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Monomeric stabilization of insulin in solution

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Dedicated to Prof. Dr. G. Zessin, Halle (Saale) on the occasion of his 65th birthday

The primary condition necessary to achieve absorption of insulin via a mucous membrane is the prevention of association occurring. Transport which is not dependent on an enzymatic degradation of the molecule is only possible in monomeric form. Various substances such as alkylsaccharide, D-(+)-glucose and urea were tested with respect to their effect on the prevention of a molecule association and on stability of the insulin molecule. It could be shown that the addition of the alkylsaccharide dodecyl-β-D-maltosid causes the desired monomeric distribution of the insulin molecules in a pH 7.4 buffer solution, and that this is also connected with a stabilization of the molecule.

1. Introduction

The success of peptide and protein oral formulations is attributed to the overcoming of two main physiological barriers in the gastrointestinal (GI) tract: the enzymatic degradation [1–3] and the absorption through the intestinal mucosa [4–8]. In the first case, enzymatic inhibitors are incorporated into the formulation as a common practice to reduce the degradation of peptides and proteins in their passage through the GI tract [9–12], while in the second case other substances, for instance surfactants with relatively strong effects, are also included in order to enhance the mucosa membrane permeability [13–15]. However, this effect is mostly based on a temporary disruption of the intestinal mucosa [16, 17].

Theoretically, if both resistances to the insulin biological activity are considered as simultaneous competitive processes, an increase in the absorption rate through the intestinal mucosa will assure a relatively important lowering of the insulin inactivation due to enzymatic degradation [18]. Although the intestinal absorption of drugs has been shown to be a biochemical process of high complexity depending on several mechanisms [19], it has also been demonstrated that the absorption of peptides and proteins through the intestinal mucosa is mainly limited with regard to the molecular weight and consequently, to the size of the molecule [20].

It is well known that insulin shows a high tendency to form aggregates [21], based on a mechanism which subsequently goes from monomer to dimer to hexamer forms [22–24]. Obviously this particular feature of the insulin molecule conspires against the absorption rate through the intestinal mucosa. Hence, in addition to the secondary and tertiary structure, insulin also shows a quaternary structure related to the self-association of the molecule, which depends mainly on insulin concentration in solution and pH, among other conditions. Insulin exists as a monomer only at low concentrations ($<0.1\ \mu M\ \sim 0.6\ \mu g\ ml^{-1}$) and dimerizes as concentration increases, where the dimeric fraction in solution stays in equilibrium with the remanent monomeric fraction. At concentrations $>2\ \mu M$ the equilibrium is shifted to the formation of hexamers as the predominant form. At the same time, other studies performed at a fixed concentration of $1\ mg \cdot ml^{-1}$ established a prevailing of dimer configurations at low pH and monomers at high pH, while at neutral pH the associational state was predominantly the hexamer form [25].

It has been also well documented that this aggregational tendency is mainly assigned to residues located in the B chain of the molecule at the C-terminal strand, identified as segment B21–B29 [26, 27], which also shows some variability in its conformation. Thus in the dimer, the two insulin molecules are held together through these residues by predominantly non-polar forces and reinforced to some extent by hydrogen bonds in an antiparallel β-sheet structure.

If a passive transport mechanism, which is paracellular or transcellular, is taken as the main route for insulin absorption, it is desirable to have the lowest association form of the insulin present at the absorption site at this process is size-dependent. In this context it has been established that the size of the insulin monomeric form is approximately 1.1–1.3 nm; 1.9 nm for the dimeric state and about 2.8 to 3.5 nm for the hexameric state while on the other hand, intestinal pore sizes have been found to range between 0.71–1.6 nm. Therefore it is important to keep the insulin molecular weight as low as possible in order to facilitate the absorption rate [28].

In the present study, trials with different substances such as alkylsaccharides, D-(+)-glucose and urea were carried out in an attempt to reduce the association tendency of insulin to a minimum via complexation in order to stabilize the molecule in subsequent oral formulations.

2. Investigations, results and discussion

As a first stage of the experiment, all flasks were at rest and at room temperature during 12 days while 4 circular dichroism (CD) measurements were performed at 4 days intervals taking 2 ml samples from each flask. In the insulin sample without stabilizer the corresponding typical CD spectrum was obtained (Fig. 1), where two peaks were observed at 210 nm ($[\theta] = -10.73$ E 03) and 221 nm ($[\theta] = -9.33$ E 03). The peak at 276 nm could be practically ignored, hence an equilibrium between the monomeric and dimeric forms was present at this concentration ($0.1\ mg \cdot ml^{-1}$) with a monomeric form predominance, which is in full agreement with the available references. In the insulin-stabilizer mixtures, the CD spectra showed no remarkable differences as compared with the typical insulin CD spectrum shape, except in the case of the N methyl lauryl acid glucamid where the 210 nm peak was missed, and a peak at 222–223 nm resulted, which could be a sign that dimers were the predominant form in any of the proportion levels.

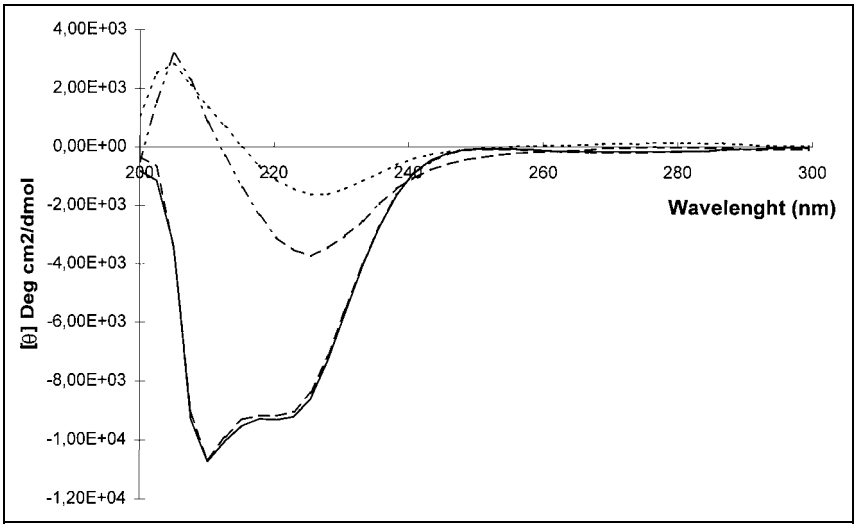

Fig. 1:
CD Spectra of insulin without any stabilizer
——— Day 0: No Shaking, Room T °C
– – – – Day 12: Before Shaking and Applying T °C
—··—·· Day 16: Shaking, 37 °C
– – – – – Day 24: Shaking, 37 °C

Using the corresponding negative peaks ratio as an indicator, it was detected in all cases that the presence of monomer relative to dimer was reduced as the proportion of the stabilizer in solution increases. This effect was less noticeable in the cases of glucose and urea than the cases of C12 and C14 maltosides and even more remarkable in the cases of glucoside 24 and N methyl lauryl acid glucamide. A possible explanation for this effect was that, as the substances could perform a change of solvent structure, a direct interaction with the insulin molecules or a complex integration of both occurred.

Surface tension measurements showed that taking the corresponding pure water parameter as a reference ($72.6 \ldots 72.8\ mN \cdot m^{-1}$), the presence of disodium hydrogen phosphate in a concentration of 0.1 M reduced this value to $59.4\ mN \cdot m^{-1}$ and the addition of insulin ($0.1\ mg \cdot ml^{-1}$) still lowered it to $46\ mN \cdot m^{-1}$. In addition a general tendency was observed that surface tension was reduced in the following order: insulin-glucose > insulin-urea > insulin-C12 maltoside > insulin-C14 maltoside > insulin-glucoside 24 > insulin-MLAglucamide. Very small differences were seen between the alkylmaltoside and between glucosid 24 and MLAglucamid (Fig. 2). Regarding the surface tension for each stabilizer from the lowest to the highest proportion level with insulin, there is no evidence of a clear dependence of the monomer/dimer presence in solution on the surface tension in each case. Hence, the decreasing amount of monomers relative to dimers in their equilibrium state could be explained as a complex integration of the change in the solvent structure influencing the surface tension as an effect, and the specific interaction of each stabilizer with the insulin, according to sterical factors associated with the molecular disposition of each stabilizer in solution and the insulin molecular structure in the medium.

In another stage of the experiment all solutions were agitated in a water bath shaker at a rate of about 180 strokes $\cdot\ min^{-1}$ and temperature was set at 37 °C in an attempt to check the stabilizers performance in conditions which can largely favor an equilibrium shift to the formation of dimers and higher aggregational forms.

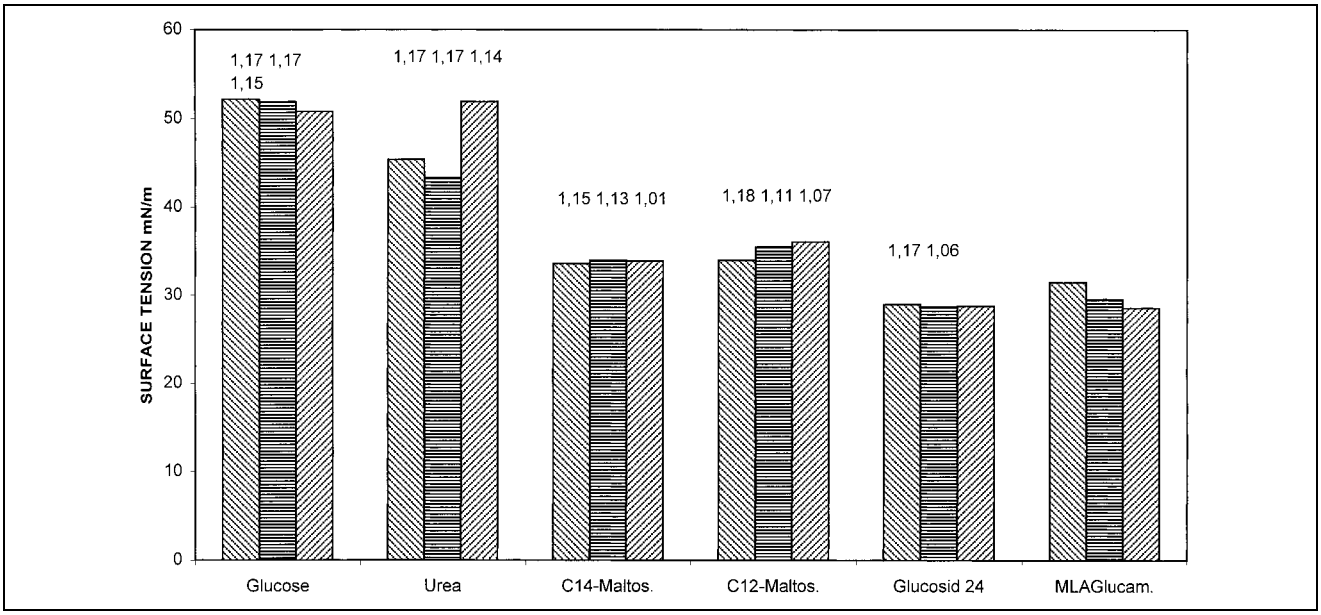

Fig. 2: Surface tension and cd spectra negative maximum ratios for insulin and stabilizer mixtures
CD Peak Ratios at Bar Top $-[q]1/-[q]2$
Low level
Middle Level
High Level
Low Level 1 : 5 w/w
Middle Level 1 : 20 w/w
High Level 1 : 50 w/w
Low Level 1 : 1 w/w
Middle Level 1 : 4 w/w
High Level 1 : 16 w/w
Insulin/Stabilizer proportions levels

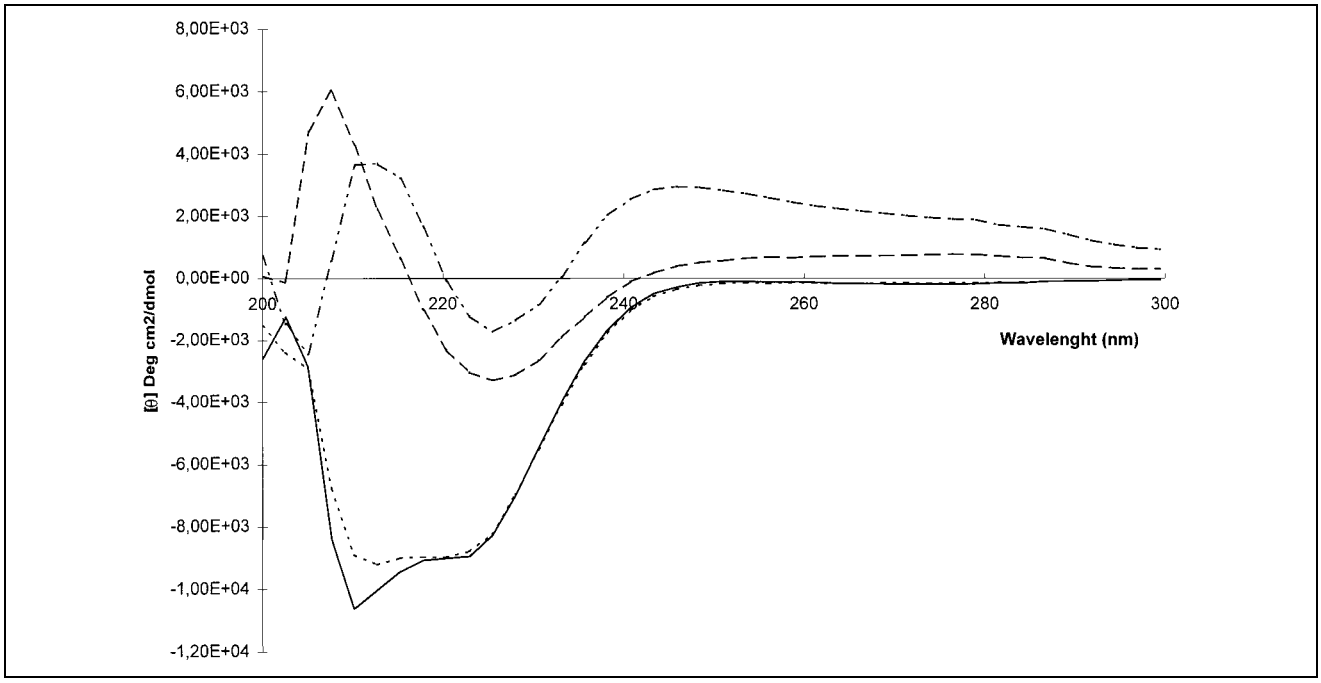

Fig. 3: CD Spectra of insulin/glucose before and after shaking and applying temperature (37 °C)
——— Day 12: Any Proportion, Before Shaking and Applying T °C
– – – – Day 16; 1 : 5 w/w
–·–·– Day 16; 1 : 20 w/w
– – – – – Day 16; 1 : 50 w/w

After several days of experiment and 12 days after all samples were submitted to shaking at 37 °C it was proved conclusively that shaking and temperature accelerate the aggregation process of insulin. This is in full agreement with all references dealing with this specific subject.
Even though a reference [29] established the stabilization effect of glucose in some proteins including insulin using similar proportions to the present experiment, it was demonstrated that no stabilization was found when shaking and temperature were applied, except in the case of the 1 : 50 w/w proportion level in which the effect was limited (Figs. 3, 4). The same was demonstrated for urea in all proportion levels. Even though a similar research paper showed that urea could stabilize the insulin in a 1 : 20 w/w proportion [30], in this case only high stirring was applied during the experiment for 7 days, hence it seems the temperature causes complete annulment of the effect.

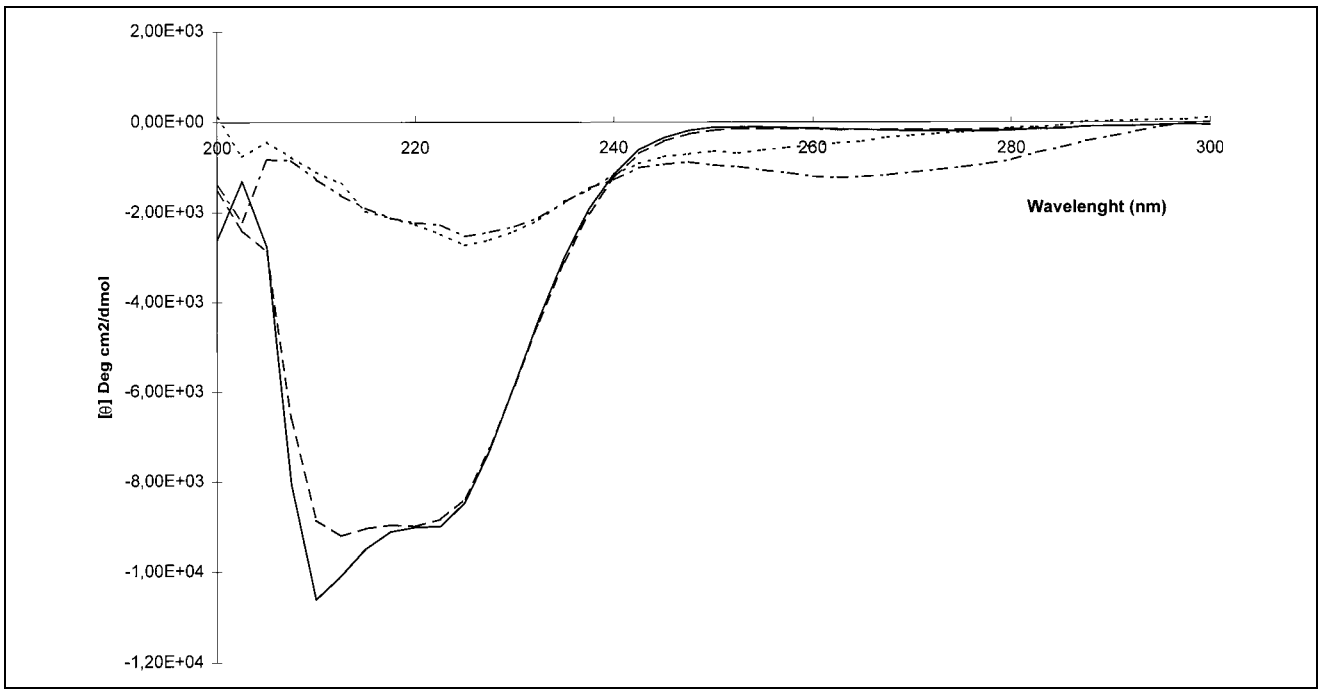

Fig. 4: CD Spectra of insulin/glucose 1 : 50 w/w before and after shaking and applying temperature (37 °C)
——— Day 12: Before Shaking and Applying T °C
– – – – Day 16
–·–·– Day 20
– – – – – Day 24

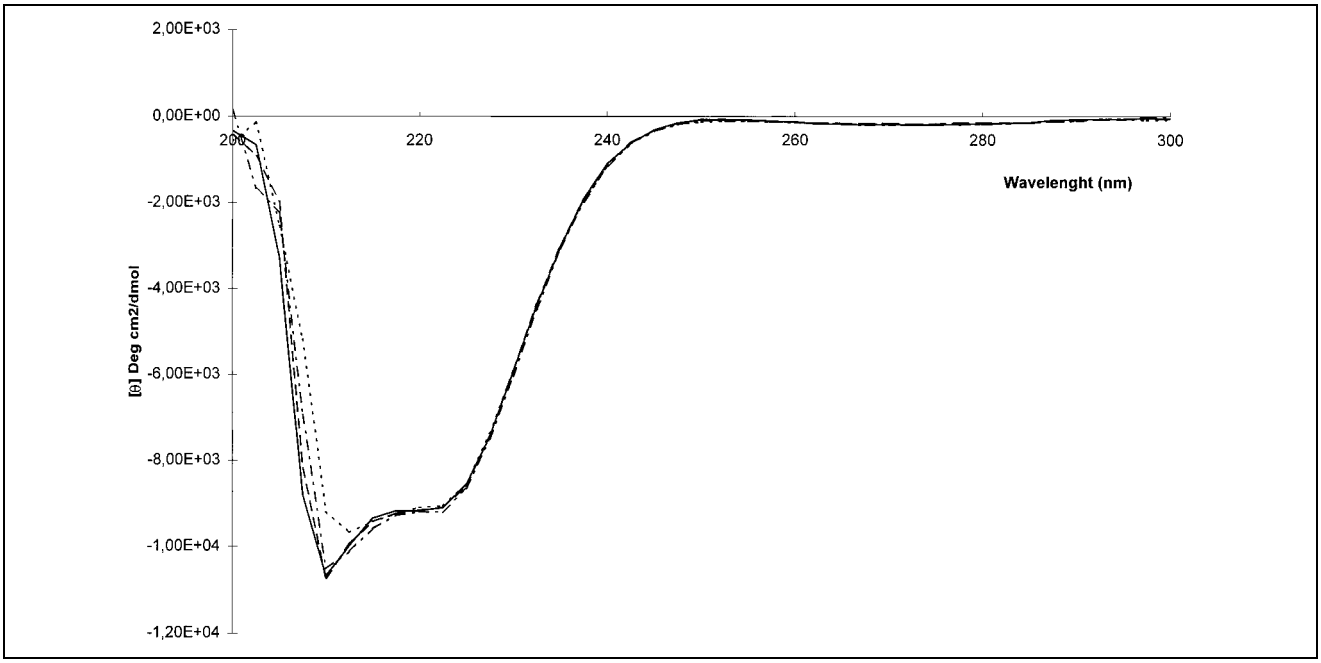

Fig. 5: CD Spectra of insulin/C12 maltoside after shaking and applying temperature (37 °C)
——— Day 12: Insulin without stabilizer, before shaking and applying T °C
– – – – Day 24; 1 : 1 w/w
–·–·– Day 24; 1 : 4 w/w
– – – – – Day 24; 1 : 16 w/w

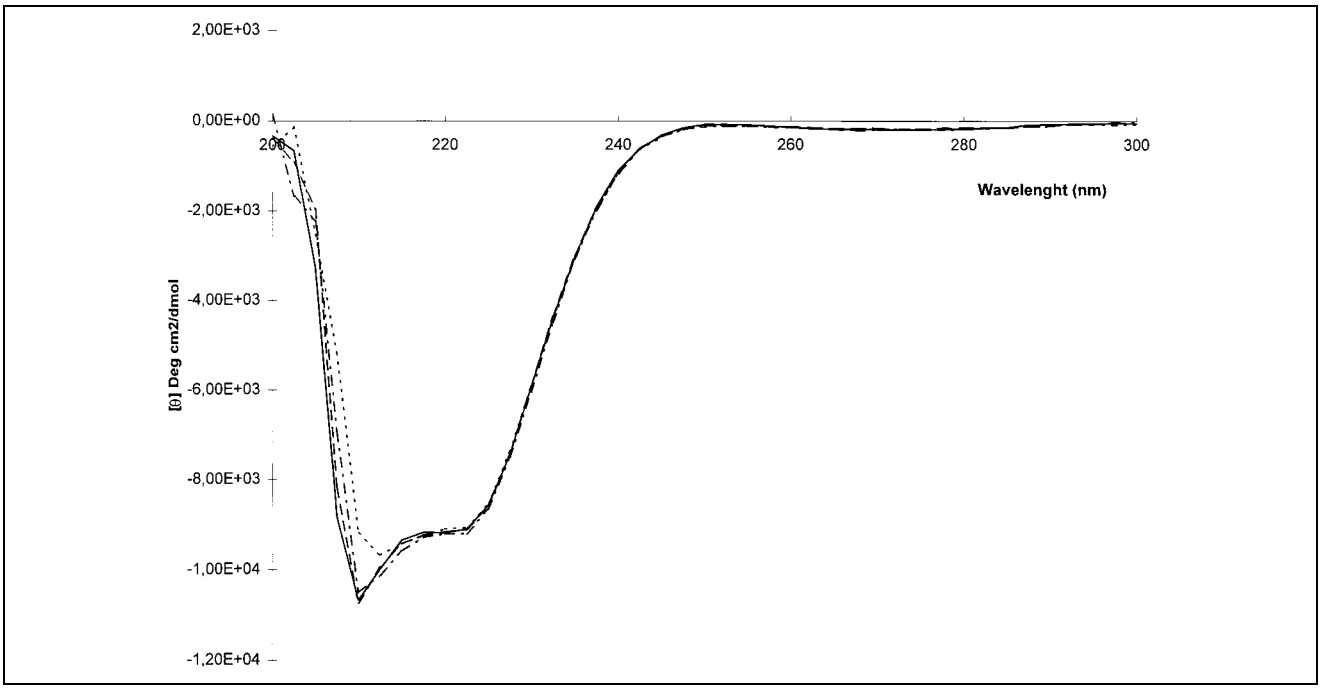

Fig. 6: CD Spectra of insulin/C14 maltoside before and after shaking and applying temperature (37 °C)
——— Day 12; 1 :1 w/w
– – – – Day 24; 1 : 1 w/w
–·–·– Day 12; 1 : 4 w/w
– – – – – Day 24; 1 : 4 w/w

The stabilizing effect of the C12 maltoside in all proportion levels under the critical conditions of this experiment (Fig. 5) was also documented. The C14 maltoside shows limitations (Fig. 6) in this sense and both results are in full agreement with similar studies recently performed [28, 31]. It is speculated that this peculiar effect of the C12 maltoside is related to the molecular structure and its ability to promote, even under critical conditions, a stable and suitable micellar system in solution which is able to penetrate the tertiary structure of insulin and to reach the residues involved in the aggregation process.

In the case of the so-called glucoside 24 and N methyl lauryl acid glucamide and as far as it is known, there are no previous references concerning the stabilization of the insulin molecule. In the first case only a limited stabilization effect was found on the 1 : 1 and 1 : 4 w/w proportion

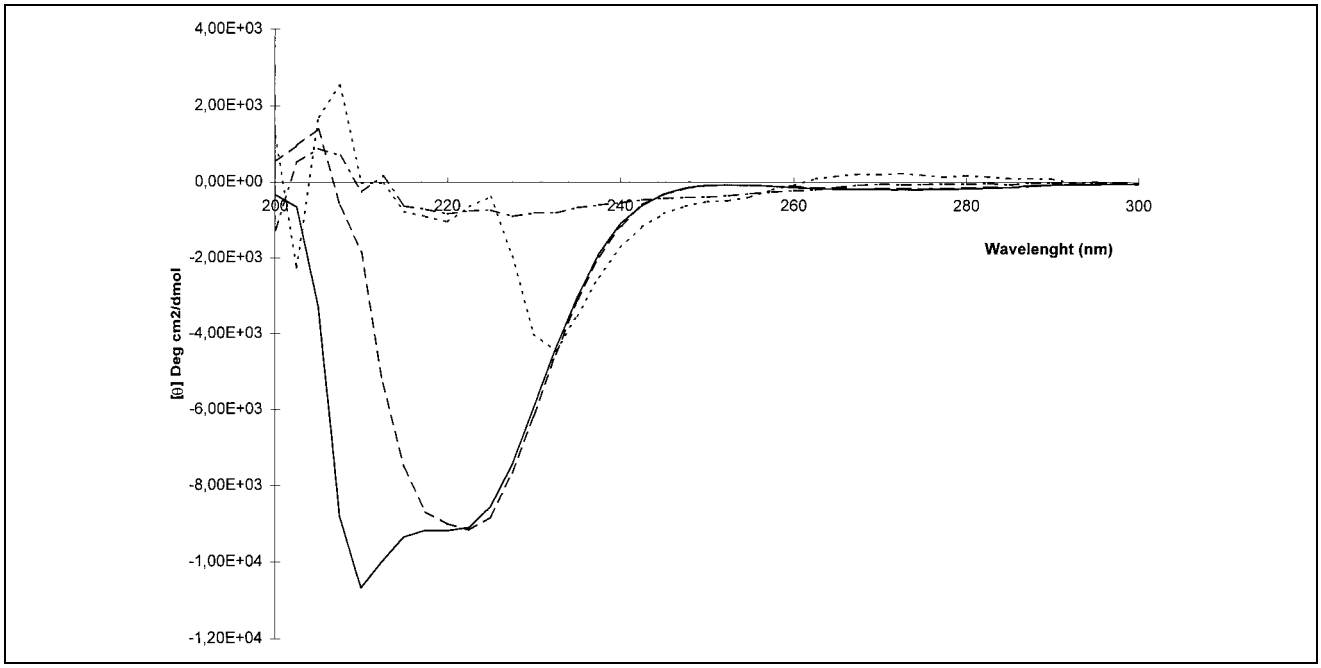

Fig. 7: CD Spectra of insulin/mlaglucamid after shaking and applying temperature (37 °C)
——— Day 12: Insulin without stabilizer, before shaking and applying T °C
– – – – Day 24; 1 : 1 w/w
—·—·— Day 24; 1 : 4 w/w
– – – – – Day 24; 1 : 16 w/w

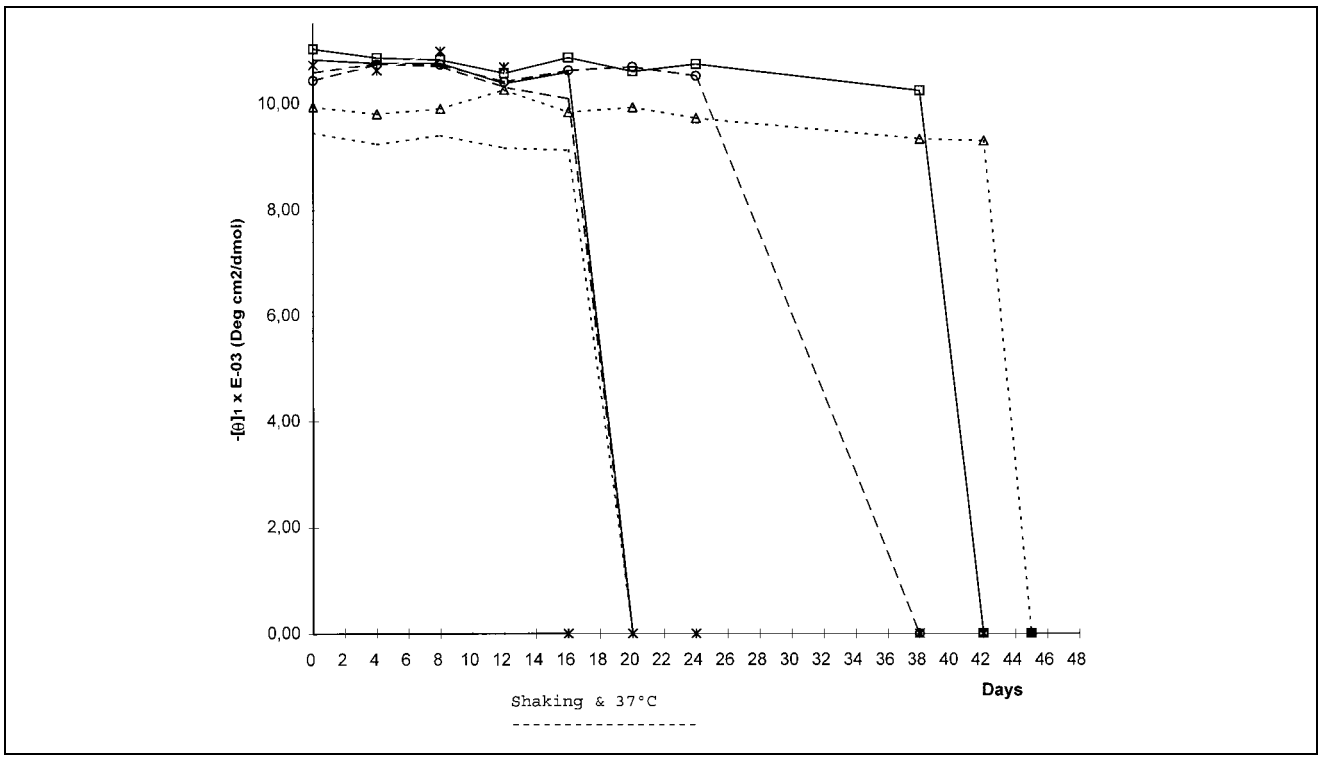

Fig. 8: Monomer peaks tendency with time for C14 & C12 maltosides
——— INS/C14 Malt. 1 : 1 w/w
– – – – INS/C14 Malt. 1 : 4 w/w
– – – – – INS/C14 Malt. 1 : 16 w/w
—□— INS/C12 Malt. 1 : 1 w/w
– –○– – INS/C12 Malt. 1 : 14 w/w
– –△– – INS/C12 Malt. 1 : 16 w/w
—✳— INS/No Stabilizer

levels, whereas in the second case the dimer form could only be specifically stabilized in the 1 : 1 proportion level (Fig. 7), as the 1 : 4 and 1 : 16 levels showed limitations. This particular stabilization effect of the dimeric form could also be explained on the basis of the specific structure of the substance molecule and the sterical interactions with the insulin terciary structure in solution, since a suitable and stable micellar system in solution is obtained.

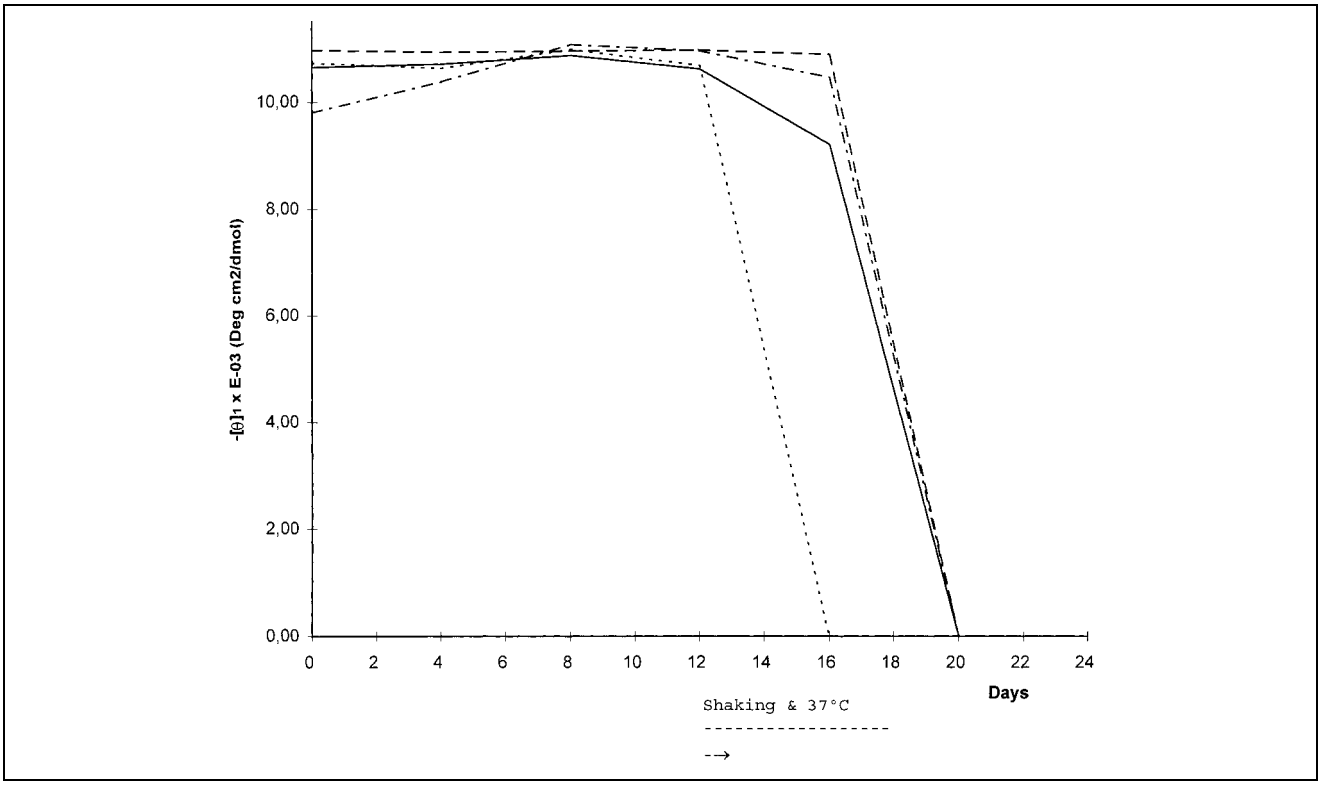

Fig. 9: Monomer peaks tendency with time for glucose and glucosid 24
——— INS/Glucose 1 : 50 w/w
– – – – INS/Glucos24 1 : 1 w/w
–·–·–· INS/Glucos24 1 : 4 w/w
– – – – – INS/No Stabilizer

A general diagram of monomer peaks during the experiment is shown in Figs. 8, 9 and the final result in Fig. 10. The C12-Maltoside shows better stabilization properties on the insulin molecule at all proportion levels and in addition it also has a nontoxic nature (Code Federal Register 21, 1986). Thus it could be considered as suitable for use in subsequent insulin oral formulations for stabilization purposes.

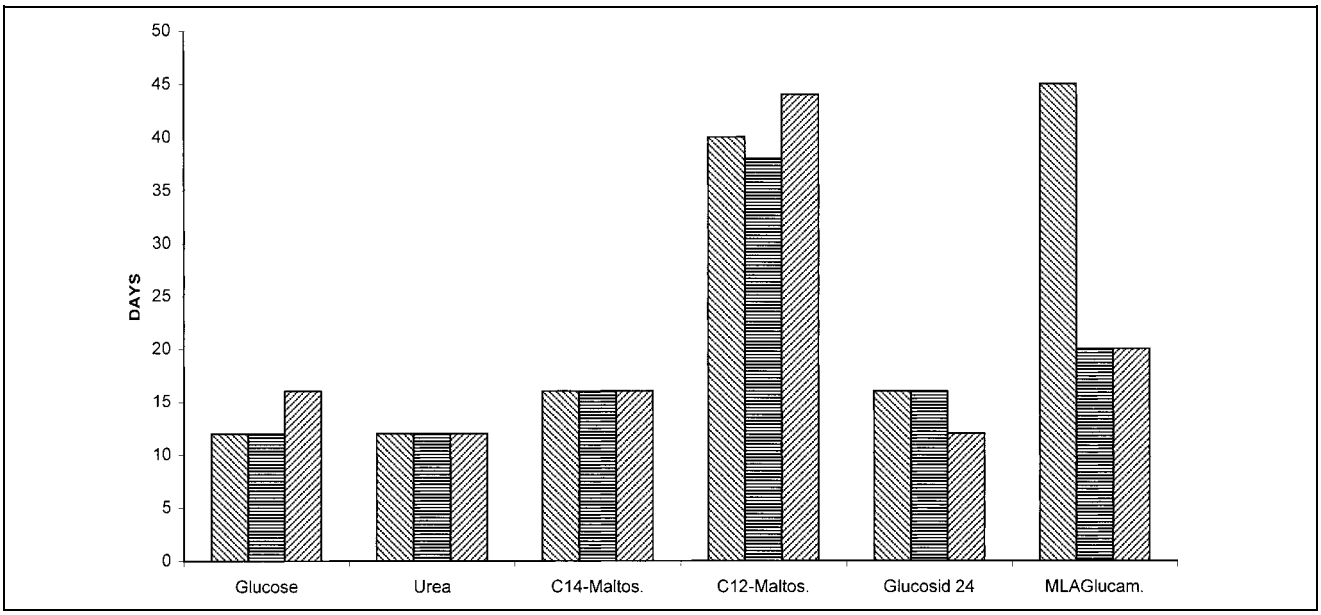

Fig. 10: Elapesed time since the experiment start and before the first sign of aggregation is detected
Low Level 1 : 5 w/w
Middle Level 1 : 20 w/w
High Level 1 : 50 w/w
Low Level 1 : 1 w/w
Middle Level 1 : 4 w/w
High Level 1 : 16 w/w
Insulin/stabilizer proportions levels
Low Level
Middle Level
High Level

N-methyl lauryl acid glucamide cannot be considered as a good alternative as it stabilizes the dimeric form in particular only in limited low proportions. In addition, there is no information about its toxicity at the present time.

The present study showed that dodecyl-β-D-maltoside (C12 maltoside) could provide a relative insulin monomeric stabilization in aqueous buffered solution at neutral pH and it could be conveniently included in oral formulations in order to improve absorption through the intestinal mucosa. This result could also be extended to other insulin administration routes, where an absorption process through a mucosa is involved and it could also include ultrarapid action parenterals destined to achieve some specific therapies. Although other means for assuring this stabilization effect are worth investigating, the complexation of insulin with substances such as C12 maltoside is preferred as a low-risk way of preserving the natural bioactivity of the molecule.

3. Experimental

3.1. Materials

The porcine insulin used (27.4 IU · mg^{-1}, 0.4% zinc) was a gift from Hoechst AG (Frankfurt am Main, Germany). The alkylglucosides: C12-glucoside (α = 97.19%; β = 1.55%), C14-glucoside (α = 52.51%; β = 22.83%), C16-glucoside (α = 65.1%; β = 32.4%); the alkylmaltosides: C12-maltoside (α = 66.35%; β = 27.50%), C14-maltoside (α = 22.93%; β = 64.12%); N-methyl lauryl-acid glucamide and glucosid 24 were kindly donated by Hüls AG (Marl, Germany). D-(+)-Glucose and urea (reagent degree) were acquired from Merck AG (Darmstadt, Germany).

3.2 Formulations

Insulin solution samples (100 ml) were prepared using a hypothetical stabilizers the different alkylsaccharides at 1:1, 1:4 and 1:16 w/w insulin to stabilizer ratio levels. Also D-(+)-glucose and urea were tested as stabilizers in 1:5, 1:20, and 1:50 w/w ratio levels. The medium for these preparations was 0.1 M aqueous phosphate buffer solution and pH was adjusted for all samples to 7.4. All solutions were prepared under aseptical conditions in order to reduce the presence of extraneous particles to a minimum and to reduce the development of bioburden avoiding the usage of additional preservative substances in the medium, as these samples were used for long-term determinations.

3.2.1. Buffer solution

The buffer was prepared dissolving 71 g of anh. Na_2HPO_4 from Merck AG (reagent degree) in 2.5 l of water for injection (WFI) under vigorous stirring and pH was primarily adjusted to 7.4. This solution was transferred to a 5 l volumetric flask, and more WFI was added to reach the exact volume. The volumetric flask was tightly closed and the buffer was kept for a week before use.

3.2.2. Stabilizer solutions

Each alkylsaccharide (12, 48 and 192 mg) was dissolved with 50 ml of buffer solution in 100 ml flasks, in an attempt to assure complete dilution with vigorous stirring and temperature as needed. In the case of urea and D-(+)-glucose 60, 240 and 600 mg were dissolved in the same way. The pH was checked and corrected in each flask to a value of 7.4 by adding 2N HCl or 2N NaOH solution. More buffer was added to reach 60 ml. During preparation, it was observed that the alkylglucosides were practically impossible to dissolve in the buffer, even by applying heat with long and vigorous stirring. Further testing at lower concentrations showed the same result. An amorphous precipitate with aggregates very close to a lamellar system was obtained. It was then decided to primarily discard these samples as insulin monomeric stabilizers due to their unsuitable behaviour in an aqueous medium. The assays were performed with the alkylmaltosides, (glucosid 24), the N methyl lauryl acid glucamide, urea and D-(+)-glucose. The remanent flasks were tightly closed, labelled and transferred to the laminar flow at the clean area for further mixtures with the insulin solution in equal proportions after filtration using syringes provided with Minisart 0.2 μm filters.

3.2.3. Insulin solution

Insulin (250 mg) was dispersed in 80 ml of buffer solution and the resulting suspension was acidified adding 2N HCL until pH 3. After complete dilution, 2N NaOH was added to obtain a pH of 7.4 and the volume was completed with additional buffer solution to 100 ml. The exact insulin concentration in the primary solution was checked using the UV method (2.5 mg · ml^{-1}) and 80 ml of this concentrated solution were diluted under stirring in about 700 ml of buffer solution. The solution pH was checked and adjusted to 7.4; then more buffer solution was added to complete 800 ml and the reservoir was tightly closed. The diluted insulin solution was transferred to a laminar flow in a clean area for sterile filtration with a Sartorius Vacuum System (Sartorius AG, Göttingen, Germany) using a 0.2 μm cellulose nitrate membrane. First the filter was rinsed with 200 ml of phosphate buffer previous to the insulin solution filtration, so both portions were mixed directly after filtration to finally obtain 1,000 ml of sterile insulin solution. This final insulin solution volume was divided in 50 ml portions for mixing with 50 ml of each stabilizer solution in an aseptical way under laminar flow, obtaining a set of samples of 100 ml volume each for testing. A final check of the actual insulin concentration in these samples was carried out, mixing in the same way 50 ml of the remnant final insulin solution volume with 50 ml of buffer solution without stabilizer and using this solution as a reference. The insulin concentration found was 0.092 mg · ml^{-1}, determined with an UV Spectrophotometer (UVIKON 930 model from Kontron Instruments, Munich, Germany). The difference from the theoretical value of 0.1 mg · ml^{-1} is 0.008, which is inside the standard deviation range of ±0.023. This is statistically determined as a result of the linear regression applied for obtaining the absorbance vs. insulin concentration dependence during the calibration procedure performed prior to the measurements. Therefore insulin concentration was assumed to be 0.1 mg · ml^{-1} in all samples.

3.2.4. Samples aseptic preparation procedure

Under a clean environment provided by a laminar flow cabinet and using 20 ml sterilized syringes with 0,2 μm Minisart filters in three times, 50 ml of the corresponding stabilizer solutions were added to 50 ml of the filtered insulin solution previously dosed in 100 ml sterilized vials with a 50 ml Eppendorf dosing pipette. Finally, 18 vials containing insulin-stabilizer buffered solutions at 7.4 pH were obtained. In an additional vial, 50 ml of buffer solution without any stabilizer were added to 50 ml of insulin solution as a means of comparison in order to determine the real stabilization action in each case. The remanent 10 ml of each stabilizer solution were mixed with 10 ml of buffer solution in 20 ml vials to use them as a blank for the baseline corrections in the circular dichroism (CD) spectra determinations. An additional 20 ml vial was also included as the blank corresponding to the insulin solution without stabilizer.

3.3. Methods

3.3.1. Surface tension determinations

In order to elucidate the hypothetical influence of the solutions surface tension as a sign of solvent structure changing, which could facilitate or inhibit insulin molecular association, measurements were performed in a K121 tensiometer (Krüss GmbH, Hamburg, Germany) using a Platinum Plaque technique at a constant temperature of 25 ± 0.1 °C. The instrument was first tested several times with pure bidistilled water, which produced the results 72.6 . . . 72.8 mN · m^{-1} which were taken as proper values.

3.3.2. Circular dichroism determinations

To evaluate the capacity of these substances to stabilize the insulin molecule, circular dichroism (CD) determinations were made. It has been stated in previous studies of this kind that insulin shows different negative maxima in the CD spectrum as described below [26]:

208–210 nm Band: Normally this negative trough has been assigned largely to an α helix structure which is a predominant feature of the monomer involving residues B10–B19 and additional contributions from residues of the A chain identified as A2–A6 and A13–A19, which are arranged in distorted helices. This negative trough is slightly affected by a decrease in concentration.

220–225 nm Band: This has been normally attributed to an antiparallel β sheet structure which is a predominant feature of the dimer form involving the residues B21–B29 of the B chain at the C terminal. This negative trough has been shown to be attenuated as concentration decreases in a certain range, and if it is combined with the appearance of the negative peak at 208–210 nm it suggests that dimers are less prevalent in solution.

270–275 nm Band: Usually this negative maximum can be detected for concentrations higher than 0.2 mg · ml^{-1} and is attributed to the tyrosine and phenylalanine aromatic residues located at B24–B26. Attenuation of this peak has been correlated commonly with disaggregation of insulin, while a strengthening has been associated normally with conditions that enhance the aggregation of the molecule. The optical activity of these aromatic residues is therefore dependent on the state of the insulin self-association.

Hence the 220–225 and 270–275 nm bands provide an indication of the insulin aggregational tendency and on this basis, the CD spectra of the different samples were determined and analyzed in a range between 200 to 300 nm. For this task a Jasco 720 Spectropolarimeter (Jasco Inc. Tokyo,

Japan) specially configured to perform CD spectra determinations was used. The instrument was adjusted before measuring using a 0.06% w/v solution of ammonium d-10-camphor sulfonate (ACS) as the standard recommended by the instrument supplier, in such a way that a maximum peak of 1.904 E 02 ± 1 mdeg should be obtained at 290.5 nm wavelength.
For the particular case of insulin solutions at 0.1 mg · ml^{-1} concentration, the following parameters were established in order to obtain good CD spectra: band width 1.0 nm (slit width was automatically adjusted during the measurement), sensitivity 200 mdeg/Full scale, step resolution 0.05 nm, scan speed 100 nm · min^{-1}, response 1 s, wavelength interval 200 to 300 nm, cell type: 1 cm, rectangular.
CD data are normally expressed in terms of the mean residual ellipticity, $[\theta] = \theta_\lambda\ M_0/C \cdot /$ in degrees · cm^2 · $decimol^{-1}$, where Θ_λ is the observed ellipticity at wavelength λ, M_0 is the mean residue molecular weight for insulin (113.3 g/residue), C is the insulin concentration (g · ml^{-1}) and / is the cell pathlength (1 cm).

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