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Development and Validation of the ELISA Method for Neutralizing Anti-trastuzumab Antibodies Detection in Human Blood Serum

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Abstract

Introduction. Trastuzumab is the first known anti-HER2 agent, which revolutionized the treatment of one of the most common cancer types – breast cancer. Despite trastuzumab being approved long time ago, further improvement of related analytical methods remains relevant primarily due to the emergence of new biosimilars. For instance, immunogenicity – adverse reaction which is usually associated with biological drugs, can still be relevant for trastuzumab. Anti-drug antibodies, including neutralizing antibodies, caused by trastuzumab therapy, can affect drug effectiveness and safety profile.

Aim. The aim of this study was to develop and validate the analytical method for neutralizing anti-trastuzumab antibodies determination in human blood serum.

Materials and methods. The neutralizing anti-trastuzumab antibody determination was carried out by the competitive ELISA method, using spectrophotometric detection in the visible range of the spectrum.

Results and discussion. The developed method was validated for cut-point, selectivity, sensitivity, specificity, precision and stability (short-term and long-term). To decrease the background noise from non-specific binding of sera components, the minimum required dilution value was determined at 0.5 % serum. The calculated value for cut-point was 14.62 %. The sensitivity of the developed method was estimated at 1985.2 ng/mL of neutralizing anti-trastuzumab antibodies.

Conclusion. The obtained results allowed us to apply the developed ELISA method for the neutralizing anti-trastuzumab antibodies determination in human blood serum during trastuzumab immunogenicity assessment in bioequivalence clinical trials.

Keywords: trastuzumab, neutralizing antibodies, anti-drug antibodies, immunogenicity, biosimilars

Conflict of interest. The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

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Разработка и валидация методики определения нейтрализующих антител к трастузумабу в сыворотке крови человека методом иммуноферментного анализа

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Резюме

Введение. «Трастузумаб» – препарат специфической анти-HER2 терапии одного из самых распространенных типов онкологических заболеваний – рака молочной железы. Несмотря на то, что препарат давно представлен на фармацевтическом рынке, дальнейшее совершенствование связанных с ним аналитических методик остается актуальным в первую очередь в свете разработки и исследования действия биоаналогов. Для трастузумаба одной из возможных нежелательных реакций со стороны иммунной системы является иммуногенность – выработка противолекарственных антител к препарату, в том числе нейтрализующих антител, которые могут влиять на эффективность и профиль безопасности препарата.

Цель. Разработка и валидация методики определения нейтрализующих антител к трастузумабу в сыворотке крови человека.

Материалы и методы. Определение нейтрализующих антител к трастузумабу проводилось с помощью метода конкурентного иммуноферментного анализа, с использованием фотометрического детектирования в видимом диапазоне спектра.

Результаты и обсуждение. Разработанная методика была валидирована по показателям: предел исключения, чувствительность, селективность, специфичность, прецизионность и стабильность (краткосрочная и долгосрочная). Для снижения интерференции компонентов биологической матрицы в анализе на этапе разработки было определено значение минимального необходимого разбавления (1 : 200). Рассчитанное значение предела исключения составило 14,62 %. Чувствительность разработанной методики составила 1985,2 нг/мл нейтрализующих антител к трастузумабу.

Заключение. Полученные при валидации методики результаты позволяют применять методику определения нейтрализующих антител к трастузумабу в сыворотке крови человека оценки иммуногенности препаратов трастузумаба при проведении клинических исследований биоаналогичности.

Ключевые слова: «Трастузумаб», нейтрализующие антитела, противолекарственные антитела, иммуногенность, биоаналоги

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. О. С. Сагимбаева, Ю. С. Борисова и М. А. Колганова участвовали в разработке и валидации методики. М. А. Колганова и Е. Е. Бекетов отвечали за написание текста статьи. И. Е. Шохин отвечал за методологию исследования и рецензирование текста статьи. Все авторы принимали участие в обсуждении результатов и написании текста статьи.

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INTRODUCTION

Oncological diseases are one of the most common causes of death worldwide. In 2020, deaths from various cancers amounted to more than 10 million cases. Notably, the most common types of cancer are: breast cancer (BC), lung, colorectal cancers and prostate tumors[1]. It is breast cancer that annually takes leading positions in morbidity and mortality among all oncological diseases, and in particular among cancers in women. Thus, it is estimated that only in 2020, breast cancer accounted for 12.5 % of all identified cancer cases in the world. Of these, breast cancer amounts to 25.8 %, if to speak about the situation in the world[2] or 21.7 % if to speak about the situation with the identification of new cases of breast cancer in Russia [1]. It should be noted that mortality rate of this cancer in the first year since the diagnosis is established, and treatment is started, is one of the lowest among all malignant neoplasms. Moreover, over the past 10 years, it has significantly decreased from 8.7 % in 2011 to 5.2 % in 2020 [2].

Standard methods of cancer therapy nowadays include surgical treatment, namely: excision of the tumor or the entire affected organ (for example, in the case of breast cancer – radical mastectomy), chemotherapy, radiation therapy, hormonal therapy, as well as innovative methods such as immunotherapy (for example, drugs targeting PD-1 or PDL-1), target biological therapy (for example, recombinant fusion proteins, monoclonal antibodies) and gene therapy. Often, the best prognosis for patients is given by a combination of several types of treatment, for example, surgical intervention combined with biological or chemotherapy in adjuvant and neoadjuvant settings. The combination of biological therapy and surgery is often used as the first line therapy in the event of breast cancer [3, 4].

[1] WHO fact sheet "Cancer". Available at: https://www.who.int/ru/news-room/fact-sheets/detail/breast-cancer. Accessed: 10.04.2023.

[2] Global cancer statistics for the most common cancers in the world. Available at: https://www.wcrf.org/cancer-trends/worldwide-cancer-data. Accessed: 10.04.2023.

Trastuzumab represents humanized monoclonal IgG1 antibodies containing mouse CDR-fragments fused with constant sites of light and heavy chains of human immunoglobulin G. Trastuzumab has become one the drugs aimed directly at HER2/neu (exactly, its IV extracellular domain) – human epidermal growth factor receptor 2 associated primarily with one of the most severe breast cancer types for patients: HER2-positive metastatic breast cancer. The type is usually associated with a high risk of metastasis and can also lead to brain metastases [5]. The introduction of trastuzumab to clinical practice has significantly reduced mortality rate of HER2-positive breast cancer. Another indication for trastuzumab as part of combination therapy has later become an advanced gastric adenocarcinoma or adenocarcinoma of the esophageal-gastric junction which is also accompanied with tumor HER2 overexpression. Moreover, Trastuzumab, as the first drug aimed at HER2 receptor, has become the starting point for the development of conjugate products "monoclonal antibody – chemotherapy agent", as well as bi- or trispecific antibodies or fusion proteins targeted simultaneously at various HER2 sub-domains (for example, combined therapy with trastuzumab or pertuzumab) or several different target receptors at once [6]. For example, at present, such drugs as Trastuzumab emtansine and Trastuzumab deruxtecan have already been registered, in which trastuzumab not only affects HER2, but also acts as a system for targeted delivery of chemotherapy agents to tumor cells. As for bi- and trispecific targeted therapies, such drugs are actively being developed in several countries [7–10].

One of the causes for the development of new trastuzumab-based drugs was not only the desire to increase treatment efficacy, but also the common trastuzumab resistance which may develop in patients taking the drug for the prolonged time. The causes of such resistance may be various ERBB2 mutations, its transcriptional and post-translations alternations, or (to a lesser extent) drug immunogenicity, and, in particular, neutralizing anti-trastuzumab antibodies [11]. Despite the fact that Trastuzumab is a drug with a rather low immunogenicity, neutralizing antibodies can bind to it in the active center, thereby preventing the binding of trastuzumab with HER2 and reducing therapy efficacy. Anti-drug antibodies can not only reduce treatment efficacy but also affect the drug safety profile [12].

Another important problem is the average cost of biological therapy, in particular, trastuzumab therapy. Considering that, on average, the steady-state concentration of trastuzumab in the body is reached after 25 weeks of therapy (with multiple administration according to the scheme once in 3 weeks), and the total time of therapy may be up to 1 year, the cost of such long-term therapy for any patient compared to other types of treatment may be higher by 300 % [13]. Thus, the development of new biosimilars of trastuzumab, market release of which will contribute to an increase in competition, and, as a result, a decrease in the cost of trastuzumab treatment [14], seems to be a promising goal. In this context, the development of new bioanalytical methods that can be used in clinical trials of trastuzumab biosimilars to obtain its pharmacokinetic and immunogenicity data seems relevant.

The aim of the work was to develop and validate a method for determination of neutralizing antibodies in human serum by the enzyme-linked immunosorbent assay (ELISA) to evaluate immunogenicity of trastuzumab biosimilar.

MATERIALS AND METHODS

Since the method was developed as part of a clinical study of the biosimilarity of trastuzumab products, originator Herceptin® and its biosimilar served as the investigational product and comparator:

- *investigational product*: "Trastuzumab". Lyophilizate for concentrate for solution for infusions. 440 mg/ 20 ml (Mabscale LLC, Russia).
- *comparator:* Herceptin®. Lyophilizate for concentrate for solution for infusions. 440 mg / 20 ml (F. Hoffmann- La Roche, Ltd., Switzerland).

Samples of the investigational product and comparator were kept in a pharmaceutical refrigerator in a dark place at temperature of 2–8 °C.

Reagents

During the validation of the method for determination of anti-trastuzumab antibodies in human serum, the following reagents were used: a set of reagents for the enzyme-linked immunosorbent assay of trastuzumab in the serum (RPC Probiotech LLC, Russia, lot 02, 03); neutralizing anti-trastuzumab antibodies (Bio-Rad Laboratories, Inc., USA, HCA177); bovine serum albumin (pure, Sigma-Aldrich, USA, A9647); polysorbate-20 (pure, pharma grade, PanReac, Spain, 142312); various salts of potassium and sodium, namely: potassium chloride (chemically pure, Aldosa LLC, Russia), sodium chloride (chemically pure, Aldosa LLC, Russia), potassium dihydrogen phosphate (AR grade, RusChem LLC, Russia), sodium hydrogen phosphate (pure, pharma grade, PanReac, Spain, 141677).

Equipment

During the development and validation of the method, a Stat Fax 3200 photometer (Awareness Technology, USA) was used for the determination of sample absorbance in ELISA plate wells. As an auxiliary equipment, the following was used: plate washer Aquamarine (BioSan, Latvia), plate thermal shaker (BioSan, Latvia), vortex Reax top (Heidolph Instruments GmbH & Co. KG, Germany), analytical balance OHAUS Pioneer PA-214C (OHAUS Corporation, USA), single- and multi-channel dispensers of various volumes (Thermo Fisher Scientific, USA), pH-meter-millivoltmeter ("NPO Aquilon" LLC, Russia) and volumetric glassware (flasks, cylinders) of various size (Shott Duran, Germany). 1 type purified water was obtained with Aqualab water treatment system AL-1 (JSC "NPK Mediana-Filter", Russia). Reagents and samples were stored at temperature of +2 °C to +8 °C in a pharmaceutical refrigerator HF-400-2 (JSC "PoZiS", Russia) and not above than –35 °C in a medical freezer MM-180 (JSC "POZIS", Russia).

Method for the determination of neutralizing anti-trastuzumab antibodies in human serum

The method for the determination of neutralizing antibodies is based on the ability of anti-trastuzumab antibodies to inhibit the drug binding to the target (HER2). During the determination of neutralizing antibodies, a set of reagents was used for the enzyme-linked immunosorbent assay of trastuzumab in the serum, which includes a plate coated with HER2 receptor and detecting reagent: goat anti-human IgG Fc-fragment polyclonal antibodies labeled with horseradish peroxidase.

According to the method, samples (control/test) containing anti-trastuzumab antibodies were incubated with trastuzumab solution (25 ng/ml), so that the minimal required dilution (MRD) was simultaneously achieved for serum samples. In addition to the control and test samples, calibration samples with a known concentration of trastuzumab, separately prepared according to the instructions for the reagent kit, were included in each cycle. After incubation, 100 µl of sample mixture was introduced to wells of a plate coated with HER2 and incubated for 1 hour (250 rpm) at room temperature (RT). At the end of incubation, the plate was washed, 100 µl of the conjugate was introduced into wells, incubated for 30 minutes (250 rpm) at RT. After incubation and plate washing, 100 µl of the substrate was introduced to all wells, and after 15 minutes of incubation, the reaction was stopped at RT. The absorbance was measured at test wavelength 450 nm and reference wavelength 630 nm.

For each cycle, a calibration curve of the dependence between trastuzumab concentration and absorbance of calibration samples was plotted. Then trastuzumab concentration for each sample was calculated, as well as the absolute value of the relative error (RE, %) which characterized the extent to which anti-trastuzumab antibodies neutralizing the drug in the mixture, inhibited its binding to the target in relation to the added (nominal) concentration of 25 ng/ml.

RESULTS AND DISCUSSION

Method development

During the method development, the main goal was to introduce changes to the analysis algorithm recommended by the instructions for the ELISA kit which would allow the use of the kit originally intended for trastuzumab assay, for the purposes of determination of the anti-drug neutralizing antibodies. The optimal MRD value was selected for the method: the best result was shown by the use of a serum concentration in 0.2 %; MRD was 1 : 200. As well, at the stage of the development, the procedure for pre-incubation of test samples containing trastuzumab was chosen, and an optimal concentration of the working solution of trastuzumab was selected (25 ng/ml). One of the stages of development was experiments on the addition of acid dissociation of samples to the analysis as a measure of the increase of the method tolerance to free trastuzumab in test samples. However, the acid dissociation to the analysis, even under mild conditions, had a negative impact on the results obtained. Due to that, we had to refuse from acid dissociation for the method, thereby reducing the resistance of the developed method to the presence of trastuzumab.

Method validation

The method for the determination of neutralizing anti-trastuzumab antibodies was validated in accordance with the FDA: Guidance for Industry: Immunogenicity Testing of Therapeutic Protein Products – Developing and Validating Assays for Anti-Drug Antibody Detection[1] and Rules for Conducting Studies of Biological Medicinal

[1] Guidance for Industry: Immunogenicity Testing of Therapeutic Protein Products – Developing and Validating Assays for Anti-Drug Antibody Detection U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER); Center for Biologics Evaluation and Research (CBER), January 2019. Available at: https://www.fda.gov/regulatory-information/search-fdaguidance-documents/immunogenicity-testing-therapeuticprotein-products-developing-and-validating-assays-anti-drug. Accessed: 12.04.2022.

Products of the European Economic Union[1] by parameters: cut-point, sensitivity, selectivity, specificity, precision and stability.

Cut-point

The cut-point was determined in the analysis of 30 individual samples of blank human serum which were analyzed by two analysts for 2 days. In addition to samples of blank serum, each cycle included positive control samples (PC – positive control; 20 000 ng/ml and 5000 ng/ml of anti-trastuzumab antibodies) and negative control samples (NC – negative control; pooled human serum). For the determination of cut-point, the obtained results were statistically processed: relative error, E, %, for 30 samples, for 2 analytics, 2 days (total N = 120). Based on the results of the statistical analysis, the fixed cut-point with the value of 14.62 % was selected for the use. In the further analysis, the value of relative error of samples, E, % (i. e. the deviation of the actual trastuzumab concentration in a sample from the nominal concentration added during the analysis – 25 ng/ml), taken by the module, was compared with the calculated value of the cut-point. Samples for which the relative error value is greater or equal to the cut-point are classified as "positive".

Method sensitivity

The method sensitivity was determined in 2 cycles, while establishing concentration of PC samples [at the upper and lower levels: HPC (high positive control) and LPC (low positive control], respectively). PC samples containing anti-trastuzumab antibodies at concentration of 1000 to 30 000 ng/ml were analyzed. Each cycle included three series of dilution, PC and NC samples.

To determine the sensitivity of the analysis and calculate LPC concentration, the nominal values of reference sample concentrations were used, the relative error value of which was higher than the value of the cut-point. Values of antibody concentrations (ng/ml) were converted into a log-form, then LPC sensitivity and concentration of the method were calculated by the formula:

$$\text{Sensitivity, ng/ml} = \log (C_{mean}) + t_{df} \times SD.$$

where C_{mean} – mean concentration; t_{df} – one-sided Student's t-test corresponding to a significance level of 0.05 for the determination of sensitivity or 0.01 for the determination of LPC concentration; SD –standard sample deviation.

The conversion of the obtained value from the logarithmic scale to the arithmetic scale made it possible to obtain a numerical value of the method sensitivity (1985.2 ng/ml). The detailed information on the calculation of the method sensitivity and LPC is given in table 1.

Table 1. Sensitivity and LPC concentration calculations

Dilution series, No.	Standard concentration. with RE, % value above cut-point, ng/mL	Standard concentration. with RE, % value above cut-point, ng/mL (log)
1	1000	3,000
2	1000	3,000
3	1000	3,000
4	2000	3,301
5	1000	3,000
6	1000	3,000
Average (log)		3,050
SD (log)		0,123
Sensitivity (log)		3,298
Sensitivity, ng/ml		1985,2
LPC (log)		3,464
LPC, ng/ml		2908,8

According to the results, the method sensitivity was 1985.2 ng/ml of anti-trastuzumab neutralizing antibodies, and the calculated LPC concentration – 2908.8 ng/ml. For the convenience and accuracy of preparation of control samples, the actual LPC concentration was selected at the level of 2909.1 ng/ml of anti-trastuzumab antibodies. HPC concentration was selected as the upper point in the linear range when plotting the calibration curve and was 20 000 ng/ml of anti-trastuzumab antibodies.

Selectivity

The method selectivity was evaluated in two cycles using 10 individual samples of blank human serum including hemolyzed samples. Each of the cycles included five individual samples of blank human serum, without

[1] Rules for conducting studies of biological medicinal products of the Eurasian Economic Union approved by the decision of the Council of the Eurasian Economic Commission № 89, dated November 3, 2016. Available at: http://pharmacopoeia.ru/wp-content/uploads/2016/11/8903111.pdf. Accessed at 12.04.2022.

Table 2. **Intra-day precision**

Sample	PC samples set No.						Mean	CV, %
	1	2	3	4	5	6		
HPC	98,60	97,52	98,52	98,36	98,91	97,86	98,30	0,52
LPC	29,91	33,49	22,28	26,73	24,45	29,91	27,79	14,76

Table 3. **Inter-day precision**

	Run No.							
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**CV, %**
HPC	98,30	92,36	93,68	98,64	98,15	98,52	96,61	2,91
LPC	27,79	29,83	23,05	31,39	32,42	27,50	28,66	11,73

the addition and with the addition of anti-trastuzumab neutralizing antibodies up to the HPC and LPC levels. The method selectivity was assessed by comparing RE, % of samples with the value cut-point. The selectivity of the method was confirmed, since 90 % of individual samples (9/10) of intact human blood serum had response below the cut-point, while 100 % positive control samples (HPC, LPC) were "positive" according to the results of the comparison calculated RE value, % with cut-point value.

Precision

To evaluate the precision, HPC and LPC samples were used, which were analyzed during 6 cycles by two analysts for three days. Each cycle included three sets control samples (HPC, LPC and NC), first cycle for the first analyst included 6 sets controls to evaluate intra-run precision. The data obtained during all six cycles, were used to evaluate the precision between cycles. Quantitative precision of the technique within and between cycles was expressed by calculating coefficient of variation (CV) for the first cycle and for all six cycles, respectively. Precision was calculated using the values RE, % for positive control samples (HPC, LPC). The results obtained are shown in Table 2 (inside the cycle) and in Table 3 (between cycles).

The calculated CV values did not exceed 20 %, which complies with the requirements of regulatory documentation[1].

[1] Guidance for Industry: Immunogenicity Testing of Therapeutic Protein Products – Developing and Validating Assays for Anti-Drug Antibody Detection U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER); Center for Biologics Evaluation and Research (CBER), January 2019. Available at: https://www.fda.gov/regulatory-information/search-fdaguidance-documents/immunogenicity-testing-therapeuticprotein-products-developing-and-validating-assays-anti-drug. Accessed: 12.04.2022.

Specificity

During the validation, the specificity was evaluated techniques in the presence of concomitant drugs chemotherapy: "Doxorubicin", "Paclitaxel", and "Cyclophosphamide". For sample preparation specificity evaluations were prepared for interfering compound samples (serum) and HPC and LPC samples (serum) at 2x concentration. After that, the samples were mixed 1:1 for obtaining samples for specificity assessment with required concentrations of components. Total 4 concentrations were tested for each of the drugs at HPC and LPC levels. The drug concentrations in the wells of the plate (after all dilutions) were 50, 25, 15 and 10 ng/ml, respectively. To control method specificity NC samples were also analyzed with the addition of interfering compounds.

The technique was recognized as specific in relation to concomitant chemotherapy drugs, since no results were observed in the NC samples, exceeding the cut-point, while the image positive control samples both at the HPC level and at LPC level demonstrated RE values, % higher cut-point.

Stability

During the validation of the method, the following was evaluated: short-term stability (bench-top stability, BTS: PC samples were stored for 20 hours at RT before analysis, 18–25 °C); freeze-thaw stability, F/T: 3 cycles each freezing for at least 12 hours); as well as long-term stability (LTS: storage under conditions of low-temperature freezing from minus 50 °C to minus 35 °C, assessment after 30 days). The cycles for evaluating various types of stability in addition to the stability samples themselves (3 sets of PC samples for each type) included in two sets of freshly prepared PC samples –

Table 4. **CV values calculated for sample stability assessment**

Sample bench-top stability (BTS)						
Sample	**1**	**2**	**3**	**Mean**	**S.D.**	**CV, %**
HPC_BTS20	96,03	96,54	97,95	96,84	0,997	1,03
LPC_BTS20	24,99	34,59	35,05	31,54	5,685	18,02
Sample freeze-thaw stability (F/T)						
Sample	**1**	**2**	**3**	**Mean**	**S.D.**	**CV, %**
HPC_F/T3	97,22	98,46	96,40	97,36	1,033	1,06
LPC_F/T3	29,05	29,60	21,35	26,67	4,612	17,29
Sample long-term stability (LTS)						
Sample	**1**	**2**	**3**	**Mean**	**S.D.**	**CV, %**
HPC_LTS30	98,77	98,43	98,64	98,62	0,171	0,17
LPC_LTS30	24,41	24,29	26,47	25,06	1,222	4,88

for the control of the system suitability. Sample stability was evaluated by calculating CV between three sets of samples for each type of stability. Samples were found to be stable as coefficients of variation did not exceed 20 %. The obtained results are given in Table 4.

CONCLUSION

The method for the determination of anti-trastuzumab neutralizing antibodies in human serum by ELISA was developed and validated. It was determined by the method of the competitive ELISA using photometric detection in the visible range of the spectrum. The method sensitivity and LPC concentration were determined at the level of 1985.2 ng/ml and 2909.1 ng/ml of anti-trastuzumab neutralizing antibodies, respectively. The obtained values of the cut-point and sensitivity of the validated method allow using the method for the determination of immunogenicity of trastuzumab products in clinical trials, including biosimilarity studies.

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