Faculty of Pharmaceutical Sciences[1], School of Health Sciences; Department of Chemistry[2], Science Institute, University of Iceland; Calor ehf.[3], Reykjavik, Iceland; Department of Pharmacy[4], College of Medicine, University of Malawi, Blantyre, Malawi

The effect of D-(+)-glucosamine, N-acetyl-D-glucosamine and tetraethylene glycol on the stability of oxytocin in aqueous solution

M. Ghasemisarabbadieh[1,2,3], S. J. Sigurdsson[1], F. V. Nu Dong[1], S. Gizurarson[1,3,4], B. R. Sveinbjörnsson[2,3,*]

Received May 15, 2021, accepted July 6, 2021

**Corresponding author: Benjamin Ragnar Sveinbjörnsson, Department of Chemistry, Science Institute, University of Iceland, Dunhagi 3, 107 Reykjavik, Iceland*
benjaminrs@hi.is

Pharmazie 76: 480-483 (2021) *doi: 10.1691/ph.2021.1081*

The aim of the present study was to identify the effect of D-(+)-glucosamine, N-acetyl-D-glucosamine, tetraethyleneglycol, and the mixture of these additives on the stability of oxytocin in phosphate and acetate buffer solutions, at pH 4.5. Our findings demonstrate that tetraethyleneglycol has a destabilizing effect on oxytocin in both phosphate buffer and acetate buffer. D-(+)-Glucosamine hydrochloride had small to negligible effect at low concentrations, yielding a slight improvement lower concentrations of the additive in the presence of the buffers used, but at higher concentrations it increased the rate of degradation. N-Acetyl-D-glucosamine showed a possibly slight improvement to the stability of oxytocin. It is hypothesized that the different effect of N-acetyl-D-glucosamine compared to D-(+)-glucosamine is a consequence of the free amine group in D-(+)-glucosamine promoting a faster degradation, while the amino group is acetylated in N-acetyl-D-glucosamine and therefore no longer reactive in the same way. While it remains unclear why tetraethyleneglycol has a destabilizing effect on oxytocin, the D-(+)-glucosamine results aid in deepening our understanding of the degradation mechanism of oxytocin.

1. Introduction

Oxytocin is a uterotonic neuropeptide that has been recommended by the World Health Organization (WHO) as the first line treatment to prevent and treat *postpartum* hemorrhage (PPH) (*WHO Recommendations Uterotonics for the Prevention of Postpartum Haemorrhage*, 2018). PPH is the main cause of maternal deaths (27.1%) in many low-income countries (Say et al. 2014). Although studies have shown that multiple cycles of freezing and thawing do not affect the oxytocin content (Nassta et al. 2013), there is a concern about the storage conditions and its quality in these countries. Oxytocin should be stored at 2–8°C and data show that it cannot tolerate more than one month at 30°C or 2 weeks at 40°C (Hogerzeil et al. 1993). Injectable formulations of oxytocin are, therefore, unstable if the storage temperature reaches 30°C or higher (Gard et al. 2002). These formulations are not suitable for use in the small villages in these countries.

Approaches to improve the stability of oxytocin have shown that it is possible to improve its stability in aqueous solution. These studies include using divalent metal ions in the presence of aspartate buffer and citrate buffer solutions (Avanti et al. 2011, 2012, 2013), as well as using dextrose and isotonic sodium chloride solutions (Trissel et al. 2006). It has been elucidated that the disulfide bridge and the N-terminal amino group are two of the structural features responsible for the low stability of oxytocin when dissolved in aqueous solutions (Wisniewski et al. 2013). Furthermore, it has been proposed that oxytocin can degrade *via* a mechanism involving a beta-elimination with R-S-S$^-$ as the leaving group (Wisniewski et al. 2013). The acidity of the buffer has been shown to play an important role, with the best stability found at pH ≈ 4.5 (Hawe et al. 2009; Wisniewski et al. 2013). Recently, we showed that 18-crown-6 decreases the degradation process in citrate/phosphate buffer. We proposed that the mechanism of protection involved the crown ether binding to oxytocin's protonated amino group, stabilizing it in its protonated form. That way, the amino group would be protected from acting as a base that could promote the beta-elimination reaction, opening the Cys1-Cys6 disulfide bridge (Ghasemisarabbadieh et al. 2021a).

Preliminary results and a recent patent suggested that a combination of poly or oligo(ethylene glycols), such as tetraethylene glycol, and select aminosugars, such as glucosamine, galactosamine, fructosamine, mannitosamine, and N-acetylglucosamine, could increase the thermal stability of peptides and proteins in aqueous solution (Gizurarson and Sigurdsson 2018). The examples provided in the patent suggest that a combination of glucosamine and tetraethyleneglycol (4EG) has beneficial effects on oxytocin's stability in 50 mM phosphate buffer at pH 4.5 (Gizurarson and Sigurdsson 2018).

Glucosamine sulfate is known to act as an antioxidant and its scavenging ability for superoxide/hydroxyl radicals has been evaluated with the results suggesting that glucosamine sulfate can be used as an additive to reduce oxidative stress (Xing et al. 2009). As degradation studies on oxytocin have also found that Tyr2 and Cys1,6 are prone to oxidation (Avanti et al. 2012) (Fig. 1), antioxidative abilities of aminosugars, like D-(+)-glucosamine or N-acetyl-D-glucosamine could play a role in the previously observed stabilization.

The aim of this study was to explore further the preliminary results described in the aforementioned patent, by examining the effect of D-(+)-glucosamine, N-acetyl-D-glucosamine and a mixture of these additives with 4EG on oxytocin stability. Previous findings have shown that acetate buffer works better than citrate/phosphate buffer on the stability of oxytocin and that lower acetate buffer concentrations are slightly better compared to higher buffer concentrations (Ghasemisarabbadieh et al. 2021b). Therefore, the aforementioned additives in this study were tested in acetate buffer. Since the examples in the patent were measured in phosphate buffer, the additives were also tested in phosphate solutions but as pH 4.5 is outside of the ideal pH range for phosphate buffers (5.8-8.0), the pH was verified upon sample preparation and after over a week's storage to ensure that pH changes were not impacting the results.

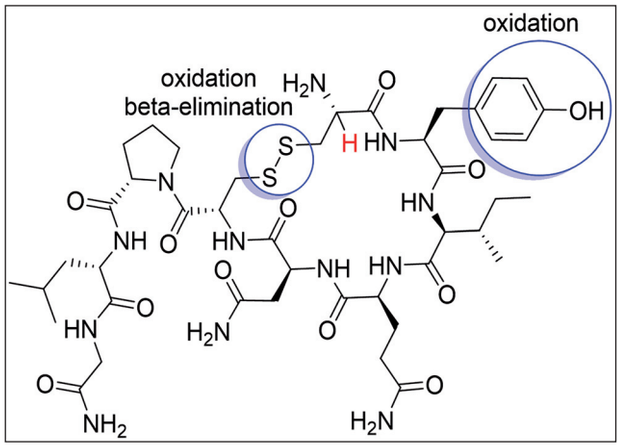

Fig. 1: The structure of oxytocin with known sites of degradation identified.

2. Investigations, results and discussion

2.1. Effect of glucosamine and a mixture of D-(+)-glucosamine with tetraethyleneglycol on oxytocin stability in solution

The effect of different concentrations (ranging from 0.04 to 14 mM) of D-(+)-glucosamine and a mixture of D-(+)-glucosamine and 4EG on oxytocin's stability was determined in phosphate buffer (0.1M) by HPLC. Analogous determinations were done in acetate buffer (50 mM) for oxytocin solutions with D-(+)-glucosamine concentrations ranging from 1.0 to 10 mM). The degradation rate constants were plotted against the concentration and the resulting plots are shown in Fig. 2, with the y-axis kept the same for better comparison.

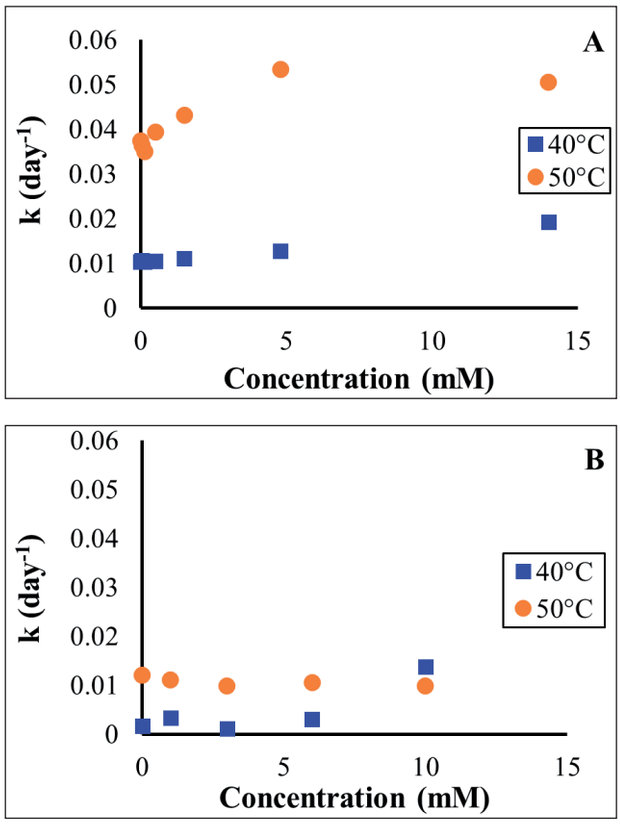

Fig. 2: Degradation rate constant (k) of oxytocin vs. different concentrations of D-(+)-glucosamine in phosphate buffer (A) and in acetate buffer (B) at 40 °C and 50 °C.

The results show a slight improvement in the presence of low or extremely low concentrations of D-(+)-glucosamine, or at <5 mM in acetate buffer and <0.5 mM in phosphate buffer. Higher concentrations showed increased degradation, especially in phosphate buffer. It was interesting to see the difference in at what concentration glucosamine starts accelerating the degradation, but that point was significantly different in phosphate buffer vs. acetate buffer. It seemed that glucosamine had less effect in acetate buffer than in phosphate buffer, especially at 50°C where not much change was observed.

Previous results have already highlighted that the choice of buffer plays an important role on oxytocin stability. When we compared acetate buffer to citrate/phosphate buffer, the degradation rate constant was almost two times less in the acetate buffer (Ghasemisarabbadieh et al. 2021b). When Figs. 2A and 2B are compared, we observe that the rate constant for oxytocin is also significantly lower in the presence of acetate buffer at pH 4.5 compared with that in phosphate buffer at the same pH. The level of stability improvement observed at low glucosamine concentrations is vastly lower than the stability improvement gained by using acetate buffer instead of phosphate. For the oxytocin control samples, the degradation rate decreased from 0.038 in phosphate to 0.012 in acetate buffer at pH 4.5 and 50°C. The lowest calculated degradation rate in samples with glucosamine present was 0.010 when the glucosamine concentration was in the range of 3.0 to 10.0 mM.

The difference in degradation rate constants in samples with low glucosamine concentration could be considered negligible, but if it is a real difference, there are a few possibilities for the different effects of D-(+)-glucosamine on oxytocin stability when it is used in different concentrations. One possibility is that hydrogen bonding interactions between the OH groups of glucosamine and the carbonyl groups of oxytocin may provide a slight protection of oxytocin at lower concentrations of D-(+)-glucosamine, while higher glucosamine concentrations may lead to more likelihood of a beta elimination promoted by the amino group of D-(+)-glucosamine grabbing the proton that is in the neighborhood of the N-terminal amino group of oxytocin and opening the C-S bond with the resulting degradation as shown in Figure 3.

Two samples were prepared with D-(+)-glucosamine and 4EG used together in acetate buffer, in case a synergistic effect would be observed there. Since the acetate buffer had given significantly better results than phosphate, the 4EG effect here was only tested in the acetate buffer. Unfortunately, the rate of degradation increased significantly in the presence of 4EG. When 1.0 mM of glucosamine was used with 0.3% (v/v) 4EG in acetate buffer at 50°C, the observed rate constant was 0.018 compared to 0.011 when 1.0 mM of glucosamine was used by itself. When the concentrations were increased to 3.0 mM for glucosamine and 1.0% (v/v) for 4EG, the observed rate constant was 0.016 compared to 0.010 when 3.0 mM of glucosamine was used by itself.

2.2. Effect of N-acetyl-D-glucosamine and a mixture of N-acetyl-D-glucosamine with tetraethyleneglycol on oxytocin stability in solution

The effect of four different concentrations of N-acetyl-D-glucosamine (1.0, 3.0, 6.0 and 10.0 mM) on the stability of oxytocin in acetate buffer at pH 4.5 was tested, both at 40°C and at 50°C. The resulting degradation rate constants were plotted against the concentration and the resulting plot is shown in Fig. 4.

Measurements at 40°C exhibited negligible difference between the results with or without N-acetyl-D-glucosamine. All these results showed more than 95% remaining amount of oxytocin in all samples after 15 days. At 50°C the measurements showed that the remaining amount of oxytocin seemed to have increased slightly with increasing concentration of N-acetyl-D-glucosamine while 4EG accelerated the degradation rate. The slight stabilization effect could however be considered negligible as well.

It was interesting to see that N-acetylglucosamine did not have the same negative effect on oxytocin as glucosamine did at the highest concentrations measured. A potential explanation for that

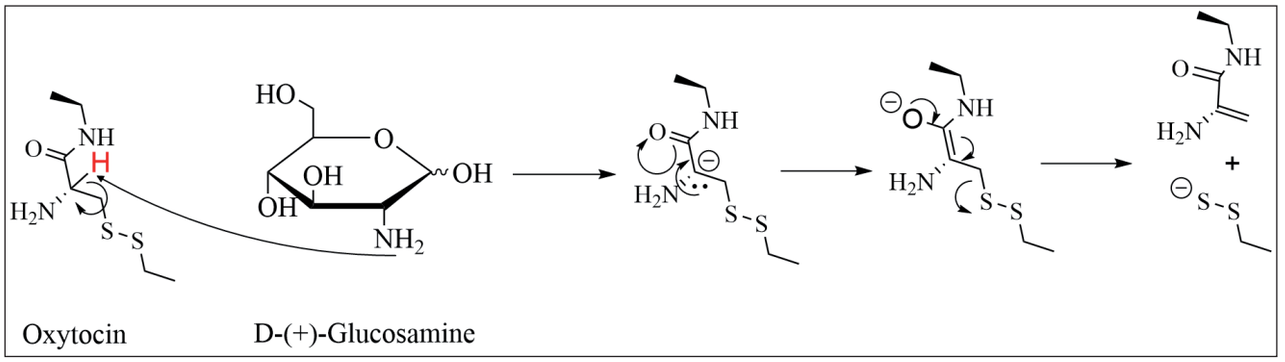

Fig. 3: Proposed mechanism of degradation of oxytocin in the presence of high glucosamine concentration.

is that the amino group could be considered as being protected as an amide in N-acetyl-D-glucosamine. This would align with the hypothesis that the amino group is a problem for the stability, at least at certain concentrations, with the potential of increasing the rate of degradation by promoting beta-elimination as a base. Here, increasing concentrations of N-acetyl-D-glucosamine may protect the carbonyl groups of the oxytocin molecule *via* hydrogen bonding, improving the stability slightly, compared with lower concentration, without the risk of having more of potentially reactive amino groups present. Nevertheless, the effect seems to be very small within the concentration range tested, especially compared to the buffer effect described here above.

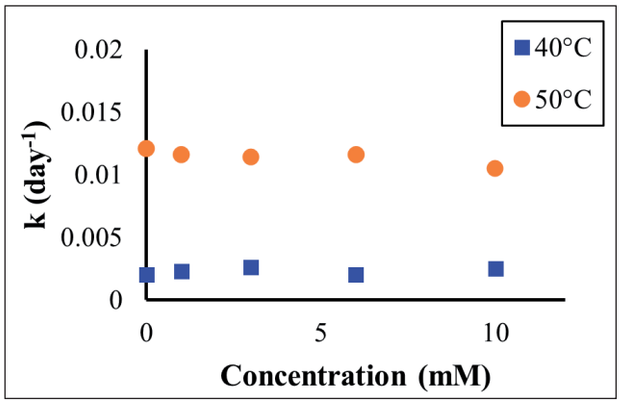

Fig. 4: Degradation rate constants (k) of oxytocin at different concentrations of N-acetylglucosamine in acetate buffer at 40 °C and 50 °C.

Again, the presence of 4EG sped up the rate of degradation. For the oxytocin control and the samples without 4EG, the half-lives were in the range of 57-66 days at 50°C. However, for the sample with 1.0 mM N-acetylglucosamine and 0.3% 4EG (v/v), the half-life was 47 days at 50°C, and when the 4EG concentration was increased to 1.0% (v/v), with the N-acetylglucosamine concentration at 3.0 mM, the half-life was shortened to 14 days. Since the N-acetylglucosamine concentration had little effect, we concluded that the shortened half-life was primarily due to the increase in 4EG concentration.

2.3. Effect of tetraethyleneglycol on the stability of oxytocin

The effect of 4EG on its own on the stability of oxytocin was also explored in phosphate solution at pH 4.5. Previous results with 18-crown-6 showed that the same additive can have drastically different effect depending on the buffer used (Ghasemisarabbadieh et al. 2021a), so it was decided to use the same buffer again as had been used in the previously mentioned patent (Gizurarson and Sigurdsson, 2018). The 4EG concentrations used ranged from 0.3% to 10% (v/v) and the degradation rates at 40°C and 50°C are plotted up against the 4EG concentration in Fig. 5. The results show that oxytocin stability did not improve but decreased in the presence of 4EG and the degradation was accelerated when higher concentrations of 4EG were used in the formulation.

These results were surprising in light of the previous results that had been obtained and reported in the patent, as well as given previous results obtained with trehalose as an additive. While the degradation mechanism of oxytocin in the presence of 4EG has not been elucidated yet, the only functional groups present are two alcohol groups and three ether groups. Ethers tend to be relatively unreactive and alcohol groups, e.g. in sugars like trehalose, seem to have a limited effect on oxytocin's stability. This could however be an interesting avenue for future studies in order to better understand how different additives might affect the degradation path of oxytocin.

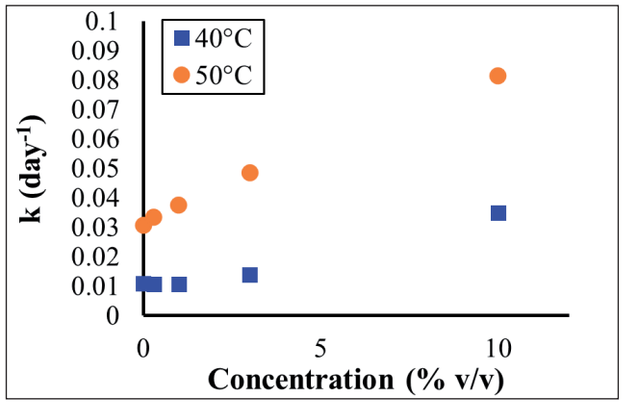

Fig. 5: A plot of the degradation rate constants (k) of oxytocin at different concentrations of 4EG in phosphate buffer at 40 °C and 50 °C.

2.4. Conclusions

In this study, the stability of oxytocin in aqueous buffer solutions was evaluated in the presence of D-(+)-glucosamine, N-acetyl-D-glucosamine, 4EG and the mixture of these additives. The buffers used were phosphate buffer and acetate buffer with the pH adjusted to 4.5. The results showed that 4EG accelerates the degradation of oxytocin. While the reasons for this destabilization are unclear at the moment, it could be the avenue of a future study where the degradation products are analyzed in more detail. D-(+)-Glucosamine had small to negligible effect at low concentrations but sped up the degradation at higher concentrations. N-Acetyl-D-glucosamine however, had small to negligible effect at all concentrations tested. One possibility for these different effects of D-(+)-glucosamine and N-acetylglucosamine could be an interaction between the amino group of D-(+)-glucosamine with

oxytocin, that may result in more degradation *via* beta elimination. The same reaction between oxytocin and N-acetyl-D-glucosamine is unlikely as the amine group is protected as an amide there. This suggests that the presence of more amino groups may be problematic for the stability of oxytocin, and reinforces a previously suggested hypothesis that the amine group on oxytocin itself is also involved in increasing the rate of its degradation.

4. Experimental

4.1. Materials

The following materials were used in this study: oxytocin, purchased from Grindeks (Latvia). Sodium phosphate monobasic, sodium phosphate dibasic, D-(+)-glucosamine hydrochloride, N-acetyl-D-glucosamine, tetraethyleneglycol (4EG), and trifluoroacetic acid (St. Louis, MO, USA) were purchased from Sigma-Aldrich. Ortho-phosphoric acid (85%) and sodium acetate were purchased from Merck (Darmstadt, Germany). Acetic acid, acetonitrile and methanol were bought from Honeywell (Germany). All water used was from a Milli-Q water purification system.

4.2. Analysis

High-performance liquid chromatography (HPLC) was carried out as previously described using Dionex UltiMate 3.0 HPLC system (Ghasemisarabbadieh et al. 2021b). The samples (20 µL) were injected using an ASI-100 autosampler. The flow rate was adjusted to 1.0 mL/min and the UV detection (VWD-3400 UV-VIS detector) was at 220 nm. The HPLC analysis for the samples in phosphate buffer consisted of mobile phase A: a 15.6 g/L solution of sodium dihydrogen phosphate, and mobile phase B: a 1:1 acetonitrile : H_2O solution, according to guidelines from the European Pharmacopoeia (*European Pharmacopoeia*, 2005). The HPLC analysis for the samples in acetate buffer was prepared according to a previous study (Ghasemisarabbadieh et al. 2021a) that consisted of mobile phase A: 0.01% TFA / H_2O and mobile phase B: 0.01% TFA / 70% MeCN : 30% H_2O. All samples were run in triplicate.

4.3. Formulation

A fixed concentration of oxytocin was used in all series tested, using 0.25 mg/mL. The samples included uric acid (saturated) to minimize oxidation of the D-(+)-glucosamine, but some discoloration had been previously observed in samples where uric acid was not included. Previous studies also suggested that uric acid has negligible effect on oxytocin's stability in aqueous solution (Ghasemisarabbadieh er al. 2021b). For the D-(+)-glucosamine series, oxytocin samples were made with six different concentrations of D-(+)-glucosamine: 0.04 mM, 0.14 mM, 0.5 mM, 1.5 mM, 4.8 mM and, 14 mM, in phosphate buffer (0.1M) at pH 4.5. For the samples in acetate buffer, D-(+)-glucosamine and N-acetyl-D-glucosamine were used at following concentrations: 1.0 mM, 3.0 mM, 6.0 mM, and 10.0 mM. Then two mixture samples of each additive (1.0 mM and 3.0 mM) with 4EG (0.3% and 1.0%) were also made. For the 4EG series, four oxytocin samples were prepared in the presence of 0.3%, 1.0%, 3.0%, and 10.0% 4EG. An oxytocin control was prepared for each series, made in the same buffer as the samples, but without the additives being studied.
The accelerated stability studies were carried out by storing the formulations in phosphate buffer at 40°C and 50°C for a period of up to 30 days and in acetate buffer at 40°C and 50°C for a period of 14 days. The samples were analyzed by HPLC several times during that period. Oxytocin standards were made in the following concentrations: 10 mg/mL, 4.0 mg/mL, 1.6 mg/mL, 0.64 mg/mL, 0.26 mg/mL, 0.10 mg/mL and 0.04 mg/mL in acetate buffer and phosphate buffer at the same pH (4.5).

Supplementary materials: Figure S1: Oxytocin remaining (%) in the presence of different concentrations of D-(+)-glucosamine and two mixture samples of this additive with tetraethyleneglycol in acetate buffer, pH 4.5 at 50°C and 40°C. Figure S2: Oxytocin remaining (%) in the presence of different concentrations of D-(+)-glucosamine in phosphate buffer, pH 4.5 at 50°C and 40°C. Figure S3: Oxytocin remaining (%) in the presence of different concentrations of N-acetyl-glucosamine and the mixture of N-acetyl-glucosamine with tetraethyleneglycol in acetate buffer, pH 4.5 at 50°C and 40°C. Figure S4: Oxytocin remaining (%) in the presence of different concentrations of 4EG at 50°C and 40°C in phosphate buffer, pH 4.5. Figure S5: Oxytocin remaining (%) in the presence of different concentrations of D-(+)-glucosamine in phosphate buffer, pH 4.5, at 6°C. Figure S6: Oxytocin remaining (%) in the presence of different concentrations of 4EG at 6°C in phosphate buffer, pH 4.5.

Author contributions: Conceptualization, S.G.; methodology, M.G., S.G., and B.R.S.; data curation, M.G., S.J.S. and F.V.N.D.; writing—original draft preparation, M.G.; writing—review and editing, M.G., S.G., and B.R.S.; supervision, B.R.S and S.G.; project administration, B.R.S.; funding acquisition, S.G. All authors have read and agreed to the published version of the manuscript.

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

Acknowledgments: We would like to thank Árni Þorgrímur Kristjánsson and Bergþóra Sigríður Snorradóttir for help with trouble-shooting with the HPLC instrument.

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.

References

Avanti C, Amorij JP, Setyaningsih D, Hawe A, Jiskoot W, Visser J, Kedrov A, Driessen AJ M, Hinrichs WLJ, Frijlink HW (2011) A new strategy to stabilize oxytocin in aqueous solutions: I. The effects of divalent metal ions and citrate buffer. AAPS J 13: 284–290.

Avanti C, Oktaviani NA, Hinrichs WLJ, Frijlink HW, Mulder FAA (2013) Aspartate buffer and divalent metal ions affect oxytocin in aqueous solution and protect it from degradation. Int J Pharm 444: 139–145.

Avanti C, Permentier HP, van Dam A, Poole R, Jiskoot W, Frijlink HW, Hinrichs WLJ (2012) A new strategy to stabilize oxytocin in aqueous solutions: II. Suppression of cysteine-mediated intermolecular reactions by a combination of divalent metal ions and citrate. Mol Pharm 9: 554–562.

European Pharmacopoeia. (2005).

Gard JW, Alexander JM, Bawdon RE, Albrecht JT (2002) Oxytocin preparation stability in several common obstetric intravenous solutions. Am J Obstet Gynecol 186: 496–498.

Ghasemisarabbadieh M, Gizurarson S, Sveinbjörnsson BR (2021a) Effect of 18-crown-6 on oxytocin stability in aqueous buffer solutions. ACS Omega 6: 5805–5811.

Ghasemisarabbadieh M, Gizurarson S, Sveinbjörnsson BR (2021b) The effect of trehalose, antioxidants, and acetate buffer concentration on oxytocin stability. J Peptide Sci: e3324.

Gizurarson S, Sigurdsson SJ (2018) Peptide or Protein Stabilizing Formulation (Patent No. US 9,968,648 B2).

Hawe A, Poole R, Romeijn S, Kasper P, Heijden R van der, Jiskoot W (2009) Towards heat-stable oxytocin formulations: analysis of degradation kinetics and identification of degradation products. Pharm Res 26: 1679–1688.

Hogerzeil HV, Walker,GJA, de Goeje MJ (1993). Stability of injectable oxytocics in tropical climates. WHO/DAP/93.6 https://apps.who.int/iris/handle/10665/59411

Nassta GC, Prankerd RJ, McIntosh MP (2013) Effect of freezing on oxytocin ampules. New Engl J Med 368: 2145–2146.

Say L, Chou D, Gemmill A, Tunçalp Ö, Moller A, Daniels J, Gülmezoglu AM, Temmerman M, Alkema L (2014) Global causes of maternal death: a WHO systematic analysis. Lancet Glob Health 2: e323–333.

Trissel LA, Zhang Y, Douglass K, Kastango E (2006) Extended stability of oxytocin in common infusion. Int J Pharm Compound 10: 156–158.

WHO recommendations Uterotonics for the prevention of postpartum haemorrhage. (2018).

Wisniewski K, Finnman J, Flipo M, Galyean R, Schteingart CD (2013) On the mechanism of degradation of oxytocin and its analogues in aqueous solution. Biopolymers (Peptide Sci) 100: 408–421.

Xing R, Liu S, Wang L, Cai S, Yu H, Feng J, Li P.(2009) The preparation and antioxidant activity of glucosamine sulfate. Chin J Oceanolog Limnol 27: 283–287.