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Chemometrics analysis of insulin aggregation induced by an antiretroviral drug (AZT)

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ABSTRACT

The analysis of the effect of drugs in insulin aggregation is interesting due to its influence on human metabolism. We present a method to analyse the effect of an antiretroviral drug (AZT) on the insulin aggregation, based on the principal components analysis of the infrared spectra recorded in the process. This method allows the analysis of the conformational changes involved in the aggregation, providing a time value associated with the aggregates formation. The analysis was performed using Zn-free insulin in buffered solution at 1.72 mM concentration. The aggregation was monitored *in situ* by FTIR at 37 °C and the dependence of the concentration of AZT was considered, contemplating three concentration levels of AZT. The interaction between AZT and insulin was proved by ^{1}HNMR spectroscopy. The aggregation states were characterised by solubility tests and microscope imaging. A comparative analysis of time aggregation led observing that the process was delayed when the concentration of AZT was lower than 1.72 mM, while it accelerated at equal or higher concentrations. This dependence was related to the concentration of the monomeric species in the medium. The analysis was performed in serum to evaluate the influence of serum proteins in the phenomena, concluding that AZT binds to insulin before than to serum proteins.

1. Introduction

Insulin is a protein that has a key role in the glucose metabolism. Although insulin functions in the bloodstream take place as a Zn-free monomer, it can assemble into dimers or hexamers, the latter in presence of Zn [1–5]. The aggregation of insulin monomers to form oligomers is an intriguing and widely studied phenomenon [6–9]. This process takes place due to the natural propensity of insulin to self-assemble and it is affected by, among others, temperature [3,5,10–12], acidity [11–13], ionic strength [5,13,14] and the presence of other molecules [15,16].

AIDS (Acquired Immune Deficiency Syndrome) patients under a prolonged treatment of AZT (Zidovudine, 3′-azido-3′-deoxy-thymidin) frequently develop insulin resistance, a physiological condition where the natural insulin become less effective [17,18]. The presence of drugs in the bloodstream may be related with this disease as long as drugs are able to modify the natural aggregation of insulin.

Bioinformatics tools applied to laborious experimental data have proved useful for timely providing deep insights on the drugs behaviour in biological medium both in basic research and drug development [19–23]. In this context, the aim of the present work was to conduct, for the first time, a chemometrics analysis of the insulin aggregation process induced by the antiretroviral drug AZT.

To investigate the insulin aggregation, many spectroscopic techniques have been employed, including circular dicroism (CD) [24,25], fluorescence [26,27], ^{1}H NMR [28,29] and Raman spectroscopy [30,31]. Infrared (IR) spectroscopy is commonly employed in the analysis of the structural changes of proteins [32–34]. This technique easily allows the monitoring *in situ* of the aggregation, so that has been selected in the present work. In this region, the amide I band is a sensitive marker of peptide secondary structure, as the vibrational frequency of each C O bond depends on hydrogen bonding and the interactions between the amide units, both of which are influenced by the secondary structure.

The analytical methodology proposed in this work is based on the Principal Components Analysis (PCA) of the recorded FTIR spectra. Chemometric methods based on factor analysis have been proved to be powerful techniques for the study of other protein processes [35–38]. Among them, PCA is a useful tool to evidence physicochemical transitions during conformation changes of proteins and other biological complex systems [39–43]. The insulin aggregation induced by AZT is studied for the first time using both techniques.

The analysis was performed using Zn-free insulin, initially in aqueous solution at 1.72 mM concentration. Then, the analysis was carried out in a serum matrix to mimic the human body conditions and to evaluate the competitiveness between insulin and serum proteins.

The aggregation was monitored *in situ* by FTIR at 37 °C and the dependence of the concentration of AZT was considered, so that three experimental runs at different insulin:AZT molar ratios (1:0.25, 1:1 and 1:2) were performed.

The time-dependent spectral changes in the amide I region, characteristic of the secondary structure of proteins, were analysed by Principal Component Analysis (PCA). The scores plots were

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representative of a two state process. The aggregates were characterised by solubility tests and microscope imaging. PCA analysis provided a time value between both stages, useful to analyse the AZT effect on the insulin aggregation. In absence of AZT, a unique transition time at 38 minutes was detected.

The interaction between AZT and insulin, proved by ^{1}H NMR spectroscopy, and the analysis of the effect of AZT in the studied phenomenon, shows that depends on its concentration. The aggregation was delayed at a concentration of AZT lower than 1.72 mM, whereas at an equal or higher concentration, this process went faster and throughout the formation of soluble or/and insoluble aggregates depending on the concentration of AZT. The analysis was also performed in a serum matrix to evaluate the influence of serum proteins in the process, concluding that AZT binds to insulin before than to serum proteins.

In our opinion, the biological significance of the results not only resides in contributing and understanding causes involved in insulin resistance observed after the prolonged treatment with this drug, which is actually a challenge, but also in controlling the intake of this drug in the human metabolism cycle.

2. Materials and methods

2.1. Chemical reagents

Zn-free human insulin in a HEPES sodium salt buffer (pH = 8.2) at a concentration of (1,72 mM) was purchased from Sigma-Aldrich (St. Quentin Fallavier, France) and used without further purification. The pharmaceutical product 3′-azido-3′-deoxythymidine (Zidovudine, AZT) was purchased in a commercial chemist. A human serum type AB was acquired from Sigma-Aldrich at stored at −20 °C. All reagents were used as received.

2.2. Experimental procedure and FTIR acquisition

The aggregation of native insulin was studied first at a concentration of 1.72 mM. This concentration value was chosen because it is within the interval of concentrations used in other aggregation studies. Secondly, insulin was mixed with AZT and the aggregation of this "altered insulin" was studied. Two sets of three experimental runs at different insulin:AZT molar ratios (1:0.25, 1:1 and 1:2) in absence or presence of human serum were monitored. In addition, three control experiments with the same AZT quantity in human serum were carried out. The experimental procedure involved mixing the necessary amounts of insulin and AZT. Immediately, a drop of the mixture was placed on a small diamond crystal in the spectrophotometer ATR cell (FTIR 680 Plus JASCO and a RS232 Control) to take the measurements, which it was continuously purged with N_2 during the FTIR analysis. The FTIR spectra were acquired *in situ* every minute during 45 minutes, in the spectral range 1550–1750 cm^{-1}. The CO_2 contribution was removed with the control software Spectra Manager before exporting the spectra into Matlab [44]. All experiments were carried out at 37 °C to mimic the human body conditions.

2.3. Chemometrics analysis

The recorded infrared spectra from experiment n were arranged as rows in matrices $\mathbf{D}_n$ of size 45×209. Firstly, Savitzky–Golay smoothing was applied to all data sets to suppress the instrumental noise. Then, spectra were normalised to have the same area. PCA of the mean-centred spectral data was used to reveal spectral trends over time. PCA is a multivariate representation technique that decomposes the spectral data matrix as the product of two reduced-size matrices: a scores matrix, which corresponds to a compressed view of the spectra, and a matrix of loadings that contains the contribution of every original variable in the compressed data. The scores and loadings plots were used to detect groups of the spectra recorded throughout time and to relate them with the spectral bands and the involved changes.

2.4. Auxiliary instrumental techniques

^{1}H NMR spectra were performed in a Varian Gemini 300 NMR spectrometer at 75.4 MHz operating with proton noise decoupling.

The optical images were collected using a Leica DM 2500 confocal microscope, with a 100 W illumination system and using a 10× objective.

3. Results

3.1. Native insulin aggregation analysis

Infrared normalised spectra of insulin (1.72 mM) acquired *in situ* over 45 minutes are presented in Fig. 1. Although in Zn-free buffered solution insulin exists mainly as monomer [2,5], its propensity to self-associate leads to a small percentage of dimer species in equilibrium with the monomer [31]. The coexistence of both forms is revealed by the 1636 cm^{-1} band, that is indicative of the amide I marker for the β-sheet modes in the dimer, and the band at 1651 cm^{-1}, that corresponds to the α-helical structure present in both the monomeric and dimeric forms [34]. Regarding the spectral evolution, a shift of the maximum absorbance from 1636 cm^{-1} to 1651 cm^{-1} suggests a partial loss of the initial β forms of insulin throughout time at the experimental temperature.

Insulin aggregation induced by the self-association of the native protein is commonly described in the literature. The pathway of this process can be represented as:

$$\text{Native insulin} \rightleftharpoons \text{Partial unfolding} \longrightarrow \text{Aggregates}$$

where the formation of aggregates requires the partial unfolding of native insulin, which is responsible of the irreversibility of this process. The extension of this process is proportional to the concentration of monomers in the native protein [7].

According to the literature, some spectral changes related to the aggregates formation are expected, specifically the transition from a α-helix (1651 cm^{-1}) to a β-sheet structure (1625 cm^{-1} and 1680 cm^{-1}) [45–49]. Although these spectral changes were not observed in Fig. 1, the decrease in intensity of the absorption bands

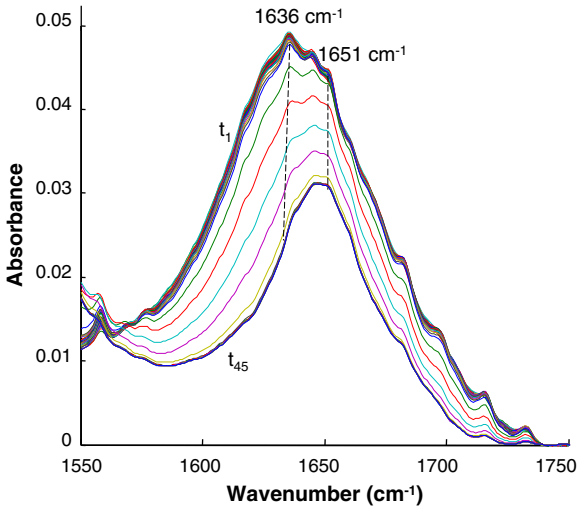

Fig. 1. Infrared spectra of the native insulin obtained every minute at 37 °C, during 45 minutes.