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Research paper

Comparison of nanosuspensions and hydroxypropyl-β-cyclodextrin complex of melarsoprol: Pharmacokinetics and tissue distribution in mice

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ABSTRACT

The aim of this work was to develop and compare two formulations of melarsoprol (nanosuspension and hydroxypropyl-β-cyclodextrin inclusion complex). The arsenic concentrations in the organs have been assessed on a mouse model. Since this organoarsenical drug has been proposed for the treatment of cerebral trypanosomiasis and refractory leukaemias, special emphasis has been put on the bone marrow and on the brain.

The organic solution of melarsoprol (Mel B, 0.039 mmol/kg), injected intravenously as control formulation, was found to concentrate significantly in the bone marrow (C_{max} = 1.64 mmol/g), though, not surprisingly, the brain concentration was quite high (C_{max} = 0. 093 mmol/g) and the LD_{50} was 0.12 mmol/kg.The hydroxypropyl-β-cyclodextrin inclusion complex was found to concentrate much more in the brain (C_{max} = 0.25 mmol/g) leading to a higher acute toxicity (i.e., lower LD_{50}; 0.056 mmol/kg). Nevertheless, even if the encephalopathy risk has to be taken in to account, this could be considered as a positive point for the treatment of the cerebral trypanosomiasis, which is the main indication for this drug.On the contrary, the use of nanosuspensions allowed us to reduce the cerebral concentration (C_{max} = 0.02 μmol/g) and the acute toxicity (LD_{50} = 0.25 mmol/kg). Moreover, nanosuspensions, especially those prepared with polxamer 407, preserved a good *in vitro* antileukemic activity (IC_{50} = 3.34 ± 0.33 after 48 h on K562) with high bone marrow concentrations (C_{max} = 1.85 μmol/g). As a consequence this formulation could be proposed for the treatment of refractory leukaemias.

1. Introduction

Human African trypanosomiasis is a slowly progressing fatal disease. In the late stage, the central nervous system is invaded by the parasites, leading, if untreated, to lethal neurological complications [1]. Patients are often treated by melarsoprol (Mel B), which is the only drug commonly available that crosses the blood–brain barrier. The treatment is long and complicated, and is hampered by severe arsenical encephalopathies that limit its use [2]. This organoarsenical compound is also very poorly soluble in water and was dissolved in propylene glycol in the only commercially available solution (Arsobal®, Sanofi Aventis) that exhibits a local intolerability after i.v. injection (severe pains, burns and necrosis). Until now, it is the only organic solvent with a good solubility level and occasionally used intravenously [3].

In 1996, Mel B has also demonstrated an antileukaemic activity [4] on a variety of leukemia cell lines [5] leading to a renewal of interest for organoarsenical As^{III} drugs. Recently, other dithiaarsanes (i.e. arsthinol), have also proved to be effective on K562 and U937 leukemia cell lines [6,7]. Although these compounds are very promising, the lack of solubility remains a major drawback for a final development among modern antileukemic cancer protocols [8].

A lot of research has been going on during the last two decades to developing adequate drug delivery systems for challenging drug candidates which belong to the classes II and IV of the biopharmaceutical classification system [9]. These works gave birth to other techniques used to solve solubility problems associated with poorly soluble drugs [10]. Among them, cyclodextrins, nanoparticles and nanosuspensions raise many hopes.

Cyclodextrins are cyclic oligosaccharides produced from starch bioconversion and have been extensively studied to improve the solubility of lipophilic drugs [11]. We have previously shown that the solubility of melarsoprol was increased 7.2×10^3-fold after complexation with randomly methylated β-cyclodextrin (RAMEβCD) or hydroxypropyl-β-cyclodextrin (HPβCD) [12]. Moreover, the complexation had a pronounced effect on the hydrolysis and the dissolution rate of Mel B.

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However, cyclodextrins should not be considered as simple solubility enhancers, since the formation of inclusion complex might also affect the bioavailability [13,14], the tissue distribution of drugs [15] and thereby therapeutic response.

Nano-sized carriers (i.e. nanoparticles, nanocapsules, nanosuspensions, and nanoemulsions) can readily allow a preferential distribution in the Mononuclear Phagocyte System (MPS) [16]. In the treatment of leukaemias by organoarsenic compounds, this can be considered as a double-advantage since polymeric nanoparticles have been shown to concentrate in the bone marrow [17–19] and hardly diffuse through the blood–brain barrier (BBB) [20] which could possibly limit the risk of encephalopathy. Nevertheless, this could be a drawback in the treatment of the cerebral stage of African trypanosomiasis.

In this work, we try to investigate whether cyclodextrins and nanosuspensions can modify the pharmacokinetics of Mel B. Special emphasis has been put on the bone marrow and on the brain, respectively, responsible for the antileukemic activity and for the trypanocidal activity on the cerebral stage of African trypanosomiasis.

2. Materials and methods

2.1. Materials

Melarsoprol (Mel B) was synthesized according to the method described by Friedheim [7,21,22]. The hydroxypropyl-β-cyclodextrin (HPβCD, degree of substitution: DS = 4.4 hydroxyl units per anhydroglucose unit) was purchased from Sigma–Aldrich (St. Quentin, France). All other chemicals were of analytical grade.

2.2. HPLC assay for melarsoprol determination

Determination of Mel B was carried out by high-performance liquid chromatography (HPLC). Fifty microlitres of sample were injected onto a C18 column (Nucleosil® AB, 5 μm, 0.46 × 25 cm, Macherey-Nagel, Eckbolsheim, France) using an autosampler (WISP 712, waters). The mobile phase was a mixture of acetonitrile and 0.6% acetic acid in water (45/55, v/v) at a flow rate of 1 ml/min (Spectra Physics P1000XR, Thermo Electron S.A., France). Detection was performed by UV spectrophotometry at 286 nm (Spectra Physics, UV 100) and peak surface was used for the quantification of the Mel B. In these analytical conditions, the retention time for Mel B was about 3.82 min and the limit of quantification was 28 μg/l. The external standard curves had excellent linearity from 1 to 20 mg/l (correlation coefficient >0.9994).

2.3. Nanosuspension preparation

In order to produce the Mel B nanosuspensions (NS-Mel B), the method proposed by Müller et al. [23] was applied. Briefly, Mel B powder (0.1–1%, granulometry: 50 ± 28 μm) was dispersed in an aqueous solution containing 1% or 2% Pluronic F-68® (poloxamer 188) or Pluronic F-127® (poloxamer 407) and 0.5% mannitol by using an Ultra Turrax stirrer T25® (Janke and Kunkel, IKA®-Labortechnik) for 1 min at 9500 rpm. The resulting pre-dispersion was then homogenised by an Avestin EmulsiFlex-B3 (Avestin, Canada): 2 cycles at 2.10^4 KPa and 2 cycles at 5.10^4 KPa were applied as premilling. Then, the dispersion was submitted to 15–30 cycles at 15.10^4 KPa to obtain nanosuspensions. As these nanosuspensions were intended for intravenous administration, there was a need to verify and exclude all particles larger than 5 μm by centrifugation (1000g for 10 min, the pellet was discarded). Afterwards, the nanosuspension (i.e. the supernant) was frozen overnight at −20 °C and freeze-dried in a SMH15 freeze-drier (Usifroid, Maurepas, France): the temperature of the sample, coming from the cold chamber, was first equilibrated with the cooling plate at −56 °C during 1 h. Then the total pressure was kept at 5 Pa at 12 °C for 24 h.

2.4. Size determination

Particle-size analysis was performed by photon correlation spectroscopy (PCS; Malvern Zeta master ZEM5000, Malvern instruments, UK). This analysis yields the mean diameter (*z*-average, measuring range: 20–1000 nm) and a polydispersity index (PI) as measure for the extent of the distribution. Additionally, laser diffractometry (LD) was used to detect the larger particles in NS-Mel B formulations (Zetasizer 3000 HS_A, Malvern instruments, UK). The diameters were calculated using the volume size distribution.

2.5. Preparation of the Mel B/HPβCD complex

The melarsoprol/hydroxypropyl-β-cyclodextrin complex (Mel B/HPβCD) was prepared and assessed as previously described [12]. Mel B (1 mmol) was incubated for 14 h in an aqueous solution of HPβCD (2 mmol) under magnetic stirring. Subsequently, the solution was filtered through a 0.22-μm filter and freeze-dried as previously described.

2.6. Drug–plasma protein binding

Heparinized blood samples were collected from 5 mice, centrifuged and pooled to obtain a standardized plasma. Mel B or Mel B/HPβCD (0.1 mg/ml) was incubated in the mouse plasma under magnetic stirring (23 and 37 °C). The drug–protein binding was assessed after ultrafiltration using a Solvent-Resistant Stirred Cell (Millipore) and a cellulose membrane (Sartorius, 10,000 Da). The free-drug concentrations were determined in the ultrafiltrate by HPLC.

The effect of HPβCD on the drug–protein binding was also assessed with increasing amounts of HPβCD: four samples of Mel B (0.1 mg/ml, 23 °C, 5 h) in mouse plasma were tested with 1, 2, 5 and 10 mg/ml of HPβCD.

2.7. In vitro antileukemic activity (K562 and U937 cell lines)

The cytotoxic activity of each formulation was estimated using K562 erythroleukemia and U937 myelomonocytic leukemia cell lines. Briefly, coarse Mel B was dissolved in DMSO leading to a final concentration of DMSO < 1% (blank controls of DMSO did not show any toxicity); NS-Mel was added as a suspension and Mel B/HPβCD was spontaneously dissolved in the culture medium.

Exponential growing cells were seeded into a 96-well plate at a final density of 4.10^4/well using different concentrations of Mel B, NS-Mel B or Mel B/HPβCD (0.01 μM to 1 mM of melarsoprol). Cells were incubated for 2 or 3 days at 37 °C in a humidified 5% CO_2 atmosphere and viabilities were determined using the classical MTT test [24].

2.8. Animal studies

Animal handling procedures were in accordance with the recommendations of the EEC (86/609/CEE) and French National Committee (décret 87/848) for the care and use of laboratory animals.

Mel B was dissolved in a mixture of propylene glycol, sodium chloride 0.9% and DMSO (33:33:33; v/v/v) and Mel B/HPβCD was dissolved in a solution of sodium chloride (0.9%). These intravenous formulations were freshly prepared and injected in the caudal vein to mice, (female CD1, Charles River Laboratories, France).

Table 1
Exposed doses for Mel B, Mel B/HPβCD and NS-Mel B used in the animal experiment

Mel B		NS-Mel B		Mel B/HPβCD	
Groups	Dose (mmol/kg)	Group	Dose (mmol/kg)	Group	Dose (mmol/kg)
M1	0.044	N1	0.079	C 1	0.0192
M2	0.063	N2	0.112	C2	0.027
M3	0.088	N3	0.158	C3	0.038
M4	0.125	N4	0.223	C4	0.054
M5	0.177	N5	0.320	C5	0.076
M6	0.249	N6	0.450	C6	0.108
M7	0.350	N7	0.630	C7	0.0192

2.8.1. Determination of LD_{50}

To provide the LD_{50} of Mel B, Mel B/HPβCD and NS-Mel B, the experiments and its intervals are designed in accordance with the method provided by the Organization for Economic Cooperation and Development (OECD, Table 1).

Mice (female CD1, 24–28 g, Charles River, Saint Germain sur l'Arbresle) were housed in cages and observed throughout the quarantine-period experiments under controlled conditions of 21 ± 1 °C temperature, 50 ± 10% relative humidity and normal photoperiod (12 h dark and light). The first animal was injected at a dose one step below the assumed estimate of the LD_{50}. If the first animal dies, the second animal receives a lower dose. The mortality was assessed at the 96th hour after injecting 0.25 ml of the respective formulation. Control mice were injected with the solvent [propylene glycol, sodium chloride 0.9% and DMSO (33:33:33; v/v/v)] in the same conditions. All animals were observed twice a day throughout the experiment and in a postmortem examination (i.e. body weights, organ weights).

The LD_{50} and 95% profile likehood (PL) are obtained by AOT program (OECD guideline 425).

For more accuracy, the conventional method was also applied to determine the LD_{50}: five mice were used at each dose level (Table 1). Results are together presented for comparison.

2.8.2. Pharmacokinetics studies

Groups of three CD1 female mice (24–28 g) were used. Mel B, Mel B/HPβCD and NS-Mel B were given as a single bolus at a dose of 0.038 mmol/kg expressed in Mel B (0.25 ml). Control mice were injected with the solvent in the same conditions. At scheduled time-points (0.5, 30 min, 1, 5, 8, 18, 24 and 48 h), heparinized blood samples were collected from anaesthetized mice via cardiac puncture. Liver, kidneys and brain were removed, weighted and stored at −20 °C until analysis. Bone marrow was flushed from femur shafts with NaCl 0.9%.

2.9. Quantification of arsenic in the tissues

The tissue concentrations of arsenic were determined using a colorimetric method [25] after digestion with nitric acid (HNO_3; 65%) and hydrogen peroxide (H_2O_2; 3%). In brief, each tissue or blood sample was weighted and placed in a digestion tube with 5 ml of HNO_3 (65%) and 5 ml of H_2O_2 (3%). The tubes were heated with a digester apparatus DK-20 (Velp Scientifica), by slowly increasing the temperature from 100 to 200 °C. The clear solution was then evaporated to dryness, the residue was taken up with 10 ml of HCl (2 N) and introduced into an arsine generator apparatus (European Pharmacopeia). The reaction was initiated by zinc powder after reduction to As^{III} with tin chloride ($SnCl_2$; 40%) and potassium iodide (KI; 15%). After 30 min, the pentavalent arsenic (As^{V}) was completely reduced to arsine (AsH_3) and the gas bubbled through a solution of silver salt of diethyldithiocarbamate in pyridine (0.5 g/100 ml). The absorbance of the brown complex was measured at 525 nm (Cary-50 spectrophotometer, Varian). A calibration curve was obtained with increasing amounts of arsenic (As_2O_3, 0–10 μg). Results were expressed as weight of total arsenic per gram of fresh tissue ± SD. The Mann and Whitney test was used, at the level of $p < 0.05$, to compare assay groups.

Table 2
Sizes of various formulations of nanosuspensions prepared with poloxamer 188 or poloxamer 407

Mel B concentration (%)	Sizes (nm)		
	Poloxamer 188 – 1%	Poloxamer 188 – 2%	Poloxamer 407 – 1%
0.1	380 ± 42	337 ± 38	ND
0.2	427 ± 45	447 ± 56	329 ± 9
0.5	648 ± 174	663 ± 129	ND
1	564 ± 182	560 ± 32	324 ± 88

ND: not determinated.

2.10. Data analysis

Arsenic plasma and organ concentration profiles were analyzed with Kaleidagraph 4.0 (Synergy software). The pharmacokinetics parameters including areas under concentration–time curves (AUC), clearance, terminal elimination half-life t1/2 (β), constant of elimination and volume of distribution were calculated subsequently.

3. Results

3.1. Nanosuspension size determination

In our conditions, no further decrease of size was obtained after 30 cycles at 15.10^4 KPa (size ranging from 324 ± 88 to 663 ± 129 nm; Table 2). Since we have compared various formulations of nanosuspension, it can be observed that the sizes of the particles prepared with poloxamer 188 increased significantly as a function of the Mel B concentrations. The formulations used for the *in vivo* experimentation (poloxamer 188 – 1%/Mel B – 0.2% and poloxamer 407 – 1%/Mel B – 1%) gave, respectively, 324 and 427 nm (i.e. 295 ± 71 and 409 ± 102 nm after lyophilisation).

3.2. Mel B/HPβCD characterization

Cyclodextrins–melarsoprol inclusion complexes have been previously developed in our laboratory to obtain an aqueous solution of melarsoprol, more convenient in clinical use [12]. Both methyl-β-cyclodextrin (Mel B/RAMEβCD) and hydroxypropyl-β-cyclodextrin (Mel B/HPβCD) have exhibited very good apparent stabilities ($50\ 761 \pm 5\ 070\ M^{-1}$ and $57\ 143 \pm 4\ 425\ M^{-1}$, respectively) and the solubility enhancement factor was about 7.2×10^3.

Since the inclusion of the Mel B in a cyclodextrin may affect the Mel B-protein interactions [44] and consequently its pharmacokinetics, the influence of the cyclodextrin on the drug–protein binding was evaluated. In the first part of the study, Mel B or Mel B/HPβCD was incubated in mouse plasma at 23 and 37 °C. The binding rate was lower for Mel B/HPβCD than for Mel B whatever the temperature was (Table 3).

Table 3
Protein binding of melarsoprol (Mel B) after 30 min and 5 h of incubation of Mel B and Mel B/HPβCD in mouse plasma – mean values ($n = 3$) ± SD

	Mel B		Mel B/HPβCD	
	Protein binding after 30 min (%)	Protein binding after after 5 h (%)	Protein binding after 30 min (%)	Protein binding after after 5 h (%)
23 °C	72 ± 3	82 ± 2	61 ± 2	78 ± 2
37 °C	81 ± 5	92 ± 3	75 ± 3	89 ± 2

Table 4
Effect of hydroxypropyl-β-cyclodextrin (HPβCD) on the plasma protein binding of melarsoprol (Mel B, 23 °C, 5 h) – melarsoprol total concentration: 0.1 mg/ml – mean values (n = 3) ± SD

HPβCD concentrations (mg/ml)	Unbound fraction of Mel B (%)
–	32 ± 1
1	50 ± 4
2	60 ± 2
5	63 ± 2
10	63 ± 3

In the second part of the study, the binding of melarsoprol, in mixed solutions Mel B/HPβCD/plasma, decreased considerably when the HPβCD concentration was increased (Table 4).

3.3. In vitro antileukemic activity

We examined the cytotoxicity effect of Mel B, NS-Mel B and Mel B/HPβCD on the human cell lines K562 and U937 by incubating with different concentrations of melarsoprol for 24 and 72 h. Cytotoxicity was determined by a MTT assay and Mel B was used as reference for the two other formulations. The IC_{50} (Table 5) values clearly showed that the NS-Mel B and the Mel B/HPβCD have kept a good antileukemic activity. Nevertheless, as compared with Mel B, a significant reduction of cytotoxicity after exposure of K562 cell line to Mel B/HPβCD was observed. To a lesser extent, a reduction of cytotoxicity was also observed after exposure to NS-Mel B.

Table 5
Cytotoxicity parameters of free melarsoprol (Mel B), melarsoprol nanosuspensions (NS-Mel B) and a melarsoprol–cyclodextrin inclusion complex (Mel B/HPβCD) on human cancer cell lines K562 and U937 (MTT method, mean ± SD, n = 3)

	Mel B		NS-Mel B		Mel B/HPβCD	
	48 h	72 h	48 h	72 h	48 h	72 h
IC_{50} (μM) K562	2.24 ± 0.08	2.65 ± 0.05	3.34 ± 0.33*	2.73 ± 0.13	16.6 ± 1.2*	9.3 ± 1.2*
IC_{50} (μM) U937	2.09 ± 0.08	2.40 ± 0.02	2.72 ± 0.40	3.33 ± 0.89*	11.6 ± 3.0*	8.9 ± 7.2*

* $P < 0.01$ vs. free melarsoprol (Mel B).

Table 6
Lethality obtained after injection of seven groups of 5 mice (Mel B/HPβCD, Mel B, or NS-Mel B)

Mel B group		NS-Mel B group		Mel B/HPβCD group	
Dose (mmol/kg)	Lethality	Dose (mmol/kg)	Lethality	Dose (mmol/kg)	Lethality
0.044	0	0.079	0	0.0192	0
0.063	0	0.112	0	0.027	0
0.088	1	0.158	0	0.038	2
0.125	3	0.223	2	0.054	2
0.177	5	0.320	4	0.076	4
0.249	5	0.450	5	0.108	5
0.350	5	0.630	5	0.152	5

3.4. LD_{50}

To determine LD_{50} of each formulation, experimental animals are dosed at different levels (Table 1). After injection, the mortality was observed and recorded in AOT425 program (AOT425). As significant differences were not achieved due to the small sample number of mice, a conventional method with 5 mice per group was also used and lethalities are presented in Table 6. Results are together presented for comparison (Table 7). For both methods, the Mel B/HPβCD was more toxic than Mel B and nanosuspensions (NS-Mel B) were less toxic than Mel B.

Symptoms exhibited by the experimental animals after injection were observed during the experimental period. In every group, mice fell into coma before dying. No other symptom of poisoning was observed.

3.5. Pharmacokinetics

The pharmacokinetics of arsenic was best described by a two-compartment model and parameters are presented in Table 8. Whatever the formulation, the apparent drug volume of distribution is relatively large (186 ± 38–463 ± 60 ml for a mouse) and consistent with very high tissue concentrations, especially in the bone marrow (Fig. 1a).

Nevertheless, significant differences between these formulations can be pointed out. Compared with Mel B, the NS-Mel B formulation tends to accumulate in the liver (C_{max} = 0.33 and 0.17 vs. 0.038 μmol/g, Fig. 1b) and the brain concentrations are very low (C_{max} = 0.026 and 0.018 vs. 0.093 μmol/g, Fig. 1c). On the contrary, the Mel B/HPβCD tends to accumulate in the brain (C_{max} = 0.25 vs. 0.093 mmol/g, Fig. 1c) and the bone marrow (C_{max} = 4.09 vs. 1.64 μmol/g, Fig. 1b) and the phase of elimination is shorter (T1/2(β) = 2.6 ± 0.4 vs. 9.1 ± 5.7, Table 8). Differences are less pronounced in the kidney (Fig. 1d).

4. Discussion

4.1. Mel B

The pharmacokinetics of Mel B has been first studied by Cristau et al. [26] on rodent models. These authors have verified by quantitative determination of arsenic in bile, the hypothesis previously

Table 8
Pharmacokinetics parameters (Mean ± SD) of arsenic after i.v. administration of Mel B and Mel/HPβCD (melarsoprol: 15 mg/kg)

Parameters	Mel	NS-Mel B (poloxamer 188)	NS-Mel B (poloxamer 407)	Mel/HPβCD
AUC (μmol h/ml)	0.035 ± 0.014	0.060 ± 0.041	0.147 ± 0.011	0.019 ± 0.004
C_0 (μmol/ml)	0.009 ± 0.003	0.009 ± 0.006	0.012 ± 0.011	0.017 ± 0.001
Cl_{tot} (ml/h)	29 ± 12	20 ± 10	6.4 ± 4.3	50 ± 89
K_{el} (h^{-1})	0.094 ± 0.059	0.044 ± 0.023	0.026 ± 0.004	0.272 ± 0.050
$V_d\beta$ (ml)	338 ± 88	463 ± 60	251 ± 35	186 ± 38
$T_{1/2}(\beta)$ (h)	9.1 ± 5.7	20 ± 13	27 ± 4	2.6 ± 0.4

Table 7
LD_{50} obtained from the AOT425 software and from the conventional method

	Mel B group		NS-Mel B group		Mel B/HPβCD group	
	AOT425 software	Conventional method	AOT425 software		AOT425 software	Conventional method
LD_{50} (mmol/kg)	0.125	0.117	0.223	0.251	0.054	0.056
95% Confidence intervals (mmol/kg)	0.072–0.192	0.112–0.122	0.163–0.533	0.228–0.273	0.031–0.082	0.045–0.067

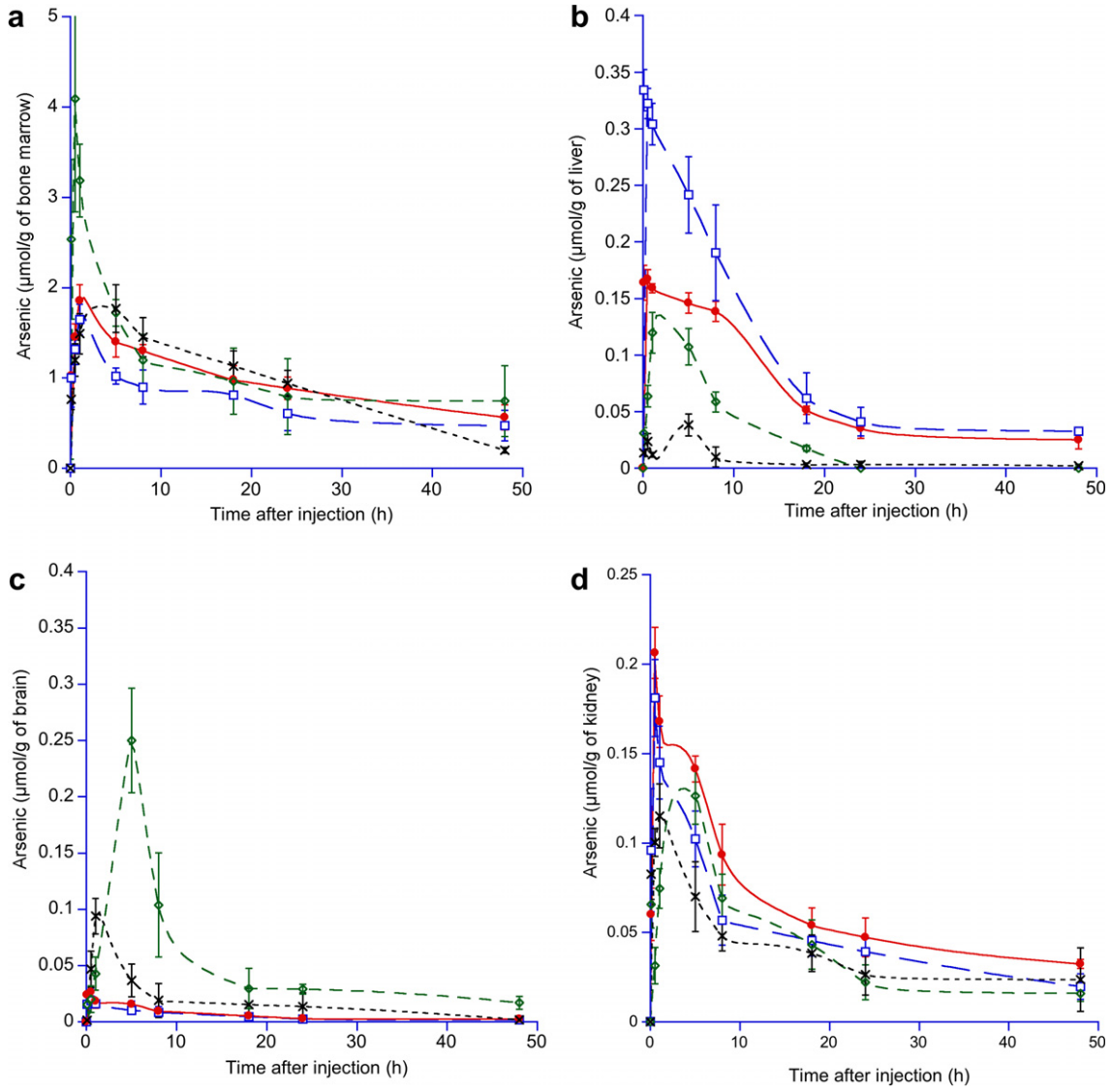

Fig. 1. Changes of concentration of melarsoprol (Mel B) as a function of time following i.v. administration of 0.038 mmol/kg (expressed in Mel B) of Mel B (-X-), Mel B/HPβCD (-◇-), NS-Mel B (poloxamer 188; -□-) and NS-Mel B (poloxamer 407; -●-). Each data-point represents the mean ± SD of the concentrations of total arsenic contained in the organs of three mice, as expressed in µg/g of fresh tissue: (a) bone marrow, (b) liver, (c) brain, and (d) kidney.

expressed about the privilege biliary elimination of some organoarsenic drugs: melarsoprol and melarsonyl. More recently, Burri et al. [27] have demonstrated that the elimination in human serum was biphasic, with a pronounced beta 1 phase. The mean terminal elimination half-life of Mel B was about 35 h, the volume of distribution was about 100 l and the total clearance was about 50 ml/min.

We have previously demonstrated that non-transformed Mel B is detected by HPLC only during the first minutes after injection [28]. This behaviour is due to a rapid hydrolysis of the dithiarsolane into the melarsen oxide [29,30]. Subsequently, in the trypanosoma, melarsen oxide forms a stable adduct with the trypanothione [31], leading to a competitive inhibition of the flavoprotein trypanothione reductase [32] in the glycolysis pathways. Besides, the antileukemic activity is mainly due to the fixation of arsenic metabolites on numerous proteins, especially apoptosis proteins [33]. The plasmatic binding of melarsoprol was quantified by Keiser et al. [29] and a total serum drug–protein binding of 79% was found.

Overall, Mel B is very lipophilic (log $P = 2.53$) and its accumulation in the nervous system has been attested. Indeed, very high concentrations of arsenic were found in the spinal cord, contrasting with undetectable levels in peripheral nerves in a patient who died after melarsoprol overdose [34]. In our experiments, the concentrations of the brain ($C_{max} = 0.093$ µmol/g of brain, Fig. 1c) were higher than those of the liver ($C_{max} = 0.038$ µmol/g of liver, Fig. 1b). Nevertheless, the major affinity has to be attributed to the bone marrow ($C_{max} = 1.64$ µmol/g of bone marrow, Fig. 1a), irrespective of the formulation administered.

4.2. NS-Mel B

In our best conditions (poloxamer 188 – 1%/Mel B), the Emulsiflex-B3 allows us to produce nanospensions of 324 ± 88 nm (Table 2). As melarsoprol is known to be very sensitive to water [36,37] this operation is necessary to preserve NS-Mel B from hydrolysis.

Since nanoparticles are likely to agglomerate during the lyophilisation process [35], particle sizing was performed immediately after freeze-drying. No significant reduction of the size was observed (295 ± 71 nm); this allowed us to exclude any aggregation. However, this preparation has to be administered immediately after reconstitution to limit hydrolysis and formation of melarsen oxide.

In vitro, as compared with Mel B, the significant albeit slight reduction of cytotoxicity after 48-h exposure of K562 cell line to NS-Mel B could be explained by a slow release of Mel B in the incubation medium, leading to a delayed formation of the active metabolite, melarsenoxide (Table 5). However, after a 72-h incubation, the IC_{50} was not different for K562 cells but remained higher for U937 cells despite both cell lines responding similarly to free Mel B. Therefore, these contradictory behaviours between both cell lines remain to be more deeply investigated.

In vivo, the ability of solid particles to reach specific tissue sites is severely limited by rapid clearance from the circulation by phagocytic cells of the MPS. Indeed, as previously described, nanoparticles were rapidly cleared from the blood stream after intravenous administration and were concentrated mainly in the MPS [16]. Actually, the Fig. 1b shows that the liver concentrations observed after injection of NS-Mel B were 5- to 9-fold higher than after injection of the free drug, confirming the preferential distribution in the MPS.

Surprisingly enough, the Fig. 1a shows that the C_{max} of NS-Mel B was similar to that of the free drug in solution. Consequently, no improvement in bone marrow distribution was obtained by the use of nanosuspensions, despite appropriate sizes and the use of poloxamer 407.

In fact, nanoparticles sized about 200 nm are known to concentrate in the mouse bone marrow [19]. In the rabbit, it has been shown that colloidal particulates (150 nm and below in diameter) could be redirected specifically to the bone marrow by coating their surface with the block co-polymer poloxamer 407 [38]. However, among three different commercially available batches of poloxamer 407, only one was found to be capable of directing polystyrene particles (60–150 nm) to sinus wall [39]. In this study, a range of varying bimodal molecular weight distribution profiles were observed following GPC analysis of the same poloxamer type from each different supplier. Hence, this homing process is strongly correlated with surface properties and apparently exclusive to the rabbit.

Due to their too large sizes (>50 nm), nanoparticulate systems are known to be unable to diffuse through the blood–brain barrier. Nevertheless, polysorbate 80-coated polyisobutylcyanoacrylate nanoparticles were successfully used for the *in vivo* administration of drugs to the brain [40]. As the mechanism of action, it was first hypothesized that the polysorbate-coated nanoparticles were transported across the BBB via endocytosis by the brain capillary endothelial cells [41]. An alternative explanation was in favour of a nanoparticle-induced nonspecific permeabilization [42].

Generally, the chemical structure of the overcoating surfactant is of paramount importance, because only polysorbates, not poloxamers (184, 188, 388 or 407), poloxamine 908, CREMOPHOR® (EZ or RH) or polyoxyethylene (23)-laurylether, led to a CNS pharmacological effect of dalargin [43]. The Fig. 1c shows that, after injection of NS-Mel B, the brain concentrations were 3- to 5-fold lower than after injection of the solution. These results confirm the assumption that poloxamers do not facilitate the passage through the BBB. As generally observed for colloidal systems [44], less NS-Mel B accumulates in the kidney and the concentrations remain low (Fig. 1d).

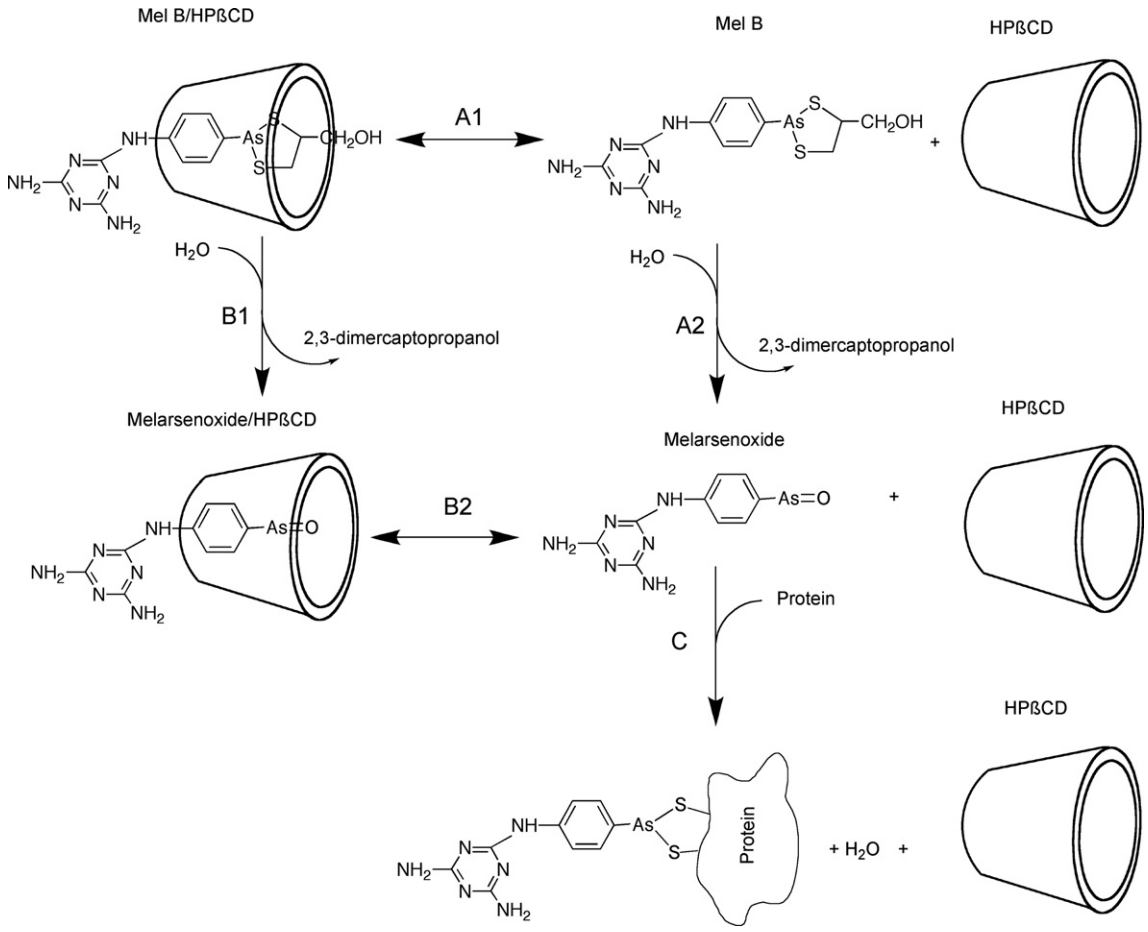

Fig. 2. Schematic reaction for dissociation and hydrolysis of melarsoprol/cyclodextrin inclusion complexes (Mel B/HPβCD). A1 and B2: dissociation – B1 and A2: hydrolysis – C: protein binding.

Finally, the lower concentrations in the brain were well-correlated with a lower acute toxicity (i.e. higher LD_{50}; 0.25 mmol/kg), as compared to Mel B (i.e. lower LD_{50}; 0.12 mmol/kg), confirming that the major cause of animal deaths is due to the arsenical encephalopathy.

4.3. Mel B/HPβCD

Cyclodextrins–melarsoprol inclusion complexes have been previously developed in our laboratory to obtain an aqueous solution of melarsoprol, more convenient in clinical use [12]. It was demonstrated that complexation of Mel B by RAMEβCD did not impair its cytotoxic properties. The slight reduction of activity was explained by the protection of Mel B from hydrolysis by its complexation and the consequent slower transformation in its active metabolite, melarsenoxide.

We have now confirmed that there is a competition between the HPβCD and the proteins to bind the drug by adding increasing amounts of HPβCD in mouse plasma with 0.1 mg/ml of Mel B (Table 4). The interaction between these different mechanisms (Fig. 2) is complex and hardly predictable; the first process is the dissociation of the complex (Fig. 2 A1 and B2) and the second process is the hydrolysis of the Mel B (Fig. 2 A2 and B1). The free reactive group (As = O) of melarsenoxide can, afterwards, bind the protein sulfhydryl groups (Fig. 2 C).

After injection of Mel/HPβCD, the pharmacokinetic parameters were significantly modified (Table 8) and the concentrations in all the organs (Fig. 1) were higher than those after injection of the free drug. This result was similar to that described for flurbiprofen complexed with HPβCD [45]. The higher concentration in the lipophilic tissues (i.e. brain and bone marrow) that we observed might seem illogical if HPβCD is considered as a simple hydrophilic drug. In fact, as suggested by Frijlink et al. [45], the cyclodextrins have to be regarded as drug carriers which are able to deliver the drug to biological membranes in a more efficient way than the plasma proteins. Consequently, the tissues were described as a «sink» causing dissociation of the complex based on simple mass action principle [46].

On the contrary, the free drug, dissolved in organic solvents, could be suspected to precipitate in the bloodstream after injection by simple dilution process, causing thrombophlebilis and likely limiting contact between the drug and the biological membranes. The use of cyclodextrins may be most relevant for poorly soluble drug administered at a site where dilution is not sufficient to avoid a precipitation.

Finally and as a consequence of the pharmacokinetic behaviour, the acute toxicity (LD_{50}, Table 7) of the Mel/HPβCD, strongly related to its cerebral toxicity, is higher (LD_{50} = 0.056 mmol/kg) than that of the free drug (LD_{50} = 0.117 mmol/kg). Nevertheless, this could be considered as a positive point for late-stage trypanosomiasis treatments.

5. Conclusion

Both formulations (i.e. Mel B/HPβCD and NS-Mel B) are essentially aqueous and thus, suitable for clinical use. The pains and necrosis occurring after i.v. injection of Arsobal®, mostly related to the propylene glycol, should be suppressed.

Additionally, our results demonstrate that these formulations can have complementary albeit opposed advantages. The administration of nanosuspension formulation of Mel B could limit access to the central nervous system, and thus, decrease the acute toxicity of this organoarsenical compound. On the contrary, the administration of cyclodextrin complexes of Mel B could concentrate the drug in the brain.

Therefore, it could be proposed to use Mel B/HPβCD, as a better tolerated and optimized treatment of the cerebral African trypanosomiasis whereas NS-Mel B could be more adapted for the treatment of refractory leukaemias. The latter indication could be extended to other cancer diseases, since melarsoprol is known to be efficient on numerous cell lines [47]. Unfortunately, until now, its use has been limited by its cerebral toxicity. Works are planned in our laboratory to compare the efficacy and the tolerance of NS-Mel B vs. free Mel B on tumor-bearing animals.

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References

[1] P.G. Kennedy, Human African trypanosomiasis-neurological aspects, J. Neurol. 253 (2006) 411–416.
[2] J. Pepin, F. Milord, C. Guern, B. Mpia, L. Ethier, D. Mansinsa, Trial of prednisolone for prevention of melarsoprol-induced encephalopathy in gambiense sleeping sickness, Lancet 1 (1989) 1246–1250.
[3] E.A.H. Friedheim, Propylene glycol solution of arsenic medicaments, U.S. Patent 2,593,434 (1952).
[4] F.W. Jennings, G.M. Urquhart, P.K. Murray, B.M. Miller, The use of 2-substituted 5-nitroimidazoles in the treatment of chronic murine *Trypanosoma* brucei infections with central nervous system involvement, Z. Parasitenkd. 70 (1984) 691–697.
[5] S.L. Soignet, W.P. Tong, S. Hirschfeld, R.P. Warrell Jr., Clinical study of an organic arsenical melarsoprol in patients with advanced leukemia, Cancer Chemother. Pharmacol. 44 (1999) 417–421.
[6] A. Astier, S. Gibaud, Use of organoarsenic or organoantimony derivatives for their anticancer activities, WO Patent 2005034935 (2005).
[7] S. Gibaud, R. Alfonsi, P. Mutzenhardt, I. Fries, A. Astier, (2-phenyl-[1,3,2]dithiarsolan-4-yl)-methanol derivatives show in vitro antileukemic activity, J. Organometallic Chem. 691 (2006) 1081–1084.
[8] S. Gibaud, A. Astier, Organoarsenicals derived from 2-phenyl-[1,3,2]dithiarsolan-4-yl)-methanol (AsIII) with antileukaemic properties: from trypanosomicides to anticancer drugs, Ann. Pharm. Fr. 65 (2007) 162–168.
[9] G.L. Amidon, H. Lennernas, V.P. Shah, J.R. Crison, A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability, Pharm. Res. 12 (1995) 413–420.
[10] B. Bittner, R.J. Mountfield, Intravenous administration of poorly soluble new drug entities in early drug discovery: the potential impact of formulation on pharmacokinetic parameters, Curr. Opin. Drug Discov. Dev. 5 (2002) 59–71.
[11] J. Szejtli, Cyclodextrin technology, Kluwer Academic Publishers, Dordrecht, Netherlands, 1988.
[12] S. Gibaud, S. Ben Zirar, P. Mutzenhardt, I. Fries, A. Astier, Melarsoprol–cyclodextrins inclusion complexes, Int. J. Pharm. 306 (2005) 107–121.
[13] M.F. Wempe, V.J. Wacher, K.M. Ruble, M.G. Ramsey, K.J. Edgar, N.L. Buchanan, C.M. Buchanan, Pharmacokinetics of raloxifene in male Wistar–Hannover rats: influence of complexation with hydroxybutenyl-beta-cyclodextrin, Int. J. Pharm. 346 (2008) 25–37.
[14] C.M. Buchanan, N.L. Buchanan, K.J. Edgar, J.L. Little, M.G. Ramsey, K.M. Ruble, V.J. Wacher, M.F. Wempe, Pharmacokinetics of saquinavir after intravenous and oral dosing of saquinavir: hydroxybutenyl-beta-cyclodextrin formulations, Biomacromolecules 9 (2008) 305–313.
[15] P.Y. Grosse, F. Bressolle, P. Rouanet, J.M. Joulia, F. Pinguet, Methyl-beta-cyclodextrin and doxorubicin pharmacokinetics and tissue concentrations following bolus injection of these drugs alone or together in the rabbit, Int. J. Pharm. 180 (1999) 215–223.
[16] L. Grislain, P. Couvreur, V. Lenaerts, M. Roland, D. Deprez-Decampeneere, P. Speiser, Pharmacokinetics and distribution of biodegradable drug-carriers, Int. J. Pharm. 15 (1983) 335–345.
[17] S. Gibaud, C. Weingarten, J.P. Andreux, P. Couvreur, Ciblage de la moelle osseuse à l'aide de nanoparticules de polyalkylcyanoacrylate, Ann. Pharm. Fr. 57 (1999) 324–331.
[18] S. Gibaud, M. Demoy, J.P. Andreux, C. Weingarten, B. Gouritin, P. Couvreur, Cells involved in the capture of nanoparticles in hematopoietic organs, J. Pharm. Sci. 85 (1996) 944–950.
[19] S. Gibaud, J.P. Andreux, C. Weingarten, M. Renard, P. Couvreur, Increased bone marrow toxicity of doxorubicin bound to nanoparticles, Eur. J. Cancer 6 (1994) 820–826.
[20] J.C. Olivier, Drug transport to brain with targeted nanoparticles, NeuroRx 2 (2005) 108–119.
[21] E.A.H. Friedheim, Substituted [1,3,5,-triazinyl-(6)]-aminophenyl-arsonic acids and process for manufacture of same, U.S. Patent 2,295,574 (1942).

[22] E.A.H. Friedheim, Triazine organometallic compounds and process for preparing same, U.S. Patent 2,659,723 (1953).
[23] R.H. Müller, R. Becker, B. Kruss, K. Peters, Pharmaceutical nanosuspensions for medicament administration as systems with increased saturation solubility and rate of solution, U.S. Patent 5,858,410 (1999).
[24] M.B. Hansen, S.E. Nielsen, K. Berg, Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill, J. Immunol. Methods 119 (1989) 203–210.
[25] S.C. Elliot, B.R. Loper, Improved absorption tube for arsenic determinations, Anal. Chem. 46 (1974) 2256–2257.
[26] B. Cristau, M. Placidi, Routes and kinetics of arsenic elimination in rats after administration of organoarsenic drugs. IV. Biliary excretion of melarsoprol, melarsonyl and related compounds, Med. Trop. 32 (1972) 477–482.
[27] C. Burri, T. Baltz, C. Giroud, F. Doua, H.A. Welker, R. Brun, Pharmacokinetic properties of the trypanocidal drug melarsoprol, Chemotherapy 39 (1993) 225–234.
[28] S. Ben Zirar, S. Gibaud, A. Camut, A. Astier, Pharmacokinetics and tissue distribution of the antileukemic organoarsenicals arsthinol and melarsoprol in mice, J. Organometallic Chem. 692 (2007) 1348–1352.
[29] J. Keiser, C. Burri, Physico-chemical properties of the trypanocidal drug melarsoprol, Acta Trop. 74 (2000) 101–104.
[30] C.F. Hiskey, F.F. Cantwell, Ultraviolet spectrum correlations with the conjugate acid–base species of acetarsone and arsthinol, J. Pharm. Sci. 57 (1968) 2105–2111.
[31] A.H. Fairlamb, G.B. Henderson, A. Cerami, Trypanothione is the primary target for arsenical drugs against African trypanosomes, Proc. Natl. Acad. Sci. USA 86 (1989) 2607–2611.
[32] M.L. Cunningham, M.J. Zvelebil, A.H. Fairlamb, Mechanism of inhibition of trypanothione reductase and glutathione reductase by trivalent organic arsenicals, Eur. J. Biochem. 221 (1994) 285–295.
[33] Y. Akao, H. Mizoguchi, S. Kojima, T. Naoe, N. Ohishi, K. Yagi, Arsenic induces apoptosis in B-cell leukaemic cell lines in vitro: activation of caspases and down-regulation of Bcl-2 protein, Br. J. Haematol. 102 (1998) 1055–1060.
[34] R.K. Gherardi, P. Chariot, M. Vanderstigel, D. Malapert, J. Verroust, A. Astier, C. Brun-Buisson, A. Schaeffer, Organic arsenic-induced guillain-barre-like syndrome due to melarsoprol: a clinical, electrophysiological, and pathological study, Muscle Nerve 13 (1990) 637–645.
[35] A. Ain-Ai, P.K. Gupta, Effect of arginine hydrochloride and hydroxypropyl cellulose as stabilizers on the physical stability of high drug loading nanosuspensions of a poorly soluble compound, Int. J. Pharm. 351 (2008) 282–288.
[36] B.J. Berger, A.H. Fairlamb, Properties of melarsamine hydrochloride (Cymelarsan) in aqueous solution, Antimicrob. Agents Chemother. 38 (1994) 1298–1302.
[37] O. Ericsson, E.K. Schweda, U. Bronner, L. Rombo, M. Friden, L.L. Gustafsson, Determination of melarsoprol in biological fluids by high-performance liquid chromatography and characterisation of two stereoisomers by nuclear magnetic resonance spectroscopy, J. Chromatogr. B 690 (1997) 243–251.
[38] C.J. Porter, S.M. Moghimi, L. Illum, S.S. Davis, The polyoxyethylene/polyoxypropylene block co-polymer poloxamer-407 selectively redirects intravenously injected microspheres to sinusoidal endothelial cells of rabbit bone marrow, FEBS Lett. 305 (1992) 62–66.
[39] S.M. Moghimi, A.C. Hunter, J.C. Murray, Long-circulating and target-specific nanoparticles: theory to practice, Pharmacol. Rev. 53 (2001) 283–318.
[40] J. Kreuter, R.N. Alyautdin, D.A. Kharkevich, A.A. Ivanov, Passage of peptides through the blood–brain barrier with colloidal polymer particles (nanoparticles), Brain Res. 674 (1995) 171–174.
[41] J. Kreuter, Nanoparticulate systems for brain delivery of drugs, Adv. Drug Deliv. Rev. 47 (2001) 65–81.
[42] J.C. Olivier, L. Fenart, R. Chauvet, C. Pariat, R. Cecchelli, W. Couet, Indirect evidence that drug brain targeting using polysorbate 80-coated polybutylcyanoacrylate nanoparticles is related to toxicity, Pharm. Res. 16 (1999) 1836–1842.
[43] J. Kreuter, V.E. Petrov, D.A. Kharkevich, R.N. Alyautdin, Influence of the type of surfactant on the analgesic effects induced by the peptide dalargin after its delivery across the blood–brain barrier using surfactant-coated nanoparticles, J. Control. Release 49 (1997) 81–87.
[44] Y. Xi-Xiao, C. Jan-Hai, L. Shi-Ting, G. Dan, Z. Xv-Xin, Polybutylcyanoacrylate nanoparticles as a carrier for mitomycin C in rabbits bearing VX2-liver tumor, Regul. Toxicol. Pharmacol. 46 (2006) 211–217.
[45] H.W. Frijlink, E.J. Franssen, A.C. Eissens, R. Oosting, C.F. Lerk, D.K. Meijer, The effects of cyclodextrins on the disposition of intravenously injected drugs in the rat, Pharm. Res. 8 (1991) 380–384.
[46] V.J. Stella, V.M. Rao, E.A. Zannou, V.V. Zia, Mechanisms of drug release from cyclodextrin complexes, Adv. Drug Deliv. Rev. 36 (1999) 3–16.
[47] K. Koshiuka, E. Elstner, E. Williamson, J.W. Said, Y. Tada, H.P. Koeffler, Novel therapeutic approach: organic arsenical melarsoprol alone or with all-*trans*-retinoic acid markedly inhibit growth of human breast and prostate cancer cells in vitro and in vivo, Br. J. Cancer 82 (2000) 452–458.