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In vitro stability of arsenic trioxide-liposome encapsulates for acute promyelocytic leukemia treatment

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ABSTRACT

In this work, we investigated the stability of arsenic trioxide (ATO) used in leukemia treatment, encapsulated with nanoliposome, with the aid of ultrasound treatment. Stability studies of As species were followed by liquid chromatography-inductively coupled plasma mass spectrometry (LC-ICP-MS), allowing for the detection of the conversion of low amounts of As(III) to As(V) or the formation of other As species. The influence of storage temperature and time on ATO was evaluated. Low amounts of As(III) to As(V) conversions were observed when the As encapsulated with nanoliposome was incubated at 25 °C and 40 °C. However, As(III) was stable if the solution was maintained at 5 °C, even after 90 days. No formation of other As species was observed, indicating good stability of the encapsulated ATO. Next step of the work will focus on spray drying of ATO nanoliposomes-encapsuleted with the aim of long term stability of As.

1. Introduction

Acute promyelocytic leukemia (APL) is characterized by the t (15;17) chromosomal translocation, leading to the formation of the promyelocytic leukemia-specific-retinoic acid receptor alpha (PML-RARα or RARA) gene and is distinguished from other forms of PML by its responsiveness to all-trans retinoic acid (ATRA, also known as Tretinoin) therapy. It is a unique subtype of APL and accounts for about 10% of all acute myeloid leukemia (AML) cases in adults [1]. Promyelocytic leukemia was first described in 1957 [2] as a fatal illness with a median survival time of less than a week. Prognoses have drastically improved and today 10 year survival rates are estimated for approximately 80% of patients. Relatively good responses to the treatment of APL were initially achieved in 1973 using anthracycline (Daunorubicin, DNR). Given the efficacy of DNR as a single agent, several investigations were carried out to identify the superiority of anthracycline drug combinations over DNR alone, but no improvement was observed. A new era in the treatment of APL emerged in 1985; ATRA was introduced and dramatically improved the outcome of the treatment of this disease. In the early 1990s, a new treatment of APL with arsenic trioxide (ATO) was introduced, which led to a high complete remission (CR) rate with relatively long-term remissions when used as a single agent. After this discovery, several investigations on treatments using single or combinations of drugs were performed, which improved the ability to cure the disease [3]. A research group observed that for patients receiving combination ATRA-ATO therapy, a statistically significant improvement in the time to achieve CR, the time for complete recovery, and a decrease in the rate of relapse was achieved [4]. Based upon results observed with ATRA-ATO [5], it has emerged as the new form of care for patients with low or intermediate risk for APL. ATRA-ATO therapy may also serve as an attractive alternative for patients who are considered unfit for conventional treatment and with severe comorbidities, such as older adults and patients with cardiac dysfunction or other severe organ dysfunctions. In general, the treatment with ATRA-ATO is done intravenously, but adverse effects are reported, including high blood arsenic levels that can lead to arrhythmia. However, a similar response was also observed using oral treatment of patients with ATO but little risk of arrhythmia [6].

Although several studies have been done on the excretion of As and total As species in blood, plasma, and cerebrospinal fluid [1,7,8], the

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mechanism of ATO action and its stability is scarcely discussed [9,10]. The efficacy of ATO is related to As(III) species, but interconversion of As species can occur. In this form, when As(III) is converted to As(V) the treatment is inefficient [3]. Furthermore, an accurate control of arsenic species is recommended because it is toxic. Organic and inorganic As (III) species can react easily with –SH groups of proteins, causing protein inhibition. The inorganic As(V) is able to change the phosphate group in many reactions [10].

In this way, the behaviour of arsenic species in human cells causes a special interest in As(III) stability and its controlled delivery in APL treatment. In this context, liposomes can represent an alternative for simulating cellular membranes since they have a structure similar to the cell membrane. The liposomal vesicles are colloidal associations of amphipathic lipids that are organized and closed spontaneously in spherical structures [11,12]. Liposomes are nanocarrier delivery systems that are extensively used for drug delivery to cancer cells to overcome the limitations of conventional chemotherapy. Liposomes protect the drug from degradation and reduce drug-related nonspecific toxicity and can be produced and formulated easily for target specific delivery [13,14].

Researchers [15,16] used the encapsulation of ATO with liposomal nanotechnology to evaluate if this could reduce drug toxicity and improve the efficacy of ATO in treating different cancers, such as human papillomavirus (HPV)-associated cancers. Evaluation of the action of ATO and As stability in treated cells was conducted. However, As species interconversion has not been studied until now, where ATO was encapsulated with liposomes. Therefore, in this work an evaluation of As(III) stability of ATO submitted to different treatment conditions is proposed. Arsenic species determination was done by liquid chromatography-inductively coupled plasma mass spectrometry (LC-ICP-MS).

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade. Nitric acid was sub-boiling purified and water was distilled and purified using a Milli-Q system (Millipore Corp., Billerica, USA). Arsenic trioxide (As_2O_3, Merck, Germany) and arsenic (V) oxide (As_2O_5, Merck, Germany) were used to prepare reference solutions of 1000 mg L^{-1} of As(III) and As(V) species, respectively. A solution of ammonium monohydrogen phosphate ($(NH_4)_2HPO_4$, Merck) was used as the mobile phase and was prepared in water and filtrated through a 0.45 µm membrane filter (Millipore) before use. Phosphate buffer saline (PBS) was prepared with NaCl, KCl, Na_2HPO_4, and Na_2HPO_4; all of -analytical grade (Merck, Germany). Phospholipids (Lipoid S100, Germany), cholesterol (Sigma-Aldrich, USA), vitamin E, ethylacetate (Merck), and polysorbate 80 (Nuclear, Brazil) were also used.

2.2. Solutions of As species and liposome preparation

Arsenic trioxide solutions were prepared in *i)* buffer solution (AsBS), *ii)* As(III) in liposome prior to centrifugation (LAs), and *iii)* As(III) in supernatant of liposome solution after centrifugation (LAsC). These solutions were used for As speciation analysis and the concentrations of As species are 5.0 µg L^{-1}.

Liposomes were prepared using the reverse phase evaporation method [17] according to Moreira et al. [18] from a mixture of phospholipids (Lipoid S100, Germany), cholesterol (Sigma-Aldrich, USA), and vitamin E. The three reagents were solubilized in ethyl acetate (40 mL) with the aid of an ultrasonic bath (37 kHz and amplitude 100%) for 5 min at 25 °C. Then, 4 mL of an aqueous phase with polysorbate 80 (Nuclear, Brazil), arsenic trioxide (1.0 mg mL^{-1}), and phosphate buffer (pH 7.4) were added to the liposome solution. The mixture was then evaporated under vacuum at 40 °C for organogel formation. For liposomal vesicle formation, 96 mL of water were added to the organogel and submitted to stirring at 200 rpm and 25 °C for 30 min. The solution was then submitted to ultrasound with an ultrasonic probe (130 W and 750 W) for 5 min to homogenize the particle sizes. These solutions contain 400 µg L^{-1} of As and were used for stability studies.

Phosphate buffer saline was prepared in water by mixing 8.0 g NaCl, 0.2013 g KCl, 1.15 g Na_2HPO_4, and 0.2041 g Na_2HPO_4. The final volume of PBS was 1000 mL and the pH was adjusted with sodium hydroxide or hydrochloric acid.

For stability studies, arsenic trioxide prepared in buffer solution (AsBS) and arsenic trioxide prepared in liposome form (LAs) was submitted to the following three different conditions: *i)* at room temperature, *ii)* at low temperature (approximately 5 °C), and *iii)* at high temperature (40 °C) with controlled humidity (60%) in a climatic chamber (TE-4001, TECNAL, São Paulo, Brazil). In addition, the supernatant of LAsC was stored at low temperature and was analysed on day 1 and day 90.

2.3. Instrumentation

Arsenic determinations were performed using an inductively coupled plasma mass spectrometer (Elan DRC II, PerkinElmer Sciex, Canada) equipped with a concentric nebulizer (Meinhard Associates, USA), a cyclonic spray chamber (Glass Expansion, Inc., Australia), and a quartz torch with a quartz injector tube (2 mm i.d.). The nebulizer gas flow rate, ion lens voltage, and torch alignment were adjusted following the manufacturer instructions using conventional nebulization. Single ion monitoring at *m/z* 75 was used to collect the data.

The liquid chromatograph system consists of a quaternary pump (Model Series 200, PerkinElmer) equipped with a Rheodyne six-port injector valve, a 200 µL sample loop, and an anion exchange separation column (Hamilton, PRP-X100, 250 x 4.1 mm i.d., Bonaduz, Switzerland). The mobile phase was transported from the separation column to the nebulizer of the inductively coupled plasma mass spectrometer by PEEK-tubing. The LC operational conditions were based on work by Moreira et al. [19]. The optimized conditions for the liquid chromatograph-inductively coupled plasma mass spectrometer system are shown in Table 1.

An ultrasonic bath (37 kHz, Elma sonic P120H, Germany) and ultrasonic systems (130 W, Vibra-Cell VCX 130, and 750 W, VCX 7500) equipped with titanium probe CV18 and CV33 (Sonics & Materials Inc., USA) were used for lipossomes preparation.

2.4. Arsenic speciation

Chromatographic conditions [19] were revaluated in order to achieve good As(III) and As(V) separations in the different solutions. The concentration of $(NH_4)_2HPO_4$ was evaluated at 1.0–20.0 mmol L^{-1}, a pH of 4.0–10.0, and a mobile phase solution flow rate of 0.80–1.25 mL

Table 1
Optimized conditions for LC-ICP-MS for total As and As speciation analysis.

Total As determination by ICP-MS	Conditions
RF power, W	1300
Plasma gas flow rate, L min^{-1}	15
Auxiliary gas flow rate, L min^{-1}	1.2
Nebulizer gas flow rate, L min^{-1}	1.14
Sampler and skimmer cones	Pt
m/z monitored	75
Dwell time, ms	25 (500 for LC-ICP-MS)
As speciation by LC-ICP-MS	
pH of mobile phase	6.0
Mobile phase concentration, mmol L^{-1}	20
Sample volume, µL	200
Mobile phase flow rate, mL min^{-1}	1.25
Mobile phase (10 mmol L^{-1})	$(NH_4)_2HPO_4$

min^{-1}. All chromatographic separations were carried out in isocratic elution mode.

3. Results and discussion

Chromatographic conditions for arsenic species separation were first evaluated using a 5.0 μg L^{-1} As(III) and As(V) reference solution prepared in water. The optimized conditions for As species separation are shown in Table 1. As can be observed, good separation of the As species was achieved at pH 6.00, 10 mmol L^{-1} of $(NH_4)_2HPO_4$ with a flow rate of 1.25 mL min^{-1}, and using isocratic elution mode. By using these conditions, As species separation was achieved in 15 min.

These conditions were re-evaluated for As species separation when ATO is prepared in PBS solution (AsBS), in the presence of liposomes (LAs) and in the supernatant of centrifuged liposomes solution (LAsC). No major influence was observed when separating the As species, except a small change in retention time, which was probably a consequence of small instrumental changes. Therefore, stability studies of As species were carried out using these conditions.

Stability studies of As species were carried out over a period of 90 days and at low (~5 °C), ambient (~25 °C), and high (~40 °C) temperatures according to recommendations described by Moraes do Carmo et al. [20].

3.1. Arsenic trioxide prepared in water and phosphate buffer solution

Arsenic(III) is the active species used for APL treatment and is used as arsenic trioxide (As_2O_3) solution. However, interconversion of As species can occur, depending on factors like storage time, pH, temperature, and solution composition. Therefore, in the present study an investigation was carried out in order to evaluate the effect of storage time and temperature of As species prepared in different mediums. Fig. 1(**a**) shows the signal intensities of As(III) and As(V), corresponding to an arsenic concentration of 5 μg L^{-1}, prepared in water from day 1 to 90, and maintained at low temperature (5 °C). It was observed that the signal intensity (peak area) of both As species changed during this time, which was related to the variation in sensitivity of the instrument and not the interconversion of As species. Therefore, the results were evaluated using the relative peak area of each As species. In this case, no differences were observed over the time of analysis, meaning that As species interconversion did not occur.

Then, As(III) as ATO (5.0 μg L^{-1}) was prepared in 20 mmol L^{-1} phosphate buffer (AsBS) at pH 7.4 and analysed. As can be observed in Fig. 1(**b**)–(**d**), no conversion of As(III) to As(V) was observed after 90 days when the solution was preserved at 5 °C, 25 °C, and 40 °C.

3.2. Arsenic trioxide encapsulated with liposome

Fig. 2(**a**)–(**f**) shows the behaviour of ATO (5.0 μg L^{-1} As) encapsulated with liposomes (LAs) from 1 to 90 days, preserved at 5, 25, and 40 °C. As can be observed, only an insignificant amount of As(III) was converted to As(V) when the solution was stored at 5 °C over the entire time period (90 days). After 15 days, at 25 °C and 40 °C, As(III) was converted to As(V). In a similar form, As(III) was progressively converted to As(V) during the time of storage. In general, an element and its species are more stable at lower temperatures. However, as observed in Figs. 1 and 2(**a**)–(**f**), the behaviour of As(III) was not the same in water, in AsBS, or when encapsulated with liposomes (LAs). It is possible to assume that in the presence of liposomes, As(III) is oxidized to As(V) when preserved at temperatures of 25 °C or 40 °C.

To verify that the ATO was released from the liposome, the solution preserved at 5 °C for 1 day and 90 days was centrifuged at 10,000 *g* for 15 min at 20 °C. As can be seen in Fig. 2(**g**), no difference was observed between the As species in the non-centrifuged As-liposome solutions (LAs) and the supernatant of As-liposome solution after centrifugation (LAsC).

Therefore, it is possible to infer that to avoid the oxidation of As(III) encapsulated with liposome the solution must be preserved at low temperature (~5 °C).

4. Conclusion

This is the first investigation of the stability of ATO when submitted to encapsulation with liposomes. The stability of As(III) was studied by LC-ICP-MS, a very sensitive technique that is able to detect low amounts of oxidation of As(III) to As(V) or formation of other As species. In this study, conversion of As(III) to As(V) was observed when the arsenic encapsulated with liposomes was incubated at 25 °C and 40 °C for 90 days. However, As(III) was more stable if the solution was maintained at 5 °C during the same period. In this case, a low amount of arsenic species conversion probably occurred due to the application of ultrasonic energy and not from the temperature or time of storage. The results of the present work are significant since they have shown that the nanoliposome-encapsulated arsenic trioxide is stable. In the next step of the study, ATO nanoliposome-encapsulated will be spray dried in order to evaluate the stability to preserve it for longer time and at least at

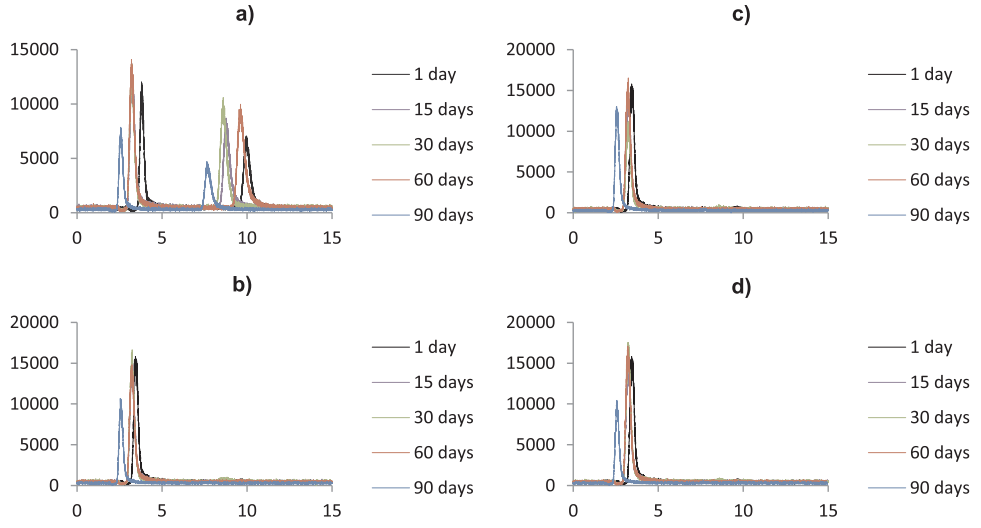

Fig. 1. Arsenic species stability in (a) water (pH 6.0), (b) phosphate buffer solution (20 mmol L^{-1} and pH 7.4) containing ATO (AsBS) at 5 °C, (c) AsBS at 25 °C, and (d) AsBS at 40 °C. Solutions with 5 μg L^{-1} As of each species. Analysis carried out according to conditions shown in Table 1.

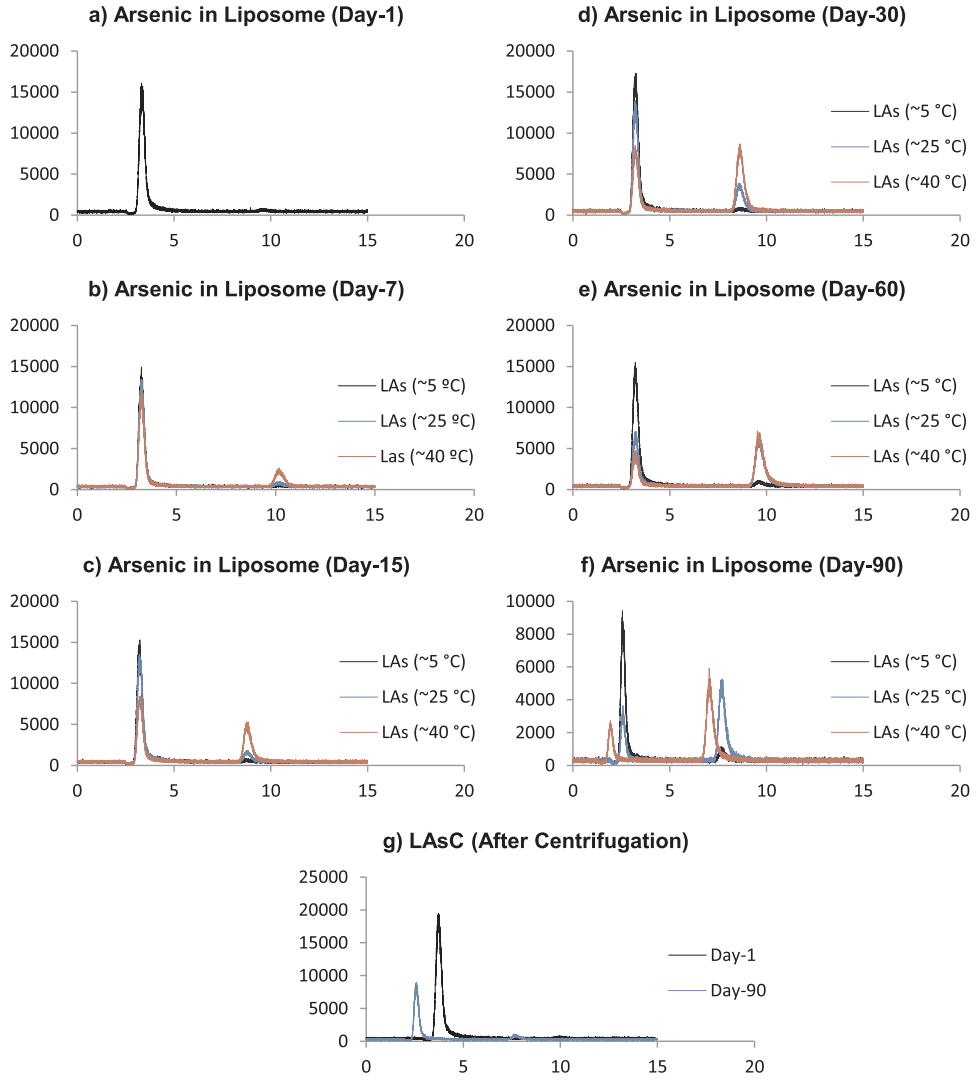

Fig. 2. Arsenic species stability of ATO encapsulated with liposomes (pH 7.4) (a–f: As-liposome solutions before centrifugation (LAs) at 5, 25, and 40 °C), and (g: supernatant As-liposome solution after centrifugation (LAsC) at 5 °C). Solutions with 5 μg L^{-1} As of each species. Analysis were carried out according to conditions shown in Table 1.

ambient temperature (25 °C).

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