Generation of Controlled Release Gelatin-NSAID Conjugates for Post-Surgical Applications

Emily Rozen

Florida Southern College

Mentor: Shameka Shelby, Ph.D.

Department of Chemistry, Biochemistry, & Physics

Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) are often prescribed after surgery to reduce inflammation and aid in pain management. NSAIDs, such as Advil, Motrin, and Aleve, are typically taken orally as over-the-counter medication. While these drugs are typically safe in small doses, sustained intake of high doses can have adverse side effects. The goal of this project was to design a product that would allow for direct application of NSAIDs to the surgical site, avoiding systemic circulation. By conjugating the NSAID to a gelatin hemostatic agent, the product can be applied to the open site and provide a subcutaneous delayed release. Type B gelatin was conjugated to the NSAID diclofenac using 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide (EDC) as the cross-linking agent. UV-Vis spectrometry confirmed concentrations eluted across different time frames to confirm the presence of a controlled release system. Use of this new technology in post-operative scenarios may help to lower NSAID intake, efficiently aid in patient pain management, and improve surgical protocols.

Introduction

Inflammation is the body's natural response to an injury. Fluid accumulates in the tissue and white blood cell counts increase to protect the area and help fight against the possibility of infection. Inflammation that occurs during and after surgery often facilitates a successful recovery. However, in some cases it can affect the body's natural stress response and cause many problems, especially if the patient has pre-existing conditions.[1] Inflammation is one of the most common side effects of surgery, and while it can be helpful in healing, it also causes a great deal of pain for the patient and has been linked to poor long term surgical outcomes.[2] This postoperative pain is present in about 80% of patients, and serves as a significant motivator for physicians to aim to reduce swelling.[3]

The signs of acute inflammation are redness, heat, swelling, pain, and sometimes impaired function. The first step of inflammation is a chemical response where inflammatory chemicals are released into the extracellular matrix by tissue cells. These chemicals each have specific roles and promote steps of the inflammation process.[4] One of these chemicals, prostaglandin, promotes vasodilation of arterioles and the production of fluid containing clotting factors and antibodies.[5] The body maintains a basal level of prostaglandin expression which increases substantially during inflammation.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are competitive active site inhibitors that block arachidonic acid from binding to both forms of the COX enzyme.[7] Due to inhibitory action, the production of prostaglandins pauses, and the inflammatory response cannot occur. Without the inflammation, the patient experiences less pain.[5] Postsurgical medications are often prescribed to help alleviate pain, including anti-inflammatories. NSAIDs are the most common type of anti-inflammatories used, typically found as over-the-counter products such as Advil,

Motrin, Aleve, and Excedrin. People take these medications for everyday pain and fever relief, as they are widely available in non-prescription strength doses.

While using NSAIDs can solve many problems, they come with many potential side effects. Many healthcare professionals caution patients to lower their NSAID intake as continued use often leads to significant complications. Some of the warnings include an increased risk of heart attack or stroke, heightened blood pressure, and gastrointestinal problems such as nausea, vomiting, severe stomach pain, and bleeding. For those with preexisting conditions such as diabetes, bleeding problems, a history of heart problems, or are over the age of 65, oral NSAIDs can be very dangerous.[7] NSAIDs can increase blood pressure, decrease platelet activity causing excessive bleeding, and reduce the amount of blood flow to the liver. [8,9] While these effects are unlikely to occur when an NSAID is taken over a short period of time, prolonged use and conditions weakening the heart or liver can greatly increase one's risk.

NSAIDs such as ibuprofen are known to have significant impact on liver function. The severity of drug-induced liver injury (DILI) ranges from asymptomatic increases in alanine aminotransferase (ALT) levels to acute liver failure. ALT is one of the many enzymes used by the body to convert food into energy and typically exists at low levels in the liver, but significant increases in ALT level can cause liver failure.[10] While the reason for the damage to the liver caused by NSAIDs is not specifically known, it is likely due to the NSAID causing an inhibition of metabolic pathways. Acute liver damage is rare, but it is a possible side effect of excessive NSAID intake. [11] While taking the smaller over-the-counter doses of ibuprofen will likely have little to no negative effect on the body, once a person starts to reach the daily maximum doses of 2,400 to 3,200 mg, this can greatly increase the risk for harmful side effects, such as hepatotoxicity.[12] Because NSAIDs are typically taken orally, the drug affects the whole body,

not just the area in pain, increasing the risk of the patient experiencing the aforementioned side effects.

An approach to minimize the potential side effects of oral delivery of NSAIDs is to apply the drug directly to the affected area. This approach minimizes the risks associated with exposing the entire body to the drug. While the risk of side effects remains, the probability is much lower than if it was taken orally as the systemic exposure decreases by almost 90%.[13,14] Treatments for arthritis have begun to utilize products that focus on local delivery, such as Voltaren and Pennsaid.[15] These two products, and other similar topical NSAIDs, are applied directly to the affected area approximately every 12 hours and are absorbed through the skin. Topical NSAIDs differ from transdermal drug delivery and therefore do not rely on systemic circulation. The drug stays localized, affecting only that area. As a result, the systemic side effects contributed to commonly used oral NSAIDs are less likely to occur.[16] A recent study at the University of Pennsylvania developed a topical NSAID by loading ibuprofen into a bilayer delivery system (BiLDS) composed of microspheres between two layers of nanofibrous scaffolds.[13] There are existing products that accomplish localized treatment options similar to the one in this study, but the goal here was to investigate the effects of a delayed release NSAID on the healing of a tendon. The study revealed that there were lower levels of proinflammatory cytokine and higher levels of anti-inflammatory cytokine over an 8-week period, indicating that the delayed release of the drug was effective in managing inflammation over an extended period of time.[17]

Hemostatic Agents

Hemostatic agents are often used to help control blood loss both during and after surgical procedures.[18] There are many types of hemostatic agents used in topical forms, including fibrin glue, gelatin sponges, and collagen.[18] While fibrin glue was the historical standard for surgical procedures, gelatin-based agents are now found in nearly every operating room.[19] Gelatin is most commonly made from porcine or bovine skin using extraction methods to perform a partial hydrolysis of collagen.[20] Collagen fibers are the main component of gelatin. During heating, the fibers are denatured and the helices lose their conformations. Once they are cooled back down, the helixes only reform partially, trapping water in the mesh which causes the gelatin to form into a gel.[20] There are two types of gelatin: type A and type B. Type A gelatin is made by using acid baths to extract proteins, while type B uses alkaline baths.[20] There are many factors that contribute to the variations in the structure of gelatin.

Gelatin has been adapted to be used as a drug delivery carrier[21]. To be the most effective in the body a drug has to be above the minimum level of effectiveness, but it also must stay relatively inert to avoid cytotoxicity.[21] As a result, when designing a drug delivery system it is important to ensure that the materials are biocompatible and that the system will not accidently release the drug prematurely. The product will also be more useful if it is easy to make and apply. Many properties of gelatin make it a good candidate as a drug delivery system, such as its biocompatibility, biodegradability, and its ability to be manipulated to fit its environment and intended use.

Gelatin has been shown to be biocompatible and its hydrophilic nature allows for an increased diffusion of bioactivated molecules. Also, modifying the type of gelatin used and the degree of crosslinking will have an effect of the rate of drug release.[22] The physiochemical

environment and material origin affect the dissolution rate of the gelatin. Cross-linking density, hydrophilicity of the drug, and drug-polymer electrical interactions all affect the drug release profile. One of the reasons gelatin is so widely used is because of its biocompatibility and biodegradability. In order to control the degradation rate and mechanical properties of gelatin, crosslinking is required.[21]

Using gelatin as a drug delivery system has led to many developments for the medical world. Studies have shown that a controlled release of drugs from gelatin can be achieved under the appropriate conditions. Gelatin has been modified to deliver anti-cancer drugs, genetic material, vaccines, ocular and pulmonary drugs, and much more.[23] One application is seen in cationized gelatin hydrogels which have been loaded with plasmid DNA through physical encapsulation, bonding of surface modifying groups, and electrostatic attraction.[22,23] The use of gelatin hydrogels has allowed for increased gene expression due to its ability to deliver a controlled release of the gene which increases the transfection rate when the delivery is targeted. In a study done by Leong et al.,[24] GNP encapsulated pDNA was expressed at a higher rate and for a longer time than pDNA administered by itself. Another major application is gelatin nanoparticles (GNPs) being used to deliver anti-cancer drugs. [23] This form of cancer treatment allows for a high local concentration of the drug at the tumor region, even if the actual dosage is lower in comparison to classical chemotherapy dosages. The specified delivery of the drug prevents the whole body from exposure, decreasing the extreme side effects that often come with cancer treatments. GNPs have also been used for gene delivery. These examples showcase the ability of gelatin to serve as an effective carrier for drug delivery that can be modified for the delivery of an assortment of drug types.

NSAID-eluting Hemostatic Agent

The proposed product of this study utilizes peptide chemistry to form cages within the gelatin and to bind the NSAID to the gelatin. A peptide bond is a type of amide bond between a carboxyl group and an amino group, releasing water. [25] While these interactions are not typically thermodynamically favorable, they can exist at a high rate compared to other types of bonds in the gelatin because of the high number of available carboxyl and amide groups on both the gelatin and the NSAID added.

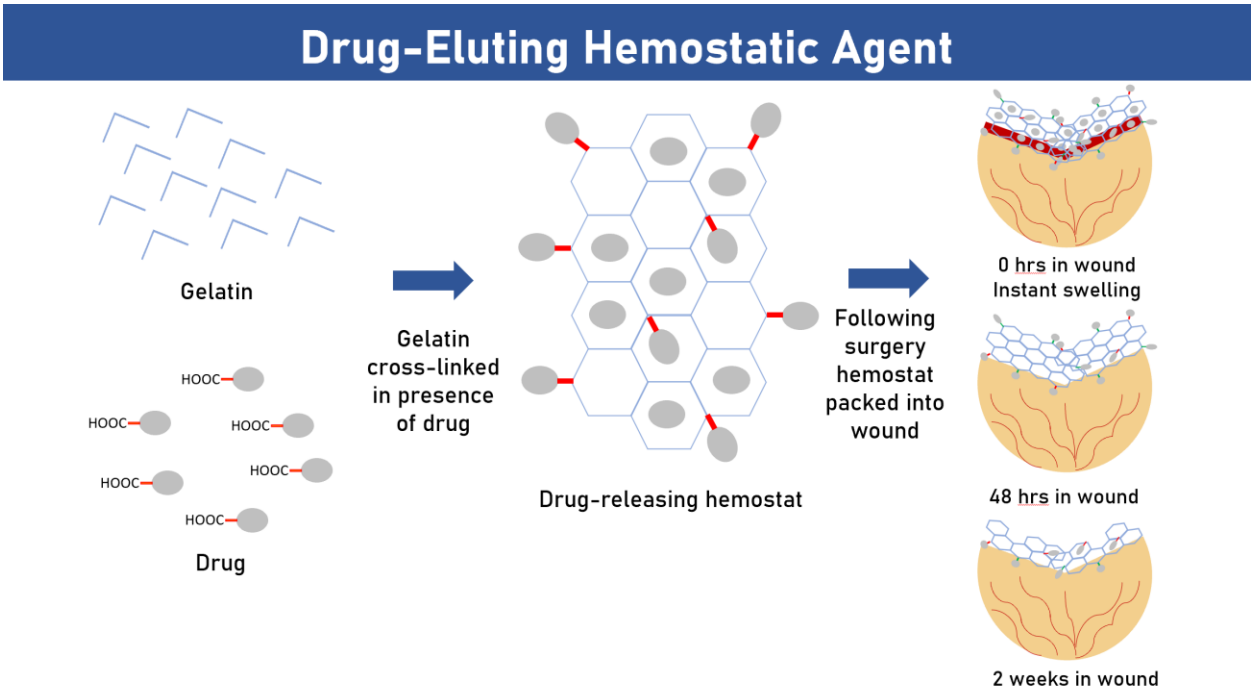

Figure 1. This figure shows the proposed mechanism for delivery of the drug-eluting hemostatic agent designed in this study.

In order to maximize the efficacy of the gelatin-NSAID conjugate, the crosslinker EDC was used. EDC, or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, is a zero-length crosslinker that activates the carboxylate group on the glutamic acid and aspartic acid residues of gelatin.[26] Once activated, these carboxylates are able to react with the gelatin's amino groups forming amide bonds, creating cages within the gelatin structure. When the NSAID is added, most of the molecules are trapped within these cages. Amide bonds also form between the gelatin and the NSAID if the carboxyl group on the NSAID comes in contact with an amide group on the gelatin. As a result, after the release of the initial burst of free drug trapped in the cages, a continuous release is expected due to the presence of the drug bonded directly to the gelatin, which will degrade over time.

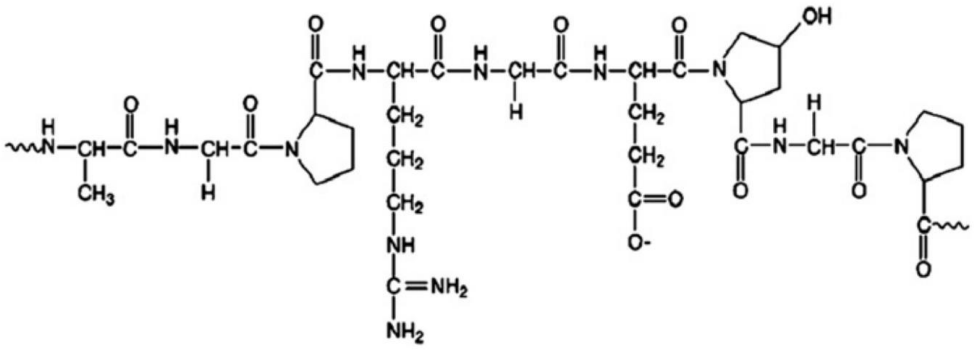

Figure 2. Generic structure of gelatin.[23]

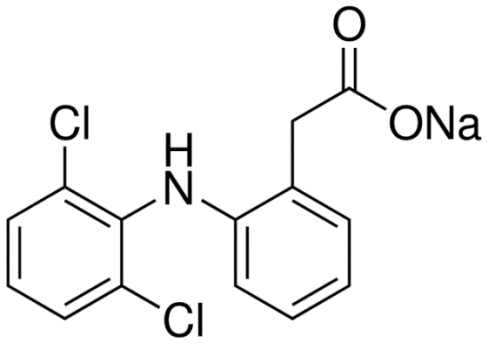

Figure 3. Structure of diclofenac sodium salt. [27]

Through utilizing the properties of gelatin as a drug delivery system, the goal of this project was to develop a gelatin product to be used in surgical settings that will allow for the controlled release of a specified NSAID. Applying this gelatin directly to the surgical site avoids many of the risks that arise from oral NSAIDs. Products such as Voltaren already exist for treating inflammation resulting from arthritis, however these products do not allow for a slow release and do not also serve as hemostatic agents. While these must be applied anywhere from two to four times a day, this new gelatin product will potentially provide release of the NSAID to the surgical site over the span of weeks. This again decreases the necessity for oral medications, limiting side effects from these drugs following surgical procedures.

Materials and Methods

Materials

Gelatin type B, diclofenac sodium, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and absolute ethanol were purchased from Fischer Chemical. NaCl was purchased from Sigma-Aldrich (St. Louis, MO). Sodium phosphate was purchased from Fisher Chemical.

Synthesis of Gelatin-NSAID Conjugates

Gelatin was prepared as a 20 mg/mL stock solution in 0.1 M phosphate buffer pH 7.4 and stirred at 60^{o}C until soluble. The conjugates were synthesized by incubating 6 mL () with diclofenac dissolved in phosphate buffer (6.29 mM) at 25^{o}C under EDC (60 mM) for 2 hours and shaking at 50 rpm. Precipitation was performed using ice-cold absolute ethanol and centrifugation at 6000x g. 1.85 mM NaCl was used to wash the precipitate, and then a second round of precipitation was performed. A soft spin was done at 2500x g to acquire the final pellet precipitate.

Determination of Delayed Release Via Elution Assay

Conjugates were immersed in 1.5 mL phosphate-buffered saline solution (PBS). These samples were incubated at 37^{o}C for a period of 4 weeks. 1 mL of PBS was removed from the elute and replaced with 1 mL of fresh PBS. The elution was run through UV-Vis to determine the concentration of diclofenac in the solution.

Analysis of Product

In order to determine the effectiveness of the delayed release, ultraviolet-visible spectroscopy (UV-Vis) is used. UV-Vis can be used to determine the concentration of a known compound in a solution by measuring the absorbance of light from that solution in the context of Beer's Law.[28] Samples of the proposed product are placed in solution to allow for drug release. Released samples are collected at various time periods ranging from 24 hours to weeks later in order to determine the drug concentration eluted from the complexes. This project uses the wavelength of 276 nm for diclofenac[29] to be compared to a calibration curve of known concentrations to determine the concentrations of the compound in various solutions.

Results

UV-Vis was performed to analyze the elution from conjugates of various concentrations. 20 mg/mL and 25 mg/mL gelatin concentrations were used for comparison and to evaluate the most efficient conditions for elution.

An extensive amount of time was spent on method development for this project. The decision to use diclofenac as the main NSAID came from problems with solubility. The diclofenac salt was discovered to be the most soluble in the solutions in the initial protocol. The maximum concentration permitted was 2 mg/mL of diclofenac. The phosphate buffer was chosen for a similar reason: it allowed for the best solubility of the diclofenac. A previous study showed that a phosphate buffer with a pH of 7.4 allowed for a significant increase in diclofenac sodium solubility.[30]

Medium	Solubility (mg/mL)
Hydrochloric acid 0.1 M	0.0012
Hydrochloric acid 0.01 M	0.0017
Hydrochloric acid 0.001 M	0.28
Acetate buffer solution pH 4.1	0.0033
Acetate buffer solution pH 4.5	0.0036
Acetate buffer solution pH 5.5	0.036
Purified water	14.18
Phosphate buffer solution pH 5.8	0.14
Phosphate buffer solution pH 6.0	0.15
Phosphate buffer solution pH 6.8	0.67
Phosphate buffer solution pH 7.0	1.36
Phosphate buffer solution pH 7.4	5.15
Phosphate buffer solution pH 7.8	12.00
Phosphate buffer solution pH 8.0	12.14
Alkaline borate buffer solution pH 8.0	17.17
Alkaline borate buffer solution pH 9.0	15.18
Alkaline borate buffer solution pH 10.0	12.08

Figure 4. A previous study showed that a phosphate buffer with a pH of 7.4 gave a significant jump in solubility of diclofenac sodium salt, and therefore was the pH used in the protocol. Too much of an increase in pH would disturb the reactions needed to occur.[30]

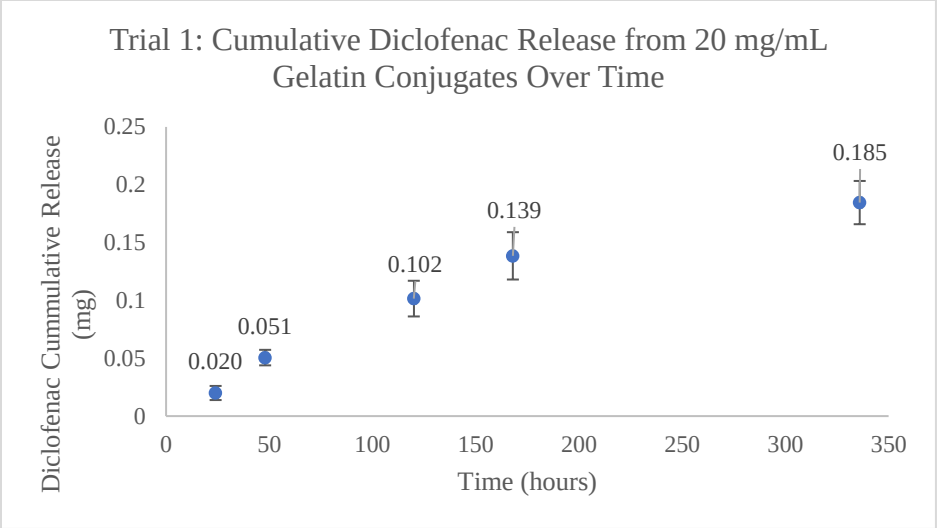

Figure 5. Cumulative diclofenac release from the first trial of 20 mg/mL gelatin loaded with 2 mg/mL of diclofenac over a 350-hour timeframe. 0.020 mg was eluted in the initial 24 hours and a total of 0.185 mg was eluted over the whole timeframe.

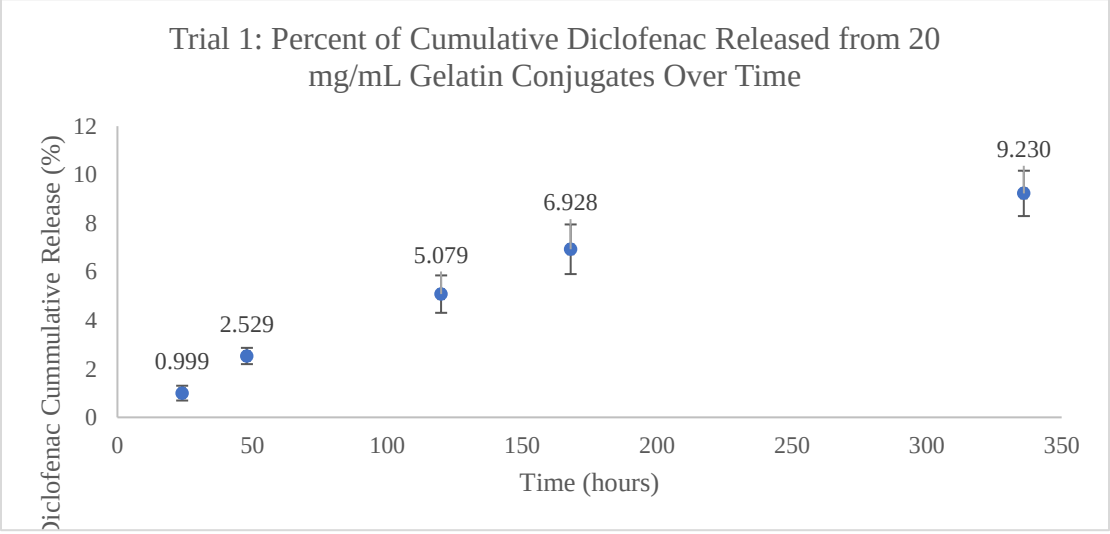

Figure 6. Percent of cumulative diclofenac release from the first trial of 20 mg/mL gelatin loaded with 2 mg/mL of diclofenac over a 350-hour timeframe. 0.999% was eluted in the initial 24 hours and a total of 9.230% was eluted over the whole timeframe.

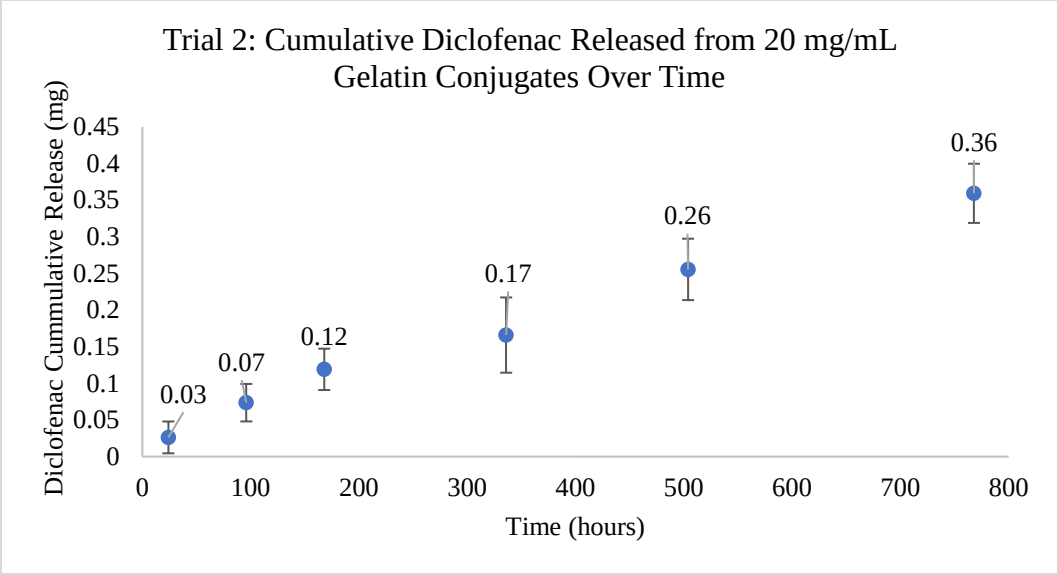

Figure 7. Cumulative diclofenac release from the second trial of 20 mg/mL gelatin loaded with 2 mg/mL of diclofenac over an 800-hour timeframe. 0.03 mg was eluted in the initial 24 hours and a total of 0.36 mg was eluted over the whole timeframe.

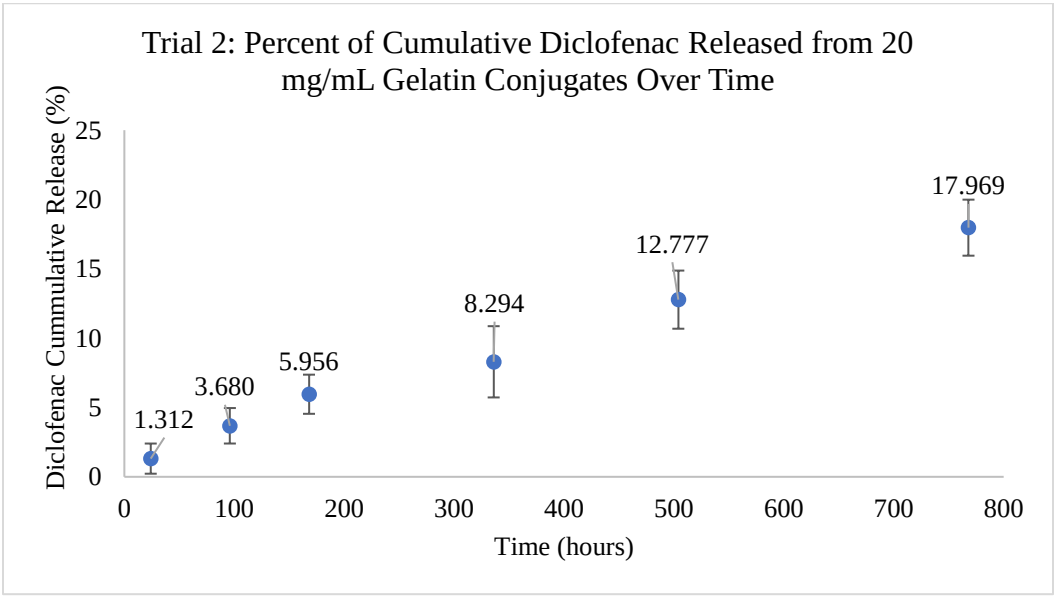

Figure 8. Percent of cumulative diclofenac release from the second trial of 20 mg/mL gelatin loaded with 2 mg/mL of diclofenac over an 800-hour timeframe. 1.312% was eluted in the initial 24 hours and a total of 17.969% was eluted over the whole timeframe.

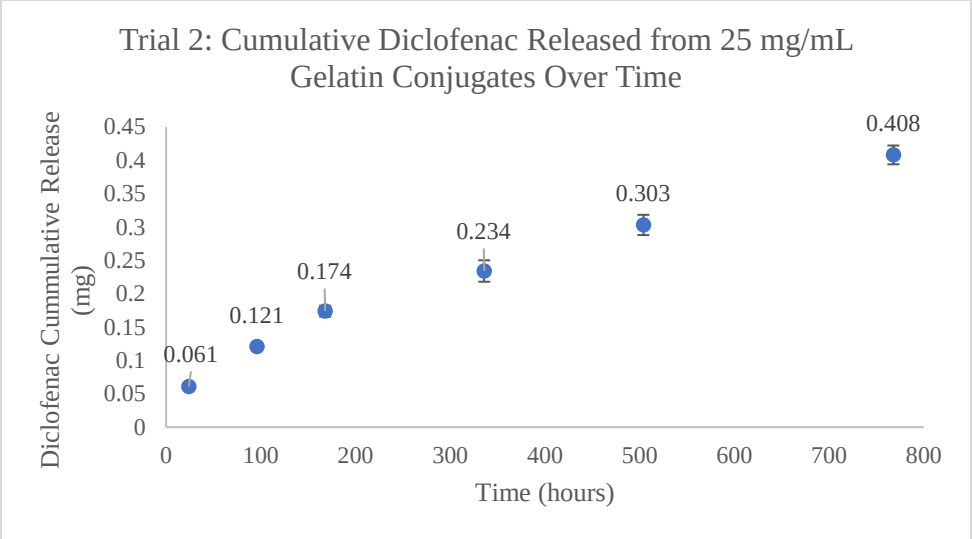

Figure 9. Cumulative diclofenac release from the second trial of 25 mg/mL gelatin loaded with 2 mg/mL of diclofenac over an 800-hour timeframe. 0.061 mg was eluted in the initial 24 hours and a total of 0.408 mg was eluted over the whole timeframe.

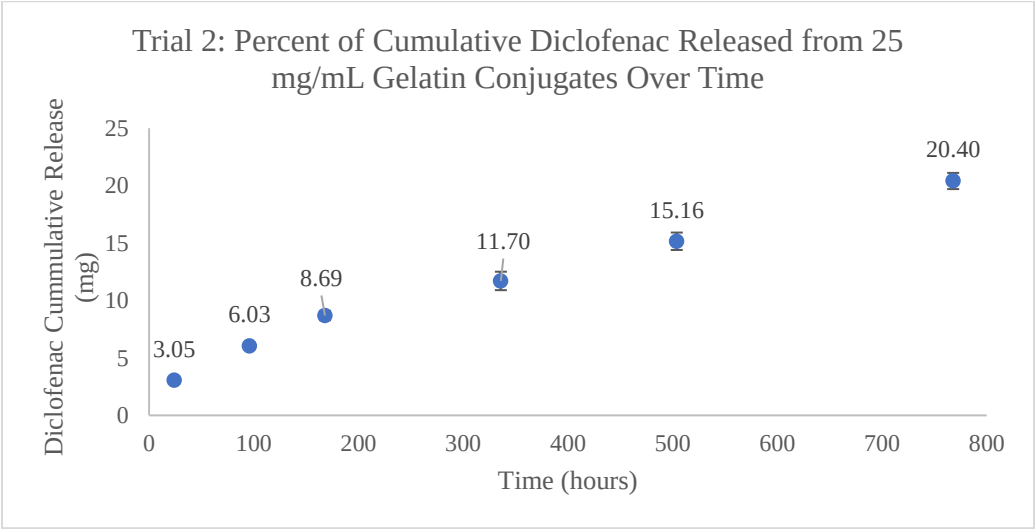

Figure 10. Percent of cumulative diclofenac release from the second trial of 25 mg/mL gelatin loaded with 2 mg/mL of diclofenac over an 800-hour timeframe. 3.05% was eluted in the initial 24 hours and a total of 20.40% was eluted over the whole timeframe.

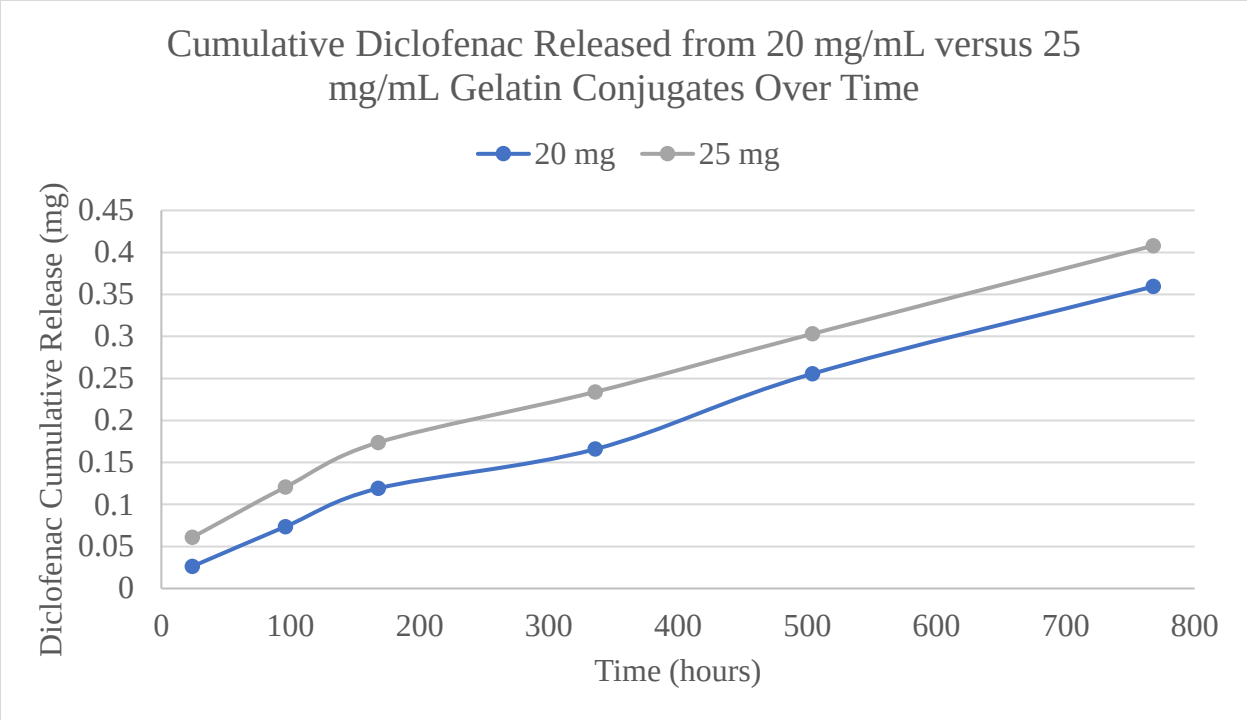

Figure 11. Cumulative concentration release profiles for diclofenac-gelatin conjugates using 20 mg/mL gelatin versus 25 mg/mL gelatin stock solutions. Both are loaded with 2 mg/mL diclofenac and were analyzed using UV-Vis and collected in PBS solution. At every timepoint, the 25mg/mL gelatin gave a higher release.

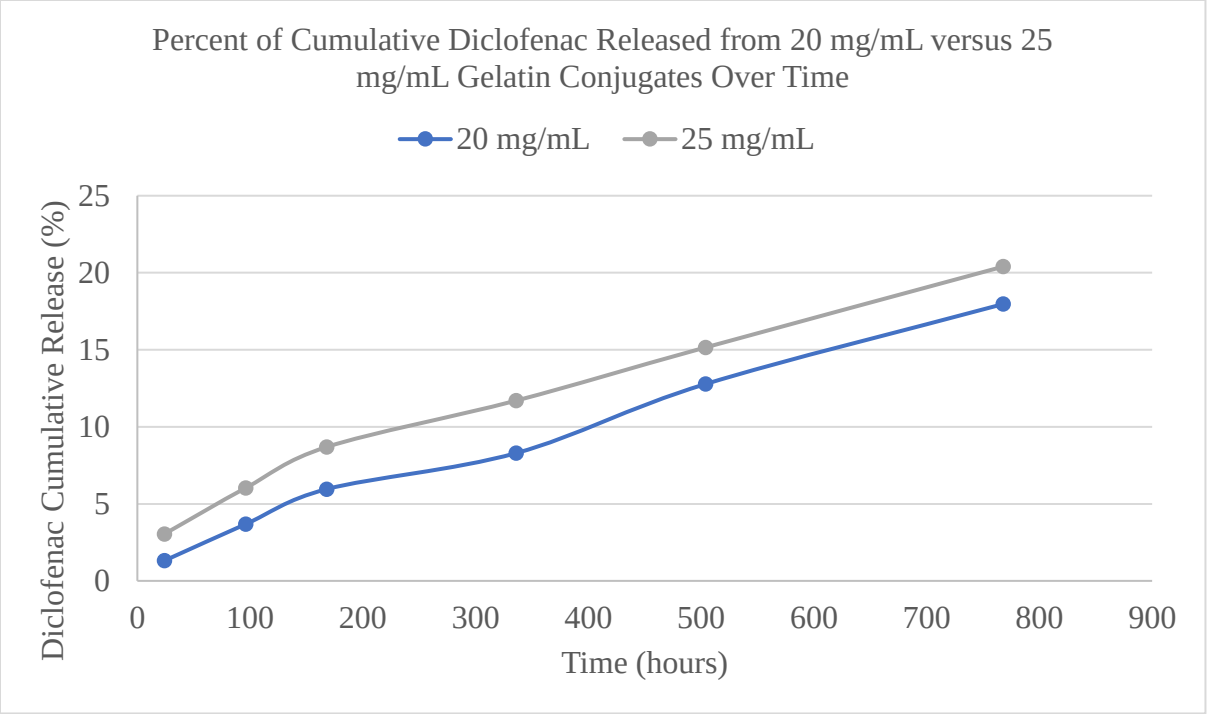

Figure 12. Cumulative percentage release profiles for diclofenac-gelatin conjugates using 20 mg/mL gelatin versus 25 mg/mL gelatin stock solutions. Both are loaded with 2 mg/mL diclofenac and were analyzed using UV-Vis and collected in PBS solution. At every timepoint, the 25mg/mL gelatin gave a higher release.

Discussion

This study developed gelatin-diclofenac conjugates to be used in surgical settings with the purpose of reducing inflammation and pain. These conjugates can be applied directly to the surgical site for direct subcutaneous delivery. While there are existing products that offer site-specific delivery, the product from this study is novel in that it uses a hemostatic gelatin agent as the drug delivery system while also providing a delayed release. Carbodiimide crosslinking using EDC allowed for an initial release burst as well as a delayed release as a result of direct binding of diclofenac to the gelatin, in addition to the entrapment of diclofenac in gelatin cages.

Release assays allowed for the determination that a higher gelatin concentration provides a higher initial burst of diclofenac release, followed by a steady delayed release. The initial burst correlates to the release of the diclofenac that was trapped in the gelatin cages. As the gelatin starts to degrade, the bonds creating the cages break causing a release of the drug. The delayed release is likely from the diclofenac bound directly to the gelatin. These bonds likely break and release the drug, causing the delayed release. Release assays using UV-Vis **(Figures 5-12)** were used to determine gelatin concentration most suitable for this delayed release.

While it can be confirmed that there was a controlled release, the percent eluted was lower than initially expected. When comparing the NSAID structure to the antibiotic structure, antibiotics have more groups available to interact with the structure of the gelatin. Because of this, it would be expected that the antibiotics would elute at lower percentages compared to NSAID elution. Also, because NSAIDs are much smaller in size than antibiotics, the NSAIDs should elute at higher rates. However, this was not seen: the diclofenac eluted at a significantly lower percentage than the antibiotics.

A probable reason as to why the diclofenac eluted at a lower rate involves the initial precipitation step using absolute ethanol. It is possible that the diclofenac dissolved in the ethanol instead of crashing out with the gelatin pellet. If this is the case, there would not be any diclofenac remaining to elute as the gelatin breaks down. Another possibility is that the diclofenac did not properly dissolve initially in the phosphate buffer, and therefore was not properly incorporated into the conjugate structure.

Efforts should be made to further optimize the precipitation protocol and careful monitoring must be done to ensure accurate results. In order to ensure that diclofenac is present at each step of the protocol, UV-Vis analysis or another form of analysis should be performed throughout the entirety of the protocol. The current protocol states that UV-Vis should be completed only at the end, but performing UV-Vis throughout will ensure that diclofenac was not lost in any step.

Further studies must be done to evaluate the accuracy of diclofenac in the final products. There are a few conflicts that may affect the accuracy of the current data. Firstly, the lambda max values of the gelatin and the diclofenac are very close to each other. Diclofenac has been shown to have a lambda max value of 276.[29] However, the lambda max of gelatin at the concentrations used in this study is between 250 and 300, creating an overlap. While it is believed that the UV-Vis is analyzing the diclofenac and the gelatin, it cannot be confirmed from the current study that the diclofenac concentration has not been confused by only gelatin absorbance values.

Acknowledgements

I would like to thank my research advisor, Dr. Shelby for all of her knowledge and guidance through my research and my college career. Her support and flexibility was a major source of support through this process. I would also like to thank the CBP Department for the resources and opportunity to push myself and further my education. I would like to thank Dr. Keith Berend for funding and originating this project. Finally, I would like to thank the Honors Program for the ability to complete my thesis and to continue to expand my research education.

References

(1) Kohl, B. A.; Deutschman, C. S. The Inflammatory Response to Surgery and Trauma: *Curr. Opin. Crit. Care* **2006**, *12* (4), 325–332. https://doi.org/10.1097/01.ccx.0000235210.85073.fc.

(2) McSorley, S. T.; Watt, D. G.; Horgan, P. G.; McMillan, D. C. Postoperative Systemic Inflammatory Response, Complication Severity, and Survival Following Surgery for Colorectal Cancer. *Ann. Surg. Oncol.* **2016**, *23*, 2832–2840. https://doi.org/10.1245/s10434-016-5204-5.

(3) Gupta, A.; Bah, M. NSAIDs in the Treatment of Postoperative Pain. *Curr. Pain Headache Rep.* **2016**, *20* (11), 62. https://doi.org/10.1007/s11916-016-0591-7.

(4) Marieb, E.; Hoehn, K. *Human Anatomy and Physiology*, Tenth.; Pearson, 2016.

(5) Griffin, R. M. Pain Relief: How NSAIDs Work https://www.webmd.com/arthritis/features/pain-relief-how-nsaids-work (accessed May 31, 2020).

(6) Ricciotti Emanuela; FitzGerald Garret A. Prostaglandins and Inflammation. *Arterioscler. Thromb. Vasc. Biol.* **2011**, *31* (5), 986–1000. https://doi.org/10.1161/ATVBAHA.110.207449.

(7) Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) https://my.clevelandclinic.org/health/drugs/11086-non-steroidal-anti-inflammatory-medicines-nsaids (accessed May 30, 2020).

(8) Davis, A.; Robson, J. The Dangers of NSAIDs: Look Both Ways. *Br. J. Gen. Pract.* **2016**, *66* (645), 172–173. https://doi.org/10.3399/bjgp16X684433.

(9) NSAIDs (Nonsteroidal Antiinflammatory Drugs) Drug Information https://www.rxlist.com/nsaids_nonsteroidal_antiinflammatory_drugs/drug-class.htm (accessed Sep 23, 2020).

(10) What Is an Alanine Aminotransferase (ALT) Test? https://www.webmd.com/digestive-disorders/alanine-aminotransferase-test (accessed Oct 15, 2020).

(11) Liver Injury Caused by Drugs - Hepatic and Biliary Disorders https://www.merckmanuals.com/professional/hepatic-and-biliary-disorders/drugs-and-the-liver/liver-injury-caused-by-drugs (accessed Aug 25, 2020).

(12) Ibuprofen. In *LiverTox: Clinical and Research Information on Drug-Induced Liver Injury*; National Institute of Diabetes and Digestive and Kidney Diseases: Bethesda (MD), 2012.
(13) Voltaren Gel: Uses, Dosage & Side Effects https://www.drugs.com/voltaren-gel.html (accessed Jul 12, 2020).
(14) Why Topical NSAIDs Could Be a Safer Option to Relieve Your Arthritis Pain https://health.clevelandclinic.org/why-topical-nsaids-could-be-a-safer-option-to-relieve-your-arthritis-pain/ (accessed Jul 23, 2020).
(15) Diclofenac Topical (arthritis pain): MedlinePlus Drug Information https://medlineplus.gov/druginfo/meds/a611002.html (accessed Jul 8, 2020).
(16) Lionberger, D. R.; Brennan, M. J. Topical Nonsteroidal Anti-Inflammatory Drugs for the Treatment of Pain Due to Soft Tissue Injury: Diclofenac Epolamine Topical Patch. *J. Pain Res.* **2010**, *3*, 223–233. https://doi.org/10.2147/JPR.S13238.
(17) Brittany L. Taylor; Dong Hwa Kim; Julianne Huegel; Harina A. Raja; Sophie J. Burkholder; Stephanie N. Weiss; Courtney A. Nuss; Louis J. Soslowsky; Robert L. Mauck; Andrew F. Kuntz; Joseph Bernstein. Localized Delivery of Ibuprofen via a Bilayer Delivery System (BiLDS) for Supraspinatus Tendon Healing in a Rat Model. *Wiley Period.* **2020**. https://doi.org/10.1002/jor.24670.
(18) Green, D.; Wong, C. A.; Twardowski, P. Efficacy of Hemostatic Agents in Improving Surgical Hemostasis. *Transfus. Med. Rev.* **1996**, *10* (3), 171–182. https://doi.org/10.1016/S0887-7963(96)80058-7.
(19) Sundaram, C. P.; Keenan, A. C. Evolution of Hemostatic Agents in Surgical Practice. *Indian J. Urol. IJU J. Urol. Soc. India* **2010**, *26* (3), 374–378. https://doi.org/10.4103/0970-1591.70574.
(20) Duconseille, A.; Astruc, T.; Quintana, N.; Meersman, F.; Sante-Lhoutellier, V. Gelatin Structure and Composition Linked to Hard Capsule Dissolution: A Review. *Food Hydrocoll.* **2015**, *43*, 360–376. https://doi.org/10.1016/j.foodhyd.2014.06.006.
(21) Foox, M.; Zilberman, M. Drug Delivery from Gelatin-Based Systems. *Expert Opin. Drug Deliv.* **2015**, *12* (9), 1547–1563. https://doi.org/10.1517/17425247.2015.1037272.
(22) Young, S.; Wong, M.; Tabata, Y.; Mikos, A. G. Gelatin as a Delivery Vehicle for the Controlled Release of Bioactive Molecules. *J. Controlled Release* **2005**, *109* (1–3), 256–274. https://doi.org/10.1016/j.jconrel.2005.09.023.
(23) Elzoghby, A. O. Gelatin-Based Nanoparticles as Drug and Gene Delivery Systems: Reviewing Three Decades of Research. *J. Controlled Release* **2013**, *172* (3), 1075–1091. https://doi.org/10.1016/j.jconrel.2013.09.019.
(24) Leong, K. W.; Mao, H.-Q.; Truong-Le, V. L.; Roy, K.; Walsh, S. M.; August, J. T. DNA-Polycation Nanospheres as Non-Viral Gene Delivery Vehicles. *J. Controlled Release* **1998**, *53* (1), 183–193. https://doi.org/10.1016/S0168-3659(97)00252-6.
(25) 18.3 Peptides | The Basics of General, Organic, and Biological Chemistry https://courses.lumenlearning.com/suny-orgbiochemistry/chapter/peptides/ (accessed Aug 27, 2020).
(26) EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) https://www.thermofisher.com/order/catalog/product/77149 (accessed Aug 27, 2020).
(27) Diclofenac sodium salt | Sigma-Aldrich https://www.sigmaaldrich.com/catalog/substance/diclofenacsodiumsalt318131530779611?lang=en&region=US (accessed May 2, 2021).

(28) 4.4: UV-Visible Spectroscopy https://chem.libretexts.org/Bookshelves/Analytical_Chemistry/Book%3A_Physical_Methods_in_Chemistry_and_Nano_Science_(Barron)/04%3A_Chemical_Speciation/4.04%3A_UV-Visible_Spectroscopy (accessed Aug 27, 2020).
(29) Naveed, S.; Qamar, F. UV Spectrophotometric Assay of Diclofenac Sodium Available Brands. **2014**, *1*, 5.
(30) Kincl, M.; Meleh, M.; Veber, M.; Vrečer, F. Study of Physicochemical Parameters Affecting the Release of Diclofenac Sodium From Lipophilic Matrix Tablets. *Acta Chim Slov* **2004**, 17.