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THE SHELF LIFE PREDICTING OF IMMUNOENZYME COMBINED TEST SYSTEMS FOR HIV1/2 DIAGNOSTICS

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The aim of the research was to determine the shelf life of the ELISA test kit DIA-HIVAg/Ab (PJSC SPC "Diaproph-Med") intended for the determination of antibodies to HIV1/2 and p24 HIV1 antigen using accelerated storage model at elevated temperatures. It is established that the thermal inactivation process is subject to a first-order kinetic law. The dependence of the rate constants of inactivation (lnK) on temperature (1/T) is described by the Arrhenius equation at 95% probability level (F-test). Calculated on the basis of this model, the activation energy (ΔEa) equals 23.27 kcal · mol^{-1}. It is established that the projected shelf life of the test kit was 2 years and 1 month when stored at 4 °C in terms of reduction of its diagnostic activity by 10%.

Isothermal method of accelerated storage based on the Arrhenius model can significantly save time by determining the expiration date of the test kit as early as at the stages of its development or modification. The obtained data can be used for confirmation of the diagnostic kit stability studies, in terms of long-term storage, correction recommended conditions, and for determination of test kit capability of withstanding exposure to adverse environmental factors, which may occur during transportation and storage.

Key words: accelerated storage method, the Arrhenius equation, immune enzyme analysis, HIV diagnosis, combined test kit.

HIV infection remains one of the main problems of world health. According to the data released by the Ukrainian Centre for Control of Socially Dangerous Diseases of the Ministry of Health of Ukraine, 7.612 new cases of infection were registered in six months of 2016, including 1.365 children below 14 years old [1]. One of the main ways to limit the disease's spread is its early diagnostics, and its efficiency is to large extent determined by the testing strategy, methods adequacy and quality of diagnostic techniques.

To identify HIV infection at an early stage, the usual choice is immune enzyme combined test-systems to simultaneously identify summary antibodies to HIV and the viral antigen p24 (viral diagnosticum of the 4th generation). High analytic sensitivity of these tests allows one to find HIV not only at early stages of the infection process, but also on every stage of the disease, and so to apply antiviral therapy in a timely manner and lower the risk of the infection's spread [2].

An important aspect of development and production of combined test systems is the stability and verification of its shelf life, which influences its functional characteristics and therefore has an impact upon the results of the patients' testing. Research of diagnostics test-systems' stability allows to obtain data about the changes in their quality with time under the influence of environmental factors (extreme temperature, humidity, illumination or vibration).

When determining the shelf life of test systems, various methods are applied: stress-testing, study of stability under accelerated and long-term storage in real time, etc. [3]. The results are used to work out recommendations on storage conditions and shelf life of a

diagnosticum. The method of accelerated determination of the test system's stability under increased temperature lets one to significantly save time establishing its shelf life during development and modification [4].

The method of accelerated storage ("accelerated aging") of a diagnosticum under increased temperature is based on applications of the laws of chemical kinetics to the processes of inactivation of diagnostic preparations. It was established that an increase in temperature speeds up the rates of chemical reactions. According to the van't Hoff equation, a 10 °C increase leads to two- to four-folds increase in chemical reactions' rates [5]. The parameter under study is the kinetics of the decomposition reaction under the high temperature. The method allows to determine the time during which the test system retains its functional characteristics at a certain temperature, within a short period of time. The data are then used to establish the optimal conditions of storage for the diagnosticum and its shelf life under certain temperature.

The isothermic method of accelerated "aging" of a test system, based on Arrhenius equation, establishes the dependency of the constant of the reaction speed on the temperature for reactions of different types, which lets one to project the shelf life of a diagnosticum under any temperature [6].

The PJSC SPC "Diaproph-Med" (Ukraine) developed the immune enzyme combined test system DIA-HIVAg/Ab that has increased sensitivity to HIV1/2 identification. Its diagnostic characteristics (sensitivity and specificity) allow us to use it to identify HIV both at early stage and during further stages of the pathological process.

The work's goal was to prognose shelf life of the developed test system at 4 °C using a model with accelerated storage at increased temperature using the Arrhenius equation.

Materials and Methods

Test system

We used the diagnostic immune enzyme test system DIA-HIV-Ag/Ab of three series (063-15, 066-15, 072-16) made by PJSC SPC "Diaproph-Med". The diagnosticum is constructed as a two-step double 'sandwich' from monoclonal antibodies to p24 HIV1 that finds the viral antigen, and a mixture of synthetic and recombinant polypeptides that determine summary antibodies to HIV1/2.

We conducted the immune enzyme analysis (IEA) following the instruction provided for the test system. The results of the analysis of the serum panel in the test kit was expressed as the ratio of mean ОП/cut off.

Human blood serum sampling

Standard industrial human blood serum panel (PJSC SPC "Diaproph-Med"), containing 13 diluted samples from HIV-infected, of which 11 have antibodies to HIV1 and 2 — to HIV2. All serum panels were confirmed in the combined immune enzyme test system Genscreen ULTRA HIV Ag-Ab («BioRad»).

Standard of the antigen p24 HIV1 ABI (USA) was diluted with healthy blood serum to 10 pg/ml, 5 pg/ml and 2.5 pg/ml.

Shelf life storage research

The shelf life of test system DIA-HIVAg/Ab was studied at 3 industrial series, each of which was simultaneously placed into dry-air sterilizers ТС-1/80 СПУ under different temperatures: (30.0±0.2) °C, (37.0±0.2) °C, (42.0±0.2) °C, (47.0±0.2) °C and (54.0±0.2) °C. For comparison, we used similar test kits stored at (4.0±0.2) °C. The periodicity of the control of the test systems' diagnostic abilities depended on the temperature regime of the storage and the observed results. For every temperature value, we counted the mean residual activity for 3 series of the test system.

Mathematical modeling of the experimental curves of thermal inactivation

The kinetics curves of the dependency of residual activity of the diagnosticum on storage time at every temperature were described using the equation of exponential function:

$$C = C_0 \cdot e^{-kt} \quad (1),$$

where C_0 — initial activity at the beginning ($t = 0$);

C — residual activity of the diagnosticum after storage, at time t;

t — time of storage (days);

k — constant of inactivation rate of the diagnosticum (days^{-1});

e — natural logarithm base, $e = 2{,}71834$.

The dependency of the constant of inactivation rates for the diagnosticum on temperature was established using the Arrhenius equation:

$$K = A \cdot e^{-E\alpha/RT} \quad (2),$$

where K — constant of inactivation rate for diagnosticum (days^{-1});

$E\alpha$ — activation energy, kcal×mol^{-1}, which is the minimal energy the molecules should have that their collision could lead to a chemical reaction;

R — universal gaseous constant, R = 0.001987 kcal·mol^{-1}·K^{-1};

A — empirical constant (days^{-1});

T— absolute temperature (K).

The data were statistically treated using common software [7]. The adequacy of Arrhenius equation was evaluated by analysis of variance using Fisher's criterion *(F-test)*. To compute the kinetic parameters of linear regression we used the "Microsoft Excel" program (Linear function).

Results and Discussion

The determination of the shelf life of the DIA-HIVAg/Ab test system at 4 °C using the accelerated storage model included:

– analyzing the kinetics of the experimental curves of the diagnosticum inactivation at different temperatures to determine the order law of the equations and to calculate the rate constants for them;

– plotting the dependency of the test system's inactivation rate constant on the temperature in the coordinates of the Arrhenius equation and testing its adequacy by statistical methods;

– calculating the activation energy and projecting the test system's shelf life at 4 °C based on the Arrhenius equation.

According to the "accelerated aging" method, the test system under investigation is kept at temperatures higher than its usual storage temperature. At the heightened temperatures the physical and chemical processes are accelerated, leading to a shortened time period during which the pertinent parameters of the diagnosticum's quality remain within acceptable limits. This allows to significantly decrease time necessary to establish the shelf life of the product while it is still being developed or modified. The obtained data are then used to confirm results of the test system's stability under long-term storage. The results also allow to see how capable is the test system of withstanding short-term unfavourable conditions which might occur during its transport and storage.

According to the laws of kinetics, the rate of a chemical reaction is determined by the change in the reagents' concentrations per unit of time and is expressed by the equation:

$$V = -dC/dt = k \cdot C_t,$$

where dC/dt is the change of the initial substance in time;

k is rate constant;

C_t is the quantity of the decomposed substance over time t.

The "minus" sign shows the decrease in the quantity of the reagent.

Integrating the equation we obtain the following formula:

$$lnC = lnC_0 - kt \quad (3),$$

where lnC — natural logarithm of the concentration;

C_0 — initial concentration at time $t = 0$.

Meanwhile, remaining quantity of the substance that remains active will be equal $C = C_0 - C_t$. If the kinetics of the reaction is a firt-order law, then the plot of the dependency of lnC on time t should be a straight line, and the tangent of its inclination angle would be $-k$. The length of the fragment of the ordinate axis cut at $t = 0$ will be lnC_0. Thus, the rate constant of a first-order reaction k is calculated by the equation:

$$k = \frac{1}{t} \ln \frac{C_o}{C_o - C_t} \quad (4).$$

Therefore, to determine the order of the reaction in our reaction in our experiments it was necessary to determine the change in diagnostic activity of the test system at certain points of time at certain temperature and to plot the dependency of $ln\ C/C_0$ on t. The unchanging value of the constant k supports the correspondence of the thermal inactivation kinetics to a first-order equation.

Fig. 1 shows the plots of the dependencies of the residual activity of DIA-HIVAg/Ab test system on storage time in the range of temperatures from 30 °C to 54 °C.

Based on the semi-log anamorphoses of kinetic curves (Fig. 2) we calculated the parameters of the linear regression and developed the equations for the trend lines for the corresponding temperature. For the calculations we used the method of least squares in the "Microsoft Excel" program. The inactivation rate constant is graphically represented as the tangent of the inclination angle of the kinetic line.

Table 1 contains theoretical values of the inactivation rate at different storage temperatures of test system DIA-HIV-Ag/Ab.

It follows from the obtained data that the thermal inactivation process follows a kinetic law of the first order (the coefficients of determination R^2 are in the range of 0.95 to 0.99), which allows to use the theoretically derived rate constant for further calculations in the coordinates of the Arrhenius equation.

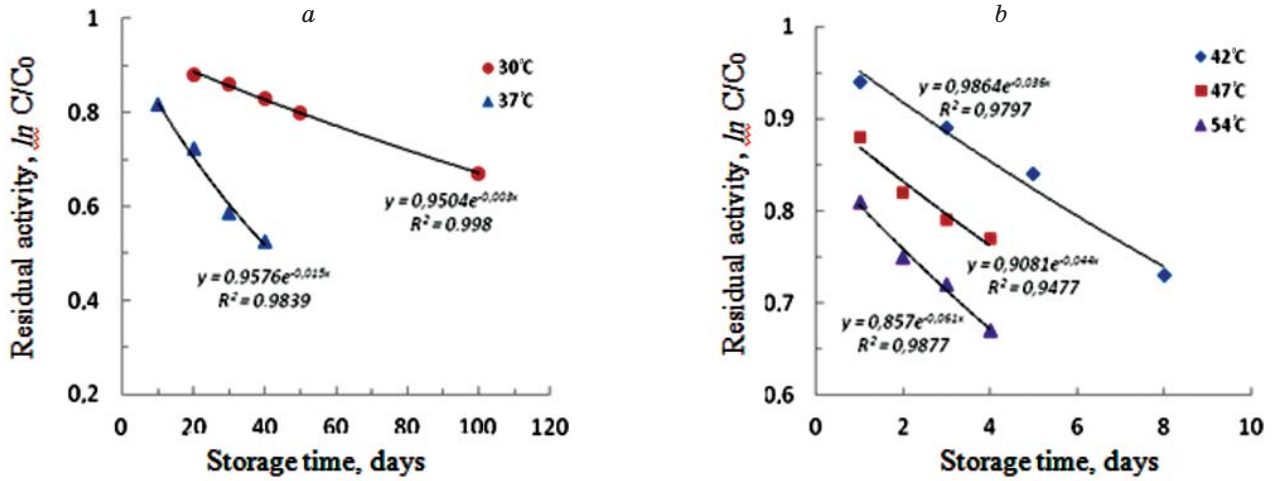

Fig. 1. **Kinetic curves o the dependency of the residual activity of the DIA-HIV-Ag/Ab test system on the storage time at different temperatures:**
a: 30 °C and 37 °C, *b*: 42 °C, 47 °C and 54 °C

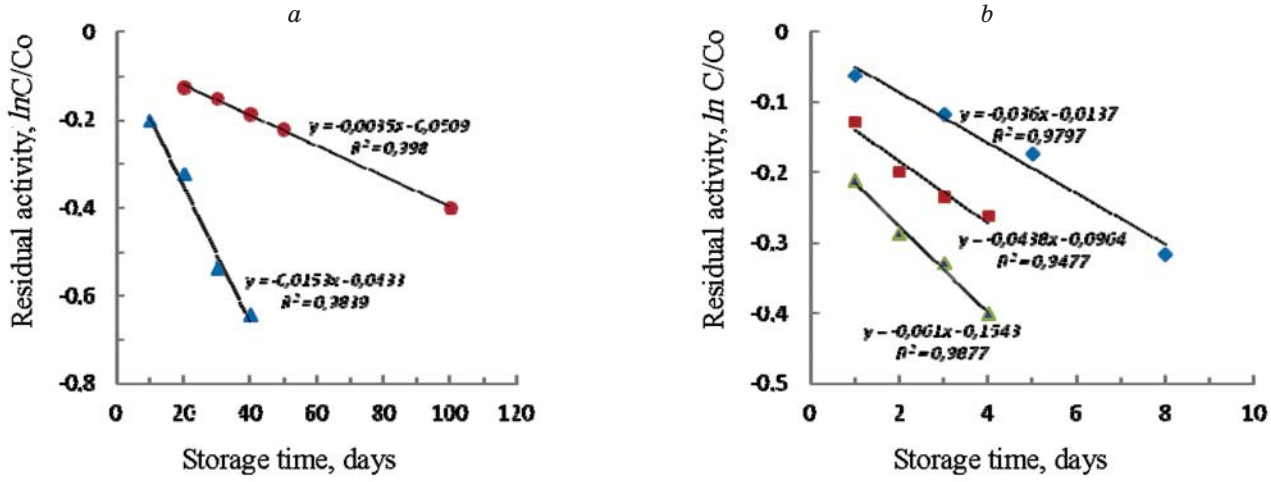

Fig. 2. **Linear anamorphoses of kinetic curves o the dependency of the residual activity of the test system DIA-HIVAg/Ab on storage time at different temperatures:**
a: 30 °C and 37 °C, *b*: 42 °C, 47 °C and 54 °C

Table 1. **Comparative evaluation o the inactivation rate constants for the test system DIA-HIV-Ag/Ab depending on storage temperature**

Storage temperature, t	Inactivation rate constant k, (day^{-1}) $M \pm m$	ln$K$	Determination coefficient, R^2
30 °C	$(3.47 \pm 0.09) \cdot 10^{-3}$	−5.6619	0.998
37 °C	$(15.35 \pm 1.39) \cdot 10^{-3}$	−4.1767	0.984
42 °C	$(36.04 \pm 3.66) \cdot 10^{-3}$	−3.3230	0.977
47 °C	$(43.79 \pm 7.28) \cdot 10^{-3}$	−3.1284	0.948
54 °C	$(61.01 \pm 4.82) \cdot 10^{-3}$	−2.7967	0.988

In the logarithmic form, the Arrhenius equation (equation 2) attains the following form:

$$\ln K = \ln A - Ea/RT \qquad (5),$$

where A is the pre-exponential factor, determined by the fragment of a line cut by the straight line on the ordinate axis at $1/T = 0$.

According to this formula, the logarithm of rate constant depends linearly on the inverse temperature.

The obtained values of the test system's inactivation rates at five different temperatures allowed us to build a graph of dependency $Y = ln$k on $X = 1/T$ (Fig. 3).

To confirm the linear dependency between the X and Y variables we used the least squares method, to determine the parameters of the linear regression, using for these calculations the Linear function in "Microsoft Excel" program. At linear regression, the sum of the squares of random deviations from the variable that depends on the arithmetic mean, is composed by the sum of squares for the regression and the random values. Dividing the obtained sums of squares on the corresponding degrees of freedom, we obtained two dispersions — one for regression and one for the random errors. Fisher's criterion *(Ffact.)* is the ratio of the former to the latter. The actual value of Fisher's criterion was compared to the table at the chosen significance level for two degrees of freedom f1 = m and f2 = (n − m −1), where n is the number of observations, and m is the number of parameters for the variable X. If *Ffact*. exceeded the critical value provided in the table, we concluded that the equation was on the whole statistically significant, which allows to postulate the linearity of the anamorphose and so the adequacy of the approximation model.

From the data provided in Table 2, the significance of the obtained results corresponds with the 95% level of true prognoses. Therefore, the variable lnK linearly depends on the variable $1/T$ (Fig. 3) and the Arrhenius kinetic model adequately describes the dependency of the inactivation process for the DIA-HIV-Ag/Ab test system on temperature.

Having found the tangent of the inclination angle for the straight line in the coordinates of the Arrhenius equation (formula 4), one can calculate the activation energy according to the formula:

$$\mathrm{tg}\,\alpha = \frac{-Ea}{R} \qquad (6).$$

Activation energy (Ea) is an important quantitative parameter of a substance's reactivity. According to the collision model, the chemical reaction between two initial substances can occur only as a result of their molecules colliding [8, 9]. However, not any collision leads to a chemical reaction. First, a certain energy barrier must be overcome so that the molecules could start reacting with each other. E.g., these molecules must have a certain minimal energy (activation energy) to overcome the barrier. According to our data, activation energy for this reaction is 23.27 kcal/mol, which agrees with results [8] for temperature inactivation of a number of medicinal preparation (12 to 24 kcal/mol).

Using the formula for linear regression for Arrhenius equation

$$y = -11713\,x + 33.393,$$

we calculated the kinetic parameters of the DIA-HIVAg/Ab test system stability required to project its shelf life if stored at 4 °C. The time at which the test system's diagnostic

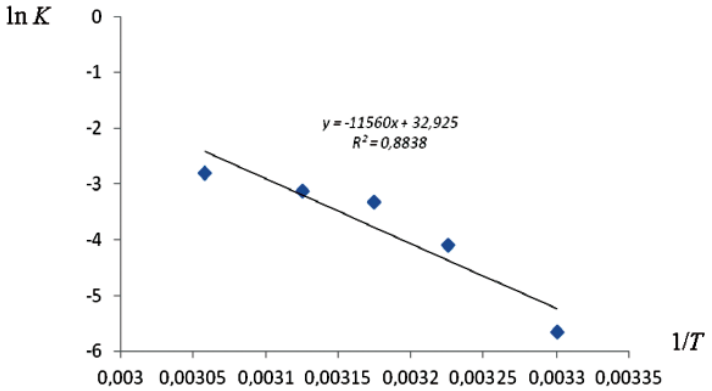

Fig. 3. **A semi-log plot of the dependency of the DIA-HIVAg/Ab diagnosticum's inactivation constant on the temperature in the Arrhenius equation coordinates**

Table 2. **Kinetic parameters of the thermal inactivation process for the DIA-HIV-Ag/Ab test system to project its shelf life at 4 °C**

Temperature		$1/T \times 10^{-3}$	ln*K*	*K*	Time of the diagnostical test system remaining active, days	*F*-test		Activation energy (Δ*Ea*), kcal/mol)
							*F*05	
t, °C	*T*, °K					$F_{fact.}$	*F*01	
4	277	3.61	−8.8907	0.000138	765	24.99	10.13	23.27
							34.12	

ability decreases by 10% – the normative parameter for immunobiological preparations [10], was calculated by the following formula:

$$\tau = \frac{1}{K_4} \cdot \ln \frac{Co}{C} = \frac{1}{K_4} \cdot \ln \frac{1}{0.90} \qquad (7).$$

According to the parameters of the diagnosticum's thermal inactivation at 4 °C (Table 2), the projected time of storage for the test system before its activity decreases 10% is 765 days (or 2 years and 1 month).

Therefore, despite the complex chemical composition of the diagnosticum's reagents, the thermal inactivation process follows a kinetic equation of the first order. The dependency of the inactivation rates on temperature is described by Arrhenius equation, allowing us to use this kinetic model to project the diagnosticum's shelf life at any set storage temperature. Such artificial modeling of the diagnosticum's storage can be used as a rationale to prolong initial shelf life of the DIA-HIVAg/Ab immune enzyme system that was found by the developer team, and to correct the storage conditions. Besides that, the model allows to determine the impact of the unfavorable factors of the environment that may occur during its transport and storage.

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ПРОГНОЗУВАННЯ ТЕРМІНУ ПРИДАТНОСТІ ІМУНОЕНЗИМНОЇ КОМБІНОВАНОЇ ТЕСТ-СИСТЕМИ ДЛЯ ДІАГНОСТИКИ ВІЛ1/2

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Метою роботи було визначення терміну придатності імуноензимної тест-системи DIA-HIVAg/Ab (ПрАТ НВК«Діапроф-Мед»), що призначена для виявлення сумарних антитіл до ВІЛ1/2 та антигену р24 ВІЛ1, методом прискореного зберігання за підвищених температур з використанням рівняння Арреніуса.

Встановлено, що процес термоінактивації підпорядковується кінетичному закону першого порядку. При цьому залежність констант швидкостей інактивації (lnK) від температури ($1/T$) описується рівнянням Арреніуса на 95%-му рівні ймовірності (*F-тест*). Розрахована на основі цієї моделі енергія активації (ΔEa) дорівнює 23,27 ккал·моль$^{-1}$. Прогнозований термін придатності тест-системи з урахуванням зниження її діагностичної активності на 10% становить 2 роки 1 міс за температури зберігання 4 °С.

Ізотермічний метод прискореного зберігання на основі моделі Арреніуса дає змогу значно заощадити час для встановлення терміну придатності тест-системи на етапах її розроблення або модифікації. Отримані дані надалі можуть бути використані для підтвердження результатів досліджень стабільності діагностикуму в умовах довгострокового зберігання, корекції рекомендованих умов, а також допоможуть визначити, наскільки тест-система здатна витримувати вплив несприятливих факторів довкілля, які можуть виникнути під час її транспортування та зберігання.

Ключові слова: метод прискореного зберігання, рівняння Арреніуса, імуноензимний аналіз, діагностика ВІЛ, комбінована тест-система.

ПРОГНОЗИРОВАНИЕ СРОКА ГОДНОСТИ ИММУНОЭНЗИМНОЙ КОМБИНИРОВАННОЙ ТЕСТ-СИСТЕМЫ ДЛЯ ДИАГНОСТИКИ ВИЧ1/2

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Целью работы было определение срока годности иммуноэнзимной тест-системы DIA-HIVAg/Ab (ЧАО НПК «Диапроф-Мед»), предназначенной для выявления суммарных антител к ВИЧ1/2 и антигена р24 ВИЧ1, методом ускоренного хранения при повышенных температурах с использованием уравнения Аррениуса.

Установлено, что процесс термоинактивации подчиняется кинетическому закону первого порядка. При этом зависимость констант скоростей инактивации (lnK) от температуры ($1/T$) описывается уравнением Аррениуса на 95%-м уровне вероятности (*F-тест*). Рассчитанная на основе этой модели энергия активации (ΔEa) равна 23,27 ккал·моль$^{-1}$. Прогнозируемый срок стабильности тест-системы с учетом снижения ее диагностической активности на 10% составляет 2 года 1 мес при температуре хранения 4 °С.

Изотермический метод ускоренного хранения на основе модели Аррениуса позволяет значительно сэкономить время установления срока годности тест-системы на этапах ее разработки либо модификации. Полученные данные в дальнейшем могут быть использованы для подтверждения результатов исследований стабильности диагностикума в условиях долгосрочного хранения, коррекции рекомендованных условий, а также дадут возможность определить, насколько тест-система способна выдерживать воздействия неблагоприятных факторов окружающей среды, которые могут возникнуть при ее транспортировке и хранении.

Ключевые слова: метод ускоренного хранения, уравнение Аррениуса, иммуноэнзимный анализ, диагностика ВИЧ, комбинированная тест-система.