influence on the extraction recoveries [8]. In addition, table 1 does not show any major differences in the chemical and physical properties of the investigated substances, other than the ascending number of hydrogen bond acceptors.

The HT value of 0.60 L/L showed lower extraction recoveries for all substances compared to the HT of 0.50 L/L. The current DBS extraction procedure 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.

The results indicate that the measurement of trough levels in DBS could be performed in a wide HT range with minimal deterioration of the recoveries for TaC, AsC, SiR, EvE and TeM. For the measurement of a wide range of concentrations such as pharmacokinetic curves of SiR, EvE and TeM, the HT dependent recovery effects should be acknowledged and the interpretation should be conducted with caution. The preparation of the calibration curve at the same HT as the pharmacokinetic curve of the patient could correct for the concentration dependant recovery effects at a certain HT value. Because the HT is normally unknown in a DBS sample, the analysis method could also be validated with a lower concentration range of for example 1.0 to 15 ng/mL. Within that framework the recoveries may not be affected, even at extreme HT values.

Based on our results, a simple correction for the HT value based on one tested concentration would not correct for all HT effects, and would have limited value. The HT and concentration dependant recovery effects require a more advanced algorithm for correction.

CONCLUSIONS

This study proved that the widely discussed HT effect in DBS analysis includes more than just the effect on the spot size and thus the punched blood volume with partial spot analysis. The influence of the number of H-Bond acceptors on the recoveries of TaC (12 H-bond acceptors), SiR (13 H-bond acceptors) and EvE (14 H-bond acceptors) seem to be confirmed with the inclusion of AsC (12 H-bond acceptors) and TeM (16 H-bond acceptors), where the substance with the highest number of H-bond acceptors showed the lowest recovery.

FUTURE PERSPECTIVE

This study showed that the number of H-bond acceptors of the analyte of interest may influence the recoveries in DBS analysis and is a relevant factor to be investigated during method development and validation. Therefore, guidelines for DBS validation should be constantly improved based on ongoing DBS research.

Future research should include the testing of a wider range of substances and H-bond acceptors in order to elaborate and substantiate the current theory.

In the near future, newly engineered DBS materials will primarily focus on limiting the HT effects regarding spot formation. However, the alternative material could also offer significant improvements regarding the substance binding to the DBS matrix, resulting in improved extraction recoveries.

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