

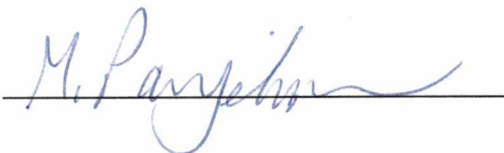
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Quantification of Phthalocyanine Concentration in Rat Brain
Using Laser-Induced Fluorescence Spectroscopy

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Charles W. Brinkley

December, 1994

ACKNOWLEDGEMENTS:

First, I would like to thank the Thompson Cancer Survival Center (TCSC) for financial support of this research project. I am grateful they gave me the opportunity to participate in research that may help in treating cancer patients in the future. I would like to thank Dr. Masoud Panjehpour, my TCSC advisor, for all his support. He always offered both his time and experience whenever I needed advice. I would also like to thank Rick Sneed for his help throughout this project.

Secondly, I would like to thank Dr. Michael J. Roberts, my UT advisor, for his supervision and support. I would also like to thank Dr. Marshall Pace for his time and effort as a member of my graduate committee. I would also like to thank Dr. Arlene Garrison, the Director of the UT Measurement and Controls Engineering Center, for their support. I would also like to thank everyone at the UT College of Veterinary Medicine. In particular, I would like to thank everyone in the Pharmacology Laboratory for their help and patience. I would especially like to thank Dr. Mark Petersen for taking his time to train me in rodent surgical techniques.

Finally, I would like to thank all my friends and family who supported me while I completed my degree. I am very fortunate to have such a great group of friends. Most importantly, I would like to thank my wife, Tonya, for her loving support and encouragement.

ABSTRACT

The goal of this project was to develop a method to non-invasively estimate chloroaluminum sulfonated phthalocyanine (CASP) concentration in rat brain using laser-induced fluorescence. To achieve this goal, three objectives had to be met. First, a system and technique for measurement of the drug fluorescence in rat brain tissue had to be developed. Next, the concentration of CASP in the brain tissue had to be approximated. This was done using standard chemical extraction methods. Once, the concentration of drug was known, a relationship between the measured drug fluorescence and concentration had to be derived. By the completion of these objectives, a non-invasive method for determining drug concentration in rat brain tissue was developed. The area under the drug fluorescence measured from the brain tissue was plotted versus the estimated drug concentration in the tissue. Using least-squares approximation, the relationship between the area under the drug fluorescence and concentration was determined. The r^2 value for this approximation was 0.97867. Due to the limitations of the chemical extraction technique, this relationship was derived over a small range of concentration. If a more sensitive chemical extraction technique were developed, the range of estimated drug concentration using laser-induced fluorescence technique would be improved.

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CHAPTER 1

INTRODUCTION

1.1 Photodynamic Therapy

Photodynamic therapy (PDT) is a cancer treatment modality which utilizes the photoactivation of a sensitizer to destroy malignant tissue. The use of photosensitive drugs for both diagnosis and treatment began in the early 1900's.¹ However, it was not until the early 1960's that Lipson and others demonstrated effectiveness of hematoporphyrin derivative (HpD) as a photosensitizer to detect and treat malignant tumors in animal models.² During the 1970's, Dougherty and his coworkers pioneered the use of photodynamic therapy in the treatment of human malignant tumors.³ Since that time, numerous human malignancies have been treated with PDT.

PDT utilizes the phototoxicity of a sensitizer to destroy the cancerous tissue. Light absorption by the photosensitizer causes it to be excited to its triplet state. Singlet oxygen, a highly reactive and short-lived excited state of oxygen, is produced by an energy transfer process from the triplet state of the photosensitizer to molecular oxygen.⁴ The exact mechanism leading to tissue destruction is currently under investigation. Many researchers believe it is caused by PDT-induced vascular damage.⁵ Therefore, three components are

required for photodynamic therapy: a photosensitizing drug, light of appropriate wavelength, and oxygen. All of these components influence the effectiveness of PDT.

1.2 Photosensitizers

Hematoporphyrin derivative (HpD) has been purified and is currently called porfimer sodium. It is manufactured by Quadra Logic Technologies (Vancouver, B. C., Canada) under the tradename of Photofrin®. Photofrin® is the first photosensitizer approved by the Food and Drug Administration (FDA) for use in human clinical trials. A disadvantage of Photofrin® is prolonged skin photosensitivity.⁶ This skin photosensitivity requires the patients to avoid direct sunlight for a period of four to six weeks after injection. In addition, the absorption peak for Photofrin® is low at a clinically-usable wavelength.⁷ Due to these disadvantages, many second-generation photosensitizers, such as chloroaluminum sulfonated phthalocyanine (CASP), are currently being investigated.^{8, 9}

Three important properties of photosensitizers are: preferential tumor uptake, phototoxicity, and fluorescence.¹⁰ An ideal photosensitizer preferentially concentrates in the cancerous tissue while clearing normal tissue completely. Typically, malignant tissue and some normal tissue margins are illuminated with laser light for tumor destruction. Since the concentration of photosensitizer in

the malignant tissue is higher, the tumor can be destroyed with little damage to the surrounding normal tissue. Localized treatment can be delivered by controlling the beam size and pattern of illumination. If the area is excited with light of the appropriate wavelength, the fluorescence of the photosensitizer can be detected. Using the fluorescence emission, the location, size, and boundaries of the tumor can be determined.

1.3 Laser-Induced Fluorescence

The idea of using laser-induced fluorescence (LIF) for diagnosis and localization of tumors was realized in the 1940's.¹¹ LIF emission is employed to examine the spatial fluorescence distribution of a photosensitizer using either an imaging system or nonimaging system. Typically, the excitation light is delivered to the tissue via an optical fiber. This excitation light is absorbed by the photosensitizer and host tissue resulting in fluorescence. Since the photosensitizer concentrates preferentially in tumor tissue, the fluorescence signal of the drug can be used to determine tumor boundaries.

Most of the previous research has focused on the detection of malignant tissue by utilizing the difference between drug fluorescence in normal and tumor tissue.^{12, 13, 14} However, it is more useful to have a quantitative estimate of the drug concentration. The standard method of determining drug concentration requires the removal and examination of the tissue. Panjehpour and others

reported using LIF to estimate drug concentration in various rat tissues.¹⁵

However, the study did not include brain tissue. Development of a non-invasive quantitative method for estimation of photosensitizer concentration in rat brain tissue would be a valuable tool for planning of PDT.

1.4 Objectives

The first objective of this study was to develop a technique for measurement of photosensitizer fluorescence in normal rat brain using laser-induced fluorescence. The second objective was to determine drug concentration in brain tissue using a chemical extraction technique. Finally, the correlation between LIF emission of the drug and its concentration was determined.

CHAPTER 2

MATERIALS AND METHODS

2.1 Photosensitizer

The photosensitive drug used for this study was chloroaluminum sulfonated phthalocyanine (CASP) (Porphyrin Products, Inc., Logan, Utah). CASP is a second-generation photosensitizer which has several advantages over Photofrin[®]. The absorption spectrum for CASP can be seen in Figure 1. The main absorption peak of CASP is at 680 nm. This wavelength is used during PDT for tissue destruction. The lower peak at 610 nm is used during LIF.

The mechanisms for both PDT and LIF can be seen in Figure 2. First, CASP is intravenously injected into the patient. Following the injection, the drug is distributed in both normal and cancerous tissues. Given sufficient time, the drug preferentially concentrates in the tumor tissue. If the area is illuminated with 610 nm light, the fluorescence of CASP can be detected at approximately 680 nm. If the area is illuminated with 680 nm light, the phototoxicity of CASP destroys the tumor.

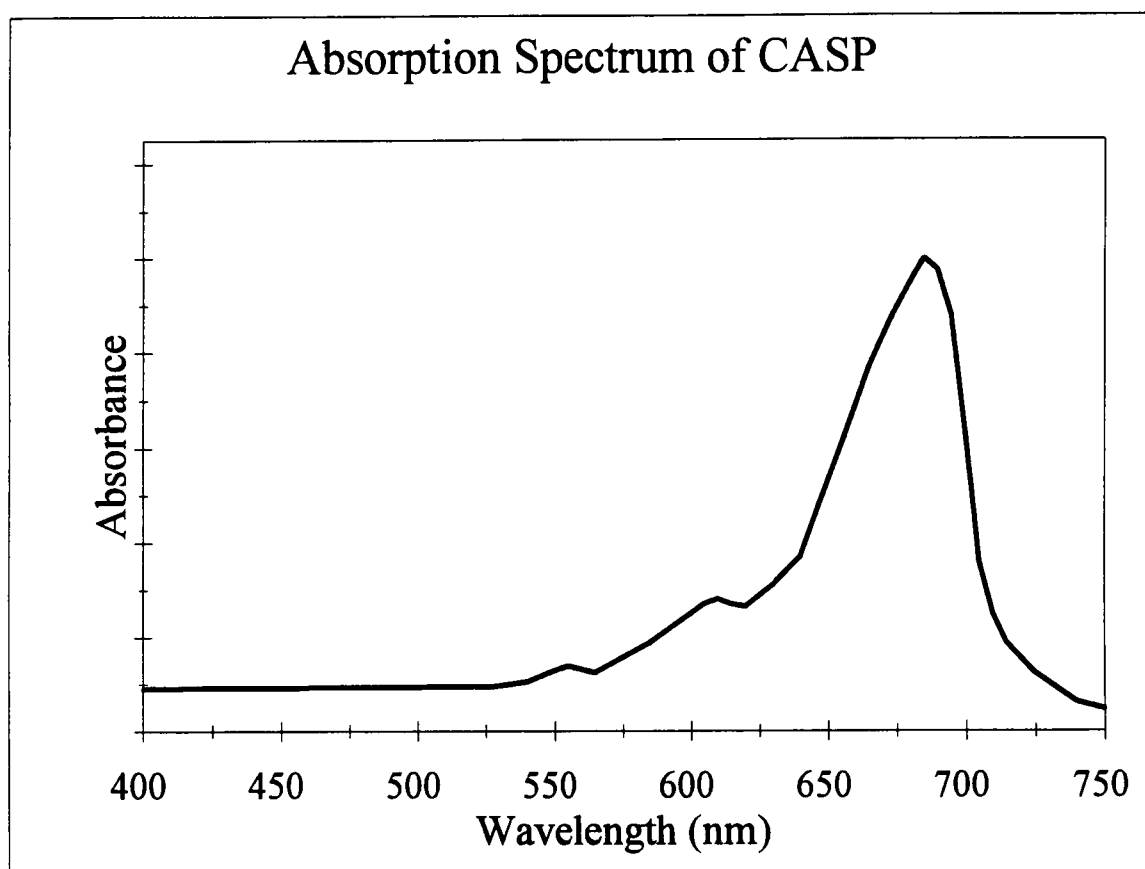


Figure 1. Absorption Spectrum of CASP. Note the small peak at 610 nm.

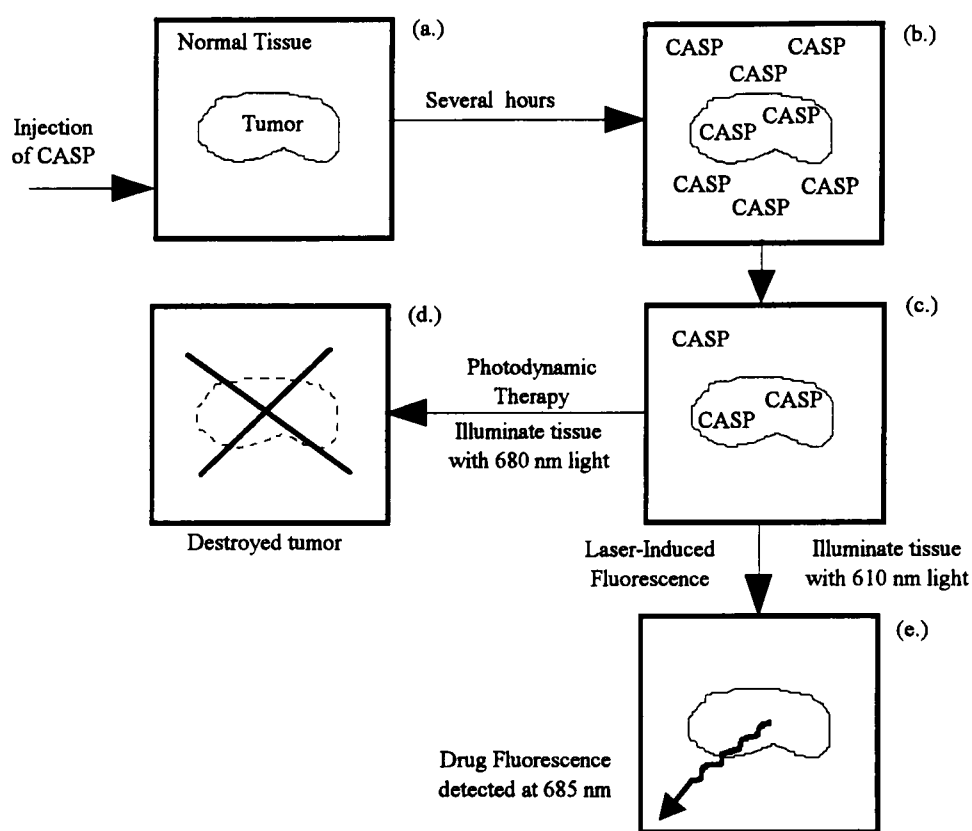


Figure 2. Mechanisms for PDT and LIF. a.) CASP is intravenously injected. b.) CASP is distributed throughout both normal and cancerous tissue. c.) Given sufficient time, CASP preferentially concentrates in the tumor tissue. d.) If the tumor and surrounding area are illuminated with 680 nm light, the tumor is destroyed. e.) If the tumor and surrounding area are illuminated with 610 nm light, the CASP fluorescence can be detected at 680 nm.

2.2 Animal Model and Tissue Preparation

Forty-eight female Sprague-Dawley rats, weighing between 275 and 375 grams, were purchased from Taconic Farms (Germantown, New York). The rats were divided into six groups with eight rats per group. One group of eight rats was used as a control group. The control group was not injected with CASP. The other groups of rats were euthanized at various times post-injection of CASP. These post-injection times were 1 hour, 4 hours, 8 hours, 24 hours, and 48 hours.

The groups of rats were anesthetized with an intramuscular injection of acepromazine and ketamine. While under anesthesia, the rats were injected with 5.0 mg/kg of CASP via the tail vein. The drug was prepared with sterile water at a concentration of 2.0 mg/ml. The drug was injected over several minutes so as not to damage the vein. Approximately 0.3 cc of heparinized saline was injected into the tail vein before and after the CASP injection.

At times ranging from 1 hour to 48 hours post injection, the rats were anesthetized, and approximately 1.5 cc of blood was removed via the tail vein. The blood was stored in a heparinized vial to eliminate clotting. The blood sample was centrifuged at 2500 RPM for 10 minutes (Model RT6000B, DuPont Industries Inc., Wilmington, Delaware) to separate the plasma from the red blood cells. The plasma was drawn off using a sterile pipette and placed in a sterile vial. The rats were then euthanized in a CO₂ chamber before removing

the brain, liver, and spleen.

To remove the brain, the skin over the entire dorsal aspect of the skull and neck was removed. Next, the ligaments connecting the dorsal aspects of the first cervical vertebra and the occipital bone were transected. The meninges and spinal cord were then transected and the head and neck were flexed ventrally exposing the foramen magnum. Beginning at the lateral aspects of the foramen magnum, the occipital, temporal, and frontal bones were cut bilaterally and the calvaria removed. The meninges were reflected, and the tentorium osseum was cut along its lateral aspects. The brain was then removed from the skull via sequential transection of the cranial nerves. The weight of the brain was used to help expose the cranial nerves for transection. The brain was not handled during the removal procedure, and was placed on a sterile wet gauze.

To remove the liver and spleen, the skin over the peritoneal cavity was removed. Next, a vertical incision approximately one inch in length was made at the midline of the peritoneal cavity. Using forceps, the muscles were gently pulled up so that further incisions would not damage the organs in the cavity. The vertical incision was then lengthened up to the sternum. At the sternum, horizontal incisions were made so the tissue could be spread back to expose the peritoneal cavity. Using forceps, a lobe of the liver was removed. The approximate weight of the liver tissue removed was 2 grams. The entire spleen was removed by cutting it away from the attached tissues.

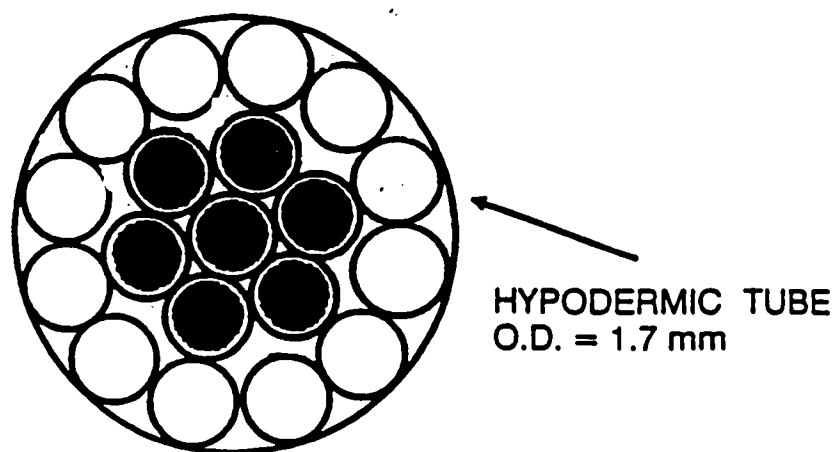
2.3 Laser-Induced Fluorescence Measurement System

2.3.1 Overall System Description

The excitation source for this study was a nitrogen-pumped dye laser (Model VSL-337ND, Laser Science, Inc., Cambridge, Massachusetts). The laser delivered 3 ns pulses of excitation light. Rhodamine 610, which has a peak wavelength at 595 nm, was used for the dye. The dye laser was tuned to 610 nm. The maximum repetition rate for this laser was 20 Hz.

The laser beam was coupled via a quartz lens to the excitation fiber of a fluorescence probe (C Technologies, Verona, New Jersey). The fluorescence probe consisted of seven 200 μ m fibers used for delivery of excitation light and twelve 200 μ m fiber used for collection of the fluorescence emission. The distal end of the probe was encased in a 10 mm long hypodermic needle with an outside diameter of 1.7 mm. Figure 3 shows the arrangement of the fibers in the fluorescence probe.

The output end of the collection fibers was arranged in a linear array. The linear array of the collection fibers formed the input to the entrance slit of an $f/3.8$, 0.28-m triple grating spectrograph (Model 1235, EG&G Princeton Applied Research, Princeton, New Jersey). A grating with 150 grooves/mm was used for this study. The spectrally-dispersed emission spectrum was imaged on a gated, intensified 1,024 diode array detector



- EMISSION FIBERS
- EXCITATION FIBERS

Figure 3. Fluorescence Probe. Cross-section of fluorescence probe showing the distribution of the excitation and emission fibers.

(Model 1456B-990-HQ). The detector was controlled by an optical multichannel analyzer (Model 1461/88 OMA[®] III, EG&G Princeton Applied Research, Princeton, New Jersey). The laser pulse and gating of the intensified diode array were synchronized by programming a 300 ns delay between the laser trigger and detector activation. The array was gated for 100 ns during which the 3 ns laser pulse was delivered to the tissue. The emission spectra were stored in a file on a personal computer (PS/2 Model 30, IBM Corp., Armonk, New York) for subsequent analysis. Figure 4 is a schematic of the entire system used to measure laser-induced fluorescence.

2.3.2 Spectral Calibration of Diode Array

To accurately determine the wavelengths of light emitted from the tissue, the diode array was calibrated using a mercury/argon spectral calibration lamp (Model 6035, Oriel Corp., Stratford, Connecticut). The software was set to detect the spectrum produced by the mercury/argon lamp. The grating of the spectrograph was rotated such that the excitation wavelength of 610 nm would be shifted 20 nm off the intensified diode array. To achieve this, the center wavelength of the spectrograph was placed at 940 nm.

Using the published spectrum of the mercury/argon lamp and the built-in calibration subroutine supplied with the software, the diode array was calibrated. The system subroutine uses a two-point linear fit method of calibration. Because linearity is greatest between the two calibration points, these points

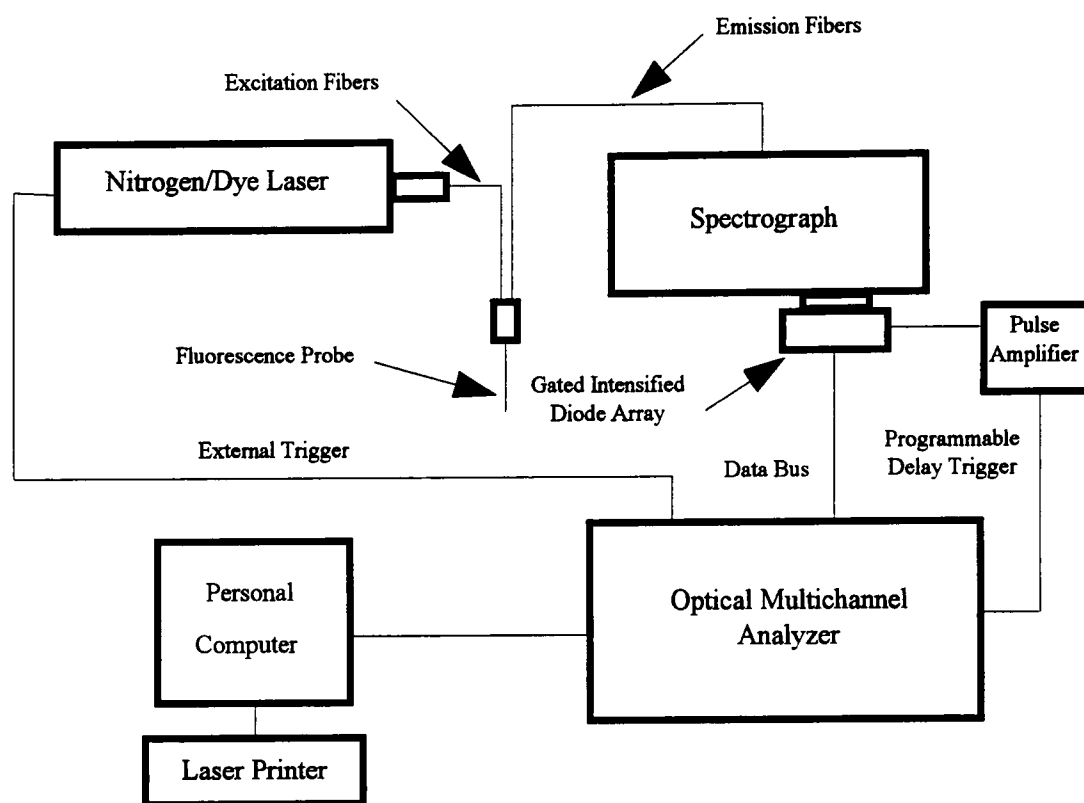


Figure 4. Schematic Diagram of LIF Measurement System.

should be as far apart as possible and should bracket the spectral lines of interest in the actual measurement. Therefore, the two peaks chosen were 696.54 nm and 912.25 nm. Since the center wavelength was set to 940 nm, the range of calibration was from 635 nm to 1,245 nm. The distance between data points was approximately 0.596 nm because the 1,024 channels of the diode array were evenly divided over this range.

To verify the accuracy of the calibration of the diode array, the spectrum emitted by the mercury/argon lamp was again detected by the fluorescence probe and stored. Figure 5 shows the spectrum obtained by the OMA[®] III system. The peak wavelengths of this spectrum agreed closely to the peaks of the standard mercury/argon lamp spectrum.

2.3.3 Laser Pulse Energy Measurement

The fluorescence emission intensity is directly proportional to the excitation light energy. Therefore, the excitation light energy should be measured to normalize all fluorescence intensities with respect to excitation energy. Normalization compensates for the daily changes in laser pulse energy.

The pulse energy from the distal end of the fluorescence probe was measured using a single channel energy meter (Model RJ-7610, Laser Precision Corp., Utica, New York). One hundred pulses were collected, and the average and standard deviation was recorded before and after completion of the laser-induced fluorescence measurements. All fluorescence intensities were

Mercury/Argon Lamp Spectrum

Spectrograph set at 940 nm

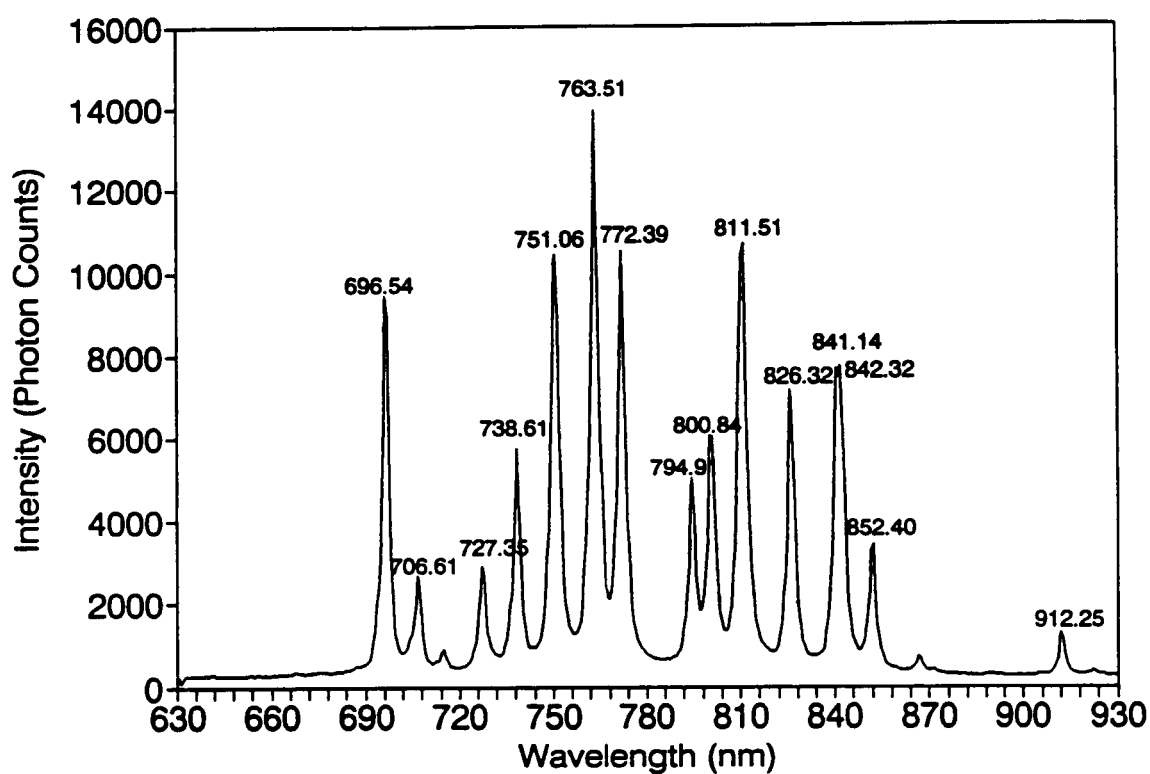


Figure 5. Spectrum of Mercury/Argon Lamp. This spectrum was obtained using the calibrated system.

normalized with respect to the average pulse energy. Since 50 pulses were used for this study, all emission intensities are given in terms of intensity per 50 μJ of excitation energy.

2.4 Measurement of Tissue Sample Fluorescence

2.4.1 Composition of Groups

Although brain, liver, and spleen tissue samples were removed from all injected rats, some tissues were eliminated from the study due to blown veins during the injection. In such cases, a large area of the tail turned blue, the color of CASP. This blue color was the result of the CASP infiltrating into the cartilage cavity within the tail rather than into the vein.

Due to the above mentioned problem, two rats were eliminated from both the 1 hour post-injection group and the 4 hour post-injection group. Three rats were also eliminated from the 8 hour post-injection group. All rats for the 24 hour post-injection group were determined to be valid. Out of the eight rats to be used for the 48 post-injection group, only three were injected and euthanized. After comparing the 24 hour post-injection results with the initial 48 hour post-injection results, it was determined that this group did not add any information. Table 1 shows the composition of each group.

Table 1. Composition of Experimental Groups.

Experimental Group	Initial Number	Number Eliminated	Actual Number
Control	8	0	8
1 hr. Post-Injection	8	2	6
4 hr. Post-Injection	8	2	6
8 hr. Post-Injection	8	3	5
24 hr. Post-Injection	8	0	8
48 hr. Post-Injection	8	5	3

2.4.2 Measurement of Fluorescence

The fluorescence was measured immediately after the tissues were removed and placed on sterile wet gauze. The distal end of the fluorescence probe was lightly touched to the tissue. The OMA[®] III system was programmed to externally trigger the nitrogen/dye laser for 50 pulses of 610 nm light. The emitted fluorescence was collected for each laser pulse. The data were digitally accumulated and stored in a file. For every pulse of laser light, a background reading was subtracted from the accumulated data. This background subtraction allowed the measurements to be performed in room light without any background interference. Each reading was completed in less than 3 seconds. The measured fluorescence intensities in each organ were reproducible. The variability in fluorescence intensities was less than 10% within each organ.

For the brain, a total of eight measurements were taken. Two measurements were taken at four sites on the brain. Two sites were done on each hemisphere, one near the anterior aspect of the cerebral cortex and one near the posterior aspect. The average of these measurements was used for the fluorescence of each brain sample. For the liver and spleen, four measurements were taken for each tissue. The average was determined and used as the fluorescence signal for each tissue sample. Four fluorescence measurements were taken of the plasma by placing the tip of the fluorescence probe against the flat bottom of the vial. The average of the four fluorescence spectra was used for the fluorescence of the plasma sample. After all measurements were completed, the tissues and plasma were stored in a -70°C freezer.

Normalization of the fluorescence emission with respect to the intensity at 650 nm was also performed. This wavelength was chosen because it represents the scattering of the excitation light within the spectrograph. This scattering was important because the fluorescence of CASP is directly related to the excitation light. Shifting the spectrograph so that the first wavelength present on the diode array was 20 nm greater than the excitation wavelength decreased this scattering. Normalizing with respect to the scattering of the excitation light compensated for changes in the scattering component of the signal.

2.5 Chemical Extraction Technique

2.5.1 General Description

The chemical extraction technique used for this study was based on techniques used by Panjehpour et al.¹⁵ and Poon et al.¹⁶. In this technique, a known amount of drug is added to tissue samples. These spiked tissues simulate known tissue concentrations. The drug is then extracted from tissue samples by tissue homogenization in sodium hydroxide. The supernatant is separated and its fluorescence spectrum is measured. The fluorescence spectrum from control supernatant is also measured. The drug fluorescence is determined by subtracting the fluorescence spectrum of the control samples from the fluorescence spectrum of the spiked tissue samples. The area under the drug fluorescence spectrum is calculated for these known concentrations. By plotting the area under the fluorescence spectrum versus the concentration, a "standard curve" is derived. The drug concentration in unknown tissue samples is determined by comparing the area under their fluorescence spectra with the "standard curve".

2.5.2 Extraction from Spiked Brain Samples

Brain tissue samples weighing 0.2 g were dissected from the cortex of control brains. These tissue samples were spiked with CASP which resulted in tissue concentrations of 2 $\mu\text{g/g}$ (μgrams of CASP per gram of tissue), 1 $\mu\text{g/g}$, 0.5 $\mu\text{g/g}$, 0.25 $\mu\text{g/g}$, 0.125 $\mu\text{g/g}$. Three brain tissue samples were spiked for

each concentration. Microliter pipettes (Model 4810, Brinkman Instruments Inc., Westbury, New York) were used to carefully measure the amount of CASP used to spike each tissue sample.

Once the tissue absorbed the CASP, it was homogenized in 7.0 ml of 0.1 M sodium hydroxide for approximately two minutes. The suspension was centrifuged at 12,000 RPM for 10 minutes at 4°C in a refrigerated superspeed centrifuge (Model RC5B, DuPont Industries, Wilmington, Delaware). Using sterile glass pipettes, 2.0 ml of the separated supernatant from each sample was drawn into a sterile glass vial for fluorescence emission measurement.

The emission fluorescence of the supernatant was measured using the LIF measurement system. The distal end of the fluorescence probe was placed against flat surface of the vial. As with the previous LIF measurements, 50 scans were performed. Three measurements from each sample were taken. The average of the three measurements was stored in a file for subsequent analysis as the LIF emission for the sample. The LIF emission spectra were normalized with respect to laser energy as previously described.

2.5.3 Area Under the Curve Approximation

The areas under the LIF emission spectra were needed so that a correlation could be made between the area under the CASP fluorescence peak and CASP concentration. Since the fluorescence spectrum was stored as a series of data points, an approximation technique was used to determine the area under

the curve. Because of the ease of calculations, the Trapezoidal approximation technique from *Numerical Analysis* by Burden and Faires¹⁷ was used. Equation 1 shows the Trapezoidal approximation of the area between two consecutive data points. By continuing these calculations and adding the result for every two data points, the total area under the LIF emission spectrum was accurately approximated.

$$\int_{\lambda_1}^{\lambda_2} f(\lambda) d\lambda \approx \frac{\lambda_2 - \lambda_1}{2} \cdot (f(\lambda_1) + f(\lambda_2)) \quad (\text{Eq. 1})$$

Since the actual area of interest was the area under the CASP fluorescence peak, this approximation technique was used to determine the area under the curve from 650 nm to 750 nm. The area under the control LIF emission spectrum from 650 nm to 750 nm was subtracted so that the actual area of the CASP fluorescence was computed.

The area under the CASP fluorescence was plotted versus the known concentration of CASP in the tissue. From this graph, a relationship between the area of the CASP fluorescence and the concentration of CASP was derived. This relationship was the "standard curve" used to determine the concentration of CASP present in the unknown tissue samples.

2.5.4 Extraction from Unknown Brain Samples

Two brain tissue samples, weighing 0.2 g each, were dissected from the cortex of the brain of each injected rat. Twelve tissue samples were obtained

from both the 1 hour post-injection and 4 hours post-injection rat brains. Ten tissue samples were obtained for 8 hours post-injection rats. Sixteen tissue samples were obtained from 24 hours post-injection rats. Six tissue samples were obtained from 48 hours post-injection rats.

The brain tissue samples were homogenized in 7.0 ml of 0.1 M sodium hydroxide and centrifuged as previously described. 2.0 ml of supernatant from each sample was placed in a sterile glass vial. Using the LIF measurement system, the emission spectrum of each supernatant sample was measured and stored. These spectra were normalized with respect to both laser energy and the intensity at 650 nm.

Again, the Trapezoidal approximation technique was used to determine the area under the normalized emission spectra from 650 nm to 750 nm. The average area under the control emission spectra was subtracted so that the actual area under the CASP fluorescence was approximated. Using the "standard curve", the concentration of CASP in the unknown tissue samples was approximated.

2.6 Correlation of Data

Once the concentration of CASP in the unknown brain tissues was approximated, a correlation between the area under the LIF emission spectrum and CASP concentration was preformed. A discrete, least squares

approximation technique¹⁷ was used to determine the relationship between area under the LIF emission spectrum and CASP concentration. This approximation technique involves determining the best approximating line where the error involved is the sum of the squares of the differences between the values on the approximating curve and the given values. The coefficient of determination, r^2 , provides a measure of goodness-of-fit. If the coefficient of determination is equal to 1, then the line-of-best-fit explains 100% of the variability of the data.

The areas under the LIF emission spectra taken from the tissue samples were also plotted with respect to the post-injection time. This plot shows that clearance of the drug from the brain tissue. Since the concentration in brain tissue was exponentially related to time, these data were also plotted on a semi-log axis. This resulted in a linear relationship. The approximate exponential rate of clearance from brain tissue was calculated using the least-squares approximation technique. The same technique was used to approximate the clearance rate of the drug in the plasma.

CHAPTER 3

RESULTS AND ANALYSIS

3.1 Tissue and Plasma Fluorescence Measurements

3.1.1 Control Brain Tissue Fluorescence

The control group was formed by taking fluorescence measurements from brain tissue in rats that were not injected with CASP. The emission spectra from these measurements shows the scattering of the excitation light and the auto-fluorescence of normal brain tissue. Eight fluorescence measurements were performed on each brain sample. The measurements were reproducible as seen in Figure 6. This graph shows the average and sample standard deviation of eight fluorescence spectra in brain. The solid line in the middle represents the average of the eight measurements within one tissue sample. The solid lines above and below the average represent the average plus or minus one standard deviation. The low standard deviation compared to the average, shows the accuracy of the measurement technique within a given sample.

Fluorescence measurements from eight rats were used to form the control group. Figure 7 shows the average fluorescence spectra for each member of the control group. Every spectrum was normalized with respect to both the average laser energy and the intensity at 650 nm.

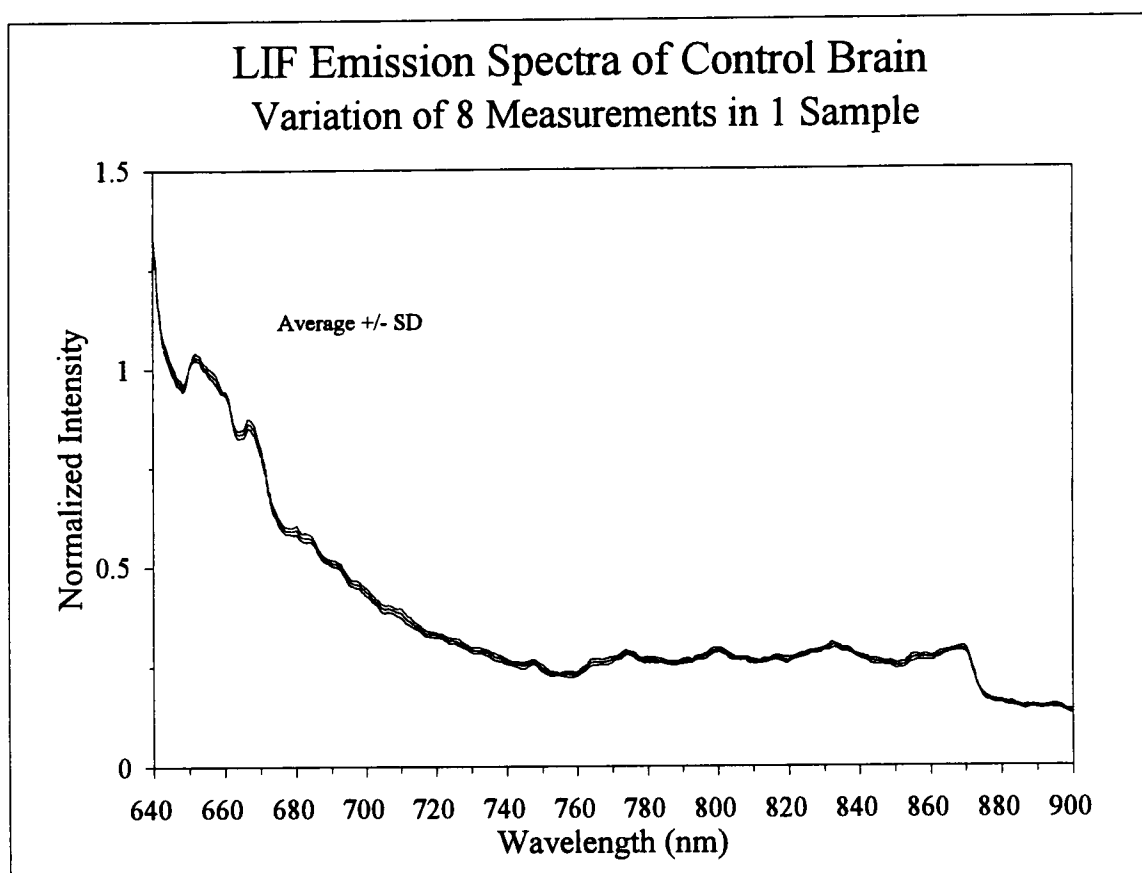


Figure 6. LIF Emission Spectra from Control Brain. This plot shows the average and standard deviation of the eight measurements from one brain sample. The standard deviation was calculated at each channel which results in the spectra above and below the average. The average of these eight measurements was used as the control spectrum for this rat.

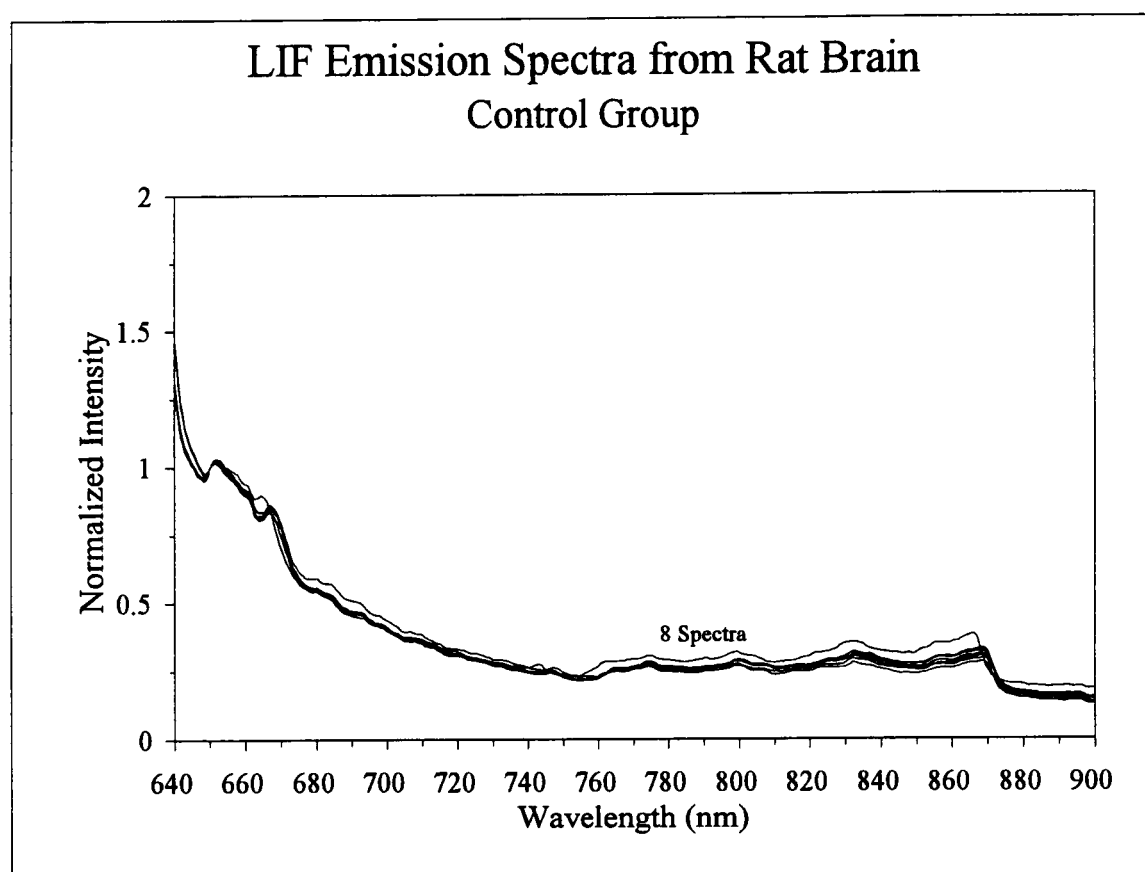


Figure 7. LIF Emission Spectra for Control Group. This plot shows the eight fluorescence spectra measured from the brain tissue of the control rats. Each spectrum is the average of eight measurements.

To show the small variation between the eight control rats, the average of the control group, the average plus one sample standard deviation, and the average minus one sample standard deviation were plotted. These three fluorescence spectra are shown in Figure 8. The average and sample standard deviation were calculated for each channel.

The control average fluorescence was used for two types of analysis. First, the entire control average spectrum was subtracted from the spectra of the CASP-injected groups. This subtraction was used to show the CASP fluorescence without the autofluorescence of the brain tissue. Secondly, the areas under the background spectra were subtracted from the areas under the CASP-injected groups so that the actual areas of CASP fluorescence could be calculated. The Trapezoidal approximation technique was used to calculate the area under the fluorescence emission spectrum. Using this technique, the area under the average control emission from 660 nm to 750 nm was calculated.

3.1.2 CASP Fluorescence in Brain Tissue

The LIF measurement technique was performed on the brain tissue of five groups of rats with post-injection times of 1 hour, 4 hours, 8 hours, 24 hours, and 48 hours. Figure 9 shows the normalized fluorescence spectra from the six brain samples removed 1 hour post-injection of CASP and the average control fluorescence emission. The peak of the CASP fluorescence occurred at approximately 685 nm. The normalized intensity at 685 nm ranged from 1.375

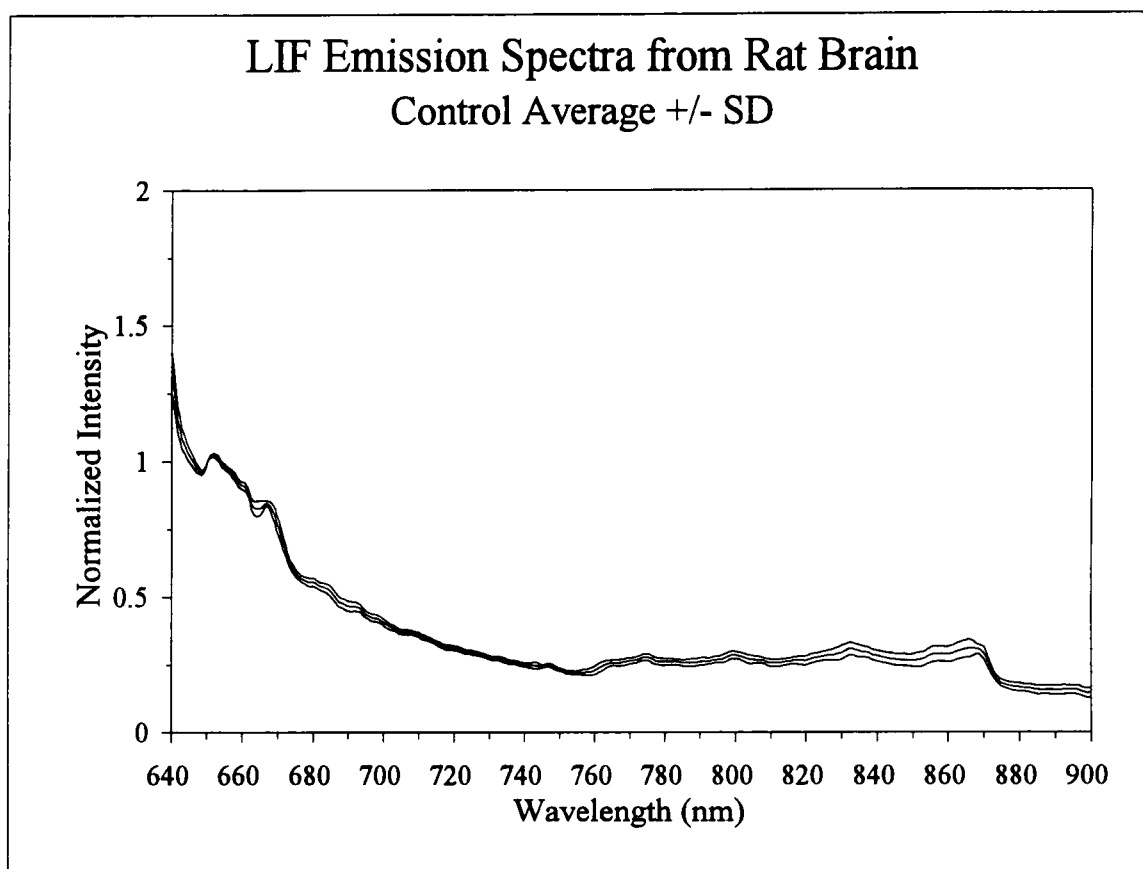


Figure 8. Average LIF Emission from Control Group. This plot shows the average fluorescence of brain tissue from the control group. Also plotted is the standard deviation for the control group.

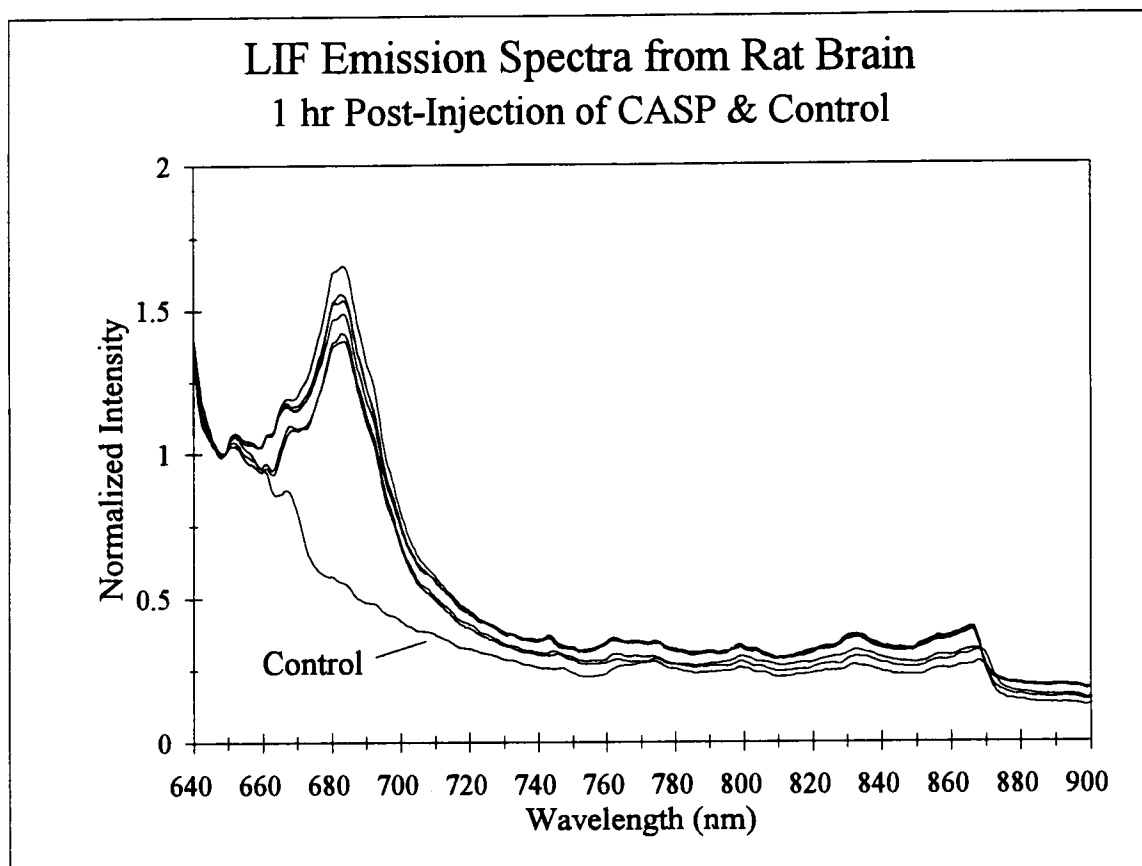


Figure 9. LIF Emission from 1 hour Post-Injection Rats. This plot shows the six fluorescence spectra from the 1 hour post-injection rats. The control average was also plotted. Each peak is located at approximately 685 nm.

to 1.7.

Figure 10 shows the normalized fluorescence spectra from the six brain samples removed 4 hours post-injection. Again, the average control emission was also plotted in this graph. The CASP fluorescence from these tissues was lower than the CASP fluorescence obtained from the 1 hour post-injection tissues. Again, the peak of the CASP fluorescence was present at 685 nm. At 4 hours post-injection, the normalized intensity at 685 nm ranged from 0.9 to 1.25. This decrease in fluorescence from 1 hour post-injection shows that the concentration of CASP in the brain tissue is decreasing with time.

Figure 11 shows the normalized fluorescence spectra from the five brain samples removed 8 hours post-injection and the average control emission. The CASP fluorescence continued to decrease, but at a slower rate. The peak was still present at 685 nm and the range of normalized intensity was from 0.74 to 0.95.

Figure 12 shows the normalized fluorescence spectra from the eight brain samples removed 24 hours post-injection of CASP. The control average was again plotted. The CASP fluorescence was detected between approximately 665 nm and 720 nm. From this graph, one can see that the CASP fluorescence signal was approaching the detection threshold. If the CASP fluorescence continues to decrease, it will be difficult to distinguish it from the control fluorescence. The peak of the CASP fluorescence was still present at 685 nm

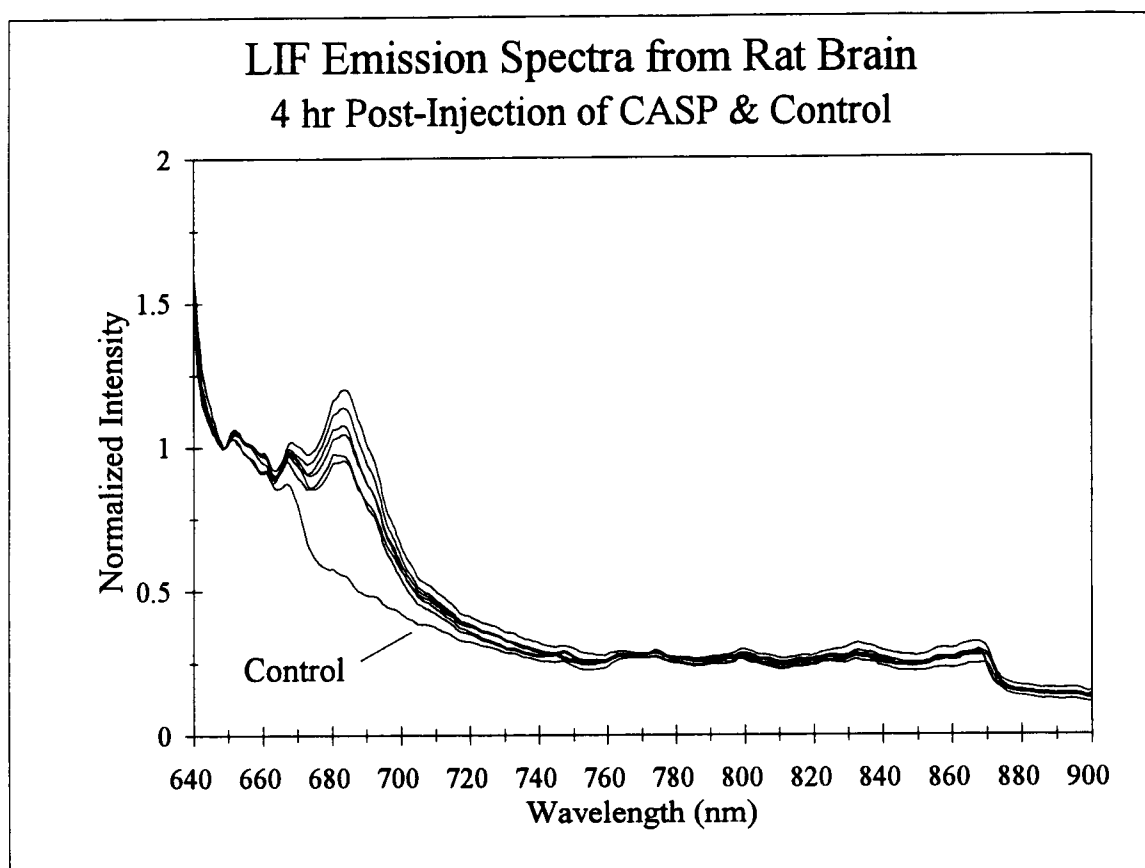


Figure 10. LIF Emission from 4 hours Post-Injection Rats. This plot shows the six fluorescence spectra from the 4 hours post-injection rats. The control average was also plotted. Each peak is located at approximately 685 nm.

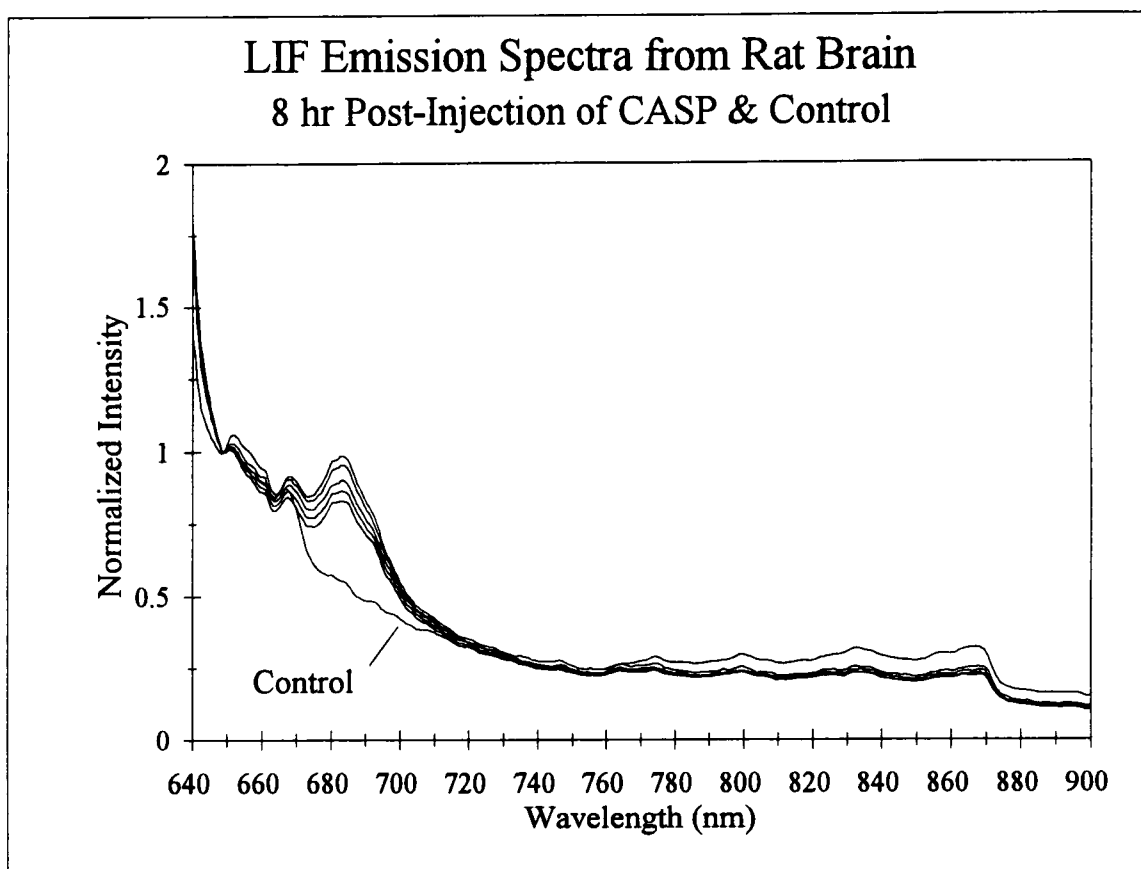


Figure 11. LIF Emission from 8 hours Post-Injection Rats. This plot shows the five fluorescence spectra from the 8 hours post-injection rats. The control average was also plotted. Each peak is located at approximately 685 nm.

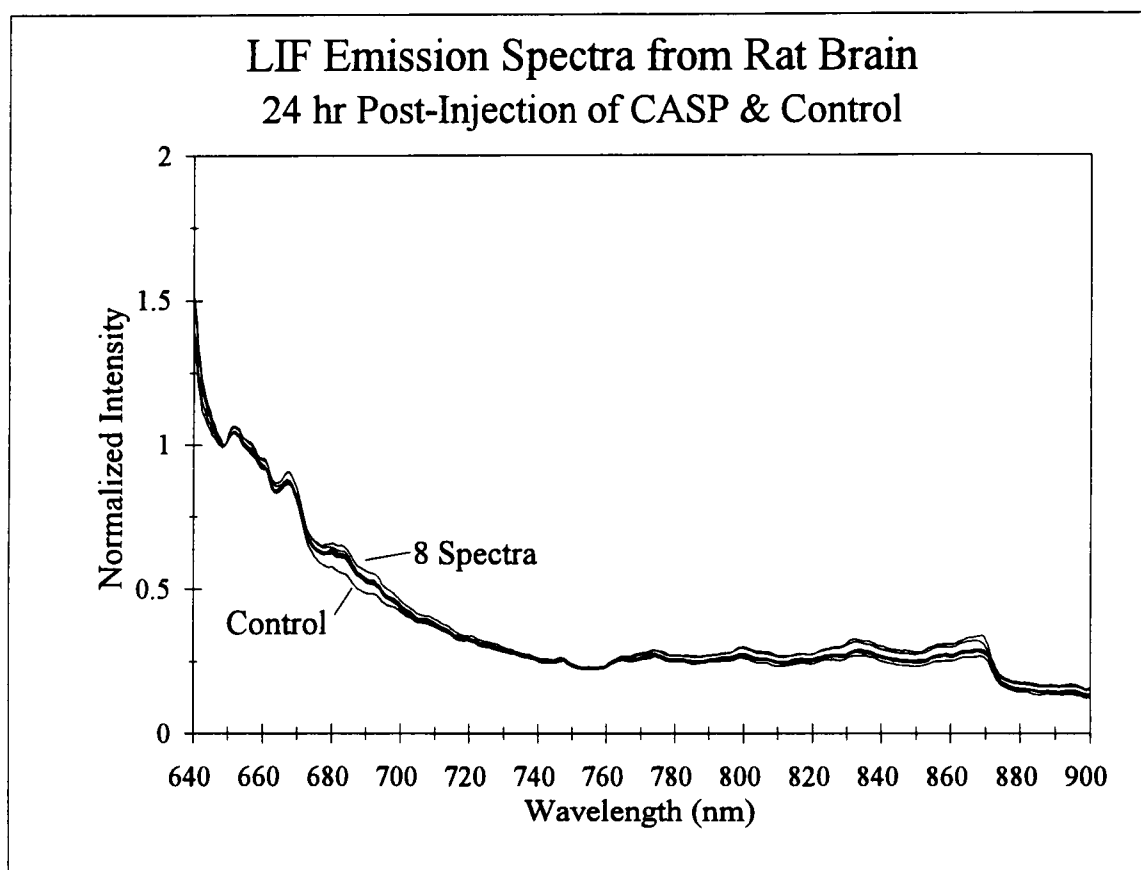


Figure 12. LIF Emission from 24 hours Post-Injection Rats. This plot shows the eight fluorescence spectra from the 24 hours post-injection rats. The control average was also plotted.

with a range of normalized fluorescence intensity from 0.6 to 0.65.

Figure 13 shows the normalized fluorescence spectra from three brain samples removed 48 hours post-injection of CASP. These fluorescence spectra were similar to those from 24 hours post-injection. The CASP fluorescence was slightly larger than the control fluorescence. A small decrease in fluorescence was seen between the 24 hours and 48 hours post-injection samples. This decrease was close to the signal variations from sample-to-sample. Only three measurements were performed since no additional information was obtained from animals in this group. Therefore, the remaining five rats initially assigned to this group were reassigned. These five rats were used as control tissues for the chemical extraction technique.

Figure 14 shows the average fluorescence spectrum from each group. The decrease in CASP fluorescence as a function of time can be seen. The average CASP fluorescence from each group was determined by subtracting the control average from the average fluorescence spectrum from each injected group. The result of this subtraction can be seen in Figure 15. This graph shows that the CASP fluorescence at 1 hour, 4 hours, and 8 hours are much stronger than at 24 hours and 48 hours. Although the CASP fluorescence was weaker at 24 hours and 48 hours, a distinct peak was still present at 685 nm.

The clearance rate of the CASP was approximated by plotting the area under the CASP fluorescence versus time. Figure 16 shows the area from each

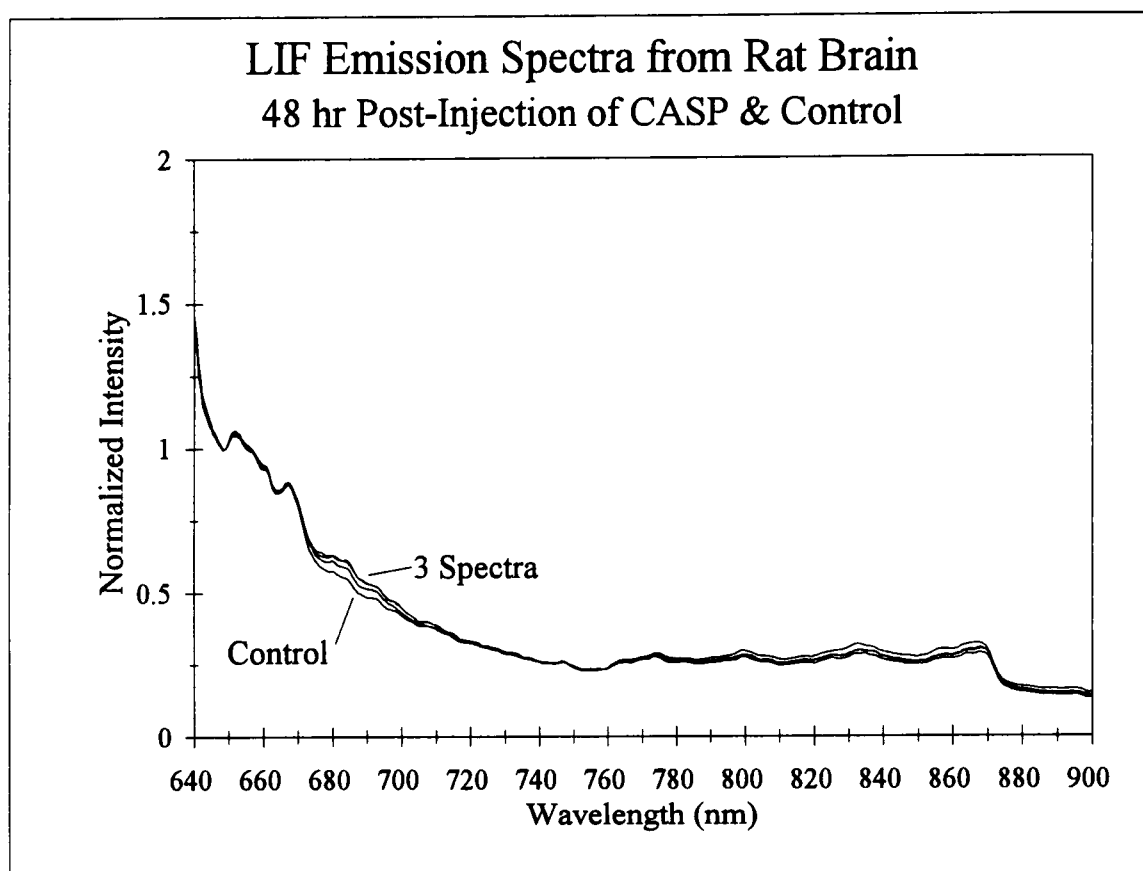


Figure 13. LIF Emission from 48 hours Post-Injection Rats. This plot shows the three fluorescence spectra from the 48 hours post-injection rats. The control average was also plotted.

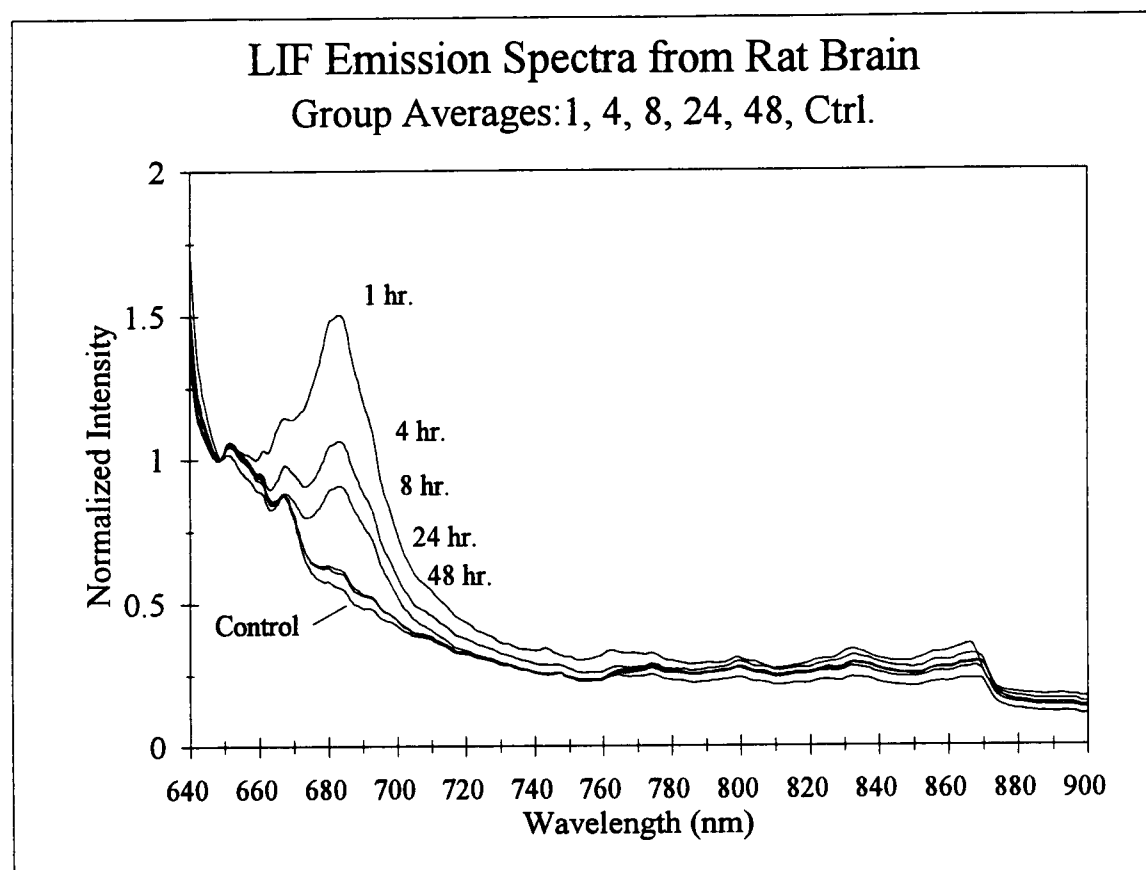


Figure 14. Group Averages of LIF Emission. This plot shows the average fluorescence emission for each group.

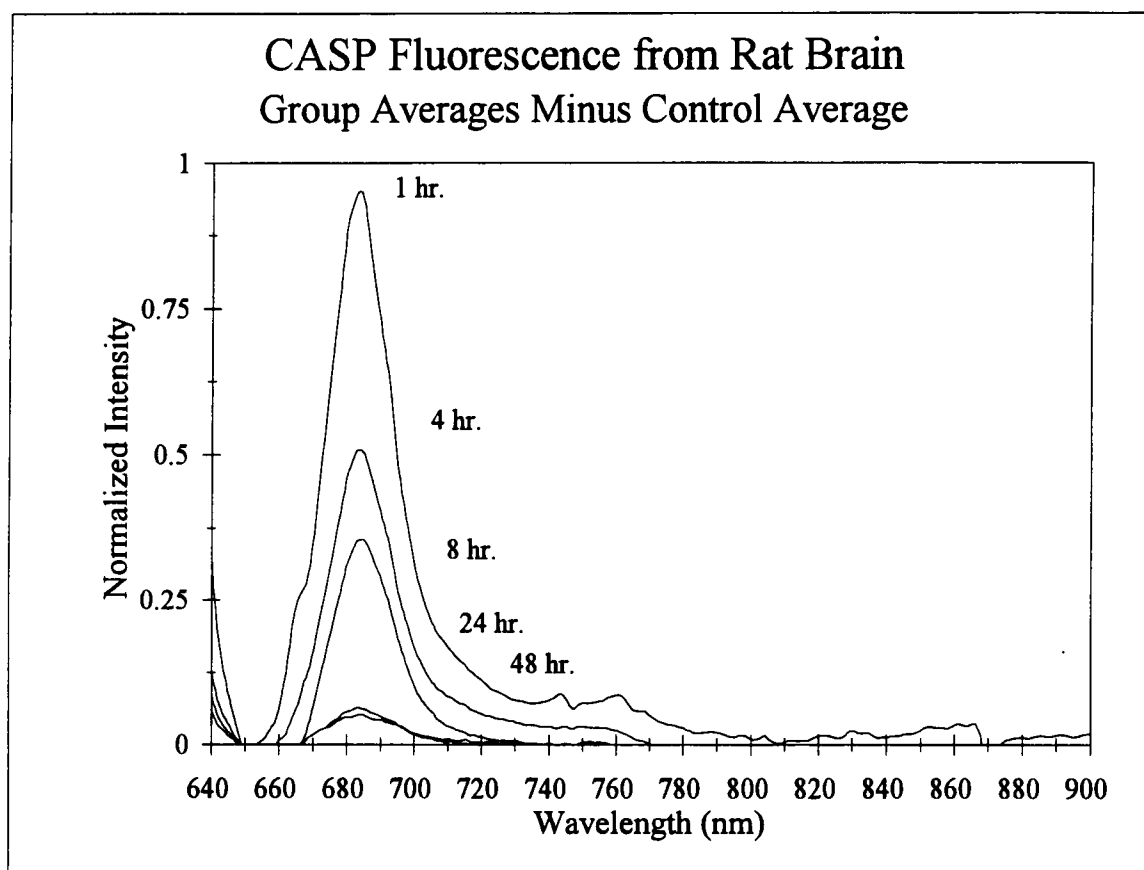


Figure 15. Average CASP Fluorescence from Each Group. This plot shows the average CASP fluorescence from each group. The CASP fluorescence was calculated by subtracting the average control fluorescence spectrum from each group average. Each peak is located at approximately 685 nm.

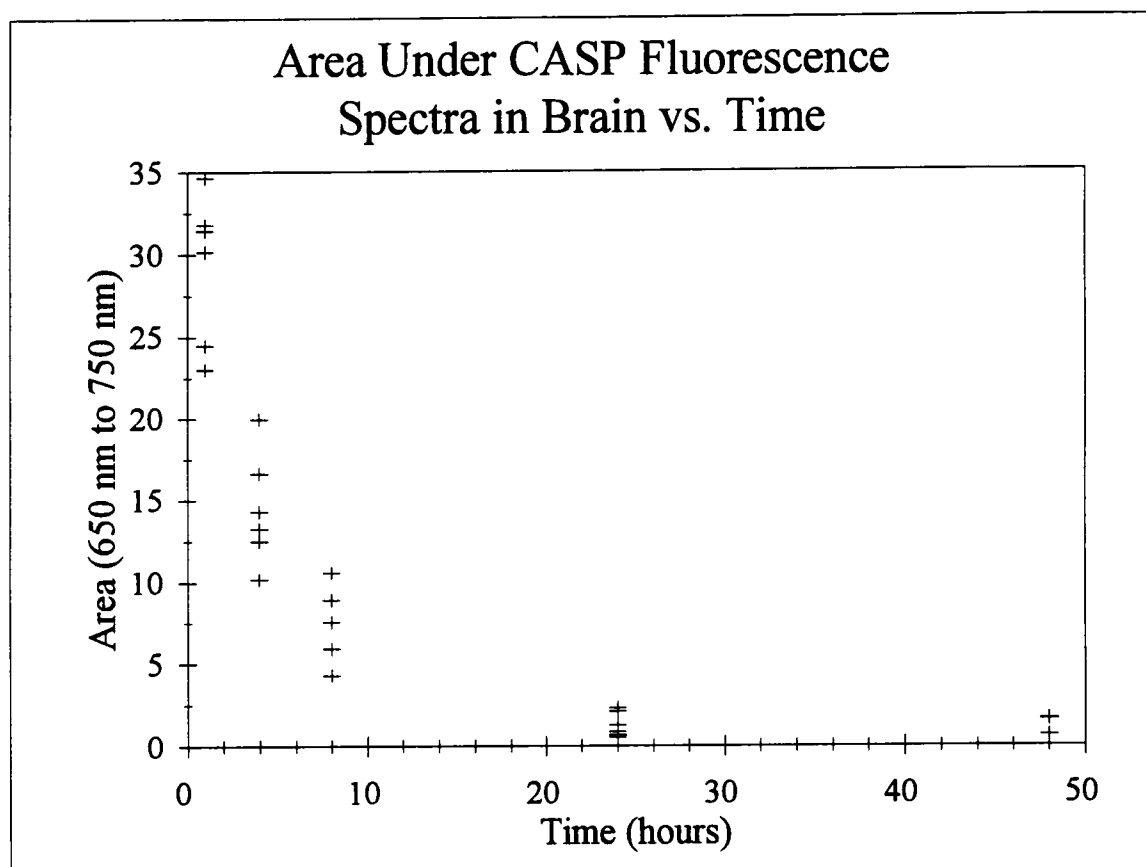


Figure 16. Area Under CASP Fluorescence in Brain versus Time. The area under the CASP fluorescence was calculated from each measured spectrum. The range of data points represent each post-injection group.

CASP fluorescence spectra at various times. The data appeared to have an exponential relationship. To determine if the clearance rate was truly exponential, the data were plotted on a semi-log axis as seen in Figure 17. From the linear relationship of the data points, it appears that the CASP fluorescence exponentially decreases over the first 24 hours. However, after 24 hours, the decrease does not appear to fit the exponential relationship. The CASP fluorescence at 48 hours is very close to the fluorescence at 24 hours.

An approximation of exponential relationship was calculated from the 1 hour, 4 hour, 8 hour, and 24 hour data. Since the data were assumed to be exponentially related, the approximating function was of the form:

$$y = b \cdot e^{a \cdot x} \quad (\text{Eq. 2})$$

The logarithm of the approximating equation becomes:

$$\ln(y) = \ln(b) + a \cdot x \quad (\text{Eq. 3})$$

The $\ln(b)$ represents the y-intercept of the data and a represents the slope of the line. Using the natural logarithm of the y data and Equation 3, solutions for $\ln(b)$ and a were be obtained. This resulted in the line-of-best-fit through the data points shown in Figure 17. From this relationship, the clearance rate of the CASP from the brain tissue was approximated. The rate was the slope of the straight line approximation and the constant coefficient was natural logarithm of the y-intercept. This rate of clearance was compared to the rate determined

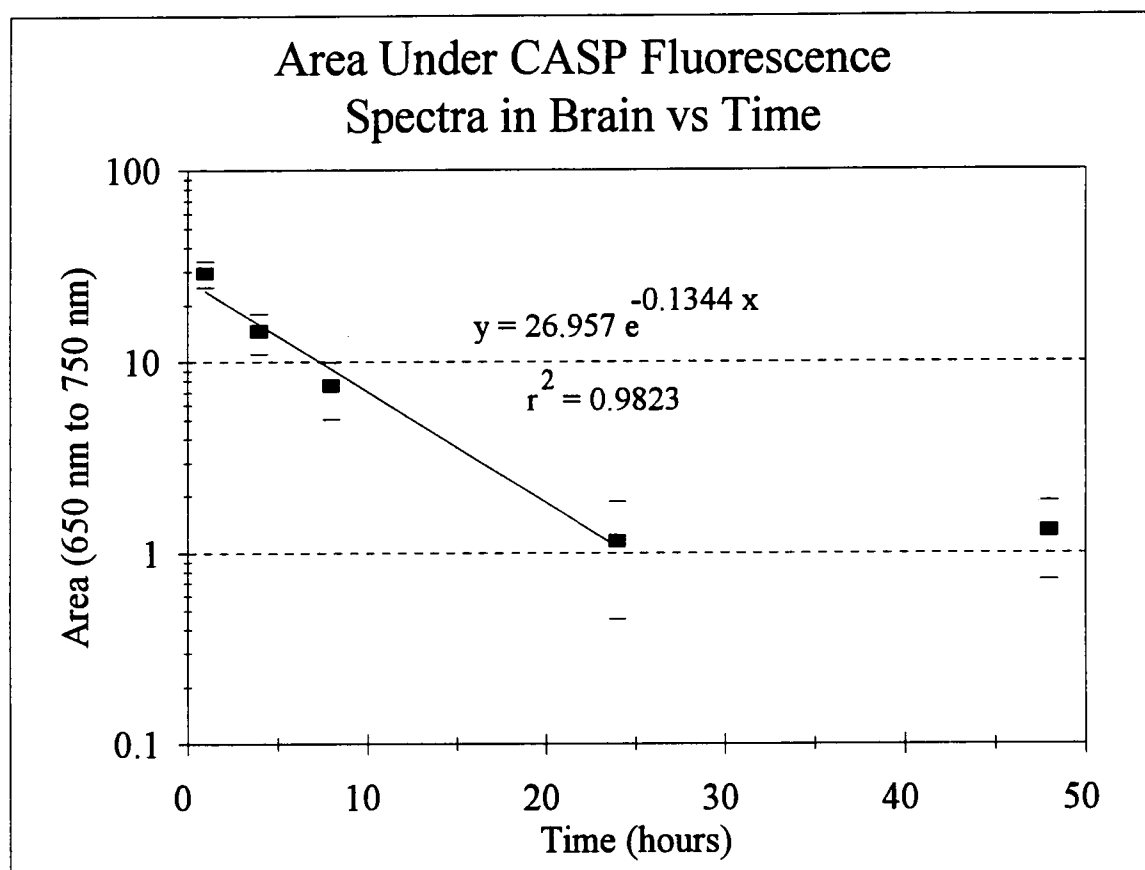


Figure 17. Area Under Average CASP Fluorescence in Brain versus Time. This semi-log plot shows the area under the average CASP fluorescence for each post-injection group. The exponential relationship between area under CASP fluorescence and time was approximated over the first 24 hours.

from the plasma fluorescence.

3.1.3 CASP Fluorescence in Plasma

The CASP fluorescence from plasma was measured by placing the fluorescence probe against the flat wall of the glass vial containing the plasma. Figure 18 shows the normalized fluorescence spectra of CASP in plasma. The four spectra are the averages of measurements from each group of samples. The average spectrum from the 48 hours post-injection group was not plotted because it showed no detectable CASP fluorescence. From Figure 18, one can see how much easier it was to detect the CASP fluorescence from the plasma.

The areas under these fluorescence spectra were approximated and plotted with respect to time. Figure 19 shows the semi-log plot of the CASP fluorescence area versus time. Again, the relationship was confirmed to be exponential by plotting a line-of-best-fit through the semi-log plot of the data. From the slope and intercept of this line, the clearance rate of CASP from the plasma was approximated. Figure 20 shows the semi-log plot of the area under the CASP fluorescence versus time for both brain tissue and plasma. Since these exponential relationships appear as approximate parallel lines on the semi-log plot, the clearance rates in brain and plasma are similar.

3.1.4 CASP Fluorescence in Liver and Spleen Tissue

Figure 21 and Figure 22 show the fluorescence of CASP in liver and spleen tissue, respectively, for various time intervals post-injection. These

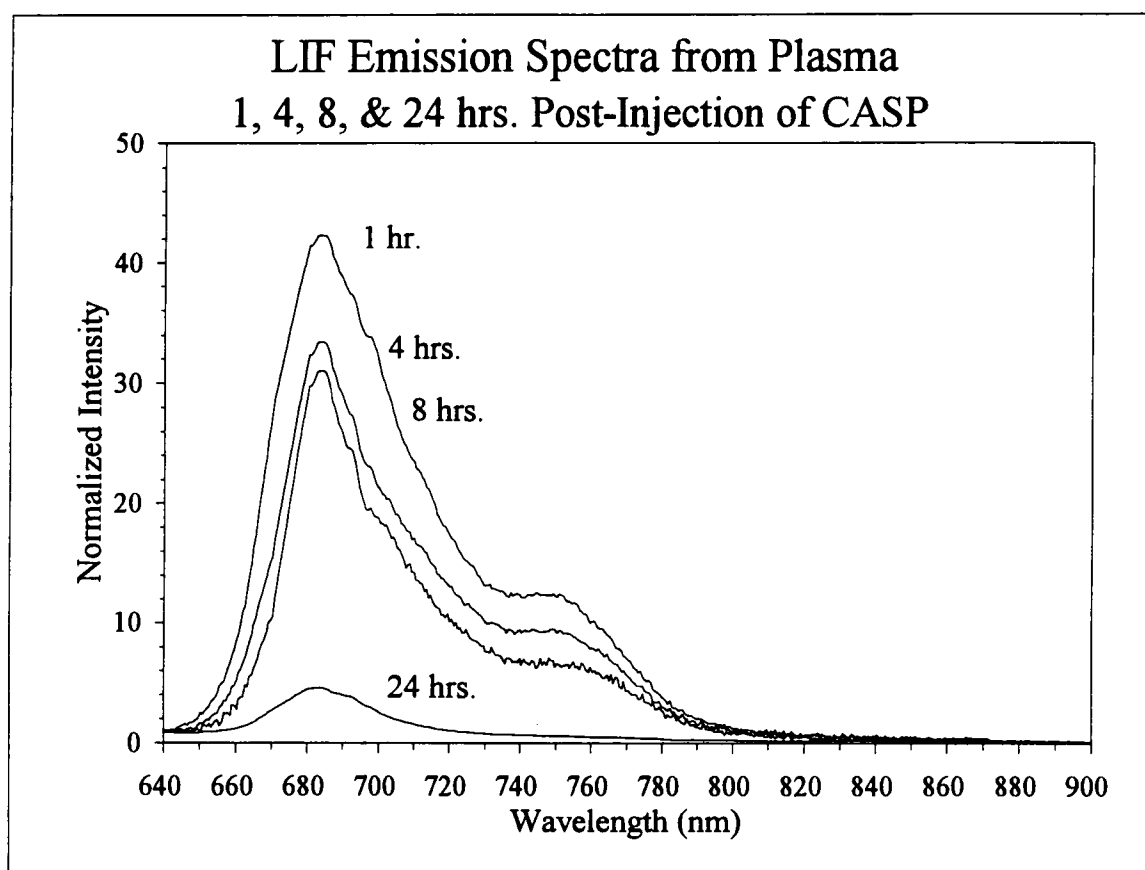


Figure 18. LIF Emission from Plasma. This plot shows the CASP fluorescence from plasma at various times post-injection. Notice how even 24 hours post-injection the CASP fluorescence is easily detectable without any subtraction.

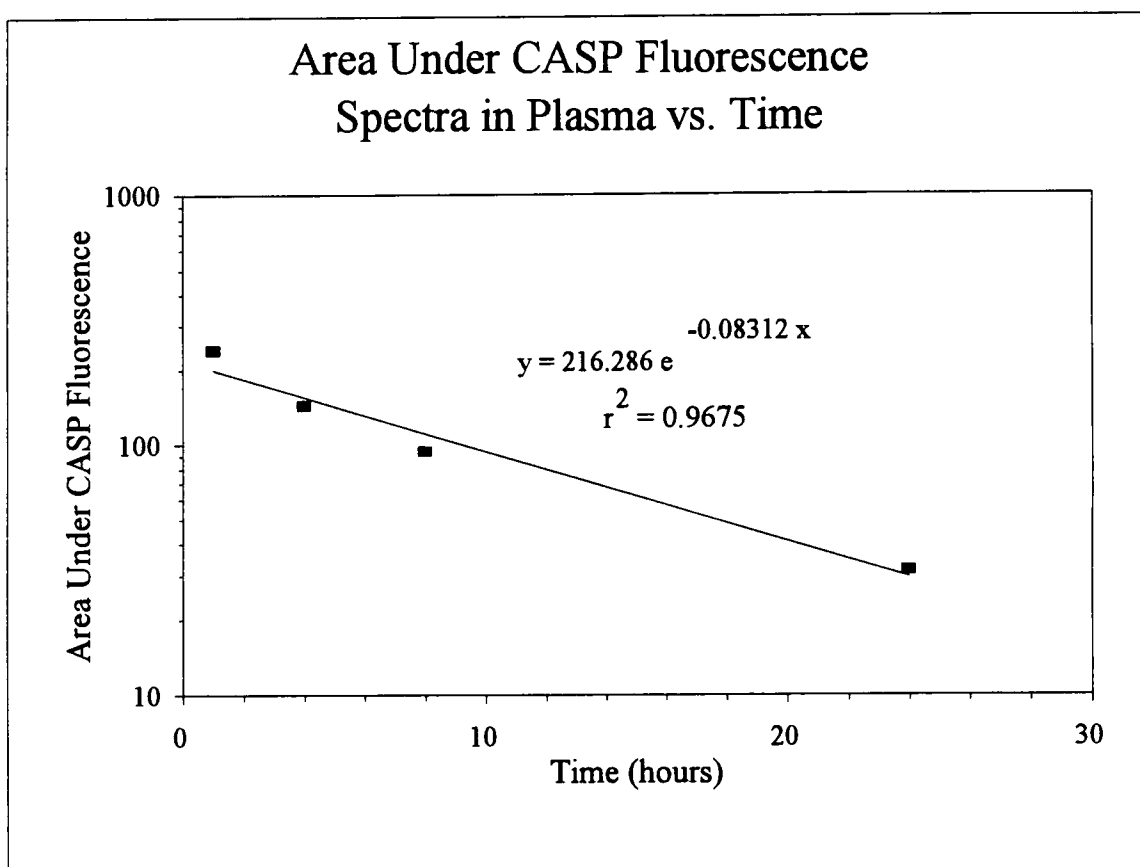


Figure 19. Area Under CASP Fluorescence from Plasma versus Time. This plot shows the area under the CASP fluorescence versus time. The time scale ends at 24 hours because the CASP fluorescence was undetectable at 48 hours post-injection.

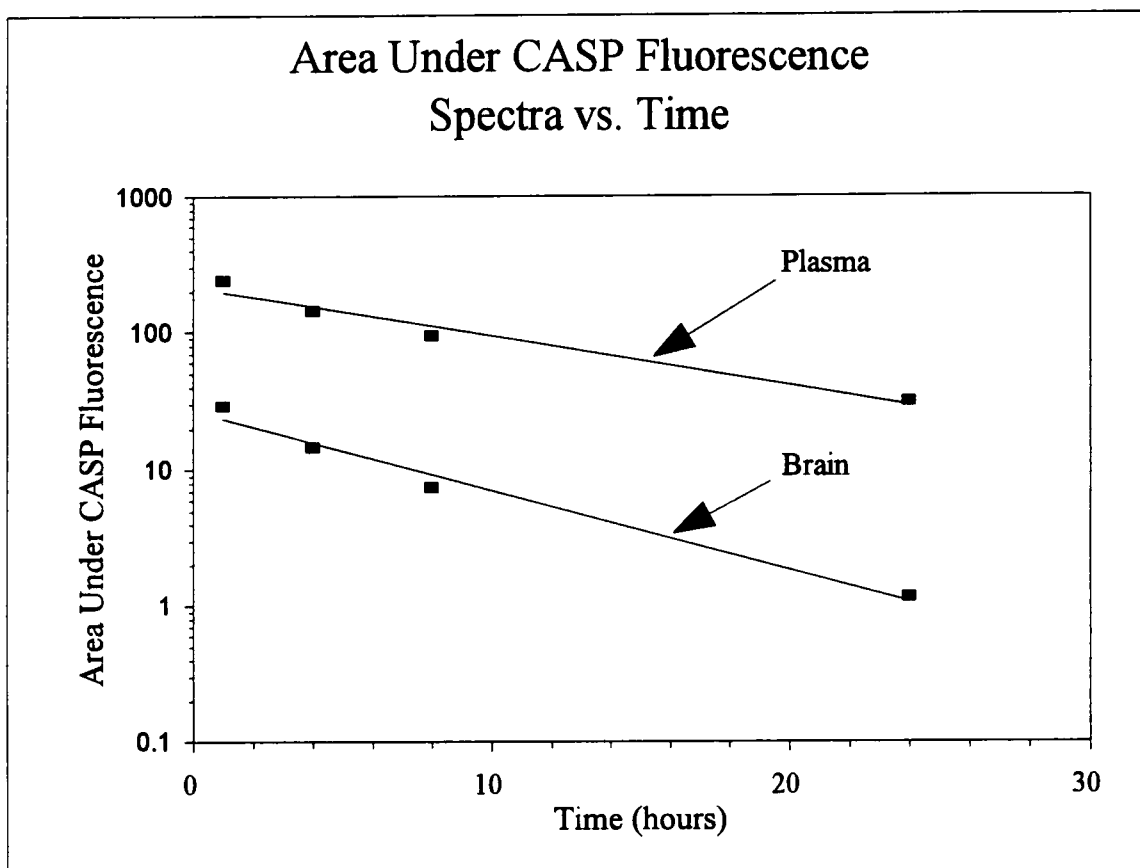


Figure 20. Area Under CASP Fluorescence versus Time. This semi-log plot shows the area under CASP fluorescence versus time from both brain tissue and plasma. Since these exponential relationships appear as approximate parallel lines, the clearance rates in brain and plasma are similar.

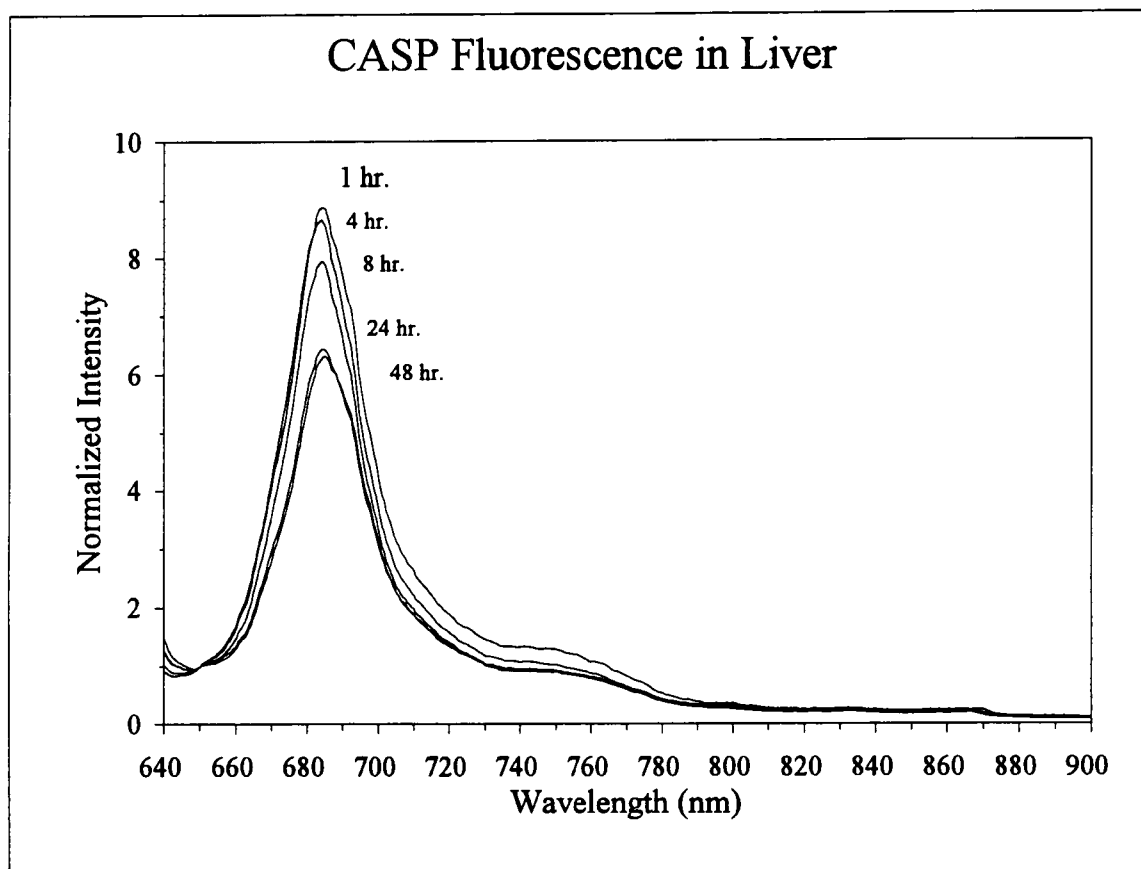


Figure 21. CASP Fluorescence in Liver. This plot shows the CASP fluorescence measured from liver tissue at various times post-injection. Each spectrum is the average for the post-injection group. Notice how easily the CASP fluorescence can be detected at 48 hours without any subtraction.

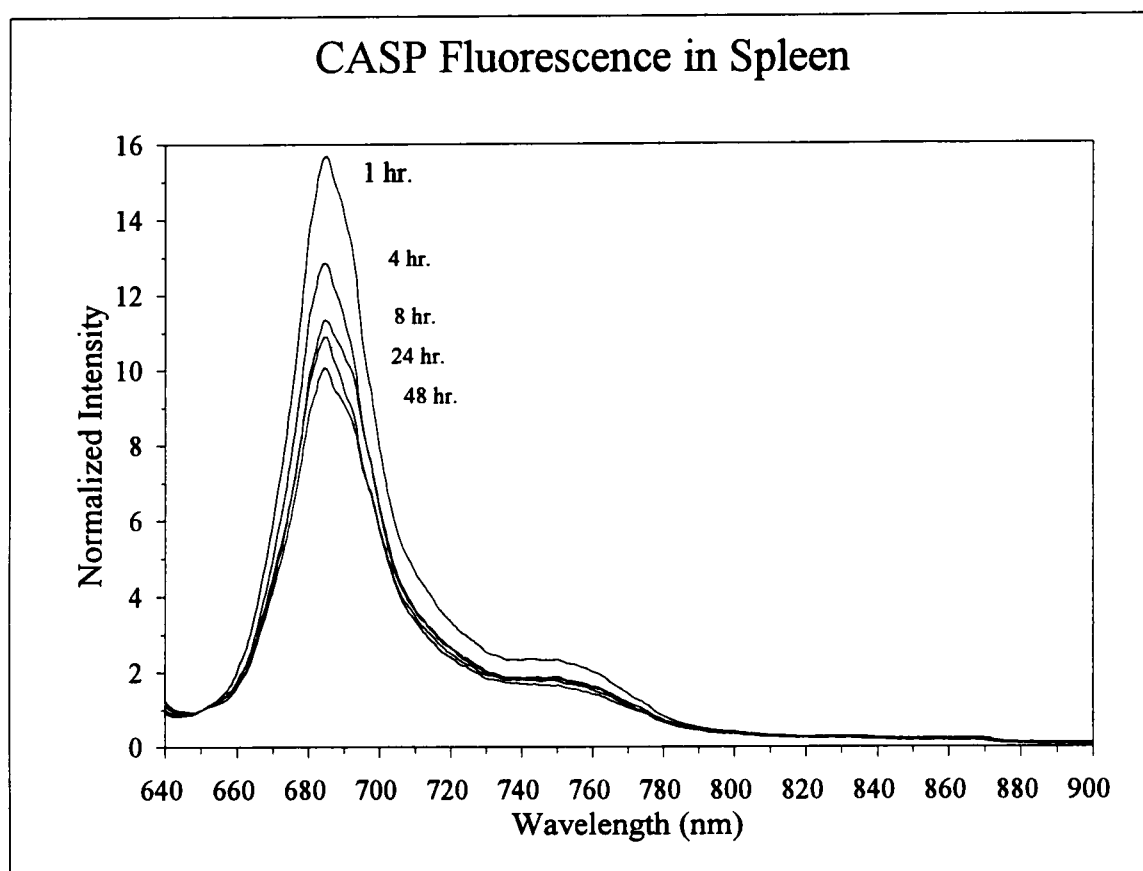


Figure 22. CASP Fluorescence in Spleen. This plot shows the CASP fluorescence measured from spleen tissue at various times post-injection. Each spectrum is the average for the post-injection group. Notice how easily the CASP fluorescence can be detected at 48 hours without any subtraction.

tissues were chosen because they accumulate and retain the highest concentrations of CASP.¹⁸ It is important that only general comparisons be made between fluorescence from different tissues. For example, a fluorescence intensity that is twenty times larger from liver tissue than from that of brain tissue does not mean the drug concentration is also twenty times larger. This is due to several variables such as the optical properties of liver, spleen, and brain tissue. Liver and spleen tissue absorb both excitation light and the fluorescence of CASP more than brain tissue. In spite of high absorption, the CASP fluorescence measured from the liver and spleen tissues was much greater than the fluorescence from brain tissue. The fluorescence of CASP in liver and spleen tissue can be seen without any control subtraction even 48 hours after injection. These measurements were performed to emphasize the ease in detection of CASP in liver and spleen tissue compared with the difficulty in brain tissue.

3.2 Fluorescence Measurements from Supernatant

3.2.1 Measurements from Known Concentrations of CASP

Figure 23 shows the fluorescence spectra measured from the supernatant of spiked brain tissue. Each spectrum is the average of three spiked samples. The spiked concentrations were 2 $\mu\text{g/g}$, 1 $\mu\text{g/g}$, 0.5 $\mu\text{g/g}$, 0.25 $\mu\text{g/g}$, 0.125 $\mu\text{g/g}$. Fluorescence measurements were also taken from the supernatant of control

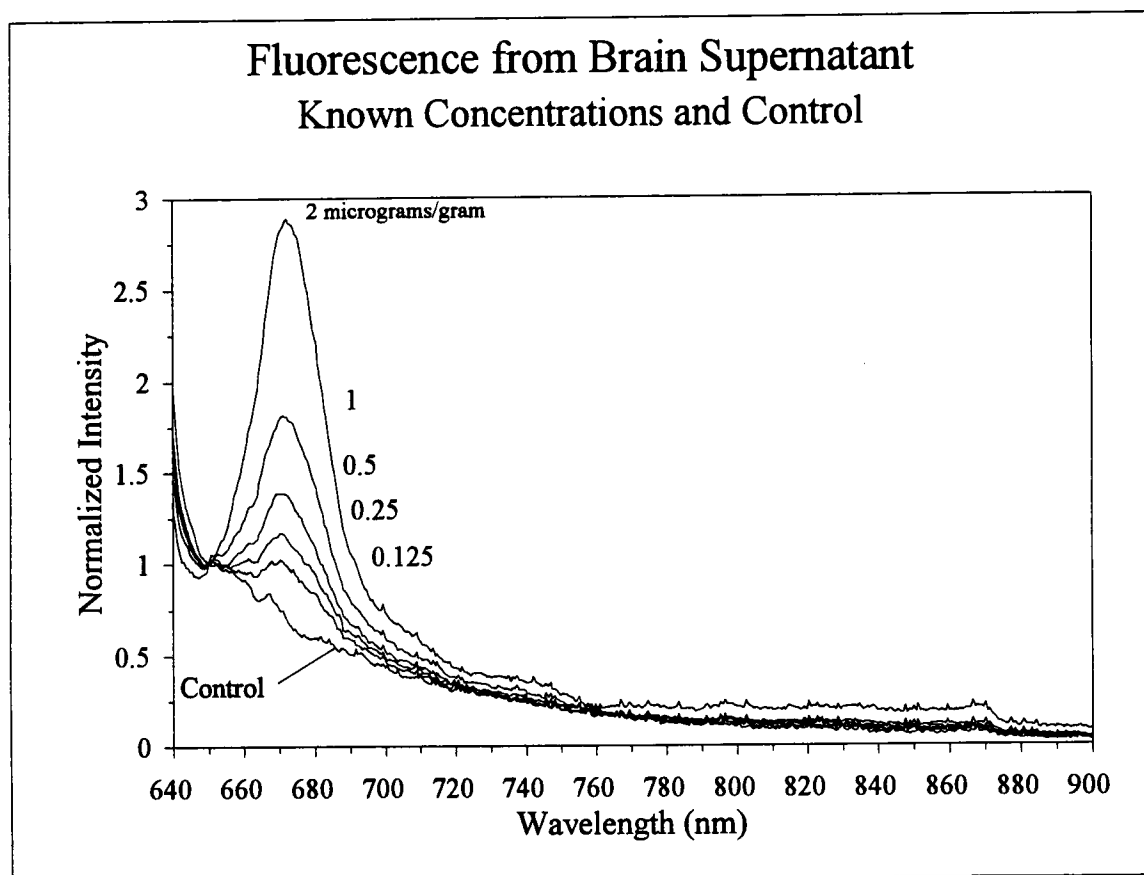


Figure 23. Fluorescence from Brain Supernatant. This plot shows the fluorescence spectra measured from brain supernatant for various spiked concentrations. These spiked concentrations range from 2 $\mu\text{g/g}$ to 0.125 $\mu\text{g/g}$. The average control fluorescence from brain supernatant was also plotted.

brain tissue. The control spectrum is also shown in Figure 23.

The areas under the CASP fluorescence spectra from the spiked tissues were calculated. The approximate area under the average control fluorescence spectrum was also calculated. The CASP fluorescence of each spectrum was calculated by subtracting the control average from the LIF emission. Figure 24 shows the area under the CASP fluorescence versus spiked concentration. This plot shows the average plus and minus one standard deviation of the area at each concentration. Using least-squares approximation, a relationship between the area under the CASP fluorescence from supernatant and the spiked CASP concentration was calculated. This relationship, seen in Equation 4, was used as the "standard curve".

$$Area = 27.87 \cdot Conc. + 2.18 \quad (Eq. 4)$$

The coefficient of determination, r^2 , for this linear approximation was 0.9961. Using this relationship, the concentration of CASP in unknown brain samples was approximated.

3.2.2 Measurements from Unknown Brain Samples

Fluorescence measurements were taken from the supernatant of two brain samples from each rat. The average fluorescence spectrum from these two samples was calculated. Figure 25 shows the 6 spectra of the 1 hour post-injection group. The average control fluorescence was also plotted. These

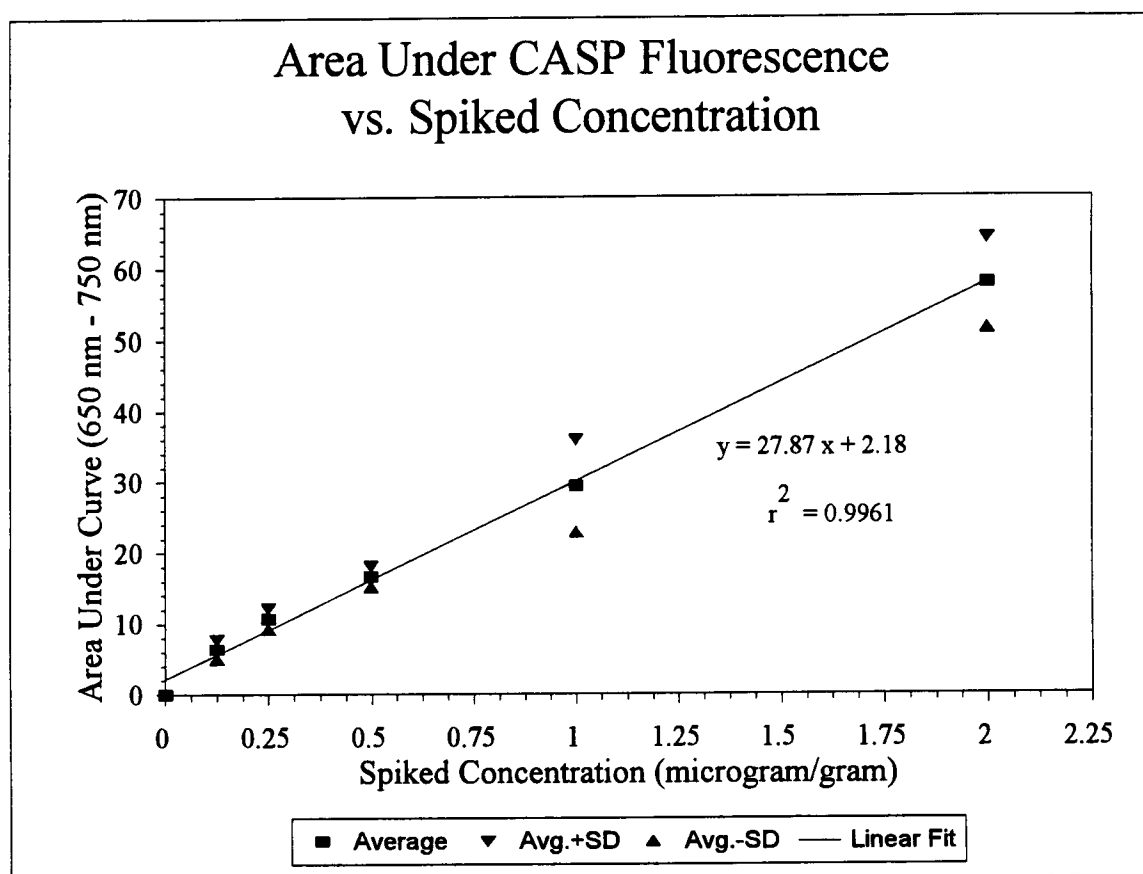


Figure 24. Area Under CASP Fluorescence in Supernatant versus Spiked Concentration. This plot shows the average area under the CASP fluorescence versus the spiked concentrations. The standard deviation of each concentration was also plotted. The data point at (0,0) represents the subtraction of the control fluorescence from each fluorescence spectrum. Using least-squares approximation, a linear relationship was fit through the data points.

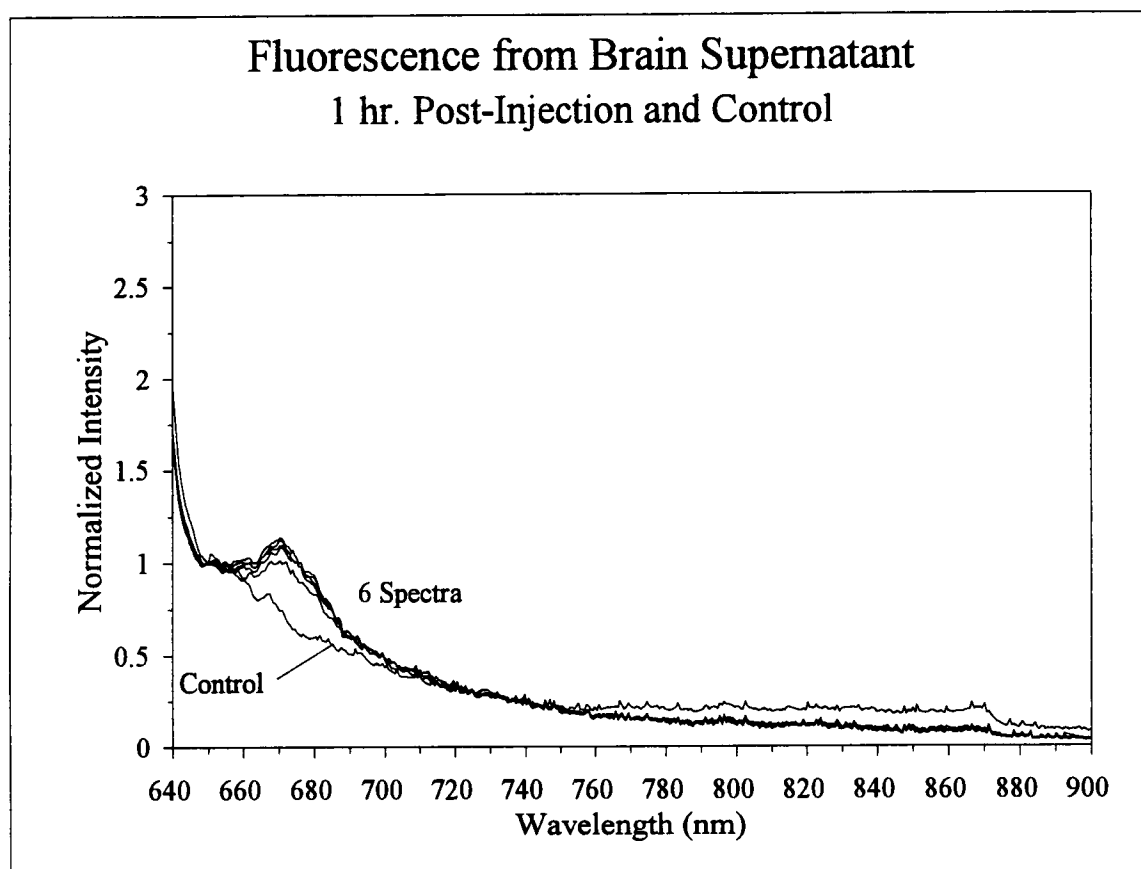


Figure 25. Fluorescence from 1 hour Post-Injection Brain Supernatant. This plot shows the six fluorescence spectra from the supernatant of unknown brain samples. The average control fluorescence was also plotted.

spectra show that the concentration of CASP from the 1 hour post-injection group is low. Figure 26 shows the 6 spectra of the 4 hours post-injection group and the average control fluorescence. From this graph, one can see that the fluorescence from the 4 hours post-injection group is very close to the detectable limit of measurement. The fluorescence spectra from the 8 hours, 24 hours, and 48 hours post-injection groups were not plotted because they all were indistinguishable from the average control fluorescence.

To approximate the concentration of CASP in each brain tissue, the area under the CASP fluorescence was calculated for each spectrum. The average control spectrum was subtracted from each LIF emission spectrum to calculate the CASP fluorescence. Using Equation 4, the corresponding concentration of CASP was calculated. Table 2 shows the area under the CASP fluorescence from the supernatant, the estimated CASP concentration for each rat, and the area under the CASP fluorescence measured directly from the brain tissue. If the area under the CASP fluorescence from the brain supernatant was less than 2.18, then the CASP concentration calculated would be negative. Since this is not possible, the CASP concentration was not calculated for the samples whose supernatant area was less than the detectable limit of 2.18.

From Table 2, the area under the CASP fluorescence measured directly from the brain tissue was plotted versus the approximate CASP concentration for the tissue. This plot can be seen in Figure 27. Figure 27 was used to

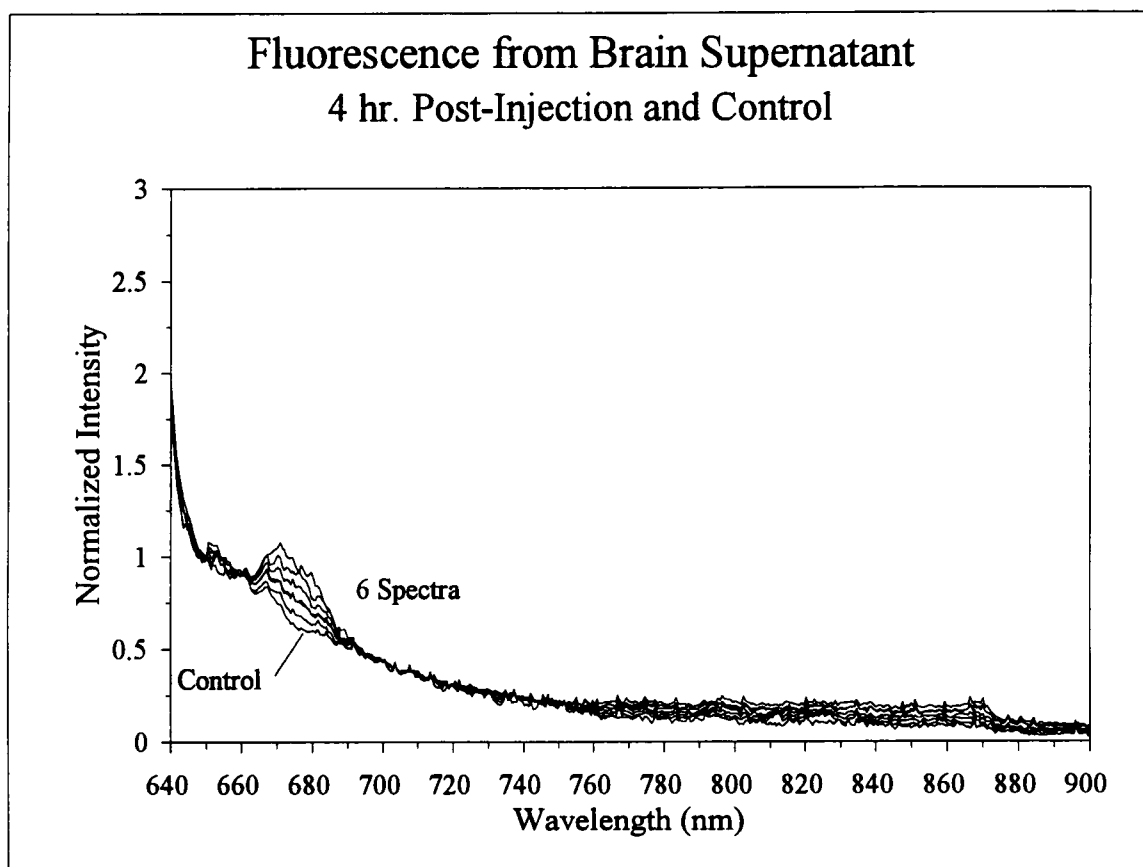


Figure 26. Fluorescence from 4 hours Post-Injection Brain Supernatant. This plot shows the six fluorescence spectra from the supernatant of unknown brain samples. The average control fluorescence was also plotted. These fluorescence spectra are approaching the detectable limit of the measurement system.

Table 2. Supernatant and Tissue Fluorescence Areas and CASP Concentration for all groups except 48 hours post-injection.

Experimental Group	Rat Number	CASP Fluorescence Area from Supernatant	Estimated CASP Concentration ($\mu\text{g/g}$)	CASP Fluorescence Area from Tissue
1 hour Post-Injection	R214	10.116	0.285	34.651
	R215	8.793	0.237	31.802
	R216	9.015	0.245	31.422
	R217	7.693	0.198	30.188
	R220	6.189	0.144	23.031
	R221	7.034	0.174	24.499
4 hours Post-Injection	R224	1.861	----	13.241
	R225	0.919	----	10.211
	R226	4.772	0.091	19.967
	R228	1.587	----	12.514
	R229	3.686	0.054	16.652
	R231	2.637	0.016	14.306
8 hours Post-Injection	R242	0.876	----	7.566
	R243	0.560	----	10.594
	R244	1.021	----	8.930
	R245	0.433	----	5.954
	R246	0.476	----	4.341
24 hours Post-Injection	R210	0.318	----	2.124
	R235	-0.164	----	0.550
	R236	0.146	----	1.286
	R237	-0.088	----	0.639
	R238	0.413	----	0.689
	R239	-0.254	----	2.337
	R240	-0.227	----	0.708
	R241	0.321	----	0.887

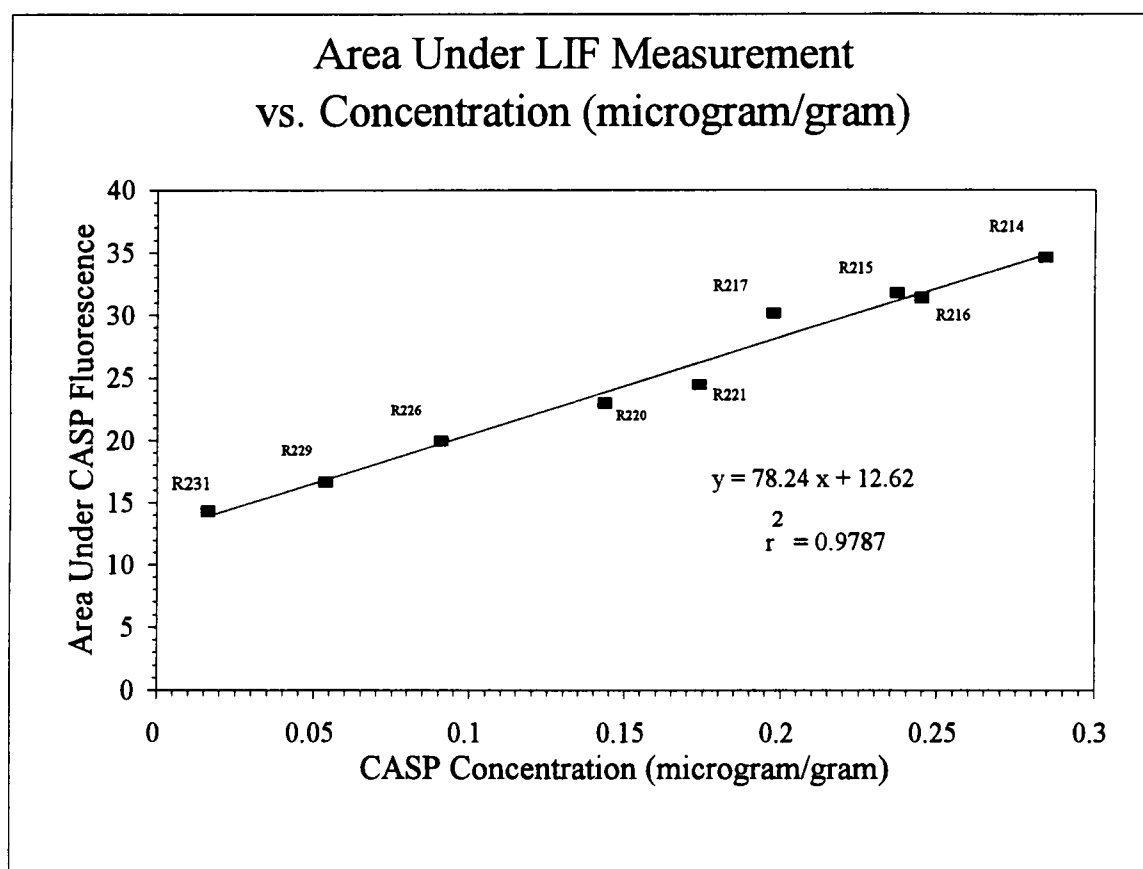


Figure 27. Area Under CASP Fluorescence from LIF versus Concentration. This plot shows the area under the CASP fluorescence measured directly from the tissue versus estimated CASP concentration in the tissue. Since the y-intercept of this relationship is 12.62, any area less than this value is below the detectable limit of the measurement system.

determine the relationship between the area under the CASP fluorescence measured from the tissue, using LIF, and the estimated concentration of CASP in the tissue. Using least-squares approximation, the linear approximation seen in Equation 5 was computed.

$$Conc. = \frac{Area - 12.62}{78.24} \quad (Eq. 5)$$

The coefficient of determination, r^2 , for this line-of-best-fit was 0.9787.

Using this equation, one can approximate the concentration of CASP in the brain tissue by measuring the area under the CASP fluorescence. Equation 5 is valid when the area of CASP fluorescence is greater than 12.62. Therefore, any area less than 12.62 should be considered below the detectable limit of the LIF approximation technique.

CHAPTER 4

DISCUSSION

4.1 Completion of Objectives

The goal of this project was to develop a method of non-invasively estimating CASP concentration in rat brain tissue using laser-induced fluorescence. To achieve this goal, three objectives had to be met. First, a system and technique for measurement of the CASP fluorescence in rat brain tissue had to be developed. Next, the concentration of CASP present in the brain tissue had to be approximated. This was done using standard chemical extraction methods. Once, the concentration of CASP was known, a relationship between the measured CASP fluorescence and concentration had to be derived. By the completion of these objectives, a non-invasive method for determining CASP concentration in rat brain tissue was developed.

4.1.1 LIF Measurement System and Technique

The first objective of this study was to develop a system and technique for measurement of the LIF emission from brain tissue. The system included a pulsed dye laser, fluorescence probe, spectrograph, gated intensified diode array, optical multichannel analyzer, and personal computer. The system was configured to automatically subtract a background reading for every emission scan. In addition, since a pulsed laser and gated detector were used, LIF

emission measurements could be made in room light with no background interference.

The measurement system was based on previous work by Panjehpour et al.¹⁵ and Poon et al.¹⁶ with minor modifications. First, the fluorescence probe used nineteen 200 μm fibers. Seven fibers were used as excitation fibers. The remaining twelve fibers were used as emission fibers to deliver the fluorescence of the tissue to the spectrograph. Panjehpour et al.¹⁵ used a single 200 μm fiber for excitation and a bundle of six fibers for collection of the fluorescence. Poon et al.¹⁶ reported using a single 600 μm fiber for both excitation and collection of the fluorescence. They also reported coupling the fluorescence from the single fiber into a circular arrangement of fibers. The output ends of these fibers were linearly arranged at the input of a polychromator.

Panjehpour reported using excitation light of 610 nm while shifting the center wavelength the spectrograph so that the lower end of the wavelength range on the diode array was 620 nm. Poon reported using excitation light of 337 nm and measured fluorescence intensities from 300 nm to 800 nm. Poon's data showed the strong autofluorescence of the brain tissue from 450 to 550 nm. Therefore, from these results, it was determined that the excitation source used for this study would be 610 nm. To reduce the effect of the scattered light, the spectrograph was set to shift the excitation wavelength 20 nm off the diode array. Using a 150 grooves per mm grating, the range of fluorescence

measured was 630 nm to 1245 nm. The range of wavelengths used for this study was from 640 nm to 900 nm.

A single measurement, consisting of 50 emission scans, was completed in less than 3 seconds. Using this technique would allow approximating the CASP concentration in tissue rapidly, a distinct advantage over present chemical extraction techniques. Present techniques require taking a tissue sample and performing time consuming and destructive tests on the sample. This system would provide a non-invasive and clinically feasible method for estimation of CASP concentration in rat brain.

To eliminate the effect of system parameters on the data, normalization of the fluorescence spectra was performed. First, the fluorescence spectra were normalized with respect to the average pulse energy. Since 50 scans were performed, the emission intensities are given in terms of intensity per 50 μJ of excitation energy. Due to the scattering of the excitation light within the spectrograph, the fluorescence spectra were also normalized with respect to the intensity at 650 nm. As seen in Figure 6 and Figure 7, the measured spectra were reproducible. Typically, the variability in fluorescence intensities was less than 10% within each organ.

The described LIF system was used to measure the CASP fluorescence emission from rat brain tissue. Figures 9 through 13 showed the fluorescence spectra from rat brain at various times post-injection. By averaging the

fluorescence spectra of each group, the decrease in fluorescence over time can be seen in Figure 14. Using the areas under the fluorescence spectra as a measure of the CASP fluorescence, the clearance rate of CASP from brain tissue was approximated.

By plotting the area on a semi-log plot, the exponential relationship between CASP fluorescence and time was determined. These results were confirmed by previous research. Peng and others¹⁸ reported that the concentration of CASP decreased exponentially with a half-life between 1.5 and 3 hours. This is consistent with the plot seen in Figure 17. The exponential decrease of concentration over the first 24 hours shows that the area under the CASP fluorescence for the 4 hour post-injection group is approximately half the area of the 1 hour post-injection group. Therefore, the half-life was approximately 3 hours.

Peng also reported that the concentration of CASP remained constant or decreased slowly from 24 hours post-injection to 72 hours post-injection. This is also consistent with the area of the CASP fluorescence seen in Figure 17. The area of CASP fluorescence at 24 hours and 48 hours are almost equal. Although the average at area 48 hours post-injection is slightly greater than the average area from 24 hours, the difference is within the range of data variability.

The CASP fluorescence measured from the plasma also corresponds to

previous research. Figure 19 shows the area under the average CASP fluorescence spectra from 1 hour to 24 hours post-injection. The CASP fluorescence present at 48 hours was not plotted because no detectable fluorescence of CASP was present. By plotting the area versus time on a semi-log plot, the exponential decrease of CASP from plasma was also calculated.

Figure 20 shows the exponential decrease of CASP in both brain tissue and plasma. Since these lines are approximately parallel, this plot indicates that the fluorescence measurements in brain tissue and plasma decrease at approximately the same rate within the first 24 hours. The CASP fluorescence measured from brain tissue prior to 24 hours may be due to the fluorescence of CASP in circulation.

These rates of clearance would have probably been closer if more data points were taken between 8 hours and 24 hours. From the data plotted in Figure 20, it appears that the inclusion of the 24 hours post-injection data affects the approximation of clearance rate of CASP from both brain tissue and plasma. Looking at the data present at 1 hour, 4 hours, and 8 hours, it appears that approximating the clearance rate of CASP in brain tissue and plasma over this range would result relationships that are more parallel. Therefore, the CASP fluorescence measured prior to 8 hours post-injection is most likely the fluorescence of the CASP present in the plasma.

It is significant to mention that at 48 hours post-injection, CASP

fluorescence was not detectable in plasma. Since the CASP was not detectable at 48 hours post-injection in plasma, but was present in brain at 48 hours, this seems to prove that the CASP fluorescence measured at 48 hours was from CASP taken up by the brain tissue. Early research with CASP in brain tissue reported that the normal brain tissue excluded the photosensitizer.¹⁹

4.1.2 Chemical Extraction Technique

To determine the CASP concentration in the brain tissue, the chemical extraction technique performed by Panjehpour et al.¹⁵ and Poon et al.¹⁶ was used. Brain tissue samples weighing 0.2 g were spiked with a known concentration of CASP. The spiked samples were homogenized in 7.0 ml of 0.1 M NaOH.¹⁵ Poon reported using 2.0 ml of NaOH. After homogenization, the samples were centrifuged at 12,000 RPM for 10 minutes. After centrifuging, the supernatant from the sample was separated and placed in a sterile vial. Panjehpour reported using a fluorescence spectrophotometer with an excitation wavelength of 610 nm to measure the fluorescence from the supernatant. Poon reported using a fluorimeter with an excitation wavelength of 610 nm to measure the fluorescence of the supernatant between wavelengths of 630 nm to 800 nm.

A disadvantage with using a fluorescence spectrophotometer is the two available gain settings, low and high. Previous use of this system showed that these two settings introduced more variables to the experiment. Normally, using

the low gain setting, the CASP fluorescence from the supernatant was too low to detect. However, at high gain, the detector saturated. To overcome this limitation, dilution of the samples is necessary. This would add more variables to the measurement of the fluorescence from the supernatant. Due to the ease of the LIF measurement system and the demonstrated accuracy, it was used to measure the fluorescence from the supernatant of the samples.

The LIF standard curve, seen in Figure 24, was constructed by plotting the area under the CASP fluorescence versus the spiked concentration. This plot demonstrates that fluorescence is directly proportional to the CASP concentration as long as self-absorption and quenching of the signal are not present. The average control fluorescence was plotted as the data point (0,0). Using these data, the least-squares approximation of this relationship was computed with a r^2 value of 0.9961. This linear relationship had a y-intercept of 2.18. This means that any sample with an area of CASP fluorescence less than 2.18 is below the detectable limit of this technique.

Once the standard curve was determined, the concentration in the unknown brain tissue samples could be approximated. Table 2 shows the results of calculating the CASP concentration in the unknown brain tissue samples. The CASP fluorescence from the supernatant was measured from two tissue samples from each brain. The average of these two measurements was used to form the fluorescence spectrum for each rat. Figure 25 and Figure 26 show the

fluorescence spectra from the 1 hour and 4 hours post-injection groups. Only these two groups were plotted because the other groups were indistinguishable from the control group.

The concentrations of CASP in the 1 hour post-injection rats were at the low end of the standard curve. The average CASP concentration for the 1 hour post-injection group was 0.214 $\mu\text{g/g}$. Only three of the six rats from the 4 hour post-injection group had a CASP fluorescence high enough to meet the minimum detectable limit. The average CASP concentration for the 4 hour post-injection group was 0.054 $\mu\text{g/g}$. The maximum CASP concentration from the 4 hour post-injection group was 0.091 $\mu\text{g/g}$. This concentration at 4 hours post-injection corresponds to previous research. Poon et al.¹⁶, whose study was done on rats with induced brain tumors, reported an average CASP concentration of 0.09 $\mu\text{g/g}$ in normal brain at 4 hours post-injection. No data were given prior to 4 hours.

Therefore, the chemical extraction technique developed for this study produced accurate results for approximating CASP concentration in rat brain tissue. This was confirmed by two methods. First, the highly linear relationship between CASP fluorescence and its concentration showed that the standard curve was a good approximation for determining CASP concentration by fluorescence. Second, the CASP concentrations determined using this method corresponded to similar previous research. One problem with this

chemical extraction technique was that the minimum detectable limit restricted the range of correlation between drug fluorescence and its concentration.

4.1.3 Correlation of LIF Emission and CASP Concentration

Once the CASP concentration of the brain samples was computed, the CASP fluorescence measured directly from the brain tissue was correlated to the CASP concentration. Due to the detectable limit of the chemical extraction technique, only nine rats could be used for the correlation. These nine rats included six rats from the 1 hour post-injection group and three rats from the 4 hours post-injection group.

Figure 27 shows the area of the CASP LIF emission measured from the brain tissue plotted versus the CASP concentration in the tissue. Using least-squares approximation, the relationship between the area of the CASP fluorescence and concentration was determined. The r^2 value for this approximation was 0.97867. The range of concentration in this relationship is from 0.016 $\mu\text{g/g}$ to 0.285 $\mu\text{g/g}$. Since the r^2 value is close to 1, this approximation technique is accurate over this range of CASP concentration.

The y-intercept for this approximation is 12.62. This means that any CASP fluorescence area less than 12.62 is below the detectable limit of the system. The areas under the CASP fluorescence, seen in Table 2, indicate a true representation of the CASP concentration decay over time. However, not all the data could be used. Due to the limitations of the chemical extraction

technique, the CASP concentrations which corresponded to these lower areas were below the detectable limit of the extraction technique.

4.2 Conclusion

In conclusion, this research study showed that the laser-induced fluorescence technique can be used to non-invasively estimate CASP concentration in rat brain. The LIF technique produced accurate fluorescence data that corresponded to previous research. Once the actual drug concentration in the brain tissue was determined, these fluorescence data were highly correlated to drug concentration in the tissue. Due to the limitations of the chemical extraction technique used here, the derived relationship between drug fluorescence and its concentration was over a small range of concentration. If a more sensitive chemical extraction technique was used, the range of estimated concentration using laser-induced fluorescence technique would be improved.

One advantage of this non-invasive technique is the rapid response of the LIF measurement system. The measurement of the drug fluorescence is completed in less than 3 seconds. Using computer software, the fluorescence data can be quickly analyzed to determine the drug concentration in the tissue. The development of a clinically feasible system to non-invasively measure drug concentration would provide the ability to promptly make treatment decisions based on the actual drug concentration in normal and malignant tissue.

4.3 Recommendations for Future Work

This study indicated that the LIF technique can be used to estimate CASP concentration in rat brain. To improve detection of drug fluorescence, one could reduce the scattered light on the detector. To achieve this, the center wavelength of the spectrograph could be shifted to set the excitation wavelength 30 nm off the detector. It was found that shifting the spectrograph helped reduce the scattered light on the detector. However, the center wavelength of the spectrograph cannot be shifted too far without losing some of the fluorescence from CASP. A filter could be used to block the excitation wavelength from entering the spectrograph. However, addition of a filter might affect the efficiency of the system. In addition, a filter with low fluorescence would be required so that the fluorescence of the filter would not interfere with the drug fluorescence.

This study may be expanded by collecting more data points between 8 hours and 24 hours post-injection. From the results of this study, it appears that at some time between 8 hours and 24 hours, the concentration of CASP decreases to constant minimum value. Similar fluorescence intensities at 24 hours and 48 hours post-injection indicated that concentration was unchanged. Therefore, more data between 8 hours and 24 hours would provide additional information on the clearance rate of CASP in normal brain tissue.

Since the chemical extraction technique proved to be a limitation in this

project, more accurate results would be obtained if a more sensitive method were developed. One factor that affects the chemical extraction technique is the dilution of the drug by homogenization in NaOH. Further research may be able to determine the optimal amount of NaOH for homogenization of brain samples. Also, it might prove beneficial to investigate if the use of a fluorescence spectrophotometer (with photomultiplier tube) would increase the detectable range of concentration. However, due to the limited gain settings available on some fluorescence spectrophotometers, accurate dilutions of the supernatant would be required.

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