ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2015, Vol. 41, No. 6, pp. 578–589. © Pleiades Publishing, Ltd., 2015.
Original Russian Text © Yu.A. Zolotarev, A.K. Dadayan, N.V. Kost, M.E. Voevodina, O.Yu. Sokolov, V.S. Kozik, S.I. Shram, V.N. Azev, E.V. Bocharov, A.P. Bogachouk, V.M. Lipkin, N.F. Myasoedov, 2015, published in Bioorganicheskaya Khimiya, 2015, Vol. 41, No. 6, pp. 644–656.

The Qualitative Analysis of the Amide of the HLDF-6 Peptide and Its Metabolites in Tissues of Laboratory Animals with the Use of Tritium-Labeled and Deuterium-Labeled Derivatives[1]

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Received March 31, 2015; in final form, June 6, 2015

Abstract—The novel method for the peptide pharmacokinetics in tissues of laboratory animals was elaborated by the example of the HLDF-6 peptide amide. This method practically completely prevented the proteolytic degradation of peptides in the course of the analysis. The HLDF-6 hexapeptide (TGENHR) is a fragment corresponding to the 41–46 sequence of the human leukocyte differentiation factor (HLDF). It exhibits a wide spectrum of nootropic and neuroprotective activity. Therapeutic agents for prevention and therapy of cerebrovascular and neurodegenerative diseases have been created on the basis of the HLDF-6 amide (TGENHR-NH_2). Pharmacokinetics and the molecular mechanism of action of the HLDF-6 peptide amide were studied using its tritium-labeled and deuterium-labeled derivatives. The labeled peptides were prepared with the use of the high-temperature solid-state catalytic isotope exchange reaction (HSCIE). The tritium-labeled [^{3}H]TGENHR-NH_2 peptide was obtained with a molar radioactivity of 230 Ci/mmol. The deuterium-labeled [^{2}H]TGENHR-NH_2 peptide was prepared with an average deuterium incorporation of 10.5 atoms per the one peptide molecule. The NMR spectroscopy confirmed a uniform distribution of the isotope label throughout the whole peptide molecule. This uniformity allowed a qualitative analysis of both the peptide itself and all the possible fragments of its biodegradation in the organism's tissues. The main TGENHR-NH_2 metabolites which were formed during its proteolytic cleavage in the blood plasma were quantitatively analyzed and pharmacokinetics of the peptide amide was investigated with the use of its tritium-labeled derivative after intravenous and intranasal administration in mice, rats, and rabbits. Values of the basic pharmacokinetic parameters were calculated, the hypothesis of pharmacokinetic linearity was checked, and metabolism of the peptide was studied on the basis of the obtained pharmacokinetic profiles of TGENHR-NH_2. The TGENHR-NH_2 peptide was shown to have extremely high bioavailability with its intranasal administration (34% for rats). The peptide was quickly disappeared from blood due to its active proteolytic degradation in organism's tissues. The TGENHR-NH_2 peptide was shown to be highly stable towards the proteolytic hydrolysis during its incubation with the blood plasma, and a quantitative analysis of the formed metabolites was performed.

Keywords: the HLDF-6 peptide, pharmacokinetics, metabolism, tritium-labeled and deuterium-labeled peptides, ^{1}H NMR spectroscopy

DOI: 10.1134/S1068162015060205

INTRODUCTION

Creation of new effective nootropic and neuroprotective drugs for treatment of cerebrovascular and neurodegenerative diseases is an urgent medico-social problem. Preparations of a peptide nature which have high efficacy and very low toxicity are especially promising for solution of this task. Peptides are subjected to biodegradation within several minutes after the introduction into an organism, and peptide fragments with their own spectra of physiological activity are formed. Therefore, quantitative analysis of the introduced peptides and all their peptide fragments is very complex and remains largely unresolved. One of the approaches to its solution is the application of peptides with an isotopic label in all their amino acid residues [1, 2]. Such uniformly tritium-labeled peptides were successfully used for a radioreceptor analysis and

Abbreviations: SH, spillover hydrogen; HSCIE, hightemperature solidstate catalytic isotope exchange; HLDF, human leukocyte differentiation factor; HLDF-6 amide, $_{41}$TGENHR$_{46}$ hexapeptide amide.

[1] The article has been adapted from the report presented at the VII Russian symposium "Proteins and Peptides", Novosibirsk, 12–17 July, 2015.

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in vitro and in vivo studies of the peptide metabolism. The high-temperature solid-state catalytic isotope exchange reaction (HSCIE) was used for the preparation of such peptides and proteins. Tne HSCIE reaction requires no solvent and occurs at high temperature under the action of spill-over hydrogen (SH) [3, 4]. The HSCIE application provides a high degree of substitution of tritium for hydrogen. A number of tritium-labeled physiologically active compounds with the 70–90% substitution degree of tritium for hydrogen was prepared by this reaction [5]. Such substitution degree is impossible to achieve by any other method [5]. Theoretical and experimental analysis of the kinetic isotope effect of the HSCIE reaction has demonstrated that its value proves to be only 1.1–1.3 [2]. At the same time, the kinetic isotope effect for the solution reactions of the hydrogen substitution varies from 3 to 30. Dramatically low values of the kinetic isotope effect result in the fact that tritium and deuterium substitute hydrogen of an organic compound with similar rates, and the distribution of the isotopic label appears to be equal for the HSCIE reactions that occur with deuterium or tritium under the same conditions [2].

Previously, we isolated new leukocyte differentiation factor (HLDF) from the HL-60 cells of promyelocytic human leukemia and identified its six-member fragment, HLDF-6 ($_{41}$TGENHR$_{46}$) which exhibited the complete differentiating activity of the full-size factor [6, 7]. Experiments on a primary culture of neuronal cells of hippocampus, cerebellum, and blood immunocompetent cells gave direct evidences of the HLDF-6 neuroprotective effect [8]. The peptide at doses of 1–50 μg/kg was shown to eliminate the pronounced cognitive deficit and to facilitate the recovery of the damaged memory on the experimental models of Alzheimer's disease, drug addiction, and ischemic stroke [9]. The conversion of the *C*-terminal carboxyl group of the HLDF-6 peptide into its amide was previously shown to increase the peptide stability in the blood plasma of rats [10]. The acidic and amide forms of HLDF-6 were also compared on the models of Alzheimer's disease and ischemic stroke. The HLDF-6 amide was shown to exhibit the higher activity and to be more promising as a therapeutic agent [11]. Thus, we chose the amide form of the HLDF-6 peptide for creation of a novel pharmaceutical preparation with high nootropic and neuroprotective activity and studied its pharmacokinetics.

RESULTS AND DISCUSSION

Synthesis of the isotope-substituted derivatives of the TGENHR-NH$_2$ peptide for the pharmacokinetic studies. The hydrogen substitution of the isotopic atoms occurs in the HSCIE reaction on new acidic centers which are formed under the SH action in the solid phase according to the electrophilic one-center synchronous mechanism. A five-coordinated carbon atom appears in the transition state of this reaction, and three-center bond is formed between carbon and the exchanging hydrogen atoms [12]. This mechanism of the HSCIE reaction is realized with the preservation of the configuration of the asymmetric carbon atom and prevents racemization in the course of substitution of isotopic atom for hydrogen. The isotopic hydrogen exchange in solution is accompanied by the configuration conversion and racemization distinct from this solid-phase reaction.

The HSCIE reaction gives peptides that are uniformly labeled with the hydrogen isotopes [1]. The use of such labeled physiologically active peptides opens up new opportunities for more correct results of an analysis of the peptide pharmacokinetics. The HLDF-6 amide was highly labeled with the hydrogen isotopes in the HSCIE reaction for the pharmacokinetic studies and investigation of mechanisms of the peptide action. The uniformly deuterium-labeled peptide, [^{2}H]TGENHR-NH$_2$, contained 10.5 deuterium atoms per one peptide molecule in average (Fig. 1). The tritium-labeled peptide, [^{3}H]TGENHR-NH$_2$, had the molar radioactivity of 230 Ci/mmol. It should be noted that the data on radioactivity of the peptide fragments that were formed from this uniformly tritium-labeled peptide did not allow the direct calculation of concentrations of products of its enzymatic reactions, because its fragments have different molar radioactivity at the uniform distribution of the label throughout the whole peptide molecule.

The data on the distribution of the deuterium atom throughout of the chain of the [^{2}H]TGENHR-NH$_2$ peptide were used for the calculation of the molar radioactivity of the fragments of the tritium-labeled [^{3}H]TGENHR-NH$_2$ peptide. It was previously shown that the HSCIE reaction with deuterium and tritium under the same conditions resulted in the same distribution of the isotope atoms in organic compounds, because deuterium and tritium reacted with organic compounds with similar rates and equal selectivity, and the rate ratio of these reactions approached a unity [2]. The deuterium distribution in the [^{2}H]TGENHR-NH$_2$ peptide was analyzed by NMR spectroscopy (Fig. 2, Table 1). The data on the deuterium distribution in the [^{2}H]TGENHR-NH$_2$ peptide were used for the calculation of molar radioactivity of the peptide fragments which were formed after the biodegradation of the [^{2}H]TGENHR-NH$_2$ tritium-labeled peptide.

Pharmacokinetics of the TGENHR-NH$_2$ peptide and its fragments in the blood plasma of rats. The biotransformation of the [^{3}H]TGENHR-NH$_2$ peptide was analyzed in heparin blood plasma by HPLC with the use of a tandem of an UV-detector and a fluid-flow radioactivity detector. The 2- to 5-member peptide fragments were synthesized as markers for an identification of the peptide fragments of the [^{3}H]TGENHR-NH$_2$ peptide biotransformation in the rat blood plasma. The formation of such peptide fragments was

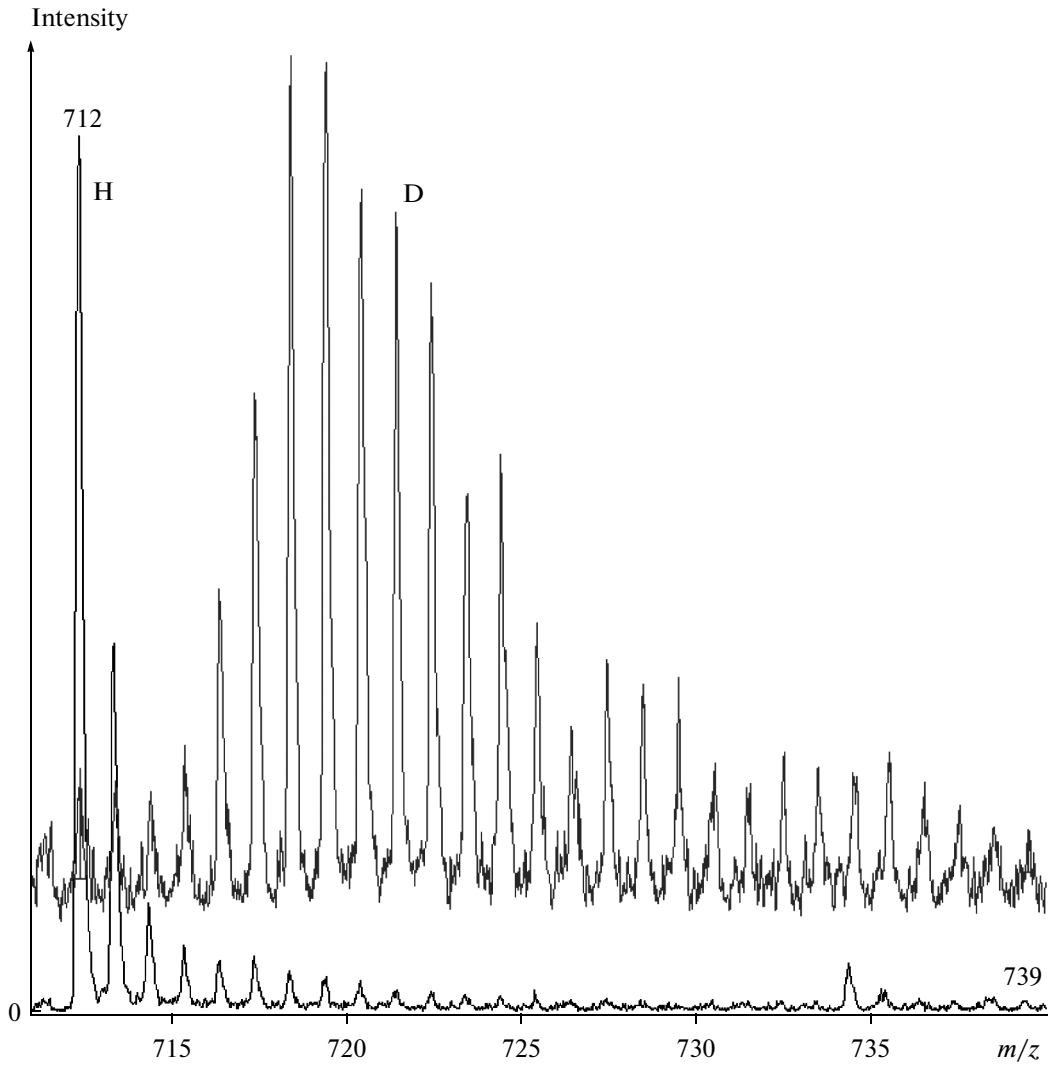

Fig. 1. Fragments of the MALDI mass spectra of (H) the TGENHR-NH_2 peptide and (D) the deuterium-labeled [^{2}H]TGENHR-NH_2 peptide.

possible as a result of the stepwise proteolytic hydrolysis from the *N*- or *C*-termini of the peptide chain. We pioneered in the quantitative in vitro analysis with the application of the tritium-labeled [^{3}H]TGENHR-NH_2 peptide for not only the TGENHR-NH_2 peptide but the fragments of its proteolytic hydrolysis as well.

Previously, the physiological activity and stability to the proteolytic hydrolysis of TGENHR and TGENHR-NH_2 peptides were compared, and the latter was shown to exhibit significantly higher nootropic activity and the stability to the hydrolysis [10]. The same paper reported quantitative analysis of these peptides after their proteolytic hydrolysis in rat blood plasma with the use of the tritium-labeled derivatives of these peptides. The peptide fragments which were formed after the proteolytic hydrolysis of these peptides were only qualitatively analyzed. The ENHR-NH_2 peptide was identified as the main product of the biodegradation of the TGENHR-NH_2 peptide and was shown to be further converted to the HR-NH_2 dipeptide.

Our study was aimed at the quantitative analysis of the peptide fragments which were formed during the proteolytic hydrolysis of the TGENHR-NH_2 peptide by the radiochromatographic method on the basis of the data on the distribution of the isotope label in this peptide. An example of the radiochromatographic HPLC analysis of the 80-min biodegradation products of the TGENHR-NH_2 peptide with the starting concentration of 2.5 μM in the rat blood plasma was illustrated in Fig. 3. The chromatographic peaks on the radiochromatogram were attributed with the application of a UV-detector and the peptide markers.

The calculation of the molar radioactivity of the peptide fragments of the biodegradation of the tritium-labeled [^{3}H]TGENHR-NH_2 peptide was based on the previously discovered fact that deuterium and

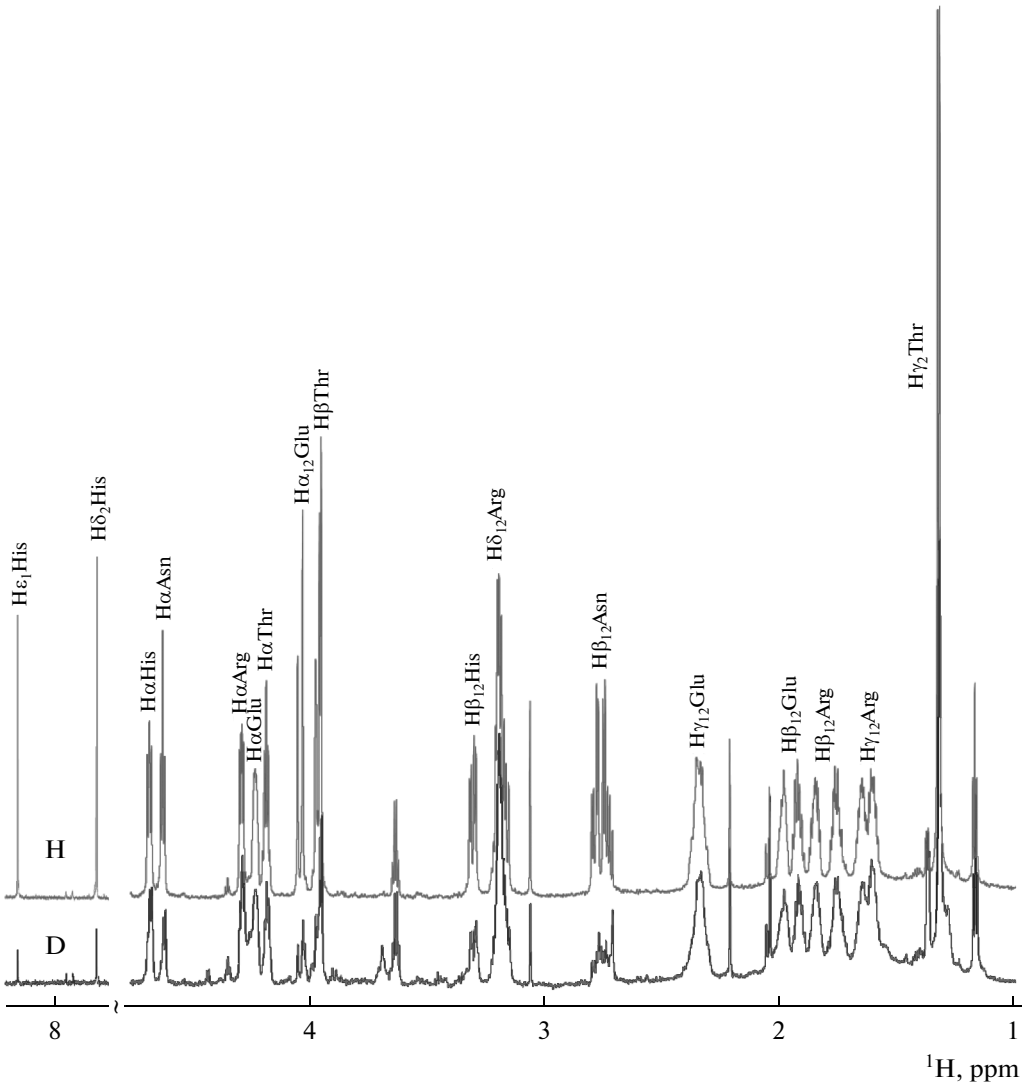

Fig. 2. The NMR spectra of (H) the nonlabeled TGENHR-NH_2 peptide and (D) deuterium-labeled [^{2}H]TGENHR-NH_2 peptide.

tritium interacted with organic compounds in the HSCIE reaction with similar rates and the same selectivity [2]. Taking into account the data on the distribution of the deuterium isotope label in the [^{2}H]TGENHR-NH_2 peptide, we calculated the deuterium incorporation into the ENHR-NH_2 and HR-NH_2 peptide fragments. The average number of the deuterium atoms in the [^{2}H]TGENHR-NH_2, [^{2}H]ENHR-NH_2, and [^{2}H]HR-NH_2 peptides proved to be 10.5, 7.2, and 4.2 per one molecule, respectively (Table 1). Then, the values of the molar radioactivity of the [^{3}H]ENHR-NH_2 and [^{3}H]HR-NH_2 peptide fragments were calculated on the basis of the data on the molar radioactivity of the tritium-labeled [^{3}H]TGENHR-NH_2 peptide and the same distribution of deuterium and tritium in these peptides. The values of the molar radioactivity for the [^{3}H]ENHR-NH_2, and [^{3}H]HR-NH_2 peptide fragments of the [^{3}H]TGENHR-NH_2 peptide (the molar radioactivity of 230 Ci/mmol) were calculated and proved to be 158 and 92 Ci/mmol, respectively. The quantitative analysis of the composition of the peptides which were formed by the biodegradation of the [^{3}H]TGENHR-NH_2 in the blood plasma was performed on the basis of these values of the molar radioactivity and the numerical values of radioactivity of the HPLC fractions.

The analysis demonstrated that the examined peptide was highly stable to hydrolysis in the blood plasma (Fig. 4). The period of its half-degradation was 8 min. This value is rather high for peptides that involved only natural amino acids. An application of the data on the molar radioactivity of the fragments allowed the refinement of the determined value of the half-degradation of the TGENHR-NH_2 peptide in the blood plasma. It was 20 min [10]. Note that the previously determined half-degradation periods of such peptides

Table 1. The degree of substitution of deuterium for hydrogen in the [^{2}H]TGENHR-NH_2 peptide

Residue	C–H-bond	D-substitution, %
Thr	$C^{\alpha}H$	44
	$C^{\beta}H$	42
	$C^{\gamma}H_3$	34
Gly	$C^{\alpha}H_2$	72
Glu	$C^{\alpha}H$	28
	$C^{\beta}H_2$	12
	$C^{\gamma}H_2$	27
Asn	$C^{\alpha}H$	62
	$C^{\beta}H_2$	66
His	$C^{\alpha}H$	39
	$C^{\beta}H_2$	49
	$C^{\delta}H$	76
	$C^{\varepsilon}H$	81
Arg	$C^{\alpha}H$	22
	$C^{\beta}H_2$	14
	$C^{\gamma}H_2$	16
	$C^{\delta}H_2$	21

as TGENHR, Semax (MEHFPGP), and Selank (TKPRPGP) without any protection on their termini by peptidases of the blood plasma varied from 2 to 3 min [3, 10]. For the first time, the investigation of the conversion of the TGENHR-NH_2 peptide in the blood plasma allowed the identification of the ENHR-NH_2 and HR-NH_2 peptides as the main products of the enzymatic hydrolysis of TGENHR-NH_2 in the blood plasma. The previously performed analysis of the nootropic activity of these peptides demonstrated that the HR-NH_2 peptide was an active metabolite with the high nootropic activity [10]. The obtained data on the quantitative content of the HR-NH_2 peptide contributed to an understanding of the mechanism of the nootropic activity of the TGENHR-NH_2 peptide.

Pharmacokinetics of the TGENHR-NH_2 peptide and its fragments in organisms of experimental animals. The most complex problem of studies of a content of peptide therapeutic agents in tissues of an organism is their fast biodegradation by peptidases. As a rule, a peptide is half-cleaved by peptidases in a few minutes. The traditional approach to the sample preparation involves the tissue homogenization, extraction, and centrifugation. This process is usually accompanied by biodegradation of examined peptides, because the tissue peptidases affect the examined peptide in the course of the whole process of the sample preparation. As a result, a quantitative content of peptides in tissues is inadequately determined by such an analysis.

Many attempts to solve the problem of the peptidase activity that complicates the determination of the quantitative content of peptides in the organism tissues have been made. The method described in paper [13] can be considered as a successful example of resolution of this problem. In this paper, a microwave device was elaborated for the analysis of the peptidome of a mouse brain. A decapitated head of a mouse was placed in the device and heated by the microwave for several seconds to the temperature close to boiling. This treatment resulted in an inactivation of tissue enzymes. Thus, the authors avoided the peptidase action and obtained reliable results of the peptide content in the tissues of the mouse brain. Unfortunately, the method was not universal despite the obvious success in this concrete case.

We proposed an alternative method for determination of the TGENHR-NH_2 peptide in tissues of experimental animals. Our method also allowed an avoidance of the effect of the peptide proteolysis on the process and results of the analysis. We can note several essential moments of this method. The [^{3}H]TGENHR-NH_2 peptide was administered to the animals, and they were decapitated after the desired time interval. The necessary organs were taken and quickly frozen in liquid nitrogen. The obtained samples were lyophilized at a low temperature to a constant weight, heated at 65°C for the peptidase inactivation, homogenized, and subjected to extraction with organic solvents. The TGENHR-NH_2, ENHR-NH_2, and HR-NH_2 peptides (10 μg of each) were added to the extraction mixture (the acetonitrile-water solution) for the fraction identification during the chromatography. The solutions which were obtained after the extraction were evaporated to dryness at a reduced pressure, subjected to the repeated extraction with methanol and evaporated. The prepared samples were examined by HPLC. Radioactivity of the fractions corresponding to the TGENHR-NH_2, ENHR-NH_2, and HR-NH_2 peptides was determined on a liquid scintillation counter.

The [^{3}H]TGENHR-NH_2 peptide (25 μCi) was added to the sample of the rat blood (400 mg) for a quantitative assessment of the significance of the obtained results. The sample was treated as described above. The HPLC analysis demonstrated that 94% and 3% of the starting radioactivity were found in the fraction corresponding to the TGENHR-NH_2 and ENHR-NH_2 peptides, respectively. Thus, the proposed method was shown to provide high extraction of the examined peptide from the tissue without the additional biodegradation.

Characteristics of the pharmacokinetic profile of HLDF-6-amide in blood at the intravenous administration were studied on males of the chinchilla rabbits. The rabbit miles were injected with the tritium-labeled [^{3}H]TGENHR-NH_2 peptide (2000 μCi) at a dose of 400 μg. The HPLC fractions corresponding to the

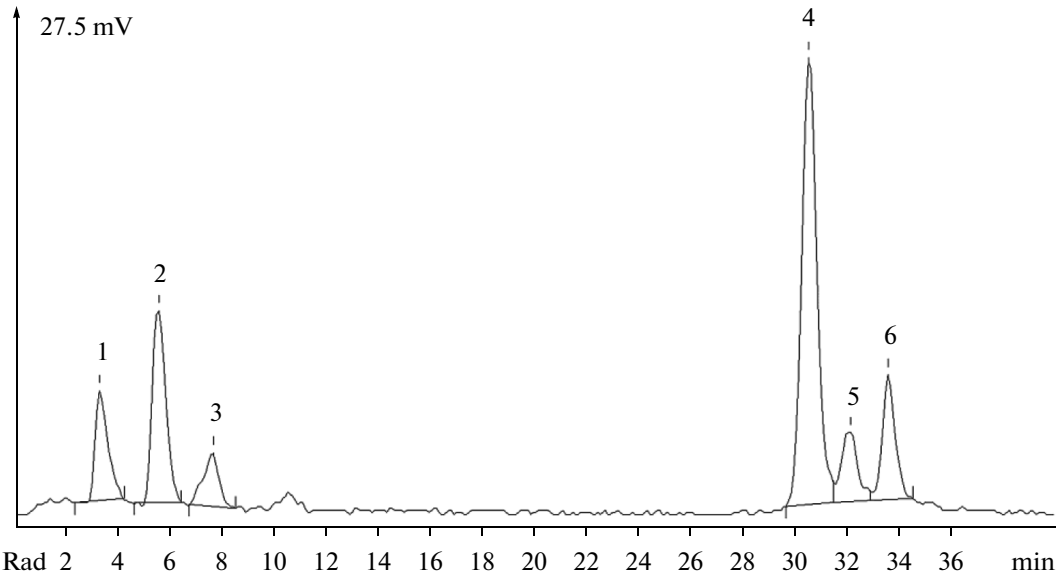

Fig. 3. The biodegradation of the TGENHR-NH_2 peptide in the blood plasma of a rat within 80 min. The radiochromatography of TGENHR-NH_2 (peak 6), ENHR-NH_2 (peak 5), and HR-NH_2 (peak 4) in the gradient (0–30%) of methanol in 0.08% TFA and 0.02% HFBA on the Kromasil C_{18} column (8 × 150 mm).

TGENHR-NH_2 peptide and its ENHR-NH_2 and HR-NH_2 fragments were collected and analyzed on a liquid scintillation counter. The radioactive concentration of the TGENHR-NH_2 peptide and its fragments which were formed in the rabbit blood under the peptidase action was determined on the basis of this analysis, and the volume concentration of the TGENHR-NH_2 peptide in blood was calculated. The pharmacokinetic data of HLDF-6-amide were given in Fig. 5. The experimental data were designated by points, and the calculated values which were obtained by the best approximation to the experimental results by the nonlinear regression were shown as a solid line. The pharmacokinetic parameters were calculated from the obtained results (Table 2). The half-excretion time of the TGENHR-NH_2 peptide was found to be 3.3 min at the intravenous injection in the rabbits. The peptide was characterized by the fast disappearance from blood due to its quick degradation in the organism's tissues.

The bioavailability of HLDF-6-amide at its intranasal administration was determined in its pharmacokinetic studies on the rat males of the Wistar line. The [3H]TGENHR-NH_2 peptide (400 μCi) was intranasally or intravenously administered to the rat males at a dose of 250 μg/kg. The chromatographic fractions corresponding to the TGENHR-NH_2 peptide and its ENHR-NH_2, and HR-NH_2 fragments were collected and analyzed by the liquid scintillation count. The radioactive concentration of the TGENHR-NH_2 peptide and its fragments in the rat blood was determined from the results of this analysis. The data on the concentration of the peptide in the rat blood were given in Figs. 6 and 7. The basic pharmacokinetic parameters which were calculated from the data on the intranasal and intravenous administration of HLDF-6-amide to the rats were evaluated from the data on the change in the peptide concentration (Table 3).

The value of the absolute bioavailability of the peptide (34%) [14] was calculated from the AUC_∞ value for the intranasal and intravenous administration of the TGENHR-NH_2 peptide to the Wistar male rats. This experiment demonstrated that the tested peptide had the extremely high bioavailability at its intranasal administration and, thus, the peptide exhibited the highest physiological activity as a neuroprotective agent. The half-excretion time of the TGENHR-NH_2 peptide at its intravenous administration to the rats proved to be 2.1 min. It was found that the TGENHR-NH_2 peptide was characterized by fast penetration and excretion from the blood of the Wistar rats given its intranasal administration. This fact was explained by the fast degradation of the peptide in the organism's tissues.

The Balb/c linear mice with a cognitive deficit are widely used for complex investigation of cognitive pathologies. Pharmacokinetics of HLDF-6-amide was studied at its intranasal administration to the mice of this line (Table 4). The results of the investigation of the peptide distribution between blood and peripheral tissues demonstrate that the highest concentration of the peptide is observed in the heart tissues. The peptide exhibits a slightly lower affinity to the tissues of kidney and omentums. We can stress that the highest concentration of the peptide is observed in the omentum tissues 15 min after the administration. This fact is possibly associated with a lower rate of the proteolytic degradation of the peptide due to lower content of

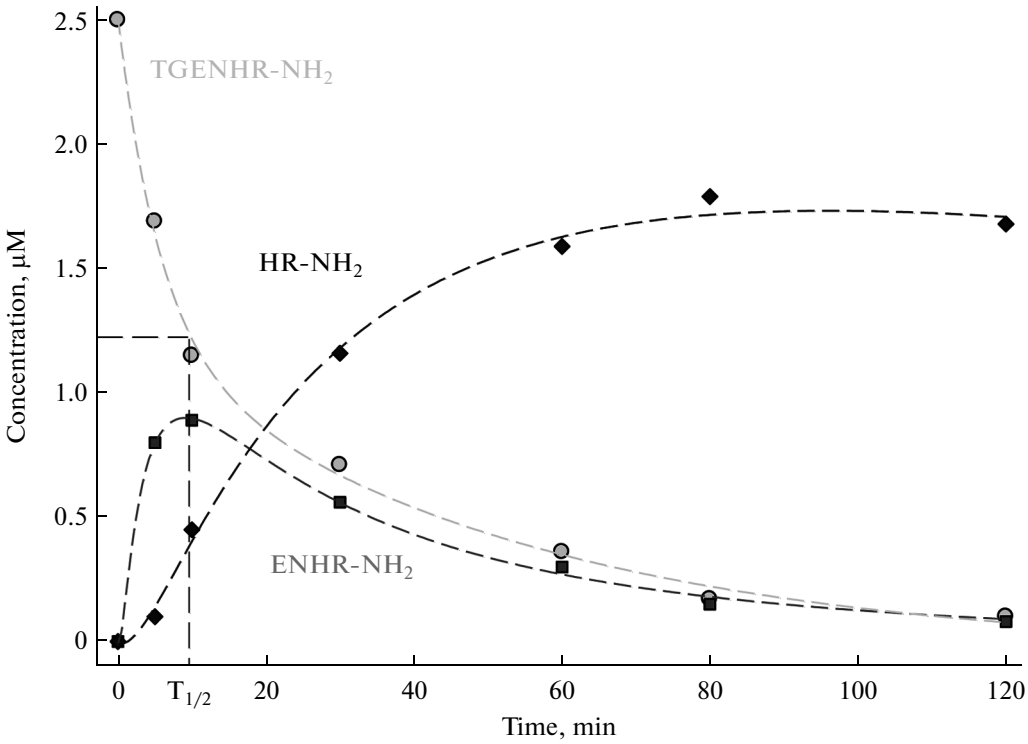

Fig. 4. Kinetics of the biodegradation of the TGENHR-NH_2 peptide (2.5 μM) in the rat blood plasma.

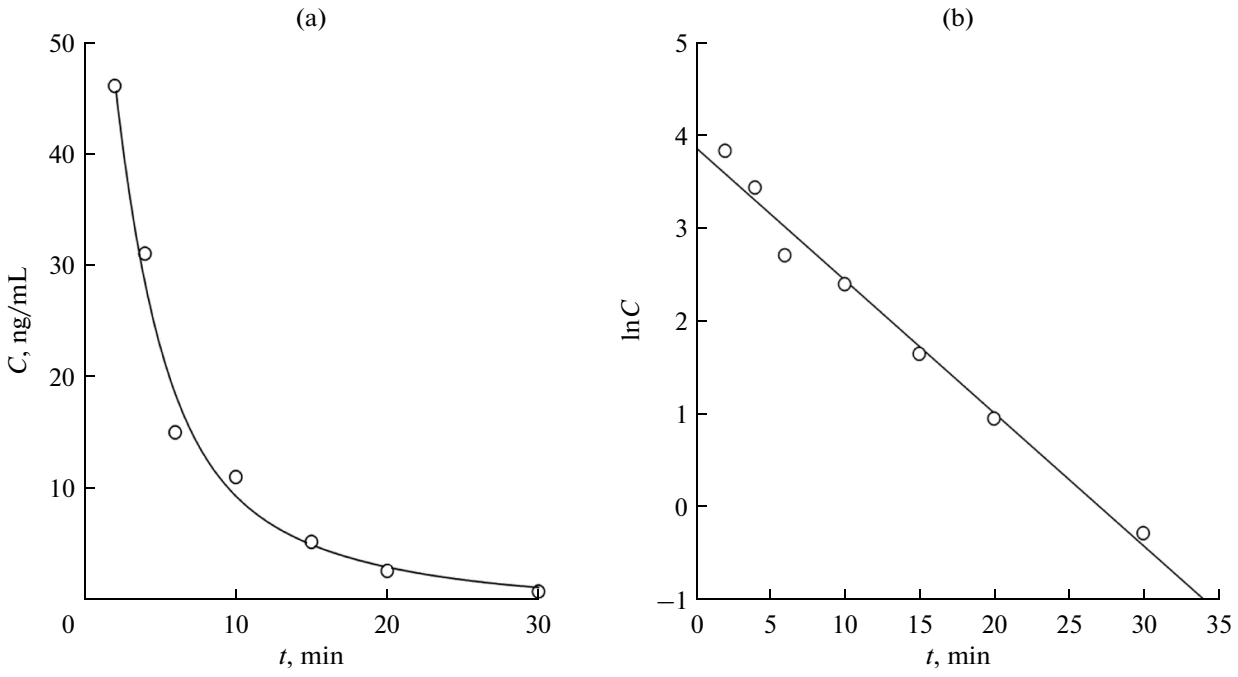

Fig. 5. The change in the concentration of the TGENHR-NH_2 peptide in the rabbit blood after its intravenous bolus dosing at a dose of 120 μg/kg. The data are presented in (a) the linear and (b) semilogarithmic coordinates.

peptidases in the adipose tissue. HLDF-6-amide does not accumulate in tissues after its administration during the prolonged time of measurements, which is the characteristic feature of peptide preparations. We can not reliably find the labeled TGENHR-NH_2 peptide (25 μCi) either in blood or in the examined tissues one hour after its intranasal introduction to the mice at a dose of 300 μg/kg. A multiple-dose intranasal intro-

duction of the peptide at doses of 300 and 1600 μg/kg for 30 days was also found to cause no changes in its natural cumulation and pharmacokinetics of the tritium-labeled TGENHR-NH_2 peptide in comparison with its single administration.

EXPERIMENTAL

Experimental Animals

Ten healthy adult male rats of the Wistar line (at the age of 180–200 days, 280–300 g), 150 male mice of the Balb/c line (at the age of 6–7 weeks, 20 ± 1 g), and five males of the chinchilla rabbits (at the age of 12 weeks, 3000 ± 300 g) were used in this study. The animals were kept according to the effective sanitary code of the provision of the necessary facilities, equipment, and housing of experimental biological clinics. The standard laboratory diet corresponded to the effective norms. The four animals were kept in one cage under the conditions of free access to water and food at a temperature of 21°C and the constant daylight of 14 hours (the light was turned on at 8 a.m.). The environmental factors (temperature, humidity, illumination, and composition of a litter) corresponded to the requirements of keeping of the laboratory animals. The investigation was performed in accordance with the Rules of Laboratory Practice of the Russian Federation, recommendation of "Handbook for Performance of Preclinical Trials of Medicines" [14], and the GLP FDA and GLP OECD international requirements for laboratory animals (experimental biomodels) of the SPF-status (free from pathogenic flora). The SPF laboratory animals were grown in the nursery for laboratory animals of the Pushchino Branch of Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences that have the AAALAC international accreditation. The quality system of the administration of the laboratory animals in this nursery was certified in accordance with the ISO 9001:2008 international requirements. All the procedures were fulfilled according to the approved protocols. The concrete protocol procedures with the use of animals were discussed and approved by the Commission on the Humane Treatment of Animals before the beginning of the studies.

The TGENHR-NH_2, ENHR-NH_2, and HR-NH_2 peptides were synthesized by the solid phase method according to the Boc/Bzl scheme and the technique of incomplete blocking of side chains of amino acid residues (the threonine residue was unprotected). The Boc-Arg(Tos)-OH was initially attached to the copolymer of styrene and 1% divinyl benzene that was modified by *p*-methylbenzhydrylamine anchoring group (MBHA-resin) [15]. The *N*-Boc protecting group was removed during the synthesis of the TGENHR-NH_2 peptide by sulfuric acid in dioxane. After the completion of the synthesis, the peptides were cleaved from the polymer by the treatment with anhydrous liquid HF with the addition of 10% (v/v) *m*-cresol. The peptides were purified by a preparative HPLC on a Phenomenex Synergy Hydro-RP RP column (50 × 250 mm). The identity of the peptides was evaluated by mass spectrometry and amino acid anal-

Table 2. Values of the basic pharmacokinetic parameters which were calculated from the data on the intravenous bolus dosing of the TGENHR-NH_2 peptide to the chinchilla rabbits

Pharmacokinetic parameter	
C_0, ng/mL	82
K_{el}, 1/min	0.211
$T_{1/2}$, min	3.3
AUC_∞, ng/mL min	390
MRT, min	7.3
V_{ss}, mL/kg	2250
Cl, mL/min kg	308
R^2	0.98

Abbreviations:
C_0, apparent concentration of the substance in blood at the initial time moment ($t - 0$ min);
K_{el}, constant of the rate of the substance elimination;
$T_{1/2}$, the half-time excretion, a period of excretion of half of the absorbed substance;
AUC_∞, the area under the concentration–time curve;
MRT, the average time of the presence of a therapeutic agent in an organism;
V_{ss}, the stationary volume of the substance distribution;
C1, the total clearance, the plasma volume that is purified from a preparation per unit time;
R^2, determination coefficient.

Table 3. The values of the basic pharmacokinetic parameters that were calculated on the basis of the data on the intranasal and intravenous administration of the TGENHR-NH_2 peptide to the rats of the Wistar line

Pharmacokinetic parameter	Administration of the peptide	
	intravenous	intranasal
Dose, μg/kg	250	250
C_0, ng/mL	60.4	43.5
K_{el}, 1/min	0.158	0.333
$T_{1/2}$, min	4.4	2.1
AUC_∞, ng/mL min	383	131
MRT, min	6.3	3.0
V_{ss}, mL/kg	4170	5700
Cl (mL/min kg)	656	1890
R^2	0.98	0.99

Abbreviations are given in the note after Table 2.

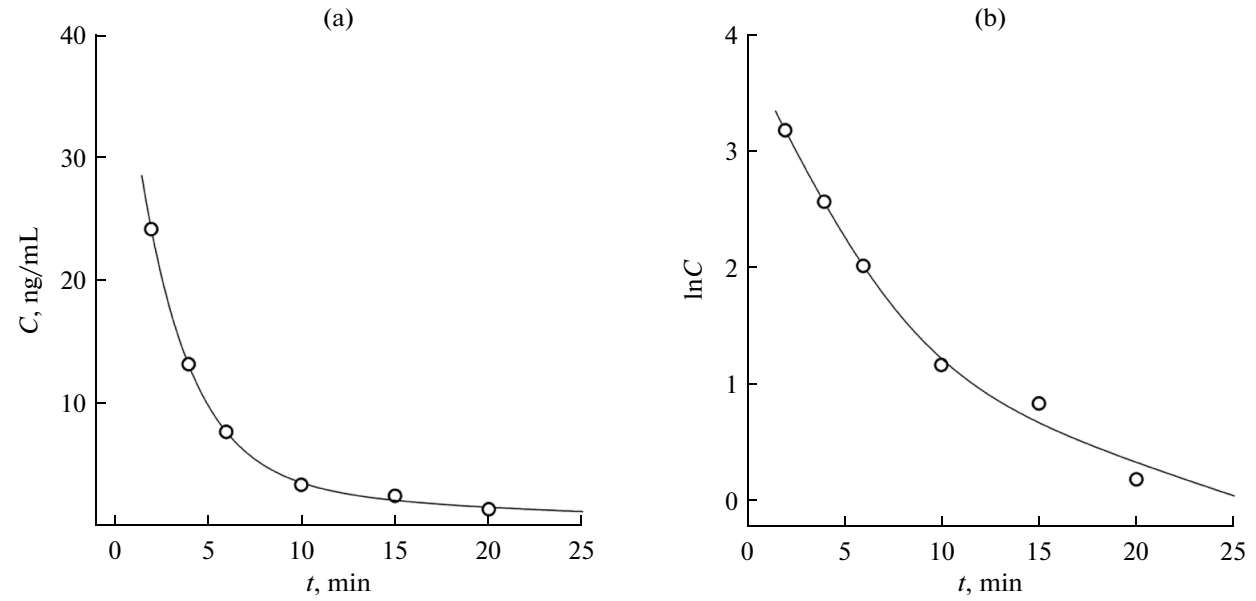

Fig. 6. The change in the concentration of the TGENHR-NH_2 peptide in the rat blood after its intranasal administration at a dose of 250 μg/kg. The data are presented in (a) linear and (b) semilogarithmic coordinates.

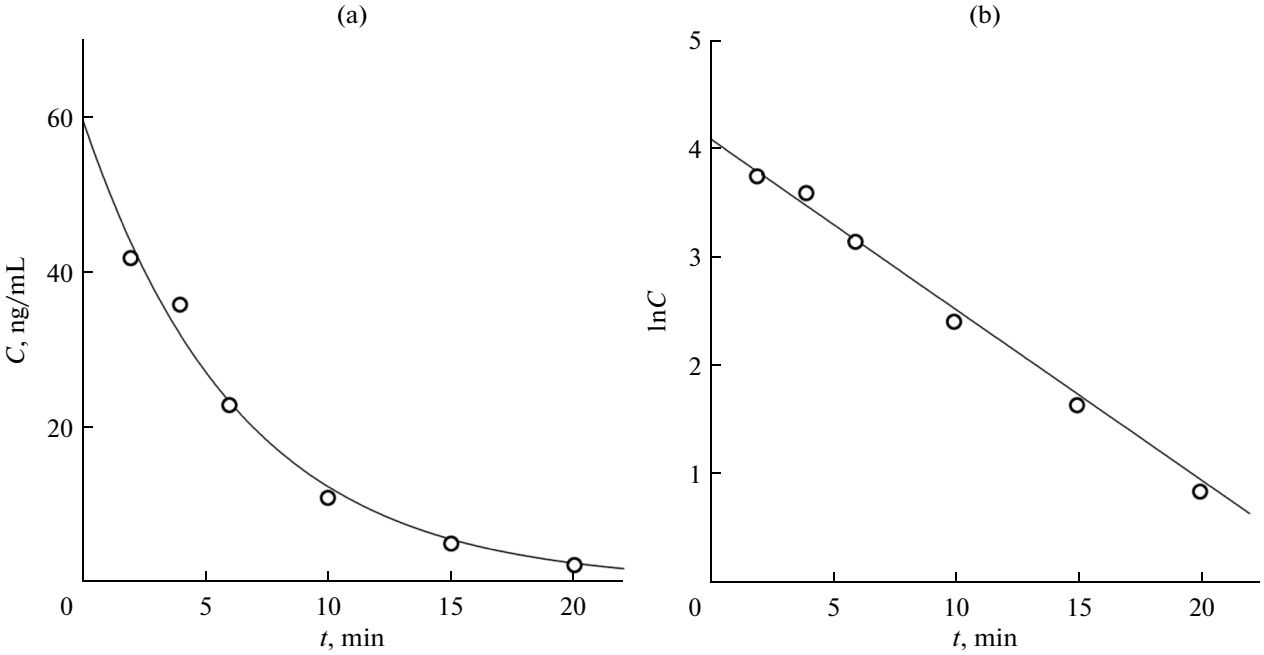

Fig. 7. The change in the concentration of the TGENHR-NH_2 peptide in the rat blood after its intravenous administration at a dose of 250 μg/kg. The data are presented in (a) linear and (b) semilogarithmic coordinates.

ysis. (A paper with the detailed description of the peptide synthesis to be published).

Preparation of the Tritium-Labeled and Deuterium-Labeled Derivatives of HLDF-6-Amide

The tritium-labeled and deuterium-labeled peptides ([^{3}H]TGENHR-NH_2 and [^{2}H]TGENHR-NH_2, repectively) were prepared in the HSCIE reaction with gaseous tritium or deuterium under the same conditions. The solid-phase isotopic hydrogen exchange of tritium or deuterium in the TGENHR-NH_2 peptide was performed in the solid mixture that was formed by the peptide on the aluminum oxide and the highly disperse heterogeneous catalyst ($Pd/BaSO_4$) in the presence of the gaseous hydrogen isotope under the same conditions. The TGENHR-NH_2 peptide (1.0 mg) was preliminary dissolved in water (0.5 mL) and mixed

Table 4. Concentration of the TGENHR-NH_2 peptide in the tissues (ng/mL) with its intranasal administration to the mice of the Balb/c line at a dose of 300 μg/kg 2–15 min after administration

Organs	Time, min				
	2	4	6	10	15
Blood	13 ± 3.2	8.5 ± 1.4	5.1 ± 0.8	1.8 ± 0.3	1.3 ± 0.2
Brain	2.1 ± 0.6	4.3 ± 1.4	3.3 ± 1.1	1.9 ± 0.6	0.9 ± 0.4
Kidneys	2.7 ± 0.7	4.5 ± 1.5	3.0 ± 1.1	1.3 ± 0.5	0.7 ± 0.3
Liver	1.3 ± 0.3	2.4 ± 0.6	1.5 ± 0.4	0.7 ± 0.3	0.4 ± 0.2
Heart	11 ± 3.4	14 ± 4.5	17.2 ± 6.7	12.7 ± 4.4	7.4 ± 3.1
Omentum	3.2 ± 1.1	4.1 ± 2.3	6.4 ± 3.4	5.3 ± 3.2	4.2 ± 2.6

with aluminum oxide (20 mg). The mixture was frozen, and water was removed by lyophilization. The obtained solid mixture was mixed with the catalyst (10 mg of 5% Pd/$Baso_4$, Fluka). This mixture was placed in an ampoule (10 mL), vacuumed, filled with the gaseous tritium or deuterium to the pressure of 30 kPa, and kept at 180°C for 10 min. The ampoule was cooled, vacuumed, and flushed with hydrogen. The peptide was desorbed with 50% aqueous ethanol. The labeled peptide was dissolved in 50% aqueous ethanol and evaporated to dryness two times for removal of labile tritium or deuterium. The peptides were purified by HPLC on a Kromasil column (8 × 150 mm) in a concentration gradient of acetonitrile in 0.1% aqueous solution of heptafluorobutyric acid (HFBA). The peptides were evaporated and dissolved in ethanol. The tritium-labeled [^{3}H]TGENHR-NH_2 peptide with the molar radioactivity of 230 Ci/mmol was prepared in the reaction with the gaseous tritium. A unique whole-soldered OBT-1 device for the hydrogen exchange of tritium (Institute of Molecular Genetics of the Russian Academy of Sciences) was used for the preparation of the peptide.

The deuterium-labeled peptide ([^{2}H]TGENHR-NH_2) was prepared by the HSCIE reaction between the starting peptide and the gaseous deuterium. The [^{2}H]TGENHR-NH_2 peptide contained 10.5 deuterium atoms per the one molecule on average. The substitution of tritium for hydrogen was evaluated from the value of the molar radioactivity of the [^{3}H]TGENHR-NH_2 peptide. The deuterium incorporation into the labeled peptide was determined by mass spectrometry. The data on the deuterium distribution in the [^{2}H]TGENHR-NH_2 peptide were obtained by NMR spectroscopy and used for the calculation of the tritium distribution in the [^{3}H]TGENHR-NH_2 peptide.

The NMR spectroscopy. The position of the isotope label and the substitution degree of deuterium for hydrogen of the C-H-bonds in the [^{2}H]TGENHR-NH_2 peptide were determined by the comparison of the peak intensities in one-dimensional and two-dimensional ^{1}H NMR spectra (^{1}H/^{1}H-TOCSY with the delay time of 60 ms). The ^{1}H NMR spectra were recorded on a Varian NMR System spectrometer (700 MHz).

MALDI-mass spectra of the deuterium-labeled [^{2}H]TGENHR-NH_2 peptide were recorded on an Ultraflex Tof/Tof spectrometer (Bruker, Germany) equipped with a nitrogen laser (337 nm). 2,5-Dihydroxybenzoic acid was used as a matrix. A sample was mixed with the matrix solution (20 mg/mL in 30% solution of acetonitrile in 0.1% TFA) in the ratio of 1 : 1. The spectra were recorded in the range from 400 to 6000 Da.

Administration of the peptides to the animals and the blood sampling. The peptide was injected to the rabbits and their blood was taken from the large auricular veins. The rabbits were fixed in a box with an opening for a head. The heparin solution (0.8 mL, 5000 MU/mL, Moscow Endocrine Factory) was injected in the large auricular vein of a right ear through an insulin syringe, and a Flexicath G22 cannula for the blood sampling was inserted to the large auricular vein of a left ear 5 min later. The [^{3}H]TGENHR-NH_2 peptide (2 μCi, at a dose of 120 μg/kg) was injected to the right auricular vein by the insulin syringe, and approximately 1 mL of the venous blood was taken through the cannula at the fixed time intervals (2, 4, 6, 10, 15, 20, and 30 min). The blood was collected into the weighted plastic tubes and frozen in liquid nitrogen.

The peptide was intravenously introduced and the blood was taken from the jugular veins in the experiments on the rats. The rats were anesthetized with chloral hydrate (300 mg/kg). Hair was cut, two vertical 3-cm incisions of skin (on the left and right) were made on the ventral side of the area near the anterior legs and neck 5-10 min after the anesthesia, and the jugular veins were prepared. A Flexicath G24 intravenous cannula was placed in the left jugular vein, and heparin (80 μL) was introduced through this cannula. Then, a Flexicath G22 cannula was placed in the right jugular vein for the blood sampling. The solution of the radioactive peptide (400 μCi, 46 μg in 200 μL) was administered into the left jugular vein within 10–15 s, and the venous blood (approximately 0.5 mL) was

taken from the right jugular vein 2, 4, 6, 10, 20, or 30 min later. The solution of the radioactive peptide (40 μL, 20 μL into each nostril) was intranasally administered within 15 s. The blood was taken as described above through the intravenous cannula. The blood was collected in the weighted plastic tubes and frozen in liquid nitrogen.

The [^{3}H]TGENHR-NH_2 labeled peptide (25 μCi) was intranasally introduced in the mice at a dose of 300 μg/kg. The animals were decapitated after the protocol-determined time, and the blood and the examined tissues were collected in the weighted plastic tubes and frozen in liquid nitrogen.

Sample preparation of the tissues and analysis of pharmacokinetic of the [^{3}H]TGENHR-NH_2 **peptide with the use of the radiochromatography.** The tubes with the weighted and frozen samples of the tissues for the HPLC analysis were lyophilized for 2 days. The lyophilized samples were heated at 65°C for 30 min, homogenized in the same plastic tubes with horizontal knifes that were rotated at a rate of 5000 rpm. The first extraction of these samples was performed with 90% aqueous acetonitrile with 1% TFA. The TGENHR-NH_2, ENHR-NH_2, and HR-NH_2 peptides (10 μg of each) were added to the aqueous-acetonitrile extracting solution for an identification of the chromatographic fractions. The solution containing the tritium-labeled peptide and the blood components was centrifuged, dried at a reduced pressure, re-extracted with methanol, and centrifuged again. The supernatant with the tritium-labeled peptide was evaporated at a reduced pressure, re-extracted with 0.1% aqueous solution of TFA, and centrifuged. The peptides were quantitatively analyzed by HPLC on a Kromasil C_{18} column (8 × 150 mm) in a gradient (from 0 to 30%) of methanol in 0.08% TFA and 0.02% HFBA. Fractions containing the TGENHR-NH_2, ENHR-NH_2, and HR-NH_2 peptides were collected, and their radioactivity was measured on a liquid scintillation counter. The peak concentration of the substances in the blood plasma (C_{max}), the time of the C_{max} achievement (T_{max}), the half-time excretion ($T_{1/2}$), and the areas of the pharmacokinetic curve (AUC_∞) were determined by the pharmacokinetic analysis using the SigmaPlot 11.0 program (Systat Software, United States). All the quantitative data were subjected to the descriptive statistics (mean, standard deviation).

CONCLUSIONS

A novel method for the quantitative analysis of peptides in organism tissues was proposed. This method allowed a practically complete avoidance of proteolysis of examined peptides in the course of the analysis. The innovative pharmacological agent on the basis of an amide of the $_{41}$TGENHR$_{46}$ peptide fragment of HLDF for prevention and treatment of cerebrovascular and neurodegenerative diseases was investigated within the bounds of creation of highly active and low-toxic peptide preparations with the neuroprotective and psychotropic activity. The [^{3}H]TGENHR-NH_2 peptide (molar radioactivity of 230 Ci/mmol) and [^{2}H]TGENHR-NH_2 peptide (the average number of deuterium atoms was 10.5 per one peptide molecule) which were uniformly labeled with tritium and deuterium, respectively, were prepared for the studies of the peptide pharmacokinetics and the mechanisms of the peptide action. The quantitative analysis of the TGENHR-NH_2 peptide in the animal's tissues was performed, the basic pharmacokinetic parameters of the peptide were calculated, and the value of its absolute bioavailability was determined with the use of these labeled compounds. The peptide was shown to have the extremely high bioavailability at its intranasal administration (34% for the rats), probably resulting in the fast appearance of the peptide in blood and its highest physiological activity by this way of introduction. The peptide quickly disappeared from blood due to its fast proteolytic hydrolysis in organism's tissues. The half-time excretion of the peptide proved to be 3.3 min for the rabbits and 2.1 min for the rats at its intranasal administration. The highest concentration of the peptide was found in the heart tissues. The peptide exhibited somewhat lower affinity to the tissues of kidneys and omentums.

ACKNOWLEDGMENTS

This study was supported by the Ministry of Education and Science of the Russian Federation, state contract no. 14N08.11.0002 and by the program of Presidium of the Russian Academy of Sciences "Fundamental Sciences to Medicine" for 2012–2014.

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Translated by L. Onoprienko