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Russian development for drug independence in endocrinology: comparative analysis of bioequivalence, safety and tolerability of the first domestic liraglutide

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Liraglutide is one of the analogues of the incretin hormone human glucagon-like peptide-1 (GLP-1) and is currently a priority treatment for diseases such as type 2 diabetes mellitus (mono- and combination therapy), obesity and overweight in the presence of at least one concomitant disease.

The aim of the work was to assess the bioequivalence and comparability of the safety and tolerability profile of the drug Enligria® (liraglutide 6 mg/ml, Promomed RUS LLC, Russia) and the drug Saxenda® (liraglutide 6 mg/ml, Novo Nordisk AS, Denmark) after a single dose in healthy volunteers.

Materials and methods. This study was an open-label, randomized, crossover comparative study to evaluate pharmacokinetic parameters, safety, tolerability and immunogenicity. The study comprised 26 healthy volunteers, 26 of whom were included in the bioequivalence assessment population. The study consisted of 2 periods, in each of which the volunteers received either the test drug (liraglutide at a single dose of 0.6 mg) or the reference drug (liraglutide at a single dose of 0.6 mg) once. The washout period between each dose was 7 days. Blood plasma samples were taken to determine the concentration of liraglutide in the range from 0 to 72 hours in each study period. Liraglutide concentrations were determined using a previously validated enzyme-linked immunosorbent assay (ELISA) method. A quantitative determination of antibodies to liraglutide in the blood serum samples was carried out using a microplate photometer and ready-made ELISA kits pre-validated by the manufacturer. The conclusion about the equivalence of the compared drugs was made based on the ratio of the parameters C_{max}, $AUC_{0\to t}$ and $AUC_{0\to t}$ of the studied drug in relation to the reference one.

Results. The pharmacokinetic parameters of the drugs were comparable to each other. The resulting 90% confidence intervals for the ratio of the values of C_{max}, $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ of the Russian test and reference drug were 87.18–110.46, 84.40–104.11 and 86.69–103.22% respectively, which satisfied the criteria for assessing bioequivalence. The tolerability of the drugs in the volunteers was notified as good. The incidence of adverse events was comparable for the test and reference drugs. No

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serious adverse events were reported throughout the study. According to the results of the immunogenicity analysis, no antibodies to russian produced liraglutide were detected in the blood serum of the volunteers, which indicated the lack of the drug immunogenicity.

Conclusion. During the study, the pharmacokinetic equivalence of the test and reference drugs was confirmed. The Russian drug Enligria® (liraglutide 6 mg/ml, Promomed RUS LLC, Russia) in comparison with a foreign drug Saxenda® (liraglutide 6 mg/ml, Novo Nordisk AS, Denmark)

Keywords: glucagon-like peptide-1; bioequivalence; pharmacokinetics; liraglutide, obesity, type 2 diabetes mellitus, Enligria

Abbreviations: T2DM – type 2 diabetes mellitus; GLP-1 – glucagon-like peptide-1; GIP – glucose-dependent insulinotropic polypeptide; CVDs – cardiovascular diseases; ASCVDs – atherosclerotic cardiovascular diseases; HbAlc – glycated hemoglobin; ELISA – enzyme-linked immunosorbent assay; BMI – body mass index; DPP-4 – dipeptidyl peptidase-4; API – active pharmaceutical substance; DNA – deoxyribonucleic acid; RNA – ribonucleic acid; GI tract – gastrointestinal tract; ARVI – acute respiratory viral infection; PCR – polymerase chain reaction; BAS – biologically active supplement; BP – blood pressure; HR – heart rate; RR – respiratory rate; ECG – electrocardiography; LDL-C – low-density lipoprotein cholesterol; HDL-C – high-density lipoprotein cholesterol; AE – adverse event; SAE – serious adverse event; CI – confidence interval; POMC – pro-opiomelanocortin, CART – cocaine-amphetamine-regulated transcript; NPY – neuropeptide Y; AgLP – agouti-like protein; GABA – gamma-aminobutyric acid, CHF – chronic heart failure; CKD – chronic kidney disease.

Российская разработка для лекарственной независимости в эндокринологии: сравнительный анализ биоэквивалентности, безопасности и переносимости первого отечественного лираглутида

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Лираглутид является одним из аналогов инкретинового гормона человеческого глюкагоноподобного пептида-1 (ГПП-1) и в настоящее время является приоритетным средством для лечения таких заболеваний, как сахарный диабет 2-го типа (в моно- и комбинированной терапии), ожирение и избыточная масса тела при наличии хотя бы одного сопутствующего заболевания.

Цель. Оценить биоэквивалентность и сопоставимость профиля безопасности и переносимости лекарственного препарата Энлигрия® (лираглутид 6 мг/мл, ООО «ПРОМОМЕД РУС», Россия) и лекарственного препарата Саксенда® (лираглутид 6 мг/мл, Ново Нордиск А/С, Дания) при однократном применении здоровыми добровольцами.

Материалы и методы. Данное исследование представляло собой открытое рандомизированное перекрестное сравнительное исследование по оценке фармакокинетических параметров, безопасности, переносимости и иммуногенности. В исследование были включены 26 здоровых добровольцев, из них в популяцию для оценки биоэквивалентности вошли все 26 участников. Оно включало 2 периода, в каждом из которых добровольцы получали либо исследуемый препарат (лираглутид в дозе 0,6 мг), либо референтный препарат (лираглутид в дозе 0,6 мг) однократно. Отмывочный период между каждым из приемов составлял 7 сут. Отбор образцов плазмы крови для определения концентрации лираглутида производили в диапазоне от 0 до 72 ч в каждом из периодов исследования. Концентрацию лираглутида определяли с помощью предварительно валидированного метода иммуноферментного анализа (ИФА). Количественное определение антител к лираглутиду в образцах сыворотки крови проводили с помощью фотометра для микропланшетов с использованием готовых предварительно валидированных производителем ИФА-наборов. Вывод об эквивалентности сравниваемых препаратов делали по отношению параметров C_{max}, $AUC_{0\to t}$ и $AUC_{0\to\infty}$ исследуемого лекарственного препарата по отношению к референтному.

Результаты. Фармакокинетические параметры препаратов были сопоставимы между собой. Полученные 90%-ные доверительные интервалы для отношения значений C_{max}, AUC_{0-t} и $AUC_{0-\infty}$ исследуемого российского и референтного препарата составили 87,18–110,46, 84,40–104,11 и 86,69–103,22% соответственно, что удовлетворяло критериям оценки биоэквивалентности. Переносимость препаратов у добровольцев была отмечена как хорошая. Частота нежелательных явлений была сопоставима для исследуемого и референтного препаратов. В течение всего исследования не было зарегистрировано ни одного серьёзного нежелательного явления. По результатам анализа иммуногенности у добровольцев не были выявлены антитела к лираглутиду российского производства в сыворотке крови, что свидетельствовало об отсутствии иммуногенности препарата.

Заключение. В ходе проведенного исследования была подтверждена фармакокинетическая эквивалентность исследуемого и референтного препаратов. Был продемонстрирован высокий профиль безопасности и отсутствие иммуногенности у российского препарата Энлигрия® (лираглутид 6 мг/мл, ООО «ПРОМОМЕД РУС», Россия) в сравнении с зарубежным препаратом Саксенда® (лираглутид 6 мг/мл, Ново Нордиск А/С, Дания).

Ключевые слова: глюкагоноподобный пептид-1; биоэквивалентность; фармакокинетика; лираглутид; ожирение; сахарный диабет 2 типа; Энлигрия

Список сокращений: СД 2 – сахарный диабет 2 типа; ГПП-1 – глюкагоноподобный пептид-1; ГИП – глюкозозависимый инсулинотропный полипептид; ССЗ – сердечно-сосудистые заболевания; АССЗ – атеросклеротические сердечно-сосудистые заболевания; HbAlc – гликированный гемоглобин; ИФА – иммуноферментный анализ; ИМТ – индекс массы тела; ДПП-4 – дипептидилпептидаза-4; АФС – активная фармацевтическая субстанция; ДНК – дезоксирибонуклеиновая кислота; РНК – рибонуклеиновая кислота; ЖКТ – желудочно-кишечный тракт; ОРВИ – острая респираторная вирусная инфекция; ПЦР – полимеразная цепная реакция; БАД – биологически активная добавка; АД – артериальное давление; ЧСС – частота сердечных сокращений; ЧДД – частота дыхательных движений; ЭКГ – электрокардиография; ХС ЛПНП – холестерин липопротеидов низкой плотности; ЛПВП ХС – холестерин липопротеидов высокой плотности; НЯ – нежелательное явление; СНЯ – серьёзное нежелательное явление; ДИ – доверительный интервал; ПОМК – проопиомеланокортин, КАРТ – кокаин-амфетамин-регулируемый транскрипт; НПУ – нейропептид Y; АПБ – агутиподобный белок; ГАМК – гамма-аминомасляная кислота., ХСН – хроническая сердечная недостаточность; ХБП – хроническая болезнь почек.

INTRODUCTION

An increased body weight is associated with metabolic disorders and is a pressing problem in modern medicine, as it leads то the development of a number of chronic diseases, including cardiovascular diseases (CVDs), type 2 diabetes mellitus (T2DM) and also has a serious impact on mental health[1,2]. T2DM is a disorder of carbohydrate metabolism caused primarily by insulin resistance and relative insulin deficiency or directly by an impaired insulin secretion[3].

The analysis of clinical practice data shows that patients often have two diseases at once: obesity and T2DM [1]. Moreover, people with T2DM have more difficulty losing weight than people without the disease. This is due to the fact that in an insulin-resistant state, skeletal muscles and the liver are the main organs responsible for glucose utilization. Hyperinsulinemia

[1] WHO European Regional Obesity Report 2022. Copenhagen: WHO Regional Office for Europe; 2022. Available from: https://www.who.int/europe/publications/i/item/9789289057738#:~:text=Overweight%20and%20obesity%20affect%20almost,in%20the%20WHO%20European%20Region.

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promotes the synthesis and accumulation of triglycerides, while inhibiting lipolysis in adipocytes; all these lead to an increase in the volume of adipose tissue [1, 2]. The result of a compensatory response to metabolic and hormonal changes that accompany an initial weight loss is an increase in the synthesis of orexigenic hormones responsible for stimulating appetite [1]. Hypoglycemic drugs, such as sulfonylureas, thiazolidinediones, and insulin, used in the treatment of patients with T2DM have a number of side effects, such as hypoglycemia, weight gain, a congestive heart failure, and osteoporosis, which prevent many patients from achieving key therapeutic goals[4].

In recent decades, the role of incretin hormones in the regulation of carbohydrate metabolism in the human body and their effect on β-cells have been actively studied. Glucagon-like peptide-1 (GLP-1) is one of the most important incretins, responsible for the production of insulin after meals, stimulating glucose-dependent insulin secretion. In addition, GLP-1 suppresses an excessively increased secretion of glucagon, slows down gastric emptying, reduces an appetite and energy consumption, and as a result, it reduces a body weight [3, 4][5,6]. The therapeutic potential of native GLP-1 is limited due to its rapid degradation by the enzyme dipeptidyl peptidase-4 (DPP-4) and short half-life (1–2 min). In this regard, liraglutide was developed – the first analogue of human GLP-1, demonstrating a persistent improvement in glycated hemoglobin (HbAlc) levels and normalization of a β-cell function in patients with T2DM, as well as reducing the body weight of overweight or obese patients regardless of the presence or absence of T2DM [3, 5]. Due to the unique structure of liraglutide, the drug half-life from plasma increases to 13 h compared to 2 min for native GLP-1. A prolonged action is ensured by three mechanisms: 1) oligomerization into heptamers through the interaction between hydrophobic palmitate residues on each liraglutide molecule - the replacement of one amino acid residue (arginine with lysine) at position 34 and an addition at position 26 to lysine of the side chain of C_{16} palmitic acid, as a result of which a slow absorption of the drug occurs; 2) binding to serum albumin in subcutaneous tissue, leading to a longer half-life after a subcutaneous administration (13 h). Taking into account the maximum concentration of the drug in the blood, which is observed after 10–14 h, the duration of the liraglutide action is 24 h[7] [3–5].

The GLP-1 receptor agonist liraglutide, like native GLP-1, has beneficial metabolic effects that include a glucose-dependent stimulation of an insulin secretion, decreased gastric emptying due to a direct effect on the hypothalamus, inhibition of food intake leading to a weight loss, increased natriuresis and diuresis, lowering total cholesterol and systolic / diastolic blood pressure[8,9] [3].

The original drug liraglutide was approved for a medical use in 2009 and is used in clinical practice under the trade names of Victoza® (Novo Nordisk AS, Denmark) and Saxenda® (Novo Nordisk AS, Denmark). In addition, the drugs based on liraglutide are currently registered in the USA, Japan and some European countries, including Russia. Phase II studies on the determination of the optimal dose have demonstrated that liraglutide has all the expected properties of GLP-1 in humans: its administration provides a glucose control throughout the day, a low incidence of hypoglycemia, and a weight loss in most patients[10].

At the doses of 1.2 and 1.8 mg/day, liraglutide has been successfully used in clinical practice for the treatment of patients with T2DM since 2010. The results of a meta-analysis of the 6-th phase of III LEAD (Liraglutide Effect and Action in Diabetes) studies demonstrated that liraglutide compared with other glucose-lowering drugs, ensures a more effective achievement of therapeutic parameters of a metabolic control HbA1c[11] [6].

Liraglutide showed benefit in the secondary prevention of atherosclerotic CVDs. The LEADER (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results) study investigated long-term cardiovascular outcomes during a long-term liraglutide use (the median of 3.5 years) in patients with T2DM and high cardiovascular risks. The study demonstrated the reduction in the likelihood of developing serious adverse events (SAEs) when using the drug at a dose of 1.8 mg compared to placebo [7].

[4] Ibid.
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[11] Ibid.

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For the treatment of obesity, liraglutide 3 mg (Saxenda®, Novo Nordisk AS, Denmark) was registered in 2014. The significant superiority of liraglutide (3 mg) over placebo in its effect on the body weight was confirmed in a series of randomized, double-blind, placebo-controlled studies that were part of the SCALE program (Satiety and Clinical Adiposity – Liraglutide Evidence in nondiabetic and diabetic individuals)[12] [8–12].

Other clinical studies have also shown that liraglutide has a unique therapeutic potential due to its combined effects on both body weight and glycemic control [13–15]. Liraglutide is one of the hypoglycemic drugs that are successfully used in patients with T2DM, including patients with cardiovascular pathology. In the SCALE Diabetes study, the proportion of patients who achieved an level of HbA1c <7% during the treatment with liraglutide (3 mg) was 69.2 *vs.* 27.2% (placebo). In the SCALE Obesity and Prediabetes study, the prevalence of prediabetes among patients diagnosed at screening after 56 weeks decreased to 30.8%, while in the placebo group in the same category of patients it decreased to 67.3% [10, 11]. During the SCALE research program, it was noted that therapy with liraglutide (3 mg) is accompanied by a decrease in the systolic blood pressure, waist circumference, total cholesterol and low-density lipoprotein cholesterol (LDL-C), and an increase in high-density lipoprotein cholesterol (HDL-C), which also proves that liraglutide therapy helps reduce a cardiometabolic risk even in patients with CVD [16, 17].

A study examining the effect of liraglutide therapy on the body weight in adolescents 12 years of age and older demonstrated that liraglutide was superior to placebo in reducing the standard deviation of BMI (95% confidence interval [CI] -0.37 to -0.08; *p*=0.002), no additional risks were identified regarding the safety of the drug [18].

At the present stage, liraglutide is included in Russian clinical guidelines for the treatment of T2DM in adults, the treatment of obesity in adults and children, the treatment of chronic kidney disease (CKD) to reduce the risk of progression in patients with CKD and T2DM, the treatment of lipid metabolism disorders in patients with T2DM and CVD, having a very high and high cardiovascular possibility to reduce the risk of both new cardiovascular complications (CVD) and the death[13] [5], which determines its demand in the Russian Federation. Accordingly, the import substitution of foreign drugs with Russian analogues and the localization of the full production cycle from the substance to the finished dosage form for liraglutide drugs is of particular relevance and importance from the point of view of ensuring the country's medicinal independence.

Liraglutide was presented on the pharmaceutical market only as a biotechnologically produced compound. However, taking into account the amino acid structure of this peptide, its lack of tertiary structure[14,15], and a number of limitations known for recombinant drugs, it is advisable to produce the active pharmaceutical substance (API) liraglutide through chemical synthesis [19]. Moreover, the production possibilities of biotechnological drugs are limited by a low productivity of the strains used, which may prevent the production of the required amount of the substance, that can be critical given the demand for this group of drugs in patients. This factor also indicates the feasibility of obtaining such drugs by chemical synthesis.

Thus, it is of interest to develop, analyze and produce synthetic liraglutide, as well as compare its physicochemical and biological properties relative to a biotechnologically produced molecule. Chemical synthesis is a high-throughput, scalable, commercially viable process [20–22]. The production of liraglutide by this method makes it possible to eliminate the spontaneous replacement of amino acids in the final product characteristic of the vital activity of microorganisms, and to obtain a product of high purity, with a minimum amount of predictably identified impurities, and a high yield [23–25]. Moreover, such a product is unchanged, homogeneous and does not contain residual impurities of producer cells, such as proteins, enzymes, DNA and RNA fragments, which improves the safety profile and reduces the risk of immunogenicity, and, consequently, the risk of a treatment failure [25].

There is a certain pool of studies to prove the effectiveness and safety of the drug. According to the FDA[16] guidelines, alpha-amino acid polymers,

[12] Register of Drugs of Russia. Instructions for medical use of the drug Saxenda. Available from: https://www.rlsnet.ru/drugs/saksenda-75258

[13] Clinical guidelines Type 2 diabetes mellitus in adults, 2022.

[14] Liraglutide (Compound). Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Liraglutide#section=3D-Status

[15] Ibid.

[16] U.S. Food and Drug Administration. ANDAs for Certain Highly Purified Synthetic Peptide Drug Products That Refer to Listed Drugs of rDNA Origin Guidance for Industry, 2021. Available from: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/andas-certain-highly-purified-synthetic-peptide-drug-products-refer-listed-drugs-rdna-origin

such as glucagon, liraglutide, etc., containing up to 40 amino acid residues, are considered not protein molecules, but peptides[17]. According to the FDA, to confirm the equivalence of a synthetic peptide and the biotechnologically derived liraglutide contained in the precursor drug, it is sufficient to demonstrate the structural identity of the API[18] using modern analytical methods. The Promomed RUS LLC company has developed its own technology for the production of API using methods of chemical synthesis and isolation of liraglutide and a finished dosage form for the treatment of both obesity and T2DM. For an additional assessment of the quality and safety of the developed drugs, its registration in our country in accordance with the Russian regulatory requirements, in addition to physicochemical methods of analysis and preclinical studies, a study of the pharmacokinetics, safety and immunogenicity of the drug Enligria® (liraglutide 6 mg/ml) was conducted in comparison with a foreign predecessor drug.

THE AIM of the work was to assess the bioequivalence and comparability of the safety and tolerability profile of the drug Enligria® (liraglutide 6 mg/ml, Promomed RUS LLC, Russia) and the drug Saxenda® (liraglutide 6 mg/ml, Novo Nordisk AS, Denmark) after a single dose in healthy volunteers.

MATERIALS AND METHODS

Study drugs

The compositions of the study drugs were identical, the composition of the domestically produced liraglutide Enligria® (Promomed RUS LLC, Russia), the solution for the subcutaneous administration, 6 mg/ml (hereinafter referred to as Russian liraglutide, test drug) corresponded to the composition of the reference drug liraglutide Saxenda® (Novo Nordisk AS, Denmark), and the solution for the subcutaneous administration of 6 mg/ml (hereinafter referred to as foreign liraglutide, reference drug).

Physical and chemical research

Spectrophotomery in the ultraviolet region (200–400 nm). When comparing the absorption spectra in the ultraviolet region of the Russian and foreign drugs of liraglutide, Russia, the test solutions of each drug was diluted with water for injection to the concentration of liraglutide in the solution of 0.03 mg/ml. The analysis was carried out on a Shimadzu UV-1800 Spectrophotometer (Shimadzu, Japan), with a spectral wave range of 190–1100 nm.

Size exclusion-high-performance liquid chromatography. Size exclusion chromatography method was used to determine the quantitative content of high molecular compounds in the original foreign drug and a synthetic Russian analogue of liraglutide. The analysis was carried out on a liquid chromatograph with a UV detection Agilent 1260 Infinity LC (Agilent Technologies, USA) using a Tosoh TSK-gel G 2000 SWXL, 7.8×300 mm, 5 µm column. The study was carried out at a wavelength of 276 nm.

Reversed-phase high-performance liquid chromatography. A reverse-phase chromatography method was used to determine the quantitative content of liraglutide, its impurities and phenol in the foreign drug and a synthetic Russian analogue, as well as a confirmation of the authenticity of the active substance (liraglutide) and preservative (phenol). The analysis was carried out using a liquid chromatograph with a UV detection Prominence (Shimadzu, Japan), at a wavelength of 215 nm. For the analysis, Jupiter 4 µm Protea 90A (Phenomenex, 250×4.6 mm, 4 µm, 90 A) and Luna RP C8 (2) (Phenomenex, 4.6×50 мм, 5 µm) columns were used.

Verification of amino acid sequence and determination of intact mass using gas chromatography-mass spectrometry (LC-MS). The confirmation of the authenticity of the target component in the original foreign drug and the Russian analogue of liraglutide was carried out using the tandem mass method – high-resolution spectrometry on a quadrupole-time-of-flight mass spectrometer maXis 4G ETD (Bruker, USA). The Amino acid sequence verification was carried out by a peptide mapping with a peptide identification by HPLC/MS/MS with an electrospray ionization (ESI) and a secondary collision-initiated ionization (CID). The peptide identification was performed by precise monoisotopic mass using high-performance liquid chromatography-high-resolution mass spectrometry (HPLC/MS) with an electrospray ionization (ESI).

Study of biological activity *in vitro*

The biological activity of the studied drugs was assessed *in vitro* on the CHO-K1/GLP-1R cell culture

[17] Ibid.
[18] Ibid.

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(GenScript, USA). This cell line has receptors for GLP-1, to which the active ingredient of the drugs, liraglutide, binds.

The cultivation of the cell line was carried out using the RPMI culture medium (PanEco, Russia) with the addition of a penicillin / streptomycin solution (1%) and fetal bovine serum (10%), under standard conditions (temperature – 37±1°C, CO_2 content – 5±1%), for 2 days. The resulting suspension was diluted to the concentration of 2.5×105 cells/ml, transferred into 96-well plates (5×103 cells/well), and incubated.

Upon the incubation completion, the medium was removed from the plates and 7.5 µl of the test samples were added, after which the plates were mixed for 30 sec and incubated at room temperature for 20 min. Then, 7.5 µl of the lysis buffer was added to the plates and incubated at room temperature for 15 min with continuous stirring.

The results were assessed using the cAMP-GloTM Assay kit (Promega, USA) in accordance with the instructions for the kit.

Assessment of bioequivalence, safety profile, tolerability and immunogenicity

This phase I clinical trial No. LIR-062022 was an open-label, randomized, crossover, two-period comparative study in healthy volunteers. The study design is presented in Figure 1.

Study conditions and duration

The study was conducted from January 23 to April 25, 2023 at the research center of the Yaroslavl Region Clinical Hospital No. 3 (Yaroslavl, Russia).

Ethical approval

The study complied with the ethical principles set forth in the Declaration of Helsinki, as recently revised, the rules of Good Clinical Practice of the Eurasian Economic Union, the Rules of Good Clinical Practice of the International Council for Harmonization (ICH E6 GCP R2), as well as other legislation applicable to this study. The clinical trial protocol was approved by the Ministry of Health of Russia (Permission No. 725 dated December 26, 2022) and the Ethics Council of the Ministry of Health (extract from Protocol No. 335 of the meeting dated May 30, 2023), as well as the local ethics committee at the research center of the state budgetary healthcare institution of the Yaroslavl region “Clinical Hospital No. 3” (extract from Protocol No. 165 of the meeting dated September 30, 2022).

Study objects and eligibility criteria

A total of 26 healthy volunteers, men aged 18 to 45 years (32.42±7.78 years), were included in the study. All participants signed an informed consent form and expressed their ability and willingness to comply with all requirements of the study Protocol. In addition, the main inclusion criteria were: body weight >50 kg; BMI 18.5–26 kg/m^2 inclusive; a verified diagnosis “healthy” according to standard clinical, laboratory and instrumental examination methods; negative results of tests for the use of alcohol, psychotropic and narcotic substances and willingness to stop drinking alcohol during the participation in the study. The participants were warned to use reliable methods of contraception and to abstain from sperm donation throughout the study and for 3 months after the end of the study.

The main non-inclusion criteria were: the presence of chronic diseases of various organ systems; mental illness; hypersensitivity to study drugs; administration of liraglutide or other analogues of human GLP-1 in past history, taking medications that have a pronounced effect on hemodynamics and/or a liver function for less than 2 months before screening; taking illicit drugs less than 4 weeks before screening; inability to perform subcutaneous injections; any history of difficulty with blood collection or any vasovagal seizures during blood collections; history of surgical interventions on the gastrointestinal tract (except appendectomy). The volunteers were not allowed to take part in the study if you had the following diseases and conditions: a history of medullary thyroid cancer, including a family history; a history of multiple endocrine neoplasia type 2; severe depression; suicidal thoughts or behavior, including a history; acute infectious diseases or ARVI symptoms for less than 4 weeks before screening; presence of a positive PCR test for SARS-CoV-2. The volunteers were excluded from the study if they refused to participate in the clinical trial, if they were taking drugs for prohibited therapy and if they were tested positive for the use of alcohol, psychotropic and/or narcotic substances, if there were gross violations of the requirements and procedures of the Protocol, if adverse events occurred, or if a volunteer had any diseases or conditions that made his further participation in the study impossible during the study. The study physician had a right to arrive at the decision to exclude a volunteer in the best interests of the volunteer.

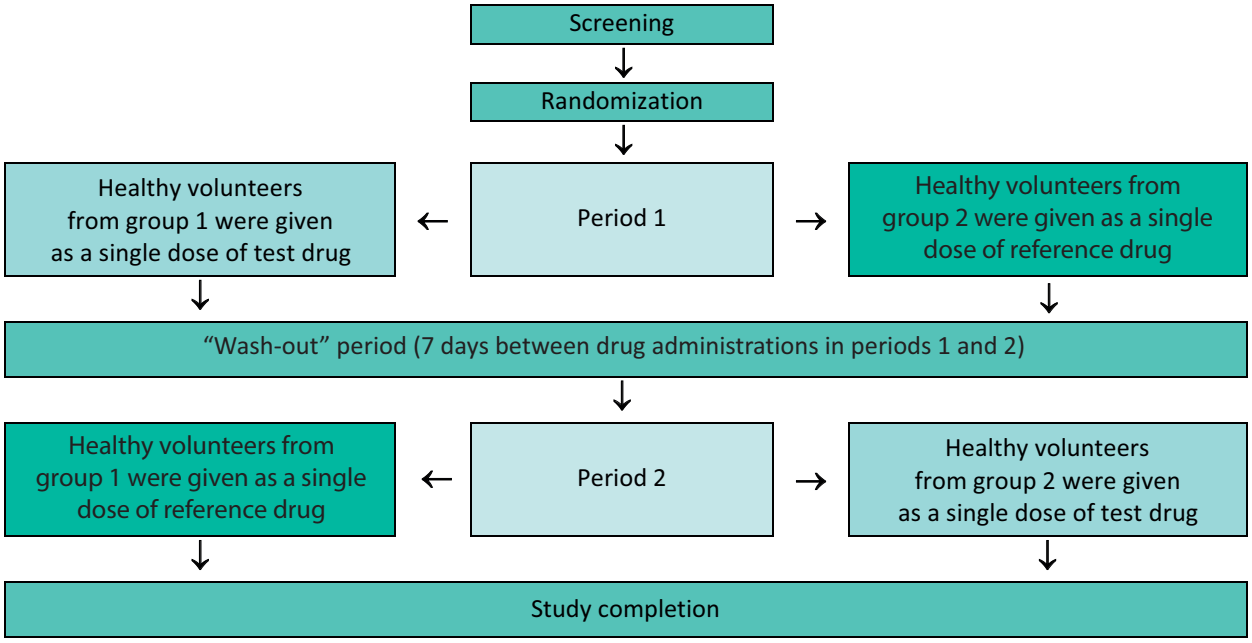

Figure 1 – Study design

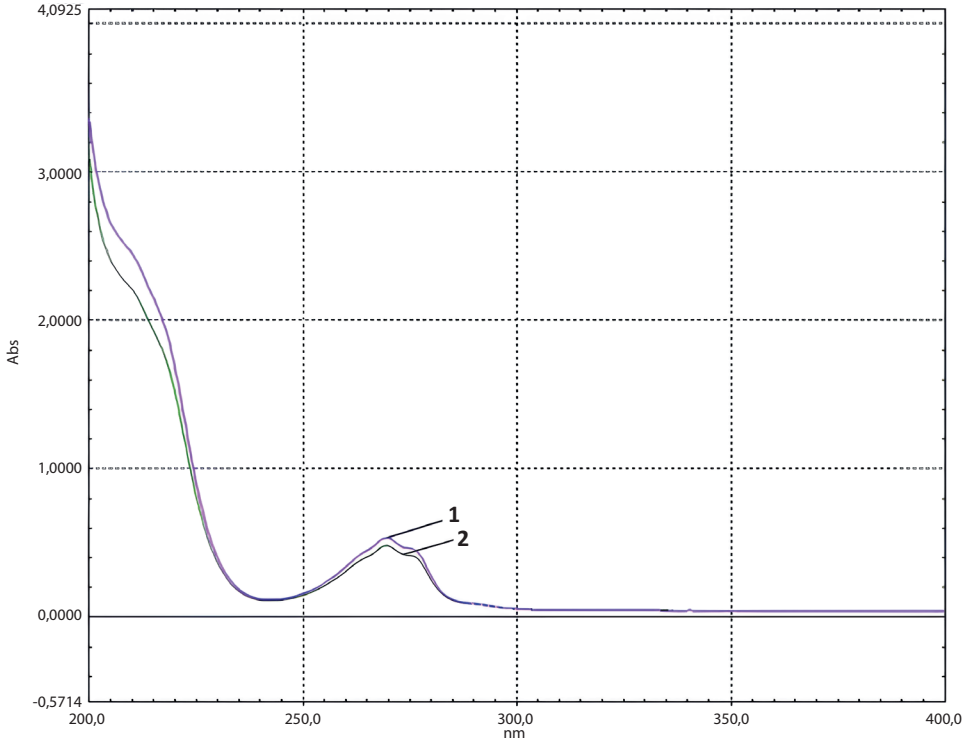

Figure 2 – Absorption spectrum of liraglutide

Note (here and in Fig. 3–6): 1 – synthesized Russian liraglutide; 2 – foreign liraglutide, an original drug.

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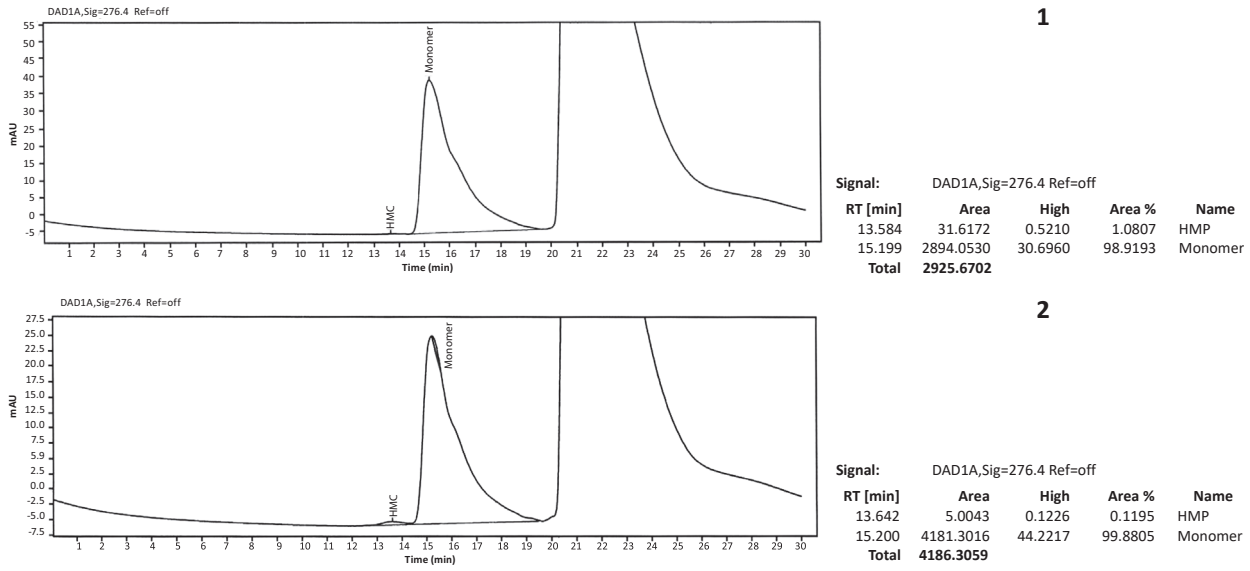


Figure 3 – Chromatograms for determining the content of high-molecular compounds in the drugs of liraglutide, a solution for subcutaneous administration of 6 mg/ml

Table 1 – Chromatographic analyses results of liraglutide drugs

Index	Saxenda®, solution for subcutaneous administration 6 mg/ml, Novo Nordisk AS, Denmark	Enligria®, solution for subcutaneous administration 6 mg/ml, Promomed RUS LLC, Russia
Quantitative determination of liraglutide, mg/ml	6.4	6.3
Amount of impurities, %	2.367	0.904
Hydrophilic impurities,%	0.164	0.072
Impurity A, %	0.614	0.515
Impurity B, %	0.877	0.241
Impurity C, %	0.346	0.076
Hydrophobic impurities,%	0.366	None
Phenol, mg/ml	5.51	5.4

Table 2 – Average values of pharmacokinetic parameters after administration of the study / reference drug

Parameter	Results, Mean±SD	
	Saxenda®, solution for subcutaneous administration 6 mg/ml, Novo Nordisk AS, Denmark	Enligria®, solution for subcutaneous administration 6 mg/ml, Promomed RUS LLC, Russia
C_{max} (ng/ml)	53.68±21.65	54.70±21.91
T_{max} (h)	11.93±4.60	13.60±4.94
$AUC_{0\to t}$ (ng*h/ml)	2313.96±671.11	2468.50±904.96
$AUC_{0\to\infty}$ (ng*h/ml)	2695.30±677.48	2849.24±905.32
$AUC_{0\to t}/AUC_{0\to\infty}$ (%)	85.84±4.57	86.63±5.00
K_{el} (h^{-1})	0.04±0.007	0.042±0.011
$T_{1/2}$ (h)	17.45±3.19	16.54±3.35
V_d (l)	5.60±2.21	5.03±2.51
$AUC_{(t-\infty)}$ (%)	13.35±4.56	12.23±5.00

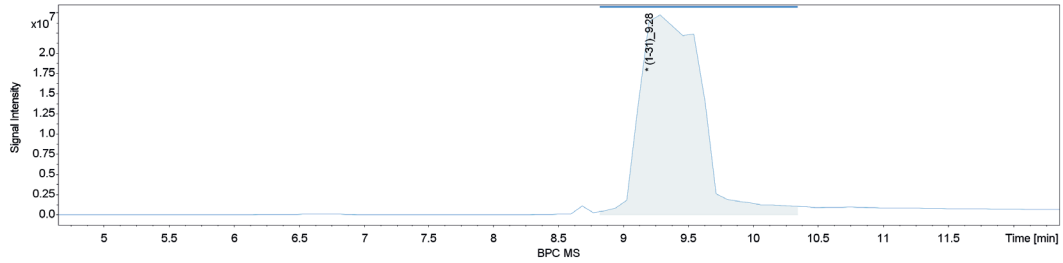
Note: C_{max} – maximum plasma concentration; T_{max} – time to reach C_{max}; $AUC_{0\to t}$ – is the area under the plasma concentration-time curve from the administration moment to the last determined concentration at time point t; $AUC_{0\to\infty}$ – area under the plasma concentration-time curve from the moment of taking the drug to infinity; K_{el} – terminal elimination rate constant; V_d – apparent volume of distribution.

Table 3 – Values of calculated 90% confidence intervals for indicators of relative liraglutide bioavailability after Russian and foreign drugs administration

Indicator	Ratio of geometric means	Calculated values 90% CI	$CV_{intra,}$ %
f'' C_{max} (T)/ C_{max} (R)	98,13±34,24	87,18–110,46	25,33
f' AUC_{0-t} (T)/ AUC_{0-t} (R)	93,74±26,73	84,40–104,11	22,38
f $AUC_{0\to\infty}$ (T)/ $AUC_{0\to\infty}$ (R)	94,60±21,78	86,69–103,22	18,55

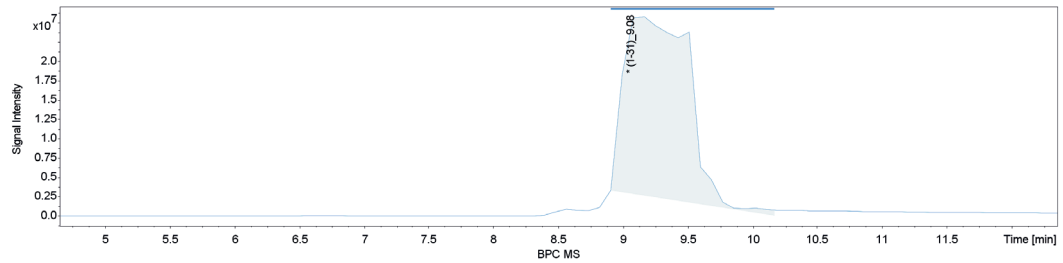
Note: C_{max} – maximum plasma concentration; $AUC_{0\to t}$ – area under the "plasma concentration – time" curve from the moment of administration to the last determined concentration at time point t; $AUC_{0\to\infty}$ is the area under the "plasma concentration – time" curve from the moment of taking the drug to infinity ; T – test drug; R – reference drug.

1



Cmpd.	m/z meas.	z	Δ m/z [ppm]	Δ m/z [Da]	Rt [min]	Score	No. of Cmpds.	P	Peptide	Sequence	Modification	Mod. Ratio [%]	Range
4	850.3762	2	0.32	0.0003	5.44	26.7	1	15		-.HAEGTFTSDVSSYLEG.Q		100.0	1-16
94	1160.6108	3	2.34	0.0027	10.14	18.4	1	27		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVR.G	Gamma-(E)_Palmitoate: 20	100.0	1-28
97	1179.615	3	-0.20	-0.0002	10.23	24.5	1	28		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVRG.R	Gamma-(E)_Palmitoate: 20	100.0	1-29
57	1250.6552	3	-0.72	-0.0009	9.37	62.7	3	30		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 20	100.0	1-31
76	1204.969	3	-0.66	-0.0008	9.71	68.6	1	29		H.AEGTFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 19	100.0	2-31
79	1181.2899	3	-0.72	-0.0009	9.71	65.3	1	28		A.EGTFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 18	100.0	3-31
81	1119.2687	3	-0.63	-0.0007	9.71	57.3	1	26		G.TFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 16	100.0	5-31
89	973.8689	3	-1.95	-0.0019	9.97	48.9	1	22		S.DVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 12	100.0	9-31

2



Cmpd.	m/z meas.	z	Δ m/z [ppm]	Δ m/z [Da]	Rt [min]	Score	No. of Cmpds.	P	Peptide	Sequence	Modification	Mod. Ratio [%]	Range
69	1160.6098	3	1.48	0.0017	10.11	40.5	1	27		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVR.G	Gamma-(E)_Palmitoate: 20	100.0	1-28
71	1179.6175	3	1.92	0.0023	10.19	33.5	1	28		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVRG.R	Gamma-(E)_Palmitoate: 20	100.0	1-29
48	1231.6495	3	0.45	0.0006	9.51	61.4	2	29		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVRGR.G	Gamma-(E)_Palmitoate: 20	100.0	1-30
38	1250.6567	3	0.48	0.0006	9.25	72.8	3	30		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 20	97.6	1-31
25	942.2424	4	-0.23	-0.0002	8.90	50.4	1	30		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 20; Oxidation: 25	2.4	1-31
51	1204.9692	3	-0.49	-0.0006	9.68	60.2	1	29		H.AEGTFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 19	100.0	2-31
53	1181.2907	3	-0.05	-0.0001	9.68	54.8	1	28		A.EGTFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 18	100.0	3-31
52	1002.881	3	-0.48	-0.0005	9.59	52.4	1	23		T.SDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 13	100.0	8-31
65	973.8703	3	-0.52	-0.0005	10.02	46.3	1	22		S.DVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 12	100.0	9-31

Figure 4 – Verification results of the amino acid sequence of liraglutide drugs samples, solution for subcutaneous administration 6 mg/ml

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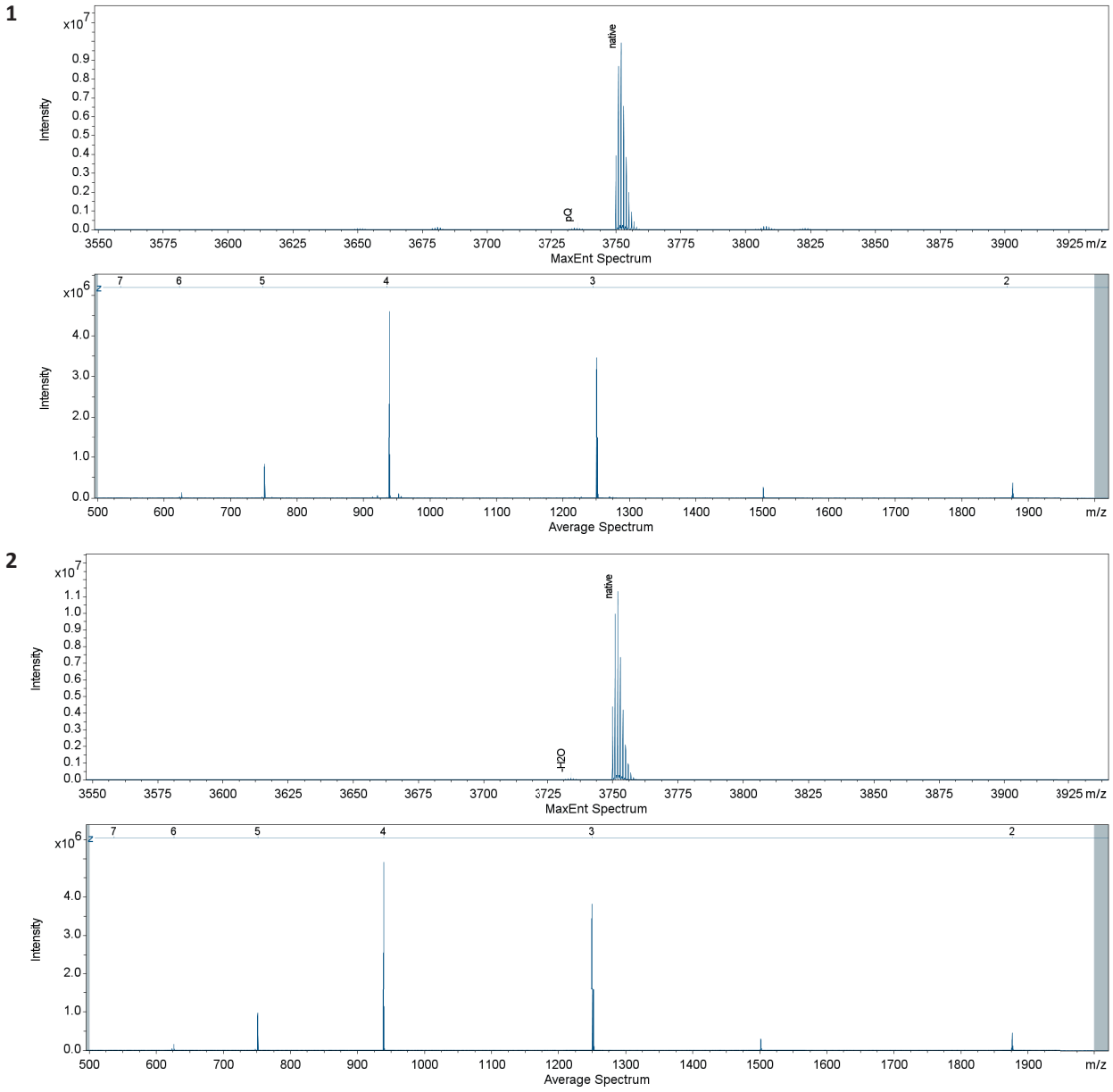

Figure 5 – Identification results of protein masses in samples of liraglutide, solution for subcutaneous administration 6 mg/ml (after deconvolution)

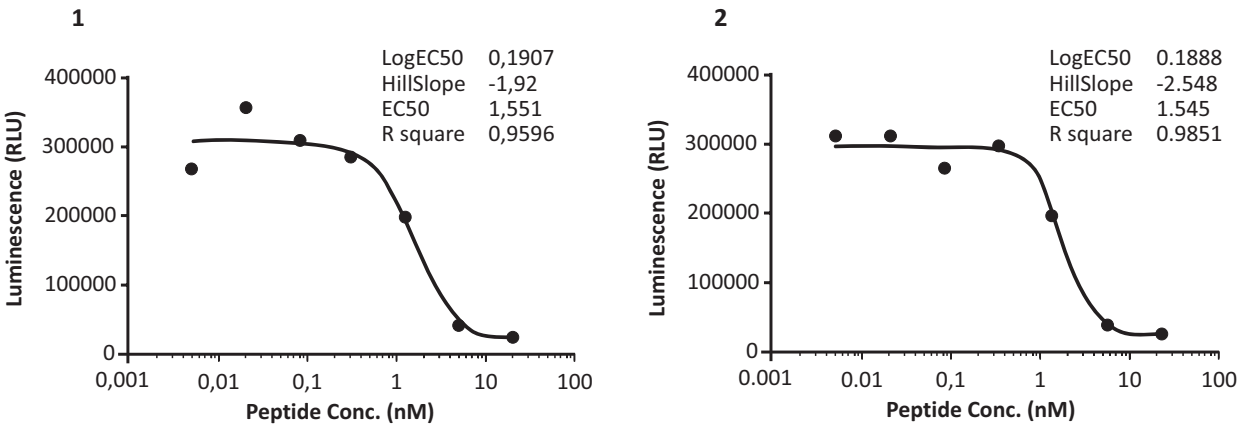

Figure 6 – Dependence graphs of RLU *vs* protein concentration, obtained as a study part of the connection with GLP-1 receptors of the active substance of liraglutide samples

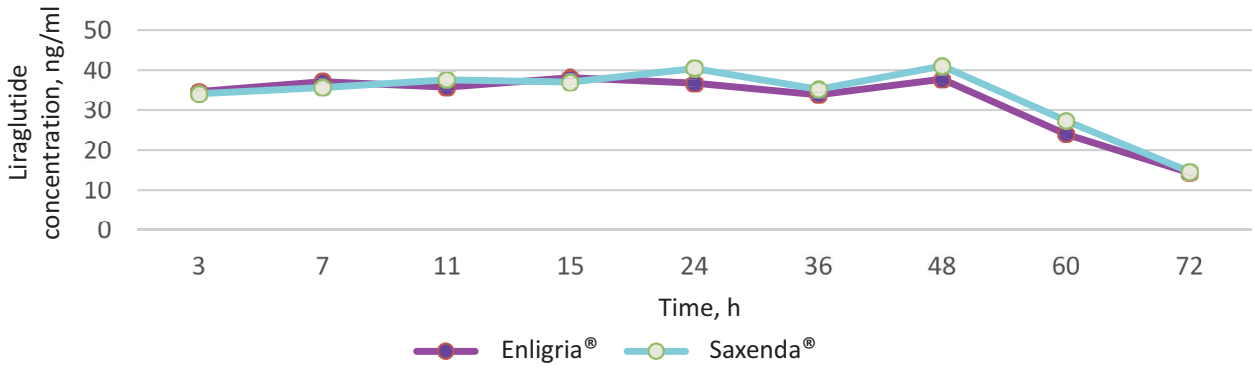

Figure 7 – Mean values (Geom Mean) of drug concentrations in linear transformation without standard deviations

The exclusion criteria were: a refusal of the volunteer to participate in the clinical trial; the research physician decided that the volunteer should be excluded in the interests of the volunteer himself; an erroneous inclusion of a volunteer who does not meet the inclusion criteria and/or meets the non-inclusion criteria; a positive test for the use of alcohol, psychotropic and/or narcotic substances; being late to the clinic for hospitalization by more than 1 h; skipping 2 or more blood samples to determine pharmacokinetic parameters in a row during one study period or three blood samples to determine pharmacokinetic parameters during one study period; a volunteer's administration of vitamins, BAS, medications, including herbal and/or homeopathic, with the exception of the test / reference drug; if a volunteer develops any diseases or conditions that make it impossible for him to further participate in the study; if a volunteer refuses to cooperate, is not disciplined, and does not comply with the rules of participation in the study.

Concomitant medications and exclusion criteria were assessed throughout the volunteer's participation in the study. The total duration of the study for each volunteer was not more than 25 days.

Randomization procedure

Each volunteer who met all the inclusion criteria and did not meet any of the non-inclusion criteria was assigned a randomization number in accordance with the randomization plan prepared for this study in the WinPepi 11.65 program (ETCETERA 3.26 module) using the random number generation method. The randomization number of the volunteer was entered by the study physician into the Screening / Randomization of Study Subjects Log. If a volunteer left the study prematurely, their randomization number was not reused and the volunteer could not subsequently return to the study.

Drugs administration

The test drug was Enligria® (liraglutide, a solution for the subcutaneous administration, 6 mg/ml, Promomed RUS LLC, Russia. The reference drug was Saxenda® (liraglutide, a solution for the subcutaneous administration, 6 mg/ml, Novo Nordisk AS, Denmark). The volunteers who had met the inclusion criteria and those who had not met the non-inclusion criteria, were randomized into 2 groups in a 1:1 ratio. Group I (*n*=13) received Russian liraglutide in the first period of the study, and a reference drug, foreign liraglutide, in the second period of the study. Group II (*n*=13) received the reference drug in the first period of the study, and the test drug in the second period. The reference / test drug was administered at a single dose of 0.6 mg subcutaneously in the abdominal area. The choice of a dose is based on the Russian and international regulatory requirements for the ethics and safety of the drugs administration in healthy volunteers[19],[20], as well as taking into account the requirements of Good Clinical Practice, within the framework of which, before the start of the study, an assessment of the ratio of foreseeable (predictable) risks and inconvenience (in in this case, the effect of the active substance on the glycemic profile and insulin production) with the expected benefit for the study subject and society, in healthy volunteers. It should be also noted that for the selected dose there is the experience in the clinical administration of the drugs biosimilar to liraglutide in healthy volunteers[21,22] [4].

[19] WMA declaration of Helsinki – Ethical principles for medical research involving human subjects. Edinburgh, Oct. 2000, 50 p.
[20] Decision of the Council of the Eurasian Economic Commission dated November 3, 2016 No. 85 (as amended on February 15, 2023) "On approval of the Rules for conducting bioequivalence studies of medicinal products within the framework of the Eurasian Economic Union". Available from: https://www.consultant.ru/document/cons_doc_LAW_207405/. Russian
[21] Register of Medicines of Russia. Instructions for medical use of the drug Enligria.
[22] Assessment report EMA/143005/2015. Saxenda, 2015.

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It is worth noting that the research results show linearity and proportionality of the kinetics with increasing doses according to the dosage regimen of liraglutide in accordance with the instructions for a medical administration, which also confirms the validity of the chosen dose in healthy volunteers and confirms the consistency of the results of pharmacokinetic parameters and safety parameters comparison regardless of the dose increase [4, 26, 27]. The washout period was 7 days and began immediately after the test / reference drug had been administered to the volunteer during period I. According to the literature, the half-life of liraglutide is about 13 h [4]. Thus, to minimize the risks of the first dose influence of drugs, the washout period should be at least 5 half-lives, i.e. at least 65 h.

To administer the test / reference drug, the volunteers were admitted to the hospital the evening before and at least 10 h before the drug administration. During the period of their stay in the hospital, the volunteers complied with the rules of their stay. The duration of hospitalization was no more than 4 days. During the study, the administration of vitamins, dietary supplements and/or medications, including herbal and homeopathic drugs, was prohibited, except as provided for in this Protocol. During the entire study, from the start of the screening examination until the completion of the final examination, the volunteers abstained from foods and drinks that may affect a circulatory function, a gastrointestinal function, a liver or kidney function, and an alcohol intake.

Preparation and sampling

After the randomization and before the administration of the test / reference drug, the volunteers were placed in a cubital heparinized catheter for no more than 16.5 h. After the removal of the catheter, the blood was collected from the volunteers by venipuncture. The blood samples were taken to determine pharmacokinetic parameters at the following time points: 10–15 min before the administration of the test / reference drug (the initial (0) sample) and then after 1, 3, 5, 7, 8, 9, 10, 11 , 12, 13, 14, 15, 16, 24, 36, 48, 60 and 72 h after the liraglutide administration. The original (0) sample was also used to assess immunogenicity. Thus, in all periods of the study, 19 blood samples were taken for each volunteer (6 ml each) for pharmacokinetic studies.

Blood samples were collected to assess the immunogenicity of the test / reference drug 10–15 min before the administration of the test / reference drug in periods I and II of the study. During screening, a blood volume of no more than 18 ml was taken for clinical, biochemical, serological tests and the determination of blood glucose levels using a glucometer. At the end of the II period of the study, the blood was also collected for clinical, biochemical tests and the determination of blood glucose levels using a glucometer (12 ml). Blood samples were collected in test tubes to obtain serum with a coagulation activator. After a clot formation, the tubes were centrifuged, and the resulting serum was carefully transferred into pre-labeled cryovials, dividing the serum into two aliquots: one for analysis (aliquot A), the other for repeat tests (aliquot B). The serum samples were frozen immediately after the receipt, transferred to cryovials and stored at the temperature not exceeding -70°C.

Analytical method

Pharmacokinetics was assessed by the concentration of liraglutide in the blood plasma and antibodies to it in the blood serum of each volunteer after the subcutaneous administration of the test / reference drug. A quantitative determination of liraglutide in serum samples was carried out using a HiPo MPP-96 microplate photometer (Biosan, Latvia). The calculation of liraglutide concentrations was carried out using the Quant Assay v0.8.2.6 Software, and the concentrations of antibodies to liraglutide – using GraphPad Prism 8.4.3. The determination of liraglutide in blood serum samples was carried out using a previously validated enzyme-linked immunosorbent assay (ELISA) method using a commercially available Enzyme-linked Immunosorbent Assay Kit For Liraglutide (LRT) Organism Species: Pan-species (General) (CEV769Ge 96 Tests)"; antibodies to liraglutide – using the KRIBIOLISATM Anti-Liraglutide ELISA kit. The sensitivity of the method was 4.64 ng/ml, the detection range was 12.35–1000.00 ng/ml. The preparation of calibration samples from the kit was carried out by diluting the standard sample. The analytical range was selected in accordance with the instructions for the kit.

Safety and tolerability assessment

During the study, a clinical observation of volunteers was carried out with the assessment of physical examination data, including a survey about the volunteer's complaints, basic vital signs (BP, HR, RR, body temperature), 12-lead ECG, laboratory parameters of clinical, biochemical blood tests, general urine analyses, determining blood glucose levels using a glucometer. Safety assessment criteria included the frequency and severity of AEs recorded based on abnormal laboratory test results, physical examinations, vital signs, and ECG; a number of cases of early participations' termination in the study due to the development of adverse events (AEs)

and/or serious adverse events (SAEs), including those related to the test / reference drug; the frequency of volunteers with detected antibodies to liraglutide; the assessment of the overall tolerability of the test / reference drug on a Likert Scale. The safety and tolerability of liraglutide were assessed for all volunteers. The identification of AEs occurred from the moment of the administration of the study drugs until the end of the volunteer's participation in the study.

Pharmacokinetic parameters: a maximum concentration of the substance in the blood serum (C_{max}); the time to reach C_{max} (T_{max}); the area under the concentration-time curve from the moment of the drug administration to the last determined concentration at the time point t ($AUC_{0\to t}$); the area under the pharmacokinetic curve, starting from the zero time value to infinity ($AUC_{0\to\infty}$); the ratio of $AUC_{0\to t}$ values to $AUC_{0\to\infty}$ ($AUC_{0\to t}/AUC_{0\to\infty}$); a terminal elimination rate constant (Ke_l); half-life ($T_{1/2}$); volume of distribution (V_d); residual (extrapolated) area under the curve, determined by the formula $AUC_{0\to\infty}–AUC_{0\to t}/AUC_{0\to\infty}$ ($AUC_{(t-\infty)}$%); the indicators of relative bioavailability and a relative degree of absorption ($f=AUC_{0\to\infty}$ (T)/$AUC_{0\to\infty}$ (R); $f'=AUC_{0\text{-}t}$ (T)/$AUC_{0\text{-}t}$ (R); $f''=C_{max}$ (T)/C_{max} (R)).

Statistical analysis

To calculate the number of participants, the data on the coefficients of intra-individual variability (CV_{intra}) of the C_{max}, $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ parameters of liraglutide were used. According to the literature sources, the intraindividual coefficient of variation for C_{max}, $AUC_{0\text{-}t}$ и $AUC_{0\text{-}\infty}$ of liraglutide does not exceed 21% [7]. In a crossover design, taking into account that the 90% CI was 80.00–125.00%, CV_{intra}=21%, α=0.05, a study power – 80%, a geometric mean ratio – 0.95, it was necessary to include at least 21 healthy volunteers (22 volunteers, taking into account an equal distribution in the study groups), who had completed the study and were included in the statistical analysis. Taking into account dropouts, the study planned to randomize 26 healthy volunteers.

For pharmacokinetic calculations, the actual time of blood sampling was used. The calculation of pharmacokinetic parameters, a statistical analysis of safety indicators and the presentation of results were carried out using statistical packages StatSoft Statistica version 10.0/13.3, IBM SPSS Statistics 22 and using the R Project program (version 3.5.1, GPL-2/GPL-3 license) with the extension *bear*, version 2.8.3-2. The indicators used to evaluate the pharmacokinetics of liraglutide are presented in Table 1.

For all pharmacokinetic parameters, the following statistical parameters were calculated: an arithmetic mean, a geometric mean, a standard deviation of the mean, a variation coefficient, median, minimum and maximum values, scatter.

A statistical analysis was carried out based on the assumption of a log-normal distribution of $AUC_{0\text{–}t}$, $AUC_{0\text{-}\infty}$, C_{max} and a normal distribution of other pharmacokinetic parameters with the exception of T_{max}. After a logarithmic transformation, these parameters were analyzed using the analysis of variance (ANOVA), with a standard significance level of α=0.05. The analysis of variance was used to test hypotheses about the statistical significance of the contribution to the observed variability of the following factors: differences between drugs, differences between healthy volunteers, sequence drug administration, study periods.

The conclusion about the equivalence of the compared drugs was made using an approach based on the assessment of 90% CI for the ratios of the geometric mean values of the C_{max}, $AUC_{0\text{–}t}$, $AUC_{0\text{-}\infty}$ liraglutide parameters.

For all the safety and tolerability indicators collected during the study, the descriptive statistics data are presented. For the analysis of frequencies, proportions, a two-sided version of Fisher's exact test or the χ^2 test was carried out. To compare quantitative continuous indicators, the Student's t-test (in the case of a normal distribution) or the Mann-Whitney test (in the case of a non-normal distribution) were used. The differences were considered statistically significant at p <0.05.

RESULTS

Physical and chemical research

Spectrophotomery in ultraviolet spectrum

The results obtained (Fig. 2) demonstrated the similarity of the absorption spectra in the UV of foreign liraglutide (min=242.4 nm; max=269.6 nm) and domestic liraglutide (min=242.2 nm; max=269, 4 nm).

Size exclusion-high-performance liquid chromatography

Typical chromatograms obtained during the determination of high-molecular compounds are shown in Figure 3.

The results obtained demonstrate the comparability of the retention time of high-molecular compounds and liraglutide, as well as the comparability of the quantitative content of impurities of high-molecular compounds in the preparation of foreign (0.131%) and Russian (0.052%) liraglutide.

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Reversed-phase high-performance liquid chromatography

Based on the results obtained (Table 1), it is shown that the retention time of the chromatographic peaks of liraglutide in the reference drug and in the test drug was about 14.7 min, the chromatogram peak profiles for liraglutide and impurities were similar. Moreover, at the time of the analysis, the content of impurities in Russian liraglutide was 3.5 times lower than in a foreign-made drug, which may be due to the technology for its production.

Moreover, the Russian liraglutide, which contains a synthetic molecule, did not contain hydrophobic impurities.

The retention time of the chromatographic peaks of phenol in the drugs was about 1.3 min, which corresponded to the retention time of phenol in the reference solution. The data obtained indicate that the phenol content in the drugs of foreign and Russian liraglutides is almost equivalent.

Verification of the amino acid sequence and determination of intact mass using gas chromatography-mass spectrometry (LC-MS)

The results of verification of the amino acid sequence of a domestically produced liraglutide sample with a chemically synthesized active substance and foreign liraglutide are presented in Figure 4.

The results of the protein masses identification taking into account possible isoforms of the sample with chemically synthesized Russian and foreign liraglutides are presented in Figure 5.

As a result of the amino acid sequence verification, a 100% coverage was obtained for all samples. The amino acid sequence of the samples fully corresponded to the declared one. As a result of the identification based on the exact mass of the protein, taking into account possible isoforms, it was found that the exact monoisotopic mass of all samples corresponded to the declared structural formula.

Biological activity *in vitro*

Dependence graph of RLU vs protein concentration are presented in Figure 6.

The results obtained demonstrate the comparability of the Russian drug (EC_{50}=1.545) biological activity with a chemically synthesized active substance and the original foreign liraglutide (EC_{50}=1.551). The range of activity of both study drugs is from 80 to 120% relative to the standard sample.

Bioequivalence and comparability of safety and tolerability profile

Population

A total of 26 male volunteers were included in the study. All the volunteers were included in the population for the safety assessment, pharmacokinetic analysis and bioequivalence assessment. The average age of the volunteers in the population was 32.42±7.78 years, the average body weight was 78.61±5.27 kg, the average height was 178.62±4.99 cm, the average body mass index (BMI) was 24.62±0.94 kg/m^2. Demographic and baseline characteristics (gender, age, race, weight, height) of the volunteers did not differ between the groups.

Pharmacokinetics and bioequivalence

The average values of the main and additional pharmacokinetic parameters after administration of the test and reference drugs are presented in Table 2.

A graph of the dynamics of average liraglutide concentrations during the administration of the test / reference drug is shown in Figure 7.

As it follows from the presented data, the mean values of both main and additional pharmacokinetic parameters obtained after the use of the test and reference drugs were comparable to each other.

According to the results of the statistical analysis, the obtained 90% CI for the ratio of the values of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ of the studied Russian and foreign drugs were 87.18–110.46, 84.40–104.11 and 86.69–103.22%, respectively. The intra-individual coefficients of variation calculated based on the ANOVA analysis were 25.33% for the C_{max} value, 22.38% for the AUC_{0-t} value and 18.55% for the $AUC_{0-\infty}$ value.

Thus, the intervals obtained during the study fully complied with the equivalence limit of 80.00–125.00% for C_{max}, AUC_{0-t} and $AUC_{0-\infty}$, clearly demonstrating the bioequivalence of the study and the reference drugs (Table 3).

The results of the ANOVA showed that the differences in the mean values of the main pharmacokinetic parameters were not statistically significant and had not been caused by the differences between the compared drugs for the factors “Sequence of the administration”, “Period” and “Drug” for the pharmacokinetic parameters of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$.

Safety

All volunteers completed the study entirely in accordance with the approved study protocol. During the study, no AEs were recorded in the volunteers. In 100% (26) cases, the tolerability among the volunteers

was rated as "good." No SAEs were identified in the volunteers during the study or after its completion. No deaths were observed. There were no cases of pregnancy of the sexual partner of a study participant during the study or after its completion. No abnormalities were found in the results of clinical and biochemical blood tests, the determination of blood glucose levels, general urinalyses, vital signs, physical examinations and ECGs.

Immunogenicity assessment

According to the results of the immunogenicity parameters analysis, no antibodies to liraglutide were detected in the blood serum of the volunteers, which indicated the absence of immunogenicity of the drug. No unexpected results were noted during the study.

Thus, the test drug Enligria® and the reference drug Saxenda® had a similar safety profile. At the same time, no cases of immunogenicity were observed for the domestic drug, which confirms a high safety profile and reduces the risk of ineffective therapy.

DISCUSSION

A wide range of liraglutide benefits makes it a strategically advantageous and attractive product for manufacturing. Based on this, the authors searched for ways to obtain this compound and analyzed the advantages and disadvantages of each method in order to fully form the evidence base in favor of this compound.

Biotechnological method for the production of liraglutide

Liraglutide, developed by Novo Nordisk (US7572884B2 [28], US7273921B2 [29]), was first introduced to the Russian market in 2010 as a hypoglycemic agent called Victoza®, a solution for the subcutaneous administration, 6 mg/ml [30]. The peptide precursor of liraglutide was obtained by the genetic recombination technology [31] using *Saccharomyces cerevisiae* to express the Arg34GLP-1 molecule (7–37), the structure of which was designed to be 97% homologous to native human GLP-1 by amino acid substitution arginine to lysine at position 34 [32, 33]. After that, liraglutide itself was obtained by adding C_{16} palmitic acid through a glutamine spacer to the ε-amino group of lysine located at position 26 [32].

Saccharomyces cerevisiae is a biologically safe strain with simple genetic manipulations and a clear mechanism for regulating gene expression [34]. Compared with the complete chemical synthesis of liraglutide, semichemical synthesis, including expression of the precursor peptide in a genetically engineered protein expression system and a further chemical modification of them, is more economical and environmentally friendly [33].

However, such eukaryotic systems have a number of disadvantages. It is known that the expression level of heterolytic proteins in *Saccharomyces cerevisiae,* especially GLP-1 polypeptides, is highly dependent on the protease during fermentation. That is why researchers attempted to knock out various protease and glycosylase genes in *S. cerevisiae* to create a protease-deficient strain in order to increase the expression and reduce the degradation of the target product [34].

Currently, the expression of foreign genes can be carried out using not only eukaryotic systems, but also prokaryotic ones [35]. *Escherichia coli (E. coli)* is one of the preferred expression systems (US10851146B2 [31], CN114807205A [36], US2020024321A1 [37]), since its genetic background and regulatory mechanism are well studied [35], it is able of reproduction and does not require expensive equipment [35, 38, 39].

Despite all the advantages of using eukaryotic and prokaryotic expression systems to produce the liraglutide precursor peptide, a biotechnological production has a number of significant disadvantages. The main challenge is to ensure a genetic stability and an adequate product yield, since the expression systems used may produce proteins with imperfect structures. When producing liraglutide using recombinant technology, it is also necessary to prove that the product is not contaminated with microorganisms and does not contain their metabolic products [40]. Based on this, there is a need to study and develop methods for obtaining liraglutide via an alternative route.

Chemical synthesis

There are two standard approaches to the chemical synthesis of GLP-1 agonists, i.e., liquid-phase peptide synthesis and solid-phase peptide synthesis. In addition, hybrid approaches can be used, as described in patents WO 2019069274 [40] and CN104650219 [41], in which the fragments are first synthesized by one of the above-mentioned methods and then are condensed together [42]. The main disadvantage of this synthesis is that the condensation reaction requires an excessive amount of peptide fragments, which in turn leads to serious losses, the formation of a large number of impurities, and as a result, complicates the purification of liraglutide, thereby preventing the obtaining of a pure target product [43]. It is possible to increase the yield of the peptide and obtain a product of a greater purity using

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a hybrid approach through the process of ultrasonic radiation and binding of fractionated peptides using an ionic liquid and a eutectic solvent [44].

The main challenge in the synthesis of a compound such as liraglutide is the introduction of a lipophilic group into the Lys^{20} lysine side chain. The creation of this branched structure can be achieved by directly introducing a lipidated dipeptide intermediate into the growing peptide chain or by using an orthogonally protected lysine. In the first case, to obtain a lipidated building block, orthogonally protected lysine is chosen as the starting material in order to selectively form a peptide bond between the ε-amino group of lysine and the Y-carboxylic group of glutamic acid [24]. For example, patents US11066439B2 [22] and WO2013/037266 [45] describe the solid-phase synthesis of liraglutide using lysine containing in its structure an allyloxycarbonyl protecting group (Alloc), the removal of which requires a metal catalyst - tetrakis(triphenylphosphine) palladium $Pd(PPh_3)_4$ [22, 43]. However, this method cannot be used for a large-scale production due to technological difficulties and high costs. In addition, the proposed $Pd(PPh_3)_4$ catalyst is sensitive to moisture, therefore, the reaction must be carried out under strictly controlled conditions, and when obtaining the final product, the content of heavy metals must be taken into account [22].

The use of copper (II) lysinate can greatly simplify the preparation of the palmitoylated intermediate, since copper complexes of trifunctional amino acids such as Lys, Asp or Glu can provide a temporary protection to selectively introduce protecting groups into the side chain. This method is commercially viable as it is widely used in the production of protected amino acids as raw materials for industrial peptide synthesis. Moreover, the procedures for storing and disposing of copper-containing waste are well known and do not pose any particular problems due to the low toxicity of copper salts [24].

In the drug Enligria®, the active substance liraglutide is obtained as a result of chemical synthesis. This method has a number of advantages in the production of peptides, ensuring the stable production of a clearly defined peptide structure, which is associated with a predictable and controllable effect and the exclusion of foreign impurities of producers (proteins and biomolecules) from entering the finished form, which ensures a high degree of purity and eliminates the risks of changes in the properties of the resulting substance; it also reduces the risk of AEs and immunogenicity [22, 44, 46, 47].

Key benefits of liraglutide

As stated earlier, liraglutide is a human GLP-1 analogue that stimulates glucose-dependent insulin secretion. However, the mechanism of a liraglutide action has a number of differences from the effects of native GLP-1. As a result of the experimental work on the animals, it was found that the drug has a predominantly central effect, exerting an effect in the arcuate nuclei of the hypothalamus. When administered peripherally, liraglutide, by binding to GLP-1 receptors, activates a pool of anorexigenic pro-opiomelanocortini and cocaine-amphetamine-regulated transcript, or otherwise POMC/CART-producing neurons. At the same time, a decrease in the orexigenic neurons activity producing neuropeptide Y (NPY) and agouti-like protein (AgLP) occurs indirectly, by inhibiting the GABA production [48, 49]. According to the studies conducted in humans, the administration of liraglutide statistically significantly increased the feeling of fullness after eating, and decreased the severity of hunger compared to placebo. At the same time, a slowdown in gastric emptying was observed only during the first hour after the drug administration; after 5 h, the difference compared to placebo, was not traced [50].

The studies have shown that liraglutide (1.8 mg), without taking into consideration other GLP-1 analogues, is one of the priority drugs in patients with T2DM and with indications of a high risk of CVD or existing CVD, CHF, CKD. Liraglutide (3 mg) was the only drug from the GLP-1 group with the indication (in Russia) of “the correction of body weight in adults and adolescents with obesity or overweight in the presence of concomitant diseases”[23]. In the Russian Federation, foreign-made liraglutide drugs have recently become commercially unavailable due to supply restrictions from the manufacturing company.

In 2023, liraglutide was included in the list of drugs that are in defect or for which there is a risk of its occurrence[24]. The company Promomed RUS LLC has developed the drug Enligria® based on liraglutide. The phase I comparative clinical study was conducted in accordance with the current legislation of the Russian Federation and the EAEU.

On September 14, 2023, the state registration of the first domestic liraglutide drug Enligria® (LP 008822) was

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[24] State register of drugs of the Russian Federation. Liraglutide. Available from: https://grls.rosminzdrav.ru/grls.aspx?s=%D0%BB%D0%B8%D1%80%D0%B0%D0%B3%D0%BB%D1%83%D1%82%D0%B8%D0%B4&m=INN

carried out, which returns the opportunity to personalize therapy for patients with obesity and overweight.

Despite some limitations of the study, in particular, the participation of only healthy male volunteers, the study was conducted in accordance with GCP standards and Russian and international recommendations developed for this group of drugs.

During the administration of the drug Enligria®, no AEs were identified in the study participants, and a good tolerability was noted. There were also no SAEs identified. Separately, it is worth noting that none of the participants had antibodies to liraglutide (Enligria®). In obese or overweight patients with at least one comorbid condition receiving the reference drug liraglutide 3.0 mg, the most commonly reported side effects were mild to moderate gastrointestinal disturbances. In this group, 2.5% of patients developed antibodies to liraglutide, which did not lead to a decrease in the effectiveness of the drug[25] [8, 9–12]. The main factors influencing the likelihood of an immune response[26] include the manufacturing process, formulation, and stability characteristics of the drug[27] [51]. The reference drug of liraglutide uses a biotechnological method of recombinant DNA in *Saccharomyces cerevisiae*[28]. With this production method, there is a potential danger of inducing an immune reaction due to the structural transformation of the active substance protein and the presence of impurities, for example, fragments of producer cells or reaction products with excipients. Adverse reactions can range from clinically insignificant, for example, the development of antibodies that do not affect the effectiveness of therapy and the severity of side effects, to serious adverse reactions when antibodies neutralize the protein of the active substance up to a complete loss of a biological activity[29] [51, 52]. The chemical synthesis-based technology used for the production of Russiam liraglutide eliminates the presence of impurities from the producer cells and, therefore, provides a high safety profile and a reduction in the above-described risks of developing immunogenicity[30,31] [51–53].

CONCLUSION

As a result of the studies, a sufficient amount of data was collected confirming the similarity of the physicochemical and biological properties of the drug with the chemically synthesized active substance liraglutide Enligria® (solution for the subcutaneous administration, 6 mg/ml, Promomed RUS LLC, Russia) with the reference drug Saxenda® (solution for the subcutaneous administration6 6 mg/ml, Novo Nordisk AS, Denmark). Based on this, it can be concluded that the quality, safety and effectiveness of the drug with a synthetic analogue of the active substance are similar to the reference drug, and in a number of parameters they even surpass them.

The reduction of medical and social damage caused by the increasing prevalence of obesity is one of the priority areas for the development of the Russian healthcare system. The inclusion of modern, high-quality, effective and safe drugs in treatment regimens for this disease is of particular importance. The entry of Russian GLP-1 receptor agonist drugs onto the market will allow patients to receive therapy that meets modern requirements. In an open, randomized, crossover comparative study assessing pharmacokinetic parameters, safety and tolerability in healthy volunteers, the equivalence of the study drug Enligria® and the reference drug Saxenda® was confirmed, and a high safety profile, tolerability of the study drug and lack of immunogenicity were demonstrated. Based on the data obtained, the drug Enligria® was registered in the Russian Federation.

The use of a chemical synthesis method in the production of the drug determines the identity of the active substance to the original product and a low risk of adverse immune reactions. It is advisable to conduct further clinical studies to assess the effectiveness and safety of therapy in patients with obesity and overweight, as well as to identify potential new possibilities for therapy with GLP-1 agonists, including the Russian analogue of liraglutide.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Alexander S. Ametov – development of a clinical trial concept, analysis and description of results, text correction; Igor E. Shokhin – organization and conduct of physical and chemical research, interpretation of results; Ekaterina A. Rogozhina – organization and conduct of physicochemical and biological properties research, discussion of the design and results of the study; Tatyana G. Bodrova – analysis and selection of literary sources, writing the text of the article, organizing and conducting physical and chemical studies, interpreting the results; Maria E. Nevretdinova – analysis and selection of literary sources, interpretation of results, writing the text of the article; Petr A. Bely – implementation of the research design, processing of research data; Kira Ya. Zaslavskaya – development of the design and concept of the study, writing the text of the article; Denis V. Kurkin – analysis and description of results; Ksenia N. Koryanova – analysis and description of results, search and analysis of literary sources; Ekaterina S. Mishchenko – analysis and description of the results; Sergey M. Noskov – development of the design and concept of a clinical trial.
All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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