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Biodistribution of Mixed Fluorocarbon–Hydrocarbon Dowel Molecules Used as Stabilizers of Fluorocarbon Emulsions: A Quantitative Study by Fluorine Nuclear Magnetic Resonance (NMR)

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^{19}F NMR spectroscopy was used to determine quantitatively the organ distribution and organ retention time in rats of the mixed fluorocarbon–hydrocarbon dowel molecule $C_6F_{13}CH=CHC_{10}H_{21}$ (F6H10E), which stabilizes highly concentrated injectable fluorocarbon emulsions destined for *in vivo* oxygen transport and delivery. The only fluorine resonances detected in the ^{19}F NMR spectra of the organs analyzed were those of the F6H10E dowel itself, indicating that metabolites, if present, have very low concentrations ($<10^{-4}$ *M*, limit of our assay). The F6H10E content in the liver peaked 1 day after administration (7 days for the spleen). At a dose of 3.6 g/kg body weight, the half-life of F6H10E in the liver was 25 ± 5 days.

KEY WORDS: blood substitutes; mixed fluorocarbon/hydrocarbons; fluorocarbon emulsion stabilization; biocompatibility; biodistribution; ^{19}F nuclear magnetic resonance.

INTRODUCTION

Oxygen-carrying fluorocarbon emulsions constitute a promising alternative to homologous blood transfusion. Their scope of applications has been extended from temporary blood substitutes for emergency oxygen delivery to numerous other biomedical applications (1–3).

The first-generation dilute injectable fluorocarbon emulsion Fluosol (Green Cross Corp., Osaka, Japan) has been approved by the FDA for use in high-risk percutaneous transluminal coronary angioplasty (PTCA). However, its poor stability makes it unfit for general use. The stem emulsion must be frozen for shipping and storage, then thawed and admixed with two annex solutions prior to administration; the reconstituted preparation must then be used within 8 hr. The second-generation emulsion Oxygent (Alliance Pharm. Corp., San Diego, CA) is much more efficacious, being considerably more concentrated (3,4). It is also much more stable and can be stored for several months at room temperature, and for over 1 year at 5–10°C; it is ready for use. It is, nevertheless, desirable that even stabler emulsions be developed to allow long-term room temperature storage and field use.

We have achieved remarkable stabilization of concentrated (90% by weight, 47% by volume) fluorocarbon emulsions formulated with egg-yolk phospholipids (EYP) by adding small amounts (typically 1 to 3% by weight) of a mixed fluorocarbon–hydrocarbon R_FR_H amphiphile such as $C_6F_{13}CH=CHC_{10}H_{21}$ (F6H10E). These mixed fluorocarbon/hydrocarbon amphiphiles act as molecular "dowels" to reinforce the binding between the fluorocarbon phase and its surrounding lipidic EYP membrane. The incorporation of such a R_FR_H dowel molecule to 90% (w/v) concentrated emulsions of perfluorooctyl bromide (perflubron) or $C_4F_9CH=CHC_4F_9$ (F-44E) results in easier emulsification, smaller initial median droplet sizes, narrower particle size distributions, and almost-complete suppression of particle growth at room temperature or even at 40°C. Similar stabilization effects have been obtained with saturated dowel molecules such as $C_6F_{13}C_{10}H_{21}$ (F6H10) (5,6).

This paper reports on the *in vivo* tolerance, biodistribution, and excretion from the reticuloendothelial system (RES) organs of this mixed fluorocarbon–hydrocarbon "dowel" molecule.

The fluorocarbons investigated for use as injectable oxygen carriers are excreted from the body by exhalation without being metabolized or chemically altered (7–9). The emulsion particles are removed from the blood stream by phagocytosis and transiently stored in the monocyte/macrophage system. The largest proportion of the accumulated fluorocarbon is found in the liver, followed by the spleen (10). The transport of fluorocarbon from the cells to the lungs is probably assured by plasmatic lipoproteins (11,12). It has been shown that the retention of a fluorocarbon in the organs depends mainly on two factors: (1) the fluorocarbon's molecular weight—its rate of excretion is most generally an exponential function of molecular weight, the acceptable mass for i.v. use being comprized between 460 and 520; and (2) its lipophilic character. It has indeed been found that linear fluorocarbons, terminally substituted by a bromine atom, depart from this rule and are excreted more rapidly than expected on the basis of their sole molecular weight (2,4,13).

The R_FR_H dowel molecules can definitely be considered as belonging to this class of lipophilic fluorocarbons. In contrast to typical fluorocarbons of comparable molecular weight, they are infinitely soluble in hexane and other fluorocarbons. Therefore they are expected to be excreted from the body at a faster rate than would be predicted on the sole basis of their molecular weight. The $C_6F_{13}CH=CHC_{10}H_{21}$ dowel molecule, which we have selected for this study, has a molecular weight of 486, which, if this compound were an ordinary fluorocarbon, would lead to predict a half-retention time in the organs in the range of 30 to 45 days (14). In view of its strong lipophilic character, a shorter organ-dwelling time was anticipated for this particular dowel molecule.

To evaluate the distribution in the organs and the rate of excretion from the RES organs of this typical mixed fluorocarbon–hydrocarbon compound, it was formulated as a 25% (w/v) emulsion, with EYP (6%, w/v) as the emulsifier. The

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latter was used because of its wide acceptance in pharmaceuticals, including concentrated fluorocarbon emulsions and lipid emulsions for parenteral nutrition (1,2,15). This F6H10E emulsion was injected intravenously into female rats at a 3.6 g/kg body weight dose. Such a dose represents 30 to 60 times the amount needed to stabilize a clinically relevant dose of a concentrated fluorocarbon emulsion (16).

The distribution of the F6H10E "dowel" molecule in the organs was determined by ^{19}F NMR at various time points. This technique has already been successfully used for analyzing biological samples for exogeneous fluorinated compounds (17,18).

MATERIALS AND METHODS

F6H10E was prepared according to the literature (19). Its purity (>99%) was checked by gas chromatography. Final purification, prior to use, included (i) washing with 10% (w/v) KOH and water for injection, followed by filtration over Whatman No. 1 SP filter paper, and (ii) treatment with activated carbon (neutralized Sigma C-5385; 1 g/100 mL of product) and filtration over a short Al_2O_3 column (70–230 mesh, Merck Inc.). EYP came from Asahi.

Biological Tolerance

Both F6H10E and EYP were tested for biological tolerance before use in emulsions. No effect of F6H10E has been observed on the growth and survival of mice after intraperitoneal administration of 35 g per kg body weight (limit of the protocol) of the dowel molecule; no death was noted among 10 animals after an observation period of 1 month. Incubation (4 days) of the molecular dowels with Namalva lymphoblastoid cell cultures did not affect their growth and viability (20).

Emulsion Preparation

The F6H10E emulsion was prepared under argon in two steps.

1. Preemulsification. $NaH_2PO_4 \cdot 2H_2O$ (0.293 g), Na_2HPO_4 (0.937 g), NaCl (1.395 g), and $Na_2EDTA \cdot 2H_2O$ (0.101 g) were transferred to a 500-mL round-bottom flask containing 243.6 g of water for injection, and the mixture was swirled to dissolve the salts. The solution was sparged with argon, and EYP (19.80 g) and (+)-α-tocopherol (0.009 g) were added, the latter in order to protect the EYP against oxidation. The mixture was dispersed for 60 sec at 8000 rpm using an Ultra-turrax mixer (Janke & Kunkel IKA-Labotechnik, rotor S-25 N). F6H10E (82.8 g) was then added at a rate of 20 mL/min while mixing. After complete addition, the rate of mixing was increased to 24,000 rpm for an additional 20 min.

2. Emulsification. The above premix was emulsified under argon using a Microfluidizer Model 110, 25–30°C; 12 passes were used under a pressure of 10,000 psi. The emulsion was then transferred to 12-mL vials, sealed with Teflon-lined stoppers and aluminum outerseals, and heat sterilized (121°C/15 min/1 bar). The final composition of the emulsion (pH 7.06; osmolarity, 265) is given in Table I. Particle size measurements (Fig. 1) were made by photosedimentation using a Horiba CAPA-700 Particle Analyzer.

Table I. Composition of the F6H10E Emulsion

Component	% (w/v)
F6H10E	25.1
Lecithin (EYP)	6.00
$NaH_2PO_4 \cdot 2H_2O$	0.089
Na_2HPO_4	0.284
NaCl	0.423
$Na_2EDTA \cdot 2H_2O$	0.030
(+)-α-Tocopherol	0.003
H_2O	q.s.ad. 100 mL

Animal Experiment

Strain OFA (Sprague–Dawley) IOPS female rats of 175 to 200 g, 9 to 10 weeks old, purchased from Iffa-Credo, Lyon, were anesthetized by inhalation of a halothane (2% by volume)/oxygen mixture for 5 min and injected with the emulsion at the F6H10E dose of 3.6 g/kg body weight (i.e., 2.5 mL of emulsion for a 175-g rat) through the right jugular vein [catheterization:Trocarth Intraflon, 0.8 mm (SODIS, Mulhouse)] at a rate of 1 mL/min. The animals were sacrificed at various intervals of time after injection. The wet organs (liver, spleen, lungs, and kidneys) were removed, weighed and immediately frozen. The control animals, of the same origin, were submitted to the same protocol but received 14.4 mL/kg body weight dose of physiological water (0.9% NaCl) instead of the emulsion. Results are expressed as mean ± SE; Student's *t* test was performed.

No halothane was detected in the ^{19}F NMR spectrum of the animals which were sacrificed 4 hr after injection.

NMR Sample Preparation

The entire organ (liver, spleen, lung, or kidney) was ground into a known amount of water for injection (ca. 2 mL) using an Ultra-Turrax (8000 t/min) mixer at a temperature of 20°C. One-fourth milliliter of the resulting preparation was then directly transferred to the 5-mm calibrated NMR tube; 0.25 mL of an aqueous solution of CF_3CH_2OH, which is used as the internal NMR standard, was added. The NMR tube was vortexed for 1 min and the ^{19}F spectrum measured. In the case of blood, the NMR samples were prepared by directly adding, in the NMR tube, 0.25 mL of blood to 0.25 mL of the aqueous solution of the NMR standard. We did check that the eventual precipitation of the heavy fluorocarbon emulsion droplets did not significantly affect the results of the NMR assay, even when the measurement was made 2 hr after preparation of the sample. We have, nevertheless, always measured the ^{19}F spectra immediately after preparation and we have limited the accumulation time, in the case of the most diluted samples, to 1 hr (ca. 7000 scans).

^{19}F NMR Determinations

The NMR spectra were measured at 188.3 MHz at room temperature on a BRUKER AC-200 spectrometer interfaced with an Aspect 3000 Computer. The measurements were

performed at 27°C on a dedicated ^{19}F probe using broadband proton decoupling. CF_3CH_2OH was used as an internal standard (−77 ppm upfield from $CFCl_3$); its concentration was chosen to be close to the fluorine content of the organ sample to be analyzed. The assay consisted of integrating the surface areas covered by the terminal CF_3 signals of the internal standard and of the F6H10E dowel molecule. We established that a pulse length of 2 μsec on a sweep width of 15,000 Hz (flip angle $\alpha = 40°$) with a relaxation delay of 0.5 sec was the optimum conditions for quantification: an increased recycle time did not modify the intensity of the ^{19}F signals. The reproducibility of the NMR assay was estimated to ±3%. Each data point corresponds to the average fluorocarbon concentration measured for three different animals, and the overall experimental error was estimated as ±10%. The smallest detectable concentration of F6H10E (signal/noise ratio) was ca. 10^{-4} *M*, which corresponds to 0.02% of the injected dose.

RESULTS AND DISCUSSION

The mixed fluorocarbon/hydrocarbon dowels, being devoid of reactive functional groups, were expected to be chemically and biologically rather inert. The biological tolerance of the compounds themselves was nevertheless tested, prior to their use in emulsion, through intraperitoneal administration to mice at 35 g/kg body weight of the dowel molecule; this dose, which corresponds to the limit of the protocol, induced no detectable effect on their growth, yet this dose represented ca. 600 times the amount of dowel that would be administered i.v. in a clinically relevant situation when used as a stabilizing adjunct.

A 25% (w/v) emulsion of the typical dowel molecule, F6H10E, was then formulated using 6% (w/v) EYP (Table I). This emulsion proved to be stable after sterilization for at least 50 days at 5°C. The emulsion consists of two populations illustrated on the histogram delivered by the analyzer (Fig. 1): lipid-coated fluorocarbon droplets, with a mean volume diameter of 0.22 μm, and small water-filled vesicles showing as a stripe at the right side of the histogram (21). To study the biodistribution, possible metabolism, and excretion rate from the RES organs of the dowel molecule, this emulsion was injected into anesthesized, catherized OFA female rats at a 3.6 g of dowel/kg body weight dose (i.e., 14.4 mL of emulsion) through the right jugular vein at a rate of 1 mL/min. The animals behaved normally after the injection and all 33 animals treated survived until the programmed date of sacrifice.

The growth of the animals which received the dowel emulsion showed only little disturbance as compared to that of the control animals, which received the same 14.4 mL/kg body weight dose of physiological water. As illustrated in Fig. 2, the animals which received F6H10E lost some weight in the first few days after injection: 4 days after injection, the weight loss for the animals that received F6H10E was 8%, compared with the control animals, which, over the same period of time, gained 2% in weight ($P < 0.005$). The difference in weight was found to be still significant at day 7 ($P < 0.005$). However, the treated animals started gaining weight again ca. 1 week after the injection, and the average weights of the animals injected with F6H10E and of the control animals were no longer significantly different after 10 days.

Figures 3 a–c illustrate the variation of the weight of the liver, spleen, and lungs with respect to time for the animals which received F6H10E as well as for the control animals. As a consequence of massive F6H10E injection, the weight of the liver increased to 6% ($\sigma = 0.28$) of the total body weight, to be compared to 3.8% ($\sigma = 0.66$) for the control animals, during the first 4 days after injection ($P < 0.005$). This effect diminished and was barely significant ($P < 0.05$) 2 months later, when the weight of the liver represented 3.6% ($\sigma = 0.09$) of the total body weight for the treated animals, compared to 2.7% ($\sigma = 0.4$) for the control animals. The relative weight of the spleen also increased as a result of F6H10E injection: after day 4, the relative weight of the spleen of the treated animals closely paralleled that of the control animals, the difference remaining significant 2 months after injection ($P < 0.005$). The effect on the lungs was significant only at day 4 ($P < 0.001$).

The amount of exogenous fluorine present in the blood and in the main organs was quantitatively determined by ^{19}F NMR after the animals were sacrificed at regular intervals (2, 4, 8, 24, and 48 hr, 4, 10, 15, and 21 days, 1, 2.5, 3, and 4 months). A typical ^{19}F spectrum measured on blood 4 hr after injection of the 25% (w/v) F6H10E emulsion is illus-

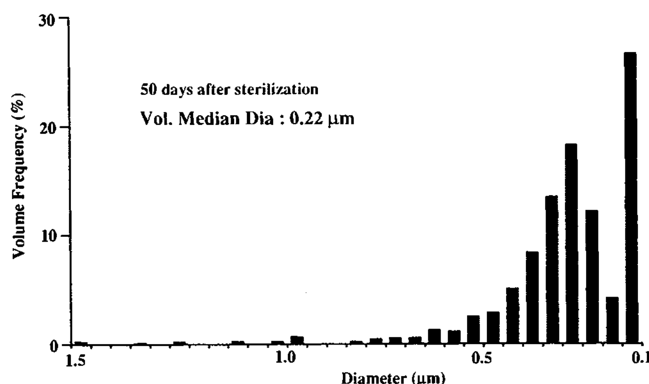

Fig. 1. Drop size distribution of F6H10E emulsion 50 days after sterilization at 121°C for 15 min.

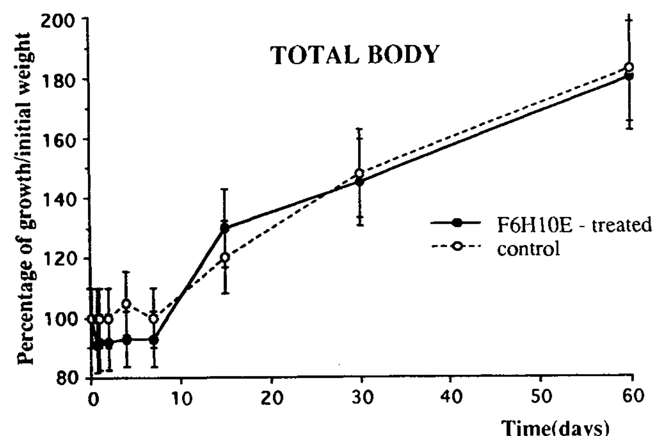

Fig. 2. Weight increase of rats injected with 14.4 mL/kg body wt of the F6H10E emulsion, i.e., 3.6 g of the dowel molecule (full line), and of control animals (dashed line) that received the same dose of saline (0.9% NaCl). Each data point is the average for three animals.

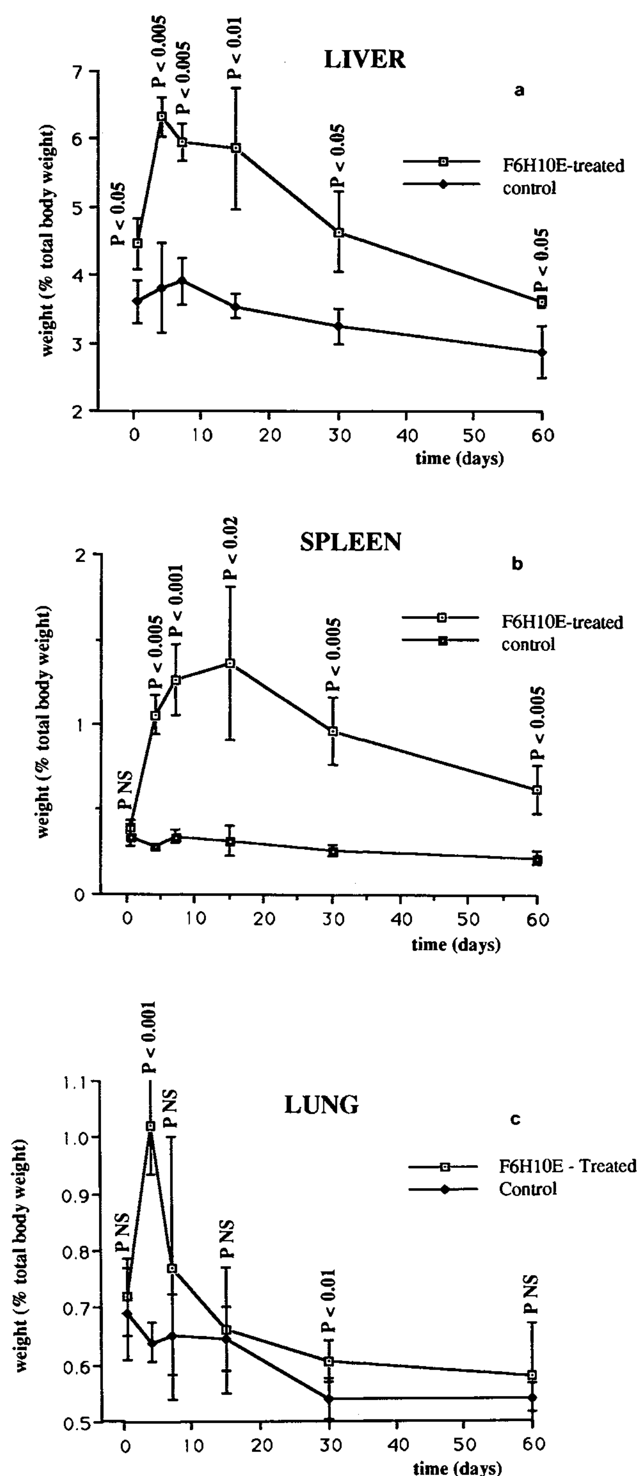

Fig. 3. Variation of the weights of liver (a), spleen (b), and lungs (c) vs time for animals that received 14.4 mL/kg body wt of the dowel emulsion, i.e., 3.6 g of the dowel molecule (open squares), and for control animals that received the same dose of saline (0.9% NaCl, black dots).

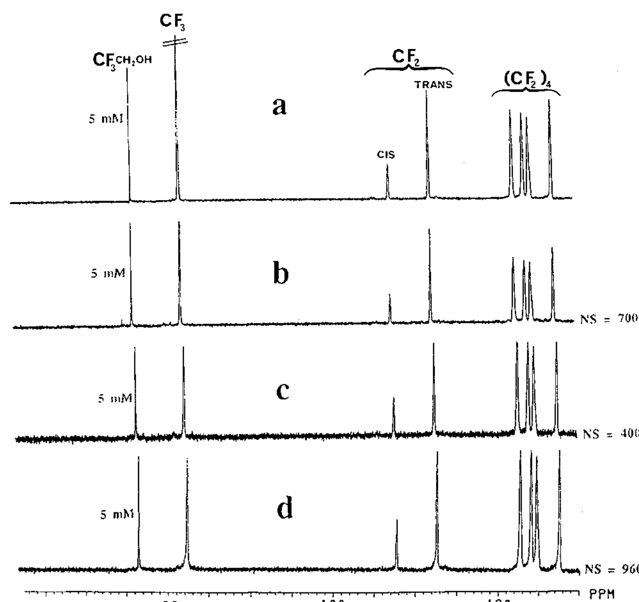

Fig. 4. Typical ^{19}F NMR spectra measured (a) on the F6H10E emulsion (CF_3CH_2OH as an internal standard) and (b–d): on various organs of rats having received a 3.6 g dose of F6H10E in the form of a 25% (w/v) emulsion (CF_3CH_2OH as an internal standard): (b) on blood 4 hr after injection, (c) on the liver 15 days after injection, and (d) on the lungs 16 hr after injection.

trated in Fig. 4b. Likewise, ^{19}F spectra measured on the liver 15 days after injection and on the lungs 16 hr after injection are shown in Figs. 4c and d.

The first important observation is that the only fluorine-containing compound detected in all the spectra recorded, whether on blood, liver, spleen, kidneys, or lungs, from 2 hr up to 4 months after injection, was F6H10E. We take this result to indicate either that F6H10E was not metabolized or that the concentration of the resulting metabolites, if present, is always very low ($<10^{-4}$ *M*, limit of our assay). This is in line with the existing documentation on fluorochemicals investigated for use in blood substitutes (2,3,7–13). It also shows that the double bond between the fluorinated and the hydrogenated chains of the dowel have no, or very limited, biological reactivity.

After 2 hr, only one-third of the injected dose was left in the bloodstream. Twenty-four hours after injection its amount was reduced to 2% of the injected dose, and only 0.1% of this dose was detected after 48 hr. It is believed that larger fluorocarbon particles are eliminated from the bloodstream more rapidly than smaller ones, which tend to stay in the circulation for a longer period of time (22). The dowels are *in fine* destined to be used as additives to stabilize fluorocarbon emulsions. The dowel emulsion used in this study, which was prepared only to allow the determination of its RES organ retention time, had not been optimized and its particle size distribution, as illustrated in Fig. 1, was rather broad. Its intravascular persistence is likely to be determined by particle sizes and size distribution in the emulsion rather than by the compound itself.

Figure 5 illustrates the amount of F6H10E, expressed as the percentage of the injected dose, which is left in the RES organs analyzed. Twenty-four hours after the injection, the dowel was distributed as follows: 70% of the injected dose in the liver, 17% in the spleen, 4% in the lungs, 2% in the kidneys, and 2% in the blood. It is noteworthy that the ^{19}F NMR determinations allowed the localization of ca. 95% of

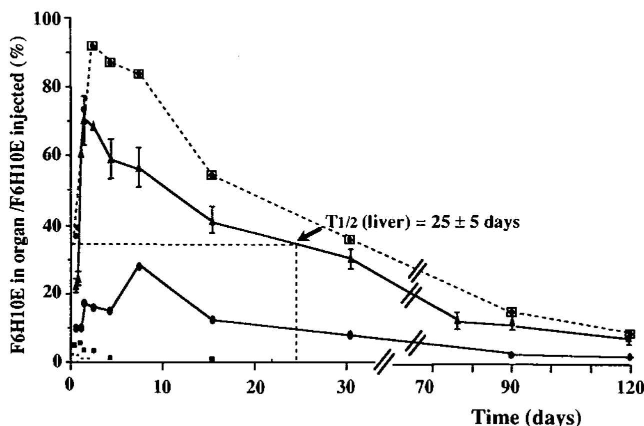

Fig. 5. Plot of F6H10E concentrations in organs vs time (% of the total injected dose). Each point is the average of three animals: liver (triangles); spleen (circles); lungs (squares); kidneys (points). Dashed line: sum for these organs.

the total injected dose. This is taken to indicate (22) that the emulsion droplets had been almost-totally captured by the macrophages in these RES organs.

The concentration of the dowel molecule reached a maximum after ca. 1 day in the liver (70% of the injected dose) and then decreased. The half-retention time in the liver can be estimated to be 25 ± 5 days (Fig. 5).

In the spleen, maximum accumulation was found after ca. 7 days and represented ca. 25% of the injected dose. The capture of the dowel by the liver, then by the spleen, is also reflected by the weight-change profile of these organs, as shown by comparing Figs. 3a and b.

The dowel content of the lungs was always low and decreased from 4 after 24 hr to ca. 1.5% after 1 month. The dowel content of the kidneys was also very low, ca. 2% of the total injected dose 2 hr after injection, and reached the limit of the method's sensitivity (10^{-4} *M,* i.e., 0.02% of the injected dose) after ca. 4 days.

The dowel molecule's organ retention time is, as expected, significantly shorter than would be predicted for a nonlipophilic fluorocarbon of the same molecular weight. It is also much shorter than that of perfluorotripropylamine [half-life, $T_{1/2}$, in the liver and spleen of 65 days (14)] and of perfluoromethylcyclohexylpiperidine [$T_{1/2}$ ~60 days (23)], the two compounds used, but in much larger proportions, as emulsion stabilizing additives in, respectively, Fluosol (which contains 8.4%, w/v, perfluorotripropylamine) and Ftorosan (which contains 7.6%, w/v, perfluoromethylcyclohexylpiperidine). The faster excretion rate of F6H10E is consistent with its lower molecular weight and much higher lipophilic character.

CONCLUSIONS

The neat mixed fluorocarbon–hydrocarbon "dowel" molecules proved to be well tolerated at at least 35 g/kg body weight i.p. in mice and showed no impact on the growth and viability of cell cultures. The emulsified dowel (25/6%, w/v, dowel/EYP) is well tolerated when injected intravenously in rats at a dose of 14.4 mL emulsion/kg body weight of animals, i.e., 3.6 g dowel/kg body weight.

The uptake by the macrophage/monocyte system of intravenously administered dowel emulsion droplets results in the enlargement of liver and spleen. This is, however, fully reversible, and the short half-life of only 25 days of the dowel analyzed, F6H10E, in the liver confirms that lipophilic fluorocarbons are excreted from such organs faster than would be anticipated on the sole basis of their molecular weight. The half-life of F6H10E is expected to be unchanged when taken as an additive to a fluorocarbon emulsion (24). This result supports its use for the stabilization of fluorocarbon emulsions destined to serve as injectable oxygen carriers or contrast agents.

Fluorine NMR, which was used for analysing the organs for the presence of fluorocarbon, showed that metabolites, if present, have concentrations lower than 10^{-4} M. These results can guide the development of new injectable fluorocarbon emulsions with high oxygen-carrying capacity and long-term room-temperature storage stability.

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