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Pharmacokinetics and bioavailability assessment of Miltefosine in rats using high performance liquid chromatography tandem mass spectrometry

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ABSTRACT

Miltefosine (MFS) is the first effective oral drug for treatment of visceral, mucosal and cutaneous leishmaniasis. In this study, liquid chromatography coupled mass spectrometry (LC–MS/MS) method of MFS was validated in rat plasma and its practical utilization to pharmacokinetic studies in rats for the first time. A rapid, selective and sensitive LC–MS/MS method for MFS in rat plasma was linear over the calibration range of 1–500 ng/mL. MFS and Phenacetin (internal standard) were separated on Phenomenex Luna 3 μ HILIC 200A (150 × 4.6 mm) column under isocratic condition using methanol: 0.1% formic acid in triple distilled water, 90:10 (v/v) mobile phase at a flow rate of 0.8 mL/min. The total chromatographic run time was 4.0 min. The intra- and inter-day assay accuracy was observed between 99.45-102.88% and 99.92-101.58%, respectively. The intra- and inter-day assay precision was observed between 2.68-5.54% and 2.35-5.94%, respectively. The validated assay was practically applied to determine the plasma concentrations after oral and intravenous administration of MFS to rats. After oral administration, MFS showed C_{max} (3200.00 ± 95.39 ng/mL) was observed at 12.00 h (t_{max}) and $t_{1/2}$ was 102.36 ± 16.65 h. The absolute bioavailability of MFS was 60.33 ± 2.32%.

1. Introduction

Leishmaniasis is a vector borne disease caused by different leishmanial species and transmitted to human subjects by phlebotomine sand flies. It contains four main clinical syndromes: cutaneous (CL), mucosal, visceral (VL, kala azar) and post kala azar dermal leishmaniasis (PKDL) [1]. Among all types of leishmaniasis, VL is systemic, most severe and fatal if untreated. VL occurs worldwide, but majority in six countries: Bangladesh, Brazil, Ethiopia, India, Nepal and Sudan [2]. According to WHO, around 0.3 million new cases and 20,000 deaths from the VL were estimated annually [3]. During the development of leishmaniasis in the human body, the amastigote forms of the parasite distributed throughout the body and multiplies within the monocytes and macrophages, resulting infiltration of the bone marrow and hepatosplenomegaly [4].

The pentavalent antimonials sodium stilbogluconate and meglumine antimoniate have been used for VL for many years, but the antimonials are toxic drugs with adverse side effects like cardiac arrhythmia and acute pancreatitis [1]. Later Amphotericin B replaced antimonials for the treatment of VL. Parenteral Amphotericin B also showed life threatening side effects like hypokalemia, nephrotoxicity and first dose anaphylaxis. Even though liposomal Amphotericin B (AmBisome) is best for the treatment of VL because of its milder toxicity, but it is an unaffordable treatment [5,6].

Miltefosine (hexadecylphosphocholine, MFS) is the first and still only oral drug for the treatment of VL and CL. It was licensed for the treatment of VL and CL as Impavido and Miltex, respectively [7]. It showed 98% cure for Indian VL patients with an oral dose of 2.5 mg/kg daily for 4 weeks [8]. Marketing authorization for the MFS

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was first granted in India in 2002, followed by Germany, Colombia; and is included in the WHO list of essential medicines [5]. Several clinical studies have been reported for MFS in VL and CL patients [9–12].

There is little data published related to preclinical and clinical pharmacokinetics of MFS. Few bioanalytical methods are available for the quantification of MFS in liquid chromatography coupled mass spectrometry (LC–MS/MS) and high performance liquid chromatography (HPLC). MFS was quantified in fetal calf serum by using HPLC with evaporative light scattering detector [13]. There have been reports of LC–MS/MS bioanalytical method of MFS in human plasma and human peripheral blood mononuclear cells with a lower limit of quantification (LLOQ), 4 ng/mL [14,15]. All these methods were utilized solid phase extraction (SPE) procedure for sample processing. An LC–MS/MS method has been reported for MFS quantification in dried blood spotting (DBS) of human samples with LLOQ, 10 ng/mL [16]. A recent article published for MFS quantification in human and hamster plasma using LC–MS/MS method using protein precipitation with LLOQ, 2.5 ng/mL [17]. Larger plasma volumes, costlier sample collection methods like DBS, costlier sample extraction procedures like SPE and longer analysis time are the disadvantages of the previous methods. There have been reports of MFS showing the oral pharmacokinetics study in rats by using HPTLC method with a densitometer [18]. To the best of our knowledge, there is no published report on LC–MS/MS method validation and stability studies in rat plasma. The main aim of the present study is LC–MS/MS method validation and stability studies in rat plasma and to explore *in-vivo* pharmacokinetic behavior and absolute oral bioavailability of MFS in Sprague Dawley (SD) rats. The present method has advantages over previous methods like smaller sample volume (50 μL), rapid analysis (4 min), sensitive (1 ng/mL), simple plasma extraction like protein precipitation.

2. Materials and methods

2.1. Chemicals and reagents

MFS was purchased from EMD Chemicals Inc. (San Diego, CA). Phenacetin (Internal Standard, I.S) and LC–MS/MS grade methanol were obtained from Sigma Aldrich (Mumbai, India). The chemical structures of MFS and I.S are represented in Fig. 1. HPLC grade Acetonitrile (ACN), Methanol, Hexane, *tert*-butyl methyl ether and Ethyl acetate were obtained from Merck Limited (Mumbai, India). Extra pure formic acid was purchased from Sisco Research Laboratories Pvt. Ltd. (SRL) (Mumbai, India). Purified triple distilled water (TDW) was obtained from MilliQ water purification system (EMD Millipore, USA). Diethyl ether was purchased from TKM Pharma (Hyderabad, India). Sodium heparin anticoagulant injection (5000 IU/mL vial) was obtained from Biological E. Ltd. (Hyderabad, India). Young male SD rats (8–10 weeks) weighing around 200–220 g were procured from the Division of Animal Laboratory, Council of Scientific and Industrial Research-Central Drug Research Institute (CSIR-CDRI) (Lucknow, India). Rats were housed in well ventilated cages at standard laboratory conditions with regular light/dark cycles for 12 h. Rats were kept for one week acclimatization prior to the experiments. Pharmacokinetic studies were conducted according to a protocol approved by the Institutional Animal Ethic Committee, CSIR-CDRI (IAEC approval no. IAEC/2012/91).

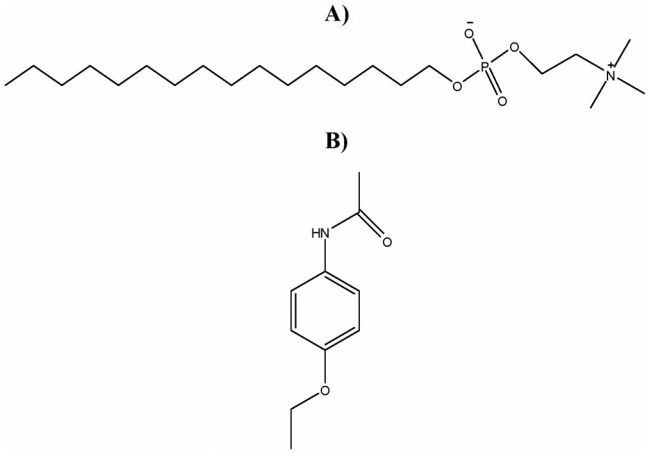

Fig. 1. Representation of chemical structures of **A)** Miltefosine (MFS) and **B)** Phenacetin (I.S).

2.2. Mass spectrometric conditions

All calibration standards (CS), quality control (QC) samples, stability samples, *in vivo* pharmacokinetic samples were analyzed on API 4000 QTRAP mass spectrometer (ABSciex, Canada) equipped with electro-spray ionization (ESI) source in a positive mode for both MFS and I.S. Quantification was performed using the multiple reaction monitoring (MRM) mode. Optimization of compound and source dependent parameters for MFS and I.S was performed by infusing the standard solutions using a Harvard infusion pump (Holliston, USA). Data acquisition and quantitation were performed using Analyst 1.6 (Applied Biosystems, MDS Sciex Toronto, Canada).

2.3. Liquid chromatographic conditions

A Shimadzu UFLC system (Kyoto, Japan) equipped with a binary pump (LC-20AD), a degasser (DGU-20A3), an auto sampler (SIL-HTc) and a column oven (CTO-20AC) was used to inject the samples. MFS and I.S were separated on Phenomenex Luna 3 μ HILIC 200A (150 × 4.6 mm) column with a mobile phase consists of methanol: 0.1% formic acid in TDW, 90:10 (v/v) at 0.8 mL/min flow rate. The injection volume was 10 μL and total run time was 4 min.

2.4. Preparation of CS and QC samples

To achieve the desired concentration of 1 mg/mL, primary stock solutions of MFS and I.S were prepared in TDW and ACN, respectively. Working stock solutions were prepared by stepwise dilutions of stock solution in ACN. The concentrations of working stock solutions of MFS for CS were prepared as 20, 40, 100, 200, 400, 1000, 2000, 4000 and 10000 ng/mL in ACN. The concentrations of working stock solutions of MFS for QC were prepared as 20, 80, 800 and 8000 ng/mL in ACN. CS and QC samples were prepared by spiking 47.5 μL of drug free plasma with corresponding working solution 2.5 μL of MFS. In the total plasma volume the organic content (ACN) was found to be 5%. The final CS were prepared as 1, 2, 5, 10, 20, 50, 100, 200 and 500 ng/mL in plasma matrix. The QC were prepared using a stock solution of MFS at four concentration levels, such as LLOQ (1 ng/mL), low QC (4 ng/mL), medium QC (40 ng/mL) and high QC (400 ng/mL). All the stock and working solutions were stored in the refrigerator until use for analysis.

2.5. Plasma sample extraction procedure

A simple protein precipitation (PPT) method was used for the processing of rat plasma. To 50 μL of CS, QC and plasma samples, 250 μL of I.S (200 ng/mL) solution in mobile phase (methanol: 0.1% formic acid in TDW, 90:10 (v/v)) was added and vortexed for 10 min at 2500 rpm on Vibramax. After protein precipitation, the mixed samples were followed by centrifugation for 10 min at 10,000 rpm