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Development and validation of ultra-performance liquid chromatography tandem mass spectrometry methods for the quantitative analysis of the antiparasitic drug DNDI-6148 in human plasma and various mouse biomatrices

Wietse M. Schouten [a], Katrien Van Bocxlaer [b], Hilde Rosing [a], Alwin D.R. Huitema [a,c,d], Jos H. Beijnen [a,e], Jadel M. Kratz [f], Charles E. Mowbray [g], Thomas P.C. Dorlo [a,h,*]

[a] Department of Pharmacy & Pharmacology, Antoni van Leeuwenhoek/The Netherlands Cancer Institute, Amsterdam, The Netherlands
[b] Skin Research Centre, Hull York Medical School, York Biomedical Research Institute, University of York, York, UK
[c] Department of Pharmacology, Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands
[d] Department of Clinical Pharmacy, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
[e] Utrecht Institute of Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands
[f] Drugs for Neglected Diseases Initiative Latin America, Rio de Janeiro, Brazil
[g] Drugs for Neglected Diseases Initiative, Geneva, Switzerland
[h] Department of Pharmacy, Uppsala University, Uppsala, Sweden

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ABSTRACT

DNDI-6148 is a promising new oral drug for the treatment of cutaneous leishmaniasis (CL), a parasitic neglected tropical disease that affects impoverished populations worldwide. Preclinical target site pharmacokinetics (PK) studies are necessary to evaluate the actual exposure to DNDI-6148 of *Leishmania* parasites in the skin. To facilitate these investigations, we have developed and validated a reversed phase ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) method to quantify DNDI-6148 in relevant target site PK samples, adhering to the relevant International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) M10 guideline on bioanalytical method validation. Full validation was performed for the surrogate biomatrices human K_2EDTA plasma, enzymatic digestion buffer and skin microdialysate. Partial validation was conducted for mouse K_2EDTA plasma and tissues. The tissue samples, including mouse skin, liver and spleen, were homogenized using a collagenase A-based enzymatic homogenization workflow. This method was found to be 2.9-fold more effective in extracting DNDI-6148 from skin than the commonly used mechanical homogenization. Protein precipitation was subsequently carried out for all biomatrices. A surrogate biomatrix was used for each method and the range was specifically developed for its intended application, resulting in a linear concentration range of 5.00–2000 ng/mL, 2.00–1000 ng/mL, and 3.00–600 ng/mL for human K_2EDTA plasma, enzymatic digestion buffer and microdialysate, respectively. Each biomatrix had intra- and inter-run accuracy and precision within 15 % for all concentration levels. Matrix effects did not affect the determination of DNDI-6148, since the stable isotopically-labelled internal standard for DNDI-6148 effectively compensated for these matrix effects. Total recovery across all methods was between 73.5 % and 81.3 % (CV $\leq$4.5 %). DNDI-6148 was stable under various conditions in all the tested biomatrices. However, a decrease in its concentration was observed during homogenization, for which the internal standard corrected adequately. The suitability of the method for use in future preclinical research involving DNDI-6148 was demonstrated in a preclinical target site PK study using a CL-infected murine model.

* Corresponding author at: Department of Pharmacy, Uppsala University, Uppsala, Sweden.
E-mail address: thomas.dorlo@farmaci.uu.se (T.P.C. Dorlo).

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1. Introduction

Cutaneous leishmaniasis (CL) is the most common clinical form of leishmaniasis, a poverty-related infectious parasitic disease caused by the unicellular protozoan *Leishmania*. Over 200,000 new cases of CL were reported in 2022, affecting the world's poorest populations [1]. However, this number is probably an underestimate. CL is clinically characterized by disfiguring skin lesions, which lead to social and psychological stigma. These lesions originate from parasites that reside and replicate in macrophages in the dermis. While there are several treatments available for CL patients, they all have considerable limitations, such as toxicity, high cost, and/or non-oral administration [2,3].

A promising lead series in the field of antileishmanial drugs is the benzoxaboroles, which is under development for the treatment of various parasitic diseases [4,5]. The new chemical entity (NCE) DNDI-6148 (Fig. 1) is the preclinical frontrunner for the treatment of CL within this class of compounds. DNDI-6148 exhibits significant *in vitro* and *in vivo* activity against multiple geographically diverse *Leishmania* strains, by inhibiting the *Leishmania* cleavage and polyadenylation specificity factor (CPSF3) endonuclease [6,7]. However, comprehensive pharmacokinetic (PK) and tissue distribution data for this drug, essential to enable further optimization of its dose regimen in the treatment of CL, are currently unavailable.

Recent research has increasingly emphasized the need to study the target site PK, underlining a growing awareness of the importance of understanding the behaviour of antileishmanial drugs in specific tissues at the cellular level [8–10]. The distribution of the drugs within the skin is critical for CL, since the *Leishmania parasites* are localized within macrophages in the dermis.

Although Mowbray *et al.* have reported plasma PK data for DNDI-6148 in animal studies [7], no details regarding the utilized bioanalytical methods have been published. Furthermore, there is a lack of data on DNDI-6148 concentrations at relevant target sites, such as the skin in the case of CL. This lack of knowledge about the target site PK of DNDI-6148 emphasizes the need for bioanalytical method development to further characterize its tissue distribution. However, to determine the target site concentration, tissues must be pre-treated and homogenized using either mechanical or enzymatic digestion methods [11]. Our group recently developed an enzymatic sample preparation method to determine the target site concentrations of the antileishmanial drugs miltefosine, paromomycin, and amphotericin B, in skin tissue biopsies [12–14]. Microdialysis techniques have also been proposed and used to assess free, unbound drug levels in skin in preclinical studies [15,16]. Taken together, these techniques may improve our understanding of the pharmacological activity of DNDI-6148 in treating CL and its distribution kinetics in the skin, where the parasites reside and replicate intracellularly [15–18].

In this paper, we present a highly sensitive ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) method for the quantification of DNDI-6148 in human K_2EDTA plasma, enzymatic digestion buffer and microdialysate together with mouse biomatrices, including K_2EDTA plasma and digested skin, liver and spleen tissues. This method was successfully applied to target site PK and tissue distribution studies in a murine infection model of CL treated with DNDI-6148.

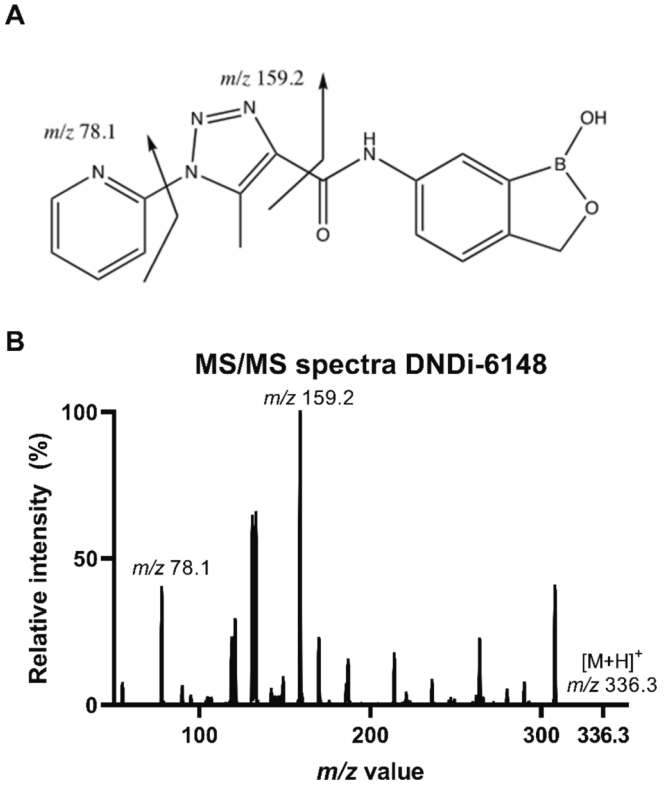

Fig. 1. (A) Chemical structure of DNDI-6148. The arrows indicate the proposed fragmentation of the product ion *m/z* 159.2, which was used for the plasma method, and the product ion *m/z* 78.1, which was used for the tissue digestion and microdialysate methods. (B) MS/MS spectrum of the parent ion of DNDI-6148 (*m/z* 336.3).

2. Materials and methods

2.1. Chemicals and biomatrices

ULC/MS grade acetonitrile, methanol, formic acid, and water were all purchased from Biosolve (Valkenswaard, The Netherlands). The salt form of DNDI-6148, arginine monohydrate (as-is purity, 62.9 %), along with the internal standard (IS) [D_4]-DNDI-6148, a stable isotopically labeled compound, were provided by DNDi (Geneva, Switzerland). Calcium chloride dihydrate and tris base were supplied by Sigma Aldrich (Zwijndrecht, The Netherlands). Dimethyl sulfoxide was purchased from Merck (Darmstadt, Germany). Bovine serum albumin fraction V and collagenase A were both purchased from Roche (Woerden, The Netherlands). Ringer's solution tablets (quarter strength) were acquired from VWR (Amsterdam, The Netherlands). Blank human K_2EDTA plasma was obtained from BioIVT (Westbury, NY, USA). The enzymatic collagenase A digestion buffer (enzymatic digestion buffer), consisting of 5 mg/mL collagenase A in a 2 % bovine serum albumin in 5 mM calcium chloride – 25 mM TRIS-buffer (pH 7.5), was used for tissue homogenization and prepared as described previously [12–14]. Skin microdialysate consisted of a mixture of 2.5 % bovine serum albumin in Ringer's solution (full strength) – acetonitrile (6:1, v/v). Blank mouse tissues (skin, liver and spleen) and mouse K_2EDTA plasma were all provided by the Biological Services Facility at the University of York (York, UK).

2.2. Stock and working solutions

DNDI-6148 stock solutions were prepared in methanol by dissolving appropriate amounts, corrected for the as-is purity, to obtain a concentration of 1.00 mg/mL. Working solutions for calibration standards and quality control (QC) samples were prepared using different stock solutions. The IS was dissolved in dimethyl sulfoxide to obtain a concentration of 1.00 mg/mL. Working solutions for both DNDI-6148 and the IS were further prepared in methanol by dilution of the stock solutions. All stock and working solutions were stored at −20 °C.

2.3. Calibration samples and quality control samples

For all the methods, calibration standards (eight calibration points) and QC samples (QC-lower limit of quantification (LLOQ), QC-LOW, QC-

Table 1
Calibration standard concentrations, back-calculated accuracy (Bias, %) and precision (CV, %) of DNDI-6148 (in ng/mL) for the three different sample preparation methods, analyzed in three consecutive analytical runs.

Calibration standard	Human K_2EDTA plasma			Enzymatic Digestion Buffer			Microdialysate		
	Conc., ng/mL	Bias, %	CV, %	Conc., ng/mL	Bias, %	CV, %	Conc., ng/mL	Bias, %	CV, %
1 (LLOQ)	5.00	0.8	4.3	2.00	0.9	6.7	3.00	2.8	6.1
2	10.0	−1.0	4.2	4.00	−1.2	5.0	6.00	−5.7	7.7
3	20.0	−1.6	1.7	20.0	−3.8	3.9	30.0	−4.4	2.4
4	100	1.0	1.2	60.0	−0.1	1.9	90.0	−3.1	3.0
5	400	2.3	1.1	200	0.4	3.2	180	2.4	1.0
6	800	−4.6	1.0	400	0.8	1.8	360	1.4	1.0
7	1,600	1.3	2.0	800	3.0	0.7	480	0.6	2.3
8 (ULOQ)	2,000	1.8	0.7	1,000	−0.2	2.1	600	5.1	2.6

Abbreviations: Conc. = concentration; CV = coefficient of variation; LLOQ = Lower limit of quantification; ULOQ = Upper limit of quantification.

MID, QC-HIGH) were made by diluting the working solutions twenty-fold in their biomatrix (Table 1 for calibration standard concentrations, Table 2 for QC sample concentrations). Spiked human K_2EDTA plasma samples were stored at −20 °C and spiked enzymatic digestion buffer and microdialysate were stored at −70 °C.

2.4. Sample preparation

For the plasma method, 10 µL aliquots of study samples, calibration standards, and QC samples were used. To each plasma sample, 90 µL of a precipitation mixture comprising 1 % formic acid in water-acetonitrile (1:8, v/v) containing the IS (at a concentration of 100 ng/mL) was added. The resulting solution was vortex-mixed for 10 s. After mixing, the samples were centrifuged for 10 min at 18,600 *g*. Following centrifugation, 75 µL of supernatant was transferred to an autosampler vial with insert.

For the tissue digestion method, preclinical tissue samples were first weighed. The enzymatic digestion buffer was then added in a 20:1 (v/w) digestion buffer to organ weight ratio. The IS (400 ng/mL in the enzymatic digestion buffer) was added in a 1:5 (v/v) IS-enzymatic digestion buffer ratio. A volume of 10 µL of the IS was added to the calibration standards and QC samples, each aliquoted in 50 µL in surrogate enzymatic digestion buffer. All samples, including the calibration standards and QC samples, were then incubated for a minimum of 16 h at 37 °C with shaking at 1200 rpm with a Thermomixer (VWR, Amsterdam, The Netherlands). Following the incubation period, the homogenized tissue mixture containing the IS was aliquoted in 60 µL portions. The remaining tissue homogenates were preserved at −70 °C for potential reanalysis. After homogenization, all samples were precipitated by adding 200 µL of a mixture of acetonitrile-methanol (1:1, v/v). The mixture was then shaken at 900 rpm for 10 min in the Thermomixer. Following mixing, the samples underwent centrifugation for 5 min at 21,000 *g*. Afterwards, 150 µL of the supernatant was carefully transferred to an autosampler vial with insert.

For the microdialysis method, 25 µL aliquots of study samples, calibration standards and QC samples were processed. To these microdialysis samples, 10 µL of IS (200 ng/mL in methanol) and 65 µL of an acetonitrile-methanol (1:1, v/v) mixture were added. Processed samples were then shaken at 900 rpm for about 10 min in the Thermomixer, followed by centrifuging for 5 min at 21,000 *g*. A volume of 60 µL of supernatant was then carefully transferred to an autosampler vial with insert.

2.5. Instrumentation and chromatographic conditions

Sample analysis was performed using an Agilent 1290 Infinity II system (Agilent Technologies, Santa Clara, CA, USA) coupled to a QTRAP6500 MS (Sciex, Framingham, MA, USA), equipped with a turbo ionspray. Chromatographic separation was achieved using a Waters ACQUITY UPLC BEH C18 column (50 x 2.1 mm, 1.7 µm) (Waters, Milford, MA, USA) at 40 °C. Separation was performed using a gradient elution with 0.1 % (v/v) formic acid in water (mobile phase A) and 0.1 % (v/v) formic acid in acetonitrile (mobile phase B) with the following gradient: start, 30 % B; 2.5 min, 60 % B; 2.6 min, 95 % B; 3.0 min, 95 % B; 3.1 min, 30 % B; 4.0 min, 30 %B; at a flowrate of 0.40 mL/min. An injection volume of 2 µL was used. The MS operated in positive mode

Table 2
Method performance data for DNDI-6148 in human K_2EDTA plasma, enzymatic digestion buffer and microdialysate. The accuracy (Bias, %) and the precision (CV, %) were analyzed at four concentrations in quintuplicate in three consecutive analytical runs (n = 15).

Matrix	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Intra-method (n = 15)		Inter-method (n = 15)	
			Accuracy (Bias, %)	Precision (CV, %)	Accuracy (Bias, %)	Precision (CV, %)
Plasma	5.00	4.84	±5.4	≤6.4	−3.3	2.5
	15.0	14.9	±4.3	≤4.6	−0.5	3.9
	150	156	±6.7	≤4.8	4.2	1.6
	1,500	1,576	±7.6	≤5.2	5.1	1.5
Enzymatic digestion buffer	2.00	2.05	±12.5	≤11.5	2.6	7.2
	6.00	5.93	±1.9	≤5.3	−1.1	–*
	80.0	81.9	±4.1	≤4.1	2.4	–*
	750	760	±3.4	≤3.1	1.3	1.6
Microdialysate	3.00	3.27	±13.9	≤9.7	8.9	6.9
	9.00	9.03	±4.1	≤5.4	0.3	3.6
	75.0	75.6	±1.7	≤5.0	0.8	–*
	450	464	±3.5	≤3.2	3.1	–*

Abbreviations: CV = coefficient of variation.
* No additional variation was found by performing the method between days (mean square between groups is less than mean square within groups).

with the following general MS parameters: Ionspray voltage: 5500 V; Nebulizer gas: 55 psi; Turbo gas: 40 psi; Curtain gas: 30 psi; Collision gas: 10 psi; Source temperature: 500 °C. Multiple reaction monitoring (MRM) was utilized for analyte and IS detection, for the following *m/z* transitions: 336.3 → 159.2 (for the plasma method), 336.3 → 78.1 (for the tissue digestion buffer and microdialysate methods) and 340.3 → 162.2 (for the IS in all methods). Both the analyte and IS were detected using the following analyte specific MS parameters: Collision energy: 66 V; Collision exit potential: 27 V; Declustering potential: 10 V; Entrance potential: 10 V; Dwell time: 100 msec. Instrument operation, data acquisition and processing were performed using Analyst™ software, version 1.7.2 (Sciex).

2.6. Validation procedure

For each method, the validation process for the bioanalytical method to quantify the DNDI-6148 concentration was conducted following the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline M10 on bioanalytical method validation [19]. The full bioanalytical validation for the quantification of DNDI-6148 in human K_2EDTA plasma, enzymatic digestion buffer and microdialysate involved the following experiments: calibration model, lower limit of quantification, accuracy and precision, carry-over, dilution integrity, selectivity (including cross-analyte/IS interference, endogenous interferences), matrix effect, recovery and stability under various conditions. Due to the limited availability of mouse plasma and tissues, partial validations were performed for these specific biomatrices, focusing on accuracy and precision, dilution integrity, endogenous interferences and stability evaluations.

2.6.1. Linearity, sensitivity and carry-over

Each run consisted of eight calibration concentration levels, prepared in the surrogate biomatrices (human K_2EDTA plasma, enzymatic digestion buffer and microdialysate) and was measured in duplicate. Linear regression was chosen as the quantification model with a weighting of $1/x^2$, where x is the analyte concentration. The back-calculated concentrations of each set of calibration standards should not exceed ±15 % of the nominal concentrations (±20 % for the LLOQ). The sensitivity was determined by assessing the signal-to-noise (S/N) ratio at the limit of detection (LOD) and the LLOQ. Carry-over was determined by injection of two double blanks after an upper limit of quantification (ULOQ) calibration standard. The peak area of DNDI-6148 in the two double blanks should not exceed 20 % of the DNDI-6148 peak area of the LLOQ.

2.6.2. Accuracy and precision

The performance of the methods was determined by measuring QC samples (QC-LLOQ, QC-LOW, QC-MID, QC-HIGH) in five-fold for three separate runs for all three surrogate biomatrices. Due to scarcity of blank mouse plasma and tissues, only the intra-method accuracy and precision were determined for these matrices. The calculation of the bias (%) from the nominal concentrations allowed for the determination of the intra- and inter-method accuracy. A one-way analysis of variance (ANOVA) was used to assess intra- and inter-method precision, expressed as the coefficient of variation (CV, %). The bias values should not exceed ±15 % (±20 % for the LLOQ) and the precision values should be ≤15 % (≤20 % for the LLOQ), respectively.

2.6.3. Dilution integrity

Dilution integrity was determined by assessing whether samples >ULOQ could be diluted 20-fold with control matrix and yet still be quantified correctly. For the tissue digestion method, the final extract of the >ULOQ samples was diluted with processed blank sample. The bias and precision values of these diluted samples should not exceed ±15 % and ≤15 %, respectively.

2.6.4. Selectivity

The cross-analyte/IS experiment examined the extent of the potential DNDI-6148 interference in the IS transition or vice versa. This was evaluated by separately spiking the DNDI-6148 analyte at the ULOQ level while maintaining the IS at its standard concentration in two separate samples. These samples were then processed as double blanks, following the procedure outlined in Section 2.4 and Section 2.5. The signal interference at the retention time of DNDI-6148, caused by the IS, was calculated as percentage of the area of the LLOQ, and should not exceed 20 %.

To investigate whether different batches of matrix influence the quantification of DNDI-6148, six different batches of control human K_2EDTA plasma were used. For the other biomatrices, only one batch was used. Double blanks and LLOQ samples were processed for each batch. The endogenous interferences were assessed by comparing the co-eluting peaks in the double blanks at the retention time of DNDI-6148 and the IS with the LLOQ peak area. The peaks in the double blanks should not exceed 20 % of the peak area of the LLOQ sample. In addition, the bias of the spiked LLOQ samples in each batch of biomatrix should fall within ±20 % of the nominal concentration.

2.6.5. Matrix effect and recovery

The matrix effect was investigated by determining the IS-normalized matrix factor (MF) for QC-LOW and QC-HIGH, which was calculated by dividing the DNDI-6148 MF by the IS MF. This was performed for all the surrogate biomatrices. The DNDI-6148 MF and the IS MF were assessed by comparing the peak area of a post-extraction spiked control matrix (matrix present sample) with the peak area of a matrix absent sample.

The recovery of the extraction method for each method was investigated in control matrix at QC-LOW and QC-HIGH levels. The sample preparation recovery was calculated by comparing the area of processed samples with the area of a matrix present sample. The overall recovery was defined as the peak area of the processed sample divided by the peak area in a matrix absent sample.

2.6.6. Stability evaluations

The stability of DNDI-6148 was investigated in surrogate biomatrices and mouse plasma under various conditions, including ambient temperature storage (24 h at nominally 20 °C), long-term storage (at nominally −20 °C or −70 °C), and after three freeze (−20 °C or −70 °C)/thaw (ambient temperature) (F/T) cycles. These surrogate biomatrices were tested at QC-LOW and QC-HIGH levels, while mouse plasma was tested at QC-MID. Each condition was analyzed in triplicate for each concentration level.

The stability of DNDI-6148 in mouse tissue homogenates was also assessed under the following conditions: ambient temperature storage (4 h at ambient temperature, nominally 20 °C), long term storage (nominally −70 °C), and after three F (−70 °C)/T (ambient temperature) cycles. Additionally, a homogenization stability test was conducted on enzymatic digestion buffer and digested mouse tissues, where spiked tissue samples were stored at 37 °C for 16 h to mimic the enzymatic digestion workflow of mouse tissue study samples.

Under the tested conditions, DNDI-6148 was deemed stable in the respective biomatrix if the mean concentration of the stability samples was within ±15 % of the nominal concentration, when quantified on freshly prepared calibration standards. For stock solution stability, concentrations should be within ±5 % of freshly prepared solutions.

2.7. Preclinical application: Target site PK study in infected mice models

The primary objective was to assess whether the developed and validated methods could facilitate PK studies, specifically focusing on target site PK and the distribution of DNDI-6148 in a *Leishmania*-infected mouse model. For this experiment, female BALB/c mice (8–10 weeks old) were purchased from Charles River Laboratories (Margate, UK) and maintained in individually ventilated cages in a controlled environment

of 56 % relative humidity, 20–21 °C and a 12:12 light: dark cycle. They were provided with tap water and a standard laboratory diet *ad libitum* and were left to acclimatize for 5 days prior to the beginning of research studies.

All animal work was conducted under project license PP1651724 at the Biological Services Facility at the University of York and was performed in accordance with the Animal (Scientific Procedures) Act 1986 under a UK Home Office project license according to the European Directive 2010/63/EU. Protocols and procedures were also examined and approved by the University of York Animal Welfare and Ethical Review Board.

2.7.1. Target site PK

BALB/c mice were first injected with stationary phase Ppy RE9H + L. major Friedlin (MHOM/IL/81/Friedlin) promastigotes. Upon the emergence of skin lesions, the diameters of the nodules were monitored until they reached 6 mm. The mice were administered with DNDI-6148 arginine monohydrate (50 mg/kg, bid) via oral gavage for 10 days. During the treatment period, plasma samples were collected from the tail vein on both day 1 and day 10 at these specific time points: 0.5 h, 1.0 h, 2.0 h, 4.0 h, 8.0 h (n = 3 per time point). After 10 days of treatment, the mice were sacrificed and two skin samples, lesional and uninfected skin, as well as the spleen and liver were excised. After collection, all samples were immediately stored at −70 °C and shipped under frozen conditions before being processed and analyzed as described above (Section 2.4 and 2.5). The area-under-the-curve (AUC) and maximal concentration (C_{max}) were both determined using Prism software, version 9.3.0 (GraphPad, Boston, MA, USA). The concentrations of DNDI-6148 (in ng/mL) measured in the lesional and uninfected skin tissue homogenates were converted to concentrations of DNDI-6148 in skin tissue (ng/g). A paired *t*-test was used to determine the differences between lesional and uninfected skin in three different mice, using Prism software, version 9.3.0.

2.7.2. Target site microdialysis

Microdialysis was performed in *Leishmania*-infected mice. Mice were anesthetized using a urethane:chlorprothixene hydrochloride (CPX) mixture (ip, 1.5 g/kg of urethane and 5 mg/kg CPX) and placed on a temperature-controlled heating pad (VetTech, Cheshire, UK) to maintain their body temperature at 32 ± 2°C. Using a 22-gauge needle, MAB 1.2.4 Cu probes (6 kDa-cutoff cuprophane membrane, Microbiotech/se AB, Sweden) were inserted into the following locations: the dermal skin layer of the cutaneous leishmaniasis lesion on the rump and the dermal skin layer of the healthy skin higher up on the back. To equilibrate the system and allow the skin to recover from the probe insertion trauma, a stabilization period of 30 min of perfusion with 2.5 % bovine serum albumin in Ringer's solution at a flow rate of 2 μL/min (CMA 402 syringe pump, Biochrom Ltd, UK) was included before the start of the experiment. At the start of the PK experiment, the mouse was administered with 50 mg/kg DNDI-6148. Samples were collected at 30-minute intervals over a 6-hour period using a refrigerated microdialysis fraction collector set at 4 °C (MAB 85, Microbiotech/se AB). To enhance solubility of the compound, 10 μL acetonitrile was added to each collected sample immediately after collection. Skin microdialysis samples were stored at −20 °C throughout the experiment and transferred to −70 °C at the end of the procedure before being processed and analyzed as described above (Section 2.4 and 2.5).

3. Results and discussion

3.1. Development of the bioanalytical methods

3.1.1. Chromatography and mass spectrometry

Triple quadrupole positive ionization MS was used to detect DNDI-6148. Direct infusion was used to determine the parent and the product ions of DNDI-6148. The MS spectra of DNDI-6148 and the proposed product ions are depicted in Fig. 1B. The parent ion $[M+H]^+$ was observed at *m/z* 336.3, which was selected as the Q1 transition. The most abundant ion in the Q3 spectrum was *m/z* 159.2, and this transition was therefore chosen for the quantification (*m/z* 336.3 → 159.2). An interfering peak was observed during the development of the tissue digestion method, which raised the baseline noise in the 159.2 transition and had a negative impact on the quantification of the lower DNDI-6148 concentrations (Fig. 2A/B). As a result, we selected a different product ion (*m/z* 78.1) to test if the interfering endogenous peak remained. Using this 78.1 transition (*m/z* 336.3 → 78.1) no interfering peak was detected, and the required LLOQ could still be achieved (Fig. 2C) with a large reduction in baseline noise (N = 25 cps versus N = 200 cps for the previous transition). The IS was observed at *m/z* 340.3, and the highest signal was observed in the *m/z* 162.2 transition. This IS transition, *m/z* 340.3 → 162.2, was used for all methods.

Separation was performed using a reversed phase ACQUITY UPLC BEH C18 column using a linear gradient from 30 % to 60 % mobile phase B (0.1 % v/v in acetonitrile) in 2.5 min to obtain a symmetrical peak with retention time of 1.3 min, and a total run time of 4.0 min.

3.1.2. Tissue homogenization optimization

Typically, mechanical homogenization is used to prepare preclinical tissue samples, disrupting the tissue structures with metal beads. A downside of this sample preparation method is the heat generated, which can negatively impact the stability of the analyte of interest. In addition, mechanical homogenization might lead to insufficient tissue disruption and analyte recovery for (human) skin tissues [11]. We recently developed a human skin tissue homogenization workflow using a collagenase A-based enzymatic digestion buffer [12], and investigated its applicability for mouse skin tissue and other organs. This involved visual inspection of the homogenates and assessment of absolute recovery of the enzymatic homogenization workflow in comparison with a mechanical homogenization workflow. Visual inspection indicated that the enzymatic homogenization method was capable of fully homogenizing the various tissues, except for skin, for which a small layer of the skin remained present. We hypothesized that this was the stratum corneum, the outer layer of the skin, mainly comprising dead keratinocytes. Generally, the stratum corneum is the limiting factor in the exposure of topical drugs to the deeper layers of the skin. Additionally, parasites are generally not present in this layer. Therefore, this layer was deemed irrelevant for drug quantification. The absolute recovery was assessed by using the two homogenization techniques on four study samples from two different dosing groups. Enzymatic homogenization resulted in a median of 2.9 (1.9–3.4) fold higher concentration than mechanical homogenization, indicating that the former extracts the compound more effectively from the skin tissue. Based on this and visual inspection, the decision was made to use the enzymatic workflow for tissue homogenization. During the development of the workflow, it was observed that DNDI-6148 was unstable under the homogenization conditions (16 h at 37 °C, in the presence of a peptide-cleaving enzyme), resulting in a loss of analyte area response of 20–40 % for both QC-LOW and QC-HIGH levels. We hypothesize that this loss is due to the presence of a peptide moiety in DNDI-6148 which could be cleaved by the enzyme collagenase A. Investigations into incubation without the enzyme and exposure to light demonstrated no degradation of the compound. This decrease in analyte area response was corrected by an equivalent decrease in IS area response, keeping the DNDI-6148/IS ratio constant. The degradation of DNDI-6148 during the homogenization did not affect the accuracy of the method if IS was added prior to the homogenization of tissues.

3.1.3. Sample preparation

Various sample preparation methods were tested during the development of the methods. A liquid–liquid extraction using *tert*-methylbutyl ether resulted in a near-zero recovery in both acidic and alkalic environments. Protein precipitation was also investigated using three

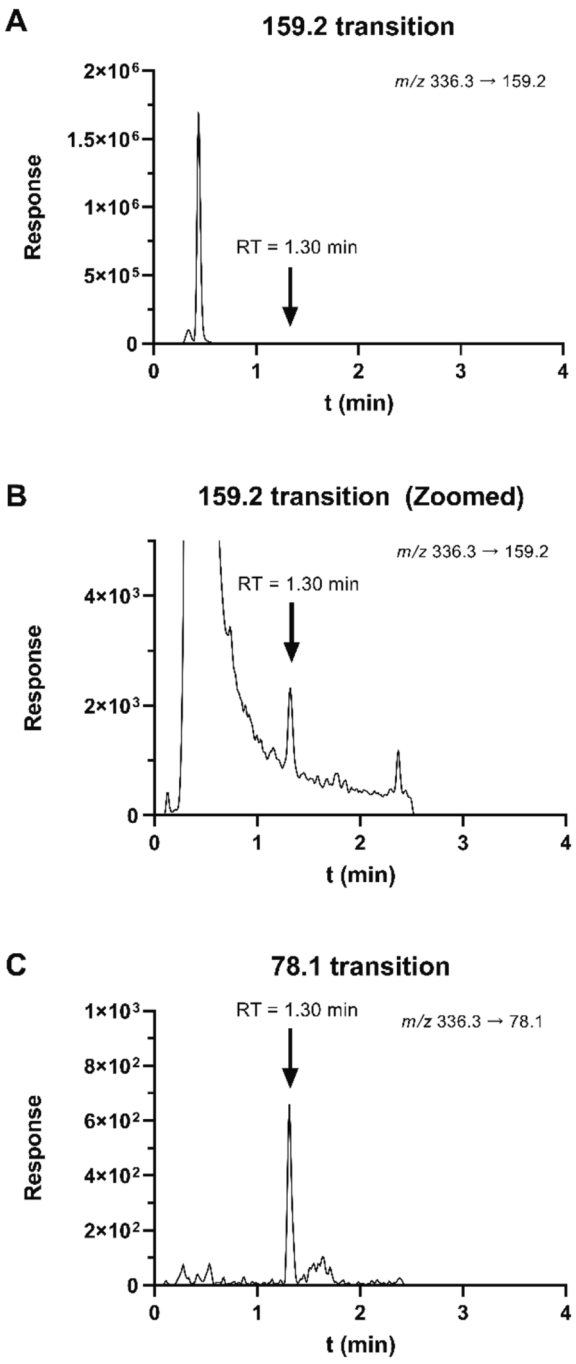

Fig. 2. Endogenous interferences in the chromatograms of DNDI-6148 in the same processed enzymatic digestion buffer sample at the LLOQ level. (A) The 159.2 transition (*m/z* 336.3 → 159.2) with an endogenous peak originating from the enzymatic digestion buffer. The arrow indicates the DNDI-6148 peak (at 1.30 min). (B) The zoomed chromatograms, highlighting the DNDI-6148 peak. An increased baseline is observed due to the endogenous peak, limiting the quantification. (C) The 78.1 transition (*m/z* 336.3 → 78.1) did not show this interfering peak and was chosen for the validation of the method in enzymatic digestion buffer and microdialysate.

organic solvent mixtures (acetonitrile, methanol, and acetonitrile-methanol (1:1, v/v)). For each surrogate biomatrix, the precipitation mixture which yielded the best recovery and the lowest variability was chosen.

3.2. Validation procedures

3.2.1. Linearity, sensitivity and carry-over

Each method was linear over the concentration range used. The back-calculated concentrations of these calibration standards all met the requirements (Table 1). A correlation coefficient of the linear fit (r^2) of 0.9975 or better were obtained for all calibration standard curves. The S/N ratio at the LLOQ sample was at least 10, 11 and 21, for the plasma, enzymatic digestion buffer and microdialysate methods, respectively. The LOD was set at an S/N ratio of 3, resulting in LODs of 1.50 ng/mL, 0.600 ng/mL and 0.400 ng/mL, respectively. Representative MRM chromatograms of DNDI-6148 and the IS in human K_2EDTA plasma are shown in Fig. 3.

Carry-over was only observed for the tissue and the microdialysate methods, which was found to be 7.2 % of the area of the LLOQ in the first double blank for both methods. The second double blank did not show any presence of a carry-over peak. Therefore, it was concluded that there was no substantial carry-over affecting the integrity of the data.

3.2.2. Accuracy and precision

A detailed overview of the accuracy and precision data for each validated method is provided in Table 2. The intra- and inter-method accuracy ranged between −3.3 % and +13.9 %, and the intra- and inter-method precision was at most ≤8.9 % for all surrogate matrices at all tested concentrations. Therefore, acceptance criteria of ±15 % for bias and ≤15 % for precision were met for all conditions.

The data on the accuracy and precision of the other biomatrices is presented in Table 3. Mouse plasma was successfully quantified using calibration standards prepared in human K_2EDTA plasma, and the concentration of DNDI-6148 was accurately determined in enzymatically digested mouse tissue homogenates using the calibration standards prepared in digestion buffer. Acceptable accuracy values (ranging from −13.4 % to 5.8 %) and precision values (≤11.8 %) demonstrated that human K_2EDTA plasma and enzymatic digestion buffer could be used as surrogate biomatrices for the quantification of mouse plasma and homogenized mouse tissues, respectively.

3.2.3. Dilution integrity

The bias and precision of the 20-fold dilution were ±2.5 % and ≤5.3 %, ±0.4 % and ≤1.5 %, ±6.1 % and ≤3.7 % for the plasma, tissue digestion and the microdialysate methods, respectively. The dilution integrity of mouse plasma and tissues was also determined. This data is summarized in Table 3 and was all within the acceptance criteria. All biomatrices were able to quantify diluted samples accurately and precisely, which means that study samples can be diluted in the appropriate biomatrix if the concentration is >ULOQ.

3.2.4. Selectivity

The cross analyte/IS experiments showed that there were no interference peaks in either the DNDI-6148 transition or for the IS, indicating that there were no cross-analyte/IS interferences.

The deviation from the LLOQ concentration was ±7.2 % in six different batches of human K_2EDTA plasma, and no interferences originating from the different batches of matrix were observed in the DNDI-6148 or IS transition. Due to the absence of different batches of enzymatic digestion buffer, microdialysate, and mouse plasma and tissues, only one batch was used for the endogenous interferences experiment. The bias of the quantification of the LLOQ did not exceed the acceptance criteria of ±20 % for each biomatrix. There was also no interference observed in the DNDI-6148 or IS transition except for the enzymatic digestion buffer, which is already described in Section 3.1.

3.2.5. Matrix effect and recovery

The enzymatic digestion buffer at QC-LOW level showed potential ion enhancement caused by the matrix (MF of 1.19). However, the IS-normalized MF for the surrogate matrices were between 0.99 and 1.06

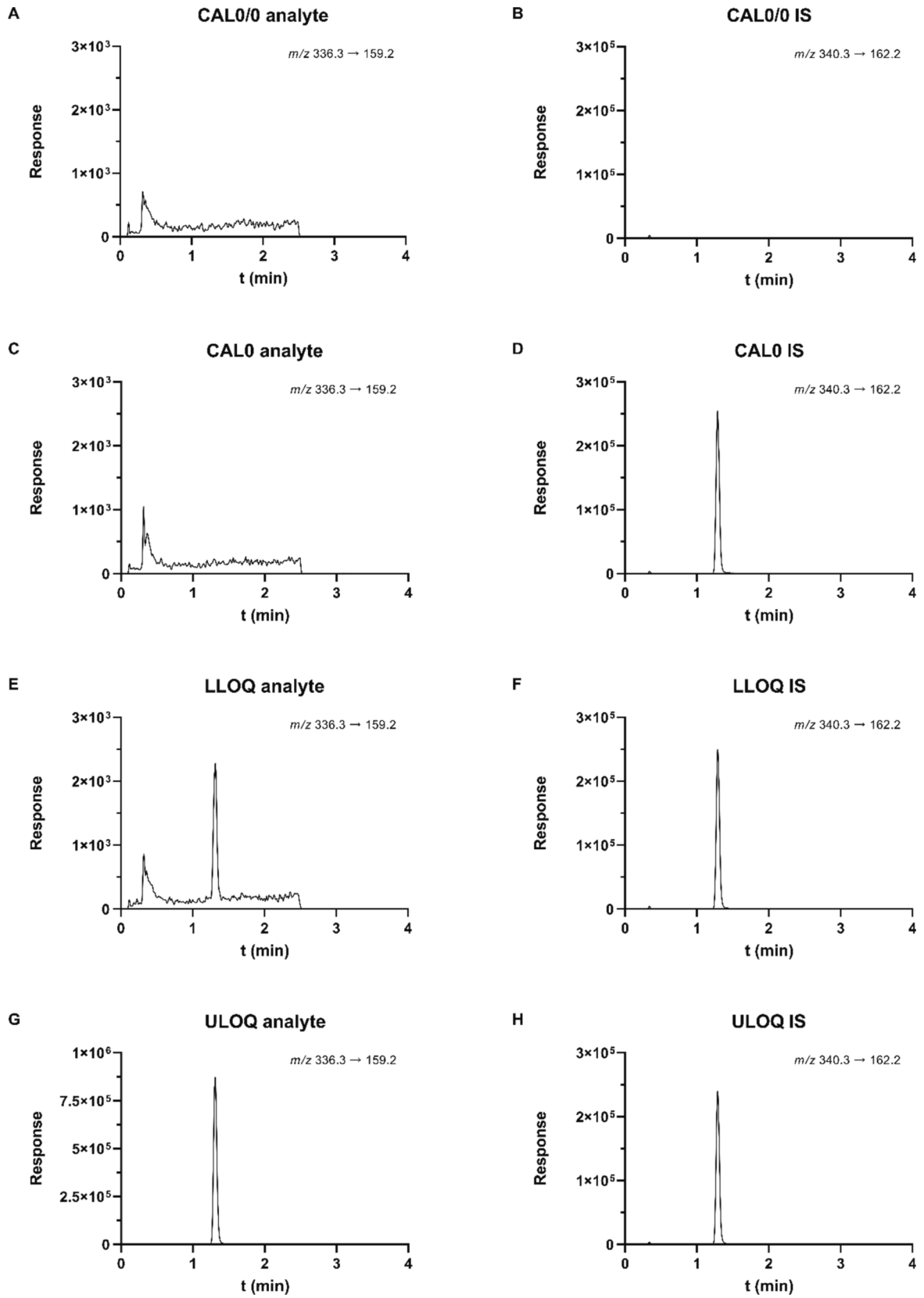

Fig. 3. Representative chromatograms of DNDI-6148 and the internal standard (IS) for the double blank (A and B), the blank (C and D), the lower limit of quantification (LLOQ) of 5.00 ng/mL (E and F) and the upper limit of quantification (ULOQ) of 2,000 ng/mL (G and H), at their respective transitions in human K_2EDTA plasma.

Table 3
Method performance data for the partial validation of DNDI-6148 in mouse K_2EDTA plasma and several digested mouse tissues. The accuracy (Bias, %) and the precision (CV, %) were analyzed at five concentrations in quintuplicate in a single run.

Matrix	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Accuracy (Bias, %)	Precision (CV, %)
Mouse K_2EDTA plasma	5.00	4.89	−2.0	6.2
	15.0	14.9	−0.5	3.5
	150	155	3.2	1.1
	1,500	1,540	2.7	0.9
	20,000	21,280	6.4	3.3
Mouse skin tissue homogenate	2.00	2.08	4.1	9.2
	6.00	6.35	5.8	3.6
	80.0	82.1	2.7	1.4
	750	753	1.4	2.3
	4,000	4,020	0.5	1.1
Mouse liver homogenate	2.00	2.02	1.2	7.5
	6.00	6.10	1.6	3.1
	80.0	80.1	0.2	2.7
	750	760	1.3	2.5
	4,000	4,020	0.5	1.8
Mouse spleen homogenate	2.00	1.73	−13.4	11.8
	6.00	6.14	2.4	4.4
	80.0	77.6	−3.0	1.8
	750	740	−1.3	1.4
	4,000	3,904	−2.4	1.7

Abbreviations: CV = coefficient of variation

(CV ≤ 4.5 %), demonstrating that the ion enhancement or suppression encountered, induced by interfering endogenous compounds in the processed matrix could successfully be overcome by IS correction and thus did not have any influence on the quantification of DNDI-6148 (Table 4).

The sample preparation recovery was 75.5–80.0 % (CV ≤ 3.8 %), 64.7–71.4 % (CV ≤ 3.4 %) and 83.9–84.9 % (CV ≤ 1.5 %), for human K_2EDTA plasma, enzymatic digestion buffer and microdialysate, respectively (Table 4). The overall recovery and the variability were comparable to that of the sample preparation recovery for human K_2EDTA plasma and microdialysate. The difference between the overall recovery and the sample preparation recovery, observed in the enzymatic digestion buffer method at QC-LOW level, can be attributed to the matrix effect, as previously explained.

3.2.6. *Stability evaluations*

The results of the stability experiments are presented in Table 5. The stability of DNDI-6148 was not affected by three F/T cycles for all biomatrices. DNDI-6148 in microdialysate was found to be unstable when stored at ambient temperature for 24 h, while the other two surrogate biomatrices did not show this tendency. Furthermore, DNDI-6148 remained stable in human K_2EDTA plasma for 34 days when stored at −20 °C. It also remained stable in microdialysate, enzymatic digestion buffer and mouse plasma for 14 days, 16 days and 65 days, respectively, when stored at −70 °C. The stock solution of DNDI-6148 (in methanol) was also stable for 180 days when stored at −20 °C.

DNDI-6148 remained stable in digested tissue homogenates after storage for 9 days at −70 °C and after undergoing three F/T cycles. Additionally, storage at ambient temperature for 6 h did not affect the stability of DNDI-6148. To mimic the homogenization conditions (37 °C for 16 h), the enzymatic digestion buffer and digested tissue homogenates were incubated, after which the IS was added. The results confirmed our aforementioned suspicion about the instability of DNDI-6148 in the enzymatic digestion buffer, with a bias of at least −18.9 % from the nominal concentration across the investigated biomatrices. The pre-incubation addition of IS effectively corrected for the instability of the analyte during incubation resulting in a maximum bias of ±2.5 % from the nominal concentration across all investigated biomatrices (Table 6). This ensured accurate determination of the total concentration of DNDI-6148 in mouse tissues, accounting for the loss of the compound during the homogenization incubation.

3.3. *Preclinical application*

The validated analytical method was used to quantify samples collected during a preclinical study including mouse plasma, mouse skin tissue and microdialysate. Extensive interpretation and discussion of the results of the preclinical study will be reported elsewhere. The PK curves

Table 4
Matrix factor and recovery (in %) for DNDI-6148 in each appropriate biomatrix, determined at QC-LOW and QC-HIGH levels.

Biomatrix	Nominal concentration (ng/mL)	MF DNDI-6148 (CV, %)	IS − normalized MF (CV, %)	Sample preparation recovery (CV, %)	Overall recovery (CV, %)
Human plasma	15.0	1.03 (4.0 %)	1.03 (3.6 %)	80.0 % (3.8 %)	79.8 % (2.5 %)
	1500	1.01 (0.7 %)	1.00 (1.1 %)	75.5 % (0.4 %)	75.0 % (0.9 %)
Enzymatic digestion buffer	6.00	1.19 (4.8 %)	1.02 (4.5 %)	64.7 % (1.4 %)	76.3 % (1.1 %)
	750	1.03 (1.4 %)	0.99 (1.7 %)	71.4 % (3.4 %)	73.5 % (3.2 %)
Microdialysate	9.00	0.97 (3.1 %)	1.06 (3.4 %)	83.9 % (1.5 %)	80.3 % (4.5 %)
	450	0.96 (0.8 %)	1.04 (1.8 %)	84.9 % (0.7 %)	81.3 % (2.5 %)

Abbreviations: MF = matrix factor; CV = coefficient of variation; IS = internal standard;

Table 5
Stability parameters for DNDI-6148 in biomatrix and stock solution with the accuracy (Bias, %) and precision (CV, %) (n = 3 samples per condition, per concentration level).

Matrix	Condition	Nominal concentration (ng/mL)	Accuracy (Bias, %)	Precision (CV, %)
Human K_2EDTA plasma	−20 °C, 34d	15.0	0.0	2.1
		1,500	6.7	0.9
	RT, 24 h	15.0	1.7	3.3
		1,500	0.2	2.0
	3 F/T	15.0	4.0	2.8
		1,500	9.6	2.9
Mouse K_2EDTA plasma	−70 °C, 65d	150	10.2	9.8
	RT, 24 h	150	3.8	2.0
	3 F/T	150	1.3	3.1
Enzymatic digestion buffer	−70 °C, 16d	6.00	5.2	5.8
		750	2.4	1.5
	RT, 24 h	6.00	−2.6	0.4
		750	−3.0	2.6
	3 F/T	6.00	11.5	2.5
		750	2.7	4.0
	37 °C, 16 h	6.00	−35.7	6.4
		750	−18.9	0.5
Mouse skin tissue homogenate	−70 °C, 9d	80.0	−5.3	2.2
	RT, 6 h	80.0	−1.4	1.2
	3 F/T	80.0	−1.9	1.6
	37 °C, 16 h	80.0	−46.1	6.0
Mouse liver homogenate	−70 °C, 9d	80.0	−3.6	4.0
	RT, 6 h	80.0	4.5	4.0
	3 F/T	80.0	−3.9	2.4
	37 °C, 16 h	80.0	−24.0	3.2
Mouse spleen homogenate	−70 °C, 9d	80.0	−2.5	0.9
	RT, 6 h	80.0	−1.6	1.5
	3 F/T	80.0	−0.4	2.5
	37 °C, 16 h	80.0	−25.9	7.1
Microdialysate	−70 °C, 14d	9.00	3.9	5.0
		450	2.6	2.0
	RT, 24 h	9.00	−33.9	3.9
		450	−14.0	3.2
	3 F/T	9.00	−2.0	4.7
		450	1.3	3.6
Methanol (Stock solution)	−20 °C, 180d	$1.00*10^6$	4.7	2.7

Abbreviations: CV = coefficient of variation; d = days; F/T = freeze/thaw cycles; h = hours;

at day 1 and day 10 of treatment are depicted in Fig. 4A. DNDI-6148 accumulated in the plasma during a 10-day treatment ($AUC_{D1,\ 0h\rightarrow8h}$ of 102.7 ± 4.28 μg·h/mL versus $AUC_{D10,\ 0h\rightarrow8h}$ of 161.8 ± 13.9 μg·h/mL, $C_{max,D1}$ of 22.3 ± 1.35 μg/mL versus $C_{max,D10}$ of 28.7 ± 4.26 μg/mL). The concentrations of DNDI-6148 in the lesional and uninfected skin tissue, collected at 24 h after a 10-day treatment, are shown in Fig. 4B. There was no significant difference between the mean DNDI-6148 concentrations in lesional and uninfected skin tissue (1,986 ± 85.0 ng/g for lesional skin versus 1,926 ± 167 ng/g for uninfected skin, p value of 0.4646).

Skin microdialysate was collected at 30-minute intervals over a 6-hour period following administration. All collected samples had quantifiable levels of DNDI-6148 within the validated concentration range. Fig. 4C shows the measured concentrations of DNDI-6148 in microdialysate obtained from both lesional and uninfected skin probes. After two hours the free drug concentration tends to reach a steady state.

Table 6
Comparison of DNDI-6148 (n = 3 samples per tested concentration level) determination in enzymatic digestion buffer and digested mouse tissues with internal standard (IS) addition pre- and post-incubation (at 37 °C for 16 h), highlighting the importance of pre-incubation addition of IS.

Matrix	Nominal concentration (ng/mL)	Addition IS post-incubation		Addition IS pre-incubation	
		Accuracy (Bias, %)	Precision (CV, %)	Accuracy (Bias, %)	Precision (CV, %)
Enzymatic digestion buffer	6.00	−35.7	6.4	−2.5	4.9
	750	−18.9	0.5	0.7	1.7
Mouse skin homogenate	80.0	−46.1	6.0	−0.5	2.2
Mouse liver homogenate	80.0	−24.0	3.2	−1.1	0.6
Mouse spleen homogenate	80.0	−25.9	7.1	1.1	1.7

Abbreviations: CV = coefficient of variation; IS = internal standard;

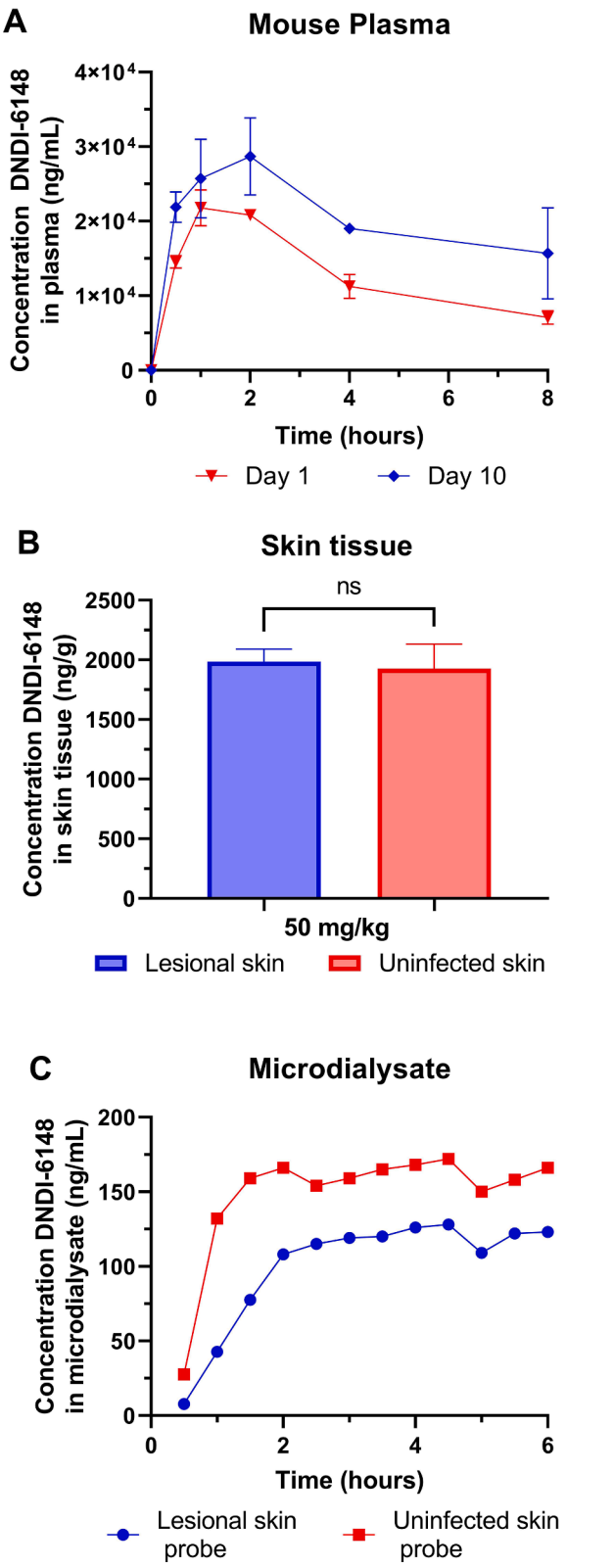

Fig. 4. Determination of DNDI-6148 in different mouse biomatrices in a preclinical experiment, in which all mice received a 50 mg/kg oral dose. (A) DNDI-6148 concentration–time profile of *Leishmania*-infected mice (n = 3) after 1 day or 10 day-treatment. (B) DNDI-6148 infected and uninfected skin tissue concentrations at 24 h after a 10 day-treatment (n = 3). (C) DNDI-6148 concentration in microdialysis samples retrieved for the lesional and uninfected skin probe (n = 1).

There seems to be a tendency for the free-drug concentration to be higher in the uninfected skin compared to the lesional skin.

All the preclinical samples measured, including mouse plasma, mouse skin and microdialysate, exceeded the LLOQ, indicating adequate sensitivity of the developed methods.

4. Conclusion

We successfully developed and validated bioanalytical methods to quantify the NCE DNDI-6148, a CL drug candidate, in mouse plasma, skin, liver, spleen and skin microdialysate within international validation acceptance criteria. To begin, mouse tissues were homogenized using a collagenase A-based enzymatic buffer. All samples were precipitated to extract DNDI-6148 from the biomatrices. The overall recovery was high, ranging from 73.5 % to 81.3 % with low variability (CV $\leq$ 4.5 %) and no significant matrix effects. The extracted samples were quantified using a fast and sensitive UPLC-MS/MS method. Stability evaluations demonstrated that DNDI-6148 was unstable under the homogenization conditions. However, this issue was mitigated by the addition of the IS pre-incubation. The developed methods were successfully used to support a preclinical target site PK exposure–response study in CL-infected murine models. The concentration of DNDI-6148 was successfully determined in samples obtained from the study, including mouse plasma, mouse skin and microdialysate. The use of these methods could further help to predict a safe, tolerable and effective in-human dose for the treatment of CL.

CRediT authorship contribution statement

Wietse M. Schouten: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Katrien Van Bocxlaer:** Writing – review & editing, Resources. **Hilde Rosing:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Alwin D.R. Huitema:** Writing – review & editing, Supervision. **Jos H. Beijnen:** Writing – review & editing, Supervision. **Jadel M. Kratz:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Charles E. Mowbray:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Thomas P.C. Dorlo:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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