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Radioiodination of Cellex®

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Abstract—The possibility of labeling Cellex® with ^{123}I was examined. Labeling was performed by oxidative iodination in the presence of Chloramine-T and Iodogen. The optimum labeling conditions were found. The target product with the radiochemical purity higher than 95% was isolated from the reaction mixture by column gel chromatography. The label distribution between constituent proteins of different molecular masses was determined by gel electrophoresis.

Keywords: Cellex®, labeling with radionuclides, iodine-123

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Modern knowledge in the field of clinical pharmacokinetics allows a physician to control the "fate" of a drug in patient's body, performing the maximum efficient and safe therapy [1]. Problems in studying the pharmacokinetics (resorption, metabolism/biotransformation, distribution, penetration through the blood–brain barrier, excretion) of drugs containing nucleic acids, proteins, and peptides are caused not only by possible hydrolysis of the components, but also by the fact that numerous similar compounds are present in the body of mammals, which complicates selective detection of the components of the drug complex.

The modern achievements of molecular biology with the development of high-sensitivity methods for in vivo imaging on the microscopic scale allow detection of ionizing radiation (PET, positron emission tomography; SPECT, single-photon emission computed tomography) with observation of the dynamics of molecular events on the whole-body, cell, and subcell levels [2–6], which, in turn, allows continuous monitoring of numerous biological and pathophysiological processes using radiolabeled proteins, peptides, and other molecules in doses exerting no pharmacological side effects.

Cellex®, an innovative formulation in the form of a solution for parenteral administration, is a complex of proteins and polypeptides of embryonal brain tissue with the molecular mass from 10 to 250 kDa with high degree of nativity. It contains growth and differentiation factors and signal molecules. Cellex® is used for treatment of acute brain perfusion disorders of ischemic and hemorrhagic character in the acute and early rehabilitation steps of the disease; it exhibits pronounced neuroprotective effect on neuron and glial cells of the brain, and also high neuroreparative potential [7].

One of the most suitable radioactive isotopes for labeling such formulations for in vivo investigations is ^{123}I. The γ-radiation energy of this isotope (159 keV) is ideal for SPECT imaging of its distribution in cells. The half-life of ^{123}I (13.2 h) allows monitoring its distribution in a living body for 2–3 days, making it possible to study pharmacokinetics of labeled preparations with relatively low dose burden.

This study was aimed at finding conditions for ^{123}I labeling of Cellex® constituent proteins into which the label can be introduced and at demonstrating that the labeled product preserves the molecular-mass distribution of the initial substrate.

The most widely used method for protein radiolabeling is direct iodination of thyrosine and histidine molecules in the presence of oxidants by the method suggested as early as 1963 [8, 9]. The most widely used oxidants are *N*-chlorotoluenesulfamide (Chloramine T) and 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen).

Introduction of an iodine atom into a protein molecule, as a rule, does not significantly alter the properties of the labeled compound. However, introduction of excess iodine [10] reduces the solubility of the protein in aqueous solution down to complete precipitation (in the case of proteins with high thyrosine content). For this study, we chose the reaction conditions in a neutral medium traditionally used for iodine labeling of proteins and thyrosine [11–13], with Chloramine-T and Iodogen as oxidants.

EXPERIMENTAL

Materials and equipment. We used chemically or analytically pure grade chemicals without additional purification. A solution of Cellex® was supplied by AO Farm-Sintez. Carrier-free ^{123}I in the form of an Na^{123}I solution in 0.01 M NaOH was produced at the Khlopin Radium Institute. Radioiodination was performed using Chloramine-T (Sigma–Aldrich) and ready reaction test tubes with Iodogen (Thermo Scientific). The materials for chromatography, Sephadex G-15 and TLC plates (silica gel on TLC Al foils), were purchased from Sigma–Adrich. The molecular-mass distribution of components of the protein–peptide complex was determined using a set of protein standards (Thermo Scientific) and the equipment for gel electrophoresis (Bio-Rad).

The radioactivity was measured with a Curiementor 3 dose calibrator (PTW Freiburg GmbH), a γ-ray spectrometer (ORTEC), and a scintillation scanning counter.

Iodination. The iodination using Chloramine-T was performed in 1.5-mL plastic test tubes with stoppers. A test tube was charged with 100 μL of a Cellex solution (200 μg of the protein), and 100 μL of a 0.001 M solution of Chloramine-T in a sodium phosphate buffer (pH 7.4) and 100 μL of an Na^{123}I solution (0.3 to 6 GBq of ^{123}I) in 0.01 M NaOH were added. Iodination with Iodogen was performed in test tubes of 12 × 75 mm size with the walls coated with Iodogen (50 μg). A test tube was charged with 100 μL of a Cellex solution (200 μg of the protein) and 100 μL of an Na^{123}I solution. The test tubes with the reaction mixtures were allowed to stand at room temperature with stirring for the required time.

The completeness of the reaction at room temperature and neutral pH values was judged from the yield of the labeled compound, which was determined by TLC. A typical radiochromatogram of the reaction mixture is shown in Fig. 1.

The peak at R_f from 0 to 0.1 belongs to labeled proteins; that with R_f = 0.9, to the residual unchanged iodide; and that at R_f 0.7–0.9, to unidentified labeled compounds. The radiochemical yield of the reaction (in our case, the ratio of the radioactivity bound with the protein to the initial radioactivity) was determined from the ratio of the peak areas.

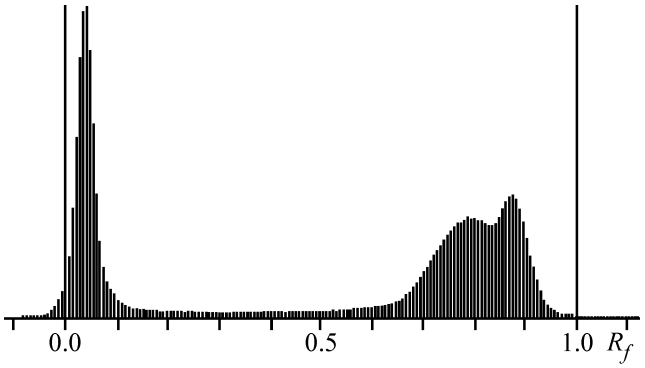

Fig. 1. Radiochromatogram of the reaction mixture.

Chromatographic isolation and analysis. After labeling, the labeled proteins and polypeptides of Cellex® were separated from the unchanged radioiodine and residual oxidant by gel chromatography [14–16]. After the iodination completion, the reaction mixture was applied onto a 10 × 200 mm column packed with Sephadex G-15. The elution was performed at a rate of 0.2 mL min^{-1} with sodium phosphate buffer (pH 7.4) and UV detection (280 nm). The eluent at the column outlet was collected in 1-mL fractions for the subsequent analysis. In the course of gel filtration, high-molecular-mass fractions pass through the sorbent bed virtually without retention, whereas low-molecular-mass impurities, salts, and, possibly, partial decomposition products are eluted later. Thus, separation of the reaction mixture on the chosen column allows isolation of fractions containing the labeled product with the radiochemical purity higher than 95%.

The reaction mixtures and fractions obtained by separation of the mixtures on a column were separated by TLC with a fluorescence indicator. After testing a series of chromatographic systems, we chose 50% methanol with the addition of 0.1% NaI. NaI was added to suppress the nonspecific sorption of microamounts of carrier-free Na^{123}I on silica gel. The activity distribution on the chromatogram was determined with a scanning scintillation counter.

Taking into account the possibility of aggregation and fragmentation of some proteins in the course of iodination in the presence of oxidants [17–19], we checked the constancy of the molecular-mass distribu-

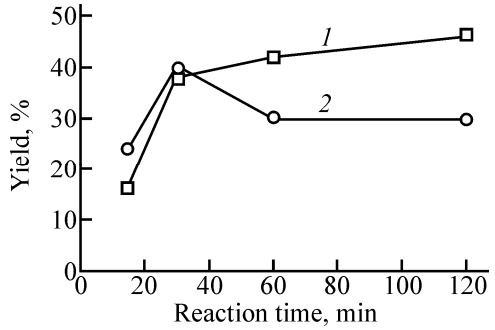

Fig. 2. Labeled product yield as a function of time with various oxidants. (*1*) Iodogen and (*2*) Chloramine-T.

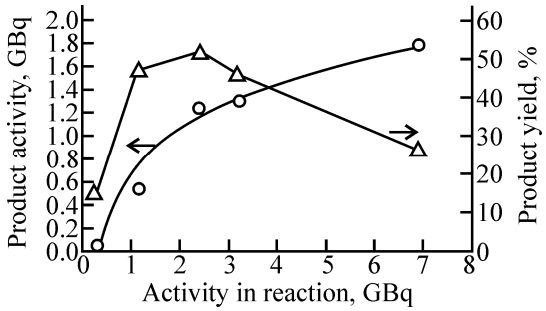

Fig. 3. Labeled product yield as a function of the activity taken into the reaction.

tion of the labeled sample. The protein concentration in the solutions obtained was determined by the Lowry method [20]. Gel electrophoresis of the protein fractions was performed by the procedure described in detail in [21].

RESULTS AND DISCUSSION

Iodination reaction. In a series of experiments, we chose the optimum time parameters of the electrophilic substitution in the presence of oxidants (Chloramine-T and Iodogen) for binding iodine atoms with thyrosine residues incorporated in protein molecules of Cellex. We have found that the labeled protein yield is directly proportional to the reaction time (Fig. 2).

The relatively low reaction rate when using Iodogen can be associated with the limited area of contact of the reaction mixture with the Iodogen deposited onto the test tube walls. The yield of the reaction performed with Chloramine-T passes through a maximum at 30 min, after which the yield of the labeled compound decreases. This fact can be attributed to side reactions occurring with such a strong oxidant as Chloramine-T with increasing reaction time (e.g., chlorination [18, 19]) or with incorporation of excess iodine (two atoms per thyrosine residue). In the latter case, an increase in the degree of protein iodination can lead to a decrease in its solubility, down to complete insolubility in aqueous solutions for proteins with high thyrosine content [10, 22].

The yield of the labeled substrate is also directly proportional to the activity taken for the reaction (at the same protein amount). With an increase in the amount of ^{123}I taken into the reaction, the activity of the product increases, and the radiochemical yield (activity fraction incorporated into the protein) after reaching the value of 50–55% starts to decrease (Fig. 3).

Thus, we have chosen the optimum ratio of the protein and ^{123}I to obtain the highest product yield.

Isolation of the labeled product. The results of the analysis of the fractions obtained in experiments on isolation of the labeled substrate from the reaction mixture (fraction volume 1 mL) are given in the table. The radiochemical purity of the labeled product was determined by TLC.

Results of analysis of the fractions obtained in one of the experiments on isolation of the labeled substrate from the reaction mixture (fraction volume 1 mL)

Fraction no.	Activity, MBq	Fraction of total activity, %	RCP, %
1	–		
2	–		
3	149	6.6	95
4	559	24.9	97
5	208	9.3	73
6	174	7.8	15
7	283	12.6	6
8	315	14.1	2
Sum from 9 to 14	553	24.7	
Total	2241	100	

The product had high radiochemical purity in the third and fourth fractions in which we were able to isolate, on the average, 25–30% of the activity applied onto the column. A part of the labeled protein was eluted later in fractions containing a large amount of unchanged iodine. The curve obtained in separation of the reaction mixture on a column with a gel sorbent and the measurement results are given in Fig. 4.

Molecular-mass distribution of the labeled substrate. It was repeatedly noted in the literature that iodination performed in the presence of oxidants can be accompanied by both fragmentation and aggregation of protein molecules. In this connection, it was necessary to make sure that the labeled product preserves to a sufficient extent the molecular-mass distri-

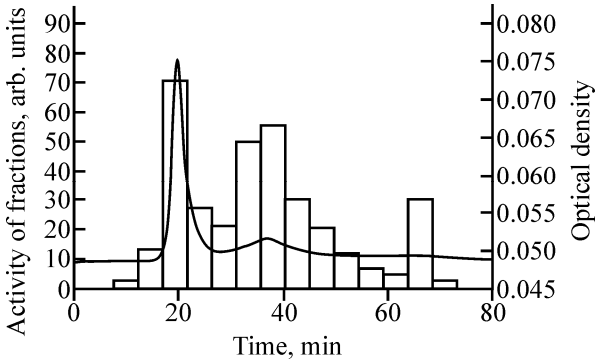

Fig. 4. Separation of the reaction mixture on a column packed with Sephadex G-15. Curve: signal of the UV absorption detector (280 nm); histogram: results of measuring the activities of 1-mL fractions.

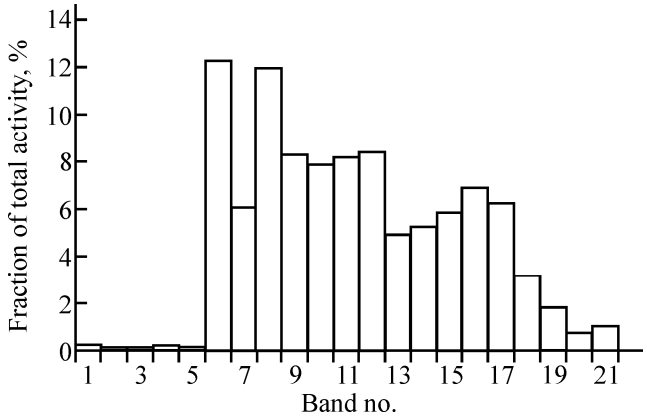

Fig. 5. Results of activity measurements after the gel electrophoresis of the labeled substrate. Molecular mass markers, kDa: band 3, ~250; band 5, ~150; band 7, ~100; band 8, ~70; band 9, ~55; band 12, ~35; band 13, ~25; and band 17, ~15.

bution of the initial substrate. Simultaneously we determined the label distribution between proteins of different molecular masses. To this end, a labeled sample was subjected to separation by gel electrophoresis in the presence of molecular mass markers to estimate the molecular masses of the bands obtained. To determine the radioactivity in the bands, the gel layer was cut into narrow strips, and their activity was measured with a γ-ray spectrometer. The results are shown in Fig. 5.

As can be seen, the labeling did not alter the molecular-mass distribution of the sample, and the activity distribution was approximately uniform for proteins of masses in the interval 15–120 kDa.

Thus, we have demonstrated the possibility of preparing ^{123}I-labeled Cellex® without altering the molecular-mass distribution of the constituent proteins. Depending on the task, the labeling conditions can vary with the aim to obtain either the highest percent yield or the maximal amount of the labeled substrate.

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