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In vitro and *in vivo* properties of usnic acid encapsulated into PLGA-microspheres

R. M. RIBEIRO-COSTA†‡, A. J. ALVES‡, N. P. SANTOS†, S. C. NASCIMENTO§, E. C. P. GONÇALVES¶, N. H. SILVA¶, N. K. HONDA and N. S. SANTOS-MAGALHÃES†¶*

†Laboratório de Imunopatologia Keizo-Asami (LIKA),
‡Departamento de Ciências Farmacêuticas, Laboratório de Tecnologia Químico-Farmacêutica (LTQF),
§Departamento de Antibióticos, Setor de Farmacologia, Laboratório de Cultura de Células e Cancerologia Experimental,
¶Departamento de Bioquímica, Laboratório de Produtos Naturais, Universidade Federal de Pernambuco (UFPE), Recife, PE, Brazil
#Universidade Federal de Mato Grosso do Sul (UFMS), Departamento de Química, Laboratório de Produtos Naturais, MS, Brazil

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Microparticles will probably play a promising role in the future of chemotherapy. These polymeric delivery systems are capable of maximizing the therapeutic activity while reducing side effects of anti-cancer agents. Usnic acid (UA) is a secondary metabolite produced by lichens, which exhibits an anti-tumour activity. In this study, PLGA-microspheres containing usnic acid from *Cladonia substellata* were prepared by the double emulsion method, with or without PEG as stabilizer. The morphology of the microspheres was examined by optical and scanning electron microscopy. The *in vitro* kinetic profile of usnic acid loaded-microspheres was carried out by dissolution testing. The usnic acid content was analysed by HPLC. The cytotoxicity of free and encapsulated usnic acid was evaluated against HEp-2 cells using the MTT method. The anti-tumour assay was performed in mice against Sarcoma-180 tumour (UA 15 mg kg^{-1} weight body/day) during 7 days. Animals were then sacrificed and tumour and organs were excised for histopathological analysis. Microspheres presented a smooth spherical surface with a mean diameter of 7.02 ± 2.72 μm. The usnic acid encapsulation efficiency was ~100% (UA 10 mg 460 mg $^{-1}$ microspheres). A maximum release of 92% was achieved at the fifth day. The IC_{50} values for free and encapsulated usnic acid were 12 and 14 μg ml^{-1}, respectively. The encapsulation of usnic acid into microspheres promoted an increase of 21% in the tumour inhibition as compared with the free usnic acid treatment. In summary, usnic acid was efficiently encapsulated into PLGA-microspheres and the microencapsulation improved its anti-tumour activity.

Keywords: Usnic acid, *Cladonia substellata*, PLGA microspheres, cytotoxicity, anti-tumour activity.

Introduction

Drug delivery systems are versatile pharmaceutical dosage forms with a promising future (Verma and Garg 2001). Microencapsulation is a very common

* To whom correspondence should be addressed. e-mail: nssm@ufpe.br

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method for preparing controlled release systems such as carriers for drugs and vaccines (Jain *et al*. 2000, Lamprecht *et al*. 2000, Barrio *et al*. 2003). The use of microspheres has a substantial appeal to enhance the therapeutic efficacy of drugs. Alternative microparticulate carriers have been developed aiming to decrease the toxic effects of anti-cancer compounds toward normal tissues and to increase their efficiency against tumours (Singh and Udupa 1997, Wang *et al*. 1997, Roullin *et al*. 2002, Le Ray *et al*. 2003).

Biodegradable polyesters such as copolymers of lactic and glycolic acid (PLGA) are attractive biomaterial for pharmaceutical and medical applications. They have been widely used as carriers in controlled-release systems due to their excellent biocompatibility and biodegradability properties (Kumar *et al*. 2001).

The usnic acid (UA) is a low-molecular weight dibenzofuran derivative (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3[2H,9bH]-dibenzo-furandione) produced by some lichen species (Müller, 2001). Usnic acid presents as a yellowish cortical pigment, which occurs in two enantiomeric forms differing in the orientation of the methyl group located in the stereogenic centre at the 9b position (Cocchietto *et al*. 2002). Usnic acid is a product from the secondary metabolism of the fungal partner and it is widely distributed in species of *Cladonia*, *Usnea*, *Lecanora*, *Ramalina*, *Evernia and Parmelia* (Ingólfsdóttir, 2002). Since its first isolation in 1844, usnic acid has become the most extensively studied lichen metabolite and one of the few that is commercially available. Usnic acid exhibits several biological and pharmacological properties such as anti-microbial activity against human and plant pathogens, anti-viral (Campanella *et al*. 2002), anti-protozoal, anti-proloferative, anti-inflammatory and analgesic activity. In addition, usnic acid presents cytotoxicity and anti-tumour activity (Cocchietto *et al*. 2002).

Nevertheless, the practical use of usnic acid in therapy has been rather limited due to its poor solubility in water. In particular, the intra-molecular hydrogen bonds contribute to the lipophilic nature of usnic acid and its lipophilicity is deeply linked do its cytotoxic effect, which can be attributed to the β-triketone moiety present in the molecule (Takai *et al*. 1979).

The challenges of the biomedical applications of usnic acid namely hepatotoxicity, water insolubility and consequently low efficacy can be surpassed by suitable pharmaceutical technology such as using drug delivery systems. In this framework, for the first time usnic acid from *Cladonia substellata* (Vainio) was encapsulated into microspheres. The goal of the present work is to develop PLGA-microspheres containing usnic acid and to evaluate their *in vitro* kinetic profile and cytotoxicity, as well as their *in vivo* anti-tumour activity.

Materials and methods

Materials

The microspheres were prepared with poly (D,L-lactic-co-glycolic) acid (PLGA, 50/50, inherent viscosity 0.44 dL g^{-1}), which was purchased from Birmingham Polymers (Alabama, USA). The emulsifiers poly (vinyl alcohol) (PVA, mw 13 000–17 000), poly (ethylene glycol) (PEG, mw 4000) and poly (vinyl pyrrolidone) (PVP, mw 40 000), the cryoprotector threalose and the

standard usnic acid were obtained from Sigma-Aldrich (St Louis, USA). Poloxamer (Pluronic® F68) was generously supplied by ICI (France). Usnic acid was extracted and purified from *Cladonia substellata* (Vainio). The lichen material was collected on sandy soils of *tabuleiro* (Savannah like vegetation of Mamanguape country, Paraíba, Northest of Brazil) and identified through morphological and chemical thalus characterization. The physicochemical characterization of the usnic acid was carried out at the Chemistry Laboratory of the Federal University of Mato Grosso do Sul (Brazil).

HPLC grade methanol, analytical grade solvents and reagents were obtained from Merck (Darmstadt, Germany). Cell culture supplies were obtained from GIBCO (New York, USA). Larynx epidermoid carcinoma cell line (HEp-2, Animal and Human Cell Catalogue, Rio de Janeiro Cell Bank, Brazil) were subcultured and seeded at 10^5 cells ml^{-1} in 96 well-plates in Dulbecco modified Eagle's medium (DMEM) containing 25 mM glucose, and supplemented with 10% bovine serum and 1% non-essential amino acids.

Preparation of usnic acid loaded-microspheres

The microspheres were prepared by a w/o/w multiple emulsion technique. Initially, PLGA (450 mg) and usnic acid (10 mg) were separately dissolved in methylene chloride (10 and 2 ml, respectively). The organic solution containing usnic acid was then added to the polymeric solution and emulsified with deionized water (5 ml) with or without PEG (5 mg). The simple emulsion (w/o) was generated by mechanical agitation (ultra-turrax T25, IKA, Germany) for 1 min at 8000 rpm using an ice bath. Afterwards, this simple emulsion was added to a continuous phase, constituted of 50 ml aqueous solution of PVA (0.5%, w/v) and emulsified for 30 s at 8000 rpm, resulting in a multiple emulsion (w/o/w). The mixture was maintained under agitation at 400 rpm (4 h) leading to the solvent evaporation and consequently the microsphere formation by hardening the polymeric wall of the particles. The microspheres were then isolated by centrifugation (Kubota KN-70 centrifuge, Japan) for 5 min at 3000 rpm and washed three times with deionized water to remove the excess of emulsifiers. Finally, the microspheres were recovered by either filtration or lyophilization procedures. Before lyophilization, microspheres were previously dispersed with 1.0% (w/v) threalose aqueous solution, frozen at −80°C overnight and placed in the lyophilizer (EZ-DRY, FTS System, New York, USA) operating at 200 bars during 16 h. Unloaded microspheres were prepared under the above described conditions. The storage of unloaded and usnic acid loaded-microspheres was performed at 4 ± 1°C in a vacuum dessicator.

Several batches of microspheres were developed by modifying the amount of polymer, drug and emulsifiers and also by evaluating the final recovering process of microspheres. Initially, formulations were prepared with Synperonic® F68 or PVP as emulsifiers for stabilizing the double emulsion. Following, microspheres were prepared with PVA at concentrations varying from 0.3–1.0% (w/v) in an attempt to obtain more stable formulations. The influence of the presence of PEG in the simple emulsion on the stability of PLGA-microspheres containing usnic acid was also investigated.

Characterization of usnic acid loaded-microspheres

Morphological analysis. The manufacturing process of microspheres was followed by optical microscopy (Olympus CH-2 microscope, USA) for evaluating the formation, homogeneity and surface properties of particles. The morphological characteristics of the microspheres were analysed by scanning electron microscopy (SEM) using an electron microscope (JSM-T200, JEOL, Japan). A sample of microspheres was suspended in distilled water to obtain an homogeneous suspension and placed on a glass surface, which was fixed on a metallic support with carbon-glue. After drying at 37°C, samples were directly coated with colloidal gold using a sputter module in a high-vacuum evaporator (JFC-1100, JOEL, Japan). Then, microspheres were examined and the mean diameter was determined by particle counting.

Stability of usnic acid loaded-microspheres. The stability of the usnic acid encapsulated into PLGA-microspheres was evaluated in a period from 7–510 days, aiming to establish formulation durability. The macroscopic and microscopic aspects were monitored and pH evolution after water redispersion was determined at regular time intervals. In addition, the usnic acid content was analysed by the High Performance Liquid Chromatography (HPLC) method described below.

Usnic acid encapsulation efficiency. The usnic acid loading into PLGA-microspheres was determined by HPLC after extraction of usnic acid from microspheres. Samples of microspheres (13.8 mg, corresponding to 0.3 mg of usnic acid) were diluted with 1 ml dimethyl sulphoxide (DMSO) under ultrasonic agitation for 5 min. Then, the volume (5 ml) was completed with methanol/phosphate buffered solution pH 7.4 (70:30 v/v). An aliquot of this solution was diluted to a theoretical concentration of 6 μg ml^{-1}, filtered through a 0.45 μm membrane filter (Millipore®) and the usnic acid content was analysed using the HPLC method, as described below.

Usnic acid assay by HPLC method. A variant of the method proposed by Venkataramana and Krishna (1992) was developed and validated to quantify the usnic acid content in PLGA-microspheres. The computer assisted HPLC system (Hewlett Packard, HP-1100, USA) is constituted by a quaternary pump, a degasser system, an UV/VIS detector and a manual injector with a 20 μl loop. The chromatographic run was performed using a μBondapack C_{18} column (10 μm particle size, 125 Å, 300 × 3.9 mm I.D., Waters, USA) and a mobile phase composed of methanol/20 mM phosphate buffered solution pH 7.4 (70:30, v/v). Sample aliquots (20 μl) were injected and eluted with the mobile phase at a flow rate of 1.5 ml min^{-1}. The usnic acid peak was verified at a wavelength of 280 nm (0.005 a.u.f.s) with a retention time of ~3 min. The amount of usnic acid into microspheres was determined through the standard calibration curve. Usnic acid standard calibration curve was prepared for concentrations varying from 1–10 μg ml^{-1}. Each experiment was performed in triplicate.

In vitro *kinetic release of usnic acid loaded-microspheres*. The *in vitro* release profile of usnic acid from PLGA-microspheres was determined according to Rafati *et al*. (1997). A sample of 25 mg of the usnic acid-loaded PLGA microspheres (0.54 mg of usnic acid) were suspended in 54 ml of 20 mM phosphate-buffered

saline (PBS, pH 7.4). The tubes were incubated at $37 \pm 1°C$ and shacked horizontally at 180 rev min^{-1}. At pre-determined time intervals, 2 ml aliquots of the dissolution medium were withdrawn and centrifuged (2800 rpm, 5 min) in order to separate any microspheres present in the sample. The supernatant was removed and the amount of the released usnic acid was measured by the HPLC. Assays were performed in triplicate and results were expressed as the percentage of the mean values and their standard deviation.

Cytotoxicity of usnic acid loaded-microspheres. The cytotoxic effect of free and encapsulated usnic acid was evaluated on cells of larynx epidermoid carcinoma (HEp-2) by the colorimetric technique with 3-(4,5-dimethyltiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Mosmann 1983, modified by Alley *et al.* 1988). Cell suspensions were diluted to 10^5 cells ml^{-1} and aliquots of 220 µl of such suspension were seeded on 96 well plates. After 24 h, samples of free and encapsulated usnic acid were placed in contact with HEp-2 cells at different concentrations ranging from 2.5–15 µg ml^{-1}. Usnic acid solution was prepared as the standard solution, as previously described. After 72 h incubation of cells, a solution of MTT (25 µl $well^{-1}$) was added and plates were incubated for 2 h. The supernatant was removed and DMSO (100 µl $well^{-1}$) was added to dissolve the formazan crystals. Plates were read at 590 nm. The maintenance of cells and all experiments were carried out at 37°C under a 5% CO_2 atmosphere. Unloaded microspheres and standard usnic acid were used at the same sample concentrations as negative and positive controls, respectively. Each dosage group was assayed in quadruplicate. Results are expressed as the mean values of viable cells.

Anti-tumour activity of usnic acid loaded-microspheres. The *in vivo* anti-tumour activity of usnic acid loaded-microspheres was evaluated in mice against Sarcoma 180 tumour. Tumour ascytic cells (5×10^6 cells ml^{-1} suspension) were subcutaneously inoculated into male Swiss mice (32–41 g body weight, 45–60 days old). Three groups of six animals were randomly established as control group and treated groups. Chemotherapy started after 24 h of tumour implantation by intraperitoneal injections of free usnic acid or usnic acid loaded-microspheres at a dose of 15 mg kg^{-1}/day during 7 days. After 1 week of treatment, animals were sacrificed and tumour weights were measured. Then, tumour inhibition percentage was calculated from the mean tumour weights of the treated animal group in relation to the untreated control group. Animal experiments were performed according to the National Cancer Institute (NCI) protocol (Geran *et al.* 1972), with the approval of the Ethic Committee for Animal Experimental Assays of the Federal University of Pernambuco (Recife, Brazil).

Histopathological analysis

The animal organs (tumour, liver, kidneys and spleen) were submitted to histopathological analysis. Sample tissues were preserved in a 10% neutral buffered-formalin solution until paraffin inclusions. Slices of sample tissues (4 µm) were prepared and a fixation with haematoxylin and eosin staining was developed, before examination by optical microscopy (Olympus BH-2, Japan).

Results and discussion

Formation of usnic acid loaded-microspheres

Pre-formulation studies were performed to guide the choice of the optimal concentration of constituents for obtaining a stable formulation of usnic acid loaded-microspheres (table 1). An amount of 400 mg of PLGA was used to prepare microspheres. Initially, an attempt to prepare microspheres using Pluronic® F68 or PVP (0.5% w/v) as emulsifiers to stabilize the simple emulsion was accomplished. However, Pluronic® was not able to stabilize the double emulsion and the use of PVP led to an aggregated final product. Therefore, those emulsifiers were discarded in the preparation of PLGA-microspheres. In a second step, PVA was chosen as a stabilizer for the double emulsion. The effect of its initial concentration (0.3, 0.5 or 1.0% w/v) on the stability of microspheres and drug entrapment was evaluated. The concentration of 0.5% PVA promoted a great homogeneity of particle size distribution and led to 99% drug entrapment efficiency for an initial amount of 10 mg usnic acid (table 2). In fact, the presence of a stabilizer in the external phase give rise to a decrease on the superficial tension between the aqueous and organic phases, which conducted to a reduction of the diameter of the multiple emulsion globules (Blanco-Pietro *et al*. 1998). Consequently, a reduction on the size of the resulting microparticles was observed.

In a second stage, the amount of PLGA (50/50) was varied from 0.40–0.55 g. As observed by optical microscopy, 0.4 g of PLGA promoted the formation of a little amount of particles. On the contrary, 0.55 g of PLGA bring upon the presence of polymer crystals detected by visual and microscopic observation (data not showed). The optimized amount of PLGA was found to be 0.45 g. However, PLGA-microspheres prepared with PVA (0.5%) as stabilizer of the double emulsion presented irregular shape, porous at the surface and a tendency to form aggregates (figure 1(*a*)). This drawback was solved by the addition of PEG, as the stabilizer of the simple emulsion.

The influence of the PEG concentration (0.1–0.4 g) on the stability of PLGA-microspheres was evaluated. The SEM morphological evaluation revealed that microspheres could be produced with or without the presence of PEG. Nevertheless, microporous were detected at the surface of microspheres prepared without PEG (figure 1(*b*)). Furthermore, it was observed that the presence of PEG improved the formation of well-defined microspheres with spherical shape, homogeneous particle size distribution and smooth surfaces. In addition, it was verified that an increase in the PEG amount promoted the formation of more stable microspheres with a better morphological aspect. The amount of 0.4 g was chosen as the optimal PEG quantity for obtaining stable microspheres (figure 1(*a*, *b*)). Therefore, the presence of PEG in the first step of the microspheres manufacturing process was clearly crucial to derive stable formulations. Despite that PEG was used as a surfactant in the aqueous phase of the simple emulsion, it can be speculated that some PEG molecules were blended to PLGA. As expected, a substantial fraction of the PEG originally dissolved in the first aqueous phase should be eliminated during the washing process. Actually, Cleek *et al*. (1997) had detected the formation of PLGA-PEG physical blends during microspheres producing when PEG was initially dissolved in the organic phase as a mixture with PLGA.

Table 1. Optimization of the formulation of UA-loaded PLGA-microspheres.

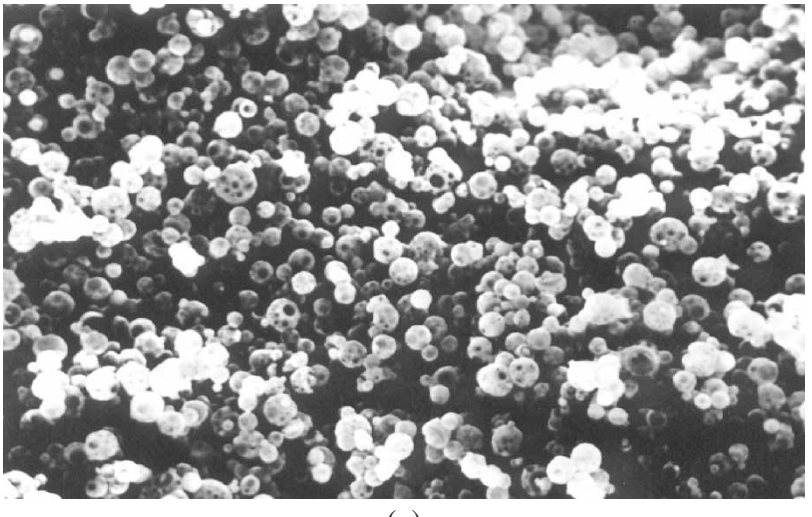
	Formulations												
Constituents	1	2	3	4	5	6	7	8	9	10	11	12	13
Usnic acid (g)	—	0.01	—	—	—	—	0.01	0.01	0.01	0.01	0.01	0.015	0.02
PLGA 50:50 (g)	0.4	0.4	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
PEG 4000 (g)	—	—	0.05	0.1	0.2	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
PVA 0.3%	—	—	—	—	—	—	—	—	50	—	—	—	—
PVA 0.5%	60	60	60	60	60	60	60	50	—	—	50	50	50
PVA 1.0%	—	—	—	—	—	—	—	—	—	50	—	—	—
Methylene chloride (ml)	10	12	10	10	10	10	12	12	12	12	12	12	12

The aqueous phase of the first emulsion consisted of different PEG quantities into 5 ml deionized water.

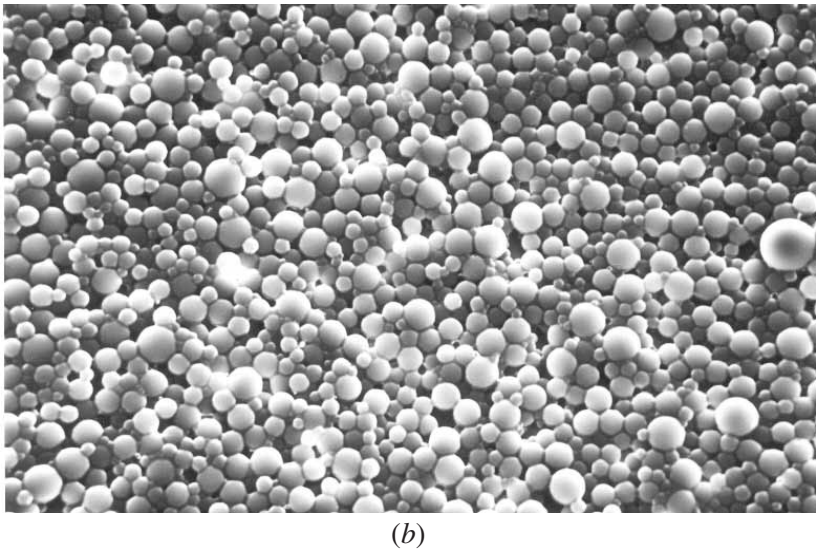
Table 2. Evaluation of the influence of the surfactant concentration on the usnic acid entrapment efficiency into PLGA-microspheres.

Initial PVA concentration (%)	UA encapsulation (%)
0.3	93 ± 1.12
0.5	99 ± 0.46
1.0	86 ± 0.95

Data shown are the mean ± SD (standard deviation) ($n = 3$). The initial amount of usnic acid was 10 mg.



(*a*)



(*b*)

Figure 1. Photomicrographs of the usnic acid-loaded PLGA microspheres prepared without (*a*) and with (*b*) PEG analysed by SEM (100×, 0.7 cm = 10 μm). The initial PEG amount (0.4 g) is expressed on dry basis for the total mass of the polymer and drug (46.5 wt%).

Table 3. Encapsulation efficiency of usnic acid into PLGA-microspheres (450 mg of polymer).

Usnic acid amount (mg)	Encapsulation efficiency (%)
10	99 ± 0.46
15	87 ± 1.79
20	81 ± 0.51

Data shown are the mean ± standard deviation ($n = 3$).

In spite of unfavourable physicochemical properties of the usnic acid, such as its photosensitive nature and chemical instability, an UA microencapsulation (10, 15 or 20 mg) was achieved into microspheres prepared with 450 mg of PLGA. At the 1:45 drug–polymer ratio, the best usnic acid encapsulation efficiency (~ 100%) and more stable microspheres were attained (table 3).

Optimization studies resulted in a typical microsphere formulation prepared with PLGA, PEG and PVA containing usnic acid from *C. substellata* (table 1, formulation 11).

Characterization of usnic acid loaded-microspheres

Lyophilized PLGA-microspheres containing usnic acid were evaluated according to their morphological characteristics, encapsulation efficiency and long-term stability. Usnic acid loaded-microspheres presented an initial macroscopic aspect as a yellowish powder. Microscopic analysis displayed spherical shaped microspheres and a relative homogeneity in the particle size distribution (figure 1(*b*)). A mean diameter of 7.02 ± 2.74 μm was estimated by particle counting.

The encapsulation efficiency of the usnic acid into PLGA-microspheres for different drug–polymer ratio is presented in table 3. The encapsulation efficiency decreased at higher usnic acid concentrations, being 87% for 15 mg and 81% for 20 mg, respectively. The highest encapsulation (99 ± 0.46%) was achieved for microspheres prepared with 10 mg of usnic acid and 450 mg of PLGA.

The content of usnic acid was 105 ± 5% after microspheres preparation, which guarantees that the drug integrity was maintained during the manufacture of the dosage form. The evaluation of the long-term stability of lyophilized PLGA-microspheres showed that acceptable levels of usnic acid content (90%) were kept until 7 months of storage at 4°C. However, a gradual decrease of usnic acid content was detected reaching 61 ± 6.7% 510 days after preparation of microspheres (table 4).

In vitro *release kinetics of usnic acid loaded-microspheres*

The *in vitro* release of usnic acid from PLGA-microspheres is characterized by a typical bimodal behaviour (figure 2). Initially, a large burst effect (35 ± 0.13%) occurred at the first hour. This effect might be associated to the presence of usnic acid crystals on or nearby the surface of microspheres. This burst step is followed by a gradual release of usnic acid reaching 92 ± 0.04% within 5 days. However, a slight decrease of the usnic acid in the dissolution medium after this period was observed. This usnic acid declining can be attributed to its photo degradation

Table 4. Stability of usnic acid encapsulated into PLGA-microspheres.

Time (days)	Usnic acid content (%)
7	105 ± 5.07
15	104 ± 7.89
72	103 ± 8.50
90	100 ± 10.97
105	97 ± 1.66
190	94 ± 4.53
210	91 ± 1.27
245	80 ± 1.06
300	70 ± 3.79
510	61 ± 6.67

Data shown are the mean ± standard deviation ($n = 3$).

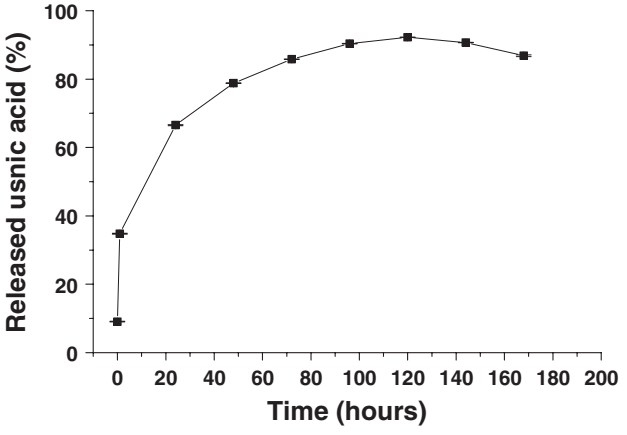


Figure 2. *In vitro* release profile of usnic acid from PLGA-microspheres. Error bars represent mean ± standard deviation for $n = 3$.

and chemical instability. The bimodal kinetic profile could be explained by the presence of a part of the drug in the external surface of the microspheres (burst effect) and a part of the drug entrapped in the polymeric matrix (plateau), which requires a longer time for degradation. Therefore, it can be postulated that the release mechanism of usnic acid from PLGA-microspheres is governed by diffusion followed by the polymeric matrix erosion through hydrolytic degradation of the copolymer. In fact, it well-known that PLGA containing a 50:50 ratio of lactic and glycolic acids are hydrolyzed faster than those containing a higher proportion of one of the two monomers (Lewis, 1990).

Kinetic profiles of drug from PLGA microspheres reported in the literature are quite controversial and fundamentally dependent on the monomeric lactic and glycolic acid ratio on the polymeric chain. In addition, the *in vitro* experimental conditions seem to play an important role on the drug release behaviour. In this way, the usnic acid kinetic profile is corroborated by that one reported for the release of taxol from PLGA 50/50 microspheres (Wang *et al.* 1997) with a faster drug release attaining 80% in 2 days. Recently, it was reported a slower constant

release of 5-fluorouracil from microspheres prepared with PLGA (75/25) for a 3-week period (Roullin *et al*. 2002).

Cytotoxicity of usnic acid loaded-microspheres

Results of cell viability for the incubation at different concentration of free and encapsulated usnic acid are shown in figure 3. Concentration required to inhibit 50% of cell proliferation (IC_{50}) were 12.6 and 14.4 $\mu g\,ml^{-1}$, for free and encapsulated usnic acid, respectively. Therefore, there was no significant difference between the cytotoxicity of free and encapsulated usnic acid, presenting the same IC_{50} magnitude order. No cytotoxic effects were observed for cells incubated with unloaded microspheres. This last result confirms the cell biocompatibility of the microspheres prepared with PLGA, PEG and PVA.

Anti-tumour activity of usnic acid loaded-microspheres

The effect of the intraperitoneal administration of free and encapsulated usnic acid on the inhibition of Sarcoma 180-bearing mice was examined (table 5). Usnic acid loaded-microspheres promoted a 63% inhibition on the tumour growth, while the inhibition was smaller for free usnic acid (42%). Therefore, an improvement of 21% on tumour inhibition was achieved with microencapsulation of usnic acid into PLGA-microspheres.

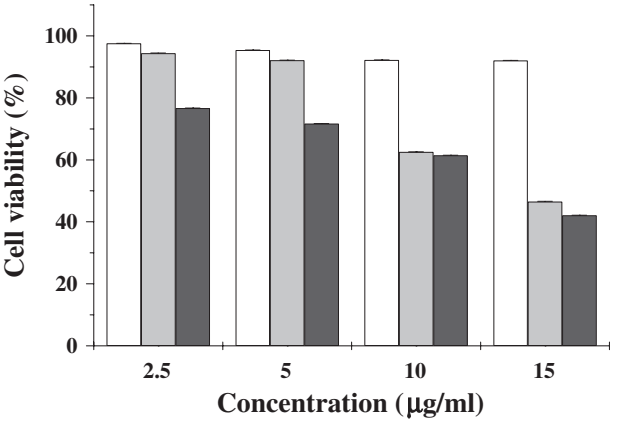


Figure 3. Cytotoxicity of free and microencapsulated usnic acid against HEp-2 cells, MTT test (mean of four wells). Usnic acid loaded-microspheres (grey); unloaded microspheres (white); usnic acid in phosphate buffered saline (pH 7.4) (black).

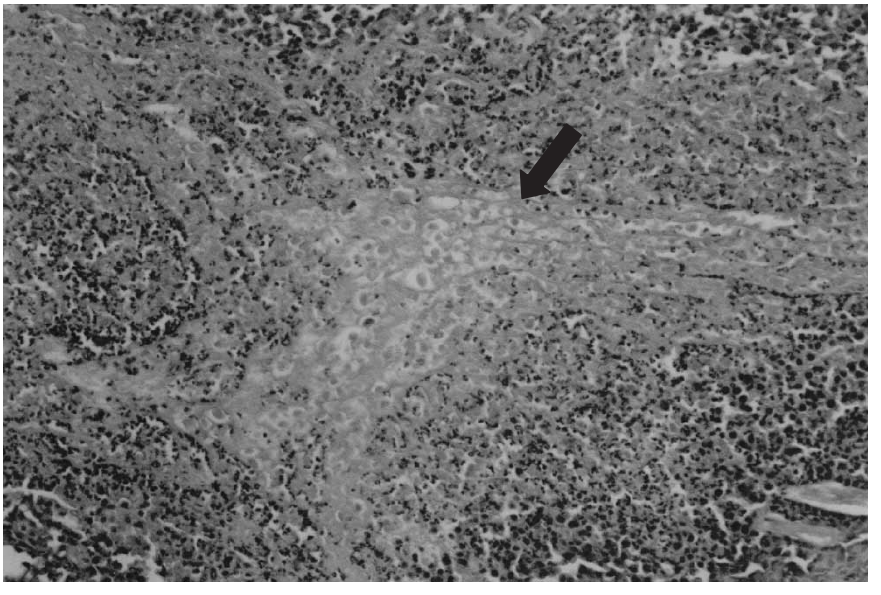
Table 5. Evaluation of the anti-tumour activity of the free and encapsulated usnic acid against Sarcoma-180.

Treatment of animals	Tumour inhibition (%)
Free usnic acid	42 ± 1.56
usnic acid loaded-microspheres	63 ± 0.47

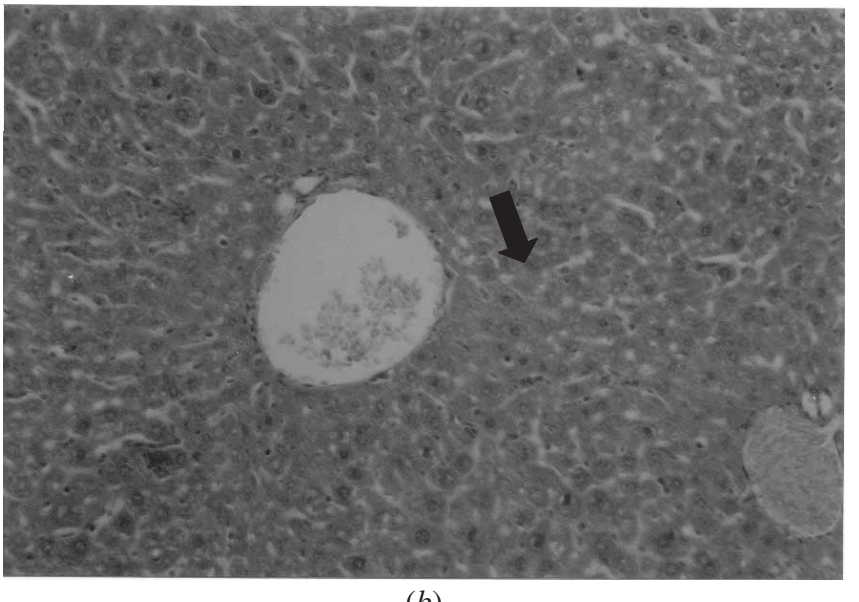
Data shown are the mean $\pm$ standard deviation ($n = 5$).

Histopathological analysis of tumour and organs

Histopathological analysis of tumour and liver of animals treated with encapsulated usnic acid are illustrated in figure 4. Microphotograph revealed extensive necrotic areas on tumour tissue after treatment with free usnic acid, while this abnormality was less with loaded-usnic acid (figure 4(*a*)). On the other hand, liver



(*a*)



(*b*)

Figure 4. Histopathological analysis (H & E) of tumour (200×) and liver (100×) of mice treated with UA-loaded PLGA microspheres. Arrows indicate areas of necrosis.

of animals treated with usnic acid loaded-microspheres presented only morphological uncharacterised hepatocytes (figure 4(*b*)). No significant abnormalities were verified for the treatment with unloaded-microspheres. No histological alterations were observed in the spleen and kidneys of all animals treated with free or encapsulated usnic acid.

Conclusions

PLGA microspheres containing usnic acid were obtained with a relative homogeneity in the size distribution of particles. The presence of PEG 4000 as a emulsifier of the simple emulsion in the first aqueous phase of the system improved the morphology of the microspheres. The encapsulation efficiency of usnic acid (10 mg) was remarkable in 460 mg of microspheres. The *in vitro* kinetic study revealed that usnic acid presented typical release biphasic behaviour. The cytotoxicity of free and encapsulated usnic acid was not significantly different. Moreover, the microencapsulation promoted an increase on tumour inhibition activity of usnic acid. Achievements in the present work were supported by those reported in the literature, which presented the potential effect of the drug microencapsulation as a promise alternative for an effective chemotherapy.

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