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In vivo distribution of arsonoliposomes: Effect of vesicle lipid composition

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Abstract

Sonicated arsonoliposomes were prepared using arsonolipid with palmitic acid acyl chain (C16), mixed with 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC-based), and cholesterol (Chol) with a molar ratio C16/DSPC/Chol 8:12:10. PEG-lipid (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine conjugated to polyethylenoglycol 2000) containing vesicles (Pegylated-arsonoliposomes) were also prepared. DSPC-based and Pegylated-arsonoliposomes, were administered by intraperitoneal injection in balb/c mice (15 mg arsenic/kg) and the distribution of As in the organs was measured by atomic absorption spectroscopy.

Results demonstrate that a high portion of the dose administered is rapidly excreted since 1 h post-injection only about 30–40% of the dose was detected cumulatively in animal tissues. After this, the whole body elimination of arsenic was a slow process with a half-life of 27.6 h for Pegylated-arsonoliposomes, and 83 h, for the DSPC-based ones. For both arsonoliposomes, arsenic distribution was greater in intestines, followed by liver, carcass + skin stomach, spleen, kidney, lung and heart. Different arsenic kinetics in blood between the two liposome types were observed.

Compared to the results obtained previously with PC-based arsonoliposomes, both the DSPC-based and Pegylated-arsonoliposomes have better bioavailability. This proves that arsonoliposome lipid composition (and consequently their integrity) influences their pharmacokinetic profile. Thus, the proper arsonoliposome composition should be used according to the intended application.

Keywords: Liposome; Arsenates; Arsenic; Distribution; Tissue; *In vivo*; Toxicity; Pharmacokinetics

1. Introduction

Arsonolipid-containing liposomes called arsonoliposomes were prepared and characterized in our laboratory (Fatouros et al., 2001). As recently summarized (Fatouros et al., 2006), promising results were obtained with the arsonoliposome types prepared, for which a differential toxicity towards cancer and normal cells was demonstrated (Gortzi et al., 2002, 2003) as well as *in vitro* antiparasitic activity (Antimisiaris et al., 2003). However, when the *in vivo* distribution (of arsenic) after i.p. injection of arsonoliposomes (composed of C16-arsonolipid mixed with phosphatidylcholine (PC) and cholesterol (Chol) [PC-based arsonoliposomes]), was studied (Antimisiaris et al., 2005) a low arsenic distribution was observed in most tissues, especially blood, and considerable amounts of arsenic were determined only in liver and spleen. The demonstrated distribution profile was attributed to the physical instability of the specific type of arsonoliposomes used in that study. As anticipated, although the low bioavailability of PC-based arsonoliposomes would not exclude the possibility of using them as antiparasitic agents (providing that they will demonstrate *in vivo* activity) since the amounts measured in liver and spleen were not too low, it highly limits the possibility of using them as anticancer therapeutics and/or carriers to deliver other cytotoxic agents to cancer cells.

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Recently, it was found that arsonoliposome membrane integrity and size stability can be influenced by their lipid composition; indeed, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)-containing arsonoliposomes [DSPC-based arsonoliposomes] were found to demonstrate increased integrity during incubation in serum, compared to the PC-based ones (Piperoudi et al., 2005). Additionally, when PEG-conjugated lipids were included in the lipid membranes of DSPC-based arsonoliposomes, forming thus Pegylated-arsonoliposomes, their integrity was further increased. The later (Pegylated-arsonoliposomes) were also stable in presence of divalent cations (e.g. Ca^{2+}) at concentrations that were previously seen to initiate aggregation and finally fusion of non-Pegylated-arsonoliposomes (Fatouros et al., 2005; Piperoudi et al., 2006). Thereby, in order to understand if arsonoliposome stability has an effect on their pharmacokinetic profile, the *in vivo* kinetics of the more stable types of arsonoliposomes (compared to PC-based arsonoliposomes) should be determined, and compared to that of the PC-based ones. Comparison of the pharmacokinetic profile of the different arsonoliposome types will allow better selection of the arsonoliposome types that should be used in future *in vivo* disease model testing of arsonoliposome anticancer activity.

Herein the *in vivo* distribution of arsenic after i.p. injection of the arsonoliposome types that were found to be more stable (DSPC-based arsonoliposomes and Pegylated-arsonoliposomes) compared to PC-based arsonoliposomes is determined under the same experimental protocol which was used previously for the later arsonoliposomes (Antimisiaris et al., 2005), for direct comparison of the results.

2. Materials and methods

2.1. Reagents

1,2-Distearoyl-*sn*-glycero-3-phosphocholine [DSPC] (synthetic, grade 1), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine conjugated to polyethylene glycol (MW 2000) [DSPE-PEG_{2000}] (synthetic, grade 1) were purchased from Avanti Polar Lipids. Cholesterol [Chol] (pure) and Triton X-100 were obtained from Sigma–Aldrich (O.M.), Athens, Greece. The water used was deionized and then distilled.

The *rac*-arsonolipid [C16] (2,3-dipalmitoyloxypropylarsonic acid) with a palmitic side chain ($R = C_{15}H_{31}$) was synthesized and characterized, as described in detail before (Tsivgoulis et al., 1991a,b; Serves et al., 1992, 1993).

2.2. Liposome composition

Using the arsonolipid C_{16} and DSPC, and in some cases DSPE-PEG, we prepared liposomes with the following lipid compositions: (a) DSPC-based arsonoliposomes (DSPC/Ars/Chol 12:8:10 [mol/mol/mol]), and (b) Pegylated-arsonoliposomes (DSPC/Ars/Chol 12:8:10 [mol/mol/mol] in which 8 mol% DSPE-PEG_{2000} lipid was incorporated).

2.3. Preparation of liposomes

Arsonolipid-containing liposomes were prepared as described previously (Fatouros et al., 2001, 2005). In brief, lipids (after removing the organic solvents with a nitrogen stream) were mixed with 5 mM phosphate buffer (pH 7.4) and 20 mM NaCl and magnetically stirred vigorously on a hot plate for 4 h at 70–80 °C. After formation of liposomes, the samples were left to anneal for at least 1 h at the liposome preparation temperature.

In order to reduce liposome size, the large liposome suspension initially produced was sonicated, using a Vibra-cell probe sonicator (Sonics and Materials, UK) equipped with a tapered tip, for at least two 5 min cycles. In all cases the initially turbid liposomal suspension was well clarified after sonication. Following sonication, the liposome suspensions were left to stand for 1 h at 65 °C (or higher than the transition temperature of the lipid used in each case), in order to anneal any structural defects. The titanium fragments and any multilamellar vesicles or liposomal aggregates were removed by centrifugation at 10,000 × *g* for 10 min.

2.4. Liposome characterization

The liposomes prepared were characterized by measuring their size by dynamic light spectroscopy (DLS) with a Malvern Zetasizer 5000 (Malvern, UK), as described before (Fatouros et al., 2001). In brief, liposome dispersions were diluted with filtered PBS pH 7.40 and sized immediately.

The arsonoliposome electrophoretic mobility was also measured at 25 °C (Zetasizer 5000 Malvern Instruments, UK), after diluting the vesicle dispersion with filtered PBS pH 7.40. Zeta potentials of the dispersions were calculated (by application of the Helmholtz-Smolowkovski equation).

2.5. In vivo distribution study

Forty-three female balb-c mice with a mean weight of 20.6 ± 2.1 g were obtained from the Pasteur Institute (Athens, Greece) animal facility. The animals were housed in an approved animal facility and maintained according to the National Institutes of Health "Guideline on the Care and Use of Laboratory Animals". The animals were provided with Rodent Chow and water ad libitum. Two hundred and fifty microlitres of a concentrated arsonoliposome dispersion (DSPC-based or PEG-arsonoliposomes) corresponding to approximately 15 mg As(V)/kg of animal body weight, were injected i.p. to each animal. At specified time periods post-injection (1, 2, 5, 12 and 24 h); animals were sacrificed in groups of four, by decapitation. Three animals were sacrificed without treatment (their tissues were used as blank controls and for preparation of control spiked samples). Blood samples were immediately taken from each animal by cardiac puncture immediately following decapitation. After this, the animal tissues [liver, kidneys, spleen, lungs, heart, intestines (small and large together), stomach and carcass + skin] were removed from the animals, washed from residual blood, air dried and

weighted. The contents of intestines and stomach were discarded.

The As content of all tissues was measured by the technique described in detail below.

2.6. Quantification of arsenic in tissues and arsonoliposomes

2.6.1. Sample preparation

The arsonolipid content of arsonoliposomes as well as the tissue concentrations of arsenic was determined using atomic absorption spectrophotometry after digestion with fuming nitric acid, as previously reported (Desaulniers et al., 1985). In brief, each tissue from each mouse after being weighted was dried and placed in a 250 ml Erlenmeyer conical flask with 10–50 ml of fuming HNO_3 (depending on the weight of each tissue). For determination of arsenic concentration of arsonoliposomes, 20 μl of the liposome dispersion was digested in 12 ml of nitric acid. The flasks were heated on hot plates placed under a hood, by slowly increasing the temperature to 90–100 °C. The solution was allowed to evaporate to dryness (but not charred), and the residue was taken up with 3 ml HNO_3 and 3 ml cold (4 °C) 30% H_2O_2. A reaction was initiated by slowly heating the mixture and the rate of decomposition of H_2O_2 was controlled by frequently removing the flask from the hot plate. The solution was then brought to a brief boil, cooled, and diluted to 50 ml with water. Blanks were taken through all steps.

2.6.2. Procedure

The total arsenic in these samples was determined by graphite furnace atomic absorption spectroscopy technique (GFAAS). A computer controlled atomic absorption spectrometer (AAnalyst 300, Perkin-Elmer) equipped with a graphite furnace (HGA-800, Perkin-Elmer) was used in this study. The absorption was measured at a wavelength of 193.7 nm and a 0.70-nm slit bandwidth. Deuterium lamp continuous background correction was used throughout the study to eliminate spectral interferences. Pyrolytic graphite coated tubes (Perkin-Elmer) were used and the atomization process was done at the tube wall. Argon at a 250 ml/min flow rate was used as a purge gas and its flow was interrupted during atomization.

Matrix modifier solution of nickel nitrate (5%, m/V) was prepared by dissolving an appropriate amount of the corresponding high purity salt (>99.999%, Aldrich) in DD-water. The addition of matrix modifier converts the As to a less volatile compound and thus the char temperature may be increased to 1400 °C. An aqueous arsenic standard solution of 1000 mg/l (Merck) was used for the preparation of aqueous calibration standards of lower concentrations (20–300 ppb). These standards were prepared daily, acidified with nitric acid and stored in polyethylene containers. The final nitric acid concentration was 0.2% (w/v). The furnace conditions during the analysis were summarized previously (Antimisiaris et al., 2005). The linear range was found to be between 0 and 400 ppb.

The reagent blanks (0.2% nitric acid) and calibration standards were measured in triplicate, while samples in quadruplicate. A 10 μl aliquot of sample or standard was transferred on to the wall of the pyrolytic graphite coated tube, followed by 10-μl of matrix modifier solution. The arsenic in the samples or standards was atomized by raising the temperature from ambient to atomization-temperature, according to the temperature program. The reliability of the measurement was assessed by measuring blank tissues (from the animals that were not injected with arsonoliposomes) and by spiking control tissue samples with known amounts of arsonoliposomes or inorganic arsenic. Recoveries in all cases ranged from 88 to 109% with coefficients of variations between 5 and 14%.

2.7. Statistical analysis and calculation of pharmacokinetic parameters

In the physicochemical characterization measurements the values calculated are mean values from at least five measurements of two independent samples. Results of the *in vivo* tissue distribution values between the different formulations tested were analyzed statistically by one-way analysis of variance and *t*-test using a statistical package for social sciences (SPSS version 14.0) software. The level of statistical significant is mentioned in each case ($p < 0.05$ [symbolized as *] and $p < 0.01$ [as **] in the graphs). All values are expressed as their mean ± S.D. (standard deviation of the mean).

The apparent total body arsenic elimination rate constant K_{el} (and the elimination rate constant from some tissues), was obtained from the terminal slope of the individual total body concentration (or tissue arsenic concentration)–time curves, after logarithmic transformation of the concentration values and application of linear regression.

3. Results

3.1. Physicochemical characteristics of arsonoliposome types prepared

The arsonoliposomes used in this *in vivo* study were characterized by measuring their size distribution and surface charge. The results of these measurements are presented in Table 1 and are in line with previous findings (Fatouros et al., 2001, 2005). For comparison, the physicochemical properties of PC-based liposomes (measured in media containing 1 mM EDTA) are also presented, however it should be mentioned that the same liposomes have different physicochemical values (mean diameter and ζ-potential) when measured in non-EDTA containing media (their mean diameter is 91.5 nm and their ζ-potential −45.3 mV), as published before (Antimisiaris et al., 2005) and explained in detail elsewhere (Fatouros et al., 2005). As seen in Table 1, in line with previous findings, when DSPC replaces PC in arsonoliposomes their mean diameter increases (from 68 to 80 nm) as it happens also when PEG-lipids are added in the arsonoliposome membrane (from 80 to 100 nm). Additionally, the ζ-potential of the Pegylated-arsonoliposomes is substantially lower (at $p = 0.01$) compared to non-Pegylated ones, proving that the arsonoliposome surface is coated with PEG-molecules.

Table 1
Physicochemical properties of the arsonoliposomes used in this study

Arsonoliposome [lipid composition]	Vesicle mean diameter (nm)[a]	Zeta-potential (mV)[a]
PC-based arsonoliposomes [PC/Ars/Chol (12:8:10)]	67.5 (2.7)	−32.5 (1.3)
DSPC-based arsonoliposomes [DSPC/Ars/Chol (12:8:10)]	79.9 (1.9)	−25.3 (1.8)
PEG-arsonoliposomes [DSPC/Ars/Chol (12:8:10) + 8 mol% DSPE-PEG2000]	99.5 (3.4)	−2.7 (1.0)

These measurements were performed in PBS buffer, pH 7.4, containing 1 mM EDTA, as described in detail in Section 2. The corresponding values of PC-based arsonoliposomes are also stated, for direct comparison.
[a] All values are mean values measured from at least two different batches of liposomes, and SD of each mean is reported.

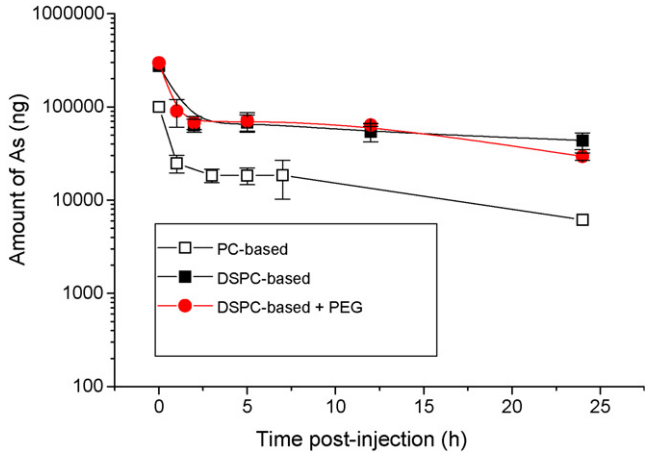

Fig. 1. Time-course of clearance of arsenic from the body of balb-c mice after intraperitoneal injection of various arsonoliposome types at a dose equivalent to 15 mg As/kg of body weight. Each point is the cumulative amount of As (ng) remaining in the body of mice (mean value plus standard deviation) calculated from the calibration curve of arsenic, which was measured by the atomic absorption technique presented in detail in Section 2.5.

3.2. *In vivo distribution of As after i.p. administration of DSPC-based and Pegylated-arsonoliposomes*

When the mean values of the cumulative amount of arsenic present in the animals (calculated by adding arsenic concentration values from all tissues) at the various time points tested, are plotted against time, it is evident from the resulting graph (Fig. 1) that arsenic (total body) clearance from mice is a bi-exponential process. This is due to the mode of injection used (intraperitoneal injection) which allows fast distribution to organs involved in the clearance of xenobiotics, i.e., liver, kidney and bile. Similar clearance kinetics were also demonstrated previously when liposomal formulations have been injected i.p. as in the case of PC-based arsonoliposomes (Antimisiaris et al., 2005) and admiamycin-loaded conventional and Pegylated-liposomes (Sadzuka et al., 1997). The results obtained previously with the PC-based arsonoliposomes were plotted in the same graph (Fig. 1, open symbols) for comparison. As seen, although for both DSPC-based and Pegylated-arsonoliposomes, at 1 h post-injection about 30–40% of the total dose administered was cleared from the body, in the case of PC-based arsonoliposomes a significantly (at $p = 0.05$) higher percent of the dose injected was cleared (from the body) at the same time point, proving that the two types of liposomes evaluated herein, were better distributed and retained in body organs, compared to PC-based arsonoliposomes. In all cases, after the rapid initial elimination of a significant amount of the administered arsenic, the elimination of the remaining portion of the dose was a very slow process.

However, there was a difference between the DSPC-based and Pegylated-arsonoliposomes in respect to this second phase of As elimination. The elimination rate constant calculated for Pegylated-arsonoliposomes was $0.0251 \pm 0.0035\ h^{-1}$ (corresponding to a half-life of 27.6 ± 4.5 h), very similar to that calculated previously for the PC-based ones ($0.023\ h^{-1}$), but for the DSPC-based arsonoliposomes it was a lot lower $0.0084 \pm 0.0017\ h^{-1}$ (corresponding to a substantially higher half-life [$t_{1/2} = 83 \pm 21$ h]).

The mean values calculated for the tissue-distribution of arsenic, presented as percentage of administered dose per tissue (%dose/tissue) at various time points after intraperitoneal administration of DSPC-based and Pegylated-arsonoliposomes are shown in Tables 2 and 3, respectively. The distribution or arsenic in blood is not presented in these tables, to avoid mistakes, since the full amount of blood could never be extracted from the animals. At the first time point at which arsenic distribution was measured, 1 h post-injection, for both types of

Table 2
Distribution of As in tissues and carcass + skin of balb/c mice at various time points following i.p. administration of DSPC-based arsonoliposomes (containing approximately 15 mg arsenic/kg body weight)

Time (h)	DSPC-based arsonoliposomes: mean% of dose/tissue (±S.D.)							
	Liver	Heart	Spleen	Lungs	Stomach	Kidneys	Intestines	Carcass + skin
1	4.04 (0.35)	0.284 (0.051)	0.844 (0.085)	0.276 (0.016)	0.73 (0.15)	0.60 (0.15)	4.71 (0.32)	2.35 (0.12)
2	3.59 (0.72)	0.130 (0.039)	0.79 (0.22)	1.07 (0.22)	1.20 (0.11)	1.57 (0.42)	7.531 (0.050)	2.1 (1.2)
5	4.199 (0.039)	0.167 (0.011)	2.66 (0.37)	0.608 (0.094)	0.60 (0.12)	1.381 (0.025)	4.929 (0.095)	3.794 (0.019)
12	3.81 (0.42)	0.396 (0.052)	2.43 (0.36)	0.751 (0.069)	0.972 (0.052)	0.73 (0.15)	4.234 (0.055)	3.107 (0.66)
24	3.74 (0.19)	0.442 (0.029)	2.165 (0.049)	0.790 (0.043)	0.398 (0.097)	1.022 (0.069)	4.343 (0.082)	1.156 (0.18)

Results are expressed as mean% of dose/tissue (±S.D.). Each point is the mean of four values.

Table 3
Distribution of As in tissues and carcass + skin of balb/c mice at various time points following i.p. administration of Pegylated-arsonoliposomes (containing approximately 15 mg arsenic/kg body weight)

Time (h)	Pegylated-arsonoliposomes: mean% of dose/tissue (±S.D.)							
	Liver	Heart	Spleen	Lungs	Stomach	Kidneys	Intestines	Carcass + skin
1	5.4 (0.55)	0.149 (0.021)	0.76 (0.24)	0.220 (0.032)	1.651 (0.310)	1.72 (0.18)	11.04 (3.7)	1.66 (0.17)
2	6.7 (1.2)	0.374 (0.064)	0.56 (0.13)	0.781 (0.094)	0.959 (0.205)	1.297 (0.086)	7.3 (1.3)	3.17 (0.14)
5	6.3 (1.8)	0.180 (0.039)	1.39 (0.28)	1.070 (0.083)	2.076 (0.39)	1.807 (0.091)	9.9 (2.1)	2.88 (0.31)
12	5.9 (1.1)	0.304 (0.072)	0.99 (0.18)	0.56 (0.11)	1.53 (0.34)	2.05 (0.39)	8.1 (0.83)	1.59 (0.25)
24	2.32 (0.51)	0.225 (0.024)	0.73 (0.120)	0.291 (0.028)	0.34 (0.11)	0.726 (0.088)	2.72 (0.35)	1.26 (0.25)

Results are expressed as mean% of dose/tissue (±S.D.). Each point is the mean of four or five values.

arsonoliposomes, the distribution of arsenic was greater in the intestines, followed by, in descending order, liver, carcass + skin, stomach, spleen, kidney, lung, heart. After this time point, the relative distribution of arsenic between different organs fluctuates, while for the full period studied (24 h) the organs in which less arsenic was distributed (%dose/tissue) are the lung and heart. Brain and tail samples from all animals were analyzed and no arsenic was detected, in any case in both of these tissues.

From the distribution results and the corresponding mean tissue weights, the concentration of arsenic in each tissue per tissue weight (%dose/g tissue) was calculated. These values are presented in Figs. 2 and 3, for DSPC-based and the Pegylated-arsonoliposomes, respectively. As observed, for both arsonoliposome types, the compartments with the highest arsenic concentration during the full study period were: blood and spleen (presented in separate graphs). Furthermore, for both arsonoliposome types the arsenic levels in abdominal cavity organs (especially liver and intestines) were high even after only 1 h from the administration time. The "i.p.-administration effect" was thus evident for both of the two arsonoliposome types evaluated.

When the concentration of arsenic in tissues (%dose/g of tissue), at the various time points evaluated after administration of the two arsonoliposome types were compared and analyzed for statistical significance, it was seen that in several cases the arsenic levels obtained by DSPC-based and Pegylated-arsonoliposomes were statistically different. Differences at p 0.05 are marked with * and at p 0.01 with ** in both, Figs. 2 and 3. Due to these later arsenic tissue concentration differences, the pharmacokinetic profile of arsenic in most tissues (and especially in those with the highest arsenic load) was significantly different between the non-Pegylated (DSPC-based) and Pegylated-arsonoliposomes, and it seems that – at least some of – the differences observed can be explained on the basis of the surface coating of arsonoliposomes with PEG molecules. In more detail, firstly arsenic levels in spleen were very high (about two times higher) after conventional (non-Pegylated) DSPC-based arsonoliposome administration compared to Pegylated-arsonoliposomes. Secondly, the arsenic levels in all other tissues, especially liver and intestines followed by stomach and kidneys, remain also at high levels for the whole period examined (up to 24 h post-injection) for the DSPC-based arsonoliposomes. Additionally, although at 2 h post-injection of DSPC-based arsonoliposomes a high arsenic amount was detected in blood, after this time point it was cleared from circulation with a high elimination rate (K_{bl}

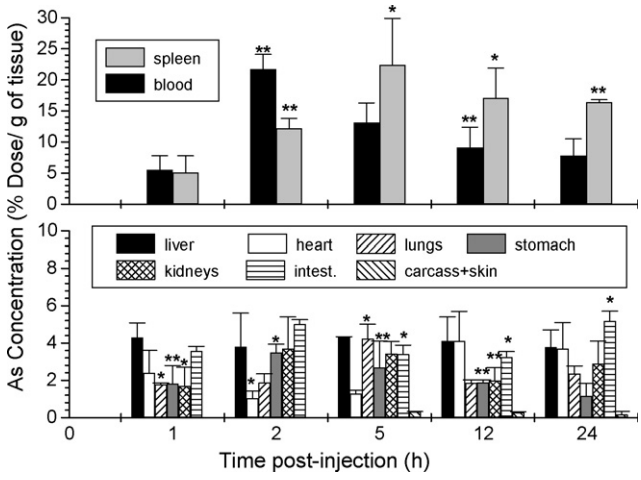

Fig. 2. Arsenic concentration in the different tissues of balb-c mice, at various time points post-injection of DSPC-based arsonoliposomes. Concentration is expressed as: %dose/g of tissue. Cases for which statistically significant differences were calculated between DSPC-based and Pegylated-arsonoliposomes are highlighted with asterisks (*significant at $p = 0.05$; **significant at $p = 0.01$).

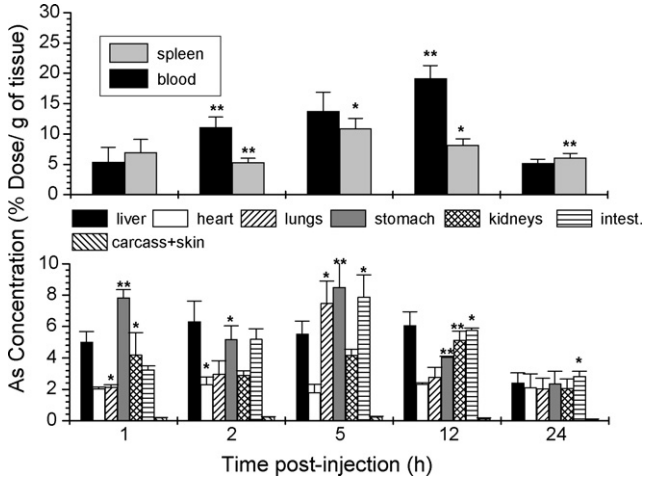

Fig. 3. Arsenic concentration in the different tissues of balb-c mice, at various time points post-injection of Pegylated-arsonoliposomes. Concentration is expressed as: %dose/g of tissue. Cases for which statistically significant differences were calculated between Pegylated and DSPC-based arsonoliposomes are highlighted with asterisks (*significant at $p = 0.05$; **significant at $p = 0.01$).

$0.0267 \pm 0.0045\ h^{-1}$) when compared to the whole body elimination rate (K_{el} $0.0084 \pm 0.0017\ h^{-1}$) for this arsonoliposome type (Fig. 2, top graph). On the other hand, after administration of Pegylated-arsonoliposomes in most organs (but not in all, due to the "i.p.-administration effect") the arsenic levels gradually increase indicating that, as Pegylated-arsonoliposomes are absorbed in the blood, they are also being distributed in the various organs until reaching a maximum level at 5 h post-injection. At this time point, it seems that the organs in which the Pegylated-arsonoliposomes are distributed become saturated and thus afterwards the arsenic levels start to decline due to elimination from most organs. In fact only blood and kidney levels continue to increase after the 5 h time point reaching maximum arsenic levels at 12 h post-injection. As for the arsenic levels in liver, for both cases (DSPC-based and Pegylated-arsonoliposomes), the levels are high already at the 1 h post-administration time point and remain high for the full study period, while no statistically significant difference (at $p = 0.05$) between the levels measured after administration of the different arsonoliposomes was calculated. However, as discussed in more detail below (in Section 4), since the tissue distribution of arsenic after administration of arsonoliposomes is most probably affected by many concurrent processes it is very difficult to fully clarify the mechanisms involved as well as the importance of each mechanism.

The arsenic levels in tissues measured for the arsonoliposome types studied here (Figs. 2 and 3) are substantially higher (differences are statistically significant at $p = 0.01$) compared to those reported previously (Antimisiaris et al., 2005) for PC-based arsonoliposomes. Most interesting is the fact that arsenic blood levels (at the period between 2 and 12 h post-injection) are more than 10 times higher after administration of the more stable arsonoliposomes (DSPC-based and Pegylated) compared to the PC-based ones. This proves that arsonoliposome stability has a significant effect on their absorption and distribution, in line with our initial hypothesis.

4. Discussion

PC-based arsonoliposomes have been previously shown to possess interesting anticancer (Gortzi et al., 2002) and antiprotozoal activity (Antimisiaris et al., 2003) in *in vitro* studies. In a later study (Antimisiaris et al., 2005) the *in vivo* distribution of arsenic after i.p. injection of these PC-based arsonoliposomes in balb-c mice was evaluated, and the instability of this arsonoliposome type (Piperoudi et al., 2005; Fatouros et al., 2005) was pinpointed as the reason for the very low tissue distribution and absorption of arsenic in blood, which was observed. In order to investigate the validity of this hypothesis, the *in vivo* distribution of arsenic after administration (by the same route) of new arsonoliposome compositions that were found to be considerably more stable (compared to PC-based arsonoliposomes): DSPC-based and Pegylated-arsonoliposomes (Piperoudi et al., 2005; Fatouros et al., 2005) was evaluated. It is essential to know how these new types of arsonoliposomes are distributed *in vivo*, before selecting the proper arsonoliposome types for development of formulations or drug delivery systems as anticancer or antiprotozoal therapeutic systems.

As explained before (Antimisiaris et al., 2005), the distribution of arsenic in tissues is a complex mechanism not fully elucidated. It should be considered as a combination of the metabolism of the arsenic-containing compound and the relative kinetics of each metabolite. Additionally, arsonolipid metabolism may be completely different from that of inorganic arsenic, as seen for arsenosugars (Devalla and Feldmann, 2003). Furthermore, the current situation is even more complicated since for arsonoliposomes, vesicle stability in the *in vivo* environment is another aspect that should also be considered.

Previously, by comparing the results of As distribution after administration of PC-based arsonoliposomes with those of disposition of inorganic arsenic taken from the literature (Hughes et al., 1999), it was concluded that for all the tissues measured, the concentration of arsenic was substantially higher after arsonoliposome administration (from 2 to 10 times higher arsenic levels in tissues), a fact which indicated increased retention of arsenic in the abdominal tissues when As was administered in the form of a lipid incorporated in vesicles. The results of the present study (Tables 2 and 3 and Figs. 2 and 3) prove that arsonoliposome lipid composition has a significant effect on the vesicle distribution after i.p. administration, and this is most probably because of the different stability that arsonoliposomes with different lipid compositions have. Indeed the levels of arsenic (not only in abdominal tissues but also in blood) were significantly higher after administration of DSPC-based arsonoliposomes compared with those measured previously after *in vivo* administration of the PC-based ones, and furthermore, when the arsonolipid vesicles were coated with PEG molecules (which has been demonstrated to result in formation of highly stable vesicles (Piperoudi et al., 2006), the Pegylated-arsonoliposomes), the pharmacokinetics of arsenic is influenced even more, as concluded by comparing the two sets of results (Tables 2 and 3, or Figs. 2 and 3). Thereby, the current results prove the validity, and further extend the conclusion made previously that administration of arsenic in the form of a vesicle-incorporated arsonoliposome has a significant influence on the distribution and retention of arsenic in the tissues that are located in the abdominal area. Furthermore, it is proven that the stability of the vesicles in the *in vivo* environment is one of the important factors which determine the arsenic tissue distribution and kinetics.

Nevertheless, when comparing the arsenic tissue distribution after administration of PC-based arsonoliposomes (reported previously) and DSPC-based or Pegylated-arsonoliposomes (obtained herein) it should be considered that in addition to stability issues as well as the influence of the PEG coating applied on the vesicles, the pharmacokinetic profile of arsenic (following arsonoliposome administration) is also influenced by the fact that the i.p.-administration route has been used. In a previous study, in which the tissue distribution of adriamycin was evaluated after i.v. and i.p. injection of conventional and Pegylated drug-encapsulating liposomes (Sadzuka et al., 1997; Sadzuka et al., 2000), the amount of drug in plasma increased between 2 and 4 h post-injection and remained more or less

constant up to 24 h. In addition, in the same previous studies it was seen that from 1 to 8 h, adriamycin disappeared slowly from the abdominal cavity organs, especially liver, after i.p. administration of conventional (PLADR) as well as PEG liposomal formulations (PEGLARD). This pattern is, more or less, similar to the blood and tissue distribution observed for arsenic (Tables 2 and 3 and Figs. 2 and 3) after administration of DSPC-based as well Pegylated-arsonoliposomes. Thereby, it may be suggested that the distribution of arsenic after administration of arsonoliposomes by i.p. injection is mainly governed by the vesicle distribution and that any effect of arsonoliposome disruption and subsequent metabolism of arsonolipids should be minimal. However, on the other hand, the fast clearance of 30–40% or higher amounts of As (in the case of the least stable PC-based arsonoliposomes) in initial stages followed by gradual increase in As content of some tissues might indicate disruption of a part of the arsonoliposomes and subsequent distribution of intact arsonoliposomes from the i.p. area to other tissues, through lymphatic system, blood, or peripheral organs. These last possibilities should not be overruled since it is clear (as stated several times before) that the stability of arsonoliposomes determines the *in vivo* distribution of arsenic after arsonoliposome administration; nevertheless it is very difficult to fully clarify the importance of each mechanism for the final distribution profile demonstrated after administration of each arsonoliposome type.

It is important to mention that no acute toxicity was observed during the present *in vivo* study and the body weight and organ weight of the mice receiving this high dose of As(V) was not altered.

Summarizing, the results presented herein indicate that arsonoliposome lipid composition has a significant effect on the *in vivo* distribution of these vesicles, at least after i.p. administration. Thereby, when designing arsonoliposome formulations for usage as antiprotozoal or anticancer therapeutics, in addition to the relative activities demonstrated by the different arsonoliposome types (lipid compositions) their biodistribution profiles should also be taken into account, in order to fine tune their structure with the goal of obtaining the best activity in conjunction with the *in vivo* distribution that would be beneficial for the specific application.

Additionally, since arsenic was not detected in the brain of the animals receiving the two arsonoliposome types studied herein, as also the PC-based arsonoliposomes studied before, another interesting challenge for future exploitation would be to coat arsonoliposomes with ligands that will improve their ability to cross over the blood brain barrier in order to kill parasites in the central nervous system or act as an anticancer agent against brain tumours.

As an ending remark, the *in vivo* distribution profiles of both, DSPC-based and PEG-arsonoliposomes, after i.p. injection look promising for the development of anticancer arsonoliposome-based formulations, especially for tumours which are located in the abdominal area.

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