Published in final edited form as:

J Appl Toxicol. 2015 December ; 35(12): 1452–1464. doi:10.1002/jat.3110.

Distribution and biomarkers of Carbon-14 labeled fullerene C₆₀ ([¹⁴C(U)]C₆₀) in female rats and mice for up to 30 days after intravenous exposure

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Abstract

A comprehensive distribution study was conducted in female rats and mice exposed to a suspension of uniformly carbon-14 labeled C₆₀ ([¹⁴C(U)]C₆₀). Rodents were administered [¹⁴C(U)]C₆₀ (~0.9 mg /kg body weight) or 5% PVP-saline vehicle alone via a single tail vein injection. Tissues were collected at 1 hour, 1, 7, 14 and 30 days after administration. A separate group of rodents received 5 daily injections of suspensions of either [¹⁴C(U)]C₆₀ or vehicle with tissue collection 14 days post exposure. Radioactivity was detected in over 20 tissues at all time period. The highest concentration of radioactivity in rodents at each time point was in liver, lungs and spleen. Elimination of [¹⁴C(U)]C₆₀ was <2% in urine and feces at any 24 hour time points. [¹⁴C(U)]C₆₀ and [¹⁴C(U)]C₆₀-retinol were detected in liver of rats and together accounted for ~99% and ~56% of the total recovered at 1 and 30 days post exposure, respectively. The blood radioactivity at 1 hour after [¹⁴C(U)]C₆₀ exposure was four-fold higher in rats than in mice; blood radioactivity was still in circulation at 30 days post [¹⁴C(U)]C₆₀ exposure in both species (<1%). Levels of oxidative stress markers increased by 5 days after exposure and remained elevated, while levels of inflammation markers initially increased and then returned to control values. The level of cardiovascular marker vWF, increased in rats, but remained at control levels in mice. This study demonstrates that [¹⁴C(U)]C₆₀ is retained in female rodents with little elimination by 30 days after i.v. exposure, and leads to systemic oxidative stress.

Keywords

Carbon-14 labeled C₆₀ ([¹⁴C(U)]C₆₀); female rodents; species variation; fullerenes; In vivo disposition; oxidative stress; cytokines; metabolomics analysis

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INTRODUCTION

New and growing applications of engineered nanomaterials in food, biomedical and consumer products lead to increasing intentional human exposures to nanomaterials. However, our understanding of the distribution, fate and biological impact of nanomaterials is often lagging behind their development. Important components in estimating risks associated with exposure include understanding the uptake, distribution, elimination, mode of action, and pharmacodynamics of nanomaterials. One engineered nanomaterial incorporated in both pharmaceutical and consumer applications is fullerene C₆₀. C₆₀ was discovered in 1985 and has gained prominence due to its highly unsaturated structure and excellent electron-receptor properties (Kroto, et al. 1985). However, one challenge for biomedical application is the insolubility of pure C₆₀ in physiologically compatible media which means that the surface of C₆₀ particles needs to be modified, for example by polymer enwrapping to increase their bioavailability and distribution (Tong, et al. 2011, Torres, et al. 2011) or by covalent modification such as hydroxylation (Grebowski, et al. 2013). With the increasing use of C₆₀, concerns regarding its possible health effects and risks have been raised. A fundamental understanding of the distribution over longer periods of time and biological impacts of C₆₀ is needed to assess the potential health for any effects and risks connected with human exposure to this nanomaterial.

Several studies have investigated the pharmacokinetics and biological impacts of C₆₀ in vivo, using different forms and formulation of C₆₀ as well as different routes of administration, such as oral delivery of a mixture of partially dissolved C₆₀ in corn oil (Takahashi, et al. 2012), pulmonary exposure to C₆₀ water suspension or polyvinylpyrrolidone (PVP)-formulated C₆₀ via intratracheal delivery (Sayes, et al. 2007, Thompson, et al. 2014) or aerosol inhalation of nano- and microparticles of C₆₀ (Baker, et al. 2008), and intravenous (i.v.) injection of an aqueous suspension C₆₀ or of PVP formulated C₆₀ (Bullard-Dillarda, et al. 1996, Kubota, et al. 2011, Sumner, et al. 2010, Thompson, et al. 2014, Vidanapathirana, et al. 2014). Orally administered C₆₀ formulated in corn oil at a dose ranging between 1–1,000 mg/kg/day showed little, if any, toxicity in vivo as shown in a study over a period of 29 days followed by a 14 days recovery (Takahashi, et al. 2012). Only discoloration of feces, stomach and large intestine for the highest exposure group of 1,000 mg/kg/day, and a slight increase in liver and spleen weights were observed after the recovery period (Takahashi, et al. 2012). Intratracheally instilled C₆₀ in rats showed limited toxicity up to 3 months post instillation (Sayes, et al. 2007). However, the long half-life of C₆₀ in the lungs after a 10 days inhalation exposure of 2.22 mg/m³ nanoparticle or 2.35 mg/m³ microparticles C₆₀ (26 and 29 days, respectively) raises the question of systemic uptake and distribution of C₆₀ and possible biotransformation. In vivo biotransformation of C₆₀ has been observed in the livers of mice administered micronized C₆₀ via intraperitoneal injection 7 days after administration. C₆₀-adducts of retinol and of retinyl-palmitate were isolated, providing evidence that C₆₀ is capable of undergoing transformation in vivo by the mouse (Moussa, et al. 1998, Tsuchiya, et al. 1996).

To understand the distribution of C₆₀ several injection studies have been conducted (Bullard-Dillarda, et al. 1996, Kubota, et al. 2011, Sumner, et al. 2010). When C₆₀ prepared and suspended in water was i. v. injected in rats it was rapidly cleared from the blood stream and

migrated to the liver, where it was detected for the 120 hours of the study (Bullard-Dillarda, et al. 1996). While different formulation of C₆₀ have been used, the overall results show a rapid clearance of C₆₀ from blood (Kubota, et al. 2011, Sumner, et al. 2010), and have indicated accumulation of C₆₀ in the liver prior to its accumulation in the spleen (Bullard-Dillarda, et al. 1996, Kubota, et al. 2011, Sumner, et al. 2010). In a time-dependent distribution study of C₆₀-liposome solution over 28 days, the levels of C₆₀ in the lungs and liver decreased between day 1 and day 28, whereas levels in the spleen over all increased in the same period of time (Kubota, et al. 2011). The same study also observed small amounts of C₆₀ in the kidneys and brain for the first seven days following exposure, but after 14 days the levels of C₆₀ were greatly diminished, to below detection limits in brain tissue (Kubota, et al. 2011). In a limited in vivo investigation, Tsuchiya et al. reported that C₆₀ in PVP administered to pregnant mice on gestation day 10 caused embryo lethality at the highest dose (137 mg/kg i.p.), and abnormalities at lower doses (Tsuchiya, et al. 1996). Even though C₆₀ has been shown to have low toxicity in vivo; the long half-life and low clearance of accumulated C₆₀ in tissues like liver, lungs and spleen does raise concerns of potential long term health effects and risks, as well as possible neonatal risks and birth defects. A distribution study (Sumner, et al. 2010) of [¹⁴C(U)]C₆₀ formulated in 5% PVP-saline suspension in pregnant rats showed radioactivity in the placenta (2.21%±0.21 of administered dose), which crossed the placental barrier and enters the fetuses (0.87%±0.21). Moreover, radioactivity was distributed from the lactating dam via the milk to the pup where [¹⁴C(U)]C₆₀ was detected in their gastrointestinal tract (2.75%±0.012 after 24 hours and 4.37%±0.52± after 48 hours) and liver (0.063%±0.04 after 24 hours and 0.051%±0.02 after 48 hours) (Sumner, et al. 2010). Together these studies support a concern for the long term health effects and risks associated with C₆₀ regardless of formulation and route of exposure, and emphasize the need to better understand the distribution and migration of C₆₀ over long periods of time.

Herein we report the results of a comprehensive investigation of the distribution of PVP-formulated [¹⁴C(U)]C₆₀ (suspended in saline to form a 5% PVP-saline suspension) in female rats and mice after administration of a single or 5 consecutive daily tail vein injections. The intravenous route was chosen as to determine tissue distribution and elimination profile of C₆₀ in two rodent species following single or multiple injections. Biomarkers of inflammation, cardiovascular injury and oxidative stress were measured to study the biological impact of C₆₀ exposure. These results are basic to an understanding of the distribution, migration, elimination and biological impacts of [¹⁴C(U)]C₆₀ in rats and mice.

METHODS

Preparation of [¹⁴C(U)]C₆₀ and dose solution

The [¹⁴C(U)]C₆₀ was prepared using the Kratschmer and co-workers (Kratschmer, et al. 1990) plasma arc discharge method as modified by Scrivens and Tour (Scrivens, et al. 1994). [¹⁴C(U)]C₆₀ was isolated by exhaustive extraction of the soot with toluene, followed by column chromatography on alumina, eluting with hexane. Final purification was by preparative high-performance liquid chromatography (HPLC) (Cosmosil Buckyprep, toluene

1 mL/min, λ 340nm). HPLC with UV and radiochemical detection were used to determine the chemical purity (98.4%) and radiochemical purity (96.3%). The specific activity was calculated using a Beer's Law dose response curve (HPLC) generated using sublimed C₆₀ (#379646, Sigma Aldrich, St. Louis, MO) to determine the C₆₀ content of a counted aliquot.

The dose formulation was prepared using a modification of the of Yamakoshi and co-workers procedure (Yamakoshi, et al. 1994). Specifically, a solution was prepared by sonicating [¹⁴C(U)]C₆₀ (2.52. mg) in toluene (3.15 mL) until dissolved, adding a solution of Polyvinylpyrrolidone (PVP, Cat. #: 234257, Lot: MKBD2366V, Aldrich, St. Louis, MO) (314.6 mg) in CHCl₃ (Cat #: 048-4L, Lot AX767, B&J, Morristown, NJ) (6.292 mL) and stirring for 30 min. The volatiles were evaporated under N₂ and the residual solid was kept in a vacuum dessicator (Edwards Model RV5, Crawley Sussex, England) under high vacuum overnight.

Dosing solution

All glassware was silanized prior to use. The dose formulation was prepared by the addition of saline (0.9% injectable, Lot J7H025, B. Braun Medical Inc., Irvine, CA) (18.0 mL) to the dried solid was sonicated for 2 minutes resulting in a suspension of. 0.14 mg/mL [¹⁴C(U)]C₆₀ with a specific activity of 5.5 mCi/mmol or 7.23 μ Ci/mg in 5% PVP-saline. [¹⁴C(U)]C₆₀ will henceforth be used to describe 5% PVP-saline suspensions of [¹⁴C(U)]C₆₀; vehicle control refers to the suspension of 5% PVP in saline.

Characterization of dosing solution

The hydrodynamic diameter of [¹⁴C(U)]C₆₀ in such suspensions, prepared based on the Yamakoshi protocol as described was determined using a Malvern Zetasizer NanoZS (Malvern Instruments, Worcestershire, UK) with a 633 nm laser source, 173° detection angle, and a clear disposable cell. To mimic the dosing conditions measurements were made 30 min after preparation of the saline suspension and each suspension was sonicated 5 sec and vortexed 5 sec prior to transfer to the cell. Three independent measurements were conducted. The hydrodynamic diameter and the polydispersity index (PDI) of the particles in the suspension were obtained using CONTIN algorithm. Further characterization by transmission electron microscopy (TEM) was obtained using an FEI Tecnai G2 Twin (Hillsboro, OR, USA) high resolution TEM. The sample was prepared by dipping TEM copper grids (prod#01822, Ted Pella, Redding, CA) followed by thorough drying in a well-ventilated fume hood before imaging.

Housing and Dosing of Female Rats and Mice

Sprague Dawley rats and C57BL/6 mice were obtained from Charles River, Raleigh, NC. Female rats arrived at 10 weeks of age and female mice arrived at 8 weeks of age. Rats and mice were housed in microisolator cages, with a 12/12 light/dark lighting cycle and provided food (Purina rodent chow 5002) and water ad libitum.

Rats and mice were dosed and sacrificed after 1 hour, 1 day, 7 days, 14 days, or 30 days. Rats and mice received a single tail vein injection of ~0.9 mg [¹⁴C(U)]C₆₀/kg body weight, formulated to afford 5% PVP-saline, and one group (n=3) of each species received an

additional dose for the next four days, for a total of five consecutive doses. Rats and mice were placed individually in all glass metabolism cages for 7 days prior to termination and 24 hour urine and feces were collected over dry ice. Urine and feces were stored at -80°C .

Necropsy and Sample Collection and Processing

Female rats and mice were sacrificed under CO_2 and blood was drawn by cardiac puncture. An aliquot of whole blood was reserved and the remainder was processed to plasma as necessary. During necropsy, utensils were cleaned with a delicate task wiper following each tissue removal. The liver, spleen, kidney, adipose near kidney (hereafter referred to as perirenal adipose), lung, heart, brown fat, brain, lymph nodes, uterus (with cervix and uterine horns), ovaries, bladder, stomach, cecum, small intestine, large intestine, digestive content, muscle (hindleg), pancreas, skin, bone, bone marrow, mammary tissue/gland, and residual carcass were collected. Wet weights of all samples were recorded at the time of collection.

Radioactivity Distribution

Radioactivity was determined using a Packard 1900CA Liquid Scintillation Counter (LSC). Urine aliquots were weighed into scintillation vials containing scintillation cocktail. Feces, carcass and digestive tract were homogenized in 2N ethanolic sodium hydroxide or water (feces only). Tissues were dissolved with Soluene-350TM, bleached by addition of perchloric acid and hydrogen peroxide and counted after addition of scintillation cocktail. Control tissue was analyzed as described above to determine background counts.

Radioactivity limit of quantitation was designated at three-fold above background. Radioactivity was calculated as percentage of the administered dose recovered and as concentration, ng/g tissue. Blood volume in rats was estimated to be 7.4% of body weight based on the literature and 4.9% in mice (Brown, et al. 1997). Adipose tissue was assumed to be 7.2% of body weight in rats and 5.9% in mice (Brown, et al. 1997), muscles was assumed to be 40.4% in rats and 38.4% in mice, bones 7.3% in rats and 11.2% in mice, and skin 19.0% in rats and 16.5% in mice (Brown, et al. 1997).

C₆₀ and C₆₀-retinol detection using LC-MS/MS

Synthetic C₆₀-retinol was used as standard to confirm the presence of C₆₀-retinol in the in vivo samples. Synthesis of C₆₀-retinol adduct was carried out as previously described (Moussa, et al. 1998). Briefly, C₆₀ and retinol were dissolved in 1,2-dichlorobenzene and stirred for 24 hours in the dark. The resulting reaction mixture was isolated via precipitation in acetonitrile, dried and dissolved in toluene. The formation of C₆₀-retinol was confirmed using high resolution mass spectrometry (Orbitrap Velos, Thermo Scientific) and NMR spectroscopy.

Rodent livers (100 mg) were homogenized in 1 mL of 0.9% aqueous NaCl solution using a sonic probe. Liver [¹⁴C(U)]C₆₀ and [¹⁴C(U)]C₆₀-adduct were extracted from homogenates using previously described method (Moussa, et al. 1998). The dried extracts were reconstituted in 50:50 acetonitrile:toluene and a 10 μL aliquot was injected into the LC-MS system for the analysis. A blank liver spiked with [¹⁴C(U)]C₆₀ in toluene was homogenized

and extracted in parallel to validate the recovery of [$^{14}\text{C}(\text{U})\text{C}_{60}$] and as a control for potential [$^{14}\text{C}(\text{U})\text{C}_{60}$]-adduct formation during the homogenization and extraction process. Similarly, 100 μL aliquots of urine samples were extracted and analyzed using LC-MS for [$^{14}\text{C}(\text{U})\text{C}_{60}$] and [$^{14}\text{C}(\text{U})\text{C}_{60}$]-adducts. All LC-MS analyses were performed using Waters Acquity UPLC (Waters Corporation) coupled with an Orbitrap Velos (Thermo Scientific) mass spectrometer. [$^{14}\text{C}(\text{U})\text{C}_{60}$] and [$^{14}\text{C}(\text{U})\text{C}_{60}$]-adducts extracted from liver were separated on a LiChroCART 125-4 column using acetonitrile and toluene as mobile phase solvents. The starting HPLC condition was 60:40 acetonitrile:toluene at a flow rate of a 0.5 mL/min with a linear gradient to 5:95 acetonitrile:toluene over the course of 13 minutes, returning to initial the condition in the last 3 minutes of a 16 min LC-MS run. MS and MS/MS data were acquired in negative APCI mode with FTMS as the detector. The following instrumental setting were used for data acquisition: needle voltage at -6.0 kV , APCI vaporizer temperature at $450\text{ }^{\circ}\text{C}$, capillary temperature at $350\text{ }^{\circ}\text{C}$, sheath gas at flow at 50 units and auxiliary gas flow at 5 units. A full MS scan over 700–2000 m/z window was acquired, followed by MS/MS data on the 6 most intense ions from the MS scan.

Analysis of Cytokines, Chemokines and Markers of Cardiovascular Injury

Analysis of cytokines, chemokines and markers of cardiovascular injury levels was conducted using plasma samples collected from rats or mice following exposure to [$^{14}\text{C}(\text{U})\text{C}_{60}$] in 5% PVP-saline or 5% PVP-saline vehicle. Plasma concentrations of eotaxin, interleukins; IL-1 β , IL-4, IL-5, IL-6, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNF- α), vascular endothelial growth factor (VEGF), were quantified utilizing Milliplex MAP Cytokine/Chemokine and Cardiovascular Immunoassay panels (RECYTMAG-65K and MPXMCYTO70K, EMD Millipore, Billerica, MA, USA). Plasma concentrations of plasminogen activator inhibitor (PAI)-1, von Willebrand factor (vWF), soluble intercellular adhesion molecule 1 (ICAM-1) and soluble e-selectin were measured quantitatively with a Milliplex MAP Cardiovascular Immunoassay Panel (RCVD1-89kMCVD77K and MCVD177AK, EMD Millipore, Billerica, MA, USA). All assays plates were run according to the manufacturer's protocol with samples run in duplicate. Assays results were analyzed using a Luminex 200 (Luminex, Austin, TX, USA) and results reported using Luminex xPONENT® software version 3.1. Results are reported as mean $\pm$ sem; statistical comparison between experimental groups was assessed by ANOVA using GraphPad Prism 5.0 (San Diego, CA) and difference were considered significant if $p < 0.05$.

Detection of 8-Oxo-2'-deoxyguanosine (8-OH-dG) in Urine

A commercially available "New 8-OH-dG Check" ELISA kit from JAICA (Japan, Supplier: Genox, MD, USA) was used in the study to determine the levels of 8-OH-dG in rat urine samples obtained from rats administered [$^{14}\text{C}(\text{U})\text{C}_{60}$] in 5% PVP-saline or 5% PVP-saline vehicle. The assays were performed following the instructions of the manufacturer (JAICA, Japan). Urine samples were centrifuged for 10 min at 5000 $\times g$ and the clarified urine samples were diluted five-fold. 50 μL of the diluted samples was used in the assay. Statistical analyses were performed using a Wilcoxon matched pairs test using GraphPad Prism 5.0 (San Diego, CA) and difference were considered significant if $p < 0.05$.

Metabolomics Analysis of Urine

Samples for NMR metabolomics analysis were prepared by mixing an aliquot (540 μ L) of rat or mouse urine with 60 μ L of Chenomx ISTD solution (Chenomx, Edmonton, Alberta, Canada) containing two internal standards [4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) and imidazole] used for line shape analysis and chemical shift referencing, pH determination, and quantifying using the library in NMR Suite 7.1 Professional software (Chenomx, Edmonton, Alberta, Canada). 500 μ L of the NMR sample was transferred into 5mm NMR tubes. ^1H NMR spectra were acquired using a Bruker Avance III 950 MHz NMR spectrometer (located at the David H. Murdock Research Institute at Kannapolis, NC, USA) using the first increment of a NOESY sequence (Beckonert, et al. 2007), with a 100 ms mixing time, 2 s relaxation delay, a spectral width of 16 ppm, 32k data points, and 128 transients. The water resonance was suppressed using resonance irradiation during the relaxation delay and mixing time. All spectra were acquired at 25°C, zero filled, and Fourier transformed after exponential multiplication with line broadening factor of 0.5. Phase and baseline of the spectra were manually corrected for each spectrum. The quality of each NMR spectrum was assessed for the level of noise and alignment of identified markers.

Metabolomics analysis was conducted as previously described (Church, et al. 2014, Pathmasiri, et al. 2012, Sumner, et al. 2009, Sumner, et al. 2010, Sumner, et al. 2010). Briefly, NMR data were processed by automated integration (increments of 0.04 ppm) over the spectral window (binning), excluding the region of water suppression, and DSS signal and each of the bins were normalized to the total integral of the bins of each spectrum. NMR binned data (bin region and integral value) were transported for data reduction and visualization using SIMCA-P+ 12.0 (Umetrics, Umeå, Sweden). Principal component analysis (PCA) and orthogonal partial least squares projection to latent structures discriminant analysis (OPLS-DA) were conducted using SIMCA-P+ 12.0 for the binned data. Data were preprocessed by mean centering and Pareto scaling prior to multivariate data analysis. Loadings plots and variable importance for projection plots were examined to determine the bins that best define the separation of study groups. Chenomx software was used to determine markers associated with specific bins that best differentiate the group were entered into a pathway mapping software package GeneGo 5.0, MetaCore (St. Joseph, MI). Student's t-Tests were conducted on the binned data to determine the statistical significance of the bins responsible for the separation of the study groups.

RESULTS

Characterization of dosing solution

The C_{60} in 5% PVP-saline suspensions were found to consist of uniformly dispersed particles with normal size distribution, and an average hydrodynamic diameter of 615- 1,718 nm. Likewise prepared blank dose formulations (5% PVP in saline) were recognized by dynamic light scattering (DLS) as unstable suspensions, presenting as large and sedimenting particles not suitable for cumulant analysis; the hydrodynamic diameter (Z-average) of the particles was 2,372 nm with a PDI of 0.306. TEM of C_{60} in 5% PVP-saline suspensions showed particles (Figure 1a). A count of 22 separate C_{60} in 5% PVP-saline suspension

agglomerates showed an average particle size of 26 ± 7 nm (Figure 1c). TEM images of vehicle did not show any particles (Figure 1b).

Distribution of radioactivity in female rats

Percentage of Recovered Radioactivity (Table 1)—Nearly 85% of the administered radioactivity was recovered in the tissue, excreta and carcass digests obtained from female rats. For rats, at 1 hour after [$^{14}\text{C}(\text{U})$]C₆₀ exposure, the highest levels of recovered radioactivity were in liver (34%), blood (27%), lung (12%), muscle (10%), spleen (6%), and skin (5%). Levels at least 3-fold above background were also detected in perirenal adipose, bladder, kidney, brain, heart, digestive tract, bone, ovaries, uterus/vagina/cervix, mammary tissue, lymph nodes and pancreas (Table 1). Radioactivity increased in the liver (55%) and spleen (10%) at 1 day after exposure, and decreased in the blood (6%) and muscle (3%). The radioactivity distribution at 7, 14, and 30 days after exposure were similar to the 1 day results. Rats administered [$^{14}\text{C}(\text{U})$]C₆₀ for 5 consecutive days with sample collection on day 14, had a distribution profile similar to that observed in the 14 days single dose study. Less than 2% of the administered [$^{14}\text{C}(\text{U})$]C₆₀ was excreted in feces and urine in any 24 hour time period. Plasma radioactivity level was nearly two times that measured for blood. Because plasma constitutes approximately $\frac{1}{2}$ of the blood volume, the potential for [$^{14}\text{C}(\text{U})$]C₆₀ binding to or uptake into red blood cells is considered negligible.

Tissue Concentrations (ng/g) (Table 2)—The concentration of [$^{14}\text{C}(\text{U})$]C₆₀ in the rat (ng/g tissue) 1 hour post exposure was highest in the spleen (10,693 ng/g), and lung (8,038 ng/g), with lower concentration in liver (2,727 ng/g), marrow (718 ng/g), digestive tract (404 ng/g), heart (288 ng/g), kidney (278 ng/g), lymph nodes (274 ng/g) and ovaries (206 ng/g) (Table 2). After 1 day the concentration of [$^{14}\text{C}(\text{U})$]C₆₀ decreased in kidney (134 ng/g), heart (91 ng/g) and brain (from 44 ng/g, after 1 hour to 13 ng/g). The concentration of [$^{14}\text{C}(\text{U})$]C₆₀ in blood was highest at 1 hour after exposure (1,035 ng/g), decreasing rapidly at 24 hours (177 ng/g), and continuing to decrease over the next 30 days (20 ng/g). On average the concentration in lymph nodes and ovary increased 2–5 fold between 1 day and 30 days after exposure. The concentrations in the digestive tract (stomach, cecum, intestine and digestive content), bone, marrow, reproductive tract were similar across all time points. On average, the concentration of radioactivity in brown fat was higher than perirenal adipose at each time point.

Distribution of radioactivity in female mice

Percentage of Recovered Radioactivity (Table 3)—Approximately 74% of the administered radioactivity was recovered in the excreta and in the tissue and carcass digests obtained from female mice. Female mice showed a different distribution when administered [$^{14}\text{C}(\text{U})$]C₆₀ through tail vein injections than female rats. Similar to rats at 1 hour after [$^{14}\text{C}(\text{U})$]C₆₀ exposure, mice had the highest level of recovered radioactivity in liver (32%). However, the rank order of recovered radioactivity in mice was lung (20%), muscle (19%), blood (8%), skin (6%), bone (5%), perirenal adipose (4%) and spleen (3%). Levels above background were detected in bladder, kidney, brain, heart, digestive tract, ovaries, uterus/vagina/cervix, mammary tissue, lymph nodes and pancreas (Table 3). On average the recovered radioactivity increased in the liver by 24 hours (44%) and continued to increase in

spleen over the 30 day study period (65%). Likewise, the percentage of recovered radioactivity increased over 30 days (8%). The radioactivity decreased over 30 days in the muscle (2%) and lungs (11%). Mice administered 5 consecutive [$^{14}\text{C}(\text{U})$] C_{60} injections showed a two-fold increase in accumulation in the spleen (11%) compared to a single dose (5%) after 14 days. Less than 2% of the recovered radioactivity was excreted in urine and feces in a 24 hour time period.

Tissue Concentrations (ng/g) (Table 4)—The concentration of [$^{14}\text{C}(\text{U})$] C_{60} (ng/g tissue) in the mice, in contrast to rats was highest in lungs (4,270 ng/g), spleen (1,711 ng/g), liver (1,300 ng/g), blood (279 ng/g), ovaries (163 ng/g), perirenal adipose (159 ng/g), lymph nodes (155 ng/g) and heart (151 ng/g) (Table 4). On average 24 hours after exposure the concentration decreased in the kidney, brain, heart, lungs, uterus/vagina/cervix and brown fat and stayed below the concentration recovered at 1 hour.

Detection of [$^{14}\text{C}(\text{U})$] C_{60} Biotransformation in Female Rat by LC-MC/MS Analysis

Good chromatographic separation of C_{60} and C_{60} -retinol was obtained in spiked rat liver homogenate (Figure 2). Both C_{60} and C_{60} -retinol were detected in the negative ion mode as radical anions, and were confirmed using the measured accurate masses of 719.999 and 1,006.228, respectively (Figure 2). Collision induced dissociation (CID) of the 1,006.228 peak, gave a product ion at 720.00 m/z and further confirmed the identity of the C_{60} -retinol adduct. This identification and confirmation of C_{60} -retinol is consistent with the literature (Moussa, et al. 1998). During LC-MS/MS analysis other parent ions appeared to be C_{60} -adducts as they fragmented to give C_{60} radical anion with a mass spectrometric peak at 720.00 m/z; however, using a series of synthetic standards, all of these adducts were confirmed to have formed at the source of the mass spectrometer and not to be present in the *in vivo* samples. Adducts observed to have formed at the source of the mass spectrometer included C_{60} -O (735.99 m/z), C_{60} -acetonitrile (760.01 m/z), C_{60} -toluene (812.06, and C_{60} -retinol-O (990.26).

Extracts of liver collected 1 day and 30 days after exposure to a single tail vein injection of [$^{14}\text{C}(\text{U})$] C_{60} showed the presence of [$^{14}\text{C}(\text{U})$] C_{60} and [$^{14}\text{C}(\text{U})$] C_{60} -retinol. The [$^{14}\text{C}(\text{U})$] C_{60} measured via LC-MS accounted for ~ 99% and ~56% of the radioactivity measured for the liver homogenate by LSC at 1 day and 30 days after exposure, respectively. The chromatographic peak area analysis of [$^{14}\text{C}(\text{U})$] C_{60} and [$^{14}\text{C}(\text{U})$] C_{60} -retinol indicated the presence of small amounts of [$^{14}\text{C}(\text{U})$] C_{60} -retinol compared to [$^{14}\text{C}(\text{U})$] C_{60} . At 1 day after exposure [$^{14}\text{C}(\text{U})$] C_{60} -retinol peak area was $5.4 \pm 0.1\%$ of [$^{14}\text{C}(\text{U})$] C_{60} total peak area, which decreased to $3.3 \pm 0.8\%$ at 30 days after exposure. [$^{14}\text{C}(\text{U})$] C_{60} -retinol peak area in liver from rats that had five consecutive doses of [$^{14}\text{C}(\text{U})$] C_{60} was 15% of [$^{14}\text{C}(\text{U})$] C_{60} total peak area.

Biomarkers of Inflammation, Cardiovascular Injury and Oxidative Stress after [$^{14}\text{C}(\text{U})$] C_{60} Exposure

Cytokine and chemokine expression—Eight cytokines and chemokines were evaluated in plasma from female rats and mice post i.v. administration of [$^{14}\text{C}(\text{U})$] C_{60} in 5% PVP-saline or 5% PVP-saline vehicle alone (Table 5 and 6).

For rats, an initial increase was measured in the levels of cytokines IL-4, IL-5 and IL-6 at 1 hour and 1 day following [$^{14}\text{C}(\text{U})\text{C}_{60}$] exposure; all returned to background levels by 30 days post exposure (Table 5). On average levels of the chemokines eotaxin, MCP-1, and VEGF decrease by 30 days post exposure compared to the 5% PVP-saline vehicle. The decrease was statistically significant for MCP-1 at 1 and 30 days post exposure, and for VEGF at 1 hour, 7 days and 14 days post exposure.

The response of IL-5 in mice was similar to that in rat, showing an average initial increase at 1 hour and at 1 day post exposure and then a decrease to levels similar to those of vehicle (Table 6). The level of the chemokine MCP-1 was significantly increase at 1 hour post exposure, and on average remained elevated compared to the level in vehicle-treated rats.

Markers of cardiovascular injury—vWF was significantly elevated in rats receiving 5 consecutive doses of [$^{14}\text{C}(\text{U})\text{C}_{60}$] compared with a single dose of vehicle, while PAI-1 decreased. For mice, levels of ICAM-1, PAI-1 and E-selectin were similar at all time points.

Levels of vWF, but not PAI-1, were increased in female rats at each time point after [$^{14}\text{C}(\text{U})\text{C}_{60}$] exposure (Table 7), while levels of ICAM-1, PAI-1 and e-selectin in mice were similar to those in rats exposed to vehicle (Table 8). The changes for markers of cardiovascular injury were only significant in rats receiving 5 daily consecutive doses.

Oxidative stress—The marker for oxidative stress, 8-oxo-2'-deoxyguanosine (8-OH-dG), which forms in the nuclear and mitochondrial DNA and is one of the predominant forms of free radical-induced oxidative stress, was measured in urine samples. On average, the level of 8-OH-dG was found to increase in female rats by 7 days post-dose and remaining elevated for up to 21 days post-dose. The differences did not test significant, but the trend showed an elevation in oxidative stress markers (Figure 3).

Metabolomics Analysis of [$^{14}\text{C}(\text{U})\text{C}_{60}$] Exposure

Urine from mice and rats administered [$^{14}\text{C}(\text{U})\text{C}_{60}$] or vehicle was analyzed using NMR metabolomics. While the urinary metabolomics expressed in rats exposed to [$^{14}\text{C}(\text{U})\text{C}_{60}$] at day 1 post exposure were closely clustered with the vehicle in PCA score plots, female rats exposed to [$^{14}\text{C}(\text{U})\text{C}_{60}$] at day 7, 14, and 21 post exposure were clustered separately (Figure 4). Analysis was conducted to determine any metabolites that best differentiated the 7 days post exposure group from the vehicle group. Twenty-three metabolites that distinguish these two groups were identified in urine; of these the levels of 6 metabolites were increased and those of 17 were decreased as a result of [$^{14}\text{C}(\text{U})\text{C}_{60}$] exposure (Table 9). Of the ten most significant pathways tricarboxylic acid (TCA) cycle, involving three of the metabolites; citrate, cis-aconitate and 2-oxoglutarate, was second to nicotine signaling in cholinergic neurons (intracellular, cytosol and extracellular choline), followed by muscle contraction regulation of (endothelial nitric oxide synthase) eNOS activity in endothelial cells (extracellular, cytosol and cytoplasm L-arginine) (Figure 5a). Three metabolites (L-arginine, choline and benzoic acid) were identified to be involved in Nrf-2 signaling pathway using GeneGo, 3-step removed analysis (Figure 5b). A four step removed GeneGo analysis showed the involvement of eight of the metabolites (2-oxoglutarate, arginine, benzoate, choline, glucose-6-phosphate, glutamate, N-nitrosodimethylamine, and proline).

DISCUSSION

To provide data for modeling of risks associated with C₆₀ exposure, the distribution of C₆₀ in female rats and mice was compared. Female rats and mice were exposed, via tail vein injection, to [¹⁴C(U)]C₆₀ (formulated as a 5% PVP suspension in saline) or to 5% PVP saline vehicle. Samples were collected for distribution analysis for up to 30 days post exposure.

Both species had the highest percentage of recovered radioactivity in the liver. The rank order for distribution at one hour post exposure, expressed as a percent of total recovered radioactivity in rats was liver>blood>lungs>muscle>spleen>skin, while the rank order in mice was liver>lungs>muscle>blood>skin>bone>spleen. The higher percentage of recovered radioactivity in the blood for rats, compared to mice, is likely attributed to a faster clearance of circulating compounds by the mice by 1 hour post exposure. Differences in distribution between species diminished, but persisted through the study, so at 30 days post exposure the rank order for rats were liver<lung<spleen<bone<muscle<skin<digestive tract, while the rank order in mice were liver<lung<spleen<skin<bone<muscle<digestive tract. In both species, the percentage of recovered [¹⁴C(U)]C₆₀ increased in the liver and spleen between 1 hour and 30 days post exposure. A high recovery from lung tissue was expected due to the filtering capacity of alveolar macrophages. On average, the percentage of radioactivity recovered from the lungs of mice decreased over time, but this was not the case in rats. Excretion of [¹⁴C(U)]C₆₀ through urine and feces was limited in both rats and mice, and accounted for less of 2% of the administered dose at any time point. The persistence of [¹⁴C(U)]C₆₀ in organs over a period of 30 days post exposure, together with the low elimination of [¹⁴C(U)]C₆₀, raise concern of long term adverse biological impact from exposure to C₆₀.

A previous study identified C₆₀-metabolites in the liver of mice by determining the presence of C₆₀-retinol in the liver (Moussa, et al. 1998). Here we confirmed the in vivo formation of a C₆₀-retinol adduct in the liver, detecting [¹⁴C(U)]C₆₀-retinol adduct in the liver collected both 1 and 30 day post exposure. However, the 2-fold reduction in the [¹⁴C(U)]C₆₀ to [¹⁴C(U)]C₆₀-retinol ratio at day 30 after a single tail vein injection compared to 1 day post exposure suggests an overall slow biotransformation of [¹⁴C(U)]C₆₀.

We have previously found that intratracheal administration of C₆₀, formulated with PVP and suspended in saline to form a 5% PVP-dosage, to both female and male rats resulted in deleterious cardiovascular consequences, including myocardial infarction following cardiac ischemia/reperfusion, and enhancement of endothelin-1 mediated stress, potentially indicative of increased coronary vascular resistance (Thompson, et al. 2014). The same study also demonstrated that the route of C₆₀ administration may generate unique cardiovascular detriments (Thompson, et al. 2014, Vidanapathirana, et al. 2014). Relevant to the Thompson et al study is our observation that in female rats the level of vWF, a glycoproteins that plays an important role in hemostasis following vascular injury, and is predictive for adverse cardiac events (Spiel, et al. 2008), was highly elevated 1 hour post [¹⁴C(U)]C₆₀ exposure and then declined at 1 day post exposure, staying above background level for the remaining 30 days of our study.

Oxidative stress has been identified as the result of nanoparticle exposure that may be involved in the generation of adverse effects (Oberdorster 2010, Usenko, et al. 2008), and may result in a tiered response, including anti-oxidant defense, inflammation and cytotoxicity (Nel, et al. 2006). Markers of oxidative damage and inflammation were included in this study, since systemic oxidative stress can be induced by obesity, cigarette smoking and diabetes and is the unifying mechanism for many cardiovascular disease risk factors (Madamanchi, et al. 2005), 8-OH-dG levels were determined. Metabolomics analysis revealed a different signature for urine collected from rats that had elevated levels of 8-OH-dG from that of urine collected from rats with normal levels of 8-OH-dG. One marker for oxidative damage is 8-OH-dG, which can be measured in DNA extracted from tissues or in urine (Pilger and Rudiger 2006, Valavanidis, et al. 2009). Recent reports suggested that gavage administration of C₆₀ to rats did not greatly influence the formation of 8-OH-dG in DNA from lung, liver, and colon 24 hours post exposure (Folkmann, et al. 2009). This is in agreement with the finding presented here for 24 hours post i.v. administration of PVP-formulated [¹⁴C(U)]C₆₀. However, at 5 days post exposure 8-OH-dG levels in our female rodents were elevated, and remained elevated. This finding indicates systemic oxidative stress occurring after just a single dose of [¹⁴C(U)]C₆₀ in 5% PVP-saline solution. While the literature contains numerous examples of gamma;-radiation induced oxidative stress, no such examples were found for β-radiation (Inano and Onoda 2002, Kasai, et al. 1986, Mori, et al. 1993). Studies with gamma;-irradiation of mice (cobalt 60 [⁶⁰Co]) at a dose of 173 krad, but not 60 krad, reported an increase in 8-OH-dG in liver (Kasai, et al. 1986). One uCi of ¹⁴C is expected to produce 1.2 rads per hour. Even if the energy and damage potential of soft β-irradiation by ¹⁴C were equivalent to gamma;-rays, 1 uCi of [¹⁴C(U)]C₆₀ would be expected to be 29 rads/day, 200 rads/week, or 800 rads over 4 weeks. This is an order of magnitude lower than the low dose gamma;-radiation used by Inano and co-workers, which had no effect on 8-OH-dG levels. In another study 300 rad gamma;-irradiation (from a ⁶⁰Co source) in rats produced a 28% increase in rat urinary 8-OH-dG at 24 hours (Inano and Onoda 2002). The [¹⁴C(U)]C₆₀ dose in our study, 1.2 rad/hr, is a magnitude lower. It appears, therefore, that the delayed oxidative stress was caused by C₆₀ administration.

Metabolomics analysis was used as a discovery tool to determine metabolites that were most important to the difference between the vehicle and day 7 exposure. These metabolites were entered into a pathway mapping software, GeneGo, and the three most significant pathways where nicotine signaling in cholinergic neurons, TCA cycle and eNOS activity in endothelial cells. All three pathways are connected to oxidative stress. eNOS is responsible for most of the vascular nitric oxide production which protects the vasculature from oxidative stress (Forstermann and Munzel 2006). Endothelial cell malfunction is associated with decreased availability of nitric oxide, which in turn leads to increased generation of ROS. In nicotine signaling in cholinergic neurons, the presence of reactive oxygen species (ROS) has been shown to inactivate nicotinic acetylcholine receptors on neurons (Campanucci, et al. 2008, Krishnaswamy and Cooper 2012). The TCA cycle in the mitochondria is an essential metabolic pathway in all oxidative organism, and an interruption in TCA cycle can cause inefficient electron transfer resulting in generating toxic ROS (Mailloux, et al. 2007). One of the major cellular defenses in oxidative stress is Nrf-2 antioxidant response element signaling pathway involved in detoxification and elimination of reactive oxidants and

electrophilic agents (Nguyen, et al. 2009). The metabolites that differentiated the female rats receiving [$^{14}\text{C}(\text{U})$] C_{60} from vehicle also included 3 metabolites involved in the oxidative stress signaling pathway Nrf-2. The oxidative stress induced by [$^{14}\text{C}(\text{U})$] C_{60} exposure and possible involvement of the Nrf-2 pathway will need further investigation, but our findings highlight the complex physiological response to nanomaterial exposure. This study indicates a potential to use metabolomics to detect biologically responses, such as oxidative stress, to nanomaterial exposure.

In an effort to understand how circulating biomarkers may be related to adverse effects of administered [$^{14}\text{C}(\text{U})$] C_{60} , the levels of a select number for cytokines and chemokines in the plasma of female rats and mice was examined. Here as well, species differences were evident, as in rats the levels of three hematopoietins cytokines, IL-4, IL-5 and IL-6, were increased 1 hour and 1 day post exposure, but in mice only IL-5 showed the same expression pattern. The levels of other measured cytokines (TNF- α and IL-1 β) and chemokines (eotaxin and MCP-1) as well as that of the signaling protein VEGF, were not elevated following [$^{14}\text{C}(\text{U})$] C_{60} exposure. Cytokines are generally categorized as messenger molecules and chemokines as inducers of chemotaxis, directing the migration of white blood cells to infected or damaged tissue. The three cytokines IL-4, IL-5 and IL-6 all belong to the family of hematopoietins, which are involved in growth and differentiation of hematopoietic cells, which are the cells that are lodged in the bone marrow, and are responsible for producing the red and white blood cells as well as platelets (Wells and de Vos 1993). It is interesting that while the levels of a group of cytokines involved in growth and differentiation of hematopoietic cells are elevated shortly after [$^{14}\text{C}(\text{U})$] C_{60} exposure, the levels of chemotaxis inducing chemokines are not.

This study demonstrated species differences in the distribution of [$^{14}\text{C}(\text{U})$] C_{60} between female rats and mice, and elevation of biomarkers reflective of cardiovascular and oxidative stress. Our findings fit well into the hierarchical oxidative stress model (Nel, et al. 2006), since on average there is an elevation in 8-OH-dG and several metabolites involved in oxidative stress pathways, one being Nrf-2. Nrf-2 is described as the signaling pathway in tier 1 level of oxidative stress preceding inflammation and cytotoxicity. This study show that [$^{14}\text{C}(\text{U})$] C_{60} exposure cause oxidative stress, and emphasize the need to understand the biological impact of exposure to C_{60} , and to determine the associated risks.

Acknowledgments

This study was supported by National Institute of Environmental Health Sciences (U19 ES019525), and National Institute of Diabetes and Digestive and Kidney Diseases (1U24DK097193-01).

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Figure 1. TEM images of the C60 in 5% PVP-saline suspensions (a) and 5% PVP-saline suspensions alone (b), (c) show distribution histogram of C60 in 5% PVP-saline suspensions particle size.

Figure 2. Representative LC-MS analysis of a rat liver homogenate extract obtained 30 days after $[^{14}C(U)]C_{60}$ exposure. Representative extracted ion chromatograms for $[^{14}C(U)]C_{60}$ -retinol and $[^{14}C(U)]C_{60}$ along with the corresponding MS spectra at 1006.228 and 719.999 m/z .

Figure 3.

8-OH-dG was measured in urine collected from the rats exposed to $[^{14}\text{C}(\text{U})\text{C}_{60}]$. On average, levels of 8-OH-dG increased by (but did not test significant) 5 days following administration of $[^{14}\text{C}(\text{U})]\text{C}_{60}$, and did not return to control levels by 21 days after exposure.

Figure 4.

Scores plot of PCA of individual urine samples obtained from female rats administered [$^{14}\text{C}(\text{U})\text{C}_{60}$] or vehicle control. Black filled circles, day 1 post 5% PVP-saline vehicle; red filled circles, day 1 post [$^{14}\text{C}(\text{U})\text{C}_{60}$]; blue filled squares, day 7 post [$^{14}\text{C}(\text{U})\text{C}_{60}$]; violet diamonds, day 14 post [$^{14}\text{C}(\text{U})\text{C}_{60}$]; dark green stars, day 14 post five consecutive [$^{14}\text{C}(\text{U})\text{C}_{60}$]; brown filled triangles, day 21 post [$^{14}\text{C}(\text{U})\text{C}_{60}$].

Figure 5. Metabolites in urine associated with $[^{14}\text{C}(\text{U})]\text{C}_{60}$ exposure involved in multiple pathways as listed in (a), but also in Nrf-2 signaling pathway (b), generated in GeneGo 5.0 software from MetaCore, 3 step removed analysis. Nrf-2 is labeled with a green, blue and red circle. Metabolites (choline, benzoic acid, and arginine) from the analyses are indicated with purple circles.

Table 1

The percentage of total recovered radioactivity (average $\pm$ standard deviation) in tissue digests and fluids collected from the female rats at 1 hour, 1 day, 7 days, 14 days and 30 days following a single tail vein injection of [$^{14}\text{C}(\text{U})$]C₆₀ and 14 days after five consecutive doses (n=3).

Tissue	1 hour	1 day	7 days	14 days	30 days	14 days 5xdoses
Adipose ^a	1.00 $\pm$ 0.74	0.361 $\pm$ 0.11	0.870 $\pm$ 0.060	0.898 $\pm$ 0.33	0.795 $\pm$ 0.46	0.532 $\pm$ 0.17
Bladder	0.00658 $\pm$ 0.004	0.004 $\pm$ 0.002	0.0105 $\pm$ 0.0028	0.00781 $\pm$ 0.00045	0.00403 $\pm$ 0.00064	0.00456 $\pm$ 0.0021
Muscle ^b	10.3 $\pm$ 11.2	2.92 $\pm$ 0.55	3.19 $\pm$ 0.66	5.61 $\pm$ 4.74	3.68 $\pm$ 0.80	3.11 $\pm$ 1.79
Blood ^c	26.6 $\pm$ 2.24	5.58 $\pm$ 0.82	0.508 $\pm$ 0.14	0.415 $\pm$ 0.39	0.884 $\pm$ 1.10	0.200 $\pm$ 0.045
Spleen	5.93 $\pm$ 1.98	9.54 $\pm$ 3.21	8.31 $\pm$ 1.56	8.38 $\pm$ 1.81	9.12 $\pm$ 2.02	9.59 $\pm$ 2.45
Liver	33.5 $\pm$ 3.95	54.6 $\pm$ 4.93	56.8 $\pm$ 2.29	57.1 $\pm$ 7.46	58.1 $\pm$ 1.36	63.6 $\pm$ 7.03
Kidney	0.657 $\pm$ 0.134	0.449 $\pm$ 0.083	0.269 $\pm$ 0.028	0.264 $\pm$ 0.13	0.252 $\pm$ 0.35	0.247 $\pm$ 0.13
Brain	0.121 $\pm$ 0.010	0.048 $\pm$ 0.018	0.0156 $\pm$ 0.0026	0.0131 $\pm$ 0.0070	0.0140 $\pm$ 0.0043	0.00758 $\pm$ 0.0035
Heart	0.407 $\pm$ 0.046	0.141 $\pm$ 0.039	0.0882 $\pm$ 0.033	0.0632 $\pm$ 0.014	0.0461 $\pm$ 0.011	0.0579 $\pm$ 0.024
Lung	12.2 $\pm$ 7.88	16.5 $\pm$ 1.63	14.4 $\pm$ 4.75	11.4 $\pm$ 7.26	15.2 $\pm$ 3.18	8.08 $\pm$ 1.24
Digestive Tract ^d	1.260 $\pm$ 0.18	1.820 $\pm$ 0.48	1.810 $\pm$ 0.18	0.558 $\pm$ 0.13	1.678 $\pm$ 0.12	1.413 $\pm$ 0.33
Bone ^e	3.05 $\pm$ 0.84	4.46 $\pm$ 0.53	3.59 $\pm$ 1.40	5.17 $\pm$ 1.53	4.27 $\pm$ 0.24	2.12 $\pm$ 0.74
Ovaries	0.0388 $\pm$ 0.0079	0.0368 $\pm$ 0.0067	0.0702 $\pm$ 0.012	0.137 $\pm$ 0.092	0.0828 $\pm$ 0.016	0.104 $\pm$ 0.032
Skin ^f	4.69 $\pm$ 4.70	3.11 $\pm$ 0.68	4.89 $\pm$ 1.22	4.98 $\pm$ 3.34	2.88 $\pm$ 0.26	4.03 $\pm$ 1.15
Uterus, vagina, cervix	0.0588 $\pm$ 0.051	0.183 $\pm$ 0.095	0.180 $\pm$ 0.11	0.151 $\pm$ 0.058	0.0758 $\pm$ 0.021	0.102 $\pm$ 0.053
Mammary Tissue	0.0170 $\pm$ 0.13	0.00616 $\pm$ 0.0013	0.0237 $\pm$ 0.020	0.0112 $\pm$ 0.0058	0.00970 $\pm$ 0.0053	0.00938 $\pm$ 0.0060
Lymph Nodes	0.0491 $\pm$ 0.0077	0.0612 $\pm$ 0.027	0.108 $\pm$ 0.067	0.196 $\pm$ 0.14	0.0710 $\pm$ 0.029	0.207 $\pm$ 0.16
Pancreas	0.110 $\pm$ 0.027	0.109 $\pm$ 0.025	0.0254 $\pm$ 0.044	0.176 $\pm$ 0.088	0.157 $\pm$ 0.10	0.197 $\pm$ 0.11

^a Adipose tissue were estimated as 7.21% of body weight.

^b Muscles were estimated as a total of 40.43% of body weight.

^c The total estimated blood radioactivity assuming 7.4% body weight as blood for rats.

^d Digestive Tract comprised of stomach, cecum, small and large intestines and digestive content.

^e Bones were assumed to be 7.3% of body weight.

^f Skin was estimated to be 19.03% of total body weight.

Table 2

Concentration (ng/g tissue) of [$^{14}\text{C}(\text{U})$] C_{60} (average $\pm$ standard deviation) in tissue digests and fluids collected from the female rats at 1 hour, 1 day, 7 days, 14 days and 30 days following a single tail vein injection of [$^{14}\text{C}(\text{U})$] C_{60} and 14 days after five consecutive doses (n=3).

Sample	1hour	1 day	7 days	14 days	30 days	14 days 5xdose
Adipose	40.8 $\pm$ 33.2	11.5 $\pm$ 1.47	22.8 $\pm$ 3.58	19.6 $\pm$ 15.9	22.2 $\pm$ 14.1	53.4 $\pm$ 8.56
Bladder	67.8 $\pm$ 50.0	29.0 $\pm$ 15.1	57.6 $\pm$ 13.7	33.3 $\pm$ 12.4	33.4 $\pm$ 2.27	97.3 $\pm$ 35.9
Muscle	77.9 $\pm$ 87.1	17.4 $\pm$ 5.61	14.7 $\pm$ 2.64	15.1 $\pm$ 1.89	17.9 $\pm$ 3.62	56.4 $\pm$ 31.3
Blood	1,035 $\pm$ 126	177 $\pm$ 24.6	12.6 $\pm$ 0.762	5.67 $\pm$ 1.16	20.4 $\pm$ 22.4	19.9 $\pm$ 0.704
Spleen	10,693 $\pm$ 1,589	10,720 $\pm$ 2,384	8,240 $\pm$ 2,212	7,765 $\pm$ 4,998	10,312 $\pm$ 895	35,859 $\pm$ 15,117
Liver	2,727 $\pm$ 580	3,584 $\pm$ 1,401	2,873 $\pm$ 464	2,461 $\pm$ 1,522	2,840 $\pm$ 453	12,506 $\pm$ 3,596
Kidney	278 $\pm$ 66.7	134 $\pm$ 18.0	67.6 $\pm$ 10.6	42.5 $\pm$ 18.6	64.6 $\pm$ 19.7	238 $\pm$ 99.7
Brain	43.5 $\pm$ 5.93	13.4 $\pm$ 1.94	3.66 $\pm$ 1.27	2.01 $\pm$ 0.668	3.34 $\pm$ 1.09	7.54 $\pm$ 3.70
Heart	288 $\pm$ 57.1	90.7 $\pm$ 15.0	39.8 $\pm$ 3.58	28.0 $\pm$ 12.5	24.1 $\pm$ 4.69	125.0 $\pm$ 51.1
Lung	8,038 $\pm$ 4,856	8,486 $\pm$ 2,619	5,616 $\pm$ 2,268	6,264 $\pm$ 7,070	7,738 $\pm$ 3,247	13,402 $\pm$ 4,774
Digestive Tract	404 $\pm$ 89.1	488 $\pm$ 72.7	487 $\pm$ 70.0	151 $\pm$ 52.0	400 $\pm$ 85.0	1,388 $\pm$ 91.3
Bone	104 $\pm$ 32.2	137 $\pm$ 30.0	59.8 $\pm$ 52.7	84.3 $\pm$ 37.2	100 $\pm$ 27.5	182 $\pm$ 47.6
Marrow	718 $\pm$ 158	412 $\pm$ 216	697 $\pm$ 201	1,190 $\pm$ 465	970 $\pm$ 115	2,618 $\pm$ 1,032
Ovaries	206 $\pm$ 15.6	137 $\pm$ 4.41	264 $\pm$ 75.2	304 $\pm$ 74.0	457 $\pm$ 115	1,396 $\pm$ 484
Skin	74.2 $\pm$ 78.6	37.7 $\pm$ 2.59	47.3 $\pm$ 4.53	31.5 $\pm$ 8.98	30.1 $\pm$ 5.17	156 $\pm$ 32.5
Uterus, vagina, cervix	45.7 $\pm$ 11.1	87.8 $\pm$ 35.4	89.4 $\pm$ 25.0	57.8 $\pm$ 27.7	51.6 $\pm$ 20.1	298 $\pm$ 140
Brown Fat	186 $\pm$ 76.6	46.8 $\pm$ 13.2	55.4 $\pm$ 32.4	29.8 $\pm$ 13.4	38.9 $\pm$ 6.01	161 $\pm$ 143
Mammary Tissue	93.4 $\pm$ 91.6	35.4 $\pm$ 11.0	59.8 $\pm$ 10.4	45.9 $\pm$ 15.1	48.4 $\pm$ 9.36	178 $\pm$ 54.1
Lymph Nodes	274 $\pm$ 68.6	542 $\pm$ 234	761 $\pm$ 779	670 $\pm$ 375	1,456 $\pm$ 1,913	5,382 $\pm$ 4,677
Pancreas	75.9 $\pm$ 17.0	49.0 $\pm$ 7.67	87.5 $\pm$ 22.5	49.8 $\pm$ 27.1	57.6 $\pm$ 38.4	260 $\pm$ 135

Table 3

The percentage of total recovered radioactivity (average± standard deviation) in tissue digests and fluids collected from female mice at 1 hour, 1 day, 7 days, 14 days and 30 days following a single tail vein injection of [¹⁴C(U)]C₆₀ and 14 days after five consecutive doses, (n=3, for day 14 n=2).

Sample	1 hour	1 day	7 days	14 days	30 days	14 days 5xdose
Adipose ^a	4.12±4.28	4.47±7.74	7.40±12.2	0.00	0.522±0.71	0.374±0.40
Bladder	0.0469±0.055	0.0757±0.11	0.0106±0.0065	0.0194±0.025	0.00370±0.0040	0.00832±0.00084
Muscle ^b	18.9±20.7	22.0±27.1	1.47±1.30	1.57±2.22	1.84±3.18	2.52±0.433
Blood ^c	7.88±2.21	2.68±0.58	0.00±0.00	0.00±0.00	0.0371±0.038	0.105±0.035
Spleen	3.25±1.40	2.30±1.32	5.17±3.20	4.71±0.78	7.74±2.56	10.7±2.90
Liver	31.8±16.1	43.6±30.5	60.1±6.03	53.4±9.31	65.0±5.10	58.4±3.14
Kidney	0.859±0.44	0.896±0.39	0.647±0.38	0.906±0.36	0.666±0.12	0.828±0.20
Brain	0.320±0.053	0.256±0.13	0.0613±0.032	0.0703±0.020	0.0542±0.018	0.0810±0.012
Heart	0.567±0.36	0.255±0.18	0.191±0.081	0.166±0.043	0.145±0.013	0.145±0.025
Lung	20.1±13.3	3.707±4.67	6.38±5.81	9.09±8.14	11.1±4.96	8.01±2.96
Digestive Tract ^d	0.935±0.40	0.660±0.24	1.451±0.14	1.557±0.83	1.579±0.09	1.342±0.34
Bone ^e	5.10±0.68	5.23±3.17	6.89±1.61	6.39±3.81	3.90±2.19	5.51±1.21
Ovaries	0.0884±0.11	0.233±0.21	0.0214±0.012	0.00767±0.011	0.0453±0.031	0.0302±0.045
Skin ^f	5.62±6.63	11.0±4.83	5.01±1.67	13.1±9.57	4.45±1.06	7.78±3.09
Uterus, Vagina, Cervix	0.190±0.18	0.264±0.12	0.230±0.15	0.501±0.39	0.143±0.035	0.215±0.021
Mammary Tissue	0.0694±0.025	0.0543±0.063	0.0639±0.0301	0.0402±0.057	0.0455±0.041	0.0843±0.020
Lymph Nodes	0.0240±0.020	0.156±0.17	0.0928±0.055	0.257±0.36	0.123±0.030	0.191±0.13
Pancreas	0.152±0.042	0.610±0.59	0.151±0.070	0.0479±0.068	0.133±0.084	0.242±0.11

^a Adipose tissue were estimated as 5.9% of body weight.

^b Muscles were estimated as a total of 38.4% of body weight.

^c The total estimated blood radioactivity assuming 4.9% body weight as blood for rats.

^d Digestive Tract comprised of stomach, cecum, small and large intestines and digestive content.

^e Bones were assumed to be 11.2% of body weight.

^f Skin was estimated to be 16.53% of total body weight.

Table 4

Concentration (ng/g tissue) of [$^{14}\text{C}(\text{U})$] C_{60} (average $\pm$ standard deviation) in tissue digests and fluids collected from the female mice at 1 hour, 1 day, 7 days, 14 days and 30 days following a single tail vein injection of [$^{14}\text{C}(\text{U})$] C_{60} and 14 days after five consecutive doses, (n=3, for day 14 n=2).

Tissue	1 hour	1 day	7 days	14 days	30 days	14 days 5xdoses
Adipose	159 $\pm$ 204	273 $\pm$ 386	16.8 $\pm$ 10.9	0.00 $\pm$ 0.00	9.77 $\pm$ 9.91	39.6 $\pm$ 40.6
Bladder	130 $\pm$ 187	168 $\pm$ 237	16.4 $\pm$ 12.7	6.76 $\pm$ 5.05	7.62 $\pm$ 11.6	86.0 $\pm$ 18.8
Muscle	113 $\pm$ 150	110 $\pm$ 188	7.34 $\pm$ 6.81	6.55 $\pm$ 9.26	5.70 $\pm$ 9.87	44.4 $\pm$ 5.09
Blood	279 $\pm$ 47.8	55.4 $\pm$ 54.1	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	1.19 $\pm$ 1.03	14.3 $\pm$ 4.07
Spleen	1,711 $\pm$ 709	721 $\pm$ 586	2,903 $\pm$ 1,818	2,599 $\pm$ 3,016	3,031 $\pm$ 1,672	31,962 $\pm$ 14,501
Liver	1,300 $\pm$ 442	441 $\pm$ 298	2,286 $\pm$ 1,187	1,562 $\pm$ 1793	1,921 $\pm$ 1149	11,203 $\pm$ 3,529
Kidney	101 $\pm$ 32.9	48.8 $\pm$ 36.9	70.2 $\pm$ 43.1	51.9 $\pm$ 39.3	79.1 $\pm$ 42.6	497 $\pm$ 150
Brain	25.7 $\pm$ 5.68	12.7 $\pm$ 17.1	5.11 $\pm$ 3.61	2.58 $\pm$ 2.10	3.42 $\pm$ 0.626	28.4 $\pm$ 4.76
Heart	151 $\pm$ 60	41.0 $\pm$ 34.8	44.9 $\pm$ 39.2	28.6 $\pm$ 26.0	49.5 $\pm$ 23.5	235 $\pm$ 20.1
Lung	4,270 $\pm$ 2,721	487 $\pm$ 762	1,572 $\pm$ 1,586	2,820 $\pm$ 3,772	2,644 $\pm$ 1,398	8,758 $\pm$ 3,652
Digestive Tract	84.9 $\pm$ 11.4	40.2 $\pm$ 20.8	163 $\pm$ 48.4	246 $\pm$ 147	508 $\pm$ 554	1,272 $\pm$ 237
Bone	83.8 $\pm$ 41.6	54.8 $\pm$ 45.0	62.6 $\pm$ 61.5	55.9 $\pm$ 54.9	66.7 $\pm$ 21.9	282 $\pm$ 26.6
Ovaries	163 $\pm$ 234	317 $\pm$ 480	35.5 $\pm$ 8.01	18.2 $\pm$ 25.7	61.6 $\pm$ 61.1	238 $\pm$ 207
Marrow	81.8 $\pm$ 0.00	2.28 $\pm$ 0.00	110 $\pm$ 41.8	10.9 $\pm$ 15.5	22.1 $\pm$ 34.6	395 $\pm$ 94.6
Skin	79.4 $\pm$ 110	92.1 $\pm$ 123	46.5 $\pm$ 30.5	46.1 $\pm$ 22.2	40.7 $\pm$ 26.5	329 $\pm$ 168
Uterus, Vagina, Cervix	88.4 $\pm$ 106	48.4 $\pm$ 60.1	98.4 $\pm$ 68.1	83.6 $\pm$ 16.0	30.2 $\pm$ 16.7	572 $\pm$ 246
Brown Fat	213 $\pm$ 308	93.3 $\pm$ 144	31.3 $\pm$ 15.9	0.0640 $\pm$ 0.0905	59.6 $\pm$ 1.94	156 $\pm$ 89.4
Mammary Tissue	51.0 $\pm$ 49.5	27.4 $\pm$ 33.0	51.2 $\pm$ 32.4	28.8 $\pm$ 40.7	50.8 $\pm$ 46.8	362 $\pm$ 126
Lymph Nodes	155 $\pm$ 84.5	118 $\pm$ 90.8	243 $\pm$ 156	942 $\pm$ 1,333	213 $\pm$ 155	2,308 $\pm$ 1,026
Pancreas	39.1 $\pm$ 5.53	25.6 $\pm$ 11.9	21.5 $\pm$ 13.5	10.0 $\pm$ 14.2	22.8 $\pm$ 22.3	285 $\pm$ 194

Cytokine/Chemokine expression in female rats 1 hour, 1 day, 7 days, 14 days and 30 days following a single tail vein injection of [$^{14}\text{C}(\text{U})$] C_{60} and 14 days after five consecutive doses (n=3). All data are reported as Mean $\pm$ SEM pg/mL of plasma of duplicates (n= 4 – 5).

Table 5

Cytokine	1 day – vehicle	1 hour	1 day	7 days	14 days	30 days	14 days 5x dose
Eotaxin	153 $\pm$ 125	114 $\pm$ 33.8	145 $\pm$ 60.1	37.4 $\pm$ 37.4	81.5 $\pm$ 81.5	10.8 $\pm$ 10.8	n.d. ^a
IL-1β	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
IL-4	23.0 $\pm$ 17.0	70.5 $\pm$ 41.9	28.5 $\pm$ 9.1	20.0 $\pm$ 20.0	24.7 $\pm$ 24.7	15.5 $\pm$ 15.5	3.6 $\pm$ 3.6
IL-5	4.9 $\pm$ 4.9	12.9 $\pm$ 12.9	12.7 $\pm$ 14.2	n.d.	n.d.	3.4 $\pm$ 3.4	n.d.
IL-6	124 $\pm$ 76.0	608 $\pm$ 428	639 $\pm$ 291	106 $\pm$ 106	130 $\pm$ 130	140 $\pm$ 140	2.1 $\pm$ 2.1
MCP-1	71.8 $\pm$ 22.3	31.0 $\pm$ 20.2	6.0 $\pm$ 0.9 ^b	19.3 $\pm$ 19.3	24.2 $\pm$ 24.2	3.9 $\pm$ 3.9 ^b	21.2 $\pm$ 21.2
TNF-α	n.d.	11.1 $\pm$ 11.1	n.d.	n.d.	2.3 $\pm$ 2.3	n.d.	n.d.
VEGF	24.9 $\pm$ 9.4	n.d. ^b	7.8 $\pm$ 2.5	n.d. ^b	n.d. ^b	12.3 $\pm$ 12.3	n.d. ^b

^a n.d. = not detected, '0' was used in the statistical analysis.

^b ANOVA 1 way analysis, between vehicle and treatment, p <0.05.

Cytokine/Chemokine expression in female mice 1 hour, 1 day, 7 days, 14 days and 30 days following a single tail vein injection of [$^{14}\text{C}(\text{U})$]C₆₀ and 14 days after five consecutive doses (n=3). All data are reported as Mean $\pm$ SEM pg/mL of plasma of duplicates (n= 4 – 5).

Table 6

Cytokine	1 day – vehicle	1 hour	1 day	7 days	14 days	30 days	14 days 5x dose
Eotaxin	1,183 $\pm$ 34.1	1,268 $\pm$ 15.8	1,264 $\pm$ 43.7	1,095 $\pm$ 64.8	1,085 $\pm$ 103	1,256 $\pm$ 48.3	1,274 $\pm$ 62.1
IL-1β	n.d. ^a	n.d.	23.4 $\pm$ 23.4	n.d.	5.9 $\pm$ 5.9	2.1 $\pm$ 2.1	n.d.
IL-4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
IL-5	1.30 $\pm$ 1.3	128 $\pm$ 104	16.0 $\pm$ 10.3	n.d.	7.2 $\pm$ 3.7	3.9 $\pm$ 3.9	22.4 $\pm$ 6.8
IL-6	64.9 $\pm$ 29.3	19.5 $\pm$ 19.5	8.3 $\pm$ 3.1	27.0 $\pm$ 14.9	47.5 $\pm$ 23.3	75.4 $\pm$ 53.9	0.8 $\pm$ 0.8
MCP-1	5.30 $\pm$ 4.3	36.6 $\pm$ 4.3 ^b	24.8 $\pm$ 9.7	11.5 $\pm$ 4.9	18.5 $\pm$ 4.6	11.9 $\pm$ 5.9	15.5 $\pm$ 9.0
TNF-α	2.40 $\pm$ 2.4	n.d.	1.8 $\pm$ 1.8	n.d.	4.8 $\pm$ 4.8	4.9 $\pm$ 3.1	0.7 $\pm$ 0.7
VEGF	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

^a n.d. = not detected, '0' was used in the statistical analysis.

^b ANOVA 1 way analysis, between vehicle and treatment, p <0.05.

Table 7

Markers of cardiovascular injury in female rats 1 hour, 1 day, 7 days, 14 days and 30 days following a single tail vein injection of [$^{14}\text{C}(\text{U})$] C_{60} and 14 days after five consecutive doses (n=3). All data are reported as Mean $\pm$ SEM ng/mL plasma of duplicates except for PAL-1 reported as pg/ml, from (n = 4 – 5).

	1 day – vehicle	1 hour	1 day	7 days	14 days	30 days	14 days 5x doses
PAL-1	1,205 $\pm$ 210	795 $\pm$ 233	963 $\pm$ 347	929 $\pm$ 329	477 $\pm$ 228	220 $\pm$ 220 ^b	n.d. ^{a,b}
vWF	140 $\pm$ 84.4	486 $\pm$ 113	268 $\pm$ 32.8	260 $\pm$ 31.1	388 $\pm$ 57.6	245 $\pm$ 51.3	419 $\pm$ 85.5 ^b

^a n.d. = not detected, '0' was used in the statistical analysis.

^b ANOVA 1 way analysis, between vehicle and treatment, p <0.05.

Table 8

Markers of cardiovascular injury in female mice 1 hour, 1 day, 7 days, 14 days and 30 days following a single tail vein injection of [¹⁴C(U)]C₆₀ and 14 days after five consecutive doses (n=3). All data are reported as Mean ± SEM ng/ml plasma of duplicates from n = 3–4.

	1 day – vehicle	1 hour	1 day	7 days	14 days	30 days	14 days 5x doses
ICAM-1	62.9 ± 7.4	91.8 ± 7.3 ^a	60.2 ± 3.5	77.3 ± 11.0	79.8 ± 18.5	94.8 ± 14.5	66.7 ± 11.6
PAI-1	3.2 ± 0.6	6.8 ± 1.4	4.5 ± 1.4	2.4 ± 0.3	4.1 ± 1.5	2.9 ± 0.7	3.1 ± 1.0
E-selectin	93.7 ± 18.7	75.8 ± 5.4	73.2 ± 19.6	83.0 ± 15.3	112 ± 62.6	100 ± 28.9	64.3 ± 10.9

^a ANOVA 1 way analysis, between vehicle and treatment, p <0.05.

Table 9

Metabolites in urine distinguishing [$^{14}\text{C}(\text{U})$] C_{60} treated rodents (day 7) from vehicle (1 day).

Metabolite	Direction
Isovalerate	$\uparrow^a$
2-Oxoglutarate	$\downarrow^b$
2-Oxovalerate	$\uparrow$
Arginine	$\downarrow$
Benzoate	$\uparrow$
Betaine	$\downarrow$
Choline	$\uparrow$
cis-Aconitate	$\downarrow$
Creatinine	$\downarrow$
Glucose-6-Phosphate	$\downarrow$
Glutamate	$\downarrow$
Hippurate	$\downarrow$
Lactate	$\uparrow$
N,N-Dimethylglycine	$\downarrow$
N6-Acetyllysine	$\downarrow$
N-Acetylaspartate	$\downarrow$
N-Acetylglutamate	$\downarrow$
N-Acetylmethionine	$\downarrow$
N-Nitrosodimethylamine	$\downarrow$
Phenylalanine	$\downarrow$
Proline	$\downarrow$
Taurine	$\downarrow$
Trimethylamine-N-oxide	$\uparrow$

$a_{\uparrow}$ = increased

$b_{\downarrow}$ = decreased