Journal of Chromatography B, 998-999 (2015) 57–62

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Quantification of miltefosine in peripheral blood mononuclear cells by high-performance liquid chromatography-tandem mass spectrometry

A.E. Kip[a,b,*], H. Rosing[a], M.J.X. Hillebrand[a], M.M. Castro[c], M.A. Gomez[c], J.H.M. Schellens[b,d], J.H. Beijnen[a,b,d], T.P.C. Dorlo[b]

[a] Department of Pharmacy & Pharmacology, Antoni van Leeuwenhoek Hospital/Slotervaart Hospital, Amsterdam, The Netherlands
[b] Division of Pharmacoepidemiology & Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences (UIPS), Faculty of Science, Utrecht University, Utrecht, The Netherlands
[c] Centro Internacional de Entrenamiento e Investigaciones Medicas (CIDEIM), Cali, Colombia
[d] Department of Clinical Pharmacology, Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 13 March 2015
Received in revised form 11 June 2015
Accepted 13 June 2015
Available online 24 June 2015

ABSTRACT

Phagocytes, the physiological compartment in which *Leishmania* parasites reside, are the main site of action of the drug miltefosine, but the intracellular pharmacokinetics of miltefosine remain unexplored. We developed a bioanalytical method to quantify miltefosine in human peripheral blood mononuclear cells (PBMCs), expanding from an existing high performance liquid chromatography-tandem mass spectrometry method for the quantification of miltefosine in plasma. The method introduced deuterated miltefosine as an internal standard. Miltefosine was extracted from PBMC pellets by addition of 62.5% methanol. Supernatant was collected, evaporated and reconstituted in plasma. Chromatographic separation was performed on a reversed phase C18 column and detection with a triple-quadrupole mass spectrometer. Miltefosine was quantified using plasma calibration standards ranging from 4 to 1000 ng/mL. This method was validated with respect to its PBMC matrix effect, selectivity, recovery and stability. No matrix effect could be observed from the PBMC content (ranging from 0.17 to 26.3 $\times$ 10^6 PBMCs) reconstituted in plasma, as quality control samples were within 3.0% of the nominal concentration (precision less than 7.7%). At the lower limit of quantitation of 4 ng/mL plasma, corresponding to 0.12 ng/10^6 PBMCs in a typical clinical sample, measured concentrations were within 8.6% of the nominal value. Recovery showed to be reproducible as adding additional pre-treatment steps did not increase the recovery with more than 9%. This method was successfully applied to measure intracellular miltefosine concentrations in PBMC samples from six cutaneous leishmaniasis patients up to one month post-treatment.

1. Introduction

The *Leishmania* parasite, causative agent of the neglected infectious disease leishmaniasis, resides and replicates within human phagocytes. These cells are therefore the main site of action of the antileishmanial drug miltefosine [1], however, the intracellular pharmacokinetics of the drug are currently unknown. Miltefosine is transported into cells both by passive incorporation in the cellular membranes (non-saturable from 20 to 200 μM/8.2 to 82 μg/mL) and by active carrier-mediated cellular transport (saturable at 50 μM/20.4 μg/mL) [2,3]. In Dutch cutaneous leishmaniasis (CL) patients, the average steady-state plasma concentration, reached only in the last week of treatment during a standard 28-day miltefosine regimen, was 30.8 μg/mL [4]. Within the treatment period, the contribution of the active (saturable) transport is thus substantial and the relative contribution of both transport mechanisms on the intracellular miltefosine accumulation *in vivo* is expected to vary during treatment. The saturability of the active transport could result in substantial between-subject variability in intracellular miltefosine concentrations.

Resident tissue macrophages are the host cells for intracellular *Leishmania* survival and replication. Thus, intracellular drug quantification is pivotal to provide a better understanding of the drug disposition within the physiological compartment in which the parasites reside. Intracellular miltefosine concentrations better represent the drug concentrations to which the parasites are

* Corresponding author at: Department of Pharmacy & Pharmacology, Antoni van Leeuwenhoek hospital/Slotervaart hospital, Amsterdam, the Netherlands, P.O. Box 90440, 1006 BK, Amsterdam, The Netherlands.
E-mail address: anke.kip@slz.nl (A.E. Kip).

http://dx.doi.org/10.1016/j.jchromb.2015.06.017
1570-0232/

exposed and will probably relate more accurately to *Leishmania* drug susceptibility and pharmacokinetic/ pharmacodynamic relationships than plasma drug concentrations.

We have previously validated an LC/MS–MS assay to measure miltefosine in plasma [5]. Here we expand this method to intracellular measurements. In this assay peripheral blood mononuclear cells (PBMCs) were used as a model to assess intracellular miltefosine accumulation within human leukocytes. The sample pre-treatment was modified and a partial validation was executed. This assay was evaluated using PBMC samples from six Colombian CL patients treated with a miltefosine monotherapy.

2. Methods

2.1. Chemicals and reagents

Miltefosine and phosphate buffered saline (PBS) were purchased from Sigma–Aldrich (Zwijndrecht, the Netherlands), and deuterated miltefosine (miltefosine-D4, Fig. 1) from Alsachim (Illkirch Graffenstaden, France). Acetonitrile, methanol and H_2O were obtained from Biosolve Ltd. (Valkenswaard, the Netherlands), ammonia 25%, triethylamine and acetic acid 99.8% from Merck (Amsterdam, the Netherlands) and Ficoll from GE Healthcare (Hoevelaken, the Netherlands). Blank Na-EDTA plasma was obtained from Bioreclamations (Baltimore, US).

2.2. Clinical sample collection and PBMC isolation

Heparin-treated blood samples (10 mL for adults, 3 mL for children) were taken from CL patients (Section 2.7) and centrifuged 10 min at 800 × *g* at room temperature. All plasma was transferred and stored at −80 °C, while the remaining blood sample was diluted 1:4 in PBS and placed over a Ficoll gradient at a 1:5 Ficoll-to-blood ratio. Samples were centrifuged 15 min at 400 × *g* at room temperature and the mononuclear leukocyte layer was isolated. Subsequently the cells were washed two times with 10 mL PBS, resuspended in 1 mL PBS and counted on a haemocytometer. Samples were centrifuged at 800 × *g*, the supernatant was removed, and the PBMC pellet stored at −80 °C. Plasma and PBMC pellets were transported on dry ice to the bioanalytical laboratory and stored at −20 °C until analysis.

The cell pellet was resuspended in 120 μL PBS, after which the cells were lysed by adding 200 μL methanol yielding a total volume of 320 μL 62.5% methanol-PBS (v/v). The sample was vortexed and centrifuged for 5 min at 20,000 × *g*. The supernatant, referred to as the "PBMC lysate" was transferred to a 1.5 mL tube. Depending on the expected concentration, the PBMC lysate volume transferred varied between 50 μL (expected concentration above upper limit of quantitation, ULOQ) and 280 μL (expected concentration close to lower limit of quantitation, LLOQ). Finally, the PBMC lysate was evaporated under a gentle stream of nitrogen and reconstituted in 250 μL of blank Na-EDTA human plasma. These so-called "reconstituted PBMC samples" were handled as normal plasma samples (Section 2.5).

2.3. Preparation of plasma calibration standards and internal standard solution

Calibration standards were prepared in plasma. A stock solution of 1 mg/mL miltefosine was prepared in methanol-water (1:1, v/v). Calibration standard working solutions were further diluted from this stock solution with methanol–water (1:1, v/v) to final concentrations of 0.08, 0.2, 0.4, 1, 2, 4, 8, 16 and 20 μg/mL.

Calibration standards were freshly prepared before each run by spiking 570 μL of blank Na-EDTA human plasma with 30 μL of working solution, yielding calibration standards of 4, 10, 20, 50, 100, 200, 400, 800 and 1000 ng/mL. Two 250 μL aliquots were prepared per calibration standard and processed for each analytical run.

An internal standard working solution was prepared by dilution of a stock solution of 1 mg/mL deuterated miltefosine (miltefosine-D4) in methanol-water (1:1, v/v) to 4000 ng/mL with methanol-water (1:1, v/v).

2.4. Preparation of PBMC quality control samples

A separate stock solution of 1 mg/mL miltefosine in methanol-water (1:1, v/v) was prepared from an independent weighing for the preparation of quality control (QC) samples. PBMC QC working solutions were diluted from this stock solution with methanol-water (1:1, v/v) to concentrations of 0.6, 15 and 37.5 μg/mL and an LLOQ working solution of 0.2 μg/mL.

To mimic the study samples, QC samples were prepared freshly by spiking 5 μL of working solution to 95 μL of blank PBMC lysate. To prepare blank PBMC lysate, blank PBMCs were isolated from human leukocyte buffy coat (∼50 mL, freshly derived from 500 mL whole blood) purchased from Sanquin (Amsterdam, the Netherlands). 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. After a 20 min 550 × *g* centrifugation at 4 °C (without brake), 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 × *g* for 5 min at 4 °C (without brake). The supernatant was discarded and the pellet was resuspended in 300 μL PBS. All aliquots were pooled and a cell count was performed with a Cell Dyn Hematology analyzer (Abbott Diagnostics, Lake Forest, IL). After a 3000 × *g* centrifugation, PBS was removed to adjust the PBMC concentration to approximately 200 × 10^6 cells per mL PBS, to obtain reconstituted PBMC QC samples containing 7.1 × 10^6 cells, close to the mean found in patient samples (see Section 2.7). Depending on the final volume of blank PBMCs in PBS, a volume of methanol was subsequently added to obtain PBMC lysate of 62.5% methanol-PBS (v/v).

After spiking, the QCs were evaporated under a gentle stream of nitrogen and reconstituted in 250 μL of blank Na-EDTA human plasma. The final miltefosine concentrations of the reconstituted PBMC QCs were 12, 300 and 750 ng/mL (low; QCL, mid; QCM and high; QCH respectively). Considering an average of 7.1 × 10^6 PBMCs in the reconstituted PBMC samples, this would correspond to concentrations of 0.42, 11 and 26 ng/10^6 cells.

2.5. Plasma sample preparation and LC-MS/MS analysis

Reconstituted PBMC samples and plasma calibration standards were further prepared as previously described [5] with slight modifications. First, 25 μL of miltefosine-D4 (4000 ng/mL) was added to each 250 μL aliquot, except for double blanks to which 25 μL methanol-water (1:1, v/v) was added. All samples were briefly vortexed and subsequently 700 μL of acetic acid buffer (1 M, pH 4.5) was added. Samples were vortexed and centrifuged 5 min at 23,100 × *g* at room temperature. The extraction of miltefosine was performed on Bond Elut PH SPE cartridges (Agilent Technologies, Amstelveen, the Netherlands), which were first conditioned with 1 mL acetonitrile and subsequently 1 mL acetic acid buffer (1 M, pH 4.5). Afterwards, samples were loaded on the SPE cartridges and the cartridges were washed with 1 mL methanol–water (1:1, v/v). The analyte was eluted with two times 750 μL of 0.1% (v/v) triethylamine in methanol. The eluate was transferred to a glass autosampler vial and 10 μL was injected on the analytical column.

The chromatographic separation and LC–MS/MS analysis was performed as described previously [5], but the more sensitive API3000 triple-quadrupole mass spectrometer was used, which