TOXICITY OF ^{13}C-LABELED LINOLEIC AND LINOLENIC ACIDS FOR DIAGNOSTIC BREATH TESTS

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Noninvasive stable isotope breath tests allow highly accurate and safe estimation of liver and biliary tract function. The aim of this study was to test ^{13}C-labeled linoleic and linolenic acids intended for diagnostic use for acute and subchronic toxicity. The acids were synthesized using the patented method. A single intragastric administration of the tested compounds to experimental BALB/c mice and Wistar rats in the amounts exceeding clinical doses 500 to 2500-fold did not cause animal death. In the subchronic toxicity test, the rats received 5 to 25 times higher doses than recommended for clinical use in humans. In a 14-day follow-up period, no significant differences were observed between the main and the control groups in terms of weight, blood count (red blood cells, white blood cells, platelets), and blood biochemistry (hemoglobin, total protein, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, bilirubin). The studied compounds are safe at doses intended for oral administration and are recommended for further preclinical and clinical trials.

Keywords: breath test, linoleic acid, linolenic acid, carbon-13

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Compliance with ethical standards: the study was approved by the regional Ethics Committee (Protocol № 3 dated February 26, 2018). Animal housing met the *Sanitary and Epidemiological Requirements for Laboratory Animal Facilities* (Guidelines 2.2.1.3218-14).

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ИССЛЕДОВАНИЕ ТОКСИЧНОСТИ ^{13}C-МЕЧЕНЫХ ЛИНОЛЕВОЙ И ЛИНОЛЕНОВОЙ КИСЛОТ, ПРЕДНАЗНАЧЕННЫХ ДЛЯ ПРОВЕДЕНИЯ ДИАГНОСТИЧЕСКИХ ДЫХАТЕЛЬНЫХ ТЕСТОВ

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Неинвазивные дыхательные тесты с применением изотопно-меченых соединений представляют собой новый высокоточный и безопасный метод функционального исследования печени и билиарной системы. Целью работы было провести биологические испытания острой и субхронической токсичности ^{13}C-меченых линолевой и линоленовой кислот, синтезированных по оригинальной методике и предназначенных для проведения диагностических дыхательных тестов. При однократном внутрижелудочном введении изучаемых соединений лабораторным мышам линии BALB/c и крысам Wistar в дозах, превышающих диагностические в 500–2500 раз, образцы соединений не вызывали смертности экспериментальных животных. При проведении субхронического эксперимента на крысах при дозировках испытываемых соединений, в 5 и 25 раз превышающих терапевтическую дозу для человека, в течение 14 суток было выявлено отсутствие достоверных изменений у животных в экспериментальных группах по сравнению с контрольной по массе тела, гематологическим показателям (содержанию эритроцитов, лейкоцитов и тромбоцитов в крови) и биохимическим показателям сыворотки крови (уровню гемоглобина, общего белка, щелочной фосфатазы, аланинаминотрансферазы, аспартатаминотрансферазы, лактатдегидрогеназы, билирубина). Исследованные меченые кислоты безвредны в дозах, планируемых для перорального введения, и могут быть рекомендованы к доклиническим и клиническим испытаниям.

Ключевые слова: дыхательный тест, линолевая кислота, линоленовая кислота, углерод-13

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Breath tests are safe and effective tools that provide diagnostic information about the internal organs of the human body. The stable ^{13}C [1] and minimally radioactive ^{14}C [2] breath tests were introduced into clinical practice in the late 20th century. Since then, they have become the gold standard in detecting *Helicobacter pylori* infection [3]. Urease abundantly produced by *H. pylori* breaks down the urea taken in by the patient into $^{13}CO_2$ or $^{14}CO_2$ and ammonia. These products of urea hydrolysis are absorbed into the bloodstream and then excreted by the lungs [4]. The exhaled isotope-labeled CO_2 can be measured using mass spectrometry or a Geiger-Mueller counter [5]. In the absence of *H. pylori* infection, the reaction described above does not occur, and the concentrations of the exhaled $^{13}CO_2$ or $^{14}CO_2$ do not fall outside the reference range.

Non-invasive breath tests are highly accurate, cheap, easy, and safe for both the doctor and the patient. They provide valuable information about a number of parameters needed to choose a treatment strategy [6].

So far, carbon isotopes have been employed as tracers in a variety of breath tests for assessing insulin resistance (those are based on ^{13}C-glucose [7], ^{13}C-metacetin [8], ^{13}C-galactose and ^{13}C-aminopyrine [9]) and in the diagnosis of chronic liver diseases, including hepatitis B and C, cirrhosis, toxic hepatitis, alcoholic hepatitis, etc. ^{13}C-octanoic acid is used to measure the rate of gastric emptying [10], and ($^{13}C3$-glyceryl) tri-octanoate helps in detecting exocrine pancreatic insufficiency [4].

We believe that extensive efforts taken to increase production output of carbon isotopes and create cost-effective equipment for measuring the isotopic composition of the exhaled breath will expedite introduction of such tests into clinical routine.

Linoleic and linolenic acids are the main active components used in breath tests designed to assess liver and biliary tract function. They are fatty acids with a carbon chain consisting of 18 carbon atoms. The acids are unsaturated: linoleic acid has two carbon-carbon bonds, whereas linolenic acid has three [11].

Tetrasodium pyrophosphate widely used in the production of radiopharmaceutical agents both in Russia and abroad serves as an excipient. In Russia, it was approved for medical applications by Order 507 of the Ministry of Healthcare dated April 14, 1985 (Certificate 85/507/13) and is now marketed as Pyrphotech ^{99m}Tc [12]. Pyrphotech has demonstrated good performance in bone scintigraphy, imaging of acute myocardial infarction, imaging of the choroid, angiocardiography, etc. [12, 13].

Patent 2630691 registered in Russia describes the original *Method of synthesis of ^{13}C and ^{14}C linoleic and linolenic acids* [14]; these fatty acids labeled with carbon isotopes are intended for use in breath tests that assess liver and biliary tract function. It is essential that therapeutic and diagnostic agents undergo a safety trial prior to being introduced into clinical practice. The aim of this work was to study acute and subchronic toxicity of ^{13}C-labeled linoleic and linolenic acids.

METHODS

The acute toxicity test of linoleic and linolenic acids labeled with ^{13}C at position 1 was conducted in strict compliance with the *Guidelines on the study of general toxicity of pharmacological agents* [15]. The test was carried out in 95 male and female BALB/C mice weighing 18 to 20 g and 45 male and female Wistar rats weighing 180 to 210 g.

The tested ^{13}C-labeled acids were synthesized as described in the *Method of synthesis of ^{13}C and ^{14}C linoleic and linolenic acids* (Patent 2630691) [14]. The structure and purity of the intermediate and end products were assessed with nuclear magnetic resonance spectroscopy using an AM 300 spectrometer operating at 300 MHz (Bruker; Germany) and a DRX-500 spectrometer operating at 500 MHz (Bruker; Germany). Mass spectra were acquired using a direct-infusion Finnigan MAT Model Incos 50, 70 eV (Finnigan MAT; UK) and a high-resolution mass spectrometer MicrOTOFII (BrukerDaltonics; Germany) (ESI).

The animals selected for the study were kept in T3 cages. Animal housing met the *Sanitary and Epidemiological Requirements for Laboratory Animal Facilities*. The animals had unlimited access to tap water supplied via 500 ml glass bottles with stainless steel stoppers. The animals had a fixed meal schedule and were fed with pellets containing a balanced composition of amino acids, minerals and vitamins. Throughout the experiment, their physical activity, body weight, appetite, hair condition, and behavior were closely monitored.

For the acute toxicity test, the samples of the tested compounds were dissolved in olive oil and the freshly prepared solutions were administered to the animals by gavage. The rats received a single dose of 100 to 500 mg; the mice, a single dose of 10 to 50 mg. The follow-up period was 3 days. The lethal dose (LD_{50}) was determined for both BALB/c mice and Wistar rats using the Deichmann–LeBlanc method [15].

The subacute toxicity test of the synthesized isotope-labeled acids was conducted in 180 Wistar rats weighing 110–135 g. The test was performed in strict compliance with the *Guidelines on the study of general toxicity of pharmacological agents* [15]. The freshly prepared solutions of the tested compounds were fed to the animals by gavage at doses specified above every day for 2 weeks. The animals were distributed into several groups consisting of 15 males and 15 females each. Group 1 was the control group. The controls received olive oil that did not contain any of the tested acids. Group 2 received 5 mg/kg ^{13}C-labeled linoleic acid, which is 5 times higher than the clinical dose in humans. Group 3 received 25 mg/kg ^{13}C-labeled linoleic acid, which is 25 times higher than the human clinical dose. Group 4 received 5 mg/kg ^{13}C-labeled linolenic acid; this dose exceeds the human clinical dose 5-fold. Group 5 received 25 mg/kg ^{13}C-labeled linolenic acid, which exceeds the human clinical dose 5-fold.

In rats, blood samples (2.0–2.5 ml) were collected from the tail vein before the subchronic toxicity test, one week after the first administration of the tested compounds and 2 weeks after the first administration. Blood count was aided by a Picoscale PS-4M automated analyzer (Medicor-Elektromedika; Hungary).

Concentration of blood glucose, total protein, creatinine, cholesterol, total bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase were determined using a discrete FP 901 analyzer (Labsystems; Finland).

The rats were sacrificed on day 14 of the subchronic toxicity experiment. Necropsy was performed straight away in order to avoid self-digestion of tissue by intracellular enzymes. A detailed report was prepared for each necropsy.

Specimens of organs and tissues were fixed in 10% neutral buffered formalin, then dehydrated, cleared, and embedded in paraffin wax. Serial sections were prepared using a sliding MS-1 microtome (Ambimed; Russia). Upon deparaffinization, the sections were stained with hematoxylin-eosin, mounted in Canada balsam and covered with a coverslip. The obtained slides were examined under a Leica CM E microscope (Leica Microsystems; German) and photographed using a Micromed DCM-510 SCOPE eyepiece camera (Nabludatelnye pribory; Russia) at ×40, ×100, ×200, and ×400 magnification. Images

were processed in the Future Win Joe software (Future Optics; Chine) supplied with the eyepiece camera. Necropsy data were compared between the rats who had received ^{13}C-labeled linoleic and linolenic acids and the controls.

The null hypothesis was tested using the nonparametric Mann–Whitney U and Fisher exact tests in *Statistica 8.0 for Windows* (Dell; USA). Means, the median, maximum and minimum values and interquartile ranges were calculated [16]. For qualitative variables, the sampling fraction was calculated and expressed as percentage, as well as the sampling error.

RESULTS

Study of acute single-dose toxicity of ^{13}C-labeled linoleic and linoleic acids

No signs of intoxication or gastric irritation were noticed in the animals following single administration of the tested compounds at the highest dose of 2.632 mg/kg for mice and 2.564 mg/kg for rats. No death cases were observed in both rats and mice. We found no differences in sensitivity to the compounds between the animals of different sex and species.

Thus, intragastric administration of ^{13}C-labeled linoleic and ^{13}C-labeled linolenic acids to small animal species at doses 2,500 times higher than the clinical dose in humans did not cause intoxication or animal death during the entire follow-up period (3 days). The manufacturer of ^{13}C-labeled linoleic acid (Science Lab; USA) specifies that its LD_{50} is 3.2 g/kg. This value was obtained after administering a significantly higher dose of the tested fatty acid to experimental animals. Therefore, we conclude that our acute toxicity test revealed no difference in toxicity between the acid synthesized by us and its commercially available analogue.

Study of subchronic toxicity of ^{13}C-labeled linoleic and linolenic acids in rats

Two-week daily administration of 5 and 25 mg/kg ^{13}C-labeled linoleic and linolenic acids did not cause any significant changes in the animals' behavior and appearance, as compared to the controls. The animals were active, their hair was smooth and appetite was good.

Body weight dynamics monitored in all experimental and control groups throughout the experiment are presented in Tables 1 and 2.

On the whole, no significant differences were observed between the experimental and control groups except for the male group that was receiving 5 and 25 mg/kg ^{13}C-linolenic acid for 2 weeks. Those male rats were gaining more weight than the controls, which suggests a stimulatory effect of the tested compound on animal growth. No detrimental effect of the acid was observed on the growth and general health of the laboratory animals.

Results of blood tests in the experimental and control groups are shown in Table 3.

Slight yet significant differences (> 0.95 confidence level) in hemoglobin concentrations were revealed in the male rats who were receiving 5 mg/kg ^{13}C-linolenic acid. In the group of female rats who receiving the same dose of ^{13}C-linolenic acid, the differences were significant for white blood cell and platelet

Table 1. Body weight dynamics of male rats that received ^{13}C-labeled linoleic and linolenic acids by intragastric gavage for 2 weeks

Before the experiment	Controls, g	Experimental group, g	*p*
Linoleic acid, 5 mg/kg	113.3	115.7	> 0.05
Linoleic acid, 25 mg/kg	113.3	114.0	> 0.05
Day 14 of the experiment			
Linoleic acid, 5 mg/kg	132.1	135.3	> 0.05
Linoleic acid, 25 mg/kg	132.1	133.6	> 0.05
Before the experiment			
Linolenic acid, 5 mg/kg	116.4	114.2	> 0.05
Linolenic acid, 25 mg/kg	116.4	117.5	> 0.05
Day 14 of the experiment			
Linolenic acid, 5 mg/kg	130.6	136.3	> 0.001
Linolenic acid, 25 mg/kg	130.6	138.2	< 0.001

Table 2. Body weight dynamics of female rats that received ^{13}C-labeled linoleic and linolenic acids by intragastric gavage for 2 weeks

Before the experiment	Controls, g	Experimental group, g	*p*
Linoleic acid, 5 mg/kg	111.9	112.2	> 0.05
Linoleic acid, 25 mg/kg	111.9	112.9	> 0.05
Day 14 of the experiment			
Linoleic acid, 5 mg/kg	129.3	132.1	> 0.05
Linoleic acid, 25 mg/kg	129.3	131.5	> 0.05
Before the experiment			
Linolenic acid, 5 mg/kg	113.8	115.4	> 0.05
Linolenic acid, 25 mg/kg	113.8	114.6	> 0.05
Day 14 of the experiment			
Linolenic acid, 5 mg/kg	127.2	134.1	> 0.05
Linolenic acid, 25 mg/kg	127.2	130.3	> 0.05

counts. However, on day 14 of the experiment, the differences faded, which may suggest they were accidental.

By contrast, the end of week 1 revealed no significant differences between the groups that were receiving higher (25 mg/kg) doses of ^{13}C-labeled linolenic acid. However, a significant decline in white blood cell count (> 0.999 confidence level) was noticed in the female rats on day 14, as well as a slight decline in platelet count in both males and females (> 0.95 confidence level). In spite of the differences between the groups, the absolute values of the measured parameters fell within the normal reference range, suggesting safety of the tested doses.

Total protein levels in the blood serum are given in Table 4.

The female rats who were receiving 5 mg/kg of the tested compound had higher total protein concentrations than the controls by the end of week 1 and week 2. A similar situation

Table 3. Blood count in rats that received ^{13}C-linoleic acid for 2 weeks

5 mg/kg			25 mg/kg		
Red blood cells					
	Males	Females		Males	Females
Week 1	$p > 0.05$	$p > 0.05$	Week 1	$p > 0.05$	$p < 0.05$
Week 2	$p > 0.05$	$p > 0.05$	Вторая неделя	$p > 0.05$	$p < 0.05$
White blood cells					
Week 1	$p > 0.05$	$p < 0.05$	Week 1	$p > 0.05$	$p > 0.05$
Week 2	$p > 0.05$	$p > 0.05$	Week 2	$p > 0.05$	$p = 0.001$
Platelets					
Week 1	$p > 0.05$	$p < 0.05$	Week 1	$p > 0.05$	$p > 0.05$
Week 2	$p > 0.05$	$p > 0.05$	Week 2	$p < 0.05$	$p < 0.05$
Hemoglobin					
Week 1	$p < 0.05$	$p > 0.05$	Week 1	$p > 0.05$	$p > 0.05$
Week 2	$p > 0.05$	$p > 0.05$	Week 2	$p > 0.05$	$p > 0.05$

Table 4. Total protein concentrations (g/L) in the blood serum of rats that received ^{13}C-linoleic acid for 2 weeks

5 mg/kg			25 mg/kg		
	Males	Females		Males	Females
Week 1	$p > 0.05$	$p < 0.05$	Week 1	$p > 0.05$	$p < 0.05$
Week 2	$p < 0.05$	$p < 0.05$	Week 2	$p < 0.05$	$p > 0.05$

Table 5. Enzymic activity and total bilirubin levels in the blood serum of male rats that received ^{13}C-linoleic acid for 2 weeks

5 mg/kg			25 mg/kg		
Alkaline phosphatase					
	Males	Females		Males	Females
Week 1	$p > 0.05$	$p < 0.05$	Week 1	$p < 0.01$	$p < 0.01$
Week 2	$p < 0.05$	$p < 0.05$	Week 2	$p < 0.001$	$p < 0.01$
Alanine aminotransferase, un/L					
Week 1	$p > 0.05$	$p < 0.05$	Week 1	$p < 0.001$	$p < 0.001$
Week 2	$p < 0.05$	$p < 0.05$	Week 2	$p > 0.05$	$p > 0.05$
Aspartate aminotransferase, un/L					
Week 1	$p > 0.05$	$p > 0.05$	Week 1	$p < 0.05$	$p > 0.05$
Week 2	$p < 0.001$	$p < 0.01$	Week 2	$p > 0.05$	$p > 0.05$
Lactate dehydrogenase, un/L					
Week 1	$p > 0.05$	$p < 0.001$	Week 1	$p < 0.001$	$p < 0.001$
Week 2	$p < 0.001$	$p < 0.001$	Week 2	$p < 0.001$	$p < 0.001$
Total bilirubin, μM					
Week 1	$p < 0.001$	$p < 0.01$	Week 1	$p < 0.001$	$p < 0.001$
Week 2	$p > 0.05$	$p < 0.001$	Week 2	$p < 0.001$	$p < 0.001$

was observed in the male rats on day 14 of the experiment. As compared to the controls, total protein levels were also higher in the female rats a week after the onset of the experiment and in the male animals on day 14. This suggests that the studied compound stimulates protein synthesis, which cannot be regarded as a sign of its toxic effect.

Hepatotoxicity of candidate drugs is traditionally inferred from elevated aspartate and alanine aminotransferases, alkaline phosphatase, lactate dehydrogenase, and total bilirubin in the blood serum (Table 5).

In week one, we witnessed a slight decline in hepatic enzymes in the animals who were receiving 5 mg/kg ^{13}C-linoleic acid. During week 2, this decline became significant in all the subjects. A drop in alkaline phosphatase was the most pronounced for 25 mg/kg doses. By contrast, the levels of aminotransferases had gone back to normal by the end of week 2.

Administered at 5 and 25 mg/kg, ^{13}C-linoleic acid caused a significant decline in total bilirubin measured in the blood serum during weeks 1 and 2 of the experiment (except for the group of male rats during week 2). This observation suggests that the tested compound is not toxic to the liver and possibly has a hepatoprotective effect.

DISCUSSION

It is hard to assess the feasibility of breath tests in small laboratory animals because collection of exhaled air samples is a technically demanding procedure. Therefore, we plan to conduct the efficacy and safety trials of ^{13}C-linoleic and ^{13}C-linolenic acids in human patients once the acids successfully pass extensive toxicity studies. A similar strategy was adopted by other researchers who developed a ^{14}C urea breath test for detecting *H. pylori* infection [2].

Acute toxicity tests of ^{13}C-linoleic and ^{13}C-linolenic acids administered to BALB/c mice and Wistar rats as a single oral dose that exceeds the clinical dose in humans 500 to 2,500-fold did not reveal any signs of general toxicity or gastric irritation. No death cases were observed.

The study of subchronic toxicity of ^{13}C-linoleic acid administered to male and female Wistar rats at 5 and 25 mg/kg, which is 5 and 25 times higher than the clinical dose in humans, on a daily basis for 2 weeks did not reveal any pronounced effect of the acids on the general health, activity and behavior of the animals.

Moreover, at such high doses the acids produced a beneficial effect on the animals reflected in their blood count and blood chemistry. White blood cells and platelets underwent a transient decline in their number but the counts were still within the normal reference range. A transient decline was also observed for alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, and bilirubin. Those effects were dose-dependent, suggesting that they will be further reduced to zero at clinical doses. There are reports of similar effects observed for linoleic and linolenic acids not labeled with carbon isotopes [17].

CONCLUSIONS

^{13}C-labeled linoleic and ^{13}C-labeled linolenic acids synthesized following the original method described in Patent 2630691 (Russia) are safe for laboratory animals at doses intended for oral intake and can be recommended for further preclinical and clinical trials.

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