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Disposition of fullerene C60 in rats following intratracheal or intravenous administration

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Abstract

1. Fullerene C60 is used in a variety of industrial and consumer capacities. As part of a comprehensive evaluation of the toxicity of fullerene C60 by the National Toxicology Program, the disposition following intratracheal (IT) instillation and intravenous (IV) administration of 1 or 5 mg/kg b.wt. fullerene C60 was investigated in male Fischer 344 rats.
2. Following IT instillation, fullerene C60 was detected in the lung as early as 0.5 h post-exposure with minimal clearance over the 168 h period; the concentration increased 20-fold with a 5-fold increase in the dose. Fullerene C60 was not detected in extrapulmonary tissues.
3. Following IV administration, fullerene C60 was rapidly eliminated from the blood and was undetectable after 0.5 h post-administration. The highest tissue concentrations of fullerene C60 occurred in the liver, followed by the spleen, lung, and kidney. Fullerene C60 was cleared slowly from the kidney and the lung with estimated half-lives of 24 and 139 h, respectively. The liver concentration of fullerene C60 did not change much with time; over 90% of the fullerene C60 remained there over the study duration up to 168 h. Fullerene C60 was also not detected in urine or feces.
4. These data support the hypothesis that fullerene C60 accumulates in the body and therefore has the potential to induce detrimental health effects following exposure.

INTRODUCTION

Nanoscale materials, or nanomaterials, are defined as materials having at least one dimension between 1 and 100 nm (European Commission, 2011; Bleeker et al., 2013). The unique properties of nanomaterials, including strength and durability, high conductivity, and reactivity, make them ideal for use in a variety of commercial and industrial applications. Many consumer goods are manufactured using nanomaterials, and nanomaterials are also

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utilized in medical applications such as drug delivery (Gwinn and Vallyathan, 2006; Nel, et al., 2006; Kessler, 2011; Contado, 2015).

Fullerene C60, a primary allotrope of carbon, is a stable aggregate of 60 carbon atoms in the form of a truncated icosahedron and is classified as a nanomaterial (Kroto et al., 1991; Terrones and Mackay, 1992; Hirsch, 2010; Johnson et al., 2010). Fullerene C60 is generated naturally following forest fires, volcanic eruptions, and the combustion of carbon-based materials (Wang et al., 1998; Murr et al., 2004; Baker et al., 2008). Fullerene C60 has also been engineered for use in different industrial and commercial capacities, including consumer products, photovoltaics, microelectronics, water treatment, and drug delivery (Halford, 2006; Singh and Lillard, 2009; Hendren et al., 2011; Cha et al., 2013). Therefore, human exposure could occur via inhalation, dermal, and/or parenteral routes (Johnston et al., 2010). Pulmonary exposure to fullerene C60 has been shown to induce inflammation, immunotoxicity and respiratory toxicity in rodents (Baker et al., 2008; Johnston et al., 2010; Park et al., 2010; Stanley et al., 2012; Sayers et al., 2016a). Fullerene C60 has been shown to accumulate in the liver following intravenous (IV) or intraperitoneal (IP) injection, and it has been reported that fullerene C60 can both induce and improve hepatotoxicity (Gharbi et al., 2005; Djordjevic et al., 2014; Johnston et al., 2010; Bogdanovic and Djordjevic, 2016; Elshater et al., 2018). Due to the widespread use of nanomaterials in consumer applications and inadequate information on possible biological interactions and toxicity following exposure, the toxicity of fullerene C60 was comprehensively evaluated as part of an extensive assessment of nanomaterials under the National Toxicology Program's (NTP) Nanotechnology Safety Evaluation (<https://ntp.niehs.nih.gov/testing/status/agents/ts-m040089.html>).

The disposition properties of nanomaterials are important for putting toxicological findings into context. Disposition properties of nanomaterials are affected by factors such as particle size, shape, and dispersion pattern. Following pulmonary exposure, retention of nanoparticles in the lung is dependent on exposure concentration and particle size; smaller particles are retained for longer, particularly at higher exposure concentrations (Ferin et al. 1990). Aggregation state and electrostatic charge can also affect absorption and distribution following exposure (Oberdörster et al., 1994; Mercer et al., 2008). Multiple clearance mechanisms have been proposed for removal of nanomaterials from the lung, including 1) uptake of particles by inflammatory cells, which then enter systemic circulation; 2) phagocytosis of particles by macrophages and clearance via mucociliary escalator; and 3) phagocytosis by macrophages and removal via tracheobronchial and pleural lymphatic systems (Harmsen et al., 1985; Mercer et al., 2013). Baker et al. (2008) assessed the half-life and deposition rate of micro- and nano- fullerene C60 in F344 rats following 10 days of exposure (3 hours/day) via nose-only inhalation. Deposition rate and fraction were increased in rats exposed to fullerene C60 nanoparticles (55 nm diameter; 2.22 mg/m³) relative to microparticles (0.93 µm; 2.35 mg/m³). Half-life was similar between the two particles. The deposition and clearance of micro- and nano-fullerene C60 following nose-only inhalation exposure was also thoroughly investigated by Sayers et al. (2016b). B6C3F1 mice and Wistar Han rats were exposed to fullerene C60 for 13 weeks and evaluated for lung clearance and lung and extrapulmonary tissue deposition. Fullerene C60 lung burden was higher in both species following exposure to fullerene C60 nanoparticles relative to

microparticles. In rats, the half-life of fullerene C60 nanoparticles was approximately double that of the microparticles at the same exposure concentration (2 mg/m³). Fullerene C60 was present in bronchial lymph nodes but was below the limit of quantitation in the liver, spleen, blood, brain, and kidney.

Little work has been done to assess the disposition of nanomaterials following non-inhalation exposure routes. It has been proposed that fullerenes likely remain at or near the site of entry (Djordjevic et al., 2014; Johnston et al., 2010; Bogdanovic and Djordjevic, 2016). The goal of the work presented here was to evaluate the disposition of fullerene C60 after intratracheal (IT) and intravenous (IV) exposure in rats.

MATERIALS AND METHODS

Chemicals and Reagents

Fullerene C60 (CAS RN 99685-96-8, Catalog # MR713) with ~ 25% [¹³C]-fullerene C60 was obtained from MER Corporation (Tucson, AZ), along with the fullerene C70 used as an internal standard (IS). High performance liquid chromatography (HPLC)-grade acetonitrile was purchased from Fisher Chemicals (Fairlawn, NJ). Ethanol was purchased from AAPER Chemicals (Shelbyville, KY). Toluene, acetic acid, phosphate buffered saline (PBS) and Tween-80 were purchased from Sigma-Aldrich (St. Louis, MO). Ammonium acetate was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Euthasol was purchased from Delmarva Laboratories, Inc. (Midlothian, VA). Isoflurane was purchased from MWI Veterinary Supply (Meridian, ID).

Test Chemical Characterization

The test chemical was identified as fullerene C60 by liquid chromatography-mass spectrometry (LC-MS) using an Agilent 1200 HPLC (Agilent, Santa Clara, CA) LC coupled to an API-5000 mass spectrometer (MS) (Applied Biosystems, Foster City, CA). The MS was scanned from 719.5 – 740.0 Da and operated in positive ion mode. A CosmosilBuckyprep column (150 × 2 mm; Nacalai Tesque, San Diego, CA) was used with an isocratic mobile phase of 100% toluene and a flow rate of 0.2 mL/min. The column temperature was set at 40 °C. The retention time was 4.3 min.

Scanning electron microscopy (SEM) electron microscopy was done on a JEOL 840A electron microscope (JEOL, Peabody, MA) at Battelle (Columbus, OH). Transmission electron microscopy (TEM) was done on a JEOL 2010F at Lovelace Biomedical and Environmental Research Institute (LBERI). Images were analyzed for the presence of fullerene C60 and other carbon structures. Particle size was determined using a Nanotracer dynamic light scattering particle analyzer (Microtrac, Inc.; North Fargo, FL). Approximately 10 mg of fullerene C60 was suspended in 5 mL of toluene and sonicated for 1 min. The sample was analyzed with a corresponding toluene blank.

Animal Studies

Animals—All studies were conducted at LBERI. All animal procedures were approved by the LBERI Institutional Animal Care and Use Committee and were in accordance with

applicable local, state, and federal regulations. Animals were housed in a facility that was fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Male Fischer 344 (F344) rats were purchased from Charles River Laboratories (Wilmington, MA) for IT studies. For IV studies, cannulated male Fischer 344 rats were obtained from Hilltop Lab Animals, Inc. (Scottsdale, PA). All rats were 8–10 weeks old upon receipt and were quarantined for 14 days prior to being used in studies. Rats were housed two per cage in shoebox cages with hardwood bedding (SaniChip®, P.J. Murphy Forest Products Corp.; Montville, NJ) upon arrival. Animal rooms were maintained at 18–26 °C and 30–70% relative humidity with a 12-hour light/dark cycle. Air circulations were 100% fresh filtered air with 10–15 air changes/hour. Animals were fed Teklad Certified Rodent Diet (W) 8728C (Harlan Teklad; Madison, WI) *ad libitum*. Unlimited municipal water was available.

Animals were weighed and randomized prior to dosing and uniquely identified with ear tags. Rats were individually acclimated to the all-glass metabolism chambers approximately 24 hours prior to dosing. Following dosing, rats were returned to the metabolism chambers to allow for separate collection of urine and feces.

At the end of all studies, rats were administered Euthasol (390 mg sodium pentobarbital/kg and 50 mg phenytoin sodium/kg) by intraperitoneal injection to induce surgical-level anesthesia. Anesthetized rats were exsanguinated and euthanized by section of the diaphragm.

Dose Formulation Preparation—Dose formulations for IT instillation and IV administration were prepared as suspensions in phosphate buffered saline (PBS)/1% Tween 80. Formulations were continually stirred at room temperature to maintain the homogeneity of the suspension. Aliquots of the dosing solutions were extracted with toluene and analyzed by HPLC with UV detection. The concentration, stability, and homogeneity of the formulation was determined on the day of dosing.

Intratracheal Instillation of Fullerene C60—Animal weights on the day of dosing were used to determine dose volumes. Rats were administered a single dose of fullerene C60 via IT instillation at either 1 mg/kg b.wt. or 5 mg/kg b.wt. (N = 30 for each dose). An additional three rats/dose group served as vehicle controls. Prior to administration, rats were anesthetized by inhalation exposure to isoflurane (5%). Anesthetized animals were placed chest-up and hung by the upper incisors on an inclined hanging instillation platform, and an IT catheter unit was inserted into the trachea. IT doses were administered at a volume of 2 mL/kg using 1-mL Luer lock gas-tight Hamilton syringes equipped with 18-gauge, 2-inch blunt tipped IV catheter placement units.

Intravenous Administration of Fullerene C60—Animal weights on the day of dosing were used to determine dose volumes. Rats were dosed with a single dose of fullerene C60 via IV administration at 5 mg/kg b.wt. (N = 20). An additional four rats served as vehicle controls. The IV doses were administered to rats via the catheters of surgically fitted indwelling jugular vein canulae. A “HepLock” solution (1000 IU heparin and 50% dextrose

in a 1:1 mixture) was utilized to heparinize the catheter and maintain patency prior to dose administration. Doses were administered at a volume of 2 mL/kg using a syringe equipped with 23-gauge needles.

Collection of Biological Samples and Determination of Fullerene C60 by LC-MS—Urine and feces were collected at 24 h intervals into receivers cooled over dry ice up to 168 h following administration, weights recorded, and stored in the dark at approximately -20°C until analyzed.

Rats were anesthetized with Euthasol as described above. Blood samples were collected by cardiac puncture into heparinized syringes from 6 animals/IT exposure group and 4 animals/IV exposure group at 0.5, 2, 6, 24 and 168 h post-dosing. An aliquot of the blood was centrifuged, and the plasma was collected. Following blood collection, the following tissues were collected: brain, lung, heart, spleen, kidney, liver, intestine and contents. All samples were stored at -20°C until analyzed.

The concentration of fullerene C60 in blood and tissues was determined using a LC-MS method. The method used was the same as that described above for test article characterization except that fullerene C70 was used as an internal standard. The analytical method was assessed for the linearity, accuracy (% relative error, RE), precision (% relative standard deviation, RSD) and recovery in biological matrices of interest over the concentration range 1 ng/mL to 2 mg/mL, depending on the matrix. Stock solutions were prepared in toluene and diluted as needed in toluene to prepare standards in the working range. Matrix standards were prepared by spiking the respective matrix (50 μg of plasma or blood and 50 μg of other tissues) with fullerene C60 in toluene (25 μL) and the fullerene C70 internal standard (IS) (50 μL) in toluene. Tissue samples were prepared prior to extraction by freeze drying the aliquot followed by grinding using a mortar and a pestle. Five-point matrix calibration curves were prepared in the range of interest depending on the matrix. Matrix standards were extracted with 0.2 mL of toluene and the extract was analyzed by LC-MS as described below. Solvent standards were prepared in the same concentration range as the matrix standards. The limit of quantitation (LOQ) is defined as the lowest concentration of the matrix standard curve. The freeze-thaw stability of fullerene C60 was investigated only in plasma: two sets of samples were prepared and one set was assayed on the day of preparation, and the second set was stored in -80°C and assayed after undergoing a freeze-thaw cycle within 24 h. Study samples were prepared and analyzed similar to matrix standards except for the addition of the fullerene C60 standard.

Samples were analyzed via LC-MS using an Agilent 1200 HPLC (Agilent, Santa Clara, CA) LC coupled to an API-5000 mass spectrometer (MS) (Applied Biosystems, Foster City, CA). A CosmosilBuckyprep column (150 $\times$ 2 mm; NacalaiTesque, San Diego, CA) was used with an isocratic mobile phase of 100% toluene and a flow rate of 0.4 mL/min. The column temperature was set at 40°C . The MS was scanned from 719.5 – 740.0 Da for fullerene C60 and from 839.3– 843.6 Da for fullerene C70 while operated in positive ion mode. An electrospray ion source in positive ion mode was used with a source temperature of 400°C . The retention times for fullerene C60 and fullerene C70 were 2.2 and 3.4 min, respectively.

A linear regression was used to relate the LC-MS peak area response ratio of the analyte to the internal standard and concentration in the matrix. The concentration of fullerene C60 was calculated using the response ratio of the analyte to the internal standard, the regression equation, the initial sample volume and the dilution when applicable. Study samples were prepared and analyzed similar to the matrix standards. Data are presented as ng/mL for plasma and blood and µg/g for other matrices.

Partitioning of Fullerene C60 *In Vitro*

To determine the partitioning of fullerene C60 between red cells and plasma, 16 mL of whole blood was spiked with 1.25 mg fullerene C60 in PBS/1% Tween 80. Aliquots (1 mL) of whole blood were taken at 0.5, 2, 6, and 24 h post-spiking and 50 µL removed for analysis. The remaining sample was centrifuged and 50 µL aliquots of plasma and erythrocytes taken for analysis. Blood, plasma, and erythrocytes were all analyzed by LC-MS for fullerene C60 as described above.

Statistical and Toxicokinetic Analysis

All summary statistics are expressed as mean ± the standard deviation of the mean (SD). All calculations, including summary statistics, were performed using Microsoft Excel (2010) and manually rounded for reporting. Tissue concentration versus time data from the IV study was evaluated using noncompartmental analysis (WinNonlin, Version 6.4, Pharsight Corporation, Mountain View, CA).

RESULTS

Test Article Characterization

TEM and SEM micrographs of fullerene C60 suspended in toluene indicated that the fullerene C60 particles existed as both chain aggregates and more tightly condensed crystal forms (Figure 1A and 1B). These aggregate crystals were several hundred nm in diameter. Due to the high affinity of fullerene C60 particles for each other, the observed agglomeration in suspension was not surprising. The identity of fullerene C60 (molecular weight = 720 Da) was confirmed using LC-MS (Figure 2A). Nanotracer particle size distribution analysis indicated that the average size of the fullerene C60 in suspension was approximately 1 micron (1 µm) (Figure 2B).

LC-MS Method for Quantitation of Fullerene C60

Performance of methods for quantitation of fullerene C60 in plasma, excreta, and tissues were assessed and the data are shown in Table 1. Methods were linear over the concentration ranges examined ($R^2 = 0.989$). The precision and accuracy, determined as RSD and RE, were 9.8% and 15%, respectively. Fullerene C60 was stable in plasma after undergoing a freeze-thaw cycle within 24 h. Overall, analytical methods were demonstrated to be acceptable for quantification of fullerene C60 in different matrices.

Partitioning of Fullerene C60 *In Vitro*

Partitioning of fullerene C60 was determined in whole rat blood, red blood cells, and plasma spiked with 1.25 mg fullerene C60 (Figure 3). Data demonstrated preferential partitioning into red blood cells over plasma. The estimated ratio of fullerene C60 in red blood cells relative to plasma was 29:1 (0.5 h), 14:1 (2 h), 11:1 (6 h), and 8:1 (24 h).

Disposition of Fullerene C60 Following Intratracheal Instillation

The disposition of fullerene C60 was investigated following a single IT instillation of 1 or 5 mg/kg b.wt. fullerene C60. Blood and tissues were collected at 0.5, 2, 6, 24 and 168 h and excreta were collected at 24 h intervals up to 168 h post-dosing. The concentrations of fullerene C60 in tissues and excreta are given in Table 2. Fullerene C60 was detected in the lung at the earliest time point of 0.5 h and remained more or less the same at all time points examined, demonstrating little or no pulmonary clearance. The levels in the lung increased 20-fold with a 5-fold increase in the dose, showing a more than dose-proportional increase. Fullerene C60 was not detected or below the LOQ in the liver (5 ng/g), kidney (0.05 ng/g), intestine (10 ng/g), spleen (0.1 ng/g), or in urine, (250 ng/g) suggesting an absence of and/or poor distribution into peripheral tissues. A small amount of fullerene C60 (< 1% of the total administered dose) was detected in the feces and intestinal contents. The lack of pulmonary clearance and lack of apparent distribution into peripheral tissues suggests that the presence of fullerene C60 in feces and intestinal contents is likely from slow removal from the lung via mucociliary clearance and subsequent ingestion.

Disposition of Fullerene C60 Following Intravenous Administration

The disposition of fullerene C60 was also investigated following a single IV administration of 5 mg/kg b.wt. Blood and tissues were collected at 0.5, 2, 6, 24, and 168 h and excreta were collected at 24 h intervals up to 168 h post-dosing. The concentrations of fullerene C60 in tissues and excreta are given in Figure 4 and Table A1. Fullerene C60 was detectable in the blood only at 0.5 h post-administration (0.14 µg/g), with levels decreasing to below the LOQ by 2 h. The highest tissue concentrations of fullerene C60 occurred in the liver, with a maximum concentration of approximately 100 µg/g determined 2 h post-administration that was fairly unchanged by 168 h post-administration (Figure 4 and Table A1). Concentrations of fullerene C60 reached a maximum in the spleen (24 h), lung (0.5 h), and kidney (2 h), but then decreased approximately 38% (spleen), 75% (lung), and 99% (kidney) of maximum concentration by the 168 h time point (Figure 4 and Table A1). Kidney and lung concentration versus time data were analyzed by NCA; half-lives and area under the concentration versus time curves ($AUC_{0-\infty}$) estimated for kidney and lung are 24 and 139 h, and 1778 and 8505 h* µg/g, respectively. For the lung, the half-life is on the order of the time point range used in the study and hence the $AUC_{0-\infty}$ may be overestimated.

DISCUSSION

Human exposure to nanomaterials is of significant concern due to their unique physicochemical properties and use in a variety of consumer goods (Gwinn and Vallyathan, 2006; Nel, et al., 2006; Kessler, 2011; Contado, 2015). Fullerene C60 has been engineered for use in a variety of industrial and commercial applications (Halford, 2006; Singh and

Lillard, 2009; Hendren et al., 2011; Cha et al., 2013). Fullerene C60 has the potential to be aerosolized, and pulmonary exposure to fullerene C60 has been shown to induce inflammation, immunotoxicity, and respiratory toxicity in rodents (Baker et al., 2008; Johnston et al., 2010; Park et al., 2010; Stanley et al., 2012). The goal of the work presented here was to evaluate the ADME of fullerene C60 following pulmonary exposure (IT administration); IV studies were also conducted to assess the fate of fullerene C60 compared to pulmonary exposure.

[¹⁴C]-labeled test articles are typically used in ADME studies; however, [¹⁴C]-fullerene C60 was not commercially available for use in these studies. An alternative approach utilizing [¹³C]-fullerene C60 and IMS was investigated, but it lacked the sensitivity and accuracy needed for quantitation. Subsequently, an LC-MS assay was used to quantify [¹²C]-fullerene C60 in biological matrices.

Following IT administration, fullerene C60 was detected only in the lung. Pulmonary concentrations of fullerene C60 reached a maximum by the earliest time point (0.5 h) with little-to-no clearance occurring through 168 h post-administration. The higher standard deviations observed for 0.5 and 2 h time points may be due to a combination of animal to animal variability in getting the material to the lung following instillation and/or the analytical measurement variability. Despite this, the pattern of clearance in the lungs over time remained the same at both doses. Fullerene C60 has previously been shown to persist in the lung following inhalation exposure (Baker et al., 2008), and it has been proposed that uptake of fullerene C60 by alveolar macrophages is one of the reasons for this phenomenon (Fujita et al., 2009). Pulmonary retention was greater than dose proportional in rats with levels increasing 20-fold with 5-fold increase in the dose. Interestingly, levels of fullerene C60 in the feces and intestinal contents were lower in rats administered 5 mg/kg b.wt. fullerene C60 relative to 1 mg/kg. Fullerene C60 was not detected (LOQ, 1–5 ng/mL) in the tissues and urine, suggesting an absence of and/or poor distribution into peripheral tissues. This suggests that the presence of fullerene C60 in the feces and intestinal contents is likely from slow removal from the lung via mucociliary clearance and subsequent ingestion. Yamago et al. (1995) reported similar results in mice and F344 rats orally administered [¹⁴C]-lipophilic fullerene C60, where absorption and tissue distribution were minimal and the majority of the administered dose was excreted in feces.

In order to evaluate whether an IV administered dose would be distributed into peripheral tissues rats were administered a 5 mg/kg b.wt. dose and blood and tissues were analyzed. Following administration, fullerene C60 was rapidly eliminated from the blood and was undetectable after 0.5 h post-administration; concomitant with this, fullerene C60 was detected in tissues. The highest concentrations were found in the liver, followed by the spleen, lung, and kidney. Although the concentrations in other tissues decreased over time, the liver concentration didn't change; over 90% of the fullerene C60 entering the liver remained there over the study duration. The phagocytic nature of Kupffer cells and hepatocytes may have also influenced the tissue retention of fullerene C60 in the liver, as phagocytosed particles that are not fully broken down will remain in the cells, and, subsequently, the tissue. Despite the low clearance from the liver, clearance from the spleen was fairly moderate (approximately 39% between 24 and 168 h), and clearance from the

lungs and kidney was approximately 75% and 98%, respectively (Table A1). Renal excretion has been reported to be an ideal pathway for nanoparticle clearance (Longmire et al., 2012). No fullerene C60 was detected in the urine. Fullerene C60 was also not detected in feces, suggesting the absence of biliary excretion. Taken collectively, these data demonstrate the tendency for tissue retention of administered fullerene C60. This was consistent with previous work reported in the literature, as both Bullard-Dillard et al. (1996) and Yamago et al. (1995) demonstrated that [^{14}C]-fullerene C60 was quickly cleared from the blood and localized in the liver following IV exposure.

In vitro partitioning studies of fullerene C60 demonstrated that it preferentially partitions into red blood cells. This may explain the lack of fullerene C60 distribution in the brain, adipose, muscle, and other non-reticuloendothelial tissues reported by Bullard-Dillard et al. (1996). This may have also contributed to the accumulation of fullerene C60 in the spleen following IV administration, as high levels of red blood cell processing occur there. Singh et al. (2006) and Riviere (2009) have previously reported that nanomaterials are phagocytosed by cells of the reticuloendothelial system.

In conclusion, the data reported herein demonstrate that pulmonary exposure (via IT administration) resulted in deposition of fullerene C60 in the lung with minimal distribution into the systemic circulation. When in circulation, fullerene C60 was rapidly cleared from the blood and retained primarily in the liver and spleen. These data support the hypothesis that fullerene C60 accumulates in the body following repeated exposure and therefore increase the concern for potential to induce detrimental health effects following exposure.

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Appendix

Appendix Table 1:

Disposition of fullerene C60 in selected tissues in male F344 rats following IV administration of 5 mg/kg b.wt.^a

Matrix	Time (h)	Fullerene C60 Concentration ($\mu\text{g/g}$)	
		Mean	SD ^b
Lung	0.5	79	8
	2	68	15
	6	50	9
	24	31	3
	168	19	2
Spleen	0.5	38	13
	2	76	18
	6	80	27

Fullerene C60 Concentration (µg/g)			
Matrix	Time (h)	Mean	SD^b
Liver	24	93	7
	168	57	30
	0.5	85	26
	2	100	23
	6	96	92
	24	92	17
Kidney	168	97	45
	0.5	22	3
	2	28	3
	6	25	10
	24	17	10
	168	0.25	0.3

^a Additional tissues and excreta were examined but were below the limit of quantification (1-5 µg/g)

^b SD = standard deviation

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Figure 1: TEM and SEM micrographs of fullerene C60.

A) Transmission electron micrographs of fullerene C60 suspended in toluene and placed on a carbon Cu grid for imaging. B) Scanning electron micrograph of pure (99.5%) fullerene C60 at 100x and 350x magnification.

Figure 2: Physicochemical characterization of fullerene C60.

Suspensions of 1 μm fullerene C60 particles were prepared in toluene for characterization.

A) Spectral confirmation of fullerene C60 identity in a toluene solution by LC-MS using the molecular weight of C60 (720 Da). B) Nanotracer particle size distribution analysis of fullerene C60 formulations.

Figure 3: Partitioning of fullerene C60 in rat blood, plasma, and erythrocytes *in vitro*. Whole blood (16 mL, representing the blood volume of a 250 g rat) was spiked with 1.25 mg fullerene C60 and aliquoted into samples of whole blood, plasma, and erythrocytes. Fullerene C60 concentrations were evaluated in each matrix and the data is presented as mean $\pm$ SD.

Tissue Distribution of [^{13}C]-Fullerene C₆₀-Derived Radioactivity

Figure 4: Tissue distribution of fullerene C₆₀ following IV administration.

Male F344 rats were administered 5 mg/kg b.wt. [^{13}C]-fullerene C₆₀ by IV administration and tissue distribution was assessed at 0.5, 2, 6, 24, and 168 h post-exposure. Data is presented as mean $\pm$ SD. Data is shown for the lung, spleen, liver, and kidney; additional tissues and excreta were examined but were below the limit of quantification (1-5 $\mu\text{g/g}$).

Table 1.
LC-MS Analytical Method Data for Fullerene C60 in Rat Plasma, Blood, and Tissues

	Plasma	Blood	Lung	Kidney	Spleen	Liver	Intestine	Intestine Contents	Urine	Feces
Concentration Range ^a (ng/mL or µg/g)	1 – 10	5 – 25	5 – 100 250 – 2000	0.05 – 1	0.1 – 2	100 – 2	10 – 1000	50 – 2000 2000 – 20000	250 – 4000	0.1 – 2
Linearity (R ²)	0.998	0.989	0.999 0.998	0.992	0.998	0.998	0.994	0.999 0.999	0.999	0.999
Accuracy (% RE)	±13.4	±11.4	±4.2 ± 4.0	±12.0	±15.0	±15.0	±11.0	±8.0 ±5.0	±15.0	±3.5
Precision (% RSD) ^a	0.74	4.9	4.3	ND ^c	ND	ND	8.6	ND	9.8	ND
Freeze-Thaw Stability (% of T = 0)	88.6 – 101.4	ND	ND	ND	ND	ND	ND	ND	ND	ND

^a ng/mL for plasma and blood and µg/g for other matrices.

^b Values reported are based on 1-3 matrix standards.

^c Not determined.

Table 2:Disposition of fullerene C60 in male F344 rats following IT administration of 1 or 5 mg/kg b.wt.^a

Fullerene C60 Concentration (µg/g)					
Tissue	Time (h)	1 mg/kg		5 mg/kg	
		Mean	SD ^b	Mean	SD
Lung	0.5	24	67	597	84
	2	21	11	513	182
	6	24	7	543	125
	24	22	15	456	52
	168	21	8	435	104
Intestinal Contents	0.5	0.2	0.2	0.6	0.9
	2	0.8	0.5	2.3	3.2
	6	0.1	0.1	2.9	3.7
	24	0.1	0.6	0.01	0.04
	168	0.2	0.09	0.2	0.09
Feces	24	0.1	0.08	0.60	0.60
	48	0.59	0.57	0.40	0.30
	72	0.17	0.13	0.06	0.05
	96	0.1	0.3	0.09	0.02
	120	0.1	0.1	0.2	0.03
	144	0.1	0.4	0.27	0.06
	168	0.20	0.27	0.23	0.06

^a Additional tissues and excreta were examined but were below the limit of quantification (1–5 µg/g)^b SD = standard deviation