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Development and Validation of the Cell-based Functional Method for Neutralizing Anti-adalimumab Antibodies Detection in Human Serum

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Abstract

Introduction. Adalimumab, a fully humanized monoclonal antibody, is a tumor necrosis factor (TNFα) inactivator that is used against a number of autoimmune diseases such as rheumatoid arthritis and other most common inflammatory arthropathies (ankylosing spondylitis, psoriatic arthritis). Despite the proven efficacy of adalimumab treatment, there is a risk of adverse events, tied up with the formation of anti-drug antibodies, including neutralizing antibodies. Currently, the evaluation and characterization of neutralizing antibodies has become an important part of clinical trials in the development of new drugs and biosimilars.

Aim. The aim of this study is to develop and validate the cell-based functional method for neutralizing anti-adalimumab antibodies determination in human serum.

Materials and methods. For determination of neutralizing anti-adalimumab antibodies, the cell line L-929 has been employed. L-929 is a cell line sensitive to the TNFα-mediated apoptosis; the neutralizing antibodies interact with adalimumab that leads to TNFα-mediated cytotoxicity. Cytotoxicity was measured using resazurin, an aromatic compound that is a redox indicator.

Results and discussion. The developed method was validated for cut point, selectivity, sensitivity, precision, specificity and stability (short- and long-term). An important part of a method development for determining neutralizing antibodies is the selection of concentrations of TNFα (4 ng/ml) and adalimumab (250 ng/ml), as well as determining the minimum required dilution – this parameter is established as 1:20. Cut point was chosen as a «floating» cut point, and a correction factor (normalization factor) was determined equal to 0,86. The sensitivity of the developed method was estimated at 108,9 ng/ml of neutralizing anti-adalimumab antibodies.

Conclusion. The obtained results can be applied for determining anti-adalimumab neutralizing antibodies in the assessment of the adalimumab immunogenicity, including clinical trials.

Keywords: adalimumab, neutralizing antibodies, anti-drug antibodies, immunogenicity, TNFα, L-929 cell line

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 на основе функционального клеточного теста для определения нейтрализующих антител к адалимумабу в сыворотке крови человека

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Резюме

Введение. Адалимумаб – рекомбинантное, полностью гуманизированное моноклональное антитело, которое связывается с высокой степенью сродства и специфичностью с растворимой и мембранной формами фактора некроза опухоли (TNFα) и используется для лечения ряда аутоиммунных заболеваний, таких как ревматоидный артрит и другие наиболее распространенные воспалительные артропатии (анкилозирующий спондилит, псориатический артрит). Несмотря на доказанную эффективность лечения адалимумабом, у части пациентов с течением времени происходит снижение клинической эффективности и возникает риск нежелательных явлений. Одним из объяснений данного эффекта является образование антилекарственных антител (ADA) к препарату, в том числе и нейтрализующих антител (NAb). В настоящее время при разработке новых препаратов и препаратов-биоаналогов неотъемлемой частью клинических испытаний стала оценка и характеристика нейтрализующих антител.

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

Материалы и методы. Определение нейтрализующих антител к адалимумабу основано на использовании клеточной линии L-929, чувствительной к индукции апоптоза посредством TNFα; нейтрализующие антитела связываются с адалимумабом, блокируя его взаимодействие с TNFα, что приводит к TNFα-опосредованной цитотоксичности. Измерение цитотоксичности проводили с помощью резазурина, ароматического соединения, представляющего собой окислительно-восстановительный индикатор.

Результаты и обсуждение. Разработанная методика была валидирована по показателям: пределу исключения, селективности, чувствительности, прецизионности, специфичности и стабильности (краткосрочной и долгосрочной). Важной частью разработки методики определения нейтрализующих антител является подбор концентраций TNFα (4 нг/мл) и адалимумаба (250 нг/мл), а также определение минимального необходимого разведения образца: данный параметр составил 1:20. По итогам валидации был выбран «плавающий» предел исключения с поправочным коэффициентом (фактор нормализации) равным 0,86. Чувствительность разработанной методики составила 108,9 нг/мл нейтрализующих антител к адалимумабу.

Заключение. Полученные при валидации методики результаты позволяют применять методику определения нейтрализующих антител к адалимумабу в сыворотке крови человека в исследовании иммуногенности препаратов адалимумаба, в том числе при проведении клинических исследований биоэквивалентности.

Ключевые слова: адалимумаб, нейтрализующие антитела, антилекарственные антитела, иммуногенность, TNFα, клеточная линия L-929

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

Вклад авторов. М. А. Никифорова и И. А. Валуев участвовали в разработке и валидации методики. М. А. Никифорова, И. А. Валуев и Е. Е. Бекетов отвечали за написание текста статьи. А. В. Петров отвечал за общий дизайн исследования. И. Е. Шохин отвечал за рецензирование текста статьи. Все авторы принимали участие в обсуждении результатов и написании текста статьи.

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INTRODUCTION

Adalimumab is a recombinant, fully humanized monoclonal antibody (IgG1) that binds with a high degree of affinity for TNFα and blocks its interaction with the TNFα p55 and p75 cell surface receptors. Adalimumab was approved by the FDA back in 2002 for the treatment of rheumatoid arthritis (RA) [1]. RA is a systemic chronic inflammatory autoimmune disease that leads to progressive joint damage and affects about 1 % of the Caucasian population [2, 3]. Over the course of the disease, most patients develop typical lesions of the small joints of the hands and feet, erosion and destruction of articular cartilage and bones occur. Adalimumab has proven to be effective in the treatment of RA, especially in the early stages of the disease [4, 5].

Adalimumab is also currently approved for the treatment of adult patients with moderate to severe chronic plaque psoriasis who are eligible for systemic therapy or phototherapy. A number of studies show the high efficacy of adalimumab in the treatment of psoriasis [6, 7].

Psoriasis is a common chronic non-infectious skin disease, the most common form of psoriasis is plaque psoriasis, accounting for more than 80 % of cases [8].

The causes of plaque psoriasis have not yet been unequivocally found, one of the main versions is considered to be a hereditary factor [9]. Nevertheless, this disease is a serious global problem, since at least 100 million people around the world suffer from it [10].

Despite the effectiveness of adalimumab treatment, some patients experience a decrease or even loss of clinical efficacy (clinical response) over time and a risk of adverse events [11]. One of the explanations for this effect is the formation of anti-drug antibodies (ADA) to therapeutic antibodies, which leads to a loss of treatment efficacy [12]. According to various studies, for adalimumab, the incidence of ADA ranges from 10 to 60 % [13].

The reason why the fully humanized adalimumab antibody is immunogenic is explained by the structure of the complementary determining regions (CDR). CDRH3 loops in immunogenic Abs are slightly shorter compared to non-immunogenic Abs, and thus this structure promotes the binding of anti-idiotypic antibodies (ADAs) to Abs [14].

The resulting ADAs may have neutralizing activity (neutralizing anti-drug antibodies – NAbs), they block the antigen-binding ability of the therapeutic molecule. NAbs are of great clinical importance because their formation may jeopardize the continuation of therapy. Currently, in the development of new drugs and biosimilar preparations, including adalimumab, the assessment and characterization of NAb has become an integral part of clinical trials. For biotechnological drugs for which a high risk of immunogenicity is shown, NAb assessment is recommended using methods based on cell tests [15].

The aim of this work was to develop and validate a method for determining neutralizing antibodies to adalimumab in human serum based on a functional cell test to assess the immunogenicity of the biosimilar drug adalimumab.

MATERIALS AND METHODS

When developing and validating the method, originator Humira® and its biosimilar were used:

- *Investigational drug*: adalimumab; solution for subcutaneous administration; 40 mg / 0.8 ml.
- *Comparator*: Humira®; solution for subcutaneous administration; 40 mg / 0.8 ml.

Samples of the study drug and the comparator were stored in a pharmaceutical refrigerator, away from light, at a temperature of 2–8 °C.

For the preparation of calibration and control (positive) samples, anti-idiotypic, neutralizing monoclonal IgG1 antibodies to adalimumab (human anti-adalimumab antibodies), with a protein concentration of 0.5 mg/ml (BioRad, USA).

Reagents

The following reagents were used to validate the method for determining antibodies to adalimumab in human serum: antibodies to adalimumab with neutralizing activity (HCA204, BioRad, USA); Wednesday RPMI1640 (S363p, NPP "PanEco", Russia); fetal bovine serum (FTS, SV3016003, HyClone, USA); recombinant human TNFα (30001A, PeproTech, USA); actinomycin D (A9415, Sigma-Aldrich, USA); DMSO (D2650, Sigma-Aldrich, USA) and resazurin sodium salt (B21187, Thermo Fisher Scientific, USA).

Equipment

For the cultivation of the L-929 cell line (Shared Research Facility "Vertebrate Cell Culture Collection", Institute of Cytology of the Russian Academy of Sciences) and for analysis, the following was used: a microbiological safety cabinet BMB-II-"Laminar-C"-1.5 (CJSC "Laminar Systems", Russia), an inverted laboratory microscope BI-200 (LLC "BiOptic", Russia) and CO_2 incubator MCO-170AC (PHCbi, Japan). To determine fluorescence in the wells of the plate, a SpectraMax® M5 spectrophotometer (Molecular Devices, USA) was used.

Method for determination of neutralizing antibodies to adalimumab in human serum

The method is based on the determination of the specific activity of the therapeutic antibody adalimumab, which is based on the assessment of the inhibition of the pro-apoptotic effect of the cytokine TNFα on L-929 cells under the action of adalimumab. Cells of this line express on the surface of TNFα receptors [16], the activation of which initiates apoptotic cell death. For the detection of neutralizing anti-adalimumab anti-Abs, an additional stage is required in which a human serum sample is preincubated with the addition of adalimumab at a fixed concentration. Neutralizing antibodies, competing with TNFα for adalimumab, contribute to the manifestation of TNFα mediated cytotoxicity.

L-929 cells were cultured in RPMI-1640 medium with the addition of 10 % FBS in a CO_2 incubator at a

temperature of 37 °C and a CO_2 content of 5 %. To determine neutralizing antibodies, cells with a density of 300 000 cells/ml were inoculated into the wells of the 96-well plate 18–24 hours before the analysis.

Samples (controls/study) were incubated in the presence of a fixed concentration of adalimumab (250 ng/mL) for 60 to 90 minutes at 25 °C (500–600 rpm), followed by the addition of TNFα solution (4 ng/mL) and incubation for 60 to 90 minutes at 25 °C (500–600 rpm) before addition to prepared (actinomycin D) L-929 cells in a 96-well plate. Then the plate was incubated for 16–20 hours in a CO_2 incubator, and a solution of resazurin (0.15 μg/ml) was added to the wells to assess cytotoxicity. Fluorescence detection parameters: excitation at a wavelength of 545 nm, detection at a wavelength of 600 nm.

RESULTS AND DISCUSSION

Development of the method

At the first stage of the development of the method, the TNFα concentration was selected, which leads to the death of 90–95 % of cells. Then an experiment was carried out with serial dilutions of adalimumab in the presence of a fixed concentration of TNFα, adding L-929 to TNFα-sensitive cells, and a concentration was selected at which a cellular response of ≥50 % (ED_{50}) would be observed. It is important to use the lowest concentration of adalimumab that will not lead to a strong cell response and will provide adequate sensitivity to the method. Based on the results of the experiments, the following concentrations were selected: for TNFα – 4 ng/ml, adalimumab – 250 ng/ml.

The next stage was to assess the matrix effect (serum) and, accordingly, determine the minimum permissible dilution (MRD) of the sample. This parameter should be selected in such a way that the serum does not affect the cell line and the results of the analysis. For this purpose, positive controls were prepared in individual blank samples of human serum, which were sequentially diluted in RPMI-1640 medium. Based on the results of development, a 5 % solution of human serum was used in RPMI-1640 medium in the further work, therefore, the MRD was 1 : 20.

Validation of the method

Validation of the method for determining neutralizing antibodies to adalimumab was carried out according to the recommendations of Guidance for Industry: Immunogenicity Testing of Therapeutic Protein Products– Developing and Validating Assays for AntiDrug Antibody Detection (FDA)[1]; Immunogenicity Assays –Design and Validation of Immunoassays to Detect AntiDrug Antibodies (USP)[2] and Rules for Conducting Studies of Biological Medicinal Products of the Eurasian Economic Union[3,4] according to the parameters: exclusion limit, selectivity, sensitivity, precision, specificity and stability.

Cut point

To determine the exclusion limit, 64 individual blank human serum samples were analyzed by two analysts in 6 independent analytical cycles. In each analytical cycle, 16 samples were analyzed in three repetitions. The exclusion limit was calculated by data normalization (conversion per % of viable cells) of the relative fluorescence units (RFUs), the formula is given below:

$$V = \frac{S - \text{TNF}}{\text{NC} - \text{TNF}} \cdot 100,$$

where V – cell viability, %; S – RFU of the sample; TNF – RFU of TNFα control (minimum RFU value, in wells only TNFα); NC – RFU of negative NC control (maximum RFU value, in wells 5 % human serum solution per RPMI-1640).

To select the type of exclusion limit (fixed, floating or dynamic), we performed an analysis of variance (ANOVA) of means and an assessment of the homogeneity of

[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/media/119788/download. Accessed: 01.11.2023.

[2] Pharmacopeia US. United States Pharmacopeia. General Chapter, <1106> Immunogenicity assays–design and validation of immunoassays to detect anti-drug antibodies. Rockville, MD: United States Pharmacopeia 2022. Available at: https://doi.usp.org/USPNF/USPNF_M99825_02_01.html. Accessed: 01.11.2023.

[3] Decision of the Council of the Eurasian Economic Commission dated November 3, 2016 No. 89 "On approval of the Rules for conducting research on biological medicinal products of the Eurasian Economic Union". Available at: https://docs.eaeunion.org/docs/ru-ru/01411954/cncd_21112016_89. Accessed: 01.11.2023.

[4] Decision of the Council of the Eurasian Economic Commission dated November 3, 2016 No. 85 "On approval of the Rules conducting bioequivalence studies of medicinal products within the framework of the Eurasian Economic Union". Available at: https://docs.eaeunion.org/docs/ru-ru/01411942/cncd_21112016_85. Accessed: 01.11.2023.

dispersions (Brown-Forsythe test) for all analytical cycles conducted by each analyst, and for all analytical cycles in total. The results are presented in Table 1.

Table 1. Data for means and variances homogeneity for normalized data values between analytical runs

Parameter	Analyst 1			Analyst 2		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Analysis of variance (ANOVA)						
F	6,274			8,643		
P-value	0,0023			0,0003		
Differences are significant ($P < 0,05$)	Yes			Yes		
F	24,37					
P-value	<0,0001					
Differences are significant ($P < 0,05$)	Yes					
Brown – Forsythe test statistics						
F	0,6588			0,1017		
P-value (significance)	0,5186			0,9034		
Differences are significant ($P < 0,05$)	No			No		
F	0,4834					
P-value (significance)	0,7887					
Differences are significant ($P < 0,05$)	No					

To assess the homogeneity of mean values and variances between analysts, the test (Student's test) and the F test were used, respectively. The results of the data analysis are presented in Table 2.

Table 2. Data for means and variances homogeneity for normalized data values between analysts

Parameter	Estimation of means homogeneity	Estimation of variances homogeneity
P-value (significance)	<0,0001	0,4446
Differences are significant ($P < 0,05$)	Yes	No

Statistical processing showed homogeneity of variances between analysts and no homogeneity of mean values. The result of the analysis, which shows the heterogeneity of mean values and the homogeneity of variances between analytical cycles and analysts, allows us to select a "floating" type of exclusion limit with a correction factor – a normalization factor, the value of which was 0.86. The correction factor was determined as 1 percentile of normalized values averaged over all 6 cycles (% of viable cells). In the further analysis for each analytical cycle, the exclusion limit was determined by the formula:

$$CUTOFF = CPF \cdot \overline{RFU}_{NC},$$

where CUTOFF – floating limit of the exception for this analytical cycle; $\overline{RFU}_{NC}$ – is the average value of the RFU of negative NC control in the analytical cycle; CPF is a normalization factor.

Samples with RFU values below the calculated exclusion limit are classified as "positive".

Sensitivity of the method

To determine the sensitivity, calibration standards were used for four levels of concentrations of antibodies to adalimumab: 200, 150, 125, 80 ng/ml. Analysis of samples was carried out by two analysts in 12 analytical cycles. Each cycle included three series of dilutions, positive and negative control samples. Sensitivity was calculated by determining the point of intersection of the exclusion limit value for each analytical cycle with the linear regression curve of RFU versus logarithm of antibody concentration. The average sensitivity value was obtained by averaging over all 12 analytical cycles. The results are presented in Table 3.

It is recommended to consider 0.5–2 μg/ml[1] as the upper cut-off sensitivity values for neutralizing antibody determination methods. The resulting sensitivity value meets this criterion. For further validation, the following control concentrations were selected: HPC – 300 ng/ml, MPC – 200 ng/ml, LPC – 150 ng/ml.

Selectivity

The selectivity of the method is the ability of the method to determine neutralizing antibodies to adalimumab in blood serum in the presence of unrelated compounds (components of the matrix, serum) as a signal below the exclusion limit. Selectivity was studied in 14 individual (including hemolyzed) human blank serum samples with the addition of control antibodies to adalimumab at the LPC, MPC, and HPC levels. For each sample the signal level was assessed relative to the exclusion limit.

According to the data obtained, the values of relative fluorescence units for more than 80 % of samples containing neutralizing antibodies to adalimumab at the level of HPC, MPC and LPC concentrations are

[1] Pharmacopeia US. United States Pharmacopeia. General Chapter. <1106> Immunogenicity assays—design and validation of immunoassays to detect anti-drug antibodies. Rockville, MD: United States Pharmacopeia; 2022. Available at: https://doi.usp.org/USPNF/USPNF_M99825_02_01.html. Accessed: 01.11.2023.

Table 3. **Sensitivity calculations**

Calibration Standards, ng/ml	Run No, RFU											
	1	2	3	4	5	6	7	8	9	10	11	12
200	12350	14086	11837	14978	14048	12314	5975	5042	5196	6642	6973	5584
150	13919	16071	13965	16263	14676	14181	8926	6600	8073	8817	8257	7592
125	14211	16828	14659	16258	15195	14158	9660	8381	7807	9147	9391	8891
80	14960	18115	15543	17637	15823	14271	11388	9345	9176	10951	9461	9331
NC	16378	18378	15609	19688	17627	16687	13286	11797	12288	10820	10878	9938
Exclusion limit	14085	15805	13424	16932	15159	14351	11426	10145	10568	9305	9355	8547
Sensitivity in each cycle, ng/ml	128,26	152,76	157,40	108,19	123,87	99,71	84,10	63,79	43,55	126,88	100,71	117,18
Average sensitivity value, ng/ml									108,87			

below the exclusion limit. For HPC and MPC of 100 % of the samples (14/14) had a response rate below the exclusion limit, and for the LPC it was 92.9 % (13/14).

Precision

Functional cell tests for the determination of neutralizing activity are characterized by the complexity of the test systems used (cell cultures), differences in methodology, the presence of a large number of stages while setting up the method, semi-quantitative presentation of data, and therefore there are no generalized acceptance criteria for assessing precision, suitable for all cell tests[1].

Accordingly, it is recommended to use an approach based on the minimum significant ratio (MSR) to estimate precision[2]. The minimum significant ratio is the smallest difference between titer values that is statistically significant ($P < 0.05$). The eligibility criterion for assessing precision in this case is formulated as follows: the largest difference between the titers within the cycle and between cycles should not exceed the MSR.

To calculate the MSR, the RFU values of calibration standards with the concentration of antibodies to adalimumab of 300, 200, 150 and 125 ng/ml were used. Samples were applied in three repetitions. The RFU values for the standards of 300, 200 and 150 ng/ml were calculated, normalized for the RFU for the 125 ng/mL standard (titers). The resulting titers were converted into a logarithmic scale (log 10) and standard deviations for each cycle and between cycles. MSR was calculated using the following formula:

$$MSR = 10^{t\sqrt{2SD}},$$

where MSR – the minimum significant ratio; t – the critical value of the Student's distribution, corresponding to the 5 % significance level. For the number of degrees of freedom 11 (number of cycles minus 1); SD – the sum of standard deviations for values within a cycle and between cycles on a logarithmic scale.

The results obtained for precision are shown in Table 4.

Table 4. **Intra- and inter-day precision**

Calibration Standards, ng/ml	Largest difference between titers within a run (intra-run precision)	Largest difference between average titers for each cycle (precision between cycles)	Acceptance criteria (≤ MSR)
300	0,86	3,36	≤4,14
200	0,38	2,18	≤3,15
150	0,35	0,30	≤1,77

In accordance with the data obtained, the greatest difference between the titers did not ever exceed MSR, which corresponds to the acceptance criterion [15,17].

Specificity

Specificity characterizes the ability of the bioanalytical method to uniquely identify the analyte in the presence of related compounds (endogenous or exo-

[1] Pharmacopeia US. United States Pharmacopeia. General Chapter. <1106> Immunogenicity assays—design and validation of immunoassays to detect anti-drug antibodies. Rockville, MD: United States Pharmacopeia; 2022. Available at: https://doi.usp.org/USPNF/USPNF_M99825_02_01.html. Accessed: 01.11.2023.

[2] Right there.

genous) in the biological sample (no cross-linking). The method is based on the binding of adalimumab to neutralizing antibodies specific to this therapeutic protein. Human antibodies to bevacizumab and denosumab were selected to investigate the possibility of cross-linking. The specificity of the method was investigated using HPC and LPC samples and the addition of antibodies to bevacizumab or denosumab at the HPC level.

The results are presented in Table 5. According to the data obtained, the absolute value of the relative error of relative fluorescence units containing structurally related molecules does not exceed 25 % of RFU of control samples.

Table 5. Method specificity

Sample, №	Sample composition	RE (abs),%
1	HPC + antibevacizumab	0,81
2	HPC + antidenosumab	14,07
3	LPC + antibevacizumab	10,01
4	LPC + antidenosumab	8,29
Eligibility Criteria		≤25 %

Stability

In the stability study of neutralizing antibodies to adalimumab in the samples, short-term stability at room temperature of the original samples, stability of up to five freeze-thaw cycles, and long-term stability for 101 days at temperatures of –80 and –36 °C were established.

To study long-term stability under natural storage conditions, the samples were stored at –80 and –36 °C, and then the relative error (RE, %) RFU of control freshly prepared samples was determined for each sample. To assess the stability during freezing-thawing, samples were thawed and kept for 1 hour at room temperature, then subjected to repeated freezing at –80 °C, there were at least 12 hours between freeze-thaw cycles. To study short-term stability, the original samples were kept at room temperature for at least 6 hours. Each sample was analyzed in three repetitions. The results are given in Table 6.

CONCLUSION

A method for the determination of neutralizing antibodies to adalimumab in human serum based on a functional cell test was developed and validated. The test was performed using the TNFα sensitive L-929 cell line; Neutralizing antibodies bind to adalimumab, blocking its interaction with TNFα, resulting in TNFα-mediated cytotoxicity. Cytotoxicity has been evaluated with rezasurin. The sensitivity of the method was 108.9 ng/mL of neutralizing antibodies to adalimumab. The limit of detection was selected as "floating" with a correction factor (normalization factor) equal to 0.86. Thus, the possibility of using the method for the determination of neutralizing antibodies to adalimumab in human serum has been proved, as well as, in the study of immunogenicity during clinical trials and bioequivalence studies.

Table 6. Results of stability study in samples human serum

Sample freeze-thaw stability (F/T), RE (abs), %			
Sample	**1**	**3**	**5**
HPC_F/T	24,79	7,96	11,74
MPC_F/T	10,36	7,07	3,09
LPC_F/T	3,60	2,23	7,65
Sample bench-top stability (BTS)			
Sample	**RE (abs), %**		
HPC_BTS	12,40		
MPC_BTS	4,36		
LPC_BTS	7,74		
Sample long-term stability (LTS), RE (abs), %			
Sample	**–36 °C**		**–80 °C**
HPC_LTS	9,43		20,74
MPC_LTS	9,81		7,84
LPC_LTS	10,81		9,28
Eligibility Criteria	≤25 %		

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