УДК 535.33

СПЕКТРОФОТОМЕТРИЧЕСКИЙ КОНТРОЛЬ ЧИСТОТЫ И СТАБИЛЬНОСТИ ФОТОСЕНСИБИЛИЗАТОРА НА ОСНОВЕ ИНДОТРИКАРБОЦИАНИНОВОГО КРАСИТЕЛЯ

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Разработана экспресс-методика контроля чистоты и стабильности фотосенсибилизатора для фотодинамической терапии на основе гидрофильного индотрикарбоцианинового красителя. Хранение фотосенсибилизатора в неблагоприятных условиях приводит к образованию в его субстанции примеси гидрофобного красителя. В спектре поглощения гидрофобного красителя проявляется новая полоса при 514 нм, нехарактерная для фотосенсибилизатора, с поглощением которого данная полоса не перекрывается, и поэтому ее наличие в спектре позволяет достоверно выявить примесь этого гидрофобного соединения в субстанции фотосенсибилизатора. Установлено, что оптимальные условия для контроля чистоты реализуются при концентрации фотосенсибилизатора ~0,8 ммоль/л, когда возможно обнаружение примеси гидрофобного красителя в количестве 0,6 мас. % и более. Методика обеспечивает оперативность проведения контроля чистоты фотосенсибилизатора и подразумевает использование доступного оборудования. Информация, полученная с помощью разработанной методики, согласуется с данными хромато-масс-спектрометрии.

Ключевые слова: фотодинамическая терапия; фотосенсибилизаторы; спектрофотометрия.

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SPECTROPHOTOMETRICAL PURITY AND STABILITY TESTING OF A PHOTOSENSITIZER BASED ON AN INDOTRICARBOCYANINE DYE

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The express method for testing purity and stability of an indotricarbocyanine dye based photosensitizer for photodynamic therapy was developed. Storage of the hydrophilic photosensitizer under unfavorable conditions resulted in contamination with a hydrophobic dye. The absorption spectrum of the hydrophobic dye was characterized by a new band at 514 nm that was absent in the absorption spectrum of the photosensitizer. There was no spectral overlap between this absorption band and the absorbance of the photosensitizer. Therefore, presence of the band at 514 nm allowed us to detect contamination of the photosensitizer substance with the hydrophobic dye. It was established that the optimal photosensitizer concentration for purity testing is *ca*. 0.8 mmol/L, when as little as 0.6 wt. % of the impurity can be detected. The developed method is not time consuming and requires accessible equipment only. Data obtained with this method was verified with the aid of liquid chromatography-mass spectrometry.

Keywords: photodynamic therapy; photosensitizers; spectrophotometry.

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Introduction

Photodynamic therapy is a promising method for cancer treatment [1–4]. The clinically approved photosensitizers are activated by radiation in the visible spectral range [5]. The visible radiation is absorbed and scattered significantly in biological tissues thus resulting in the tumor damage depth of only 14 mm [5]. Alternatively, a novel indotricarbocyanine dye is suggested as a photosensitizer for photodynamic therapy [6; 7]. The dye is characterized by an absorption band in the transparency window of biological tissues and a high molar absorption coefficient. Activation of the dye molecules is achieved with laser or light-emitting diode radiation in the spectral range between 700 and 800 nm [4], where intrinsic absorbance of biomolecules is negligible [2; 8]. The dye molecules in electronically excited states generate active intermediates that damage malignant cells. Photodynamic activity of this dye was demonstrated *in vivo* [6]. The high molar absorption coefficient of the dye ($2.5 \cdot 10^5$ L $\cdot$ mol^{-1} $\cdot$ cm^{-1} in the peak of the absorption band) allows us to extract information about microenvironment and stability of the dye molecules from spectral data.

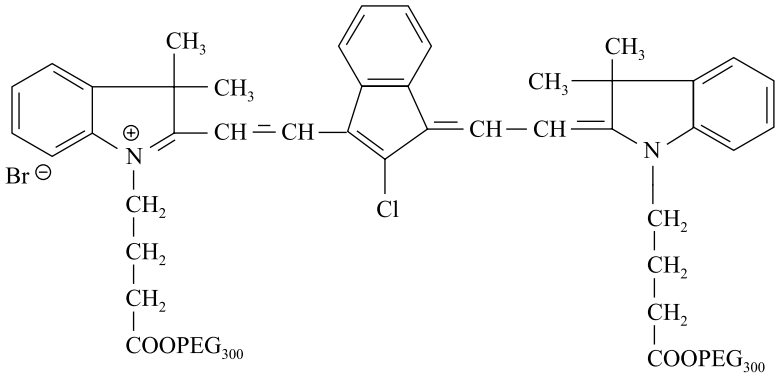
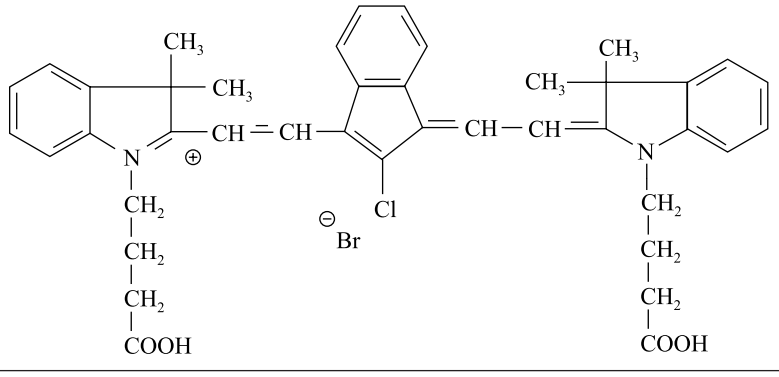
One of the most important properties of a therapeutic substance is the stability of its chemical composition upon storage. Proper water solubility (>1 mmol/L) of this indotricarbocyanine dye is due to presence of polyethylene glycol substituents linked to the carboxyl groups of a hydrophobic dye via ester bonds. Long-term storage of the hydrophilic dye at room temperature results in hydrolysis of the ester bonds. Purity testing is carried out for each batch of freshly synthesized photosensitizer with the aid of liquid chromatography-mass spectrometry (LC-MS). Although this method provides high detectability of impurities, it requires specific equipment and a large amount of time to carry out the testing. At the same time, direct purity testing of infusion solutions of the photosensitizer is of great importance. For that reason, it was decided to develop a method for testing purity of the indotricarbocyanine dye that would not be time consuming and would require accessible equipment only.

We report on research data that allowed us to develop the express method for spectrophotometrical purity testing of the indorticarbocyanine dye based photosensitizer.

Materials and methods

The compounds under study are symmetrical cationic indotricarbocyanine dyes (the structural formulae are shown in the table), that differ in their water solubility.

Structural formulae of the indotricarbocyanine dyes studied

Dye	Structural formula
1	
2	

The dye 1 is water soluble (>1 mmol/L) due to presence of hydrophilic polyethylene glycol substituents with the molar mass of 300 g/mol. The molar mass of the dye 1 was confirmed with LC-MS to be 1225 g/mol, in agreement with the structural formula. This compound is the active component of the photosensitizer for photodynamic therapy under development [6].

The hydrophilic dye 1 was synthesized by symmetrical substitution of the both carboxyl groups of the hydrophobic dye 2 with polyethylene glycol via ester bonds [6]. Molar mass of the compound 2 was confirmed by LC-MS to be 742 g/mol, in agreement with the structural formula. Aqueous solutions of the dye 2 were prepared using the 2 following methods: 1) ethanolic stock solution of the dye was injected into aqueous medium [9]; 2) water suspension of the dye was subjected to prolonged ultrasonic treatment and the dye solution was decanted after sedimentation of undissolved dye particles.

Absorption spectra were measured with the aid of SOLAR PV1251 spectrophotometer (Belarus) in quartz cells with the optical path length of 0.2 to 50.0 mm.

LC-MS measurements were carried out on the Agilent 1200 Rapid Resolution LC system with the Agilent 6410 Triple Quadruple LC/MS mass detector (the USA).

Results and discussion

Low concentrated (0.8 μmol/L) aqueous solutions of the dye 1 are characterized by the absorption band peaked at 706 nm with a shoulder in the range between 600 and 670 nm (fig. 1, curve *1*). The full width at half maximum (FWHM) of the spectrum is 64 nm (1300 cm^{-1}). Increasing the dye concentration up to 8 μmol/L results in growth of absorbance in the spectral range between 600 and 670 nm (see fig. 1, curve *2*), the FWHM of the spectrum being 100 nm (2130 cm^{-1}). An additional increase in the dye concentration up to 80 μmol/L leads to appearance of a new absorption peak at 650 nm, a decrease of the peak at 706 nm (see fig. 1, curve *3*), and growth of the spectrum FWHM up to 136 nm (3120 cm^{-1}). A further increase in the dye concentration up to 800 μmol/L is accompanied by a hypsochromic shift of the absorption peak to 610 nm (see fig. 1, curve *4*) and a reduction of the spectrum FWHM to 73 nm (1850 cm^{-1}). The observed changes in the absorption spectrum are due to aggregation of the dye molecules [10]. Only dye monomers with the absorption peak at 706 nm are present in the low concentrated solution (0.8 μmol/L). Increasing concentration facilitates aggregation of the dye molecules thereby resulting in an increase of the spectrum FWHM and a hypsochromic shift of the absorption peak. Both monomers and dye aggregates are present in the solutions of intermediate concentration (80 μmol/L),

which leads to the highest FWHM value of the spectrum. The high concentrated solution (800 μmol/L) is characterized by a lower FWHM value owing to the fact that the fraction of the monomers decreases and the solution contains predominantly dye aggregates. In addition to the monomers absorption band at 706 nm, distinct peaks at 650 nm (80 μmol/L solution) и 610 nm (800 μmol/L solution) are present in the spectrum. When the concentration increases, aggregation of the dye is intensified, with dimers and then trimers being formed.

Storage of the dye 1 in a non-airtight container at room temperature for 3 months and longer results in significant changes of the spectral properties. For example, the absorption spectrum of the dye after storage at room temperature for 2 years is shown in the fig. 2. A new absorption band at 514 nm is observed in the spectrum. Moreover, the FWHM of the main band increases significantly and intense unstructured absorption is present in the whole spectral range. The presence of the band at 514 nm in the absorption spectrum may be due to impurities that are formed upon long-term storage of the dye 1.

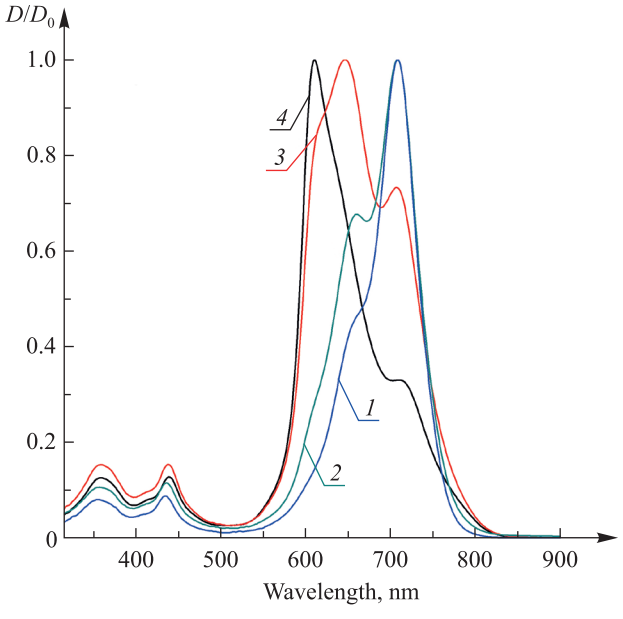


Fig. 1. Normalized absorption spectra of the dye 1 aqueous solutions at different concentrations: 0.8 μmol/L (*1*), 8 μmol/L (*2*), 80 μmol/L (*3*), 800 μmol/L (*4*)

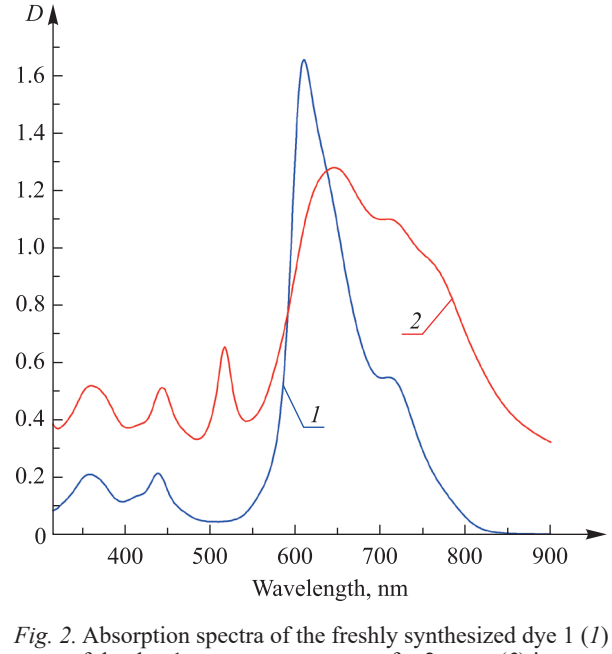


Fig. 2. Absorption spectra of the freshly synthesized dye 1 (*1*) and after storage of the dye 1 at room temperature for 2 years (*2*) in aqueous solution

In order to elucidate the reasons for the described changes in spectral properties, LC-MS analysis was carried out for the dye 1 samples that had been stored under different conditions. After storing of the photosensitizer 1 in a non-airtight glass or plastic container for 3 months at 2–6 °C virtually no changes in the mass spectrum were observed. Storage in a non-airtight container at 22–26 °C caused significant degradation of the dye 1 as soon as after 3 months. According to the LC-MS analysis, upon storage at the room temperature ester bonds in the dye 1 molecules are cleaved. It is evidenced by presence of the peaks corresponding to the dye 2 and free polyethylene glycol in the mass spectra. Hence, storage temperature affects the hydrolysis rate of the dye 1 ester bonds.

Purity testing of the dye 1 with the aid of LC-MS is not always possible due to limited access to the necessary equipment and a relatively large amount of time required for the testing. When the photosensitizer 1 is stored under unfavorable conditions, it is contaminated with the dye 2. The compounds 1 and 2 differ in water solubility and, consequently, in spectral properties of their aqueous solutions. It is thus suggested to identify the contamination of the photosensitizer 1 with the dye 2 by analyzing absorption spectra of the aqueous solutions. In order to determine the impurity concentration that can be identified with the aid of spectrophotometry, spectral properties of the dye 1 and 2 solutions of varying concentration were examined.

The dye 2 is virtually water insoluble at room conditions. Aqueous solutions of this hydrophobic dye can be prepared by addition of the ethanolic stock solution into aqueous medium as well as by a prolonged (*ca*. 2 h) ultrasonic treatment. The former method allows us to control the dye concentration and study the changes in spectral properties of freshly prepared solutions. The latter method does not require the addition of organic solvents and thus creates conditions similar to infusion solutions of the photosensitizer 1.

Spectral properties of the dye 2 aqueous solutions with ethanol admixture were studied thoroughly in [9]. It was shown that a narrow band at 514 nm with a FWHM of 21 nm (797 cm^{-1}) appears in the absorption spectrum, when the dye 2 concentration exceeds 2 μmol/L. This absorption band corresponds to H^*-aggregates, i. e. nanostructures containing many molecules of the dye 2.

Alternatively, aqueous solutions of the dye 2 were prepared by ultrasonication of an aqueous dye suspension. The absorption spectrum of the suspension immediately after ultrasonication is characterized by a broad long-wave band peaked at 706 nm and a narrow short-wave band peaked at 514 nm (fig. 3, curve *1*). The long-wave band decreases over time, whereas, the short-wave band increases (see fig. 3, curves *2*, *3*). The shape of the spectrum stays unchanged after *ca*. 10 days after preparation.

Absorbance in the spectral range between 850 and 1000 nm decreases over time and 14 days after ultrasonic treatment is virtually indiscernible (see fig. 3, curve *3*). The decrease in absorbance is accompanied by sedimentation of undissolved particles of the dye 2. Hence, light scattering on the undissolved particles contributes to the shape of the spectrum in this range. Absence of absorbance between 850 and 1000 nm 14 days after preparation of the suspension indicates that sedimentation of the undissolved particles is complete at this point. The homogeneous aqueous solution of the dye 2 can thus be decanted and used in further experiments.

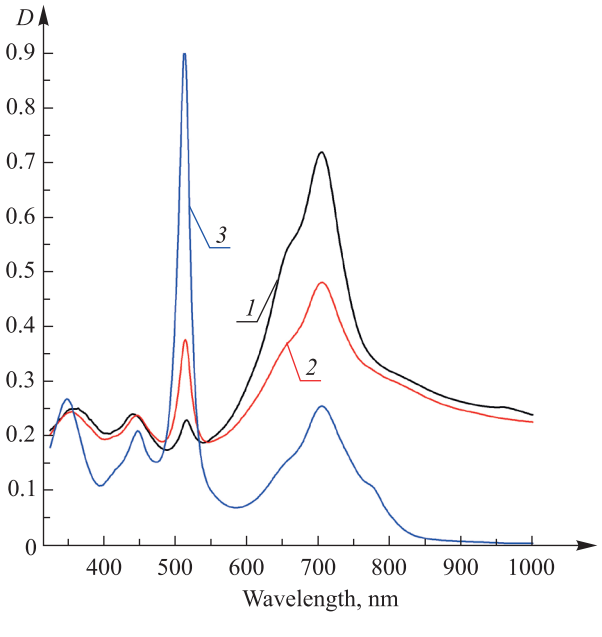


Fig. 3. Absorption spectra of the dye 2 aqueous suspensions 3 min (*1*) and 30 min (*2*) after ultrasonication; absorption spectrum of the dye 2 aqueous solution decanted 14 days after ultrasonication (*3*)

Solutions of the dye 2 prepared by the two described methods have similar shape of the absorption spectra. Consequently, the band at 514 nm can be ascribed to the H*-aggregates of the dye 2. The broad long-wave band corresponds to both the monomers and the dimers with absorption peaks at 706 and 658 nm, respectively. The H*-aggregates are formed in the aqueous solutions with ethanol admixture, when the dye 2 concentration exceeds 2 μmol/L. In the aqueous solutions prepared with the aid of ultrasonication the H*-aggregates remain stable, when the concentration is decreased to as low as 0.5 μmol/L.

Thus, the presence of the band at 514 nm in the absorption spectrum of the dye 1 after long-term storage (see fig. 2) is explained by contamination with the dye 2, whose molecules form the H*-aggregates. It is important to understand, why the molecules of the hydrophobic dye 2 are present in such aqueous solution, where no organic solvents are present and no ultrasonic treatment was applied. The reason for that is probably solubilization of a fraction of the dye 2 molecules with polyethylene glycol. Polyethylene glycol is covalently bonded to the dye 1 and is formed in its free form as a result of hydrolysis as well. Undissolved particles of the dye 2 are responsible for the unstructured absorbance present in the whole absorption spectrum of the contaminated dye 1 (see fig. 2, curve *2*).

The influence of polyethylene glycol on the dye 2 was studied in a thin layer (0.2 mm quartz cuvette) of the contaminated dye 1 solution. The band at 514 nm is initially present in the absorption spectrum. As water evaporates and the dye molecules are transferred to the cuvette walls, the absorption band at 514 nm decreases and is eventually indiscernable. The increase in polyethylene glycol concentration upon evaporation of water is probably the reason for dissolution of the dye 2 H*-aggregates.

The contamination of the photosensitizer 1 substance with the hydrophobic dye 2 can be identified by the presence of the H*-aggregate absorption band at 514 nm. Absence of spectral overlap of this band with the long-wave absorbance of the photosensitizer allows efficient detection of the impurity.

Purity testing of the dye 1 has to be conducted in a certain concentration range favorable for the H*-aggregate formation. In order to determine this concentration range, the mixtures of the dyes 1 and 2 with purity assured by LC-MS were prepared and their absorption spectra were analyzed. The dye 2 aqueous solutions were prepared with the aid of ultrasonication to avoid the influence of ethanol and then mixed with the dye 1 aqueous solutions. The peak at 514 nm is readily discernible (fig. 4, *a*) in the spectrum of the solution containing 792 μmol/L of the dye 1 and 8 μmol/L of the dye 2 (0.01 molar fraction of the dye 2). Absorbance at 514 nm is present in the spectrum of the aqueous solution containing 198 μmol/L of the dye 1 and 2 μmol/L of the dye 2 (0.01 molar fraction of the dye 2) as well, however, it is not distinct (see fig. 4, *b*).

As a result, in the 800 μmol/L solution of the contaminated dye 1 the peak at 514 nm is distinct, whereas, the influence of polyethylene glycol on the H*-aggregates stability is negligible. In such a solution contamination with the dye 2 can be detected, when the impurity molar fraction is as low as 0.01, which corresponds to 0.6 wt. %. Decreasing the concentration leads to a decrease in the H*-aggregate band and, consequently, to lower detectability.

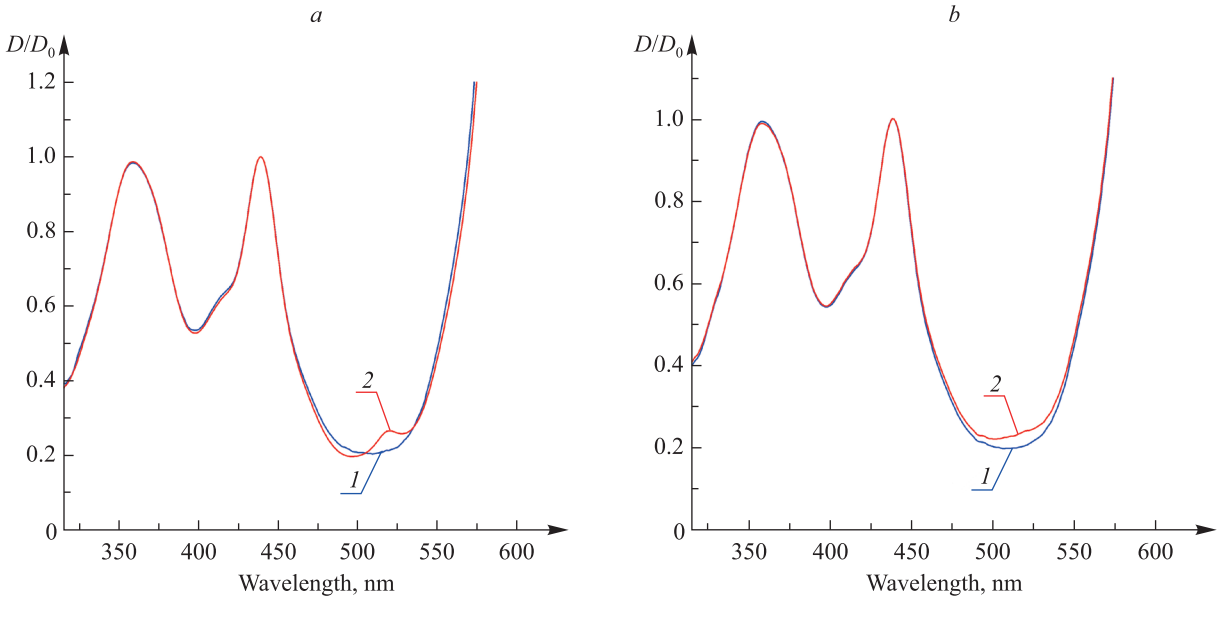


Fig. 4. Absorption spectra of the 800 μmol/L aqueous solution of the dye 1, curve *1*, and of the aqueous solution containing 792 μmol/L of the dye 1 and 8 μmol/L of the dye 2, curve *2* (*a*); absorption spectra of the 200 μmol/L aqueous solution of the dye 1, curve *1*, and of the aqueous solution containing 198 μmol/L of the dye 1 and 2 μmol/L of the dye 2, curve *2* (*b*). All spectra are normalized at 439 nm

Summary

The express method for testing purity of the photosensitizer based on the indotricarbocyanine dye 1 was developed. This method allows for detection of contamination with the hydrophobic dye 2, when as low as 0.6 wt. % of the impurity is present. The method requires accessible equipment only (a spectrophotometer) and can be performed in a short space of time providing a possibility to directly test infusion solutions of the photosensitizer. The developed method was verified with LC-MS.

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