



Development, validation, and clinical application of a high-performance liquid chromatography-tandem mass spectrometry assay for the quantification of total intracellular β -decitabine nucleotides and genomic DNA incorporated β -decitabine and 5-methyl-2'-deoxycytidine

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ABSTRACT

DNA hypermethylation is an epigenetic event that is commonly found in malignant cells and is used as a therapeutic target for β -decitabine (β -DEC) containing hypomethylating agents (eg Dacogen[®] and guadecitabine). β -DEC requires cellular uptake and intracellular metabolic activation to β -DEC triphosphate before it can get incorporated into the DNA. Once incorporated in the DNA, β -DEC can exert its hypomethylating effect by trapping DNA methyltransferases (DNMTs), resulting in reduced 5-methyl-2'-deoxycytidine (5mC) DNA content. β -DEC DNA incorporation and its effect on DNA methylation, however, have not yet been investigated in patients treated with β -DEC containing therapies. For this reason, we developed and validated a sensitive and selective LC–MS/MS method to determine total intracellular β -DEC nucleotide (β -DEC-XP) concentrations, as well as to quantify β -DEC and 5mC DNA incorporation relative to 2'-deoxycytidine (2dC) DNA content. The assay was successfully validated according to FDA and EMA guidelines in a linear range from 0.5 to 100 ng/mL (β -DEC), 50 to 10,000 ng/mL (2dC), and 5 to 1,000 ng/mL (5mC) in peripheral blood mononuclear cell (PBMC) lysate. An additional calibrator at a concentration of 0.1 ng/mL was added for β -DEC to serve as a limit of detection (LOD). Clinical applicability of the method was demonstrated in patients treated with guadecitabine. Our data support the use of the validated LC–MS/MS method to further explore the intracellular pharmacokinetics in patients treated with β -DEC containing hypomethylating agents.

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Abbreviations: 2dC, 2'-deoxycytidine; 5mC, 5-methyl-2'-deoxycytidine; ACN, acetonitrile; β -DEC, β -decitabine; β -DEC-XP, β -decitabine mono-, di- and triphosphate; CpG, 5'-cytosine-phosphate-guanosine-3'; CV, coefficient of variation; DFAM, deferoxamine mesylate; DMSO, dimethyl sulfoxide; DNMT, DNA methyltransferase; DP, diphosphate; EDTA, ethylenediaminetetraacetic acid; IS, internal standard; LLOQ, lower limit of quantification; LOD, limit of detection; MeOH, methanol; MP, monophosphate; MRM, multiple reaction monitoring; m/z, mass-to-charge ratio; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; QC, quality control sample; THU, tetrahydrouridine; TP, triphosphate.

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1. Introduction

Aberrant DNA methylation of cytosine residues within 5'-cytosine-phosphate-guanosine-3' (CpG) dinucleotides has been described in cancer cells, and might contribute to tumorigenesis by blocking the expression of tumor suppressor genes [1,2]. The process of DNA methylation is regulated by DNA methyltransferases (DNMTs), which form a therapeutic target for hypomethylating agents such as β -decitabine (β -DEC, Dacogen[®], Fig. 1), azacitidine, and guadecitabine. Guadecitabine, a dinucleotide of β -decitabine (β -DEC) and deoxyguanosine, is a novel nucleoside analogue

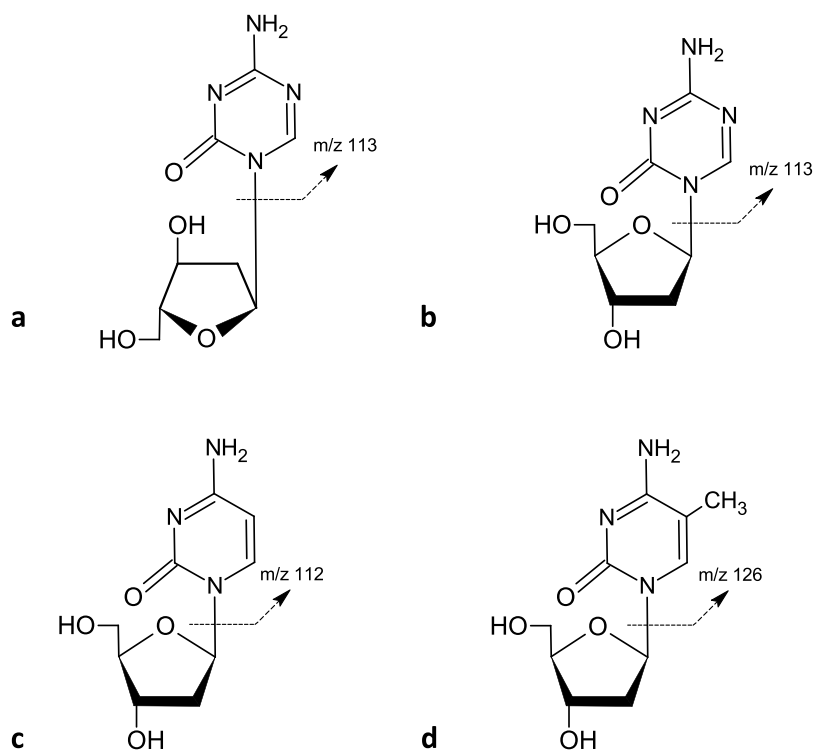


Fig. 1. Chemical structures of (a) α -decitabine (α -DEC), (b) β -decitabine (β -DEC), (c) 2'-deoxycytidine (2dC), and (d) 5-methyl-2'-deoxycytidine (5mdC), with proposed location of fragmentation.

hypomethylating agent that is under clinical evaluation for the treatment of hematological malignancies and solid tumors [3].

After release of β -DEC from guadecitabine via enzymatic cleavage, β -DEC undergoes subsequent intracellular conversion into its mono-, di-, and triphosphate nucleotide (β -DEC-MP,-DP,-TP, Fig. 2a). The ultimate active metabolite β -DEC-TP is in competition with endogenous 2'-deoxycytidine (2dC, Fig. 1) triphosphate (2dC-TP) for incorporation into DNA. Once incorporated, adducts are formed between DNA incorporated β -DEC and DNMT. These adducts are degraded by the proteasome, after which the DNA is restored. DNA synthesis is then resumed in the absence of DNMT, resulting in a passive loss of methylation [4]. Global DNA methylation can be monitored by analysis of 5-methyl-2'-deoxycytidine (5mdC, Fig. 1) DNA content. This biomarker holds potential for becoming a valuable tool in monitoring efficacy of β -DEC containing therapies [2]. Intracellular β -DEC nucleotide concentrations can be monitored as the ultimate active metabolite β -DEC-TP only, or as a total concentration of β -DEC-MP, -DP, and -TP (DEC-XP).

At the moment, research is still ongoing to find the optimal dosing regimen for β -DEC containing therapies [5,6]. In order to optimize these therapies, a predictable correlation between drug exposure and efficacy with acceptable safety is required. Unfortunately, plasma concentrations of β -DEC are not predictive of tumor site activity due to multiple activation steps required prior to manifestation of the pharmacodynamic effect. Based on the mechanism of action, the most direct technique would be to measure intracellular β -DEC-XP concentrations and β -DEC and 5mdC incorporation in tumor DNA.

Previously published work, including our own, suggests a relationship between DEC-TP levels in peripheral blood mononuclear cells (PBMCs) and response to therapy [7,8]. Unfortunately, the LC-MS/MS assay that is used for DEC-TP analysis is limited because of the large sample volumes (15–20 mL of whole blood) required to obtain acceptable lower limits of quantification (LLOQ) and more importantly, because of endogenous 2dC-TP interfering with the

quantification of DEC-TP levels [7]. Although a correction factor can be used to account for this bias, the LLOQ is affected negatively. Furthermore, it is uncertain if DEC-TP levels in PBMCs are predictive of β -DEC DNA incorporation and clinical effect.

DEC DNA incorporation content has been investigated in human cell lines before by making use of a radiolabel [9]. Recently, the direct measurement of DEC genomic DNA incorporation, combined with a global DNA methylation assessment in human cell lines has been described [10]. This LC-MS/MS assay has been utilized in pre-clinical experiments, but its sensitivity was deemed insufficient for use in clinical trials. Furthermore, the assay provides no separation of β -DEC from the inactive anomer α -DEC, which might be of clinical importance [11,12]. More recently, the intracellular dynamics of DEC were successfully described in patients treated with azacitidine [13]. However, this method makes use of a high resolution mass spectrometer, which is less sensitive than a triple quadrupole mass spectrometer and is less commonly used in clinical laboratories for routine drug monitoring.

To date, all published methods to clinically assess the intracellular pharmacokinetics of β -DEC are limited for at least one of two reasons: 1) insufficient selectivity to separate β -DEC from the inactive anomer α -DEC and from interfering endogenous nucleosides such as 2dC, and 2) insufficient sensitivity to analyze intracellular β -DEC-XP and DNA-incorporated β -DEC in patient samples. Furthermore, no assays have been described in literature that can assess both intracellular pharmacokinetics and pharmacodynamics, i.e. quantify the total intracellular β -DEC-XP concentration as well as genomic DNA incorporated β -DEC and 5mdC content, at the same time.

For this reason, we developed and validated a new, more sensitive and selective assay for the quantitative determination of intracellular β -DEC-XP in human PBMCs and genomic DNA-incorporated β -DEC and 5mdC in whole blood. Global DNA methylation grade, expressed as the 5mdC content per thousand 2dC (DNA-incorporated ratio), may be used as a pharmacodynamic

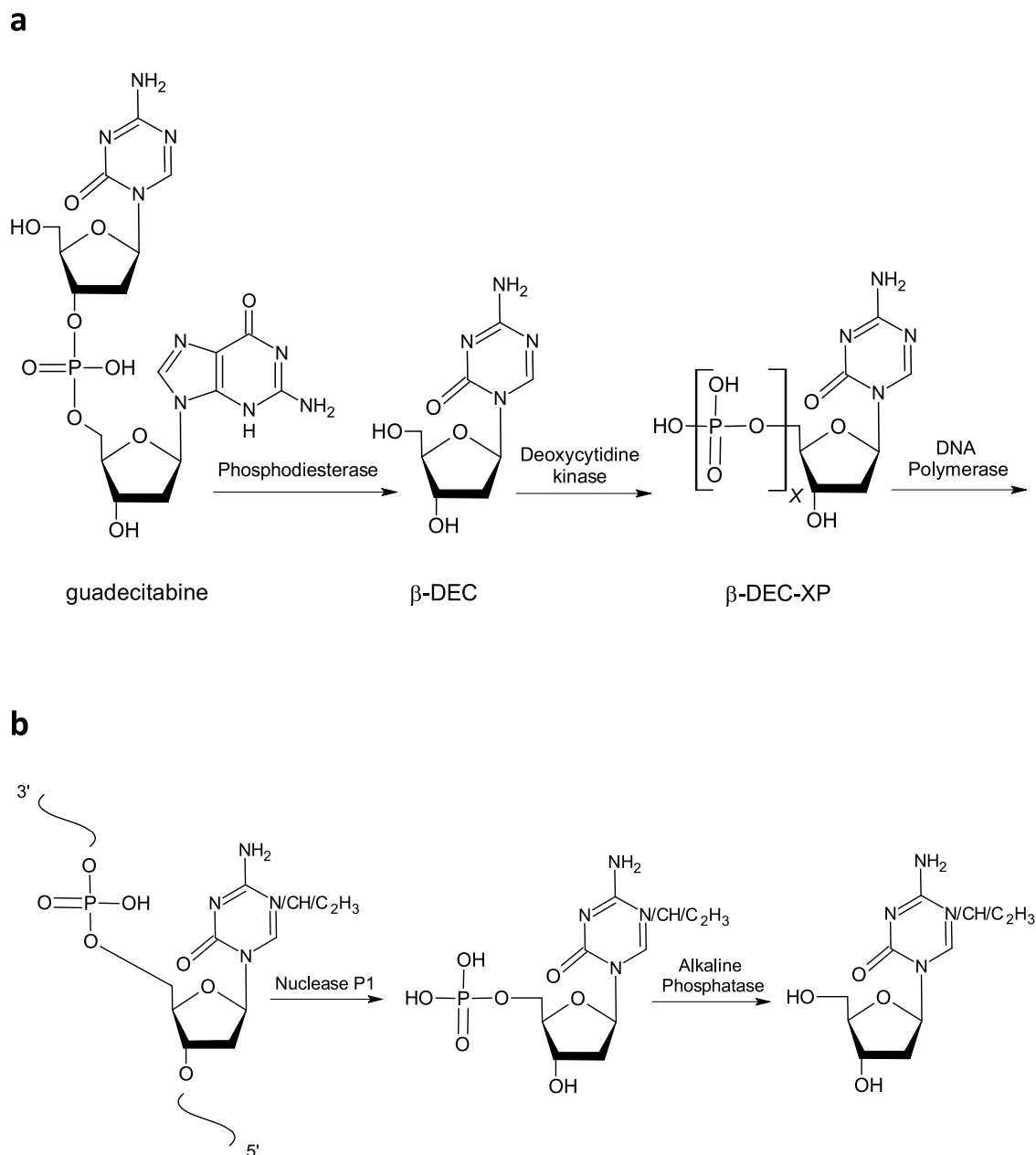


Fig. 2. (a) Subsequent in-patient enzymatic conversion steps of guadecitabine to β -decitabine (β -DEC) to DNA incorporated β -DEC ($X=1, 2,$ or 3 for mono-, di-, and triphosphate, respectively) and (b) the enzymatic degradation of DNA incorporated β -DEC (N), 2dC (CH), or 5mdC (C_2H_3) monophosphate to their respective nucleosides during sample processing.

marker to assess the hypomethylating effect of β -DEC containing therapies. Proof of clinical applicability was provided by the quantification of intracellular β -DEC-XP, as well as β -DEC and 5mdC genomic DNA incorporation relative to the 2dC content in cancer patients treated with guadecitabine as part of a clinical mass balance trial conducted at our institute.

2. Materials and methods

2.1. Materials

All reference standard analytes purchased had a purity greater than 98%. 5mdC [$^{13}\text{C},^{15}\text{N}_2$], 5mdC-d3 and α -DEC were purchased from Toronto Research Chemicals (Toronto, ON, Canada). β -DEC was purchased from AlsaChim (Illkirch, France). 2dC originated from Sigma-Aldrich (St. Louis, MO, USA). DEC [$^{13}\text{C}_2,^{15}\text{N}_4$] was

purchased from Asclep Pharmard (Newark, DE, USA). A reference standard containing a mixture of DEC mono-, di-, and triphosphate (DEC-XP) was synthesized in-house as described by Jansen et al. [14]. ULC-grade acetonitrile (ACN), water, and ammonium formate, and HPLC-grade methanol (MeOH) and isopropylalcohol were purchased from Biosolve Ltd (Valkenswaard, The Netherlands). Dimethyl sulfoxide (DMSO) and formic acid were from Merck (Darmstadt, Germany). Ammonium acetate, deferoxamine mesylate (DFAM), Ficoll paque plus, phosphate buffered saline (PBS), Tris(hydroxymethyl)aminomethane (Trizma[®] base), zinc chloride, ethylenediaminetetraacetic acid (EDTA), alkaline phosphatase, and nuclease P1 were all of molecular biological grade or higher and were obtained from Sigma-Aldrich (St. Louis, MO). K_2EDTA whole blood was obtained from the Medical Center Slotervaart (Amsterdam, The Netherlands). Buffy coats for PBMC isolation were purchased from Sanquin (Amsterdam, The Netherlands).

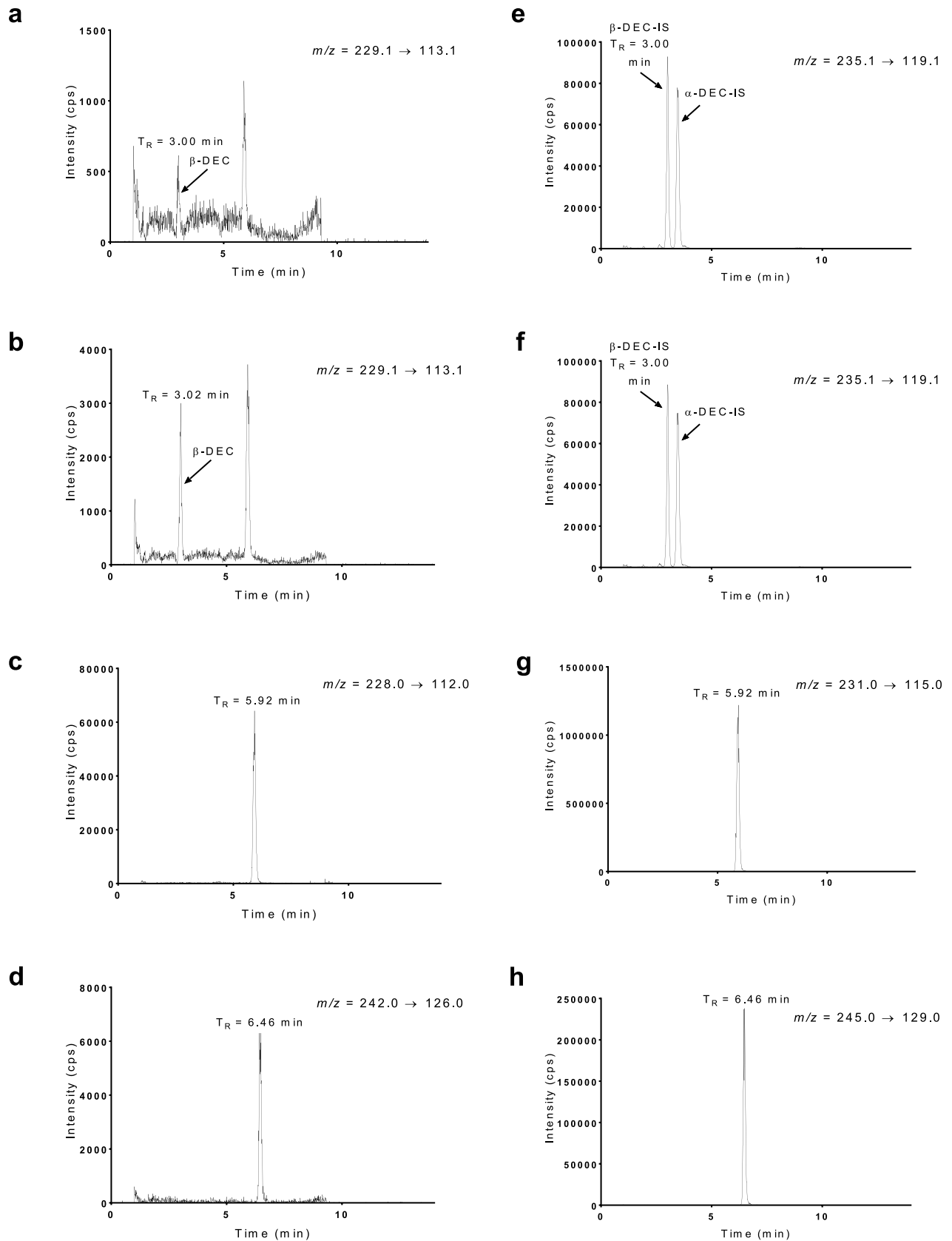


Fig. 3. Representative ion chromatograms of β -decibabine (β -DEC) spiked at (a) the LOD of 0.1 ng/mL, (b) β -decibabine spiked at the LLOQ of 0.5 ng/mL, (c) 50 ng/mL 2'-deoxycytidine, (d) 5 ng/mL 5-methyl-2'-deoxycytidine and (e-h) internal standards monitoring decibabine [$^{13}\text{C}_2, ^{15}\text{N}_4$], 2'-deoxycytidine [$^{13}\text{C}, ^{15}\text{N}_2$] and 5-methyl-2'-deoxycytidine-d3 in PBMC lysate.

Table 1
General and analyte specific mass spectrometric settings.

General settings						
Run duration (min)	14					
Ion spray voltage (V)	5500					
Ion Source Gas 1 (au)	80					
Ion Source Gas 2 (au)	20					
Curtain gas (au)	45					
Collision gas (au)	10					
Temperature (°C)	500					
Dwell time (msec)	50					
	Decitabine	Decitabine-IS	2'-deoxycytidine	2'-deoxycytidine-IS	5-methyl-2'-deoxycytidine	5-methyl-2'-deoxycytidine-IS
Parent mass	229.1	235.1	228	231	242	245
Product mass	113.1	119.1	112	115	126	129
Collision energy (V)	21	21	21	21	21	21
Collision exit potential (V)	14	14	14	14	14	14
Declustering potential (V)	96	96	96	96	96	96
Retention time (min)	3	3	5.92	5.92	6.46	6.46

2.2. Instrumentation

For quantification, the LC–MS system used consisted of a QTRAP 5500 tandem mass spectrometer (Sciex, Framingham, MA, USA) coupled to an HPLC Acquity I Class pump (Waters, Milford, MA, USA). The HPLC system was equipped with a FTN I-Class autosampler and a I-Class column oven (Waters). Data acquisition was performed using Analyst 1.6.2. software (Sciex).

2.3. Chromatography

Chromatographic separation of β -DEC, α -DEC, 2dC, and 5mdC (Fig. 1) was carried out using a Waters Nova-Pak Silica column (150 x 3.9 mm, 4 μ m). Analytes were separated using gradient elution with 5 mM ammonium acetate in water (mobile phase A) and 5 mM ammonium acetate in water – ACN (2:98, v/v) (mobile phase B). A flow rate of 1.4 mL per minute was applied and the column oven was set to 30 °C. The elution gradient was as follows: mobile phase B: 97% (0–3.2 minutes), 97 → 90% (3.2–7.2 minutes), 90 → 2% (7.2–7.3 minutes), 2% (7.3–11.3 minutes), 2 → 97% (11.3–11.4 minutes), 97% (11.3–14.0 minutes). The divert valve was set in place to direct the flow to the mass spectrometer from 2.0 to 7.5 minutes and to the waste for the remainder of the acquisition time to protect the MS from contaminants.

2.4. Mass spectrometry

The MS (Sciex QTRAP 5500) was operated in the positive electrospray ionization mode by multiple reaction monitoring (MRM), using the following mass-to-charge ratio (m/z) transitions: 229.1 → 113.1 for DEC, 235.1 → 119.1 for DEC [$^{13}\text{C}_2, ^{15}\text{N}_4$], 228.0 → 112.0 for 2dC, 231.0 → 115.0 for 2dC [$^{13}\text{C}^{15}\text{N}_2$], 242.0 → 126.0 for 5mdC, and 245.0 → 129.0 for 5mdC-d3. Source and gas parameters were optimized for the DEC transition window, according to Table 1.

2.5. Blank PBMC and DNA isolation

Blank PBMC lysates were prepared from six batches of donor buffy coat individually as previously described [7]. In short, PBMCs were isolated from human leukocyte buffy coat (~50 mL, freshly derived from 500 mL whole blood), 200 mL PBS was added to the buffy coat, and 25 mL aliquots of this suspension were each carefully added to 12.5 mL of high density centrifugation medium ficoll paque plus. After a 20 min 550 x g centrifugation at 4 °C (without break), the interface containing the PBMCs was transferred to a clean tube. Subsequently, the PBMCs of each aliquot were washed with 35 mL PBS and centrifuged at 1500 x g for 5 min at 4 °C.

The supernatant was discarded and the pellet was resuspended in 300 μ L PBS. All aliquots were pooled (for each batch separately) and a cell count was performed with a Cell Dyn Hematology analyzer (Abbott Diagnostics, Lake Forest, IL). After a 3000 x g centrifugation, PBS was removed or added to adjust the PBMC concentration to approximately 150×10^6 cells, close to the mean found in cancer patient samples from the guadecitabine mass balance trial (unpublished data). Depending on the final volume of blank PBMCs in PBS, a volume of methanol was subsequently added to obtain PBMC lysate in methanol – PBS (62.5:37.5, v/v).

Genomic DNA was extracted from 400 μ L tetrahydrouridine (THU)-stabilized human whole blood using the QIAamp DNA Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol.

2.6. Sample preparation

Stock solutions were prepared for each analyte independently. The stock solutions for β -DEC, 2dC and 5mdC and all internal standards (IS) were prepared at a concentration of 1.0 mg/mL in DMSO. All stock solutions were stored at –20 °C.

Working solutions were prepared in DMSO and spiked into blank PBMC lysate to obtain calibration standards and quality control (QC) samples. The calibration curve consisted of 8 calibration standards at the following concentrations: 0.5, 1, 5, 12.5, 25, 50, 80, 100 ng/mL for β -DEC; 50, 100, 500, 1250, 2500, 5000, 8000 and 10,000 ng/mL for 2dC; and 5, 10, 50, 125, 250, 500, 800 and 1000 ng/mL for 5mdC. For β -DEC, an extra calibration standard was added at 0.1 ng/mL to serve as a limit of detection (LOD) level. The QC samples were made at 4 different concentration levels for validation: a lower limit of quantification (LLOQ), low, mid and high QC levels. The QCs were made at the following concentration levels: 0.5, 1.5, 12.5 and 75 ng/mL for β -DEC; 50, 150, 1250 and 7500 ng/mL for 2dC; and 5, 15, 125 and 750 ng/mL for 5mdC. Blank calibration standards (with and without addition of the IS) were also analyzed to check for potential endogenous interferences and potential conversion of the IS to their non-labelled forms (analytes). These calibration standards were not included in the calibration curve and were made from blank digest PBMC lysate.

2.7. Method validation

The validation of this method includes the establishment of the calibration model, accuracy and imprecision, specificity and selectivity, LLOQ and LOD, matrix effect and recovery, carryover and stability under various conditions. All acceptance criteria described

in the latest FDA and EMA guidelines on bioanalytical method validation were followed [15,16].

2.8. Patient sample collection and processing - PBMCs

We quantified intracellular β -DEC-XP concentrations in PBMCs isolated from THU-stabilized whole blood collected from four cancer patients participating in a guadecitabine mass balance trial (EudraCT 2015-003083-36), conducted at our institute. Patients were treated with a dose of 45 mg/m² guadecitabine once daily from day 1–5 of a 28-day treatment cycle via subcutaneous injection. Whole blood samples from Day 6 (24 h post Day 5 injection) of cycle 1 were used for PBMC isolation and the analysis of intracellular β -DEC-XP concentrations.

PBMCs were isolated from 20 mL THU-stabilized human whole blood and counted using a Cell Dyn Hematology analyzer (Abbott Diagnostics, Lake Forest, IL). The PBMCs were lysed subsequently using the procedure described by Jansen et al. [7]. To 100 μ L of PBMC lysate, 10 μ L IS (1000 ng/mL DEC [¹³C₂, ¹⁵N₄] and 5mdC-d₃, and 10,000 ng/mL 2dC [¹³C¹⁵N₂] in DMSO) was added, after which samples were vortex-mixed for 10 s. If available, 20 μ L of the sample was used for β -DEC analysis prior to dephosphorylation. The rest of the sample was evaporated to dryness at 40 °C using a gentle stream of nitrogen and reconstituted in 100 μ L of 5 mM ammonium acetate in water by vortex-mixing for 1 min. To each sample, 20 μ L of Trizma[®] base, pH 8.5 and 4 units of alkaline phosphatase were added. Samples were then vortex mixed for 10 s, followed by incubation for 1 h at 37 °C, and evaporated to dryness at 40 °C using a gentle stream of nitrogen. To the dried samples, 50 μ L of mobile phase B was added and samples were vortex mixed for approximately 1 min. For each sample, 10 μ L was injected onto the LC–MS/MS system.

The amount of DEC-TP was quantified as well using the previously developed method by Jansen et al. [7]. For this, PBMCs were lysed and injected onto the LC–MS/MS system directly after adding a separate IS.

2.9. Patient sample collection and processing – genomic DNA

From the same patients genomic DNA was extracted from 400 μ L THU-stabilized human whole blood using the QIAamp DNA Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. The amount of isolated DNA per sample was quantified using a NanoDrop[®] ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Whole blood samples from predose, Day 1 (2 h post injection), Day 5 (2 h post injection), and Day 6 (24 post Day 5 injection) were used for DNA isolation and analysis of genomic DNA-incorporated β -DEC and 5mdC, relative to the amount of 2dC incorporated in the DNA.

Genomic DNA eluted in 200 μ L of distilled water was first digested into single nucleotides by adding 4 units of Nuclease P1 (Sigma-Aldrich, St. Louis, MO) and 100 μ L of digest buffer (containing 0.04 mM DFAM, 3.25 mM ammonium acetate pH 5.0 and 0.5 mM zinc chloride) and incubated at 65 °C for 10 min. Following incubation, the nucleotides were converted to nucleosides by adding 20 μ L of 100 mM Trizma[®] base, pH 8.5 and 4 units of alkaline phosphatase (Roche Life Science, Indianapolis, IN) and incubated at 37 °C for 1 h. To stop the digest, 20 μ L of 300 mM ammonium acetate, pH 5.0 was added to the sample. To each sample, 10 μ L of IS (1000 ng/mL DEC [¹³C₂, ¹⁵N₄] and 5mdC-d₃, and 10,000 ng/mL 2dC [¹³C¹⁵N₂] in DMSO) was added, after which the sample was vortex mixed and evaporated to dryness at 40 °C using a gentle stream of nitrogen. Samples were reconstituted in 50 μ L of mobile phase B by vortex-mixing for 1 min. Samples were centrifuged for 5 min at 15,000 rpm,

5 °C, and transferred to an autosampler vial. For each sample 10 μ L was injected onto the LC–MS/MS system.

2.10. Quantification of intracellular β -DEC-XP and genomic DNA incorporated β -DEC and 5mdC

β -DEC-XP, meaning the total amount of β -DEC mono-, di-, and triphosphate, was quantified as β -DEC after sample dephosphorylation, and expressed as a concentration in femtomol per million PBMCs. The DEC-TP concentrations, quantified using the assay of Jansen et al. [7], were also expressed in femtomol per million PBMCs.

Ratios of β -DEC/2dC and 5mdC/2dC were determined for each digested DNA sample. β -DEC, 2dC, and 5mdC concentrations were quantified within the validated calibration range after sample processing. Using the obtained concentrations, molar ratios were calculated and expressed as the amount of β -DEC or 5mdC per thousand 2dC (DNA incorporated ratio).

3. Results and discussion

3.1. Method development

The LC–MS/MS method was developed with the aim to quantify intracellular β -DEC-XP concentrations after nucleotide to nucleoside conversion in PBMC lysate, and to quantify β -DEC, 2dC, and 5mdC in digested genomic DNA isolated from whole blood.

DEC exists in two forms: the active anomer β -DEC, and the inactive anomer α -DEC. After administration of β -DEC, interconversion can take place under physiological conditions where β -DEC is converted into α -DEC, indicating the need to develop a selective assay [11,12]. Separation of α - and β -DEC was achieved using a Silica Nova-Pak column. By using a mixture of stable isotope-labeled α - and β -DEC IS, separation of the anomers was demonstrated during each run (Fig. 3). Reference standards of α - and β -DEC were used to confirm the order of elution of the anomers during the method development.

As the molecular structures and masses of β -DEC and 2dC are highly similar (Fig. 1), isotope interference occurs, and therefore complete chromatographic separation of the analytes is required. Endogenous interferences were encountered in our previously developed DEC-TP assay, limiting its LLOQ [7]. By changing the sample processing method and chromatographic system, successful separation of the analytes was achieved (Fig. 3). β -DEC-XP was dephosphorylated using alkaline phosphatase, making it possible to quantify the intracellular β -DEC-XP level with increased selectivity, an approach that has been used successfully in the past [17]. By analyzing the nucleoside content prior to the dephosphorylation step, the intracellular β -DEC concentration can be distinguished from the β -DEC-XP concentration. From previous work it is already known that DEC-TP is the main intracellular nucleotide in patients treated with β -DEC [7]. Quantification of total β -DEC-XP concentrations may serve as a novel way to express the intracellular pharmacokinetics of intracellular β -DEC nucleotides, without limiting the LLOQ by endogenous interferences.

For the analysis of genomic DNA-incorporated β -DEC and 5mdC, the sample processing method consists of sequential degradation steps (Fig. 2b). In the first step, isolated DNA is degraded into single monophosphate nucleotides. During the second step, the nucleotides are converted into nucleosides by cleavage of the phosphate bond. By expressing the DNA content of β -DEC and 5mdC to the amount of the endogenous nucleoside 2dC, a molar ratio could be determined that is independent of the total amount of isolated and digested DNA within the validated range.

Table 2
Assay performance data for the analysis of β -decitabine (β -DEC), 2'-deoxycytidine (2dC), and 5-methyl-2'-deoxycytidine (5mdC) in human PBMC lysate.

Analyte	Nominal concentration (ng/mL)	Intra-assay		Inter-assay	
		Bias (%)	C.V. (%)	Bias (%)	C.V. (%)
β -DEC	0.5	0.2–5.2	5.1–10.8	2.5	– ^a
	1.5	–4.9 to 2.1	1.9–8.5	–0.4	2.9
	12.5	–6.2 to –0.8	2.2–4.9	–3.7	2.4
	75	–4.9 to –1.2	1.0–4.5	–3.2	1.4
2dC	50	–2.4 to 5.0	4.4–5.0	1.9	3.2
	150	3.5–7.5	2.0–7.9	5.8	– ^a
	1250	1.3–9.9	0.9–4.8	5.1	3.9
	7500	–5.2 to 0.9	2.0–6.0	–2.0	2.7
5mdC	5	3.2–8.4	3.1–7.3	6.5	1.6
	10	5.7–6.4	2.0–9.1	6.0	– ^a
	125	2.4–9.0	1.8–12.6	5.2	– ^a
	750	0.6–5.3	2.5–6.0	2.3	1.7

^a MS_{WC} is greater than MS_{BC}: there is no significant additional variation due to the performance of the assay in different runs.

Table 3
Stability data for β -decitabine (β -DEC), 2'-deoxycytidine (2dC), and 5-methyl-2'-deoxycytidine (5mdC) in PBMC lysate and final extract. All experiments were performed in triplicate in QC low and QC high samples.

Analyte	Matrix	Conditions	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Bias (%)	C.V. (%)
β -DEC	final extract	2–8 °C, 4 days	1.5	1.5	0.2	4.8
			75	75	0.0	1.4
	PBMC lysate	0 °C, 4 hours	1.5	1.7	12.0	2.1
			75	82	8.9	2.8
PBMC lysate	–70 °C, 49 days	1.5	1.4	–5.8	2.3	
		75	78	3.6	2.5	
2dC	final extract	2–8 °C, 4 days	150	168.7	12.4	4.4
			7500	7697	2.6	0.9
	PBMC lysate	0 °C, 4 hours	150	172	14.7	2.7
			7500	7757	3.4	1.3
5mdC	final extract	2–8 °C, 4 days	15	16.5	10.0	6.3
			750	796	6.1	3.2
	PBMC lysate	0 °C, 4 hours	15	17.0	13.3	4.2
			750	843	12.4	1.2

During method development, HPLC-grade chemicals were used for the preparation of the eluents but high noise levels were observed in the DEC transition window. To increase the sensitivity of the method, noise levels were successfully reduced by using ULC-MS grade solvents and additives (ACN, water and ammonium formate).

General source and gas parameters were optimized for the low abundant analyte β -DEC to maximize the sensitivity for this analyte. General and analyte specific MS instrument settings are presented in Table 1.

3.2. Method validation

Eight non-zero calibration standards with a concentration range of 0.5–100 ng/mL (β -DEC), 50 – 10,000 ng/mL (2dC), and 5–1000 ng/mL (5mdC) were prepared in blank human PBMC lysate. Linear regression with a weighting factor of $1/x^2$ was applied, where x equals the concentration of the analyte. The calibration curves were acceptable if 75% of all non-zero calibration standards were within $\pm 15\%$ of the nominal concentration, or $\pm 20\%$ for the LLOQ. These acceptance criteria were met in three separate runs, with correlation coefficients of at least 0.995 for all three analytes.

Five replicates of QC LLOQ, QC low, QC mid, and QC high were analyzed in three analytical runs for all three analytes to assess the accuracy and precision of the method. The concentration of each QC sample was calculated using the calibration standards that were analyzed in duplicate in the same analytical run. Table 2 summarizes the results. The difference between the calculated and the nominal concentration was used to determine the bias. The bias was within the acceptance criteria of $\pm 15\%$ for low, mid and high concentration levels and within $\pm 20\%$ for the LLOQ level. The intra-

and inter-assay imprecision (expressed as CV) was for all analytes at each tested concentration level within the criteria ($\leq 15\%$ and $\leq 20\%$ at the LLOQ).

The response at the LLOQ level was at least 5 times the response compared to a blank response in three validation runs for each of the analytes. Fig. 3 shows representative MRM ion chromatograms of QC LOD for β -DEC, and LLOQ samples for all three analytes.

All β -DEC LOD standards had a signal-to-noise ratio of at least 3. To determine concentrations between the LOD and the LLOQ, the LOD could be included in the calibration range. The calibration model (linear fit with a weighting factor of $1/x^2$) remained the same and all calibration standards and QC samples remained within the acceptance criteria. In the application phase of the method it is feasible to include the LOD in the calibration model to quantify β -DEC concentrations between the LLOQ and the LOD as long as the signal-to-noise ratio is at least 3.

To assess endogenous interference for the β -DEC-XP processing method, six different batches of control PBMC lysate were spiked at the LLOQ level and processed together with double blank samples. Single determinations were performed. HPLC-MS/MS ion chromatograms of the blanks and LLOQ samples were monitored and compared for chromatographic integrity and potential interferences. LLOQ samples were within $\pm 20\%$ of their nominal concentrations for at least 2/3 of the samples for all three analytes. No interferences from endogenous material at the retention time of the analytes with areas $>20\%$ (or $>5\%$ for the IS) of the LLOQ and LOD areas were observed in the blanks. Therefore, the tests for endogenous interferences were considered acceptable.

Cross-analyte and IS interference were tested by spiking control human PBMC lysate at ULOQ level with 5mdC, DEC and DEC [$^{13}\text{C}_2$, $^{15}\text{N}_4$] at nominal concentration separately. Cross-analyte

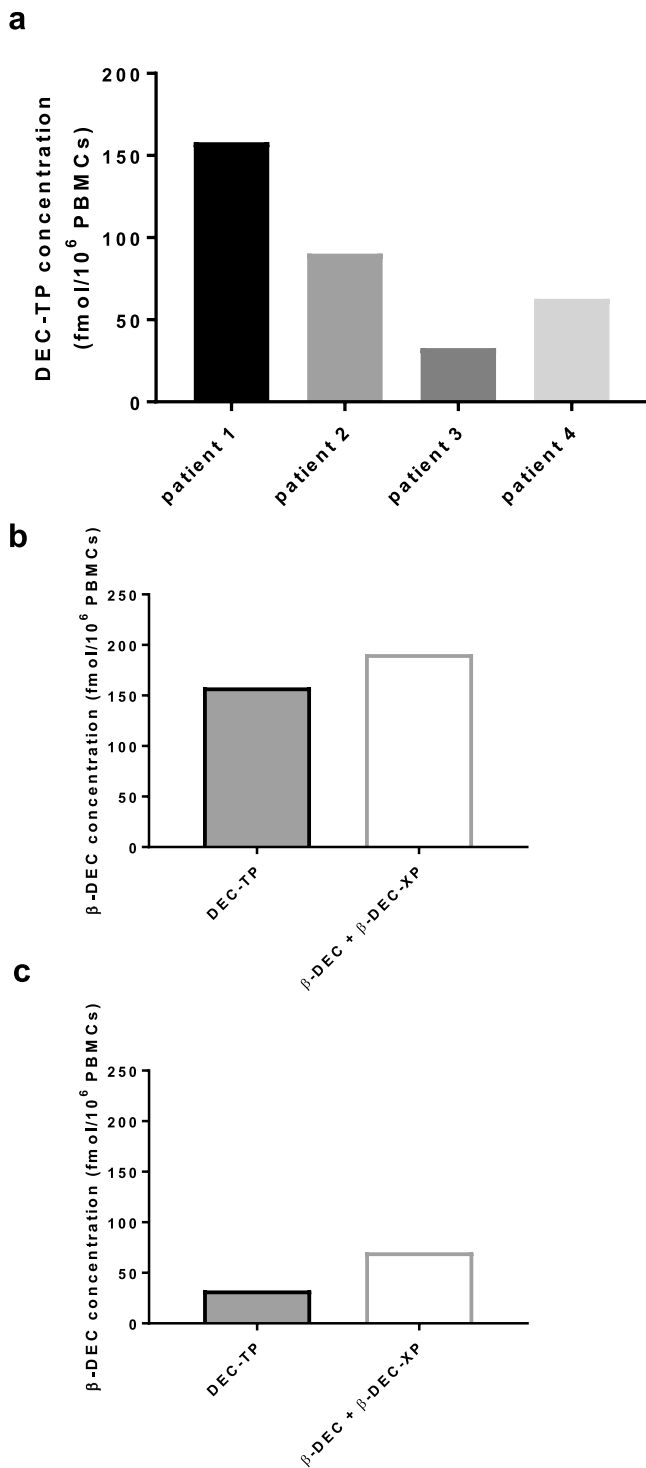


Fig. 4. (a) Individual intracellular PBMC concentrations of decitabine triphosphate (DEC-TP) and (b,c) the amount of DEC-TP (direct analysis) versus β -DEC + β -DEC nucleotides (β -DEC -XP)(analysis after sample dephosphorylation) in (b) patient 1 and (c) patient 3 receiving guadecitabine treatment. Samples were analyzed for Day 6, 24 h post Day 5 injection. All concentrations are expressed in femtomol (fmol) per million PBMCs.

interference of guadecitabine was tested as well, at a concentration of 200 ng/mL, which is over two times the maximum plasma concentration as seen in patients treated with guadecitabine at a dose level of 60 mg/m² [3]. The cross-analyte and IS interference at the retention time of the analytes and IS were $\leq 20\%$ of the peak area of the LLOQ and LOD level. For the IS the interference was $\leq 5\%$. The

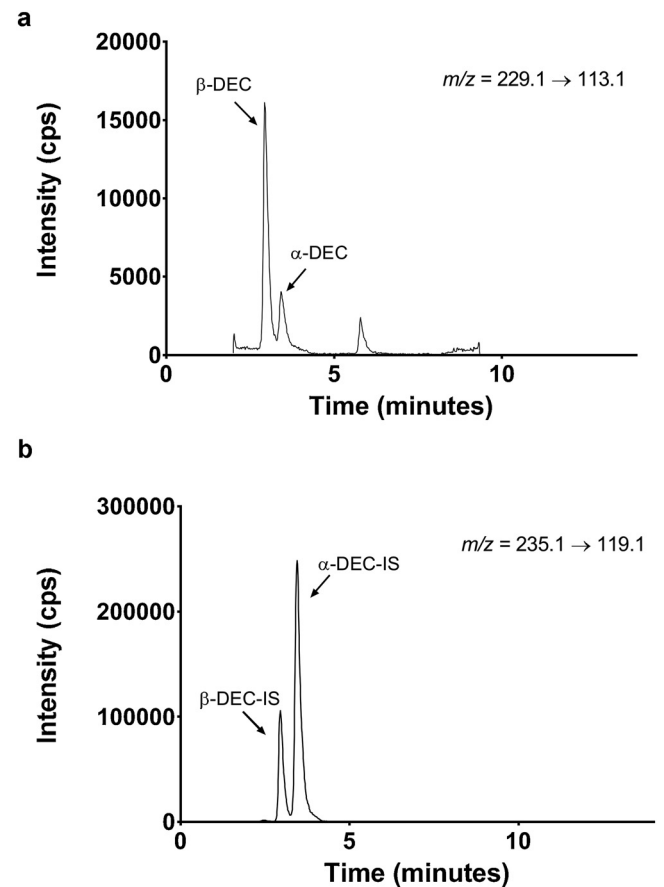


Fig. 5. Representative ion chromatogram monitoring (a) decitabine and (b) decitabine [¹³C₂, ¹⁵N₄] transition windows in PBMC-lysate after sample dephosphorylation from patient 1 after receiving guadecitabine treatment. Chromatograms are shown for Day 6, 24-hour post Day 5 injection.

cross-analyte and IS interference was therefore considered acceptable.

Carry-over was determined by analyzing two processed control PBMC lysate samples after an ULOQ sample in three separate runs. Eluting peaks with areas $>20\%$ or $>5\%$ of the LOD and LLOQ were not observed in the blank samples injected directly after ULOQ samples for all three analytes and IS, respectively, and therefore, the criteria for carry-over were met.

Matrix effects were assessed to determine suppression/enhancement for β -DEC, 2dC and 5mdC in PBMC-lysate and DNA digest matrix (processed water samples). To determine the matrix effects, blank QCs were processed to dried extract and spiked with neat solution at QC mid concentration. These QCs were analyzed next to samples in absence of matrix in triplicate for each experiment. The IS-normalized MF was 1.01, 0.99, and 0.97 for β -DEC, 2dC and 5mdC, respectively. The CV for the IS-normalized matrix factor was $\leq 15\%$ at the tested concentration for all three analytes, for both processing methods. These results show that the IS effectively minimizes the influence of matrix effects for both sample pretreatment procedures.

Recovery was assessed to determine the conversion of β -DEC-XP into β -DEC in PBMC lysate after dephosphorylating the nucleotide mixture with alkaline phosphatase. Based on the concentration and molar weights of β -DEC mono-, di- and triphosphates in a reference mixture (synthesized in-house), it was determined how much β -DEC could theoretically be formed out of the β -DEC-XP mixture. β -DEC concentrations were measured in triplicate at QC mid-level before and after dephosphorylation and recovery was calculated accordingly. After dephosphorylation

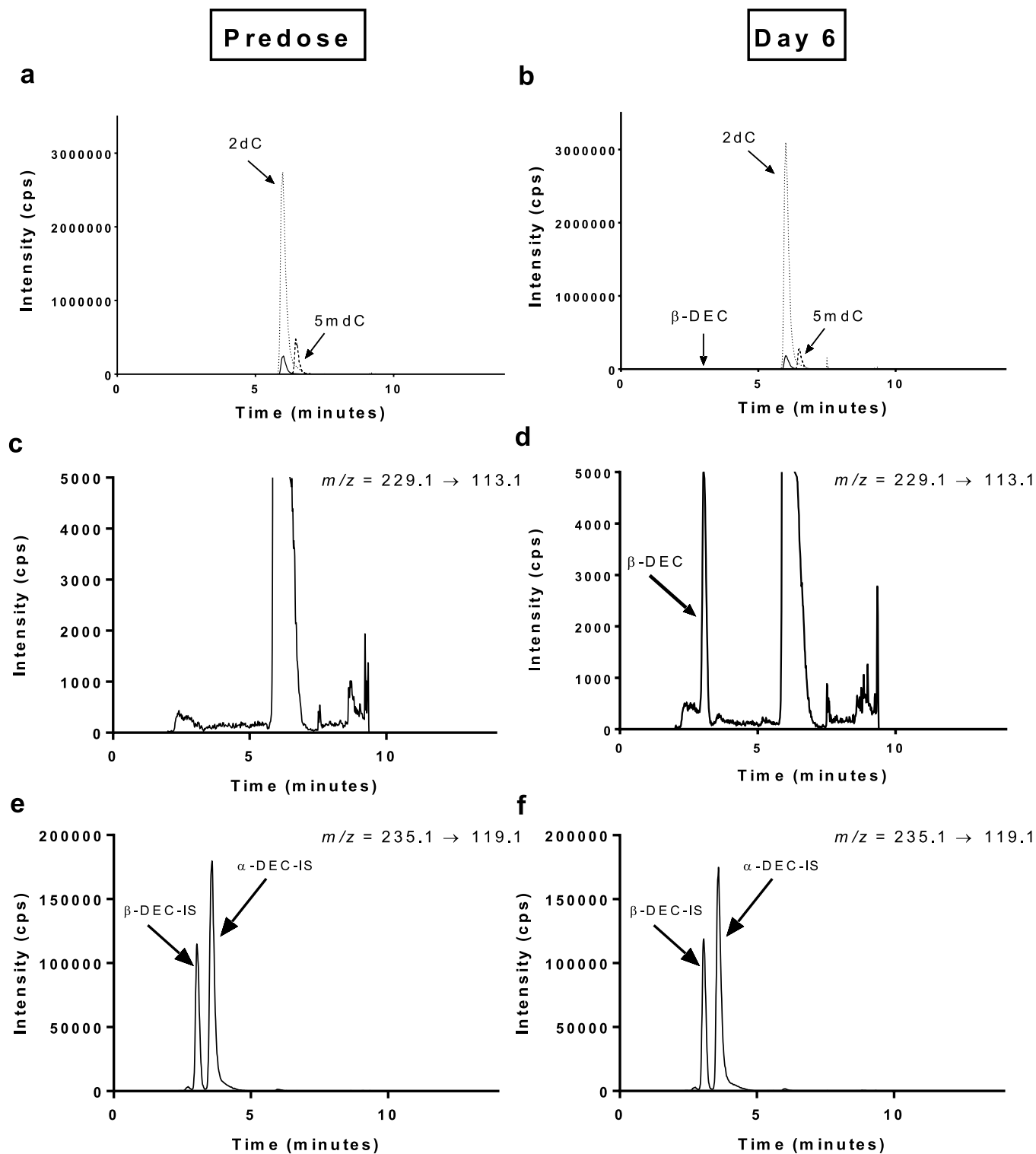


Fig. 6. (a,b) Representative ion chromatograms monitoring decitabine (DEC), 2'-deoxycytidine (2dC), and 5-methyl-2'-deoxycytidine (5mdC) transition windows after injection of digested whole blood genomic DNA from patient 1 receiving guadecitabine treatment. Chromatograms are shown for (a,c,d) predose and (b,d,e) Day 6, 24-hour post Day 5 injection. Chromatograms of (b,c) decitabine and (d,e) decitabine [$^{13}\text{C}_2, ^{15}\text{N}_4$] are enlarged to demonstrate chromatographic separation of α -decitabine (α -DEC) and β -decitabine (β -DEC).

of the spiked PBMC lysate, a recovery of $89 \pm 2.8\%$ as compared to the calculated nominal concentration was achieved, indicating that the dephosphorylation step was reproducible yielding acceptable recoveries.

Determination of the sample pretreatment recovery was also performed for β -DEC, 2dC and 5mdC at QC mid-level. The recovery

was calculated by dividing the peak area of processed QC samples by the peak area of blank processed PBMC lysate spiked with reference standards at equal concentrations. The overall recovery was 58.9% for β -DEC, 49.9% for β -DEC [$^{13}\text{C}_2, ^{15}\text{N}_4$], 31.0% for 2dC, 32.3% for 2dC [$^{13}\text{C}, ^{15}\text{N}_2$], 21.9% for 5mdC, and 24.4% for 5mdC-d3. The coefficient of variation was below 15% for all three analytes. Despite

relatively low recoveries, the method was able to reach an LOD of 0.1 ng/mL for β -DEC, and an LLOQ of 50 ng/mL and 5 ng/mL for 2dC and 5mdC, respectively. These results indicate that the sample pretreatment is adequate.

Stock solution stability was tested at storage conditions of -20°C , by comparing stored solutions to freshly prepared stock solutions. Acceptance criteria were met when the deviation from the fresh stock was within $\pm 5\%$. As this was the case, it was concluded that 2dC and 5mdC stock solutions were stable for at least 245 days in DMSO at -20°C . β -DEC was stable for at least 167 days in DMSO at -20°C . Stability data for all three analytes in human PBMC lysate and final extract are summarized in Table 3. Analytes were considered stable under specified conditions when 85–115% of the initial concentration were recovered. All three analytes were found to be stable at the indicated conditions. Long-term stability testing at -70°C revealed that β -DEC was stable in PBMC lysate for up to 49 days, and these tests are still ongoing.

3.3. Clinical application

3.3.1. Intracellular β -DEC-XP concentrations in patients treated with guadecitabine

To test the applicability of our assay to clinical samples, intracellular β -DEC-XP concentrations were quantitated in PBMCs from four patients treated with 45 mg/m²/day guadecitabine s.c. for 5 days, 24 h after the last injection.

Using the assay developed by Jansen et al. [7], DEC-TP levels were detectable in all four patients at corrected concentrations ranging from 32.7 to 158 fmol / million PBMCs (Fig. 4a), with an estimated interference of 55–66% caused by endogenous 2dC-TP. This percentage was determined by quantification of the 2dC-TP signal, and by subtracting 5.5% of this signal from the DEC-TP signal. The 5.5% interference is based on naturally occurring isotopes in the 2dC-TP molecule, and is in line with our observations in previously performed experiments [7]. Because all four sample levels were around the DEC-TP LLOQ of the LC–MS/MS method, the high percentage of endogenous interference results in a less accurate quantification, underlining the need for a more selective and sensitive assay.

After sample dephosphorylation, the concentration of β -DEC in PBMC lysate was analyzed. This was done for two patients only (due to availability of samples). β -DEC levels were higher in the dephosphorylated samples than expected based on the DEC-TP count (Fig. 4b). This is probably due to the fact that the dephosphorylated samples account for all β -DEC related material in the cell, meaning the nucleoside, as well as the mono-, di- and triphosphate nucleotides. We were not able to measure the intracellular nucleoside concentration before the dephosphorylation step, because of the low sample volumes that were available. Although it is known that DEC-TP is a highly abundant metabolite detected in PBMCs, the nucleoside and other nucleotides may add to the total quantified signal [7]. Interestingly, α -DEC was also seen in patient samples, indicating that both anomers are present inside the PBMCs (Fig. 5). This might be of interest for future studies, where it can be determined if the source of the α -DEC is nucleoside or nucleotide based. The possibility to separate β -DEC from α -DEC might be of clinical relevance, and adds important selectivity to the assay.

3.3.2. β -DEC and 5mdC DNA incorporation in patients treated with guadecitabine

In the same four patients, we assessed the degree of β -DEC incorporation and the global methylation grade in genomic DNA isolated from whole blood. THU-stabilized whole blood samples from predose, Day 1 (2 h post injection), Day 5 (2 h post injection), and Day 6 (24 h-post Day 5 injection) of cycle 1 were analyzed for β -DEC and 5mdC DNA incorporation. The amount of processed DNA

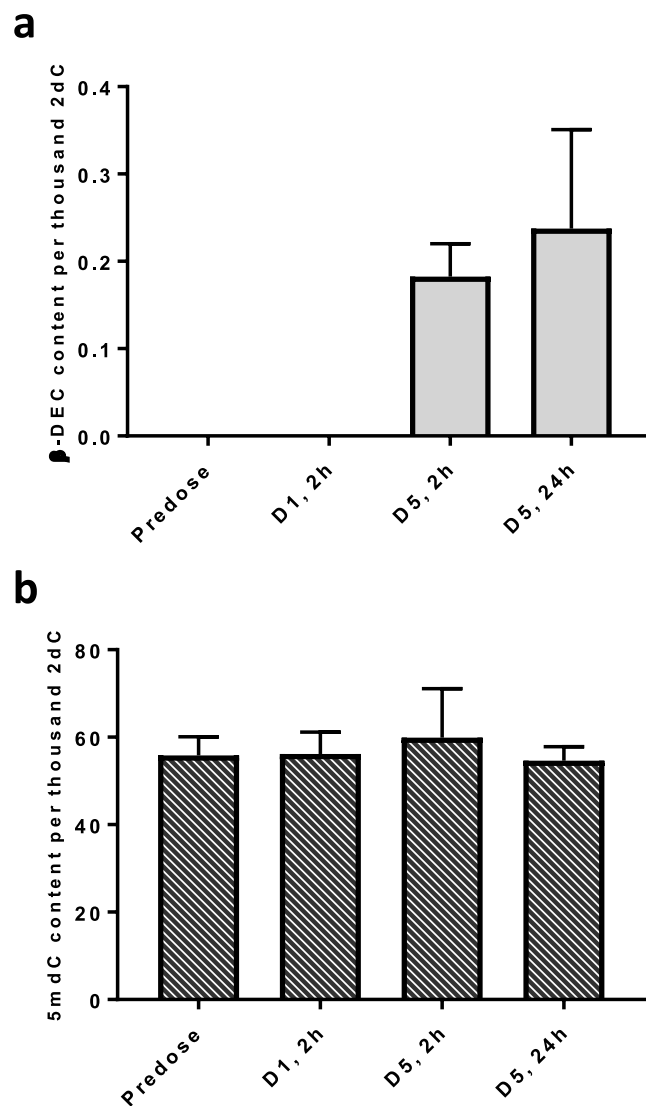


Fig. 7. (a) Genomic DNA β -decitabine (β -DEC) and (b) 5-methyl-2'-deoxycytidine (5mdC) incorporation content, normalized to 2'-deoxycytidine (2dC) content, after isolation and degradation of DNA obtained from whole blood from patients ($n=4$) receiving guadecitabine treatment (45 mg/m² once daily, day 1–5, 28-day treatment cycle). Samples were analyzed at predose, Day 1 (2 h post dose), Day 5 (2 h post dose), and Day 6 (24 h post Day 5 dose) and data is expressed as means with standard deviations.

per sample ranged from 3.5 to 12.4 μg , after isolation from 400 μL of whole blood. A representative patient ion chromatogram is shown in Fig. 6, where β -DEC DNA incorporation is clearly demonstrated after treatment with guadecitabine.

This can also be seen in Fig. 7a. β -DEC incorporation levels were not detectable in any of the predose and Day 1 samples from four patients, while having peak β -DEC plasma concentrations around 2 h post-dose (unpublished data). On Day 5, β -DEC incorporation levels were detectable in all four patients. On Day 6, 24 h after the last dose of guadecitabine, β -DEC incorporation levels were also detectable in all four patients, at even higher levels than on Day 5, indicating a prolonged intracellular effect. DNA methylation, expressed as the amount of 5mdC per thousand 2dC incorporated in the DNA, remains constant during the first treatment cycle (Fig. 7b). Since β -DEC incorporation into DNA is cell cycle-dependent and occurs during the S-phase, it may require prolonged administration or extended exposures to achieve maximum benefit [5,18]. In this study, patient samples were only collected during the first treatment cycle, which might be too early in therapy to diagnose changes

in DNA methylation status by measurement of 5mdC incorporation. It may also be possible that a more specific marker is needed to detect changes in DNA methylation over time (eg LINE1), instead of a global methylation marker.

There were no samples with a concentration above the ULOQ for all three analytes, confirming that the calibration ranges were selected correctly. For the β -DEC samples that had quantifiable concentrations, 5 out of 8 samples were between the LOD and the LLOQ. This shows that the addition of the LOD calibrator provides essential additional information for use in a clinical setting. Incorporation of concentration data below the LLOQ results in increased performance in terms of bias and precision in population pharmacokinetic analysis, especially when the percentage of data below the LLOQ is above 10% [19]. α -DEC was not detected in digested DNA in any of patient samples, as illustrated by the ion chromatogram for patient 1 in Fig. 6. This further confirms that β -DEC is indeed the active anomer incorporated into the DNA.

4. Conclusion

An LC-MS/MS method was developed and validated to quantify the amount of β -DEC-XP in PBMC-lysate and β -DEC and 5mdC incorporation levels in whole blood genomic DNA. Using this method, intracellular β -DEC-XP concentrations as well as the amount of genomic DNA-incorporated β -DEC and 5mdC relative to 2dC were quantified in patients treated with guadecitabine. To our knowledge this is the first assay that can be used to quantify β -DEC and 5mdC incorporation in patients treated with a β -DEC containing therapy without making use of a radiolabel. In addition, the assay can be used to analyze intracellular β -DEC-XP levels with increased sensitivity and selectivity. Because of the low required sample volumes for DNA incorporation analysis, this assay can easily be applied for future clinical experiments to investigate optimal dosing schedules for β -DEC containing therapies.

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Declarations of interest

Aram Oganessian is an employee of Astex Pharmaceuticals, Inc. The other authors declare no conflict of interest.

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