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Short communication

Quantitative analysis of C_{60} fullerene in blood and tissues by high-performance liquid chromatography with photodiode-array and mass spectrometric detection

Fathi Moussa[a,*], Monique Pressac[b], Eric Genin[c], Stéphane Roux[d], François Trivin[a], André Rassat[d], René Céolin[a], Henri Szwarc[e]

[a]*Jeune Equipe 1990, Faculté de Pharmacie, 31, Avenue Monge, 37200 Tours, France*
[b]*Service d'Endocrinologie, Faculté de Pharmacie, 4, Avenue de l'Observatoire, 75006 Paris, France*
[c]*Finnigan MAT Sarl, Parc Club Orsay Université 2, Rue J. Monod, F-91893 Orsay Cédex, France*
[d]*URA 1110 du CNRS, Ecole Normale Supérieure, 23, rue Lhomond, F-75 Paris, France*
[e]*URA D 1104 du CNRS, Bâtiment 490, Université Parix XI, F-91405 Orsay Cédex, France*

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Abstract

A high-performance liquid chromatographic (HPLC) assay method for C_{60} fullerene, in blood, liver and spleen using photodiode-array detection or mass spectrometric detection (LC–MS) and C_{70} fullerene, as the internal standard, is described. The recovery from mouse blood and tissues spiked with micronized C_{60} exceeds 90%. The method is linear from 0.05 to 200 mg of C_{60} per liter of blood and from 0.05 to 5.00% of C_{60} per tissue weight. The limit of detection of the method is 0.1 ng of C_{60} per injection. This method was applied to mouse blood and tissue samples after intraperitoneal administration of a micronized C_{60} suspension. © 1997 Elsevier Science B.V.

Keywords: C_{60} Fullerene

1. Introduction

Water-soluble derivatives of C_{60} fullerene show interesting properties in various biological systems [1]. However, the biological properties of C_{60} itself have not been widely studied [1]. In order to check its possible acute toxicity in vivo, we have injected micronized C_{60} intraperitoneally into Swiss mice [2]. Under these conditions, C_{60} accumulates preferentially in spleen and liver without any acute toxic effects [2]. In order to study the kinetics of accumulation and possible elimination of C_{60} in these tissues, it is necessary to develop a sensitive method to measure the concentration of C_{60} in biological samples. Several chromatographic separations of fullerenes have been described [3–6], but only one of them addresses C_{60} determination in plasma [6].

We describe here a high-performance liquid chromatographic (HPLC) method with internal calibration for assays of blood, spleen and liver. The experiments were carried out on live Swiss mice

*Corresponding author.

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treated with a micronized C_{60} suspension, as described previously [2].

2. Experimental

2.1. Reagents

Toluene, acetonitrile and methanol were of HPLC grade from Merck (Darmstadt, Germany). Diethyl ether and glacial acetic acid were purchased from Carlo Erba (Milan, Italy) and sodium dodecyl sulphate (SDS) was from Merck.

"Ultra" C_{60}, "Ultra" C_{70} (used as standards) and type M micronized C_{60} (C_{60m}) were purchased from Technocarbo (Plan de Grasse, France). Standard solutions of C_{60} (S_1=130 mg/l and S_2=13 mg/l) and stock standard solutions of C_{70}, used as internal standard (IS_1=500 mg/l and IS_2=50 mg/l), were prepared in toluene and stored in the dark.

The concentration of the standard solutions of C_{60} in toluene was determined, after equilibration at room temperature, by measuring the absorbance (after suitable dilution) at 328 nm (molar absorptivity=512 381 mol^{-1} cm^{-1}; [7]).

An aqueous suspension of C_{60m} (SC_{60}=100 mg/ml) was prepared as described previously [2]. The SC_{60} was diluted with suspending media [2] as required for method validation.

2.2. Apparatus and columns

2.2.1. HPLC with photodiode-array detection (LC–PDA)

The chromatograph featured a Waters 600E multisolvent delivery system and a Waters 991 photodiode-array detector (Waters, Milford, MA, USA). The analytical column was a RP-18 5 μm LiChrocart 125×4 mm I.D. HPLC Cartridge (Merck) protected by a PSF 25 C_{18}, 5 μm, 20×4.6 mm I.D. precolumn (SFCC Shandon, Eragny, France). The mobile phase was acetonitrile–toluene (40:60, v/v) at a flow-rate of 1.0 ml/min.

2.2.2. HPLC with mass spectrometric detection (LC–MS)

We used a P4000 HPLC pump (Thermo Separation Products, Courtaboeuf, France) equipped with a column, as described above, connected to a Finnigan MATLCQ mass spectrometer with an atmospheric pressure chemical ionization (APCI) source. The temperatures of the vaporizer and the heated capillary were set at 450 and 220°C, respectively. The needle for corona discharge was maintained at 5 mA and the multiplier voltage was set at 800 V (giving a gain of $3 \cdot 10^5$). Full scan negative ion mass spectra were monitored from m/z 150 up to 1000 th in order to identify the C_{60}. Corresponding UV chromatograms were recorded before MS detection using a TSPUV 1000 apparatus (Thermo Separation Products). The mobile phase was methanol–toluene (45:55, v/v) or acetonitrile–toluene (45:55,v/v) at a flow-rate of 1.0 ml/min.

All chromatographic analyses were performed at ambient temperature.

A 1 ml glass-pulverizer with dual action (Touzart et Matignon, Vitry-sur-Seine, France) was used to homogenize tissues.

Absorbance measurements were carried out with a Shimadzu UV-160 A spectrophotometer (Shimadzu, Kyoto, Japan).

2.3. Animal preparation and samples

Six specific pathogen-free male Swiss mice, weighing 20±2 g (purchased from Charles Rivers, France), received 0.5 ml per 20 g body weight of SC_{60}, intraperitoneally. On days one (D_1), two (D_2) and six (D_6), two animals were sacrificed, after anesthesia with diethyl ether, for collection of blood and tissues. Blood specimens (approximately 1.0 ml) were collected by intracardiac puncture. Blood samples (100 μl per dry tube), livers and spleens were stored at −20°C until analysis.

2.3.1. Preparation of calibration graphs

Five 5 ml glass tubes were filled with aliquots of standard C_{60} solution (0.00, 0.25, 0.5, 1.0 and 2.0 ml of S_1 for liver and spleen analysis or 0.00, 0.01, 0.02, 0.05 and 0.10 ml of S_2 for blood analysis). A 1.5 ml volume of IS_1 was pipetted into each tube for tissue analysis or 0.1 ml of IS_1 was used for blood analysis, and the samples were evaporated to dryness under a stream of nitrogen. These dry residues were treated under the same conditions as the corresponding biological samples.

2.3.2. Assay of C_{60} in tissues

A 1.5 ml volume of IS_1 was pipetted into a 5 ml glass tube and evaporated under a stream of nitrogen. About 50 mg of liver (taken from the right lobe) or about 10 mg of spleen (taken from right extremity), determined accurately, was placed in a tissue pulverizer and 0.5 ml of a 0.1 *M* SDS solution was added. After homogenization, the contents of the pulverizer were transferred to a glass tube containing the internal standard (I.S.). Then the pulverizer was rinsed with 0.5 ml of acetic acid and the rinsing liquid was transferred to the same glass tube. After shaking for 2 min and sonication for 15 min, 4 ml of toluene were added and the mixture was agitated for 12 h (overnight) at room temperature in the dark. After centrifugation at 5000 *g* for 10 min, the supernatant was collected into a conical glass tube, 4 ml of toluene were added and sonication was repeated for 15 min, followed by agitation for 2 h. After centrifugation, the supernatant was collected into the same conical tube and the extraction procedure was repeated under the above conditions. After complete extraction, the color of the precipitate became milky white. Then the supernatants were evaporated under a stream of nitrogen and the dry residue was dissolved in 3 ml of toluene. Finally, the toluene solution was diluted with mobile phase (1:9, v/v) before 0.05 ml were injected into the chromatograph.

2.3.3. Assay of C_{60} in blood

To a 5 ml glass tube containing 0.1 ml of blood, a 1.0 ml volume of 0.1 *M* SDS solution was added. After shaking for 2 min, the contents was transferred to a second glass tube containing the dry residue of 0.1 ml of IS_1 or IS_2 (depending on the C_{60} concentration in the blood sample). The first tube was rinsed with 1.0 ml of acetic acid and the rinsing liquid was transferred to the second tube. After shaking for 2 min and sonication for 15 min, 3 ml of toluene were added and the mixture was agitated for 1 h at room temperature in the dark. After centrifugation at 5000 *g* for 10 min, the supernatant was collected into a conical glass tube and the extraction was repeated under the same conditions. The supernatants were evaporated to dryness under a stream of nitrogen and the residue was rinsed twice with 3 ml of methanol. The dry residue was dissolved in 0.1 ml of toluene and diluted with 2.9 ml (extract containing IS_1) or 0.2 ml (extract containing IS_2) of mobile phase before 0.05 ml were injected into the chromatograph.

2.3.4. Validation of the method

For livers and spleens, linearity, accuracy and recovery of the method were studied by spiking pulverized and homogenized samples (about 50 mg of liver sample or about 10 mg of spleen sample+ 0.5 ml of 0.1 *M* SDS), collected from untreated animals, with 0.05 ml of an appropriate dilution of the SC_{60}.

In the same way, untreated blood samples were hemolysed (0.10 ml of blood+1.0 ml of 0.1 *M* SDS) and spiked with an appropriate dilution of the SC_{60}.

3. Results

The chromatograms obtained by the LC–PDA method are shown in Fig. 1. The identity and purity of the C_{60} peak were ascertained by UV–Vis (A_1) and MS spectra (Fig. 2, A_1). Chromatograms of liver, spleen and blood samples, collected from untreated animals, did not exhibit any peak interfering with C_{60} or the I.S. (not shown here). The limit of detection of the method (signal-to-noise ratio=3) was 0.1 ng of C_{60} injected.

Fig. 2 shows MS (A) and corresponding UV (B) chromatograms of C_{60}. The MS spectra obtained from chromatograms (Fig. 2) are identical to those obtained by flow injection analysis and exhibit the usual negative ion peak at *m/z* 720, accompanied by satellites at 721, 722 and 723. The latter correspond to ^{13}C-containing C_{60} and the main peaks at 751 (A1), 760 (C) and 811 (not shown here) correspond to deprotonated negative ions formed from the addition of methanol, acetonitrile and toluene, respectively, on the C_{60}. The covalent nature of these complexes was confirmed by their extreme stability when submitted to collision-induced dissociation, either in the source or in the analyzer (MS–MS). Under our conditions, the signal-to-noise ratio is twice as high on the reconstructed mass chromatogram of the ion at *m/z* 720 than on the UV trace.

As MS spectra have definitely identified C_{60}, and as the sensitivity of the PDA mode is sufficient in

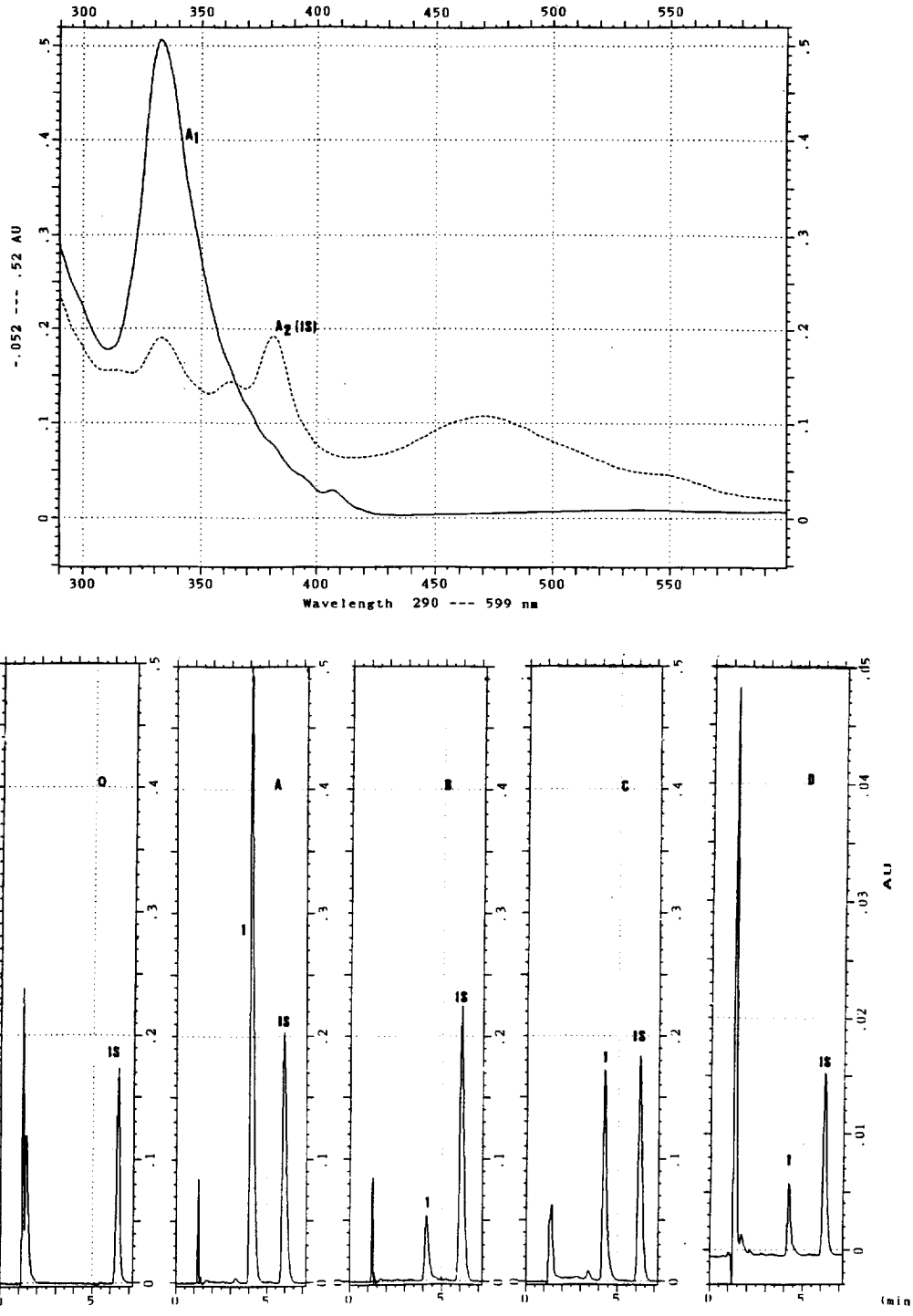

Fig. 1. Chromatograms using photodiode-array detection (λ=333 nm) of mouse tissue extracts: (○), untreated liver. (A) liver, (B) spleen, and (C) blood extracts obtained at D_1, after SC_{60} administration (0.5 ml/20 g); (D) blood extract obtained at D_6 (peak 1=C_{60}, IS=C_{70}). [A_1 and A_2=UV–Vis spectra of peak 1 and I.S. of (A), respectively].

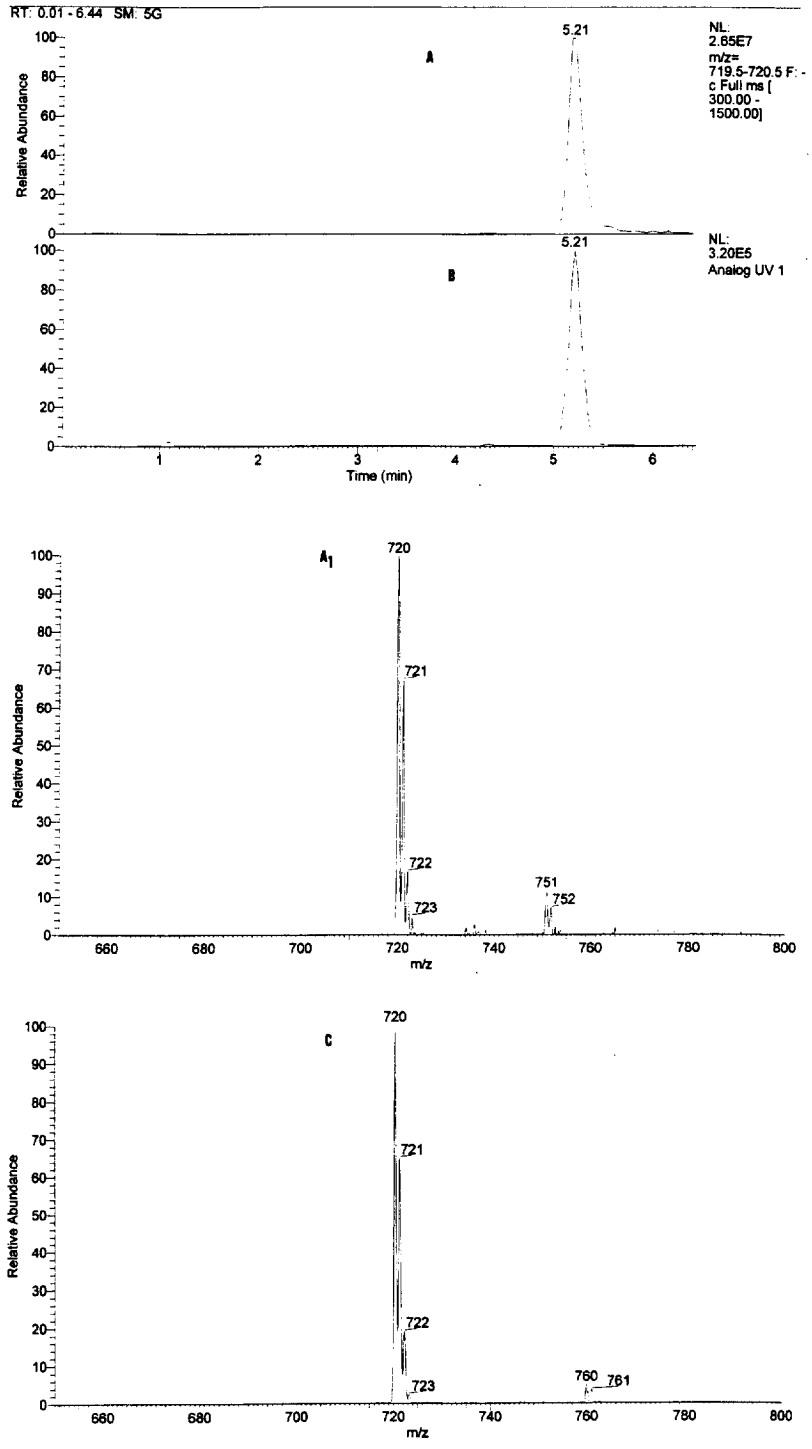

Fig. 2. Selected ion monitoring (A) and corresponding UV (B) chromatograms of C_{60}. Mass spectra obtained with a mobile phase (A_1) of methanol–toluene (45:55, v/v) or (C) of acetonitrile–toluene (42:58, v/v). (See text for chromatographic conditions).

Table 1
Precision of the method

Spiked sample	Concentration added	Concentration determined			
		Within-assay ($n=5$)		Between-assay ($n=3$)	
		Mean	R.S.D. (%)	Mean	R.S.D. (%)
$Liver_1$	0.050%	0.051%	4.6	0.048%	7.8
$Liver_2$	5.000%	5.018%	2.2	4.960%	3.9
$Spleen_1$	0.050%	0.052%	5.2	0.052%	8.1
$Spleen_2$	5.000%	5.005%	3.1	5.115%	5.3
$Blood_1$	0.100 mg/l	0.103 mg/l	4.8	0.097 mg/l	6.2
$Blood_2$	5.000 mg/l	5.020 mg/l	2.1	4.493 mg/l	4.2
$Blood_3$	200.000 mg/l	200.350 mg/l	0.8	210.060 mg/l	3.6

our case, the latter method was used to determine the concentration of C_{60} in tissues and blood.

For liver and spleen samples, the linearity of the PDA method was checked from 0.05 to 5.00% of C_{60}, expressed as the % of tissue weight ($y=0.870x+0.005$, $r=0.998$, $n=6$ for livers, and $y=0.867x+0.003$, $r=0.995$, $n=6$ for spleens, where y is the area ratio, C_{60}/IS, and x is the concentration of the C_{60} extract). For blood, the linearity was checked for two concentration ranges: (i) from 0.05 to 5.00 mg/l ($y=0.910x+0.003$, $r=0.999$, $n=6$) and (ii) from 5.00 to 200.00 mg/l ($y=0.916x+0.001$, $r=0.999$, $n=7$), where y is the area ratio, C_{60}/IS, and x is the concentration of C_{60} in blood).

The accuracy of the PDA method is shown in Table 1. The relative standard deviation (R.S.D.) does not exceed 8.1%.

The recovery of C_{60} was determined by spiking samples collected from untreated animals at two levels (0.05 and 200.00 mg/l for blood; 0.05 and 5.00% for tissues) and by comparing their peak areas with those obtained after direct injection into the chromatograph of pure solutions of the two compounds. The recovery of C_{60} ($n=3$ for both samples) was 85 and 91% for liver, 84 and 90% for spleen, and 87 and 92% for blood, respectively, for lower and upper values. Under the same conditions, the recovery of I.S. was 88% for liver, 87% for spleen and 92% for blood. The recovery calculated with respect to the I.S. for both upper and lower values exceeded 90% in all instances.

The results obtained with SC_{60}-treated mice are summarized in Table 2. Each result represents the mean of two mice. To visualize the heterogeneity of organ C_{60} distribution, C_{60} determinations were made in triplicate. R.S.D. values recorded for liver, spleen and blood were 15.2, 11.5 and 1.1%, respectively.

4. Discussion

The chromatographic separation of C_{60} from solutions is usually easy, unlike the biological sample preparation step prior to injection into the chromatograph. To extract C_{60} quantitatively from

Table 2
C_{60} concentrations in liver, spleen and blood after SC_{60} administration to mice (0.5 ml per 20 g body weight)

Day	Blood (mg/l)		Liver (% of weight)		Spleen (% of weight)	
	Mean	Interval	Mean	Interval	Mean	Interval
1	179	152–206	0.7	0.5–0.9	0.5	0.2–0.8
2	87.5	62–113	1.0	0.7–1.2	2.4	1.5–3.2
6	1.10	0.90–1.30	0.4	0.1–0.6	2.4	1.4–3.3

biological samples, such as liver, spleen and blood, several steps of sonication and stirring are needed. C_{60} is difficult to redissolve in its usual solvents when it is suspended in aqueous media. When toluene and SC_{60} are brought into contact, several hours of stirring are necessary to obtain significant concentrations of C_{60} in the organic phase. This phenomenon has already been described by Andrievsky et al. [8] for C_{60} solutions in polyvinyl pyrrolidone (PVP). The method of Santa et al. [6] for the determination of C_{60} in plasma gives yields of only 62.1%. This emphasizes the fact that extracting C_{60} from aqueous colloidal media is difficult.

Knowing that C_{60} in blood and tissues is intracellular [2], it is necessary to lyse cellular membranes by means of appropriate enzymes or with detergents, such as SDS, as we propose in our method. Once extracted, C_{60} can easily be separated from the co-extracted lipophilic substances, which are generally eluted with the solvent front.

The extraction procedure that we propose is rather time-consuming, but it is simple and accurate. The use of C_{70} as an internal standard results in greater precision.

Photodiode-array detection is sufficient to identify and to quantity C_{60} concentrations in biological media, but LC–MS will be necessary to study possible C_{60} metabolites.

The results show that the concentration of C_{60} in the different organs varies strongly from one animal to another (Table 2). Fluctuations in the injection procedure (injection site, depth of injection, etc.) may cause the variations of the SC_{60} absorption efficiency. Because of the strong individual variations and of the distribution heterogeneity of C_{60} in each organ, it will be necessary to study a great number of animals in order to get a better knowledge of the C_{60} distribution. It will also be necessary to standardize the injection procedure. This work is under way.

Nevertheless, these preliminary results show that SC_{60}, injected intraperitoneally, is well absorbed and is progressively picked up by organs that are rich in reticulo-endothelial tissue.

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