Thermal stabilization of oxytocin and fibroblast growth factor 2

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Ágrip

Oxýtósin er legæða taugapeptíð sem Alþjóðaheilbrigðismálastofnunin ráðleggur að sé notað sem fyrsta stigs vörn til að fyrirbyggja og meðhöndla blæðingar eftir fæðingu. Blæðingar eftir fæðingu eru aðal dánarorsök mæðra (27.1%) í mörgum löndum þar sem tekjur eru lægri. Basíski fibroblast vaxtaþátturinn (FGF2) er hluti af fjölskyldu fibroblast vaxtaþátta. Það tekur þátt í að stýra ýmissi frumustarfsemi, þar á meðal frumufjölgun, flæði, aðgreiningar, sem og æðamyndunar í ýmsum vefjum svo sem húð, æðum, vöðvum, figu, sinum/liðböndum, brjóski, bein, tennum og taugum.

Bæði FGF2 og oxýtósin eru óstöðug og brotna hratt niður við stofuhita. Sýnt hefur verið fram á að FGF2 vatnslausnir eru einungis stöðugar í um viku við 4°C og gögn hafa sýnt að oxýtósin þolir ekki geymsu við 30°C í meira en mánuð eða 2 vikur við 40°C. Markmið þessa verkefnis var að auka hitastöðugleika oxýtósins og gera frumrannsóknir á FGF2 í vatnslausnum til að auka geymsluþol þessara efna.

D-(+)-glúkósamín hýdróklóríð, tetraetýlenglýkól (4EG), N-acetýl-D-glúkósamín og blanda af þessum viðbótarefnum voru prófuð til að sjá áhrif þeirra á stöðugleika oxýtósins og FGF2. Niðurstöður okkar sýna að öll þessara efna gerðu FGF2 óstöðugra í fosfat buffer salín (PBS) lausn. 4EG og D-(+) glúkósamín hýdroklóríð gerðu oxýtósin óstöðugra í bæði fosfat og asetat stuðpúðalausnum. N-acetýl-D-glúkósamín sýndi hverfandi eða hugsanlega örlítið stöðgandi áhrif á oxýtósin.

Áhrif trehalósa á stöðugleika FGF2 og oxýtósins í vatns stuðpúðalausnum var líka prófað. Þrátt fyrir að trehalósi sýndi lítil sem hverfandi áhrif á stöðugleika oxýtósin jafnvel við allt að 1,0 M styrk í asetat stuðpúðalausn, þá hægði það á niðurbrotshraða FGF2 í viðurvist allar stuðpúðalausnanna sem voruð notaðir (HEPES, TRIS, sítrate/fosfat og PBS).

Áhrif mismunandi stuðpúðalausna voru líka metin. Oxýtósin reyndist vera stöðugra í acetat stuðpúðalausn heldur en sítrat/fosfat eða fosfat stuðpúðalausn og við sáum að stöðugleikinn er líka stykháður, þar sem asetat stuðpúðalausnir með styrk 0,025 M eða lægra er betra. FGF2 var stöðugast í PBS í samanburði við aðra stuðpúða sem voru notaðir.

Áhrif kórónuetera á stöðugleika oxýtósins og FGF2 í vatnslausnum var líka kannað. 18-kóróna-6 og 12-kóróna-4 höfðu neikvæð áhrif á stöðugleika FGF2. Við sáum að á

meðan 12-króna-4 og 15-króna-5 höfðu ekki stöðgandi áhrif á oxýtósin, þá hafði 18-króna-6 stöðgandi áhrif á óxýtósin í sítrate/fosfat stuðpúðalausn við pH 4,5. Hins vegar hafði viðurvist 18-króna-6 neikvæð áhrif á stöðugleikann í asetat stuðpúðalausn við sama pH, og leiddi mögulega til annars niðurbrotsferlis.

Áhrif andoxunarefna, svo sem þvagsýru, bútýlað hýdroxýtóluen (BHT) og L-ascorbic sýru var líka prófað fyrir stöðugleika oxýtósins í lausn. Þrátt fyrir að þekktar niðurbrotsleiðir fyrir oxýtósin innihaldi oxun á ákveðnum amínósýrum, þá höfðu andoxunarefnin þvagsýra og BHT hverfandi áhrif á stöðugleika oxýtósins á meðan L-ascorbic sýran leiddi til hraðara niðurbrots.

Áhrif mismunandi sýrustiga á stöðugleika FGF2 í sítrat/fosfat stuðpúðalausn við pH 6-8 og asetat stuðpúðalausn við pH 4,5 og 5,5 sýndi að líkt og önnur prótín í FGF fjölskyldunni, þá er FGF2 stöðugra við sýrustig sem er nálægt hlutlausu eða örlítið basískt í samanburði við súrt pH.

Abstract

Oxytocin is a uterotonic neuropeptide. It has been recommended by the World Health Organization (WHO) as the first line treatment to prevent and treat postpartum hemorrhage (PPH). PPH is the main cause of maternal deaths (27.1%) in many low-income countries. Basic fibroblast growth factor (FGF2) is one of the family members of fibroblast growth factors. It has been shown to regulate many cellular functions including cell proliferation, migration, and differentiation, as well as angiogenesis in a variety of tissues, including skin, blood vessel, muscle, adipose, tendon/ligament, cartilage, bone, tooth, and nerve.

Both FGF2 and oxytocin are unstable and degrade rapidly at room temperature. It has been shown the reconstituted FGF2 solutions are stable for only about one week at 4 ℃ and data shows that oxytocin cannot tolerate being stored at 30°C for more than one month or 2 weeks at 40°C. The aim of this project was to enhance the thermal stability of oxytocin and do preliminary studies on FGF2 in aqueous solutions, which could improve the products shelf-lives.

D-(+)-glucosamine hydrochloride, tetraethyleneglycol (4EG), N-acetyl-D-glucosamine and the mixture of these additives were tested on the stability of oxytocin and FGF2. Our findings showed that all these additives had destabilizing effect on FGF2 stability in PBS. Tetraethylene glycol and D-(+)- glucosamine hydrochloride had a destabilizing effect on oxytocin in both phosphate buffer and acetate buffer. N-acetyl-D-glucosamine showed a negligible or possibly slight improvement to the stability of oxytocin.

The effect of trehalose on stability of FGF2 and oxytocin in aqueous buffer solutions was also tested. Although trehalose showed small to negligible effect on oxytocin stability even at a concentration of 1.0 M in acetate buffer, it slowed down the degradation rate of FGF2 in the presence of all buffers used (HEPES, TRIS, citrate/phosphate and PBS).

The effect of different buffers was evaluated as well. Oxytocin was found to be more stable in acetate buffer than citrate/phosphate buffer or phosphate buffer and we found that the stability is also concentration dependent with acetate buffer concentrations of 0.025 M or less being more beneficial. FGF2 showed the most stablility in PBS buffer compared with the other buffers used.

The effect of crown ethers on the stability of oxytocin and FGF2 in aqueous solution was also explored. 18-crown-6 and 12-crown-4 had destabilizing effect on FGF2. We explored that while 12-crown-4 and 15-crown-5 did not stabilize oxytocin, 18-crown-6 showed to enhance significantly oxytocin stability in citrate/phosphate buffer at pH 4.5. However, in acetate buffer at the same pH, the presence of 18-crown-6 had a destabilizing effect, possibly leading to a different degradation pathway.

The effect of antioxidants such as uric acid, butylated hydroxytoluene, and L-ascorbic acid was also tested on oxytocin stability in solution. Despite known degradation pathways of oxytocin including oxidation of certain amino acids, the antioxidants uric acid and butylated hydroxytoluene had negligible effect on the oxytocin stability while L-ascorbic acid led to significantly faster degradation.

The effect of different pH on FGF2 stability in citrate/phosphate buffer at a pH range (6-8) and acetate buffer at pH 4.5 and 5.5 showed that like other FGF family members, FGF2 is more stable in near-neutral to basic pH compared with acidic pH.

Keywords:

Oxytocin, FGF2, stability, formulation, buffer solutions, crown ethers, trehalose, aminosugars, tetraethyleneglycol, antioxidants, pH effect

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Contents

List of abbreviations

List of Figures

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List of original papers

This thesis is based on the following original publications:

1. M. Ghasemisarabbadieh; S. Gizurarson; Sveinbjörnsson, B.R. The effect of 18-crown-6 on oxytocin stability in aqueous buffer solutions. ACS Omega. 2021, 6, 5805-581.

2. M. Ghasemisarabbadieh, S. Gizurarson and B.R. Sveinbjörnsson. The Effect of trehalose, Antioxidants, and Acetate Buffer Concentration on Oxytocin Stability. J Pept Sci. 2021;. https://doi.org/10.1002/psc.3324

3. M. Ghasemisarabbadieh, S. Gizurarson and B.R. Sveinbjörnsson. The stability effect of Glucosamine, N-acetylglucosamine and Tetraethyleneglycol on oxytocin in Aqueous Solution (Die Pharmazie, accepted).

4. M. Ghasemisarabbadieh, S. Gizurarson and B.R. Sveinbjörnsson. FGF2 stability in aqueous buffer solutions (In preparation).

Declaration of contribution

The doctoral student, Mostafa Ghasemisarabbadeih planned the research work, conducted research, designed the experimental analysis, drafted the manuscripts and wrote this thesis- under the sound guidance of his supervisors, the doctoral committee, and worked in close co-operation with the co-authors of each study

1 Introduction

Peptides and proteins have gained high popularity as drugs and therapeutic agents due to their properties such as high specificity, affinity, and efficacy. As they continue to enter the pharmaceutical market, their stability becomes an important issue for the manufacturers, and the users, and a scientific puzzle for pharmaceutical scientists.

The instabilities of peptides/proteins can be classified into chemical instability and physical instability. Chemical instability refers to the formation or destruction of covalent bonds within the polypeptide or protein molecule. Common reasons for chemical instability are deamidation, racemization, hydrolysis, beta-elimination, oxidation, and cystine destruction/disulfide exchange (Volkin & Ahern, 1992; Manning M. P., 1989). Physical instability refers to any process that changes the physical state of the biomolecule without any change in its chemical composition. Physical instabilities consist of denaturation, aggregation, precipitation, adsorption to surface, and protein unfolding (Manning M. P., 1989; Brange & Frokjaer, 2000; Volkin & Ahern, 1992; Randolph & Carpenter, 2002). Chemical instability, such as deamidation and disulfide bond cleavage, may also lead to physical instabilities, and inversely.

The integrity of the peptide/protein structure can often be very temperature dependent. Therefore, thermal stability of these biomolecules is important, and needs to be closely monitored to maintain the pharmaceutical potency of the formulation. The importance of thermal stability of biomolecules has become even more apparent following the COVID-19 pandemic, as one of the main problems with some of the SARS-CoV-2 vaccines is storage. Although these vaccines are not peptides and proteins, they include very fragile biomolecules.

As a result of thermal instability, the use of peptides and proteins is often limited in areas that do not have easy access to proper storage units (such as refrigerators) or stabilization techniques to prevent them from chemical or physical changes. Of special interest in this thesis was the peptide oxytocin, but initial screenings with the protein fibroblast growth factor 2 (FGF2) were also conducted.

1.1 Oxytocin

Oxytocin is a nonapeptidic hormone with a molecular weight of 1,007.19 g/mol. The oxytocin structure was elucidated in 1951 and the characterization and synthesis were reported in 1954 (Winkler & Rath, 1999; Davoli, Turner, Pierce, & du Vigneaud, 1951; du Vigneaud, Ressler, & Trippett, The Sequence of Amino Acids in Oxytocin, with a Proposal for the Structure of Oxytocin, 1953). It is a cyclic nonapeptide that includes a ring composed of six amino acids, with a disulfide bridge closing the ring (Figure 1). Oxytocin is recommended by the World Health Organization (WHO) for the prevention and treatment of postpartum hemorrhage, the primary cause of maternal deaths around the globe (Winkler & Rath, 1999; WHO, 2018; Say, et al., 2014; Van Dongen, Van Roosmalen, De Boer, & Van Rooij, p. 1991). Oxytocin is also used therapeutically to induce labor and has numerous other biological and psychological functions including a role in lactation and relationships (Olff, et al., 2013). More recently, efforts have been made towards using oxytocin as a drug to treat conditions such as alcoholism (Lee & Weerts, 2016), autism (Young & Barrett, 2015), and schizophrenia (Averbeck, Bobin, Evans, & Shergill, 2012; Rubin, et al., 2010), as well as being studied for its potential modulatory role in antinociception (Kang & Park, 2000).

Unfortunately, oxytocin is unstable in aqueous solutions and degrades rapidly when kept at temperatures above 30℃ (de Groot, Vree, Hogerzeil, & Walker, 1994). In addition to that, we must face the fact that oxytocin products in low and middle income countries, are often of poor quality (Torloni, Gomes Freitas, Kartoglu, Metin Gülmezoglu, & Widmer, 2016). This may be because of poor manufacturing quality, inadequate transport conditions and/or storage, such as limited cold chain systems in resource-constrained settings, or due to both (Davoli, Turner, Pierce, & du Vigneaud, 1951; du Vigneaud, Ressler, & Trippett, The Sequence of Amino Acids in Oxytocin, with a Proposal for the Structure of Oxytocin, 1953). According to a report from the Reproductive Health Supplies Coalition, there are about 300 different oxytocin products on the market, with most of them being labeled for refrigerated storage, but some being labeled for non-refrigerated storage (Schocken, 2014). A recent study showed that some of these products can tolerate exposure to room temperature (25-30°C) for a few months with little effect to the oxytocin. However, that same study found that some of the products labeled for non-refrigerated storage had in fact lower stability than the high-quality products intended for storage at 2-8°C (Hagen, 2020).

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 (Malm & Kjellström, 2018; Widmer, et al., 2018). 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 (Wisniewski, Finnman, Flipo, Galyean, & Schteingart, 2013). 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 (Malm & Kjellström, 2018). 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 (Briones, Talungchit, Thavorncharoensap, & Chaikledkaew, 2020).

Several approaches have been explored to enhance the stability of oxytocin. In these studies the effect of different buffer solutions was evaluated, including citrate, phosphate, citrate/phosphate, acetate, and aspartate buffers as well as lactated Ringer‘s solution (Hawe, et al., 2009; Avanti, et al., 2011; Avanti, et al., 2012; Avanti, Oktaviani, Hinrichs, Frijlink, & Mulder, 2013; Wisniewski, Finnman, Flipo, Galyean, & Schteingart, 2013; Kumar, et al., 2007; Trissel, L. A.; Zhang, Y.; Douglass, K.; Kastango, E. Extended Stability of Oxytocin in Common Infusion, 2006; Gard, Alexander, Bawdon, & Albrecht, 2002).

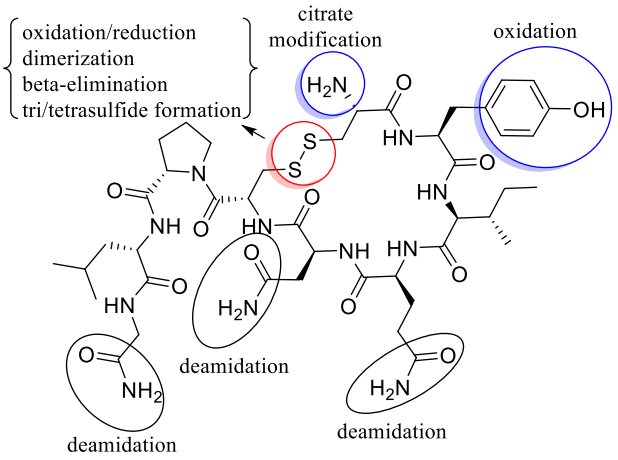


Figure 1. The structure of oxytocin, with sites known to be susceptible to degradation circled.

Citrate buffer was shown to accelerate the degradation of oxytocin, forming amide- and imide-linked degradation products (Figures 2 and 3) (Avanti, et al., 2012; Poole, Kasper, & Jiskoot, 2011). The main reaction site for this degradation process has been shown to be the N-terminal primary amine. The authors showed that although the degradation profiles are similar in acetate, phosphate, and citrate buffers, there is a series of peaks that appear before the main oxytocin peak (which appears at approximately 6 min) and that are present only for the sample formulated in citrate buffer, indicating that additional degradation products are being formed only in that buffer (Figure 3).

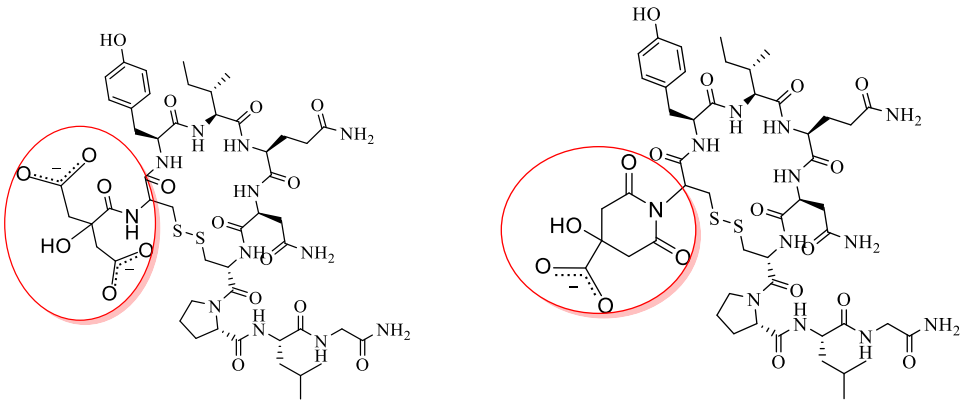


Figure 2. Molecular structures of oxytocin N-citryl amide (left) and N-citryl imide (right) from the degradation of stressed oxytocin in citrate buffered solution (Reproduced with permission from (Avanti, et al., 2012)).

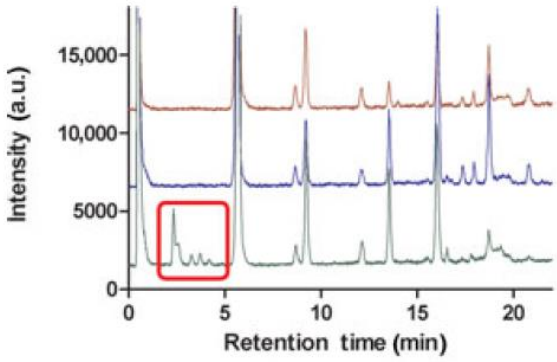


Figure 3. LCMS chromatograms of heat-stressed oxytocin (0.1 mg/mL, 48 h at 70°C) in 10 mM acetate (red, upper), phosphate (blue, middle), and citrate (green, lower) pH 4.5 (Reproduced with permission from (Poole, Kasper, & Jiskoot, 2011)).

The acidity of the buffer has also been found to play an important role, with the best stability found at pH ≈ 4.5 (Figure 4) (Hawe, et al., 2009; Wisniewski, Finnman, Flipo, Galyean, & Schteingart, 2013). It has been shown that at strongly acidic (pH = 2.0) conditions, the primary degradation mechanism of oxytocin changed and was found to be deamidation (Asn, Gln or Gly-NH_2) as evidenced by the change in peaks and their intensity in the high-performance liquid chromatography (HPLC) chromatograms (Figure 5). The deamidation of Asn and Gln residues under acidic conditions takes place by direct

hydrolysis of the Asn or Gln side chains, to form the free carboxylic acid, yielding Asp and Glu, respectively (Figure 6) (Manning M. P., 1989). Various deamidated oxytocin molecules were identified at pH 2.0 and pH 9.0 (Figure 5).

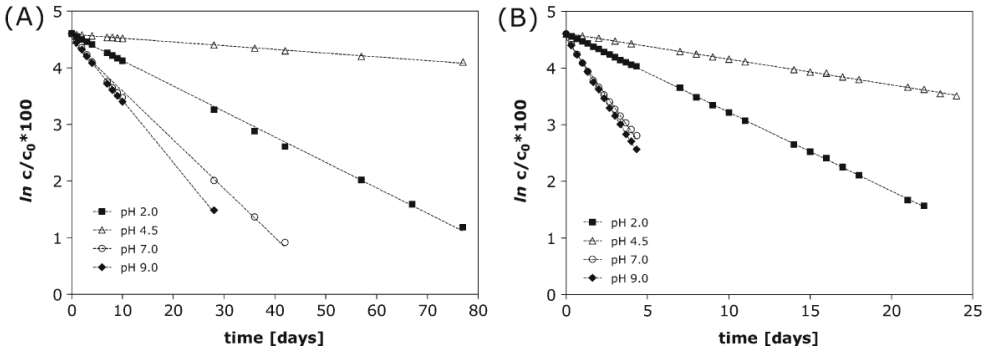


Figure 4. Degradation of oxytocin over time at 40°C (A) and 55°C (B), for formulations with 0.1 mg/ml oxytocin at pH 2.0, 4.5, 7.0 and 9.0, determined by RP-HPLC (Reproduced with permission from (Hawe, et al., 2009)). Note the time difference in x-axis when comparing these two graphs.

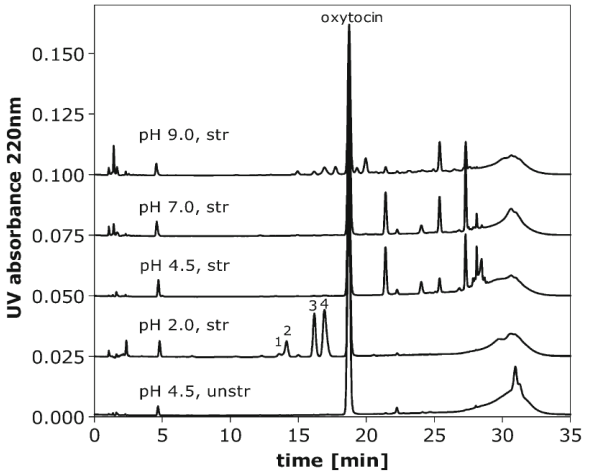


Figure 5. Example RP-HPLC chromatograms of 0.1 mg/ml oxytocin standard and stressed formulations in 50 mM phosphate buffer at pH 2.0, 4.5, 7.0, and 9.0 (UV signal at 220 nm). The peaks eluting before 5 min are not related to oxytocin, but to the buffer (Reproduced with permission from (Hawe, et al., 2009)*).*

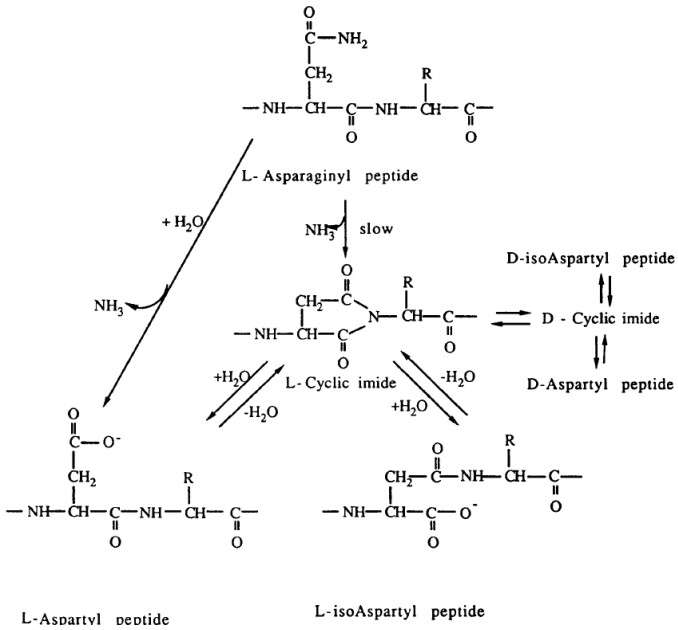


Figure 6. Deamidation mechanism of Asn residues and isomerization of Asp to isoAsp (reproduced with permission from (Manning M. P., 1989)). Direct hydrolysis occurs below pH 4 while the cyclic imide pathway predominates at pH 6 and above.

At pH 4.5, 7.0 and 9.0 the formation of tri- and tetrasulfide-containing monomeric oxytocin, different oxytocin dimers, and larger aggregates were identified (Manning M. P., 1989). The interchange mechanism of disulfide bond has been found to be different in acidic and alkaline solutions (CECIL, 1959; Manning M. P., 1989; Benesch, 1958). Under neutral and alkaline conditions, the reaction is catalyzed by thiolate ions that carry out a nucleophilic attack on a sulfur atom of the disulfide (Figure 7). Under acidic conditions, the interchange takes place via a sulfonium cation, which is formed by protonation of one of the sulfurs in the disulfide bond (Manning M. P., 1989; CECIL, 1959; Benesch, 1958), followed by the disulfide bond breaking. The sulfonium cation carries out an electrophilic displacement on a sulfur atom of the disulfide (Figure 8). Addition of thiols can stop such exchange by scavenging the sulfonium cations.

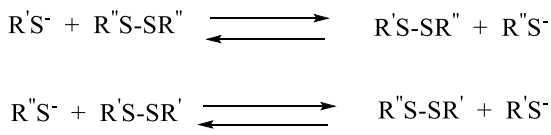


Figure 7. The mechanism of disulfide exchange under neutral and alkaline conditions.

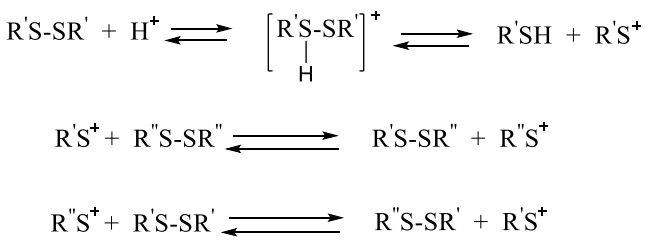


Figure 8. The mechanism of disulfide exchange under acidic conditions.

The proposed mechanism of degradation at near-neutral pH suggests that the first step of degradation process appears to be C-S bond breakage by beta-elimination, expelling R-S-S^- as the leaving group (Figure 9) (Wisniewski, Finnman, Flipo, Galyean, & Schteingart, 2013). The mechanism of beta-elimination is shown in Figure 10. The experimental support for this hypothesis included testing oxytocin analogs with minor modifications for stability at 40°C in phosphate buffer solution at pH 7.4. For unaltered oxytocin, only 12.6% remained after 28 days, but when the sulfur atoms in the disulfide bridge were substituted with CH_2 units, 97.5% of this new analog remained. Those results support the idea that an important early degradation step is the expulsion of R-S-S^- as a leaving group (Wisniewski, Finnman, Flipo, Galyean, & Schteingart, 2013). Another interesting result was obtained when the only free amino group was substituted for a hydrogen, resulting in 90.1% of that analog remaining after 28 days under the above-mentioned conditions (Wisniewski, Finnman, Flipo, Galyean, & Schteingart, 2013). A potential explanation for this observation is that the free amine could act as the base, thus promoting the beta-elimination reaction. This hypothesis is further supported by oxytocin showing the greatest stability around pH 4.5 (Hawe, et al., 2009; Wisniewski, Finnman, Flipo, Galyean, & Schteingart, 2013), at which pH more of the amino group is significantly protonated and thus unable to act as a base. Furthermore, oxytocin has shown faster degradation kinetics when stored at higher concentrations at pH 4.5, suggesting a potential intermolecular degradation pathway with another oxytocin molecule (Hawe, et al., 2009). If the process was primarily intramolecular or between oxytocin and the solvent, the oxytocin concentration should have a more limited effect on the kinetics of degradation. This concentration dependence was not observed at pH 2.0 which can be explained by another degradation mechanism taking over. As the RP-HPLC chromatograms for stressed oxytocin at pH 7.0 and 4.5 are very similar (*Figure 5*), this may also be the primary mechanism of degradation at that pH.

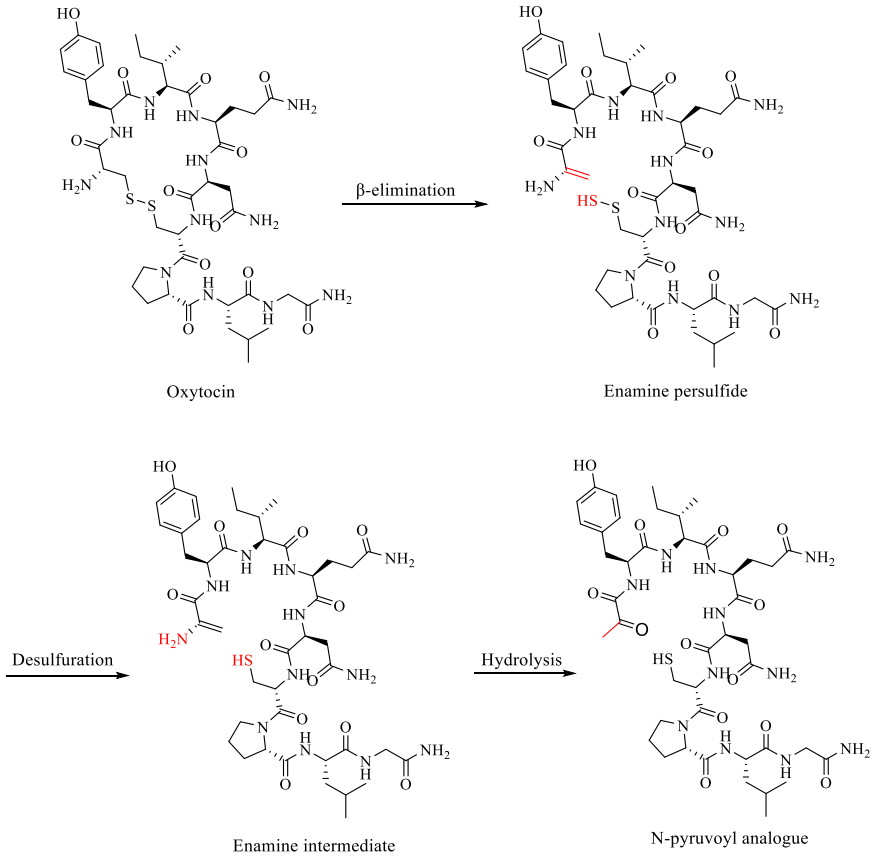


Figure 9. Proposed degradation of oxytocin in solution at near-neutral pH.

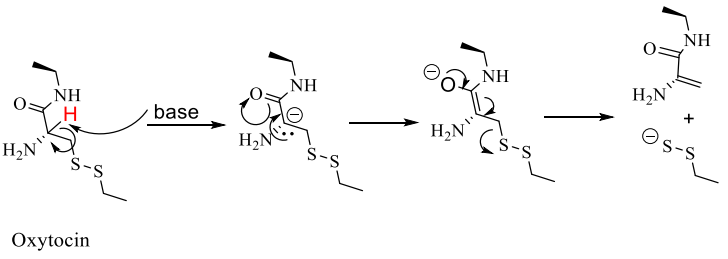


Figure 10. Beta-elimination mechanism of oxytocin.

The effect of divalent metal ions in citrate and aspartate buffer solutions has also been evaluated. These studies showed that oxytocin stability increased in aqueous formulations in the presence of citrate buffer (10 mM) or aspartate buffer (10 mM) with divalent salts such as: $CaCl_2$, $MgCl_2$, or $ZnCl_2$ (Figures 11 and 12) (Avanti, et al., 2011; Avanti, et al.,

2012; Avanti, et al., 2012; Avanti, Oktaviani, Hinrichs, Frijlink, & Mulder, 2013). Oxytocin remaining in the presence of 10 mM of Ca^{2+}, Mg^{2+} and Zn^{2+} after 5 days at 70 °C showed that oxytocin is more stable in the presence of Zn^{2+} than in the presence of Ca^{2+} or Mg^{2+} in both aspartate buffer and citrate buffer. These results also showed that not only was the amount of oxytocin remaining after 5 days significantly different in aspartate buffer (25 %) compared with that in citrate buffer (35 %), but the effect of divalent metal ions was more noticeable where aspartate buffer was used, with Zn^{2+} resulting in 45% oxytocin remaining while in citrate buffer this amount was 73%.

Dextrose and isotonic sodium chloride solutions have similarly been tested in an attempt to enhance the stability of oxytocin in solution (Catinean, 2019). Recently, chlorobutanol has also shown promise as a stabilizing agent for oxytocin (Hagen, 2020). Likewise, dry powder formulations have been tested with excipients such as trehalose, isoleucine, polyvinylpyrrolidone in the presence of citrate and zinc salts to make the solid state oxytocin stable in extreme hot climates (Fabio, et al., Heat-Stable Dry Powder Oxytocin Formulations for Delivery by Oral Inhalation, 2015). Despite some of these efforts having resulted in the enhancement of oxytocin stability, there is still room for further improvement.

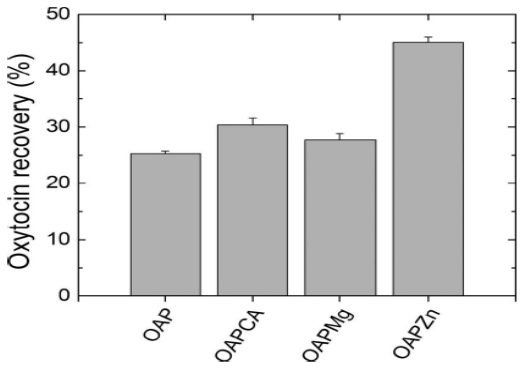


Figure 11. Recovery of oxytocin in the absence (OAP) and presence of 10 mM Ca^{2+} (OAPCa), Mg^{2+} (OAPMg), and Zn^{2+} (OAPZn) in 10 mM aspartate-buffered solution at pH 4.5 under stressed conditions at a temperature of 70℃ for 5 days (Reproduced with permission from (Avanti, et al., 2012)).

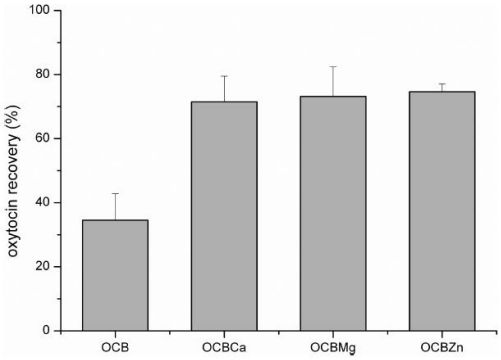


Figure 12. *Recovery of oxytocin in the absence (OCB) and presence of 10 mM Ca^{2+} (OCBCa), Mg^{2+} (OCBMg) and Zn^{2+} (OCBZn) in 10 mM citrate-buffered solution at pH 4.5 under stressed conditions at a temperature of 70 °C for 5 days (Reproduced with permission from (Avanti, et al., 2012)).*

1.2 Basic fibroblast growth factor (FGF2)

Fibroblast growth factor 2 (FGF2) is one of the best characterized members of the family of fibroblast growth factors (Figure 13). FGF2 is a 17 kDa heparin binding protein that plays an important role in stem cell culture medium (Catinean, 2019; Nur-E-Kamal, 2008), various healing processes (Okabe, Hayashi, Aramaki-Hattori, Sakamoto, & Kishi, 2013; Nagayasu-Tanaka, et al., 2015), has protective properties (Beenken, 2009; Katsouri, 2015), and has been used in cancer treatments (Ilkow, et al., 2015), as well as treatment of mood disorders (Turner, 2011). FGF2 also plays an important role in tissue repair, bone growth, angiogenesis, and neuroregeneration (Chen B. L., 1996). Unfortunately, FGF2 is not very stable, with the half-life of native FGF2 being less than 8 hours at 37°C (Levenstein, 2006; Furue MK, 2008), and the instability of FGF2 as a therapeutic protein medicine is of concern when it is stored and transported (Edelman, 1991; Whalen, 1989). Methods to enhance the thermostability of FGF2 in a formulation to improve the product's shelf-life would therefore be beneficial.

There have been a wide range of stability studies with different strategies to enhance the stability of FGF2 in solution. These studies can be classified into ionic interactions between FGF2 and heparin, or heparin-mimicking polymers, or polycations to form the complexation of the protein with additives that lead to reduction of the structural energy at the heparin-binding site, stabilize the FGF-2 native conformation, and prolong its

bioactivity in aqueous solution (Chu, Gao, Chen, Huard, & Wang, 2011; Paluck, Nguyen, Lee, & Maynard, 2016; Wu, Mao, Hong, Han, & Gao, 2013), chemical protection of FGF2 based on binding the protein to another molecule in order to slow down or retard the active conformation of the FGF2 molecule, preventing those residues of the FGF2 molecule from being involved in undesired reactions (Chu, Gao, Chen, Huard, & Wang, 2011; Paluck, Nguyen, Lee, & Maynard, 2016; Wu, Mao, Hong, Han, & Gao, 2013; Macdonald, Rodriguez, Shah, & Hammond, 2010; Nguyen, et al., 2013; Dvorak, et al., 2017; Moon, 2015), and physical protection of FGF2 with strategies including the encapsulation of FGF2 within a polymer matrix, and the fabrication of a mixture of the protein with polymers into hydrogels or other composite scaffolds (Ali, 2018; Galderisi, et al., 2013; Layman, et al., 2007; Lotz, et al., 2013; Yoon, Kim, El-Fiqi, Jang, & Kim, 2017; Patent No. 5482929, 1996). In this project we were looking for physical protection of FGF2 and although some of these physical protection studies have shown very promising data, they have not yielded much information about the degradation mechanism. Little information is provided on the stability of the protein and factors such as pH, ionic strength and buffer effect which are known to affect the stability of proteins were not reported for FGF2. Therefore, we endeavored to conduct preliminary tests on FGF2 stability such as the effect of buffer and pH and also, we decided to test potential stabilization effect of some additives in order to enhance FGF2 stability.

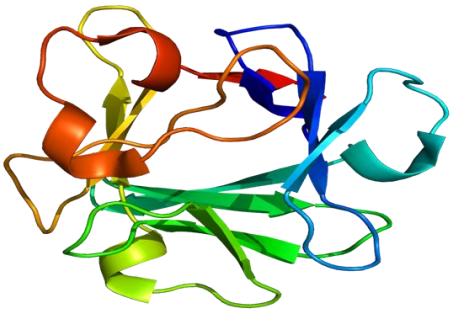
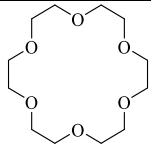
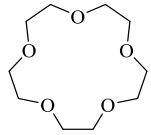
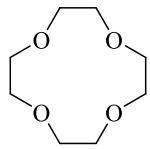
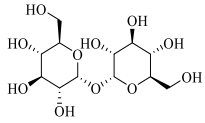
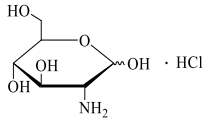
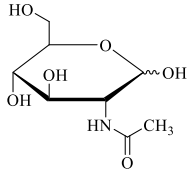
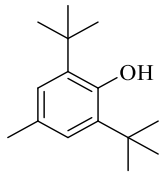


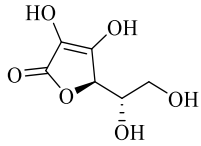
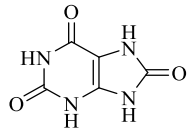
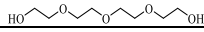
Figure 13. The structure of fibroblast growth factor 2 (FGF2) (Reproduced from Protein Data Bank in Europe (PDBE)).

1.3 Additives

In this thesis the potential stabilization effect of a few additives on oxytocin and FGF2 in different buffers, using non-covalent interactions, was tested. Table 1 shows the additives and compounds that were used. Additionally, the optimal pH for FGF2 stability in the presence of different buffers was also examined.

Table 1. The structure, molecular weight, and solubility in water of the additives tested.

Additive	Chemical structure	Molecular weight	Solubility in water (g/L)
18-crown-6		264.122	75
15-crown-5		220.265	Miscible
12-crown-4		176.21	Miscible
Trehalose		342.296	689
D-(+)-Glucosamine hydrochloride		215.63	100
N-Acetyl-D-glucosamine		221.21	50
Butylated hydroxytoluene (BHT)		220.35	0.011

L-ascorbic acid		176.12	0.33
Uric acid		168.1103	0.06
Tetraethyleneglycol (4EG)		194.23	Miscible

1.3.1 Trehalose

Trehalose is generally known to have a stabilizing effect on certain biochemicals, such as proteins and lipids, and can be especially important for anhydrobiotic organisms (Crowe J. H., 1984). The role of trehalose as a stabilizer and protective agent against environmental stresses for various biochemicals, in particular lipids and proteins has been studied (Fabio, et al., Heat-Stable Dry Powder Oxytocin Formulations for Delivery by Oral Inhalation, 2015; Nagase, et al., 2008; Olgenblum, Sapir, & Harries, 2020; Patent No. US 9,968,648 B2, 2017). It has been shown to enhance the stability of these molecules both under anhydrous conditions (Crowe J. H., 1984; Fabio, et al., Heat-Stable Dry Powder Oxytocin Formulations for Delivery by Oral Inhalation, 2015; Nagase, et al., 2008; Olgenblum, Sapir, & Harries, 2020; Patent No. 01572, 2017; Crowe, Carpenter, & Crowe, 1998), and specially relevant for this thesis, in aqueous solutions, with higher concentrations yielding greater stability enhancing effect (Xie & Timasheff, 1997; Kaushik & Bhat, 2003; Sola-Penna & Meyer-Fernandes, 1998; Carninci, et al., 1998; Lins, Pereira, & Hünenberger, 2004 ; Lin & Timasheff, 1996). There are two hypotheses about the stabilization mechanism that can be applied to both conditions: The water-replacement hypothesis in which hydrogen bonding between trehalose and the biomolecule is believed to play a key role (Fabio, et al., Heat-Stable Dry Powder Oxytocin Formulations for Delivery by Oral Inhalation, 2015; Nagase, et al., 2008; Olgenblum, Sapir, & Harries, 2020; Sola-Penna & Meyer-Fernandes, 1998; Lins, Pereira, & Hünenberger, 2004), and the high viscosity hypothesis (Xing, et al., 2009; Ames, Cathcart, Schwiers, & Hochstein, 1981), which suggests the reduction of solvent molecular motion by the formation of a glassy matrix of trehalose and water with high viscosity, but the solvent molecular motion can lead to structural loss and denaturation. In the water-replacement hypothesis, one could envision

trehalose being able to form a protective layer around a water layer that encircles the biomolecule (Figure 14), providing a protection against chemical degradation by minimizing undesired degradation reactions, while in the high viscosity hypothesis, one could envision that slowing down the solvent molecular motion could lead to a slowing down of chemical reactions as a consequence.

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 FGF2 and oxytocin in aqueous solutions. Therefore, we tested its potential stabilization effect on oxytocin and FGF2 in different buffer solutions.

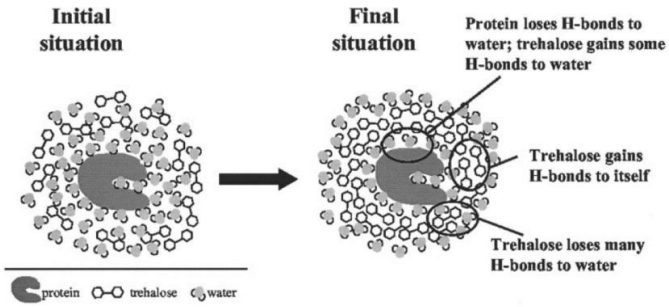


Figure 14. Model for trehalose–protein interaction in aqueous solution on the nanosecond timescale (Reproduced with permission from (Lins, Pereira, & Hünenberger, 2004)).

1.3.2 Aminosugars and tetraethyleneglycol

It has been recently suggested that a combination of a low molecular poly or oligo(ethylene glycols) such as tetraethyleneglycol and select aminosugars such as glucosamine, galactosamine, fructosamine, mannitosamine, and N-acetylglucosamine, could increase the thermal stability of peptides and proteins in aqueous solution (Patent No. US 9,968,648 B2, 2017). The examples provided suggest that a combination of glucosamine and tetraethyleneglycol (4EG) may have a beneficial effect on oxytocin's stability in 50 mM phosphate buffer at pH 4.5 (Patent No. US 9,968,648 B2, 2017).

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 (Chang L, 2005). Biomolecules that contain His, Met, Cys, Tyr and Trp amino acids are prone to be

damaged by reactions with any of a number of reactive oxygen species (ROS) (Sampedro JG, 2004; Simic & Jovanovic, 1989; Yehye, et al., 2015; J. D. Lamb, 1980; Paul, 2003). Oxidation of these reactive sidechains in a biomolecule can take place during any stage of protein production, purification, formulation and storage (Chen B. L., 1996; ER., 1993; Hovorka SW, 2001; Hawkins, 2001).

The degradation studies on oxytocin have also found that Tyr^{2} and $Cys^{1,6}$ can undergo oxidation (Avanti, et al., 2012) (Figure 1). It was, therefore, decided to explore the effect of aminosugars, like D-(+)-glucosamine or N-acetyl-D-glucosamine and a mixture of them with 4EG on the stability of oxytocin and FGF2.

1.3.3 Antioxidants

Antioxidants were first tested in order to see if they could protect against a color change that was observed in oxytocin samples containing D-(+)-glucosamine (the solutions turned yellow/red-brown), and was hypothesized to be due to oxidation of the aminosugar in solution. A commonly used antioxidant, butylated hydroxytoluene (BHT) prevented the color from developing in these samples to some extent. Oxytocin degradation studies have also shown that Tyr^{2} and $Cys^{1,6}$ are susceptible to oxidation-reduction reactions, amongst other degradation pathways (Avanti, et al., 2012). For this reason, it was decided to explore the effect of a few antioxidants on oxytocin's stability, hoping it could be improved using this approach. 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. Although it has 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 (Li, 1995; J., 2005). BHT is one of the most commonly used antioxidants in various industries, including the food and pharmaceutical industry (Kerwin BA, Protect from light: photodegradation and protein biologics , 2007). 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 and success in preventing the color change in the glucosamine samples. 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.

1.3.4 Crown ethers

Crown ethers are small, cyclic polyethers and known cation chelators (Lamb JD, 1980). They have found wide-spread use in phase-transfer catalysis and the activation of proteins in organic solvents (Paul D, 2003). Certain crown ethers, including 18-crown-6, are known to have a binding affinity for protonated ammonium groups (Angela F. Danil de Namor, 1991; Steed JW, 2013), and also positively charged amino acids and N-termini in small peptides (Chen Y. &., 2012). Amino acids have also been shown to form complexes with 18-crown-6 (A .F. Danil de Namor, 1991; A .F. Danil de Namor, 1991). The utility of cyclic polyether 18-crown-6 as non-covalent protecting group in peptide synthesis was already illustrated (C.B Hyde, 1990; M. A, 1989; C.B Hyde, 1990). In contrast, smaller crown ethers, such as 15-crown-5 and 12-crown-4 do not show a strong binding affinity to ammonium groups due to the smaller ring sizes (Pagni, 2006).

As mentioned earlier, the N-terminal amine of oxytocin is prone to amide- and imide-linked degradation (Poole, Kasper, & Jiskoot, 2011), and might be involved in the beta-elimination reaction that can initiate the degradation process for oxytocin. We hypothesized that if the amino group could be further inhibited from acting as a base, it would retard the degradation process of oxytocin (Figure 15) and possibly provide similar protection to proteins such as FGF2. To test this hypothesis, it was decided to use 18-crown-6 ether, and smaller crown ethers, such as 15-crown-5 and 12-crown-4 as controls. It should be noted however, that 18-crown-6 may not be a suitable excipient in pharmaceutical formulations, as it also has a strong binding affinity for potassium ions, and studies have shown oral toxicity in dogs and mice (Takayama, Hasegawa, & Sasagawa, 1977; Hendrixson, Mack, Palmer, Ottolenghi, & Ghirardelli, 1978). Still, the goal of using crown ethers was to explore a mechanistically guided approach to the oxytocin and FGF2 thermostability challenge for which it could provide valuable insight.

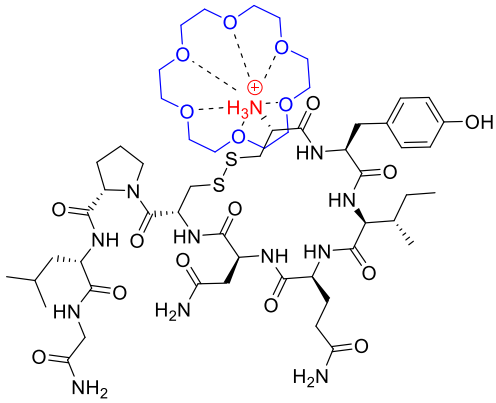


Figure 15. Hypothesized stabilizing interaction between 18-crown-6 (blue) and the protonated ammonium group (red) on the oxytocin molecule (Reproduced with permission from (Ghasemisarabbadieh, Gizurarson, & Sveinbjörnsson, 2021)).

2 Aim

Oxytocin and FGF2 are both susceptible to degradation at elevated temperatures (room temperature and above). Oxytocin should generally be stored between 2-8 ℃ and it can not tolerate being stored for more than one month at 30 ℃ or two weeks at 40 ℃, although some products claim to be stable at higher temperatures and a more thermostable formulation of carbetocin, an oxytocin analog, has been developed (Hagen, 2020). FGF2 needs to be stored as a lyophilized powder at -20℃, and the reconstituted FGF2 solutions are stable for only one week at 4 ℃. FGF2 solutions at a concentration of 72 µg/mL lose 50% functionality after only 46 minutes at 25 ℃. The functional half-life was decreased to 37, 33 and 10 minutes respectively, as the storage temperature was increased to 37 ℃, 42 ℃ and 50 ℃. Instability of these medicines is problematic for hot climate where daytime temperature can exceed to 40 ℃ and a reliable cold chain storage is not always achievable.

The purpose of this thesis was to enhance the thermal stability of oxytocin in aqueous solution which could improve the products shelf-life and start analogous stability enhancement studies with FGF2.

The following aims represent each paper /manuscript enclosed or planned within the project:

1. To test the effect of trehalose, antioxidants, and acetate buffer concentration on oxytocin stability in aqueous solutions.
2. To protect the N-terminal amino group of oxytocin which is susceptible to participate in beta-elimination, the first step of oxytocin degradation, using 18-crown-6.
3. To test the effect of glucosamine, N-acetylglucosamine and the mixture of these additives with tetraethyleneglycol on oxytocin stability.
4. To test the stability of FGF2 at different pH and the effect of buffers such as PBS, HEPES, TRIS and citrate/phosphate buffer. Additionally, to test the effect of the additives that were tested on oxytocin.

3 Materials and Methods

3.1 Materials

Oxytocin was purchased from Grindeks (Latvia). The following chemicals were bought from Sigma-Aldrich, with the country of origin indicated in brackets as well as the city for the compounds where that information was provided: 18-crown-6 (India), 15-crown-5 (Switzerland), 12-crown-4 (Germany), potassium chloride (Germany), sodium hydrogen phosphate (Germany), sodium dihydrogen phosphate (Germany), trifluoroacetic acid (France), potassium dihydrogen phosphate (Germany), D-(+)-glucosamine hydrochloride (UK), N-acetyl-D-glucosamine (China), tetraethyleneglycol (St. Louis, MO, USA), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (St. Louis, MO, USA), butylated hydroxytoluene (Russia), uric acid (Hungary), L-ascorbic acid (China), ammonium acetate (St. Louis, Missouri, USA) and D_2O (Canada). Citric acid anhydrous, methylparaoxybenzoate and propylparaoxybenzoate were bought from Pernhofen (Jungbunzlauer, Austria). Ortho-phosphoric acid (85%), sodium acetate and TRIS base (tris(hydroxymethyl)aminomethane) were obtained from Merck (Darmstadt, Germany). Acetic acid, acetonitrile, sodium chloride, hydrogen chloride and methanol were purchased from Honeywell (Hannover, Germany). Trehalose from Pfanstiehl Inc. (Waukegan, USA) was kindly provided by Alvotech (Reykjavik, Iceland). An FGF2 sample was kindly provided by ORF Genetics (Reykjavik Iceland) and additional FGF2 was purchased from R&D systems along with ELISA kits for the FGF2 studies. The ELISA kits (Human FGF basic/FGF2/bFGF DuoSet ELISA and DuoSet ELISA Ancillary Kit 2) consisted of capture antibody, detection antibody, recombinant standard, streptavidin conjugated to horseradish-peroxidase (Streptavidin-HRP), color reagents A and B, stop solution, wash buffer and reagent diluent concentrate. All water used in this study was purified, using a Milli-Q water purification system.

3.2 Quantitative analysis

3.2.1 Quantitative analysis of oxytocin

High performance liquid chromatography system (HPLC) from Dionex Softron GmbH (Germering, Germany) was used in this study. It included a Dionex UltiMate 3.0 HPLC system, controlled by Dionex Chromeleon software v7.2. The equipment consisted of a column oven compartment, an autosampler with temperature control, an UltiMate 3000 pump, and an UltiMate 3000 photo- diode array detector. The columns used were Phenomenex Luna® 5µm C18(2), 250 x 4.6 mm, 100Å, LC column with a Security Guard Cartridge (C18 4 x 3.0 mm) and Phenomenex Luna® 5µm C18(2), 150 x 4.6 mm, 100Å, LC column with a Security Guard Cartridge (C18 4 x 3.0 mm). The injection volume was 20 µL and wavelength was 220 nm. The compositions of isocratic mobile phases are shown in Table 2. All HPLC methods showed good selectivity and were operated at 1 mL/min flow rate.

Table 2. Chromatographic conditions

Method	Mobile phase (A) [a]	Mobile phase (B) [a]	Ratio [b]	pH [c]
1	0.01%TFA/H_2O	0.01%TFA/70%AC/30%H_2O	70:30	3.02
2	0.1% TFA/H_2O	0.01%TFA/70%AC/30%H_2O	70:30	2.04
3	Acetic acid	20 mM Ammonium acetate	25:75	5.8
4	50% Acetonitrile	0.1M Sodium dihydrogen phosphate	30:70	5.7

[a] TFA: trifluoroacetic acid

[b] volume ratio of the phases before mixing.

[c] pH of the aqueous phase before mixing with the non-aqueous phase.

Methods 1 and 2: At minutes 0-17, mobile phase A went from 70 to 40% in a linear gradient elution. From minute 17-19 the mobile phase went back to 70%. The equilibration time was 11 minutes.

Method 3: At minutes 0-8, mobile phase A was constant at 75%. From minute 8-10.5 mobile phase A went down to 10% in a linear gradient elution, followed by an isocratic step at this concentration until minute 11. From minute 11-12.5, mobile phase A percentage was increased again to 75%. The equilibration time was 3.5 minutes.

Method 4: At minutes 0-17, mobile phase A went from 70 to 40 % (v/v) in a linear gradient elution. From minute 17-19, mobile phase A went back to 70%. The equilibration time was 11 minutes.

UV-Vis spectrophotometric analyses were performed on a Genesys 150 UV-visible spectrophotometer using 1 cm quartz cells. The range of wavelengths measured was 200-300 nm.

^{1}H NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer. Deuterated water was used as solvent.

3.2.2 Quantitative analysis of FGF2

The method used for FGF2 analysis was a sandwich ELISA using Human FGF basic/FGF2/bFGF DuoSet ELISA along with the DuoSet ELISA Ancillary Reagent Kit 2 from R&D systems. The protocol provided by R&D systems was followed according to instructions provided by the manufacturer in the preparation of the samples (BioTechneBrands, 2021). The color was analyzed using a BioTeK Synergy/LX multi-mode plate-reader instrument (Vermont, USA), at λ = 450 nm, 540 nm or 570 nm. All the washings were carried out using a microplate washer 50/TS. The samples were prepared in triplicate and the concentrations of the samples were calculated according to a plot of concentration vs. absorption for the FGF2 standards.

3.3 Formulation of oxytocin samples

The oxytocin concentration was kept constant at 0.25 mg/mL in all samples of all series. The pH of all series of samples was adjusted to 4.5 and all series included an oxytocin control that was prepared in the same buffer as the samples in the series, but without any additives. Oxytocin standards were made at a range of concentrations (10 mg/mL, 4.0 mg/mL, 1.6 mg/mL, 0.64 mg/mL, 0.26 mg/mL, 0.10 mg/mL, 0.04 mg/mL) in a buffer at pH 4.5.

3.3.1 Preparation of buffers samples

For buffer series, oxytocin samples were prepared in phosphate buffer (0.1 M), citrate/phosphate buffer (0.1 M/0.2 M) as well as acetate buffer at a range of concentrations (0.01 M/0.025 M/0.05 M/0.1 M/0.2 M). The samples were stored at 50°C and analyzed by HPLC two times per week over a two-week period using method 1.

3.3.2 Preparation of trehalose samples

Oxytocin samples were prepared with trehalose added at a range of concentrations (10 mM, 20 mM, 30 mM, or 40 mM) in acetate buffer (0.1 M) and citrate/phosphate buffer (0.1 M/0.2 M). Additional samples were prepared with trehalose at higher concentrations (0.1 M, 0.5 M and 1.0 M) in acetate buffer. The formulations were stored at 50°C for a period of up to 16 days and were analyzed by HPLC (method 2) several times during their storage periods.

3.3.3 Preparation of D-(+)-glucosamine, tetraethyleneglycol and N-acetyl-D-glucosamine samples

For the D-(+)-glucosamine series, oxytocin samples were prepared in six different concentrations of D-(+)-glucosamine (0.04 mM, 0.14 mM, 0.5 mM, 1.5 mM, 4.8 mM or 14 mM) in phosphate buffer (0.1M). Two series of oxytocin samples with the same range of concentrations of D-(+)-glucosamine and N-acetyl-D-glucosamine (1.0 mM, 3.0 mM, 6.0 mM or 10.0 mM) were also made in acetate buffer (50 mM). The accelerated stability study was carried out by storing the samples in phosphate buffer at 40°C and 50°C for a period of up to 30 days and the samples in acetate buffer at 40°C and 50°C for a period of 14 days. The samples were analyzed by HPLC (methods 4 or 2) several times during their storage periods.

For the tetraethyleneglycol series, oxytocin samples were made with four different concentrations of 4EG (0.3%, 1.0%, 3.0% or 10.0%) as an additive in phosphate buffer (0.1 M) and the samples were then stored at 40°C and 50°C for a period of up to 30 days. Two mixture samples of each sugar (1.0 mM and 3.0 mM) with 4EG (0.3% and 1.0%) were also made and stored at 40°C and 50°C for a period of 14 days. The samples of all series were analyzed by HPLC (method 4) several times during their storage periods.

3.3.4 Preparation of antioxidant samples

Antioxidant samples were prepared in a 50 mM acetate buffer at pH 4.5. The different samples in this series included either butylated hydroxytoluene (saturated conc.), uric acid (saturated conc.), or L-ascorbic acid at a range of concentrations (1, 2, 3, and 4 mg/mL). The samples were stored at 50°C for a period of 14 days and analyzed by HPLC (method 4 or 3) several times during that period.

3.3.5 Preparation of crown ether samples

Six series of crown ether samples were prepared. The first four series were analyzed regularly via HPLC (according to the methods mentioned in Table 2 (methods 1, 2 or 3)) over the storing period time of the samples, the fifth series was analyzed via UV-visible spectrophotometry and the last series was prepared for NMR analyzes.

The first series of samples was made in citrate/phosphate buffer (0.1 M/0.2 M). These samples contained 12-crown-4, 15-crown-5, or 18-crown-6. The crown ether concentration was kept constant at 1% *w/v*. These samples were then stored at 40°C or 50°C for a period of 47 days and were analyzed according to method 1.

For the second series, eight oxytocin samples were made with 18-crown-6 as the additive in a range of concentrations (0.1%, 0.3%, and 2% *w/v*). Four of the samples were prepared in citrate/phosphate buffer (0.1 M/0.2 M) and the other four samples were prepared in acetate buffer (0.1 M). These samples were stored at 50°C for two weeks and were analyzed according to method 2.

The third series of oxytocin samples was prepared in acetate buffer (0.1M) with 18-crown-6 in a range of concentrations (0%, 0.01%, 0.05%, 0.1% and 1.0%) and were analyzed according to method 1.

The fourth series of samples were made in both acetate buffer (0.1 M) and citrate/phosphate buffer (0.1 M/0.2 M) with 18-crown-6 added in a range of concentrations (0%, 0.1%, 0.5% or 1.0% *w/v*). Furthermore, two oxytocin samples were also prepared in each of the buffers, one with 18-crown-6 (1.0% *w/v*) and KCl (2.0% *w/v*), the other sample with only 18-crown-6 (1.0% *w/v*). The samples were analyzed according to method 2.

For the fifth series, samples included pure oxytocin (0.25 mg/mL), pure 18-crown-6 (4.0% *w/v*), a mixture of oxytocin with 18-crown-6, and a mixture of oxytocin with 18-crown-6 and potassium chloride (4.0% *w/v*). The samples were prepared in a solvent that included 70% of mobile phase A (0.01% TFA in water) and 30% of mobile phase B (0.01% TFA in a 70% MeCN : 30% H_2O solution).

The last series included two NMR samples in D_2O solvent. One of the samples only contained oxytocin and the other one contained oxytocin and 18-crown-6. The spectra were both calibrated to the NMR solvent, D_2O, at 4.79 ppm, to enable a better comparison of the peak locations.

3.4 FGF2 formulations

The initial concentration of FGF2 was kept in the range of 500-1000 pg/mL in all samples of all series and the series included an FGF2 control that was prepared in the same buffer as the samples in the series but had no additives. FGF2 standards were prepared at a range of concentrations (1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL or 15.6 pg/mL) in the buffer used. The samples were analyzed using an ELISA-sandwich assay.

3.4.1 Preparation of FGF 2 samples for pH study

FGF2 samples were made at a range of pH (6.4, 7.2, 7.4, 7.7 or 8.0) in citrate/phosphate buffer (10 mM/20 mM) and at two additional pH values (4.5 or 5.4) in acetate buffer (0.1 M). The samples were divided into three Eppendorf vials, and the first series was stored at -80°C immediately after preparation, and the next two series were stored at 25°C for 4 and 7 hours, respectively before being moved to a -80°C storage.

3.4.2 Preparation of FGF2 samples for buffer study

Eight FGF2 samples were prepared in the presence of four different buffers: HEPES (1.0 M), TRIS (1.0 M), phosphate buffered saline (PBS) and citrate/phosphate (10 mM/20 mM). The pH of all buffers was adjusted to pH 7.2-7.4. Four of the samples included trehalose (1.0 M) and the other four samples had no trehalose. The samples were divided into three series and the first series was stored at -80°C immediately after preparation and the other two series were stored at 25°C for 4 and 8 hours, respectively, before being moved to -80°C storage.

3.4.3 Preparation of FGF2 samples with trehalose, aminosugars and tetraethyleneglycol

FGF2 samples were prepared in PBS buffer at pH 7.2-7.4 and with different additives at different concentrations. The samples included trehalose (0.1 M or 0.5 M), N-acetyl-D-glucosamine (0.1 M or 0.5 M), D-(+)-glucosamine (0.05 mM or 0.15 mM) and a mixture sample of D-(+)-glucosamine (0.05 M) with tetraethyleneglycol (0.3%). The samples were divided into three portions, the first portion was stored at -80°C immediately and the other two portions were first stored at 25°C for 11 and 23 hours, respectively, and then stored at -80°C.

3.4.4 Preparation of FGF2 samples with crown ether additives

For the crown ether series, FGF2 samples were prepared in PBS buffer at pH 7.2-7.4. Crown ethers used were 18-crown-6 (1.0% and 3.0%), 12-crown-4 (3.0%) and two additional samples were prepared with tetraethyleneglycol (1.0% and 3.0%). All the samples were divided into three Eppendorf vials, the first vials were stored at -80°C immediately and the other two vials were stored first at 25°C for 11 and 23 hours, respectively, and then moved to -80°C storage.

3.5 Calculations

All samples were run in triplicate and standard deviations are reported. Evaluation of the statistical significance of improvement or declination in stability was carried out using Student's t-test. p-values equal to or below 0.05 were regarded as significant.

The degradation rate of oxytocin and FGF2 was tested for zero, first and second order kinetics, where most of the results followed first order kinetics (Hawe, et al., 2009; Ding, 2021), using equation (1), where $[A]_t$ is the concentration of oxytocin or FGF2 at a given time, $[A]_0$ is the concentration at the beginning, t is the time (in days for oxytocin and hours for FGF2) and k is the slope of the equation and shows the degradation rate constant.

$$\ln[A]_t = \ln[A]_0 - kt \quad (1)$$

4 Result and Discussion

4.1 Oxytocin stability in the presence of additives in solution

The concept in the first part of project was to find suitable additives which effectively stabilize oxytocin and to investigate if and how the stability of oxytocin could be increased while various additives were added to the solution.

4.1.1 Effect of trehalose

The effect of trehalose on oxytocin stability in solution was determined. Oxytocin samples were prepared with different trehalose concentrations (10, 20, 30, and 40 mM), in either acetate buffer (0.1 M) or citrate/phosphate buffer (0.1 M/0.2 M), respectively. The solutions included a small amount of BHT (saturated concentration). The samples were stored at 50°C and the oxytocin amount remaining was analyzed using HPLC. Results are shown in Table 3. In both acetate buffer and citrate/phosphate buffer trehalose did not enhance the stability of oxytocin. There was no significant difference in oxytocin remaining when comparing solutions with and without trehalose, suggesting that the stability of oxytocin is unaffected by trehalose although in the citrate/phosphate buffer, all of the measurements at days 10 and 14 showed a very slight and statistically significant improvement (0.6-1.3%) according to the *t*-test assuming equal variance.

Table 3. Oxytocin remaining (%) in the presence of different concentrations of trehalose. Standard deviation is shown in brackets below each value. (M. Ghasemisarabbadieh et al., 2021).

Sample	Buffer	Trehalose conc. (mM)	Oxytocin remaining (%) at day 0	3	7	10	14
OTA-1	Acetate (0.1M)	0	100.0 (1.1)	92.6 (1.2)	87.2 (0.6)	82.7 (0.8)	74.2 (1.3)
TR1A		10	100.0 (0.3)	92.2 (0.9)	87.8 (1.0)	81.5 (0.7)	75.0 (0.5)
TR2A		20	100.0 (0.4)	92.6 (0.7)	87.5 (1.0)	82.7 (0.1)	75.1 (0.4)
TR3A		30	100.0 (0.7)	93.8 (1.1)	87.9 (1.1)	83.1 (0.1)	75.0 (1.1)
TR4A		40	100.0 (0.7)	92.3 (0.8)	87.8 (0.1)	81.3* (0.1)	72.5 (0.6)
OTC	Citrate/phosphate (0.1M/0.2M)	0	100.0 (0.4)	84.8 (0.7)	71.9 (0.0)	61.6 (0.1)	51.9 (0.1)
TR1C		10	100.0 (0.5)	85.3 (0.8)	71.5 (0.9)	62.3* (0.4)	53.1*** (0.3)
TR2C		20	100.0 (0.1)	84.6 (0.6)	70.9* (0.7)	62.5*** (0.2)	53.1*** (0.1)
TR3C		30	100.0 (0.3)	85.3 (0.3)	71.7 (0.9)	62.5* (0.5)	53.2*** (0.2)
TR4C		40	100.0 (0.3)	85.2 (0.3)	72.0* (0.1)	62.2* (0.4)	52.5* (0.3)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.005$

The negligible effect of trehalose was surprising to us, as it is generally viewed as a good stabilizer for certain biomolecules such as proteins in solution (Xie & Timasheff, 1997; Kaushik & Bhat, 2003; Sola-Penna & Meyer-Fernandes, 1998; Carninci, et al., 1998; Lins, Pereira, & Hünenberger, 2004 ; Lin & Timasheff, 1996). In order to understand the lack of effect, the degradation kinetics of oxytocin were evaluated. It was suggested already that the degradation kinetics of oxytocin follow (pseudo) first order (Hawe, et al., 2009). The calculated degradation rate constants for all acetate buffer samples were $1.8 \pm 0.1 \times 10^{-2}$ 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 16). 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 for oxytocin in citrate buffers (Poole, Kasper, & Jiskoot, 2011).

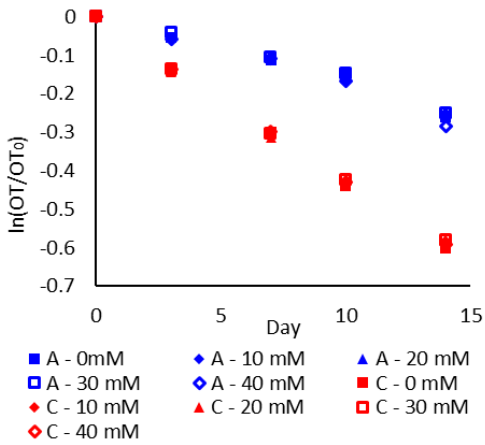


Figure 16. A plot of ln($[OT]_t$ /$[OT]_0$) vs. day. The A samples (blue) were in acetate buffer with different concentrations of trehalose as indicated in the legend. The C samples (red) were in citrate/phosphate buffer (M. Ghasemisarabbadieh, et al., 2021).

The stabilizing effect of trehalose has been reported to increase as the trehalose concentration increases (Xie & Timasheff, 1997; Kaushik & Bhat, 2003; Sola-Penna & Meyer-Fernandes, 1998), although it has also been suggested that trehalose has a small to negligible impact on small peptides. We decided to test the mechanical-entrapment hypothesis of trehalose. That hypothesis refers to entrapment of the biomolecules' conformation in high viscosity trehalose glass. Trehalose's effect on oxytocin stability was therefore explored with the preparation of several more formulations with higher trehalose concentrations (0.1, 0.5, and 1.0 M). 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 showed no stabilizing effect, even at these higher concentrations of trehalose (Table 4). In fact, all but one of these new samples gave a rate constant in the same range as before $1.8 \pm 0.1 \times 10^{-2}$ day^{-1}, or a half-life of 39 days. The only sample with a different rate constant was the one with trehalose with the highest concentration, or 1.0 M. That sample had a statistically significantly lower amount of oxytocin remaining after both 7 and 10 days and gave a rate constant of 2.2×10^{-2} day^{-1} (half-life of 32 days), seemingly degrading slightly faster than the other samples at 50°C (Figure 17).

These results fit with the literature that suggests that trehalose has negligible impact on small peptides (Kaushik & Bhat, 2003). It is surprising that at 1.0 M concentration, trehalose, which is considered to have a chemically inert nature, seemed to have a slightly destabilizing effect on oxytocin in acetate buffer. These results indicate however, that

although simulations have shown that trehalose can promote preferential hydration of biomolecules, yielding a coating by trapped water layer around biomolecules (Lins, Pereira, & Hünenberger, 2004), 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 (Kaushik & Bhat, 2003; Sola-Penna & Meyer-Fernandes, 1998; Carninci, et al., 1998; Lins, Pereira, & Hünenberger, 2004 ; Lin & Timasheff, 1996). 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? The answer to this question may be solvophobic (osmophobic) interaction between the peptide backbone of proteins and trehalose which is the key force to bring the protein to a more compact state (Kaushik & Bhat, 2003).

Table 4. Oxytocin remaining (%) in the presence of different concentrations of trehalose. (M. Ghasemisarabbadieh et al., 2021).

Sample	**Trehalose conc. (M)**	**Oxytocin remaining (%) at day**		
		0	**7**	**10**
OTA-2	0	100.0 (0.5)	88.0 (0.4)	85.8 (0.9)
TR5A	0.1	100.0 (0.4)	88.3 (0.3)	86.9 (0.1)
TR6A	0.5	100.0 (0.5)	87.6 (0.3)	85.7 (0.3)
TR7A	1.0	100.0 (0.6)	85.1*** (0.6)	83.4*** (0.7)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.005$

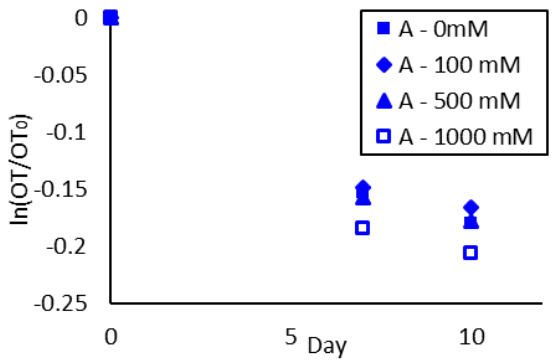


Figure 17. *A plot of ln($[OT]_t$ /$[OT]_0$) vs. day. All samples were made in acetate buffer and with different concentrations of trehalose as indicated in the box (M. Ghasemisarabbadieh et al., 2021).*

4.1.2 Effect of buffer composition and concentration

Trehalose results (Table 3) showed significantly more oxytocin remaining in 0.1 M acetate buffer after 14 days (74.2% oxytocin remaining) compared to that in the citrate/phosphate buffer (0.1 M/0.2 M) (51.9% oxytocin remaining), indicating that acetate buffer is a significantly better buffer for oxytocin storage than citrate/phosphate. We decided to further explore the buffer effect by studying the concentration effect of acetate buffer on oxytocin stability. Pure oxytocin samples (0.25 mg/mL) were made in acetate buffers at a range of concentrations (0.01, 0.025, 0.05, 0.1 or 0.2 M), keeping the pH constant at 4.5. The results showed a slight improvement at lower buffer concentrations (see Table 5), with 82.2% oxytocin remaining after 13 days at 50°C in the most dilute buffer tested (0.01 M) compared to 78.8% in the most concentrated buffer tested (0.2 M). At day 13, there was a statistically significantly greater amount of oxytocin remaining in the lowest buffer concentration compared to all other buffer concentrations except for the one at 0.05 M. This suggests that while the choice of buffer is important, its concentration should also be considered. For acetate buffer, the concentration should be kept low.

Table 5. Amount of oxytocin remaining in acetate buffers of different concentrations (M. Ghasemisarabbadieh et al., 2021.

Sample	Acetate buffer conc. (M)	Oxytocin remaining (%) at day				
		0	**3**	**6**	**10**	**13**
A1	0.01	100.0 (0.4)	96.6 (2.1)	91.3 (1.0)	85.1 (0.5)	82.2 (0.2)
A2	0.025	100.0 (0.8)	94.8 (0.4)	91.0 (0.8)	85.0 (0.6)	81.4* (0.6)
A3	0.05	100.0 (1.5)	95.8 (1.6)	91.6 (0.6)	84.3 (1.3)	81.0 (1.2)
A4	0.1	100.0 (1.5)	95.4 (0.3)	90.9 (0.3)	82.9* (1.4)	79.2** (1.3)
A5	0.2	100.0 (1.5)	95.5 (1.3)	91.2 (0.2)	83.3*** (0.1)	78.8* (1.9)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.0005$

Figure 18 shows the variation in the rate of degradation dependent on the acetate concentration. The rate is lowest at concentrations below 0.025 M, but above that concentration the degradation rate increases and reaches a plateau. The maximal half-life in acetate buffer with 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. A possible reason for this is if the buffer components, such as the acetate anion, interact or react with oxytocin in a degradation-promoting manner.

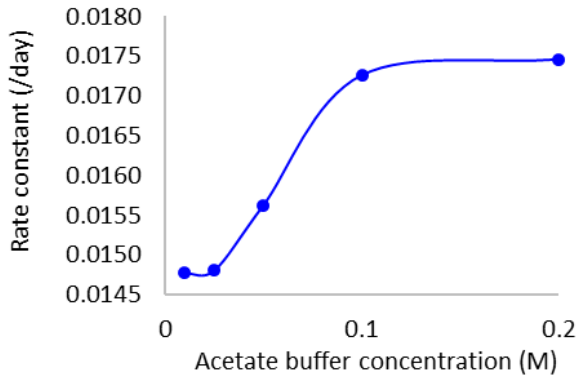


Figure 18. A plot of acetate buffer concentration against the oxytocin degradation rate constants (M. Ghasemisarabbadieh et al., 2021).

4.1.3 Effect of D-(+)-glucosamine and a mixture of D-(+)-glucosamine with 4EG

The effect of different concentrations (ranging from 0.04 (OTGL-1) to 14 mM (OTGL-6)) of D-(+)-glucosamine on oxytocin's stability was determined in phosphate buffer (0.1 M) via HPLC. Analogous determinations were done in acetate buffer (50 mM) for oxytocin solutions with D-(+)-glucosamine concentrations (ranging from 1.0 (Glu 1) to 10 mM (Glu 4)) and a mixture of D-(+)-glucosamine and 4EG. The results shown in Figures 19 and 20 demonstrate a slight improvement in the presence of low concentrations of D-(+)-glucosamine in both acetate buffer and phosphate buffer while higher concentrations showed increased degradation. When D-(+)-glucosamine and 4EG were used together the degradation was more noticeable.

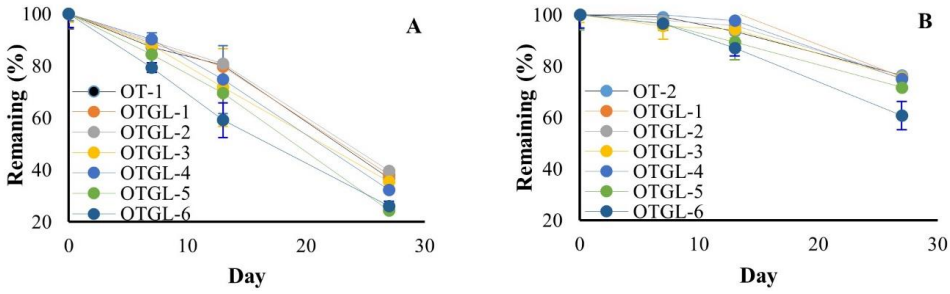


Figure 19. Oxytocin remaining (%) in the presence of different concentrations of D-(+)-glucosamine in phosphate buffer at 50 ℃ (A); and 40 ℃ (B) (M. Ghasemisarabbadieh et al., 2021).

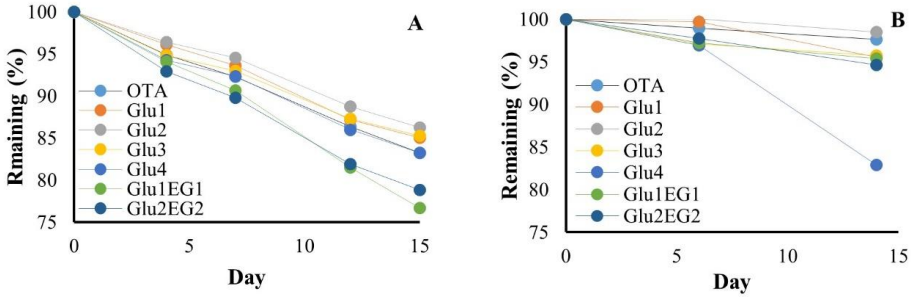


Figure 20. Oxytocin remaining (%) in the presence of different concentrations of D-(+)-glucosamine and two mixture samples of this additive with 4EG (0.3 %, 1.0 %) in acetate buffer, pH 4.5 at 50 ℃ (A), 40 ℃ (B) (M. Ghasemisarabbadieh et al., 2021).

To understand the results better, the degradation rate constants were plotted up against the concentration of D-(+)-glucosamine and the resulting plots are shown in Figure 21, with the scale of the y-axis kept the same for better comparison.

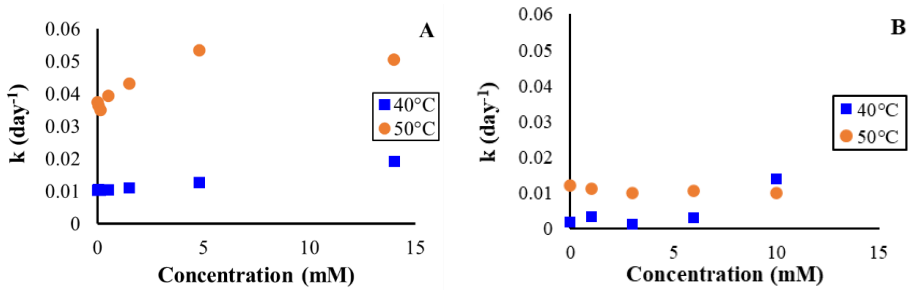


Figure 21. The plot of degradation rate constant (k) of oxytocin vs. different concentrations of D-(+)-glucosamine in phosphate buffer (A) and in acetate buffer (B) at 50 ℃ and 40 ℃ (M. Ghasemisarabbadieh et al., 2021).

Previous results have already highlighted that the choice of buffer plays an important role on oxytocin stability (Ghasemisarabbadieh M. G., 2021). When we compared acetate buffer to citrate/phosphate buffer, the degradation was significantly less than that in the acetate buffer (Ghasemisarabbadieh M. G., 2021). When Figures 21A and 21B 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. For the oxytocin control samples, the degradation rate decreased from 0.038 in phosphate to 0.012 in acetate buffer at pH 4.5. 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 constant 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 subtracting the Cys^1 α-proton that is adjacent of the N-terminal amino group of oxytocin and opening the C-S bond, resulting in degradation as

shown in Figure 22. Another possibility might be the interaction of glucosamine with free H^+ in solution or more important the action of D-(+) glucosamine as a reducing agent. We already mentioned a color change that was observed in oxytocin samples containing D-(+) -glucosamine and was hypothesized to be due to oxidation of D-(+) glucosamine. It raised the possibility that D-(+) glucosamine acts as a reducing agent and breaks the disulfide bond ($Cys^{1,6}$), resulting in faster degradation of oxytocin although there is no evidence to prove these hypotheses.

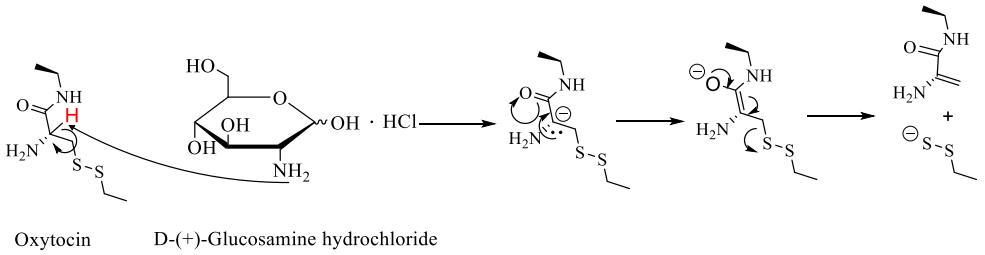


Figure 22. Proposed mechanism of degradation of oxytocin in the presence of D-(+)-glucosamine (M. Ghasemisarabbadieh et al., 2021).

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 1.8×10^{-2} day^{-1} (half-life of 39 days) compared to 1.1×10^{-2} day^{-1} (half-life of 63 days) 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 1.6×10^{-2} day^{-1} (half-life of 43 days) compared to 1.0×10^{-2} day^{-1} (half-life of 69 days) when 3.0 mM of glucosamine was used alone.

4.1.4 Effect of N-acetyl-D-glucosamine and a mixture of N-acetyl-D-glucosamine with 4EG

The effect of four different concentrations of N-acetyl-D-glucosamine (1.0 (Ac1), 3.0, 6.0 and 10.0 mM (N-Ac4)) and a mixture of N-acetyl-D-glucosamine with 4EG were tested in acetate buffer at pH 4.5. The results are shown in Figure 23. Measurements at 40°C

exhibited negligible difference between the results with or without N-acetyl-D-glucosamine. All these results showed more than 95 percent 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 increase could however be considered negligible as well.

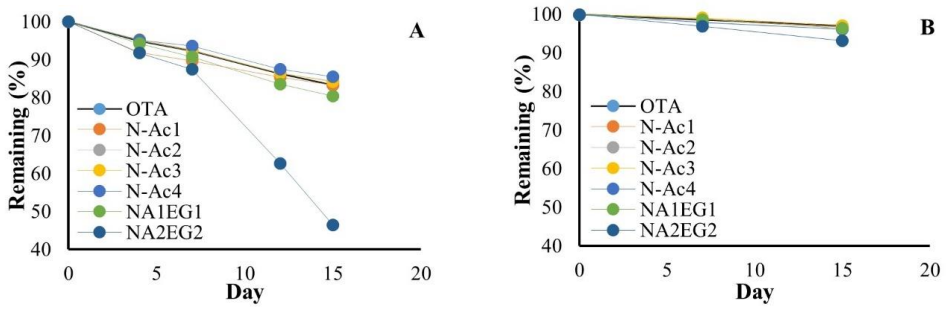


Figure 23. Oxytocin remaining (%) in the presence of different concentrations of N-acetylglucosamine and the mixture of N-acetylglucosamine with 4EG in acetate buffer pH 4.5 at 50 ℃ (A) or 40 ℃ (B) (M. Ghasemisarabbadieh et al., 2021).

It was interesting to see that N-acetylglucosamine did not have the same negative effect on oxytocin stability as glucosamine did at the highest concentrations tested. A potential explanation for that 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 of D-(+)-glucosamine is detrimental for the OT 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, compared with lower concentration, without the risk of additional potentially reactive amino group present. Nevertheless, the effect seems to be very small within the concentration range tested, especially compared to the buffer effect described above (Figure 24).

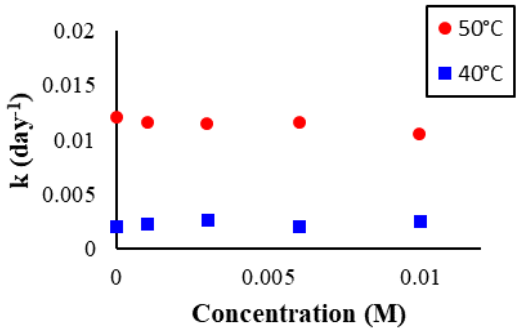


Figure 24. The plot of degradation rate constant (k) of oxytocin vs. different concentrations of N-acetylglucosamine in acetate buffer at 40 °C and 50 °C (M. Ghasemisarabbadieh et al., 2021).

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 had little effect, we concluded that the shortened half-life was primarily due to the increase in 4EG concentration.

4.1.5 Effect of tetraethyleneglycol (4EG)

As described above, the effect of 4EG on the stability of oxytocin was explored in phosphate buffer at pH 4.5. Previous results with 18-crown-6 showed that the same additive can have drastically different effect on stability depending on the buffer used, so it was decided to use the same buffer again as had been employed in the patent (Patent No. US 9,968,648 B2, 2017) on formulations containing glucosamines and various tetraethyleneglycols. The results show that 4EG had a negative impact on oxytocin stability as the degradation was accelerated with increasing concentration of 4EG (Figure 25).

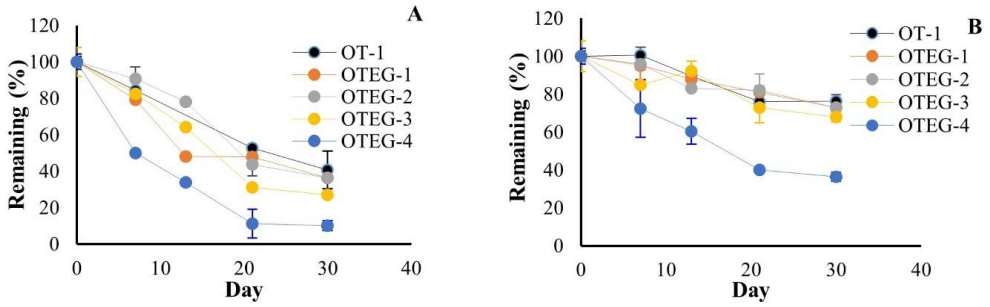


Figure 25. Oxytocin remaining (%) in the presence of different concentrations of 4EG at 50 ℃ (A) and 40 ℃ (B) in phosphate buffer, pH 4.5 (M. Ghasemisarabbadieh et al., 2021).

The degradation rates at 40°C and 50°C are also plotted up against the 4EG concentration in Figure 26. The rate constants for all samples at 40 ℃ and 50 ℃ were higher than oxytocin control, and the rate constant was raised with increasing the concentration of 4EG at both 40℃ and 50℃. These findings were surprising, in light of previous results that had been obtained and reported in the previously mentioned patent (Patent No. US 9,968,648 B2, 2017), as well as given previous results obtained with trehalose as an additive (Ghasemisarabbadieh M. G., 2021). 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. Alcohol groups in sugars like trehalose, also seem to have a limited effect on oxytocin's stability. This could however be an interesting avenue for future studies, in order to understand better how different additives might affect the degradation path of oxytocin.

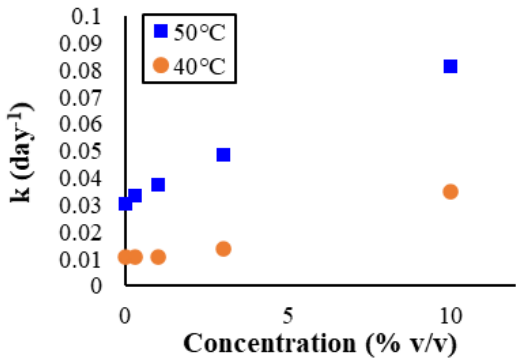


Figure 26. The plot of degradation rate constant (k) of oxytocin vs. different concentrations of 4EG in phosphate buffer at 50 ℃ and 40 ℃ (M. Ghasemisarabbadieh et al., 2021).

4.1.6 Effect of antioxidants

During initial experiments with D-(+)-glucosamine as an additive in formulations without any BHT, a color change was observed in the samples. In an attempt to prevent this color change, BHT and uric acid were tested and the results showed some improvement, possibly due to the antioxidative or radical-scavenging properties of these antioxidants. The samples discussed in section 4.1.4 and 4.1.5 included antioxidant. On the other hand, as previously mentioned, degradation studies on oxytocin have also found that Tyr^{2} and $Cys^{1,6}$ are susceptible to oxidation (Avanti, et al., 2011). Knowing that, it was hypothesized that antioxidants could also improve the stability of oxytocin. Therefore, BHT and uric acid were tested, in addition to L-ascorbic acid, to see if they could slow down oxytocin degradation in solution. BHT was chosen as it is one of the best known antioxidant and has been used in various industries, including as an antioxidant food additive. Uric acid is an antioxidant found in human blood and L-ascorbic acid, or vitamin C, is a common antioxidant as well.

Since L-ascorbic acid is water-soluble, it was tested at a range of concentrations (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 50 mM acetate buffer was used at a constant pH of 4.5 and the samples stored at 50°C. The results (Table 6) showed that BHT and uric acid did not have a statistically significant effect on the oxytocin stability, despite the literature having suggested that one of oxytocin's degradation pathways is due to oxidation (Tyr).[24] Although one measurement had a difference that was statistically significant (at $p < 0.05$), BHT after 4 days, the measurements after 8 and 15 days were not statistically significant, so it can be concluded that these two antioxidants had negligible effect. This could still be beneficial if other, prone to oxidation excipients were used. L-ascorbic acid, however, showed a clear negative impact and accelerated the degradation rate of oxytocin significantly in solution. This effect was concentration dependent, showing half-life of less than 4 days at 50°C, at concentrations ranging from 1-4 mg/mL with the lowest oxytocin stability being with L-ascorbic acid at a concentration of 2 mg/mL and the stability increasing with both lower and higher concentrations of L-ascorbic acid.

It is not clear yet why L-ascorbic acid is impacting the oxytocin stability so negatively and so drastically. High solubility of L-ascorbic acid in water compared with BHT and uric acid on one hand, and its character as a reducing agent on the other hand, raised the question of whether L-ascorbic acid is interacting with the disulfide bond as a reducing

agent and accelerating the breakage of the disulfide bond, yielding faster degradation of oxytocin. 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. Since the rate of degradation seems to slow down a bit when the L-ascorbic acid concentration is increased from 2 mg/mL up to 4 mg/mL (the observed rate constants were 3.3×10^{-1} day^{-1}, 2.2×10^{-1} day^{-1}, and 1.6×10^{-1} day^{-1} assuming pseudo first order kinetics), it might also be interesting to see how significantly higher concentrations of L-ascorbic acid would affect the oxytocin stability.

Table 6. Oxytocin remaining (%) in the presence of different antioxidants in 50 mM acetate buffer. (M. Ghasemisarabbadieh et al., 2021).

Sample	Antioxidant	Conc. (mg/mL)	Oxytocin remaining (%) at day			
			0	4	8	15
OTA-3	None	-	100.0 (0.4)	93.5 (0.1)	88.0 (0.1)	78.8 (0.6)
OTB	BHT	saturated	100.0 (0.7)	94.1* (0.4)	87.9 (0.3)	80.4 (1.6)
OTU	Uric acid	saturated	100.0 (0.5)	96.7 (4.0)	88.2 (0.3)	79.6 (0.3)
OTL-1	L-ascorbic acid	1	100.0 (0.8)	66.5*** (1.2)	32.7*** (1.4)	10.2*** (0.3)
OTL-2		2	100.0 (0.2)	62.9*** (0.5)	33.3*** (1.7)	0.4*** (0.5)
OTL-3		3	100.0 (0.5)	66.2*** (0.3)	35.5*** (1.7)	3.7*** (0.2)
OTL-4		4	100.0 (1.2)	73.4*** (0.3)	46.6*** (2.0)	5.0*** (7.0)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.0005$

4.1.7 Effect of crown ethers

Oxytocin solutions (0.25 mg/mL) were made in citrate/phosphate buffer (0.1 M/0.2 M) at pH 4.5, and with three different crown ethers along with one with only oxytocin as a control. The crown ethers included were 12-crown-4, 15-crown-5, and 18-crown-6 and the crown ether concentration was kept constant across the samples at 1.0 % *w/v*. The samples were then each divided into two portions with one half being stored at 40°C and the other at 50°C for a period of up to 47 days. The HPLC results showed the remaining amount of oxytocin was similar for the control sample and the samples with 15-crown-5 or 12-crown-4 (statistically insignificant for the majority of those samples) while a greater amount of oxytocin remained in samples with 18-crown-6, both at 40°C and at 50°C (Table 7). At

40°C the increased remaining amount of oxytocin was statistically significant, with $p < 0.005$ for all samples with 18-crown-6 present. At 50°C, three out of four measurements showed statistically significantly greater remaining amount of oxytocin with 18-crown-6 in the samples ($p < 0.05$ for 2 samples and $p < 0.005$ for 1 sample). The measurements showed that the rate constant of oxytocin was clearly decreased in the presence of 18-crown-6. For oxytocin alone, the rate constant was 1.2×10^{-2} day^{-1} with a half-life of 56.6 days. For oxytocin in the presence of 1 % (w/v) 18-crown-6 was 0.09×10^{-2} day^{-1} and a half-life of 76.6 days. The rate constants for the oxytocin samples with 12-crown-4 and 15-crown-5 were 1.2×10^{-2} day^{-1} (a half-life of 58 days) and 1.3×10^{-2} day^{-1} (a half-life of 53.7 days) respectively. These results fit with the hypothesis that 18-crown-6 would be the only one of these crown ethers to have a positive effect on the oxytocin stability because of its binding affinity with ammonium groups (Figure 14). Since 15-crown-5 and 12-crown-4 have a smaller ring size, the ring size is not large enough to have a significant binding affinity for ammonium groups and they were thus not expected to affect the oxytocin stability. Indeed, their effect was negligible at the crown ether concentration tested. Therefore, these results provided further evidence that the free amine group plays a role in oxytocin's degradation pathway and reinforced the importance of understanding the degradation mechanism, in order to better stabilize the relevant sites.

Table 7. Remaining amount of oxytocin (%) in a citrate/phosphate buffer (0.1 M/0.2 M) in the presence of different crown ethers (1.0 % w/v) as well as by itself (M. Ghasemisarabbadieh et al., 2021).

Sample	Temperature	Remaining oxytocin (%) at day				
		0	**14**	**25**	**40**	**47**
OT	40°C	100.0 (0.1)	74.1 (0.4)	62.4 (1.5)	57.4 (0.7)	56.3 (0.3)
OT-12C4		100.0 (6.3)	76.3*** (0.1)	68.4 (4.5)	59.3 (1.0)	57.3*** (0.2)
OT-15C5		100.0 (0.3)	72.4** (0.3)	66.8 (4.4)	58.1 (2.1)	54.6* (0.6)
OT-18C6		100.0 (0.6)	78.3*** (0.6)	71.4*** (2.1)	68.4*** (0.2)	65.4*** (0.3)
		0	**14**	**18**	**29**	**40**
OT	50°C	100.0 (0.1)	47.2 (1.0)	40.0 (2.4)	26.7 (4.3)	21.1 (0.0)
OT-12C4		100.0 (6.3)	47.2 (0.2)	40.2* (0.2)	26.0 (3.2)	18.7* (8.1)
OT-15C5		100.0 (0.3)	47.7 (3.7)	37.9 (1.1)	24.0 (5.2)	18.7*** (0.4)
OT-18C6		100.0 (0.6)	53.5 (9.1)	44.8* (1.1)	33.3* (2.1)	25.4*** (0.7)

* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.005$

Concentration effect of 18-crown-6 on oxytocin stability

To investigate the concentration effect of 18-crown-6 on oxytocin stability, eight solutions of oxytocin (0.25 mg/mL) were prepared with different concentrations of 18-crown-6 (0%, 0.1%, 0.3% and 2.0% *w/v*), four of these samples were made in citrate/phosphate buffer (0.1 M/0.2 M) and the other four samples in acetate buffer (0.1 M). The pH of samples was kept at 4.5. The results illustrated that the stability of oxytocin was improved with higher concentrations of the crown ether in citrate/phosphate buffer, with all of the samples with 2.0% 18-crown-6 showing statistical significantly more oxytocin remaining (Table 8). Surprisingly, the measurements of samples in acetate buffer (0.1 M) showed the oxytocin stability decreasing as the crown ether concentration increased (Table 8). The acetate results showed statistical significance except one with the lowest concentration of 18-crown-6 (18C6-A1) that did not show statistical significance on the day 3 measurement.

Table 8. Remaining amount of oxytocin in the presence of different concentrations of 18-crown-6 (18C6) in either citrate/phosphate buffer (C) or acetate buffer (A) at 50°C (M. Ghasemisarabbadieh et al., 2021).

Sample	Buffer	Crown ether conc. (% *w/v*)	Remaining oxytocin (%)				
			Day 0	Day 3	Day 7	Day 10	Day 14
OT-C	Citrate/ phosphate (0.1 M/0.2 M)	0	100.0 (0.4)	84.8 (0.7)	71.9 (0.1)	61.6 (0.2)	51.9 (0.2)
18C6-C1		0.1	100.0 (1.4)	85.8* (0.1)	68.7*** (0.3)	58.9*** (0.1)	49.5*** (0.5)
18C6-C2		0.3	100.0 (0.2)	86.6*** (0.2)	70.8* (0.7)	60.9*** (0.1)	51.3*** (0.0)
18C6-C3		2.0	100.0 (1.2)	88.5*** (0.2)	75.6*** (0.3)	67.8*** (0.0)	59.6*** (0.3)
OT-A	Acetate (0.1 M)	0	100.0 (1.1)	92.6 (1.3)	87.2 (0.7)	82.7 (0.9)	74.2 (1.8)
18C6-A1		0.1	100.0 (0.6)	90.5 (0.0)	83.2** (1.1)	76.2*** (0.0)	67.5** (0.0)
18C6-A2		0.3	100.0 (0.5)	87.3* (1.0)	82.5** (1.1)	73.4*** (0.0)	62.1*** (0.7)
18C6-A3		2.0	100.0 (0.1)	84.2* (1.3)	72.1*** (1.4)	62.5*** (2.4)	51.2*** (0.2)

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.005$

[1] OT-A gave significantly better results at all time points ($p = 0.02$, 0.0004, 0.0003, 0.0007, respectively for days 3, 7, 10, and 14), compared with OT-C.

The results in citrate/phosphate buffer fit with what was expected as the higher concentration of 18-crown-6 increases the probability of it binding to a protonated ammonium group, thus preventing it from being involved in undesired reactions. It is not clear, however, why 18-crown-6 has the opposite effect on oxytocin stability in acetate buffer, seemingly destabilizing it. It has already been shown that citrate buffer produces amide- and imide linked citrate derived degradation products of oxytocin (Poole, Kasper, & Jiskoot, 2011). The degradation also involves the amine group, in that case reacting with citric anhydride. The question then becomes whether the stabilizing effect of 18-crown-6 in citrate/phosphate is because it slows down the amide- and imide-linked degradation pathway, or if it retards the beta-elimination pathway that includes the disulfide bridge, or a bit of both. To understand better what was happening, the HPLC traces were analyzed further, in order to see if a variation in degradation products could be observed, depending on the presence or absence of 18-crown-6. After 14 days at 50°C, oxytocin showed a similar degradation pattern, based on the HPLC trace, in both acetate and citrate/phosphate

buffer, as well as for the sample that included 18-crown-6 (2% *w/v*) in citrate/phosphate buffer. This suggests that 18-crown-6 may be slowing down the main degradation pathway in citrate/phosphate buffer (Figure 27). When oxytocin was stored in acetate buffer in the presence of 2 % *w/v* of 18-crown-6, the HPLC trace did not show differences with any of other HPLC traces, although a new peak around minute 10.5 was observed (Figure 27). The presence of 18-crown-6 in acetate buffer may lead to a complex formation of 18-crown-6 and Na^+ or different degradation pathway for oxytocin. Further studies will be required to better understand why 18-crown-6 has such a different effect on oxytocin stability in acetate buffer compared to in citrate/phosphate buffer.

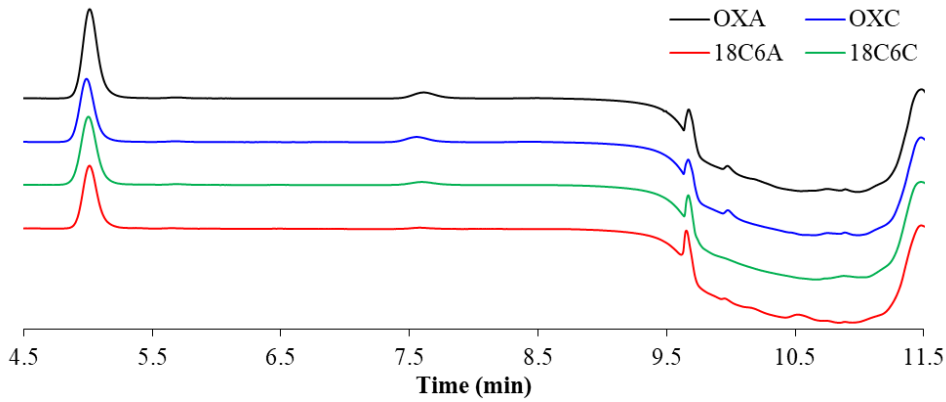


Figure 27. HPLC traces of oxytocin after 14 days of storage at 50°C in the presence and absence of 2 % w/v of 18-crown-6 in both acetate and citrate/phosphate buffer (M. Ghasemisarabbadieh et al., 2021).

The effect of 18-crown-6 on oxytocin retention time-acidic mobile phase and short column

Several samples were prepared with the oxytocin concentration kept constant at 0.25 mg/mL, while the 18-crown-6 concentration ranged from 0% up to 1% *w/v*, with the 0% sample serving as a control. In these HPLC runs, the mobile phase was changed to a more acidic one (A: 0.01% TFA in H_2O and B: 0.01% TFA in 70% MeCN: 30% H_2O), allowing most of the amino group of oxytocin to remain protonated throughout the run. A partial shift was observed when 18-crown-6 was included in the samples (Figure 28). The oxytocin amount in all these samples was kept constant and the overall area under both peaks was close to constant. The amount of relative peak area that was shifted was dependent on the 18-crown-6 concentration, suggesting that its presence in the solution

caused the peak shift. These results further support the hypothesis that a guest/host complex is being formed between oxytocin and 18-crown-6 and we hypothesize that the new peak may be due to this guest/host complex (Table 9).

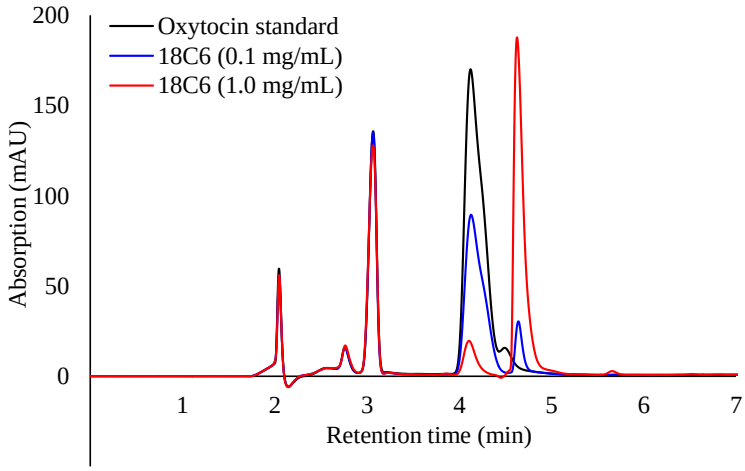


Figure 28. *UV trace from HPLC runs of an oxytocin standard (black), oxytocin samples with 0.1 % w/v and 1.0 % w/v of 18-crown-6 added (blue and red respectively). These samples were prepared in acetate buffer (M. Ghasemisarabbadieh et al., 2021).*

Table 9. Relative peak area of the peaks showing up at 4.1 min and 4.6 min for freshly prepared oxytocin samples with different concentrations of 18-crown-6 (M. Ghasemisarabbadieh et al., 2021).

Sample	**18-crown-6 conc. (% *w/v*)**	**% of area under peak at 4.1 min**	**% of area under peak at 4.6 min**
OT	0	100	0
OT-A-18C6-1	0.01	95	5
OT-A-18C6-2	0.05	92	8
OT-A-18C6-3	0.1	89	11
OT-A-18C6-4	1.0	12	88

18-crown-6 effect on the retention time - acidic mobile phase and a longer column

Several more samples were made with the oxytocin concentration kept constant at 0.25 mg/mL and the 18-crown-6 concentration ranged again from 0% up to 1% *w/v*. In this series, an additional sample with a mixture of oxytocin (0.25 mg/mL), 18-crown-6 (1.0% *w/v*), and potassium chloride (2.0% *w/v*) was included as well as a sample with 18-crown-6 only. These samples were prepared in both acetate and citrate/phosphate buffers at pH 4.5. When a longer column was used (250 mm instead of 150 mm), a complete shift was observed in the retention time of all oxytocin samples containing 18-crown-6, regardless of buffer used (Figure 29). It is unclear why the length of the columns, which should be otherwise identical, would have such a drastic effect (partial vs. complete peak shift). Nevertheless, these results were reproducible in our lab with these columns. A future study could be needed to provide an adequate explanation for this observation. We hypothesize that the new peak observed here is also for the oxytocin/18-crown-6 complex shown in Figure 14. No peak was observed for 18-crown-6, as it does not absorb light at the detection wavelength (220 nm), and KCl did not prevent a peak shift from being observed in the samples. In the samples that included KCl, the buffer region of the HPLC trace (2.5-5.5 min) was significantly altered (Figure 29), suggesting that the potassium and/or chloride ions were interacting with the buffer components (acetate or citrate/phosphate) and came out of the column alongside them. If the potassium ion is binding more strongly to the buffer components than to 18-crown-6 in these HPLC runs, that would explain why its presence did not prevent the peak shift from being observed.

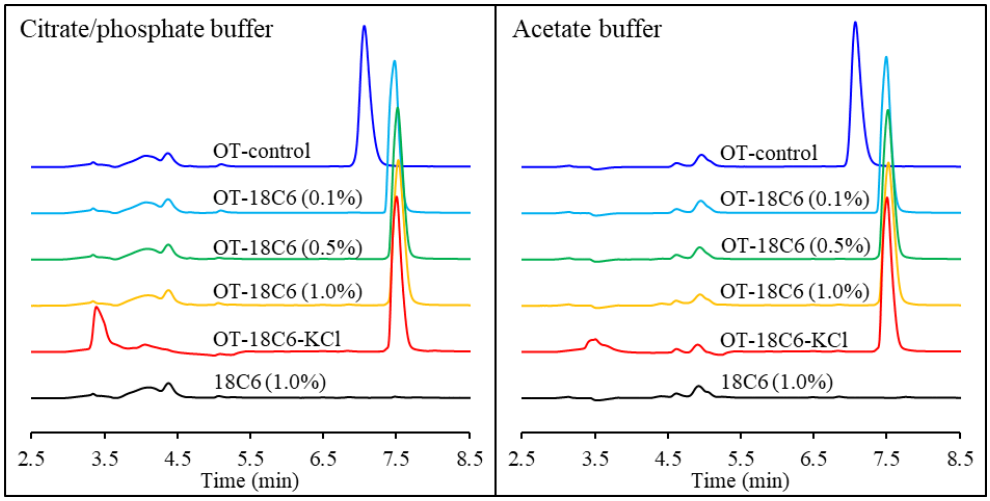


Figure 29. HPLC traces of samples showing an oxytocin peak shift in citrate/phosphate buffer (left) and in acetate buffer (right) in the presence of 18-crown-6. Mobile phase was A: 0.01% TFA in H_2O and B: 0.01% TFA in 70% MeCN: 30% H_2O (M. Ghasemisarabbadieh et a., 2021).

When analyzing these results there are two important things to keep in mind. First, these samples were prepared fresh so no degradation product was expected to be observed at this point in time. Second, this peak shift had not been observed in previous HPLC runs, so this phenomenon was directly dependent on the mobile phase being used. Still, the possibility remained that a fast reaction was taking place between the mobile phase and oxytocin yielding a new product with a new retention time. To test this possibility, we changed the TFA concentration in the mobile phase and re-analyzed the samples. The new mobile phase composition had increased TFA concentration and was A: 0.1% TFA in H_2O and B: 0.1% TFA in 70% MeCN: 30% H_2O. When this mobile phase was used, all of the oxytocin samples showed up at the same retention time, regardless of whether 18-crown-6 was present or not (Figure 30). It remains unclear why a more acidic mobile phase leads to 18-crown-6 no longer having any observable effect on the retention time. It could have been expected that the more acidic medium would lead to greater complexation yielding a potential peak shift, which is not what we observed. For the 0.1% TFA mobile phase, the pH was close to 2, and for the 0.01% TFA mobile phase it was closer to 3. 18-crown-6 can also bind hydronium ions, and at the lower pH, we have an order of magnitude more hydronium ions that might also bind to the 18-crown-6. It is difficult to tell whether this would be enough to affect the complexation or not, and the difference seems low to

completely negate the observed peak shift. These results do suggest however, that a reaction is unlikely to be causing the previously observed peak shift, but rather a pH or mobile phase dependent guest-host complex interaction.

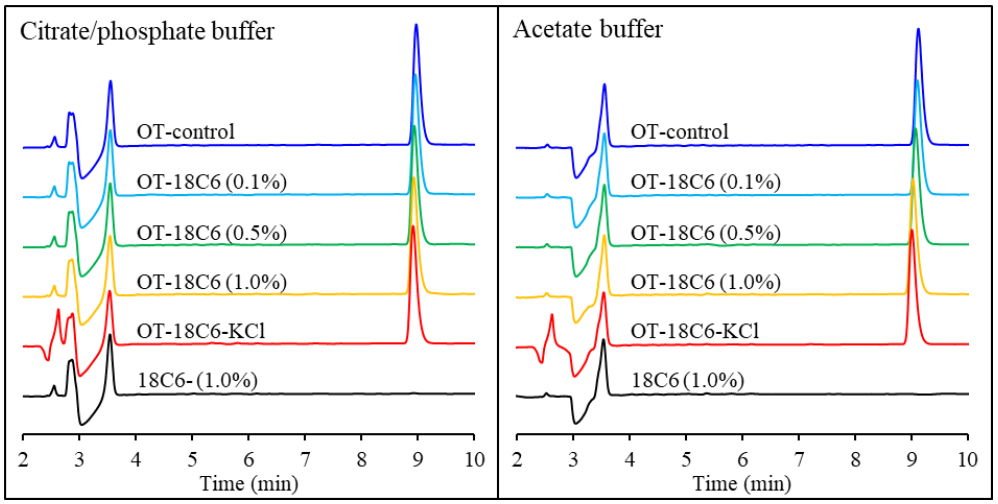


Figure 30. HPLC traces of samples showing no oxytocin peak shift regardless of the presence of 18-crown-6. The samples were prepared in citrate/phosphate buffer (left) and acetate buffer (right). Mobile phase was A: 0.1% TFA in H_2O and B: 0.1% TFA in 70% MeCN: 30 H_2O (M. Ghasemisarabbadieh et a., 2021).

18-crown-6 effect on the UV absorption spectrum

To better understand the results, samples were made to see if any changes would be observed in the UV absorption spectrum (200-300 nm) of oxytocin in the presence of 18-crown-6 (Figure 31). 18-crown-6 (4% *w/v*) on its own did not absorb much. The trace of the mixture of oxytocin (0.25 mg/mL), 18-crown-6 (4% *w/v*), and KCl (4% *w/v*) together was very similar to the UV trace of oxytocin by itself. For oxytocin/18-crown-6 sample without KCl, there was a noticeabe right shoulder observed around 230 nm. The solvent used here was a 70/30 mixture of mobile phases A and B that were already employed in the part of the 18-crown-6 study where a peak shift had been observed in the HPLC traces. These results suggest that in this solution mixture, potassium may have bound more strongly to the 18-crown-6 and prevented the crown ether from interacting as strongly with the oxytocin and affecting its UV trace.

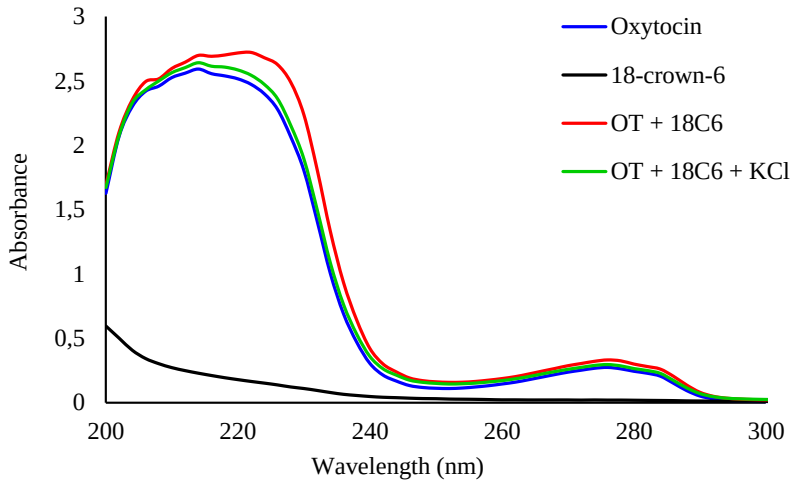


Figure 31. UV absorption spectra for oxytocin (blue), 18-crown-6 (black), a solution with both oxytocin and 18-crown-6 (red), and a solution with oxytocin, 18-crown-6, and potassium chloride (green) (M. Ghasemisarabbadieh et al., 2021).

18-crown-6 effect on the NMR spectrum

Two NMR samples were prepared in D_2O, to see if any interaction could be observed using that approach. One of the samples only contained oxytocin whereas the other contained oxytocin and 18-crown-6. The spectra were both calibrated to the NMR solvent, D_2O, at 4.79 ppm, to enable a better comparison of the peak locations. The NMR showed a clear peak shift for many of the oxytocin peaks when 18-crown-6 is present (Figure 32). This is especially noticeable for the aromatic protons on tyrosine, where the distance between the chemical shifts of the aromatic peaks has increased in the presence of 18-crown-6. There are, however, many other peaks that have shifted slightly as can be seen in Figure 32. While it is difficult to identify exactly what type of interaction exists between the two compounds based on the NMR spectrum, the observed peak shifts of certain protons, such as the aforementioned aromatic protons on tyrosine, as well as the Cys^1 (originally at 4.25 ppm) and Cys^6 (originally at 4.9 ppm) alpha protons, do support the possibility of a guest/host interaction between the 18-crown-6 and the oxytocin, even in pure D_2O.

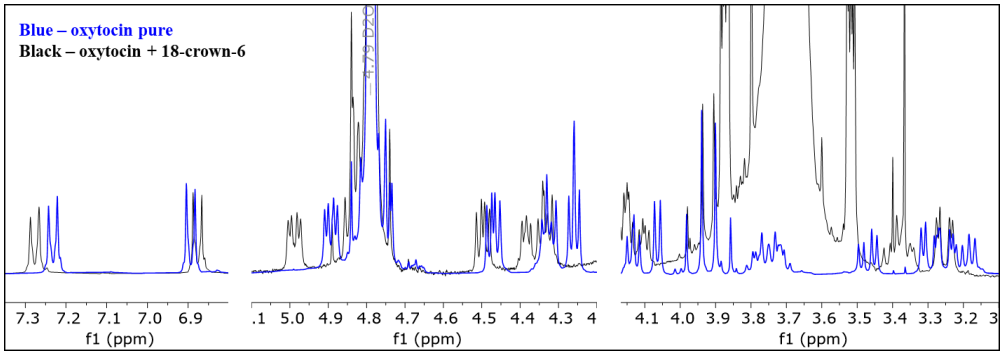


Figure 32. Stacked NMR spectra of pure oxytocin (blue) and a mixture of oxytocin and 18-crown-6 (black) in D2O (M. Ghasemisarabbadieh et al., 2021).

4.2 FGF2 stability in solution

The second part of the thesis involved studying the stability of a large protein, fibroblast growth factor 2 (FGF2), in solution. In this part some of the additives and solutions used in the oxytocin part of the study were tested in order to see if they could enhance the stability of FGF2 in solution. The additives tested were trehalose, N-acetyl-D-glucosamine, D-(+)-glucosamine, 18-crown-6, 12-crown-4 and 4EG. Additionally, the following buffers were tested: citrate/phosphate, HEPES, TRIS and PBS, as well as the pH effect.

4.2.1 Effect of pH on FGF2 stability in solution

It has already been shown that changing the pH can induce unfolding of proteins and denaturation, which can be accelerated below pH 5 for acidic conditions and at pH 10 and higher for basic conditions (Konermann, 2003), but to the best of our knowledge, the effect of different pH on FGF2 stability has not yet been studied extensively, although it is known that it is an important factor for the stability of biomolecules such as proteins. To investigate FGF2 stability at different pH at 25 ℃, samples were prepared in a manner to try to fix the initial FGF2 concentration at approximately 500 pg/ml in citrate/phosphate buffer (10 mM/20 mM) at a range of pH (6.4-8.6) and in acetate buffer (0.1 M) at pH 4.5 and 5.4. The preliminary results showed that FGF2, like other FGF family members, showed the best stability at near-neutral to slightly basic pH. The initial concentrations of samples were different from the FGF2 concentration (500 pg/mL) and it is more noticeable for samples at pH 5.4 and pH 4.5 where these FGF2 samples showed much lower initial

concentration and faster degradation whereas samples in the pH range of 7.2 to 8.0 showed no initial drop in concentrations (Table 10, Figure 33). The difference between actual initial concentrations of FGF2 samples and the intended FGF2 concentration used in the samples can be explained by FGF2 sensitivity and its very low stability. The results also conform with the literature, which reports that changing the pH of solution to acidic (pH 5) or strong basic (pH 10) condition can cause the denaturation and unfolding of proteins (Konermann, 2003).

Table 10. FGF2 remaining (%) in the presence of citrate/phosphate buffer (6.4-8.6) and acetate buffer (4.5-5.4) at different pH (M. Ghasemisarabbadieh et al., 2021).

Sample	**Initial FGF2 conc. (pg/mL)**	**Remaining FGF2 (%) at hour**		
		0	**21**	**29**
pH 8.0	793.4	100(2.4)	99.4(18.5)	88.0(7.4)
pH 7.7	834.6	100(3.9)	93.5(13.0)	82.3(16.2)
pH 7.4	745.1	100(1.3)	105.7(12.8)	86.1(24.1)
pH 7.2	692.1	100(4.7)	95.1(4.3)	75.2(10.5)
pH 6.4	537.7	100*(5.8)	66.8**(9.9)	56.1*(9.2)
pH 5.4	192.6	100***(3.6)	30.2***(14.6)	24.2***(19.3)
pH 4.5	28.8	100***(11.3)	4.62***(3.3)	5.54***(15.1)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.0005$

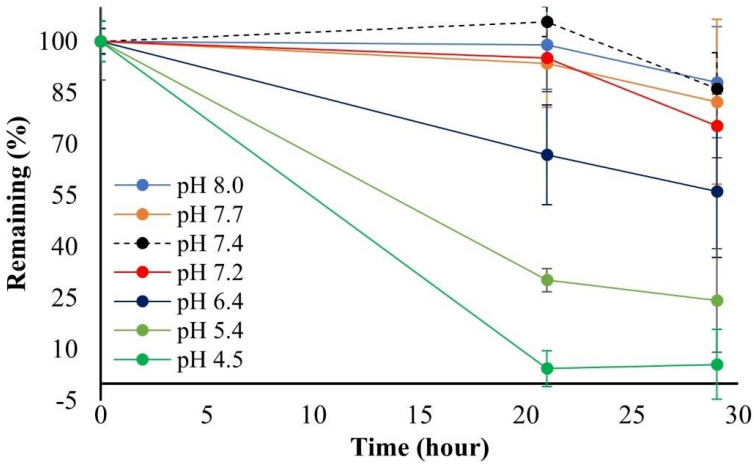


Figure 33. FGF2 remaining (%) in the presence of citrate/phosphate buffer (6.4-8.6) and acetate buffer (4.5-5.4) at different pH (M. Ghasemisarabbadieh et al., 2021).

4.2.2 Effect of different buffers on FGF2 stability

The effect of different buffers on FGF2 stability was also examined. The buffers tested were HEPES (1.0 M), TRIS (1.0 M), PBS, and citrate/phosphate (10 mM/20 mM). The pH of all buffers was adjusted to physiological pH (7.4) because no statistically significant differences between the % FGF2 remaining at pH 7.2-8.0 were observed. The samples were prepared in the presence and absence of trehalose (1.0 M). FGF2 showed the most stability in PBS buffer compared with other buffers. The FGF2 amount remaining in PBS after 8 hours, at 25 °C, was 86.3%, or a half-life of 38 hours, but in citrate/phosphate buffer FGF2 showed the lowest stability where the analogous amount of FGF2 remaining was 68.7 %, or half-life of 15 hours. The half-life of FGF2 in TRIS and HEPES were 31 hours and 16 hours respectively (Table 11). The degradation rate of FGF2 in PBS ($-2.0 \pm 1.4 \times 10^{-2}$ $hour^{-1}$) was significantly lower than that in citrate/phosphate ($-5.8 \pm 1.8 \times 10^{-2}$ $hour^{-1}$), HEPES ($-4.9 \pm 0.5 \times 10^{-2}$ $hour^{-1}$) and Tris ($-3.0 \pm 1.1 \times 10^{-2}$ $hour^{-1}$). Lower stability of FGF2 in the presence of other buffers compared with PBS needs more studies. One possibility can be relating to the ionic effect of Na^{+}, K^{+}, and Cl^{-} in PBS buffer solutions on the salt structure of the protein, keeping this structure more stable, resulting in higher stability of FGF2 in this buffer compared with other buffers. Lower FGF2 stability in HEPES buffer compared with PBS can be related to the reducing effect of HEPES buffer.

It has been advised to keep HEPES-containing solutions in darkness as much as possible to prevent oxidation and production of hydrogen peroxide (Zigler JS, 1985).

The degradation rate was decreased in the presence of trehalose for all samples except HEPES that showed unreliable data and initial concentration of FGF2 in HEPES buffer was lower than that in hours 4 or 8. The rate constants of the buffers PBS, TRIS, and citrate/phosphate in the presence of trehalose after 8 hours were ($-1.6 \pm 0.3 \times 10^{-2}$ $hour^{-1}$), ($-6.0 \pm 0.3 \times 10^{-3}$ $hour^{-1}$), and ($-2.5 \pm 0.3 \times 10^{-2}$ $hour^{-1}$) respectively. The stabilizing effect of trehalose for biomolecules in solution was already reported with higher trehalose concentration showing more improvement (Crowe, Carpenter, & Crowe, 1998; Xie & Timasheff, 1997; Kaushik & Bhat, 2003; Sola-Penna & Meyer-Fernandes, 1998; Carninci, et al., 1998). We already discussed the mechanism of action of trehalose as a stabilizer in the oxytocin part. We hypothesized that trehalose's stabilizing effect is primarily a structural stabilization while it has limited to no impact on smaller biomolecules such as peptides that only have a primary structure, it has more protective effect on proteins that typically have well-defined secondary or tertiary structure (Ghasemisarabbadieh M. G., 2021). The results in Table 11 and Figure 34 showed the degradation rates of all FGF2 solutions were decreased in the presence of trehalose and the corresponding FGF2 half-lives in PBS, TRIS, and citrate/phosphate buffers and were 52 hours, 134 hours and 29 hours respectively. Although FGF2 showed the most stability in PBS compared with other buffers, the most profound stabilizing effect of trehalose was observed in TRIS buffer.

The initial concentrations of FGF2 samples in the presence of trehalose were decreased. In the absence of trehalose, PBS sample showed the highest initial concentration of FGF2 compared with other buffers. One possible explanation for this lower initial concentration of FGF2 in the presence of trehalose can be the viscosity or osmolality of the solutions which are increased in the presence of trehalose. This might result in decreased solubility of the protein in the beginning with slight precipitation and/or denaturation of FGF2 samples. This potential effect, along with reducing the difference in osmolality and viscosity of the different buffer solutions should be considered for next studies.

Table 11. FGF2 remaining (%) in the presence of different buffers and trehalose (M. Ghasemisarabbadieh et al., 2021).

Sample	Initial FGF2 conc. (pg/mL)	Remaining FGF2 (%) at hour		
		0	4	8
PBS	473	100 (5.4)	97.0 (5.8)	86.3 (10.7)
Tris	402	100 (5.8)	83.6 (6.1)	83.4 (4.1)
HEPES	379	100 (3.1)	93.5 (7.5)	71.1 (8.1)
Citrate/phosphate	357	100 (4.8)	94.6 (13.1)	68.7 (10.1)
PBS + Trehalose	294	100 (5.4)	94.3 (1.3)	89.9 (1.6)
Tris + Trehalose	225	100 (4.4)	97.4***(2.7)	95.9***(1.8)
HEPES + Trehalose	195	100 (4.5)	121.2***(3.6)	114.7***(1.8)
Citrate/phosphate + Trehalose	260	100 (6.3)	98.8 (4.4)	82.7 (3.2)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.0005$

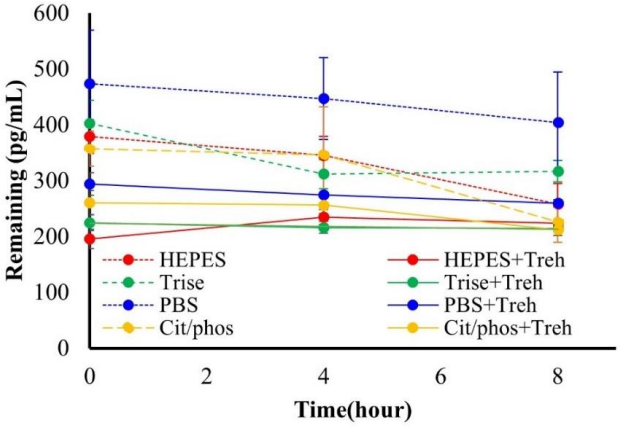


Figure 34. A plot of FGF2 remaining concentration vs time (hour) in different buffers in the presence or absence of trehalose (M. Ghasemisarabbadieh et al., 2021).

4.2.3 Effect of trehalose, D-(+)-glucosamine hydrochloride, 4EG and a mixture of D-(+)-glucosamine hydrochloride with 4EG on FGF2 stability

To understand better why trehalose showed different effect on FGF2 stability in all buffers used at the beginning and after a few hours (Table 11), two more samples of trehalose with lower concentration than 1.0 M (500 mM and 100 mM) were tested. D-(+)-Glucosamine hydrochloride, 4EG along with a mixture of these additives were also tested to see the effect of these additives on FGF2 stability in solution. FGF2 concentration was kept constant at 500 pg/mL in all solutions and PBS buffer at pH of 7.2-7.4 was used and results showed in Table 12. Initial concentration of FGF2 in the presence of lower concentrations of trehalose was increased. The degradation rate for trehalose samples with concentrations of 100 mM and 500 mM were ($-1.9 \pm 0.1 \times 10^{-2}$ hour^{-1}) with a half-life of 37 hours and ($-1.6 \pm 0.2 \times 10^{-2}$ hour^{-1}) with a half-life of 44 hours while for FGF2 sample without trehalose was ($-2.2 \pm 0.1 \times 10^{-2}$ hour^{-1}) and a half-life of 31 hours (Figure 36). The measurements showed that D-(+)-glucosamine, 4EG, and a mixture of D-(+)-glucosamine with 4EG had a negative effect on the FGF2 stability.

Table 12. FGF2 remaining (%) in the presence of different additives in PBS buffer (M. Ghasemisarabbadieh et al., 2021).

Sample	Additives Con. (mM)	Initial FGF2 conc. (pg/mL)	Remaining FGF2 (%), (hours)		
			0	11	23
FGF2	500 pg/mL	829	100 (3.0)	91.7 (0.1)	61.1 (2.6)
Treh 1	100	685	100 (1.6)	84.3 (1.1)	64.8 (2.9)
Treh 2	500	600	100 (1.9)	75.7***(0.3)	71.4*(3.0)
Glu 1	5	427	100 (4.6)	71.3*(1.0)	36.0***(0.8)
Glu 2	15	93	100 (3.1)	71.8*(2.0)	27.0***(3.0)
4EG (1)	1.0 %	467	100 (1.5)	81.8*(2.1)	28.2***(1.7)
4EG (2)	3.0 %	358	100 (1.0)	81.4*(2.9)	40.9***(3.1)
Glu1+4EG1	-	412	100 (1.7)	69.8*(2.5)	34.9***(2.0)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.0005$

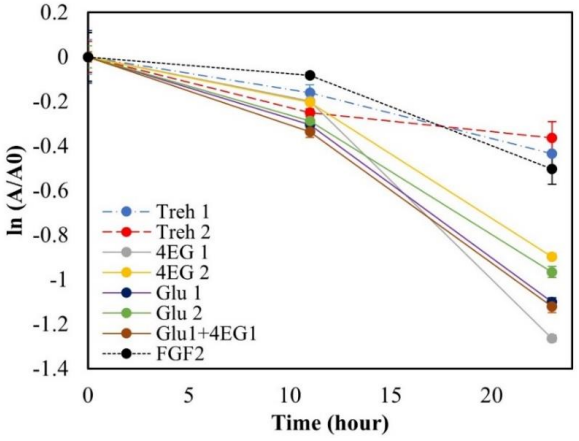


Figure 35. A plot of ln([A/A0]). Vs time (hour) (A0 = FGF2 initial concentration; A= FGF2 concentration at different time points (M. Ghasemisarabbadieh et al., 2021).

4.2.4 Effect of crown ethers and N-acetylglucosamine on FGF2 stability

We previously reported that 18-crown-6 enhanced the stability of oxytocin in solution in citrate/phosphate buffer (Ghasemisarabbadieh, Gizurarson, & Sveinbjörnsson, 2021). Amino acids have been reported to form complexes with 18-crown-6 as well (A .F. Danil de Namor, 1991). The ability of the cyclic polyether 18-crown-6 as non-covalent protecting group in peptide synthesis has also been illustrated (P. Mascagni, 1987; C. B. Hyde, 1989; C.B Hyde, 1990; P. Botti, 1995). Therefore, we decided to test the effect of 18-crown-6 and a small crown ether like 12-crown-4 to see if they are able to enhance FGF2 stability. FGF2 samples (500 pg/mL) were made at different concentrations of 18-crown-6 (0.5%, 1.0% or 2.0%) and 12-crown-4 (1.0% and 3.0%), the effect of N-acetylglucosamine (0.3M and 0.7M) was also tested. All samples were prepared in PBS buffer at pH 7.4 and one sample was included with only FGF2 without any additive. The results in Table 13 show that FGF2 stability was not enhanced in the presence of these additives and raising the concentration of the additives caused further degradation. The degradation rate of FGF2 in the presence of the additives showed in Figure 36. The degradation rate of all samples in the presence of the additives was more than FGF2 control without any additive, suggesting N-acetylglucosamine, 18-crown-6 and 12-crown-4 destabilize FGF2 in solution. One possibility for raising degradation rate of FGF2 in the presence of 18-crown-6 and 12-crown-4 can be the formation of complex of these crown ethers with ions like Na^+ and K^+ in PBS buffer. As we hypothesized in section 4.2.2, the presence of these ions appears to improve the stability of FGF2 in PBS buffer compared with other buffers used. The results of this part could support the hypothesis that ions Na^+, K^+ have a positive impact on FGF2 stability in solution.

Table 13. FGF2 remaining (%) in the presence of different additives in PBS buffer (M. Ghasemisarabbadieh et al., 2021).

Sample	Additives Con. (%)	Initial FGF2 conc. (pg/mL)	Remaining FGF2 (%) at hour		
			0	24	43
FGF2	0	758	100 (3.5)	85 (20.0)	55 (1.5)
18C6 (1)	0.5	721	100 (15.7)	80 (7.8)	55 (0.9)
18C6 (2)	1.0	796	100 (9.0)	69 (3.5)	46***(0.7)
18C6 (3)	2.0	723	100 (6.1)	51*(0.9)	35***(0.9)
12C4 (1)	1.0	737	100 (3.1)	54*(6.0)	36***(0.8)
12C4 (2)	3.0	660	100 (7.0)	75 (7.7)	52 (2.2)
N-acet 1	0.3M	730	100 (6.1)	75 (7.4)	42***(1.8)
N-acet 2	0.7M	631	100 (2.7)	60*(2.9)	36***(0.3)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.0005$

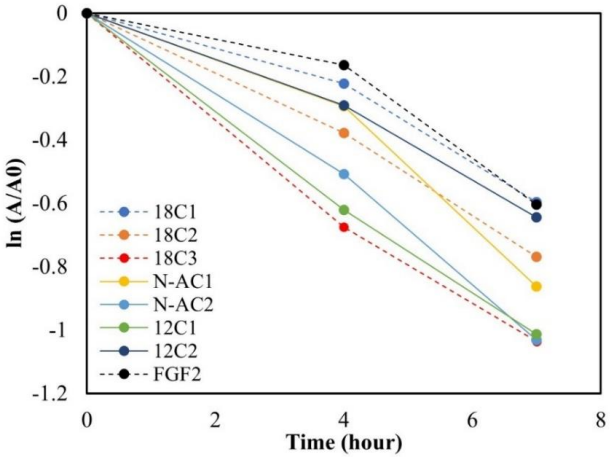


Figure 36. *A plot of Ln([At/A0]). Vs hour (A0 = FGF2 initial concentration; At= FGF2 concentration at different time points (M. Ghasemisarabbadieh et al., 2021).*

5 Summary and conclusions

This thesis was designed to evaluate the thermal stability of oxytocin and FGF2 in the presence of different excipients and solution compositions and can be divided into two parts. The main work in the first part was to test the effect of different additives and solutions with the objective of enhancing oxytocin stability in aqueous solutions. The focus of the second part of the thesis was preliminary studies on FGF2 in order to improve the FGF2 stability in aqueous solution and screen the stability of FGF2 at different pH along with evaluation of the effect of different buffers and excipients.

Trehalose effect

The effect of trehalose on oxytocin in aqueous solutions showed that although trehalose is often considered an excellent stabilizer for a wide range of biomolecules, it 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 raised 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. To answer this question the trehalose effect on FGF2 stability was investigated in aqueous solutions. The results showed that trehalose slowed down the rate of degradation for FGF2 but the initial amount of FGF2 in the samples seemed to decrease with increasing trehalose concentration.

The effect of aminosugars such as glucosamine and N-acetylglucosamine, as well as the effect of 4EG and a mixture of these additives

The stability of oxytocin and FGF2 in aqueous buffer solutions was evaluated in the presence of D-(+)-glucosamine, N-acetyl-D-glucosamine, 4EG and the mixture of these additives. The results showed that 4EG accelerates the degradation of both oxytocin and FGF2. While the reasons for this destabilization are unclear at the moment, it could be an avenue for a future study where the degradation products are analyzed in more detail. D-(+)-glucosamine had small to negligible effect on oxytocin at low concentrations but sped

up the degradation at higher concentrations. N-acetyl-D-glucosamine however, had small to negligible effect on oxytocin 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. Both D-(+)-glucosamine and N-acetylglucosamine had negative effect on FGF2 stability.

Buffer effect

Oxytocin was found to be more stable in acetate buffer than in citrate/phosphate buffer or phosphate buffer and we found that the stability is also concentration dependent with acetate buffer concentrations of 0.025 M or less being more beneficial, at pH of around 4.5. The effect of four different buffers, citrate/phosphate, HEPES, TRIS, and PBS, on FGF2 stability was evaluated. The results showed that FGF2 was more stable in PBS buffer compared with the other buffers used.

Crown ethers effect

The effect of different crown ethers on oxytocin and FGF2 in solutions was investigated. FGF2 stability in the presence of 18-crown-6 in PBS buffer was decreased and raising the concentration of the crown ethers led to faster degradation. 12-crown-4 at 1% (v/v) concentration decreased the FGF2 stability, while the effect observed at 3% concentration was statistically insignificant. We found that 15-crown-5 and 12-crown-4 did not have any stabilizing effect on oxytocin while we clearly demonstrated positive effect of 18-crown-6 on oxytocin stability in citrate/phosphate buffer but negative effect in acetate buffer.

Evidence of potential 18-crown-6 binding interactions was observed in HPLC experiments where a potential guest-host complex could be observed at a new retention time only when 18-crown-6 was present and only in certain mobile phases. Additional evidence of potential binding interactions was observed in the UV trace of oxytocin compared to oxytocin with 18-crown-6 in it. In the UV experiment, KCl seemed to negate the 18-

crown-6 effect on the UV trace, likely due to K^+ being able to bind to 18-crown-6. The presence of KCl did not, however, negate the HPLC peak shifting effect of 18-crown-6 in citrate/phosphate buffer samples, possibly due to the K^+ ions separating efficiently from the 18-crown-6 on the column and exiting it alongside the buffer. Lastly, NMR experiments showed that an NMR peak shift can be observed when oxytocin is in the presence of 18-crown-6 in D_2O, further supporting the possibility of binding interactions between the crown ether and oxytocin. These results will hopefully aid in developing more efficient mechanistically guided stabilization approaches for oxytocin as well as for other thermolabile molecules.

Antioxidants effect

Three antioxidants were tested on oxytocin stability in solutions. We observed 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 increased the rate of oxytocin degradation in solution significantly. One possibility for the different effect of antioxidants can be their solubilities in water, as L-ascorbic acid has a good solubility in water compared with uric acid and BHT, it has this opportunity to participate in undesired reactions, such as acting as a reducing agent, breaking the disulfide bond, instead of protecting the tyrosine part of oxytocin which is susceptible to oxidation. Disulfide bond is obtained via linkage of two cysteine residues by the oxidation of two sulfhydryl groups.

pH effect

The effect of different pH on FGF2 stability in citrate/phosphate buffer at a pH range (6-8) and acetate buffer at pH 4.5 and 5.5 was evaluated and the results showed that FGF2 is more stable in near-neutral to basic pH compared with acidic pH.

In conclusion, FGF2 showed to be more stable in PBS buffer at a range of pH 7.2-7.4. Oxytocin was more stable in acetate buffer than in phosphate or citrate/phosphate buffer, with greater stability at lower buffer concentrations (<0.025 M). We observed that trehalose slowed down the rate of degradation of FGF2 but had negligible effect on oxytocin stability in solution. Glucosamine, and 4EG accelerated the degradation rate for both oxytocin and FGF2. N-acetylglucosamine had negligible effect on oxytocin but increased the rate of degradation for FGF2. We also found that 18-crown-6 affected

stability of both oxytocin and FGF2, while it significantly enhanced oxytocin stability in citrate/phosphate buffer, it had a negative effect on the degradation of FGF2. Antioxidants such as BHT and uric acid had negligible effect on oxytocin stability, but L-ascorbic acid caused faster degradation of oxytocin in solution.

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Paper I

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Article

Effect of 18-Crown-6 on Oxytocin Stability in Aqueous Buffer Solutions

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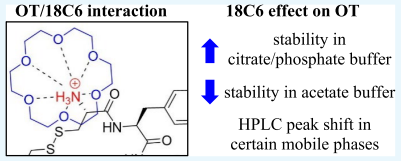
ABSTRACT: In this study, the effect of 18-crown-6 on the stability of oxytocin in aqueous solution was explored. The study found that while 12-crown-4 and 15-crown-5 do not stabilize oxytocin, 18-crown-6 does have a stabilizing effect in citrate/phosphate buffer at pH 4.5. However, in acetate buffer at the same pH, the presence of 18-crown-6 had a destabilizing effect, possibly leading to a different degradation pathway. Both the stabilizing and destabilizing effects, depending on the buffer used, are concentration dependent where a higher concentration of 18-crown-6 is linked to a stronger effect. It is hypothesized that this effect may be linked to 18-crown-6 binding to the protonated ammonium group of oxytocin. Upon changing the mobile phase used in high-performance liquid chromatography experiments, we observed evidence supporting this binding hypothesis. When an acidic mobile phase was used (0.01% trifluoroacetic acid (TFA)), a partial shift in oxytocin retention time was observed for samples in acetate buffers in the presence of 18-crown-6 when using a 150 mm column (C18). The amount of the peak that shifted depended on the 18-crown-6 concentration used. A similar shift in oxytocin peak retention time was observed for samples in both acetate and citrate/phosphate buffers when using a 250 mm column (C18), but the peak completely shifted in those samples. When using an even more acidic mobile phase (0.1% TFA), the oxytocin peaks all had the same retention time again. Ultraviolet and nuclear magnetic resonance spectroscopy experiments also showed that the presence of 18-crown-6 has an observable effect on the resulting oxytocin spectra.



INTRODUCTION

Oxytocin is a nonapeptidic hormone, often used as a drug to combat postpartum hemorrhage, the primary cause of maternal deaths around the globe.[1−4] Oxytocin also has other numerous biological and psychological functions including a role in lactation and relationships.[5] Additionally, efforts have been made toward using oxytocin as a drug to treat conditions such as alcoholism,[6] autism,[7] and schizophrenia.[8,9]

Unfortunately, oxytocin, in aqueous solutions, degrades rapidly when kept at temperatures above 30 °C.[10] Although it is not entirely clear why oxytocin samples in low and middle income countries are often of poor quality, oxytocin's thermostability has been suggested to be a contributing factor to the poor quality.[11] Methods for enhancing oxytocin's shelf life would therefore be beneficial. For this reason, an understanding of the degradation process can help in the development of successful stability-enhancing methods.

The oxytocin structure was elucidated by du Vigneaud et al. and reported in 1953.[12−14] It includes a six amino acid cyclic unit, with a disulfide bridge closing the ring. Wiśniewski et al. have proposed that the first step in the degradation mechanism of oxytocin is beta elimination, expelling R−S−S$^-$ as the leaving group. The experimental support for this hypothesis included experiments where they altered the oxytocin structure in small ways and measured how much remained after 28 days of storage at 40 °C in phosphate buffer solution at pH 7.4. For unaltered oxytocin, only 12.6% remained after 28 days, but when the sulfur atoms in the disulfide bridge were substituted with CH_2 units, 97.5% of this new analog remained. Those results support the idea that an important early degradation step is the expulsion of R−S−S$^-$ as a leaving group.[15]

Another interesting result was obtained when the only free amino group was substituted for a hydrogen, resulting in 90.1% of that analog remaining after 28 days under the above-mentioned conditions.[15] A potential explanation for this observation is that the free amine could act as the base, thus promoting the beta elimination. This hypothesis is further supported by oxytocin showing the greatest stability around pH 4.5[15,16] at which pH more of the amino group is significantly protonated and thus unable to act as a base. Furthermore, oxytocin has shown faster degradation kinetics when stored at higher concentrations at pH 4.5, suggesting a potential intermolecular degradation pathway with another oxytocin molecule.[16] If the process was primarily intramolecular or between oxytocin and the solvent, the oxytocin concentration should have a more limited effect on the kinetics

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of degradation. This concentration dependence was not observed at pH 2.0, which can be explained by another degradation mechanism taking over.

We hypothesized that if the amino group could be further inhibited from acting as a base, it would retard the oxytocin degradation process (Figure 1). To test this hypothesis, we

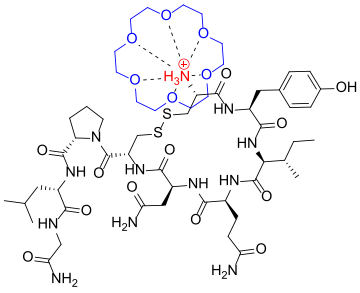

Figure 1. Hypothesized stabilizing interaction between 18-crown-6 (blue) and the protonated ammonium group (red) on the oxytocin molecule.

decided to use 18-crown-6 ether, which is known to have a binding affinity for protonated ammonium groups.[17] In contrast, smaller crown ethers, such as 15-crown-5 and 12-crown-4, do not show a strong binding affinity to ammonium groups because of the smaller ring sizes. It should be noted however, that 18-crown-6 may not be a suitable excipient in pharmaceutical formulations, as it also has a strong binding affinity for potassium ions, and studies have shown oral toxicity in dogs and mice.[18,19] However, the goal of this study was to explore a mechanistically guided approach to the oxytocin thermostability challenge and for that it can provide valuable insights.

RESULTS AND DISCUSSION

Effect of Different Crown Ethers on Oxytocin Stability in Citrate/Phosphate Buffer. Oxytocin solutions (0.25 mg/mL) were prepared in citrate/phosphate buffer (0.1 M/0.2 M) at pH 4.5. Four samples were made, one with only oxytocin as a control, and the others with different crown ethers for each one. The crown ethers used were 12-crown-4, 15-crown-5, and 18-crown-6 and the crown ether concentration was kept constant across the samples at 1.0% w/v. The samples were then each divided into two portions with one half being stored at 40 °C and the other at 50 °C for a period of up to 47 days. During this time period the remaining amount of oxytocin was regularly measured by high-performance liquid chromatography (HPLC) analysis. The results are shown in Table 1. While the remaining amount of oxytocin was similar for the control sample and the samples with 15-crown-5 or 12-crown-4 (statistically insignificant for the majority of those samples), a greater amount of oxytocin remained in the solutions with 18-crown-6, both at 40 °C and at 50 °C. At 40 °C the increased remaining amount of oxytocin was statistically significant, with $p < 0.005$ for all samples with 18-crown-6 present. At 50 °C, three out of four measurements showed that there was statistically significantly greater remaining amount of oxytocin when 18-crown-6 was present in the samples ($p < 0.05$ for 2 samples and $p < 0.005$ for 1 sample).

Table 1. Remaining Amount of Oxytocin (%) in a Citrate/Phosphate Buffer (0.1 M/0.2 M) in the Presence of Different Crown Ethers (1.0% w/v) As Well As by Itself[a]

		remaining oxytocin (%)				
sample	temperature	day 0	day 14	day 25	day 40	day 47
OT	40 °C	100.0 (0.1)	74.1 (0.4)	62.4 (1.5)	57.4 (0.7)	56.3 (0.3)
OT-12C4		100.0 (6.3)	76.3[d] (0.1)	68.4 (4.5)	59.3 (1.0)	57.3[d] (0.2)
OT-15C5		100.0 (0.3)	72.4[c] (0.3)	66.8 (4.4)	58.1 (2.1)	54.6[b] (0.6)
OT-18C6		100.0 (0.6)	78.3[d] (0.6)	71.4[d] (2.1)	68.4[d] (0.2)	65.4[d] (0.3)
		day 0	day 14	day 18	day 29	day 40
OT	50 °C	100.0 (0.1)	47.2 (1.0)	40.0 (2.4)	26.7 (4.3)	21.1 (0.0)
OT-12C4		100.0 (6.3)	47.2 (0.2)	40.2[b] (0.2)	26.0 (3.2)	18.7[b] (8.1)
OT-15C5		100.0 (0.3)	47.7 (3.7)	37.9 (1.1)	24.0 (5.2)	18.7[d] (0.4)
OT-18C6		100.0 (0.6)	53.5 (9.1)	44.8[b] (1.1)	33.3[b] (2.1)	25.4[d] (0.7)

[a]Standard deviation is shown in brackets behind each value (OT = oxytocin, 12C4 = 12-crown-4, 15C5 = 15-crown-5, 18C6 = 18-crown-6). [b]= $p \leq 0.05$. [c]= $p \leq 0.01$. [d]= $p \leq 0.005$.

These results fit with the hypothesis that 18-crown-6 would be the only one of these crown ethers to have a positive effect on the oxytocin stability because of its binding affinity with ammonium groups. Since 15-crown-5 and 12-crown-4 do not have as significant binding affinity to ammonium groups because of a smaller ring size, they were not expected to impact the oxytocin stability and indeed, their effect was negligible at the crown ether concentration tested. Therefore, these results provide further support that the free amine group plays a role in oxytocin's degradation pathway and reinforced the importance of understanding the degradation mechanism in order to stabilize better the sites of importance.

Concentration Effect of 18-Crown-6 on Oxytocin Stability. Oxytocin solutions (0.25 mg/mL) were also prepared in citrate/phosphate buffer (0.1 M/0.2 M) at pH 4.5 with different concentrations of 18-crown-6 as an additive (0.1, 0.3, and 2.0% w/v). The results showed that the oxytocin stability was enhanced with higher concentrations of the crown ether (Table 2). All of these results showed statistical significance. Analogous samples were prepared in acetate buffer (0.1 M). Surprisingly, in this sample series, the oxytocin stability diminished as the crown ether concentration increased (Table 2). Regarding the acetate results, all of them showed statistical significance except one. The only one that did not show statistical significance on the day 3 measurement was the sample with the lowest concentration of 18-crown-6 (18C6-A1).

The results in citrate/phosphate buffer fit with what was expected. As the concentration of 18-crown-6 increases the probability of it binding to a protonated ammonium group, thus preventing it from being involved in undesired reactions. It is not clear, however, why 18-crown-6 has an opposite effect on oxytocin stability in acetate buffer, seemingly destabilizing it. It is known that citrate buffer may produce amide- and imide-linked citrate derived degradation products of oxytocin.[20] That degradation also involves the amine group, in that case reacting with citric anhydride. The question then becomes whether the stabilizing effect of 18-crown-6 in

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Table 2. Remaining Amount of Oxytocin in the Presence of Different Concentrations of 18-Crown-6 (18C6) in either Citrate/Phosphate Buffer (C) or Acetate Buffer (A) at 50 °C

			remaining oxytocin (%)				
sample	buffer	crown ether conc. (% w/v)	day 0	day 3	day 7	day 10	day 14
OT-C	citrate/phosphate(0.1 M/0.2 M)	0	100.0 (0.4)	84.8 (0.7)	71.9 (0.1)	61.6 (0.2)	51.9 (0.2)
18C6-C1		0.1	100.0 (1.4)	85.8[a] (0.1)	68.7[c] (0.3)	58.9[c] (0.1)	49.5[c] (0.5)
18C6-C2		0.3	100.0 (0.2)	86.6[c] (0.2)	70.8[a] (0.7)	60.9[c] (0.1)	51.3[c] (0.0)
18C6-C3		2.0	100.0 (1.2)	88.5[c] (0.2)	75.6[c] (0.3)	67.8[c] (0.0)	59.6[c] (0.3)
OT-A[d]	acetate (0.1 M)	0	100.0 (1.1)	92.6 (1.3)	87.2 (0.7)	82.7 (0.9)	74.2 (1.8)
18C6-A1		0.1	100.0 (0.6)	90.5 (0.0)	83.2[b] (1.1)	76.2[c] (0.0)	67.5[b] (0.0)
18C6-A2		0.3	100.0 (0.5)	87.3[a] (1.0)	82.5[b] (1.1)	73.4[c] (0.0)	62.1[c] (0.7)
18C6-A3		2.0	100.0 (0.1)	84.2[a] (1.3)	72.1[c] (1.4)	62.5[c] (2.4)	51.2[c] (0.2)

[a]$p \le 0.05$. [b]$p \le 0.01$. [c]$p \le 0.005$ [d]OT-A gave significantly better results at all time points (p = 0.02, 0.0004, 0.0003, and 0.0007, respectively, for days 3, 7, 10, and 14), compared with OT-C.

citrate/phosphate is because it slows down the amide- and imide-linked degradation pathway, or if it retards the beta elimination pathway that includes the disulfide bridge, or a bit of both.

In an attempt to understand better what was happening, the HPLC traces were analyzed further in order to see if a variation in degradation products could be observed, depending on the presence or absence of 18-crown-6. After 14 days at 50 °C, oxytocin showed a similar degradation pattern, based on the HPLC trace, for oxytocin in both acetate and citrate/phosphate buffer, as well as for the sample that included 18-crown-6 (2% w/v) in citrate/phosphate buffer. This suggests that 18-crown-6 may be slowing down the main degradation pathway in citrate/phosphate buffer (Figure 2).

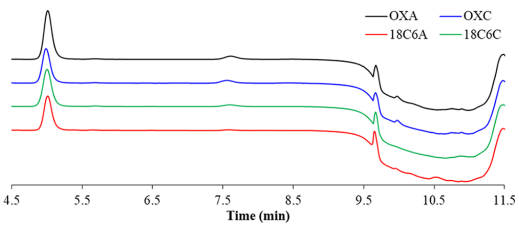

Figure 2. HPLC traces of oxytocin after 14 days of storage at 50 °C in the presence and absence of 2% w/v of 18-crown-6 in both acetate and citrate/phosphate buffer.

When oxytocin was stored in acetate buffer in the presence of 2% w/v of 18-crown-6, the HPLC trace showed more significant differences, most notably a new peak around minute 10.5 that was not observed in any of the other HPLC traces (Figure 2). This suggests that the presence of 18-crown-6 in acetate buffer may lead to a different degradation pathway for oxytocin. Further studies will be required to better understand why 18-crown-6 has such a different effect on oxytocin stability in acetate buffer compared to in citrate/phosphate buffer.

18-Crown-6 Effect on the Retention Time—Acidic Mobile Phase and Short Column. In a different HPLC run, the mobile phase was changed to a more acidic one (A: 0.01% trifluoroacetic acid (TFA) in H_2O and B: 0.01% TFA in 70% MeCN: 30% H_2O), allowing most of the amino group of oxytocin to remain protonated throughout the run. Several samples were prepared with the oxytocin concentration kept constant at 0.25 mg/mL, while the 18-crown-6 concentration ranged from 0% up to 1% w/v, with the 0% sample serving as a control. In these HPLC runs, a partial shift was observed when 18-crown-6 was included in the samples (Figure 3). The

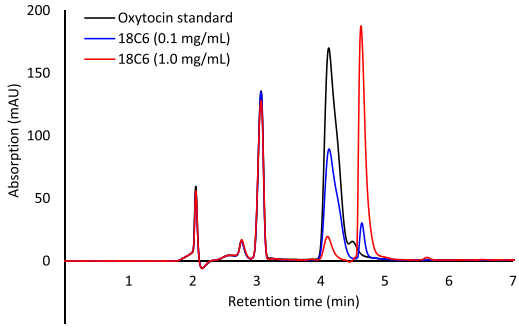

Figure 3. Ultraviolet (UV) trace from HPLC runs of an oxytocin standard (black), an oxytocin sample with 0.1% w/v of 18-crown-6 added (blue), and an oxytocin sample with 1.0% w/v of 18-crown-6 added (red). These samples were prepared in acetate buffer.

oxytocin amount in all of these samples was kept constant and the overall area under both peaks was close to constant. The amount of relative peak area that was shifted was dependent on the 18-crown-6 concentration, suggesting that its presence in the solution caused the peak shift. These results further support the hypothesis that a guest/host complex is being formed between oxytocin and 18-crown-6 and we hypothesize that the new peak may be due to this guest/host complex (Table 3).

Table 3. Relative Peak Area of the Peaks Showing up at 4.1 and 4.6 min for Freshly Prepared Oxytocin Samples with Different Concentrations of 18-Crown-6

sample	18-crown-6 conc. (% w/v)	% of area under peak at 4.1 min	% of area under peak at 4.6 min
OT	0	100	0
OT-A-18C6−1	0.01	95	5
OT-A-18C6−2	0.05	92	8
OT-A-18C6−3	0.1	89	11
OT-A-18C6−4	1.0	12	88

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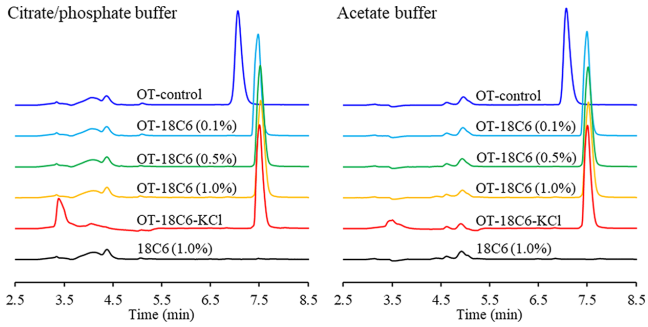

Figure 4. HPLC traces of samples showing an oxytocin peak shift when in the presence of 18-crown-6. The samples were prepared in citrate/phosphate buffer (left) and in acetate buffer (right). The mobile phase used was A: 0.01% TFA in H_2O and B: 0.01% TFA in 70% MeCN: 30% H_2O.

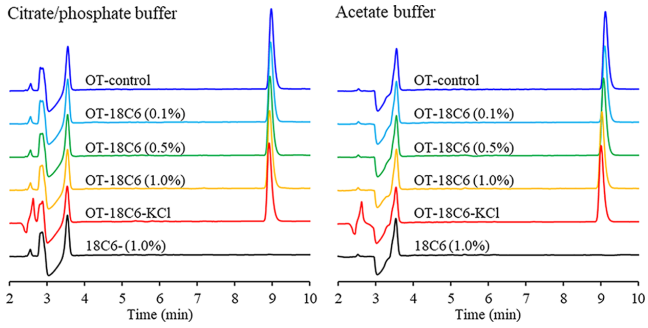

Figure 5. HPLC traces of samples showing no oxytocin peak shift regardless of the presence of 18-crown-6. The samples were prepared in citrate/phosphate buffer (left) and acetate buffer (right). The mobile phase used was A: 0.1% TFA in H_2O and B: 0.1% TFA in 70% MeCN: 30% H_2O.

18-Crown-6 Effect on the Retention Time—Acidic Mobile Phase and a Longer Column. Several more samples were prepared with the oxytocin concentration remaining constant at 0.25 mg/mL, while the 18-crown-6 concentration ranged again from 0% up to 1% w/v. In this set of samples, an additional one with oxytocin (0.25 mg/mL), 18-crown-6 (1.0% w/v), and potassium chloride (2.0% w/v) was included as well as a sample with only 18-crown-6. These samples were prepared in both acetate and citrate/phosphate buffers at pH 4.5. When a longer column was used (250 mm instead of 150 mm), a complete shift was observed in the retention time of all of the oxytocin samples containing 18-crown-6, regardless of buffer used (Figure 4). It is unclear why the length of the columns, which should be otherwise identical, would have such a drastic effect (partial vs complete peak shift). Nevertheless, these results were reproducible in our lab with these columns. A future study could be needed to provide an adequate explanation for this observation. We hypothesize that the new peak observed here is also for the oxytocin/18-crown-6 complex shown in Figure 1. No peak was observed for 18-crown-6, as it does not absorb light at the detection wavelength (220 nm), and KCl did not prevent a peak shift from being observed in the samples. In the samples that included KCl, the buffer region of the HPLC trace (2.5−5.5 min) was significantly altered (Figure 4), suggesting that the potassium and/or chloride ions were interacting with the buffer components (acetate or citrate/phosphate) and came out of the column alongside them. If the potassium ion is binding more strongly to the buffer components than to 18-crown-6 in these HPLC runs, that would explain why its presence did not prevent the peak shift from being observed.

When analyzing these results there are two important things to keep in mind. First, these samples were fresh so no degradation product is to be expected to be observed at this point in time. Second, this peak shift had not been observed in previous HPLC runs, so this peak shift/complexation was directly dependent on the mobile phase being used. Still, the possibility remained that a fast reaction was taking place between the mobile phase and oxytocin yielding a new product with a new retention time. To test this possibility, we changed the TFA concentration in the mobile phase and re-analyzed the samples. The new mobile phase composition had increased TFA concentration and was A: 0.1% TFA in H_2O and B: 0.1% TFA in 70% MeCN: 30% H_2O. When this mobile phase was used, all of the oxytocin samples showed up at the same retention time, regardless of whether 18-crown-6 was present or not (Figure 5). It remains unclear why a more acidic mobile phase leads to 18-crown-6 no longer having any observable effect on the retention time. It could have been expected that the more acidic medium would lead to greater complexation yielding a potential peak shift, which is not what we observed. For the 0.1% TFA mobile phase, the pH was close to 2, and for the 0.01% TFA mobile phase it was closer to 3. 18-crown-6 can also bind hydronium ions, and at the lower pH, we have an

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order of magnitude more hydronium ions that might also bind to the 18-crown-6. It is difficult to tell whether this would be enough to affect the complexation or not, and the difference seems low to completely negate the observed peak shift. These results do suggest however, that a reaction is unlikely to be causing the previously observed peak shift, but rather a pH or mobile phase dependent guest−host complex interaction.

18-Crown-6 Effect on the UV Absorption Spectrum. Samples were prepared to see if changes would be observed in the UV absorption spectrum (200−300 nm) of oxytocin depending on the additives present (Figure 6). 18-crown-6

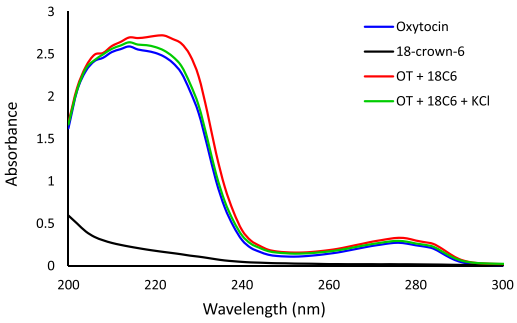

Figure 6. UV absorption spectra for oxytocin, 18-crown-6, a solution with both oxytocin and 18-crown-6, and a solution with oxytocin, 18-crown-6, and potassium chloride.

(4% w/v) on its own did not absorb much. When oxytocin (0.25 mg/mL), 18-crown-6 (4% w/v), and KCl (4% w/v) were all together, the UV trace was very similar to the UV trace of oxytocin by itself. When oxytocin and 18-crown-6 were in the solution by themselves, there was a noticeably bigger right shoulder observed around 230 nm. The solvent used here was a 70/30 mixture of mobile phases A and B that were used in the third part of this study. These results suggest that in this solution mixture, potassium may have bound more strongly to the 18-crown-6 preventing the crown ether to interact as strongly with the oxytocin and affecting its UV trace.

18-Crown-6 Effect on the Nuclear Magnetic Resonance Spectroscopy Spectrum. Two nuclear magnetic resonance spectroscopy (NMR) samples were prepared in D_2O to see if any interaction could be observed using that approach. One of the NMR samples only contained oxytocin whereas the other contained oxytocin and 18-crown-6. The spectra were both calibrated to the NMR solvent, D_2O, at 4.79 ppm, to enable a better comparison of the peak locations. The NMR shows that there is a clear peak shift for many of the oxytocin peaks when 18-crown-6 is present (Figure 7). This is especially noticeable for the aromatic protons on tyrosine, where the distance between the chemical shifts of the aromatic peaks has increased in the presence of 18-crown-6. There are, however, many other peaks that have shifted slightly as can be seen in Figure 7. While it is difficult to identify exactly what type of interaction is between the two compounds based on the NMR spectrum, the observed peak shifts do support the possibility of a guest/host interaction between the 18-crown-6 and the oxytocin, even in pure D_2O.

CONCLUSIONS

In conclusion, this study explored how 18-crown-6 affects oxytocin stability in both acetate and citrate/phosphate buffer solutions, yielding positive effect in citrate/phosphate buffer but negative effect in acetate buffer. Evidence of potential 18-crown-6 binding interactions was observed in HPLC experiments where a potential guest−host complex could be observed at a new retention time only when 18-crown-6 was present and only in certain mobile phases. Additional evidence of potential binding interactions was observed in the UV trace of oxytocin compared to oxytocin with 18-crown-6 in it. In the UV experiment, KCl seemed to negate the 18-crown-6 effect on the UV trace, likely because of K^+ being able to bind to 18-crown-6. The presence of potassium chloride did not, however, seem to negate the HPLC peak shifting effect of 18-crown-6 in citrate/phosphate buffer samples, likely because of it binding more strongly to the buffer and exiting the column along with the buffer. Lastly, NMR experiments showed that an NMR peak shift can be observed when oxytocin is in the presence of 18-crown-6 in D_2O, further supporting the possibility of binding interactions between the crown ether and oxytocin. These results will hopefully aid in developing more efficient mechanistically guided stabilization approaches for oxytocin as well as for other thermolabile molecules.

MATERIALS AND METHODS

MATERIALS

Oxytocin was purchased from Grindeks (Latvia). The following chemicals were bought from Sigma-Aldrich, with the country of origin indicated in brackets: 18-crown-6 (India),

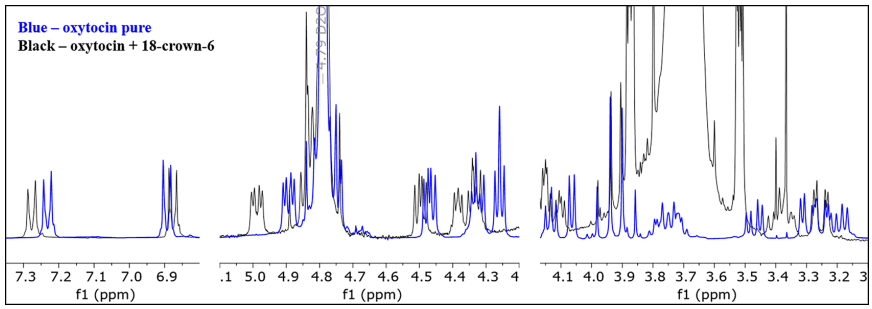

Figure 7. Stacked NMR spectra of pure oxytocin (blue) and a mixture of oxytocin and 18-crown-6 (black) in D_2O.

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15-crown-5 (Switzerland), 12-crown-4 (Germany), potassium chloride (Germany), sodium hydrogen phosphate (Germany), sodium dihydrogen phosphate (Germany), and TFA (France). Citric acid anhydrous was obtained from Pernhofen (Austria). Ammonium acetate was purchased from Riedel-de Haën (Serbia/Montenegro). Sodium acetate was bought from Merck (Germany). Acetic acid, acetonitrile, and methanol were purchased from Honeywell (Germany). All water used in this study was obtained from a Milli-Q water purification system.

Formulation. Oxytocin standards for HPLC runs were generally prepared in the same buffer as samples being run in each part of the study.

Section 1: Four oxytocin samples with a constant concentration of 0.25 mg/mL were prepared in a citrate/phosphate buffer (0.1 M/0.2 M) at pH 4.5. One of these samples had no other additives, while the other three samples contained 12-crown-4, 15-crown-5, or 18-crown-6. The crown ether concentration was kept constant at 1% w/v. These samples were then stored at 40 or 50 °C and analyzed regularly via HPLC over a period of 47 days.

Section 2: Eight oxytocin samples were prepared with the oxytocin concentration kept constant at 0.25 mg/mL. Four of these samples were prepared in a citrate/phosphate buffer (0.1 M/0.2 M) and the other four were prepared in acetate buffer (0.1 M), all at pH 4.5. For the four samples in each buffer, one sample had no additives, while the others had 18-crown-6 at a range of concentrations (0.1%, 0.3% or 2% w/v). These samples were stored at 50 °C and analyzed regularly over a period of two weeks.

Section 3: Oxytocin samples (0.25 mg/mL) were prepared in acetate buffer (0.1 M) with 18-crown-6 added in a range of concentrations (0, 0.01, 0.05, 0.1, and 1.0%).

Section 4: Oxytocin samples (0.25 mg/mL) were prepared in acetate buffer (0.1 M) on the one hand, and citrate/phosphate buffer (0.1 M/0.2 M) on the other hand, with 18-crown-6 added in a range of concentrations (0, 0.1, 0.5, and 1.0% w/v). Furthermore, an oxytocin sample with 18-crown-6 (1.0% w/v) and KCl (2.0% w/v) was prepared in each of the buffers, as well as a sample with pure 18-crown-6 (1.0% w/v).

Section 5: For the UV−vis part of the study, the additives were at the same concentration whenever present in a sample. The samples in this series included: pure oxytocin (0.25 mg/mL), pure 18-crown-6 (4.0% w/v), a mixture of oxytocin with 18-crown-6, a mixture of oxytocin with 18-crown-6 and potassium chloride (4.0% w/v). The solvent for this series was the same as the mobile phase used in the third part of this study, that is 70% of mobile phase A (0.01% TFA in water) and 30% of mobile phase B (0.01% TFA in a 70% MeCN: 30% H_2O solution). These samples were analyzed directly by UV−visible spectrophotometry.

Analysis. Analytical HPLC used in this study included a 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 photodiode array detector. The UV detection was set at 220 nm. All HPLC samples were run in triplicate and the averages and standard deviation of the calculated remaining amount of oxytocin are reported.

Section 1: A Phenomenex Luna 5 μm C18(2), 150 × 4.6 mm, 100 Å, LC column was used with a SecurityGuard Cartridge (C18 4 × 3.0 mm). The chromatographic procedure of the European pharmacopoeia was followed. Mobile phase A was a 15.6 g/L solution of sodium dihydrogen phosphate. Mobile phase B was a 1:1 acetonitrile:water solution. The flow rate was 1 mL/min. At minutes 0−17, mobile phase A went from 70 to 40% (v/v) in a linear gradient elution. From minute 17−19, mobile phase A went back to 70%. The equilibration time was 11 min. As the high salt concentration could more easily lead to clogging in the instrument, new mobile phase constitutions were sought.

Section 2: A Phenomenex Luna 5 μm C18(2), 250 × 4.6 mm, 100 Å, LC column was used with a SecurityGuard Cartridge (C18 4 × 3.0 mm). Mobile phase A was an aqueous 20 mM ammonium acetate solution and mobile phase B was pure acetonitrile. The flow rate was 1 mL/min. At minutes 0−11, mobile phase A was constant at 75%. From minute 8−10.5 mobile phase A went down to 10% in a linear gradient elution followed by an isocratic step at this concentration until minute 11. From minute 11−12.5, mobile phase A percentage was increased again to 75%. The equilibration time was 3.5 min.

Section 3: A Phenomenex Luna 5 μm C18(2), 150 × 4.6 mm, 100 Å, LC column was used with a SecurityGuard Cartridge (C18 4 × 3.0 mm). The mobile phase combination was A: 0.01% TFA in H_2O and B: 0.01% TFA in 70% MeCN: 30% H_2O. The flow rate was 1 mL/min. At minutes 0−17, mobile phase A went from 70 to 40% in a linear gradient elution. From minute 17−19 the mobile phase went back to 70%. The equilibration time was 11 min.

Section 4: A Phenomenex Luna 5 μm C18(2), 250 × 4.6 mm, 100 Å, LC column was used with a SecurityGuard Cartridge (C18 4 × 3.0 mm). The first mobile phase combination was A: 0.01% TFA in H_2O and B: 0.01% TFA in 70% MeCN: 30% H_2O. The flow rate was 1 mL/min. At minutes 0−17, mobile phase A went from 70 to 40% in a linear gradient elution. From minute 17−19 the mobile phase went back to 70%. The equilibration time was 11 min. The second mobile phase combination was A: 0.1% TFA in H_2O and B: 0.1% TFA in 70% MeCN: 30% H_2O.

Section 5: UV−vis spectrophotometric analyses were performed on a Genesys 150 UV−visible spectrophotometer using 1 cm quartz cells. The range of wavelengths measured was 200−300 nm. ^1H spectra were recorded on a Bruker Avance 400 MHz spectrometer in deuterated water as the solvent.

Statistical Analysis. Evaluation of the statistical significance of improvement or declination in stability was carried out using Student's t-test. The *p*-values equal or below 0.05 were regarded as significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c06248.

HPLC traces and NMR spectra (PDF)

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Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c06248

Author Contributions

Conceptualization, S.G.; methodology, M.G., S.G., and B.R.S.; data curation, M.G..; writing − original draft preparation, M.G. and B.R.S.; 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. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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Supplementary Information

The effect of 18-crown-6 on oxytocin stability in aqueous buffer solutions

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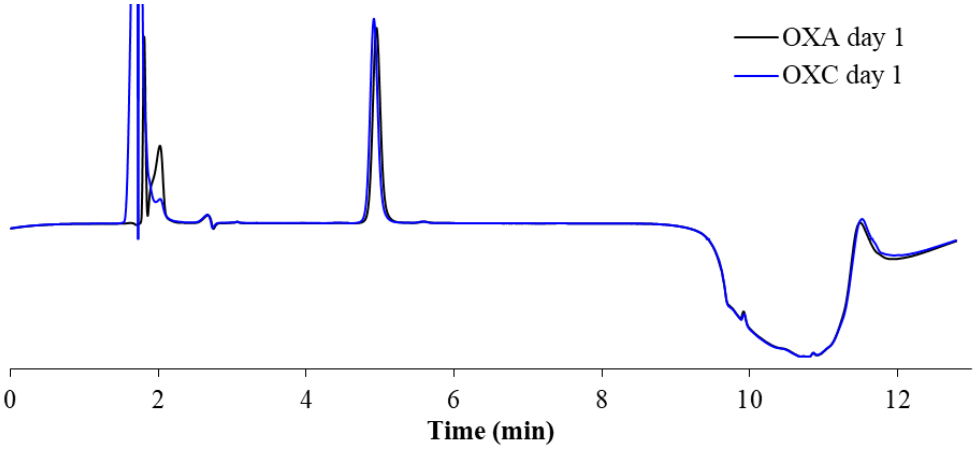

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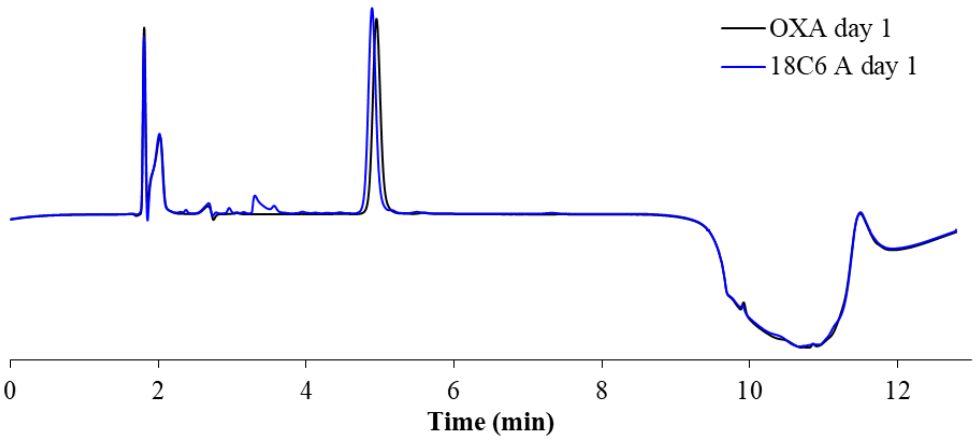

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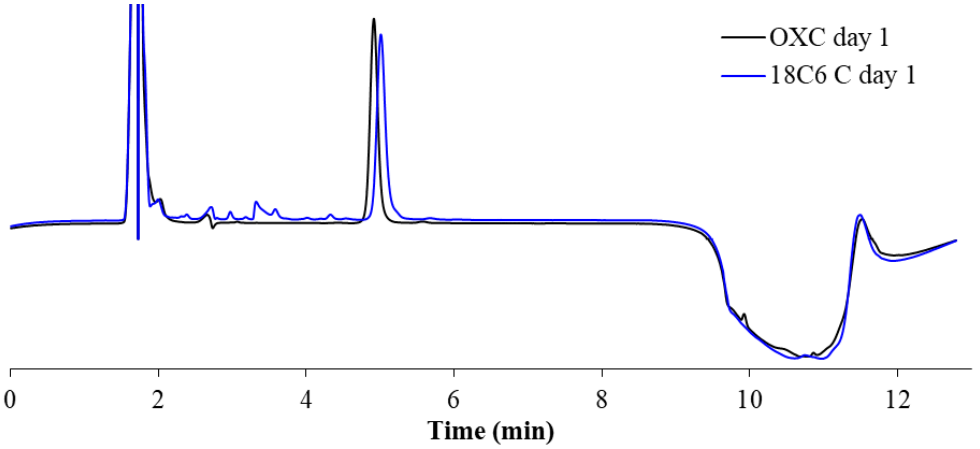

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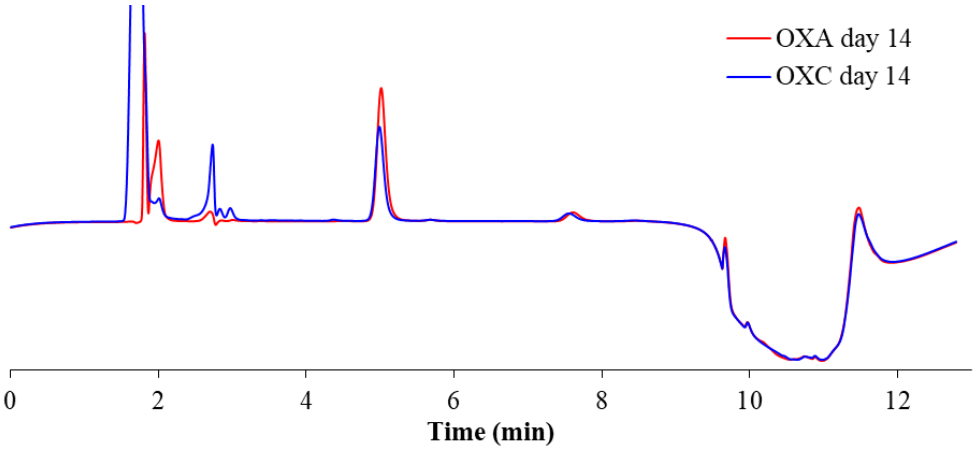

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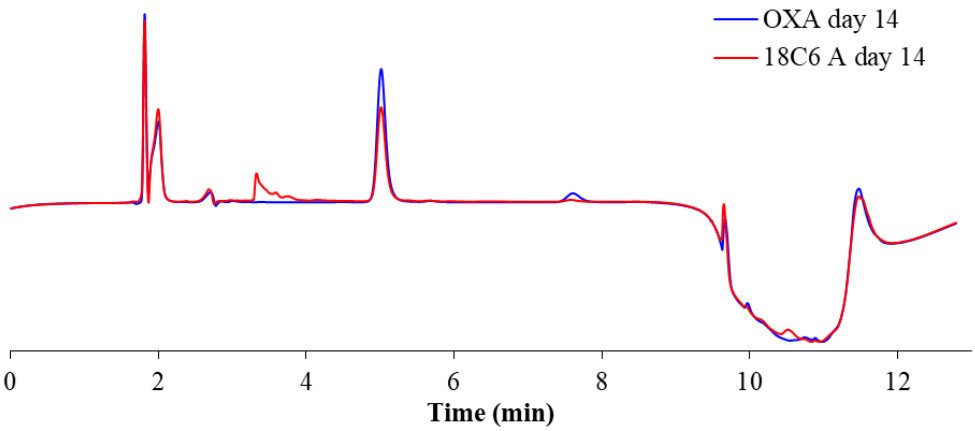

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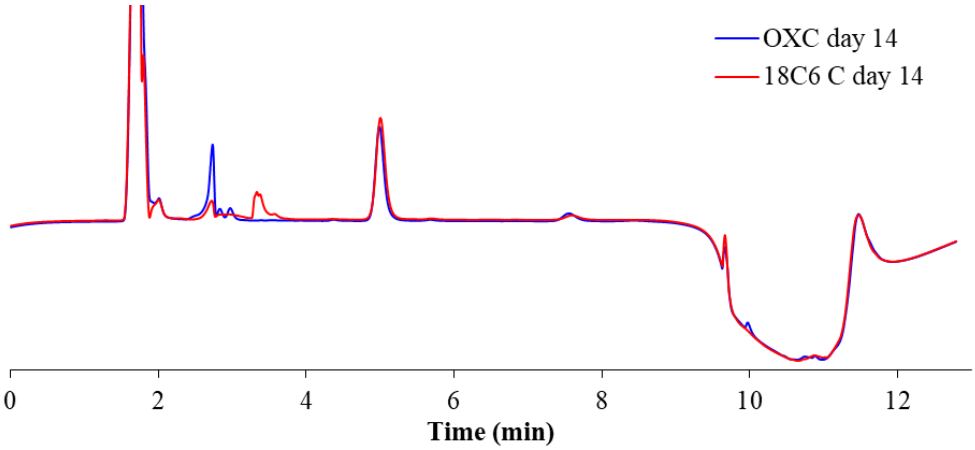

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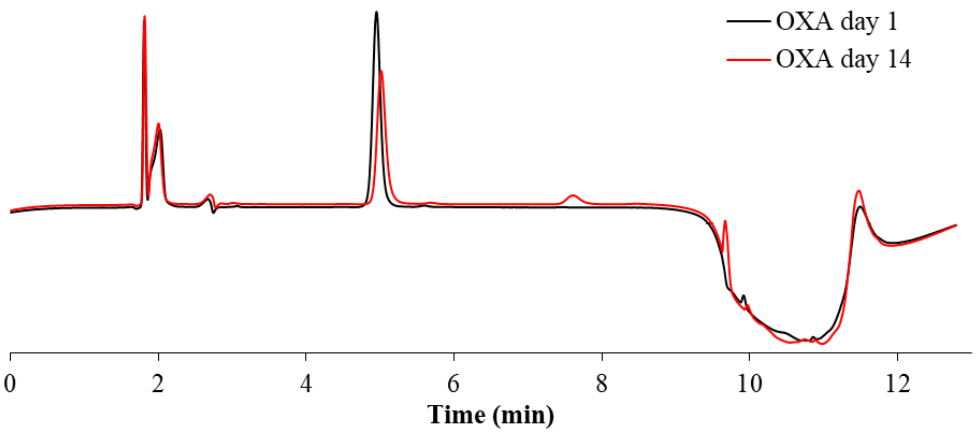

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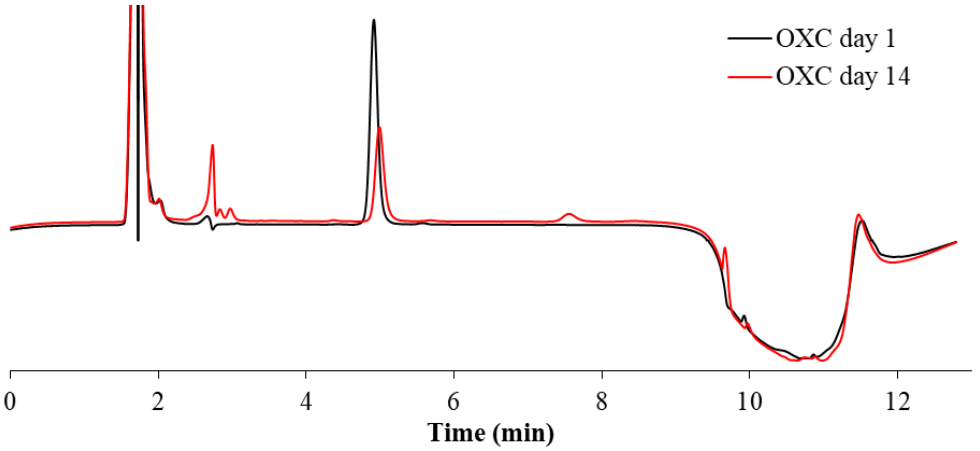

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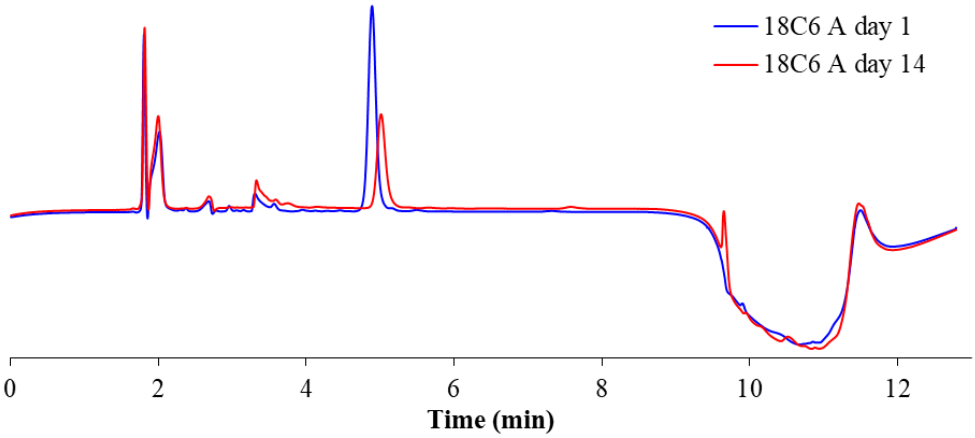

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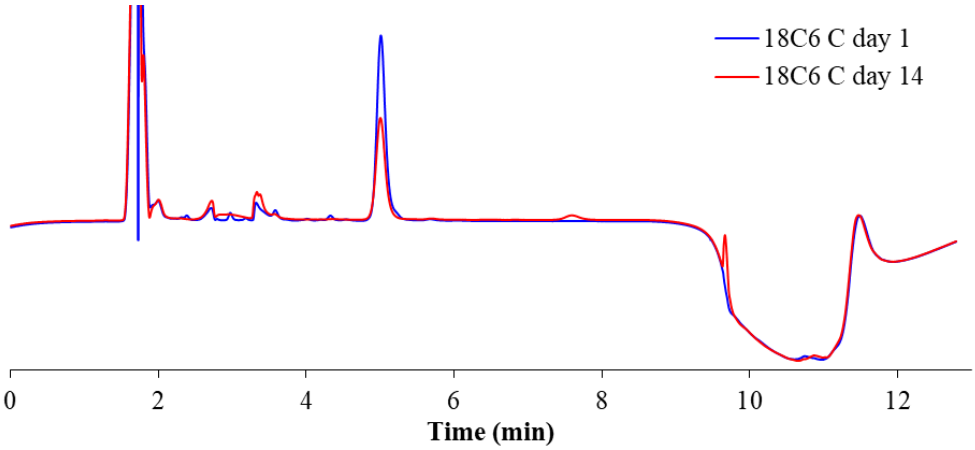

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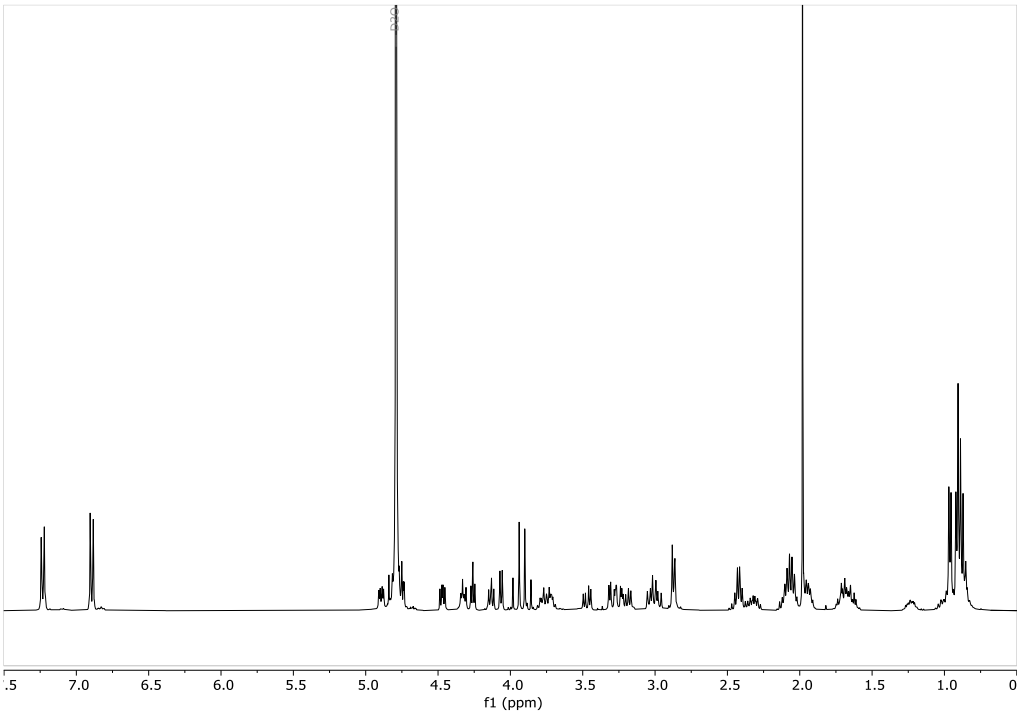
NMR spectra



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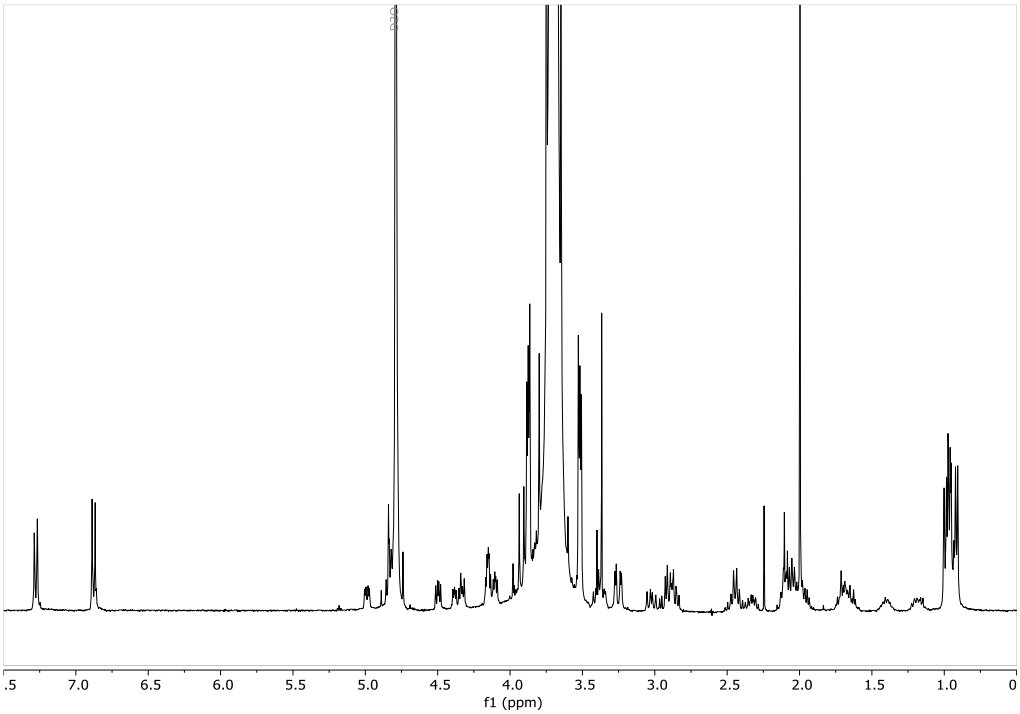

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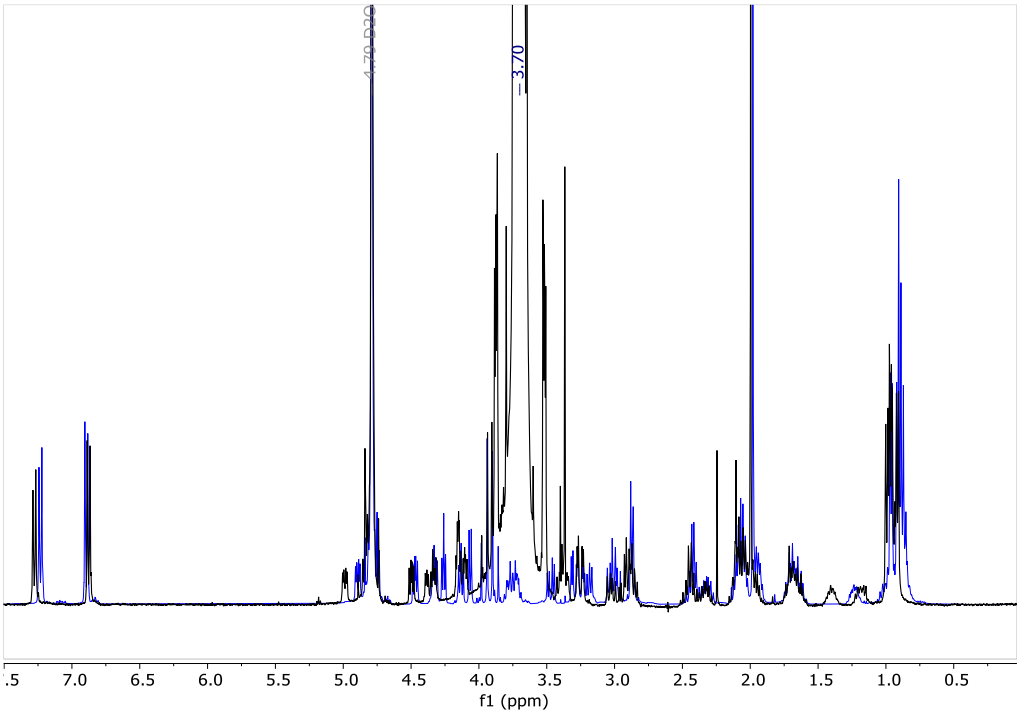

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Paper II

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RESEARCH ARTICLE

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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 compared to 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
acetate buffer, antioxidants, formulation, oxytocin, stability, trehalose

1 | 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 and 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] and 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 with 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, and 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 hypotheses 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

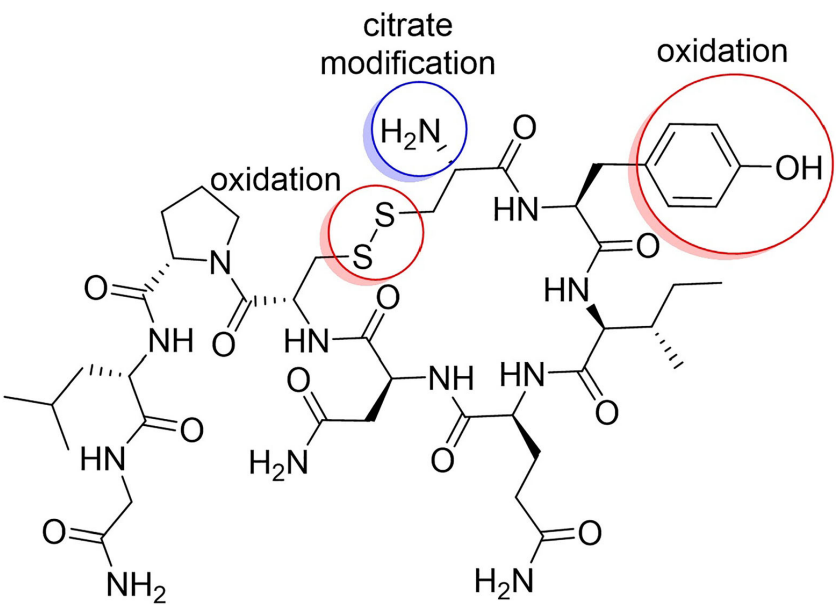

FIGURE 1 The structure of oxytocin with known sites of degradation identified

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

2 | MATERIALS AND METHODS

2.1 | Materials

Oxytocin was purchased from Grindeks (Latvia). Trehalose came from Pfanstiehl Inc. (Waukegan, USA) and was kindly provided by Alvotech (Reykjavik, Iceland). BHT, 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.

2.2 | Analysis

Analytical high-performance liquid chromatography (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® 5 μm C18 (2), 250 × 4.6 mm, 100-Å, LC column was used with a SecurityGuard Cartridge (C18 4 × 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[43] 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.

2.3 | 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 (0.1 M/0.2 M) at pH 4.5 was prepared as well as acetate buffers at a range of concentrations (0.01 M/0.025 M/0.05 M/0.1 M/0.2 M) 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 BHT (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 (0.1 M/0.2 M), and four different concentrations of trehalose (10, 20, 30, or 40 mM) were used for the samples made in each of the buffers used. The additional trehalose samples (0.1, 0.5, and 1.0 M) 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, 4.0, 1.6, 0.64, and 0.26 mg/ml) in acetate buffer at pH 4.5.

3 | 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 (0.1 M) or citrate/phosphate buffer (0.1 M/0.2 M), 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

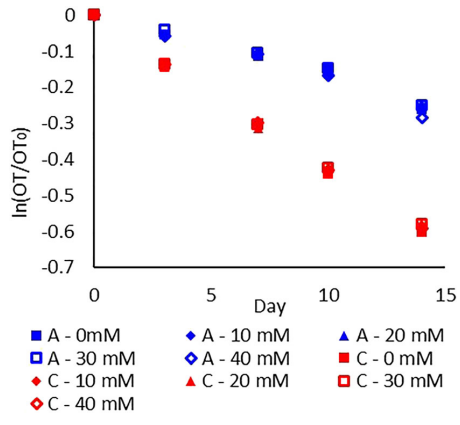

FIGURE 2 A plot of $\ln([OT]t/[OT]_0)$ versus day. The A samples (blue) are in acetate buffer with different concentrations of trehalose as indicated in the legend. The C samples (red) are in citrate/phosphate buffer

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.[44]

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, may 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?

As oxytocin was significantly more stable in 0.1 M acetate buffer, than in citric/phosphate buffer (0.1 M/0.2 M), the importance of the acetate concentration was also studied by storing oxytocin in 0.01, 0.025, 0.05, 0.1, and 0.2 M acetate buffer, keeping the pH constant at 4.5. When the rate constants were plotted up versus 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 0.025 M. 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 with a half-life of 40 days when the acetate buffer concentration was 0.1 or 0.2 M.

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. Because 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 50-mM 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 whereas L-ascorbic acid accelerated the degradation rate significantly, showing half-life of less than 4 days at concentrations ranging from 1 to 4 mg/ml (see supporting 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.

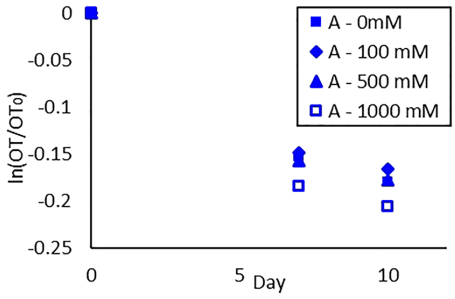

FIGURE 3 A plot of ln([OT]t/[OT]$_0$) versus day. All samples were made in acetate buffer and with different concentrations of trehalose as indicated in the box

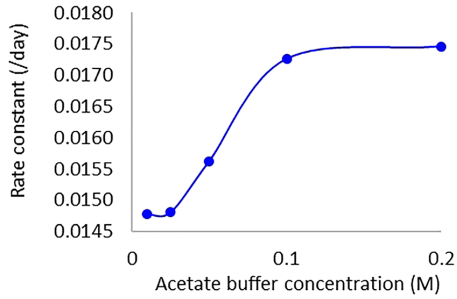

FIGURE 4 A plot of acetate buffer concentration against the oxytocin degradation rate constants

4 | 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 to 1000 mM, as well as negligible effect on oxytocin stability in citrate/phosphate buffers at concentrations ranging from 10 to 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 when considering whether to utilize trehalose as a stabilizer.

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CONFLICT 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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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Supplementary Information

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

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Table 1. Oxytocin remaining (%) in the presence of different concentrations of trehalose. Standard deviation is shown in brackets behind each value. (OTC = oxytocin in citrate/phosphate buffer; OTA = oxytocin in acetate buffer; TRA = trehalose in acetate buffer; TRC = trehalose in citrate/phosphate buffer).

Sample	Buffer	Trehalose conc. (mM)	Oxytocin remaining (%) at day				
			0	3	7	10	14
OTA-1	Acetate (0.1M)	0	100.0 (1.1)	92.6 (1.2)	87.2 (0.6)	82.7 (0.8)	74.2 (1.3)
TR1A		10	100.0 (0.3)	92.2 (0.9)	87.8 (1.0)	81.5 (0.7)	75.0 (0.5)
TR2A		20	100.0 (0.4)	92.6 (0.7)	87.5 (1.0)	82.7 (0.1)	75.1 (0.4)
TR3A		30	100.0 (0.7)	93.8 (1.1)	87.9 (1.1)	83.1 (0.1)	75.0 (1.1)
TR4A		40	100.0 (0.7)	92.3 (0.8)	87.8 (0.1)	81.3* (0.1)	72.5 (0.6)
OTC	Citrate/phosphate (0.1M/0.2M)	0	100.0 (0.4)	84.8 (0.7)	71.9 (0.0)	61.6 (0.1)	51.9 (0.1)
TR1C		10	100.0 (0.5)	85.3 (0.8)	71.5 (0.9)	62.3* (0.4)	53.1*** (0.3)
TR2C		20	100.0 (0.1)	84.6 (0.6)	70.9* (0.7)	62.5*** (0.2)	53.1*** (0.1)
TR3C		30	100.0 (0.3)	85.3 (0.3)	71.7 (0.9)	62.5* (0.5)	53.2*** (0.2)
TR4C		40	100.0 (0.3)	85.2 (0.3)	72.0* (0.1)	62.2* (0.4)	52.5* (0.3)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.005$

Table 2. Oxytocin remaining (%) in the presence of different concentrations of trehalose. Standard deviation is shown in brackets behind each value. (OTA = oxytocin in acetate buffer; TRA = trehalose in acetate buffer).

Sample	Trehalose conc. (M)	Oxytocin remaining (%) at day		
		0	7	10
OTA-2	0	100.0 (0.5)	88.0 (0.4)	85.8 (0.9)
TR5A	0.1	100.0 (0.4)	88.3 (0.3)	86.9 (0.1)
TR6A	0.5	100.0 (0.5)	87.6 (0.3)	85.7 (0.3)
TR7A	1.0	100.0 (0.6)	85.1 (0.6)***	83.4 (0.7)*

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.005$

Table 3. Oxytocin remaining (%) in the presence of different antioxidants in 50 mM acetate buffer. (OTA = pure oxytocin; OTB = oxytocin with BHT; OUT = oxytocin with uric acid; OTL = oxytocin with L-ascorbic acid).

Sample	Antioxidant	Conc. (mg/mL)	Oxytocin remaining (%) at day			
			0	4	8	15
OTA-3	None	-	100.0 (0.4)	93.5 (0.1)	88.0 (0.1)	78.8 (0.6)
OTB	BHT	saturated	100.0 (0.7)	94.1* (0.4)	87.9 (0.3)	80.4 (1.6)
OTU	Uric acid	saturated	100.0 (0.5)	96.7 (4.0)	88.2 (0.3)	79.6 (0.3)
OTL-1	L-ascorbic acid	1	100.0 (0.8)	66.5*** (1.2)	32.7*** (1.4)	10.2*** (0.3)
OTL-2		2	100.0 (0.2)	62.9*** (0.5)	33.3*** (1.7)	0.4*** (0.5)
OTL-3		3	100.0 (0.5)	66.2*** (0.3)	35.5*** (1.7)	3.7*** (0.2)
OTL-4		4	100.0 (1.2)	73.4*** (0.3)	46.6*** (2.0)	5.0*** (7.0)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.0005$

Table 4. Amount of oxytocin remaining in acetate buffers of different concentrations.

Sample	Acetate buffer conc. (M)	Oxytocin remaining (%) at day				
		0	3	6	10	13
A1	0.01	100.0 (0.4)	96.6 (2.1)	91.3 (1.0)	85.1 (0.5)	82.2 (0.2)
A2	0.025	100.0 (0.8)	94.8 (0.4)	91.0 (0.8)	85.0 (0.6)	81.4* (0.6)
A3	0.05	100.0 (1.5)	95.8 (1.6)	91.6 (0.6)	84.3 (1.3)	81.0 (1.2)
A4	0.1	100.0 (1.5)	95.4 (0.3)	90.9 (0.3)	82.9* (1.4)	79.2** (1.3)
A5	0.2	100.0 (1.5)	95.5 (1.3)	91.2 (0.2)	83.3*** (0.1)	78.8* (1.9)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.0005$

Paper III

The effect of D-(+)-glucosamine, N-acetyl-D-glucosamine and Tetraethylene glycol on the stability of Oxytocin in Aqueous Solution

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Abstract: 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 in lower concentration 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.

Keywords: Oxytocin; stability; N-acetyl-D-glucosamine; D-(+)-glucosamine; tetraethyleneglycol; formulation

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 between 2–8°C and data shows 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 in the small villages in these countries.

Approaches that have been used 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 in 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 & Sigurdsson, 2018). The examples provided in the patent suggest that a combination of glucosamine and tetraethyleneglycol (4EG) has beneficial effect on oxytocin's stability in 50 mM phosphate buffer at pH 4.5 (Gizurarson & 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) (Figure 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.

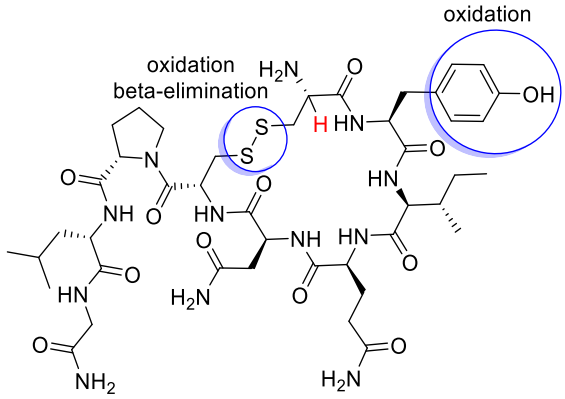

Figure 1. The structure of oxytocin with known sites of degradation identified.

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) via 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 Figure 2, with the y-axis kept the same for better comparison.

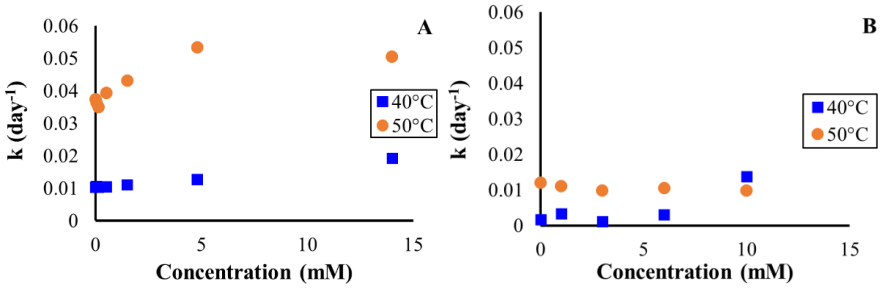

Figure 2. The plot of degradation rate constant (k) of oxytocin vs. different concentrations of D-(+)-glucosamine in phosphate buffer (A) and in acetate buffer (B) at 50 °C and 40 °C.

The results showed 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 Figures 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.

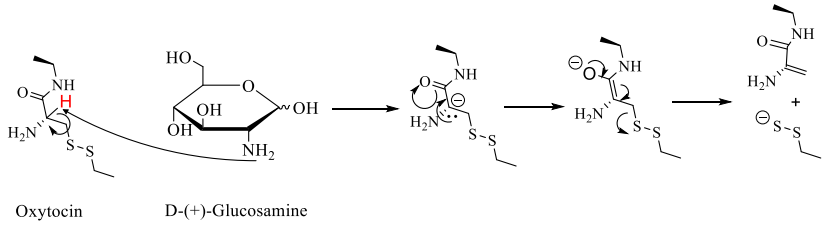

Figure 3. Proposed mechanism of degradation of oxytocin in the presence of high glucosamine concentration.

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 Figure 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 percent 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 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.

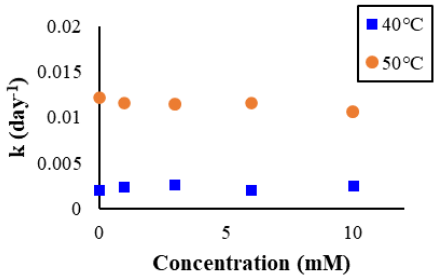

Figure 4. A plot of the degradation rate constants (k) of oxytocin at different concentrations of N-acetylglucosamine in acetate buffer at 40 °C and 50 °C.

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 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 & 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 Figure 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.

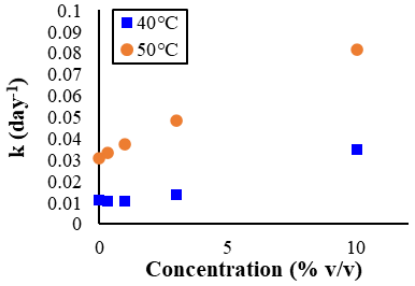

Figure 5. A plot of the degradation rate constants (k) of oxytocin at different concentrations of 4EG in phosphate buffer at 50 °C and 40 °C.

3. 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 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 suggest 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 or 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 or 10.0 mM. Then two mixture samples of each additive (1.0 mM or 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% or 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: The following are available online at www.mdpi.com/xxx/s1, 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.

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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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Supplementary Information

The effect of D-(+)-glucosamine, N-acetyl-D-glucosamine and Tetraethylene glycol on the stability of Oxytocin in Aqueous Solution

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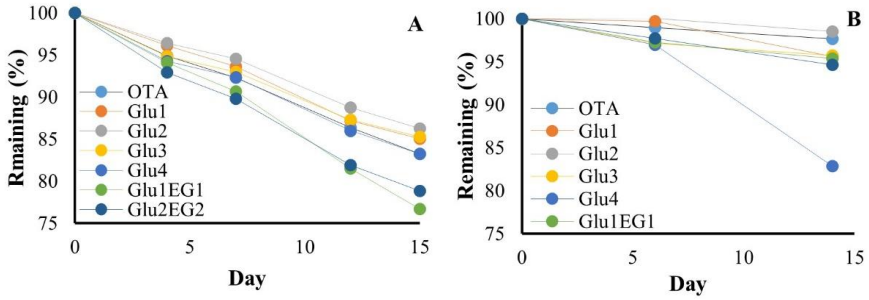

Figure S1. Oxytocin remaining (%) in the presence of different concentrations of D-(+)-glucosamine and two mixture samples of this additive with tetraethyleneglycol (0.3 %, 1.0 %) in acetate buffer, pH 4.5 at 50 °C (A), 40 °C (B) (OTA = oxytocin in acetate buffer; Glu 1 = oxytocin with glucosamine (1.0 mM); Glu 2 = oxytocin with glucosamine (3.0 mM); Glu 3 = oxytocin with glucosamine (6.0 mM); Glu 4 = oxytocin with glucosamine (10 mM); Glu1EG1= oxytocin with glucosamine (1.0 mM) and tetraethyleneglycol (0.3 %); Glu2EG2= oxytocin with glucosamine (3.0 mM) and tetraethyleneglycol (1.0 %).

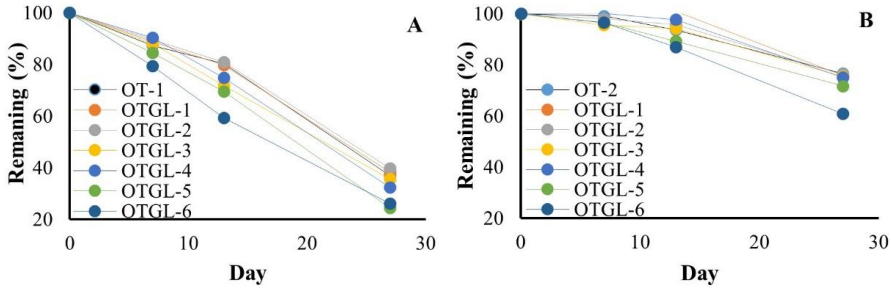

Figure S2. Oxytocin remaining (%) in the presence of different concentrations of D-(+)-glucosamine in phosphate buffer at 50 °C (A); and 40 °C (B). OT-1 = oxytocin at 50 °C; OT-2 = oxytocin at 40 °C; OTGL-1 = oxytocin with glucosamine (0.04 mM); OTGL-2 = oxytocin with glucosamine (0.14 mM); OTGL-3 = oxytocin with glucosamine (0.5 mM); OTGL-4 = oxytocin with glucosamine (1.5 mM); OTGL-5 = oxytocin with glucosamine (4.8 mM); OTGL-6 = oxytocin with glucosamine (14 mM).

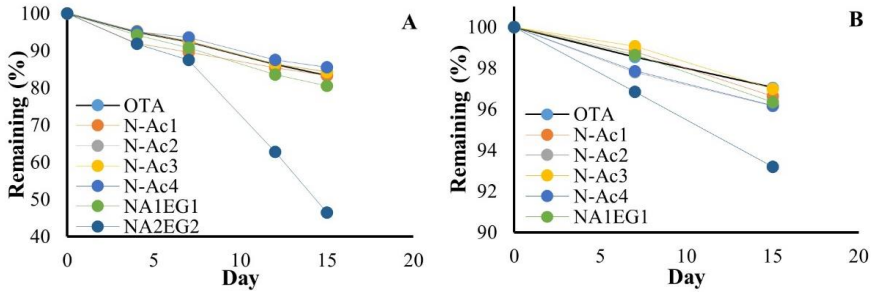

Figure S3. Oxytocin remaining (%) in the presence of different concentrations of N-acetyl-glucosamine and the mixture of N-acetyl-glucosamine with tetraethyleneglycol in acetate buffer, pH 4.5 at 50 °C (A) or 40 °C (B) (OTA = oxytocin in acetate buffer; N-Ac1= oxytocin with N-acetylglucosamine (1.0 mM); N-Ac2 = oxytocin with N-acetylglucosamine (3.0 mM); N-Ac3= oxytocin with N-acetylglucosamine (6.0 mM); N-Ac4= oxytocin with N-acetylglucosamine (10.0 mM); NA1EG1 = oxytocin with N-acetylglucosamine (1.0 mM) and tetraethyleneglycol (0.3 %); NA2EG2 = oxytocin with N-acetylglucosamine (3.0 mM) and tetraethyleneglycol (1.0 %).

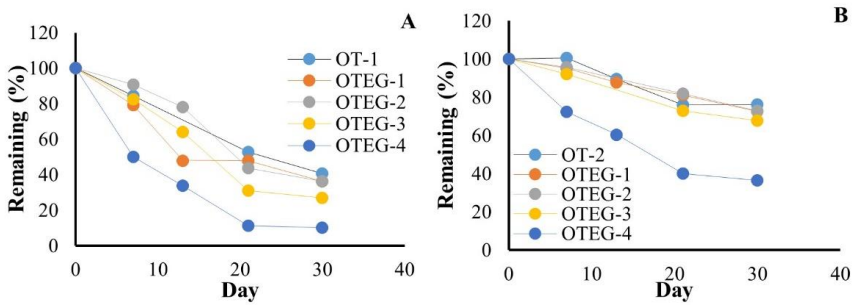

Figure S4. Oxytocin remaining (%) in the presence of different concentrations of 4EG at 50 ℃ (A) and 40 ℃ (B) in phosphate buffer, pH 4.5 (OT-1 = oxytocin at 50 ℃; OT-2 = oxytocin at 40 ℃; OTEG-1 = oxytocin with 4EG (0.3 %); OTEG-2 = oxytocin with 4EG (1.0 %); OTEG-3 = oxytocin with 4EG (3.0 %); OTEG-4 = oxytocin with 4EG (10.0 %).

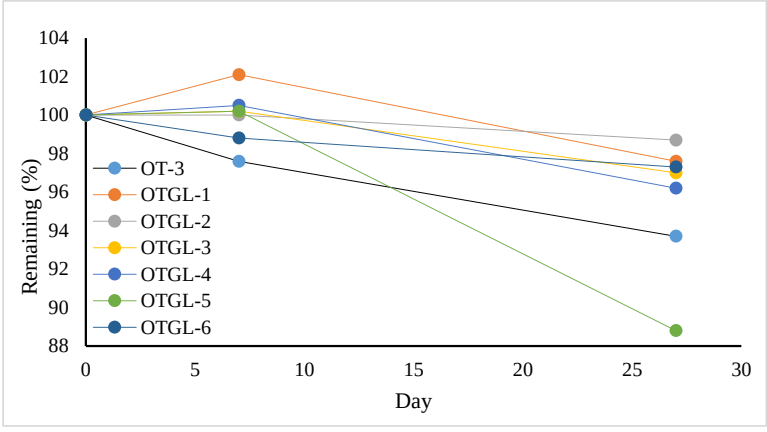

Figure S5. Oxytocin remaining (%) in the presence of different concentrations of D-(+)-glucosamine in phosphate buffer, pH 4.5, at 6 ℃ (C) (OT-3 = oxytocin at 6 ℃; OTGL-1 = oxytocin with glucosamine (0.04 mM); OTGL-2 = oxytocin with glucosamine (0.14 mM); OTGL-3 = oxytocin with glucosamine (0.5 mM); OTGL-4 = oxytocin with glucosamine (1.5 mM); OTGL-5 = oxytocin with glucosamine (4.8 mM); OTGL-6 = oxytocin with glucosamine (14 mM).

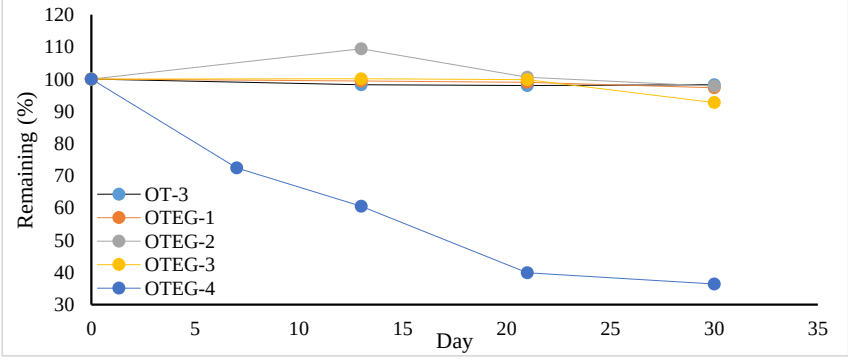

Figure S6. Oxytocin remaining (%) in the presence of different concentrations of 4EG at 6 ℃ (C) in phosphate buffer, pH 4.5 (OT-3 = oxytocin at 6 ℃; OTEG-1 = oxytocin with 4EG (0.3 %); OTEG-2 = oxytocin with 4EG (1.0 %); OTEG-3 = oxytocin with 4EG (3.0 %); OTEG-4 = oxytocin with 4EG (10.0 %).

Paper IV

Stability of fibroblast growth factor 2 (FGF2) in aqueous solution

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Abstract

Basic fibroblast growth factor (FGF2) is a protein involved in many cellular functions like cell proliferation, migration and differentiation, as well as tissue regeneration including skin, muscle, blood vessel and nerve. These multiple functions make FGF2 an attractive component for its use as a therapeutic, however the native protein is unstable during storage and delivery. In this study, the effect of pH on FGF2 stability in aqueous solutions was explored. The study found that FGF2 is more stable in near-neutral and slight basic pH. The effect of buffer composition was also studied. PBS buffer showed the best stability compared with other buffers used. This improvement was further enhanced in the presence of trehalose, while other additives such as N-acetyl-D-glucosamine, D-(+)-glucosamine hydrochloride, tetraethyleneglycol, 18-crown-6 and 12-crown-2 decreased FGF2 stability.

Introduction

Fibroblast growth factor 2 (FGF2) belongs to a family of fibroblast growth factors. Other known receptor factors are FGF1, FGF3 and FGF4. FGF2 is a 17 kDa heparin binding protein that controls a plethora of cellular processes including cell proliferation, cell survival, migration, vasculogenesis and cell differentiation (Yun, et al., 2010; Beenken, 2009; Ornitz, 2015). FGF2 also plays an important role in tissue repair, bone regrowth, angiogenesis, and neuroregeneration (Biological roles of fibroblast growth factor-2, 1997). FGF2 instability as a therapeutic protein medicine is of concern, when it is stored and transported (Edelman, 1991; Whalen, 1989). It has been reported FGF2 degrades rapidly at 37℃ (Levenstein, 2006; Furue, 2008), and FGF-2 solutions at a concentration of 72 Mg/mL have been shown to lose 50% functionality after just 46 min at 25 ℃ (Shah, 1998). The functional half-life was decreased to 37, 33 and 10 min, respectively, as the storage temperature was increased to 37 ℃, 42 ℃ and 50 ℃ (Shah, 1998; Chen G. G., 2012). Methods to enhance the thermostability of FGF2 in a formulation and to improve the product's shelf-life would, therefore, be beneficial. The stability and activity of FGF2 has been studied to find ways to improve its stability. It has been shown that heparin and heparinoid complexes may increase the biological activity of FGF2 (Crowe, Crowe, & Chapman, Preservation of Membranes in Anhydrobiotic Organisms: The Role of Trehalose , 1984; Xie & Timasheff, 1997), as well as improving the stability of the protein (Olgenblum, Sapir, & Harries, 2020). Although heparin and heparinoids showed positive effects on FGF2 stability, they are prone to *in vivo* degradation and desulfation by heparinases (Wu, Conjugation of basic fibroblast growth factor on a heparin gradient for regulating the migration of different types of cells. , 2013). By covalent conjugation of FGF2 to a heparin-mimicking polymer, it was found to increase the stability as well as the activity of the protein (Nguyen, A heparin-mimicking polymer conjugate stabilizes basic fibroblast growth factor. , 2013). Multiple studies have been carried out to enhance the stability of FGF2 in solution to some extent. These studies can be classified into ionic interactions between FGF2 and additives, with strategies like the complexation of the protein with its internal stabiliser, heparin, or heparin-mimiking polymers, or polycation (Chu, 2011; Paluck, 2016; Wu, Conjugation of basic fibroblast growth factor on a heparin gradient for regulating the migration of different types of cells , 2013), chemical protection based on binding FGF2 to another molecule in such a way as to

slow down the active conformation of the FGF2 molecule or preventing those residues of the FGF2 molecule from being involved in undesired reactions (Chu, 2011; Paluck, 2016; Wu, Conjugation of basic fibroblast growth factor on a heparin gradient for regulating the migration of different types of cells , 2013; Macdonald, Rodriguez, Shah, & Hammond, 2010; Nguyen, A heparin-mimicking polymer conjugate stabilizes basic fibroblast growth factor. , 2013; Dvorak, 2018; Moon, 2015), physical protection with strategies include the encapsulation of FGF2 within a polymer matrix and the fabrication of a mixture of the protein with polymers into hydrogels or other composite scaffolds (Ali, 2018; Galderisi, 2013; Layman, 2007; Lotz, 2013; Yoon, Kim, El-Fiqi, Jang, & Kim, 2017; Patent No. 5482929, 1996). There have been a wide range of stabilization studies with different strategies for FGF2 that are reported already, some of which have shown very promising data, but they have not yielded much information regarding degradation mechanism of FGF2, and little information is provided on stability of the protein. Factors such as pH, the effect of different buffers and ionic strength that are known to effect on protein stability, have not been reported for FGF2.

The purpose of this study is to examine the optimal pH for the stability of FGF2 in the presence of different buffers. Additionally, we are using non-covalent interactions rather than covalent interactions between FGF2 and the additives and compounds that have been shown to affect the stability of proteins and peptides, such as different carbohydrates, will be studied together with FGF2. Several carbohydrates are regarded as protein stabilizers (Andreas Bikfalvi, 1997; Chen & Arakawa, 1996; Tsai, et al., 1993), and trehalose is known as an excellent stabilizer for certain biomolecules, in particular lipids and proteins. It has been showed the stability of these biomolecules is enhanced in the presence of trehalose both under anhydrous conditions (Fabio, et al., 2015; Crowe, Crowe, & Chapman, Preservation of Membranes in Anhydrobiotic Organisms: The Role of Trehalose , 1984; Crowe, Carpenter, & Crowe, The Role of Vitrification in Anhydrobiosis , 1998; Nagase, et al., 2008; Olgenblum, Sapir, & Harries, 2020), and more importantly for this project, in aqueous solution, with higher concentrations yielding greater stability enhancing effect (Xie & Timasheff, 1997; Kaushik & Bhat, 2003; Sola-Penna & Meyer-Fernandes, 1998; Carninci, et al., 1998; Lins, 2004; Lin, 1996). More recently, crown ethers such as 18-crown-6 has shown interesting results. We previously reported that 18-crown-6 can bind to a protonated amino group of cysteine of oxytocin and inhibit it from acting as a bas an taking part in undesired reactions (Mostafa Ghasemisarabbadieh, 2021).

Therefore, the stability effect of trehalose, aminosugars like N-acetyl-D-glucosamine, D-(+)-glucosamine hydrochloride and crown ethers such as 18-crown-6 and 12-crown-4 were also tested.

Materials and methods

Materials

Trehalose from Pfanstiehl Inc. (Waukegan, USA) was kindly provided by Alvotech (Reykjavik, Iceland). 18-crown-6, 15-crown-5, 12-crown-4, sodium phosphate dibasic, potassium dihydrogen phosphate, glucosamine hydrochloride, tetraethyleneglycol and HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)) were purchased from Sigma-Aldrich (Germany (Darmstadt), UK and USA). Sodium acetate and TRIS base (tris(hydroxymethyl)aminomethane) were obtained from Merck (Darmstadt, Germany) and anhydrous citric acid from Pernhofen (Jungbunzelauer Austria). Acetic acid, sodium chloride, hydrogen chloride and potassium chloride were purchased from Honeywell (Hannover, Germany). An FGF2 sample was kindly provided by ORF Genetics (Reykjavik, Iceland) and additional FGF2 was purchased from R&D systems along with ELISA kits for the FGF2 studies. The ELISA kits (Human FGF basic/FGF2/bFGF DuoSet ELISA and DuoSet ELISA Ancillary Kit 2) consisted of capture antibody, detection antibody, recombinant standard, streptavidin conjugated to horseradish-peroxidase (Streptavidin-HRP), color reagents A and B, stop solution, wash buffer and reagent diluent concentrate.

Formulation

The concentration of FGF2 was kept in the range of 500-1000 pg/mL in all samples of all series and the series included an FGF2 control that was prepared in the same buffer as the samples in the series but had no additives. FGF2 standards were prepared at a range of concentrations (1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL or 15.6 pg/mL) in the buffer used. The samples were analyzed using an ELISA-sandwich assay.

Preparation of FGF2 samples for pH study

FGF2 samples were made at a range of pH (6.4, 7.2, 7.4, 7.7 or 8.0) in citrate/phosphate buffer (10 mM/20 mM) and at two additional pH values (4.5 or 5.4) in acetate buffer (0.1 M). The samples were divided into three Eppendorf vials, and the first series was stored at -80°C immediately after preparation, and the next two series were stored at 25°C for 4 and 7 hours, respectively before being moved to a -80°C storage.

Preparation of FGF2 samples for buffer study

Eight FGF2 samples were prepared in the presence of four different buffers: HEPES (1.0 M), TRIS (1.0 M), phosphate buffered saline (PBS) and citrate/phosphate (10 mM/20 mM). The pH of all buffers was adjusted to pH 7.2-7.4. Four of the samples included trehalose (1.0 M) and the other four samples had no trehalose. The samples were divided into three series and the first series was stored at -80°C immediately after preparation and the other two series were stored at 25°C for 4 and 8 hours, respectively, before being moved to -80°C storage.

Preparation of FGF2 samples with trehalose, aminosugars and tetraethyleneglycol

FGF2 samples were prepared in of PBS buffer at pH 7.2-7.4 and with different additives at different concentrations. The samples included trehalose (0.1 M or 0.5 M), N-acetyl-D-glucosamine (0.1 M or 0.5 M), D-(+)-glucosamine (0.05 mM or 0.15 mM) and a mixture sample of D-(+)-glucosamine (0.05 M) with tetraethyleneglycol (0.3%). The samples were divided into three portions, the first portion was stored at -80°C immediately and the other two portions were first stored at 25°C for 11 and 23 hours, respectively, and then stored at -80°C.

Preparation of FGF2 samples with crown ether additives

For the crown ether series, FGF2 samples were prepared in PBS buffer at pH 7.2-7.4. Crown ethers used were 18-crown-6 (1.0% and 3.0%), 12-crown-4 (3.0%) and two additional samples were prepared with tetraethyleneglycol (1.0% and 3.0%). All the samples were divided into three Eppendorf vials, the first vials were stored at -80°C immediately and the other two vials were stored first at 25°C for 11 and 23 hours, respectively, and then moved to -80°C storage.

Analysis

The analysis used in this work was a sandwich ELISA system using Human FGF basic/FGF2/bFGF DuoSet ELISA along with the DuoSet ELISA Ancillary Reagent Kit 2 from R&D systems, where the wells were coated overnight with FGF2 capture antibody (mouse anti-human FGF basic capture antibody) followed by the addition of either 100 µL of FGF2 standards or the FGF2 samples. After adding the detection antibodies (biotinylated mouse anti-human FGF basic detection antibody) and washing, the reagent streptavidin-HRP was added to each well. After about 20 min incubation, with a mixture of peroxide (50%, H_2O_2) and 50% tetramethylbenzidine (1:1) the color developed was stopped using 2N sulfuric acid (H_2SO_4). The color was analyzed using multi-mode reader instrument (BioTeK Synergy/LX) (Vermont, USA), at λ = 450 nm, 540

nm or 570 nm. All the washings were carried out using microlate washer 50/TS (BioTeK). The samples were prepared in triplicate and the concentration of the samples were calculated according to a plot of concentration vs. absorption for the FGF2 standards.

Results and Discussion

Effect of pH on FGF2 stability

It has already been shown that changing the pH can induce unfolding of proteins and denaturation, which can be accelerated below pH 5 for acidic conditions and at pH 10 and higher for basic conditions (Chu, 2011), but to the best of our knowledge, the effect of different pH on FGF2 stability has not yet been studied extensively, although it is known that it is an important factor for the stability of biomolecules such as proteins. To investigate FGF2 stability at different pH, samples were prepared in a manner to try to fix the initial FGF2 concentration at approximately 500 pg/ml in citrate/phosphate buffer (10 mM/20 mM) at a range of pH (6.4-8.6) and in acetate buffer (0.1 M) at pH 4.5 and 5.4. The preliminary results showed that FGF2, like other FGF family members, showed the most stablility at near-neutral to slightly basic pH. Adjusting the pH of samples to values of 7.2 to 8.0 caused a greater amount of FGF2 to remain in the solutions. The initial concentrations of samples were different from the FGF2 concentration (500 pg/mL) and it is more noticeable for samples at pH 5.4 and pH 4.5 where these FGF2 samples showed much less initial concentration and faster degradation (Table 10, Figure 33). The difference between initial concentrations of FGF2 samples and FGF2 concentration used in the samples can be because of FGF2 sensitivity and its very low stability. The results were match with the literature, which was already reported that changing the pH of solution to acidic (pH 5) or strong basic (pH 10) condition causes the denaturation and unfolding of proteins (Chu, 2011).

Table1. FGF2 remaining (%) in the presence of citrate/phosphate buffer at a range of pH (6.4-8.6) and acetate buffer at pH (4.5-5.4).

Sample	Initial FGF2 conc. (pg/mL)	Remaining FGF2 (%) at hour		
		0	21	29
pH 8.0	793.4	100(2.4)	99.4(18.5)	88.0(7.4)

pH 7.7	834.6	100(3.9)	93.5(13.0)	82.3(16.2)
pH 7.4	745.1	100(1.3)	105.7(12.8)	86.1(24.1)
pH 7.2	692.1	100(4.7)	95.1(4.3)	75.2(10.5)
pH 6.4	537.7	100*(5.8)	66.8**(9.9)	56.1*(9.2)
pH 5.4	192.6	100***(3.6)	30.2***(14.6)	24.2***(19.3)
pH 4.5	28.8	100***(11.3)	4.62***(3.3)	5.54***(15.1)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.0005$

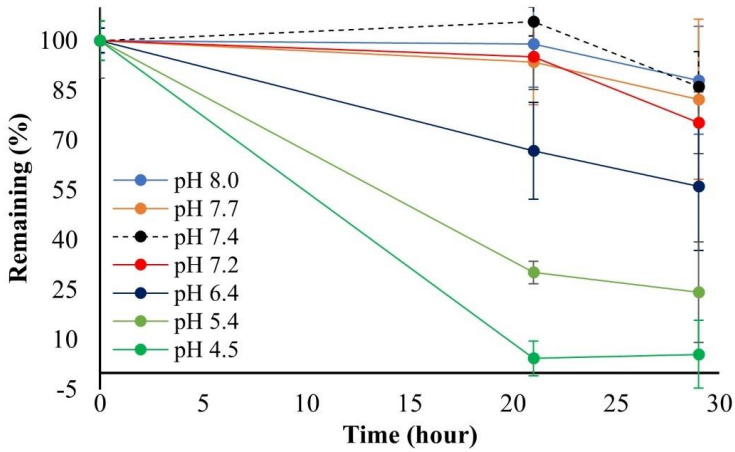

Figure 1. FGF2 remaining (%) in the presence of citrate/phosphate buffer (6.4-8.6) and acetate buffer (4.5-5.4) at different pH.

Effect of different buffers on the stability of FGF2

The effect of different buffers on FGF2 stability was also examined. The buffers tested were HEPES (1.0 M), TRIS (1.0 M), PBS, and citrate/phosphate (10 mM/20 mM). The pH of all buffers was adjusted to 7.4 which is biological pH and there was not a statistically significant difference between the % FGF2 remaining at pH 7.2-8.0. The samples were prepared in the presence and absence of trehalose (1.0 M). FGF2 showed the most stability in the presence of PBS buffer compared with other buffers. The FGF2 amount remaining in PBS after 8 hours, at 25 ℃, was 86.3%, or a half-life of 38 hours, but in citrate/phosphate buffer FGF2 showed the lowest stability where the analogous amount of FGF2 remaining was 68.7 %, or half-life of 15 hours. The half-life of FGF2 in TRIS and HEPES were 31 hours and 16 hours respectively (Table 2). The degradation rate of FGF2 in PBS ($-2.0 \pm 1.4 \times 10^{-2}$ hour^{-1}) was significantly less than that in

citrate/phosphate ($-5.8 \pm 1.8 \times 10^{-2}$ $hour^{-1}$), HEPES ($-4.9 \pm 0.5 \times 10^{-2}$ $hour^{-1}$) and Tris ($-3.0 \pm 1.1 \times 10^{-2}$ $hour^{-1}$). Lower stability of FGF2 in the presence of other buffers compared with PBS needs more studies, but one possibility can be relating to the ionic effect of Na^+, K^+, and Cl^- in PBS buffer solutions on the salt structure of the protein, keeping this structure more stable, resulting in higher stability of FGF2 in this buffer compared with other buffers. Lower FGF2 stability in HEPES buffer compared with PBS can be related to the reducing effect of HEPES buffer. It has been advised to keep HEPES-containing solutions in darkness as much as possible to prevent oxidation and production of hydrogen peroxide (Zigler JS, 1985).

In the presence of trehalose the degradation rate was decreased for all samples except HEPES that showed unreliable data and initial concentration of FGF2 in HEPES buffer was lower than that in hours 4 or 8. The rate constants of the buffers PBS, TRIS, citrate/phosphate and HEPES in the presence of trehalose after 8 hours were ($-1.6 \pm 0.3 \times 10^{-2}$ $hour^{-1}$), ($-6.0 \pm 0.3 \times 10^{-3}$ $hour^{-1}$), ($-2.5 \pm 0.3 \times 10^{-2}$ $hour^{-1}$) and ($+1.7 \pm 0.6 \times 10^{-2}$ $hour^{-1}$) respectively. The stabilizing effect of trehalose for biomolecules in solution was already reported with higher trehalose concentration showing more improvement (Crowe, Carpenter, & Crowe, The Role of Vitrification in Anhydrobiosis , 1998; Kaushik & Bhat, 2003; Sola-Penna & Meyer-Fernandes, 1998; Carninci, et al., 1998; Lins, 2004). We already discussed the mechanism of action of trehalose as a stabilizer in the oxytocin part. We hypothesized that trehalose's stabilizing effect is primarily a structural stabilization while it has limited to no impact on smaller biomolecules such as peptides that only have a primary structure, it has more protective effect on proteins that have at least a clear secondary or tertiary structure (Ding, 2021). The results in Table 2 showed the remaining amount of FGF2 in all solutions was decreased in the presence of trehalose and the measurements showed the half-lives of the samples were increased significantly where the half-life of PBS, TRIS, and citrate/phosphate were 52 hours, 134 hours and 29 hours respectively. Although FGF2 showed the most stability in PBS compared with other buffers, FGF2 showed more stability in TRIS buffer compared with PBS buffer in the presence of trehalose.

Although in the presence or absence of trehalose, PBS sample showed the highest initial concentration of FGF2 compared with other buffers, the initial concentrations of FGF2 samples in the presence of trehalose were decreased. One possible explanation for this lower initial concentration of FGF2 in the presence of trehalose can be the viscosity of the solutions which are increased in the presence of trehalose. This might result in decreased solubility of the protein in

the beginning with slight precipitation and/or denaturation of FGF2 samples. This potential effect, along with reducing the difference in osmolarity and viscosity of the different buffer solutions should be considered for next studies.

Table 1. FGF2 remaining (%) in the presence of different buffers and trehalose.

Sample	Initial FGF2 conc. (pg/mL)	Remaining FGF2 (%) at hour		
		0	4	8
PBS	473	100 (5.4)	97.0 (5.8)	86.3 (10.7)
Tris	402	100 (5.8)	83.6 (6.1)	83.4 (4.1)
HEPES	379	100 (3.1)	93.5 (7.5)	71.1 (8.1)
Citrate/phosphate	357	100 (4.8)	94.6 (13.1)	68.7 (10.1)
PBS + Trehalose	294	100 (5.4)	94.3 (1.3)	89.9 (1.6)
Tris + Trehalose	225	100 (4.4)	97.4***(2.7)	95.9***(1.8)
HEPES + Trehalose	195	100 (4.5)	121.2***(3.6)	114.7***(1.8)
Citrate/phosphate + Trehalose	260	100 (6.3)	98.8 (4.4)	82.7 (3.2)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.0005$

Effect of trehalose, D-(+)-glucosamine hydrochloride, 4EG and a mixture of D-(+)-glucosamine hydrochloride with 4EG on FGF2 stability

To understand better why trehalose showed different effect on FGF2 stability in all buffers used at the beginning and after a few hours (Table 2), two more samples of trehalose with lower concentration than 1.0 M (500 mM and 100 mM) were tested. D-(+)-Glucosamine hydrochloride, 4EG along with a mixture of these additives were also tested to see the effect of these additives on FGF2 stability in solution. FGF2 concentration was kept constant at 500 pg/mL in all solutions and PBS buffer at pH of 7.2-7.4 was used and results showed in Table 3. Initial concentration of FGF2 in the presence of lower concentrations of trehalose was increased. The degradation rate for trehalose samples with concentrations of 100 mM and 500 mM were ($-1.9 \pm 0.1 \times 10^{-2}$ hour^{-1}) with a half-life of 37 hours and ($-1.6 \pm 0.2 \times 10^{-2}$ hour^{-1}) with a half-life of 44 hours while for FGF2 sample without trehalose was ($-2.2 \pm 0.1 \times 10^{-2}$ hour^{-1}) and a half-life of 31 hours (Figure 2). The measurements showed that D-(+)-glucosamine, 4EG, and a mixture of D-(+)-glucosamine with 4EG had a negative effect on the FGF2 stability.

Table 2. FGF2 remaining (%) in the presence of different additives in PBS buffer. (Treh = trehalose; Glu = glucosamine; 4EG = teteraethyleneglycol).

Sample	Additives Con. (mM)	Initial FGF2 conc. (pg/mL)	Remaining FGF2 (%), (hours)		
			0	11	23
FGF2	500 pg/mL	829	100 (3.0)	91.7 (0.1)	61.1 (2.6)
Treh 1	100	685	100 (1.6)	84.3 (1.1)	64.8 (2.9)
Treh 2	500	600	100 (1.9)	75.7***(0.3)	71.4*(3.0)
Glu 1	5	427	100 (4.6)	71.3*(1.0)	36.0***(0.8)
Glu 2	15	93	100 (3.1)	71.8*(2.0)	27.0***(3.0)
4EG (1)	1.0 %	467	100 (1.5)	81.8*(2.1)	28.2***(1.7)
4EG (2)	3.0 %	358	100 (1.0)	81.4*(2.9)	40.9***(3.1)
Glu1+4EG1	-	412	100 (1.7)	69.8*(2.5)	34.9***(2.0)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.0005$

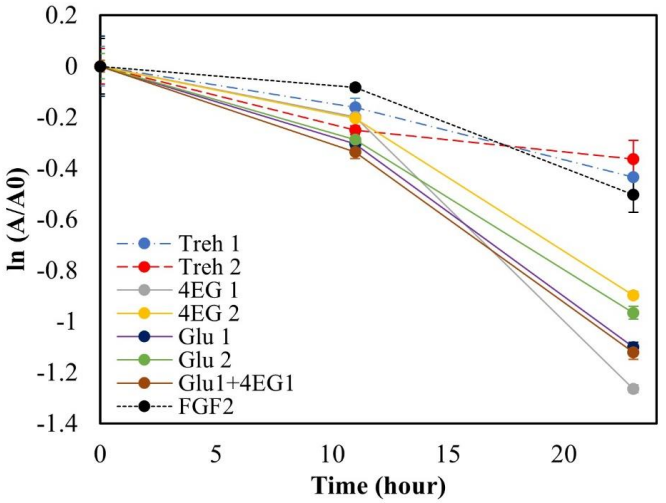

Figure 2. A plot of ln([A0/A]). Vs time (hour) (A0 = FGF2 initial concentration; At= FGF2 concentration at different time points.

Effect of crown ethers and N-acetylglucosamine on the stability of FGF2

We previously reported that 18-crown-6 enhanced the stability of oxytocin in solution in citrate/phosphate buffer.103 Amino acids have been reported to form complexes with 18-crown-6

as well (A.F. Danil de Namor M. R.-W.-N., 1991; A.F. Danil de Namor M.-C. R.-J.-W.-N., 1991). The ability of the cyclic polyether 18-crown-6 as non-covalent protecting group in peptide synthesis has also been illustrated (P. Mascagni, 1987; C. B. Hyde, 1989). Therefore, we decided to test the effect of 18-crown-6 and a small crown ether like 12-crown-4 to see if they are able to enhance FGF2 stability or not. FGF2 samples (500 pg/mL) were made at different concentrations of 18-crown-6 (0.5%, 1.0% or 2.0%) and 12-crown-4 (1.0% and 3.0%), the effect of N-acetylglucosamine (0.3M and 0.7M) was also tested. All samples were prepared in PBS buffer at pH 7.2-7.4 and one sample was included with only FGF2 without any additive. The results in Table 3 show FGF2 stability was not enhanced in the presence of these additives and raising the concentration of the additives caused further degradation. The degradation rate of FGF2 in the presence of the additives showed in Figure 3. The degradation rate of all samples in the presence of the additives was more than FGF2 control without any additive, suggesting N-acetylglucosamine, 18-crown-6 and 12-crown-4 destabilize FGF2 in solution. One possibility for raising degradation rate of FGF2 in the presence of 18-crown-6 and 12-crown-4 can be the formation of complex of these crown ethers with ions like Na^+ and K^+ in PBS buffer solution which we already hypothesized in buffer section help to enhance the stability of FGF2 in PBS buffer compared with other used buffers. The results of this part can support this hypothesis that ions Na^+, K^+ are responsible for improving FGF2 stability in PBS buffer.

Table 4. FGF2 remaining (%) in the presence of different additives in PBS buffer. (18C6 = 18-crown- 6; 12C4 = 12-crown-4; N-acet = N-acetylglucosamine).

Sample	Additives Con. (%)	Initial FGF2 conc. (pg/mL)	Remaining FGF2 (%) at hour		
			0	24	43
FGF2	0	758	100 (3.5)	85 (20.0)	55 (1.5)
18C6 (1)	0.5	721	100 (15.7)	80 (7.8)	55 (0.9)
18C6 (2)	1.0	796	100 (9.0)	69 (3.5)	46***(0.7)
18C6 (3)	2.0	723	100 (6.1)	51*(0.9)	35***(0.9)
12C4 (1)	1.0	737	100 (3.1)	54*(6.0)	36***(0.8)
12C4 (2)	3.0	660	100 (7.0)	75 (7.7)	52 (2.2)
N-acet 1	0.3M	730	100 (6.1)	75 (7.4)	42***(1.8)
N-acet 2	0.7M	631	100 (2.7)	60*(2.9)	36***(0.3)

*= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.0005$

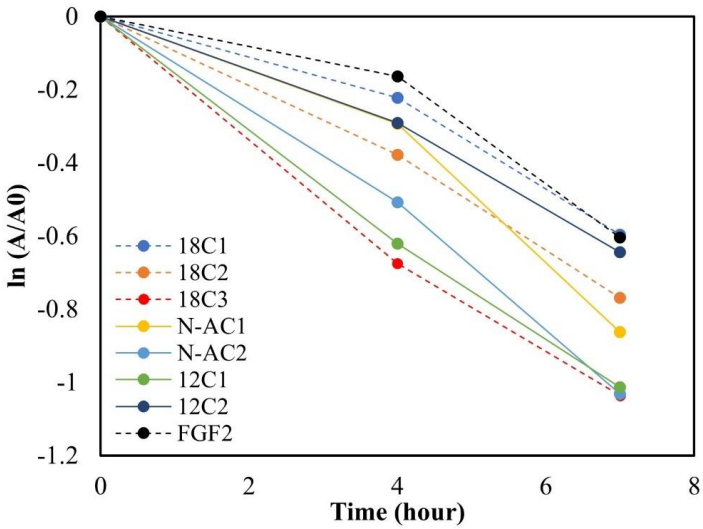

Figure 4. A plot of Ln([At/A0]). Vs hour (A0 = FGF2 initial concentration; At= FGF2 concentration at different time points.

Conclusion

In conclusion, this study showed pH plays an important role on FGF2 stability in solutions, FGF2 showed the most stability at near-neutral and slight basic pH. We also explored that FGF2 was more stable in PBS buffer compared with the other buffers used. This improvement was further enhanced in the presence of trehalose, while aminosugars like N-acetyl-D-(+)-glucosamine, D-(+)-glucosamine and tetraethyleneglycol, 18-crown-6 and 12-crown-4 increased the degradation rate of FGF2 in solution. We also found that the reason for stability of FGF2 in PBS can be the presence of ions Na^+ or K^+ in this buffer.

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