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Quantitative analysis of NIM811, a cyclophilin inhibitor, in human dried blood spots using liquid chromatography–tandem mass spectrometry

Wenkui Li*, Shannon M. Williams, Harold T. Smith, Francis L.S. Tse

Department of Drug Metabolism and Pharmacokinetics, Novartis Institutes for Biomedical Research, One Health Plaza, East Hanover, NJ 07936, USA

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ABSTRACT

A high-performance liquid chromatography–tandem mass spectrometric (LC–MS/MS) method has been developed and validated for the quantitative analysis of NIM811, a cyclophilin inhibitor, in human dried blood spot (DBS) samples, which were produced by spotting 20 μl whole blood onto FTA cards. A 3 mm disc was cut from the DBS samples and extracted using methanol, followed by liquid–liquid extraction with MTBE. The reconstituted extracts were chromatographed using a Halo C_{18} column and gradient elution for MS/MS detection. The possible impact of hematocrit, blood sample volume and punching location on DBS sampling was investigated. The results showed that blood sample volume or punching location has no impact on assay performance, but the presence of a high hematocrit resulted in significantly increased analyte concentrations measured from the high QC samples. The current method was fully validated over the range of 10.0–5000 ng/ml with correlation coefficients (r^2) for three validation batches equal to or better than 0.991. The accuracy and precision (CV) at the LLOQ were −0.7 to 6.0% bias of the nominal value (10.0 ng/ml) and 10.2–2.3%, respectively. For the balance of QC samples (20.0, 50.0, 750, 1500 and 3750 ng/ml), the precision (CV) ranged from 3.2 to 11.7% and from 5.6 to 10.2%, respectively, for the intra-day and inter-day evaluations. The accuracy ranged from −6.8 to 8.5% and −0.2% to 2.7% bias, respectively, for the intra-day and inter-day batches. NIM811 is stable in the DBS samples for at least 24 h at room temperature and 4 h at 60 °C. Interestingly, the long term stability (LTS) assessment showed that the stability of the analyte is better when the DBS samples were stored at a lower storage temperature (e.g. ≤−60 °C) compared to storage at room temperature. This is probably due to the interaction of the additives and/or other materials (e.g. cellulose, etc) on the DBS card with NIM811, a cyclic peptide. The current methodology has been applied to determine the NIM811 levels in DBS samples prepared from a clinical study.

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1. Introduction

The use of dried blood spots (DBS) obtained from simple pricks and spotted onto filter paper for the collection and analysis of human blood has a history of about 50 years [1]. This approach of blood collection offers a number of advantages over conventional whole blood, plasma or serum sample collection. One of the advantages is that DBS samples could be collected directly by patients themselves or their guardians with a minimum of training. This opens up the possibility of collecting clinical pharmacokinetic samples not only from various in-patients, but also from out-patients, especially those living in remote areas. DBS samples can be promptly taken as needed and directly sent to laboratories for analysis. The method has been increasingly used to support therapeutic drug monitoring [2].

NIM811 (SDZ211-811, MeIle4-cyclosporin) is a natural cyclosporine analog characterized by a lack of immunosuppressive activity compared to cyclosporine A (CsA). Similar to CsA, NIM811 is a potent cyclophilin inhibitor with multiple effects, including antiviral effect in a number of virus–host systems, such as hepatitis C virus (HCV) [3,4]. Like CsA and other macrolide immunosuppresants, NIM811 is mainly distributed in the erythrocytes. Thus, whole blood rather than plasma or serum has been the matrix used for the measurement of its exposure in human subjects. A previously reported LC–MS/MS method has been used for the quantitative analysis of NIM811 in human blood in support of various NIM811 studies [5]. The objective of the present work was to develop and validate a simple DBS based LC–MS/MS method to determine NIM811 in human blood in support of therapeutic drug monitoring. The developed assay method was assessed against internationally accepted validation

* Corresponding author. Tel.: +1 862 778 4255; fax: +1 973 781 7579.
E-mail address: wenkui.li@novartis.com (W. Li).

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criteria. Additional investigations on the effect of collected sample volume, DBS punching location (chromatographic effect) and hematocrit and stability of the analyte at a higher temperature, etc., were conducted as part of the current evaluation.

2. Experimental

2.1. Chemicals and materials

All reagents were used without further purification. HPLC grade acetonitrile, methanol, isopropanol and methyl *tert*-butyl ether (MTBE) along with certified acetic acid, formic acid, ammonium acetate, sodium carbonate and sodium bicarbobate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water was deionized and purified via an ELGA PureLab Ultra Water System (Warrendale, PA, USA). NIM811 and the internal standard, d_{12}-CsA (Fig. 1), were synthesized at Novartis (East Hanover, NJ, USA). Fresh human blood (sodium EDTA as the anticoagulant) blank was obtained from Bioreclamation (Westbury, NY, USA) and used to prepare calibration and quality control samples. Whatman FTA® was obtained from GE Whatman (Piscataway, NJ, USA).

2.2. Chromatographic conditions

The HPLC system consists of two LC-20AD pumps, a CBM-20A system controller, a CTO-20A column oven, a DGU-20A_5 on-line solvent degasser and a SIL-20ACHT autosampler with a rack changer from Shimadzu (Columbia, MD, USA). Chromatography was performed on a MAC-MOD Halo C_{18} (2.1 mm × 50 mm, 2.7 μm particle size) column at a flow rate of 0.3 ml/min with mobile phases consisting of 10 mM ammonium acetate in water (A) and acetonitrile (B). An optimal chromatographic separation was achieved by running 65% B for 0.4 min isocratically, from 65 to 95% B over the next 0.3 min in gradient and 95% B isocratically for the next 0.95 min. This was followed by a sharp gradient from 95% to 65%B within 0.05 min and the 65%B was maintained for the next 2.3 min for column re-equilibrium prior to each next injection. The column was maintained at 60 °C with the column effluent between 0.6 and 2.0 min delivered to the mass spectrometer interface without splitting.

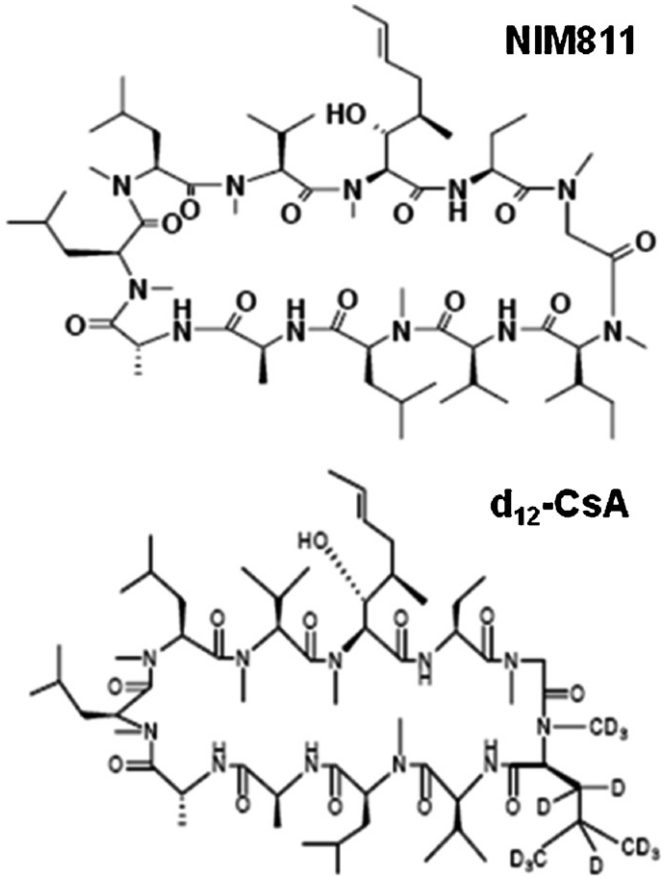

Fig. 1. Structures of NIM811 and d_{12}-CsA (ISTD).

2.3. Mass spectrometric conditions

A Sciex API4000 triple quadrupole mass spectrometer (AB Sciex, Concord, Ontario, Canada) with a Turboionspray (TIS) interface operated in the positive ionization mode was used for the multiple reaction monitoring (MRM) LC–MS/MS analyses. The optimized instrument conditions were as follows: TIS source temperature, 300 °C; TIS voltage, 5000 V; Curtain gas, 30 units; Nebulizing gas (GS1), 70 units; TIS gas (GS2), 50 units; Collision-associated dissociation (CAD) gas, 7 units; Collision energy (CE), 35 eV for NIM811 and 25 eV for d_{12}-CsA; Declustering potential (DP), 30 V for NIM811 and 20 V for d_{12}-CsA; Collision cell exit potential (EXP), 25 V; Entrance potential (EP), 10 V. The following precursor → product ion transitions were monitored in the MRM: NIM811, m/z 1219.8 → 1202.9; d_{12}-CsA, m/z 1232.8 → 1215.8. The dwell time was set at 200 ms for both NIM811 and d_{12}-CsA. The mass spectrometer was operated at unit mass resolution (half-height peak width set at 0.7 Da) for both the first and the third quadrupole.

2.4. Preparations of standards and quality control (QC) samples

Two separate primary stock solutions (stock A and stock B) of NIM811 were prepared in methanol at a concentration of 1.0 mg/ml each in 20-ml vials. The two stocks must have a difference ≤5% in the LC–MS/MS responses of each other from the stock solution comparison experiment. A NIM811 plasma intermediate at a concentration of 25,000 ng/ml was prepared by spiking an appropriate amount of the stock solution into human plasma with Na-EDTA as the anticoagulant. This was followed by preparation of human blood calibration standards at concentrations of 10.0, 20.0, 40.0, 200, 500, 1000, 2000, 4000 and 5000 ng/ml and QC samples at concentrations of 10.0 (LLOQ), 20.0, 50.0, 750, 1500 and 3750 ng/ml using fresh human blood with a hematocrit of 0.35. A 20 μl aliquot of each calibration standard and QC sample was spotted onto the circled area of FTA® cards. The cards were left on the laboratory bench for a minimum of 2 h for complete drying of the blood spots.

2.5. Sample preparation

Using a Harris UNI-CORE™ puncher, a 3-mm single punch was manually made for each calibration standard, QC, blank or unknown DBS sample. The obtained disc was placed into the appropriate well of the 2-ml 96-well assay plate. The internal standard working solution (25 μl of 200 ng/mL d_{12}-CsA in 50% aqueous methanol, v/v) was added to all wells except the blanks, to which a 25 μl aliquot of 50% aqueous methanol was added. To all wells, a 100 μl aliquot of methanol was added and the plate covered, followed by a 10 min sonication using a sonicator and a 10 min vortex-mixing using a pulse vortex-mixer at a speed setting of ~50 cycles per min. The plate was centrifuged at ~3000 rpm at room temperature for 5 min. Using a TomTec, a 100 μl volume of each resulting extract was transferred into the appropriate well of a new 1-mL 96-well plate. The extracts were evaporated to dryness using a 96-well evaporator under a stream of nitrogen at ~45 °C. To the residues, a 100 μl aliquot of 100 mM sodium bicarbonate buffer (pH ~10) was added, followed by pulse

vortex-mixing at a speed setting of ~50 cycles per min for 1 min at room temperature. A 300 μl aliquot of MTBE was added to each well and the plate covered, followed by pulse vortex-mixing for 10 min at the same speed setting as above. The plate was centrifuged at 3000 rpm for 5 min at room temperature. The supernatant (200 μl) from each well was transferred via TomTec to another new 1-ml 96-well plate. The extract was made to dryness and the residues were reconstituted using 100 μl of 50% aqueous methanol and the plate covered, followed by brief pulse vortex-mixing and centrifugation at the same setting as above. A 20 μl aliquot of the reconstituted sample extract was injected onto the LC–MS/MS system.

2.6. Validation procedures

2.6.1. Selectivity, sensitivity and linearity

The selectivity of the method was assessed by analyzing DBS samples ($n = 1$) prepared from the fresh blank blood collected from six individual human subjects. The assay selectivity was also evaluated by analyzing the zero samples (blank + internal standard, $n = 1$) from the above DBS samples.

The lower limit of quantification (LLOQ) was defined as the lowest concentration that exhibited an accuracy of ±20% bias and precision of ≤20% during method development. Nine non-zero calibration standards were analyzed in duplicates (one in the beginning and the other in the end of the assay sequence) in 3 separate validation runs. The analyte/IS peak area ratio against nominal NIM811 concentration was employed for calibration regression with a weighting factor of $1/x^2$.

2.6.2. Accuracy and precision

The inter- and intra-day accuracy and precision of the assay method were assessed from the analysis of six replicates of QCs at concentrations of 10.0, 20.0, 50.0, 750, 1500 and 3750 ng/ml along with calibration standards on each of the three validation days. The accuracy was expressed as the difference of the measured analyte concentrations from the nominal values (bias %) and the precision as the coefficient of variation (CV %). Bias (%) of within ±15% and CV of ≤15% was considered acceptable at all concentration levels except the LLOQ, for which a ±20% bias and ≤20% CV were considered acceptable.

2.6.3. Impact of blood spreadability on the accuracy of determination

The possible effect of blood spreadability (or distribution) due to possible interaction of blood and/or the analyte with the materials of DBS card was assessed by punching the DBS discs (×3) from the center and edge area of DBS QC samples at concentrations of 50.0, 750 and 3750 ng/ml, followed by analysis along with calibration standards. The measured NIM811 concentrations from both the center and edge discs of the above QCs were compared with the nominal values and each other. Bias values within ±15% of the nominal concentrations and within ±15% of each other for the QC sample results from both the central and edge discs would suggest no apparent chromatographic effect (or distribution effect).

2.6.4. Impact of blood spot size on the accuracy of determination

The relationship between the area of dried blood spot and the volume of the blood spotted on the DBS card was examined by spotting increasing volumes (10, 20 and 40 μl, $n = 3$) of DBS QC samples at three concentrations (low, median and high) onto the cards. After drying, three replicates of 3-mm discs were taken from the center of each DBS QC sample and analyzed along with calibration standards. The measured NIM811 concentrations from the above DBS QC samples were compared with the nominal values. A bias within ±15% of the nominal values would suggest no apparent difference for the DBS samples made with different blood volumes.

2.6.5. Hematocrit effect

Hematocrit (HT or HCT), packed cell volume (PCV) or erythrocyte volume fraction (EVF), is the proportion of blood volume that is occupied by red blood cells. Independent of body size, hematocrit is considered an integral part of a subject's complete blood count results [2]. Since hematocrit is directly proportional to the viscosity of blood, it may affect the diffusion/distribution properties of the blood spotted onto the DBS card and therefore the amount of drug measured. This is important to consider as the hematocrit level could change considerably among subjects, which may produce unexpected effects [6–8]. To determine the influence of hematocrit on the assay performance, fresh blood samples with adjusted hematocrit values of 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 were obtained from a commercial source (Bioreclamation). These fresh blood samples were each used to prepare DBS QCs at concentrations of 50.0, 750 and 3750 ng/ml. After drying, each of the above DBS QC samples was analyzed in three replicates along with the calibration standards that were prepared using the fresh blood with a hematocrit value of 0.35. A difference within ±15% of the nominal values for the measured analyte concentrations from above QC samples would suggest the hematocrit effect is negligible.

2.6.6. Dilution integrity

To demonstrate the method is suitable for the analysis of DBS samples with analyte concentrations exceeding the upper limit of quantification (ULOQ), the dilution integrity was assessed by diluting an extracted dilution DBS QC (DQC) sample with extracted zero samples (containing ISTD only), followed by analysis with the calibration standards and regular QCs. The obtained bias (%) from the three replicates of the DQCs should be within ±15% of the nominal value.

2.6.7. Matrix effect

NIM811 neat solutions were spiked into three replicates of extracted blank DBS samples with theoretical concentrations the same as the regular DBS QC samples at concentrations of 50.0, 750 and 3750 ng/ml. The LC–MS/MS responses from the above were compared to those of respective neat solutions. The matrix effect was calculated using the following equation: matrix effect (%) = [1-peak area of blank DBS extract post-fortified with the analyte/peak area of analyte in neat solutions] × 100.

2.6.8. Recovery of NIM811 from dried blood spot

To determine the assay recovery, a 3-mm disc for three replicates of each DBS QC sample at concentrations of 50.0, 750 and 3750 ng/ml was extracted. The analytical results from the extracted samples were compared to those from the extracted blank DBS samples post-fortified with the analyte at the same concentrations as above. Recovery was calculated using the following equation: recovery (%) = peak area of DBS extract/peak area of standard post-fortified × 100.

2.6.9. Stability

DBS samples at concentrations of 20.0, 750 and 3750 ng/ml were analyzed each in three replicates following storage at room temperature, 2 to 8 °C and ≤60 °C. The measured analyte concentrations were compared with the nominal values. The obtained bias (%) from the stability QCs should be within ±15% of the nominal values.

To mimic the possible situation where the DBS samples are collected and/or transported at a high temperature, a set of DBS QCs were stored at 60 °C for at least 4 h, followed by analysis with calibration standards and regular QCs. Bias within ±15% of the nominal

values would suggest the analyte is stable in the DBS samples under such an environment.

2.6.10. Carryover

Carry-over was evaluated by injecting two extracted blank DBS samples sequentially immediately after an upper limit of quantification (ULOQ) sample injection. The response in the first blank matrix injection at the retention time region of the analyte or internal standard should be less than 20% of the mean response of the LLOQ samples for the analyte and less than 5% of the mean response for the internal standard from the same assay sequence.

3. Results and discussion

DBS sample extraction often is carried out by adding a known volume of extraction solvent containing the internal standard(s) for off-line extraction [2]. In early feasibility tests, aqueous methanol was used for extraction of NIM811 DBS discs, followed by transfer and drying of the resulting sample extract and reconstitution of the sample residues prior to LC–MS/MS analysis. However, matrix effect led to a deteriorated peak shape for both the analyte and ISTD (Fig. 2, top panel). This result possibly was due to the materials, especially the proprietary chemicals, on the FTA card, as this phenomenon was not observed in the analysis of human whole blood samples (figure not shown). Liquid–liquid extraction using MTBE was subsequently incorporated in the sample preparation procedure and this approach resulted in a much improved LC–MS/MS chromatogram of the LLOQ sample (Fig. 2, bottom panel).

3.1. Selectivity, sensitivity and linearity

Under the current LC–MS/MS conditions, NIM81 was well separated from interferences in the matrix blank. No interference was observed in either drug-free DBS (matrix blank) or drug-free DBS spiked with the internal standard (zero sample). The current assay has a LLOQ of 10.0 ng/ml NIM811 in blood based on a single 3-mm punch. Reliable precision (CV% <12.3%) and accuracy (bias% within ±6.0%) was obtained by analyzing three sets of six replicate LLOQ samples (Table 1) along with the calibration standards over a dynamic range of 10.0–5000 ng/ml and the balance of the QC sample concentrations. The calibration curves from nine none-zero standards were obtained by plotting the peak area ratio of NIM811 and d_{12}-CsA against the corresponding concentrations of NIM811 in blood. Excellent linearity was achieved with correlation coefficients (r^2) greater than 0.990 for all validation batches using quadratic regression.

3.2. Accuracy and precision

The intra- and inter-day performance of the assay method was evaluated by analyzing six replicates each of NIM811 DBS QC samples at concentrations of 20.0, 50.0, 750, 1500 and 3750 ng/ml on the 3 separate validation days. As shown in Table 1, the obtained precision (CV) ranged from 3.2 to 11.7% and from 5.6 to 10.2%, respectively, for the intra-day and inter-day evaluations. The accuracy ranged from −6.8 to 8.5% and −0.2 to 2.7% bias, respectively, for the intra-day and inter-day batches.

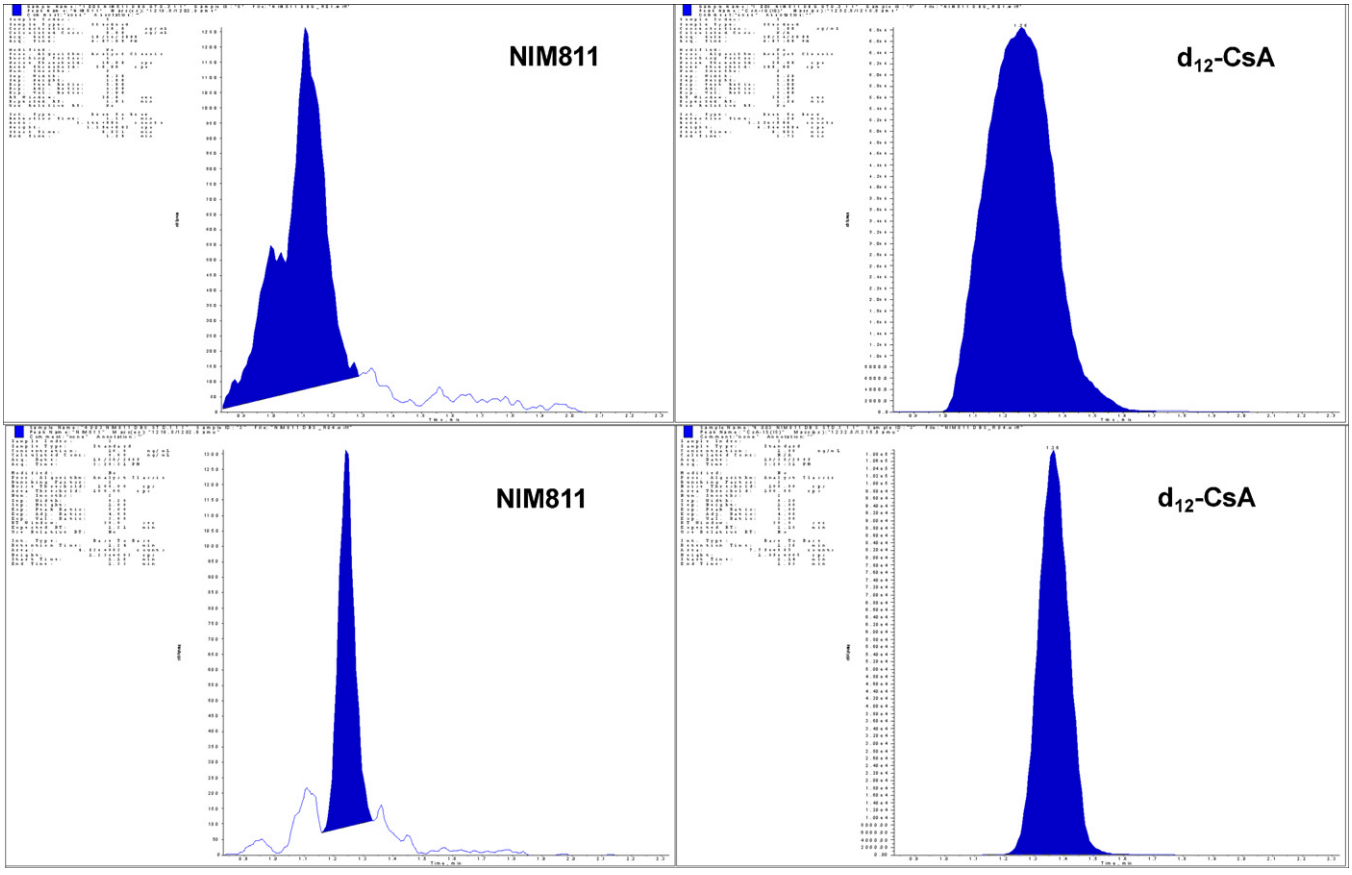

Fig. 2. Representative LC–MS/MS chromatogram of LLOQ sample with methanol extraction (top panel) and methanol extraction in combination with liquid–liquid extraction using MTBE (bottom panel).

Table 1
Summary of QC sample results from three-day validation runs.

	Day	LLOQ (10.0 ng/ml)	LQC (20.0 ng/ml)	MQC (50.0 ng/ml)	MQC (750 ng/ml)	MQC (1500 ng/ml)	HQC (3750 ng/ml)	DQC (3750 ng/ml)
Intrarun Mean		10.6	20.5	50.7	810	1570	4030	
Intrarun SD		1.25	1.80	5.93	32.5	50.8	348	
Intrarun % CV	Day 1	11.8	8.8	11.7	4.0	3.2	8.6	
Intrarun % Bias		6.0	2.5	1.4	8.0	4.7	7.5	
n		6	6	6	6	6	6	
Intrarun Mean		10.3	21.7	52.4	749	1560	3820	
Intrarun SD		1.05	1.91	5.13	34.0	90.9	172	
Intrarun % CV	Day 2	10.2	8.8	9.8	4.5	5.8	4.5	
Intrarun % Bias		3.0	8.5	4.8	−0.1	4.0	1.9	
n		6	6	6	6	6	6	
Intrarun Mean		9.93	19.5	46.6	750	1460	3610	3740
Intrarun SD		1.22	1.30	2.29	53.3	68.0	174	213
Intrarun % CV	Day 3	12.3	6.7	4.9	7.1	4.7	4.8	5.7
Intrarun % Bias		−0.7	−2.5	−6.8	0.0	−2.7	−3.7	−0.3
n		6	6	6	6	6	6	3
Overall mean		10.3	20.5	49.9	770	1530	3820	3740
Inter-run SD		1.14	1.85	5.09	48.4	85.3	291	213
Inter-run % CV		11.1	9.0	10.2	6.3	5.6	7.6	5.7
Inter-run % Bias		3.0	2.5	−0.2	2.7	2.0	1.9	−0.3
n		18	18	18	18	18	18	3

3.3. Impact of blood spreadability on the accuracy of determination

A comparison of the accuracy of NIM811 quantification using the discs punched from the center and edge areas of the DBS samples was conducted for three QC concentration levels (50.0, 750 and 3750 ng/ml) using three replicates for each concentration level. As shown in Fig. 3, the bias (%) values for the measured analyte concentrations from the discs of both the center and edge areas of the DBS samples compared with the nominal concentrations and compared with each other (center vs. edge) were all within ±15%, confirming no apparent chromatographic or distribution effect.

3.4. Impact of blood spot size on the accuracy of determination

As shown in Fig. 4, the measured analyte concentrations from the DBS samples at 50.0, 750 and 3750 ng/ml NIM811 prepared using 10, 20 and 40 μl blood volumes were all within ±15% compared with the nominal values, indicating that the amount of blood spotted in the range of 10 to 40 μl did not affect the distribution of NIM811 across the DBS card.

3.5. Hematocrit effect

As shown in Fig. 5, the observed difference in the accuracy of determination was within ±15% when compared with the nominal values for all QC samples (low, mid and high) prepared using blood with hematocrit values between 0.3 and 0.5, the range for a majority of human subjects. However, the hematocrit effect is noticeable for the results of the mid and high QC samples but not the low QC samples prepared using blood with a hematocrit value of ≥0.5. The higher hematocrit values of the blood were associated with a higher bias (%) for the obtained higher concentration QC sample results. This phenomenon suggested the presence of some kind of blood–analyte interaction, the magnitude of which is proportional to both the blood hematocrit value and the concentration of the analyte in the blood. This interaction resulted in an altered spreadability or diffusion of the blood with a higher hematocrit value (i.e. > 0.5) and a higher analyte concentration across the DBS card compared to 'normal' blood with regular hematocrit values (0.37–0.51) [2]. Apparently, the blood became more viscous and blood spot size became smaller (data not shown) with increased analyte concentrations in blood with hematocrit value ≥0.5. Similar observations have been reported by others [6–8] who also suggested the hematocrit effect might be influenced by paper type and the chemical properties of the compound of interest. Taking the above into consideration, it is suggested to include hematocrit assessment in the patient screening phase to determine whether DBS sampling via simple pricks (finger or toe) or whole blood collection via conventional venous bleeding should be used for PK assessment

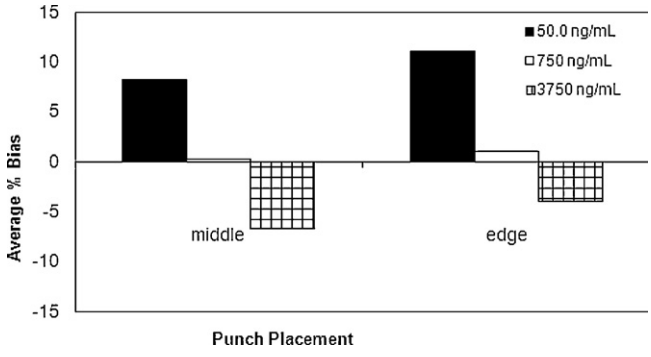

Fig. 3. Bias (%) of the measured NIM811 concentrations against the nominal values for the DBS discs punched from the center and edge areas of the QC samples.

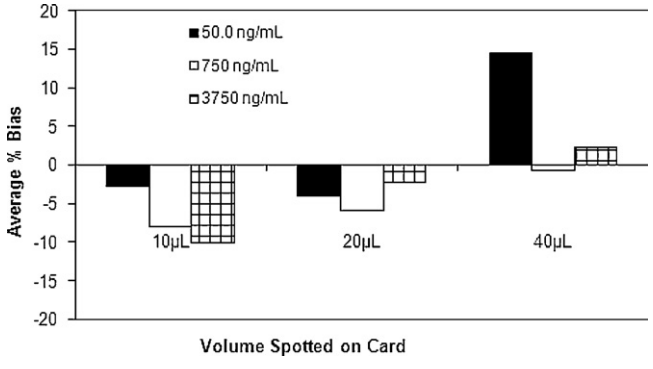

Fig. 4. Bias (%) of the measured NIM811 concentrations against the nominal values for the DBS QC samples prepared using 10, 20 and 40 μl blood volumes.

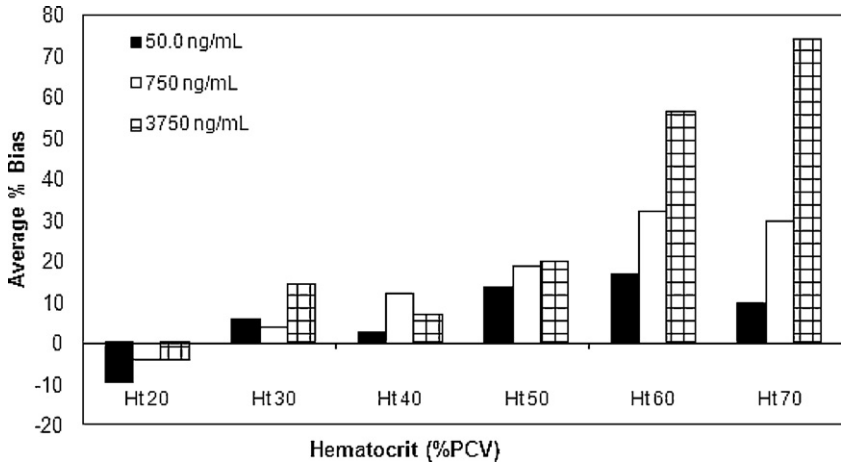

Fig. 5. Bias (%) of the measured NIM811 concentrations against the nominal values for the DBS QC samples prepared using fresh blood with hematocrit values of 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7.

or therapeutic drug monitoring for NIM811. Alternatively, DBS calibration standards and QC samples prepared using blood with a high hematocrit value (i.e. >0.5) should be employed for the analysis of the DBS samples collected from patients with hematocrit value >0.5.

3.6. Dilution integrity

Dilution integrity was assessed by 10-fold dilution of the 3750 ng/ml DBS QC sample extracts and analysis in three replicates along with calibration standards and regular QCs in one of the three validation runs. The obtained bias (%) and CV (%) was −0.3% and 5.7%, respectively.

3.7. Matrix effect

The matrix effect was estimated by spiking neat solutions of the analyte (50.0, 750 and 3750 ng/ml, $n = 3$) into blank DBS sample extracts and comparing the mean analyte responses (peak areas) with those from the corresponding neat solutions. The overall matrix effect ranged from 23.4 to 10.5% across the three concentration levels tested.

3.8. Recovery

The recovery was estimated by analyzing QC samples at 50.0, 750 and 3750 ng/ml ($n = 3$) and blank DBS sample extracts post-fortified with NIM811 at the same concentrations as above. Results were calculated by comparing the mean peak areas of NIM811 in the extracted QC samples with those of extracted blank samples post-fortified with NIM811. The overall recovery ranged from 87.4 to 93.2% for the three concentration levels tested.

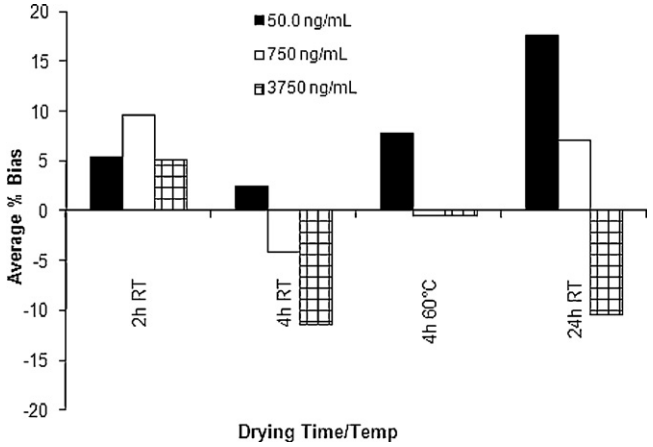

Fig. 6. Bias (%) of the measured NIM811 concentrations against the nominal values for the DBS QC samples stored at room temperature for 2, 4 and 24 h and at 60 °C for 4 h.

3.9. Stability

The bench-top stability of NIM811 in DBS was evaluated at ambient temperature (~22 °C) over 2, 4 and 24 h using QC samples at 20.0, 750 and 3750 ng/ml each in triplicate. As summarized in Fig. 6, NIM811 was stable for at least 24 h in human DBS when stored at ambient temperature. On the other hand, one set of DBS QCs (low, median and high) was placed at 60 °C for a minimum of 4 h to mimic the possible situation of sample collection and transportation at a higher temperature. The obtained results for those QCs were compared with the nominal values with bias (%) less than 10%, indicating that analyte is stable.

The long term stability of NIM811 in human DBS was evaluated at QC low, median and high concentration levels. After preparation, these QCs were stored at room temperature (~22 °C), 2–8 °C

Table 2
Summary of QC sample results after 62 days of storage under various temperatures.

Nominal concentration of NIM811 in DBS samples (ng/ml)	Bias (%) compared to nominal concentration		
	RT (~22 °C)	2–8 °C	≤−60 °C
20.0	−18.5	−24.0	7.5
750	−2.5	9.3	5.1
3750	−10.7	−9.6	−3.7

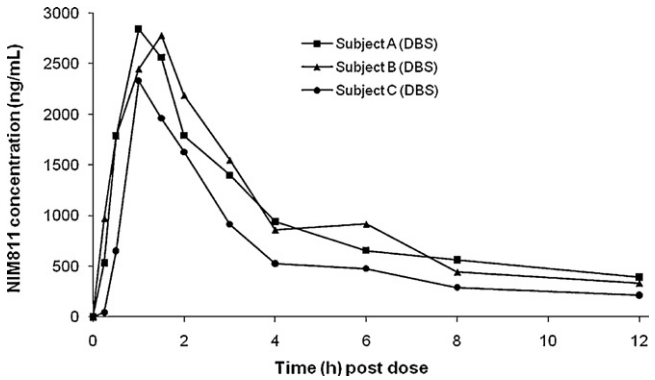

Fig. 7. Representative NIM811 concentration versus time profiles for three randomly selected subjects after oral administration of NIM811.

and ≤−60 °C. After 62 days of storage, three replicates of each of the above QC samples were analyzed along with freshly prepared DBS calibration standards and QCs. The measured NIM811 concentrations in the LTS QCs were compared with the nominal values. As summarized in Table 2, the best stability results were seen for the QCs stored at ≤−60 °C. With the increasing storage temperature to room temperature or 2–8 °C, the measured analyte concentrations, in general, decreased slightly, specifically for the low QCs. One possible reason that caused this 'instability' of the analyte in DBS may be related to the additive on the FTA card. According to the information from GE Whatman, the FTA card is chemically treated with proprietary reagents that lyse cells upon contact causing the release of nucleic acids [9]. Although the exact cell lysing reagents on the FTA card are not known, the reagent may have some kind of temperature dependent reaction with NIM811, a relatively large cyclic peptide. Another possible reason could be due to the altered extraction efficiency for the analyte as a result of the altered humidity of the DBS samples or altered interaction of the compound with DBS card materials (e.g. cellulose, etc) during this storage period. More details are pending on further exploration.

3.10. Carryover

To minimize carryover from the autosampler system, multiple washes of the autosampler system were incorporated in the assay by using two wash solvents (wash solvent 1: acetonitrile/isopropanol/water/formic acid/acetic acid at 60/30/10/0.1/0.1, v/v/v/v/v, and wash solvent 2: acetonitrile/2-propanol/water, 20/20/60%, v/v/v). The carryover was negligible (details not shown).

4. Application of assay

The present LC–MS/MS method was used to analyze the DBS samples prepared from venous blood samples collected in a clinical trial. Blood concentrations of NIM811 obtained for three randomly selected patients after an oral dose of 600 mg bid are illustrated in Fig. 7. The present method allowed for the determination of NIM811 up to 12 h (last sampling time) from all subjects.

5. Conclusion

A simple LC–MS/MS method has been developed and validated for the quantitative analysis of NIM811 in human dried blood spot (DBS) samples. The validated method is accurate and precise with bias within ±15% and CV ≤15% at all tested concentrations. The possible impact of the collected blood volume, hematocrit of the blood and the DBS punching location was carefully evaluated. There was hematocrit effect as a higher hematocrit level (≥0.5) was associated with an increased NIM811 concentration measured using the same sample size (i.e. 3 mm disc) for a QC sample with a higher nominal analyte concentration (i.e. 750 and 3750 ng/ml). Stability of NIM811 in the DBS samples was evaluated during which storage at room temperature for up to 24 h and at 60 °C for at least 4 h. The DBS method has been successfully implemented using venous blood samples collected in a clinical study. Requiring only a 20 μl blood sample, the method is well suited for application to therapeutic drug monitoring.

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