SLOW QUASIKINETIC CHANGES IN WATER-LACTOSE COMPLEXES DURING STORAGE

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ABSTRACT

Objective: To investigate kinetic changes in the spectral characteristics by Fourier Transform Infrared spectroscopy (FTIR) of water-lactose complexes (SMC), derived during the manufacturing process of the drug, containing release-active forms of antibodies.

Methods: Lactose monohydrate substance, saturated with release-active forms of affinity-purified polyclonal rabbit antibodies to recombinant human interferon-gamma (RA forms of Abs); tablets produced from this substance by direct compression after the addition of excipients (microcrystalline cellulose, magnesium stearate). Powdered and tableted placebo samples saturated with technologically processed water or phosphate-buffered saline, as well as with intact ethanol were used as control. Kinetic changes in SMC were studied using an Agilent Cary 630 FTIR spectrophotometer with a diamond ATR accessory (Agilent Technologies, USA). We used the method of X-ray fluorescence spectroscopy (EDX-7000 Shimadzu energy dispersive X-ray fluorescence spectrometer) to track changes in the fluorescence signal at certain wavelengths. The range of measured elements were Na⁺–U²⁺.

Results: Control of some technological characteristics of the obtained active substance (moisture, flowability) and dosage form (mean mass, disintegration rate) was used as indirect indicators of quality, but they did not allow reliably distinguishing intact lactose from the saturated one. Long-period oscillations on FTIR spectra were characteristic for all types of samples; oscillations occur at approximately two-week intervals; S/N indices were more stable for samples of RA forms of Abs than for placebo samples. On some days, the substance saturated with RA forms of Abs significantly differed from the intact lactose powder. The kinetics of the X-ray fluorescence intensity at certain wavelengths indicates the possibility of a periodic cooperative trigger transition of the system. Reversible conformational transitions are observed for powders on the 30th and 130th days (Kα 3.313 keV). For tablets at Kα 3.313 keV and Kα 1.740 keV small changes were visualized on those days (100–110th day) when hysteresis phenomena were recorded in the IR spectra of these samples.

Conclusion: As a result, the evidence for a long-period dramatic conformational mobility of the water-lactose complex was obtained. Based on the data on the semianual kinetics of IR spectra, a universal criterion for the identity of lactose powder saturated with RA forms of Abs was obtained. Also, it was confirmed that the lactose conformation state was changed by saturation with RA forms of Abs.

Keywords: Release-active forms of antibodies, Slow kinetic transitions, X-ray fluorescence spectroscopy, FTIR, Lactose monohydrate, Supramolecular complexes

INTRODUCTION

For more than ten years among the antiviral drugs duly registered in the State Register of the Russian Federation, there have been several drugs of the so-called release-active forms of antibodies. Anferon for Children (reg. number LP-N (000035)-(RG-RU)) is widely used in pediatric practice for the successful treatment of various infectious diseases not only in Russia but also in many countries. The effectiveness has been proven in preclinical and clinical studies and noted in many publications [1-3].

Our previous works were devoted to the study of the influence of sample preparation conditions (grinding, saturation with a solvent, etc.) on the physicochemical characteristics of lactose monohydrate powder, which is used as a carrier of the active substance in these drugs [4]. The presence of critical differences in the differential IR spectra, in the spectra within the terahertz region between the sample of intact lactose substance and the powder, which underwent the fluidization procedure with the introduction of the active substance in an aerosol chamber, was shown [5]. We substantiated the results obtained by the possibility of the formation of supramolecular structures of water-lactose complexes in the amorphous and quasicrystalline state (fig. 1).

In addition to the special spectral characteristics that present the specific organization, the supramolecular water-lactose complex (SMC) also demonstrates the biological activity relative to the unicellular model of S. ambiguus [6]. It is noteworthy that all of the above properties disappear upon drying of powdered preparations, which directly indicates the role of water in the formation of release-active SMCs.

Fig. 1: The formation of a water-lactose complex (SMC) and reversible transitions between the conformational states of the amorphous RA forms of Abs system (L–Lactose, W–H₂O, L–W⁺–RA forms of Abs)
The clinical efficacy of drugs based on RA forms of Abs can be interpreted as a test of their biological activity at the population level [3]. Since the concentration of Abs proper after potentiation is zero, therefore, it is necessary to detect the formation of a specific supramolecular water-lactose complex during physicochemical standardization and quality control.

In preliminary experiments, we showed that SMCs undergo slow conformational transformations with monthly kinetics. The observed kinetic transitions suggest the existence of a free-energy barrier between the states of the supramolecular structure. The process can be compared with the kinetic trapping phenomenon, well studied, and described for protein molecules, associated with conformational differences between the long-lived transitional and stable final states [7,8].

This paper presents the results of a study of the multi-month kinetics of RA forms of Abs: considering the possible oscillations of the supramolecular structure, we have registered characteristic changes in the FTIR spectra. Bearing in mind that the energy-saturated supramolecular complexes must undergo hysteresis and trigger conformational transitions (fig. 1), we also used trace elements as a kind of sensors for changes in the dielectric constant inside the SMCs. By analogy with the use of RCS-tags in the study of conformational transitions of high molecular systems [9], we used the method of X-ray fluorescence spectroscopy [10] to track changes in the fluorescence signal at certain wavelengths.

**MATERIALS AND METHODS**

**Samples under investigation**

All samples for the research were obtained directly from the manufacturer (Matra Medica Holding, Russia)-a lactose powder, as well as the experimental tablets. The lactose powder we used presented the powder of lactose monohydrate \( C_{12}H_{22}O_{11} \cdot H_2O \) (Super Tab 30G1, DFL Pharma, Germany), which was saturated with release-active forms of affinity-purified polyclonal rabbit antibodies to recombinant human interferon-gamma using the technology of applying substances in a fluidized bed [4]. Flat-cylindrical tablets were produced from this substance by direct compression after the addition of excipients (microcrystalline cellulose, magnesium stearate) and were not commercially available drugs. RA forms of Abs was produced by the technology of ultra-high dilutions as described in [4] and in the US patent 5 356 644 with the initial substance dilution factor \( (10^{10}) \).

A control, we used powdered and tableted placebo samples obtained by the same method, from the same series of lactose and excipients, but only saturated during fluidization with technologically processed (i.e. underwent the same technological dilution process as the antibodies) water (saturation control), technologically processed phosphate-buffered saline (control of antibody solvent) and intact ethanol (process control). Thus, a total of 5 types of powdered and tableted samples was used in the work—intact substance of lactose monohydrate, lactose monohydrate saturated with RA forms of Abs, and 3 types of control.

The samples received from the manufacturer were stored for the entire observation period at a temperature not exceeding 25 °C, away from the source of natural and artificial lighting and heating devices, in undisturbed packaging. The powders for the study were packaged in 0.5 ml eppendorf; each eppendorf was sealed in paraffin film. After opening the package and carrying out measurements, the powder was disposed of; for re-analysis, each time a new packed eppendorf with the substance was taken. The tablets were packaged in polymeric jars of 20 ml (with tamper-evident caps), each tablet was measured once, after that the sample was disposed of and the jar was tightly sealed with paraffin film.

**Determination of technological characteristics of samples**

The flowbility measurements were carried out under the requirements of the European Pharmacopoeia [11]: test powder mass was 100 g; the flowability was measured in five repetitions using PTG S4 powder and granule flow tester (Pharma Test Apparatebau AG (Germany)).

The residual moisture content was determined in lactose monohydrate powders using an Ohaus MB45 moisture content analyzer (OHAUS Corporation, Switzerland); analyzing mass 5 g, heating 80 °C, heating duration 15 min.

The mean mass was calculated as a result of the weighing of 10 tablets using Ohaus Scout Pro SPUI23 laboratory balance (Ohaus Instruments (Shanghai) Co., Ltd., China).

**Determination of tablets disintegration time**

Disintegration time was carried out on a disintegration tester PTZ AUTO 2 (Pharma Test Apparatebau AG, Germany). The test was carried out following with the requirements of the European Pharmacopoeia “Disintegration of tablets and capsules” [11]. The device for determining disintegration consists of two collecting baskets, two glass vessels for liquid with a capacity of 1 l a thermostatic device that maintains the temperature of the liquid within \( 37\pm 2 \) °C, and two electromechanical devices that impart reciprocating movement to the baskets at a distance of at least less than 50 and not more than 60 mm in the vertical plane at a frequency of 28–32 cycles per minute. Disintegration time measurement was performed in six replicates.

**Fourier-transform IR spectroscopy**

Kinetic changes in SMC of release-active drugs were studied using an Agilent Cary 630 FTIR spectrophotometer with a diamond ATR accessory (Agilent Technologies, USA). The spectral range is 4000–750 cm\(^{-1}\). The resolution is less than 2 cm\(^{-1}\); the correctness of the wavenumber is 0.05 cm\(^{-1}\); the reproducibility of the wavenumber is 0.005 cm\(^{-1}\). The thickness of the absorbing layer is 0.5 mm (the clamping device guarantees the setting of optimal and reproducible pressure). Before placing the attachment on the crystal, all powdered samples were controlled by weight (average weight 5.5 mg). The tablets were also fixed with the clamping device, ensuring their position with the smooth side to the crystal. The spectra of the samples were recorded weekly for 6 mo.

The standard Agilent MicroLab Expert software was used to control the device, measure the data, and evaluate the quality of the obtained spectra; in this case, the FTIR spectra visualized in the wave number in the coordinates, cm\(^{-1}\)-reflection, %. All subsequent mathematical transformations of the spectral data array were performed using the OriginPro 2015 software (OriginLab, USA).

For samples containing only intact lactose, the average spectrum was calculated based on the results of measurements of 6 samples. The spectral data of all saturated lactose samples were further transformed as follows: the corresponding “background”—the spectrum of intact lactose averaged over the results of 6 measurements—was subtracted from the spectrum of each sample (for example, the spectrum of sample 1p averaged over 6 measurements was subtracted from the spectrum of the first specimen of sample 2p). The obtained results—difference spectra—for each sample were averaged over the number of replicates \( (n = 6) \) and the standard deviation was calculated. The results of data analysis presented the characteristic changes in the coordinates, cm\(^{-1}\)—the ratio of the average difference spectrum to the standard deviation spectrum (signal-to-noise ratio S/N).

Such S/N spectra were recorded once a week during the entire study period for all 10 samples divided into groups—powders and tablets. On certain days of the study, significant (S/N>2) differences were visualized between the RA forms of Abs and placebo samples. To estimate the frequency of SMC oscillations in powders and tablets for the entire observation period, kinetic curves of the weekly change of the S/N value were plotted at points 1700 (oscillation frequencies of the O-H bond).

**X-ray fluorescence spectroscopy**

An EDX-7000 Shimadzu energy dispersive X-ray fluorescence spectrometer was used in the study. The range of measured elements-\(^{11}Na-^{92}Zr\); X-ray generator–a tube with an Rh-anode, air-cooled; voltage 4–50 kV, current 1–1000 μA; irradiated area–a circle of 10 mm in diameter; silicon drift detector (SDD), counting method–a digital counting filter; the content of elements according to the value of intensity; automatic change of filters emitting the wavelengths of the corresponding elements; chamber size 300 mm x 275 mm x 100 mm.
The kinetics of the signal intensity obtained at certain wavelengths of each of the 10 samples submitted for analysis was investigated once a week throughout the entire study period (6 mo).

The initial sample of tablets or powder did not require special sample preparation.

The content of a microtube (eppendorf) or a tablet was placed in a closed-type cuvette and covered with mylar (polyethylene terephthalate) film. The irradiated area was controlled by a collimator and was 10 mm for the powder and 5 mm for the tablet.

The investigated wavelengths were K Ka 3.313 keV (3.7424 Å); Si Ka 1.740 keV (7.1255 Å). The measurement time was 50 seconds at each wavelength.

The results obtained using the XRF method are presented in values of irradiation intensity expressed in cps/µA (cps is the line intensity in standard units (counting per second), µA is the current intensity).

The results obtained using the XRF method are presented in values of irradiation intensity expressed in cps/µA (cps is the line intensity in standard units (counting per second), µA is the current intensity).

For each of 10 samples, 6 spectra were recorded weekly. The kinetic curves of changes in the signal intensity at a certain wavelength were plotted based on averaged values.

RESULTS

In the production of release-active drugs, the stage of saturation of the lactose monohydrate substance with RA forms of Abs is critical [12]. Control of some technological characteristics of the obtained active substance—moisture and flowability—are used by the manufacturer as indirect indicators of its quality, but they do not allow reliably distinguishing intact lactose from the saturated one. A similar problem concerning the finished dosage form—tablets—seems to be unsolvable, taking into account the presence of excipients. The technological characteristics of the samples under investigation were determined directly by the manufacturer and are presented for powders—in table 1, for tablets—in table 2.

### Table 1: Technological characteristics of powdered samples

| Sample | Composition                                                                 | Loss on drying, % | Flowability±SD*, s per 100 g |
|--------|-----------------------------------------------------------------------------|-------------------|-----------------------------|
| 1p     | Lactose monohydrate                                                         | 0.20              | 17.1±0.7                    |
| 2p     | Lactose monohydrate saturated with RA forms of Abs                          | 0.18              | 15.7±1.0                    |
| 3p     | Lactose monohydrate saturated with technologically processed phosphate-buffered saline | 0.16              | 17.6±2.4                    |
| 4p     | Lactose monohydrate saturated with technologically processed water          | 0.20              | 16.5±3.6                    |
| 5p     | Lactose monohydrate saturated with ethanol                                  | 0.16              | 12.9±0.7                    |

'n = 5, SD-standard deviation

### Table 2: Technological characteristics of tableted samples

| Sample | Composition                                                                 | Mean mass±SD*, mg | Disintegration±SD*, s |
|--------|-----------------------------------------------------------------------------|-------------------|-----------------------|
| 1t     | Lactose monohydrate                                                         | 296.1±3.3         | 246.7±44.7            |
| 2t     | Lactose monohydrate saturated with RA forms of Abs, microcrystalline cellulose, magnesium stearate | 299.8±4.0         | 286.7±55.0            |
| 3t     | Lactose monohydrate saturated with technologically processed phosphate-buffered saline, microcrystalline cellulose, magnesium stearate | 302.9±3.2         | 366.2±68.5            |
| 4t     | Lactose monohydrate saturated with technologically processed water, microcrystalline cellulose, magnesium stearate | 301.5±1.6         | 339.5±62.6            |
| 5t     | Lactose monohydrate saturated with ethanol, microcrystalline cellulose, magnesium stearate | 301±2.2           | 270.5±60.3            |

'n = 10, SD-standard deviation, "n = 6, SD-standard deviation

However, we showed in previous works that differential IR spectroscopy could be a useful tool in assessing the degree of lactose fluidization [5]. Considering that chemically intact and saturated lactose are the same substance, their IR spectra do not differ in the frequency arrangement of absorption ranges, however, the formation of water-lactose SMCs should be presented in the values of their intensity. If we consider the supramolecular structure of the water-lactose complex as a quasicrystalline system with excess bond energy, then its conformational changes can be predicted, and the signal in the IR spectra should also change with time.

Results of the study of long-period kinetics of water-lactose SMC by IR spectroscopy

For 6 mo, once a week, we recorded and processed the FTIR spectra of powdered and tableted lactose samples in the infrared range according to the method described above. The differential spectra of the samples presented graphically in the "wave number-S/N" coordinates did not coincide with each other from week to week (fig. 2), which confirms the assumption about the conformational variability of SMCs.

![Fig. 2: Examples of S/N spectra on each day of measurements: A—powders on day 82 from the date of production, B—powders on day 131 from the date of production (red—sample 2p, green—3p, dark gray—4p, gray—5p), n=6](image-url)
According to generally adopted analytical approaches, the presence of an instrumental response can be said when the signal-to-noise ratio is from 3:1 to 2:1. Thus, we see that on some days of measurements (fig. 2B), the substance saturated with RA forms of Abs, according to the data of spectral analysis, significantly differed from the intact lactose powder. Similar results were obtained for tablets, but the number of measurements, when it was possible to reveal spectral differences, turned out to be significantly lower in comparison with powders.

Fig. 3 shows graphs presenting changes in time of the averaged spectrum of intact lactose at the frequency of 3250 cm\(^{-1}\). It can be seen that the reflection value for intact lactose remains stable throughout the entire study period. It can be assumed that the variations in the signal-to-noise (S/N) spectra (fig. 2) are not caused by an error of the device, by the dispersity or other characteristics of the intact lactose powder, but by the long-period conformational mobility of the water-lactose complex of RA forms of Abs and placebo samples.

Fig. 3: Signal stability control for intact lactose–kinetics of reflection signal values at 3250 cm\(^{-1}\); (A–powders, B–tablets), n=6

Below are some of the obtained kinetic curves of changes in the S/N value for the samples under investigation at points 1700, 3250, and 3450 cm\(^{-1}\), the results of the kinetic study of powders in fig. 4, tablets–in fig. 5.

Fig. 4: Kinetics of S/N values for powders at 1700 cm\(^{-1}\) (A) and 3450 cm\(^{-1}\) (B), red–sample 2p, green–3p, dark gray–4p, gray–5p, n=6. The arrows indicate the time moments when the SMCs of RA forms of Abs exhibit oscillations in antiphase relative to the controls

Fig. 5: Kinetics of S/N values for tablets at 1700 cm\(^{-1}\) (A) and 3250 cm\(^{-1}\) (B), red–sample 2t, green–3t, dark gray–4t, gray–5t, n=6. The arrows indicate the time moments when the SMCs of RA forms of Abs exhibit oscillations in the antiphase relative to the controls
A detailed examination of the dependences obtained (fig. 4, fig. 5) allows drawing some general conclusions: for example, long-period oscillations are characteristic for all types of samples—powders of the substance and the finished dosage form, regardless of the type of saturating solution; oscillations occur at approximately two-week intervals; S/N indices are more stable (have a lower average amplitude) for samples of RA forms of Abs than for placebo samples. For powders, the 30th and 130th days are critical—oscillations in the SMCs of RA forms of Abs are in antiphase relative to other controls, which allows registering the fact of saturation of the lactose powder (fig. 4). According to the results of kinetic studies (fig. 5), on the 110th day, there are observed differences between the tableted RA forms of Abs and placebo samples, which correlates with the results obtained by the XRF method.

Having paid attention to the difference in the oscillation amplitudes of the samples saturated with the active substance and control solutions, we calculated the median values of S/N magnitudes (at 1700, 3250, and 3450 cm⁻¹) based on the data of the spectra recorded weekly for each of the samples throughout the entire study period. In the group of powders, this value has the smallest meaning for sample 2p (API substance). In the group of tableted samples at the specified frequencies, it always takes the highest value for sample 2t (RA forms of Abs tablets). An example of fulfilling this identity criterion based on measurements for 23 w is shown in table 3.

| Powder samples | Oscillation recording frequency | Tablet samples | Oscillation recording frequency |
|----------------|---------------------------------|----------------|---------------------------------|
| 2p             | 1700 cm⁻¹ 3250 cm⁻¹ 3450 cm⁻¹    | 2t             | 1700 cm⁻¹ 3250 cm⁻¹ 3450 cm⁻¹    |
| 3p             | 0.65 0.47 0.73                   | 3t             | 0.19 0.15 0.004                  |
| 4p             | 0.92 0.61 1.01                   | 4t             | -0.21 -0.15 -0.23                |
| 5p             | 1.25 1.01 1.09                   | 5t             | -0.40 -0.46 -0.74                |

Results of the water-lactose SMC study by X-ray fluorescence spectroscopy

The XRF method in the framework of this study was used as a marker of the transition of conformational states of the supramolecular complex of the water-lactose conglomerate. The elemental composition of the samples could not change qualitatively and quantitatively during the experiment. However, the kinetics of the fluorescence intensity at certain wavelengths indicates the possibility of a periodic cooperative trigger transition of the system, which was recorded during the study. Experimentally, the wavelengths that are sensitive to conformational transitions between the energy states of the amorphous system and, accordingly, reflecting the kinetic changes of the samples in terms of the intensity of fluorescence signals were established: Kα 3.313 keV (3.7424 Å) and Si Kα 1.740 keV (7.1255 Å).

Thus, according to the data presented in fig. 6, such reversible conformational transitions are observed for powders on the 30th and 130th days (Kα 3.313 keV). For tablets, no pronounced transitions were observed, but at Kα 3.313 keV and Kα 1.740 keV, small changes were visualized just on those days (100–110th day) when hysteresis phenomena were recorded in the IR spectra of these samples (fig. 5).

![Fig. 6: Kinetics of changes in the intensity of X-ray fluorescence in the studied samples of powders at the wavelength of Kα 3.313 keV (3.7424 Å), n=6](image)

Note once again that a change in the fluorescence signal of the same sample is not associated with a change in the content of an element (K or Si), but shows a cooperative trigger transition of the amorphous system, in which the trace elements included in the water-lactose cluster change their position relative to a surface.

**DISCUSSION**

The manufacture of high-quality drugs of release-active antibodies requires effective incoming control of raw materials, assurance of the consistency of technological parameters of production, as well as objective characteristic of the saturated substance of lactose, which is used to obtain finished dosage forms. The fold of initial antibodies dilution is extremely high and even taking into account the presence of the initial substance as nanoadsorbs in RA forms of Abs showed before [13, 14] it is necessary to detect not a change in the chemical composition of the substance, but the formation of a specific supramolecular water-lactose complex, which is characterized by several properties [5, 6]. The phenomenon of the formation of water-lactose complexes has been studied by the broadband dielectric spectroscopy technique in the frequency range of 10⁻¹⁻¹⁰ Hz and the temperature range of 176–230 K [15] and is beyond doubt—lactose is one of the main solid (after desiccation) components in milk and it strongly interacts with water, forming a water-clustered structure with ~123 affected water molecules per lactose molecule [16].
Functions of supramolecular systems are an expression of the collective system, therefore, traditional characterization methods, such as the determination of the structure of one supermolecule at a time is not sufficient to characterize them. Kinetic studies are essential for such systems because they are dynamic [17]. Time-resolved spectroscopic techniques, such as infra-red spectroscopy can be useful for the study of supramolecular dynamics [18]. Moreover in [19] authors utilize Fourier transform infrared spectroscopy to investigate kinetics of a series of five DNA sequences to clarify the commonly observed physical processes. As a result of our study, it was shown that SMCs in the composition of drugs undergo hysteresis phenomena, exhibiting long-period conformational mobility, which probably should be taken into account when the drug is tested in vitro [8, 12]. Hysteresis phenomena in water-lactose SMCs illustrated by the results of two spectral methods of analysis are most likely associated with the influence of environmental factors such as variations in relative humidity, temperature, background variations in the level of natural radioactivity, including the flux of thermal neutrons [20, 21], which can be a source of energy for conformational rearrangements [22, 23]. Based on the results of long-period observations of FTIR spectra, data on the transfer of energy from high-potentiated clusters of RA forms of Abs to “poutage material” (intact lactose powder) were obtained, which indicates the possibility of “dry” potentiation—activation of water-lactose complexes in the absence of water.

CONCLUSION

It was shown that differential IR spectra of the saturated lactose substance and the finished dosage form of RA forms of Abs revealed kinetic changes reflecting the energy transitions of the supramolecular system and appearing at two-week intervals. The XRF data, where the fluorescence signal was used as a tag of conformational mobility, correlates with the results obtained. At the same time, it was shown that in case of long-term (>10 w) observation of changes in the IR spectra, the median S/N values (at 1700, 3250, and 3450 cm⁻¹) of RA forms of Abs are always minimal in comparison with placebo samples for powders and vice versa are maximal for tablets. Thus, the features of kinetic changes confirm that lactose state is changed by saturation with the RA forms of Abs and it should be considered when the product is tested in vitro by different analytical tools.

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AUTHORS CONTRIBUTIONS

All the author has contributed equally.

CONFLICT OF INTERESTS

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