

Development and validation of a quantitative assay for the measurement of miltefosine in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

A sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay for the quantification of miltefosine is presented. A 250 μ L human EDTA plasma aliquot was spiked with miltefosine and extracted by a solid-phase extraction method. Separation was performed on a Gemini C18 column (150 mm \times 2.0 mm I.D., 5 μ m) using an alkaline eluent. Detection was performed by positive ion electrospray ionization followed by triple-quadrupole mass spectrometry. The assay has been validated for miltefosine from 4 to 2000 ng/mL using 250 μ L human EDTA plasma samples. Results from the validation demonstrate that miltefosine can be accurately and precisely quantified in human plasma. At the lowest level, the intra-assay precision was lower than 10.7%, the inter-assay precision was 10.6% and accuracies were between 95.1 and 109%. This assay is successfully used in a clinical pharmacokinetic study with miltefosine.

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1. Introduction

Miltefosine (hexadecylphosphocholine, D-18506, Miltex[®], Impavido[®], Fig. 1) is the first oral drug registered for the treatment of both the visceral and the cutaneous form of leishmaniasis, which is an infectious disease caused by parasites of the genus *Leishmania*. The protozoan parasites are transmitted to a mammal host through the bite of a female sandfly [1,2]. Leishmaniasis is one of the diseases designated by the World Health Organization (WHO) as a ‘neglected disease’, a term reserved for diseases in mainly developing countries for which there is a lack of effective, affordable, or easy to use drug treatments [3].

For long, the treatment of leishmaniasis was based on antimony containing salts, like meglumine antimoniate or sodium

stibogluconate. However, in many parts of the world and particularly India the parasite has become resistant to antimony [4,5]. An alternative treatment is liposomal amphotericin B, which has the disadvantages that it has to be administered parenteral and is very expensive [5].

Miltefosine was originally developed in the early 1980s as an antitumor agent, however its anticancer activity was shown to be limited due to unacceptable gastrointestinal toxicity and low activity when given by the oral route [6,7]. The antiprotozoal activity of miltefosine appeared more promising for the treatment of leishmaniasis [5,8]. Miltefosine is considered highly effective in the treatment of visceral leishmaniasis [2,5,9]. The efficacy in cutaneous leishmaniasis in South America appeared to be comparable to historic efficacy rates of the standard antimony treatment, although the efficacy depended on the causative *Leishmania*-strain [10]. A trial with mucocutaneous leishmaniasis patients showed the good applicability of miltefosine in the treatment of this difficult to treat form of leishmaniasis [11].

The pharmacokinetics of miltefosine have scarcely been studied and a definitive treatment regimen based on pharma-

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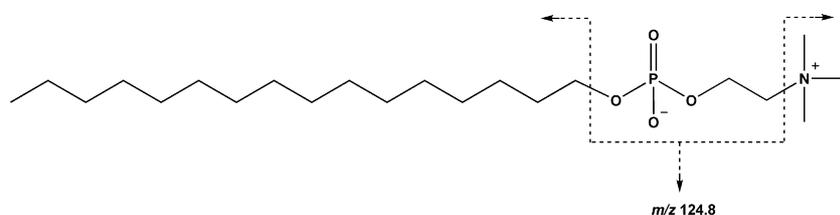


Fig. 1. Structure of miltefosine and the proposed m/z fragmentation.

cokinetic/pharmacodynamic data is lacking. Only one detection method specifically for miltefosine has previously been reported using an evaporative light scattering detector [12]. However, this method was not validated properly, was not very sensitive (a detection limit of 340 ng/mL in 5% fetal calf serum) and seems more appropriate for the analysis of pharmaceutical preparations. A sensitive and validated bioanalytical assay specifically for miltefosine was not reported hitherto. Two methods are described in the literature to measure perifosine, a structural analog of miltefosine, in human plasma using liquid chromatography coupled tandem mass spectrometry (LC–MS/MS) with miltefosine as an internal standard, and either automated solid-phase extraction (SPE) or protein precipitation as sample pretreatment techniques [13,14]. However, when we tried to employ these methods in our laboratory for the quantitative analysis of miltefosine, both methods demonstrated a high variability in results due to both the sample pretreatment as well as the LC system. Therefore, we developed a sensitive and accurate LC–MS/MS method to quantitate miltefosine in human plasma. Validation of the method was performed according to the FDA guidelines [15]. To illustrate the usefulness of the current assay in a clinical setting, we present the quantitative results of miltefosine measurements in blood plasma samples of a patient

with cutaneous leishmaniasis treated for 28 days with miltefosine.

2. Experimental

During the development and validation of this assay, perifosine was used and added to each sample as an internal standard. However, a high intra-assay variability of the internal standard response was noticed during the validation. By leaving out the internal standard response from the back-calculation of the miltefosine concentrations, better intra-assay accuracies and precisions were achieved (see Table 1 and Section 3.2). Therefore, all miltefosine concentrations were eventually back-calculated from the peak area of miltefosine alone. Hence, experimental, performance, recovery and stability data of the internal standard were omitted in both Sections 2 and 3 of this report.

2.1. Chemicals and reagents

Miltefosine was kindly supplied by Zentaris GmbH (Frankfurt, Germany). Acetonitrile and methanol (both HPLC supra gradient grade) were obtained from Biosolve Ltd. (Valkenswaard, the Netherlands). Ammonia 25%, acetic acid

Table 1
Assay performance data for miltefosine

Run	Nominal concentration (ng/mL)	Mean calculated concentration ^a (ng/mL)	Accuracy ^a (%deviation)	Precision ^a (%CV)	Accuracy ^b (%deviation)	Precision ^b (%CV)	No. of replicates
1	4.05	3.90	−3.64	8.33	7.70	7.92	5
2	4.05	4.39	8.54	10.7	27.2	12.2	5
3	4.05	4.09	0.985	9.39	3.34	4.36	5
Average	4.05	4.12	1.96 ^c	10.6 ^d	12.8 ^c	12.0 ^d	15
1	12.1	11.7	−3.96	6.33	5.23	5.29	5
2	12.1	11.6	−4.67	7.11	4.60	12.1	5
3	12.1	12.2	0.198	5.29	1.34	8.88	5
Average	12.1	11.8	−2.81 ^c	5.82 ^d	3.73 ^c	9.07 ^d	15
1	303	289	−4.87	3.73	6.59	4.21	5
2	303	302	−0.353	4.43	10.8	7.44	5
3	303	318	4.88	2.18	6.87	3.73	5
Average	303	303	−0.117 ^c	5.15 ^d	8.09 ^c	5.51 ^d	15
1	1520	1460	−3.89	6.14	−2.14	2.43	5
2	1520	1490	−1.78	3.78	11.1	6.20	5
3	1520	1560	3.11	2.84	1.81	1.70	5
Average	1520	1500	−0.851 ^c	5.14 ^d	3.58 ^c	6.61 ^d	15

^a Back-calculated from analyte peak area alone.

^b Back-calculated from analyte/internal standard peak area ratio.

^c Inter-assay accuracy (%).

^d Inter-assay precision.

99.8% and triethylamine (all analytical grade) were from Merck (Amsterdam, the Netherlands). Distilled sterile water (Aqua B. Braun, Oss, the Netherlands) was used throughout the analyses. Drug-free human EDTA plasma was obtained from Bioreclamation Inc. (Hicksville, NY, USA).

2.2. Equipment

Chromatographic separation of miltefosine was carried out using an HP1100 liquid chromatograph system (Agilent Technologies, Palo Alto, CA, USA) consisting of a binary pump, degasser and autosampler. Attached to this system was a pre-column (Gemini C18 pre-column, 4.0 mm × 2.0 mm I.D.; Phenomenex, Torrance, CA, USA) and an analytical column (Gemini C18, 150 mm × 2.0 mm I.D., 5 μm particle size; Phenomenex).

The HPLC system was connected to an API-2000 triple-quadrupole mass spectrometer equipped with a turboionspray source (Sciex, Thornhill, ON, Canada). The quadrupoles were operated with unit resolution in the positive ion mode. Data were processed using AnalystTM software (version 1.2; Sciex).

For sample pre-treatment by SPE Bond Elut PH (phenyl) cartridges (Varian Inc., Bergen op Zoom, the Netherlands) were used, containing 100 mg sorbent.

2.3. LC-MS/MS conditions

Mobile phase A consisted of 10 mM ammonia in water and mobile phase B consisted of 10 mM ammonia in methanol. An isocratic eluent (ratio A:B = 5:95, v/v) was applied for chromatographic separation, with a flow-rate of 0.3 mL/min and an injection volume of 10 μL. The autosampler temperature was set at 10 °C.

The mass transition of m/z 408.4 to 124.8 was optimised for miltefosine with a dwell-time of 200 ms. Nebuliser and turbo gas (zero air) were set at 30 psi (207 kPa) and 60 psi (414 kPa), respectively. Curtain gas (nitrogen grade 5.0) was set at 35 psi (241 kPa) and the collision gas (nitrogen grade 5.0) at a pressure setting of 4 psi (28 kPa). The ionspray voltage was set at 3500 V, while the source temperature was 400 °C.

2.4. Preparation of calibration standards and quality control samples in human plasma

All stock and working solutions were prepared in methanol–water (1:1, v/v). A miltefosine stock solution was prepared at a concentration of 1 mg/mL for the preparation of calibration standards. This solution was further diluted to obtain working solutions with concentrations of 0.08, 0.2, 0.4, 1, 2, 4, 8, 20, 30 and 40 μg/mL.

A volume of 25 μL of a working solution was spiked into 475 μL drug-free human EDTA plasma prior to sample pre-treatment to yield 250 μL duplicates of 10 non-zero calibration standards of 4, 10, 20, 50, 100, 200, 400, 1000, 1500 and 2000 ng/mL. Calibration standards were prepared freshly before each analytical run.

Another separate miltefosine stock solution (1 mg/mL) was prepared from an independent weighing and dissolution in methanol–water (1:1, v/v) for the preparation of quality control samples. This solution was further diluted with methanol–water (1:1, v/v) into four working solutions (WQ1–4) containing 0.1, 0.5, 30 and 200 μg/mL of miltefosine, respectively. A volume of either 0.4 mL of WQ1, 0.24 mL of WQ2, 0.1 mL of WQ3 or 0.075 mL of WQ4 was added to a volumetric flask and diluted up to 10 mL with drug-free human EDTA plasma, to yield quality control samples at the LLOQ, low, mid and high level with concentrations of 4, 12, 300 and 1500 ng/mL, respectively. The quality control samples were divided into replicate aliquots of 250 μL and stored at –20 °C until analysis. A dilution test sample above the upper limit of quantification (>ULOQ sample) was prepared in pentuplicate by directly spiking the same stock solution of miltefosine (1 mg/mL) used to prepare WQ1 to 4, into drug free human EDTA plasma, yielding spiked plasma samples containing 30,000 ng/mL.

All calibration standards and quality control samples contained only 5% (v/v) or less working solution, to limit the amount of methanol and to simulate a ‘real’ sample as much as possible.

2.5. Sample preparation

Each 250 μL aliquot of spiked plasma was diluted with 750 μL of acetic acid (0.9 M, pH 4.5), followed by brief vortex mixing and centrifugation (5 min at 23,100 × *g* at room temperature). Extraction of miltefosine was achieved by performing SPE. Bond Elut PH (phenyl) SPE cartridges were first conditioned with 1 mL of acetonitrile and 1 mL of 0.9 M aqueous acetic acid (pH 4.5), followed by loading of approximately 1 mL of diluted plasma, washing with 1 mL of methanol–water (1:1, v/v). The analyte was eluted with 2 times 0.75 mL of 0.1% (v/v) triethylamine in methanol. A 200 μL aliquot of the eluate was transferred to a glass autosampler vial with an insert and 10 μL was injected on to the analytical column.

2.6. Validation

Validation of the method was performed according to FDA guidelines for Bioanalytical Method Validation [15,16].

2.6.1. Linearity

Ten non-zero calibration standards ranging from 4 to 2000 ng/mL were prepared in duplicate in drug-free human EDTA plasma and analysed in three separate runs.

For determination of miltefosine, the linear regression of the peak areas of the analyte versus the concentration was weighted by $1/x^2$ (the reciprocal of the squared concentration). For every constructed calibration curve the calibration concentrations were back-calculated from the responses. Deviations from the nominal concentrations should be within ±20% for the LLOQ and within ±15% for other concentrations, with coefficient of variation (CV) values less than 20 and 15%, respectively.

The limit of detection (LOD) was defined as the concentration of miltefosine at which the response of the analyte is two times higher than the response of a blank sample. The LLOQ is the

concentration of miltefosine at which the response of the analyte is at least five times higher than the response of a blank sample and should be reproducible with a precision and accuracy as stated above.

2.6.2. Precision and accuracy

To determine precision and accuracy of the method, quality control samples were prepared with concentrations of 4, 12, 300 and 1500 ng/mL as described in Section 2.4. Five replicates of each quality control sample were analysed in three analytical runs. The concentration of each quality control sample was calculated, using the calibration curve that was analysed in the same run. Accuracies were determined as the percentage difference of the calculated concentration from the nominal concentration and the CV was used to report the precision. The intra- and inter-assay accuracies (% bias) should be within $\pm 20\%$ at the LLOQ level and within $\pm 15\%$ at the other concentrations. The intra- and inter-assay precisions should be less than $\pm 20\%$ at the LLOQ level and less than $\pm 15\%$ at the other concentrations [15].

The ability to dilute samples originally above the ULOQ of the calibration curve was demonstrated by analysing validation samples containing 15-fold the concentration of the ULOQ of miltefosine (30,000 ng/mL, >ULOQ sample). This dilution test sample was prepared in pentuplicate and these were diluted 20 times in control human EDTA plasma to obtain samples with a final nominal concentration of 1500 ng/mL and were analysed in one analytical run.

2.6.3. Specificity and selectivity

Six individual batches of control drug-free human EDTA plasma were used to assess specificity and selectivity of the method. Samples containing no analyte (double blank) and samples spiked at LLOQ level with miltefosine were prepared for each individual batch of plasma and subsequently processed and analysed according to the above described procedures, to determine whether endogenous constituents in the matrix interfere with the assay.

Areas of peaks co-eluting with miltefosine should be within 20% of the peak area of the LLOQ sample of miltefosine for each of the six batches of control drug-free human EDTA plasma. Peak areas co-eluting in the double blank samples with perifosine should be within 5% of the peak area of the normal IS response. The LLOQ samples themselves must have accuracies within $\pm 20\%$ boundaries of the nominal concentration.

2.6.4. Recovery and ion suppression

Ion suppression of miltefosine by matrix constituents was determined by comparing the areas of processed blank samples spiked with working solutions of miltefosine to areas of dilutions of the miltefosine stock solution with methanol–water (1:1, v/v), where the loss of signal represents the ion suppression. The recovery after sample pre-treatment (SPE) was determined by comparing areas of processed quality control samples to areas of processed blank samples reconstituted with working solutions of miltefosine. The total recovery was determined by comparing the areas of unprocessed samples to areas of processed quality control samples. Recovery and ion suppression experiments

were done in triplicate, with quality control samples at three concentration levels (12, 300, 1500 ng/mL).

2.6.5. Stability

The stability of miltefosine was investigated in the stock and working solutions during storage and processing. In human plasma the stability of miltefosine was examined during storage and after three freeze–thaw cycles at $-20\text{ }^{\circ}\text{C}$ with a minimal interval of 24 h. The quality control samples that had been frozen and thawed three times were compared to freshly prepared quality control samples. Furthermore, stability was tested in human plasma during processing for 6 h at room temperature and in the final SPE eluate for 2 and 4 days at $2-8\text{ }^{\circ}\text{C}$.

Also, the reinjection reproducibility after storage of the samples in glass vials in the auto sampler for 24 h at nominally $10\text{ }^{\circ}\text{C}$ was determined. Long term stability of miltefosine was determined after 3 months of storage at $-20\text{ }^{\circ}\text{C}$. Further long term stability experiments are ongoing.

Stability experiments were at least performed at two concentrations (12 and 1500 ng/mL) in triplicate. Miltefosine is considered stable in stock and working solutions when 95–105% of the original concentration is found and in the biological matrix or extract when 85–115% of the initial concentration is recovered.

2.7. Analysis of patient samples

The described bioanalytical method was used to determine miltefosine concentrations in plasma from a cutaneous leishmaniasis patient to show the applicability of the method. The patient had a *Leishmania major* infection, contracted in Afghanistan, for which he received intralesional pentavalent antimony treatment prior to the oral miltefosine treatment. The patient was treated with 50 mg oral miltefosine 3 times daily for a total of 28 days.

3. Results and discussion

3.1. Method development

3.1.1. Sample pre-treatment

Different methods of sample pre-treatment were investigated. In an earlier study of perifosine as analyte and miltefosine as internal standard, protein precipitation has been presented as a simple and quick sample pre-treatment technique [14]. However, when trying to reproduce the results achieved in that study, we encountered several problems. Protein precipitation of plasma samples containing miltefosine was performed with several precipitation agents: acetonitrile, methanol and acetonitrile–methanol mixtures, all of which were tested in various concentrations, ratios and volumes (data not shown). Nevertheless, protein precipitation resulted in nonreproducible recoveries, nonconsistent peak areas and dirty samples, resulting in interferences from the sample matrix with the chromatography of the analyte. Robustness tests showed a strong reduction in analyte response in time.

Liquid–liquid extraction was tested as sample pre-treatment using diethyl ether, ethyl acetate and *tert*-butyl methyl ether as

organic phase (data not shown). However, liquid–liquid extraction also resulted in nonsatisfying recoveries and high variability in the analysis.

Subsequently, SPE was investigated as sample pre-treatment technique, which has also been used in a previous study to isolate perifosine [13]. In that study, Knebel et al. reported a sample pre-treatment using a neutral solution (0.8 M aqueous ammonium acetate set at pH 6.5) for dilution of the plasma samples and conditioning the SPE cartridges. To further optimise the SPE, several dilution, conditioning, washing and elution reagents were tested. Use of a more acidic buffer solution for dilution of the samples and conditioning of the SPE cartridges, resulted in a slightly better recovery and, more importantly, highly reproducible results. Best results were achieved with a 0.9 M aqueous acetic acid-buffer set at pH 4.5 with ammonium hydroxide for diluting the samples and conditioning the SPE cartridges. To elute the analyte from the SPE cartridge, 0.1% triethylamine in methanol was used, similar to the method of Knebel et al.

Several types of SPE cartridges were evaluated for the extraction of miltefosine from human plasma. Cartridges that were investigated included polar ones, such as plain silica based extraction cartridges and silica bonded cyanopropyl (CN) and aminopropyl (NH₂) sorbent material; nonpolar ones, such as silica bonded C18 or phenyl (PH) sorbent material; and various copolymer based cartridges, such as Oasis HLB and MCX. Extraction recovery of miltefosine was highest, combined with a good reproducibility, with the use of PH cartridges and these were eventually chosen for further use in the assay. Due to the multiple characteristics of miltefosine, having both apolar and polar moieties, the bonding interactions between miltefosine and the sorbent are difficult to predict. The secondary ionic interactions between the silica substrate and any unbonded silanols remaining on the surface may play an important role in the retention of miltefosine on the silica bonded phenyl sorbent and might explain the observed differences between the several apolar sorbents.

After SPE, the eluate was directly transferred to glass autosampler vials with inserts and injected into the LC–MS/MS system. No further concentrating step was performed, since evaporation and concentration of the eluate led to non-reproducible results. Adhesion to the surface of the polypropylene container could be the cause of this variability when using an evaporation step, since alkylphosphocholines are reported to have adhesive properties to surfaces [13,14]. The presence of triethylamine in the sample proved to be of no negative influence on the MS-detection of miltefosine.

The here reported sample pre-treatment procedure is more rapid and simple without a time-consuming concentrating step and a satisfying sensitivity of the analytical method was achieved, enabling us to readily measure miltefosine in patient samples taken at even 5 months after the end of treatment.

3.1.2. MS/MS optimisation

The structure of miltefosine lacks any chromophores, which makes ultraviolet or fluorescence detection very difficult. Therefore MS detection was chosen, offering a sufficiently high sensitivity to measure miltefosine concentrations in patient sam-

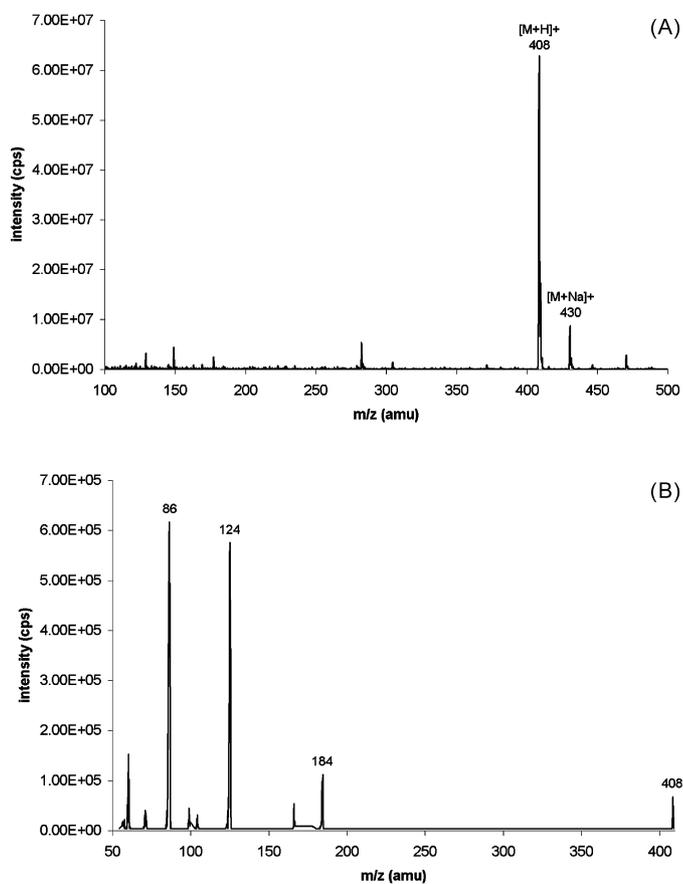


Fig. 2. Q1 (m/z 100–500) mass spectrum of miltefosine (panel A) and MS/MS product ion scan (m/z 50–410, precursor ion m/z 408.4) (panel B).

ples even taken at 5 months after end of treatment (see Section 3.3).

Fig. 2A shows the Q1 mass spectrum of miltefosine. Besides the protonated ion $[M+H]^+$ at m/z 408, a sodium adduct of miltefosine (m/z 430) is also visible in the Q1 mass spectrum. The protonated molecular ion of miltefosine was induced to fragment in the collision cell.

The proposed fragmentation pattern of the protonated ion of miltefosine is depicted in Fig. 1 and the resulting product ion spectrum is presented in Fig. 2B. The fragment ion at m/z 184 in the spectrum of miltefosine corresponds with the loss of the C16-alkyl chain. Further cleavage of the trimethyl amine from this precursor fragment ion results in the product ion at m/z 124.8, which is eventually used for quantitative MRM [13].

3.1.3. Liquid chromatography

Reversed-phased HPLC columns have been reported to provide improved selectivity in the separation of phospholipids [17]. However, a report of the quantitative analysis of perifosine described that perifosine was either unretained or subjected to severe peak-tailing when “commonly applied ‘reversed-phased’ silica columns” were used [13]. In contrast, Woo et al. reported that retention of perifosine was possible when they used a very unusual C30 reversed-phase column, probably because of the strong hydrophobicity of the alkyl-chain [14]. Here we report that miltefosine is retainable on a generally avail-

able reversed-phase column when the appropriate mobile phase is used.

In the pursuit of a symmetric peak shape and retention times of 2–4 min, liquid chromatography was investigated with acetonitrile or methanol as organic modifiers of the LC and under several alkaline conditions.

While varying the aqueous and organic components in the mobile phase, best results were obtained with a mobile phase consisting of an aqueous:organic phase ratio of 5:95 (v/v). Shifting the organic modifier in this mobile phase or applying a gradient did not improve the retention of miltefosine, whereas an isocratic mobile phase, consisting for the most part of organic phase, produced a mediocre peak.

However, a great improvement of the peak shape and retention times was seen when the mobile phase was alkaline. The effects of the mobile phase pH on the peak shape of miltefosine were investigated subsequently. Kim et al. already suggested a high mobile phase pH for the separation of phospholipids [18]. Because of the quaternary amine group in the molecule of miltefosine, total suppression of the charge of miltefosine is impossible. An increase of the pH of the eluent resulted in a sharp decrease of the retention time and an improved peak shape. Best peak shapes were obtained with a final mobile phase pH of ≈ 10.5 , which was impossible to use in previous methods due to the limited stability under alkaline conditions of the columns available then [14]. In order to apply such alkaline eluent we used a Gemini reversed-phase C18 column, suited for alkaline conditions up to pH 13.

Typical MRM chromatograms of miltefosine in a double blank, LLOQ and median level sample are shown in Fig. 3. Total LC run time was 7 min.

3.2. Method validation

As already stated in Section 2, the validation was performed with perifosine added to all the samples as an internal standard. However, all runs performed better in terms of intra-assay accuracy and precision when concentrations were back-calculated from the peak area of miltefosine alone, than when they were back-calculated from the analyte/internal standard peak area ratio (see Table 1). Hence, all concentrations in this reported method were back-calculated solely based on the peak areas of miltefosine, leaving out the internal standard peak area from the calculation.

3.2.1. Linearity

Calibration standards were prepared in duplicate in control human EDTA plasma and were analysed in three separate analytical runs with a quantifiable dynamic range from 4 to 2000 ng/mL of miltefosine in human EDTA plasma. A weighing factor of the inverse squared concentration ($1/x^2$) appeared the most appropriate weighing factor for a linear fit of the data. A correlation coefficient of 0.9951 or higher was obtained in the three analytical validation runs. Concentrations were back-calculated from nominal concentrations and are summarised in Table 2. Deviations of the back-calculated concentrations from the nominal concentrations were between -2.20% and 2.59%

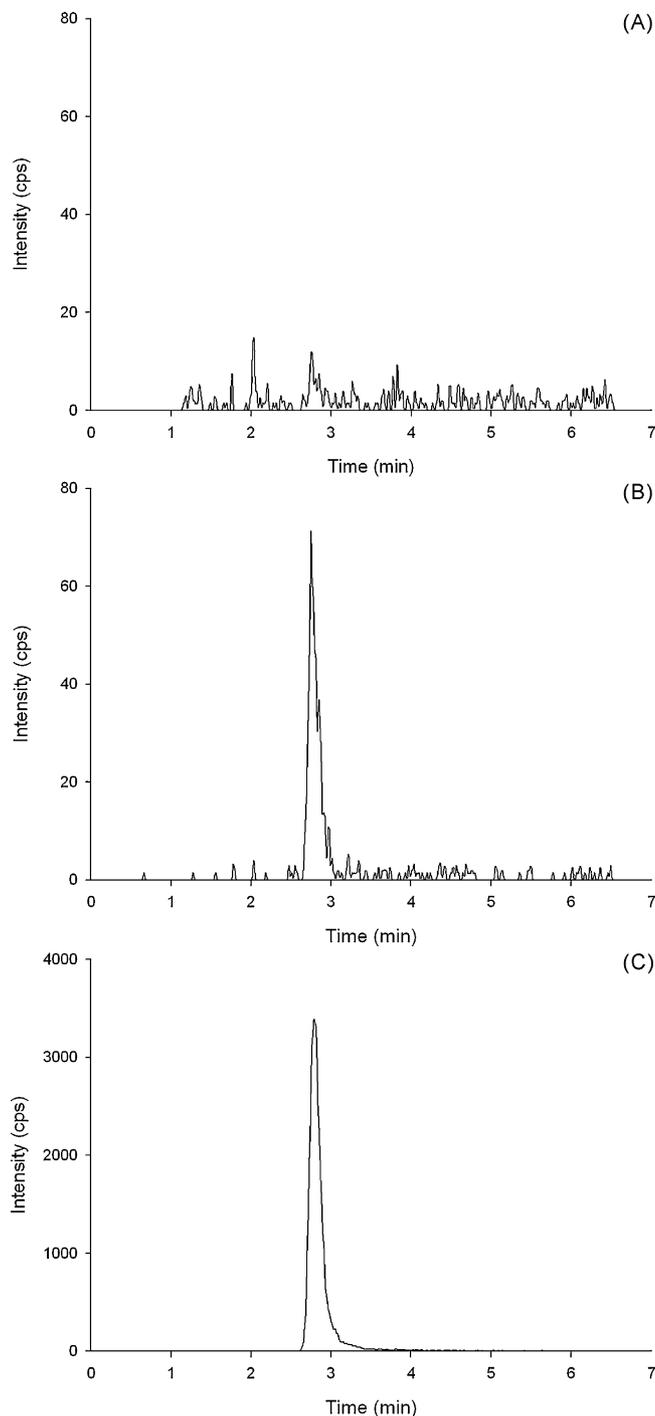


Fig. 3. Representative MRM chromatograms of a double blank sample (A), a LLOQ sample (B, 4 ng/mL) and a median level sample (C, 300 ng/mL) of miltefosine in human plasma.

with variation coefficients between 4.74% and 11.8% for all concentrations in human plasma. The mean LOD of miltefosine in our assay is estimated at 1 ng/mL with a signal-to-noise ratio of 2:1. The LLOQ has been established at 4 ng/mL of miltefosine in human EDTA plasma with reproducible good accuracy and precision (Table 1) and a mean signal-to-noise ratio of 8:1.

Table 2
Calibration standards of miltefosine in human plasma calibration curves prepared in duplicate

Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Deviation (%)	CV (%)
4.07	4.08	0.42	11.8
10.2	9.94	−2.20	5.66
20.3	20.7	1.97	6.97
50.8	51.5	1.40	6.98
102	100	−1.64	8.28
203	203	0.05	7.33
407	417	2.59	4.74
1020	1020	0.74	5.62
1530	1500	−1.66	5.64
2030	2000	−1.65	6.70

CV = coefficient of variation.

3.2.2. Precision and accuracy

Assay performance data for miltefosine are summarised in Table 1. Intra- and inter-assay accuracies are defined in terms of relative error with a precision in terms of relative standard deviation, all determined at four concentration levels. The intra-assay accuracy was within $\pm 8.54\%$ and the inter-assay accuracy was 1.96% for the LLOQ, with precisions of maximally 10.7% and 10.6% , respectively. For all other concentrations, the intra-assay accuracy was within $\pm 4.88\%$ and the inter-assay accuracy was within $\pm 2.81\%$, with precisions less than 7.11% and 5.82% , respectively.

Samples with a concentration above the ULOQ of 2000 ng/mL can be safely diluted 20 times, as the intra-assay accuracy for diluted $>\text{ULOQ}$ samples ($30,000\text{ ng/mL}$ miltefosine in human plasma diluted with a factor 20 in control human plasma resulting in a miltefosine concentration of 1500 ng/mL) was -2.83% with a precision of 3.09% .

Table 3
Stability data for miltefosine

Conditions	Matrix	Nominal concentration (ng/mL)	Dev. (%)	CV (%)	No. of replicates
Ambient, 6 h	Plasma	12.1	−9.68	3.41	3
		1520	−11.0	4.13	3
3 Freeze (-20°C)/thaw cycles	Plasma	12.1	4.95	2.64	3
		1520	2.63	2.83	3
2–8 $^\circ\text{C}$, 2 days	SPE eluate	12.1	2.78	5.31	3
		303	2.11	4.11	3
		1520	10.1	3.00	3
2–8 $^\circ\text{C}$, 4 days	SPE eluate	12.1	1.28	9.77	3
		303	8.99	4.65	3
		1520	6.00	5.57	3
Reinjection reproducibility, 10°C , 24 h	SPE eluate	12.1	1.42	4.54	3
		303	−6.37	0.832	3
		1520	3.43	2.57	3
Long term stability, -20°C , 3 months	Plasma	12.1	0.901	4.72	3
		303	7.70	3.34	3
		1520	5.06	0.758	3

Dev. = deviation, CV = coefficient of variation, SPE = solid-phase extraction. All temperatures mentioned are nominal.

3.2.3. Specificity and selectivity

MRM chromatograms of double blank, blank and LLOQ samples prepared in six batches of control human plasma did show minor deviations from the baseline that co-eluted with the analyte; two samples showed a relative interference in the blank samples of 20.4 and 20.8%, while the interference in the other four batches of plasma remained under the 20%. However, deviations from the nominal concentration at the LLOQ level were between -18.2 and 4.62% and thus acceptable.

3.2.4. Ion suppression and recovery

The mean ion suppression of miltefosine at three concentrations (12, 300, 1500 ng/mL) in human plasma was 60.4% with a CV value of 8.3% . Mean extraction recovery of miltefosine at three concentration levels after the SPE process was $82.1 \pm 3.5\%$ compared to a spiked blank processed sample representing 100% .

While ion suppression of miltefosine is high, it proved to be reproducible and acceptable accuracies and precisions were achieved with the method. Furthermore, it did not affect the desired sensitivity of the assay with a LLOQ of nominally 4 ng/mL . A more sensitive assay was not needed, since no patient sample had a miltefosine concentration lower than the demonstrated LLOQ (data not shown).

The mean extraction recovery of 82.1% was higher than the approximate recovery from the SPE cartridges as reported by Knebel et al. [13]. Because sample pre-treatment methods are quite similar in the two assays, probably the use of an acidic buffer solution to dilute the samples and neutralize the SPE cartridge, enabled us to obtain higher yields of the analyte. Possibly, the neutralisation of the negatively charged group within the miltefosine molecule is responsible for this difference, although the interactions between the sorbent material inside the cartridge and the miltefosine molecule are supposedly non-polar and based on the hydrophobicity of the long alkyl-chain of miltefosine.

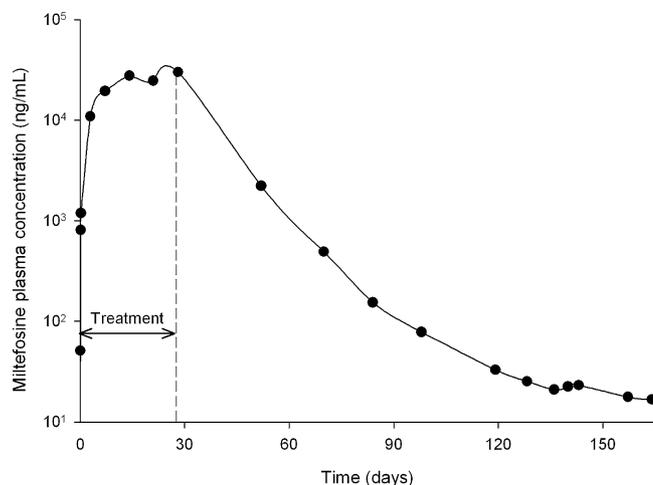


Fig. 4. Concentration of miltefosine in human plasma vs. time in a patient treated orally with 150 mg/day of miltefosine for 28 days. Plasma samples were collected during and up to 4 months after the end of treatment with miltefosine.

3.2.5. Stability

An overview of stability experiments and results is presented in Table 3. Miltefosine is stable in human plasma after at least three freeze/thaw cycles from nominally -20°C to ambient temperature and is also stable in human plasma when stored at ambient temperature for at least 6 h. The analytical run can be reinjected after at least 24 h of storage in the autosampler (at 10°C) in glass autosampler vials, while reinjection reproducibility was within $\pm 15\%$. Stability of miltefosine in the final SPE eluate is assessed at both 2 and 4 days, while kept at nominally $2-8^{\circ}\text{C}$, longer storage of the SPE eluate probably leads to higher deviations (data not shown).

Miltefosine is found stable in human plasma up to 3 months of storage at -20°C . Further long term stability experiments are ongoing.

3.3. Analysis of patient samples

Concentrations of miltefosine in plasma in samples from cutaneous leishmaniasis patients were analysed with the described method. Fig. 4 shows a concentration versus time profile obtained from a patient treated orally with 50 mg of miltefosine 3 times daily for 28 days. The administered daily total dose of 150 mg is considered to be the maximum tolerated dose, due to gastrointestinal side effects following oral administration. Plasma samples were taken from the patient at various time points during and until 4 months after treatment ($n = 19$). Due to strong accumulation and the extremely long elimination half-life (approximately 7 days) of miltefosine in the body, concentrations of miltefosine were still above the LLOQ at 4 months

after the end of treatment. These data show the applicability of the quantitative method in leishmaniasis patients.

4. Conclusion

An accurate, reproducible and fast LC–MS/MS assay for the quantitation of miltefosine in human plasma has been developed and validated. The assay quantifies a range of 4–2000 ng/mL of miltefosine using 250 μL aliquots of human plasma, providing accurate and precise quantifications of the miltefosine concentration in the described validation. With a LLOQ of 4 ng/mL we were able to measure miltefosine concentrations in human plasma samples taken from patients treated with 150 mg per day for 28 days until at least 4 months after the end of treatment. With this method we have now analysed over 400 patient samples. These data show that the presented assay has adequate specifications to reliably conduct pharmacokinetic studies of miltefosine.

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