Chromatographic Determination of C$_{70}$ Fullerene in Animal Organs and Tissues


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Abstract—A method has been developed for the determination of C$_{70}$ fullerene in animal organs and tissues using reversed-phase high-performance liquid chromatography with ultraviolet detection combined with sample preparation consisting in the homogenization of samples, treatment of the homogenates with acetic acid, and extraction of fullerene with toluene. The recovery of C$_{70}$ fullerene from various organs was ~89–93%, the limit of detection is 75 ng/mL.

Keywords: fullerenes, extraction, high-performance liquid chromatography

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C$_{70}$ fullerene together with other fullerenes is a representative of a new allotropic form of carbon. Along with C$_{60}$ fullerene, it forms a group of so-called light fullerenes, which are the main components of fullerene black (products of synthesis) manufactured on an industrial scale. Fullerenes are virtually insoluble in polar solvents, acetone, and tetrahydrofuran, and are poorly soluble in n-alkanes (pentane, hexane, and decane). They are best soluble in benzene and toluene; the solubility of C$_{70}$ fullerene in these solvents is 1.3 and 1.4 mg/mL, respectively [1].

Much attention is given to fullerenes because of their properties, such as size, molecular structure (presence of internal volume), lipophilicity, chemical and photophysical peculiarities, as well as a variety of new physicochemical phenomena occurring with their participation [2–6]. Moreover, interest in fullerenes is associated with their high biological activity. Compounds containing fullerene structures have actively been studied in recent years as potential therapeutic agents. There are evidences of their effectiveness in the treatment of neurodegenerative, immune, infectious, oncological, and other diseases. The development of theranostic agents based on fullerenes and ensures the simultaneous diagnostics of diseases and the targeted delivery of the therapeutic agent to the affected organs seems to be very promising [6, 7]. The complex characterization of their effect on living organisms and the evaluation of the degree of their biosafety are important both for the wider industrial production of fullerenes and for the practical application of fullerene-containing pharmaceuticals. Naturally fullerenes can enter living organisms through skin, respiratory organs or orally; intravenous and intraperitoneal injection must be additionally characterized in the evaluation of their medical application.

For the characterization of the biological action of fullerenes, information on their accumulation and localization in various organs and tissues is important, which causes the necessity in a highly sensitive, reproducible, and productive method of the quantitative determination of fullerenes in biological objects. The most promising is the use of HPLC for these purposes. Chromatographic methods of the determination of C$_{60}$ fullerene in soil [8–10], surface and ground water [11], and biological matrices [12–18] are known. However, only few works are dealing with the use of HPLC for the detection of C$_{70}$ fullerene in soils [9, 10] and cosmetics [19].

The aim of the present work was the development of a method of sample preparation of a biological material (animal tissues) for the quantitative determination of C$_{70}$ fullerene after its intraperitoneal injection in experimental animals (rats).

EXPERIMENTAL

Reagents and materials. Toluene and acetonitrile (LabScan, Poland), glacial acetic acid (Khimmed, Russia), C$_{70}$ fullerene (UTiM, Russia), Tween-80 (Ferak, Germany) carboxymethylcellulose (Sigma, United States), and deionized water purified on a Simplicity system (Millipore, United States) were used in the work.
Equipment. A high-pressure liquid chromatograph (Avilon, Russia), a T25 digital Ultra Turrax immersion sonicator with a S 25 N–18 G supersonic element (IKA Werke GmbH & Co. KG, Germany), a 5004R centrifuge (Eppendorf AG, Germany), an IR-1M2 rotary vacuum evaporator (Khimlabpribor, Russia), and a m08 ultrasonic bath (FinnSonic, Finland) were used in the work.

Preparation of solutions. For the preparation of a stock solution of C70 fullerene with the concentration 0.4 mg/mL, a precisely weighed portion of fullerene (2 mg) was dissolved in 5 mL of toluene, sonicated for 1 h, and filtered through a fluoroplastic filter (pores size 0.20 µm). The obtained stock solution was stored at 4° C in amber glass vials for more than 24 h and used for the calibration of a chromatographic column.

To build a calibration curve, solutions were prepared by the sequential dilution of the stock solution used for the calibration of a chromatographic column. To build the calibration curve, stock solutions prepared from three independently weighed portions of fullerene were used. Each solution was analyzed in triplicate.

HPLC of C70 fullerene. Separation was performed on a Diapsher-110 C18 chromatographic column protected with a Kromasil 100-5C18 guard column (BioKhimMak-ST, Russia). Chromatographic conditions were as follows: mobile phase toluene–acetonitrile (60 : 40, vol), mobile phase flow rate 1 mL/min, column thermostat temperature 25° C, and detection wavelength 380 nm. Peak areas were calculated using the MultiChrom 2.0 software.

To evaluate the concentration of fullerene in a biomaterial, the average value for 10 samples was used. Each sample was chromatographed in triplicate. The recovery of C70 fullerene was determined as the ratio of the calculated amount of analyte in the samples after sample preparation to the added amount of analyte; the accuracy of the determination was determined as the ratio of the average value of the found amount of C70 fullerene to the added amount of analyte.

Preparation of biosamples to the determination of C70 fullerene using the added—found method. Toluene (8 mL) was added to a weighed portion of C70 fullerene (6.5 mg), stirred, and sonicated for 40 min. The solution was placed into test tubes (150 µL into each) and dried in air. For each experiment, six test tubes with C70 fullerene were used; homogenates of organs and tissues were added into five test tubes, and 200 µL of a 50 mM of phosphate buffer solution (pH 7.4) was added into the sixth tube (control). After stirring for 20 min with a magnetic stirrer, 1 mL of glacial acetic acid was added to the test tubes; the mixture was vigorously stirred and placed in an ultrasonic bath for 20 min. Then 5 mL of toluene was added, the mixture was stirred at room temperature for 1 h, centrifuged for 10 min at 11500 g, and the toluene fraction containing fullerene was separated. Extraction with toluene was repeated two times more. All toluene fractions were combined and dried in a rotary evaporator at 60° C. The obtained dry residue was dissolved in 0.5 mL of toluene and filtered through a fluoroplastic filter (0.2 µm). To perform a chromatographic analysis, 0.2 mL of acetonitrile was added to 0.3 mL of the obtained solution.

Preparation of a dispersion of C70 fullerene for intraperitoneal injection was done according to the method [14] with some modifications. Carboxymethylcellulose (1 g) was gradually added to 50 mL of a 0.9% NaCl solution containing 0.02% of a Tween-80 surfactant under constant stirring; the mixture was stirred for 1.5 h at room temperature, kept for 12 h at 4° C, and then stirred for 1.5 h at room temperature and 1 h at 50° C. The obtained hot carboxymethylcellulose solution (5 mL) was added to a precisely weighed portion (5 or 50 mg) of C70 fullerene and stirred for 30 min at 50° C. The dispersion was treated in an ultrasonic bath for 20 min and sterilized for 1 h in an autoclave.

Injection of the C70 fullerene dispersion in experimental animals. Three month–old Wistar line male rats of the weight 180–200 g were used in the work. The freshly prepared dispersion of C70 fullerene was injected in the abdominal space of 6 rats in a single dose of 5 mg and in 6 rats in a dose of 50 mg. A solution of carboxymethylcellulose without fullerene was injected in two rats of the control group.

Organ harvesting and preparation of homogenates. Organ harvesting from the experimental animals was performed on the 7-th, 14-th, and 28-th day (on the 28-th day for the control animals) after the injection of C70 fullerene. Harvesting of lungs, liver, spleen, and kidneys, as well as blood was performed as described in [18]. The organs were twice washed with a physiological solution, dried on a filter paper, weighed, cut very small with a scalpel, and homogenized with a disper-gator (1 min, 16000 rpm) after the addition of 1 mL (6 mL in the case of liver) of a 50 mM phosphate buffer solution (pH 7.4). The homogenates were stored at −18° C. Not later than in 2 h after the harvesting, the tubes with blood were placed in a thermostat (37° C, 30 min) and then centrifuged (10 min, 11500 g); serum was separated without touching the thrombus. The specimens were stored at −18° C.

Sample preparation of the homogenates of organs and tissues. An equal volume of glacial acetic acid was added to an accurately measured volume (1–2 mL) of a homogenate or serum, vigorously stirred (10 min), and placed in an ultrasonic bath for 20 min. The further sample preparation of brain, heart, lungs, kidneys, and blood serum was performed as described above. For spleen and liver, the dried samples were dissolved in 1 and 5 mL of toluene, respectively, then 0.99 mL of a toluene–acetonitrile mixture