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Spectrochimica Acta Part A 59 (2003) 3177–3183
SPECTROCHIMICA ACTA PART A
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# Spectroscopic study of antileishmanial drug incubated in the promastigotes of *Leishmania mexicana*

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Received 29 October 2002; received in revised form 3 March 2003; accepted 4 March 2003

**Abstract**

In this work we present spectroscopic study of Boldine (aporphine alkaloid) that possesses important biological activities, in particular, in interaction with the promastigotes of *Leishmania mexicana*. The results show the applicability of autofluorescence of this drug to determinate the possible mechanism of its biological action. The blue shift and hyperchromic effect in the emission spectrum of the drug in interaction with the parasite cells indicate an energy transference process between them. The morphological change of cell shape of the promastigotes treated with the drug is observed using confocal microscopy. This morphological cell-shape transformation evidences an important interaction between the drug studied and some protein of the parasite cell. Here we describe for the first time the fluorescence properties of the Boldine in the promastigotes of *L. mexicana*.


*Keywords:* Boldine; *Leishmania mexicana*; FRET

## 1. Introduction

The Leishmaniasis is considered by World Health Organization (WHO) as one of the six priority diseases of its Special Program for Research and Training in Tropical Diseases (WHO, 2002). The flagellated protozoan parasite *Leishmania mexicana* is the causative agent of localized and diffused cutaneous leishmaniasis. The promastigote form of the parasite is transmitted to man as a flagellated extracellular single-celled organism, which is inoculated into its victim by the sand fly as it takes a blood meal [1]. More than 12 million people suffer from this disease, many of them even die as a result of the lack of a successful therapy. New therapy strategies such as vaccination and creation of a different type of drugs are an urgent priority to combat against this illness.

During the course of our study in search of antileishmanial agents of plant origin, an aporphine alkaloid Boldine showed significant effect on the promastigotes of *L. mexicana* [2,3]. Typically,

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doi:10.1016/S1386-1425(03)00136-7

one of the ways to study possible action mechanism of a given drug in the cell involves locating the drug molecule in the cell, labeling it with some fluorescent dye [4–6]. However, the addition of any foreign substance to the drug may alter the drug biological properties in the living system [7,8]. In this sense, the use of nature chromospheres such as drugs with fluorescent property is important to minimize any possibility of perturbation induced by outside agent or interaction with the normal native cellular environment.

Fluorescence resonance energy transfer (FRET) is a spectroscopic tool widely used in structural biology to study the interaction or conformational changes of molecule within a cell [9,10]. In general, FRET occurs between fluorescent dyes and macromolecules such as DNA, RNA or proteins [11]. It is well known that FRET occurs when the emission band of one fluorophore overlaps the absorption band of a second fluorophore. A donor molecule absorbs light at some frequency that temporarily places this molecule into an energetically higher electronic state. Before the electron can decay back down to its ground state, the close proximity of another molecule results in a transfer of energy from the donor molecule to an acceptor molecule by a dipole-induced dipole interaction. Thus, the donor molecule transfers its absorbed energy in a non-radiative manner to the acceptor, which can now radiatively decay at its characteristic fluorescence emission wavelength.

The reason why FRET is a valuable tool for a variety of biological applications is that the efficiency of energy transfer has a strong inverse dependency on the distance between the donor and the acceptor. In other words, FRET is based on the strong distance dependence of non-radiative energy transfer from an excited donor molecule to an acceptor molecule. Thus, in the case of biological system, the appearance of FRET is a highly specific indicator of the active interaction of a fluorescent molecule (donor) in macromolecules such as proteins (acceptor).

This phenomenon can generate multiphoton induced fluorescence, without the requirement of high local intensities [12,13], being alternative to use low-power light sources for generation of a two-photon fluorescence exciting the molecules in the infrared region and detecting the emission in the visible range.

The main aim of the present article is to describe the fluorescent properties of Boldine solution in interaction with the promastigotes of *L. mexicana*, without the need of foreign markers. The results show important differences in the emission spectra between the drug solution and the drug solution interacted with parasites. These results reveal valuable information about FRET process between the drug studied (fluorescent donor) and some protein (acceptor) of parasite. This phenomenon is possible to see by a blue shift of fluorescence maximum and its enhanced intensity in the emission spectrum observed when Boldine solution interacted with parasites.

Additionally, the morphological change of the promastigote cell shape due to the effect of Boldine was observed with confocal microscopy [14–16]. These studies may be considered as a first step for further in vivo studies on the drug action in a given biological system.

## 2. Materials and methods

### 2.1. Parasite culture and sample preparation

The promastigotes of *L. mexicana* strain NR were cultivated in Lit Medium supplemented with 10% fetal bovine serum (FBS) according to the method described in the literature [3]. The final concentration used in the experiments was $2 \times 10^6$ promastigotes $ml^{-1}$. A total of 10 ml of the solution were incubated with 50–175 μg $ml^{-1}$ alkaloid solutions in dimethyl sulfoxide (DMSO).

For microscopic observation the promastigotes of *L. mexicana* were fixed onto glass slides without previous sample treatment.

### 2.2. Absorption measurements

Absorption spectra of samples were obtained at room temperature using an Ocean Optics 2200 UV-Vis spectrometer.

### 2.3. Confocal microscopy

The schematic description of the confocal microscope is presented in Fig. 1. The confocal microscope is homemade equipment, and the entrance and outlet of the microscope are equipped with an SMA fiber adaptor to handle them flexibly.

The light emerging from the fiber (f1) is expanded using a beam-expander configuration with spatial filter (P1), in order to fill the entrance pupil of a 40 ×, 0.65 NA microscope objective lens. The objective lens focuses the light to a small spot (0.5 μm in diameter) on the sample, at the focal plane of the objective lens. The light reflected back from the illuminated spot on the sample is collected by the same objective lens and is reflected directly by a beam-splitter (BS) to the detection system. The microscope is equipped with a CCD camera (640 × 480 pixels) and a spectrometer system (Ocean Optics, INC). Reflected light can be directed to a CCD camera in order to acquire total image, an aperture placed before the CCD produces the confocal effect and permits an image to be obtained with enhanced spatial resolution of less than 500 nm.

For samples of relatively high concentration a 10 × NA objective and 150 μm pinhole is used to obtain fluorescent spectra. Spectra from promastigotes with and without drugs were taken with a 40 × NA objective and 100 μm pinhole. The signal is an average from approximately 1 $\mu m^2$ area.

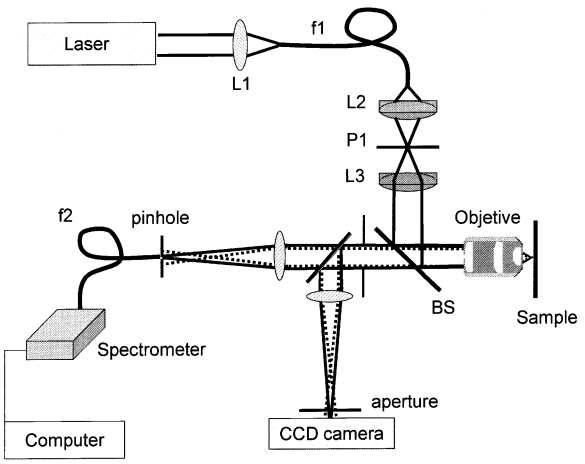


Fig. 1. Experimental setup. f1 and f2: single mode fibers; L1, L2, L3: lens; BS: beam-splitter.

### 2.4. Measurement of the fluorescence emission spectrum

The fluorescence measurements were performed using a Coherent argon-ion laser (Innova 300) at 514.5 nm. The excitation power at the sample was 20 mW.

Fluorescence emission spectra of the samples were carried out on the microscope previously described. The spectra were recorded on an optic fiber spectrometer (Ocean Optics, Inc model 2200). Spectra were digitally transferred from spectrometer to a computer for data analysis and graphics. The wavelength resolution is set by the monochromator slit to 1 nm.

We also have measured the fluorescence intensity as a function of excitation power to validate whether the fluorescence process between the drug studied and the parasites is due to fluorescence energy transfer or involves other processes. The incident power was controlled by an attenuator filter.

## 3. Results and discussion

### 3.1. Spectroscopic characterization of Boldine and Lit Medium

The absorption spectra and the structure of Boldine are shown in Fig. 2. As illustrated in this figure, the absorption maximum of the compound in the longer wavelength region is at 400 nm. Excitation was done at a longer wavelength than the absorption maximum, using a 514 nm laser, in order to minimize the risk of photodynamic perturbation.

The fluorescence emission spectra of Boldine in DMSO at a concentration of 50 μg $ml^{-1}$ show a maximum wavelength of emission at 585 nm. Under the experimental conditions of this study, the fluorescence yield of Boldine in DMSO, measured as integrated intensity of the emission band is 0.27.

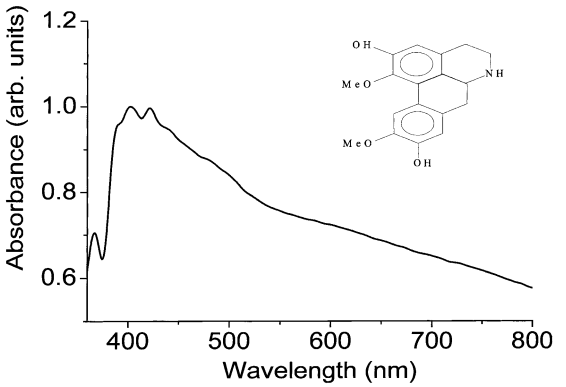


Fig. 2. Absorption spectra (0.01 mM in DMSO) and molecular structure of the Boldine.

The fluorescent properties of Boldine in solvents of different polarity than the DMSO are given in Fig. 3.

In ethanol and acetone solvent, the spectrum of Boldine shows its fluorescence maximum at shorter wavelengths up to 400 $cm^{-1}$ than in DMSO solvent. It is expected that from a polar solvent to a non-polar solvent, the energy difference between the excited state and the ground state is increased due to the inability of the non-polar solvent to stabilize the excited state. This results in a Blue shift of the emission maximum [17]. However, the fluorescence spectrum of a compound depends also on the orientation polarizability $\Delta f$ and dipole moments $\mu$ of the solvent [17]. The orientation polarizability $\Delta f$ is given by:

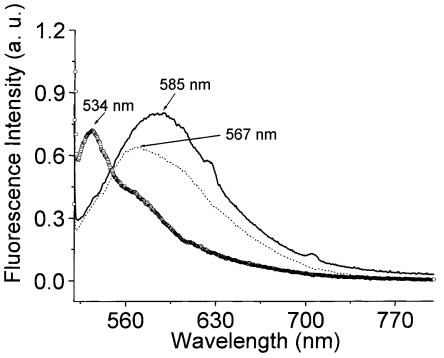


Fig. 3. Fluorescence spectra of the alkaloids in (a) dimethyl sulfoxide (-), (b) acetone (- -), and (c) ethanol (○).

$$\Delta f \approx \left(\frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right) \quad (1)$$

$n$ is the refractive index and $\varepsilon$ is the dielectric constant of the solvent.

As can be seen in Table 1, the orientation polarizability for these solvents is practically the same, but the dipole moment for ethanol and acetone is 1.69 and 2.88, respectively, and 3.96 for DMSO. Therefore, the Stoke shift of Boldine in the three solvents is a result of the change in dipole moment which occurs upon excitation, causing the shifted emission spectra of Boldine in different solvents. As a result, the emission spectra of Boldine in different solvents are sensible to solvent polarity.

To our knowledge, no previous work has been reported about the spectroscopic properties of Boldine solutions.

The Lit medium used as a culture medium in this experiment presents absorption maxims at 398 nm and a maximum of low intensity at 571 nm is observed in the fluorescence spectrum using a laser at 514 nm (Fig. 4). The fluorescence spectra of the parasites incubated in this medium are shown in Fig. 5. The spectra were acquired directly from sample parasites fixed to a glass slide. The fluorescence spectrum of Boldine in the culture medium shows a maximum at 580 nm with low intensity (Fig. 6). This fact means a small effect of the culture medium on Boldine in DMSO solution.

### 3.2. Effect of the interaction drug-parasite in the emission spectra

Fig. 6 shows the fluorescence spectra of Boldine in the culture medium and in the culture medium with promastigotes. Corresponding normalized

Table 1
Orientation polarizability of the solvents studied

| Solvent | $\varepsilon$ | $n$ | $\Delta f$ |
|---|---|---|---|
| DMSO | 47.2 | 1.4770 | 0.27 |
| Acetone | 20.7 | 1.3591 | 0.29 |
| Ethanol | 24.3 | 1.3651 | 0.29 |

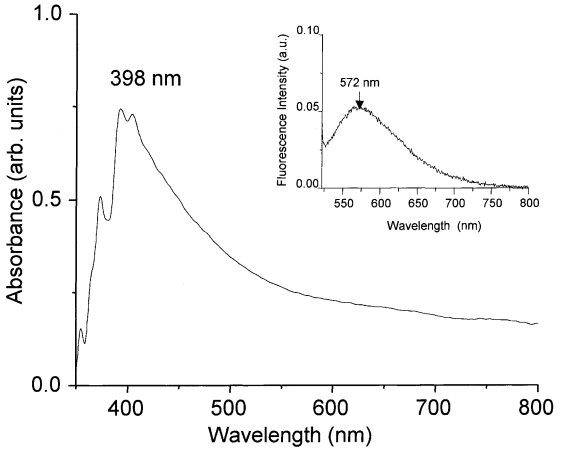


Fig. 4. Absorption and fluorescence spectra (inserted) of Lit medium.

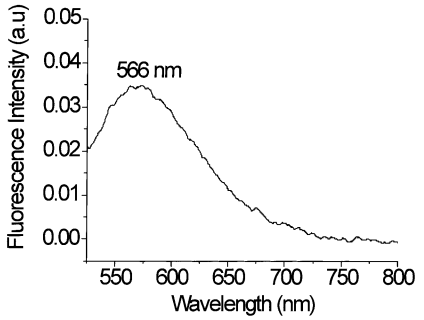


Fig. 5. Fluorescence spectra of solutions-loaded promastigotes suspension.

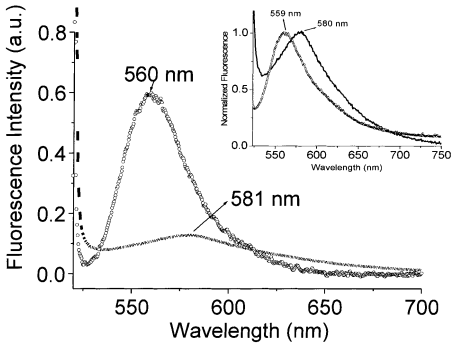


Fig. 6. Fluorescence spectra of Boldine solution in culture medium (- - -) and in solutions-loaded promastigotes suspension (○). Additionally, the Normalized fluorescence spectra of the Boldine solution in the culture medium (-) and in solutions-loaded promastigotes suspension (○) is inserted.

fluorescence spectra is shown in the inset of Fig. 6. A blue shift by about 20 nm (300 $cm^{-1}$) in the fluorescence spectra of the sample of Boldine in the medium with promastigotes was observed. This blue shift in the emission spectra of the sample can be attributed to energy transfer between the fluorophore of Boldine and some protein in the parasite. Previous reports indicate that the absorption maximum of the parasites is at 560 nm [2]. The fluorescence maximum of Boldine in the medium with promastigote (559 nm) is approximately the same that the absorption maximum of parasites (560 nm), this makes possible the energy transfer process. In this case, the drug studied (fluorescent donor) absorbs the laser radiation and transfers its electronic energy non-radiatively to some molecules (acceptor) in the parasites, Fig. 6 shows the fluorescence spectra of a sample with boldine in the media (dashed line) and Boldine in the media loaded with promastigotes (circles). It is clear that when the Boldine is in contact with the promastigotes a channel of dissipation of its fluorescence energy is activated for the energy transfer to the molecule (acceptor) of parasites. Therefore, the fluorescence intensity of the sample (Boldine + promastigotes) suffers a change in the emission maximum and is shifted to wavelengths corresponding to the fluorescence maximum of the molecules in parasites and additionally the fluorescence intensity of the sample is enhanced.

Fig. 7 shows a nonlinear excitation power dependence of the fluorescence intensity of Bol-

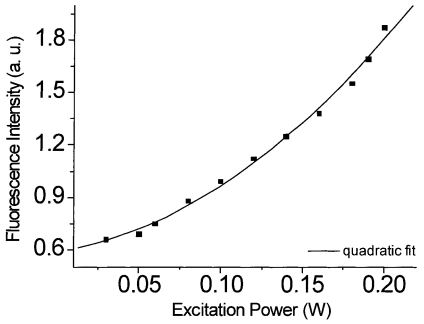


Fig. 7. The fluorescence emission intensity as a function of laser excitation power of the Boldine in the medium with promastigotes.

dine in the medium with promastigotes, indicating that the emission observed arose from the fluorescence energy transfer process between Boldine and parasites. The fluorescence energy transfer takes place without the appearance of a photon and is a result of direct dipole–dipole interaction between the molecules at a close spatial distance and is a consequence of the fact that the donor enhanced the emission fluorescence of the acceptor under the condition in which two (visible) photons are absorbed simultaneously. Therefore, the resultant fluorescence intensity should be approximately proportional to the product of the two excitation intensities [12,13]. Confocal images show changes in the morphology of the parasite after the incubation with Boldine. Fig. 8(a) shows an image of the normal promastigote.

Change in the parasite cell morphology from an elongated shape to an amastigote-like one with flagellum (Fig. 8b) is observed. Previous biological studies showed citotoxic effect ($LC_{50} = 122$ mM), concomitant with morphological change of Boldine on the promastigote cell. These results support previous conclusions [2] that the morphological transformation can be attributed to the dynamics of the cytoskeleton components, due to the steric variation of polypeptide linkage of protein.

The results presented here provide evidence that the citotoxic and antileishmanial effects of Boldine on the promastigotes of *L. mexicana* is a consequence of the interaction between the drug (Boldine) and some proteins of the parasite. This interaction resulted from energy transference between them.

## 4. Conclusions

The fluorescence properties of Boldine in the presence of the parasite promastigotes in the culture medium showed important biochemical characteristics. These properties may contribute to elucidate the biochemical action mechanism of the drug. Changes in the fluorescence maxima suggest that energy transference occurs between

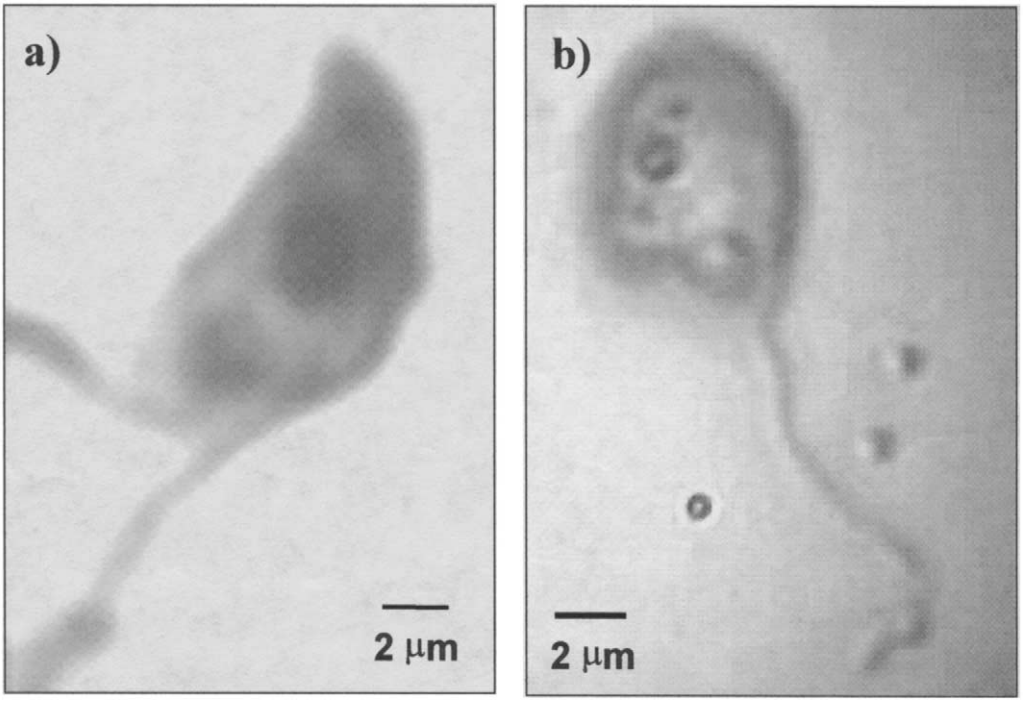


Fig. 8. Confocal Image of normal promastigote *Leishmania mexicana* (a) and after to be incubated with Boldine (b)

the fluorescent molecule and some protein of the parasite, in this way inhibiting the parasite growth.

Further studies based on this methodology are in progress to determinate the drug distribution in the cell.

## Acknowledgements

We gratefully acknowledge a Lic. Vincent Piscitelli from the University Central de Venezuela for his assistance with the construction of the Confocal Microscope. We want to express a special acknowledgement to Dra. Fraceuli Dagger from Lab. de Bioquímica de Parásitos, Escuela de Biología, UCV for supplying the parasites samples. This work was supported by the FONACIT-Venezuela through grants G-97000722, S1-95000574, G97000593, CDCH grant 03.12.4384/99.

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