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A dried blood spots technique based LC–MS/MS method for the analysis of posaconazole in human whole blood samples

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ABSTRACT

A rugged and robust liquid chromatographic tandem mass spectrometric (LC–MS/MS) method utilizing dried blood spots (DBS) was developed and validated for the analysis of posaconazole in human whole blood. Posaconazole fortified blood samples were spotted (15 μL) onto Ahlstrom Alh-226 DBS cards and dried for at least 2 h. Punched spots were then extracted by using a mixture of acetonitrile and water containing stable labeled internal standard (IS). Posaconazole and its IS were separated from endogenous matrix components on a Kinetex™ C18 column under gradient conditions with a mobile phase A consisting of 0.1% formic acid and a mobile phase B consisting of 0.1% formic acid in acetonitrile/methanol (70/30, v/v). The analyte and IS were detected using a Sciex API 4000 triple quadrupole LC–MS/MS system equipped with a TurboIonSpray™ source operated in the positive ion mode. The assay was linear over the concentration range of 5–5000 ng/mL. The inter-run accuracy and precision of the assay were −1.8% to 0.8% and 4.0% to 10.4%, respectively. Additional assessments unique to DBS were investigated including sample spot homogeneity, spot volume, and hematocrit. Blood spot homogeneity was maintained and accurate and precise quantitation results were obtained when using a blood spot volume of between 15 and 35 μL. Human blood samples with hematocrit values ranging between 25% and 41% gave acceptable quantitation results. The validation results indicate that the method is accurate, precise, sensitive, selective and reproducible.

1. Introduction

Dried blood spot (DBS) screening has been used as far back as the 1960s for the early detection of metabolic disorders in infants such as phenylketonuria [1–3]. The technique is increasingly viewed as a viable alternative to venous blood sampling for large-scale pediatric clinical trials primarily because of the small sampling volume required (usually between 10 and 30 μL). Other advantages of DBS include simplified sample collection, easy storage and less expensive sample shipment under ambient conditions [3–7]. These benefits also make DBS a practical alternative for pre-clinical toxicokinetic studies because it facilitates multiple sampling of the same animal, thus significantly reducing the number of animals per study [8–10].

In recent years, the DBS technique has extended beyond pediatric screening to monitoring concentrations of xenobiotic drugs in biological matrices [7,8,11–15]. A variety of small molecule drugs such as acetaminophen, tacrolimus, metformin and dextromethorphan have been analyzed using DBS. In addition, clinical trials for anti-malarials, anti-retrovirals and anti-epileptics in developing countries have used DBS to mitigate issues with limited resources [8,11,16–19]. In this article we will expand on the application of DBS for compassionate use in immuno-compromised patients worldwide.

Posaconazole, marketed in the United States as Noxafil®, is a triazole antifungal compound used to treat life threatening invasive fungal infections in immune suppressed patients [20]. Triazoles, such as posaconazole, are selective inhibitors of 14a-demethylase (CYP51) enzyme, leading to depletion of ergosterol and accumulation of 14a-methyl-sterols, thereby adversely affecting the fungal membrane synthesis [21]. Posaconazole is clinically effective against a variety of yeasts and molds such as Aspergillus, Candida, Cryptococus, Coccidioides and Fusarium. It is orally administered and its bioavailability is increased by co-administering it with food and liquid nutritional supplements such as Boost Plus® [22–29]. Clinical trials have established that posaconazole is safe and effective at an 800 mg/day dose, although, it is does have inter- and intra-individual variability of up to 68% in its pharmacokinetic values [30]. Posaconazole is an inhibitor of the CYP3A4 enzyme system and therefore, the potential exists for interactions with drugs also metabolized by the same system [28]. However, unlike other azole antifungals, posaconazole does not inhibit the isoforms CYP1A2, 2C8/9, 2D6, or 2E1 [28]. Nevertheless, because of variable

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gastrointestinal absorption, the potential for adverse side effects, and suspected drug–drug interactions, therapeutic drug monitoring (TDM) of posaconazole is recommended to ensure effective exposure while avoiding risks of toxicity.

To this extent, several HPLC-UV methods have been published in recent years to measure the therapeutic drug concentrations of posaconazole and other azoles in human plasma [31–37]. Unfortunately, these methods suffer the drawbacks of limited sensitivity and specificity and lengthy cycle times. Our laboratory previously developed and published several methods that resolved these drawbacks by using an LC–MS/MS platform for the determination of posaconazole concentrations in human plasma [38–40]. A number of investigators have reported LC–MS/MS based methods for TDM of posaconazole (and other triazoles) that use various sample preparation schemes with the most common being protein precipitation [41–45]. These methods support the TDM that is presently accomplished by collecting blood draws from patients. The whole blood is processed to generate plasma and frozen for shipment for analysis. The current sampling process is onerous in terms of time given that the physicians require rapid data turnaround in order to make relevant clinical decisions regarding very ill patients. It is important to note that collection methods that utilize separator gel tubes to harvest serum have the potential to underestimate exposure because of adsorption to the polymeric gel [46–50].

The use of DBS based sampling will allow for rapid sample collection, shipment and analysis. This in turn facilitates the rapid generation of results required in the clinical setting. Further, the use of the DBS sampling technique allows for a relatively small volume of blood from patients. Overall, this technique under the clinical setting results in patient comfort and a rapid generation of clinically required drug concentration results and is generally preferred to intravenous blood sampling. We now report the development and validation of a high throughput dried blood spots based LC–MS/MS method for the determination of posaconazole concentrations in human whole blood samples. The present method offers the simplicity and convenience inherent to the DBS technique, faster run time (~1 min) and the sensitivity and specificity afforded by MS/MS detection.

2. Experimental

2.1. Reference materials

Posaconazole and its internal standard (IS), ^{13}C $^{15}N_2$-posaconazole (Fig. 1) were synthesized at Schering-Plough Research Institute (Union, NJ) with purities of 99.4% and 96.8%, respectively.

The Ahlstrom Ahl-226 uncoated DBS cards were purchased from ID Biological Systems (Greenville, SC). A comparative experiment was performed using FTA DMPK-C DBS cards purchased from the GE Healthcare division of Whatman (Sanford, USA). All spots were punched using Harris Uni-Core punches (3 and 6 mm) on cutting mats purchased from the Whatman division of GE Healthcare (Sanford, USA).

2.2. Biological matrix

Human blood with K_2 EDTA anti-coagulant was purchased from Bioreclamation (Westbury, NY) and was used for the preparation of calibration standards (STD) and quality control (QC) samples without further treatment. The blood was stable for 1 month per the manufacturer's C of A and stored refrigerated (2–8 °C).

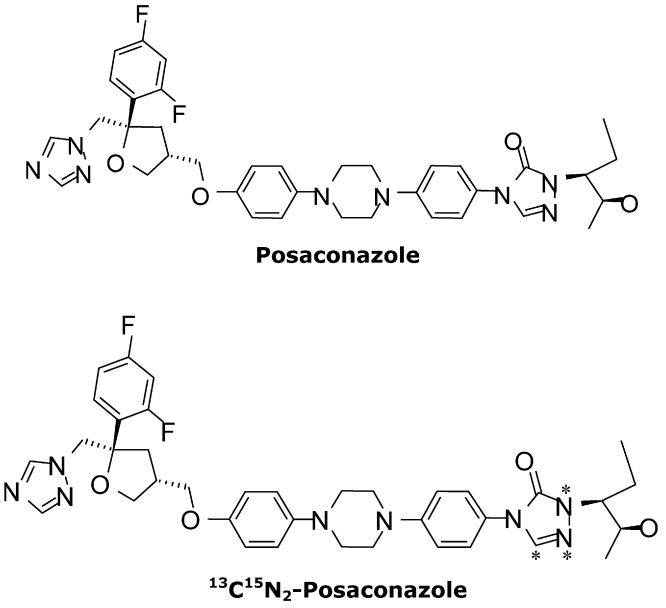

Fig. 1. Chemical structures of posaconazole and its stable-labeled internal standard (IS).

2.3. Reagents

HPLC grade acetonitrile, methanol, isopropanol and acetone were purchased from Fisher Scientific (Fair Lawn, NJ). ACS grade formic acid was also obtained from Fisher Scientific (Fair Lawn, NJ). Ultra-pure water was generated by using a Millipore Milli-Q® water system (Bedford, MA).

2.4. Preparation of stock and spiking solutions

Fresh stock solutions of posaconazole and its stable labeled internal standard (IS) were prepared in methanol at concentrations of 1.00 mg/mL for each stock. A series of posaconazole spiking solutions were prepared in methanol/water (50/50, v/v) from the stock solution. These spiking solutions ranged in concentration from 50.0 to 50,000 ng/mL. A IS working solution was prepared in acetonitrile/water (90/10, v/v) at a concentration of 50.0 ng/mL. All preparations were conducted in the spirit of the GLP guidelines for bioanalytical method validation.

2.5. Preparation of calibration standards and quality control samples

On each day of analysis, 10 posaconazole calibration standards were prepared in human K_2 EDTA blood by adding 50 μL of each spiking solution into a 450 μL aliquot of blood. The resulting standard calibration concentrations of posaconazole were 5.00, 10.0, 20.0, 50.0, 100, 250, 500, 2000, 4250, and 5000 ng/mL. In addition, four levels of posaconazole quality control (QC) samples were prepared by adding 50 μL of the corresponding spiking solution into a 450 μL aliquot of human blood. The resulting QC concentrations were 5.00 (LLOQ), 15.0 (QCL), 400 (QCM), and 4000 (QCH) ng/mL. All spiked blood samples were gently vortexed to facilitate mixing and then immediately used. All preparations were conducted in the spirit of the GLP guidelines for bioanalytical method validation.

2.6. DBS sample preparation and extraction

Validation experiments were performed by spotting a 15 μL aliquot of the appropriate posaconazole sample onto the center of

each printed circle on the Ahl-226 DBS card. The blood spots were dried for at least 2 h under ambient room temperature conditions. DBS cards spiked with QC samples designated for the long-term stability assessment were stored under ambient room temperature conditions in a sealed plastic bag with a small amount of Drierite desiccant (W.A. Hammond Drierite Co. Ltd., Xenia, OH). A 6-mm disc was punched from each dried blood spot sample using a Harris Uni-Core punch and transferred to individual wells of a polypropylene 2-mL 96-well plate (Analytical Sales & Products, Inc., Pompton Plains, NJ) containing 20 μL of Milli-Q® water. (The addition of water to the DBS card was done in order to normalize the potential wetness states of the DBS card s arising from exposure to varying environmental conditions during sample and shipment stages.) Then, a 200-μL aliquot of IS working solution was added to all samples, except matrix blanks, to which a 200-μL aliquot of acetonitrile/water (90/10, v/v) was added. Next, the samples were capped with a silicone ultra thin resealable cap mat (Analytical Sales & Products, Inc., Pompton Plains, NJ) and gently mixed on a titer-plate shaker (Lab Line Instruments, Melrose Park, IL) for 30 min. Finally, 100 μL of each extract was transferred to individual wells of a fresh polypropylene 2-mL 96-well plate using a Tomtec Quadra96® automated liquid handling system (Tomtec Corp, Hamden, CT). The 96-well plate was capped and transferred to the LC–MS/MS system for analysis.

The same procedure was executed using FTA DMPK-C dried blood spots cards for a comparative analysis.

2.7. LC–MS/MS conditions

All experiments were performed on an AB Sciex (Concord, Ontario, Canada) API 4000 triple quadrupole mass spectrometer with a Waters (Milford, MA) ACQUITY UPLC™ system. After sample preparation, 10–15 μL aliquots were injected onto a 50 mm × 2.1 mm C18, 2.6 μm analytical column (Phenomenex Kinetex®, Torrance, CA) for chromatographic separation. The column temperature was held at ambient. The mobile phase had a flow of 0.8 mL/min and consisted of 0.1% formic acid (mobile phase A) and 0.1% formic acid in acetonitrile/methanol (70/30, v/v). The following gradient was run, time 0–0.1 min: 60% mobile phase A and 40% mobile phase B, time 0.1–0.6 min: 5% mobile phase A and 95% mobile phase B, time 0.6–0.61 min: 100% mobile phase B, time 0.61–0.80 min: 100% mobile phase B, time 0.80-0.81 min: 60% mobile phase A and 40% mobile phase B.

The AB Sciex API 4000 mass spectrometer was operated in TurboIonSpray™ positive ionization mode and performed multiple reaction monitoring (MRM). The TurboIonSpray™ voltage and temperature were set at 2500 V and 500 °C, respectively. The collision gas (ultra pure nitrogen) was set to maintain a pressure of approximately 3.5×10^{-5} torr. The declustering potential was set to 90 V and the collision energy was set to 47 eV. The mass spectrometer was operated in unit mass resolution and the MRM transitions used to detect posaconazole and its IS were m/z 701 → 683 and m/z 705 → 687, respectively (The mass of the parent IS transition was increased by 1 amu to minimize cross-talk and accurately quantitate posaconazole concentrations.) A dwell time of 50 ms was used for each MRM transition.

2.8. Data analysis

Peaks areas were integrated using AB Sciex Analyst® 1.4.2 software (Concord, Ontario, Canada) and calculated concentrations were determined using Watson LIMS software version 7.3.0.1 (Thermo LabSystems Inc., Philadelphia, PA). Quadratic regression with weighting of $1/x^2$ using peak area ratios was used to generate calibration curve data.

3. Validation study design

Presently, no specific regulatory guidelines exist for the validation of bioanalytical methods using DBS cards. Hence, the present validation was conducted based on FDA guidelines and subsequent 2006 Bioanalytical Methods Validation Workshop white paper on bioanalytical method validation [51–53]. In addition, supplemental validation experiments were conducted taking into account the unique circumstances encountered with the use of DBS cards.

The validation strategy consisted of three core analytical runs which were used to establish precision, accuracy, selectivity, matrix effect and reproducibility parameters. Additional analytical runs were performed to establish analyte stability (i.e., autosampler, delayed acquisition and long-term ambient storage), LC–MS/MS ruggedness, dilution integrity, recovery, effect of hematocrit, effect of variable spotting volume, sampling homogeneity of dried blood spots, and equivalence of two different brands of DBS cards.

Each core run was comprised of two sets of calibration standards ($n = 10$) which bracketed four levels of QC samples ($n = 6$ at each level). Blanks with IS were positioned after the highest concentration standard samples and matrix blanks without IS ($n = 4$) were interspersed throughout the run.

For a core run to be considered acceptable, at least 3/4 of the calibration standards must have individual accuracy that is within ±15% of nominal concentration (±20% at the LLOQ). Additionally, each core run must have at least one of the two calibration standards at both the LLOQ and the upper level of quantitation (ULOQ) must meet the above criterion. In the event a calibration standard fails to meet these criteria, it is omitted from the calibration curve regression. The minimum acceptable coefficient of determination (r^2) for each run must be ≥0.98. In addition to calibration standard criteria, at least 2/3 of the overall number of QC samples must have individual accuracy with ±15% of nominal concentration (±20% at the LLOQ) for each core run. Furthermore, at least 50% of each QC level must meet this criterion per core run. The intra-run and inter-run precision for each core run should be ≤15% (≤20% at the LLOQ).

The selectivity of the method was evaluated by screening six individual lots of blank human blood for interference at the retention time of posaconazole and its IS. To be acceptable, the response at the expected retention time of posaconazole must be less than 20% of mean peak response of LLOQ QC samples ($n = 6$). The selectivity of the method was also evaluated to show that there was no interference between posaconazole and its IS as the response at the expected retention time of IS was less than 5% of mean peak response of the IS in the LLOQ QC samples ($n = 6$).

Dilution integrity was assessed using six replicates of QCs prepared at the posaconazole concentration of 10,000 ng/mL and extracted from 3-mm discs compared to 6-mm discs for calibration standards and QCs. To be acceptable, the mean accuracy of these samples must be within ±15% of the nominal concentration and the mean precision must be ≤15%.

The recovery was evaluated by analyzing extracted QC samples at three levels (QCL, QCM, QCH) along with neat samples made at identical concentrations in blank extraction solvent (acetonitrile/water, 90/10, v/v). The entire blood spot from each extracted sample was captured using a 6-mm punch.

Long-term stability of posaconazole in human whole blood was established by analyzing DBS cards stored for specific durations under ambient conditions. To be acceptable, the stability QC samples must have a mean accuracy within ±15% of the nominal concentration and a mean precision must be ≤15%.

Autosampler stability was assessed to demonstrate that sample integrity of a re-injected sample is maintained if re-injected after a specified number of hours when extracts are stored refrigerated. The experiment required re-injection of extracted QCL and QCH

samples and concentrations determined using the initial standard regression. The acceptance criteria were identical to that for long-term stability.

Likewise, delayed acquisition stability (post-preparative stability) was evaluated to show that the integrity of an entire run is maintained if re-injected after a specified number of hours (usually >72 h) while extracts are stored under refrigerated conditions. This was performed by re-injecting an analytical run that had previously been analyzed immediately following sample processing. The batch size for an average clinical study run was also evaluated in a similar fashion. The LC–MS/MS ruggedness experiment was performed by re-injecting an analytical run in triplicate to mimic a batch size of approximately 200 samples. The acceptance criteria for calibration standards and QCs were the same as for a core run.

The acceptance criteria for any additional experiments including evaluation of spotting volume, sample homogeneity, hematocrit and the functional equivalence between Alh-226 and FTA DMPK-C cards were the same as for stability assessments. The samples were spotted and extracted using the experimental conditions listed in sections 2.6 and 2.7.

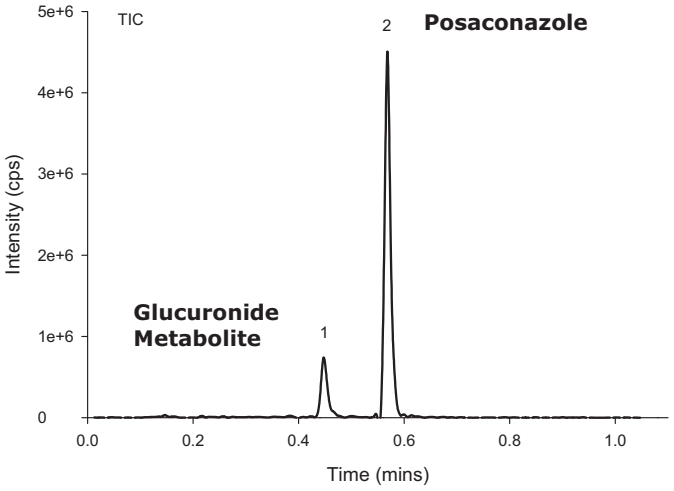

Fig. 2. Chromatographic separation of glucuronide metabolite and posaconazole.

4. Results

4.1. Method development

Prior to developing the DBS assay described here, our lab had conducted several DBS experiments using proprietary compounds of varying chemistries on coated and uncoated papers. The results of these experiments lead us to believe that uncoated paper would yield the best results for posaconazole as it is a very stable molecule. The Ahlstrom Alh-226 DBS cards were chosen simply based on them being a popular and cost-effective brand used within industry. Optimization of the extraction procedure with respect to sample volume, punch size, extraction solvent and mixing vs. sonicating needed to be further investigated, in addition to the projected application of this method in clinics worldwide.

Varying sample volumes between 10 and 35 μL were tested for precision and accuracy because the clinics would most likely be spotting directly from patient to paper. Importantly, area of spotting volume relative to punch size was calculated for the potential need of a correction factor if the blood spot area was less than that of the punched disc. And of course, sensitivity at the LLOQ of these variables was also evaluated. We found no correction factor to be necessary if volumes between 15 and 35 μL were used in conjunction with a 6 mm puncher as the DBS area was completely captured in the 6 mm disc. In addition, this combination provided better sensitivity at the LLOQ when compared with a 3 mm disc.

Having developed several plasma based posaconazole methods we knew that an acetonitrile/water solution would provide adequate recovery if used as the extraction solvent. Recovery experiments were performed using acetonitrile/water solutions between 50/50 (v/v) and 90/10 (v/v), resulting in 90/10 (v/v) having the highest recovery. Using this solvent, we investigated whether it was more advantageous to sonicate the samples to complete the extraction or gently mix them. Duration of this step was also tested at different intervals. We found that using a plate shaker to gently mix the samples for 30 min provided the easiest means to achieve the most recovery.

Up to this point, all experiments had been performed under controlled conditions, but it is likely that patient sampling may be performed at varied humidity levels in regions, like the tropics, where refrigeration is near absent. Alternatively, DBS cards bearing dried samples (prepared under controlled conditions) may arrive at a laboratory under varied humidity levels. Clearly there exists the potential to have variable water content on the DBS cards so it was essential to eliminate this variable and devise a mean of normalizing the water content of the DBS cards between controlled lab samples and "field" samples. We found that the addition of an aliquot of water to the punched DBS disc did just that, and in the case of posaconazole, it had the added benefit of doubling the analyte recovery.

The Waters ACQUITY UPLC™ system (Milford, MA) was selected as the platform to separate posaconazole and its IS because of its ability to optimize sensitivity and resolution at higher flow rates. Moreover, because the DBS extraction requires no sample cleanup, the quality of the results would improve using the ACQUITY UPLC™ to ensure that any residual endogenous interferences present in the sample extracts would not interfere with the selectivity of the assay. The chromatographic system utilized a Kinetex™ C18 (2.1 mm × 50 mm, 2.6 μm, 100 Å) column at ambient temperature, with mobile phases of 0.1% formic acid and 0.1% formic acid in acetonitrile/methanol (70/30, v/v) selected for analyte and IS separation. A ballistic gradient was constructed eluting posaconazole and its IS at ~0.65 min, followed by high organic cleanup and adequate column equilibration within a cycle time of 1.52 min. Adequate resolution of posaconazole from endogenous components and the major glucuronide metabolite was achieved (Fig. 2).

Selection of the Kinetex™ column was based on its core-shell particle technology touted to ensure reproducible and robust results comparable or better than traditional sub-2 μm columns. While sub-2 μm columns have provided superb results for several methods developed in our lab, they also presented challenges when used for clinical methods where an acetonitrile-based mobile phase and ambient conditions were optimal for separation. One such method used a flow rate of 0.8 mL/min with a starting pressure of ~8000 psi and over-pressured after a single injection. Similar results were obtained from different sub-2 μm columns regardless of the extraction procedure used for analysis. The presumption here is that endogenous matter in the clinical extracts is not soluble in acetonitrile which clogs the frit or column head resulting in over-pressuring. The addition of methanol to the organic mobile phase prevents this over-pressuring as it is a better solublizing agent, but also increases the back pressure at ambient temperature. Using the Kinetex™ 2.6 μm column in conjunction with 0.1% formic acid in acetonitrile/methanol (70/30, v/v) allowed us to perform the DBS validation runs at a comfortable, steady pressure and eliminated potential of over-pressuring altogether.

Furthermore, the column proved to be rugged and maintained consistent results. The validation experiments were performed using a single Kinetex™ C18 column. The performance of the

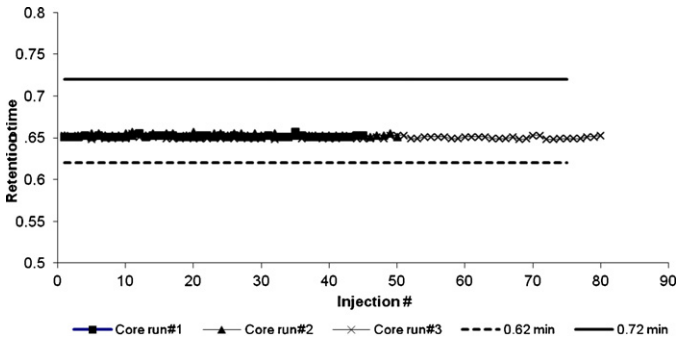

Fig. 3. Retention times of core runs.

column was monitored on a per run basis by tracking the retention times of the analyte. Posaconazole eluted between 0.62 and 0.72 min in each run. The retention time plot of the core runs is listed in Fig. 3. In addition, the column performance was monitored by overlaying the first STD_1 injection performed at the beginning of the validation vs. the last STD_1 injection performed at the end of validation over 1000 injections later (Fig. 4). The column showed excellent reproducibility of retention time and peak shape after the analysis of over a thousand DBS extracted samples.

The sample extracts were maintained in a refrigerated autosampler and there was little to no carryover present in the validation runs as an acetonitrile/water (10/90, v/v) weak wash and acetonitrile/isopropanol/acetone (60/30/10, v/v/v) strong wash combination was employed.

4.2. Method validation

4.2.1. Linearity

The validation established that the calibration standards were proportional to the nominal posaconazole concentrations ranging from 5.00 ng/mL to 5000 ng/mL. The calibration curves were quadratic and were well described by a least-squares regression. A weighting factor of 1/concentration^2 was chosen to achieve homogeneity of variance. The slopes, intercepts and coefficients of determination from all three core runs are summarized in Table 1. The inter-run precision (%CV) for the slopes of the three core runs was 4.1% and the overall mean of r^2 was 0.9956.

4.2.2. Accuracy and precision

For the calibration standards, the inter-run precision and accuracy from all three core runs are listed in Table 1. The inter-run accuracy (%Diff) ranged from −2.4% to 2% and the inter-run precision (%CV) ranged from 2.6% to 10.1%. For QC samples, the inter-run and intra-run precision and accuracy from all three core runs are summarized in Table 2. The inter-run accuracies were 0.8%, 0.7%, −0.8% and −1.8% for LLOQ, QCL, QCM and QCH (n = 18), respectively. The inter-run precision was 10.4%, 7.5%, 4% and 5.9% for LLOQ, QCL, QCM and QCH (n = 18), respectively.

4.2.3. Assay performance and reproducibility

The assay performance was evaluated by performing analysis of variance (ANOVA) calculations using the quality controls standards of the three core runs. The resulting data showed that both within run and between run precision was less than 15%. The ANOVA data is listed in Table 3.

4.2.4. Selectivity and matrix effect

Blank human whole blood samples from six different sources were screened and found to be free from potential endogenous interferences or other sources at the same mass transitions and retention times of posaconazole and its IS. The method also proved to be selective between analyte and IS. Typical chromatograms of extracted blank human blood along with a posaconazole LLOQ spiked with IS spiked are shown in Fig. 5a and b.

Evaluation of samples at the QCL concentration (n = 6) prepared from six different sources also proved to be free of enhancement or suppression of posaconazole from the presence of endogenous interferences. The matrix effect results are presented in Table 4.

4.2.5. Dilution integrity

The dilution integrity was assessed using the QCD samples (n = 6) made at 2× ULOQ. The dilution factor calculated based on the difference in the areas (πr^2) of the discs (3 mm for QCDs vs 6 mm for standards) is equal to 4. The dilution factor in terms of difference in the concentration of ULOQ and QCD is 2. Data for DBS dilution integrity presented in Table 5.

4.2.6. Recovery

Posaconazole is a strongly hydrophobic molecule and it is presumed that the addition of a polar solvent, like water, to the spotted

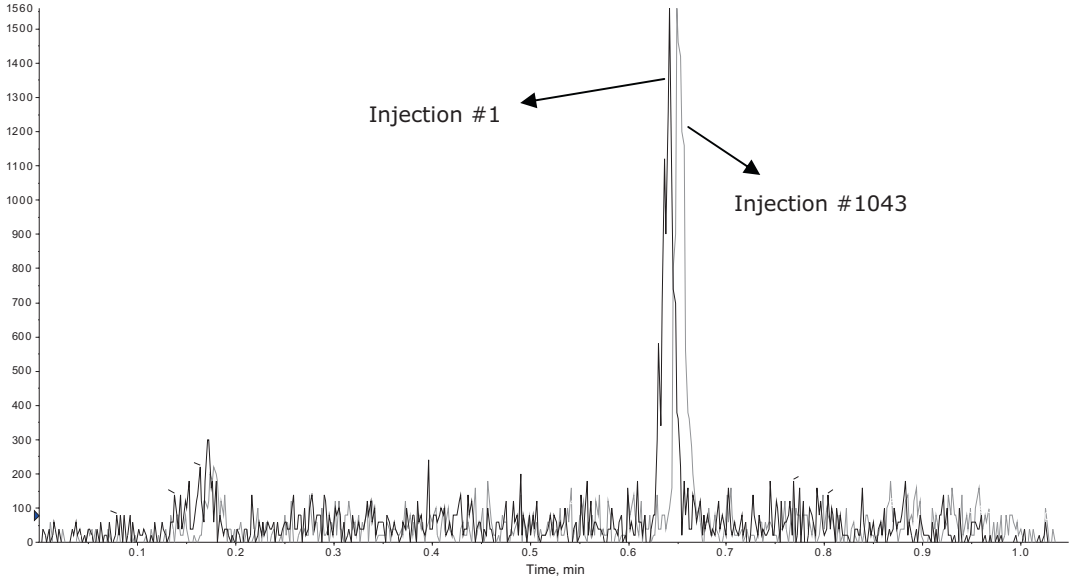

Fig. 4. Retention time reproducibility (injection 1 and 1043).

Table 1
Back-calculated posaconazole DBS concentrations in human blood, calibration standards and calibration curve parameters.[a]

Conc. (ng/mL)	STD1 5.00	STD2 10.0	STD3 20.0	STD4 50.0	STD5 100	STD6 250	STD7 500	STD8 2000	STD9 4200	STD10 5000	*A*	*B*	*C*	(r^2)
Core run 1	5.19	9.97	20.7	53.1	102	243	488	2060	4440	5180	−6.9E−08	0.00233	0.0032	0.9957
	4.71	9.55	22.9	48.2	95.7	244	484	1830	4480	4490				
Core run 2	3.96[b]	10.1	18.5	49.4	82.2	236	525	1980	3700	4760	−5.9E−08	0.00226	0.00314	0.9931
	4.98	10.4	10.6[b]	51.8	111	247	522	2270	4260	5390				
Core run 3	4.71	11.3	20.1	53.4	100	255	542	1990	4760	5500	−2E−08	0.00225	0.00107	0.9933
	5.05	10.1	17.9	49.3	94.4	242	492	1860	3740	4530				
n	5	6	5	6	6	6	6	6	6	6	3	3	3	3
Overall mean	4.93	10.2	20	50.9	97.6	245	509	2000	4230	4980	−4.9E−08	0.00228	0.00247	0.994
S.D.	0.213	0.589	1.97	2.19	9.55	6.28	24	158	426	440	2.58E−08	4.36E−05	0.00121	0.0014
%CV	4.3	5.8	9.9	4.3	9.8	2.6	4.7	7.9	10.1	8.8	−52.3	1.9	49	0.1
%DIFF	−1.4	2	0	1.8	−2.4	−2	1.8	0	0.7	−0.4				

[a] $y = Ax^2 + Bx + C$, weighted 1/concentration^2, where y is the peak area ratio of posaconazole to IS, x is the concentration of posaconazole and A, B, and C are calibration curve parameters.
[b] Outside ±15% of nominal concentration and hence not included in the calculations.

Table 2
Analytical performance of posaconazole quality control samples in human blood.

Run date	Curve number	LLOQ 5.00 ng/mL	%DIFF	QCL 15.0 ng/mL	%DIFF	QCM 400 ng/mL	%DIFF	QCH 4000 ng/mL	%DIFF
20-May-2010	Core run 1	4.76	−4.8	14.4	−4	383	−4.3	4100	2.5
		5.07	1.4	15.4	2.7	396	−1	3930	−1.8
		4.97	−0.6	13.4	−10.7	381	−4.8	3980	−0.5
		4.99	−0.2	15.3	2	408	2	3970	−0.8
		4.13	−17.4	15	0	401	0.3	4190	4.8
		4.45	−11	16.5	10	401	0.3	4100	2.5
Intrarun Mean		4.73	15		395		4050		
Intrarun SD		0.369	1.04		10.8		100		
Intrarun %CV		7.8	6.9		2.7		2.5		
Intrarun %DIFF		−5.4	0		−1.3		1.3		
n		6		6		6		6	
01-Jun-2010	Core run 2	5.98	19.6	15.7	4.7	441	10.3	4110	2.8
		4.75	−5	17.8[a]	18.7[a]	398	−0.5	4300	7.5
		5.53	10.6	13.3	−11.3	404	1	4140	3.5
		5.64	12.8	15.8	5.3	394	−1.5	4130	3.3
		4.17	−16.6	14.2	−5.3	418	4.5	3840	−4
		5.77	15.4	15.6	4	403	0.8	3590	−10.3
Intrarun Mean		5.31	15.4		410		4020		
Intrarun SD		0.697	1.54		17.4		257		
Intrarun %CV		13.1	10		4.2		6.4		
Intrarun %DIFF		6.2	2.7		2.5		0.5		
n		6		6		6		6	
21-Jun-2010	Core run 3	5.16	3.2	13.7	−8.7	371	−7.3	3490	−12.8
		4.73	−5.4	15.6	4	397	−0.8	3840	−4
		4.83	−3.4	15.8	5.3	385	−3.8	3840	−4
		5.15	3	14.3	−4.7	389	−2.8	4000	0
		5.64	12.8	15.1	0.7	391	−2.3	3600	−10
		4.99	−0.2	14.4	−4	382	−4.5	3630	−9.3
Intrarun Mean		5.08	14.8		386		3730		
Intrarun SD		0.322	0.818		8.91		191		
Intrarun %CV		6.3	5.5		2.3		5.1		
Intrarun %DIFF		1.6	−1.3		−3.5		−6.8		
n		6		6		6		6	
Mean concentration found (ng/mL)		5.04	15.1		397		3930		
Inter-run SD		0.523	1.13		15.8		233		
Inter-run %CV		10.4	7.5		4		5.9		
Inter-run %DIFF		0.8	0.7		−0.8		−1.8		
n		18		18		18		18	

[a] Outside ±15% of nominal concentration and hence not included in the calculations.

Table 3
Assay performance (ANOVA) of posaconazole quality control samples in human blood.

	LLOQ 5.00 ng/mL	QCL 15.0 ng/mL	QCM 400 ng/mL	QCH 4000 ng/mL
Mean observed conc.	5.04	15.1	397	3930
%DIFF	0.8	0.7	−0.8	−1.8
Between run precision (%CV)	4.2	0	2.7	3.9
Within run precision (%CV)	9.8	7.8	3.2	4.9
Total variation (%CV)	10.6	7.4	4.2	6.3
n	18	18	18	18
Number of runs	3	3	3	3

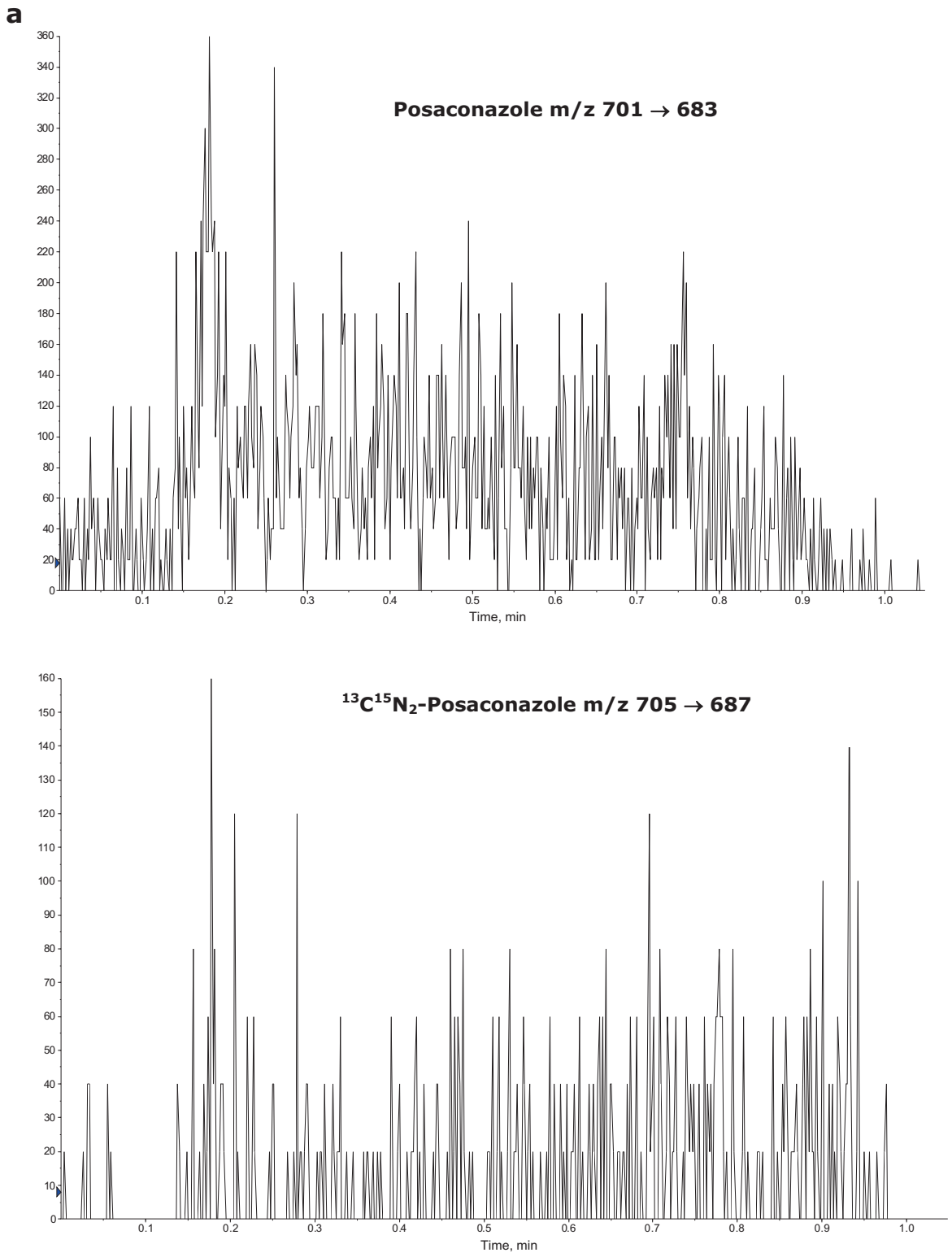

Fig. 5. (a) Chromatograms of extracted human blood samples showing MRM transitions of posaconazole and its IS. (b) Chromatograms of extracted LLOQ human blood samples spiked with IS showing MRM transitions of posaconazole and its IS.

6 mm punched disc would further diminish the existing weak interactions between it and the hydroxyl group present on the cellulose polymer. This presumed displacement enhanced the analyte recovery from the paper upon addition of the organic solvent present in the IS working solution. Unpublished experiments confirmed the improved extraction efficiency of posaconazole resulting from the addition of 20 μL of water to the punched disc prior to extraction with the organic solvent.

The recovery of posaconazole was determined by comparing the mean peak area responses of the extracted samples vs. the mean peak area responses of the neat samples prepared using the blank extraction solvent. The mean absolute recoveries for the QCL, QCM

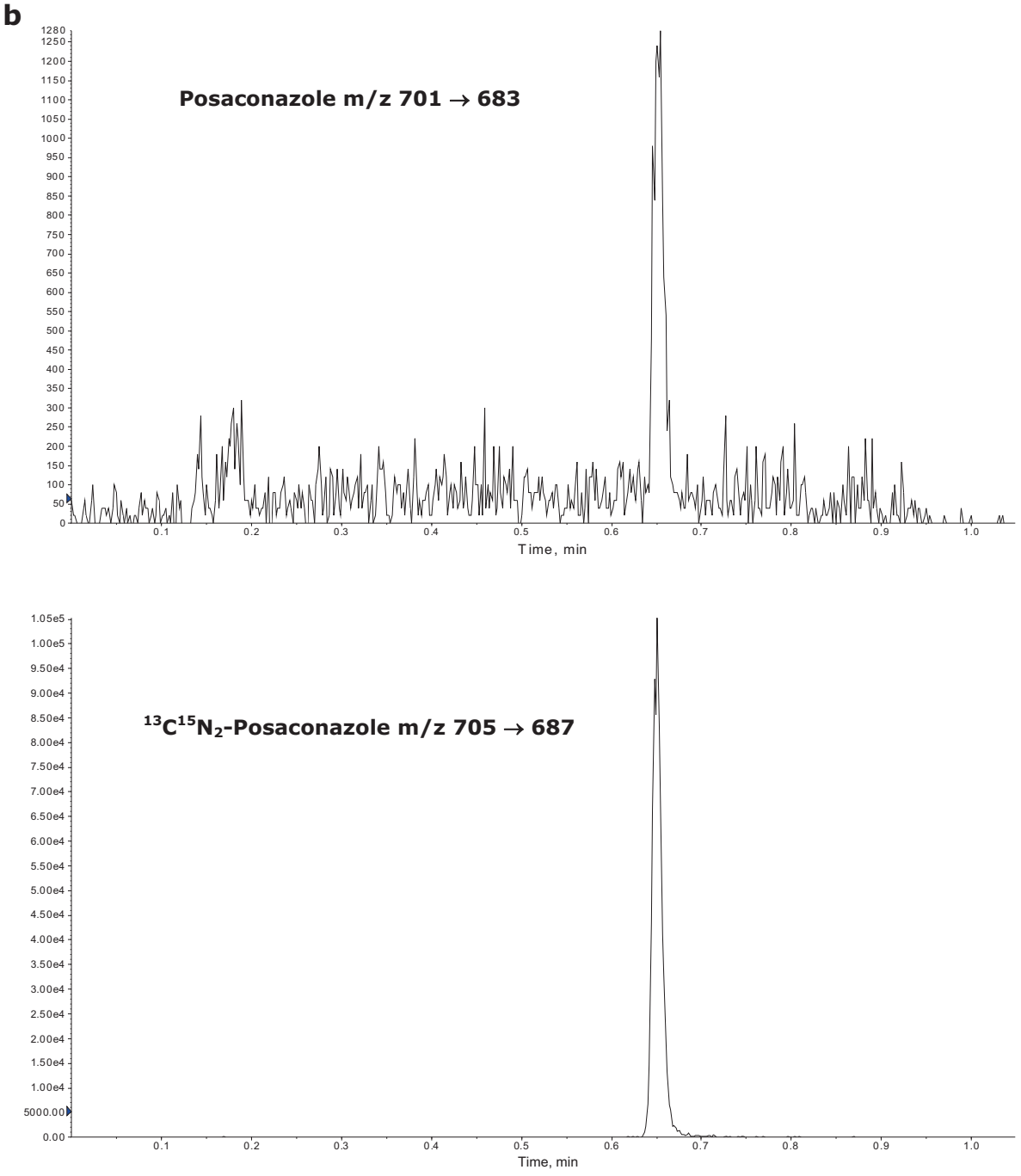

Fig. 5. (*Continued*)

and QCH were 85.4%, 85.5% and 63.3%, respectively. The results are presented in Table 6.

4.2.7. Long-term stability

Long-term stability of posaconazole QC dried blood spots ($n = 6$, QCL and QCH, respectively) stored at room temperature and under ambient conditions was established for 13 days. Table 7 presents the precision and accuracy data from this experiment.

4.2.8. Autosampler stability

Autosampler stability of the sample extracts was evaluated by reanalyzing the QCL and QCH samples ($n = 6$) stored refrigerated and quantifying the data from the reinjections against the original calibration curve. The precision and accuracy data is presented in Table 8. Refrigerated autosampler stability of 92 h was established.

4.2.9. Delayed acquisition stability

Delayed acquisition stability (post-preparative stability) was established by reanalyzing a previously injected batch of refrigerated posaconazole DBS extracts. Table 9 presents the accuracy and precision data that established 91 h of refrigerated delayed acquisition stability for this method.

4.2.10. MS ruggedness

MS ruggedness was established by injecting a set of extracted samples in triplicate to mimic a typical batch size for clinical analysis. The standard curve was built by using the first and sixth set of

Table 4
Matrix effect QCL summary.

	QCL 1 15.0 ng/mL	QCL 2 15.0 ng/mL	QCL 3 15.0 ng/mL	QCL 4 15.0 ng/mL	QCL 5 15.0 ng/mL	QCL 6 15.0 ng/mL
1	13.2	15.1	15.1	16.8	14	17.4[a]
2	17.3[a]	16.5	16.6	16.4	16.9	13.7
3	14.2	16.3	15.3	15.9	15.5	15.7
4	13.5	18.0[a]	16.5	17.2	13.8	15.6
5	15	16.2	8.00[b]	16.2	15.9	14.9
6	14.2	13.2	15.6	13	15.5	14.5
Mean	14.6	15.9	15.8	15.9	15.3	15.3
S.D.	1.48	1.61	0.691	1.5	1.18	1.27
%CV	10.1	10.1	4.4	9.4	7.7	8.3
%Theoretical	97.3	106	105.3	106	102	102
%DIFF	−2.7	6	5.3	6	2	2
n	6	6	5	6	6	6

[a] Otside ±15% of nominal concentration
[b] Grubbs statistical outlier.

Table 5
Dilution integrity QC summary.

Sample	QCD 10,000 ng/mL
1	9430
2	10,500
3	9080
4	9150
5	9810
6	8880
Mean	9480
S.D.	597
%CV	6.3
%Theoretical	94.8
%DIFF	−5.2
n	6

standards. Two samples from each QC set were chosen in a sequential order to evaluate the run. The precision and accuracy data is presented in Table 10 from a 188-sample run. Furthermore, the internal standard variability for this run was evaluated. The individual internal standard peak areas were within ∼±25% of mean internal standard peak area (Fig. 6).

4.3. Effect of blood volume spotted on the DBS card

An experiment comparing a range of blood spotting volumes from 10 to 35 μL (in 5 μL increments) was performed as a means of demonstrating whether an exact spotting volume is absolutely required to retrieve an accurate end result. The experiment assessed single replicates of two different QC levels (QCL and QCH) across the stated range and established that acceptable precision and accuracy can be obtained between the spotting range of 15–35 μL. Data from this experiment is presented in Table 11.

Table 7
Long-term stability of posaconazole from extracted human blood at ambient room temperature (13 days).

	Sample	QCL LT 15.0 ng/mL	QCH LT 4000 ng/mL
	1	14.8	3650
	2	16.2	3520
	3	13.2	3540
	4	16.3	3470
	5	14.3	3360[a]
	6	15.6	3460
Mean		15.1	3500
S.D.		1.2	96.5
%CV		7.9	2.8
%Theoretical		100.7	87.5
%DIFF		0.7	−12.5
n		6	6

[a] Outside ±15% of nominal concentration.

Table 8
Refrigerated autosampler stability of posaconazole from extracted human blood (92 h).

	Sample	QCL AS 15.0 ng/mL	QCH AS 4000 ng/mL
	1	14.8	4370
	2	14.2	4430
	3	15.8	4300
	4	14.8	4180
	5	14.2	4230
	6	13.1	4060
Mean		14.5	4260
S.D.		0.895	134
%CV		6.2	3.1
%Theoretical		96.7	106.5
%DIFF		−3.3	6.5
n		6	6

Table 6
Recovery results of extracted posaconazole samples versus neat samples.

Recovery – posaconazole ID:	QCL_Neat	QCL	QCM_Neat	QCM	QCH_Neat	QCH
Conc. (ng/mL)	60.0	60.0	1500	1500	15,000	15,000
Analyte peak area	6080	4610	140,000	116,000	1,550,000	921,000
	5710	n/a	136,000	115,000	1,570,000	894,000
	5690	4820	129,000	119,000	1,480,000	960,000
	5190	4580	132,000	115,000	1,490,000	923,000
	4920	4340	144,000	115,000	1,320,000	983,000
	5220	5000	131,000	114,000	1,460,000	935,000
Mean	5468	4670	135,333	115,667	1,478,333	936,000
n	6	6	6	6	6	6
%CV	7.85	5.37	4.27	1.51	5.98	3.36
Recovery (%)	N/A	85.4	N/A	85.5	N/A	63.3

Table 9
Refrigerated delayed acquisition stability of posaconazole extracted from human blood (91 h).

	Sample	LLOQ 5.00 ng/mL	QCL 15.0 ng/mL	QCM 400 ng/mL	QCH 4000 ng/mL
	1	5.81	14.4	384	4020
	2	5.59	15.6	388	4060
	3	5.37	13.6	404	4140
	4	4.56	16.1	394	3870
	5	4.67	15.8	416	4340
	6	5.2	15.5	391	4060
Mean		5.2	15.2	396	4080
S.D.		0.499	0.961	11.8	155
%CV		9.6	6.3	3	3.8
%Theoretical		104	101.3	99	102
%DIFF		4	1.3	−1	2
n		6	6	6	6

Table 10
MS ruggedness data of posaconazole from extracted human blood.

	Sample	QCL 15.0 ng/mL	QCM 400 ng/mL	QCH 4000 ng/mL
	1	11.3[a]	384	3030[a]
	2	14.3	−0.535[b]	4230
	3	14.5	392	4000
	4	15.9	361	4260
	5	16.4	365	4190
	6	16.2	374	4100
Mean		14.8	375	3970
S.D.		1.91	12.9	469
%CV		12.9	3.4	11.8
%Theoretical		98.7	93.8	99.3
%DIFF		−1.3	−6.3	−0.8
n		6	5	6

[a] Outside ±15% of nominal concentration
[b] Grubbs statistical outlier.

4.4. Effect of sampling location on the DBS card

The effect of sampling location of the dried blood spot was assessed to demonstrate whether or not the diffusivity of the blood spotted on Ahl-226 DBS cards was uniform throughout the spotting region. Failure to achieve uniform diffusivity of the blood spot could result in imprecision. The experiment involved removing two 3-mm sized discs (one centrally located and the other peripheral to the first) from each dried blood spot of QCL and QCH samples ($n = 3$ each) and extracting them separately for comparison. The data is summarized in Table 12 and indicates that acceptable precision and accuracy can be obtained regardless of punching location.

Table 11
Effect of spotting volume on accuracy and precision of posaconazole from extracted human blood.

Sample	Spotting volume (μL)	QCL 15.0 ng/mL	QCH 4000 ng/mL
1	10	11.0[a]	2990[a]
2	15	15.2	4250
3	20	15.2	3750
4	25	16	4090
5	30	17	4220
6	35	16	4060
Mean		15.1	3890
S.D.		2.1	477
%CV		13.9	12.3
%Theoretical		100.7	97.3
%DIFF		0.67	−2.8
n		6	6

[a] Outside ±15% of nominal concentration.

4.5. Effect of hematocrit

The effect of hematocrit on DBS analysis was evaluated as hematocrit values differ between sick and healthy subjects. [54] Given that posaconazole is administered to immuno-compromised patients it was essential to establish whether DBS extraction is suitable for monitoring levels of posaconazole in sick patients accurately. The experiment was executed by preparing QC samples (QCL and QCH) using human whole blood with varied hematocrit values (25% and 65% for both QC levels, respectively). The calibration standards in this experiment were prepared using human whole blood with a normal hematocrit value (41%). Results indicate that human whole blood samples with hematocrit values ranging between 25% and 41% gave acceptable quantitation results. Table 13 presents the accuracy and precision data from this experiment.

4.6. Ahl-226 vs FTA DMPK-C DBS card comparison

Uncoated DBS cards are currently supplied by two vendors *viz*., ID Biological Systems and Whatman (GE Healthcare Division). An experiment was performed in order to establish functional equivalence of DBS cards obtained from these two vendors. An identical set of human blood samples were spotted on both Ahl-226 and FTA DMPK-C cards and extracted. The samples were analyzed and the data was evaluated within and across the runs. The individual runs met the acceptance criteria. The standard and QC data from both runs were regressed against each other. The coefficient of determination of the resulting curve is 0.9983 (Fig. 7). In addition, the QC samples from the Ahl-226 cards were quantified against the calibration curve from the FTA DMPK-C extracted standards and *vice versa*. This data is presented Tables 14a and 14b. Based on these results it

Table 12
Effect of sampling location on accuracy and precision of posaconazole from extracted human blood.

	Punch size (mm)	QCL 3.75 ng/mL	QCH 1067 ng/mL
DB Spot-1 (Punch - 1)	3	3.97	981
DB Spot-1 (Punch - 2)	3	3.39	1090
DB Spot-2 (Punch - 1)	3	4.58[a]	1050
DB Spot-2 (Punch - 2)	3	4.05	1110
DB Spot-3 (Punch - 1)	3	4.13	1110
DB Spot-3 (Punch - 2)	3	4.27	1120
Mean		4.07	1077
S.D.		0.394	53.2
%CV		9.69	4.94
%Theoretical		108.4	101
%DIFF		8.4	0.922
n		6	6

[a] Outside ±15% of nominal concentration.

Table 13
Effect of hematocrit (HC) on accuracy and precision of posaconazole from extracted human blood.

	Sample	QCL 25%HC 15.0 ng/mL	QCL 65%HC 15.0 ng/mL	QCH 25%HC 4000 ng/mL	QCH 65%HC 4000 ng/mL
	1	14.8	19.6[a]	3930	4700[a]
	2	14.4	19.2[a]	4010	4620[a]
	3	14.9	17.8[a]	3900	4810[a]
	4	16.8	21.5[a]	4420	4910[a]
	5	14.2	17.4[a]	4100	4610[a]
	6	14.1	20.8[a]	3710	5180[a]
Mean		14.9	19.4	4010	4810
S.D.		0.999	1.61	239	217
%CV		6.7	8.3	6	4.5
%Theoretical		99.3	129.3	100.3	120.3
%DIFF		0.7	−29.3	−0.3	−20.3
n		6	6	6	6

[a] Outside ±15% of nominal concentration.

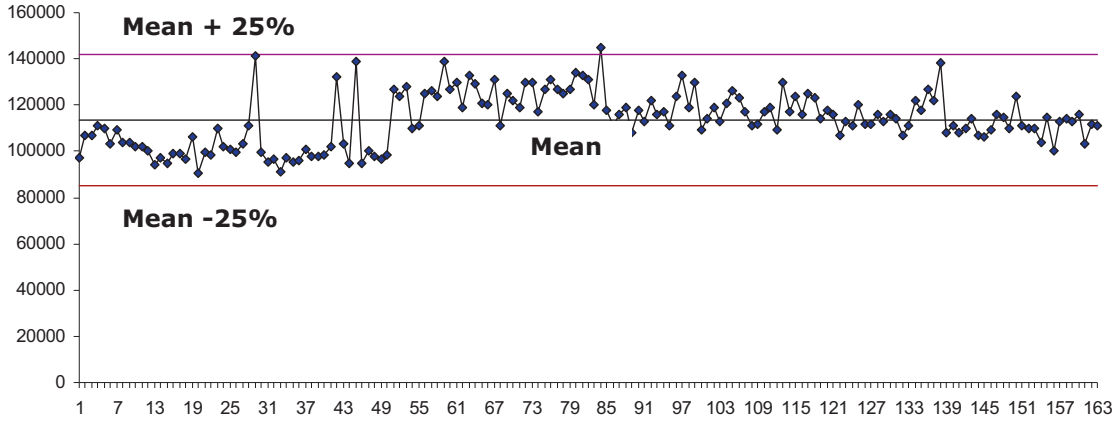

Fig. 6. IS peak area response of MS ruggedness run (188 samples).

Table 14a
Quantification of extracted Ahl-226 QCs against extracted FTA DMPK-C calibration standards.

	LLOQ 5.00 ng/mL	QCL 15.0 ng/mL	QCM 400 ng/mL	QCH 4000 ng/mL
1	4.63	14.2	381	3920
2	4.92	15.2	394	3770
3	4.81	13.2	379	3820
4	4.88	15.1	406	3810
5	4.01	14.9	399	4010
6	4.32	16.4	399	3920
Mean	4.60	14.8	393	3875
S.D.	0.361	1.07	10.8	90.1
%CV	7.86	7.22	2.75	2.32
%Theoretical	91.9	98.9	98.3	96.9
n	6	6	6	6

Table 14b
Quantification of extracted FTA DMPK-C QCs against extracted Ahl-226 calibration standards.

	LLOQ 5.00 ng/mL	QCL 15.0 ng/mL	QCM 400 ng/mL	QCH 4000 ng/mL
1	4.38	13.4	370	4140
2	4.68	14.6	397	4260
3	4.18	15.6	415	4210
4	4.48	14.6	395	4330
5	4.66	14.7	378	4510
6	5.13	14.8	403	4350
Mean	4.59	14.6	393	4300
S.D.	0.325	0.705	16.5	129
%CV	7.09	4.83	4.19	2.99
%Theoretical	91.7	97.4	98.3	107.5
n	6	6	6	6

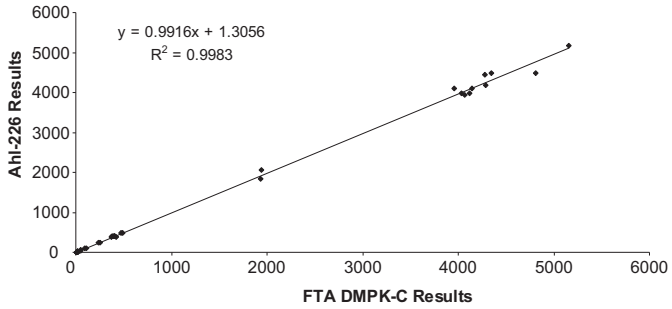

Fig. 7. Linear regression plot of Ahl-226 versus FTA DMPK-C dried blood spots cards analysis of posaconazole extracted from blood.

can be concluded that both Ahl-226 and FTA DMPK-C can be considered functionally equivalent for the DBS analysis of posaconazole in human blood.

5. Discussion

Posaconazole whole blood analysis has been performed at Schering-Plough Research Institute (SPRI, part of Merck Research Laboratories) for several years using different extraction techniques, means of detection and chromatographic separation. The methods have evolved over the years as new technologies have become available to perform high-throughput clinical analyses more efficiently. To this extent, several publications from SPRI have used posaconazole as their model compound and executed their experiments in controlled matrix. While some of these published methods have been used in the clinical realm for the quantification of posaconazole, not all papers have focused on the known metabolites, namely glucuronides, present only in clinical samples. Hence, it was important to evaluate any potential impact these metabolites had on the quantification of posaconazole via this LC–MS/MS method as it relates to therapeutic drug monitoring (TDM).

After obtaining a plate of clinical posaconazole extracts from colleagues within SPRI who conducted their analysis by means of a previously published posaconazole LC–MS/MS method [39], we tested our chromatographic separation versus theirs and found them to be comparable – they both nicely separated posaconazole from the glucuronides. It is important to note that these two means of chromatographic separation employed similar mobile phase compositions, gradients and column chemistries. The differences in column selection were strictly a result of observed robustness issues from the manufacturer in recent months, while the others were in line with historical means used. Fig. 2 shows the chromatographic separation of posaconazole from the glucuronides by the LC–MS/MS method described here, thus, demonstrating it yet again as a viable tool for TDM of posaconazole in sick patients.

6. Conclusions

A rugged and robust dried blood spots assay coupled with an LC–MS/MS platform was developed and validated for the extraction and analysis of posaconazole in human whole blood over a dynamic range of 5–5000 ng/mL. The validation data demonstrated acceptable linearity, precision, accuracy, selectivity, sensitivity and recovery. Results also indicate the assay is highly reproducible between 15 and 35 μL of spotting volume. The assay demonstrated sufficient long-term stability of posaconazole dried blood spots stored under ambient conditions. This suggests that dried blood samples from patients treated with posaconazole are stable for a minimum of 13 days under ambient storage. In addition, the homogeneity of blood spotting was also established confirming that sampling location does not adversely affect precision and accuracy. These results segued into applying dilution integrity to DBS by varying the diameter of the punched disc.

It should be mentioned, however, that while the hematocrit values between 25 and 41% demonstrated acceptable precision and accuracy, unacceptable results were initially observed at a value of 65%. This blood was observed to be more viscous than the other samples and since the viscosity of blood is proportional to its hematocrit value, then the viscosity of the blood will directly affect the diffusion property of blood when spotted on the DBS card [4]. Therefore, an investigation was conducted under the hypothesis that the high viscosity of this blood (hematocrit = 65%) adversely affected the homogeneity of spotting on the DBS and resulted in inaccurate quantification of analyte. Unpublished results obtained in our group suggest that dilution of human blood samples with high hematocrit values using phosphate buffered saline solution to yield samples with hematocrit values of ~40% offers a viable means to improve precision and accuracy within the acceptable limits.

The DBS extraction technique was successfully utilized for the quantitation of posaconazole in control human whole blood. The DBS technique does not require accurate pipetting, and hence, lends itself to potential implementation at clinical sites. Given the convenience and ease of the DBS technique and the short analysis time of posaconazole (<2 min/sample), it is believed that this dried blood spots LC–MS/MS based assay could be a viable tool for the rapid analysis of posaconazole in clinical laboratories for therapeutic drug monitoring of sick patients.

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