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Molecular Dynamics in Supercooled Liquid and Glassy States of Antibiotics: Azithromycin, Clarithromycin and Roxithromycin Studied by Dielectric Spectroscopy. Advantages Given by the Amorphous State

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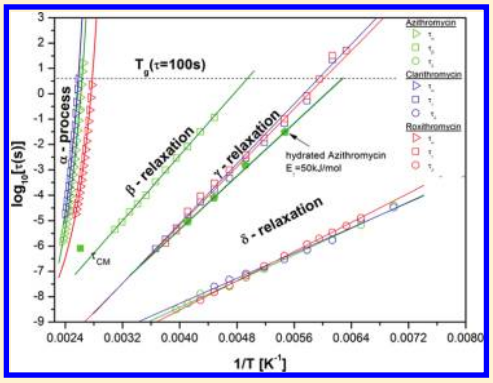
ABSTRACT: Antibiotics are chemical compounds of extremely important medical role. Their history can be traced back more than one hundred years. Despite the passing time and significant progress made in pharmacy and medicine, treatment of many bacterial infections without antibiotics would be completely impossible. This makes them particularly unique substances and explains the unflagging popularity of antibiotics within the medical community. Herein, using dielectric spectroscopy we have studied the molecular mobility in the supercooled liquid and glassy states of three well-known antibiotic agents: azithromycin, clarithromycin and roxithromycin. Dielectric studies revealed a number of relaxation processes of different molecular origin. Besides the primary α-relaxation, observed above the respective glass transition temperatures of antibiotics, two secondary relaxations in the glassy state were identified. Interestingly, the fragility index as well as activation energies of the secondary processes turned out to be practically the same for all three compounds, indicating probably much the same molecular dynamics. Long-term stability of amorphous antibiotics at room temperature was confirmed by X-ray diffraction technique, and calorimetric studies were performed to evaluate the basic thermodynamic parameters. Finally, we have also checked the experimental solubility advantages given by the amorphous form of the examined antibiotics.



KEYWORDS: *dielectric spectroscopy, molecular dynamics, glass transition, antibiotics, amorphous active pharmaceutical ingredients*

INTRODUCTION

When a liquid is cooled avoiding crystallization, a glassy state can be reached. Due to their dual nature (i.e., mechanical properties of solids and molecular disorder characteristic for liquids), glasses are very interesting materials for practical applications such as optical fibers, electrical transformers, structural materials, food and pharmacy.[1−4] Transformation from liquid into glassy state occurs at so-called the glass transition temperature, T_g, where molecular motions slow down to the time scale of hundred seconds. Below this temperature the molecular rearrangement is extremely slow and the molecules cannot reach their equilibrium positions. In order to find out more about molecular mobility in the vicinity of the glass transition it is necessary to perform relaxation dynamics studies. This is usually done by means of dielectric spectroscopy, temperature modulated differential scanning calorimetry, nuclear magnetic resonance or mechanical spectroscopy. The former technique is especially useful, since it provides information about molecular dynamics in supercooled and glassy states in a wide range of frequency, temperature and even pressure.

For a typical glass-former near the glass transition the α-relaxation process is observed, which reflects molecular rearrangements of a cooperative character and is directly related to the liquid−glass transition. As temperature decreases and the glassy state is reached, different types of motions occur. They are more local, generally termed as secondary relaxations. In fact, there are two classes of secondary processes of different molecular mechanisms. The slower one (Johari−Goldstein β-relaxation) involves motion of the entire molecule, thus has intermolecular character, while the faster ones (γ, δ, ..., termed in order of decreasing time scale) originate from a trivial rotational motion of small isolated parts of a molecule and have no connection with the α-relaxation.[5,6]

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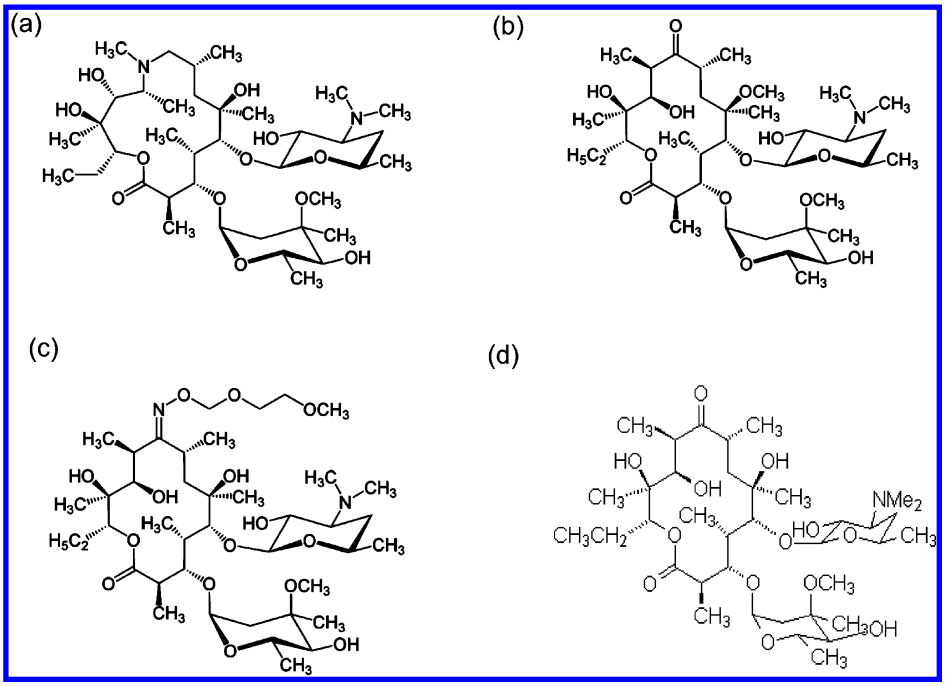

Figure 1. The chemical structure of (a) azithromycin, (b) clarithromycin, (c) roxithromycin and (d) erythromycin.

Studies of the molecular dynamics in the vicinity of the glass transition are of great interest not only in the context of deeper insight into the liquid−glass transition itself but also from practical applications. For example, in food and pharmaceutical industry the glassy state (or more generally amorphous state) is known to have greater solubility and dissolution rate than the crystalline.[7,8] Unfortunately, there is enormous disproportion between the amount of Active Pharmaceutical Ingredients prepared in the amorphous state and that successfully launched to the market. One of the most important difficulties is certainly physical and chemical instability of the amorphous state and consequences of many unwanted changes that might occur during manufacturing, processing and storage of such drugs.[9,10]

Since molecules need to rearrange in order to incorporate into crystal lattice, researchers relate often greater physical instability of the amorphous state to their mobility.[11−13] However, the types of molecular motions that are responsible for the instability issue are still very controversial and hotly debated within the research society. For example, in many cases crystal growth rate couples with global mobility (α-relaxation), and by storing glasses at least 50 K below T_g (where molecular mobility exceeds years) long-term stability can be ensured. On the other hand, the Johari−Goldstein β-relaxation was proposed by Oguni et al. to be directly responsible for recrystallization of amorphous substances.[14] Therefore, only complete understanding and characterization of molecular dynamics in the amorphous state might provide essential information for successfully designing amorphous pharmaceuticals with a desired stability and solubility profile. It should be mentioned here that this case is not so trivial since amorphous substances prepared using different amorphization routes possess different structures, enthalpies, physical stabilities and even solubility (i.e., refs 15 and 16).

Here, we investigate the molecular mobility in supercooled and glassy states of widespreadly used antibacterial agents: azithromycin, clarithromycin and roxithromycin. They are semisynthetic macrolide antibiotics derived from erythromycin, and they inhibit bacterial protein synthesis by binding to the bacterial 50S ribosomal subunit.[17] They exhibit approximately the same antibacterial spectrum. Azithromycin, clarithromycin and roxithromycin contain the same 14-membered lactone ring. However, there are some slight differences. For example, azithromycin differs in chemical structure from erythromycin in methyl-substituted nitrogen atom incorporated into the lactone ring, while in the case of roxithromycin an *N*-oxime side chain is attached to the lactone ring. Unlike erythromycin,[18] the studied antibiotics revealed improved acid-stability and can therefore be taken orally without protection from gastric acids. The chemical structures of the examined antibiotics and their original counterpart, erythromycin, are presented in Figure 1. In this paper, on the basis of collected information about thermodynamic parameters and molecular dynamics of supercooled and glassy antibiotics, we discuss their long-term physical stability proven by X-ray diffraction. Additionally, solubility studies were performed to show the benefits given by the glassy state of antibiotics at different pH conditions. This issue should be particularly interesting, since crystalline forms of azithromycin, roxithromycin and clarithromycin are characterized by poor solubility in aqueous media. In order to increase the oral absorption of examined antibiotics it is important to improve their solubility in the gastrointestinal tract, enabling rapid and complete absorption from the stomach (low pH media). Hence, in the past several attempts have been made to improve their solubility profile and bioavailability, including amorphization techniques as well. For example, Yonemochi et al.[19] obtained amorphous clarithromycin by grinding and spray drying. Biradar et al.[20] obtained amorphous roxithromycin by freeze-drying and spray drying. However it should be noted that, until now, no comprehensive studies of physicochemical properties and molecular dynamics of amorphous antibiotics have been performed.

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EXPERIMENTAL SECTION

Material. The tested samples, azithromycin (IUPAC name (2*R*,3*S*,4*R*,5*R*,8*R*,10*R*,11*R*,13*S*,14*R*)-11-{[(2*S*,3*R*,4*S*,6*R*)-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy}-2-ethyl-3,4,10-trihydroxy-13{[(2*R*,4*R*,5*S*,6*S*)-5-hydroxy-4-methoxy-4,6-dimethyloxan-2-yl]oxy}-3,5,6,8,10,12,14-heptamethyl-1-oxa-6-azacyclopentadecan-15-one, molecular formula $C_{38}H_{72}N_2O_{12}$, MW = 748.984 g/mol), clarithromycin (IUPAC name (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-6-{[(2*S*,3*R*,4*S*,6*R*)-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy}-14-ethyl-12,13-dihydroxy-4-{[(2S,4S,5R,6R)-5-hydroxy-4-methoxy-4,6-dimethyloxan-2-yl]oxy}-7-methoxy-3,5,7,9,11,13-hexamethyl-1-oxacyclotetradecane-2,10-dione, molecular formula $C_{38}H_{69}NO_{13}$, MW = 747.953 g/mol), and roxithromycin (molecular formula $C_{41}H_{76}N_2O_{15}$, MW= 837.047 g/mol), were were kindly donated by Pol-Nil (Warsaw, Poland) and were of European Pharmacopoeia grade. The water content in supplied materials was determined by the Karl Fischer (CRISON TitroMatic KF) method. We have found ∼4%, 1.5% and 2% of water in azithromycin, clarithromycin and roxithromycin respectively.

Methods. *Thermogravimetric Studies.* The thermogravimetric measurement of the crystalline forms of azithromycin, clarithromycin and roxithromycin were carried out with a Perkin-Elmer TGA 1 thermal analyzer in a platinum measuring cell, with the use of Pyris program for data handling. Measurements were performed in a nitrogen atmosphere with the heating rate 10 K/min. The samples were heated up to 873 K, starting from room temperature.

Ultraperformance Liquid Chromatography (UPLC). Chromatographic analyses of antibiotics under investigation were performed on the Waters Acquity UPLC system (Millford, MA, USA), equipped with reserved phase column, ACQUITY UPLC BEH Shield RP18, 1.7 μm, 2.1 × 100 mm (Waters, Wexford, Ireland). The column was thermostated at 40 °C. The ACQUITY UPLC system consisted of a binary solvent manager, sample manager, column manager, and photodiode array eλ detector (PDA). Analysis data were acquired and calculated using the Empower Pro 2 software (Waters, Milford, MA, USA). The developed chromatographic methods were applied in determination of related substance of examined antibiotics, as well as in drug substance solubility study.

The chromatographic separation of azithromycin and its impurities was achieved with a mobile phase consisting of methanol and 15 mM H_3PO_4 adjusted to pH 7.5 with triethylamine mixed in ratio 70:30 (v/v). The solvent mixture was delivered at a flow rate of 0.3 mL/min. The isocratic separation was achieved within run time of 8 min. The UV absorbance data were collected at 210 nm with a data collection rate of 40 points per second.

For analyses of roxithromycin and clarithromycin the mobile phase consisted of acetonitrile (mobile phase A) and 15 mM H_3PO_4 adjusted to pH 7.5 with triethylamine (mobile phase B). The separation was obtained within 7 min at a flow rate 0.3 mL/min with a linear gradient program that ran from 50% to 90% of mobile phase A in 5 min, followed by washing for 1 min at 90% of mobile phase B and equilibration at starting mobile phase ratio. The UV absorbance data were collected at λ = 210 nm for roxithromycin and at λ = 287 nm for clarithromycin with a data collection rate of 40 points per second.

Dielectric Spectroscopy (DS). Isobaric measurements of the dielectric permittivity $\varepsilon^*(\omega) = \varepsilon'(\omega) - i\varepsilon''(\omega)$ were carried out using the Novo-Control Alpha dielectric spectrometer over frequency range from 1×10^{-2} to 3×10^6 Hz at ambient pressure. Each of the tested samples was placed between two stainless steel electrodes (diameter 20 mm, gap 0.1 mm) and mounted on a cryostat. During measurement each sample was maintained under dry nitrogen gas flow. The temperature was controlled by Quatro System using a nitrogen gas cryostat, with stability better than 0.1 K. Dielectric measurements of antibiotics were performed after fast cooling of the liquid to the glassy state, and carried out from 133 K even up to 430 K in different steps: every 10 K in the glassy state and every 2 K above the glass transition temperature.

Differential Scanning Calorimetry (DSC). Thermodynamic properties of crystalline and amorphous forms of considered antibiotics were investigated by the DSC technique. Calorimetric measurements were performed with Mettler-Toledo DSC apparatus equipped with a liquid nitrogen cooling accessory and a HSS8 ceramic sensor (heat flux sensor with 120 thermocouples). Temperature and enthalpy calibrations were carried out using indium and zinc standards while heat capacity C_p calibration was performed using a sapphire disk. The amorphous form of each antibiotic was prepared in an open aluminum crucible (40 μL) outside the DSC apparatus. First, the crystalline sample in the crucible was heated on the heating plate (CAT M 17.5), and next it was immediately cooled to vitrify the liquid sample. Crucibles with such prepared glassy samples as well as with their crystalline counterparts were sealed with the top with one puncture. Crystalline and amorphous samples were scanned at a rate of 10 K/min over a temperature range of 298 K to well above the respective glass transition points.

X-ray Diffraction (XRD). The X-ray diffraction measurements were performed at ambient temperature on Rigaku-Denki D/MAX RAPID II-R diffractometer (Rigaku Corporation, Tokyo, Japan) with a rotating anode Ag Kα tube (λ = 0.5608 Å), an incident beam (002) graphite monochromator and an image plate in the Debye−Scherrer geometry. The pixel size was 100 × 100 μm. All samples were placed inside Lindemann glass capillaries (2 mm in diameter), measurements were performed for the sample filled and empty capillaries and the intensity for the empty capillary was then subtracted. The beam width at the sample was 0.1 mm. The two-dimensional diffraction patterns were converted into the one-dimensional intensity data using suitable software.

Solubility Studies. The solubility of crystalline and amorphous forms of antibiotics was determined in different media such as purified water, hydrochloric acid (0.1 M), acetate buffer pH 4.5 and phosphate buffer pH 7.5. Solubility measurement was performed at specific pH values using the shake-flask method (37 °C ± 0.5 °C, 1 h).

RESULTS AND DISCUSSION

Thermal Stability and Purity of Vitrified Antibiotics. Formulation of drugs with amorphous active pharmaceutical ingredients (APIs) requires successfully passing many rigorous criteria, such as the highest purity standards of drug substances. It is obvious that drugs, especially antibiotics, must be free of any impurities, which might grow within the shelf life, resulting in many serious or unwanted consequences. Because of that reason, it is important to make sure that the amorphization process itself will not cause the degradation of APIs. This issue is particularly important in the case of substances for which crystalline forms are thermally unstable or melt with

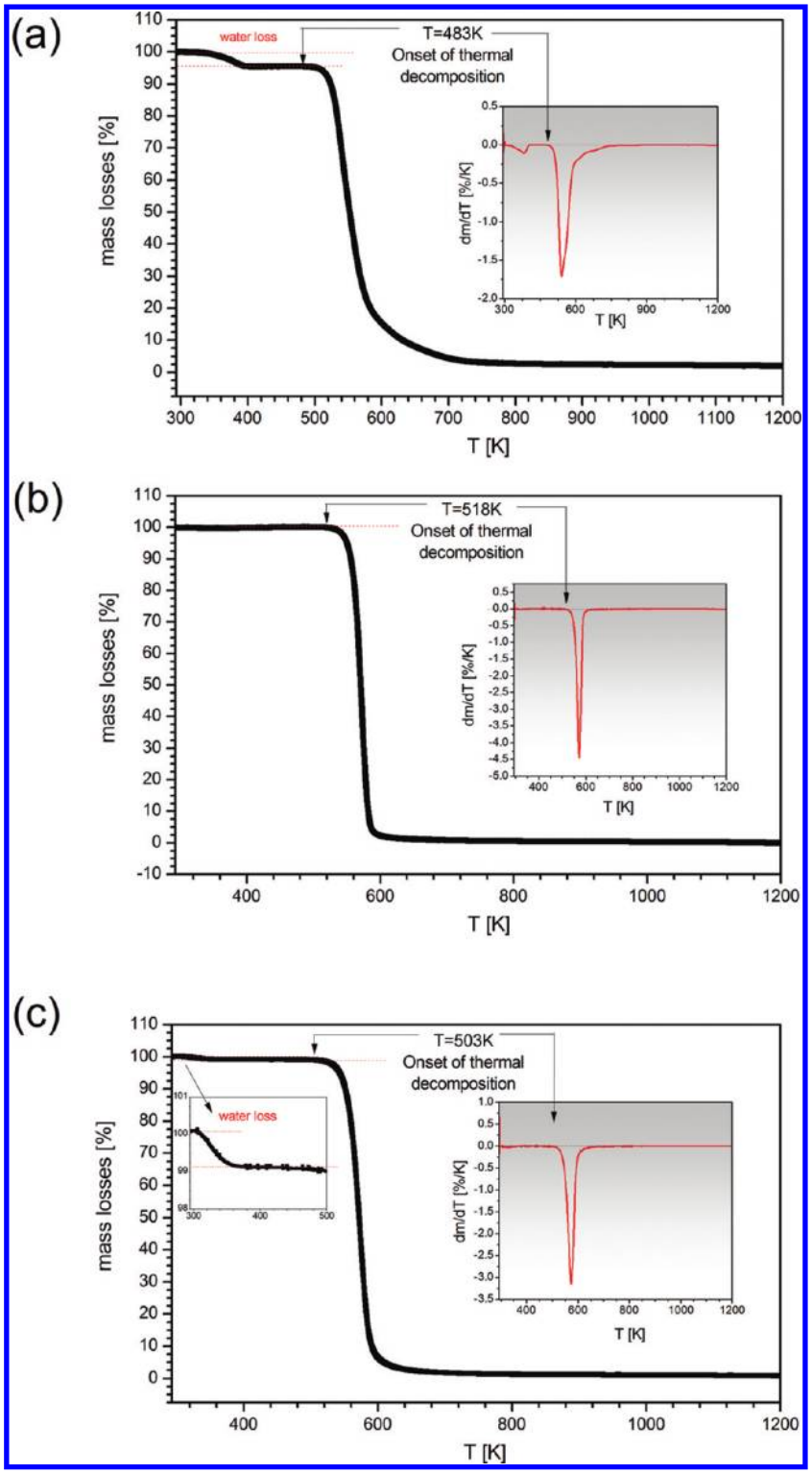

Figure 2. Thermogravimetric weight loss curves and derivative weight loss curves (insets) of crystalline azithromycin (a), clarithromycin (b) and roxithromycin (c).

decomposition (e.g., carbomazepine, furosemide or ziprasidone hydrochloride), so vitrification is simply not possible. Since in the literature there is no precise information about the extent of thermal stability of the crystalline forms of examined antibiotics as well as purity profile of their amorphous forms obtained using the vitrification method, detailed studies on that case were performed. In order to test if examined antibiotics undergo thermal degradation at higher temperatures thermogravimetric measurements were carried out. Thermogravimetric and derivative curves of respective antibiotics are shown in Figure 2. As can be seen they are stable up to 484 K (azithromycin), 518 K (clarithromycin) and 503 K (roxithromycin). At these temperatures thermal decomposition of examined samples begins and is nearly completed at about 700 K (azithromycin) and 600 K for the two other antibiotics (mass loss 97%). All samples decompose in a single weight loss.

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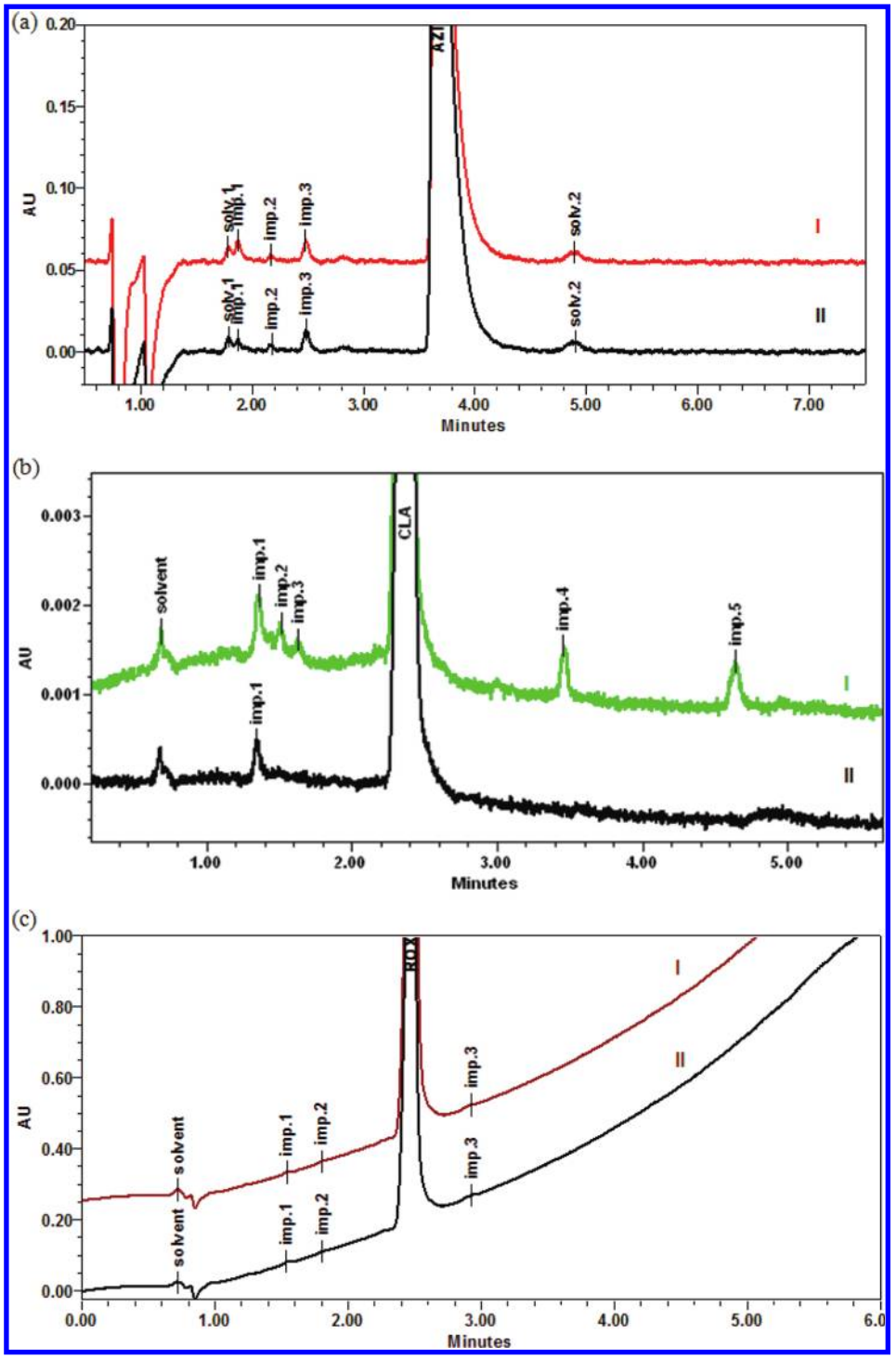

Figure 3. Comparison of impurity profiles of crystalline (II) and amorphous (I) forms of antibiotics: (a) azithromycin, (b) clarithromycin and (c) roxithromycin.

This is supported by the derivative curves which exhibit only single peaks (Figure 2 insets). From results presented above it is evident that by ably control of the temperature they can be safely vitrified. However, this fact does not mean that amorphous samples obtained in this way are free of any impurities. It is obvious that it is simply not possible to prepare active pharmaceutical ingredients without even a trace of impurity. Conversion of the crystalline pharmaceutical into the amorphous form is particularly very risky due to their greater chemical reactivity. Thus, using ultraperformance liquid chromatography we have analyzed crystalline as well as amorphous antibiotics in order to provide additional information about their purities. Herein, it is worth noting that UPLC is one of the commonly used analytical techniques for controlling the quality or consistency of active substances and final dosage forms. Chromatograms of crystalline antibiotics were used for further analysis as a reference. They are displayed in Figure 3, panels a, b and c (lower chromatogram on each panel), and consist of very well pronounced peaks ascribed to azithromycin, clarithromycin and roxithromycin,

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respectively. Few impurities were found. The analysis of area under the peak revealed that the total sum of impurities in crystalline samples does not exceed more than 0.5% in case of clarithromycin and roxithromycin, and 2% for azithromycin. This confirms that the initial crystalline materials were pure. The UPLC chromatograms of glassy azithromycin, clarithromycin and roxithromycin are also presented in Figure 3, panels a, b and c (upper chromatograms), respectively. In all three cases, it is clearly visible that the main peak ascribed to each antibiotic has exactly the same retention time as that observed for the crystalline one. During amorphization of antibiotics by quenching, a small increase in total amount of impurities can be observed: for azithromycin, about 0.23%, for clarithromycin, about 0.45% and for roxithromycin, about 0.09%, as presented in Tables 1−3. These results suggest that appearance of unwanted impurities is an unavoidable event occurring during amorphization, but able control of this process enables us to obtain glassy materials with impurity amounts insignificant for further studies.

Table 1. Comparison of Impurity Profiles of Crystalline and Amorphous Azithromycin

		amount of analyte [%]	
analyte identification	rel retention time	crystalline	amorphous
impurity 01	0.51	0.56	0.94
impurity 02	0.59	0.23	0.23
impurity 03	0.68	1.12	0.97
azithromycin	1.00	98.09	97.86
total impurities:		1.91	2.14

Table 2. Comparison of Impurity Profiles of Crystalline and Amorphous Clarithromycin

		amount of analyte [%]	
analyte identification	rel retention time	crystalline	amorphous
impurity 01	0.57	0.17	0.17
impurity 02	0.64		0.09
impurity 03	0.68		0.07
clarithromycin	1.00	99.83	99.38
impurity 04	1.46		0.14
impurity 05	1.95		0.15
total impurities:		0.17	0.62

Table 3. Comparison of Impurity Profiles of Crystalline and Amorphous Roxithromycin

		amount of analyte [%]	
analyte identification	rel retention time	crystalline	amorphous
impurity 01	0.62	0.14	0.19
impurity 02	0.73	0.10	0.12
roxithromycin	1.00	99.55	99.46
impurity 03	1.19	0.21	0.23
total impurities:		0.45	0.54

Long-Term Physical Stability of Amorphous Antibiotics. In order to investigate the long-term stability of glassy antibiotics we carried out repeatedly the X-ray diffraction (XRD) measurements at ambient conditions. The XRD patterns were recorded every month for up to 13 months. In Figure 4, panels a, b and c present diffraction patterns of crystalline (given here as a reference), as well as amorphous antibiotics. Very broad peaks observed in the latter case confirmed their amorphous nature. As can be seen, all three samples are physically stable for more than one year from the preparation date. This is a very important finding, since it points out potential usage of the amorphous forms of examined antibiotics and should be verified on the further stages of handling with these substances. More so, the glassy states of examined antibiotics were subjected to a temperature 312 K at 85% relative humidity for 7 days. Again, no signs of crystallization were reported after exposure to the above conditions. Extended physical stability of the glassy state of antibiotics might have potential application in the pharmacy, but it is also interesting from another point of view, namely, to investigate the role and relative importance of molecular mobility and thermodynamic factors in governing the physical stability of these systems.

Characterization of Molecular Dynamics. Dielectric relaxation studies performed above and below the glass transition are important sources of information about the molecular dynamics of the examined materials. In Figure 5 we present the imaginary part of dielectric permittivity plotted as a function of frequency during heating of amorphous antibiotics from 133 K even up to 421 K. To show the whole sets of data more clearly, dielectric spectra of each antibiotic were divided into two panels presenting the relaxation dynamics above and below the glass transition temperatures. Here, the glass transition temperature is defined as a temperature at which dielectric relaxation time τ_α is equal to 100 s. In the supercooled region, as typical for glass-formers, the structural relaxation process was observed, providing valuable information about changes in the cooperative reorientational dynamics of investigated samples. This process shifts toward higher frequencies with increasing temperature, indicating the increase in global molecular mobility of the system. The spectra collected above T_g's show the presence of dc-conductivity (σ_{dc}) at low frequencies. Large dc contribution to the loss spectra is commonly observed in many supercooled systems (including other pharmaceuticals, e.g. ref 21) and will be discussed carefully in the further part of this paper. In order to estimate correct values of structural relaxation times as well as determine the shape of the α-peak in the supercooled regime of examined antibiotics (especially for azithromycin and roxithromycin), it was necessary to subtract the dc-contribution from the total dielectric loss.

In the temperature region above T_g's except for the α-process an excess wing appears on the high frequency flank of the α-loss peak of clarithromycin and roxithromycin. For azithromycin, secondary β-relaxation in the vicinity of the glass transition is clearly visible. This type of secondary relaxation is called Johari−Goldstein relaxation. It is present in all glass-forming liquids, although not always well-separated from the α-relaxation.[22,23] The β-relaxation is believed to be the precursor of the cooperative structural relaxation and originates from local motion of the entire molecule.

A very interesting issue is the fact that during heating from the glassy state clarithromycin began to crystallize at temperature $\sim T_g$ + 31.5 K, whereas the other two antibiotics revealed no signs of cold-crystallization. As we found, even very slow cooling (1 K/min) of liquids azithromycin and roxithromycin results in complete amorphization without crystallinity. Because of that they are very good glass-formers. Completely different resistance against crystallization in supercooled region of antibiotics is quite surprising, especially if we take into account similarity in chemical structure of considered antibiotics.

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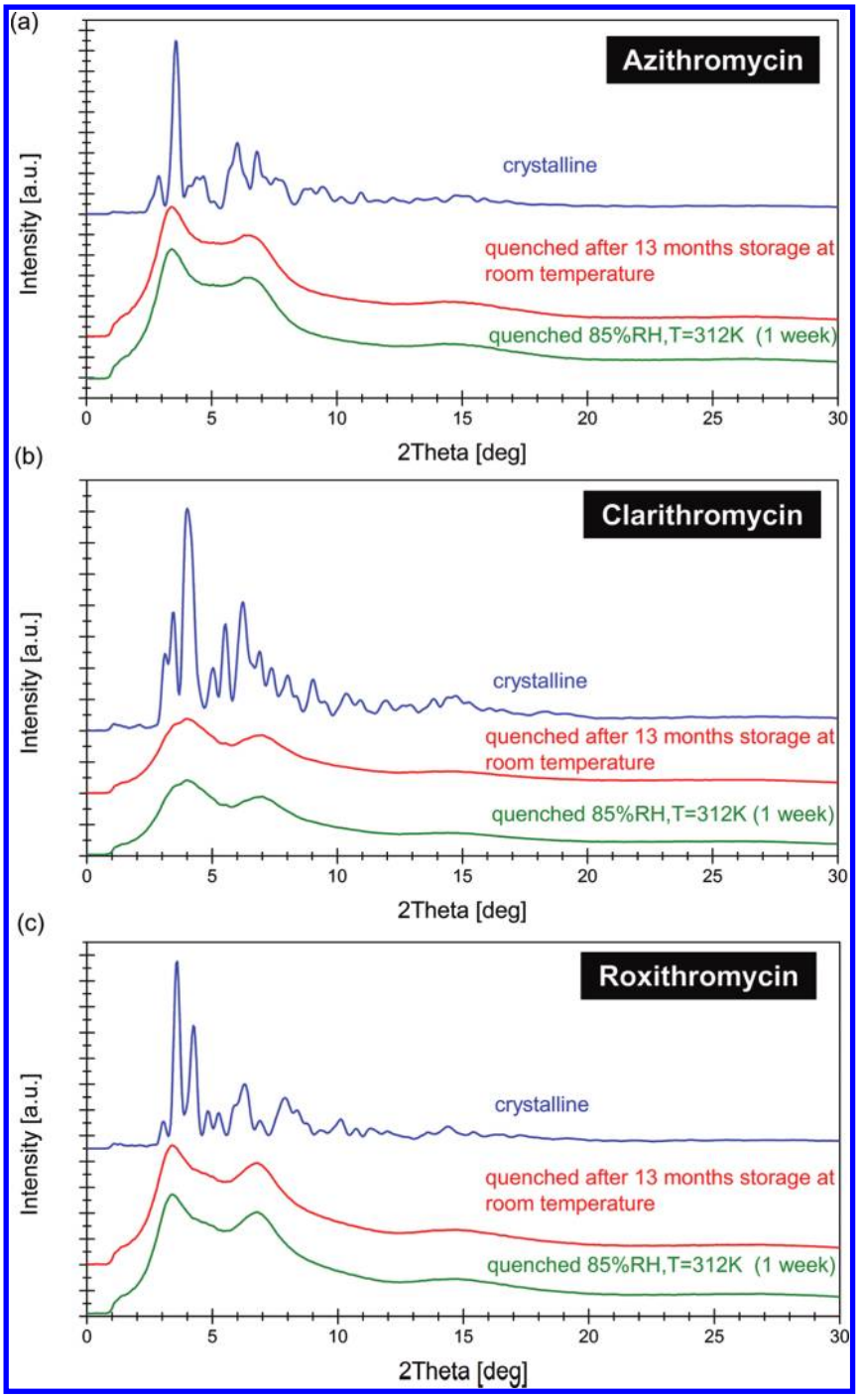

Figure 4. Representative X-ray diffraction patterns of crystalline and glassy antibiotics recorded at room temperature. Vitrified samples were stored at room temperature for 13 months and subjected also to 85% RH at 321 K for 7 days. No signs of crystallizations were recorded. Panels correspond to (a) azithromycin, (b) clarithromycin and (c) roxithromycin.

In Figure 5 panels d, e and f dielectric response of examined antibiotics below their glass transition temperatures is presented. In this region the maxima of α-peaks are out of measurable frequency range and the main source of information about molecular dynamics in the glassy state becomes secondary modes. For clarithromycin and roxithromycin two secondary processes were observed: the slowest one, i.e. the γ-process, and the faster one, the δ-process. These secondary loss peaks are broad and well-defined and move toward higher frequencies with increasing temperature, but their sensitivity to the temperature is definitely lower than the primary relaxation's. On the other hand, in dielectric spectra of azithromycin we observed only the β-relaxation and the δ-relaxation (no γ-relaxation was detected in the glassy state of vitrified azithromycin). This issue will be clarified later on.

In the next step recorded dielectric spectra were analyzed by an empirical Havriliak–Negami (HN) function plus a term from dc-conductivity[24]

$$\varepsilon^*_{\mathrm{HN}}(\omega) = \varepsilon_\infty + \frac{\Delta\varepsilon}{(1 + (\mathrm{i}\omega\tau_{\mathrm{HN}})^a)^b} + \frac{\sigma_{\mathrm{dc}}}{\mathrm{i}\omega\varepsilon_0} \quad (1)$$

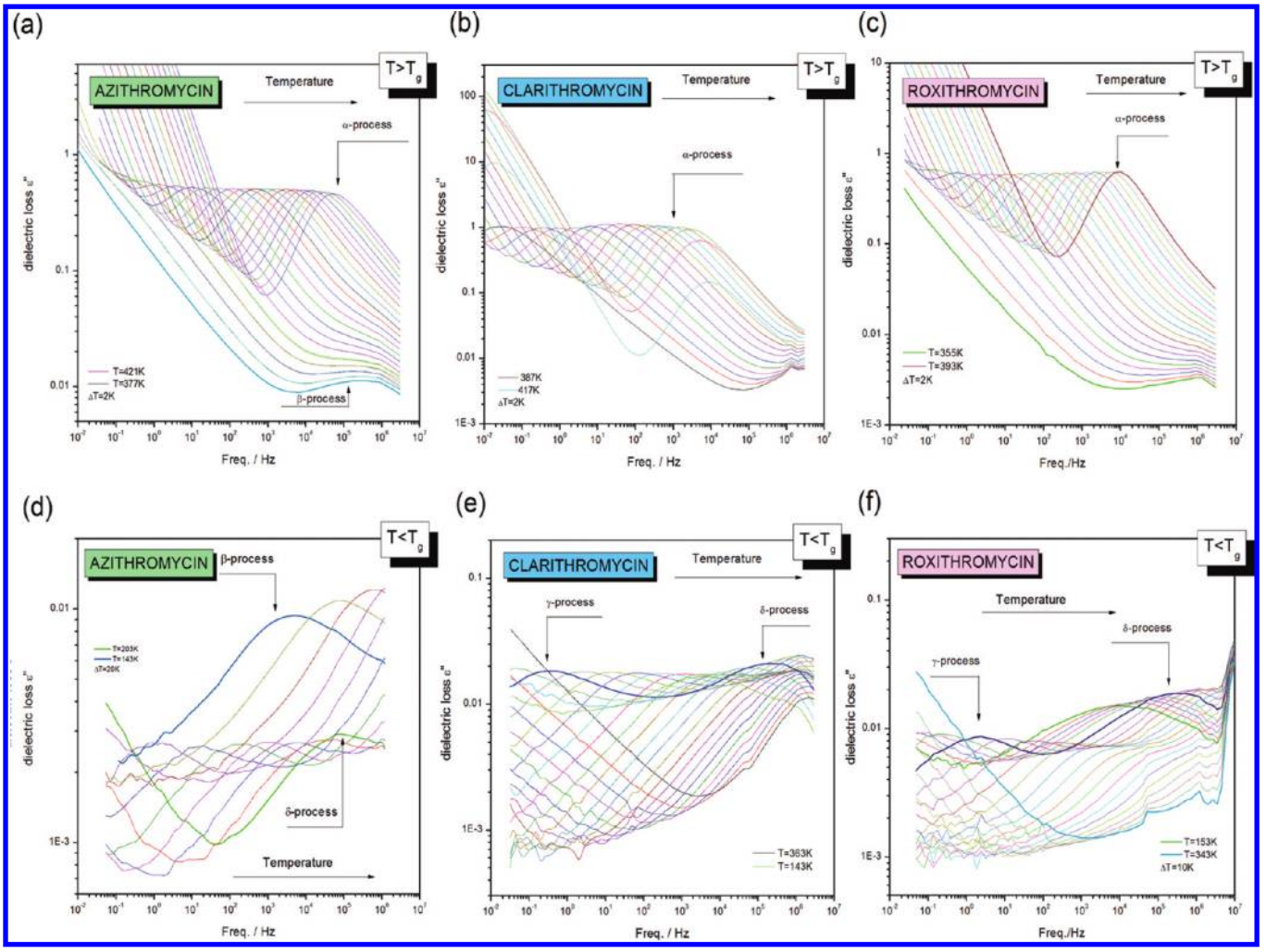

Figure 5. Dielectric loss spectra of anitbiotics: azithromycin, clarithromycin and roxithromycin measured above (panels a, b and c) and below (panels d, e and f) their glass transition temperatures.

where a and b are parameters representing symmetric and asymmetric broadenings of the dielectric loss curve, $\Delta\varepsilon$ is relaxation strength, ω is an angular frequency ($\omega = 2\pi f$), τ_{HN} is HN relaxation time, ε_∞ is high frequency limit permittivity. When b is fixed to be equal to unity, the HN function turns into the Cole−Cole function (CC). The contribution of dc-conductivity was then subtracted from fitted spectra.

In glass forming liquids the relation between structural relaxation times and dc-conductivity is usually discussed in terms of the Debye−Stokes−Einstein equation (DSE),

$$\tau_\alpha \sigma_{dc} \cong \text{const} \quad (2)$$

which describes the relationship between translational motions of ions and rotational motions of molecules. However, for many molecular liquids eq 2 fails as approaching T_g.[25] In this case, the relationship between conductivity and rotational relaxation time is well characterized by fractional Debye−Stokes−Einstein equation (FDSE),

$$\tau_\alpha{}^s \sigma_{dc} \cong \text{const} \quad (3)$$

where s is fractional exponent less than 1.

In order to describe the correlation between σ_{dc} and τ_α we plotted dc conductivity versus structural relaxation time in double logarithmic scale, as shown in Figure 6. The experimental data for clarithromycin fall on a straight line, indicating that FDSE equitation with $s = 0.67$ is suitable to describe the relation between σ_{dc} and τ_α. As a result, in supercooled liquid state of clarithromycin the enhancement of translational motion over rotational takes place. One can add that a similar trend is observed for other glass formers.[26−30] On the other hand a very interesting phenomenon was observed for azithromycin and roxithromycin where a change in the slope of the log $\tau_\alpha = f(-\log \sigma)$ dependence is observed. At higher temperatures the FDSE equation with $s = 0.66$ (azithromycin) and $s = 0.69$ (roxithromycin) describes well the correlation between σ_{dc} and τ_α. As temperature decreases and τ_α reaches $0.005s$ ($\log \tau_\alpha = -2.3$), the FDSE equation with $s = 0.46$ (azithromycin) and $s = 0.49$ (roxithromycin) turns out to describe better the relationship between conductivity and rotational motions. It should be noted here that such a change in the slope of the log $\tau_\alpha = f(-\log \sigma)$ dependence is not commonly reported in the literature. To explain this unusual finding we may only speculate that H-bonded networks start to form in both antibiotics with lowering temperature. Consequently, new pathways for the proton hopping have been created in both systems (the same as that known for saccharides[31]). As a result, the enhancement of translational motions over rotational is observed again. However, it must be pointed out that such behavior was reported only for two out of three examined antibiotics, which is quite surprising. The fact that the change in the σ_{dc} versus τ_α dependence was reported for two out of three antibiotics studied

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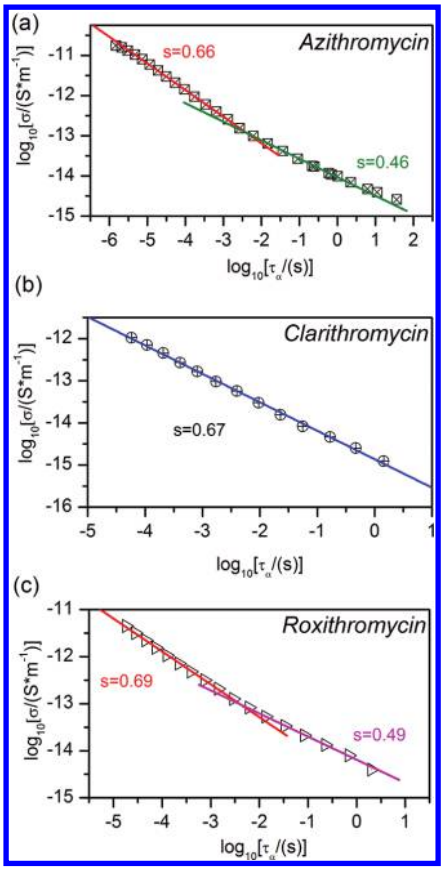

Figure 6. The dc-conductivity plotted as a function of α-relaxation time on a log–log scale for azithromycin (a), clarithromycin (b) and roxithromycin (c).

herein is very surprising. In Figure 7, panels a, b and c, we present master curves constructed by horizontal shift of several spectra taken at different temperatures and normalized in accordance with frequency–temperature superposition rules. Application of this procedure enables to extend a frequency range and look over the whole set of measured data within one figure. The master curves were made in the following way. As a reference, we have chosen spectra collected in supercooled liquid state at 383 K, 387 K and 363 K for azithromycin, clarithromycin and roxithromycin, respectively. Then, arbitrarily selected spectra above and below T_g were shifted horizontally so that their low-frequency side superimposes with the high frequency side of our reference ones. As can be seen with decreasing temperature the α-process of antibiotics slightly broadens. However, near the glass transition the shape of the structural relaxation peak for each antibiotic was found to be essentially independent of temperature. It should be also noted that presentation of dielectric data in this way for clarithromycin and roxithromycin clearly indicates the presence of an excess wing, demonstrated as a change in slope of the high frequency flank of structural relaxation peaks. For azithromycin, after subtraction of the β-relaxation from the total curve, no signs of excess wing were noticed.

The breadth of the structural relaxation peak was described using β_{KWW} ($0 < \beta_{KWW-M} \leq 1$) stretch exponent which can be determined by fitting the α-peak in the frequency domain by the one-sided Fourier transform of the KWW function[32]

$$\varepsilon_\alpha''(\omega) = \Delta\varepsilon_\alpha \int_0^\infty \left[-\frac{d\Phi}{dt}\right] \sin(\omega t)\, dt \tag{4}$$

where $\Delta\varepsilon_\alpha$ is dielectric strength of the α-relaxation and the stretch exponent is a fraction of unity. The value of $\beta_{KWW} = 1$ implies Debye-type relaxation. If $\beta_{KWW} = 1$ decreases, the distribution of molecular relaxation becomes more nonexponential. We found the following stretching exponents: $\beta_{KWW} = 0.52$ for azithromycin and $\beta_{KWW} = 0.62$ for clarithromycin and roxithromycin indicating asymmetric and broader than that for classical Debye response distribution of relaxation times (see insets in Figure 7). It was suggested that narrowing of the α-peak should improve stability of amorphous materials.[33] Based on this approach azithromycin, with the broadest distribution of relaxation times, should be the least stable among all antibiotics studied herein. However, as shown previously amorphous azithromycin reveals long-term stability, identical to that found for other samples. It also does not crystallize above T_g, so no correlation between the shape of structural relaxation peak and stability of examined pharmaceuticals can be put forward in this case.

To test whether secondary relaxation is the Johari–Goldstein β-relaxation or not the coupling model (CM)[34] was used. The CM predicts that the JG-relaxation time τ_{JG} should correspond well to so-called primitive relaxation time τ_o, and the following relation is valid:

$$\tau_{JG}(T) \cong \tau_o(T) = t_c^{\,n}\tau_\alpha(T)^{1-n} \tag{5}$$

where t_c is equal to 2 ps for molecular glass formers and ($n = 1 - \beta_{KWW}$) is the coupling parameter. For antibiotics the values of primitive relaxation times were calculated using previously determined β_{KWW} parameters and structural relaxation times at temperatures 383 K, 387 K and 364 K for respectively azithromycin, clarithromycin and roxithromycin. In panels a, b and c of Figure 7 the corresponding primitive relaxation times are indicated by black arrows (τ_{CM}). In the case of clarithromycin and roxithromycin τ_{CM} were found to fall within the range of the excess wing which supports identification of the excess wing as the unresolved JG β-relaxation. For azithromycin τ_{CM} is located close to the maximum of the β-peak, which suggest its intermolecular origin. The calculated value of the JG β-relaxation for azithromycin is also displayed in the relaxation map.

Based on estimated values of fitting parameters a, b and τ_{HN} from the HN equation (eq 1) the structural relaxation times related to loss maximum ($\tau = \tau_{max} = 1/2\pi f_{max}$) were calculated as[35]

$$\tau = \tau_{HN} \times \left[\sin\left(\frac{a\pi}{2+2b}\right)\right]^{-1/a}\left[\sin\left(\frac{ab\pi}{2+2b}\right)\right]^{1/a} \tag{6}$$

To determine the secondary relaxation times, the loss spectra were fitted by superposition of the two Cole–Cole functions.

For all materials, the temperature dependencies of the primary and secondary relaxation times are well described respectively by

- the Vogel–Fulcher–Tammann (VFT) equation[36] for the α-relaxation

$$\tau_\alpha(T) = \tau_\infty \exp\left(\frac{D_T}{T - T_0}\right) \tag{7}$$

where τ_∞, D_T, T_0 are parameters determined by fitting the experimental data. The temperature T_0 is often referred to the temperature where molecular mobility associated with instability of glass should approach to zero.

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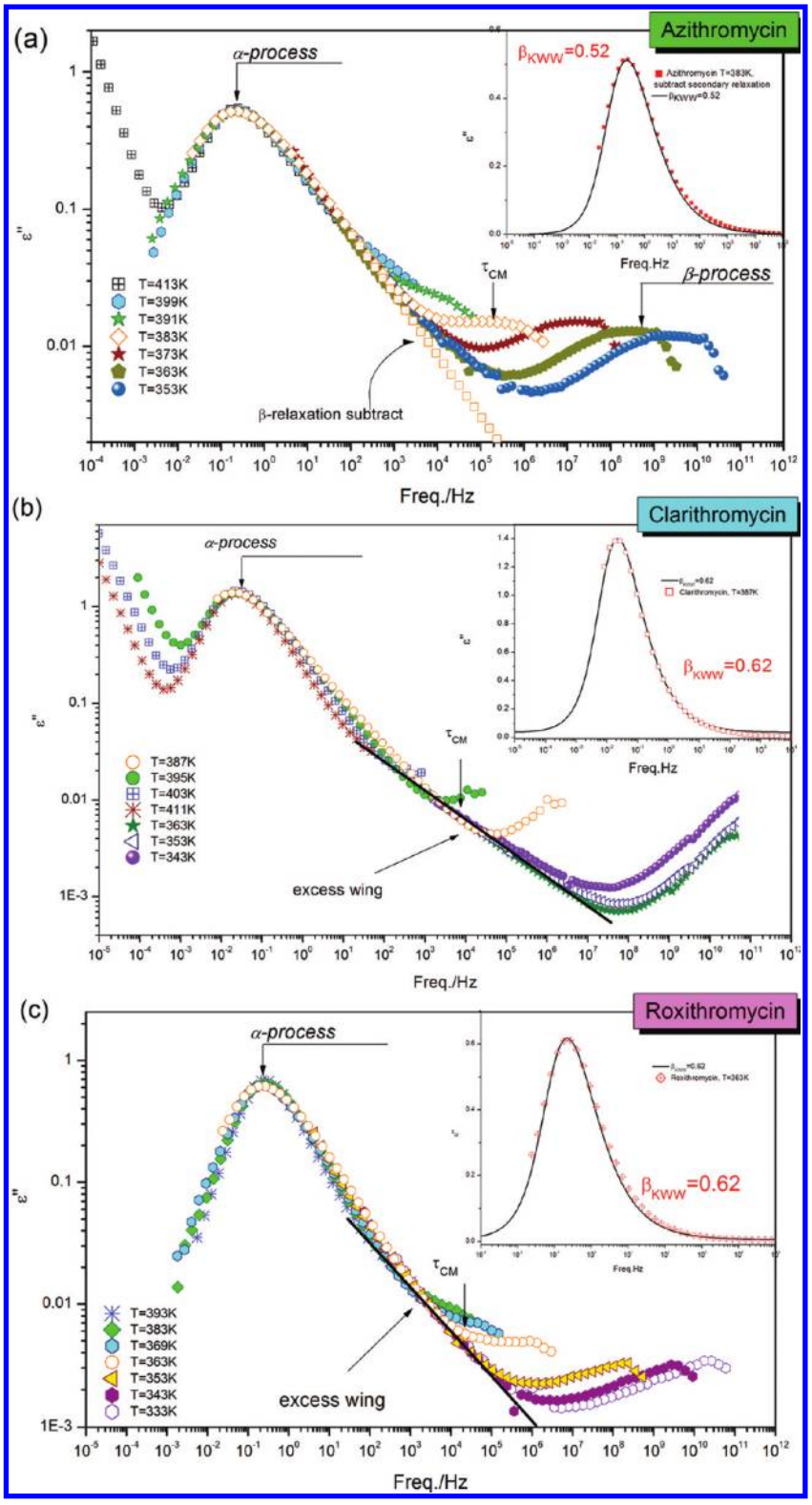

Figure 7. Superimposed dielectric spectra of antibiotics taken at different temperatures near T_g's of respective antibiotics. Solid lines represent the KWW fits with β_{KWW} = 0.62 for clarithromycin and Roxithromycin and β_{KWW} = 0.52 for azithromycin.

- the Arrhenius equation in the case of secondary relaxations

$$\tau(T) = \tau_\infty \exp\left(\frac{\Delta E}{k_B T}\right) \quad (8)$$

where τ_∞ is the pre-exponential factor and ΔE is the activation energy for concerned relaxation process.

The experimental data along with the fitting curves are presented in the relaxation map, see Figure 8. The corresponding parameters from the VFT and Arrhenius equations are collected in Table 4. From the VFT fits the kinetic glass transition

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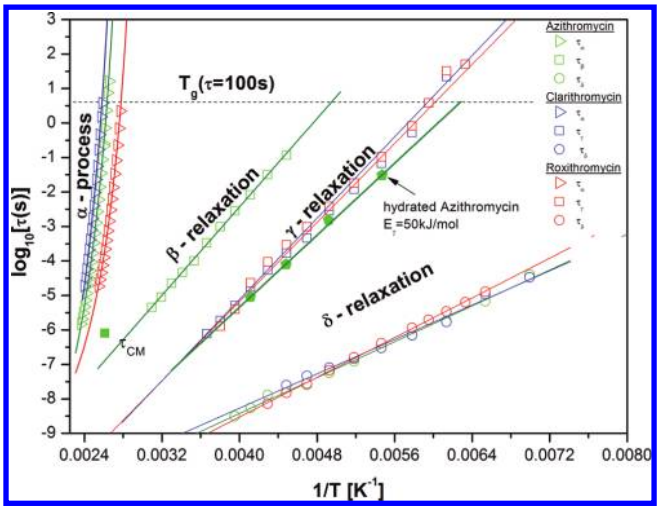

Figure 8. The relaxation map of antibiotics. Temperature dependences of structural relaxation times were fitted to the VFT equation, while the temperature dependences of secondary relaxations were fitted to the Arrhenius equation. These fits are denoted as solids lines.

temperature were estimated and are equal to 374.8 K, 382.3 K and 355.6 K for respectively azithromycin, clarithromycin and roxithromycin. Herein, it is worth pointing out that the glass transition temperatures of examined antibiotics are considerably higher than room temperature, i.e. more than 50 K in the case of roxithromycin, for the two other materials >75 K. This suggests that at ambient conditions the global molecular mobility in the glassy state of examined materials should be negligible. This supposition is additionally confirmed by the values of the zero temperature mobility, T_0, found for all investigated systems to be located above room temperature (Table 4).

From the temperature dependences of the structural relaxation times the steepness index (fragility) m[37] was calculated as

$$m = \left.\frac{\mathrm{d}\log_{10}\tau_\alpha}{\mathrm{d}(T_g/T)}\right|_{T=T_g} \quad (9)$$

We obtained practically the same steepness index values m = 117 for azithromycin, m = 118 for clarithromycin and m = 121 for roxithromycin. These values of fragility index classify examined antibiotics as fragile systems, which means that the temperature dependences of the structural relaxation times deviate significantly from the Arrhenius law. Large values of isobaric fragility usually suggest that considered glass-former should be structurally less stable than strong one, due to considerable changes in molecular mobility near the glass transition. As can be concluded from Tanaka's two order parameter model,[38] fragile glass-formers should be less stable against crystallization than strong ones, since their frustrations against crystallization are weaker. According to this criterion, fragile antibiotics should reveal great ease of crystallization. However, as confirmed by the X-ray diffraction their glassy states are long-term. In supercooled liquid state only clarithromycin crystallized, while the two other antibiotics having practically the same fragility index did not. Because of that it is evident that large fragility does not actually correlate with crystallization tendencies of antibiotics.

Now, we focus on the analysis of the secondary relaxation processes that occur in the glassy state. The values of activation barriers for β-, γ- and δ- relaxations obtained from the fitting of experimental data to the Arrhenius equation are equal to 61.3 kJ/mol (β-relaxation) and 24.8 kJ/mol (δ-relaxation) for azithromycin, 56.4 kJ/mol (γ-relaxation) and 23.8 kJ/mol (δ-relaxation for clarithromycin, 54.8 kJ/mol (γ-relaxation) and 27.5 kJ/mol (δ-relaxation) for roxithromycin. It is interesting to point out that practically the same values of the activation energies of the γ- and δ-relaxations were found. However, the most important aspect that should be considered is the origin of the γ-relaxation. To find out what might be the reason of the lack of the γ-relaxation in the glassy state of vitrified azithromycin we have performed the following experiment. Glassy azithromycin (prepared by vitrification of liquid) was stored at 70% RH 25 °C for 5 days. Then dielectric measurements in the glassy state were performed. Comparison of dielectric spectra for freshly made anhydrous glass and hydrated one revealed significant difference, see Figure 9. As can be seen, in the glassy state of hydrated azithromycin another relaxation appears. It was denoted as the γ-relaxation because the peak position as well as its activation energy agrees quite well with that found for the two other antibiotics. From that it becomes evident that this relaxation process is somehow related to the dynamics of water confined. It should be mentioned here that heating of the hydrated glassy azithromycin to the region well above T_g, and then cooling it again to the glassy state, results in the relaxation dynamics of the vitrified anhydrous sample. One can also add that our detailed studies on azithromycin suggest that heating above 373 K enabled us to obtain completely anhydrous glassy sample while in the case of the other two antibiotics complete dehydration was impossible. Analogous storage of vitrified roxithromycin at 70% RH 25 °C for 5 days did not result in significant change of the relaxation processes in the glassy state. We found that there was always some residual water in clarithromycin and roxithromycin. Thus, it seems to be reasonable to suppose that γ-relaxation in antibiotics is connected to the relaxation of water confined.

It is interesting to point out that practically the same values of the activation energies of the δ-relaxations were found for all investigated samples, which suggests involvement of the same molecular motions. To make this issue more evident, in

Table 4. Fitting Parameters from the VFT Equation, Values of the Glass Transition Temperatures, Activation Energies of the Secondary Relaxations and Fragilities for Examined Antibiotics Which Were Calculated on the Basis of Dielectric Measurements

							activation energy [kJ/mol]		
material	log τ_∞	D	T_0 [K]	T_g [K] (dielectric, τ_α = 100 s)	T_g [K] (DSC)	fragility m	β-relaxation	γ-relaxation	δ-relaxation
azithromycin	−14.6	2.7	321.5	374.8	378	117	61.3	50 (hydrated sample)	24.8
clarithromycin	−16.2	3.3	323.2	382.3	385	118	excess wing	56.4	23.8
roxithromycin	−12.2	1.8	314.3	355.6	359	121	excess wing	54.8	27.5

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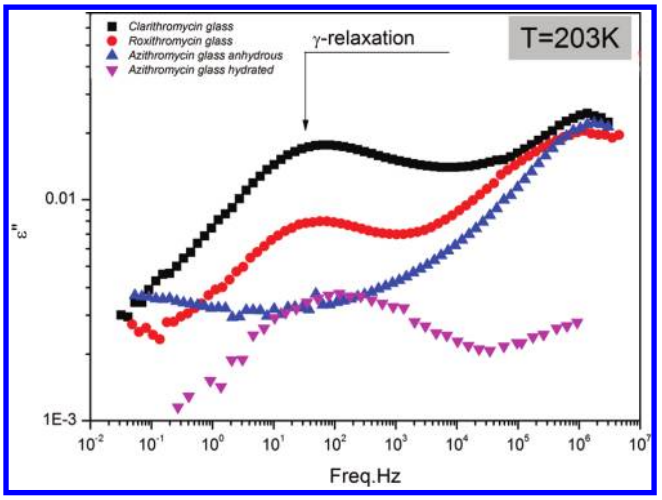

Figure 9. Comparison of the relaxation processes in the glassy state of antibiotics at T = 203 K.

Figure 10 we have plotted on the same graph δ-relaxation peaks at the same temperature (T = 143 K) that were recorded for

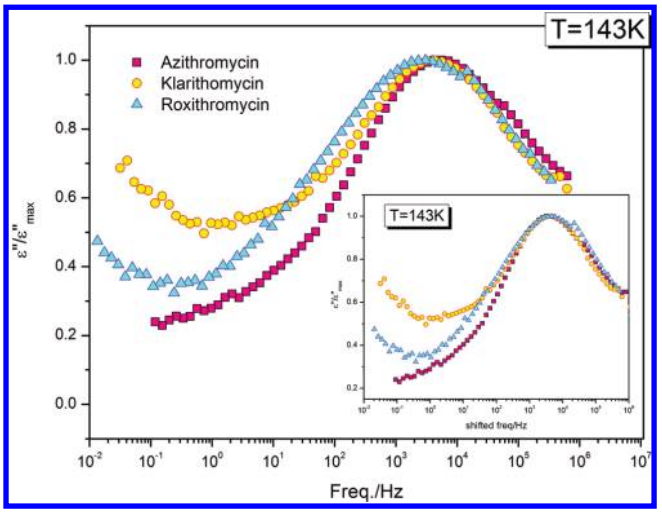

Figure 10. Comparison of the δ-relaxation in the glassy states of antibiotics at T = 143 K. The inset presents the same, superimposed dielectric loss spectra.

each herein studied antibiotic. As can be seen the shapes and relaxation times of the δ-peaks in the glassy state of examined antibiotics were found to be almost the same. From that comparison it becomes evident that this relaxation process observed for the examined group of compounds has most probably the same molecular origin.

As presented by us previously, long-term stability of amorphous APIs can be monitored using X-ray diffraction technique, which is in that case a very sensitive tool in detecting even few percentages of crystallinity. This way of studying the physical stability of amorphous materials especially at storage temperature is certainly needed. On the other hand, it might be very tiring especially when long-term studies are required. However, as shown in some of our previous papers, the physical stability of pharmaceuticals in their glassy state can be predicted based on dielectric data, with no need of prolonged X-ray diffraction studies.[39−41] It is worth mentioning that very good correlation was found between both methods.

Prediction of the physical stability of a system in the glassy state based on dielectric data requires the obvious assumption that molecular mobility is the key factor responsible for the devitrification process. From dielectric measurements we get information about the global mobility (structural relaxation) of examined material, considered by many scientists as one of the most important parameters determining the physical stability of amorphous drugs and biomaterials. As presented by us in the previous part of this paper, direct measurement of structural relaxation times below the glass transition temperature is fairly impossible due to time scale exceeding years. Nevertheless, we are still able to estimate the degree of global molecular mobility in the glassy state. This can be done by construction of a master plot, i.e. horizontal shift of the α-relaxation peak from the region above T_g, to lower temperatures in order to overlap with dielectric spectra collected below T_g for which only the high frequency flank of the α-peak is visible. This approach required assumption that the time−temperature superposition of the α-peak is valid from the region just above T_g to the region below T_g. The structural relaxation times of amorphous antibiotics determined in this way were added on the relaxation map and are presented in Figure 11. As illustrated, for all

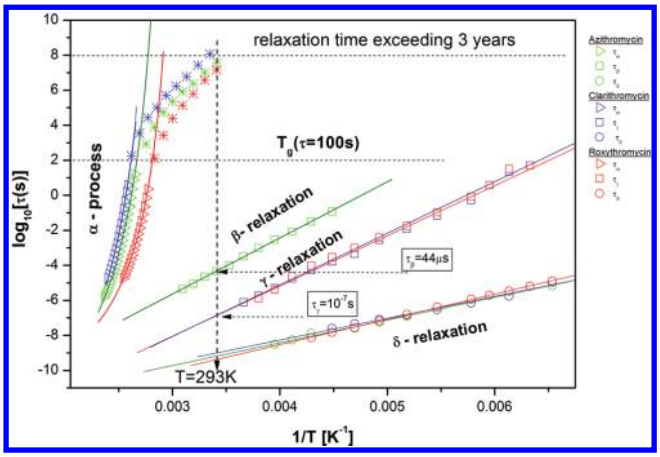

Figure 11. Prediction of the time scale of molecular motion in the glassy state of antibiotics at storage temperature T = 298 K. The α-relaxation times in the glassy state were estimated on the basis of the master plot, i.e. by horizontal shift of the α-peak from the region aboveto the temperatures below T_g (star points).

investigated materials at room temperature the time scale of molecular motions associated with structural relaxation exceeds more than one year. Consequently amorphous antibiotics prepared using vitrification method should be physically stable during their typical shelf life. This supposition is still verified by us regularly.

Finally, we would like to refer to some of the suggestions that the local mobility can play a potential role in recrystallization from the amorphous state. As can be found in the literature, in some of the cases good correlation between crystal growth rate and local mobility was found. However, if this is true and simple relationship between degree of local mobility and devitrification of amorphous materials exists, antibiotics should immediately recrystallize at 295 K, since their local motions are significantly faster than global mobility, i.e τ_β = 44 μs (azithromycin) and τ_γ = 10^{-7} s (clarithromycin and roxithromycin).

Thermal Properties of Amorphous Antibiotics. Figure 12 presents DSC thermograms of amorphous antibiotics recorded

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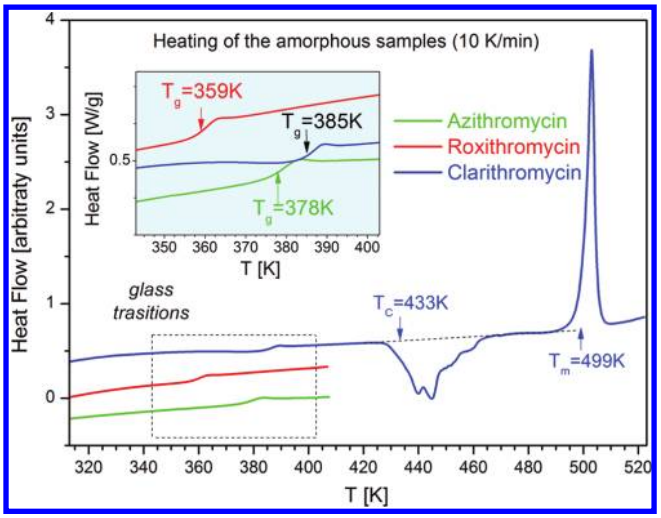

Figure 12. DSC thermograms of antibiotics performed on heating of the amorphous samples (10 K/min). The inset enlarges the glass transition region.

at a heating rate of 10 K/min. A characteristic for the glass transition event jump in the heat flow was observed. The glass transition temperatures of antibiotics (determined as a midpoint of the glass transition step) are as follows: azithromycin T_g = 378 K, clarithromycin T_g = 385 K and roxithromycin T_g = 359 K. These values are slightly higher than that obtained from dielectric measurements, although such a difference (~3 K) is nothing unusual.

Upon heating of amorphous samples non-isothermal crystallization occurred only in the case of clarithromycin, at 433 K. Then, at higher temperatures the melting endotherm (T = 499 K) was observed. It is worth remembering that non-isothermal cold-crystallization occurring during heating of amorphous clarithromycin was confirmed also from dielectric studies. The increment of heat capacity in the glass transition, $\Delta C_p(T_g)$, i.e. the difference between heat capacity of liquid and glass, was also calculated. The following values were obtained 0.38 $J \cdot g^{-1} \cdot K^{-1}$, 0.29 $J \cdot g^{-1} \cdot K^{-1}$, 0.37 $J \cdot g^{-1} \cdot K^{-1}$ for azithromycin, clarithromycin and roxithromycin, respectively. The heat capacity step at the glass transition is often correlated with fragility of the glass-former. Fragile glass-formers should have higher values of the $\Delta C_p(T_g)$. Unfortunately, this correlation does not work properly for antibiotics, because from dielectric studies we know that all three samples are fragile materials, while DSC measurements revealed that instead of high values of $\Delta C_p(T_g)$ they are characterized by relatively low values of $\Delta C_p(T_g)$. A similar result (large kinetic fragility and low value of heat capacity step at T_g) was reported in the past for decaline (m = 146, of $\Delta C_p(T_g)$ = 0.35[42]) or celecoxib (m = 110 [38], $\Delta C_p(T_g)$ = 0.26[43]). Moreover, $\Delta C_p(T_g)$ provides relative measure of number of accessible molecular conformations at T_g and as suggested by Gupta et al. might be helpful in predicting crystallization abilities of amorphous materials.[43] As claimed based on flopropione data, large values of heat capacity change at T_g lead to more stable glass. However, amorphous antibiotics having low values of heat capacity steps at T_g revealed extended physical stability confirmed by X-ray diffraction. Because of that prediction of physical stability and determination of fragilities of glass-forming materials on the basis of simple calorimetric parameters is not as obvious, and does not apply well to all investigated systems.

Solubility Studies. As mentioned in the Introduction, the antibiotics examined herein are poorly water-soluble, which results in their lower bioavailability. Because of that reason relatively large quantities must be administered in order to achieve the desired therapeutic effect, which might result in potential increase in the side effects. The use of large quantities of drugs is certainly unwanted due to production and manufacturing costs. Because of that reason amorphous pharmaceuticals having greater solubility profile attract considerable attention nowadays. For the antibiotics examined here we have also checked the experimental solubility gain given by the amorphous form. Results are presented in Figures 13−15 and collected in Tables 5−7.

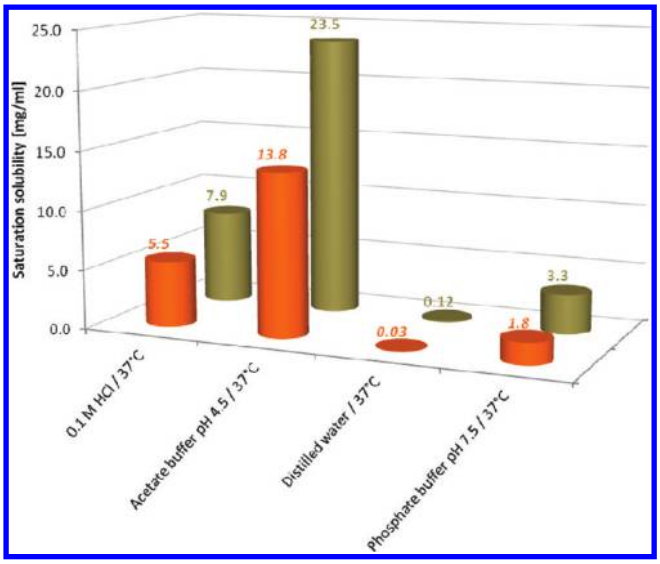

Figure 13. Comparison of saturated solubility of various forms of azithromycin in different media at 37 °C. Orange bars visible in the front of the chart represent solubility of crystalline azithromycin, whereas gray bars visible in the back of the chart represent solubility of its amorphous counterpart.

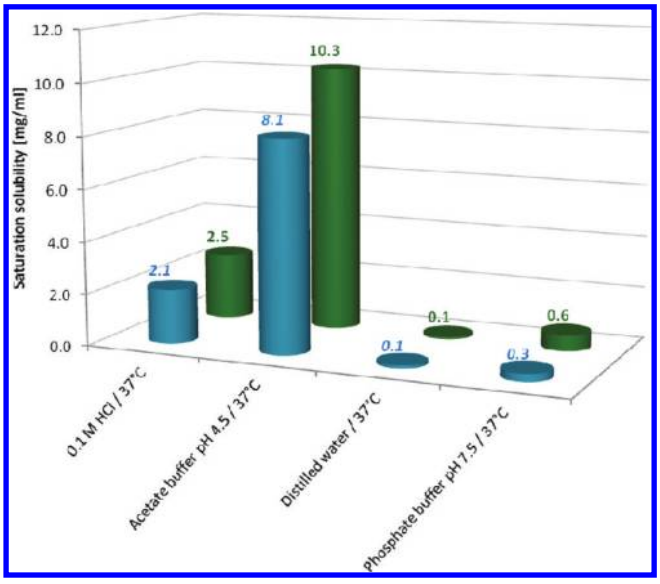

Figure 14. Comparison of saturated solubility of various forms of clarithromycin in different media at 37 °C. Blue bars visible in the front of the chart represent solubility of crystalline clarithromycin, whereas green bars visible in the back of the chart represent solubility of its amorphous counterpart.

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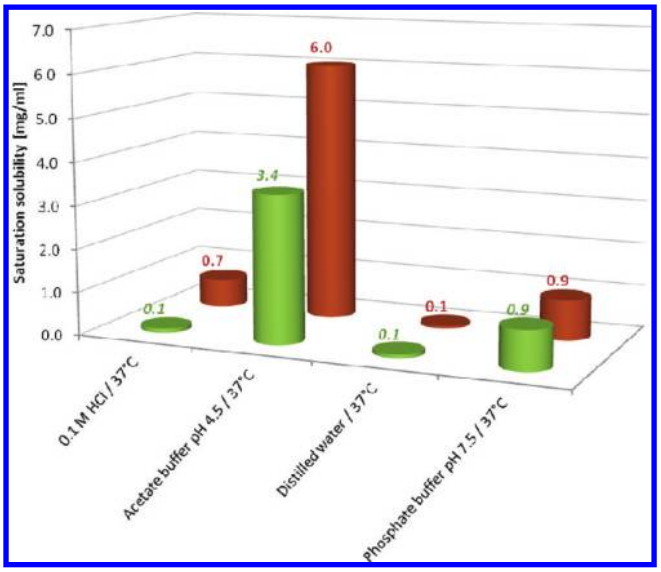

Figure 15. Comparison of saturated solubility of various forms of roxithromycin in different media at 37 °C. Green bars in the front of the chart represent solubility of crystalline roxithromycin, whereas brown bars in the back of the chart represent solubility of its amorphous counterpart.

Table 5. Comparison of Saturated Solubility of Crystalline and Amorphous Azithromycin in Different Media at 37 °C

	saturation solubility [mg/mL]	
media	crystalline	amorphous
0.1 M HCl	5.5	7.9
acetate buffer pH 4.5	13.8	23.5
distilled water	0.03	0.12
phosphate buffer pH 7.5	1.8	3.3

Table 6. Comparison of Saturated Solubility of Crystalline and Amorphous Clarithromycin in Different Media at 37 °C

	saturation solubility [mg/mL]	
media	crystalline	amorphous
0.1 M HCl	2.1	2.5
acetate buffer pH 4.5	8.1	10.3
distilled water	0.1	0.1
phosphate buffer pH 7.5	0.3	0.6

Table 7. Comparison of Saturated Solubility of Crystalline and Amorphous Roxithromycin in Different Media at 37 °C

	saturation solubility [mg/mL]	
media	crystalline	amorphous
0.1 M HCl	0.1	0.7
acetate buffer pH 4.5	3.4	6.0
distilled water	0.1	0.1
phosphate buffer pH 7.5	0.9	0.9

Generally solubility of all antibiotics under investigation was found to change with the pH of medium. The saturation solubility of the drug substances was maximum in slightly acidic conditions (pH 4.5). Considering the form of antibiotics grater solubility in physiological pH range was observed for amorphous forms than for crystalline forms, which is an interesting result for further biopharmaceutical considerations. Comparison of the amorphous form of azithromycin with raw material (crystalline) revealed 1.5-fold improvement in solubility in acidic medium, 1.7-fold improvement in pH 4.5 medium and even 4-fold improvement in distilled water. Referring to clarithromycin data (Figure 14 and Table 6), the solubility of its amorphous form in 0.1 M HCl is 1.2-fold greater and 1.3-fold greater in pH 4.5 medium compared to crystalline. Finally, the glassy state of roxithromycin shows also a significant improvement of solubility, even 7-fold in 0.1 M HCl and 1.8-fold in acetate buffer as a medium.

SUMMARY AND CONCLUSION

In this paper the molecular dynamic in the supercooled and glassy state of antibiotics azithromycin, clarithromycin and roxithromycin was thoroughly analyzed. Dielectric relaxation measurements revealed multiple relaxation processes: α, β (well-pronounced only in the case of azithromycin), γ and δ. The presence of an excess wing in the supercooled liquid state of clarithromycin and roxithromycin indicates for secondary β-relaxation of intermolecular origin. Primary and secondary relaxations provided us with complete information about the molecular mobility in the supercooled liquid and glassy states of antibiotics. From analyzing the temperature dependences of structural relaxation times it was found that antibiotics are fragile glass-formers ($m > 100$).

In the glassy states of the samples examined herein two secondary relaxations of most probably intramolecular origin were identified. Approximately the same activation energies of the δ-relaxations found for all materials investigated herein indicates involvement of the same molecular motions. We have also shown that the γ-relaxation that occurs in the glassy state of antibiotics must be of the same origin, and is in some way related to the water confined dynamics. The γ-relaxation, originally invisible in the glassy state of vitrified azithromycin, becomes well pronounced in the hydrated glassy sample. Moreover activation energy of the γ-relaxation of antibiotics is close to that found for the relaxation of water confined.[44,45]

In the supercooled liquid state of antibiotics decoupling between dc-conductivity and α-relaxation takes place. The fractional Debye−Stokes−Einstein (FSDE) equation with fractional exponent $s = 0.67$ satisfactorily describes the relationship between rotational and translational motions of supercooled clarithromycin. For azithromycin and roxithromycin as temperature decreases and τ_α reaches $0.005s$ ($\log \tau_\alpha = -2.3$), change in the slope of the $\log \tau_\alpha$ verus $\log \sigma$ occurs, i.e. s decreases from $s = 0.66$ to $s = 0.46$ (azithromycin) and $s = 0.69$ to $s = 0.49$ (roxithromycin). This type of behavior was never reported for any glass forming liquid before. Thus, we may only speculate that it might be related to the developing of the H-bonded network structures in both antibiotics with lowering temperature. Consequently, besides the translation of the ions, additional proton conductivity starts to play a key role.

Results presented in this work clearly demonstrate that universal description of crystallization abilities of glass-forming liquids based only on dynamical properties is further more complicated than expected. First, there is no correlation between fragility and physical stability below as well as above T_g. Second, from the width of the α-relaxation peak one cannot get any valuable information concerning physical stability of antibiotics. Thus, for antibiotics we do not observe the common pattern of behavior postulated in the past for less complicated systems. It can be also concluded that dynamics alone cannot be responsible for recrystallization, but can facilitate it.

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Thus, there must be other factors of greater importance that governs stability of glass-formers such as thermodynamic fator or molecular conformations.

We suppose that antibiotics are characterized by complex chemical bonding which might be related to the difference in their physical stability. Out of all antibiotics investigated by us clarithromycin is the only one which may reveal hemiacetal tautomerism, analogous to that known for erythromycin.[46] Roxithromycin and azithromycin are not able to create cyclic hemiacetals, which implies that there are more hydroxyl groups available to create hydrogen bonding networks. We suppose that the differences in crystallization tendencies of antibiotics in the liquid state might be related to the differences in hydrogen bonding patterns in 14-membered lactone ring. Nevertheless, we do not exclude the prominent role of thermodynamic factor as the least stable antibiotic in the supercooled state, clarithromycin, is characterized by the lowest value of $\Delta C_p(T_g)$. Unfortunately, we were unable to extract more detailed information about thermodynamic properties of antibiotics, especially azithromycin and roxithromycin, because they become amorphous as a result of dehydration of crystals at temperatures much below expected melting points. This makes it impossible to evaluate proper values of configurational parameters as well as heat of fusion values. Nevertheless, in the context of crystallization abilities considerably more attention should be paid to thermodynamic properties and chemical bonding of investigated materials. In this paper we have also made an attempt to predict the physical stability of amorphous antibiotics on the basis of the time scale of molecular motions in the glassy state. We found that structural relaxation times of antibiotics at the storage temperature should exceed years, while the local motions are significantly faster. Thus, we presume that long-term physical stability of amorphous antibiotics is related rather to global than local mobility.

Finally, we have also performed solubility studies, which unquestionably showed that amorphous antibiotics might be significantly better soluble then their poorly soluble crystalline counterparts. Considering the form of antibiotics greater solubility in physiological pH range was observed for amorphous forms than for crystalline forms. In some cases even 7-fold improvement of solubility was obtained. This is certainly a very promising result for further pharmaceutical considerations.

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Notes

The authors declare no competing financial interest.

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