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Clinical research

Direct quantitation of five immunosuppressant drugs in volume-controlled dried whole blood spots by a fully automated Transcend DSX-1 system

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Keywords

Transcend DSX-1 system, TSQ Altis Plus triple quadrupole mass spectrometer, Aria MX software, TraceFinder software, dried blood spot (DBS), dried spot analysis, therapeutic drug monitoring (TDM), immunosuppressant drugs, TurboFlow technology, 2D-LC-MS, Hypersil GOLD UHPLC column

Application benefits

- Demonstration of a complete and fully automated quantitative workflow for the dried blood spot analysis of five immunosuppressant drugs using a Thermo Scientific™ Transcend™ DSX-1 system
- Consistent and reproducible volume-controlled sample collection using HemaXis™ technology
- Flow-through desorption (FTD™) technology enables analyte extraction without the need for manual disc-punching
- Thermo Scientific™ TurboFlow™ technology allows fast matrix clean-up as part of a 2-D liquid chromatography (LC) method for analyte separation

Goals

A complete workflow is demonstrated that accurately quantitates five immunosuppressant drugs from volume-controlled dried blood spots collected using HemaXis™ DB10's precise sample collection technology. The automated and online workflow of the Transcend DSX-1 system combines direct analyte extraction by flow-through desorption with a 2-dimensional LC that utilizes TurboFlow technology for online sample clean-up.

Introduction

Therapeutic drug monitoring (TDM) of immunosuppressant drugs is vital for recipients of organ transplants. Thus, quantitation of the prescribed drugs is essential in such cases as it is imperative to ensure concentrations are high enough to prevent transplant rejection and low enough to avoid toxicity. Different immunosuppressant drugs may be prescribed, such as cyclosporin A, everolimus, mycophenolic acid (MPA), sirolimus, and tacrolimus. This means the developed methods must be able to detect a panel of immunosuppressant drugs.

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TDM of immunosuppressant drugs has traditionally been carried out by immunoassays. However, a shift towards mass spectrometry-based techniques is occurring due to their superior sensitivity and selectivity.

Dried matrix spot analysis is an emerging technology for clinical research and toxicology applications, such as anti-doping,[1] newborn screening,[2] and forensic toxicology.[3] The collection of dried blood spots (DBS) only requires small sample volumes that may be collected by an easy and relatively noninvasive procedure that results in stable, easy to store and transport samples. In addition, collection devices are commercially available that ensure precision and accuracy in collection volumes, reducing the potential for human error in the sample collection and preparation process.

Previously, one of the major problems with DBS analysis was the speed of the workflow. Manual disc-punching is usually followed by multiple sample preparation steps before downstream analysis by techniques such as LC-MS, resulting in processes ranging from 1 to 3 hours. The Transcend DSX-1 system incorporates a dried spot module (DSM) that utilizes flow-through desorption (FTD) technology to eliminate the need for disc-punching and performs online extraction that delivers extract directly to a UHPLC system. The UHPLC utilizes TurboFlow technology to clean up samples and deliver the eluants to an analytical column for the targeted analytes separation, resulting in quick and fully automated workflows with minimal opportunities for human error. The process uses an intelligent vision camera (IVC™) to capture images of each card both pre- and post-extraction, ensuring a clear chain of custody. Additionally, inline standards may be automatically added to allow absolute quantitation by a downstream mass spectrometer.

This technical note demonstrates a fully automated workflow for the extraction, clean-up, separation, detection, and quantitation of five immunosuppressant drugs from dried blood spots using a Transcend DSX-1 system and a Thermo Scientific™ TSQ Altis™ Plus triple quadrupole mass spectrometer (Figure 2). Samples are processed in 5 minutes, with LLODs and LLOQs demonstrated to be in the low nanogram-per-milliliter range. Strong linearity was demonstrated with $R^2 > 0.99$. Accurate and precise volumes of each spot were ensured by using volume-controlled DBS cards with HemaXis technology (Figure 1).

Figure 1. HemaXis DB10 blood collection device precisely collects 10 µL blood via capillary action.

Figure 2. Transcend DSX-1 system and TSQ Altis Plus triple quadrupole mass spectrometer

Experimental

Sample preparation

Cyclosporin A, everolimus, MPA, sirolimus, tacrolimus, and corresponding isotope-labeled standards were purchased from Cerilliant (Round Rock, TX, USA) and Toronto Research Chemicals (North York, ON, Canada). Pooled K2-EDTA stabilized normal human whole blood was purchased from BioIVT (Westbury, NY). Standards were resuspended in methanol, and calibrants were spiked into the blood for final concentrations between 0.313 and 80.0 ng/mL (for tacrolimus, everolimus, and sirolimus), or 6.25 and 1,600 ng/mL (for cyclosporin A and MPA). All samples were agitated on a thermomixer at room temperature for 2 hours for homogenous mixing. Fifty microliters of each calibrant was spotted onto aluminum foil to mimic a droplet of blood at the fingertip. HemaXis DB10 collection devices were used to collect and spot precisely 10 µL of the calibrant on each card in duplicate. Four DBS were analyzed for each calibration point. The other two spots of each card were reserved for DSM clamp cleaning. The sample cards were air-dried at room temperature for 3 hours before being transferred to the Transcend DSX-1 autosampler cardholder for analysis.

Fully automated extraction from DSM cards

The Transcend DSX-1 dried spot module (DSM) is located upstream of the UHPLC and was configured with an 8 mm clamp head for the desorption of the entire 10 µL blood spot. The internal standard mix (20 µL) was delivered via the built-in IS loading pump in the DSM to apply a precise amount onto each spot. Extraction was performed using 0.1% formic acid in 10% acetone at 2.0 mL/min for 30 seconds with HotCap™ enabled at 100 °C, followed by 25 seconds of drying to remove the residual mobile phase. Each individual spot was photographed with the IVC before and after each analysis to confirm the presence of the card and prove the chain of custody for each spot. The "Full Spot" mode located the actual positions of the DBS samples and accurately positioned the sample spot in the center of the extraction clamp (Figure 3). After extraction had occurred, the clamp head was rinsed with 0.1% formic acid in water (wash 1), 0.1% formic acid in acetonitrile (wash 2), and isopropanol/acetonitrile/acetone, 9/9/2 (v/v/v) (wash 3).

2-dimensional liquid chromatography

Fully automated online 2-dimensional sample clean-up and chromatographic separation were performed on the Transcend DSX-1 system's UHPLC component (Thermo Scientific™ Transcend™ TLX-1 UHPLC), which was equipped with TurboFlow technology and configured in Focus mode. Extracted samples were loaded onto a Thermo Scientific™ Cyclone-P™ TurboFlow column (0.5 × 50 mm) with 0.1% formic acid in 10% acetone, before analytes were eluted with 0.1% formic acid in methanol with 10 mM ammonium formate. Analytes were then refocused on a Thermo Scientific™ Hypersil GOLD™ C8 (2.1 × 50 mm, 3 µm) analytical column at 70 °C, while the TurboFlow column was washed by rapid organic/aqueous cycles. A ramped gradient (Table 1) was used to achieve chromatographic separation of targeted analytes. In preparation for the next injection, the analytical column was then washed and re-equilibrated while the TurboFlow transfer loop was filled with eluant and the TurboFlow column was re-equilibrated.

Mass spectrometry

All mass spectra were acquired using a TSQ Altis Plus triple quadrupole mass spectrometer, equipped with a Thermo Scientific™ OptaMax™ NG ion source with a heated electrospray ionization probe in positive ion mode. Source conditions and MS parameters are listed (Table 2). A selected reaction monitoring (SRM) method was used for targeted analysis (Table 3).

Data analysis

All data were acquired and processed using Thermo Scientific™ TraceFinder™ software, version 5.1.

Figure 3. Images of 10 µL dried blood spots collected by the HemaXis DB10 device before and after extraction. The Intelligent Vision Camera (IVC) recognizes the actual location of the blood spot and positions the sample spot to the center of the extraction clamp.

Table 1. Liquid chromatography conditions

Time (min)	TurboFlow column						Analytical column				
	Flow rate (mL/min)	% A	% B	%C	Tee	Loop	Divert	Flow rate (mL/min)	Gradient	%A	%B
0.00	2.0	100	-	-	====	out	Waste	0.5	Step	70	30
0.10	0.1	100	-	-	====	out	Waste	0.5	Step	70	30
0.20	2.0	100	-	-	====	out	Waste	0.5	Step	70	30
0.60	0.1	-	100	-	====	out	Waste	0.5	Step	70	30
0.68	0.1	-	100	-	T	in	Det*	0.4	Step	70	30
1.68	2.0	-	-	100	====	in	Det*	0.5	Step	70	30
1.93	2.0	-		100	====	in	Det*	0.5	Ramp	30	70
2.18	1.5	100	-	-	====	out	Det*	0.5	Ramp	25	75
2.43	2.0	-	-	100	====	in	Det*	0.5	Ramp	20	80
2.68	1.0	100	-	-	====	out	Det*	0.5	Ramp	15	85
2.93	1.0	-	100	-	====	in	Det*	0.5	Ramp	5	95
3.43	1.0	100	-	-	====	out	Det*	0.5	Step	5	95
3.93	1.0	100	-	-	====	out	Det*	0.5	Step	70	30
5.00	1.0	100	-	-	====	out	Det*	0.5	Step	70	30
Clamp washes	• Wash 1: 0.1% formic acid in water • Wash 2: 0.1% formic acid in acetonitrile • Wash 3: Isopropanol/acetonitrile/acetone, 9/9/2 (v/v/v)										
Mobile phases	• A: 0.1% formic acid, 10% acetone in water • B: 10 mM ammonium formate, 0.05% formic acid in methanol • C: Isopropanol/acetonitrile/acetone, 9/9/2 (v/v/v)						• A: 10 mM ammonium formate, 0.05% formic acid in water • B: 10 mM ammonium formate, 0.05% formic acid in methanol				
Columns	Cyclone-P TurboFlow column, 50 × 0.5 mm at room temperature						Hypersil GOLD C8 column, 50 × 2.1 mm, 3 µm, 70 °C				

*Det: flow diverts to TSQ Altis Plus MS

Table 2. Mass spectrometer source settings

Parameters	Value
Polarity	(+)
Sheath gas (Arb)	50
Aux gas (Arb)	10
Sweep gas (Arb)	0
Ion transfer tube temp. (°C)	400
Vaporizer temp. (°C)	300
Spray voltage (V)	4,000
Dwell time (ms)	15
Q1 resolution (FWHM)	0.7
Q3 resolution (FWHM)	0.7
Source fragmentation	0
CID gas (mTorr)	1.5

Table 3. SRM conditions for each targeted analyte

Analytes	Precursor		Quantifier		Qualifier	
	m/z	RF Lens (V)	*m/z*	CE (V)	*m/z*	CE (V)
Mycophenolic acid	338.2	80	207.1	23	275.1	20
Mycophenolic acid-$^{2}H_{3}$	341.1	80	210.1	23	278.1	20
Tacrolimus	821.5	175	768.4	21	786.4	17
Sirolimus	931.5	173	864.5	17	882.4	12
Sirolimus-$^{2}H_{3}$	934.6	173	864.4	17	882.4	12
Everolimus	975.6	176	908.6	16	926.4	12
Everolimus-$^{2}H_{4}$	979.6	177	912.5	16	930.4	12
Cyclosporin A	1219.9	226	1202.9	17	1184.8	32
Cyclosporin A-$^{15}N_{11}$	1230.9	230	1213.8	17	1195.8	32

Results and discussion

Dried blood spots were analyzed directly, with a total of 5 minutes from analyte extraction to mass spectrometric detection. Integrated Thermo Scientific™ Aria™ MX software (v. 2.7) was used to control each step of the process.

Limits of detection (LODs) were defined to be the lowest concentration that all calibrants display clear chromatographic peaks. Linear calibration curves were generated using a weighing factor of 1/x from LOD to the upper limits of quantification (ULOQ – Table 4). Strong linearity was obtained with R^2 values > 0.99. The limit of quantification (LOQ) value was defined at the lowest concentration with %RSD and %CV < 15, average %Diff < ±15, and relative ion ratio < 20% (N = 4). The reproducibility of the analysis was assessed by the %RSD of the internal standard peak area across all samples (N = 36), which in all cases were below 9%. The calibration curves, the %RSD of the internal standard response, and representative extraction ion chromatogram at the LOQ levels of each analyte are shown in Figure 4.

Table 4. Retention time, LODs, LLOQs, and R^2-values of line of best fit for each targeted immunosuppressant drug

Analytes	t_R (min)	Internal standards	LOD	ULOQ	R^2	LOQ	LOQ %RSD	LOQ %CV	LOQ Ave. %Diff
Mycophenolic acid	2.19	Mycophenolic acid-$^2H_3$	6.25	1,600	0.9941	12.50	3.11	2.16	12.80
Tacrolimus	2.60	Everolimus-$^2H_4$	0.31	80	0.9925	1.25	8.67	6.80	5.30
Sirolimus	2.65	Sirolimus-$^2H_3$	1.25	80	0.9926	5.00	9.84	7.36	-0.66
Everolimus	2.67	Everolimus-$^2H_4$	0.63	80	0.9918	2.50	10.28	6.81	3.68
Cyclosporin A	2.91	Cyclosporin A-$^{15}N_{11}$	6.25	1,600	0.9944	12.50	12.58	6.85	-2.92

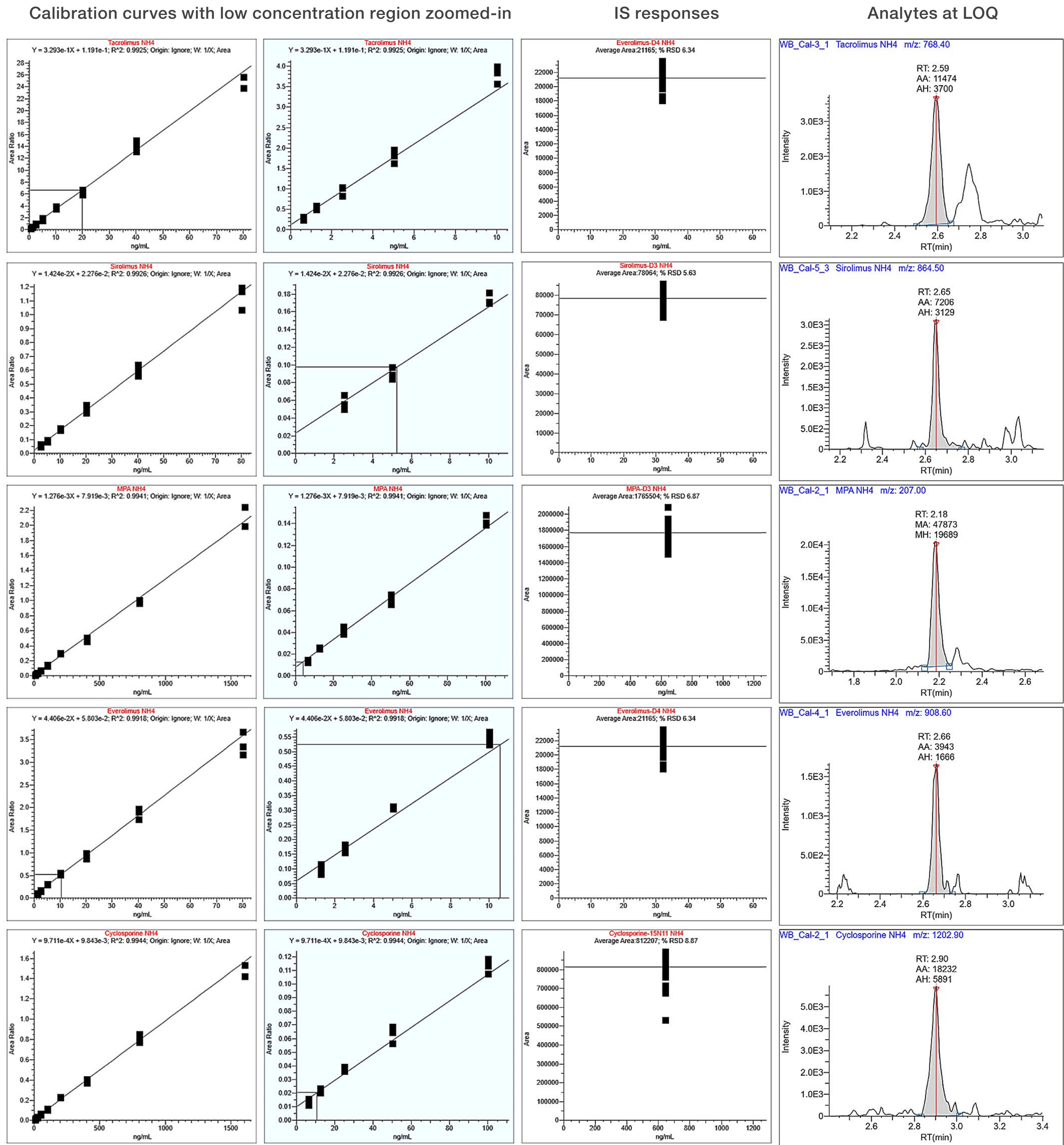

Figure 4. Calibration curves of each analyte with zoomed-in figure on the low-concentration region, the internal standard responses across all samples, and representative extracted ion chromatograms of analyte at the LOQ levels

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Conclusion

Five immunosuppressant drugs have been reliably quantified from DBS for clinical research. DBS has numerous advantages, such as quick and easy sampling, efficient sample transport and storage, and good analyte stability. Traditional downsides to DBS analysis, such as labor and resource-intensive manual disc-punching and offline sample clean-up, have been avoided by the Transcend DSX-1 system's ability to utilize flow-through desorption and TurboFlow technology to provide a quick, robust, and fully automated online platform. In addition, the workflow securely maintains chain of custody for each dried spot throughout, with images provided from before and after each extraction.

The successful quantitation was shown to be reproducible across a wide dynamic range, with strong linearity demonstrated. LODs and LOQs were in the low/sub nanogram per milliliter range, with upper ULOQs above whole blood toxicity levels.

References

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