ORIGINAL ARTICLES

STUDY OF PHARMACOKINETICS OF LIPOSOMAL PHOTOSENSITISER BASED ON HYDROXYALUMINIUM TETRA-3-PHENYLTHIOPHTHALOCYANINE ON MICE

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Abstract

The present work is devoted to the study of pharmacokinetics of infrared photosensitizer (PS) based on hydroxyaluminium tetra-3-phenylthiophthalocyanine in a sterically stabilized liposomal form. The study was carried out on adult female mice. The PS was administered once intravenously at a dose of 6 mg / kg. Evaluation of the PS accumulation dynamics in the mice tissues and organs was performed at time intervals from 5 minutes to 7 days using spectral-fluorescent method. The maximum accumulation of the PS photoactive form was recorded in lungs (32 μg / g in the interval of 5–30 minutes after introduction), liver (20.8 μg / g in the interval of 4–24 hours after introduction) and spleen (28 μg / g 4 hours after introduction). At the same time, by the end of the observation period (7 days after administration), trace amounts of the PS photoactive form were still detected in the liver and the spleen at a calculated concentration of 0.5-1 μg / g. The PS accumulated the least in muscles and skin. The fluorescent signal from the PS accumulated in skin was detectable almost immediately, and its concentration remained at the same level (1.2-1.5 μg / g) for up to 3 days of observation. In the muscles, the concentration of the PS reached 1.5 μg / g 15 minutes after administration, and then gradually decreased until 0.25 μg / g at 24 hours.

Data on the pharmacokinetics of PS in blood, basic organs and tissues of animals were obtained, pharmacokinetic parameters were calculated. 7 days after the administration, the PS concentration in the skin and muscles was below the detection limit. The studies confirmed that PEGylation of the PS liposomal form slows down the process of its capture by reticulo-endothelial system. It was shown that the PS circulates in blood and organs of mice for a long time and it completely distributes only when 4 hours pass after administration.

Keywords: photosensitizer, pharmacokinetics, fluorescence.

For citations: Budko A.P., Deichman Z.G., Meerovich G.A., Borisova L.M., Meerovich I.G., Lantsova A.V., Kulbachevskaya N.Yu. Study of pharmacokinetics of liposomal photosensitizer based on hydroxyaluminium tetra-3-phenylthiophthalocyanine on mice, *Biomedical photonics*, 2018, vol. 7, no. 4, pp. 16–22. (in Russ.) doi: 10.24931/2413–9432–2018–7–4–16–22.

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ИЗУЧЕНИЕ ФАРМАКОКИНЕТИКИ ФОТОСЕНСИБИЛИЗАТОРА НА ОСНОВЕ ЛИПОСОМАЛЬНОЙ ФОРМЫ ТЕТРА-3-ФЕНИЛТИОФТАЛОЦИАНИНА ГИДРОКСИАЛЮМИНИЯ У МЫШЕЙ

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Резюме

Настоящая работа посвящена исследованию фармакокинетики фотосенсибилизатора инфракрасного диапазона на основе тетра-3-фенилтиофталоцианина гидроксиалюминия в стабилизированной липосомальной лекарственной форме. Исследования проводили на половозрелых мышах-самках. Фотосенсибилизатор вводили мышам однократно внутривенно в дозе 6 мг/кг. Оценку динамики накопления фотосенсибилизатора в тканях и органах мышей проводили в интервалах времени от 5 мин до 7 сут с использованием спектрально-флуоресцентного метода. Максимальное накопление фотоактивной формы фотосенсибилизатора было зарегистрировано в легких (32 мкг/г в интервале 5–30 мин после введения), печени (20,8 мкг/г в интервале 4–24 ч после введения) и селезенке (28 мкг/г через 4 ч после введения). При этом в печени и селезенке к концу срока наблюдения (7 сут после введения) продолжали определяться следовые количества фотоактивной формы фотосенсибилизатора – расчетная концентрация составляла 0,5–1 мкг/г. Хуже всего фотосенсибилизатор накапливался в мышцах и коже. При этом в коже флуоресценция фотосенсибилизатора определялась практически сразу, и концентрация его оставалась на одном уровне (1,2–1,5 мкг/г) до 3 сут наблюдения. В мышцах концентрация фотосенсибилизатора достигала значения 1,5 мкг/г через 15 мин после введения, после чего постепенно снижалась и к 24 ч составила 0,25 мкг/г. Через 7 сут после введения, значения концентрации фотосенсибилизатора в коже и мышцах находились ниже предела детектирования. Исследования подтвердили, что ПЭГилирование липосомальной лекарственной формы фотосенсибилизатора замедляет процесс его захвата ретикуло-эндотелиальной системой. Показано, что фотосенсибилизатор длительно циркулирует в крови и органах мышей, распределение заканчивается только к 4 ч после введения.

Ключевые слова: фотосенсибилизатор, фармакокинетика, флуоресценция.

Для цитирования: Будько А.П., Дейчман З.Г., Меерович Г.А., Борисова Л.М., Меерович И.Г., Ланцова А.В., Кульбачевская Н.Ю. Изучение фармакокинетики фотосенсибилизатора на основе липосомальной формы тетра-3-фенилтиофталоцианина гидроксиалюминия у мышей // Biomedical photonics. – 2018. – Т. 7, № 4. – С. 16–22. doi: 10.24931/2413–9432–2018–7–4–16–22.

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Introduction

Photodynamic therapy (PDT) is widely used for the treatment of malignant tumors, especially in cases of their surface and intracavitary location. The desired effect on the deep layers of large tumors is achieved with the use of photosensitizers (PS) of near infrared (IR) range [1]. The use of liposomal dosage forms (LLF) makes it possible to use new effective hydrophobic and hydrophilic substances in PDT, to increase the selectivity of PS accumulation in the tumor compared to the surrounding tissues and the effectiveness of the technique as a whole [1, 2].

For preclinical studies of the developed dosage form, including that on the basis of PS, it is necessary to study the pharmacokinetics [1–3]. This study is to determine the concentration of the active substance in various organs, tissues and body fluids at certain times after the administration and provides information on the duration of circulation of the PS in the body, target organs, which makes it possible to correlate the concentration and dosage of the PS with the pharmacological effect [4].

One of the most important requirements for pharmacokinetic studies is a wide dynamic range of the measurement method and tools, which must be at least three orders of magnitude.

Chromatographic, spectrophotometric, flame emission [5], atomic absorption [6], optical-spectral and spectral-fluorescent [7–11], and a number of other methods are used to determine the concentration of drugs in biological media. However, in the study of pharmacokinetics of PS, serious issues arise in connection with the use of many conventional methods of quantitative determination of the substance. Thus, when performing chromatography and spectrophotometry, it is necessary to extract the studied substance as fully as possible from the organs and tissues, including skin (it is the accumulation of PS in the skin that leads to negative side effects), which is quite difficult, especially in the case of quantitative determination of tetrapyrroles, to which the majority of PS belong. The task is even more complicated in the case when nanostructured PS based on hydrophobic substances is used (including the PS studied), since at different time after the introduction, some of the active substance molecules remain in the nanocarriers, whereas the other part is already transferred to the cell structure.

Flame emission and atomic absorption methods of elemental analysis [5,6], which can be used for the analysis without extraction of the studied pharmaceutical substances from tissues, have a limited dynamic range, especially for substances that consist of the same chemical elements as the body tissues (H, N, C, O) and do not contain rare elements, for example, metal atoms. The atomic absorption method was used by P. H. Brun et al. to study the pharmacokinetics of the Tookad photosensitizer in the blood and major internal organs, and the dynamics of its content in organs and tissues was evaluated by the intensity of the line of palladium included in Tookad [6].

At present, optical-spectral and spectral-fluorescent methods are used to determine the concentration of active substances in biological samples, in particular PS with characteristic absorption and fluorescence bands [7–11]. The use of equipment with high spectral resolution provides high sensitivity of such methods [10].

The purpose of this study was to investigate PS pharmacokinetics on the basis of tetra-3-phenylthiophthalocyanine hydroxylamine (3-$(PhS)_4$-PcAlOH) in LLF when administered intravenously to mice at a dose of 6 mg/kg.

Materials and methods

The studies of pharmacokinetics of PS were conducted on the basis of the substance developed in FSBI «N. N. Blokhin Russian Cancer Research Centre» of the Ministry of Healthcare of the Russian Federation LLF hydrophobic photoactive substance of tetra-3-phenylthiophthalocyanine hydroxyaluminium (abbr. 3-$(PhS)_4$-PcAlOH) (Fig. 1), created in FSUE «SRC «NIOPIK». The spectral absorption maximum of 3-$(PhS)_4$-PcAlOH corresponds to a wavelength of 717 nm. Stabilized LLF liposomes included 3-$(PhS)_4$-PcAlOH, lecithin lipids (USP30-NF25, P. 1145, Lipid GmbH, Germany) and cholesterol (USP30-NF25, P. 1101, Avanti Polar Lipids, Inc., USA), sucrose (FS.2.1.0034.15 GOST 5833–75, KHIMMED, Russia) as a cryoprotector, PEG-2000-DSPE ((1,2-dystearoyl-*sn*-glycero-3-phosphoethanolamine-[methoxy-(polyethylene-glycol)-2000] ammonium salt), Avanti Polar Lipids, Inc., USA). for the reduction of liposome capture by the reticuloendothelial system and a longer period of their circulation in blood [12–16].

Studies were carried out on 50 sexually mature female mice hybrids (C57Bl/6_J×DBA/2) F1, weighing 20–22 g, bred at FSBI «N. N. Blokhin Russian Cancer Research Centre» of the Ministry of Healthcare of the Russian Federation. The animals were randomly arranged into 10 groups of 5 animals in each.

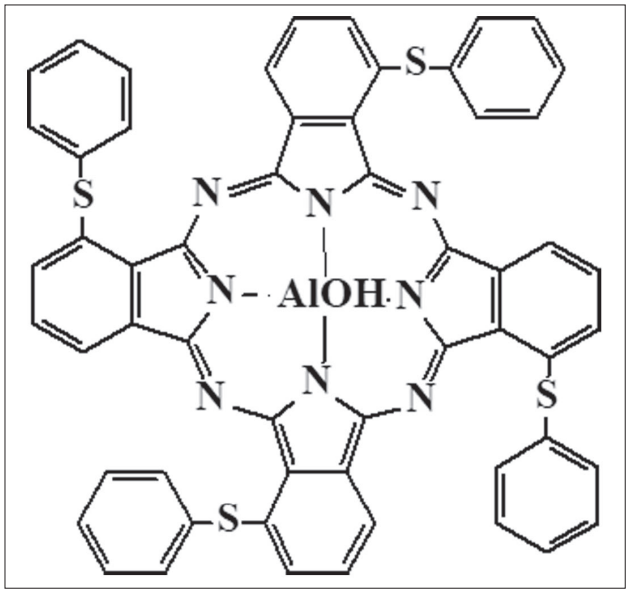

Рис. 1. Химическая формула тетра-3-фенилтиофталоцианина гидроксиалюминия
Fig. 1. Chemical formula of hydroxyaluminium tetra-3-phenylthiophthalocyanine

All animals were healthy and had a veterinary certificate of quality and of the health status. The animals were kept at an air temperature of 20–23 °C and relative humidity of 60–65% in the conditions of natural light and forced ventilation, on a litter of wood chips sterilized in a dry-air sterilizer. Animals were given standard industrial certified briquetted, fixed shelf life, feed for rodents. Feeding was done at the same time. Animals had free access to food and water.

All the experiments were performed in accordance with the recommendations of Good Clinical Practice [17].

Lyophilisate LLF 3-$(PhS)_4$-PcAlOH was redispersed with injection-grade water, 5.8 ml per vial, so that the content of 3-$(PhS)_4$-PcAlOH in the dispersion was 0.25 mg/ml. The volume of the dispersion for the injection was calculated based on the data on the body weight of the animal, and the dispersion was injected once by stream infusion into the tail vein, at a dose of 6 mg/kg.

The equipment and materials included a mechanical homogenizer (GlasCol, USA), Heildolph Reax Top vortex (Heidolph, Germany), Microman 1000 positive-displacement pipettes (Gilson, France), 24-hole plates with 16-millimeter holes 3424 MACG II for tissue culture (Costar, USA), distilled water.

The study of fluorescence was performed with the use of a modified laser electronic spectrum analyzer to LESA-01-«Biospec» (OOO «BIOSPEC», Russia). The dynamic range of the fluorescence signals recorded by the spectroanalyzer was extended to 3.5 orders of magnitude thanks to an additional algorithm for automatic control of the photosensor accumulation time. The linearity of response of the spectroanalyzer was established on samples of liposomal dispersions of 3-$(PhS)_4$-PcAlOH in distilled water with concentrations of 0.01 mg/ml 0.05 mg/ml; 0.1 mg/ml; 0.5 mg/ml; 2 mg/ml; 10 mg/ml; 25 mg/ml; 50 mg/ml and 0 (water as the control sample).

The statistical analysis was performed with the use of regular tools of Excel 2003 for Windows.

The preparation for the study of pharmacokinetics was performed as follows. The animals were killed by decapitation after 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 24 h, 48 h, 72 h and 168 h after the administration of the PS, after which blood was extracted from the jugular vein and heparin was added as an anticoagulant.

Samples of tissues and internal organs of mice (liver, kidneys, lungs, spleen, heart, muscle and skin) were obtained surgically. Liver, kidneys, and muscles were separated, reduced to a small size with eye scissors in a Petri dish on ice and divided into samples weighing 300g±1 mg, to which 1.5 ml of distilled water was added, followed by homogenization in glass on a mechanical homogenizer. For the spleen, lungs, heart and skin (skin samples were separated from subcutaneous tissue by scraping), the sample

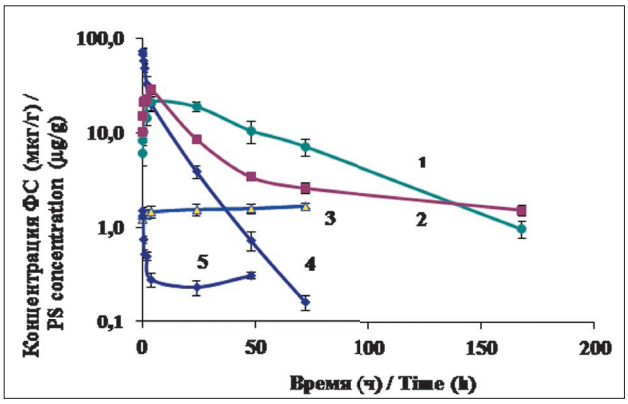

Рис. 2. Зависимость концентрации 3-(PhS)$_4$-PcAlOH от времени после его внутривенного введения в дозе 6 мг/кг в разных органах и тканях мышей: 1 – печень; 2 – селезенка; 3 – кожа; 4 – кровь; 5 – мышцы
Fig. 2. The dependence of 3-(PhS)$_4$-PcAlOH concentration on time after its intravenous administration at a dose of 6 mg/kg in different organs and tissues of mice: 1 – liver; 2 – spleen; 3 – skin; 4 – blood; 5 – muscle

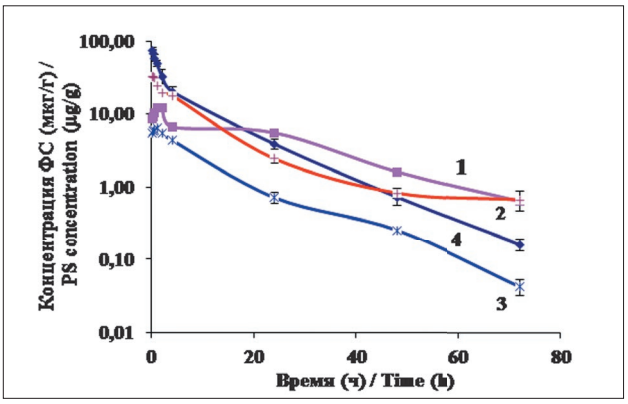

Рис. 3. Зависимость концентрации 3-(PhS)$_4$-PcAlOH от времени после его внутривенного введения в дозе 6 мг/кг в разных органах и тканях мышей: 1 – почки; 2 – легкие; 3 – сердце; 4 – кровь
Fig. 3. The dependence of 3-(PhS)$_4$-PcAlOH concentration on time after its intravenous administration at a dose of 6 mg/kg in different organs and tissues of mice: 1 – kidneys; 2 – lungs; 3 – heart; 4 – blood

weight was 100g±1 mg, the amount of water added decreased proportionally. The amount of homogenate equivalent to 100 mg of tissue was pipetted and transferred to the wells of the plates for subsequent measurement of fluorescence. Water was added in proportion to 100 mcl of blood.

Fluorescence of 3-(PhS)$_4$-PcAlOH in homogenates of samples was excited by laser radiation with a wavelength of 633 nm and recorded in the spectral range of 720–770 nm, and its concentration was determined by the integral intensity of fluorescence normalized by the intensity of the exciting laser radiation signal. To convert the values of fluorescence intensity into the values of PS concentration in the tissue, calibration curves were constructed by adding a known amount of PS to biological samples. Experimental and calibration samples underwent the same treatment.

Calibration showed that the dependence of the normalized integral fluorescence intensity on the concentration of 3-(PhS)$_4$-PcAlOH in samples within a wide range (for blood, within 0.1 to 129 μg/ml) is linearly dependant on the concentration, and with an accuracy of 6% for all organs it can be described by the function

$$I = k \times C,$$

where k = 27.99 $(\mu g/g)^{-1}$ for the measurement conditions recorded in the study, I is the normalized integral fluorescence intensity, and C is the concentration.

The ratio obtained from the calibration was used to quantify the concentration of 3-(PhS)$_4$-PcAlOH in the organs and tissues under examination.

Results and discussion

The dependences of the concentration of 3-(PhS)$_4$-PcAlOH in the blood and major organs and tissues (liver, spleen, kidneys, muscle, skin, heart, lungs) on the duration of the time interval between the introduction of the PS and the measurement of fluorescence in organs and tissues are shown in Fig. 2, 3.

The values of PS concentrations presented in the figures are the arithmetic mean of the obtained data and are accompanied by a confidence interval (p=95% at n=5).

The concentration in the blood 5 minutes after administration is 73.4 μg/l, going down to 3.9 μg/l over 24 h.

In the lungs, the maximum concentration of 32 μg/g is reached after 5 min of observation, does not change for 30 min, and then slowly decreases: the concentration drops to 2.45 μg/g over 24 hours. In the lungs, heart and kidneys there is no phase of growth of concentration 3-(PhS)$_4$-PcAlOH at the beginning of observation.

In the liver, the concentration of 3-(PhS)$_4$-PcAlOH increases from 6.2 μg/g after 5 min of observation to 20.8 μg/g after 4 h, remains at this level for up to 24 h and then slowly decreases to 0.5 μg/g for 168 h.

In the spleen, the concentration increases from 8.2 μg/g after 5 min of observation to 28 μg/g after 4 hours, then rapidly decreases to 8.5 μg/g to 24 hours, but is observed for up to 168 h.

In muscles, the concentration of 3-(PhS)$_4$-PcAlOH is maximal by 15 min after administration and is 1.5 μg/g, after which it decreases to 24 h to 0.25 μg/g.

In the kidneys, the maximum concentration of 12.5 μg/g is achieved by 2 h of observation, then by 4 h it is reduced 2 times to 6.64 μg/g, and by 72 h, to 0.65 μg/g.

In the skin (homogenized, without subcutaneous tissue), the concentration of 3-(PhS)$_4$-PcAlOH gradually increases from 1.2 μg/g after 4 h to 1.7 μg/g after 72 h after administration.

Таблица 1
Фармакокинетические параметры
Table 1
Pharmacokinetic parameters

Орган, ткань Organ, tissue	Параметры Parameters						
	C_0 мкг/мл μg/ml	V_1 мл ml	V_b мл ml	AUC мкг×ч/мл μg×h/ml	Cl_{tot} мл/ч ml/h	$T_{0,5a}$ час h	$T_{0,5b}$ час h
Кровь Blood	76,9	1,73	4,32	411,43	0,32	0,96	9,76

By 168 h of observation, the concentration values of 3-$(PhS)_4$-PcAlOH in the skin, kidneys, lungs, heart, and muscles are below the detection limit.

The obtained results allowed to calculate the following pharmacokinetic parameters in accordance with [18] for the dependence of PS concentration/time in the blood of mice after intravenous administration of 3-$(PhS)_4$-PcAlOH at a dose of 6 mg/kg: C_0 is the calculated concentration in the blood at the time of observation: 0 h; V_1 is the apparent estimated volume of dose distribution at the time of observation0 h;

V_b : kinetic volume of distribution;

AUC – area under the curve of «concentration – time»;

Cl_{tot}: total clearance, i. e. the volume of blood cleared from the pharmaceutical product over the time unit;

$T_{0.5a}$ is the distribution half-life of the pharmaceutical agent, the fast phase of the concentration decline;

$T_{0.5b}$ is the elimination half-life of the drug, the slow phase of the concentration decline.

It follows from the obtained data that the concentration-time dependence for blood is described by the two-component model equation. The distribution phase, with its rapid decrease in the concentration of 3-$(PhS)_4$-PcAlOH in the blood, is characterized by a high value of $t_{0.5a}$=0.96 h. The analysis of the data (first of all, of the high value of $T_{0.5a}$) points in the long circulation of LLF 3-$(PhS)_4$-PcAlOH in the blood, the distribution ends up to 4 hours after its introduction. High concentrations of 3-$(PhS)_4$-PcAlOH in the organs of the reticuloendothelial system (liver, spleen) are achieved only 4 h after administration. These data are consistent with the findings [14–16] that PEGylation reduces the capture of liposomes by the organs of the reticuloendothelial system, and extravasation of liposomes through the defects of the endothelial layer of neovascularization due to prolonged circulation leads to an increase in the level and selectivity of PS accumulation in the tumor. This correlates with the results obtained in the study of the level and selectivity of accumulation of the studied PS on tumor models [11], where the highest values of the level and selectivity of accumulation were observed 4–7 hours after its introduction (depending on the selected tumor model), and this time interval was recognized as the appropriate timeframe for the start of irradiation as a part of PDT.

The excretion phase with the slow decrease in the concentration of PS in the blood continues to 72 hours after administration: $T_{0.5b}$= 9.76 h.

Based on the value of V_1, the apparent volume distribution at the initial time for LLF, PS is distributed only in the blood. The value of the kinetic volume of V_b distribution is about 21% of the body volume of the animal.

The highest values of the area under the AUC curve were obtained in the liver and the spleen (2.3 and 1.6 times, respectively, higher than in the blood); in the kidneys, the ratio of AUC_{tissue}/AUC_{blood} was 0.7. The organs of FS accumulation are the spleen, the liver and the lungs.

Таблица 2
Площадь под кривой зависимости концентрация-время AUC для тканей мышей, соотношение площадей $AUC_{ткани}/AUC_{кровь}$
Table 2
Area under curve AUC for tissue, area ratio AUC_{tissue}/AUC_{blood}

Орган, ткань Organ, tissue	AUC мкг×ч/мл AUC μg×h/ml	$AUC_{ткани}/AUC_{крови}$ AUC_{tissue}/AUC_{blood}
Печень Liver	959,7	2,3
Почки Kidneys	275,4	0,7
Легкие Lungs	342,8	0,8
Селезенка Spleen	669,4	1,6
Мышцы Muscles	22,1	0,1
Сердце Heart	87,7	0,2
Кожа Skin	111	0,3

The kidneys and probably the liver are the organs responsible for PS excretion.

Conclusion

A method was developed for the preparation of biological samples for quantitative determination of concentration of PS.

The study examined the pharmacokinetics of the photosensitizer based on tetra-3-phenylthiophthalocyanine hydroxyaluminium in liposomal dosage form.

High (more than 20 μg/g) concentration values are observed in the liver, spleen, and, in the first hour of observation, in the lungs. After 24 hours of observation, the values of PS concentration are high enough in all organs. Traces of PS are detected in the liver and spleen in 168 h.

It was confirmed that the PEGilation of the liposomal drug form of PS slows down the process of its capture by the reticuloendothelial system. PS long circulates in the blood and organs of mice, with the distribution ending only 4 hours after administration.

The study was carried out with the financial support of the Russian Federal Property Fund (No. 17–07–01568) .

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