The Effect of Trehalose, Antioxidants, and Acetate Buffer Concentration on Oxytocin Stability

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Abstract

Oxytocin is a cyclic nonapeptide used to induce labor and prevent bleeding after childbirth. Due to its instability, storage and transport of oxytocin formulations can be problematic in hot/tropical climates. The aim of this study was to investigate the effect of trehalose and select antioxidants (uric acid, butylated hydroxytoluene, and L-ascorbic acid) on oxytocin stability in solution. The effect of buffer composition and acetate buffer concentration was also studied. Acetate buffer was found to work better than citrate/phosphate buffer for the oxytocin stability. Lower acetate buffer concentrations (0.025 M or less) were also found to yield improved oxytocin stability than higher concentrations. Although known degradation pathways of oxytocin include oxidation, the antioxidants uric acid and butylated hydroxytoluene had negligible effect on the oxytocin stability while L-ascorbic acid led to significantly faster degradation. Despite trehalose's reputation as a great stabilizer for biomolecules, it also had small to negligible effect on oxytocin stability at concentrations up to 1 M in acetate buffer. These results were surprising given the present literature on trehalose as a stabilizer for various biomolecules, including proteins and lipids.

Keywords: Oxytocin; trehalose; antioxidants; stability, acetate buffer, formulation

Introduction

Oxytocin is recommended by the World Health Organization (WHO) for the prevention and treatment of post-partum hemorrhage, which is the leading direct cause of maternal deaths worldwide.[1,2] Oxytocin is a cyclic nonapeptide. Its structure was elucidated in 1951 and the characterization and synthesis were reported in 1954.[3–6] Oxytocin is used therapeutically to induce labor, stimulate lactation and it is the drug of choice to prevent postpartum hemorrhage.[1,3,7] More recently, oxytocin has also been tested for the treatment of social phobia,[8] autism,[9] alcoholism,[10] schizophrenia,[11,12] as well as being studied for its potential modulatory role in antinociception[13]. It is therefore an important peptide with diverse pharmaceutical applications.

Unfortunately, oxytocin samples in low and middle income countries are often of poor quality.[14] This may be because of low manufacturing quality, inadequate transport and/or storage conditions, such as limitations to cold chain systems in resource-constrained settings, or due to both.[14–16] In injectable formulations, it is known that oxytocin degrades rapidly when stored at temperatures above 30°C, so it is important to have adequate storage conditions for oxytocin, especially in tropical climates.[16] Consequently, there is a definite need for a thermostable oxytocin formulation, which could improve the product's shelf-life.

One approach that has been taken to circumvent the instability of oxytocin, is to use the analog carbetocin instead of oxytocin, at least for post-partum hemorrhage.[17,18] Carbetocin's structure deviates from oxytocin's in three ways. First, it has a CH_2 group instead of one of the cysteine sulfur atoms. Second, the free amino group on that former cysteine unit has been replaced by a hydrogen atom. Third, instead of a hydroxy group on the tyrosine unit, it has a methoxy group. Studies have shown that the first two aforementioned structural changes slow down the degradation process significantly.[19] Recently, a heat-stable formulation of carbetocin was also developed in sodium succinate buffer at pH 5.45 and with mannitol and methionine in the formulation.[17] Although this is an exciting development, it is still feasible to develop a heat-stable formulation for oxytocin as well, as oxytocin is the naturally occurring hormone and carbetocin may cause hyper-excitation of the oxytocin receptors in addition to being more expensive than oxytocin and its use is subsequently not always cost-effective.[20]

The stability and degradation pathway of oxytocin have been studied in order to find ways to improve its stability. Buffer solutions used in these stability studies have included citrate, phosphate, citrate/phosphate, acetate, and aspartate buffers as well as lactated Ringer's solution.[19,21–27] The acidity of the buffer has been found to play an extremely significant role, with the best stability found at pH ≈ 4.5.[19,21]

Several potential excipients have been tested and found to enhance the stability of oxytocin in solution to some extent. These include divalent metal ions in citrate and aspartate buffer solutions,[22–24,28] and dextrose and sodium chloride injection solutions[26]. Dry powder formulations have also been tested with excipients including trehalose, isoleucine, polyvinylpyrrolidone along with citrate and zinc salts in an effort to make the solid state oxytocin stable in extreme hot climates.[29] Although some of these excipients have resulted in the enhancement of oxytocin stability, there is still room for further improvement. Therefore, we endeavored to study the potential stabilization effect on oxytocin of a few more additives, in particular trehalose and several antioxidants.

Trehalose is known to be an excellent stabilizer and protective agent against environmental stresses for various biochemicals, in particular lipids and proteins. It has been shown to enhance the stability of these molecules both under anhydrous conditions,[29–33] and more importantly for this project, in aqueous solutions, with higher concentrations yielding greater stability enhancing effect[34–39]. In both instances, hydrogen bonding is believed to play an important role in the stabilization mechanism, with one of the main hypothesis being water-replacement via hydrogen bonding.[30–33,36,38] One could thus envision trehalose being able to form a protective layer around the biomolecule, providing a protection against chemical degradation by minimizing undesired degradation reactions. In light of trehalose's reputation and the prior literature indicating that trehalose is an excellent stabilizer for biomolecules in solution, we hypothesized that it would improve the stability of oxytocin in aqueous solutions. Therefore, we tested its potential stabilization effect on oxytocin in both acetate and citrate/phosphate buffer solutions.

Furthermore, oxytocin degradation studies have found that Tyr^2 and $Cys^{1,6}$ are prone to oxidation, amongst other degradation pathways (**Figure 1**).[24] For that reason, we decided to also explore the effect of a few antioxidants on oxytocin's stability, hypothesizing that it would improve the peptide's stability. The antioxidants chosen were uric acid, butylated hydroxytoluene (BHT), and L-ascorbic acid (also known as vitamin C). Uric acid is one of the major antioxidants in humans, with plasma urate levels of about 300 μM. Despite its low solubility in water, it has nevertheless shown powerful antioxidant activity in aqueous solutions and is believed to provide defense against oxidant- and radical-caused aging and cancer.[40,41] BHT is one of the most commonly used antioxidants, being used in various industries, including the food and pharmaceutical industry.[42] Encouraged by uric acid's powerful antioxidant activity at very low concentrations, we decided to include BHT in our tests given its prevalence as an antioxidant in the various industries. As L-ascorbic acid is also a well-known antioxidant and a

common and affordable dietary supplement, we decided to include that as well as one of our antioxidants to be tested.

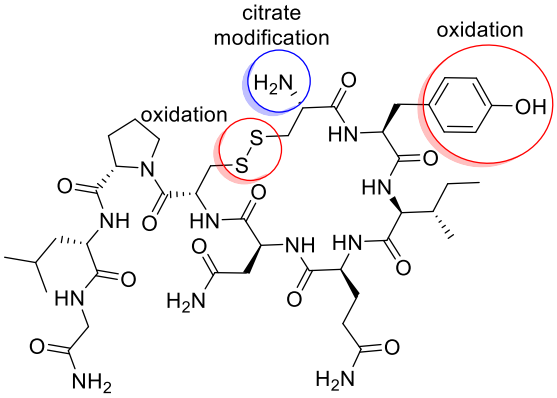

Figure 1. The structure of oxytocin with known sites of degradation identified.

Results and Discussion

The effect of trehalose on oxytocin stability in solution was determined. Samples with oxytocin concentration of 0.25 mg/mL were made with varied trehalose concentrations (10, 20, 30, and 40 mM), in either acetate buffer (0.1M) or citrate/phosphate buffer (0.1M/0.2M), respectively. The solutions also included a small amount of BHT (saturated concentration). The samples were stored at 50°C and the oxytocin amount remaining was analyzed using HPLC at a several day interval.

At these concentrations, trehalose did not have any effect on the stability of oxytocin. There was no significant difference in oxytocin degradation rate when comparing solutions with and without trehalose, suggesting that the degradation rate is unaffected by trehalose. The choice of buffer solution, however, had a dramatic effect. Prior literature suggests that the degradation kinetics of oxytocin are (pseudo) first order.[21] The degradation rate constants for all of the acetate buffer samples was $1.8 \pm 0.1 \times 10^{-2}$ day^{-1} at 50°C. Meanwhile the rate constant for the citrate/phosphate buffer samples was measured to be $4.2 \pm 0.1 \times 10^{-2}$ day^{-1} at 50°C or roughly twice that of oxytocin in acetate buffer (**Figure 2**). The faster degradation of oxytocin in the citrate/phosphate buffer may be the result of an amide- and imide-linked citrate derived degradation products of oxytocin, as has been previously reported on for oxytocin in citrate buffers.[43]

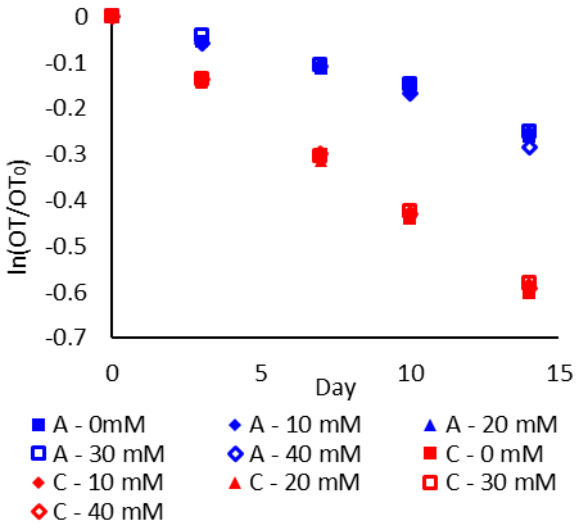

Figure 2. A plot of ln([OT]t/[OT]0) vs. day. The A samples (blue) are in acetate buffer with different concentrations of trehalose as indicated in the legend. The C samples (red) are in citrate/phosphate buffer.

The lack of effect of trehalose was surprising given its reputation and the prior literature showing that it is a good stabilizer for biomolecules in solution.[34–37] However, as the stabilizing effect has been reported to increase as the trehalose concentration increases,[34–36] several more formulations with higher trehalose concentrations (100, 500, and 1000 mM) were tested along with a fresh control sample. As the acetate buffer had yielded significantly better results than the citrate/phosphate buffer, these new samples were only analyzed in the acetate buffer. The results did not show any stabilizing effect from trehalose, even at these higher concentrations, with all but one of the new samples giving a rate constant in the same range as before ($1.8 \pm 0.1 \times 10^{-2}$ day^{-1}). The sample with the highest trehalose concentration (1000 mM) was the only acetate sample with a different rate constant (2.2×10^{-2} day^{-1}), seemingly degrading slightly faster than the other samples at 50°C (**Figure 3**). These results were especially surprising as we expected the highest trehalose concentration to be the most likely to improve the peptide's stability given the existing literature.

These results suggest however, that although simulations have shown that trehalose can promote preferential hydration of biomolecules, yielding a coating by trapped water layer around biomolecules, this coating is not sufficient enough to provide chemical protection, at least not for oxytocin in solution. Rather, this coating, along with increased surface tension resulting from the presence of trehalose, seems to primarily limit conformational fluctuations, thus protecting the three-dimensional structure of proteins, as has been previously discussed in the literature.[35,38,39] This means that for small peptides like oxytocin (MW = 1007 g/mol), that only have a primary structure, trehalose would have limited to no impact in solution. Trehalose

could however start having more protective effect on proteins that have at least a clear secondary or tertiary structure. This raises the question of at what point (size and/or structural) does trehalose start showing measurable protective effect on biomolecules?

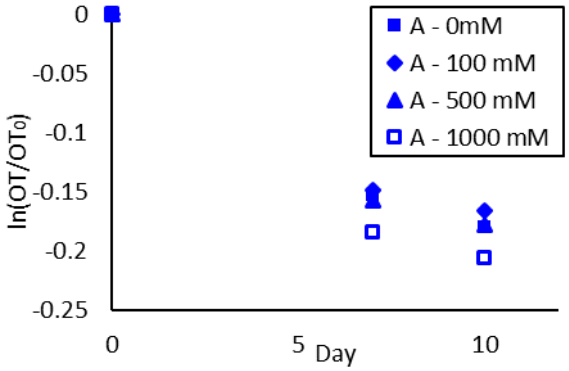

Figure 3. A plot of ln([OT]t/[OT]0) vs. day. All samples were made in acetate buffer and with different concentrations of trehalose as indicated in the box.

As oxytocin was significantly more stable in 0.1 M acetate buffer, than in citric/phosphate buffer (0.1M/0.2M), the importance of the acetate concentration was also studied by storing oxytocin in 0.01, 0.025, 0.05, 0.1 and 0.2M acetate buffer, keeping the pH constant at 4.5. When the rate constants were plotted up vs. the strength of the buffer, an S-curve was observed as shown in Figure 4, indicating that the optimal storage conditions for oxytocin is in an acetate concentration at or below 0.025M. Above that concentration, the degradation rate increases and reaches plateau. The maximal half-life at 50°C for acetate buffer concentrations of 0.01 and 0.025 M was found to be 47 days compared to a half-life of 40 days when the acetate buffer concentration was 0.1 or 0.2 M.

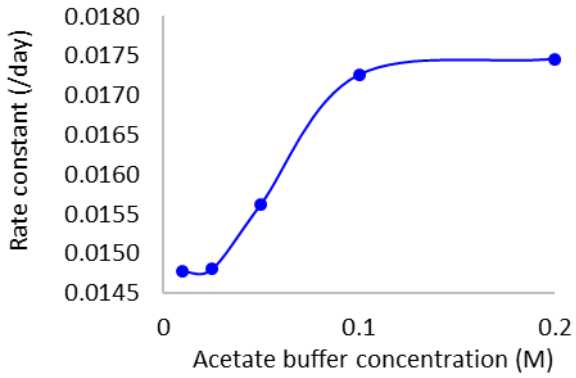

Figure 4. A plot of acetate buffer concentration against the oxytocin degradation rate constants.

As previously mentioned, degradation studies on oxytocin have found that Tyr^2 and $Cys^{1,6}$ are prone to oxidation.[24] Therefore, three antioxidants were tested to see if they could slow down oxytocin degradation in solution. Uric acid is a powerful antioxidant found in human blood, BHT was chosen as it is one of the best known antioxidants and has been used in various industries, including as an antioxidant food additive, and L-ascorbic acid was tested as it is also a common antioxidant. Since L-ascorbic acid is very water soluble, it was tested in concentrations ranging from 1 to 4 mg/mL. BHT and uric acid, however, have a low water solubility (< 1 mg/mL) so they were only tested at saturated concentrations. The oxytocin concentration of the samples was kept constant at 0.25 mg/mL and 50mM acetate buffer was used at a constant pH of 4.4. The samples were then stored at 50°C and sampled over a 15-day period. Although the literature has suggested that one of oxytocin's degradation pathways is due to oxidation, none of the antioxidants exhibited any stabilizing effect. Uric acid and BHT had no observable effect while L-ascorbic acid accelerated the degradation rate significantly, showing half-life of less than 4 days at concentrations ranging from 1-4 mg/mL (see *Supplementary Information*).

It is not clear yet why L-ascorbic acid is impacting the oxytocin stability so negatively, but this could be an avenue for further studies. One aspect of such a future study would be to analyze the degradation products in order to better understand how L-ascorbic acid affects the degradation mechanism.

Conclusions

In conclusion, we confirmed that oxytocin is more stable in acetate buffer than in citrate/phosphate buffer and we found that the stability is also concentration dependent with buffer concentrations of 0.025 M or less being more beneficial, at pH of around 4.5. We also found that the antioxidants uric acid and BHT had negligible effect on the oxytocin stability and should therefore be safe to use from a stability standpoint, while L-ascorbic acid increases the rate of oxytocin degradation in solution significantly. Finally, we found that trehalose had small to negligible effect on oxytocin stability in acetate buffers at concentrations ranging from 10-1000 mM, as well as negligible effect on oxytocin stability in citrate/phosphate buffers at concentrations ranging from 10-40 mM. This raises the question of whether trehalose's stabilizing effect is primarily a structural stabilization, and thus may have a limited effect on smaller biomolecules such as peptides. As trehalose is often considered a go to stabilizer for a wide range of biomolecules, it is important to be aware of these limitations and concentration importance when considering whether to utilize trehalose as a stabilizer.

Materials and Methods

Materials

Oxytocin was purchased from Grindeks (Latvia). Trehalose came from Pfanstiehl Inc. (Waukegan, USA) and was kindly provided by Alvotech (Reykjavik, Iceland). Butylated hydroxytoluene, uric acid, L-ascorbic acid, sodium phosphate dibasic, ammonium acetate, and trifluoroacetic acid were purchased from Sigma-Aldrich. Sodium acetate was purchased from Merck and citric acid anhydrous from Pernhofen (Austria). Acetic acid, acetonitrile, and methanol were bought from Honeywell. All water used in this study was obtained from a Milli-Q water purification system.

Analysis

Analytical HPLC used in this study included, Dionex UltiMate 3.0 HPLC system, controlled by Dionex Chromeleon software v7.2. The HPLC system consisted of a column oven compartment, an autosampler with temperature control, an UltiMate 3000 pump, and an UltiMate 3000 photo- diode array detector. A Phenomenex Luna® 5µm C18(2), 250 x 4.6 mm, 100Å, LC column 4.6 × 250 mm was used with a SecurityGuard Cartridge (C18 4 x 3.0 mm). The mobile phase for all samples, except those testing the antioxidant effect, consisted of A: ammonium acetate 20 mM and B: acetonitrile. The flow rate used was 1.0 mL/min and the UV detection was at 220 nm. The method used was as follows: for 8 min, 25% B, then 25-90% B over 2.5 min, followed by 90% B over 0.5 min, and then 90-25% B over 1.5 min and finally constant at 25% B for 3.5 min. The HPLC analysis for the sample series with antioxidants was prepared according to a previous study[44] using the section 3 protocol where mobile phase A was 0.01% TFA / H_2O and mobile phase B was 0.01% TFA / 70% MeCN : 30% H_2O. All samples were run in triplicate.

Formulation

The oxytocin concentration was kept constant at 0.25 mg/mL in all samples of all series. For the buffer only series, citrate/phosphate buffer (0.1M/0.2M) at pH 4.5 was prepared as well as acetate buffers at a range of concentrations (0.01M/0.025M/0.05M/0.1M/0.2M) and at pH 4.5. For the antioxidant series, all samples were prepared in a 50 mM acetate buffer at pH 4.4. The different samples in this series included either butylated hydroxytoluene (saturated), uric acid (saturated), or L-ascorbic acid at different concentrations (1 to 4 mg/mL). For the trehalose series, oxytocin samples were prepared in either acetate buffer (0.1 M) or citrate/phosphate buffer (0.1M/0.2M) and four different concentrations of trehalose (10 mM, 20 mM, 30 mM, or

40 mM) were used for the samples made in each of the buffers used. The additional trehalose samples (0.1M, 0.5M and 1.0M) were made in acetate buffer as well. An oxytocin control was used for each series, with the control being prepared in the same buffer as the samples in the series but without any additives. The accelerated stability study was carried out by storing the formulations at 50°C for a period of up to 16 days and the samples were analyzed by HPLC several times during that period. Oxytocin standards were prepared at a range of concentrations (10 mg/mL, 4.0 mg/mL, 1.6 mg/mL, 0.64 mg/mL, 0.26 mg/mL) in acetate buffer at pH 4.5.

Supplementary Information is available with information about observed oxytocin concentrations at each time point and results of *t*-tests to see which results were statistically significant.

Acknowledgments: We would like to the Icelandic Technology Development Fund for funding, and Árni Þorgrímur Kristjánsson and Bergþóra Sigríður Snorradóttir for help with trouble-shooting with the HPLC instrument.

Funding: This research was funded by the Icelandic Technology Development Fund (grant number 164072-0613).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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