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Development and validation of a LC/MS/MS method for quantifying the next generation calcineurin inhibitor, voclosporin, in human whole blood

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ABSTRACT

A rapid, accurate, and reproducible liquid chromatography electrospray tandem mass spectrometry (LC/ESI–MS/MS) method was developed and validated for the therapeutic drug monitoring of voclosporin in human whole blood. Sample aliquots of 100 μL were processed utilizing a protein precipitation procedure that contained a mixture of methanol, 0.2 M $ZnSO_4$, and deuterated voclosporin internal standard. Supernatant was injected onto a Zorbax SB-C8, 2.1 × 12.5 mm column (at 60 °C), and washed with water–acetonitrile, supplemented with 0.02% glacial acetic acid and 0.02 mM sodium acetate, to remove poorly retained components. After washing, water–MeOH (with 0.02% glacial acetic acid and 0.02 mM sodium acetate) was used to elute the voclosporin and internal standard to the Applied Biosystems/MDS-Sciex API3000 mass spectrometer for detection in multiple reaction monitoring. Analytical performance was assessed in the range of 1–200 ng/ml in whole blood. This method has been used to quantify concentrations of voclosporin in whole blood from healthy volunteers participating in a pharmacokinetic study.

1. Introduction

Voclosporin (ISA247) is a next generation calcineurin inhibitor being developed for the prevention of organ graft rejection and the treatment of autoimmune diseases. Voclosporin is structurally similar to cyclosporine A, except for a modification to the terminal carbon of amino acid 1 (Fig. 1). Although cyclosporine A is an effective and widely used immunosuppressive drug, numerous adverse effects are associated with therapy [1]. This led to the development of voclosporin, which exhibits greater potency than cyclosporine A *in vitro* [2,3], as well as in *in vivo* animal models of transplantation [2,4] and autoimmunity [5] (arthritis). Clinical studies have shown voclosporin to be effective for preventing rejection in stable kidney transplant patients [6], and effective for treating moderate to severe psoriasis [7]. Voclosporin presents the possibility of a calcineurin inhibitor with an improved safety profile.

An important aspect to the dosing of immunosuppressive drugs is therapeutic drug monitoring (TDM), following organ transplantation. Narrow therapeutic indices and variable pharmacokinetics of the current immunosuppressive drug therapies [8] necessitate rapid and reliable analytical methods to monitor immunosuppressive drug levels. By using TDM to monitor immunosuppressive trough blood levels (12 h-pre-dose levels) individual dose adjustments can be made, thus, potentially improving patient clinical outcomes [9]. The aim of this work was to validate a rapid and reliable method for quantifying voclosporin in human whole blood. This paper describes the development and validation of an LC/MS/MS method for the therapeutic drug monitoring (TDM) of voclosporin concentrations in human whole blood.

2. Experimental

2.1. Chemicals

Voclosporin and the internal standard, a deuterated form of voclosporin, were obtained from F. Hoffman-La Roche, Ltd. (Basel, Switzerland). Reagents and solvents were of analytical grade. The internal standard was a mixture of deuterated forms of voclosporin (6% D2, 69% D3, and 25% D4), with the major isotope having a mass shift of 4 Daltons. The positions of the added deuteriums are shown in Fig. 1. The internal standard also contained ~0.4% unlabelled voclosporin. Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Caledon Laboratories, Ltd. (Georgetown, Ontario, Canada). Glacial acetic acid (sequencing grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA), sodium acetate trihydrate was purchased from EMD Chemicals, Inc. (Darmstadt, Germany), and zinc sulphate heptahydrate was purchased

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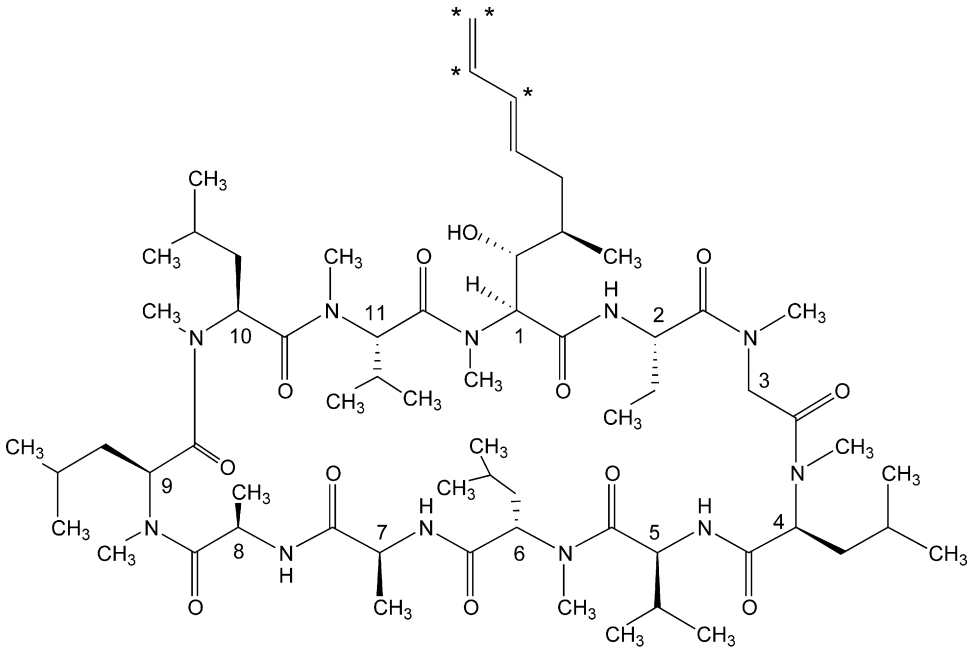

Fig. 1. Chemical structure of voclosporin.

from Alfa Aesar (Ward Mill, MA, USA). Water was purified in-house using a Milli-Q Gradient A-10 system (Millipore Corporation, Bedford, MA, USA).

2.2. Apparatus

An Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a binary pump (with micro-volume degasser), a quaternary pump (with a standard degasser), a wellplate autosampler, and a column oven compartment was used. Detection was performed using an Applied Biosystems/MDS-Sciex API3000 (Concord, Ontario, Canada) equipped with a Turbo Ion-Spray (TIS) interface and a 10 port 2 position valve from Valco (Houston, Texas, USA). A Zorbax SB-C8 2.1 × 12.5 mm, 5 μm column was purchased from Agilent Technologies (Palo Alto, CA, USA). The configuration of this setup is presented in Fig. 2.

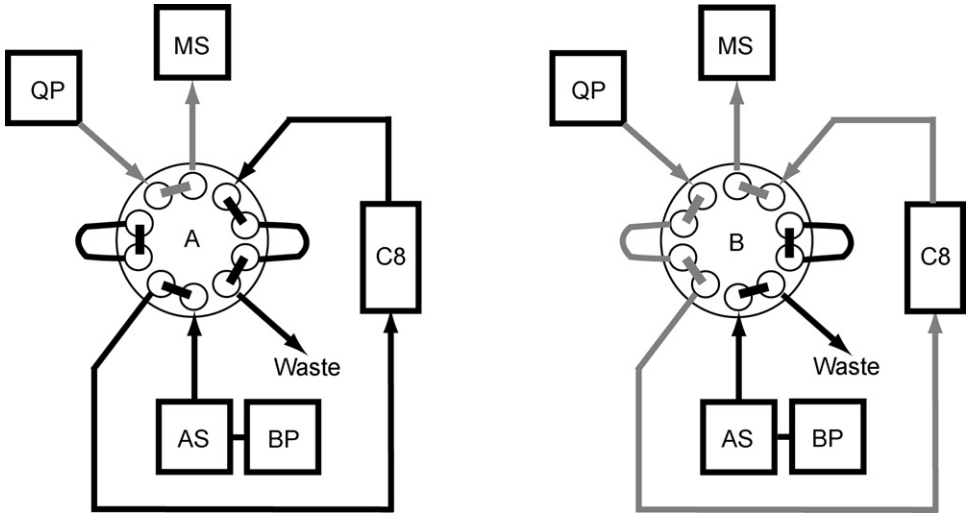

Fig. 2. Experimental setup for on-line solid phase extraction. Binary pump (BP), autosampler (AS), quaternary pump (QP), mass spectrometer (MS), and C8 column (C8).

2.3. Preparation of standards and controls

Voclosporin stock solutions were prepared in methanol at a concentration of 1 mg/mL, and further dilutions were also prepared in methanol. Stock solutions for the calibration standards and the quality controls (QCs) were made from two separate preparations of the same lot of voclosporin. After obtaining informed consent, K_2EDTA human whole blood was collected and pooled from six volunteers and frozen prior to use. Preparation of all standards and control samples utilized stock solutions added to aliquots of previously frozen whole blood. For method validation, whole blood standards and control samples were prepared fresh daily prior to extraction. The deuterated internal standard stock solution was prepared in methanol at a concentration of 1 mg/mL, and was further diluted to 0.001 mg/mL in methanol for routine use. All stock solutions were stored at −20 °C.

2.4. Sample preparation

Blood samples were extracted utilizing a protein precipitation procedure. Precipitation reagent was prepared as a mixture of methanol and 0.2 M $ZnSO_4$ in an 8:2 ratio (v/v). To this, internal standard was added to a final concentration of 3.5 ng/mL. The precipitation reagent was prepared fresh daily with an internal standard concentration of 3.5 ng/mL. A low internal standard concentration was utilized to keep the contribution of non-labeled voclosporin, from the internal standard, at <10% of the peak area of the extracted 1 ng/mL voclosporin standard (i.e., the LLOQ).

Standards and QCs were prepared by adding 10 μL of the appropriate methanolic spiking standard to 100 μL of blood to make the correct nominal whole blood concentration of voclosporin. After a brief vortex mixing, 400 μL of the precipitation reagent was added. Tubes were then capped, vortex mixed, and allowed to sit on the bench top for 10 min. They were then vortex mixed again, and the precipitate was pelleted by centrifugation at 3300 rpm for 10 min. The supernatant was then transferred to an amber vial with a glass insert and capped with Teflon lined silicone septa caps.

2.5. LC/MS/MS conditions

All LC solvents contained 0.02% (v/v) glacial acetic acid and 0.020 mM sodium acetate. The LC program details are presented in Table 1 and a schematics of the analytical setup is presented in Fig. 2. Initially, 20 μL of extract was injected onto the column at 60 °C and washed with 50% acetonitrile (2.5 mL/min for ~0.45 min) to remove poorly retained components. After the washing step, the valve position was switched, and 80% MeOH at 0.5 mL/min was used to elute the voclosporin and internal standard to the mass spectrometer for detection. After analyte elution, the valve was switched back, and the column was then flushed with 95% ACN and re-equilibrated with 50% ACN for the next injection. The total run time was 2 min, and the total cycle time (including autosampler time) was about 3 min. Note that the binary pump used has a delay volume of approximately 1 mL, and the program in Table 1 is optimized for this. Purified N_2 gas was used for all required gasses. The Turbo IonSpray was optimized at 550 °C with a capillary voltage of 5 kV for detection of the sodium adduct of voclosporin. For quantitation, the acquisition was performed in positive ionization mode with multiple reaction monitoring (MRM), monitoring the transitions of m/z 1236.8 → 1112.8 for the sodium adduct of voclosporin and 1240.8 → 1112.8 for the sodium adduct of the deuterated voclosporin internal standard. Dwell times of 350 ms for each transition were used. The Q1 was at unit resolution, and the Q3 was at low resolution. The declustering potential (DP) and the fragmentation potential (FP), were 60 and 400 V, respectively, and the Q2 collision gas setting (CAD) was CAD = 12. All data was acquired using Analyst 1.4.1.

Table 1
LC program details

Quaternary pump (for chromatographic analysis)	Isocratic, 80% methanol, 0.5 mL/min, 2.00 min	
Binary pump: (for sample clean-up and column flushing)		
Time (min)	Acetonitrile (%)	Flow rate (mL/min)
0.00	50	0.5
0.01	50	2.5
0.45	50	2.5
0.46	50	0.5
0.53	50	0.5
0.54	95	1.5
1.30	95	1.5
1.31	50	2.5
2.00	50	2.5
Injection volume		20 μL
Autosampler temperature		20 °C
Column temperature		60 °C
Valco valve program		
Time (min)		Position
0.00		A
0.50		B
1.30		A

2.6. Validation

The analytical method was validated within the guidelines established by the FDA for bioanalytical method validation [10]. A total of five different analytical runs were performed, each on a different day. In addition to any of the stability test samples, each inter-assay batch consisted of a blank, blank with internal standard, eight calibration standards, three replicates of each QC level (3, 80, and 175 ng/mL), and three samples at the LLOQ (1 ng/mL). The LLOQ defined in this assay is based on the expected range for voclosporin in the samples, as opposed to minimum criteria of response or precision. The intra-day batch was performed similar to the inter-assay batches, except that five replicates of all the QC and LLOQ samples were analyzed. Tests for stability were done using all QC levels in replicates of three, and for recovery in replicates of five. An assessment of matrix effects was done using matrix-free (solvent) samples and post-extraction addition of voclosporin to blank whole blood extracts.

3. Results and discussion

3.1. LC/MS/MS optimization

Post-column infusion of voclosporin was used to optimize the Turbo IonSpray interface and the MS ion sampling for the Na^+ adduct of voclosporin. Under "typical" LC eluant conditions, the major voclosporin species observed is the Na^+ adduct, as shown in the MS spectrum in Fig. 3(a). The addition of low micromolar levels of Na^+ (e.g., 0.020 mM as sodium acetate) to the mobile phase further shifts the observed species in favor of the Na^+ adduct. This improves sensitivity and linearity, as compared to the H^+ adduct. The LC/MS/MS conditions can be optimized for the monitoring of the NH_4^+ adduct, similar to that commonly used for cyclosporine A [11], however, the Na^+ adduct provided better sensitivity and improved specificity (data not presented). The isotopic composition of the deuterated voclosporin internal standard was such that

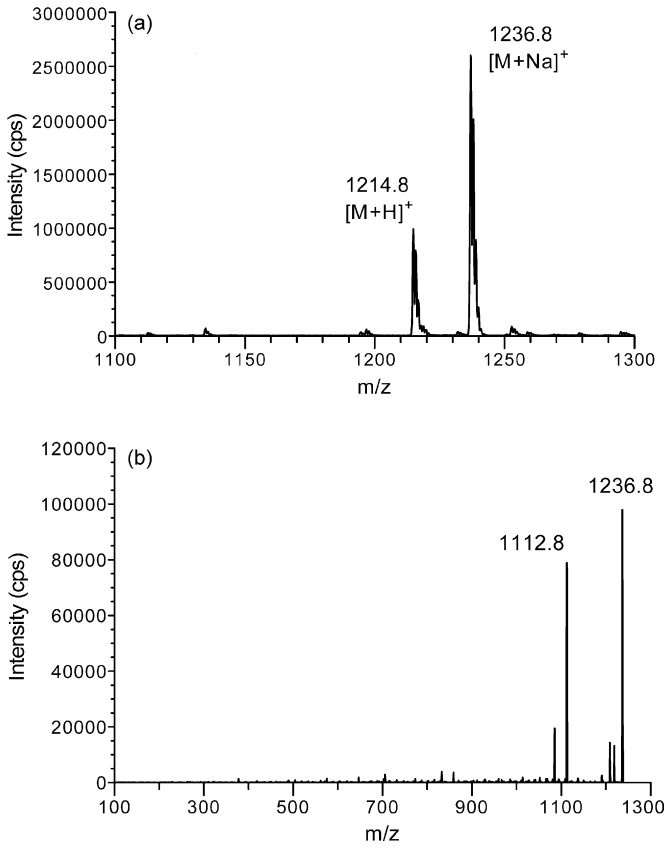

Fig. 3. Voclosporin spectrum scans. (a) Q1 scan, and (b) product ion scan for *m*/*z* 1236.8, collision energy = 87 V.

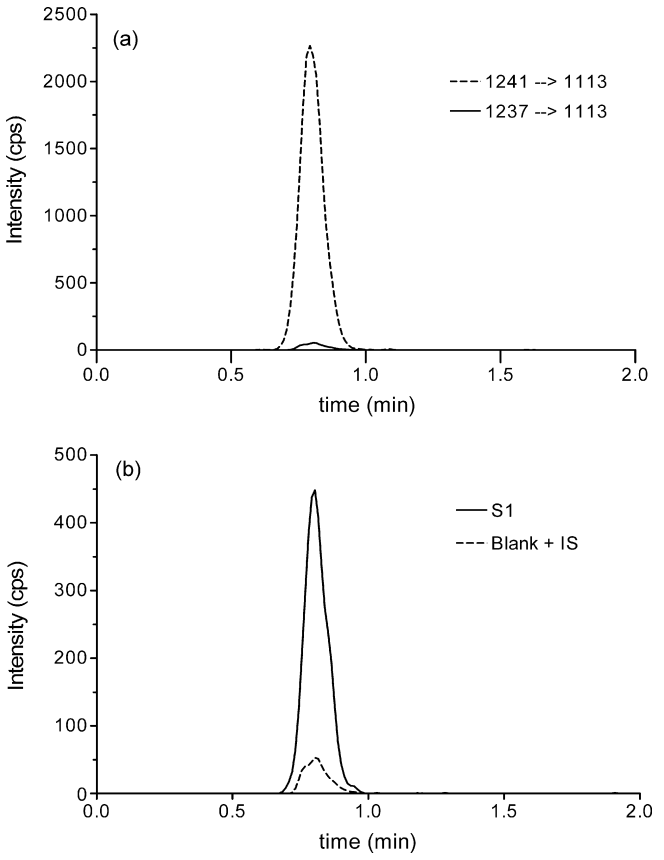

Fig. 4. Representative chromatograms for (a) an extracted human blood blank with internal standard (Blank + IS), and (b) overlaid chromatograms of the extracted blank with internal standard and the 1 ng/mL standard (S1), with only the voclosporin signals (MRM 1237 → 1113) shown.

the major isotope of the Na^+ adduct was observed at *m*/*z* 1240.8. Since all of the added deuterium labels were on the amino acid 1 side chain, the major fragment of *m*/*z* 1240.8 was observed to be *m*/*z* 1112.8, the same as the unlabelled voclosporin.

The LC solvent composition was optimized to provide some selective elution of matrix components and the analytes, to prevent excessive matrix ionization effects. A more detailed discussion of matrix effects follows in Section 3.10.

3.2. Specificity

The MS/MS spectrum of the voclosporin Na^+ adduct is presented in Fig. 3(b). The Na^+ adduct produces a major species at nominal *m*/*z* = 1113, which is the Na^+ adduct of the ring portion of voclosporin (i.e., the amino acid 1 side chain has been removed, −124 Da). By tuning the conditions to optimize the formation of the Na^+ adduct, the optimized MS/MS transition was determined to be 1236.8 → 1112.8 (precursor → product), with both the precursor and product being the major observed species in each respective spectrum. Using LC/MS/MS for the analysis of a high molecular weight compound substantially eliminates the possibility of interference due to other drugs (and endogenous compounds). Furthermore, the added specificity in monitoring the $[M+Na]^+$ adduct (and producing a structurally specific product ion) further adds to the specificity of detection.

A chromatogram of an extracted matrix blank with internal standard is presented in Fig. 4(a). A small peak for the MRM transition 1237 → 1113 is present due to the non-deuterated voclosporin in the internal standard. Fig. 4(b) is the overlaid chromatograms of the voclosporin 1237 → 1113 MRM transition from the extracted 1 ng/mL standard (i.e., the LLOQ) and the blank with internal standard. This demonstrates that the voclosporin introduced from the internal standard is low compared to the LLOQ response, and that the LLOQ can be detected with very good signal to noise. The voclosporin response at this LLOQ exceeds the blank response by >5 times.

3.3. Linear dynamic range and calibration curve summary

Eight point standard curves were generated over the range of 1–200 ng/mL, and the resultant curves of area ratio (area voclosporin/area internal standard) *versus* nominal voclosporin concentration were interpolated using a least-squares linear regression with 1/*x* curve weighting. The curve regression parameters are presented in Table 2, and the curve precision and accuracy are presented in Table 3. Precision was expressed as the coefficient of variation (CV) while accuracy was measured as percent analytical recovery relative to the nominal concentration. For the 5 curves presented, the calibration standards had analytical recoveries ranging from 92.5% to 104.8%, and the CV for each calibration standard level

Table 2
Regression parameters for the calibration curves used in the analytical validation for voclosporin in human whole blood

Batch	Slope (*m*)	*y*-Intercept (*b*)	*r*
1	0.159	0.0395	0.9986
2	0.175	0.0133	0.9981
3	0.164	0.0265	0.9992
4	0.170	0.0487	0.9995
5	0.167	0.0221	0.9990

Table 3
Precision and accuracy of the calibration samples used in the analytical validation for voclosporin in human whole blood

Nominal concentration (ng/mL)	Mean calculated concentration, $n = 5$ (ng/mL)	CV (%)	Accuracy (% bias)
1.00	1.01	5.2	0.7
5.00	5.24	2.3	4.8
25.0	26.0	3.0	4.1
50.0	46.3	1.6	−7.5
75.0	73.1	3.7	−2.5
100	95.6	2.4	−4.4
150	152	3.5	1.6
200	206	2.6	3.2

Table 4
Intra- and inter-assay precision and accuracy of the quality control samples used in the analytical validation for voclosporin in human whole blood

Nominal concentration (ng/mL)	Intra-assay			Inter-assay		
	Mean calculated concentration, $n = 5$ (ng/mL)	CV (%)	Accuracy (% bias)	Mean calculated concentration, $n = 15$ (ng/mL)	CV (%)	Accuracy (% bias)
1.00	0.993	4.8	−0.7	0.989	9.6	−1.1
3.00	3.16	4.9	5.3	3.12	4.3	4.1
80.0	75.1	2.3	−6.1	77.6	4.5	−3.0
175	174	1.9	−0.3	177	3.2	1.3

ranged from 1.6% to 5.2%, indicating good precision and accuracy for curves used in the validation.

3.4. Accuracy and precision

Between run precision and accuracy were determined from quality control (QC) samples. Four different concentrations of voclosporin were spiked into human whole blood. The lower limit of quantitation (LLOQ), low, medium and high QC concentrations used for voclosporin were 1 ng/mL (LLOQ), 3 ng/mL (LQC), 80 ng/mL (MQC), and 175 ng/mL (HQC), respectively. A total of 15 replicates of each QC concentration for voclosporin were assayed on five different instances, from five different calibration curves. As presented in Table 4, the coefficients of variation for voclosporin QCs ranged from 3.2% to 9.6% and the analytical recovery obtained for the LQC, MQC and HQC were 104.1%, 97.0%, and 101.3%, respectively.

Within-run precision and accuracy was determined similarly using a total of five replicates of the LQC, MQC, and HQC whose concentration was determined from one calibration curve (Table 4). The coefficients of variation ranged from 1.9% to 4.9%, and the analytical recovery obtained for the LQC, MQC and HQC were 105.3%, 93.9% and 99.7%, respectively.

The lower limit of quantitation utilized for this procedure was 1 ng/mL (0.1 ng/100 μL) for voclosporin. The LLOQ displayed a coefficient of variation of 9.6% and an analytical recovery of 98.9% over the five curves utilized to determine accuracy and precision (Table 4). In addition, the LLOQ QC samples displayed a coefficient of variation of 4.8% and an analytical recovery of 99.3% over the five replicates for the within-run precision and accuracy (Table 4).

3.5. Short and long-term stability of voclosporin

Short-term stability of voclosporin was assessed with three replicates of samples kept at room temperature (nominally 22 °C) for 23 h *versus* three replicates of freshly prepared comparison samples analyzed in the same analytical run. Concentrations at the LQC, MQC, and HQC levels were used, and the results are presented in Table 5. The percent difference in calculated concentration from the comparison sample varied between −7.0% and 0.4%. The results indicate that the short-term stability of voclosporin is not adversely compromised under the specified conditions. Current testing indicates that voclosporin in methanolic stocks is stable at −20 °C in excess of 12 months, and voclosporin in whole blood stored at −20 °C is stable in excess of 12 months.

3.6. Freeze–thaw stability

Freeze–thaw stability was examined by analyzing three replicates of each QC level thawed thrice *versus* three replicates of freshly prepared comparison samples. For each freeze–thaw cycle, QC test samples were frozen at −20 °C and thawed unassisted at room temperature (nominally 22 °C). As indicated in Table 5, the percent difference from the comparison samples for the calculated concentrations varied from −7.0% to −3.3%, indicating that the stability of voclosporin in human whole blood is not significantly compromised after being thawed thrice.

3.7. Post-extraction stability of voclosporin

The drug concentrations in freshly extracted QC samples were determined and later compared to the measured drug concentration for the same QC samples re-analyzed after 144 h (~6 days) in the autosampler (nominally 22 °C). The original calibration results were used to determine the analyte concentration in the re-analyzed samples. Evaluation involved all three QC concentrations of voclosporin. As indicated in Table 5, the percent difference of the re-analyzed samples as compared to the comparison samples range from −1.6% to 1.6%, indicating that the

Table 5
Short-term, freeze–thaw, and post-preparative stability of the quality control samples used in the analytical validation for voclosporin in human whole blood

	Difference in calculated concentration *versus* comparison sample, $n = 3$ (%)		
	LQC (3.00 ng/mL)	MQC (80.0 ng/mL)	HQC (175 ng/mL)
Short-term, 23 h	0.4	−2.6	−7.0
Freeze–thaw, 3 times	−3.3	−7.0	−6.1
Post-preparative, 144 h	−1.6	1.6	0.4

extracted sample is not significantly compromised after 144 h in the autosampler.

3.8. Extraction yields of voclosporin and deuterated voclosporin from human whole blood

Peak areas of voclosporin and deuterated voclosporin extracted from human whole blood were compared to their non-extracted counterparts under identical chromatographic conditions, and were analyzed within the same analytical run. The LQC, MQC and HQC and internal standard were assayed in replicates of five. Solvent-only samples of voclosporin (or deuterated voclosporin) were prepared at a concentration determined to be the same as the theoretical concentration for the extracted QC sample. The extraction yields of voclosporin and the internal standard in human whole blood were >95% for all the levels tested.

3.9. Ion suppression due to extracted sample matrix

For methods which employ a rapid elution step, the possibility of co-eluting matrix components with the analytes is likely greater than for methods with better chromatography. The presence of severe signal suppression or enhancement may lead to compromised detection limits, poor accuracy and poor precision depending on the internal standard's ability to correct for differing matrix effects between calibrants and analytical test samples. While use of isotope-labeled analogues, as internal standards (such as the deuterated voclosporin used here), has been generally reported to eliminate matrix liability [12], the relative matrix ionization effect was further characterized for the method.

The extent of matrix effects on electrospray ion suppression was assessed by comparing voclosporin peak areas from blank whole blood with voclosporin added post-extraction and matrix-free extracts. Whole blood samples were extracted in triplicate, and a measured volume (250 μL for each) of supernatant was spiked with 5 μL of an 800 ng/mL voclosporin standard solution. The final voclosporin concentration was designed to be at the MQC level. For a control, water rather than whole blood was extracted, and the measured voclosporin peak area from this was presumed to be interference-free. Measured peak areas from the whole blood extracts were then reported as percent difference relative to the interference-free response. Matrix suppression would be observed as a decrease in the measured voclosporin peak area, and positive matrix effects would be observed as an increase in the voclosporin peak area, relative to the comparison sample. The results are presented in Table 6. For extracted human whole blood, differences (i.e., ionization suppressions) of −1.5% were determined. These results are well within the analytical variation observed for the replicates (with coefficient of variation ranging from 4.9 to 8.3%), indicating that there are no significant differences with respect to matrix ionization effects between a solvent sample and extracted human whole blood. These results indicate that this method has effectively controlled the matrix ionization effects for this particular source of matrix. True robustness of the method to matrix ionization effects in a clinical population is difficult to address (even when using several different sources of matrix during development), so the method then must rely on the internal standard to make any necessary corrections. While isotopically labeled internal standards are regarded as the best scenario [12], often structural analogues must be used instead. As an example, cyclosporine A has been used as an internal standard with this basic method, and provides similar retention time and method performance (data not presented). With respect to routine monitoring of voclosporin in patients, this means that a good internal standard for voclosporin analysis is commercially available.

Table 6
Matrix-related ionization suppression for voclosporin spiked into extracted matrix blanks

	Solvent	Human whole blood
Mean peak area, $n = 3$ (cps)	1.94E+05	1.91E+05
CV (%)	8.3	4.9
Difference (%)	n/a	−1.5

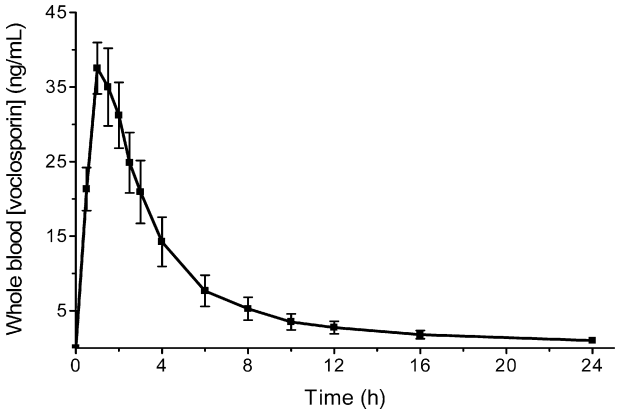

Fig. 5. Mean blood concentration-time curve from healthy subjects ($n = 6$) after a single oral dose of voclosporin at 0.25 mg/kg. Data presented as mean ± S.D.

3.10. Application of the method

This method was successfully applied to the analysis of samples from a single escalating dose clinical research protocol, approved by a local ethics committee (SFBC Anapharm, Montreal), where human whole blood samples from healthy subjects ($n = 6$) were analyzed. Results from a single oral dose of 0.25 mg/kg of voclosporin are presented in Fig. 5 as a concentration-time curve for voclosporin. Each point is a mean of six healthy subjects, and the error bars represent one standard deviation. Results indicate this method is applicable to the analysis of blood samples for both trough and pharmacokinetic TDM at this dose.

4. Conclusions

The data presented demonstrate that the LC/MS/MS method developed for voclosporin analysis in human whole blood is accurate and reproducible, and can be validated within the guidelines established by the FDA for bioanalytical method validation [10]. The dynamic range of 1–200 ng/mL is suitable for therapeutic drug trough monitoring of voclosporin, and the method is relatively free from matrix interferences. The ease of sample preparation and rapid cycle time make this method suitable for a high sample throughput work environment, especially if combined with automated sample preparation and handling. Other future enhancements to this method may include using an internal standard free from voclosporin contamination, or possibly using a structural analogue to avoid contamination issues (e.g., cyclosporine A).

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