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Development and evaluation of an oral quinine sulphate sustained release formulation for the management of visceral leishmaniasis

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Abstract

Background: Visceral Leishmaniasis is a systemic disease caused by the invasion of reticuloendothelial cells in the liver, spleen, and bone marrow by *Leishmania spp.* parasites. Current medications have numerous adverse effects and some resistance has been reported. Most treatments are administered parenterally, leading to non-compliance and treatment failure. This study aimed to develop an oral formulation of quinine sulfate to address these challenges.Methods: Quinine sulfate was encapsulated in hydroxypropylmethylcellulose using the spray-drying method and evaluated for particle size, zeta potential, drug content, entrapment efficiency, and in vitro release properties. In the in vivo study, laboratory mice infected with *L. donovani* promastigotes were treated for two weeks.Results: The average size of the quinine sulfate microparticles ranged from 2.6 to 14.2 µm, with an entrapment efficiency of 93%. Oral formulations effectively cleared parasites from the organs.Conclusion: Orally administered quinine sulfate efficiently cleared parasites from the blood of infected mice and resolved infections in their organs.

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INTRODUCTION

The oral route of drug delivery has numerous advantages and is the most preferred by patients. Sanchez Martinez et. al reported an increase in patient compliance and quality of life for patients with multiple sclerosis when an injectable medication was changed to an oral one, that is, the route of administration was changed [1][2].

Leishmaniasis is considered an important neglected tropical disease [3][4]. The three most common antileishmanial drugs: Amphotericin B, Paromomycin, Pentamidine are administered parenterally. Administration of medications

parenterally at treatment centres over prolonged periods of time is not convenient and usually not cost effective to patients.

This often leads to noncompliance. Non-adherence to therapy is one of the major causes of drug resistance by the parasites and eventually leads to treatment failure. Miltefosine is currently, the only oral drug for Leishmaniasis. Unfortunately, it is very expensive, causes gastrointestinal toxicity and has teratogenic effects [1].

Currently, about 12 million people are suffering from Leishmaniasis in 98 countries across the tropics and subtropics, more than 350 million people stand a high chance of infection and approximately, 20,000 to 40,000 deaths each year. The clinical manifestations are Cutaneous, Mucocutaneous and Visceral Leishmaniasis [5].

Among the three forms, visceral leishmaniasis is the most aggressive and often fatal without treatment [6].

This is mainly due to the parasites (*L. donovani* and *L. infantum*. *L. donovani*) invading and proliferating within the reticuloendothelial cells of the liver, spleen, and bone marrow. Quinine sulphate which has rapid schizonticidal action against malaria parasites was highly efficient in killing leishmania parasites in tissues after IP administration [7].

Since patient compliance is paramount to the effective management of chronic diseases, this study sought to develop a controlled release oral formulation of quinine sulphate, which will reduce dosing regimen, increase patient compliance and be highly efficacious against the leishmania parasite.

MATERIALS AND METHODS

Test Organism and Reagents

Leishmania donovani (WHO strain DD8) was donated by Dr. Neelo Singh of the Leishmania Research Society, India. Quinine sulphate standard was obtained courtesy the Centers for Disease Control and Prevention, Atlanta, GA, U.S.A. Ernest Chemist Ghana, donated

Hydroxypropylmethylcellulose (HPMC) polymer used. All other reagents including glutaraldehyde, cell culture media (M199), sodium bisulfite were purchased from VWR international (Radnor, PA, U.S.A).

Formulation of Quinine Sulphate Microparticles

Preparation of Microparticles

The spray-drying method was used to prepare the quinine sulphate microparticulate formulations as described by Allotey-Babington et al. with slight modifications [7]. Firstly, 8 mg of HPMC was dissolved distilled water to obtain a 160 mL of HPMC solution. 40 mL of quinine sulpahte solution was prepared in a separate beaker having a concentration of 0.05 g/mL. The two solutions were mixed in a ratio of 1:4 (drug to polymer) to produce a homogenous mixture which was then spray dried to produce a fluffy powder.

The second formulation: chitosan-coated microparticles were prepared in a similar way, however in this instance, 0.05% w/v chitosan solution was added to a homogenous mixture of quinine and HPMC amist stirring. The mixing was allowed to continue for an hour prior to spray-drying.

Zeta Potential Determination

About 2 mg of the powdered microparticulate formulation prepared were weighed individually into separate beakers containg 10 mL of distilled water. It was ensured that the particles were evenly distributed in the mix. The suspension was diluted ten-fold after which the zeta potential was determined. Each experiment was performed a minimum of three times using a Malvern Zetasizer Nano-ZS

Size and Surface Morphology of Microparticles

A scanning electron microscope was used to characterize the microparticles by size and surface morphology. Microparticles were captured on carbon sheets and observed at 5 kV and at 1550x.

Drug Loading of Microparticles

40 mg of the formulation were weighed (in triplicate) and crushed in a mortar. Phosphate-buffered saline (PBS, pH 6.8) was added, the contents transferred into Eppendorf tubes and centrifuged at 10,000 rpm for 10 minutes.

The supernatant was diluted in PBS to obtain an expected drug concentration of 20 µg/mL. The amount of drug in the microparticles was estimated using a UV/Vis spectrophotometer at a wavelength of 334 nm.

The effectiveness of the polymer in entrapping quinine sulphate was determined by calculating the encapsulation efficiency mathematically, which it is estimated as:

$$\% \text{ Encapsulation (Entrapment)} = \frac{\text{Actual drug loading}}{\text{Expected drug loading}} \times 100$$

In-vitro Drug Release

To affirm that the formulated microparticles modified the release of the drug (quinine sulphate), an *in-vitro* drug release assay was conducted. Hard gelatin capsule shells were loaded with about 30 mg of the prepared microparticulate formulation.

Another set of hard gelatin capsule shells were filled with quinine sulphate powder (not microparticles) to be used as positive control. The amount of quinine in all shells (loaded with microparticles or the unformulated powder) were equivalent.

The release of the formulated capsules was determined using a dissolution apparatus. To mimick the stomach environment in the fasted state, 0.1 M HCl, pH 1.87 was used. 500 mL of the acid was put into three out of the six vessels of the apparatus. Phosphate buffer, pH 6.8 (500 mL) was poured into the other 3 vessels to represent the intestinal fluid. Following that, 3 capsules were put in the baskets of each vessel and lowered into the medium. Thus, each formulation was run in 3 vessels containing HCl and 3 vessels containing phosphate buffer.

This was done for the 2 microparticulate formulations and the positive control.

Conditions of the apparatus was 100rpm and 37.1^{o}C. Samples were taken at predetermined time points and assayed for quinine content using a UV spectrophotometer. To maintain sink conditions, each time a sample was drawn, the same mount of fresh buffer was added to the medium in the vessel as replacement.

Treatment of Leishmania donovani Infected Mice

Ethics Statement for Animal Use

The protocol for the study was approved by the Centre for Plant Medicine Research (CPMR), Mampong, Ghana (Approval number: CPM/A.95/SF.6/111). The European Community guidelines, as accepted principles for the use of experimental animals, were adhered to.

Animal care

45 male Institute of Cancer Research (ICR) mice, about six-weeks old weighing between 24 – 30 g were procured from CPMR, Mampong, Ghana for the study. The animals were handled according to the institutional guidelines. They were kept in a facility with room temperature of about 22°C under a light/dark cycle lasting 12 hours each. They were fed with standard mouse chow and had free access to water. All experiments commenced after animals had acclimatized for one week.

L. Donovani infection of laboratory animals and Treatment

To infect the mice, forty mice (40) were inoculated intraperitoneally with 0.2 mL suspension of 2 x 10^7 cells of *Leishmania donovani*. To guarantee infection, blood was taken from the tail veins of mice to prepare thin smears and observed for the presence of parasites weekly for 3 weeks. In the fourth week, the parasite count was determined after which the mice were randomly divided into 8 groups of five for the commencement of treatment. Group 1 comprised of the five animals which were not infected (control), Group 2 and 8 mice were infected but not treated. Medications currently used for treatment of leishmaniasis: Amphotericin B and Pentamidine were used as positive controls. They were administered to animals in Groups 3 and 4 respectively at a dose of were 3 mg/kg. Group 5 received quinine sulphate solution administered orally (10 mg/kg). Animals in groups 6 and 7 were administered quinine sulphate microparticles and chitosan-coated microparticles respectively, equivalent to 10 mg/kg body weight of entrapped quinine.

For each group, blood smears were prepared from tail vein blood samples prior to treatment.

Both the controls and test formulations were administered every other day for 2 weeks.

On day fourteen, blood smears were again prepared from tail vein blood samples after which mice were euthanized. The organs of interest (liver and spleen) were removed, washed and processed for further analysis. The efficacies of the various formulations were assessed by establishing the presence or absence of infection in histological sections of the organs.

RESULTS

Characterization of Quinine Sulphate Microparticle

Average Size of particles, Zeta Potential and Entrapment Efficiency

The average size of the modified release quinine sulphate ranged between 2.6-14.2 µm. The other parameters of the particles determined are reported in **Table 1**.

Table 1 Characteristics of Quinine Sulphate Microparticles

Characteristics of Formulated Microparticles		
	Uncoated	Chitosan coated
Average particle size	6.5 µm	7.7 µm
Zeta potential	-0.189 mV	13.3 mV
Drug loading	37.2 of 40 mg	33.2 of 40 mg
Entrapment efficiency	93 %	83 %

Addition of chitosan to the formulation resulted in an increase in average size of the microparticles from 6.5 microns to 7.7 microns. Increases in particle size do affect the release of the drug as solubility is decreased. Thus, a release profile was conducted to evaluate the release of the drug from the matrix.

In Vitro Release pattern of Quinine Sulphate from various Formulations

In the pH 1.87 buffer, the formulated microparticles (plain and chitosan-coated) had burst releases of 18% and 12 % respectively within the first thirty minutes of the study, following which a constant release was observed over the next 24-hour period.

Similarly, in the intestinal fluid, both microparticles within the first half-hour, had approximately 15 % burst release and a cumulative drug release of approximately 97% in the next 24 hours.

Release of quinine sulfate from the various formulations was conducted over a 24-hour period in (a) simulated gastric fluid [0.1M HCl (pH 1.87)] and in (b) simulated intestinal fluid [Phosphate Buffered Solution (pH 6.8)].

The release profile was faster for the quinine sulphate powder than for the microparticles. Microparticles coated with chitosan had a slower release (which was expected) for the first 12 hours in both simulated fluids.

Following development and characterization of the formulations, their ability to clear intracellular leishmania parasites were evaluated in mice.

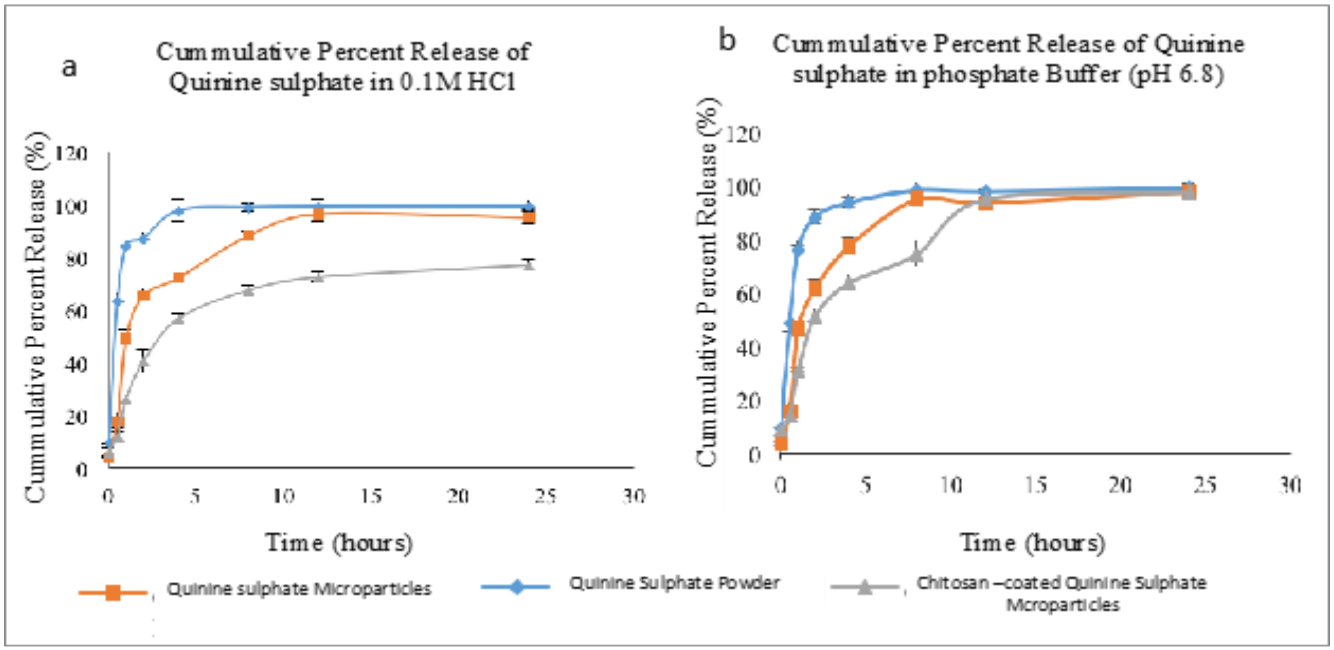

Figure 1 Cumulative drug release of the various formulations

Drug Effect on Infected Mice

Mice were inoculated with leishmania donovani suspension, following which thin blood smears slides were prepared using blood from the tail vein of infected animals. This was done to establish infection.

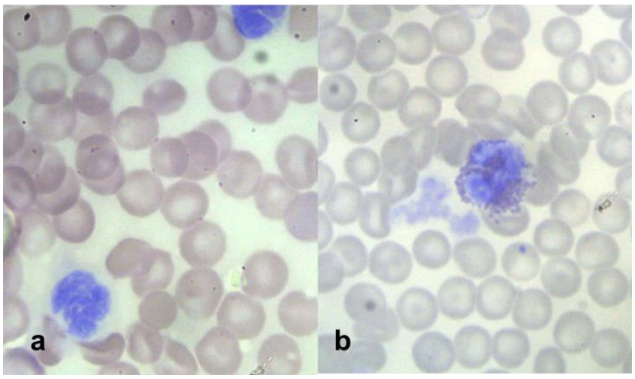

Figure 2 Thin blood films stained from uninfected mice (a) and infected mice (b) stained with Giemsa

Slides of samples from uninfected mice (fig 2a) – well-lobbed nucleus in cells) while slides of samples from infected animals (fig 2b) had leishmania parasites inside the cells and in the extracellular space.

Histological studies performed on the organs of interest (liver and spleen) revealed a well-organized cell structure with no accumulation of mononuclear cells (**Figure 3 a and 3x**) for mice in group one (uninfected). Sections prepared from organs of animals in group eight (infected but not treated) showed disorganized cell structures with extensive accumulation of mononuclear cells (Figures 3b and 3Y). These served as reference points and baselines for treatment groups.

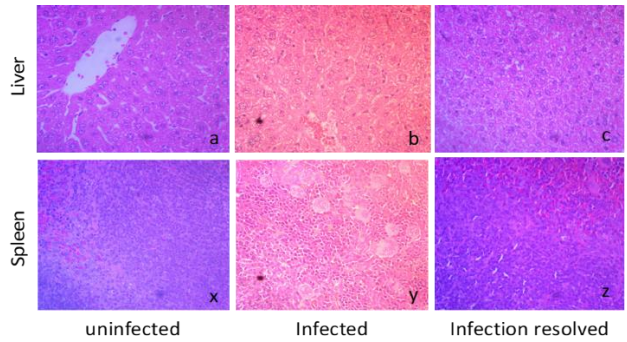

Figure 3 : Giemsa-stained liver and spleen sections

Figure 3 3a, 3b and 3c are liver sections while 3x, 3y and 3z are spleen sections

(a and x) uninfected mice, (b and y) infected mice and (c and z) infected and treated with quinine sulphate formulations.

a. occasional mononuclear cells (kupffer cells) – characteristic of uninfected sample

b. proliferating mononuclear cells (Kupffer cell hyperplasia) – confirming infection present. (Arrow pointing to intracellular parasites)

c. occasional mononuclear cells (Kupffer) and plasma cells – infection resolved.

x. occasional macrophages with no granulomata – uninfected spleen samples

y. disorganized architecture with increased macrophages and multinucleated cells - infected (Arrows pointing to intracellular parasites).

z. Lymphoid follicle with well-defined borders, no granuloma or multinucleated giant cells – infected resolved.

For the uninfected mice samples, the cells of the liver and spleen had organized structures which were well defined and contained no granulomata (**Figure 3 a and 3x**). The cells of same organs of infected animals revealed extensive hyperplasia of the kupffer cells while the spleen tissue were disorganized and contained numerous multinucleated cells and macrophages (**Figure 3 b and 3y)**

The observations made from the histological sections of organs from mice in the various groups studied are summarized in **Table 2**.

KEY: Org - Organized; DO- Disorganized; MNC-AC - Mononuclear cell accumulation;

NO-MNC- No mononuclear cell; QS - Quinine sulphate; IP – Intraperitoneal

Sections obtained from groups 2 (untreated), 3 and 4 (treated with Amphotericin B and Pentamidine) showed unresolved infections while those from

Table 2 : Histological characteristics of liver and spleen tissue cells

Group No	Group Description	Organs		Infection post treatment
		Spleen	Liver	
1	Uninfected Mice	Org	NO – MNC	Absent
2	Negative Control (NS/Water - Oral)	DO	MNC – AC	Present
3	Amphotericin B (control 1)	DO	MNC – AC	Present
4	Pentamidine (control 2)	DO	MNC – AC	Present
5	Quinine sulphate Powder (unformulated)	Org	NO – MNC	Absent
6	Quinine Sulfate Microparticles (uncoated)	Org	NO – MNC	Absent
7	Chitosan-coated Quinine Sulphate Microparticles	Org	NO - MNC	Absent
8	Non- treated Infected mice (baseline)	DO	MNC – AC	Present

groups 5, 6 and 7 (treated with the various quinine sulphate formulations) showed resolved infections.

DISCUSSION

Microparticles were produced by the spray-dryer method. Particle size is a key parameter that determines the release of drug from a formulation. Mathematically, the particle size and rate of drug release are inversely related [8]. The sizes of coated and uncoated microparticles ranged between 2.6 and 14.2 µm. The particles were highly irregular in shape, however, chitosan coating produced more spherical particles. The uncoated particles had a negative zeta potential while the coated particles were highly positively charged. The mucosal membrane is known to have a negative charge, thus, the coated particles, due to the charge they possess will be attracted to the mucosal surface. This muco-adhesion will prolong the residence time of microparticles in the intestine leading to an extension in the release of quinine [9]. A high encapsulation yield of approximately 93% and 83% for uncoated and chitosan-coated microparticles respectively was achieved affirming the high efficiency of the spray-drying technique used.

The release of quinine sulphate in 0.1M HCl (pH 1.87) mimicked gastric conditions, while phosphate buffer (pH6.8) was used for the intestinal environment. In the HCl, 85% of the quinine sulphate was released which was well above the tolerance value of 75%, indicating that the drug will be highly bioavailable.

The release of quinine from the microparticles into solution followed a biphasic pattern: a sharp burst release of about 17% was observed within the first 30 minutes, followed by a slower release over a 24-hour period (**Figure 1**). Generally, the mean residence time of drugs is about 60 - 120 minutes in the stomach when the stomach is empty (fasted state) [10]. The use of simulated gastric fluid at a very low pH in the study assumes the fasted state, hence it is desirable for less drug to be released in the first hour and most of the drug to be released in the intestinal fluid, where most absorption is likely to take place.

A high release of quinine in simulated intestinal fluid (where generally, drugs taken in the fasted state are expected to be after one hour) for the two formulations, although it was observed that, the chitosan-coated microparticles showed a much-improved drug release compared to its release in the simulated gastric fluid.

The efficacy of the formulated medication was compared with current anti-leishmanial medications Amphotericin B and Pentamidine. The presence of parasites within and outside the

phagocytic cells was a clear indication that the mice were infected (**Figure 2**b).

The groups treated with the positive controls showed signs of unresolved infection even though it is an established fact that both Amphotericin B [11]and Pentamidine [12]are efficient in clearing the parasites from the organs. It must be stated however, that if the study had been conducted for twenty days (the standard recommended treatment period) the two positive controls would have cleared all parasites from the organs studied [12]. A previous study by authors demonstrated that quinine sulphate administered intraperitoneally (IP) was efficient at clearing the parasites from organs in 14 days. Thus, for this study, the researchers wanted to investigate if oral administration instead of IP would produce the same effect in 14 days.

From the histological studies conducted, the effect of the test formulations was more apparent. Liver micrographs of treated mice showed absent or occasional presence of kuppfer cells within sinusoids, which could be interpreted as absence or resolution of infection (**Table 2** and **Figure 3**). In the spleen sections also, similar parterns were observed. Within the treatment period (14 days of therapy), all the formulations of quinine sulphate used, were more effective in the treatment of visceral leishmaniasis in mice.

LIMITATIONS

The study was designed to have a single end point. Therefore, after treatment (day 14), all animals were sacrificed, organs harvested and sectioned. Unfortunately, the infection was resolved in all the groups treated with the new formulation, making it practically impossible to find differences between the groups. It is recommended for future studies, some animals should be sacrificed at different time points before the 14th day.

CONCLUSION

Previous studies demonstrated that parenterally administered quinine sulphate was efficient in killing intracellular *L. donovani*. This study has demonstrated that orally administered formulations of quinine sulphate were equally efficient in the elimination of intracellular parasites. Quinine sulphate, an anti-parasitic drug currently used for the treatment of severe malaria, can be repurposed for the effective management of visceral leishmaniasis in a dosage form which is convenient and widely acceptable.

Ethical Approval

No ethical approval was necessary for this study.

Author Contribution

All authors made substantial contributions to the conception, design, acquisition, analysis, or interpretation of data for the work. They were involved in drafting the manuscript or revising it critically for important intellectual content. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work, ensuring its accuracy and integrity.

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

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