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Characterization and Trypanocidal Activity of Nifurtimox-containing and Empty Nanoparticles of Polyethylcyanoacrylates

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Abstract

The aim of this study was to evaluate the utility of nanoparticles of polyalkylcyanoacrylate as a targeted delivery system for nifurtimox against *Trypanosoma cruzi*, responsible for Chagas' disease. Ethylcyanoacrylate nanoparticles were prepared by an emulsion polymerization process and formulations containing different concentrations of nifurtimox, polyethylcyanoacrylates and surfactants were investigated and analysed for size and drug content.

The nanoparticles obtained were less than 200 nm in size, as measured by electron microscopy and cytometry. The peak percentage of nifurtimox uptake into the nanoparticles was 33·4% for use of 500 μL polyethylcyanoacrylate, 200 μL surfactant (Tween 20) and 10 mg nifurtimox in 50 mL polymerization medium. The highest release of nifurtimox from the nanoparticles was 65·4% after 6-h incubation at pH 7·4. In-vitro studies using cultures of *T. cruzi* epimastigotes revealed considerably increased trypanocidal activity compared with a standard solution of nifurtimox. Studies of cell cultures previously infected with metacyclic forms of the parasite showed that only 2-h treatment with solutions of 0·001% of the nanoparticle suspension reduced parasitism by 87–94% both when the nanoparticles were loaded with nifurtimox and when unloaded. Electron-microscopic examination revealed processes of degeneration and lysis, suggesting apoptotic processes, in intracellular amastigotes and free amastigotes treated with the nanoparticles. It was demonstrated that unloaded nanoparticles, by mechanisms not completely elucidated, have trypanocide activity similar to that of a standard solution of nifurtimox. It is concluded that the nanoparticles loaded with nifurtimox constitutes a good carrier of the drug against *T. cruzi*. The loaded-nanoparticles significantly increase trypanocidal activity.

Chagas' disease is considered by the World Health Organization (WHO) to be the world's most important protozoan illness; more than twelve million people are affected (WHO 1960). High morbidity and mortality from this disease raise very important health, social and economic problems in Latin-American countries. In the temperate zone of Chile, Chagas' disease has endemic-enzootic characteristics, with both rural and suburban distribution. The prevalence of infections in man is estimated to be 15% (Schenone et al 1980). No ideal drug is available for treating *Trypanosoma cruzi* infections. Nifurtimox and benznidazol are the only drugs available to treat Chagas' disease. Nifurtimox is associated with frequent vomiting, anorexia, weight loss, weakness, loss of memory, sleep disturbances, tremor and polyneuritis (Martindale 1993). If the dose of these drugs could be reduced by use of mechanisms that increase their activity, it would enable their use with fewer risks and greater safety. In these circumstances the development of new compounds or new dosage forms as alternatives to those currently used is a research area of great interest because of the economic, social, and political impact that control of trypanosomiasis would have. Moreover, because of

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the lack of success in the treatment of third-world endemic parasitic disease (Meshnick 1984), drug carriers have been considered as a means of achieving a better specificity of commonly used anti-parasitic compounds (Alving et al 1980).

Among the polymeric carriers recently proposed as drug-delivery systems, polyalkylcyanoacrylate nanoparticles have been extensively studied because of their biodegradability and efficiency of entrapping some biologically active compounds (Couvreur & Vauthier 1991; Couvreur et al 1991a).

The aim of this study was to investigate how preparation conditions could influence nifurtimox loading-capacity, nanoparticle size, and release profiles. We also studied the anti-proliferative activity of these nanoparticles, using cultures of epimastigotes of *T. cruzi*, and the activity of the nanoparticles on the intracellular amastigote forms of the parasite.

Materials and Methods

Chemicals

Nifurtimox (lampit; 4-[(5-nitrofurfurylidene)-amino]-3-methyl-thiomorpholine-1,1-dioxide) was a gift from Bayer Laboratories (Buenos Aires, Argentina). Ethylcyanoacrylate used as the monomer for polymerization was obtained from Sigma (St Louis, MO). Solvents were HPLC grade and other reagents were purchased from Merck Química Chilena, Santiago, Chile.

Preparation of nanoparticles

Polyethylcyanoacrylate nanoparticles were prepared by polymerization according to the method described by Couvreur et al (1979). The desired amount of nifurtimox (1 mg mL^{-1}), solubilized with a minimum amount of dimethylsulphoxide, was dissolved in aqueous HCl (0·01 M; 50 mL) containing the non-ionic surfactant tween 20 (0·20 mL). Ethylcyanoacrylate monomer was added dropwise over 10 min with mechanical stirring (1000 rev min^{-1}). When polymerization was complete (usually 3 h) the colloidal suspension was adjusted to pH 7·0 with 0·2 M NaOH. Unbound nifurtimox was separated by centrifugation (Sorvall Superspeed RC2-B; Sorvall, Newtown, CT). Finally, the nanoparticles were suspended in water and centrifuged again to remove drug residue from the inter-particle space.

Several concentrations of nifurtimox, ethylcyanoacrylate and surfactant were investigated to determine which led to the greatest entrapment of nifurtimox by the nanoparticles.

Nifurtimox assay

The amount of nifurtimox bound to the nanoparticles was determined by UV. Dried nanoparticles loaded with nifurtimox (normally 40 mg) were dissolved in acetone (12 mL) and the solution was analysed by UV absorbance at 401 nm with a Milton Roy Spectronic 3000 spectrophotometer. Response was linearly dependent on concentration in the range 3–12 $\mu g\ mL^{-1}$. The other components of the nanoparticles (polyethylcyanoacrylate, Tween 20 and dimethylsulphoxide) did not interfere with the analysis. The amount of drug adsorbed was expressed as the percentage of the total weight.

Morphological characterization and size analysis

Scanning electron microscopy (Jeol JSM-25-S II, 30 kV; Faculty of Biology Sciences of Catholic University of Chile, Santiago) was used to study the morphology of the nanoparticles. Nanoparticle size and homogeneity were determined by use of a FACS Vantage flow cytometer (Becton and Dickinson, Immunochemistry System, San Jose, CA) fitted with an Enterprise coherent laser, 160 mW at 488 nm and 60 mW at 535 nm. Data were collected from 10 000 particles for each sample, at a flow rate of approximately 500 particles s^{-1}. Readings were obtained at an output power of 160 mW at 488 nm. The fluorescent signal was collected with a 530/30BP band-pass filter (Fl 1 FITC), the signal being connected in logarithmic mode with the PTM Fl 1 = 555V, and analysed using the LEASES II program (Becton and Dickinson).

Nifurtimox release from nanoparticles

Nifurtimox release from nanoparticles was determined in isotonic phosphate buffer of pH 7·4 and in HCl at pH 1·2, according to the method described by Cicek et al (1994). For each pH drug-loaded nanoparticles (40 mg) were suspended in phosphate buffer (10 mL) at 37°C (thermostatted bath) with constant agitation. Samples were withdrawn every 30 min and the nifurtimox concentrations were assayed by UV. Each experiment was performed in quadruplicate.

Parasite

T. cruzi was isolated from a chronic Chagasic patient from northern Chile. Multiple clones were prepared and, after several passages in rats, one clone (CA-1) was finally adapted for axenic culture. Epimastigote forms were used to evaluate activity against *T. cruzi*.

Culture medium

Minimal essential medium (MEM; Gibco-BRL 072-110) with Hank's salts (Gibco-BRL 041-011575) supplemented with 10% calf bovine serum was used to maintain the epimastigote forms. Cultures were maintained in T-25 culture flasks (Sterilin) at 28°C and then sub-passaged at 10^4 cells mL^{-1} every third day. For sensitivity determinations, *T. cruzi* in the logarithmic growth phase were taken from these stock cultures.

Metacyclic forms were obtained in-vitro in modified Grace's medium (Osuna et al 1990). After 9 to 11 days of culture, when the proportion of metacyclic forms reached 85%, Percoll-gradient centrifugation was used to purify the infectious forms in the fraction between 1·115 and 1·120 g mL^{-1} (Castanys et al 1984). These trypomastigotes were washed and used for the infection of vero cells.

The amastigote forms were obtained from the infected vero cell culture as described previously (Sánchez-Moreno et al 1995).

Cell culture

The cells used, from the vero cell-line ATCC CCL-81, were cultured, at 37°C in a moist atmosphere supplemented with 5% CO_2, in Dulbecco's modified Eagles medium (DMEM) at pH 7·2 with 10% foetal bovine serum previously inactivated (IFCS) at 56°C for 30 min. Once formed the cell monolayer was separated by treatment with phosphate buffer solution plus 0·05% EDTA, the cells then being centrifuged and the pellet re-suspended in culture medium. Borosilicate glass cover slips (2 cm × 0·7 cm) were used as supports for the cell cultures. The number of cells used in each experiment was 1×10^6, the inoculation being made after 36 h of cell sub-culture.

Anti-proliferative study in epimastigote forms

Unloaded nanoparticles, nanoparticles loaded with nifurtimox equivalent in concentration to a standard solution of nifurtimox, and the standard solutions of nifurtimox (25 μL) were placed in each well of row A to D of a microtiter plate (Nunc, InterMed, Denmark). All the remaining wells of the plate received 50 μL medium. For three-fold serial dilution, 25 μL from each well of rows A to D were transferred to the corresponding well of rows B to E and this was continued to the wells of rows C to F; 25 μL was discarded from row F. Columns A11 and A12 served as controls. Medium (50 μL) containing 10^6 cells mL^{-1} was added to all wells to give a final concentration of 5×10^5 cells mL^{-1}. Medium alone (50 μL) was added to the wells of the remaining rows, which served as additional cell-free controls. The microtiter plate was placed in an incubator at 28°C in a 5% CO_2 atmosphere for 72 h; the plates were kept in a humidity chamber to prevent evaporation. Parasites were counted in a Neubauer chamber (Boeco, Germany) under a microscope (Cambridge Instruments, Galen III).

Inoculation of the cultures and evaluation of infected cell cultures

Before inoculation the medium was removed from the cell cultures and a suspension of the metacyclic forms in DMEM medium (pH 7·2) without serum was inoculated at a ratio of two metacyclic trypomastigotes per cell. Four hours after inoculation the cultures were washed to remove any trypomastigotes that had not penetrated the cells and the medium was replaced by DMEM with 10% IFCS (pH 7·2), which was then incubated for 72 h.

Twelve hours after inoculation the cells were treated for 2 h with the loaded-nanoparticle suspension at concentrations of 0·1, 0·01 and 0·001% in the culture medium. Control cultures were treated with unloaded nanoparticles at the same concentrations of the loaded suspension. An infected culture was used as a control of the infection and multiplication of the intracellular parasite.

After 24, 48 and 72 h of infection, three slides of the different cultures were fixed in methanol and stained with Giemsa. The percentage parasitization and the parasitization (number of amastigotes per cell) were determined for at least 600 cells. The experiment was repeated three times.

To study the alterations induced by the nanoparticles in the parasitized cells, 48 h after infection the cultures were treated for 4 h with the loaded and unloaded nanoparticles at 0·1% as described above. This treatment was followed by washing in fresh medium and fixation with glutaraldehyde at 2·5% in 0·1 M cacodillate buffer containing 0·1 M saccharose (pH 7·2). The fixer was eliminated after 2 h and replaced by fresh fixer; the mixture was then maintained at 4°C for 12 h. After fixation the culture was removed with a rubber policeman and centrifuged at 10 000 *g* for 1 min. The pellet was processed as described by Osuna et al (1993) and ultra-thin sections were examined by means of a Zeiss (6EM902) electron microscope. In other instances the treatment was performed with isolated amastigote forms for 4 h with 0·1%-nifurtimox-loaded nanoparticles.

Calculation of activity

Percentage trypanocidal activity was calculated by the use of the equation:

$$\text{Percentage activity} = (N_b - N_w)/N_b \quad (1)$$

where N_b is the number of live parasites in the blank and N_w is the number of parasites in each well.

Percentage growth inhibition for each time period and each dosage was calculated from:

$$\text{Percentage growth inhibition} = [(T_c - T_p)/T_c] \times 100 \quad (2)$$

where T_c is the number of parasites mL^{-1} in the control wells and T_p is the average number of parasites mL^{-1}.

Statistical analysis

Student's *t*-test was used to compare the trypanocidal activity of the loaded and unloaded nanoparticles and nifurtimox standard.

Results

Figure 1 shows a scanning electron micrograph of the spherical 200-nm-diameter nanoparticles. The best adsorption of nifurtimox by the nanoparticles (33·4 ± 2·1%) was obtained with a 500-μL volume of cyanoacrylate. Use of 200, 250 or 300 μL of surfactant did not lead to significant differences in the absorption of nifurtimox. Better adsorption was obtained for low concentrations of nifurtimox, entrapment efficiency decreasing as the concentration of the drug in the medium was increased. The amount of nifurtimox adsorbed mg^{-1} nanoparticles was 8·2 ± 2·1 μg.

Figure 2 shows the curves of release of nifurtimox from the nanoparticles at pH 7·4 and pH 1·2. At pH 1·2, the amount of nifurtimox released from the nanoparticles reached 20% after 6 h incubation in the acid medium. On the other hand, at pH 7·4 65% of the nifurtimox was released within 6 h of incubation in the buffer. The release of nifurtimox from the nanoparticle suspensions was characteristically biphasic, with initial fast release followed by a second, much slower, first-order release phase.

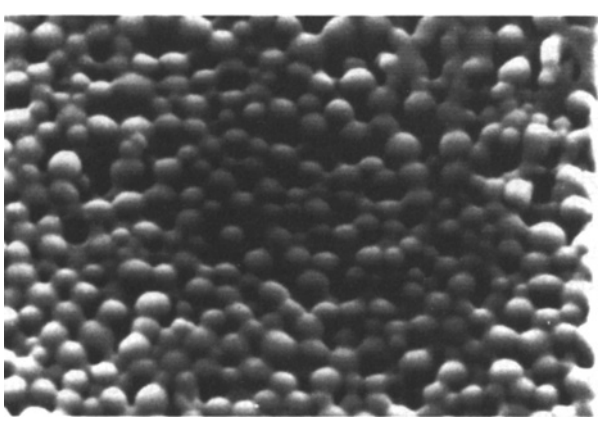

Figure 1. Scanning electron micrograph of nifurtimox-loaded nanoparticles.

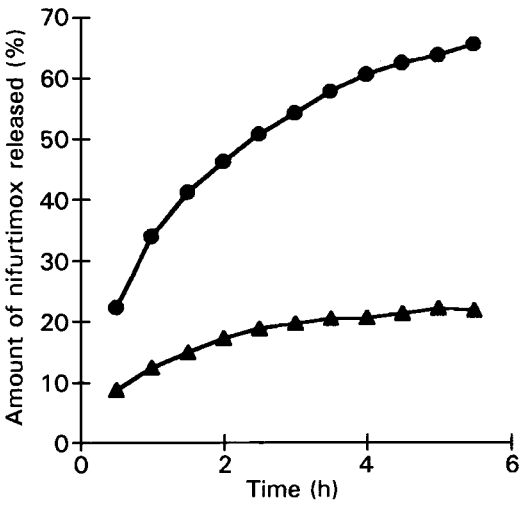

Figure 2. The release profiles of nifurtimox-loaded nanoparticles in simulated gastric fluid (pH 1·2; ▲) and physiological pH (pH 7·4; ●).

The trypanocidal activity of the nanoparticles with and without nifurtimox was calculated and was compared with that of a standard solution of nifurtimox equivalent to the concentration of the drug in the nanoparticles. Figure 3 shows the results obtained. The action on the infected cells is shown in Table 1. The electron-microscopy studies revealed that the intracellular parasites underwent a series of degenerative processes with electrodense accumulation of nuclear chromatin, autolysis of the

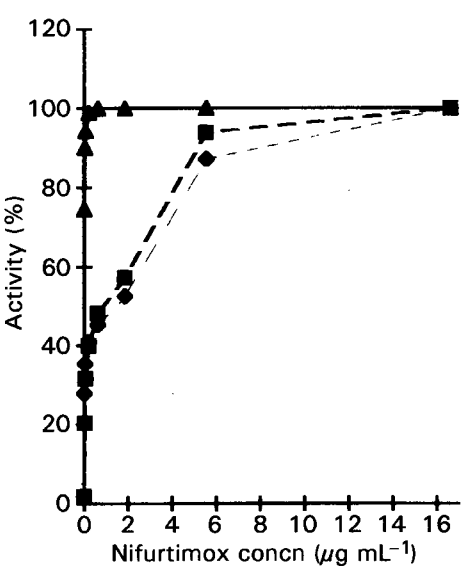

Figure 3. Trypanocidal activity profile of nifurtimox standard (■) and of nifurtimox-loaded (▲) and unloaded nanoparticles (◆) in free epimastigotes culture.

Table 1. Growth inhibition for each time period (24, 48 and 72 h incubation) and each dose of nifurtimox-loaded nanoparticles, unloaded nanoparticles and nifurtimox standard solution in infected cell cultures.

Treatment	Concentration	Amount of growth inhibition (%)		
		24 h	48 h	72 h
Nifurtimox-loaded nanoparticles	0·1%	91·4	95·8	97·8
Unloaded nanoparticles	0·1%	87·5	87·5	91·4
Nifurtimox-loaded nanoparticles	0·01%	91·6	91·8	94·3
Unloaded nanoparticles	0·01%	66·5	85·7	88·7
Nifurtimox-loaded nanoparticles	0·001%	83·6	85·7	94·1
Unloaded nanoparticles	0·001%	63·2	76·7	86·6
Nifurtimox standard solution	1 μg mL^{-1}	23·7	34·5	54·6
Nifurtimox standard solution	10 μg mL^{-1}	24·8	40·7	91·7
Nifurtimox standard solution	100 μg mL^{-1}	73·0	88·3	96·7

cytoplasm of the parasite, degeneration of the kinetoplast and lysis of the parasite's membranes (Figures 4–6). We found no alterations in the host cells or in the surrounding cells.

Alterations similar to those in the intracellular parasites were observed in the treated amastigotes isolated (Figure 6).

Differences were observed in the activity of the three preparations investigated. The greatest trypanocidal activity was recorded with the nifurtimox-loaded nanoparticles; at concentration of 0·2 μmL^{-1} activity was 98·9% whereas the activity of the same concentration of nifurtimox in the standard solution was about 40%. The trypanocidal activity of unloaded nanoparticles was, paradoxically, similar to that of the standard nifurtimox solution. The 50% inhibitory concentration (IC50) obtained from the activity–concentration curves was 0·683 ± 0·269 μg mL^{-1} for nifurtimox standard solution and of 0·0015 ± 0·0006 μg mL^{-1} for the suspension of nanoparticles loaded with nifurtimox ($t = 5·1$; $P < 0·002$).

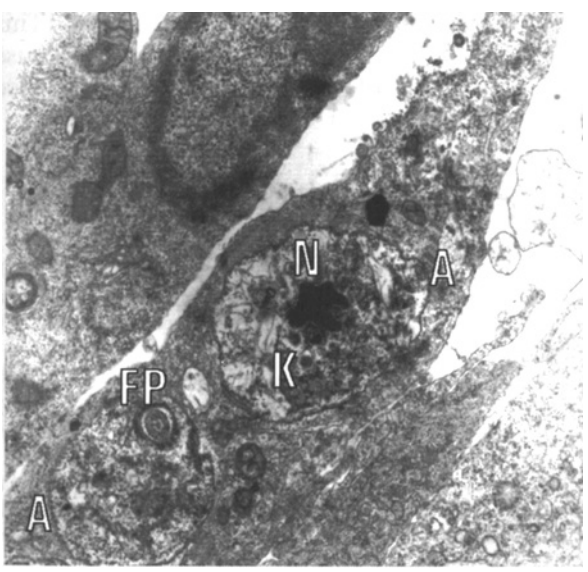

Figure 4. Effect of nifurtimox on *Trypanosoma cruzi*. Inside the cell two amastigote forms (A) are visible. The nucleolus (N) in each appears electrodense with accumulations of chromatin and disorganization of the cytosol (FP, flagellar pocket; K, kynetoplast).

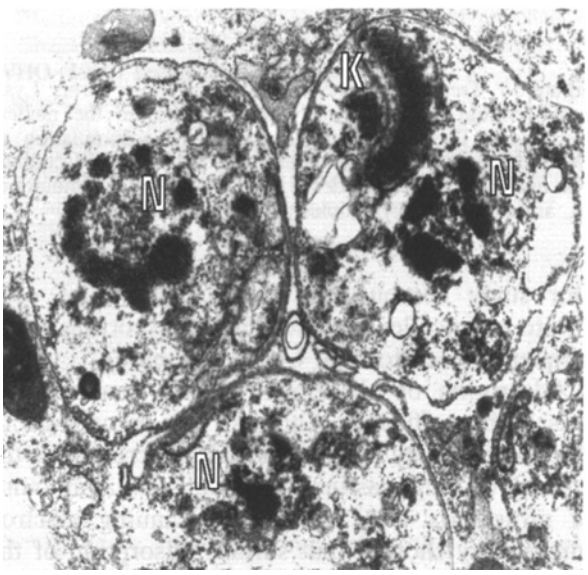

Figure 5. Intracellular amastigote forms. The cytoplasm is disorganized with great hyperchromicity of the cell nucleolus (N), and the peripheral chromatin and the kynetoplast (K) appear electrodense. Chromatin disaggregation is visible.

Discussion

Previous studies have shown the potential usefulness of polycyanoacrylate nanoparticles as colloidal carriers of drugs (Couvreur et al 1979, 1982). These ultra-fine particles have been shown to entrap a wide variety of drugs (e.g. antibiotics, hormones and cytostatics) in their polymeric network (Couvreur et al 1991b; Grislain et al 1983).

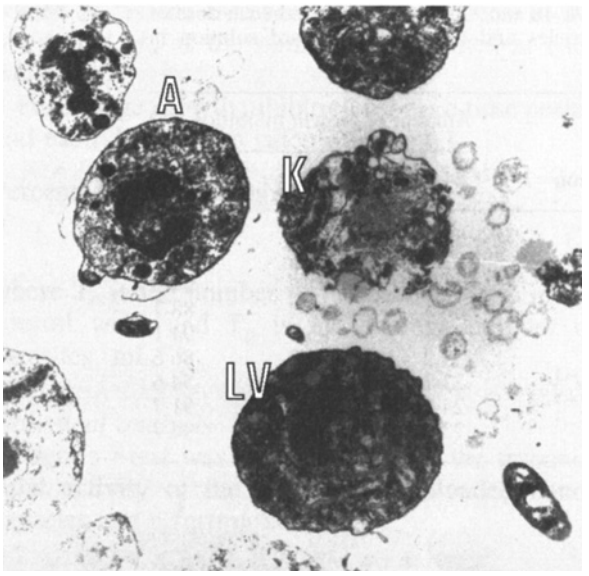

Figure 6. Treatment of the in-vitro extracellular forms. Lysis is discernible in the forms with a rupture in the nuclear membrane. The cytoplasm shows strong accumulation of electrodense vacuoles and others with possible lipid content. The nuclei appear electrodense with chromatin accumulations (A, amastigote; K, kynetoplast; LV, lipid vacuole).

Our study demonstrates that nanoparticles constitute an excellent medium for transporting drugs frequently used to treat Chagas' disease.

Adsorption of nifurtimox by the nanoparticles was highly efficient. Although the percentage of drug adsorbed by the nanoparticles decreased as the amount of drug was increased, substantial amounts of nifurtimox were adsorbed. Scanning electron microscopy showed that surface adsorption of the drug induces no morphological modification of the nanoparticles (Figure 1). On the other hand, the release kinetics of the adsorbed drugs changed according to the nature of the polymer. In our study of ethylcyanoacrylate, a short-chain monomer, we found that the release of nifurtimox from the nanoparticles differed according to the pH of the dissolution medium. Thus, in acid medium (at the pH of the stomach) release was no greater than 20% after 6-h incubation whereas at pH 7·4 nifurtimox release reached 60%. Cicek et al (1994), studied the effect of the pH on the release of dinitrophenylhydrazine from ethylcyanoacrylate nanoparticles within the pH range 1·2 to 7·4 and noted a significant increase in the release of the drug as the pH increased; this is in accordance with the results obtained in our study.

The most significant result of our study was the high trypanocidal activity of nifurtimox-loaded nanoparticles in comparison with nifurtimox standard. Thus, dilutions as low as 7·6 and 23 ng mL^{-1} resulted in trypanocidal activities of 75 and 90%, respectively. These trypanocidal activities were similar on infected cells and on amastigote forms of the parasite. Although no similar studies of nanoparticles loaded with nifurtimox are reported in the literature, Youssef et al (1988) observed strong anti-microbial activity of nanoparticles loaded with ampicillin against *Lysteria monocytogenes*; Fresta et al (1995) reported that entrapment of pefloxacine and ofloxacin in nanoparticles resulted in a two- to fiftyfold decrease in the minimum inhibitory concentrations of the drugs against some Gram-positive and Gram-negative bacteria.

Another important finding in our study was that the trypanocidal activity of the unloaded nanoparticles was 4–5 times that of a standard solution of nifurtimox. This same finding was obtained by Lherm et al (1987), who found that unloaded polyisobutylcyanoacrylate nanoparticles had activity against *Trypanosoma brucei*. The mechanism of the trypanocidal activity of these unloaded nanoparticles has not been elucidated. It is possible that enzymatic degradation of the nanoparticles produces formaldehyde (Couvreur et al 1984) which could be responsible for the cellular lysis. Other authors have demonstrated the anti-microbial activity of these nanoparticles in a culture of *Bacillus subtilis* (Youssef et al 1988). However, this feature must be investigated more deeply.

Conclusions

Nanoparticles loaded with nifurtimox constitute a good carrier of drugs against *Trypanosoma cruzi*. The trypanocidal activity of the loaded-nanoparticles is significantly greater than that of a standard solution of the same concentration. This finding is of great clinical importance, because reducing the dose of nifurtimox would also reduce the adverse effects of the drug.

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