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Nanoparticles of polyisohexylcyanoacrylate (PIHCA) as carriers of primaquine: formulation, physico-chemical characterization and acute toxicity

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Summary

Nanoparticles of polyisohexylcyanoacrylate with bound primaquine were prepared by an emulsion polymerization method and an i.v. formulation was obtained after freeze-drying. Such formulation is intended for drug targeting in visceral leishmaniasis. Drug binding seemed largely dependent on pH and formulation conditions were optimized to obtain a polymeric colloidal suspension containing particles in the 200–250 nm size range and 80–90% drug binding (final content of 10 mg of drug per vial). Aspects such as polymer-drug interaction, in vitro drug release and physicochemical stability were studied. A significant reduction in acute toxicity was demonstrated by using the targeted formulation in NMRI mice.

Introduction

Primaquine has been used for its antimalarial activity (Brossi et al., 1987); meanwhile, as with other 8-aminoquinolines, it possesses a potential activity against visceral leishmaniasis (Peters et al., 1980; Berman and Lee, 1983; Berman, 1985). However, because of its lack of specificity for the target organs and its toxicity (Tarlov et al., 1962; McChesney et al., 1987) it is not widely used in clinical treatment of visceral leishmaniasis.

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Drug targeting with colloidal systems is a useful approach for experimental chemotherapy of visceral leishmaniasis, improving delivery of drugs to the specific intracellular localization of the parasite in the reticuloendothelial system (RES) (Alving, 1982). Taking advantage of the common localization of the parasite and colloidal drug carriers, several researchers intended to target drugs with carriers to reduce their toxicity and increase their efficacy as antileishmanial drugs (Alving and Swartz, 1984).

Liposomes can increase the therapeutic index of pentavalent antimonials in animal models (Alving et al., 1978; New et al., 1978). Other researchers performed experiments using lipoproteins (Hart et al., 1987), resealed erythrocytes

('ghosts') (Berman et al., 1986), niosomes (Baillie et al., 1986), microparticles (Stjärnkvist et al., 1987) or nanoparticles (Fouarge et al., 1989) as carriers.

In the group of polymeric synthetic colloidal systems, nanoparticles of polyalkylcyanoacrylate (PACA) are well characterized as far as their physicochemical properties (size, molecular weight), biodegradability, low toxicity and biodistribution are concerned (Kante et al., 1982; Grislain et al., 1983; Lenaerts et al., 1984; Couvreur, 1988). They can easily be prepared on an industrial scale (Verdun et al., 1986). Their affinity to the RES and pharmaceutical advantages when compared with other drug delivery systems make them a good candidate for targeting drugs against visceral leishmaniasis.

Our objective is to improve the chemotherapy of visceral leishmaniasis by targeting drugs with nanoparticles of PACA to reduce their toxicity and/or increase their therapeutic efficacy.

A formulation of nanoparticles loaded with primaquine was developed. Their physicochemical properties and acute toxicity were characterized.

Materials and Methods

Preparation of polyisohexylcyanoacrylate (PIHCA) nanoparticles:

Nanoparticles of PIHCA were prepared by an emulsion polymerization method described previously (Couvreur et al., 1982). Briefly, we used anionic polymerization in which the monomer (isohexylcyanoacrylate, Sopar Pharma, 6328 Sart-Dames-Avelines, Belgium) was added in a concentration of 12 mg/ml with mechanical stirring to the polymerization media. The polymerization occurs in aqueous media, at acidic pH, containing 5% glucose and 1% dextran 40 (Pharmacia A.B., Uppsala, Sweden) as isotonic, cryoprotector and colloidal stabilizer agents. Polymerization was performed for 16 h.

For biological experiments (e.g. acute toxicity) the nanoparticle preparations were made under sterile conditions as previously described (Verdun et al., 1986). Monomer and glass material were sterilized by dry heat before use and the polymerization medium was submitted to sterile filtration with a 0.22 μm Millipore filter. All chemicals were pyrogen free.

Primaquine dosage

The primaquine content was determined by an HPLC method (Parkhurst et al., 1984) using a Waters chromatographic system (M-45 solvent delivery system, model; U6-K universal LC injector, Waters Associates, Milford, U.S.A.) equipped with a μ-Bondapak-CN chromatographic column (Waters), a mobile phase of acetonitrile : formate buffer (11 : 89) and UV spectrophotometric detection (M480 LC Spectrophotometer, Waters) at 254 nm. As internal standard 8-aminoquinoline (Janssen Chimica, Beerse, Belgium) was introduced in all dosages at a concentration of 1 μg/ml.

Adsorption of primaquine

Adsorption of primaquine (Janssen Chimica, Beerse, Belgium) was performed under different experimental conditions. The influence of pH, drug concentration, time of adsorption and freeze-drying procedure was studied as detailed. To study the effect of pH, primaquine stock solutions of 25 mg/ml were prepared at different pH values and added to unloaded nanoparticles neutralized with NaOH (0.1 N). Since primaquine at such a concentration is insoluble in an aqueous medium at pH higher than 5, a cosolvent containing 40% ethanol and adjusted to the desired pH with NaOH was used. Under the assay conditions for the adsorption procedure (drug concentration 1–4 mg/ml) primaquine is completely soluble. Primaquine solutions were protected from light.

The adsorption procedure was performed to obtain a maximum drug load and nanoparticles loaded with primaquine were freeze-dried using a Lyovac GT2 or GT15 (Leybold Heraeus) under vacuum (6×10^{-2} mbar). Before use they were redispersed in sodium bicarbonate (0.02 M).

Nanoparticle loading capacity was calculated after ultracentrifugation (Beckman centrifuge, Model J21C, Beckman Instruments, Palo Alto, CA, U.S.A.) at 20 000 rpm for 2 h. Free primaquine was determined as described in the supernatant and bound primaquine after dissolution of the precipitate in dimethylformamide (UCB, Leuven, Belgium). The level of drug binding is expressed

as the percentage of drug associated with the carrier in comparison with the sum of bound and free drug.

Physico-chemical characterization

Granulometric determinations were performed by measuring laser light scattering with a Coulter Nano-Sizer (Coulter Electronics Limited, Harpenden, U.K.) to evaluate the average size and polydispersity index or a Coulter N4 (Coulter Electronics, Hialeah, FL, U.S.A.) to evaluate size distribution profile.

In vitro release kinetics were determined with a 1/5 dilution of the primaquine-loaded nanoparticles in an aqueous (PBS: 0.01 M phosphate and 0.15 M NaCl at pH 7.4) or biological medium (heat-inactivated fetal calf serum; HIFCS, Gibco, Paisley, U.K.). The quantity of released primaquine at various time intervals was measured as described.

The molecular weight of the polymer was determined with an HPLC-GPC method (Vansnick et al., 1985). A gel permeation chromatograph (M-45 solvent delivery system, model; U6-K universal LC injector, Waters) fitted with refractive index detector (R-401, differential refractometer, Waters) and UV spectrophotometric detector (M480 LC Spectrophotometer, Waters) coupled for simultaneous double detection were used. The chromatograms were recorded with double simultaneous detection at a wavelength of 334 nm.

Columns of μ-styragel of 100, 500, 1000 and 10000 Å (Waters) were used simultaneously. The eluant was tetrahydrofuran (THF, Carlo Erba, Milano, Italy) at a solvent flow rate of 2 ml/min. The molecular weight calculation was carried out automatically after calibration with poly(ethylene oxide) standards ranging from 106 to 86000 in molecular weight (Polymer Laboratories, Shropshire, U.K.). Nanoparticles or standards were dissolved in a 5‰ concentration. These solutions in THF were filtered through a 0.45 μm filter (Acrodisc CR-PTFE; Gelman Sciences, Ann Arbor, MI, U.S.A.) and 150 μl were injected into the chromatographic system. The chromatograms were registered and the peak surfaces integrated on a printer fitted with GPC calculation capacity (M730 Data module, Waters).

Stability testing

A study of physicochemical stability of freeze-dried nanoparticles, light-protected, in vials closed under vacuum and stocked in a freezer at $-30\,^{\circ}C$, was performed. The free and bound drug were determined as described to evaluate the percentages of recuperation and fixation; the granulometric stability was also determinated by size distribution profiles with a Coulter N4 after redispersion of the colloidal system.

Acute toxicity

A study of acute toxicity by determination of LD_{50} was performed with NMRI mice divided into groups of 10 animals, which were injected in the tail vein with free primaquine, nanoparticle-bound primaquine, equivalent doses of unloaded nanoparticles or the vehicle (0.02 M sodium bicarbonate). LD_{50} was determined by a conventional method (Lichtfield and Wilcoxon, 1949).

Results

Adsorption of primaquine

Preliminary experiments showed that when primaquine is added to the polymerization medium before the monomer, large size particles are formed, suggesting that the loading of primaquine

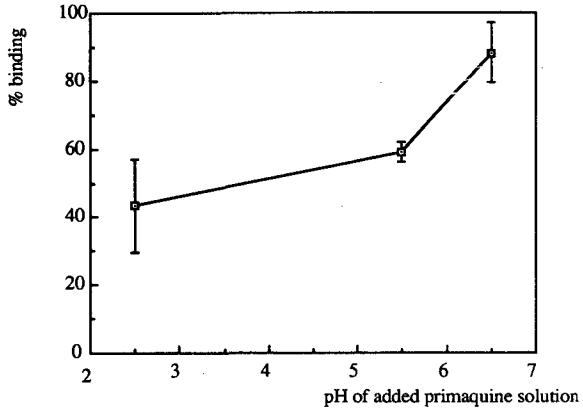

Fig. 1. Influence of pH on the binding of primaquine to the nanoparticles of PIHCA. Primaquine solution at different pH values was added to nanoparticles previously neutralised with NaOH (0.1 N).

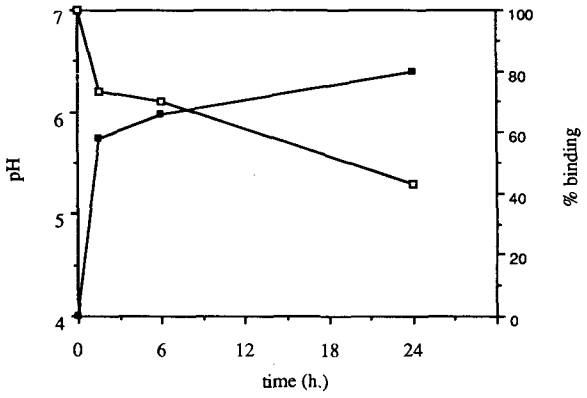

Fig. 2. Relation between variation of pH and drug binding kinetics. (□, pH, ■, % binding). Primaquine at pH 6.5 was added in a concentration of 1 mg/ml, to PIHCA nanoparticles previously neutralized with NaOH (0.1 N).

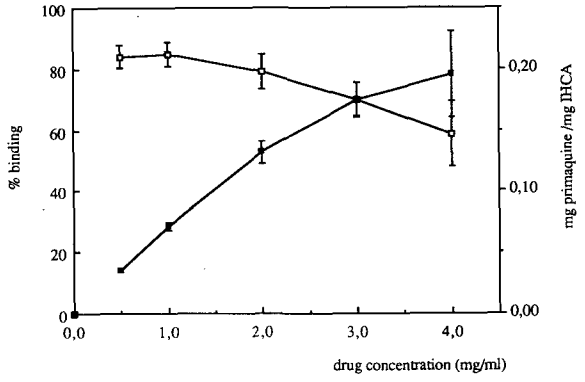

Fig. 3. Influence of primaquine concentration (pH 6.5) on its adsorption to PIHCA nanoparticles in buffered media (phosphate buffer, pH 7.0, 0.001 M) after 2 h adsorption. Results ($n = 5$) are expressed in percentage of binding and ratio between bound primaquine and initial concentration of monomer. (□, % binding; ■, mg primaquine/mg IHCA).

should be carried out by adding the drug after polymerization (data not shown).

Addition of a primaquine solution at different pH values to a neutralised PIHCA suspension showed a significant effect of pH on the binding of drug to the nanoparticles (Fig. 1). A pH values of 6.5 seemed to be appropriate for a high fixation level. By studying the variation of pH in suspension according to the binding kinetics (Fig. 2) it was concluded that the use of a buffer improved the fixation rate.

To a suspension of unloaded nanoparticles of PIHCA freeze-dried and redispersed in phosphate buffer at pH 7.0 (0.001 M) various concentrations of primaquine ranging from 0.5 to 4 mg/ml were added (Fig. 3). The relation between bound drug and polymer concentration was shown to be in accordance with an adsorption procedure with a 'saturation' level.

Concentrations of primaquine from 0.5 to 2 mg/ml resulted in a good degree of adsorption (bound drug of 79–85%) but a concentration of 1 mg/ml always gave a better binding level and faster binding kinetics than did 2 mg/ml (Table 1). The aggregation of nanoparticles observed for a concentration of 2 mg/ml was not a consequence of drug precipitation, as shown by controls of free drug dissolved in the same medium. This might be a consequence of colloidal instability resulting from the binding of drug at such a concentration. The follow-up study of adsorption at 1 mg/ml for 24 h did not yield results differing from the initial data.

TABLE 1

Adsorption kinetics of primaquine at 1 mg/ml (a) and 2 mg/ml (b) (n = 5)

		Adsorption time (h)				
		1/2	1	2	3	4
Binding (%)	(a)	83	84	88	89	89
	(b)	70	74	77	78	80
Size (nm)	(a)	227 ± 83	219 ± 84	226 ± 88	234 ± 89	243 ± 89
	(b)	215 ± 70	216 ± 74	229 ± 77	580 ± 78	1226 ± 80
Polydispersity index	(a)	1	0	0	2	1
	(b)	1	0	1	5	8

The first procedure (Fig. 4, procedure A) resulted in a colloidal dispersion of nanoparticles measuring around 200 nm and in a drug binding level of 81–87% at a drug concentration of 1 mg/ml. However, the redispersion of the final freeze-dried formulation containing bound primaquine was difficult in phosphate buffer.

Therefore, an alternative procedure (Fig. 4, procedure B) promoting contact between drug and polymer in aqueous non-buffered acidic media followed by freeze-drying and redispersion in sodium bicarbonate (0.02 M) yielded a final nanoparticle suspension with around 88% bound drug and unimodal granulometric distribution profile in the range of 200–250 nm. Comparison between both procedures showed no significant differences from the viewpoint of granulometry or binding level. However, primaquine-loaded nanoparticles prepared by procedure B can more easily be freeze-dried and redispersed.

Therefore, procedure B was chosen. It yielded a formulation of PIHCA nanoparticles containing 10 mg drug per 120 mg monomer for each vial, for redispersion in 10 ml of sodium bicarbonate (0.02

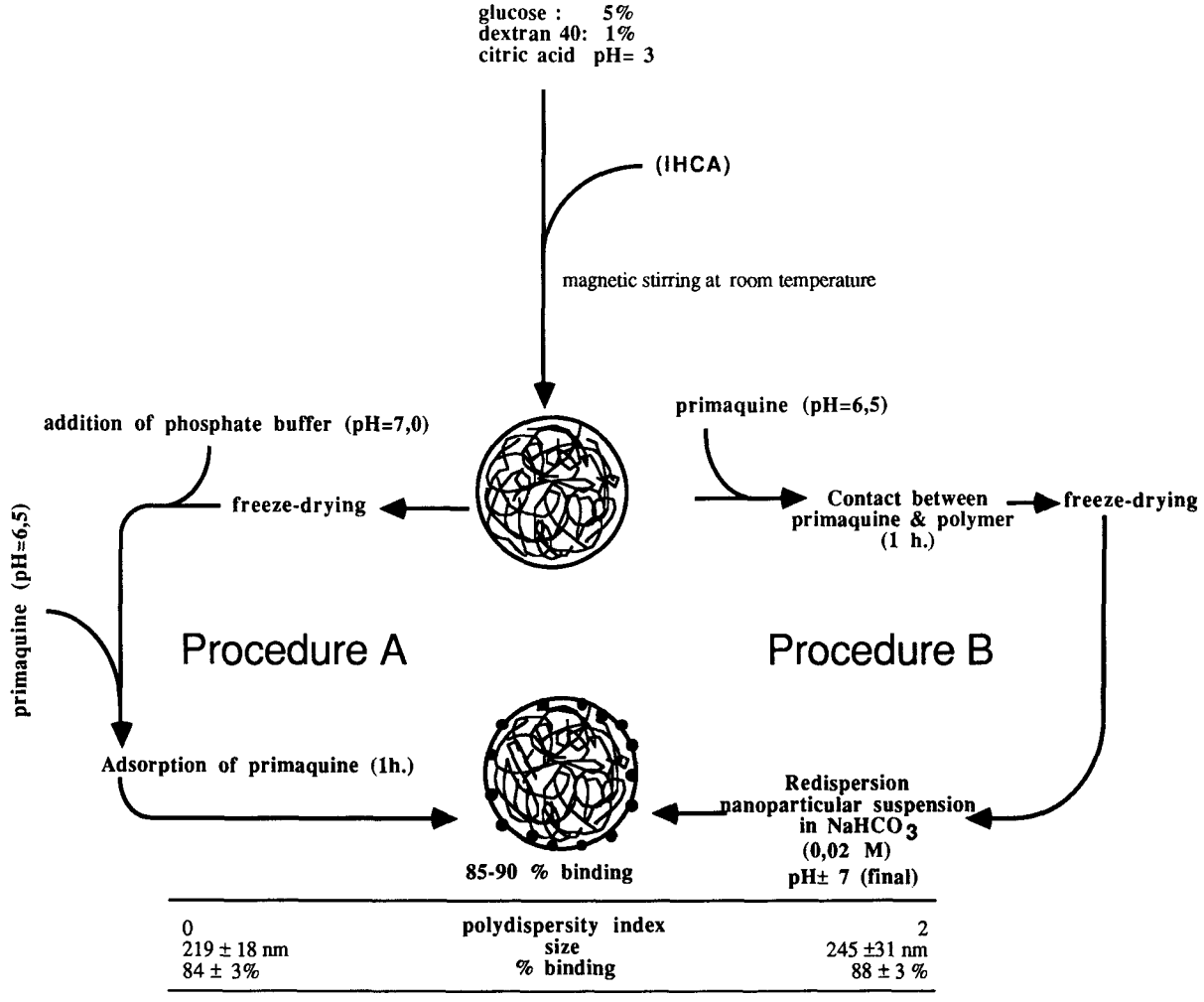

Fig. 4. Schematic representation of procedures used for preparing primaquine-bound nanoparticles ($n = 5$).

TABLE 2

Evolution of the granulometry of primaquine-loaded nanoparticles prepared by procedure B

	Unloaded nanoparticles of PIHCA	Primaquine bound nanoparticles			
		$t=0$	$t=1$ h	$t=2$ h	$t=3$ h
Size (nm)	256	257	238	218	279
Standard deviation	76	64	45	26	72

M). All subsequent experiments were performed with primaquine-loaded nanoparticles prepared by procedure B.

Physico-chemical characterization

The granulometric stability of the colloidal suspension was followed until 3 h after redispersion in sodium bicarbonate (0.02 M) by determining the size distribution profile. No significant variations were observed (Table 2).

The in vitro release kinetics in PBS (pH 7.4) showed a rapid 'burst-like' release of primaquine followed by a slow-release process. In vitro release kinetics in HIFCS showed a considerably different type of release with no burst effect (Fig. 5).

When the primaquine-loaded nanoparticles were dissolved in THF and an HPLC-GPC chromatogram was run using simultaneous double detection (see Materials and Methods), an interaction/association between the polymer and primaquine was shown (Fig. 6), since the polymer

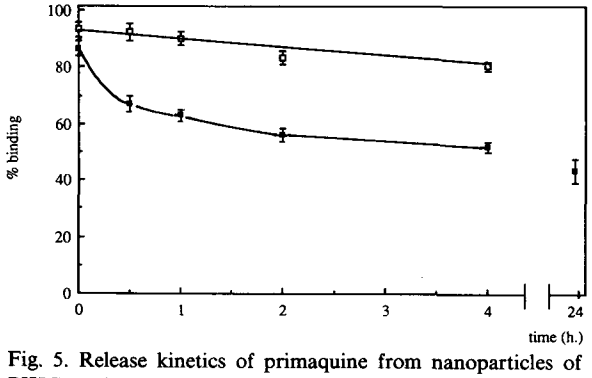

Fig. 5. Release kinetics of primaquine from nanoparticles of PIHCA when incubated (1/5) in (■) PBS (pH 7.4) or (□) HIFCS ($n = 5$).

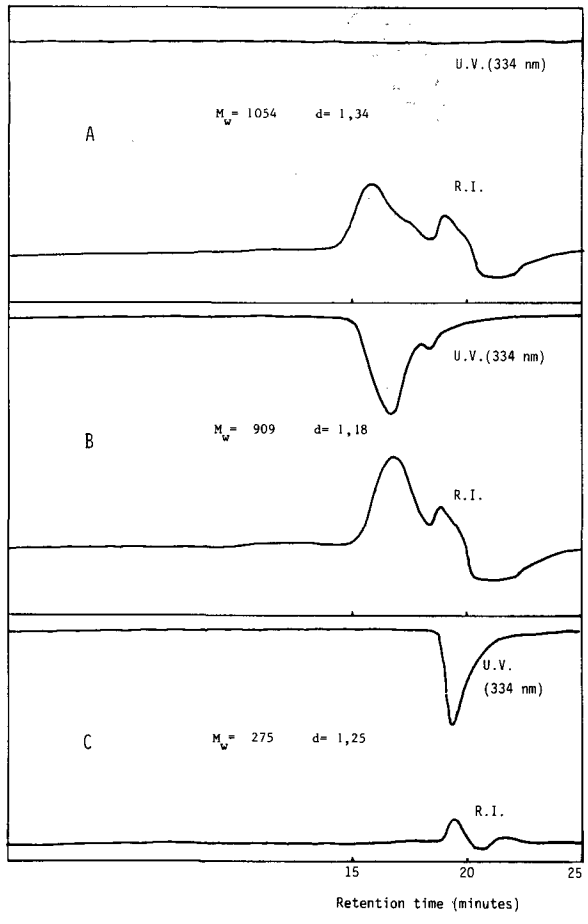

Fig. 6. Chromatograms showing the distribution (d, polydispersity index) of molecular weights (M_w) obtained by HPLC-GPC with double detection (refractive index and UV). (A) Unloaded nanoparticles of PIHCA, (B) primaquine-loaded nanoparticles, (C) free primaquine.

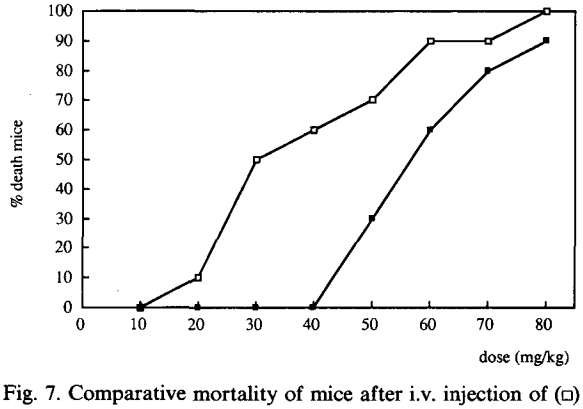

Fig. 7. Comparative mortality of mice after i.v. injection of (□) free primaquine and (■) primaquine-loaded nanoparticles at different doses.

TABLE 3

Physico-chemical stability of freeze-dried primaquine-loaded nanoparticles, stored in freezer (− 30 °C) (results are the average of n = 5 experiments)

Time (months)	Average size (nm)	Recovery (%)	Binding (%)
0	280 ± 97	104.3 ± 7.4	85.3 ± 0.8
1	213 ± 34	100.0 ± 1.5	89.1 ± 0.8
2	224 ± 26	102.0 ± 5.0	91.2 ± 1.2
3	222 ± 40	99.0 ± 9.4	88.4 ± 4.0
4	225 ± 26	99.9 ± 1.5	86.8 ± 0.8
5	194 ± 46	99.9 ± 4.1	86.5 ± 1.7
6	214 ± 33	96.1 ± 3.7	87.5 ± 0.3
9	221 ± 35	98.0 ± 6.3	87.3 ± 2.6
12	202 ± 43	99.9 ± 4.1	88.7 ± 0.4

peak detected via the refractive index had the same retention time as primaquine. Moreover, a chromatogram of primaquine alone indicates a much higher retention time (lower molecular weight) for free primaquine than for primaquine-loaded nanoparticles.

Stability testing

As shown in Table 3, the freeze-dried primaquine-loaded nanoparticles kept in a freezer appeared to be stable, since the granulometric distribution, recovery and binding level did not change.

Acute toxicity

The LD_{50} was significantly different between the free and polymer-coupled drug (33.1 mg/kg for free primaquine vs 57.5 mg/kg for primaquine-loaded nanoparticles). Thus, the binding of drug to PIHCA reduced the acute toxicity of primaquine almost 2-fold (Fig. 7).

Discussion

We developed a formulation of primaquine-loaded nanoparticles to be used against visceral leishmaniasis. Our aim was to target primaquine to the RES (site of action) and therefore reduce its toxicity and increase its anti-leishmanial efficacy.

The dissolution of the drug in the polymerization medium before addition of monomer yielded large particles, which are probably the result of a rapid polymerization. Indeed, primaquine contains a primary amine group, which is known to have a marked catalytic effect upon the polymerization of cyanoacrylates.

Therefore, an adsorption procedure had to be used. It is based mainly on the affinity of the drug to preformed polymeric particles, and is modulated by medium and drug hydrophobicity (Davis et al., 1986), even if reaction between amines and excess monomer cannot be ruled out.

Primaquine binding to the carrier seemed largely dependent on pH. Results obtained from the study of the influence of pH suggest that the reduction of the ionic character of primaquine diphosphate, at higher pH values, increased its affinity to the polymeric particles. The HPLC-GPC chromatogram indicates that the increased drug affinity to the polymer may induce strong affinity or even covalent binding. Such a close association could not result only from hydrogen bonding or another weaker type of bond. Moreover, a plot of adsorption data according to a hypothetical Langmuir adsorption model (fig. 8) showed good accordance of experimental data with the linear form of the Langmuir equation (Florence and Attwood, 1988).

In vitro drug release showed a linear relation in HIFCS which suggests an opsonization-like phenomenon common with colloidal systems in the

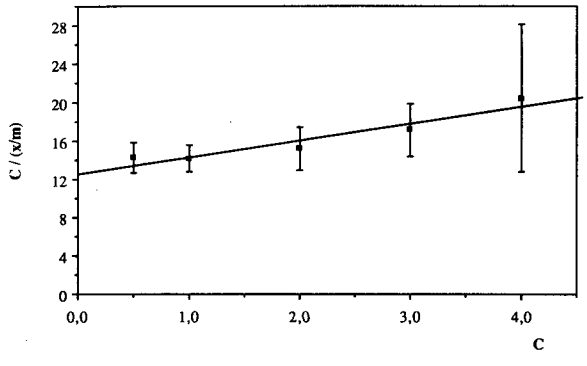

Fig. 8. Plot of data contained in Fig. 3 according to the linear form of the Langmuir equation (c, added primaquine (mg/ml); x, bound primaquine (mg); m, monomer (mg)).

presence of serum proteins (Eldem and Speiser, 1989). The serum could create a diffusion layer around the nanoparticles limiting the type and rate of drug release from the carrier to the surrounding media. Such a conclusion is reinforced when results are compared to in vitro drug release in PBS, where a burst effect was clear, indicating that drug release from the carrier might be a two-step process: first, release of surface-adsorbed drug, followed by a slow release probably resulting from erosion of the polymer. These results for in vitro release contradict the hypothesis of covalent binding but do not eliminate the possible existence of drug adsorption on the nanoparticle surface together with a closer interaction, as suggested by the HPLC-GPC data and the slow-release phase for results in PBS. These results are consistent with adsorption at the surface of the carrier together with drug dispersed in the polymeric matrix.

The decrease in acute toxicity of primaquine-loaded nanoparticles as compared to free primaquine could be explained by the different biodistribution of primaquine induced by the carrier which itself is known to be rapidly cleared in plasma and phagocytosed by the Kupffer and other cells of the RES (Grislain et al., 1983; Lenaerts et al., 1984). This formulation of primaquine-loaded nanoparticles could be useful for drug targeting in experimental chemotherapy of visceral leishmaniasis.

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