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Development and validation of LC-MS/MS methods for the quantification of the novel anticancer agent guadecitabine and its active metabolite β-decitabine in human plasma, whole blood and urine

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ABSTRACT

Guadecitabine (SGI-110), a dinucleotide of β-decitabine and deoxyguanosine, is currently being evaluated in phase II/III clinical trials for the treatment of hematological malignancies and solid tumors. This article describes the development and validation of bioanalytical assays to quantify guadecitabine and its active metabolite β-decitabine in human plasma, whole blood and urine using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Since β-decitabine is rapidly metabolized further by cytidine deaminase, plasma and whole blood samples were kept on ice-water after collection and stabilized with tetrahydrouridine (THU) directly upon sample collection. Sample preparation consisted of protein precipitation for plasma and whole blood and dilution for urine samples and was further optimized for each matrix and analyte separately. Final extracts were injected onto a C6-phenyl column for guadecitabine analysis, or a Nova-Pak Silica column for β-decitabine analysis. Gradient elution was applied for both analytes using the same eluents for each assay and detection was performed on triple quadrupole mass spectrometers operating in the positive ion mode (Sciex QTRAP 5500 and QTRAP 6500). The assay for guadecitabine was linear over a range of 1.0–200 ng/mL (plasma, whole blood) and 10–2000 ng/mL (urine). For β-decitabine the assay was linear over a range of 0.5–100 ng/mL (plasma, whole blood) and 5–1000 ng/mL (urine). The presented methods were successfully validated according to the latest FDA and EMA guidelines for bioanalytical method validation and applied in a guadecitabine clinical mass balance trial in patients with advanced cancer.

1. Introduction

Guadecitabine (SGI-110) is a novel hypomethylating agent that is synthesized as a dinucleotide of β-decitabine (Dacogen®) and deoxyguanosine linked by a phosphodiester bond (Fig. 1). Guadecitabine was designed to prolong the exposure to its active metabolite β-decitabine in-vivo by gradual enzymatic cleavage of the phosphodiester bond. [1] In contrast to β-decitabine, guadecitabine is resistant to enzymatic inactivation by cytidine deaminase, resulting in further improvement of the in-vivo exposure window to β-decitabine. [2] Guadecitabine is currently under evaluation in phase II/III clinical trials for the treatment of hematological malignancies and solid tumors, including a clinical mass balance trial conducted in our institute.

Abbreviations: ACN, Acetonitrile; AML, Acute myeloid leukemia; CAL, Calibration standard; CV, Coefficient of variation; DMSO, Dimethyl sulfoxide; HILIC, Hydrophilic interaction liquid chromatography; HPLC-MS/MS, High-performance liquid chromatography tandem mass spectrometry; IS, Internal standard; LLOQ, Lower limit of quantification; MDS, Myelodysplastic syndrome; MeOH, Methanol; MF, Matrix factor; MRM, Multiple reaction monitoring; *m/z*, Mass-to-charge ratio; RS, Reconstitution solvent; THU, Tetrahydrouridine; QC, Quality control sample

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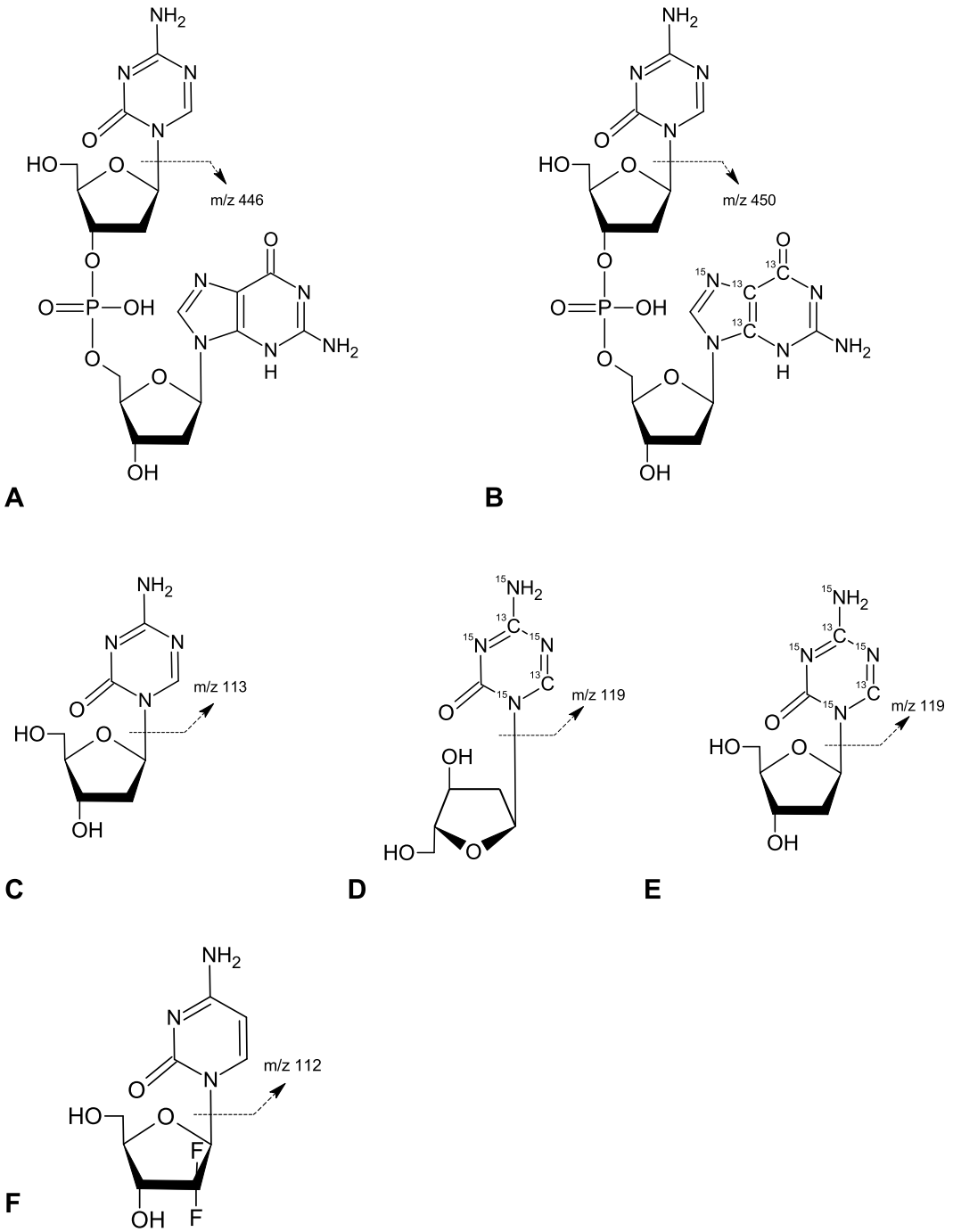

Fig. 1. Chemical structures of (A) guadecitabine, (B) guadecitabine [$^{13}C_3$,^{15}N], (C) β-decitabine, (D) α-decitabine [$^{13}C_2$, $^{15}N_4$], (E) β-decitabine [$^{13}C_2$, $^{15}N_4$], and (F) gemcitabine with their proposed fragmentation products.

One of the aims of this clinical mass balance trial is to assess the pharmacokinetics of guadecitabine and β-decitabine in plasma, whole blood and urine. To achieve this, validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods to analyze drug concentrations in different matrices are required for reliable sample analysis. To date, only one validated LC-MS/MS assay has been described for the selective analysis of β-decitabine in human plasma after β-decitabine treatment. [2] Methods for the analysis of β-decitabine in human whole blood and urine, and for guadecitabine in general, have not been published. To be able to achieve the objectives of the clinical mass balance trial, LC-MS/MS assays to quantify guadecitabine and β-decitabine in these matrices need to be developed and validated.

LC-MS/MS method development for β-decitabine analysis has shown to be challenging due to several reasons. [3–6] In the past, β-decitabine was administered at significantly higher concentrations than it is today. [7] To be able to measure lower concentrations, increased sensitivity is required, which has proven to be a challenge. [6] In addition to this, structural similarity of β-decitabine to endogenous nucleosides requires an LC-MS/MS method that is able to distinguish β-decitabine from interfering components that are present in biological matrices. Furthermore, β-decitabine is unstable at physiological temperature and pH and can be degraded in many degradation products. [8,9] As some degradation products are isomers to β-decitabine (e.g. α-decitabine), chromatographic separation to distinguish β-decitabine

from its degradation products is essential. To overcome these issues, assay development should focus on the selectivity and sensitivity of the LC-MS/MS method.

In this article, we describe the development and validation of highly sensitive and selective LC-MS/MS methods for the quantification of guadecitabine and β-decitabine in human plasma, whole blood and urine. To stabilize β-decitabine in human whole blood and plasma, tetrahydrouridine (THU) was added to collection tubes as a cytidine deaminase inhibitor at a concentration of 100 μg/mL prior to sample collection. Selected concentration ranges were based on previously published data where guadecitabine was administered to patients. [1] Using the validated methods, guadecitabine and β-decitabine can be quantified in plasma, whole blood, and urine samples collected from patients treated with guadecitabine in the clinical mass balance trial.

2. Materials and methods

2.1. Chemicals and reagents

β-Decitabine, gemcitabine, and guadecitabine [$^{13}C_3$, ^{15}N] (guadecitabine-IS) were purchased from Alsachim (Illkirch, France). Guadecitabine, and decitabine [$^{13}C_2$, $^{15}N_4$] (decitabine-IS) were provided by Astex Pharmaceuticals, and manufactured by Clauson-Kaas (Farum, Denmark) and Asclep Pharmard (Newark, DE, USA), respectively. Acetonitrile (ACN), isopropylalcohol, methanol (MeOH) and LiChrosolve water (LC-MS grade) originated from Biosolve Ltd. (Valkenswaard, The Netherlands). Ammonium formate and ammonium acetate were purchased from SigmaAldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), formic acid and tetrahydrouridine (THU) were from Merck (Darmstadt, Germany). K_2EDTA plasma was purchased from BioreclamationsIVT (Hicksville, NY, USA). Blank urine as well as K_2EDTA whole blood was obtained from the Medical Center Slotervaart (Amsterdam, The Netherlands).

2.2. Stock solutions, calibration standards and quality control samples

Standard stock solutions of β-decitabine and the internal standards (IS) gemcitabine, guadecitabine-IS, and decitabine-IS were prepared by dissolving the analytes and IS in DMSO, obtaining concentrations of 1.0 mg/mL for β-decitabine, decitabine-IS, and guadecitabine-IS and of 0.1 mg/mL for gemcitabine. Guadecitabine was already dissolved as a reference standard in MeOH-DMSO (50:50, v/v) at a concentration of 2.0 mg/mL. Working solutions for calibration standards (CALs) and quality control samples (QCs) were prepared by diluting two individual stock solutions of guadecitabine and β-decitabine with DMSO. Working solutions contained both guadecitabine and β-decitabine, except for working solutions used for stability and cross-analyte interference experiments. For plasma analysis, a combined IS working solution was prepared (5 ng/mL gemcitabine, 1000 ng/mL decitabine-IS). For whole blood analysis, separate working solutions were prepared (2000 ng/mL guadecitabine-IS, or 1000 ng/mL decitabine-IS), because of interfering levels of β-decitabine in the guadecitabine-IS. For urine analysis, a working solution containing only decitabine-IS (10,000 ng/mL) was prepared, as no IS for guadecitabine was used for this matrix. All stock solutions and working solutions were stored at −20 °C.

Fresh CALs were prepared for each run by spiking 10 μL of working solution to 190 μL blank matrix to obtain concentrations of 0.5–100 ng/mL (β-decitabine) and 1.0–200 ng/mL (guadecitabine) in THU-stabilized plasma and whole blood, and of 5–1000 ng/mL (β-decitabine) and 10–10,000 ng/mL (guadecitabine) in urine. QCs were prepared by adding 10 μL of working solution to 190 μL of THU-stabilized plasma and whole blood to obtain concentrations of 0.5 and 1.0 (QC LLOQ), 1.5 and 3.0 (QC Low), 12.5 and 25.0 (QC Mid), and 75 and 150 (QC High) ng/mL for β-decitabine and guadecitabine, respectively. The same procedure was followed for urine, obtaining 10-fold higher concentrations of 5 and 10 (QC LLOQ), 15 and 30 (QC Low), 125 and 250 (QC Mid), and 750 and 1500 (QC High) ng/mL for β-decitabine and guadecitabine, respectively. The QCs were stored at −70 °C in aliquots of 200 μL for the duration of the validation.

Plasma and whole blood samples were stabilized with THU at a final concentration of 0.1 mg/mL by dilution of a 10 mg/mL of THU in water to the biomatrix.

2.3. Sample preparation

Whole blood samples were obtained by venipuncture using K_2EDTA tubes pretreated with THU. Part of the sample was directly used for whole blood analysis, whereas the other part was centrifuged (2000 *g*, 4 °C, 10 min) prior to plasma isolation. Urine was collected in urine containers of which aliquots were stored. All collected samples were stored at −70 °C prior to sample analysis. Before analysis, samples were thawed on an ice-water bath to prevent analyte degradation.

For all samples, 200 μL was transferred to a 1.5 mL Eppendorf tubes after which 20 μL of IS working solution was added (different working solution for each matrix, see Section 2.2), except to double blank calibration standards. All samples were mixed, after which 500 μL of MeOH was added to plasma and whole blood samples for erythrocyte and/or plasma protein precipitation. After this, all samples were mixed and centrifuged (15,000 rpm, 5 °C, 5 min). For guadecitabine plasma analysis, 50 μL of the supernatant was transferred to Eppendorf tubes containing 100 μL of 5 mM ammonium acetate in water. The remainder of the supernatant (at least 600 μL for β-decitabine analysis) was evaporated to dryness (40 °C) in a clean 2.0 mL Eppendorf tube under a stream of nitrogen. Dry extracts were reconstituted using 100 μL of 5 mM ammonium formate – ACN (2:98, v/v). For whole blood analysis, the complete supernatant was evaporated to dryness (40 °C) in a clean 2.0 mL Eppendorf tube under a stream of nitrogen. Dry extracts were reconstituted using 100 μL of 5 mM ammonium acetate in water (guadecitabine analysis) or 5 mM ammonium formate – ACN (2:98, v/v) (β-decitabine analysis). For urine analysis, 50 μL was directly transferred to 1.5 mL Eppendorf tubes containing 200 μL of 5 mM ammonium acetate in water (guadecitabine analysis) or 5 mM ammonium formate – ACN (2:98, v/v)(β-decitabine analysis).

All samples were mixed and centrifuged again (15,000 rpm, 5 °C, 5 min) before being transferred to autosampler vials with inserts, prior to sample analysis. Volumes of 10 μL of the final extracts for guadecitabine and β-decitabine were injected onto the chromatographic systems.

2.4. Instrumentation and operating conditions

2.4.1. Chromatography

Guadecitabine was chromatographically separated using an HPLC system with a binary pump, a degasser, column oven (kept at 30 °C), and autosampler (Nexera 2 series, Shimadzu Corporation, Kyoto, Japan, kept at 4 °C). Gradient elution of guadecitabine was achieved using 5 mM ammonium formate in water (mobile phase A) and 5 mM ammonium formate in water – ACN (2:98, v/v) (mobile phase B). Analyses were performed at a flow rate of 1.0 mL/min through a Gemini C6-phenyl column (50 × 4.6 mm, 3 μm) attached to a Securityguard C18 (4 × 2.0 mm) pre-column (Phenomenex, Torrance, CA, USA). For plasma and whole blood analysis, the following gradient was applied: mobile phase B: 1 ➔ 20% (0–2.1 min), 20 ➔ 100% (2.1–2.5 min), 100% (2.5–5.5 min), 100 ➔ 1% (5.5–5.6 min), 1% (5.6–7.7 min). The flow was directed to the mass spectrometer from 0.7 to 3.0 min to protect the MS from contaminants during the remainder of the analytical runtime. For urine analysis, the following gradient was applied: mobile phase B: 1.5% (0–5.5 min), 1.5 ➔ 90% (5.5–6.2 min), 90% (6.2–7.7 min), 90 ➔ 1.5% (7.7–7.8 min), 1.5% (7.8–11.0 min).

β-decitabine was chromatographically separated using an HPLC Acquity I Class binary pump, a degasser, column oven (kept at 30 °C), and autosampler (Waters, Milford, MA, USA, kept at 4 °C). Mobile

Table 1
General and analyte specific mass spectromic settings.

	General settings		
Analyte	**guadecitabine (plasma & whole blood)**	**guadecitabine (urine)**	**ß-decitabine**
Mass spectrometer	QTRAP6500	QTRAP6500	QTRAP5500
Run duration (min)	7.7	11.0	10.8
Ionspray voltage (V)	5000	5000	5000
Nebulizer gas (au)	50	50	-
Turbo gas/heater gas (au)	40	40	-
Ion Source Gas 1 (au)	-	-	50
Ion Source Gas 2 (au)	-	-	40
Curtain gas (au)	10	10	24
Collision gas (au)	10	10	10
Temperature (°C)	400	450	450
Dwell time (ms)	50	250	150

Analyte specific settings					
Analyte	**ß-decitabine**	**decitabine [$^{13}C_2$, $^{15}N_4$]**	**guadecitabine**	**guadecitabine [$^{13}C_3$, ^{15}N]**	**gemcitabine**
Parent mass (Da)	229.1	235.1	558.1	562.1	264.1
Product mass (Da)	113.1	119.1	446.1	450.1	112.0
Collision energy (V)	20	20	17	17	17
Collision exit potential (V)	10	10	16	16	16
Declustering potential (V)	125	125	26	26	26

Table 2
Assay performance data for the analysis of guadecitabine and β-decitabine in human plasma, whole blood and urine.

Matrix	Analyte	Nom. conc. (ng/mL)	N	Intra-assay		Inter-assay	
				Bias (%)	CV (%)	Bias (%)	CV (%)
Plasma	Guadecitabine	1.00	15	−2.4–6.9	6.5–8.9	1.9	3.9
		3.00	15	0.8–12.3	2.3–8.6	7.0	4.5
		25.0	15	−2.7–13.0	2.6–8.1	4.2	7.3
		150	15	−1.2–7.9	2.0–5.4	3.4	4.0
	β-decitabine	0.500	15	−9.5–16.5	3.7–7.5	5.3	12.1
		1.50	15	2.8–6.3	2.5–8.4	4.0	-[a]
		12.5	15	9.8–12.3	1.4–1.7	10.8	1.0
		75.0	15	−1.8–7.0	1.2–2.0	1.8	4.4
Whole blood	Guadecitabine	1.00	15	0.1–6.9	5.6–9.8	3.5	-[a]
		3.00	15	−5.2–0.3	2.8–7.9	−2.1	1.7
		25.0	15	−6.5–3.4	2.8–8.1	0.1	4.9
		150	15	−6.0–2.3	1.8–7.5	−2.0	3.6
	β-decitabine	0.500	15	2.4–7.0	4.4–10.9	5.4	-[a]
		1.50	15	−10.0–3.1	2.8–5.2	−2.5	6.7
		12.5	15	−6.2 to −0.5	0.4–4.9	−2.8	2.7
		75.0	15	−5.4–2.5	0.8–5.7	−0.6	4.0
Urine	Guadecitabine	10.0	15	−0.1–3.4	1.3–1.8	0.2	3.0
		30.0	15	−3.7–1.7	1.2–2.2	−1.3	2.7
		250	15	−7.5–0.6	0.5–3.9	−3.3	4.1
		1500	15	−8.7 to −2.7	1.4–2.7	−5.7	3.1
	β-decitabine	5.00	15	−18.2–9.5	2.7–5.6	−1.0	15.0
		15.0	15	−5.5–3.1	2.3–4.8	−0.6	4.1
		125	15	−0.2–8.6	1.1–1.7	3.9	4.2
		750	15	−1.1–11.4	0.5–0.9	4.5	6.1

[a] The inter-run precision could not be calculated because there is no significant additional variation owing to the performance of the assay in difference runs.

phases were the same as for guadecitabine analysis. Analyses were performed using a Nova-Pak Silica column (150 × 3.9 mm, 4 µm, Waters) attached to a 0.2 µm inline filter using a flow rate of 1.4 mL per minute. The following gradient was applied for the analysis of all three matrices: mobile phase B: 97% (0–3.2 min), 97 ➔ 90% (3.2–7.2 min), 90 ➔ 2% (7.2–7.3 min), 2% (7.3–11.3 min), 2 ➔ 97% (11.3–11.4 min), 97% (11.3–14.0 min). The flow was directed to the mass spectrometer from 1.0 to 5.0 min to protect the MS from contaminants during the remainder of the analytical runtime. Volumes of 10 µL of the final extracts for guadecitabine and β-decitabine were injected onto the chromatographic systems for all three matrices.

2.4.2. Mass spectrometry

For guadecitabine analysis, a QTRAP 6500 tandem mass

spectrometer (Sciex, Framingham, MA, USA) was used as a detector. The mass spectrometer was operated in the positive ionization mode using a turbo ionspray interface. For plasma analysis, gemcitabine was used as an IS. For whole blood analysis, guadecitabine-IS was used as an IS. For urine analysis, no IS was used.

For β-decitabine analysis, a QTRAP 5500 tandem mass spectrometer (Sciex) was used as a detector. The mass spectrometer was operated in the positive ionization mode using a turbo ionspray interface. Decitabine-IS was used as an IS for all three matrices.

For both assays, data acquisition was performed in the multiple reaction monitoring (MRM) mode using Analyst 1.6.2. Software (Sciex) to acquire and process the chromatograms. General and analyte specific mass spectromic parameters are listed in Table 1 and the structures and the proposed fragmentation patterns of the analytes and IS are depicted in Fig. 1.

2.5. Validation procedures

The methods were validated for the quantification of guadecitabine and β-decitabine in THU-stabilized plasma and whole blood, and urine, according to the latest FDA and EMA guidelines for bioanalytical method validation. [10,11] Conducted validation experiments include calibration curve, accuracy and precision, lower limit of quantification, dilution integrity, carry-over, specificity and selectivity, matrix effect, and stability.

3. Results and discussion

3.1. Method development

3.1.1. Mass spectrometry

From literature it is known that decitabine is easily ionized in the positive ionization mode. [4–6,8] The protonated ions of guadecitabine and β-decitabine were observed after mass spectrometry infusion at *m/z* 558.1 and 229.1, respectively. These ions were selected for fragmentation to generate product ions. The most abundant product ions of selected precursor ions were then optimized for MRM, according to Table 1. In both cases the selected product ions are suggested to result from cleavage of the glycosidic bond in the β-decitabine molecule (Fig. 1).

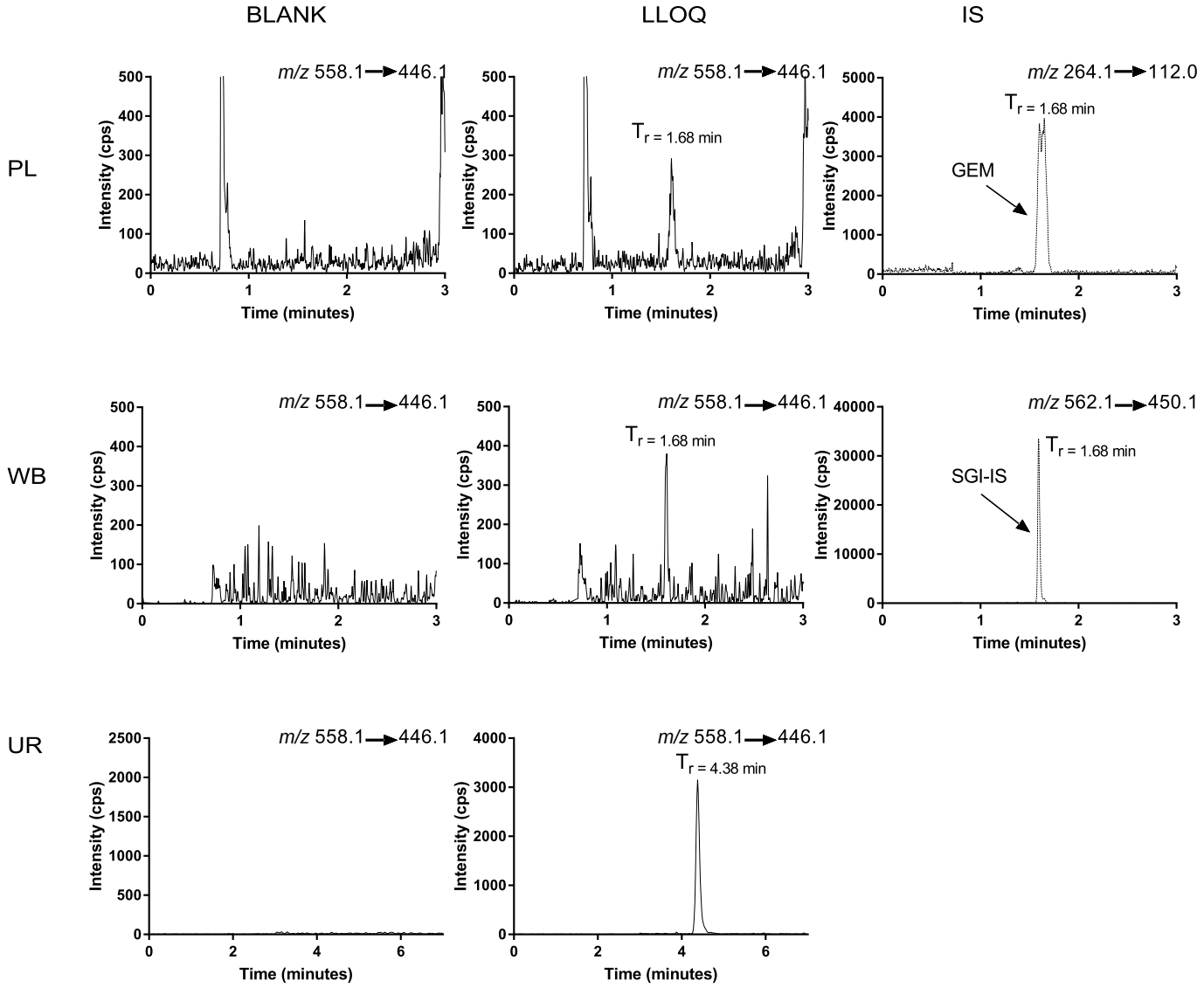

Fig. 2. Representative MRM chromatograms of guadecitabine (*m/z* 558.1 ➞ 446.1) in blank matrix and LLOQ calibration standard in plasma (PL), whole blood (WB), and urine (UR). Gemcitabine (GEM) (*m/z* 264.1 ➞ 112.0) and SGI-IS (*m/z* 562.1 ➞ 450.1) was used as an IS for PL and WB analysis, respectively. For UR analysis, no internal standard (IS) was used.

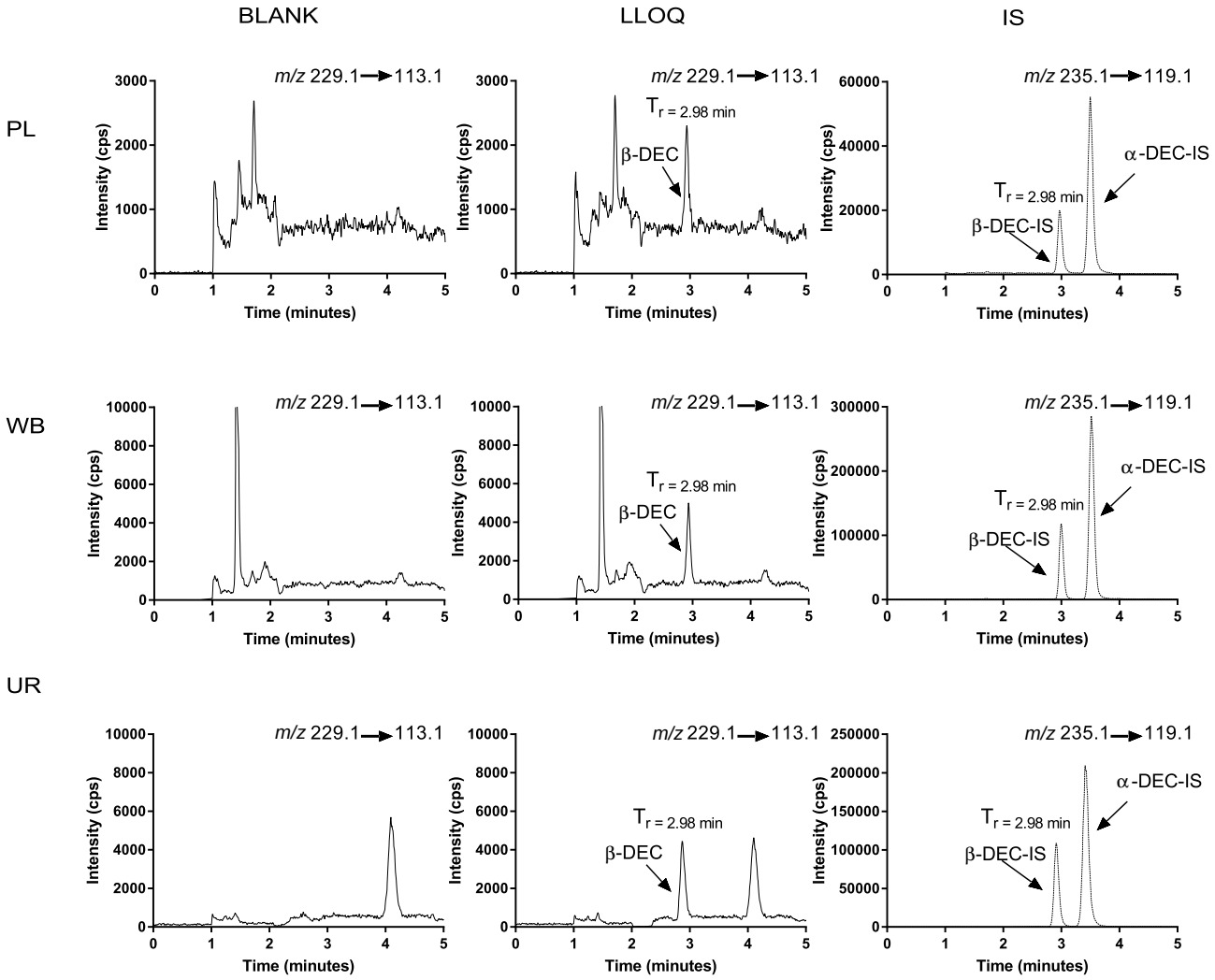

Fig. 3. Representative MRM chromatograms of decitabine (*m/z* 229.1➔113.1) and decitabine-IS (*m/z* 235.1➔119.1) in blank matrix and LLOQ calibration standard in plasma (PL), whole blood (WB), and urine (UR).

3.1.2. Chromatography

Initially, we evaluated possibilities for the combined analysis of guadecitabine and its active metabolite β-decitabine using a single LC-MS/MS method. The two main challenges to accomplish this were 1) the difference in polarity and molecular structure between guadecitabine and β-decitabine, and 2) the separation of β-decitabine from structurally related compounds.

Several columns, eluents, gradients, and mass transitions were tested to achieve combined analysis, however, without success. On the other hand, use of a Hydro-RP column, suitable for separation of both hydrophobic and polar compounds, provided the most promising results, making it possible to elute guadecitabine and β-decitabine with good peak shape. However, this system resulted in insufficient sensitivity for β-decitabine, and interfering signals at the same retention time as β-decitabine, originating from endogenous components. Use of another reversed-phase column (Gemini C6-phenyl) showed best results for guadecitabine analysis, but was not suitable for β-decitabine analysis, due to similar reasons.

Hydrophilic interaction liquid chromatography (HILIC), which has been described as a suitable separation technique for decitabine analysis, was tested next. [2] Using a Waters Nova-Pak Silica column (150 × 3.9 mm, 4 µm), β-decitabine eluted with good peak shape and high sensitivity, due to the high percentage of ACN at the time of elution, promoting the evaporation of the eluent and formation of analyte ions. It is known that β-decitabine can undergo anomerization to its α-, and other isoforms at physiological temperature and pH. [8,9] α-Decitabine is thought to be inactive, and therefore chromatographic separation of active β-decitabine from inactive isoforms is pivotal and using the Nova-Pak Silica column, we confirmed baseline separation of α- and β-decitabine (Fig. 3). Therefore, it was concluded to validate the developed HILIC system for β-decitabine analysis because it provided excellent sensitivity and selectivity.

HILIC was tested for guadecitabine analysis as well, but resulted in bad peak shape and low sensitivity for guadecitabine analysis. The structural differences of guadecitabine and β-decitabine made it extremely challenging to come up with a single LC-MS/MS system for the combined analysis of both compounds. From a practical point of view, two separate systems for the analysis of guadecitabine and β-decitabine were then developed and validated, HILIC (Nova-Pak Silica) for β-decitabine analysis and reversed-phase chromatography (Gemini C6-phenyl) for guadecitabine analysis.

3.1.3. Sample processing and analysis

In-vivo, β-decitabine is rapidly deaminated by cytidine deaminase. [12] THU is a competitive inhibitor of cytidine deaminase that can be used to prevent unwanted degradation of β-decitabine after sample collection. THU provides optimum inhibitory effect of cytidine deaminase at a concentration of 100 µg/mL. [5] For this reason, K_2EDTA tubes were spiked with THU prior to sample collection to prevent enzymatic degradation of β-decitabine ex-vivo. After collection, samples

Table 3
Stability of guadecitabine and β-decitabine in tested matrices.

Matrix	Conditions	Analyte	Nom. conc. (ng/mL)	Measured conc. (ng/mL)	Bias (%)	CV (%)	n
Plasma							
Biomatrix	4 freeze-thaw cycles (−70 °C/Ambient)	β-decitabine	1.5	1.52	1.3	1.7	3
			75.0	78.6	4.8	1.6	3
		Guadecitabine	3.0	3.33	11.1	1.4	3
			150	147	−1.8	2.4	3
Biomatrix	4 h, Ambient	β-decitabine	1.5	1.21	−19.3	2.2	3
			75.0	60.1	−19.9	1.5	3
		Guadecitabine	3.0	2.45	−18.3	5.9	3
			150	123	−17.8	1.2	3
Biomatrix	4 h, ice-water	β-decitabine	1.5	1.49	−0.4	2.2	3
			75	73.2	−2.4	0.8	3
		Guadecitabine	3	3.19	6.4	8.4	3
			150	160	6.7	4.1	3
Dry extract	3 days, 2–8 °C	β-decitabine	1.5	1.54	2.4	4.6	3
			75.0	77.8	3.7	0.3	3
Final extract	3 days, 2–8 °C	β-decitabine	1.5	1.57	4.4	1.3	3
			75.0	77.9	3.8	0.7	3
Final extract	24 h, 2–8 °C	Guadecitabine	3.0	2.82	−6.0	8.9	3
			150	131	−12.4	1.2	3
Final extract	48 h, 2–8 °C	Guadecitabine	3.0	1.99	−33.7	13.2	3
			150	105	−30.0	9.0	3
Whole blood							
Biomatrix	3 freeze-thaw cycles (−70 °C/Ambient)	β-decitabine	1.5	1.7	11.8	2.3	3
			75.0	77	2.5	2.1	3
		Guadecitabine	3.0	2.9	−2.6	4.6	3
			150	153	1.8	1.0	3
Biomatrix	4 h, ice-water	β-decitabine	1.5	1.3	−12.9	2.3	3
			75.0	67	−10.7	1.0	3
		Guadecitabine	3	3.1	4.2	3.8	3
			150	148	−1.6	5.7	3
Dry extract	24 h, 2–8 °C	β-decitabine	1.5	1.6	5.8	1.3	3
			75	75	−0.6	1.1	3
		Guadecitabine	3	3.2	7.8	2.4	3
			150	164	9.6	3.4	3
Final extract	24 h, 2–8 °C	β-decitabine	1.5	1.6	3.6	1.6	3
			75	76	0.7	0.7	3
		Guadecitabine	3	3.3	8.7	2.7	3
			150	168	11.8	3.0	3
Urine							
Biomatrix	3 freeze-thaw cycles (−70 °C/Ambient)	β-decitabine	15.0	14.5	−3.3	1.2	3
			750	690	−8.0	1.8	3
		Guadecitabine	30.0	32.3	7.6	1.4	3
			1500	1283	−14.4	7.1	3
Biomatrix	4 h, Ambient	β-decitabine	15.0	12.4	−17.6	7.7	3
			750	786	4.8	0.5	3
Biomatrix	2.5 h, Ambient	Guadecitabine	30.0	28.6	−4.7	10.8	3
			1500	1393	−7.1	3.0	3
Biomatrix	4 h, ice-water	β-decitabine	15.0	15.4	2.7	4.5	3
			750	858	14.4	5.5	3
Biomatrix	2.5 h, ice-water	Guadecitabine	30.0	27.4	−8.7	4.1	3
			1500	1690	12.7	5.6	3
Final extract	24 h, 2–8 °C	β-decitabine	15.0	14.5	−3.3	0.0	3
			750	704	−6.1	0.7	3
		guadecitabine	30.0	26.9	−10.4	3.9	3
			1500	1373	−8.4	1.7	3

were further processed on ice-water to prevent ex-vivo hydrolytic degradation of guadecitabine and β-decitabine. [8]

Combined sample processing for guadecitabine and β-decitabine analysis is preferred to increase processing efficiency. For plasma analysis, guadecitabine and β-decitabine final extracts were prepared using a single sample. Although signals for quantification were low for β-decitabine, sample dilution was adequate to reach the intended LLOQ. In whole blood final extracts, signals were much lower for both analytes, making it impossible to reach the LLOQ using the plasma sample processing method. This can be explained by the results of the matrix factor experiments (Section 3.2.6), where ionization of guadecitabine and β-decitabine was significantly lower as compared to processed plasma samples. Using sample evaporation and reconstitution, whole blood samples were concentrated and the LLOQ could be reached for both analytes. As urine CALs and QCs were 10-fold higher for both analytes to begin with, sample dilution was adequate to reach the required LLOQ in this matrix.

At the beginning of method development, a stable isotopically labeled IS of guadecitabine was not available. Gemcitabine was used as an IS for guadecitabine plasma method validation, but was not suitable for method validation in whole blood and urine. Guadecitabine structure related IS were tested, but without success. Guadecitabine could adequately be quantified in urine without use of an IS by adjusting the gradient of the method (Section 2.4.1), minimizing matrix effects that were observed using the plasma method. Validation experiments demonstrated that accuracy and precision improved without use an IS, compared to using gemcitabine as an IS. For whole blood analysis, a stable isotopically labeled IS was commercially synthesized and

successfully implemented for whole blood assay validation.

3.2. Validation procedures

3.2.1. Calibration curve

Plasma and whole blood calibration standards stabilized with 0.1 mg/mL THU ranged from 0.5 to 100 ng/mL (β-decitabine) and 1–200 ng/mL (guadecitabine). Urine calibration standards ranged from 5 to 1000 ng/mL (β-decitabine) and 10–2000 ng/mL (guadecitabine). Eight to nine non-zero calibration standards were used for each calibration curve and least-squares linear regression was applied with a weighting factor of 1/x, where x equals the concentration of the analyte. At least 75% of the non-zero calibration standards were within ± 15% of the nominal concentrations, or ± 20% for the LLOQ, in three separate runs for each matrix. Therefore, the calibration curves were accepted.

3.2.2. Accuracy and precision

QCs were prepared at four different concentration levels (QC LLOQ, QC Low, QC Mid, QC High) for each matrix. Five QC replicates at each concentration level were analyzed in three separate runs.

Accuracy values (expressed as the bias) and precision values (expressed as the coefficient of variation (CV)) were within ± 15% of the nominal concentration, or ± 20% for the LLOQ. Results are presented in Table 2 and from these data it can be concluded that the assays for all three matrices met the criteria for accuracy and precision.

3.2.3. Specificity and selectivity

To assess the selectivity of the assay, double blank and LLOQ spiked samples in THU-stabilized plasma and whole blood, and urine were prepared and processed using six different batches of each matrix. For both analytes, at least 2/3 of the samples were within ± 20% of their nominal concentrations in all three matrices. In processed double blank samples, no interferences at the retention time of the analytes with areas > 20% (or > 5% for the IS) of the LLOQ were observed. Based on these results, the criteria for endogenous interferences were met.

THU-stabilized plasma and whole blood and blank urine samples were separately spiked with guadecitabine and β-decitabine at their respective ULOQ, and with IS only (if applicable), to test for cross-analyte interference. Cross-analyte interference was not observed (0%) for any of the analytes or IS in all three matrices.

3.2.4. Dilution integrity

Five replicates of the samples with analyte concentrations around 2 times the ULOQ were prepared in K_2EDTA plasma and whole blood spiked with 0.1 mg/mL THU and blank urine and diluted 10 and 100 (urine only) times with blank matrix. In all three matrices the bias was within ± 15% and the CV were ≤ 15% for both analytes. Therefore, clinical samples exceeding the ULOQ can be diluted 10 times for all three matrices, as well as a 100 times for urine.

3.2.5. Lower limit of quantification

To assess the lower limit of quantification, the absolute signal at the LLOQ was compared to the signal in a blank sample for each analyte in all three matrices. For each matrix, the signal at the LLOQ level was at least 5 times as high as the signal in the blank sample. For guadecitabine, the signal-to-noise ratio was at least 6, 18 and 26 in THU-stabilized plasma and whole blood, and urine, respectively. For β-decitabine, the ratio was at least 5, 13 and 15 in THU-stabilized plasma and whole blood, and urine, respectively. Representative chromatograms of blank and LLOQ samples can be found in Figs. 2 and 3.

3.2.6. Matrix effect

Six different batches of THU-stabilized human K_2EDTA plasma and whole blood and blank urine, as well as neat solution, were spiked with guadecitabine and β-decitabine at QC Low and QC High concentration levels. The matrix factor (MF) was calculated by dividing the peak area in matrix present sample to the peak area in neat solution (matrix absent). Furthermore, the IS-normalized MF was calculated for β-decitabine and ranged from 1.01 to 1.12 in plasma, 0.94 to 1.02 in whole blood, and 0.96 to 1.10 in urine. The IS-normalized MF for guadecitabine ranged from 1.11 to 2.06 in plasma and from 0.98 to 1.00 in whole blood. The absolute MF for β-decitabine ranged from 1.69 to 2.07 in plasma, 0.93 to 1.14 in whole blood, and 1.48 to 1.68 in urine, indicating ion enhancement in plasma and urine. The absolute MF for guadecitabine ranged from 1.24 to 1.87 in plasma, 0.15 to 0.48 in whole blood, and 1.02 to 1.27 in urine, indicating ion enhancement in plasma and urine, but significant ion suppression in whole blood, however, these matrix effects could be compensated by the use of a stable isotopically labeled IS.

The CV of the IS-normalized MF for the six batches of guadecitabine spiked THU-stabilized plasma and whole blood was lower than 4.7% and 0.9%, respectively. For the six batches of β-decitabine spiked THU-stabilized plasma and whole blood, and urine, the IS-normalized MF was lower than 1.8%, 3.4%, and 4.5, respectively. The CV of the IS-normalized MF was ≤15% for both analytes, at all tested concentration levels in all matrices were an IS was used. Based on these results, it was concluded that the matrix has no effect on the precision of the methods for all three matrices.

3.2.7. Carry-over

For each matrix, two double blank samples were injected directly after an ULOQ sample to determine carry-over effects for both analytes.

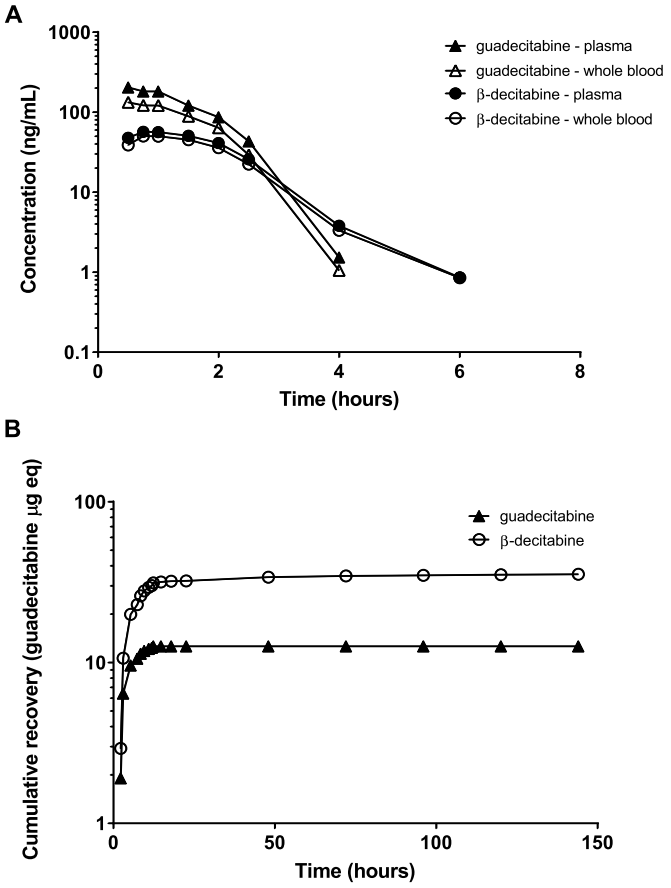

Fig. 4. Representative concentration-time curves of (A) guadecitabine & β-decitabine in plasma and whole blood and (B) cumulative recovery of guadecitabine and β-decitabine (converted to guadecitabine microgram equivalents) in urine in a patient receiving a subcutaneous injection of 45 mg/m^2 guadecitabine.

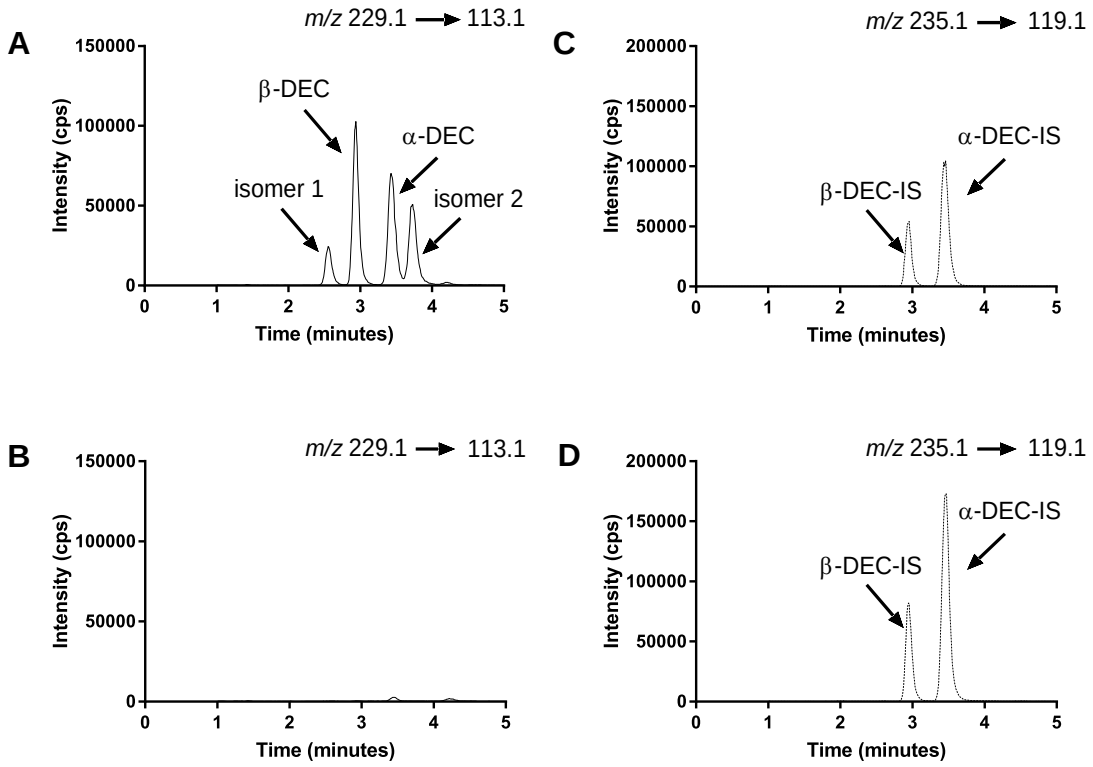

Fig. 5. MRM chromatograms of a patient urine sample collected approximately (A) 4-hours and (B) 48-hours after administration of subcutaneous guadecitabine 45 mg/m^2 monitoring (A, B) decitabine (*m/z* 229.1 ➔ 113.1) and (C, D) decitabine-IS (*m/z* 235.1 ➔ 119.1) mass transitions.

In all samples, the first double blank response showed that no peaks (0% of the LLOQ) were present at the retention time of guadecitabine and β-decitabine in plasma, whole blood and urine, indicating that there is no carry-over effect for both analytes in all three matrices.

3.2.8. Stability

Stability experiments for all three matrices were performed using separately spiked stock solutions and working solutions for both guadecitabine and β-decitabine.

Short-term stability of β-decitabine in stock solution was tested at −20 °C (storage condition), and at ambient temperature (processing condition), at different time points. Stored solutions were compared to freshly prepared stock solutions and considered stable when the deviation from the fresh stock was within ± 5%. β-decitabine stock solutions were found stable for at least 167 days at −20 °C and for at least 4 h at room temperature.

Guadecitabine was provided as a diluted stock solution including validated storage conditions at −20 °C, therefore stock stability experiments were only performed at ambient temperature. Guadecitabine stock solution was found stable for at least 4 h at ambient temperature. Guadecitabine and β-decitabine working solutions were stable for at least 45 days at −20 °C and for at least 4 h at ambient temperature.

Long-term stability in biomatrix was established up to 12 months for both analytes in all three matrices at −70 °C. Short-term stability data for both analytes in (processed) biomatrix are presented in Table 3. Stored and processed samples were compared to freshly prepared QCs at a High and Low concentrations level and considered stable when the recovery was ± 15% of the initial concentration.

4. Clinical application

The presented method was developed and validated to support a clinical mass balance study (EudraCT 2015-003083-36), where patients receive guadecitabine on five consecutive days, with the last dose being radiolabeled ^{14}C-guadecitabine. In this study, THU-stabilized plasma and whole blood, and urine samples were collected during multiple days. All samples were processed and analyzed for guadecitabine and β-decitabine concentrations using the validated bioanalytical methods presented in this article. A representative plasma/whole blood concentration-time curve and an excretion profile in urine resulting from pharmacokinetic analysis in one of the trial participants are presented in Fig. 4. The pharmacokinetic curves demonstrate the rapid absorption of guadecitabine and subsequent in-vivo conversion into β-decitabine. Excretion of unchanged guadecitabine and β-decitabine is low (< 1%), and takes place mostly during the first 24 h after administration.

Using the validated method, it was demonstrated in patient samples that metabolites were detected in the β-decitabine transition window. These metabolites were formed over time in biological matrices after administration of guadecitabine. This is most clearly illustrated in a patient urine sample, collected approximately 4 h after administration of guadecitabine (Fig. 5A) versus a sample that was collected approximately two days after the last administration of guadecitabine (Fig. 5B). Fig. 5 demonstrates that the validated method has great selectivity to separate β-decitabine from in-vivo formed isomers of β-decitabine.

5. Conclusion

Two highly sensitive and selective LC-MS/MS methods for the quantification of guadecitabine and β-decitabine in human plasma, whole blood, and urine were successfully validated according to the latest FDA and EMA guidelines. THU was added to plasma and whole blood samples to prevent ex-vivo enzymatic degradation of β-decitabine. The β-decitabine assay was validated in a range of 0.5–100 ng/mL in THU-stabilized plasma and whole blood, and in a range of 5–1000 ng/mL for urine. For guadecitabine, the assay was validated in a range of 1–200 ng/mL in THU-stabilized plasma and whole blood, and in a range of 10–2000 ng/mL for urine. Samples with

concentrations above the ULOQ can reliably be diluted 10 (all matrices) or 100 times (urine only) to quantify the analytes in the validated concentration ranges. The presented assays are currently used to support a clinical guadecitabine mass balance trial (EudraCT 2015–003083-36).

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