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OVERVIEW

Current Problems, Previous Studies, and Final Goals

Current Problems

Benefits of Peptide Drugs

- Proteins are highly specific
- Unlikely to cause adverse effects
- Unlikely to illicit an immune response
- Safer alternative to gene therapy treatments
- More likely to be approved by FDA

Market Benefits

- Lupron™ made over 2.3 billion USD
- Annual growth rate of 10.3 percent
- Projected to make 47 billion USD in revenue by 2025

75%

of peptide drugs are administered by the parenteral route

- Only 24.4% of patients with Type 2 diabetes were very willing to receive insulin therapy
- 50 % of patients surveyed anticipated pain and restrictiveness

Potential Benefits of Oral Peptide Therapy

- Called peroral route
- Patients are 65 percent more likely to accept treatment
- Less checkups by doctors
- Less risk
- Extended Release

Barriers to Development

- Stomach contains proteolytic enzymes
- Cannot passively diffuse through intestinal membrane

Potential Solutions

- emulsions, nanoparticles, microspheres, and liposomes

Can carboxymethyl chitosan/sodium alginate microspheres serve as a potential drug delivery system by demonstrating extended release properties and maintaining structural integrity?

THE EXPERIMENT

Materials, Preparing the Microspheres, Studying Protein Release, Analyzing Protein Degradation

Analysis of Novel Carboxylmethyl Chitosan/Sodium Alginate Microspheres as a Potential Drug Carrier

Materials

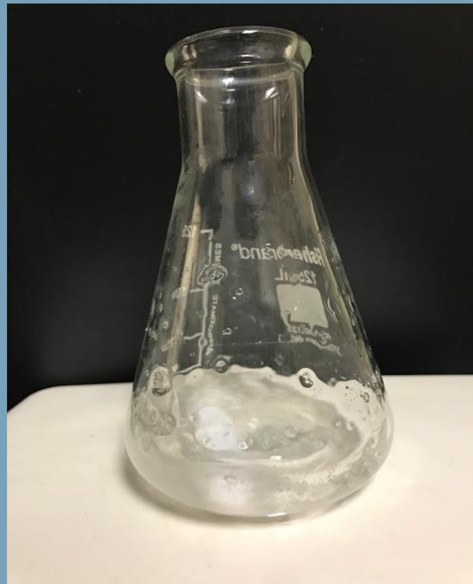
- Sodium Alginate
- Carboxymethyl chitosan
- TWEEN® 20
- Tris-hydrochloride
- Albumin, Human Serum.
- Paraffin oil
- Calcium Chloride ($CaCl_2$)
- Acetic acid (CH_3COOH)
- Ethanol (C_2H_6O)
- Hydrochloric acid (HCl)
- Deionized water (diH_2O
- Trifluoroacetic acid ($C_2HF_3O_2$)
- Acetonitrile (C_2H_3N)
- Beckman Coulter DU-650 Spectrophotometer
- Aglient 1100 Series High Performance Liquid Chromatography (HPLC)
- Beckman Coulter Allegra 6 High Speed Centrifuge
- Corning® LSE™ High Speed Microcentrifuge

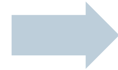
Microsphere Components

Carboxymethyl Chitosan	Sodium Alginate
• Derivative of chitin • High bioavailibitly • Low toxicity • Can open tight junctions in epithelial cells • Carboxymethyl group increases water solubility	• Derivative of algae • Nontoxic and biodegradable • Shrinks at low pH • High swelling degree

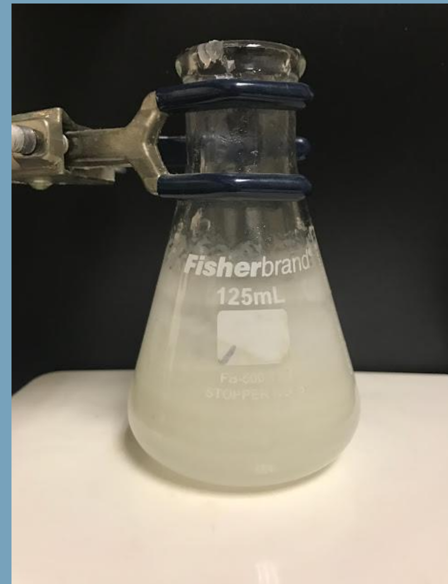
Preparing Microspheres

5% CMCS/8% SA with 250 mg HSA

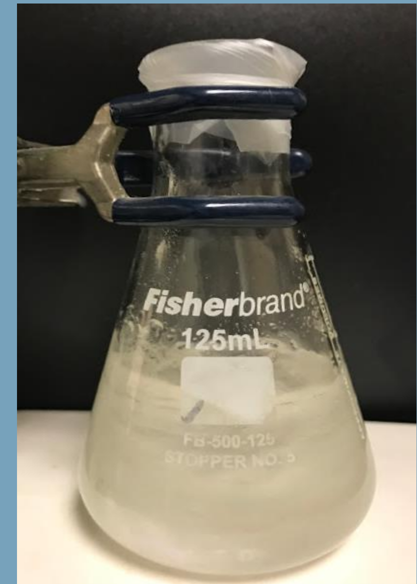




Add paraffin oil with 1% Tween-20

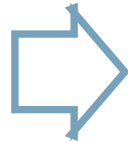


Spin for 2 hours at high speed



Preparing Microspheres Cont.

Hardening solution slowly dropped in emulsion



Centrifuged at 3000 rpm for 10 minutes

Placed into treatment groups

Morphological Analysis

- Viewed under microscope to determine size and morphology
- Determined encapsulation efficiency with equation:

$$Encapsulation\ efficiency = \frac{Amount\ of\ HSA\ released}{Loading\ amount\ of\ HSA} \times 100$$

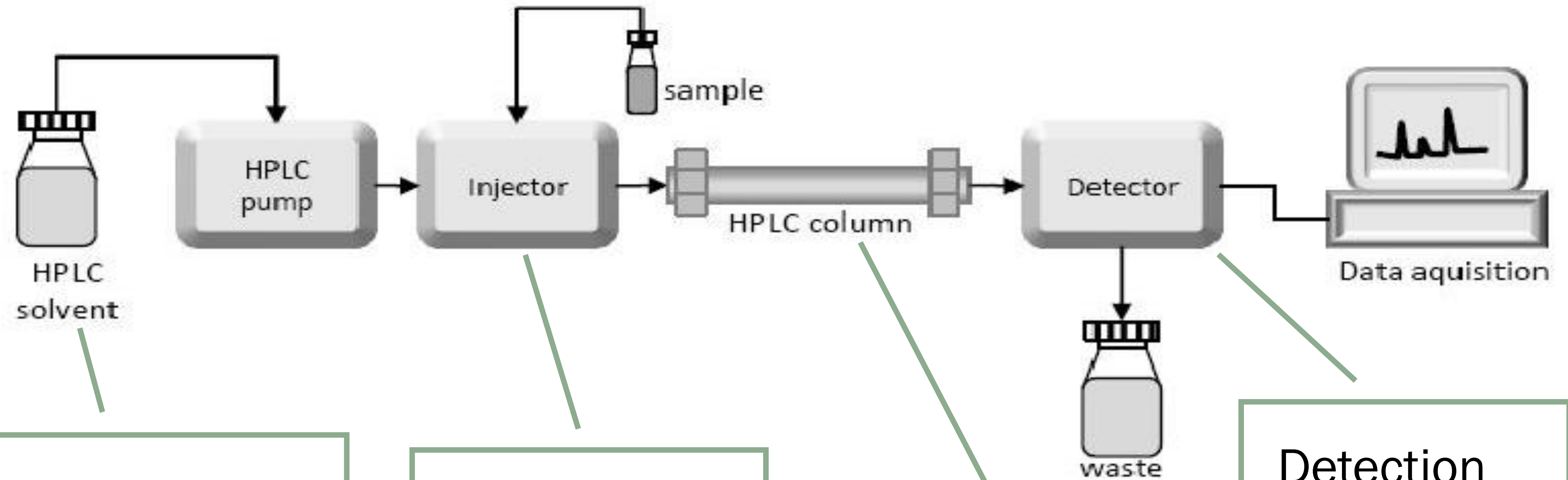
Studying the Release from Microspheres

500 uL into 1 mL media

Collected supernatant at selected intervals

Supernatant assayed by UV spectroscopy

Degradation of Protein



Solvent A : 0.1% TFA in H_2O
Solvent B: 0.1% TFA in acetonitrile

100 uL injection with flow rate of 0.9 mL/min

Reverse Phase C_{18} column
Temp: 40°C

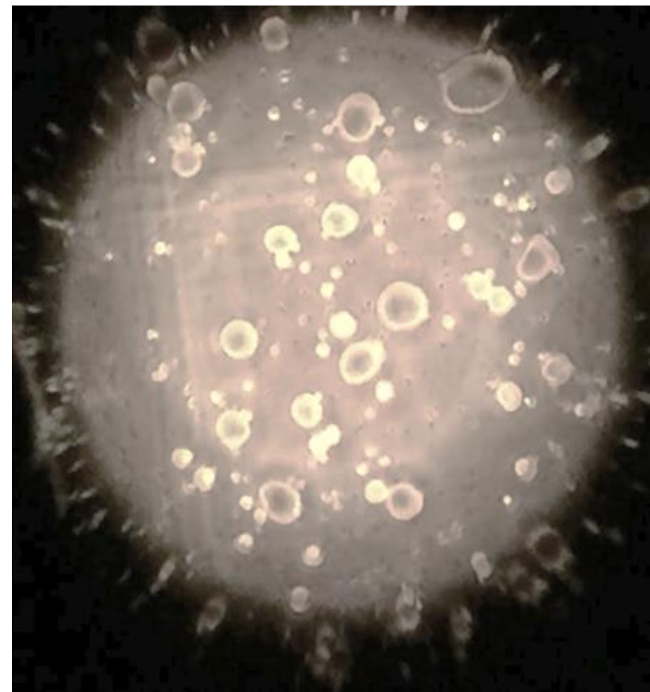
Detection at 280 nm for tryptophan groups

RESULTS

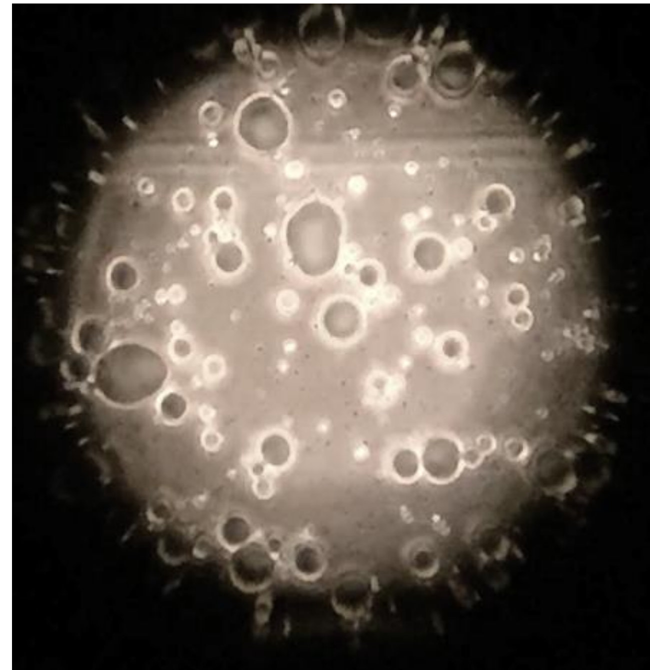
Physical Appearances of Microspheres, Release Behavior, Degradation Patterns

Physical Appearance of Microspheres

- Smooth surface
- Sometimes irregular shape
- Before treatment average size 4 μm



After 48 hours in pH 2: average size 6.8 μm

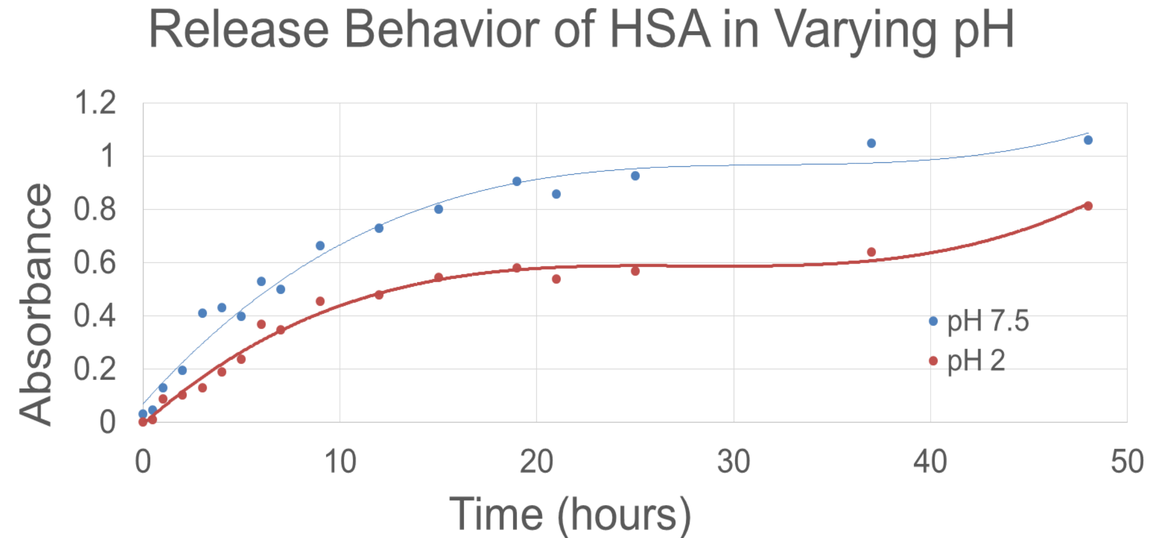


After 48 hours in pH 7.5: average size 9.2 μm

Release Behavior

- The average absorbance of two trials was graphed. The microspheres demonstrated an extended release of protein throughout its treatment in varying pH (Figure 4). As time went on the increase of protein concentration also increased. Both also showed a quick initial release since the absorbance quickly increased, but slowed down near the end of the time course. Microspheres treated in pH 7.5 had a higher overall absorbance at 280 nm compared to those treated in pH 2. Both treatments resulted in a plateau in protein concentration increase at 20 hours. After 20 hours, microspheres in pH 2 demonstrated a greater increase in HSA absorbance over the 20 hour to 48 hour time period.

- Assuming that when the absorbance was above 1.0 all the HSA had been released, the release curve shows that most of the HSA was observed to have a quick release. In the microspheres treated in pH 7.5, about 60% of the protein was released within the first 10 hours. In comparison, those treated in pH 2, only about 40% of the protein was released.



Degradation

- To determine whether HSA had maintained its structural integrity though the making of microspheres and the treatment in varying pH, the last part of the experiment was designed to determine whether HSA had been broken down. The wavelength used was 280 nm, which detects the tryptophan groups of HSA. A standard solution of HSA with the calculated expected concentration (1 mg/mL) was ran and it showed two peaks with the height of 2600 and 1480 mAU (Figure 5). The treated samples were compared to the standard to analyze integrity. Figure 6 shows a chromatogram with data from 0, 24, and 48 hours of treatment in pH 2 overlayed. At all time points within the acidic pH treatment, there were two distinct peaks present, although the baseline contained noise. The presence of peaks signifies the maintenance of protein structure. The largest increase in peak height was between hours 24 and 48. At 48 hours, where protein concentration is the greatest, the peak height was 230 and 80 mAU, which was significantly lower than the standard. In the chromatogram from the basic environment treatment, there were also two distinct peaks, showing that there were tryptophan groups intact. The baseline was also unclear, with many peaks of its own. In the overlayed chromatogram of samples treated in pH 7.5, there is little increase in peak height within the three samples (Figure 7). The peak height of the sample from time 48 was 10 mAU, which was lower than the standard.

DISCUSSION

Application of Findings

Morphological Analysis

- The microspheres maintained their small size due to the high speed spinning in their formation. The pH dependence of swelling is shown in Figure 3a and 3b. The microspheres in the acidic environment had a lower degree of swelling as seen by looking at their shape compared to the microspheres treated in the neutral environment. Therefore, the swelling ability of the microspheres decreased in the 0.1 M HCl solution. Those treated in pH 2 had a lower swelling degree because of the dense structure created by the protonated amino groups of CM-CS (Zhang, Guo, Peng, & Jin, 2003). The degree of dissociation in the chitosan was restricted, resulting in a loss of swelling. Some research shows that this occurred because the ability of CM-CS to swell may have been suppressed and the chitosan may have formed some loops according to previous studies by Zhang et al. (2003). A less dense structure resulted due to loop formation and enhancement of the electrostatic interaction between –COOH groups of the SA and $–NH_2$ groups of the CM-CS in low pH (Zhang et al., 2003). Therefore, the microspheres treated in pH 2 tended to have a smaller diameter than those treated in pH 7.5. In comparison, microspheres treated in neutral pH had a higher swelling degree because the biopolymers maintained their structure and swelling properties under a pH similar to that of water. Therefore, microspheres in the Tris-HCl buffer were less dense after treatment.
- The encapsulation efficiency observed was low. This may have been because the biopolymers were unable to capture the protein. A contributing source of error for the low encapsulation efficiency to occur is that the ratio of oil to solution may have been too high. Since the HSA is trapped in the ionized lattice of CM-CS/SA when a $CaCl_2$ solution is added according to Lemoine et al. (1998), the initial concentration of the biopolymers may not have been sufficient if over half of the protein was unable to be captured. The encapsulation efficiency must be improved if the CM-CS/SA microspheres were to be used as a potential drug carrier since many peptide drugs are expensive and the production cost would be high.

Application of Release

- The results agreed with the expected variance in swelling degree between microspheres treated in pH 2 and pH 7.5. HSA was released at a slower rate at pH 2 due to the lower swelling degree. HSA was released at a faster rate at pH 7.5 since CM-CS/SA have higher permeability due to higher swelling ability in neutral conditions. The process for the drug release was through diffusion across the CM-CS/SA membrane, so the higher the swelling, the faster the release. At pH 2, the drug diffused slower because the protein could not pass through the membrane due to the stronger interaction between CM-CS and SA in the acidic environment (Leach & Schmidt, 2004). In neutral conditions, the swelling was much higher and this resulted in a large amount of HSA being released from the microspheres by diffusion through the less dense membrane.
- In application to the microspheres' potential as a drug delivery system, the low swelling and release in low pH is beneficial. In the GI tract, the peptide drug must pass through the acidic environment of the stomach (pH 1-2) and later pass through the intestines (pH 7.4) where absorption occurs. Since the stomach contains many protease enzymes that break down protein, the drug deliverer cannot release the drug into the stomach, or else the protein will be broken down. It instead should be released when in the small intestines, where the peptides can be absorbed into the blood stream. Accordingly, the release kinetics of the CM-CS/SA microspheres show the potential to be useful in drug delivery. There was a lesser release in low pH and a greater one is neutral pH. But, some protein was still released into the stomach, where structural integrity cannot be ensured. Also, the release kinetics showed an extended release which is beneficial to patients. Drugs with an extended release stay in the body longer and result in a more controlled administration of drugs, especially in patients who must repeatedly administer therapy for metabolic disorders such as diabetes (Neutel, Zhao, & Karyekar, 2013).

Structural Integrity

- Even if the CM-CS microspheres have the proper release kinetics to form a drug delivery system, the structural integrity of the protein must be maintained. Since a protein's structure is directly related to its function, the protein must remain intact. HSA structures were detected at 0, 24, and 48 hours in both acidic and neutral environments. The wavelength of 280 nm was used to measure tryptophan groups in HSA.
- In both acidic and neutral environments, the structural integrity of the tryptophan group was maintained due to the initial stability of HSA and the biopolymer coating. In all test groups, the tryptophan was able to be detected. The standard for HSA showed much higher peaks than the treatment groups. Additionally, the treatment groups did not have clean baselines, there was a lot of noise. This may have been because portions of the microsphere were broken down and portions of it were detected in the HPLC. It also may have been because a portion of the HSA was broken down, leading to the unclear baseline. This would also account for the difference in concentration between the expected calculations from the UV spectrophotometer and the concentrations shown on the HPLC.
- The peak height also shows that the concentration increased in each time period. Therefore, the release of protein also matches the data shown in the readings from the UV spectrophotometer. The sudden jump in increase in low pH could be attributed to the slow release of protein due to the low swelling factor. In comparison, the peaks of the samples treated in neutral pH did not have as significant change because most of the protein was released within the first 24 hours since its swelling capacity is much larger than the microspheres in the low pH.

CONCLUSION

Conclusions and Further Work

Conclusions

- The current study showed that CM-CS/SA microspheres prepared by a water in oil emulsion has the potential to be a peptide drug delivery system through the parental route. The slower release of HSA in low pH due to the lower swelling degree is important for the development of oral protein therapeutics because the drug must be released in the small intestine where it is absorbed. Therefore, if there is a slower release, then the drug has a lower chance of being released into the stomach and begin degraded by proteolytic enzymes. The release kinetics of the HSA also showed an extended release, which is a more advantageous drug design. Because the microspheres maintained faster release kinetics in neutral pH, most of the protein would be released in the intestines where it would be absorbed. The CM-CS microspheres were also able to preserve the structural integrity of HSA over a 48 hour period without being degraded in both pH 2 and pH 7.5. Since the structural integrity was maintained, the drug would still be useful and create a response in the human body if the microspheres were used as a drug carrier. Orally administered insulin is still far from a reality, but it is closer than it seems. Since the CM-CS/SA was able to coat HSA, it shows that biopolymer microspheres have release kinetics that align with physiological conditions and may maintain protein structure in low pH.

Further Work

- A source of error that may have factored into the absorbance of the protein while analyzing using UV spectroscopy was that the microspheres residues may have entered the sample. When the treated samples of microspheres were centrifuged to collect the supernatant, the microspheres actually were less dense than the solutions and collected on top of the supernatant. To collect the solution carrying the released protein, a micropipette was used. The tip was submerged under the sample and then the sample was collected. Because of this technique, some of the treated microspheres may have been collected in the samples that were read by the UV spectrophotometer, making the absorbance higher than it actually should be. To combat this flaw, the microspheres should be further hardened using isopropyl alcohol because previous studies have shown that the density increases with the isopropyl alcohol's addition (Zhang et al., 2003). Then, when retrieving the supernatant, only released protein would be collected, providing more accurate results.
- Because the experiment did not factor in enzymes that degrade protein, the potential use of the CM-CS/SA microsphere is limited. In the future, I would like to repeat the experiment with treatment of microspheres under enzymatic conditions while adding a protease inhibitor cocktail to microspheres.
- Additionally, the encapsulation efficiency was low. Repeating the experiment and trying to improve the efficiency is necessity because much of the protein is going to waste and would not be resourceful if the microsphere was to be continually studied. A potential solution could be to decrease the amount of paraffin solution added when making the microspheres.
- Testing intestinal permeability of HSA after treatment of microspheres for absorption into bloodstream would also show if the microspheres should be continually studied. A possible testing method would be to see if the microspheres can release the protein across a skin like membrane. Then, the sample could be run through HPLC to see if any HSA was detected in the solution that passes through the membrane.

END SLIDE