



# Validation of high-performance liquid chromatography–tandem mass spectrometry assays quantifying omacetaxine mepesuccinate and its 4'-des-methyl and cephalotaxine metabolites in human plasma and urine



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## ABSTRACT

Omacetaxine mepesuccinate (hereafter called omacetaxine) is a modified cephalotaxine and is registered (Synribo®) for the treatment of adult patients with chronic myeloid leukemia (CML) with resistance and/or intolerance to two or more tyrosine kinase inhibitors (TKIs). To evaluate the pharmacokinetics of omacetaxine, sensitive high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) assays for the quantification of omacetaxine and its inactive 4'-des-methyl (4'-DMHHT) and cephalotaxine metabolites in human plasma and urine were developed and validated.

Since omacetaxine is mainly metabolised by esterases, the plasma samples were immediately stabilised after collection with an esterase inhibitor and stored at a nominal temperature of  $-80^{\circ}\text{C}$ . Urine samples were stored at  $-80^{\circ}\text{C}$  immediately after collection. Protein precipitation was applied as the sample pretreatment method for the plasma samples, and urine samples were processed using solid-phase extraction (SPE). For both assays, the dried and reconstituted extracts were injected on a XBridge BEH Phenyl column for analysis of all analytes. Gradient elution was applied with 0.1% formic acid in water and methanol as mobile phases. Analytes were ionised using a turbospray ionisation source in positive mode and detected with a triple quadrupole mass spectrometer.

The validated plasma assay quantifies all analytes in the concentration range of 0.1–100 ng/mL and the urine assay in the range of 0.1–50 ng/mL. At all concentrations, the accuracies were within  $\pm 15\%$  of the nominal concentrations and precisions were  $\leq 15\%$ . The developed methods have successfully been applied in a human mass balance study of omacetaxine.

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## 1. Introduction

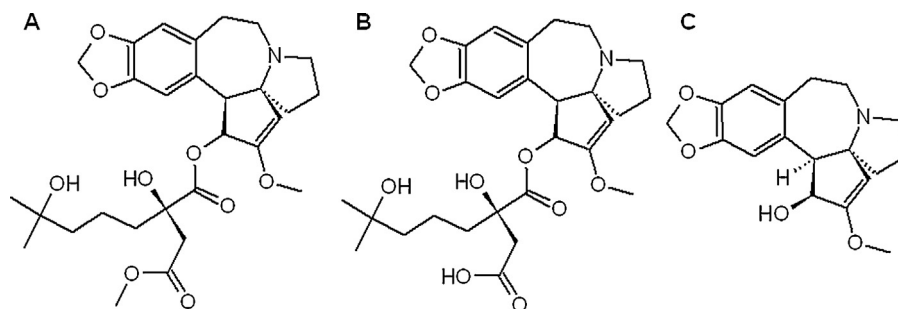
Omacetaxine mepesuccinate (hereafter called omacetaxine) is a modified cephalotaxine and is registered (Synribo®) for the treatment of adult patients with chronic myeloid leukemia (CML) with resistance and/or intolerance to two or more tyrosine kinase

inhibitors (TKIs). Omacetaxine is a semi-synthetic product from the leaves of the *Cephalotaxus fortunei*, which is identical to the natural product homoharringtonine [1]. Homoharringtonine was first extracted from the bark of this tree, and the alcoholic extract showed effects on various types of acute leukemia by inhibition of protein synthesis. Subsequently a semi-synthetic process was developed to produce large quantities of highly purified omacetaxine [2,3]. Omacetaxine is mainly metabolised by esterases into its inactive 4'-des-methyl(4'-DMHHT) and cephalotaxine metabolites.

To support clinical pharmacological studies an assay for quantification of omacetaxine and its metabolites is needed. Since

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**Fig. 1.** Chemical structures of (A) omacetaxine, (B) 4'-DMHHT and (C) cephalotaxine.

**Table 1**

Mass spectrometric parameters for the analysis of omacetaxine, 4'-DMHHT and cephalotaxine with the internal standards  $^2\text{H}_5$ -omacetaxine,  $^2\text{H}_5$ -4'-DMHHT and  $^2\text{H}_5$ -cephalotaxine.

General settings		QTrap 5500				
Run duration		5.6 min				
Ion spray voltage		+4.0 kV				
Turbo gas temperature		500 °C				
Nebuliser gas		40 psi				
Heater gas		40 psi				
Curtain gas		40 psi				
Collision gas		9 psi				
Analyte specific parameters	Omacetaxine	Omacetaxine- $^2\text{H}_5$	4'-DMHHT	4'-DMHHT- $^2\text{H}_5$	Cephalotaxine	Cephalotaxine- $^2\text{H}_5$
Parent mass	546.0 <i>m/z</i>	551.0 <i>m/z</i>	532.0 <i>m/z</i>	537.0 <i>m/z</i>	316.0 <i>m/z</i>	321.0 <i>m/z</i>
Product mass	298.0 <i>m/z</i>	303.0 <i>m/z</i>	298.0 <i>m/z</i>	303.0 <i>m/z</i>	229.0 <i>m/z</i>	286.0 <i>m/z</i>
Dwell time	50 ms	50 ms	50 ms	50 ms	50 ms	50 ms
Collision energy	45 V	45 V	45 V	45 V	41 V	41 V
Collision exit potential	10 V	10 V	10 V	10 V	24 V	24 V
Declustering potential	141 V	141 V	186 V	186 V	121 V	121 V
Entrance potential	10 V	10 V	10 V	10 V	10 V	10 V
Typical retention time	2.2 min	2.2 min	1.8 min	1.8 min	0.8 min	0.8 min

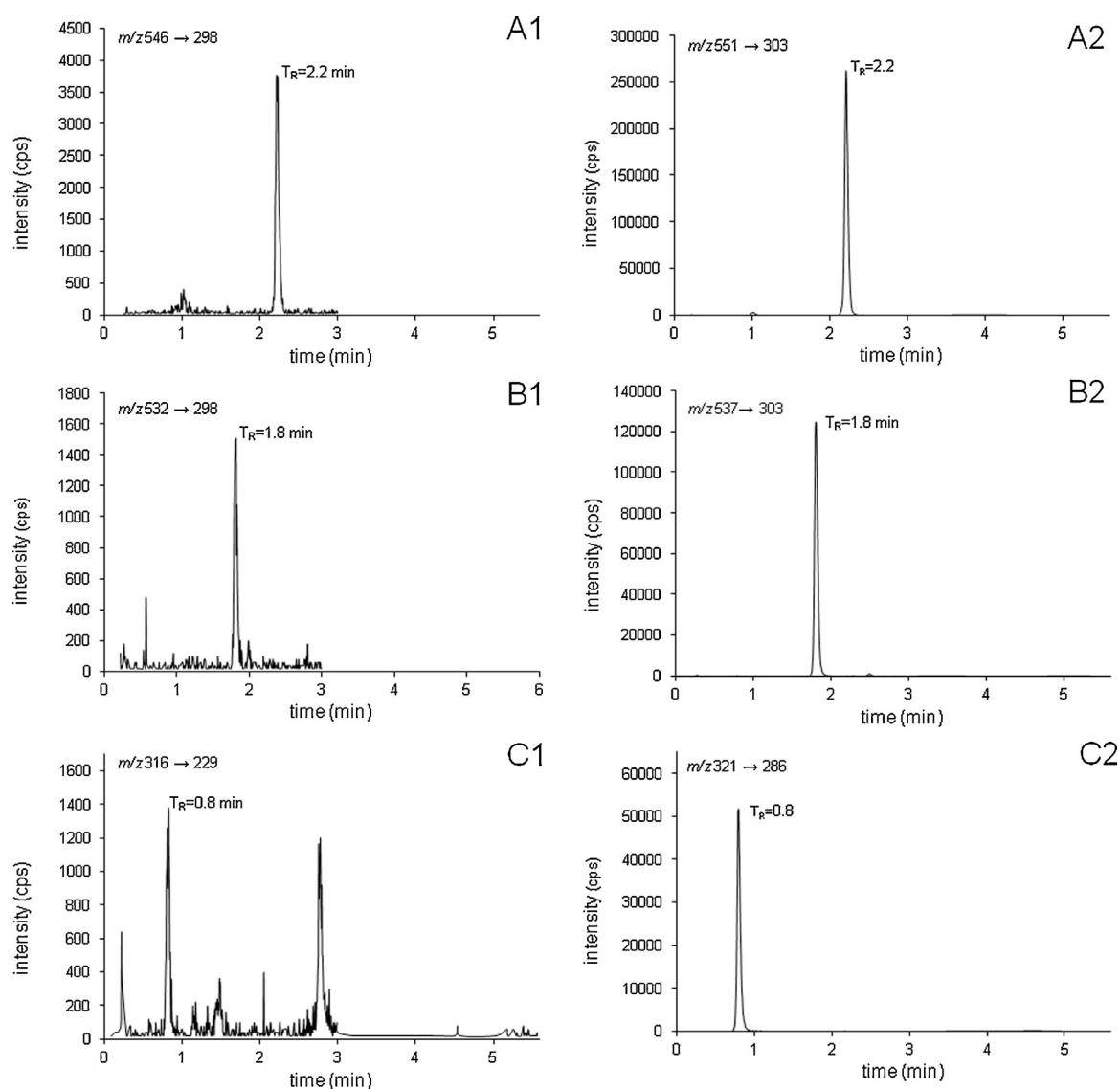
**Table 2**

Assay performance data for the analysis of omacetaxine, 4'-DMHHT and cephalotaxine in human plasma and urine. A bold number of replicates indicates that there was an outlier in one or more runs.

Matrix	Analyte	Nom. conc. (ng/mL)	<i>n</i>	Intra-assay		Inter-assay	
				Bias <sup>a</sup> (%)	CV <sup>a</sup> (%)	Bias (%)	CV (%)
Plasma	Omacetaxine	0.102	15	−1.1–6.9	1.5–5.1	5.7	6.1
		0.305	15	5.7–7.0	0.9–2.9	6.2	1.9
		45.7	15	4.6–6.0	1.1–1.7	5.4	1.5
		76.2	15	4.7–5.3	0.9–1.2	5.1	1.0
	4'-DMHHT	0.0987	15	4.5–10.8	4.4–7.6	6.8	6.3
		0.296	15	6.6–7.6	2.1–2.5	7.0	2.2
		44.4	15	5.1–7.7	0.9–1.3	6.7	1.5
		74.0	15	6.4–8.3	1.7–1.7	7.5	1.8
	Cephalotaxine	0.993	<b>14</b>	−9.8–12.4	4.7–7.9	5.0	11.0
		0.298	15	3.6–8.3	4.3–5.1	6.6	4.8
		44.7	15	3.4–6.3	1.2–1.5	5.1	1.7
		74.5	15	3.7–7.2	1.7–2.3	5.7	2.3
Urine	Omacetaxine	0.102	<b>14</b>	0.8–4.8	1.2–3.2	−0.7	4.0
		0.307	15	−0.1–0.1	1.6–1.9	0.0	1.6
		20.5	15	−0.6–0.6	0.8–2.0	0.0	1.4
		38.4	15	−2.4–0.0	0.4–2.3	−1.1	1.9
	4'-DMHHT	0.103	<b>14</b>	−10.3–0.6	1.9–3.8	−4.0	5.1
		0.308	15	−1.9–1.0	2.0–3.0	−0.8	2.6
		20.5	15	−1.1–1.7	0.5–1.1	0.2	1.4
		38.5	15	1.4–3.5	0.7–1.5	2.1	1.6
	Cephalotaxine	0.104	<b>13</b>	−0.7–3.6	2.7–8.5	1.8	5.7
		0.311	15	−1.5–5.2	0.5–1.9	0.7	3.5
		20.7	15	−1.4–0.7	0.9–2.3	−0.5	1.7
		38.9	15	0.2–2.0	0.9–1.3	1.2	1.3

Nom.: nominal; conc.: concentration; *n*: number of replicates; CV: coefficient of variation. Bold: statistical outlier.

<sup>a</sup> The range of accuracy and precision during the 3 validation runs is presented.



**Fig. 2.** MRM chromatograms of omacetaxine (A1), 4'-DMHHT (B1) and cephalotaxine (C1) in extracted plasma at LLOQ level (0.100 ng/mL) and the corresponding internal standard signals of  $^2H_5$ -omacetaxine (A2),  $^2H_5$ -4'-DMHHT (B2) and  $^2H_5$ -cephalotaxine (C2) at 20 ng/mL.

**Table 3**  
Effect of hemolysis on the quantification of omacetaxine, 4'-DMHHT and cephalotaxine in human plasma. All experiments were performed in triplicate at QC low and QC high concentration levels.

Analyte	Nominal conc.(ng/mL)	Measured conc.(ng/mL)	Bias(%)	C.V.(%)	n
Omacetaxine	0.305	0.320	4.8	1.8	3
	76.2	78.7	3.3	0.1	3
4'-DMHHT	0.296	0.303	2.3	1.0	3
	74.0	78.5	6.1	1.7	3
Cephalotaxine	0.298	0.322	8.2	4.2	3
	74.5	75.6	1.4	2.3	3

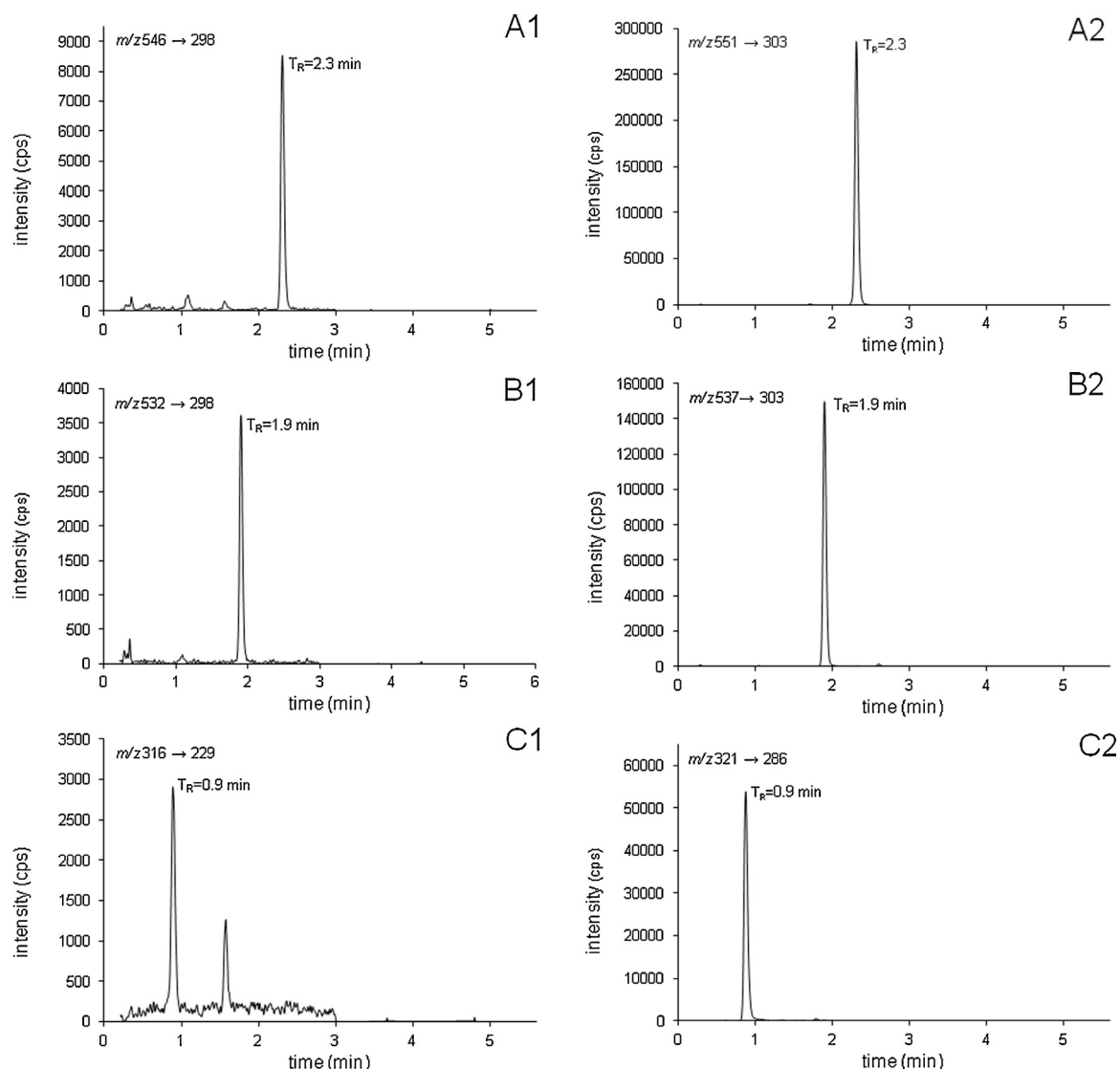
the activity of *Cephalotaxus* alkaloid extracts such as homoharringtonine and cephalotaxine have been under investigation for approximately 40 years, several assay procedures have already been described using different detection methods [4–7]. Only one method describes the quantification of 4'-DMHHT, but without bio-analytical details [8]. However, methods for quantitation of the parent drug and the two circulating metabolites in human plasma and urine were needed for use in support of a human mass balance study. Full validation of the developed assays was performed in compliance with the OECD principles of good laboratory practice (GLP) [9] and according to the FDA and latest EMA guidelines on bio-

analytical method validation [10,11]. Additionally, the applicability of the assay in clinical sample analysis was demonstrated.

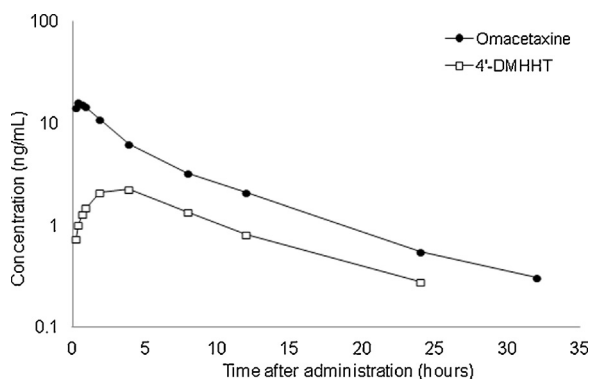
## 2. Experimental

### 2.1. Chemicals

Omacetaxine, 4'-DMHHT and cephalotaxine (Fig. 1) were manufactured by Novasep (Pompey, France) and provided by Teva Pharmaceuticals (North Wales, PA, USA). Deuterated stable isotope-labeled ( $^2H_5$ ) analogues of all compounds were used



**Fig. 3.** MRM chromatograms of omacetaxine (A1), 4'-DMHHT (B1) and cephalotaxine (C1) in urine at LLOQ level (0.100 ng/mL) and the corresponding internal standard signals of  $^2H_5$ -omacetaxine (A2),  $^2H_5$ -4'-DMHHT (B2) and  $^2H_5$ -cephalotaxine (C2) at 20 ng/mL.



**Fig. 4.** Representative plasma concentration-time curves of omacetaxine and its metabolite 4'-DMHHT following a single 1.25 mg/m<sup>2</sup> subcutaneous injection of omacetaxine administered to a patient suffering from cancer. Cephalotaxine was not detected in the plasma samples.

as internal standards, and these were manufactured by Chemtos (Austin, TX, USA) and provided by Teva Pharmaceuticals. Sigma-Aldrich (St. Louis, MO, USA) supplied paraoxon which

served as esterase inhibitor to stabilise the analytes in plasma. Methanol (UPLC grade and Supra-Gradient grade), water (UPLC grade), formic acid (UPLC grade), acetonitrile (UPLC grade) and isopropyl alcohol (UPLC grade) were obtained from Biosolve Ltd. (Valkenswaard, The Netherlands). Dimethyl sulfoxide (DMSO), potassium dihydrogen phosphate and ammonia solution (25%) were purchased from Merck (Darmstadt, Germany). Water (distilled) used for sample preparation originated from B. Braun Medical (Melsungen, Germany). K<sub>2</sub>EDTA plasma was obtained from the Slotervaart Hospital (Amsterdam, The Netherlands) and stabilised with 5% of a 0.4% ethanolic paraoxon solution. Drug-free control human urine was obtained from healthy volunteers.

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

### 2.2.1. Stock solutions

Separate 1-mg/mL stock solutions for calibration standards and quality control samples (QC samples) were prepared in DMSO for each analyte: omacetaxine, 4'-DMHHT and cephalotaxine. These stock solutions, stored at  $-20^{\circ}C$ , were further diluted with

**Table 4**  
Stability data for omacetaxine, 4'-DMHHT and cephalotaxine in stock/working solutions, plasma and urine. All experiments in the biomatrices were performed in triplicate at low and high concentrations.

Conditions	Matrix	Analyte	Nominal conc. (ng/mL)	Measured conc. (ng/mL)	Bias (%)	C.V. (%)	n		
Stock solutions									
Ambient, 4 hours	DMSO	Omacetaxine	1.00E+06	1.04E+06	3.6	4.6	3		
		4'-DMHHT	1.00E+06	1.01E+06	1.3	3.1	3		
−20 °C, 2 months	DMSO	Cephalotaxine	1.00E+06	9.62E+05	−3.8	2.2	3		
		Omacetaxine	1.00E+06	9.98E+05	−0.2	0.8	3		
		4'-DMHHT	1.00E+06	9.92E+05	−0.8	6.7	3		
		Cephalotaxine	1.00E+06	1.02E+06	1.7	7.8	3		
Working solutions									
Ambient, 16 hours	50% MeOH	Omacetaxine	2.02	2.03	0.3	0.9	3		
	MeOH		10100	10118	0.2	1	3		
4'-DMHHT		1.99	2.03	2.1	0.5	3			
		9940	10043	1	1.5	3			
Cephalotaxine		2.13	2.07	−3	0.8	3			
−20 °C, 2 months	50% MeOH		10600	10684	0.8	2.3	3		
		Omacetaxine	2.02	2.1	3.8	1.5	3		
			10100	10298	2	2.5	3		
		4'-DMHHT	1.99	1.99	0	3.3	3		
			9940	9789	−1.5	1.8	3		
		Cephalotaxine	2.13	2.18	2.5	1.4	3		
			10600	10544	−0.5	0.8	3		
Plasma 5 freeze-thaw cycles (−70 °C/ambient)	Biomatrix	Omacetaxine	0.305	0.317	3.8	0.7	3		
			76.2	79.3	4	0.5	3		
		4'-DMHHT	0.296	0.301	1.7	3.5	3		
			74	77.7	5	1.7	3		
		Cephalotaxine	0.298	0.297	−0.3	3.8	3		
			74.5	76.8	3.1	0.4	3		
		Ambient, 24 hours	Biomatrix	Omacetaxine	0.305	0.311	1.9	1.9	3
			76.2	77.9	2.2	1.8	3		
2−8 °C, 9 days	Final extract	4'-DMHHT	0.296	0.329	11.1	3.6	3		
			74	81.5	10.1	1	3		
		Cephalotaxine	0.298	0.31	3.9	3.5	3		
			74.5	81.1	8.9	1.9	3		
		Omacetaxine	0.305	0.34	11.4	0.9	3		
			76.2	82.5	8.3	0.6	3		
		4'-DMHHT	0.296	0.333	12.4	1.7	3		
			74	80.1	8.3	2.4	3		
Whole blood Ambient, 1 hours	Biomatrix	Cephalotaxine	0.298	0.324	8.7	2.4	3		
			74.5	80.4	7.9	1.9	3		
		Omacetaxine	0.341	0.304	−11.3	1	3		
			85.3	78.2	−8.7	1.4	3		
		4'-DMHHT	0.453	0.465	2.5	3.1	3		
			114	115	0.6	0.9	3		
		Cephalotaxine	0.275	0.275	−0.2	7.5	3		
Ice-water, 1 hours	Biomatrix		66.6	69.1	3.6	3.4	3		
		Omacetaxine	0.341	0.33	−3.1	2.7	3		
			85.3	82.3	−3.6	1	3		
		4'-DMHHT	0.453	0.466	2.8	3.3	3		
			114	116	1.2	2.8	3		
		Cephalotaxine	0.275	0.26	−5.7	3.4	3		
			66.6	68.4	2.7	0.5	3		
Urine 5 freeze-thaw cycles (−70 °C/2−8 °C)	Biomatrix	Omacetaxine	0.307	0.304	−1.1	1.3	3		
			38.4	38	−1	1.6	3		
		4'-DMHHT	0.308	0.303	−1.5	5.6	3		
			38.5	38.4	−0.2	1.3	3		
		Cephalotaxine	0.311	0.301	−3.1	2.5	3		
			38.9	37.9	−2.7	0.4	3		
		Ambient, 24 h	Biomatrix	Omacetaxine	0.307	0.299	−2.7	1.7	3
			38.4	37.7	−1.7	0.2	3		
2−8 °C, 15 days	Final extract	4'-DMHHT	0.308	0.309	0.2	4.1	3		
			38.5	39.1	1.6	1.6	3		
		Cephalotaxine	0.311	0.301	−3.2	5.8	3		
			38.9	38.7	−0.5	1.1	3		
		Omacetaxine	0.307	0.306	−0.2	0.9	3		
			38.4	38.5	0.2	1.7	3		
		4'-DMHHT	0.308	0.294	−4.7	1.1	3		
			38.5	38.3	−0.6	4.5	3		
Cephalotaxine	0.311	0.324	4.1	3.5	3				
	38.9	40	2.8	1.5	3				

methanol–water (50:50, v/v) to obtain working solutions. The working solutions were also stored at  $-20^{\circ}\text{C}$ .

Stock solutions of the internal standards  $^2\text{H}_5$ -omacetaxine,  $^2\text{H}_5$ -4'-DMHHT and  $^2\text{H}_5$ -cephalotaxine were also prepared at a concentration of 1 mg/mL in DMSO. The internal standard working solution in methanol–water (50:50, v/v) contained 20 ng/mL of each internal standard. Internal standard stock solutions were stored at  $-20^{\circ}\text{C}$  and the working solutions at a nominal temperature of  $2$ – $8^{\circ}\text{C}$ .

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

To minimise degradation of omacetaxine to 4'-DMHHT and cephalotaxine, the plasma was stabilised with 5% of a 0.4% ethanolic paraoxon solution. With this stabilised plasma, to which we will refer as control human plasma, calibration standards were prepared freshly before each validation run by adding 20  $\mu\text{L}$  of calibration standard working solution to 380  $\mu\text{L}$  of control human plasma. Calibration standards with the following concentrations were prepared for omacetaxine, 4'-DMHHT and cephalotaxine: 0.100, 0.200, 1.00, 10.0, 20.0, 50.0, 80.0 and 100 ng/mL. Aliquots of 100  $\mu\text{L}$  were used for sample preparation.

The QC samples were prepared by spiking control human plasma with the appropriate QC working solution. Final concentrations at the lower limit of quantification (QC LLOQ), QC low, QC mid, QC high and QC above the upper limit of quantification (>ULOQ) contained 0.100, 0.300, 45.0, 75.0 and 2000 ng/mL of omacetaxine, 4'-DMHHT and cephalotaxine, respectively. QC samples were stored in aliquots of 100  $\mu\text{L}$  at a nominal temperature of  $-70^{\circ}\text{C}$ .

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

For the urine assay, calibration standards were prepared by adding 20  $\mu\text{L}$  of calibration working solution to 380  $\mu\text{L}$  of control human urine. Calibration standards with the following concentration were prepared for omacetaxine, 4'-DMHHT and cephalotaxine: 0.100, 0.200, 0.500, 1.00, 5.00, 20.0, 40.0 and 50.0 ng/mL. A sample volume of 400  $\mu\text{L}$  was processed.

The QC samples were prepared by spiking control human urine with appropriate QC working solutions. Final concentrations at the lower limit of quantification (LLOQ), QC low, QC mid, QC high and QC above the upper limit of quantification (>ULOQ) contained 0.100, 0.300, 20.0, 37.5 and 1000 ng/mL of omacetaxine, 4'-DMHHT and cephalotaxine. QC samples were stored in aliquots of 400  $\mu\text{L}$  at a nominal temperature of  $-70^{\circ}\text{C}$ .

## 2.3. Sample preparation

Immediately after sample collection in the clinic by venipuncture, the plasma samples were put on ice–water for approximately 5 min and centrifuged for 15 min at  $1800 \times g$  at  $4^{\circ}\text{C}$ . After centrifugation, the plasma was stabilised with 5% of 0.4% ethanolic paraoxon solution and stored at  $-80^{\circ}\text{C}$ . Urine samples were immediately stored at  $-80^{\circ}\text{C}$  after collection.

### 2.3.1. Plasma

Plasma samples were thawed, vortex-mixed and centrifuged (5 min at  $900 \times g$ ) prior to processing, and a 100- $\mu\text{L}$  aliquot was used for analysis. Fifty microliters of internal standard working solution (20 ng/mL) and 500  $\mu\text{L}$  of acetonitrile–methanol (80:20, v/v) were added to precipitate the plasma proteins. After 10 min shaking at 1250 rpm on an automatic shaker, the samples were centrifuged for 5 min at  $23,100 \times g$ . The supernatant was transferred to a clean tube and evaporated to dryness under a gentle stream of nitrogen gas (approximately 30 min at  $40^{\circ}\text{C}$ ). The dry extract was then reconstituted with 400  $\mu\text{L}$  of acetonitrile–0.1% formic acid

in water (90:10, v/v). After vortex-mixing for approximately 10 s and centrifuging for 5 min at  $23,100 \times g$ , the clear supernatant was transferred to an autosampler vial. A volume of 5  $\mu\text{L}$  was injected onto the HPLC column.

### 2.3.2. Urine

Urine samples were prepared by solid-phase extraction (SPE). Urine samples were thawed in an ice–water bath, and 100  $\mu\text{L}$  of internal standard working solution (20 ng/mL) and 400  $\mu\text{L}$  of 50 mM potassium phosphate (pH 3) were added to a volume of 400  $\mu\text{L}$  of urine. After vortex-mixing, SPE was performed using Strata SCX 100-mg cartridges (Phenomenex, Torrance, CA). The cartridges were conditioned and equilibrated with 400  $\mu\text{L}$  of methanol and 400  $\mu\text{L}$  of 50 mM potassium phosphate (pH 3), respectively. Subsequently, the acidified samples were loaded, and the cartridges were washed with 400  $\mu\text{L}$  of 50 mM potassium phosphate (pH 3) and 400  $\mu\text{L}$  of methanol and dried. The analytes were eluted with 600  $\mu\text{L}$  of 3% ammonium hydroxide in methanol, and the eluates were evaporated to dryness under a gentle stream of nitrogen at  $40^{\circ}\text{C}$ . The dry extract was then reconstituted with 400  $\mu\text{L}$  of acetonitrile–0.1% formic acid in water (90:10, v/v). After vortex-mixing for approximately 10 s and centrifuging for 5 min at  $23,100 \times g$ , the clear supernatant was transferred to an autosampler vial. A volume of 5  $\mu\text{L}$  was injected onto the HPLC column.

## 2.4. Liquid chromatography–tandem mass spectrometry

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  $30^{\circ}\text{C}$ . The mobile phase A consisted of 0.1% formic acid in water and mobile phase B was methanol. Gradient elution was applied at a flow rate of 0.50 mL/min through an XBridge BEH Phenyl column ( $50 \times 2.1$  mm internal diameter, particle size 5  $\mu\text{m}$ ; waters). The following mobile phase gradient was applied: 10–15% B (0.0–0.1 min), 15% B (0.1–0.5 min), 15–50% B (0.5–2.5 min), 50% B (2.5–3.5 min), 50–80% B (3.5–3.6 min), 80% B (3.6–4.5 min), 80–10% B (4.5–5.6 min). The divert valve directed the flow to the mass spectrometer between 0.2 and 3.0 min; the eluent during the remainder of the run was sent to the waste container.

Omacetaxine, 4'-DMHHT and cephalotaxine were analysed on a QTrap 5500 mass spectrometer (MS) (AB Sciex, Thornhill, ON, Canada). This instrument is equipped with a turbo ion spray 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 summarises the MS operating parameters. For  $^2\text{H}_5$ -cephalotaxine a product mass ( $m/z$  286) that was not analogous to the non-labeled cephalotaxine fragment ( $m/z$  229) was chosen because this fragment was the most abundant.

## 2.5. Validation procedures

The assay validation was performed in accordance to the OECD principles of good laboratory practice (GLP) [9]. Calibration model, accuracy and precision, selectivity, dilution integrity, lower limit of quantitation, matrix effect, carry-over, effect of hemolysis and stability under various conditions were established according to the latest US FDA and EMA guidelines on bioanalytical method validation [10,11].

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

Within-run bias(%)

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



Between-run 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 equations were used:

Within-run CV(%)

$$= 100\% \times \frac{(\text{SD of the measured conc. per run})}{(\text{mean measured conc. per run})} \quad (3)$$

Between-run CV(%)

$$= 100\% \times \frac{(\text{SD of the overall measured conc.})}{(\text{overall mean measured conc.})} \quad (4)$$

### 3. Results and discussion

#### 3.1. Calibration model

Calibration standards were prepared and analysed in duplicate during three analytical runs. The linear regression of peak area ratios versus the concentrations was weighted  $1/x^2$  to obtain the lowest total bias over the range. The calibration range of omacetaxine, 4'-DMHHT and cephalotaxine in plasma was 0.1–100 ng/mL and in urine was 0.1–50 ng/mL. Calibration curves were accepted if 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 0.9994 or better were obtained.

#### 3.2. 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 during three analytical runs.

Table 2 summarises the intra- and inter-assay accuracies and precisions of the assay. The biases and CVs were within the acceptance criteria (i.e. within  $\pm 20\%$  and  $\leq 20\%$ , respectively, at the LLOQ level and within  $\pm 15\%$  and  $\leq 15\%$  at the other QC levels).

#### 3.3. Selectivity

Six different batches of control plasma were spiked at the LLOQ level with omacetaxine, 4'-DMHHT and cephalotaxine to investigate the selectivity. The mean deviations from the nominal concentrations for the plasma assay were  $-8.4\%$ ,  $0.3\%$  and  $-11.5\%$  with CV values of  $4.9\%$ ,  $4.7\%$  and  $2.1\%$ , respectively. For the urine assay, the mean deviations were  $0.5\%$ ,  $-2.4\%$  and  $2.0\%$  with CV values of  $1.8\%$ ,  $2.5\%$  and  $5.2\%$ , respectively. There were no peaks observed with areas  $> 20\%$  of the LLOQ in double-blank samples of these batches for any of the analytes and also no interferences were detected at the retention times of the internal standards for both assays. Selectivity was therefore considered acceptable.

Cross-analyte and internal standard interference was tested by spiking control human plasma at ULOQ level with all analytes and all internal standards separately at the nominal concentration (one level). The cross-analyte and internal standard interference at the retention time of omacetaxine was less than the allowable  $20\%$  of the peak area of the LLOQ level. For the internal standard the interference was less than  $5\%$  and thus also within the acceptance criteria.

In the plasma assay, the interference at the retention time of 4'-DMHHT from omacetaxine at the ULOQ was 7 times the area of the LLOQ of 4'-DMHHT and the interference at the retention time of cephalotaxine from 4'-DMHHT at the ULOQ was 10 times of the area of the LLOQ of cephalotaxine. The cross-analyte interference was investigated and no conversion was found to have occurred during sample processing for sample analysis. However, the reference standards contained impurities: a relative interference of  $0.34\%$  4'-DMHHT in the omacetaxine reference standard and  $0.96\%$  cephalotaxine in the 4'-DMHHT reference standard. Since the relative interference is less than  $1\%$ , this will not have consequences for the accuracy of the method since all calibration standards are spiked with analytes at equal concentration levels. Therefore, analyte and internal standard interferences for these analytes were considered acceptable. The same interferences were detected during validation of the urine method, since the same batches of reference standards were used.

#### 3.4. Dilution integrity

Five replicate plasma and urine samples of omacetaxine, 4'-DMHHT and cephalotaxine at a concentration level of  $2000 \text{ ng/mL}$  for plasma and  $1000 \text{ ng/mL}$  for urine were diluted 25-fold with control human plasma and control human urine, respectively ( $20 \mu\text{L}$  of sample was added to  $480 \mu\text{L}$  of control human matrix). For the plasma assay,  $100 \mu\text{L}$  was processed, and for the urine assay,  $400 \mu\text{L}$ . In the plasma samples, the maximum bias was  $-5.9\%$  and the precisions were less than  $2.0\%$ ; in the urine samples, the maximum bias was  $-10.6\%$  and the precisions were less than  $1.7\%$ , all within the required limits [10,11].

#### 3.5. Lower limit of quantitation

The analyte responses at the LLOQ were at least 5 times the response of a blank in three validation runs for both assays. In the plasma assay, the signal-to-noise ratio was at least 13.3, and in the urine assay, the signal-to-noise ratio was at least 10.3. Fig. 2 shows representative chromatograms of omacetaxine, 4'-DMHHT and cephalotaxine in QC LLOQ samples and double-blank samples in plasma; Fig. 3 shows chromatograms for the corresponding samples in urine.

#### 3.6. Carry-over

Carry-over was investigated by injecting two double-blank samples after a ULOQ sample in three analytical runs. Eluting peaks with areas  $> 20\%$  of the LLOQ were not observed in the blank samples injected directly after ULOQ samples, and therefore the criteria for carry-over were fulfilled.

#### 3.7. Matrix factor

The matrix factor (MF) was determined in six plasma batches and six urine batches at low and high concentration levels of omacetaxine, 4'-DMHHT and cephalotaxine and the internal standards. Single determinations were performed. Processed blank samples were spiked with neat solutions and compared to matrix-free neat solutions. The matrix factor was determined by using the following equation:

$$\text{MF} = \frac{\text{Area of blank sample spiked with neat solution (matrix present)}}{\text{Area of neat solution (matrix absent)}} \quad (5)$$

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 internal standard-normalised MF calculated from the six plasma batches

for the low and high concentration were less than 4.4% and 0.8%, respectively, fulfilling the acceptance criteria. The matrix factor in plasma ranged from 0.927 to 1.08. 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 4.4% and 0.8%, respectively, and fulfilled the criteria. The matrix factor in urine ranged from 0.917 to 1.16. These results (MF around 1) indicate that the use of the stable isotope-labeled compounds as internal standards is effectively minimising the influence of matrix effects.

### 3.8. Effect of hemolysis

The effect of hemolysis was evaluated by spiking hemolysed control human plasma. Hemolysed human plasma was obtained by adding 5% (v/v) of whole blood that had undergone one freeze (–20 °C)/thaw cycle to control human plasma. Concentrations equal to low and high QC levels were tested and analysed in triplicate. The results are shown in Table 3. No effect of hemolysis was demonstrated since the bias of the mean concentration was within  $\pm 15\%$  of the nominal concentration for all analytes.

### 3.9. Stability

The results of the stability experiments are shown in Table 4. In DMSO stock solution omacetaxine, 4'-DMHHT and cephalotaxine are stable for at least 4 h at ambient temperature and for at least 2 months at –20 °C. In working solution in methanol–water (50:50, v/v) all analytes are stable for at least 16 h at ambient temperature and for at least 2 months at –20 °C. Omacetaxine, 4'-DMHHT and cephalotaxine are stable in plasma when using the esterase inhibitor paraoxon to stabilise the compounds, with a demonstrated stability at –70 °C for almost 4 years (data not shown). After an hour at room temperature there is a substantial decrease measured in the omacetaxine concentration in whole blood. Therefore the stability of the analytes in whole blood was tested on ice–water, and under these conditions the analytes were stable for at least one hour. The compounds are stable in urine at –70 °C for at least 3.5 years (data not shown) and for at least 24 h at room temperature.

Reinjection reproducibility experiments demonstrated that processed plasma and urine samples can be successfully reinjected after 6 and 3 days, respectively, when stored at 2–8 °C (data not shown).

### 3.10. Clinical application

The purpose of the validated assays was to support clinical pharmacokinetic studies of omacetaxine. To demonstrate their applicability, we present the concentration profiles over time of omacetaxine, 4'-DMHHT and cephalotaxine in plasma of a representative patient treated with omacetaxine.

A 1.25-mg/m<sup>2</sup> dose of omacetaxine was administered as a subcutaneous injection to a cancer patient as part of a clinical phase I study investigating the metabolism and excretion of omacetaxine in patients with relapsed and/or refractory hematologic malignancies or advanced tumors. Blood samples were collected at pre-selected time points using tubes containing K<sub>2</sub>EDTA and put on ice. Within 5 min after collection, samples were centrifuged for 10 min at 1800  $\times$  g and 4 °C. Plasma was isolated and stabilised with

5% of a 0.4% ethanolic paraoxon solution, vortex-mixed and stored at –80 °C pending analysis. Fig. 4 shows the plasma concentration–time curves for omacetaxine and 4'-DMHHT in plasma of a patient. The metabolite cephalotaxine was quantifiable in urine but only in one plasma sample from a single patient.

## 4. Conclusion

A sensitive LC–MS/MS assay for the quantification of omacetaxine, 4'-DMHHT and cephalotaxine in human plasma and urine was developed and validated. The validated range for all analytes in plasma is from 0.1 to 100 ng/mL using 100- $\mu$ L plasma aliquots and in urine from 0.1 to 50 ng/mL using 400- $\mu$ L urine aliquots. Dilution integrity experiments show that samples can be diluted 25-fold in control matrix prior to analysis. The extended concentration range for plasma is therefore from 0.1 to 2000 ng/mL and for urine, from 0.1 to 1000 ng/mL. Omacetaxine, 4'-DMHHT and cephalotaxine are stable at –70 °C in plasma stabilised with paraoxon for almost 4 years and in urine for almost 3.5 years. The assays are considered fit to support clinical pharmacologic studies of omacetaxine.

## Footnote

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