



## Short communication

## Liquid chromatography-tandem mass spectrometry assay to quantify plitidepsin in human plasma, whole blood and urine



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## ABSTRACT

Plitidepsin is an anti-cancer drug currently evaluated in phase I/II/III clinical trials. This article describes the development and validation of a bioanalytical assay to quantify plitidepsin in human plasma, urine and whole blood using HPLC–MS/MS. The analyte was extracted from the matrix by liquid–liquid extraction using *tert*-butyl methyl ether. Final extracts were injected onto a C18 column, gradient elution was applied for chromatographic separation and detection was performed on a triple quadrupole mass spectrometer operating in the positive ion mode. The assay was linear over the range 0.1–100 ng/mL, with acceptable accuracy and precision values. This is the first reported bioanalytical assay quantifying plitidepsin using a stable isotopically labelled standard, achieving a lower limit of quantification of 0.1 ng/mL in all three matrices, allowing the quantification of trace levels of plitidepsin, and accomplishing this in an analysis time of two minutes only. The presented method was successfully applied in a mass balance study with plitidepsin in patients with advanced cancer.

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## 1. Introduction

Plitidepsin (Aplidin<sup>®</sup>) is an anti-cancer drug currently investigated in phase I/II/III clinical trials. The compound was originally isolated from the Mediterranean tunicate *Aplidium albicans* but is now obtained by total synthesis [1,2]. It has been proven to have anticancer activity in a wide variety of cancer cells, including lung carcinoid, melanoma, neuroblastoma, leukemia, myeloma and lymphoma [3–10]. Plitidepsin exerts its antitumor activity by targeting eEF1A2, one of the isoforms of the alpha subunit of the eukaryotic Elongation Factor 1, which is overexpressed in human tumors and is endowed with oncogenic properties, favoring tumor cell proliferation while inhibiting apoptosis [1].

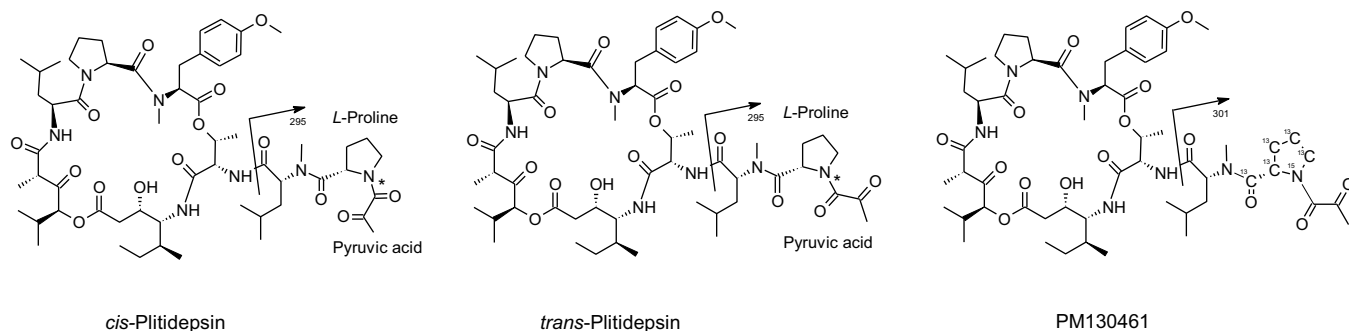
Clinical investigation into plitidepsin started in the late '90s [11]. Since then a few bioanalytical methods to quantify plitidepsin in human biological matrices have been described [12–15]. Additional methods developed at ICON Development Solutions

(currently at Whitesboro, NY) for the quantification of plitidepsin in plasma and whole blood in seven phase II studies and one phase III study have not been published yet. The published methods typically use didemn B as an internal standard, which is a depsipeptide structurally similar to plitidepsin, with the only difference being a lactyl-proline residue instead of the pyruvoyl-proline residue. One of these methods was developed for plasma, whole blood and urine, and a lower limit of quantification (LLOQ) of 1 ng/mL was achieved in this case [12]. Other methods were developed for one biological matrix only [13–15].

Part of a novel medicinal product registration, required by regulatory agencies world-wide, is the characterization of absorption, distribution, metabolism and excretion (ADME) of the compound in a mass balance study [16–18]. This mass balance study has been carried out and published recently [19]. For this purpose various biological matrices were analyzed, and to enable accurate quantification of plitidepsin in these matrices validated bioanalytical assays were essential. It is well known that plitidepsin distributes more in red blood cells as compared to plasma [5,20–22], which is why plitidepsin was also quantified in whole blood. The aim of the present work was therefore the development and optimization of a liquid chromatography – tandem mass spectrometry (LC–MS/MS) method, which was suitable for plasma, whole blood and urine

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**Fig. 1.** Structure and proposed fragmentation of plitidepsin (both conformers are shown) and the stable isotopically labelled plitidepsin internal standard PM130461. The asterisk indicates the isomerism around the terminal Pro-Pyr-amide bond.

samples collected in the mass balance study, using the stable isotopically labelled internal standard of plitidepsin (PM130461,  $C_{52}^{13}C_5H_8N_6^{15}NO_{15}$ ) as opposed to didemn B, and an API5500 mass spectrometer as the detector. The advantage of this assay over methods previously published is the short analysis time of 2 min, its applicability for not only plasma samples, but also urine and whole blood samples, the use of the stable isotopically labelled internal standard and the low LLOQ. Combining these properties results in a highly suitable, very sensitive and robust assay. This method was validated in compliance with the OECD principles of Good Laboratory Practice (GLP) [23] and in accordance with the Food and Drug Administration (FDA) and latest European Medicines Agency (EMA) guidelines on bioanalytical method validation [24,25].

## 2. Materials and methods

### 2.1. Chemicals and reagents

Acetonitrile, methanol and LiChrosolve water (all Supra-Gradient grade) were purchased from Biosolve Ltd (Valkenswaard, The Netherlands). Ammonia 25%,  $\geq 98\%$  formic acid (analytical grade) and *tert*-butyl methyl ether (TBME) were purchased from Merck (Amsterdam, the Netherlands). Reference standards of plitidepsin (purity of 97.6%) and the stable isotopically labelled internal standard (IS) of plitidepsin, PM130461 (purity of 92.2%), were supplied by Pharma Mar, S.A. (Colmenar Viejo, Madrid, Spain). Sodium heparinized (NaHe) human plasma originated from Bioreclamations (Hicksville, NY, USA). Blank urine as well as whole blood was supplied by healthy volunteers from the MC Slotervaart hospital (Amsterdam, the Netherlands).

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

Standard stock solutions of plitidepsin and the internal standard PM130461 (Fig. 1) were prepared by dissolving the analytes in acetonitrile, obtaining concentrations of 0.1 mg/mL for both plitidepsin and its IS. Working solutions for calibration standards (CS) and quality control (QC) samples were prepared by diluting two individual stock solutions of plitidepsin with acetonitrile. Diluting the IS stock solution 200 times, again using acetonitrile, resulted in the IS working solution with a concentration of 500 ng/mL. Stock solutions and working solutions were stored at  $-20^\circ\text{C}$  and  $-70^\circ\text{C}$ , respectively.

For each validation run, fresh CS were prepared by spiking 10  $\mu\text{L}$  of working solution to 190  $\mu\text{L}$  blank matrix to obtain concentrations of 0.1, 0.25, 1, 5, 10, 50, 80 and 100 ng/mL plitidepsin. QC samples were prepared in larger quantities by adding 125  $\mu\text{L}$  of working solution to 2,375  $\mu\text{L}$  human NaHe plasma and whole blood to obtain concentrations of 0.1 (QC LLOQ), 0.3 (QC Low), 5 (QC Mid) and 75

(QC High) ng/mL for plitidepsin. The QC samples were stored at  $-70^\circ\text{C}$  in aliquots of 200  $\mu\text{L}$  for the duration of the validation.

QC samples in urine were not prepared in larger volumes. Due to unselective adsorption to the container wall it was not possible to transfer aliquots from one tube to the other without losing some of the analyte. QC samples were therefore prepared by adding 10  $\mu\text{L}$  QC working solution to 190  $\mu\text{L}$  urine. These aliquots were also stored at  $-70^\circ\text{C}$ . For future studies it should be considered to store larger quantities of urine in glass containers as opposed to polypropylene containers, since this adsorptive effect was not observed in glass.

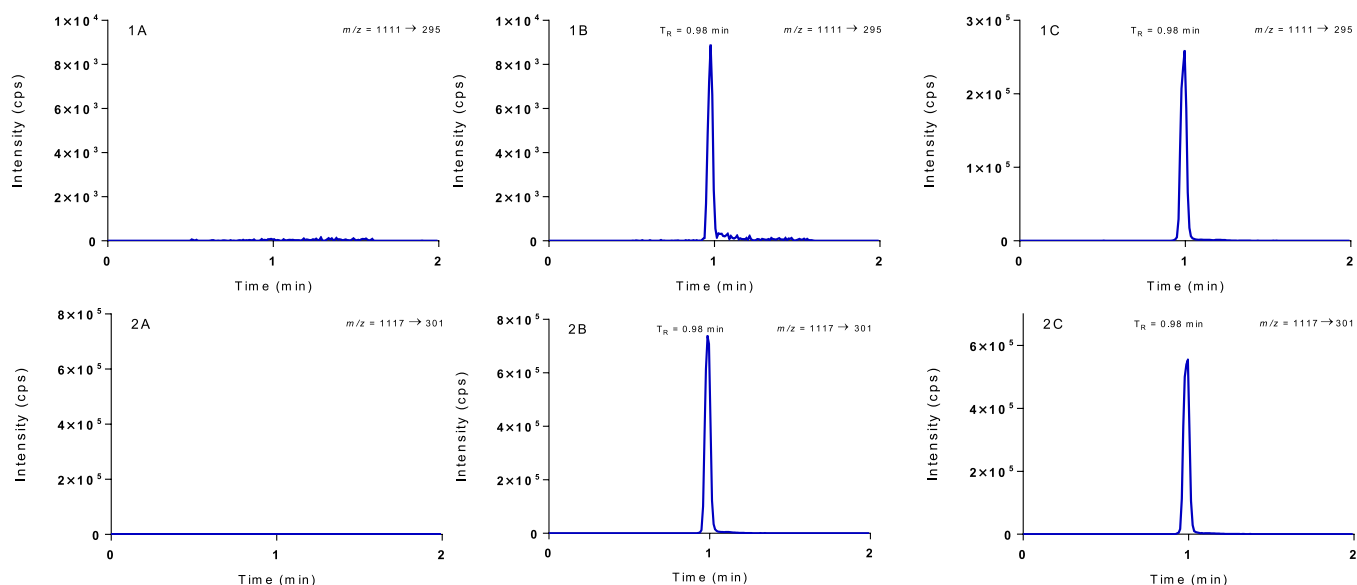
### 2.3. Sample preparation

Plitidepsin was extracted from 200  $\mu\text{L}$  plasma, urine or whole blood using liquid–liquid extraction (LLE). In 2.0 mL eppendorf tubes 10  $\mu\text{L}$  of IS working solution and 40  $\mu\text{L}$  of 1 M aqueous ammonia was added. Extraction under both acidic (0.1% formic acid) and basic (1 M aqueous ammonia) conditions had been tested, but the latter increased extraction recovery 1.5-fold. Samples were mixed vigorously, after which TBME was added to extract the analyte. After extraction, the samples were mixed, shaken on an automatic shaker (5 min at 1,250 rpm) and centrifuged for 5 min at 14,000 rpm. The samples were snap-frozen in an ethanol-dry ice bath and the organic layer was transferred to a clean 1.5 mL eppendorf tube prior to evaporating to dryness (at  $40^\circ\text{C}$ ) under a nitrogen flow. 100  $\mu\text{L}$  0.1% Formic acid in acetonitrile–water (50:50, v/v) was used to reconstitute the dry extract. Samples were centrifuged again at 14,000 rpm for 5 min and transferred to autosampler vials with inserts before analysis. 5  $\mu\text{L}$  of the final extract was injected into the chromatographic system.

### 2.4. Instrumentation and operating conditions

#### 2.4.1. Liquid chromatography

Analyses were performed using an HPLC Acquity I Class pump (Waters, Milford, MA, USA). Samples were injected using a Class I HPLC autosampler (Waters) (thermostated at  $8^\circ\text{C}$ ). Chromatographic separation was carried out on a SunFire C18 column (50 mm  $\times$  2.1 mm, 5  $\mu\text{m}$ , Waters) and separation was achieved using gradient elution with 0.1% formic acid in 5 mM ammonium acetate (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The addition of ammonium acetate to mobile phase A had been shown to considerably improve peak shape. A flow rate of 400  $\mu\text{L}$  per minute was applied through the column and the oven was set to  $60^\circ\text{C}$ . The elevated temperature was required to elute the *cis*-isomer and *trans*-isomer simultaneously, whereas lower temperatures caused the conformers to elute separately. This phenomenon has been described elsewhere [15,26]. The elution gradient was set as follows: 50% B (0.0–0.3 min), 95% B (0.3–1.8



**Fig. 2.** MRM chromatograms of plitidepsin (1A) in a blank plasma sample, (1B) in a spiked plasma sample at LLOQ level (0.1 ng/mL), (1C) in a patient plasma sample drawn at the end of infusion, and MRM chromatograms of PM130461 (2A) in a blank plasma sample, (2B) in a spiked plasma sample at LLOQ level (500 ng/mL) and (2C) spiked to a patient sample drawn at the end of infusion.

min), 50% B (1.8–2.0 min). The divert valve directed the flow to the mass spectrometer from 0.5 to 1.6 min and to the waste for the remainder of the acquisition time.

#### 2.4.2. Mass spectrometry

An API5500 tandem mass spectrometer (Sciex, Framingham, MA, USA) was used. Analyses were done in the positive ion mode by multiple reaction monitoring (MRM), selecting precursor ion  $m/z$  1110.8 and product ion  $m/z$  295.1 for plitidepsin, and precursor ion  $m/z$  1116.8 and product ion  $m/z$  301.1 for the internal standard PM130461. These  $m/z$  values of the product ions correspond to the sidechain of the molecule (Fig. 1). Collision gas was set at 8 arbitrary units (a.u.) and curtain gas (nitrogen) flow was set at 20 a.u. The ion source was heated to 650 °C and the ion spray voltage was +5500. The dwell time was 15 msec for both plitidepsin and PM130461. The declustering potential was 20 V and the entrance potential was 13 V for both the analyte and IS. Data acquisition was performed using Analyst 1.5.2 software (Sciex). Figs. 2–4 shows representative LC–MS/MS chromatograms for plitidepsin ( $t_R$ : 0.98 min), which eluted as a single chromatographic peak, and its IS ( $t_R$ : 0.98 min) in plasma, whole blood and urine, respectively.

#### 2.5. Validation procedures

A complete validation of the bioanalytical assay for plasma, urine and whole blood was carried out according to regulatory guidelines, including calibration curve, accuracy and precision, carry-over, selectivity, dilution integrity, matrix effect and stability [24,25]. Validation was performed on the instruments with the settings as mentioned above.

### 3. Results and discussion

#### 3.1. Validation procedures

The method was fully validated according to the FDA and EMA guidelines for bioanalytical method validation using calibration standards and quality control samples [24,25]. The method for plitidepsin determinations were carried out for plasma, urine and

whole blood separately. The same acceptance criteria applied to all matrices.

##### 3.1.1. Calibration curve

CS (8 non-zero) with a concentration range of 0.1–100 ng/mL were prepared in blank human NaHe plasma, urine and whole blood. Linear regression with a weighting factor of  $1/x^2$  was applied, where  $x$  equals the concentration of plitidepsin. The calibration curves were acceptable if 75% of all non-zero calibration standards were within or equal to 15% of the nominal concentrations, or 20% for the LLOQ [24,25]. These acceptance criteria were met and thus the calibration curves were accepted for all three matrices.

##### 3.1.2. Accuracy and precision

Five replicates of QC LLOQ (0.1 ng/mL), QC Low (0.3 ng/mL), QC Mid (5 ng/mL) and QC High (75 ng/mL) were analyzed in 3 analytical runs for plasma, urine and whole blood.

The intra-run accuracy, expressed as the bias, was calculated by dividing the difference between the mean measured concentration per run and the nominal concentration by the nominal concentration. The overall bias was calculated similarly, by using the overall mean measured concentration. Intra-run precision, expressed as the coefficient of variation (CV), was calculated by dividing the standard deviation of the measured concentration per run by the mean measured concentration per run. To calculate the inter-run precision a one-way ANOVA was used.

The acceptance criteria were met if two-third of the accuracy and precision calculations were within or equal to  $\pm 20\%$  for the LLOQ and 15% for the other levels. Accuracy calculations ranged from 0.8% to 4.2% for plasma, from  $-2.5\%$  to 6.0% for urine and from  $-5.2\%$  to 10.5% for whole blood. Maximum precision was observed to be 5.8% for plasma, 3.9% for urine and 4.9% for whole blood (Table 1).

##### 3.1.3. Specificity and selectivity

Six different batches of blank human NaHe plasma, urine and whole blood were spiked at the LLOQ level and were processed together with double blank samples to assess the selectivity of the assay. The maximum deviation from the nominal concentration was 8.3% for plasma, 7% for urine and 9.2% for whole blood. The

**Table 1**

Assay performance data for the analysis of plitidepsin in human plasma, urine and whole blood.

Matrix	Analyte	Nom.conc. (ng/mL)	n	Intra-assay		Inter-assay	
				Bias (%)	CV (%)	Bias (%)	CV (%)
Plasma	Plitidepsin	0.100	15	−2.5–4.2	3.0–4.1	1.3	3.2
		0.299	15	−0.6–0.9	2.4–5.8	0.3	−*
		4.99	15	−0.1–1.9	2.0–3.8	0.9	−*
		74.8	15	−2.6–3.9	1.4–3.0	0.3	3.1
Whole blood	Plitidepsin	0.100	<b>14</b>	−10.5–−1.5	3.3–4.9	−5.2	4.6
		0.300	15	−3.9–1.7	2.1–3.3	−0.3	2.9
		5.00	15	0.2–2.8	2.3–4.4	1.2	−*
		75.0	15	−1.1–0.9	1.7–3.0	−1.1	0.5
Urine	Plitidepsin	0.100	15	−6.0–−0.4	3.1–3.9	−2.5	2.7
		0.300	15	−5.9–0.4	0.8–2.5	−2.4	3.2
		5.01	15	−2.1–0.7	1.1–1.5	−0.6	1.3
		75.1	15	−1.4–1.8	1.1–1.9	−0.1	1.5

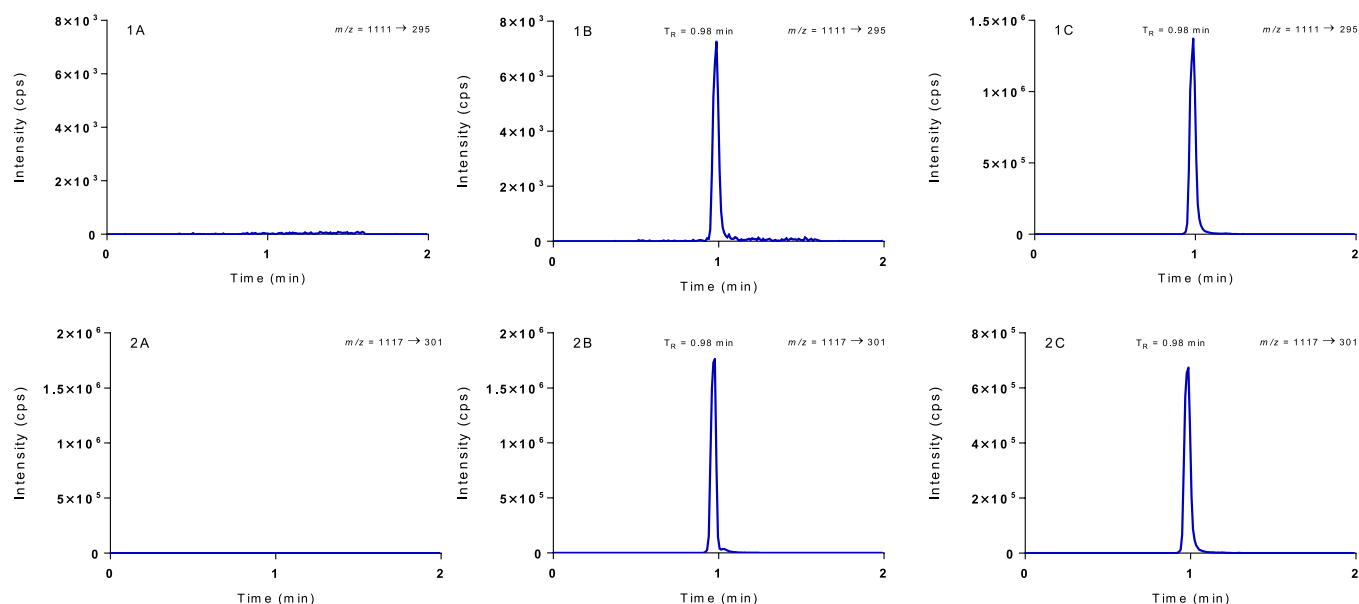
Nom.: nominal; conc.: concentration; n: number of replicates; CV: coefficient of variation. \*Inter-run precision could not be calculated (mean square between group is less than mean square within groups) Bold: statistical outlier.

**Table 2**

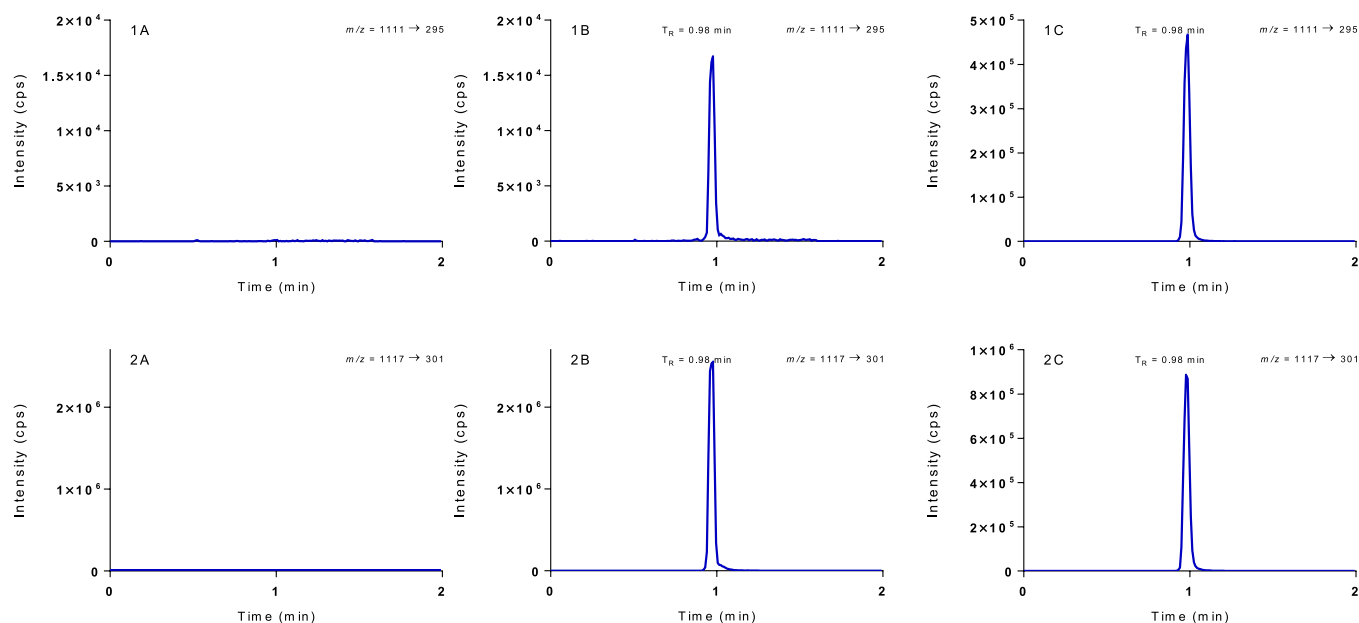
Stability data for plitidepsin in plasma, urine and whole blood. All experiments in the biomatrix were performed in triplicate at low and high concentrations.

Conditions	Matrix	Analyte	Nominal conc. (ng/mL)	Measured conc. (ng/mL)	Bias (%)	C.V. (%)	n
Plasma							
5 freeze-thaw cycles (−20 °C/ambient)	Biomatrix	Plitidepsin	0.299	0.262	−12.4	4.1	3
			74.8	68.3	−8.7	2.1	3
2 h at ambient temperatures	Biomatrix	Plitidepsin	0.299	0.278	−7.1	3.6	3
			74.8	69.6	−6.9	1.5	3
4 h at ambient temperatures	Biomatrix	Plitidepsin	0.299	0.260	−12.9	3.6	3
			74.8	67.7	−9.5	0.9	3
4 h in ice-water	Biomatrix	Plitidepsin	0.299	0.275	−7.9	3.3	3
			74.8	70.2	−6.2	1.8	3
120 h at 2–8 °C	Final extract	Plitidepsin	0.299	0.289	−3.2	4.3	3
			74.8	70.3	−6.1	5.4	3
2–8 °C, 4 days	Final extract (re-injection)	Plitidepsin	0.299	0.303	1.3	8.4	3
			74.8	71.3	−4.6	3.5	3
Whole blood							
1 h on ice-water	Biomatrix	Plitidepsin	45.2	48.5	7.2	4.2	3
1 h at ambient temperatures	Biomatrix	Plitidepsin	45.2	48.0	6.2	6.5	3
2 h at ambient temperatures	Biomatrix	Plitidepsin	0.300	0.295	−1.6	0.2	3
			75.0	70.9	−5.5	4.8	3
2 h on ice-water	Biomatrix	Plitidepsin	0.300	0.298	−0.7	2.4	3
			75.0	75.4	0.5	2.1	3
5 freeze-thaw cycles (−20 °C/ambient)	Biomatrix	Plitidepsin	0.300	0.102	−66.1	12.8	3
			75.0	33.5	−55.4	10.0	3
1 freeze-thaw cycle (−20 °C/ambient)	Biomatrix	Plitidepsin	0.300	0.313	4.2	0.9	3
			75.0	82.5	10.0	2.4	3
70 h at 2–8 °C	Final extract	Plitidepsin	0.300	0.282	−6.0	2.5	3
			75.0	71.0	−5.4	1.6	3
70 h at 2–8 °C	Final extract (re-injection)	Plitidepsin	0.300	0.309	2.9	3.0	3
			75.0	76.6	2.2	2.3	3
Urine							
5 freeze-thaw cycles (−20 °C/ambient)	Biomatrix	Plitidepsin	0.300	0.309	2.9	1.6	3
			75.1	77.3	3.0	1.0	3
2 h at ambient temperatures	Biomatrix	Plitidepsin	0.300	0.288	−4.1	0.9	3
			75.1	72.9	−2.9	0.8	3
2 h in ice-water	Biomatrix	Plitidepsin	0.300	0.287	−4.3	2.5	3
			75.1	72.3	−3.8	1.2	3
114 h at ambient temperatures	Final extract	Plitidepsin	0.300	0.290	−3.2	0.9	3
			75.1	73.2	−2.5	1.1	3
78 h at 2–8 °C	Final extract	Plitidepsin	0.300	0.309	3.1	1.8	3
			75.1	77.8	3.6	1.8	3
20 h at ambient temperatures	Final extract (re-injection)	Plitidepsin	0.300	0.295	−1.7	2.1	3
			75.1	75.9	1.1	2.4	3
65 h at 2–8 °C	Final extract (re-injection)	Plitidepsin	0.300	0.294	−2.1	4.1	3
			75.1	73.7	−1.9	0.7	3
26.5 h at ambient temperatures	Dried extract	Plitidepsin	0.300	0.300	0.0	2.6	3
			75.1	75.1	0.0	0.7	3

Nom.: nominal; conc.: concentration; n: number of replicates; CV: coefficient of variation.



**Fig. 3.** MRM chromatograms of plitidepsin (1A) in a blank whole blood sample, (1B) in a spiked whole blood sample at LLOQ level (0.1 ng/mL), (1C) in a patient whole blood sample drawn at the end of infusion, and MRM chromatograms of PM130461 (2A) in a blank whole blood sample, (2B) in a spiked whole blood sample at LLOQ level (500 ng/mL) and (2C) spiked to a patient sample drawn at the end of infusion.



**Fig. 4.** MRM chromatograms of plitidepsin (1A) in a blank urine sample, (1B) in a spiked urine sample at LLOQ level (0.1 ng/mL), (1C) in a patient urine sample taken 48–72 h after infusion, and MRM chromatograms of PM130461 (2A) in a blank urine sample, (2B) in a spiked urine sample at LLOQ level (500 ng/mL) and (2C) spiked to a patient sample taken 48–72 h after infusion.

maximum interference from plitidepsin in the double blank samples was 7.2% in plasma, 11.4% in urine and 5.4% in whole blood. No interference from the IS was observed in any of the matrices, meaning that the selectivity of this assay is sufficient for its intended purpose.

Cross-analyte interferences were tested by spiking blank NaHe plasma and whole blood with plitidepsin at its ULOQ and spiking a blank sample with IS only. The interference of plitidepsin in the IS transition was 0.0% in both matrices. The interference of the IS in the plitidepsin transition was found to be 1.3% in plasma and 3.4% in whole blood.

### 3.1.4. Dilution integrity

Five replicates of plitidepsin in plasma, urine and whole blood were spiked at a concentration of 2,000 ng/mL. Plasma and urine samples were diluted 25 times and whole blood samples were diluted 20 times with blank control matrix. Bias and CV were 2.7% and 2.0% for plasma, −1.9% and 3.0% for urine and −10.8% and 1.6% for whole blood, meaning all results fell within the acceptance criteria of  $\pm 15\%$  and  $\leq 15\%$  for accuracy and precision, respectively. This means that study samples with concentrations above the upper limit of quantification (ULOQ) can be diluted with acceptable accuracy and precision values.



### 3.1.5. Lower limit of quantification

The analyte response at the LLOQ was at least 15.4, 12 and 26 in plasma, urine and whole blood respectively, compared to a blank response. This was deemed sufficient as the values were all above the acceptance criterion of 5.

### 3.1.6. Matrix effect

Plitidepsin was spiked to six different batches of blank NaHe human plasma, urine and whole blood at QC Low and QC High concentrations and the analyte response in these samples were compared to those in unprocessed samples. The matrix factor (MF) was calculated for each batch by calculating the ratio of the peak area in the presence of matrix to the peak area in absence of matrix. Furthermore, the internal standard normalized MF was calculated. The coefficient of variation of IS-normalized MF from the 6 batches appeared to be below 2.8% for plasma and whole blood, and below 1.5% for urine. All these values were lower than the acceptance criteria of  $\leq 15\%$ . Therefore, it was concluded that the matrix effect by analyzing different matrix batches has no effect on the accuracy of the method.

### 3.1.7. Carry-over

Carry-over was assessed by injecting a double blank after the ULOQ. The first double blank response in plasma, urine and in whole blood was less than 20% of the mean response of the LLOQ for all matrices (17.0, 13.1 and 9.7%, respectively) and was consequently considered acceptable.

### 3.1.8. Stability

Short term stability of plitidepsin was tested in stock solutions, working solutions, final extracts and in biological matrices. An overview of all tested conditions and results is shown in Table 2. Before analysis, the stock solutions (0.1 mg/mL) and working solutions (1,000 ng/mL) were diluted in triplicate to concentrations of 50 ng/mL and 5 ng/mL. Stability was assessed at various time points by comparing these solutions to the concentration of freshly prepared stock solutions and working solutions, which were diluted, in triplicate, in a similar fashion. Final extract stability was assessed by storing the final extracts from plasma, urine and whole blood samples at nominally 2–8 °C for at least 48 h. The response of these extracts was compared to the response of freshly prepared plasma, urine and whole blood samples.

Short-term stability of plitidepsin was assessed in all three matrices at different conditions. Spiked plasma samples were stable for at least 4 h at room temperature. Spiked urine samples were stable at least 2 h, both at room temperature and on ice-water and the same was true for plitidepsin in whole blood samples.

The freeze/thaw (F/T) stability was assessed for plitidepsin in all three matrices. QC Low and QC High samples were subjected to 5 F/T cycles by storing them at –20 °C and thawing them at room temperature without assistance. The analyte response was compared to freshly spiked QC samples. Both plasma and urine samples showed deviations  $<15\%$  after 5 F/T cycles. The bias for F/T cycle 2 to F/T cycle 5 was outside  $\pm 15\%$  of the nominal concentration for plitidepsin in whole blood. Therefore, it was concluded that plitidepsin is stable in human NaHe whole blood after at most 1 F/T cycle.

### 3.1.9. Re-injection reproducibility

Re-injection reproducibility was tested by injecting QC Low and QC High samples a second time after leaving them in the autosampler (thermostated at 8 °C) for at least 24 h. Plasma final extracts were stable for at least four days after processing, urine final extracts for at least 65 h and whole blood final extracts for at least 45 h at autosampler temperature. This means that in case of

instrument failure, samples can be resubmitted again before these time points.

## 4. Conclusion

Quantification methods for plitidepsin in plasma, urine and whole blood were validated for all validation parameters. The quantifiable range for plitidepsin was 0.1–100 ng/mL, with the possibility to dilute plasma and urine samples 25 times, and whole blood samples 20 times with control matrix, enabling the quantification of samples outside of the calibration range. Advantages of this assay are the low LLOQ (0.1 ng/mL) and the short analysis time, which allows for reliable quantification of trace levels of plitidepsin in biological matrices with run times of only 2 min. Plitidepsin adsorbs to plastic containers when present in the urine matrix, but this problem can easily be circumvented by using glass containers. This method is applicable for the analysis of plitidepsin in all three matrices and it has been shown that it can be used to support clinical pharmacological studies.

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