



# Quantification of vosaroxin and its metabolites N-desmethylvosaroxin and O-desmethylvosaroxin in human plasma and urine using high-performance liquid chromatography-tandem mass spectrometry



C.M. Nijenhuis<sup>a,\*</sup>, L. Lucas<sup>a</sup>, H. Rosing<sup>a</sup>, G. Jamieson<sup>b</sup>, J.A. Fox<sup>b</sup>, J.H.M. Schellens<sup>c,d</sup>, J.H. Beijnen<sup>a,d</sup>

<sup>a</sup> Department of Pharmacy & Pharmacology, Antoni van Leeuwenhoek/The Netherlands Cancer Institute and MC Slotervaart, Amsterdam, The Netherlands

<sup>b</sup> Sunesis Pharmaceuticals, Inc. South San Francisco, CA, United States

<sup>c</sup> Division of Clinical Pharmacology, Department of Medical Oncology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

<sup>d</sup> Division of Pharmacoepidemiology and Clinical Pharmacology, Faculty of Science, Department of Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

## ARTICLE INFO

### Article history:

Received 29 January 2016

Received in revised form 9 May 2016

Accepted 10 May 2016

Available online 13 May 2016

### Keywords:

Vosaroxin

N-desmethylvosaroxin

O-desmethylvosaroxin

HPLC-MS/MS

GLP

Pharmacokinetics

Clinical pharmacology

## ABSTRACT

Vosaroxin is a first-in-class anticancer quinolone derivative topoisomerase II inhibitor that is currently in development in combination with cytarabine for the treatment of acute myeloid leukemia (AML). To investigate vosaroxin pharmacokinetics (PK) in patients, liquid chromatography tandem mass spectrometry (LC-MS/MS) assays to quantify vosaroxin and the two metabolites N-desmethylvosaroxin and O-desmethylvosaroxin in human plasma and urine were developed and validated.

Immediately after collection the samples were stored at  $-80^{\circ}\text{C}$ . Prior to analysis, the plasma samples were subjected to protein precipitation and the urine samples were diluted. For both assays the reconstituted extracts were injected on a Symmetry Shield RP8 column and gradient elution was applied using 0.1% formic acid in water and acetonitrile-methanol (50:50, v/v). Analyses were performed with a triple quadrupole mass spectrometer in positive-ion mode. A deuterated isotope of vosaroxin was used as internal standard for the quantification.

The validated assays quantify vosaroxin and N-desmethylvosaroxin in the concentration range of 2–500 ng/mL in plasma and urine. For O-desmethylvosaroxin the concentration range of 4–500 ng/mL in plasma and urine was validated. Dilution integrity experiments show that samples can be diluted 25 fold in control matrix prior to analysis. The expanded concentration range for plasma and urine for vosaroxin and N-desmethylvosaroxin is therefore from 2 to 15,000 ng/mL and in plasma for O-desmethylvosaroxin from 4 to 15,000 ng/mL.

© 2016 Elsevier B.V. All rights reserved.

## 1. Introduction

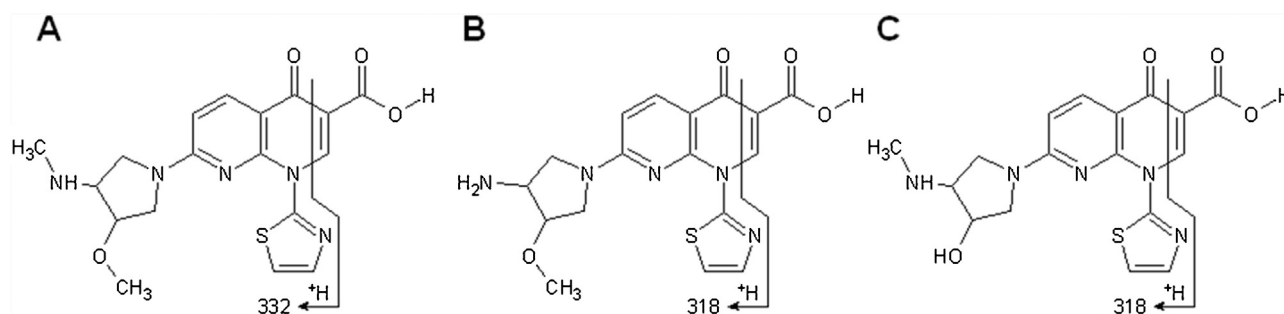
Vosaroxin is a first-in-class anticancer quinolone derivative that intercalates deoxyribonucleic acid (DNA) and inhibits topoisomerase II, inducing replication-dependent DNA damage that leads to apoptosis [1]. A recently completed pivotal phase 3 clinical trial evaluating vosaroxin plus cytarabine in relapsed or refractory acute myeloid leukemia (AML) suggested that this combination may benefit patients 60 years of age or older [2]. In preclinical

studies, the presence of the metabolites N-desmethylvosaroxin and O-desmethylvosaroxin was found in rat urine and bile [3].

To support clinical pharmacology studies, assays for quantification of vosaroxin and its metabolites in plasma and urine are needed. This article is the first to describe the validation of bioanalytical assays for these compounds, although assays were reported previously [4–7]. Herein we describe a full validation of two assays quantifying vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin in plasma and in urine. The validation was performed in compliance with the OECD principles of Good Laboratory Practice (GLP) [8] and according to the FDA and latest EMA guidelines on bioanalytical method validation [9,10]. These assays were used in a human mass balance study (manuscript in preparation) and passed ISR.

\* Corresponding author at: Antoni van Leeuwenhoek/The Netherlands Cancer Institute, Department of Pharmacy & Pharmacology, Louwesweg 6, 1066 EC Amsterdam, The Netherlands.

E-mail address: [Cynthia.Nijenhuis@slz.nl](mailto:Cynthia.Nijenhuis@slz.nl) (C.M. Nijenhuis).



**Fig. 1.** Chemical structure of the analytes ((A) vosaroxin, (B) N-desmethylvosaroxin and (C) O-desmethylvosaroxin) and their proposed fragmentation pathways.

## 2. Experimental

### 2.1. Chemicals

Vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin (Fig. 1) were manufactured by Albany Molecular Research, Inc. (AMRI, Albany, NY, USA) and provided by Sunesis Pharmaceuticals, Inc. (South San Francisco, CA, USA). A deuterated stable isotope ( $^2\text{H}_6$ -vosaroxin) was used as internal standard for all analytes and was manufactured by Syngene International (Bangalore, India) and provided by Sunesis Pharmaceuticals, Inc. All reference standards have been certified by AMRI. Methanol (HPLC grade), water (HPLC grade) and acetonitrile (HPLC grade) were obtained from Biosolve Ltd (Valkenswaard, the Netherlands). Formic acid, ammonium acetate (98%) and sodium hydroxide (50%) were purchased from Merck (Darmstadt, Germany). Water (distilled) used for sample preparation came from B. Braun Medical (Melsungen, Germany).  $\text{K}_2\text{EDTA}$  plasma and drug-free control human urine was obtained from healthy volunteers (MC Slotervaart).

### 2.2. Stock solutions, calibration standards and quality control samples

#### 2.2.1. Stock solutions

Separate 100  $\mu\text{g/mL}$  stock solutions for calibration standards and quality control (QC) samples were prepared for each analyte (corrected for potency using the chemical purity): vosaroxin in 0.1 N sodium hydroxide and N-desmethylvosaroxin and O-desmethylvosaroxin in acetonitrile–5 mM sodium hydroxide (20:80, v/v). Since all analytes are light sensitive, light exposure was minimized with amber colored vials and polypropylene (PP) tubes. These stock solutions were stored at  $-20^\circ\text{C}$  in silanized amber glass vials. The stock solutions of the different analytes were mixed and further diluted in serial with acetonitrile–5 mM sodium hydroxide (20:80, v/v) to obtain working solutions. The working solutions were stored at  $-20^\circ\text{C}$  in silanized amber glass vials since adsorption to polypropylene material was observed. Long-term stability in the working solutions could not be demonstrated under these conditions; therefore, fresh working solutions were made for the preparation of calibration standards and QC samples in plasma and urine for all analyses.

The stock solution of the internal standard  $^2\text{H}_6$ -vosaroxin (IS) was prepared at a concentration of 100  $\mu\text{g/mL}$  in 0.1 N sodium hydroxide. The IS working solution, was 200 ng/mL IS in methanol–10 mM ammonium acetate (50:50, v/v). The IS stock solution was stored at  $-20^\circ\text{C}$  and the IS working solution was stored at  $2-8^\circ\text{C}$ . This internal standard was used for all analytes since no stable isotopes were available for N-desmethylvosaroxin and O-desmethylvosaroxin.

#### 2.2.2. Calibration standards and quality control samples of the plasma assay

Human  $\text{K}_2\text{EDTA}$  plasma was used as control plasma. During the validation, calibration standards and QC samples were prepared freshly before each validation run by adding 10  $\mu\text{L}$  of working solution to 190  $\mu\text{L}$  control human plasma. Calibration standards were prepared in amber colored PP tubes; concentrations were 2, 5, 25, 50, 125, 250, 400 and 500 ng/mL for vosaroxin and N-desmethylvosaroxin; and 4, 10, 25, 50, 125, 250, 400 and 500 ng/mL for O-desmethylvosaroxin. Aliquots of 50  $\mu\text{L}$  were used for sample preparation. Final concentrations for the QC samples at the lower limit of quantification (LLOQ), QC low, QC mid and QC high were 2, 6, 150 and 375 ng/mL for vosaroxin and N-desmethylvosaroxin; for O-desmethylvosaroxin the final concentrations were 4, 12, 150, and 375 ng/mL. Calibration standards and QC samples were stored in aliquots of 50  $\mu\text{L}$  at  $-70^\circ\text{C}$  in amber 1.5 mL PP tubes.

The QC sample above the upper limit of quantification ( $>\text{ULOQ}$ ) was prepared by evaporating 30  $\mu\text{L}$  of each stock solution, under a gentle stream of  $\text{N}_2$  at  $30^\circ\text{C}$ , (vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin) and reconstituting the evaporated stocks with 200  $\mu\text{L}$  human control plasma creating a sample with a final concentration of 15,000 ng/mL.

#### 2.2.3. Calibration standards and quality control samples of the urine assay

During the validation calibration standards and QC samples were prepared freshly before each validation run by adding 10  $\mu\text{L}$  of working solution to 190  $\mu\text{L}$  human urine. Calibration standards with the following concentrations were obtained for vosaroxin and N-desmethylvosaroxin: 2, 5, 25, 50, 125, 250, 400 and 500 ng/mL; and 4, 10, 25, 50, 125, 250, 400 and 500 ng/mL for O-desmethylvosaroxin. Aliquots of 50  $\mu\text{L}$  were used for sample preparation.

Final concentrations for the QC samples at the lower limit of quantification (QC LLOQ), QC low, QC mid and QC high were 2, 6, 150 and 375 ng/mL for vosaroxin and N-desmethylvosaroxin; for O-desmethylvosaroxin the final concentrations were 4, 12, 150, and 375 ng/mL. Also, urine calibration samples and QC samples were stored in aliquots of 50  $\mu\text{L}$  at  $-70^\circ\text{C}$  in amber 1.5 mL PP tubes. The QC  $>\text{ULOQ}$  (15,000 ng/mL for all analytes) was prepared as described in the quality control samples of the plasma assay.

### 2.3. Sample preparation

#### 2.3.1. Sample collection

Whole blood samples were obtained by venipuncture and placed immediately on ice prior to centrifugation at 2000g at  $4^\circ\text{C}$  for 10 min to obtain plasma. Plasma samples were stored at  $-80^\circ\text{C}$  for a maximum of three months after which they were transferred to a  $-70^\circ\text{C}$  freezer pending analysis. Urine samples were collected and stored at  $2-8^\circ\text{C}$ . Samples were pooled and then homogenized

**Table 1**Mass spectrometric parameters for the analysis of vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin with the internal standard vosaroxin-<sup>2</sup>H<sub>6</sub>.

General settings	API4000		API5500		
Run duration	10.0 min		9.50 min		
Ion spray voltage	4500 V		4000 V		
Nebulizer gas	70 au		40 au		
Turbo gas/Heater gas	60 au		40 au		
Curtain gas	20 au		40 au		
Collision gas	80 au		9 au		
Temperature	500 °C		500 °C		
Analyte specific parameters	Vosaroxin	Vosaroxin- <sup>2</sup> H <sub>6</sub>	N-desmethyl vosaroxin	O-desmethylvosaroxin	O-desmethylvosaroxin
Parent mass	402 m/z	408 m/z	388 m/z	388 m/z	388 m/z
Product mass	332 m/z	338 m/z	318 m/z	318 m/z	318 m/z
Dwell time	100 ms	100 ms	100 ms	100 ms	5 ms
Collision energy	43 V	43 V	45 V	43 V	49 V
Collision exit potential	22 V	22 V	20 V	22 V	32 V
Declustering potential	66 V	66 V	66 V	66 V	161 V
Typical retention time	2.70 min	2.70 min	2.75 min	1.75 min	3.4 min

by shaking; 1 mL aliquots were stored at  $-80^{\circ}\text{C}$  and were also transferred to a  $-70^{\circ}\text{C}$  freezer pending analysis.

### 2.3.2. Plasma and urine pretreatment

Plasma or urine samples were thawed prior to processing and 50  $\mu\text{L}$  aliquots were placed in amber colored PP tubes on ice during pretreatment for analysis. The sample pretreatment procedure for both matrices was identical. Twenty-five  $\mu\text{L}$  of IS working solution (200 ng/mL) were added and the samples were vortex mixed for 30 s. Subsequently, 150  $\mu\text{L}$  of 0.1% formic acid in acetonitrile were added, the samples were mixed again (30 s) and were then centrifuged for 5 min at 23,100g. A volume of 100  $\mu\text{L}$  was transferred to an amber colored PP tube and 300  $\mu\text{L}$  of water was added. Samples were vortex mixed for 10 s and transferred to an amber autosampler vial with insert. A volume of 10  $\mu\text{L}$  was injected into the HPLC column.

### 2.4. Liquid chromatography-tandem mass spectrometry

The chromatographic separation of vosaroxin and N-desmethylvosaroxin in plasma and urine and of O-desmethylvosaroxin in plasma was carried out using a HP1100 binary pump, a degasser, a HP1100 autosampler and a switching valve (Agilent technologies, Palo Alto, CA, USA). The autosampler temperature was kept at  $4^{\circ}\text{C}$  and the column oven at  $50^{\circ}\text{C}$ . The mobile phase A consisted of 0.1% formic acid in water and mobile phase B was acetonitrile-methanol (50:50, v/v). Gradient elution was applied at a flow rate of 0.35 mL/min through a Symmetry Shield RP8 (100 Å,  $150 \times 2.1$  mm ID, particle size 3.5  $\mu\text{m}$ ; Waters, Etten-Leur, The Netherlands). The following gradient was applied: 20% B (0.00–1.50 min), 20–95% B (1.5–1.51 min), 95% B (1.51–6.00 min), 95–20% B (6.00–6.01), 20% B (6.01–9.00). The divert valve directed the flow to the mass spectrometer between 0.1 and 8.8 min.

These samples were analysed using an API4000 quadrupole mass spectrometer (MS) (Sciex, Framingham, MA, USA). The instrument is equipped with a Turbo IonSpray (TIS) interface, operating in positive mode and configured in multiple reaction monitoring (MRM). The LC-MS/MS data were acquired and processed with Analyst<sup>TM</sup> software (Sciex). Table 1 summarizes the MS operating parameters.

For the analysis of O-desmethylvosaroxin in urine a separate method was developed, due to initial failed ISR with the method described above for O-desmethylvosaroxin. This will be further discussed in the results. The chromatographic separation was carried out using a UPLC I Class pump, column oven and autosampler (Waters, Etten-Leur, The Netherlands).

The autosampler temperature was kept at  $5^{\circ}\text{C}$  and the column oven at  $40^{\circ}\text{C}$ . The mobile phase A and mobile phase B were the same as described above. Gradient elution was applied at a flow rate of 0.30 mL/min through an Synergi Hydro-RP C18 ( $150 \times 2.0$  mm internal diameter, particle size 4  $\mu\text{m}$ ; Phenomenex). The following mobile phase gradient was applied: 20% B, 0.3 mL/min (0.0–1.0 min); 20–85% B, 0.3 mL/min (1.0–5.6 min); 85–100% B, 0.3 mL/min (5.6–5.61 min); 100% B, 0.3–0.7 mL/min (5.61–5.63 min); 100% B, 0.7 mL/min (5.63–8.00 min); 100–20% B, 0.7 mL/min (8.00–8.01 min); 20% B, 0.7 mL/min (8.01–8.50 min); 20% B, 0.7–0.3 mL/min (8.50–8.51 min); 20% B, 0.3 mL/min (8.51–9.50). The divert valve directed the flow to the mass spectrometer between 2.0 and 5.5 min; the eluent during the remainder of the run was sent to the waste container.

O-desmethylvosaroxin in urine was analysed on a QTrap 5500 mass spectrometer (MS) (AB Sciex, Thornhill, ON, Canada). This instrument is equipped with a TIS interface, operating in positive mode and configured in multiple reaction monitoring (MRM) mode. The LC-MS/MS data were acquired and processed with Analyst<sup>TM</sup> software version 5.1.2 (AB Sciex). Table 1 summarizes the MS operating parameters.

### 2.5. Validation procedures

The validation of the assays was performed in accordance to the OECD principles of Good Laboratory Practice (GLP) [8]. Calibration model, accuracy and precision, selectivity, dilution integrity, lower limit of quantitation, matrix effect, carryover, and stability under various conditions were established according to the US FDA and latest EMA guidelines on bioanalytical method validation [9,10].

The accuracy is expressed as the bias and the following equations were used:

$$\text{Intra-run bias}[\%] = 100 \times \frac{(\text{mean measured conc. per run} - \text{nominal conc.})}{(\text{nominal conc.})} \quad (1)$$

$$\text{Overall bias}[\%] = 100 \times \frac{(\text{overall mean measured conc.} - \text{nominal conc.})}{(\text{nominal conc.})} \quad (2)$$

The precision is expressed as the coefficient of variation (CV) and the following equation was used for intra-run variation:

$$\text{Intra-run CV}[\%] = 100 \times \frac{(\text{s.d. of the measured conc. per run})}{(\text{mean measured conc. per run})} \quad (3)$$

To calculate the inter-run variation a one-way ANOVA was used.

**Table 2**  
Assay performance data for the analysis of vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin in human plasma and urine. A bold number of replicates indicates that there was an outlier in one run.

Matrix	Analyte	Nom. conc. (ng/mL)	n	Intra-run		Inter-run	
				Bias <sup>a</sup> (%)	Precision <sup>a</sup> (%)	Bias(%)	Precision(%)
Plasma	Vosaroxin	2.00	15	−4.1–0.0	6.0–8.8	−2.5	— <sup>b</sup>
		6.00	15	−4.0–1.2	2.4–5.8	−1.0	1.9
		150	15	2.5–5.5	1.3–4.9	3.9	0.2
		375	15	0.5–3.5	2.0–5.8	2.5	— <sup>b</sup>
	N-desmethyl vosaroxin	2.00	15	−6.6–10.4	6.0–8.4	3.9	8.3
		6.00	15	−3.4–4.4	4.5–6.5	−1.0	4.0
		150	15	6.1–8.9	2.0–2.4	7.2	1.0
		375	15	5.8–10.1	2.5–4.5	8.0	1.0
	O-desmethyl vosaroxin	4.00	15	−3.4–6.2	2.8–8.9	2.7	4.3
		12.0	15	2.8–6.0	2.9–5.3	4.3	— <sup>b</sup>
		150	15	0.0–10.0	2.9–5.0	3.3	5.3
		375	15	−2.2–8.9	5.6–7.8	4.4	4.6
Urine	Vosaroxin	2.00	15	1.7–9.0	7.2–11.2	4.5	— <sup>b</sup>
		6.00	15	1.3–2.9	3.3–6.3	2.3	— <sup>b</sup>
		150	15	−2.8–4.7	2.5–7.3	1.6	3.3
		375	15	0.2–4.3	2.2–5.7	1.9	1.3
	N-desmethyl vosaroxin	2.00	15	0.5–4.3	4.3–11.8	2.3	— <sup>b</sup>
		6.00	15	4.3–9.2	5.9–8.8	6.8	— <sup>b</sup>
		150	15	3.1–10.0	1.7–4.3	7.3	3.2
		375	15	5.3–10.8	0.7–3.6	7.5	2.4
	O-desmethyl vosaroxin	4.00	5	−9.4	6.8	NA	NA
		12.0	5	1.7	5.1	NA	NA
		150	5	−0.3	6.3	NA	NA
		375	5	−4.3	0.5	NA	NA

Nom.: nominal; conc.: concentration; n: number of replicates; NA: not applicable.

<sup>a</sup> The range of accuracies and precisions is listed if more than one run was performed.

<sup>b</sup> Inter-run precision could not be calculated (mean square between the groups is less than the mean square within the groups), meaning that there is no additional significant variation in performing the assay in different runs.

### 3. Results and discussion

#### 3.1. Development

##### 3.1.1. Chromatography

Initially a method was developed using a Synergi Hydro RP column (80 Å, 150 × 2.0 mm ID, particle size 4.6 µm; Phenomenex, Utrecht, The Netherlands) with the same eluents and a slightly adjusted gradient. However, during development and pre-validation high carryover was observed in both plasma and urine. Carryover was investigated by injecting two double blank samples after a ULOQ sample. In plasma, for vosaroxin up to 301% of the LLOQ value was detected in the first blank and up to 113% in the second blank. For N-desmethylvosaroxin up to 374% of the LLOQ value was detected in the first blank and up to 137% in the second blank. For O-desmethylvosaroxin no specific carryover peak was detected however, very high noise was observed in a double blank after an ULOQ level sample. This high carryover, or memory effect, indicated that the analytes have a very high affinity for the stationary phase that is used in the Synergi Hydro RP column. Therefore, a column with a lower affinity for the compounds was chosen (Symmetry Shield RP8 column). When using this column and with adjustment of the gradient the carryover decreased to approximately 20% of the LLOQ in the first blank as is described in the carryover validation results in Table 3. This result was considered adequate for proceeding with method validation. To achieve separation within a reasonable run time, a steep block gradient was developed. This gradient was chosen because the delay volume of the Agilent system is considerable, which causes a delay in the eluent composition and in the retention time of the analytes. In addition the divert valve was mainly directed to the mass spectrometer to maintain a consistent source spray.

The method that was developed for O-desmethylvosaroxin in urine using a Symmetry Shield RP8 column finally resulted in a failed ISR. The initial failed ISR was due to the combina-

**Table 3**

Carryover (maximum measured responses compared to the LLOQ) data observed during the plasma and urine method validation in the first blank and second blank after the ULOQ.

Analyte	Plasma carryover (%)		Urine carryover (%)	
	1st blank	2nd blank	1st blank	2nd blank
Vosaroxin	18.8	6.6	22.0	7.4
NDM-vosaroxin	21.3	16.4	22.0	10.9
ODM-vosaroxin	31.5	12.0	24.7	13.5

NDM-vosaroxin: N-desmethylvosaroxin; ODM-vosaroxin: O-desmethylvosaroxin.

tion of endogenous interferences, the use of an analogue internal standard for O-desmethylvosaroxin instead of a stable isotope and ion suppression effects of vosaroxin on the internal standard response, since the clinical samples contained very high concentrations of vosaroxin compared to O-desmethylvosaroxin. The study samples were only diluted to quantify vosaroxin and N-desmethylvosaroxin. Therefore the concentration of vosaroxin was especially high in the undiluted samples, to quantify O-desmethylvosaroxin, causing ion suppression of the internal standard. Therefore a separate O-desmethylvosaroxin in urine assay was developed and validated using a Synergi Hydro RP column since for O-desmethylvosaroxin no specific carry over was detected previously.

##### 3.1.2. Solubility and stability in neat solutions

Vosaroxin stock solutions were prepared in 0.1 N sodium hydroxide; N-desmethylvosaroxin and O-desmethylvosaroxin stock solutions were prepared in acetonitrile–5 mM sodium hydroxide (20:80, v/v). The analytes were not soluble in methanol and DMSO.

To prevent absorption of the analytes to the surface of the vials and to reduce the influence of light, stock solutions and working solutions are stored in silanized amber glass vials. When the stock



solutions are stored at  $-20^{\circ}\text{C}$  analytes were stable for at most 43 days (Table 4). However, the analytes in working solutions were not stable when stored at  $-20^{\circ}\text{C}$ . Low concentrations (40 ng/mL for vosaroxin and N-desmethylvosaroxin and 80 ng/mL for O-desmethylvosaroxin) of working solution in acetonitrile–5 mM sodium hydroxide (20:80, v/v) showed decreased concentrations over time (bias of >15% after 30 days). High concentration working solution (10,000 ng/mL) appeared to be stable at  $-20^{\circ}\text{C}$  (bias of <5%). This indicates that at low concentrations absorption of the analytes still plays a significant role although silanized glass was used. Therefore, working solutions were freshly prepared out of the stock solutions before use.

### 3.2. Validation procedures

#### 3.2.1. Calibration model

Calibration standards were prepared and analysed in duplicate in three analytical runs and in one analytical run for O-desmethylvosaroxin in urine. The quadratic regression of peak area versus the concentration  $1/x^2$  was weighted to obtain the lowest total bias across the range with the simplest model since non-linearity was observed. The observed non-linearity might be caused by observed adsorption of the analytes at low concentrations in the working solutions. For the calibration standards this was however not further investigated.

The calibration range of vosaroxin and N-desmethylvosaroxin in plasma and urine was 2–500 ng/mL and for O-desmethylvosaroxin the range in plasma and urine was 4–500 ng/mL. This range was chosen to cover the concentration range in the study samples in combination with extended range which was investigated in the dilution integrity experiments. A wider calibration range was not desirable, since non-linearity was already observed within this range.

Calibration curves were accepted if at least 75% of the non-zero calibration standards and 50% of each calibration level, including a LLOQ and an ULOQ, had a deviation within  $\pm 15\%$  of the nominal concentration ( $\pm 20\%$  at the LLOQ). All calibration curves of the analytes met these criteria and correlation coefficients ( $r^2$ ) of 0.998 or better were obtained.

#### 3.2.2. Accuracy and precision

To assess the accuracy and precision of the assays, five replicates of QC LLOQ, QC low, QC mid and QC high in plasma were analysed in three analytical runs.

Table 2 summarizes the intra- and inter-run accuracy and precision values of the assay. Assays met the acceptance criteria (within  $\pm 20\%$  and  $\leq 20\%$ , respectively, at the LLOQ level and within  $\pm 15\%$  and  $\leq 15\%$  at the other QC levels).

#### 3.2.3. Selectivity

Six different batches of control  $\text{K}_2\text{EDTA}$  plasma and urine were spiked at the LLOQ level with vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin to investigate the selectivity. The mean deviations from the nominal concentrations for the plasma assay were  $-1.1\%$ ,  $1.9\%$  and  $-1.3\%$  with CV values of  $8.2\%$ ,  $10.0\%$  and  $16.3\%$ , respectively. For the urine assay, the mean deviations for vosaroxin and N-desmethylvosaroxin were respectively  $-3.8\%$  and  $-2.8\%$  with CV values of  $6.3\%$  and  $5.0\%$  respectively. For the assay of O-desmethylvosaroxin in urine the mean deviation was  $-7.1\%$  and the CV  $7.2\%$ . Selectivity was therefore considered acceptable.

Cross-analyte and IS interference were tested by spiking control plasma or urine at ULOQ level with all analytes and with IS separately at the nominal concentration (one level). The cross-analyte and IS interference at the retention time of vosaroxin were less than 20% of the peak area of the LLOQ level. For the IS, interference

was less than the required 5%. No cross-analyte interference was detected in either matrix.

#### 3.2.4. Dilution integrity

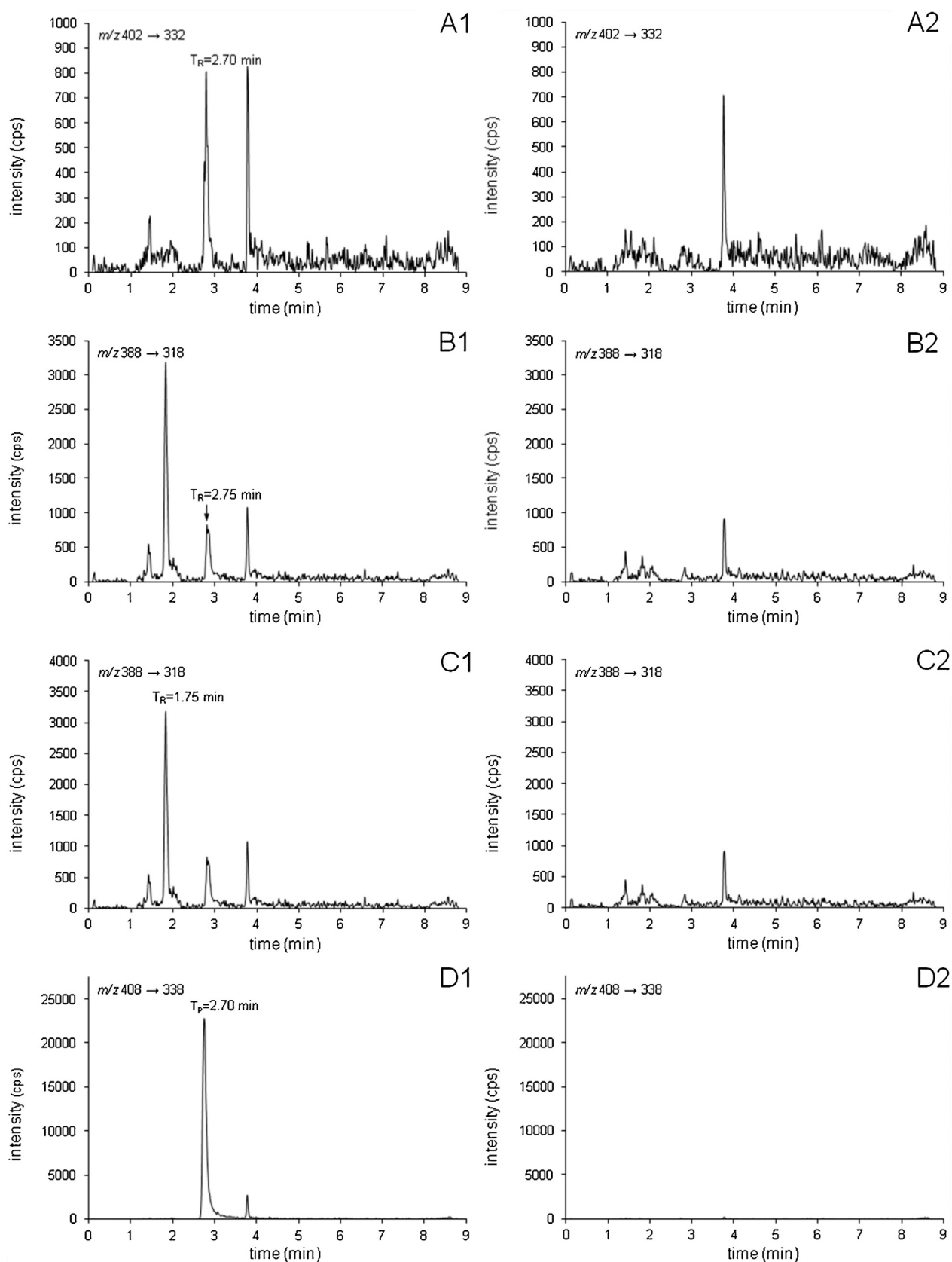
Five replicate plasma samples of vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin at QC > ULOQ level were diluted 25-fold with control human plasma (20  $\mu\text{L}$  of sample was added to 480  $\mu\text{L}$  control human matrix). This experiment was also performed for vosaroxin and N-desmethylvosaroxin in urine with the same concentrations as described for plasma. For the plasma and urine assay 50  $\mu\text{L}$  was processed. The mean bias for the three analytes was in plasma  $-7.3\%$ ,  $-3.5\%$  and  $-3.6\%$  and the precision  $4.3\%$ ,  $1.3\%$  and  $2.7\%$  respectively. The mean bias for the two analytes in urine were  $-9.7\%$  and  $-2.7\%$  and the precision  $3.2\%$  and  $1.6\%$ . The bias and precision were within  $\pm 15\%$  and  $\leq 15\%$  which indicates that the study samples can be diluted and maintain adequate accuracy and precision values.

#### 3.2.5. Lower limit of quantitation

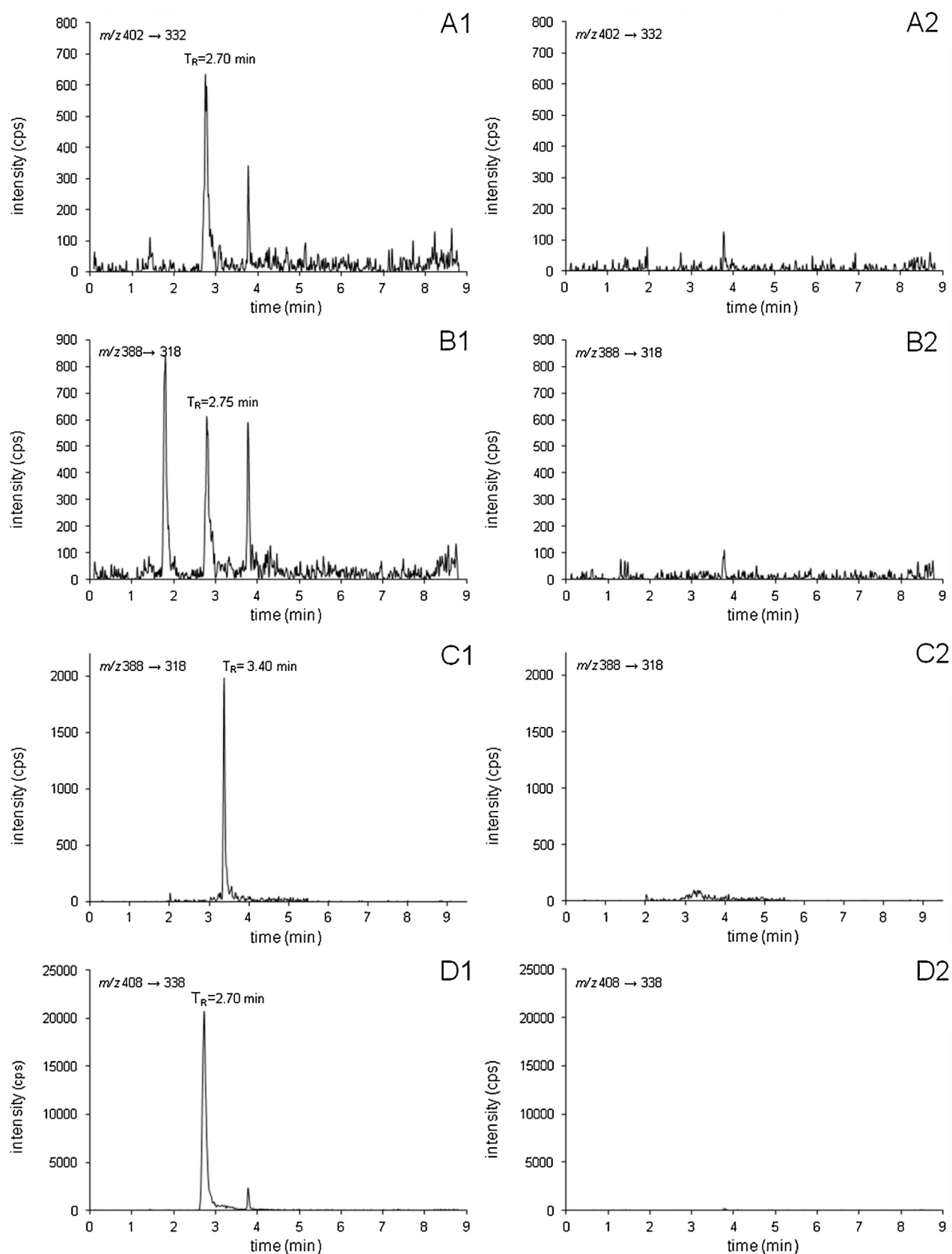
The analytes responses at the LLOQ were at least 5 times the response compared to a blank response in three validation runs for both assays. During the validation the lowest signal to noise for vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin were 7.5, 8.0 and 11.5 in plasma, respectively. In urine the signal to noise was at least 7.8, 13.1 and 19.0 for vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin, respectively. Fig. 2 shows representative chromatograms of vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin in QC LLOQ samples and double blank samples in plasma and Fig. 3 in urine.

#### 3.2.6. Carryover

Carryover was investigated in all analytical runs and the highest observed values are displayed in Table 3. In plasma, up to 18.8% of the LLOQ for vosaroxin was detected in the first blank and up to 6.6% in the second blank. For N-desmethylvosaroxin up to 21.3% (of the LLOQ) was found in the first blank and up to 16.4% in the second blank. For O-desmethylvosaroxin up to 31.5% (of the LLOQ) for O-desmethylvosaroxin was found in the first blank and up to 12.0% in the second blank. During the urine validation peaks with areas >20% of the LLOQ were also found in the blanks injected after the ULOQ. For vosaroxin up to 22.0% (of the LLOQ) was detected in the first blank and up to 7.4% in the second blank. Up to 22.0% (of the LLOQ) for N-desmethylvosaroxin in the first blank and up to 10.9% in the second blank. For O-desmethylvosaroxin up to 24.7% in the first blank and up to 13.5% in the second blank. During the application of the validated method to study sample analysis, an extra blank should be injected after samples with an expected high concentration before the analysis of the next study sample (e.g. ULOQ samples and QC high samples). During the validation a carryover evaluation was conducted to determine if carryover affects any of the outcomes of the validation results. The evaluation demonstrated that carryover did not have an impact on the results. Although carryover was already minimized by using the Symmetry Shield RP8 column there was still carryover observed during the validation. The observed carryover is most likely due to the affinity of the column for the analytes, which was also observed during the method development. Vosaroxin and its metabolites seem to have the tendency to strongly bind to the material of the column, which might be due to the functional groups of the molecule (Fig. 1). This type of column carryover, also referred to as memory effect, can be reduced by the selection of a different column (end-capped column), the selection of different mobile phase pH and the adjustment of the gradient.



**Fig. 2.** MRM chromatograms of vosaroxin (A1), N-desmethylvosaroxin (B1), O-desmethylvosaroxin (C1) and  $^2H_6$ -vosaroxin (D1) in plasma at LLOQ level (2 ng/mL for vosaroxin and N-desmethylvosaroxin, 4 ng/mL for O-desmethylvosaroxin and 200 ng/mL for  $^2H_6$ -vosaroxin) and chromatograms in a blank sample of vosaroxin (A2), N-desmethylvosaroxin (B2), O-desmethylvosaroxin (C2) and  $^2H_6$ -vosaroxin (D2).



**Fig. 3.** MRM chromatograms of vosaroxin (A1), N-desmethylvosaroxin (B1), O-desmethylvosaroxin (C1) and  $^2\text{H}_6$ -vosaroxin (D1) in urine at LLOQ level (2 ng/mL for vosaroxin and N-desmethylvosaroxin, 4 ng/mL for O-desmethylvosaroxin and 200 ng/mL for  $^2\text{H}_6$ -vosaroxin) and chromatograms in a blank sample of vosaroxin (A2), N-desmethylvosaroxin (B2), O-desmethylvosaroxin (C2) and  $^2\text{H}_6$ -vosaroxin (D2).

**Table 4**  
Stability data for vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin in plasma and urine. All experiments in the biomatrix were performed in triplicate in QC low and QC high samples.

Conditions	Matrix	Analyte	Nominal conc. (ng/mL)	Measured conc. (ng/mL)	Bias (%)	C.V. (%)	n
Stock solutions							
Ambient, 20 h	0.1N NaOH	Vosaroxin	1.00E+05	1.01E+05	1.3	4.9	3
	ACN:5 mM NaOH <sup>a</sup>		1.00E+05	9.57E+04	−4.3	2.1	3
	ACN:5 mM NaOH <sup>a</sup>	ODM-vosaroxin	1.00E+05	9.79E+04	−2.1	2.5	3
−20 °C, 51 d	0.1N NaOH	Vosaroxin	1.00E+05	9.99E+04	−0.1	1.1	3
−20 °C, 43 d	ACN:5 mM NaOH <sup>a</sup>	NDM-vosaroxin	1.00E+05	9.96E+04	0.5	2.5	3
−20 °C, 113 d	ACN:5 mM NaOH <sup>a</sup>	ODM-vosaroxin	1.00E+05	1.02E+05	2.2	0.4	3
Working solutions							
−20 °C, 7 d	ACN:5 mM NaOH <sup>a</sup>	Vosaroxin	40.0	38.1	−4.7	4.0	3
			10,000	10,241	2.4	2.8	3
		NDM-vosaroxin	40.0	31.9	−20.2	2.6	3
			10,000	7886	−21.1	1.7	3
		ODM-vosaroxin	80.0	83.8	4.7	6.6	3
			10,000	9552	−4.5	1.6	3
Plasma							
5 freeze-thaw cycles (−70 °C/2–8 °C)	Biomatrix	Vosaroxin	6.00	6.72	11.9	1.9	3
			375	399	6.5	4.2	3
		NDM-vosaroxin	6.00	6.44	7.4	9.4	3
			375	400	6.6	3.9	3
		ODM-vosaroxin	12.0	11.9	−0.6	9.9	3
			375	341	−9.0	6.2	3
Ambient, 24 h	Biomatrix	Vosaroxin	6.00	5.41	−9.9	6.1	3
			375	376	0.3	4.3	3
		NDM-vosaroxin	6.00	6.08	1.4	9.0	3
			375	388	3.5	3.0	3
		ODM-vosaroxin	12.0	12.3	2.5	5.6	3
			375	373	−0.4	4.8	3
2–8 °C, 34 days	Final extract	Vosaroxin	6.00	6.02	0.3	4.2	3
			375	363	−3.3	5.1	3
		NDM-vosaroxin	6.00	5.7	−5.1	3.4	3
			375	351	−6.3	5.3	3
		ODM-vosaroxin	12.00	12.6	4.7	5.6	3
			375	351	−6.5	5.5	3
Whole blood							
Ambient, 1 h	Biomatrix	Vosaroxin	2.25	2.45	9.1	11.0	3
		NDM-vosaroxin	2.15	2.11	−1.7	8.3	3
		ODM-vosaroxin	3.84	3.84	−0.2	1.8	3
Ice-water, 1 h	Biomatrix	Vosaroxin	2.25	3.02	−10.0	4.2	3
		NDM-vosaroxin	2.15	1.83	−14.9	2.3	3
		ODM-vosaroxin	3.84	3.97	3.4	2.3	3
Urine							
5 freeze-thaw cycles (−70 °C/2–8 °C)	Biomatrix	Vosaroxin	6.00	6.72	11.9	1.9	3
			375	399	6.5	4.2	3
		NDM-vosaroxin	6.00	6.14	2.3	6.9	3
			375	383	2.1	3.1	3
		ODM-vosaroxin	12.0	12.2	1.9	10.0	3
			375	370	−1.2	1.5	3
Ambient, 24 h	Biomatrix	Vosaroxin	6.00	6.12	2.1	6.2	3
			375	340	−9.3	1.1	3
		NDM-vosaroxin	6.00	5.41	−9.8	6.4	3
			375	359	−4.4	4.5	3
		ODM-vosaroxin	12.0	13.0	8.3	4.7	3
			375	425	13.2	4.4	3
2–8 °C, 22 days	Final extract	Vosaroxin	6.00	5.28	−11.9	6.5	3
			375	327	−12.8	3.2	3
		NDM-vosaroxin	6.00	5.52	−8.1	2.1	3
			375	346	−7.6	4.6	3
		ODM-vosaroxin	12.0	12.5	3.9	5.3	3
			375	415	10.6	4.3	3

NDM-vosaroxin: N-desmethylvosaroxin; ODM-vosaroxin: O-desmethylvosaroxin; Conc.: concentration; C.V.: Coefficient of Variation; n: number of replicates; **Bold**: not accepted;

<sup>a</sup> ACN:5 mM NaOH (2:8, v/v).

### 3.2.7. Matrix factor

The matrix factor (MF) was determined in six plasma batches and six urine batches (12 urine batches for O-desmethylvosaroxin), at low and high concentration levels of vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin in singlicate. Processed blank samples were spiked with working solu-

tions and compared to matrix free working solutions using the following equation:

$$MF = \frac{\text{Area of blank sample spiked with neat solution (matrix present)}}{\text{Area of neat solution (matrix absent)}} \quad (4)$$

In addition to the MF the internal standard-normalised MF was calculated by dividing the MF of the analyte through the MF



of the internal standard. The coefficients of variation (CV) of the standard-normalised MF calculated from the six batches plasma for the low and high concentrations were less than 11.5% and 12.9%, respectively, fulfilling the acceptance criteria. The matrix factor (low and high concentration levels combined) in plasma for vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin ranged from 0.926 to 1.00, 1.16–1.27 and 1.78–2.88, respectively. The maximum coefficient of variation (CV) of the internal standard-normalised MF calculated from the six urine batches for the low and high concentration was 11.3% and 9.8%, respectively, and also fulfilled the criteria. The matrix factor in urine (low and high concentration levels combined) for vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin ranged from 0.861 to 1.02, 0.991 to 1.36 and 1.10 to 1.80, respectively.

An MF of around 1 indicates that the stable isotope as IS is most effective minimizing the influence of matrix effects. The used IS minimizes the matrix effects for vosaroxin and N-desmethylvosaroxin, but less effective for O-desmethylvosaroxin. However, for all analytes the CV of the IS-normalised MF was less than the required 15% indicating that the MF was constant over the tested six batches and two matrices.

### 3.3. Stability

The results of the stability experiments are displayed in Table 4. Vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin are stable in plasma and urine for at least 665 days and 236 days at  $-70^{\circ}\text{C}$ . Immediately after sample collection the samples were stored at  $-80^{\circ}\text{C}$  on the clinical site for a maximum period of three months, since there was no  $-70^{\circ}\text{C}$  freezer available in the clinic, after which the samples were transferred to a  $-70^{\circ}\text{C}$  freezer. Since the study samples were stored at  $-80^{\circ}\text{C}$  for a specified period and since the temperature of  $-80^{\circ}\text{C}$  is lower than the  $-70^{\circ}\text{C}$  at which the stability was investigated, it was concluded that the data integrity was not compromised.

In whole blood vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin are stable for at least 1 h at room temperature and in ice-water. Therefore, no special precautions need to be taken during sample collection. Reinjection reproducibility experiments demonstrated that runs with plasma and urine can be reinjected after respectively 37 and 34 h when samples are kept at  $4^{\circ}\text{C}$  in case of an instrumental failure.

## 4. Clinical application

These assays were developed to support clinical pharmacology studies of vosaroxin. To demonstrate their applicability, concentration profiles over time of vosaroxin and N-desmethylvosaroxin in plasma of a representative patient treated with vosaroxin is presented. Blood and urine samples were collected as described previously and analysed with the described validated methods.

Fig. 4 displays the plasma concentration-time curves for vosaroxin and N-desmethylvosaroxin in plasma of this patient. In plasma, O-desmethylvosaroxin could not be quantified because possible levels were below the LLOQ.

ISR was performed on 5% of the clinical plasma and urine samples. In these samples the vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin concentrations were determined and compared with the original accepted values. At least 2/3 (66.7%) of the ISR samples needed to be within  $\pm 20\%$  difference to pass. The following equation was used to calculate the ISR:

$$\% \text{Difference} = \frac{(\text{ISR} - \text{original})}{\text{mean of ISR and original}} \times 100\% \quad (5)$$

ISR herein is the concentration obtained after re-assay of the sample and original is the original accepted concentration after the

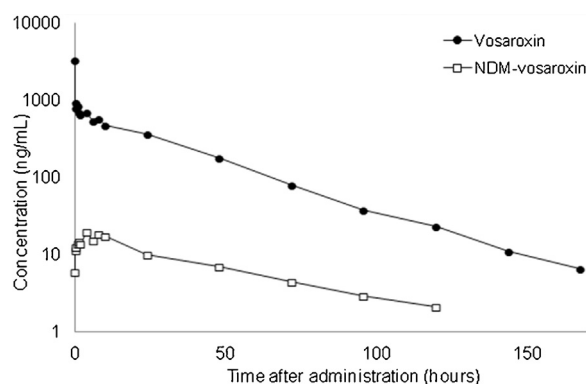


Fig. 4. Representative plasma concentration-time curves of vosaroxin and its metabolite N-desmethylvosaroxin following a single  $60 \text{ mg/m}^2$  intravenous injection of vosaroxin administered to a patient with advanced solid tumor cancer. O-desmethylvosaroxin was not detected in the plasma samples.

first analysis. All the ISR results were accepted and within  $\pm 20\%$  difference for vosaroxin and N-desmethylvosaroxin in plasma and urine. O-desmethylvosaroxin concentrations in plasma samples were all below the LLOQ value. For O-desmethylvosaroxin in urine 70% of the reanalysed samples were within  $\pm 20\%$  difference and the other samples deviated between 26.6% and 55.3%, which is acceptable according to the used guidelines. When the chromatographic peaks of O-desmethylvosaroxin in spiked samples were compared to the peak of study samples, it became clear that in the study samples another compound co-eluted with O-desmethylvosaroxin. This peak was not detected in the blank study samples. An explanation for this observation might be the formation of an O-desmethylvosaroxin related metabolite. Since O-desmethylvosaroxin is a minor metabolite, the observed deviations during ISR do not have clinical implications.

## 5. Conclusion

Assays for the quantification of vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin in human plasma and urine were validated. The validated range vosaroxin and N-desmethylvosaroxin in plasma and urine is from 2 to  $500 \text{ ng/mL}$  and for O-desmethylvosaroxin in plasma and urine from 4 to  $500 \text{ ng/mL}$ . It is possible to dilute study samples containing higher concentrations 25-fold with control human plasma prior to analysis. With this additional dilution step the range expands to  $15,000 \text{ ng/mL}$  for all analytes in both matrices. These assays were used in a human mass balance study of vosaroxin.

## Acknowledgment

This work was financially supported by Sunesis Pharmaceuticals.

## References

- [1] R.E. Hawtin, D.E. Stockett, J.A. Byl, R.S. McDowell, T. Nguyen, M.R. Arkin, A. Conroy, W. Yang, N. Osheroff, J.A. Fox, Voreloxin is an anticancer quinolone derivative that intercalates DNA and poisons topoisomerase II, *PLoS One* 5 (2010) e10186.
- [2] F. Ravandi, E.K. Ritchie, H. Sayar, J.E. Lancet, M.D. Craig, N. Vey, S.A. Strickland, G.J. Schiller, E. Jabbour, H.P. Erba, A. Pigneux, H.A. Horst, C. Recher, V.M. Klimek, J. Cortes, G.J. Roboz, O. Odenike, X. Thomas, V. Havelange, J. Maertens, H.G. Derigs, M. Heuser, L. Damon, B.L. Powell, G. Gaidano, A.M. Carella, A. Wei, D. Hogge, A.R. Craig, J.A. Fox, R. Ward, J.A. Smith, G. Acton, C. Mehta, R.K. Stuart, H.M. Kantarjian, Vosaroxin plus cytarabine versus placebo plus cytarabine in patients with first relapsed or refractory acute myeloid leukaemia (VALOR): a randomised, controlled, double-blind, multinational, phase 3 study, *Lancet Oncol.* 16 (2015) 1025–1036.

- [3] M.J. Evanchik, D. Allen, J.C. Yoburn, J.A. Silverman, U. Hoch, Metabolism of (+)-1,4-dihydro-7-(trans-3-methoxy-4-methylamino-1-pyrrolidinyl)-4-oxo-1-(2-thiazolyl)-1,8-naphthyridine-3-carboxylic acid (voreloxin; formerly SNS-595), a novel replication-dependent DNA-damaging agent, *Drug Metab. Dispos. Biol. Fate Chem.* 37 (2009) 594–601.
- [4] R.H. Advani, H.I. Hurwitz, M.S. Gordon, S.W. Ebbinghaus, D.S. Mendelson, H.A. Wakelee, U. Hoch, J.A. Silverman, N.A. Havrilla, C.J. Berman, J.A. Fox, R.S. Allen, Adelman D.C. Voreloxin, a first-in-class anticancer quinolone derivative, in relapsed/refractory solid tumors: a report on two dosing schedules, *Clin. Cancer Res.* 16 (2010) 2167–2175.
- [5] J.E. Lancet, F. Ravandi, R.M. Ricklis, L.D. Cripe, H.M. Kantarjian, F.J. Giles, A.F. List, T. Chen, R.S. Allen, J.A. Fox, G.C. Michelson, J.E. Karp, A phase Ib study of vosaroxin, an anticancer quinolone derivative, in patients with relapsed or refractory acute leukemia, *Leukemia* 25 (2011) 1808–1814.
- [6] J.E. Lancet, G.J. Roboz, L.D. Cripe, G.C. Michelson, J.A. Fox, R.D. Leavitt, T. Chen, R. Hawtin, A.R. Craig, F. Ravandi, M.B. Maris, R.K. Stuart, J.E. Karp, A phase 1b/2 study of vosaroxin in combination with cytarabine in patients with relapsed or refractory acute myeloid leukemia, *Haematologica* 100 (2015) 231–237.
- [7] R.K. Stuart, L.D. Cripe, M.B. Maris, M.A. Cooper, R.M. Stone, S.R. Dakhil, F. Turturro, W. Stock, J. Mason, P.J. Shami, S.A. Strickland, L.J. Costa, G. Borthakur, G.C. Michelson, J.A. Fox, R.D. Leavitt, F. Ravandi, REVEAL-1, a phase 2 dose regimen optimization study of vosaroxin in older poor-risk patients with previously untreated acute myeloid leukaemia, *Br. J. Haematol.* 168 (2015) 796–805.
- [8] Organisation for Economic Co-operation and Development OECD Principles on Good Laboratory Practice, Chemicals Group and Management Committee and Organisation for Economic Co-operation and Development, (1998) [http://search.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/mc/chem\(98\)17&doclanguage=en](http://search.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/mc/chem(98)17&doclanguage=en).
- [9] US Food and Drug Administration FDA Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research, (2001) <http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf>, (accessed 02.12.15).
- [10] European Medicines Agency Guide to bioanalytical method validation, Committee for Medicinal Products for Human Use and European Medicines Agency, (2011) [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/Scientific\\_guideline/2011/08/WC500109686.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf), (accessed 01.12.15).