Measurement of 8-Methoxypsoralen Concentration Using Fluorescence

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The thesis "Measurement of 8-Methoxypsoralen Concentration Using Fluorescence" by Scott D. Robinson has been examined and approved by the following Examination Committee:

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Dedication

To my wife, Pamela Robinson, and my parents, Dr. and Mrs. David Robinson, for their enduring love and strong support.

Acknowledgements

I thank all the staff and students at the Oregon Medical Laser Center for their kind support and helpful ideas. It was a journey that should not be traversed alone! Special thanks are given to Dr. Kenton Gregory and Illumenex Corporation for the valuable internship. I would like to thank Nicole Ewing who made me one of the few graduate students to have a research assistant. I am most deeply appreciative of my adviser, Dr. Scott Prahl, who coached me through various periods when the game looked like a defeat.

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Measurement of 8-Methoxypsoralen Concentration Using Fluorescence

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A new method of measuring the level of 8-methoxypsoralen in blood serum was developed for the reasons of speed, accuracy, and cost. This new method uses laser induced fluorescence of the psoralen to determine the concentration in serum. The fluorescence is analyzed with an optical multichannel analyzer coupled to an intensified photodiode array detector. Research was first attempted on samples with ethanol as the solvent to confirm that the method would work. Sample concentrations of 8-methoxypsoralen in serum are determined by comparing the fluorescence signal obtained from previously known concentrations. Levels down to 200 ng per milliliter of serum can be measured with this technique.

Chapter 1

Introduction

Since its invention in 1960, the laser has had extensive application in the medical field. Lasers have been used for surgically removing various undesirables including: tattoos, tonsils, vocal-cord nodules, blockages in arteries, polyps, and cysts [1]. The laser has also been used as a diagnostic tool for detecting such things as arteriosclerosis, oxygen shortage in the brain, caries in teeth, and jaundice in babies [2]. This thesis is concerned with using a laser to quantify a drug, psoralen, based on its fluorescence.

1.1 Fluorescence

Fluorescence is the process in which light is emitted by atoms or molecules that have been excited by the absorption of light [3]. At room temperature, most molecules are in the lowest vibrational level of the ground electronic state [4]. When an incoming photon of energy $h\nu_{absorbed}$ is absorbed, $\nu_{absorbed}$ is the frequency of the incoming photon by a molecule, that molecule gains an excess of energy. Transitions of electrons from the ground state energy level (S_0) to a higher energy level (S_n) can occur because of the excess energy (see figure 1.1). The electrons in the higher energy levels can then go through non-radiating transitions. Going from high singlet states to a lower singlet states is termed internal conversion. When the transition is from a higher singlet to a lower triplet state, the transition is called intersystem crossing and will result in phosphorescence rather than fluorescence. The electrons may end up at the first excited singlet state ($S_{absorbed}$). When the electrons undergo a transition from the first excited singlet to the ground state

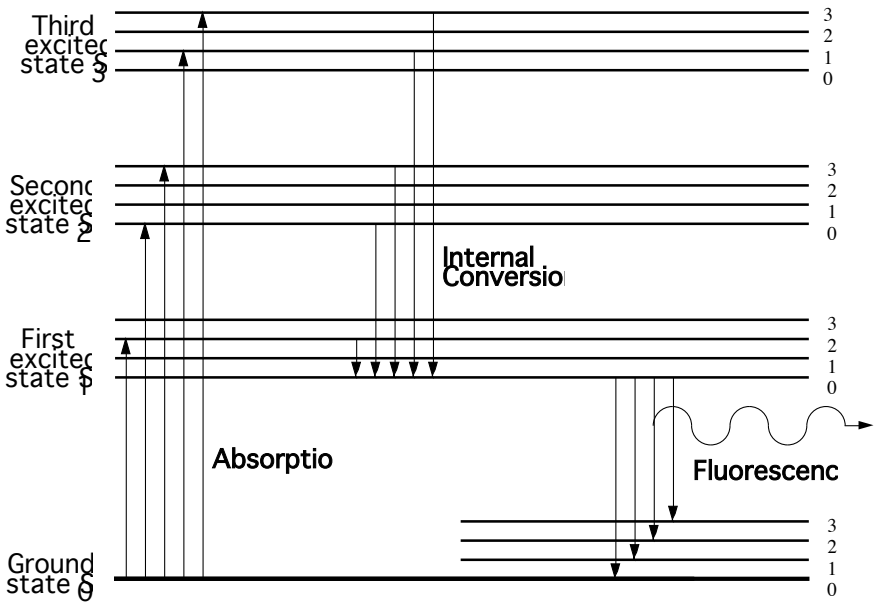

Figure 1.1: Absorption of a photon produces transitions in the energy level diagram which can produce fluorescence.

level there is often a release of radiation in the form of a photon of energy $h\nu_{emitted}$. This released energy is fluorescent light.

Within each energy level there can also be vibrational energy levels and rotational energy levels that allow a greater number of possible transitions. There is a spectrum of wavelengths absorbed by the fluorophore (a fluorescent molecule) and an accompanying spectrum of wavelengths emitted by the fluorophore (see figure 1.2). The photon emitted contains less energy than that of the absorbed photon ($E_{absorbed} > E_{emitted}$). Consequently, the frequency emitted is less than that which is absorbed ($\nu_{absorbed} > \nu_{emitted}$) and the wavelength of the photon emitted must be greater than the excitation photon ($\lambda_{absorbed} < \lambda_{emitted}$) (see equation 1.1). The incoming photon that is absorbed is referred to as the excitation photon because it excites the electrons of that molecule into higher states. Light absorption is just one way to produce fluorescent light. Fluorescence can also be obtained electrically or chemically.

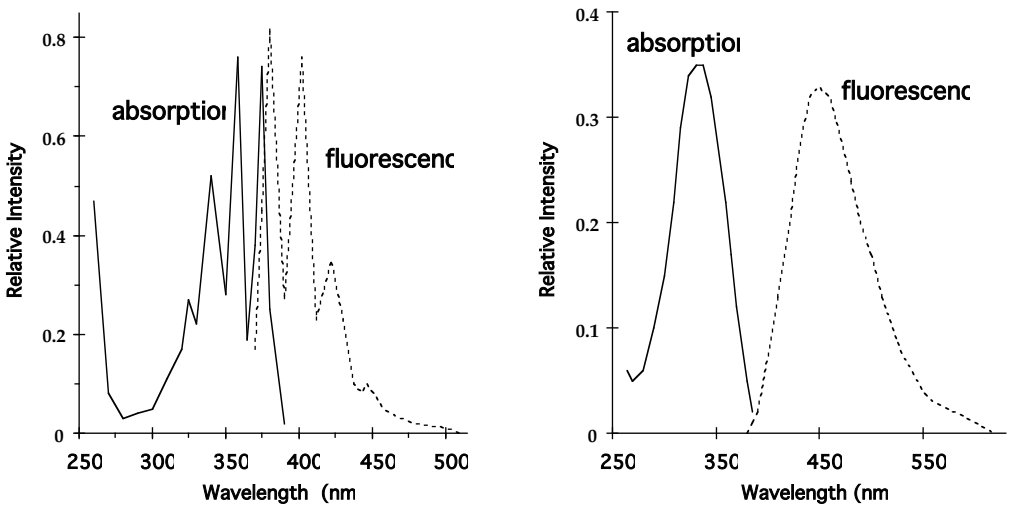

Figure 1.2: Typical absorption (solid line) and fluorescence (dashed line) spectra (adapted from *Photoluminescence of Solutions* by C. A. Parker).

$$E_{absorbed} = h\nu_{absorbed} = hc/\lambda_{absorbed} \qquad E_{emitted} = h\nu_{emitted} = hc/\lambda_{emitted} \quad (1.1)$$

Planck's constant is represented by h and the speed of light is shown as c. The time of the fluorescence transition is very short, on the order of 10^{-7} to 10^{-10} seconds — this transition is much quicker than the transition from the first triplet state to the ground state resulting in phosphorescence.[1]

1.1.1 How is fluorescence measured?

Fluorescence can be measured using any photometer. Photometers specifically designed to measure fluorescence intensity are called fluorimeters, while the term fluorometer usually refers to a device used to measure fluorescence lifetimes. Spectrofluorimeters are photometers that are designed to measure the spectrum of fluorescence emitted or the spectrum of absorbed excitation light needed to create fluorescence. A simple spectrofluorimeter usually contains four basic elements : 1) a source of light to excite

[1]Phosphorescence transitions last between 10^{-3} and 10 seconds.

the fluorophore, 2) a sample holder, 3) a device to separate the light into different wavelengths (a diffraction grating or a prism), and 4) a device to detect the emitted fluorescence.

The excitation source can be a lamp, such as a mercury, deuterium, xenon, or argon lamp, or it can be a laser, such as a nitrogen, helium cadmium, xenon chloride, or titanium:sapphire laser. Absorbed photons must have shorter wavelengths than the emitted photons; therefore, if visible fluorescence is to be seen (400 nm to 700 nm), then the excitation light may need to be in the ultraviolet region (200 nm to 400 nm). Ultraviolet light separates into three categories: UV-A is from 320 nm to 400 nm, UV-B is from 280 nm to 320 nm, and UV-C is from 100 nm to 280 nm (see figure 1.3).

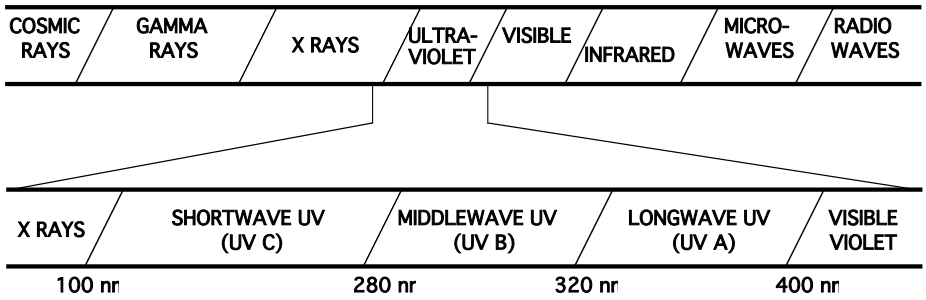

Figure 1.3: Electromagnetic spectrum showing ultraviolet classification (not drawn to scale).

Fluorescence can be measured with a photodetector or a set of photodetectors, such as a diode array. The fluorescence spectrum can be examined using a spectrograph in conjunction with a diode array. A spectrograph uses either a diffraction grating or a prism to separate input light into an organized spectrum (see figure 1.4). Lenses, mirrors, and slits can be implemented into the system to channel the correct range of wavelengths into the detector. The detector can be connected to a device, such as an optical multichannel analyzer, to observe the output.

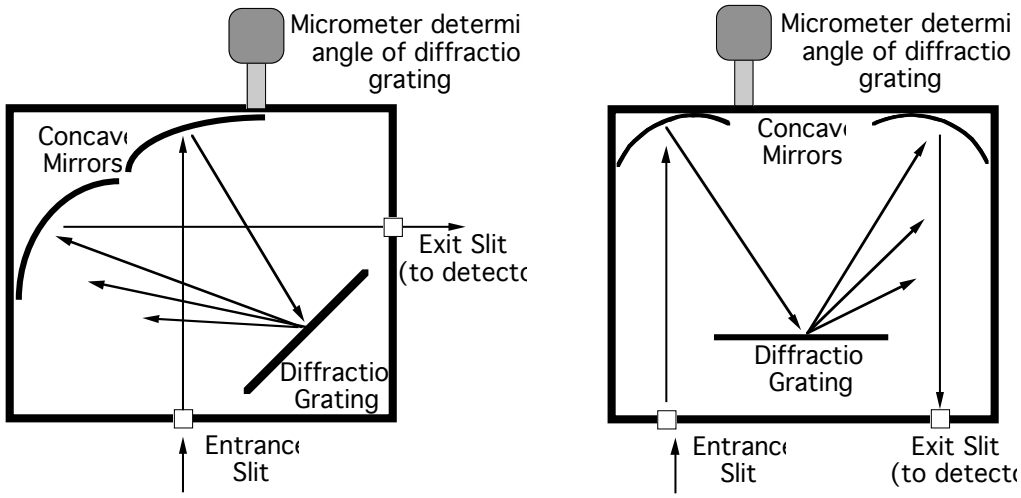

Figure 1.4: Two typical spectrograph set ups.

1.1.2 Medical applications using fluorescence

Applications using fluorescence are rapidly emerging in the medical field. Fluorescence can be used to differentiate neoplastic tissue (tissue that has excessive growth on it such as a tumor) from normal tissue [5]. The averaged normalized fluorescence intensity of histologically normal tissue is greater statistically than that of histologically abnormal tissue [6]. Research on the colon [7, 8], cervix [6], breasts [9, 10], lungs [9, 11, 12], and gastro-intestinal tract [8] have been published. Imaging of tumors has been done *in vivo* [13]. Fluorospectral studies of the rat brain may allow a better method of defining boundaries of a glioma (malignant tumor of the brain) [14]. Methods other than fluorescence, such as computerized tomography and magnetic resonance imaging, allow the glioma to be seen; however, differentiating the glioma from surrounding normal brain tissue is difficult. Laser induced fluorescence spectroscopy can be used to discriminate between normal and atherosclerotic tissue [15,16]. Myocardial reperfusion injury can be monitored using fluorescence [2] [17].

[2] After a deficiency of blood in the heart, reperfusion takes place (blood is allowed to flow again) and injury can occur.

Another application using fluorescence in the medical field is the quantification of certain drugs or chemicals. Quantification of chemicals by fluorescence is a theory that has been around for over 40 years. In 1955, Robert Bowman wrote about experiments using fluorescence to measure concentrations of 0.1 to 0.4 μg/ml of 5-hydroxyindole [18]. Bowman discussed the possibility of using fluorescence to determine 5-hydroxytryptamine in blood. In 1961, R. Brandt authored an article where he used fluorescence to determine the different proportions of morphine and codeine in various solvents [19]. Recently, this application of fluorescence has been applied to such chemicals as phthalocyanine [20], hematoporphyrin [21], and, in this study, 8-methoxypsoralen (also called 8-MOP or psoralen).

1.2 Psoralen

1.2.1 Background

The therapeutic powers of sunlight and natural products containing the class of drugs known as furocoumarins (psoralens and angelicins) have been known for thousands of years. Ancient Hindus, Turks, Egyptians, and Orientals have known about some of the biological effects of furocoumarins for over three-thousand years [22]. Early medical writings dating back to the *Ebers Papyrus* (ca. 1550 BCE) refer to the use of extracts from the leaves *Ammi majus* (Umbillifrae), which grows along the Nile River, to treat vitiligo, a depigmentation of the skin [23]. Historical references indicate that psoralen was derived from the plant *Psoralea corylifolia* in India as early as 1400 BCE [24]. Deleterious effects of the furocoumarins were mentioned in an 18th century German fairy tale where the central character, "Little Muck," suffered from photodermatitis after ingesting figs (which have furocoumarins in them) and exposing himself to sunlight [22].

Although 5-methoxypsoralen was first isolated in 1834, it took until 1931 before it was demonstrated that sunlight was necessary to activate the compound [25]. In 1948, 8-MOP was isolated and characterized as the active ingredient of the *Ammi majus* [25]. Exposure of the skin to sunlight after ingestion of the purified compound led to

repigmentation. In 1953, purified 8-MOP was administered safely to humans at low doses to manage vitiligo [26]. The excitation spectrum of the drug was recorded around this same time showing an optimal wavelength of 365 nm. Italian researchers in the 1950's demonstrated a 2+2 photocycloaddition occurred between psoralens and pyrimidines in DNA [27]. These studies also were the first to show the mutagenic nature of 8-MOP photoadducts. Finally, in 1972, the crystal structure of 8-MOP was established [22].

1.2.2 How does psoralen work?

Psoralen is a drug which creates a crosslink between DNA strands, specifically between adenine and thymine bases [28]. This crosslink prevents DNA strands from separating, consequently preventing cellular replication. Psoralen is a photosensitive (light activated) drug. In the absence of light, psoralen has no effect on the cell. Using a combination of light and a chemical to produce a medically beneficial result is called photochemotherapy. Lifetimes of psoralen fluorescence range from one to five nanoseconds with 8-methoxypsoralen having a lifetime of 1.8 ns [29]. The quantum yields of the photoaddition of psoralen and 8-MOP to DNA have been established to be 0.030 and 0.084 respectively [30]. Quantum yield is defined as the number of quanta emitted per exciting quantum absorbed. The quantum yield of the photoaddition of 8-MOP to DNA, which depends on both the amount of light absorbed by the intercalated (slipped between stack base pairs) 8-MOP and the irradiation time, can be as high as 0.19.

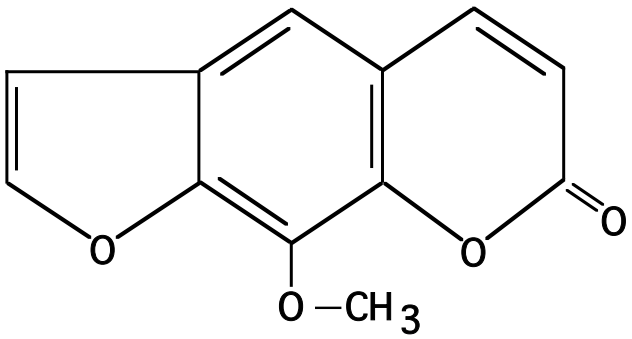

Figure 1.5: Diagram of 8-methoxypsoralen

1.2.3 Applications of psoralen

Psoralen has a number of medical applications. Psoralens were originally used to treat vitiligo, a skin condition in which the pigmentation melanin is lost from areas of otherwise normal skin [30]. Ultraviolet light-activated psoralen is a common treatment for psoriasis [31].[3] The procedure implementing a combination of psoralen with an ultraviolet A light dose is termed PUVA. Another effective use of psoralen is clearing cutaneous [4] infiltrates of cutaneous T cell lymphoma [32].[5] Mycosis fungoides may also be treated by a combination of psoralen and UV light.

PUVA therapy may be effective in treating cellular proliferation caused by restenosis following coronary balloon angioplasty [33]. A common way of removing a blockage in an artery is by inserting a balloon and inflating it. The disadvantage to this method is that the artery is also expanded. The cells in the artery may proliferate to compensate for this expansion. If the cells overcompensate, then the artery may close again so that no improvement has been made from the artery's original state. This closing of the artery, called restenosis [6], is seen in about 30–40 % of the cases following balloon angioplasty [34]. Photochemotherapy that combines UV-A light with psoralen can combat restenosis because it can stop the cells in the artery from replicating [33, 34].

The goal of this PUVA photochemotherapy is to get the best combination of light and drug. Cell culture studies have shown that a 365 nm light dose of 600 mJ/cm^2 accompanied by a drug dose of 1000 ng/mL yields therapeutic results, and has a double product of 600,000 (mJ/cm^2·ng/mL) [33]. If the combination is much higher than the double product of 600,000 (mJ/cm^2·ng/mL), the therapy can be toxic; if the combination is much lower then the therapy will have no effect (see figure 1.6). For example, when a patient has a 1500 ng/mL drug concentration, a light dose of 400 mJ/cm^2 should be delivered. It is therefore crucial to know the amount of psoralen in the blood before

[3] Psoriasis involves hyperproliferation of the stratum corneum. It can be recognized by scaly patches on the skin.

[4] Cutaneous simply refers to the skin.

[5] A lymphoma is a tumor from cellular elements of the lymphatic glands.

[6] Restenosis comes from the word restitution.

delivering a light dose.

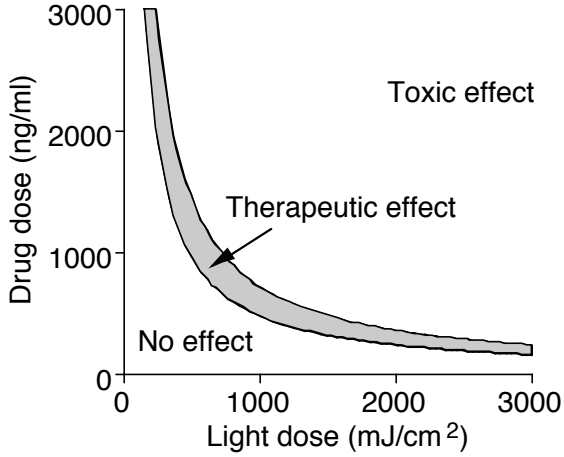

Figure 1.6: This figure is illustrative of the reciprocal relationship between light dose and drug dose for therapeutic results.

1.2.4 Measuring psoralen

Doctors can know how much psoralen is administered, but it is difficult to determine how much stays in the body. The body will naturally remove the delivered drug, but uptake and elimination varies from patient to patient. Presently samples of blood can be taken and analyzed using reverse-phase high-pressure liquid chromatography analysis [31,35,36]. This method of measurement is problematic because samples must be sent to labs with advanced equipment to do the analysis. The concentration of psoralen in the blood may not be determined for several weeks, and the cost is high. Ideally, the results need to be determined quickly (within 15 minutes) if PUVA is to be tailored to the drug dose. Speed, cost, accuracy, and simplicity are a few of the reasons that a different means of determining the concentration of psoralen in blood samples was sought. It is easy to adjust either the intensity of light delivered to the psoralen or the time that the light is delivered so that a therapeutic combination is obtained; however, it is difficult to adjust the amount of psoralen present.

Because of the fluorescent properties of psoralen, it seems logical to measure the concentration of psoralen using fluorescence. Higher concentrations of psoralen will produce more fluorescent light and a fluorescence-concentration relationship can be derived. The goal is to take a sample of blood, centrifuge it, place it in a spectrofluorimeter, analyze the fluorescence signal, and compute the psoralen concentration. To be effective, this device needs to be accurate down to 100 ng of 8-MOP per milliliter of blood. Accuracy of ± 50 ng/ml or lower is desired. Eventually, we hope to be able to quantify psoralen concentrations *in vivo* rather than *in vitro*. Before we can look to the future we have to start with the basics, psoralen fluorescence.

This thesis explores the possibility of using fluorescence to detect 8-MOP concentrations. The research began with detecting 8-MOP in clear solutions of ethanol or propylene glycol. After success was achieved using a solvent that did not absorb UV-A light, the experiments used serum as the solute. Ultimately it was possible to detect psoralen in serum at levels of 200 ng per ml.

Chapter 2

Methods and Materials

2.1 Equipment

Three tools were used throughout my experiment: a spectrograph, a photodiode array detector, and an optical multichannel analyzer. Each of these is discussed in turn. These three subsystems each play a different role in the system as a whole. The spectrograph organizes the fluorescent signal in order of wavelength. The detector measures the magnitude of the different wavelengths. The optical multichannel analyzer arranges the data as a plot comparing wavelength versus photon counts.

2.1.1 Spectrograph

The spectrograph (EG&G model 1232) disperses the incoming light across the attached detector. The range of wavelength falling on the detector is controlled by rotating the diffraction grating (300 lines per mm) with the attached micrometer. Upon hitting the diffraction grating in the spectrograph, light is dispersed into multiple orders (see figure 2.1). For simplicity, consistency, and efficiency, the first order is used almost exclusively. Two wavelengths that are ten pixels apart in the first order would be twenty pixels apart in the second order. Figure 2.2 shows the minimum and maximum wavelengths captured by the detector for various micrometer settings. The micrometer setting determines the spectrum of wavelengths reaching the detector. For example, with the 300 lines/mm grating, a setting of 0.120 lets 343.1 nm to 614.8 nm reach the detector. The spectrograph is a cube 65 by 65 mm and has a focal length of 156 mm.

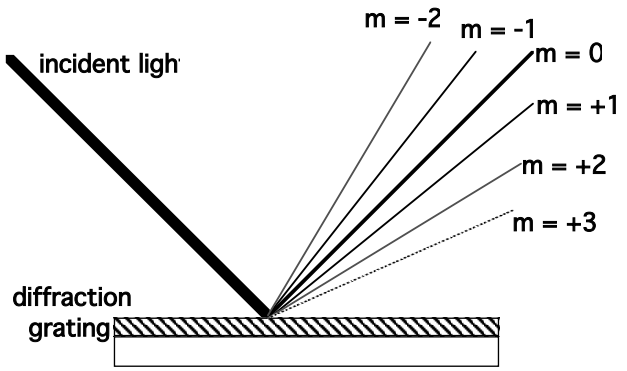

Figure 2.1: A diffraction grating splits light into different orders.

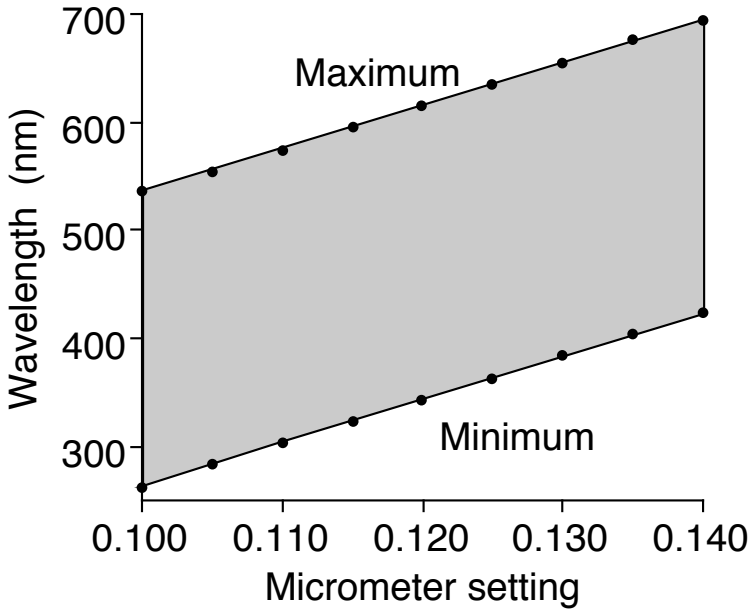

Figure 2.2: The relationship between the micrometer setting and the range of wavelengths distributed onto the diode array (represented by the shaded area).

Slits

All spectrographs (monochromators) have the following components: an entrance slit, a collimator (mirror or lens), a dispersing element (prism or diffraction grating), a mirror or lens to focus the dispersed light, and an exit slit [4]. As is the case for most spectrographs, the one used in these experiments had mirrors with identical focal lengths ($= f$). The angular dispersion, α is defined as the difference in angle between two rays leaving the diffraction grating that differ by 1 nm. The distance, m, between the two rays leaving the exit slit is given by equation 2.1.

$$m = \alpha f \tag{2.1}$$

A wavelength leaving leaving the spectrograph will be partially overlapped by adjacent wavelengths because of the angular dispersion. Consequently, adjacent photodiodes in the detector will measure common wavelengths. A narrow entrance slit will reduce some of this wavelength overlapping because the light begins at a common point. If the light at the entrance slit is spread out, as in the case of a wide slit, then the overlapping of wavelengths at the detector will be increased.

An important optical measurement to consider for a spectrograph is the resolving power. Suppose light of wavelengths λ and $(\lambda + \Delta\lambda)$ is diffracted in a certain direction Θ by a diffraction grating containing N lines and of a total width D. The path difference between portions of the wavefront which reach the focal point from opposite ends of the grating is $Dsin\Theta$. If this direction corresponds to the principal maximum of order m for wavelength λ, and to one of the two neighboring minima for $(\lambda + \Delta\lambda)$, then equation 2.2 must hold true. The resolving power R_g is defined in equation 2.3 [37].

$$m\,N\,\lambda = D\,sin\Theta = (mN - 1)\,(\lambda + \Delta\lambda) \tag{2.2}$$

$$R_g = \lambda/(\Delta\lambda) = m\,N \tag{2.3}$$

Narrow slits only permit light that is approximately perpendicular to the spectrograph's entrance, thereby decreasing the contamination from other light sources in the

room. Unfortunately, narrow slits also diminish the detected signal simply because they allow less light to enter the spectrograph. Wider slits maximize the observed fluorescence, but also maximize the contamination of other signals. Different slit widths were tested at the spectrograph entrance in an attempt to maximize fluorescence input while minimizing unwanted signal. Slits of 25 μm, 50 μm, 100 μm and 1 mm were analyzed.

Filters

Filters were used to eliminate the bright excitation light and, consequently, prevent saturation of the detector. The excitation light's intensity is of a magnitude three to five times greater than that of the fluorescence signal being observed. Two glass-plastic laminated, non-fluorescing filters were examined. The KV 370 (Schott Glass Technologies, Inc.) eliminated practically all light below wavelength of 370 nm and the KV 389 (also

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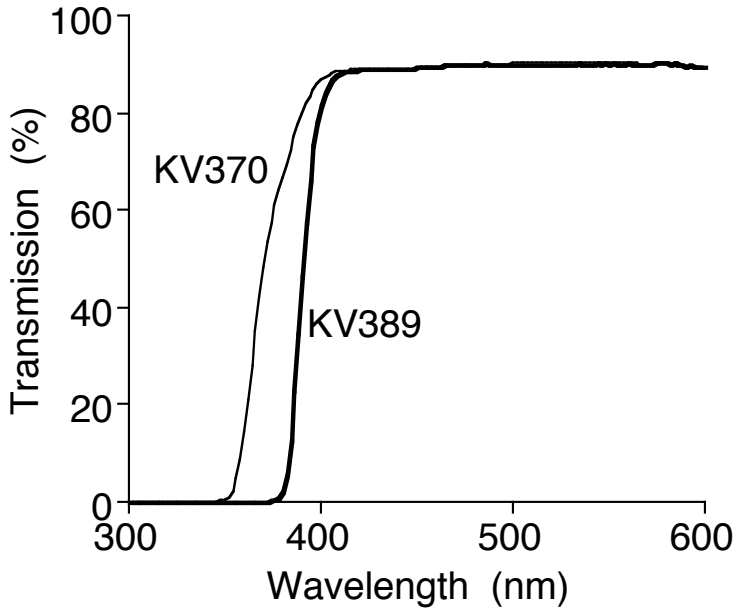

Figure 2.3: Transmission spectra of the two UV filters.

from Schott) eliminated practically all light below 389 nm (refer to figure 2.3). These could be placed at the entrance slit inside the spectrograph. Only visible fluorescent light

was observed in this way, not the ultraviolet (excitation). Non-fluorescing glass filters are available that may be a better alternative to the filters used in this experiment. It was extremely important not to touch the diffraction grating in the spectrograph when attaching the filter to the inside entrance. The filters were attached using black electrical tape to reduce the scattering of light and because the black tape does not fluoresce.

2.1.2 Detector

The intensified photodiode array detector (EG&G model 1420 UV) contains an array of 512 photodiodes so that light may be collected across an entire spectrum simultaneously. With the 300 lines/mm grating being used in this system, each photodiode in the detector measures approximately 0.5 nm of the spectrum; the entire array collectively spans about 273 nm.

Another important factor affecting the fluorescence signal was thermal noise in the detector. The detector was cooled with a thermoelectric cooler[1]. The OMA will display "Cooler Locked" as long as the detector stays within 5°C of the set temperature (20°C). This amount of thermal variation can cause readings to vary significantly. The optical multichannel analyzer manual states that it may take thirty to forty-five minutes of running before reaching thermal stabilization; however, it was determined that one hour of "warm up" time was required to establish a desired consistency.

The detector is intensified because the fluorescent signal being analyzed is small compared to other lights. The fluorescent emission can be observed in a dark room, but normal room light emission is significantly more intense. The intensified detector enlarges the observed signal of photon counts by amplifying each photon's converted electronic signal. This amplification allows dim light to be seen. Bright lights such as lasers or lamps can damage the intensified detector through saturation; such light must be substantially diffused/attenuated before reaching the detector.

[1]Condensation on the detector was prevented by passing nitrogen gas over it at a rate of about 1000 cc per minute (see Appendix B).

2.1.3 Optical Multichannel Analyzer

Information is read from the detector by the optical multichannel analyzer (EG&G model 1460 with option 1463), referred to henceforth as the OMA. The OMA graphs photon counts by each diode. Each pixel on the screen of the OMA represents one photodiode of the array.

Data acquisition programming mode

Data acquisition mode nine was selected on the OMA when the pulse laser was used. This mode sent an external trigger, gathered data, paused, and then repeated itself. The trigger caused the nitrogen laser to fire. This excited fluorescence that was collected by the OMA. A replica of the program used on the OMA to gather each set of data is shown in figure 2.4. The acquisition of a spectrum of data from the diode array was repeated one hundred times. A pause was inserted into the program by making the OMA skip over twenty scans in an inner loop after each scan of data. This pause was necessary to comply with the repetition rate of the nitrogen laser by staying below a rate of twenty Hertz. The OMA takes 17.7 seconds to acquire the one-hundred scans constituting one set of data.

```
Do I: 100
      Trigger Out
      Do J: 1
            Add 1 Scan
            Do K: 20
                  Ignore 1 Scan
            Loop
            Inc. Memory
      Loop
Loop
```

Figure 2.4: This is the program used by the OMA to trigger the system and capture each set of data.

2.1.4 Cuvettes

The plastic disposable cuvettes (Labcraft lot number: Q5999) originally used to contain the samples were discovered to have some fluorescent properties and were therefore unacceptable for accurate measurements. Switching to glass cuvettes gave a fantastic reduction in background noise. UV quartz cuvettes (NSG Precision Cells type 1 ES) were discovered to be excellent sample containers because they do not absorb any UV A light (see figure 2.5) and therefore cannot fluoresce. Unfortunately, at around sixty dollars per cuvette, quartz cuvettes are not disposable.

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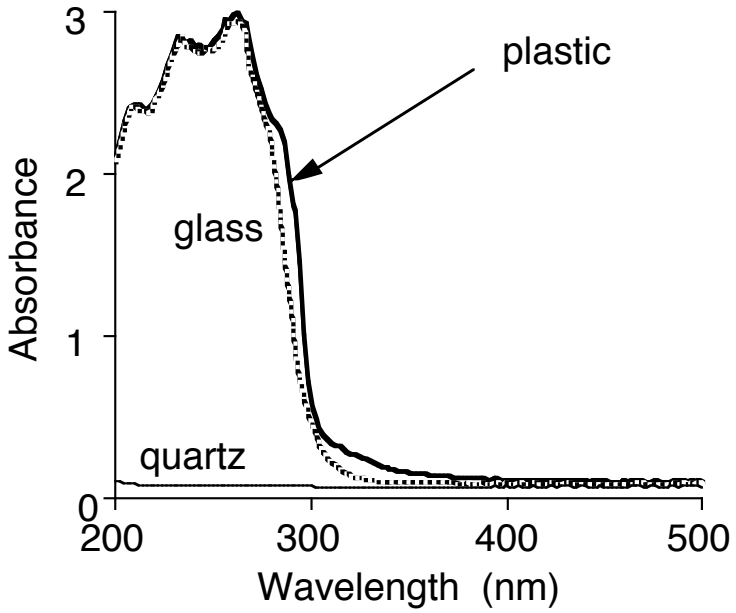

Figure 2.5: Absorption of UV light for different cuvettes.

The cuvettes had to be carefully washed after each use. Because oil smudges are difficult to remove, caution had to be taken in handling the cuvettes so that the optical surfaces were not touched. A high-grade, phosphate-free detergent (Neutrad), with neutral pH and containing no oils or insoluble materials, was mixed with distilled water to form a cleaning solution. After the cuvettes had been thoroughly rinsed with distilled water, the cleaning solution was squirted over the inside and outside of the quartz cell.

Cuvettes were then re-rinsed and excess water was shaken off. Exterior surfaces of the cuvettes were wiped dry with soft, non-abrasive tissue wipes. In drying the cuvettes, evaporation of excess water was quickened using suction. It was important to avoid blowing air into the cuvettes because this can leave unwanted debris on the interior surfaces.

2.1.5 Chemicals

The 8-methoxypsoralen (Sigma Chemical Co. M-3501, Lot: 53H0736) used in this research was a dry powder that had to be dissolved in the solvent being used. Ethanol (Chempure Brand Lot: M018 KJHA), propylene glycol (Sigma Chemical Co. P-1009, Lot: 123H1072), and type I distilled water were the primary solvents used to dilute the 8-MOP. The 8-MOP had to be dissolved in a small amount of ethanol first because it would not dissolve directly in plain water or propylene glycol. Figure 2.6 shows how UV light is absorbed by 8-MOP in water as a function of wavelength.

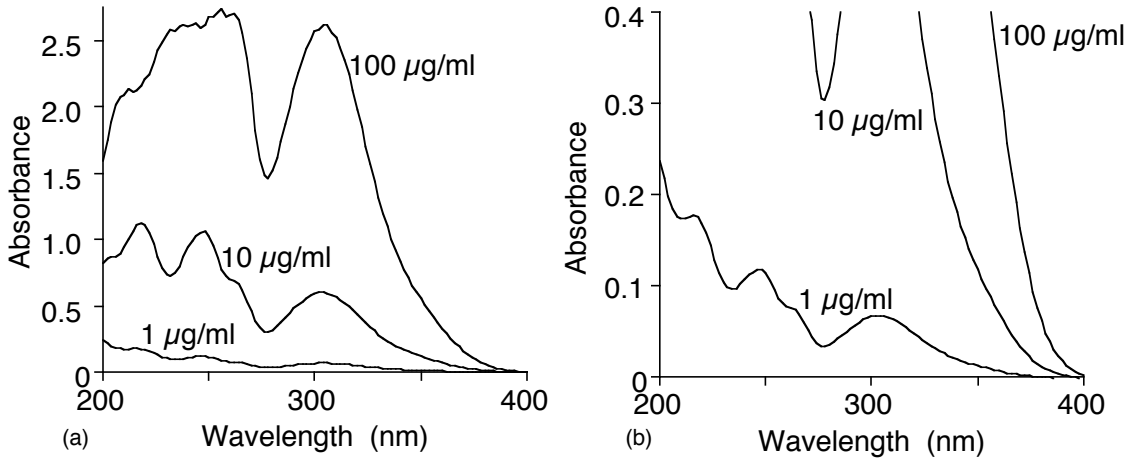

Figure 2.6: (a)The absorbance spectra for three concentrations of 8-MOP in water. (b) Enlargement of the graph on the left.

2.1.6 Timing the equipment

To improve fluorescence sensitivity, a triggering system was employed so the detector would be gathering data during the short fluorescence activity. Triggering is initiated by the OMA. The 2.0 V trigger signal travels into a four channel delay/pulse generator (Stanford Research Systems, Inc. model: DG535) which sends out a 5.0 V signal to the laser and a gated pulse amplifier (refer to figure 2.7). The delays of the laser and detector

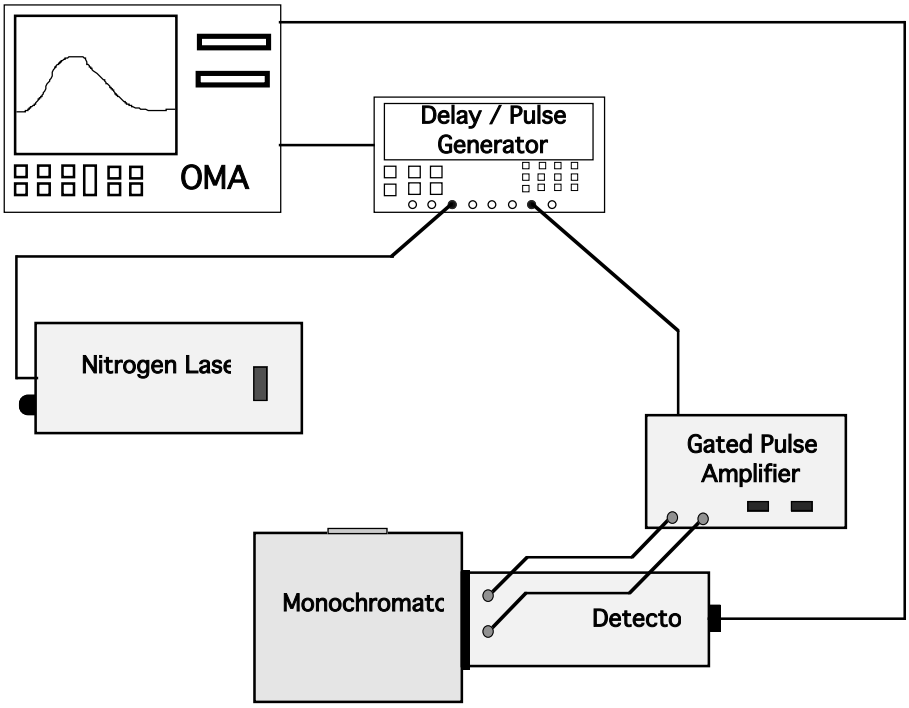

Figure 2.7: Set up of the triggering system.

were measured using a digitizing signal analyzer (Tektronix model: DSA 602A). The nitrogen laser pulse length is 3 ns and there is a 700 ns delay from the time it is triggered until the time it fires (see figure 2.8). The gated pulse amplifier (EG&G model: 1304) was connected to the diode array detector so that it could be turned on or off when desired. The triggering system was arranged so that the laser fired a pulse when the detector was reading data; consequently, 8-MOP fluorescence occurred while the detector was on. The detector was activated for a minimal length of time (approximately 70 ns) so the

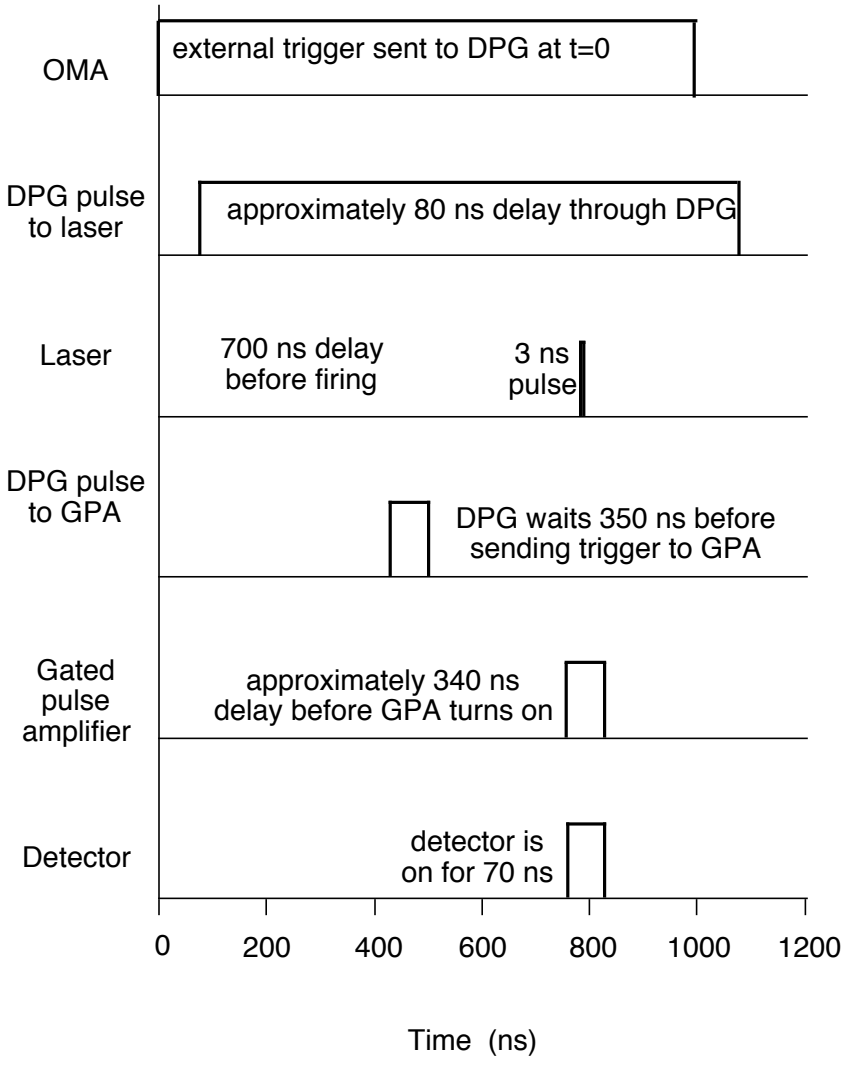

Figure 2.8: Timing diagram of the triggering system. Recall that the 8-MOP fluorescence lasts about 2 ns after the laser. Note that the detector is turned on when the laser fires. DPG represents the delay/pulse generator and GPA stands for gated pulse amplifier.

ratio of fluorescent light to background light was maximized; the delay/pulse generator was unable to turn the detector on for times less than 70 ns.

2.1.7 General measurement issues

Optical fibers

Optical fibers are an excellent way of delivering light to a specific place where it is needed. Fibers were used in this experiment to deliver the excitation light to the sample and to deliver the fluorescent light to the spectrograph. It was important to use a fiber that did not change or diminish the fluorescence signal or excitation light. There will naturally be some loss in the optical fiber; however, this should be minimized. Because much of this experiment occurred in the UV spectrum, it was important to use a fiber that transmitted light down to 300 nm. Fused silica was primarily used because it has low UV absorption. A lens was used to couple the laser light into the fibers. Later in the experiments, a bifurcated fiber was substituted for the ordinary optical fibers.

Room lights

Another important aspect was the room lighting. The detector obviously cannot differentiate between a photon originating from psoralen fluorescence and one that comes from the overhead lights. With the detector on for only 70 ns, the room lights were not a major factor; however, for scientific integrity, overhead room lights were turned off during data acquisition. If the room lights were on for one scan and off for another, it could produce inconsistent results. Several precautions were taken to eliminate extra light from entering the spectrograph. A black-felt-lined box was placed over the sample where fluorescence occurred so that only excitation light entered the sample. Furthermore, the box prevented room light from entering the detection fiber and being transferred back to the detector. Black electrical tape was used to minimize further leakage of unwanted light.

Consistency

It was extremely important to keep the set up consistent. The slightest alteration of the coupling lens into the bifurcated fiber would give a reduced intensity of excitation light. The positioning of the cuvette had to be firm and reproducible. A good set up is one where the sample can be measured, picked up, and remeasured obtaining duplicate results. Delivery of fluorescence into the detector needed to be steady and precise.

2.2 Calibration of equipment

Spectrograph's micrometer calibration

Since the wavelength of light falling on a particular detector in the diode array depends on the grating, the micrometer setting of the spectrograph, and the geometric alignment of the detector, the system must be calibrated. Because the spectrograph, detector, and OMA work as a team, they were calibrated as a team. A red helium-neon laser (Uniphase model: 1101, $\lambda = 632.8\,\text{nm}$) and a green helium-neon laser (Melles Griot model: 05-LGP-173, $\lambda = 543.5\,\text{nm}$) were diffused [2] into the entrance slit of the spectrograph to test the relation of wavelength on the micrometer setting of the spectrograph and the pixel position of the screen on the OMA. The spectrograph is aligned so that output of the zeroth-order (unseparated white-light) falls on the center of the diode array (detector 256 out of the 512) when the micrometer reads 0.000. Calibration was performed by plotting the movement of laser lines as the grating was rotated with the micrometer.

Calibration with the two lasers gave a consistent relationship between the wavelength of light, the micrometer's digital display setting, and the photodiode detectors in the detector array (this is the same as the pixels on the display screen of the OMA). Changing the wavelength of detected light by one nanometer results in a change of approximately two pixels on the OMA ($1.88 \pm 0.02\,\text{nm/pixel}$). If 400 nm light is observed at pixel 200, then 420 nm light should be seen around pixel 238. Because the screen

[2] The lasers were diffused into the spectrograph to prevent damaging the detector.

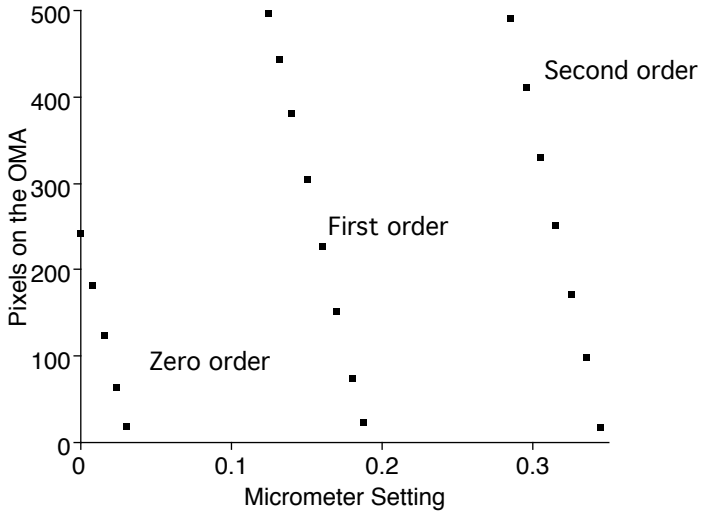

Figure 2.9: The relationship between pixel number, micrometer setting, and the orders of a certain wavelength (632.8 nm).

of the OMA represents 512 photodiodes, one can analyze a spectrum of approximately 273 nm of light. For example, a micrometer setting of 0.140 produced a wavelength range from 423.3 to 694.7 nm on the detector. One nanometer of wavelength is also equal to 0.000248 ± 0.000001 on the spectrograph's micrometer display. Thus if the 400 nm line fell on pixel 200 and the micrometer display is rotated by 0.025, then the 500 nm line will end up on pixel 200.

Because the peak of fluorescence emission of 8-MOP in ethanol is around 470 nm, it was important to place 470 nm near the center of the spectrum. The spectrograph was also used as a band pass filter, to eliminate signal contamination from the 337 nm excitation light. For these reasons the spectrograph micrometer setting was primarily set to 0.125 for the 8-MOP fluorescence measurements. This setting allows a wavelength range from approximately 363 to 636 nm to be observed through the spectrograph.

OMA calibration

The OMA is equipped with a calibration feature that allows the operator to calibrate the equipment for a particular micrometer reading. After calibration the OMA will display photon counts as a function of wavelength. A mercury lamp (Oriel model 66806) was used to calibrate the OMA. The mercury lamp has peaks at the following wavelengths: 312.6, 365, 404.7, 435.8, 546.1, and a double peak at 577 nm and 579 nm[3] (see figure 2.10). The micrometer was set to a certain position, 0.118 for example, and calibrated by following the procedure described in section 14.2 of the OMA manual. Each calibration was saved as a file that could be retrieved when the spectrograph was turned to that setting. Calibration files were saved for micrometer settings from 0.100 to 0.140 in increments of 0.005.

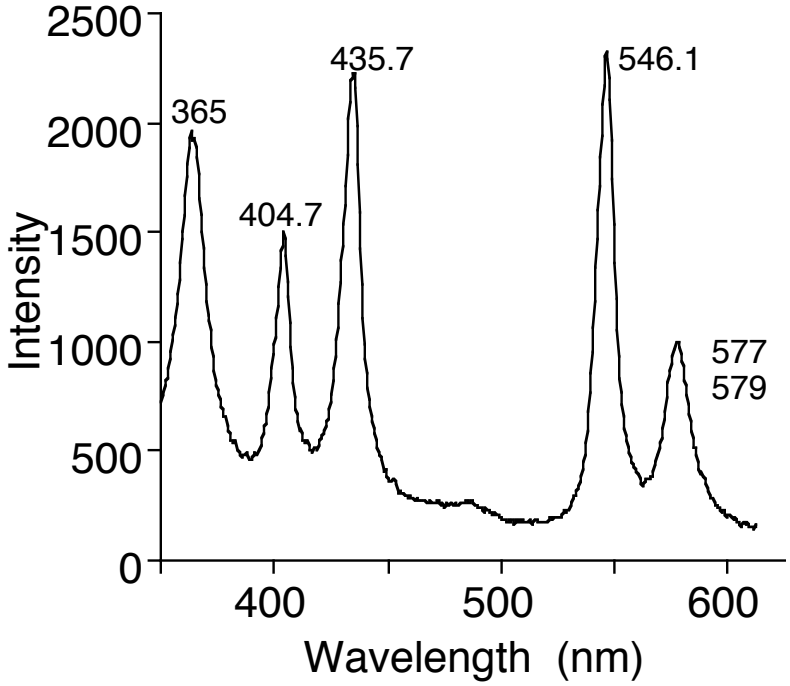

Figure 2.10: Mercury lamp output on the OMA with a spectrograph setting of 0.118.

[3]These peaks cannot be isolated with the 300 grooves/inch grating.

2.3 8-MOP in clear solutions

The initial fluorescence experiments of 8-MOP were done in ethanol, propylene glycol, and water. These solvents were chosen because they did not have fluorescence properties of their own (unlike serum). These solvents do not change the chemical composition or purity of the 8-MOP. The relevant range of concentrations were from 50 to 5000 ng of 8-MOP per ml of solvent because these are typical clinical 8-MOP concentrations in serum.

The 8-MOP dissolves in ethanol with only a minor amount of stirring and heat. Water is a more difficult solvent to use because 8-MOP does not dissolve well in water. The 8-MOP had to be dissolved in a small amount of ethanol before it could be mixed with water; 0.014 g of 8-MOP was added to 5 ml of ethanol before being mixed with water to create the proper solution. A pipette (Eppendorf 100 μl to 1000 μl) was used to get the desired concentration by adding part of the solvent to a previously made, already known concentration. If 4 μg/ml was needed as a sample, then 800 μl of 10 μg/ml solution could be added to 1200 μl of the solvent to obtain the desired concentration.

The nitrogen laser (Laser Science, Inc. model: VSL-337) was used because its beam has a relatively small divergence angle and a single appropriate wavelength ($\lambda = 337$ nm)[4]. The nitrogen laser delivered 120 μJ per pulse to the sample. A fiber optic probe with forty fibers was used to guide the fluorescence signal from the sample to the spectrograph. The forty fibers at the input of the probe were organized in a circular arrangement while the output fibers going to the spectrograph were arranged in a vertical rectangle of two fibers wide by twenty fibers high. The lens used to couple the laser into the fiber had a focal length of approximately 3 cm.

The original experiment was in the perpendicular orientation used by most fluorimeters. The excitation light from the nitrogen laser illuminated one cuvette face, while the detection probe was placed on an adjacent side (see figure 2.11). Plastic cuvettes were

[4]The first tests used a filtered mercury lamp, but this light was difficult to control because it diverged rapidly and the band pass filter allowed too much long wavelength excitation light through.

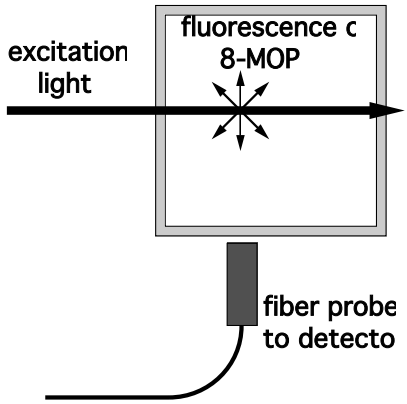

Figure 2.11: The original set up used perpendicular arrangement.

filled with different concentrations of 8-MOP in ethanol. The laser was set to pulse at twenty Hertz (its maximum repetition rate). The OMA collected ten data runs, each lasting 300 milliseconds. Although an 8-MOP fluorescence signal was obtained, results were inconsistent due to the inner filter effect which will be further discussed in chapters 3 and 4.

The original experiment was altered in several ways. The excitation source was arranged so that it came from the same direction that the fluorescence was collected. One way of accomplishing frontal illumination and detection is by using a bifurcated fiber (Oriel model: 77559). The bifurcated fiber had the laser coupled into one of its

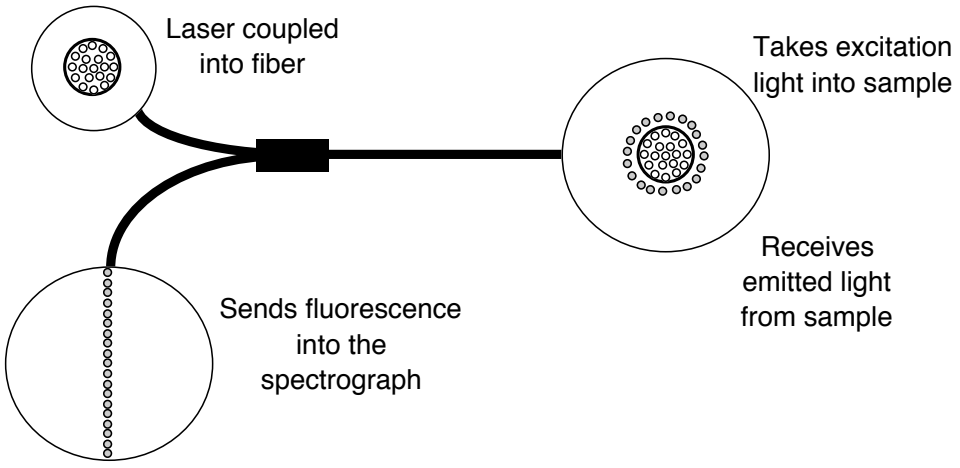

Figure 2.12: A close-up diagram of the ends of a bifurcated fiber.

three ends (see figure 2.12) using a lens. The excitation laser light traveled through the fiber to the sample where fluorescence occurs; the emitted fluorescence then travels through another set of fibers that terminate at the spectrograph. Plastic cuvettes were replaced by quartz and the triggering system described earlier was used. These changes improved results significantly.

2.4 8-MOP in serum

2.4.1 Preparation of 8-MOP in serum samples

After optimizing the set up for 8-MOP fluorescence in solvents of ethanol and propylene glycol, 8-MOP concentrations were measured in serum. Pigs were given 8-MOP rectally so the liver had less chance of removing it from the blood. Blood taken from pigs before and after administration of 8-MOP was placed in test tubes. Test tubes were spun at 1500 rpm for fifteen minutes in a centrifuge (Damon/IEC HN-SII) to remove the heavier red blood cells. Translucent serum was taken from the centrifuged test tube, placed in a separate test tube, labeled, and refrigerated until needed.

2.4.2 Light sources for 8-MOP in serum

Serum samples with and without 8-MOP could not be differentiated using the nitrogen laser's 337 nm excitation because the serum had stronger fluorescence than the psoralen. It was necessary to use a different excitation source because the nitrogen laser did not produce consistent results; 365 nm was chosen as an alternate wavelength because serum absorbs 365 nm light much less than 337 nm. The nitrogen laser was equipped with a dye module (Laser Science, Inc. model: DLM-120) so that other wavelengths could be generated. Laser dyes PBD and BPBD-365 (Exciton dyes) both have a lasing maximum at 365 nm when pumped with a 337 nm nitrogen laser source. PBD (Exciton 03660) was dissolved to obtain a $4 \cdot 10^{-3}$ molar concentration in a 7:3 toluene:ethanol solvent. This correlates to 0.00125 g of dye per 100 ml of solvent. A measurement (using a Mettler scale) of 0.0013 g of PBD dye was added to a mixture of 70 ml toluene and 30 ml of

ethanol. Approximately 3 ml of the dye solution was placed in a special four clear-sided quartz cuvette with stopper. Later, more dye was added to the solution (molar concentration was approximately doubled) because the dye did not lase. This work was all done under a fume hood because of the dangerous toluene vapor.

The dye module of the laser must be aligned precisely. A coumarin 480 dye (Exciton 04800) solution was used to line up the module mirrors and diffraction grating because this dye is more efficient and emits in the visible. A micrometer on the dye module allowed the laser to be tuned to the desired wavelength output completing the necessary alignment. The PBD dye solution was then substituted for the coumarin 480 into the dye module. Although not nearly as bright as the original 337 nm output a beautiful ultraviolet 365 nm output was produced.

Because the nitrogen dye laser output was relatively broad and extended into the region where fluorescence measurements were made and because the output was such a low intensity, the nitrogen-pumped dye laser did not produce good data for the 8-MOP tests in serum. A new option was tried; the 364 line of an argon laser. A continuous wave argon laser (Coherent Enterprise model: 652) with strong UV output was used to complete the experiments. A quartz prism separated the 351 nm and 364 nm lines of the laser. A circular aperture selected the 364 nm line, which was coupled into the bifurcated fiber using a quartz lens. A photometer measured the fiber output to be about 28 mW of power. Good results were obtained with the spectrograph set to 0.125 and attaching the KV 389 filter to the inside of the spectrograph.

2.4.3 Obtaining data from 8-MOP in serum

The set up for 8-MOP fluorescence in serum remained similar to the previous set up of 8-MOP in clear solutions except that no triggering was required (see figure 2.13). The data was acquired using the "Live" setting on the OMA. This mode of operation continually takes new photodiode measurements to replace the old; one spectra is taken instead of many. Without the triggering system, the set up is simple; however, there must be some consistency in the time of exposure to the sample from the high power of the argon laser.

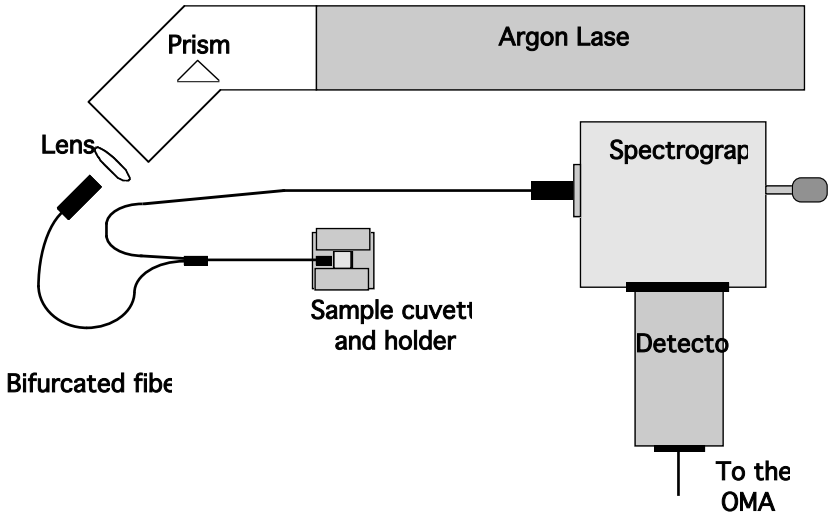

Figure 2.13: The set up used for 8-MOP measurements in serum.

The samples were each exposed for one to three seconds before the fluorescence spectrum was captured and saved in an effort to improve the consistency and reproducibility of the experiment. The exposure time was quick so that the bleaching effects of the sample did not dominate results. There was a spot size of approximately 2 mm in diameter on the front face of the cuvette. The 389 nm filter was again used for the 8-MOP in serum measurements to prevent saturation of the detector.

Spectra were taken from cuvettes containing 100 μg 8-MOP per ml of water, 10 μg 8-MOP per ml of water, plain water and nothing (i.e., an empty cuvette). The slit width was varied to evaluate the trade-off between sensitivity and resolution. Measurements were then made of serum containing no 8-MOP, diluted with various 8-MOP/water concentrations (0 to 100 μg 8-MOP per ml liquid). The cuvette must contain more than 1200 μl so that the liquid level would be above the fiber probe that received the fluorescence signal. Typically, 500 μl of serum was diluted with 1000 μl of solvent for the measurements. Testing of a range from 50 to 1000 ng/ml of 8-MOP in serum produced the most encouraging results. These tests all used serum from the same pig.

To determine if the experiment was a success, serum was tested from different pigs that had been given 8-MOP. Some of these pigs had had two serum samples taken: one

before administration and one after administration. The spectra obtained from these different samples was then compared to results from the pig's serum used to establish a fluorescence/concentration relationship.

Chapter 3

Results

The results of the experiments described in the previous chapter will be presented in chronological order. When this research began, it was not known whether the idea of using fluorescence to measure 8-MOP concentration was possible. To determine if drug levels could be analyzed by exciting a sample with a laser, the experiment was performed on 8-MOP dissolved in a solution of ethanol because it does not absorb UV-A light. Testing was also done on propylene glycol and water for the same reason. After obtaining successful results with the non-fluorescing solvents, the 8-MOP was tested in serum. Because serum does absorb the UV-A excitation light, significant changes had to be made to the experiment.

3.1 8-MOP in ethanol experiments

The first 8-MOP fluorescence measurements were made on 8-MOP dissolved in ethanol. The following different concentrations of 8-MOP were placed in plastic, disposable cuvettes: 10 ng/ml, 100 ng/ml, 1 μg/ml, 10 μg/ml, 100 μg/ml, 1 mg/ml, and plain ethanol. With the nitrogen laser pulsing at its maximum repetition of twenty Hertz, the OMA was set to its "Acc.–B." data acquisition mode.[1] Ten runs, each with a 300 ms exposure, are added together for each spectrum. The spectrograph's setting of 0.120 captured wavelengths from 343.5 to 621.8 nm. A cuvette containing only ethanol was used to obtain the background. All spectra had this background subtracted.

[1]This OMA mode automatically subtracts background after a set number of runs are made.

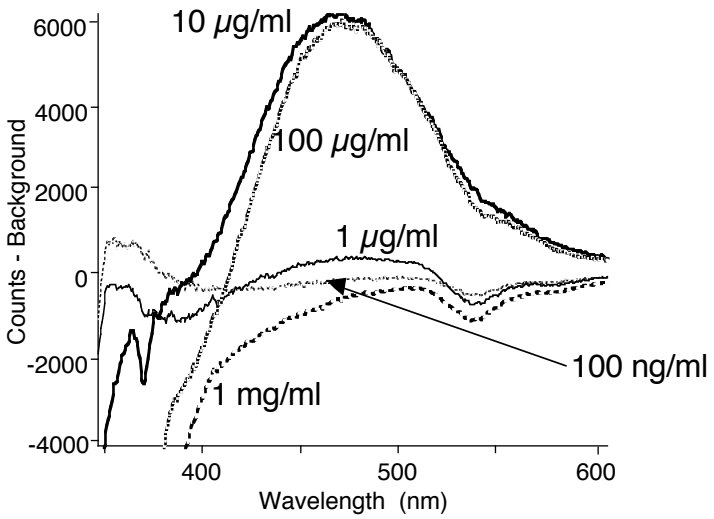

Figure 3.1: One of the first experimental results of 8-MOP fluorescence in ethanol. The untriggered nitrogen laser is perpendicular to the detection probe and the micrometer is set at 0.120.

Low 8-MOP concentrations were similar to the ethanol-only spectrum; consequently, the background subtraction produced numbers close to zero (see figure 3.1). The 10 μg/ml sample shows a fluorescence peak near 480 nm. The 8-MOP peak was thought, and later confirmed, to be at 470 nm for ethanol as a solvent. The 100 μg/ml concentration also demonstrated 8-MOP fluorescence, but the fluorescence peak's magnitude was not as great as the peak of the 10 μg/ml concentration. The highest concentration analyzed (1 mg/ml) did not show a strong fluorescence peak; and actually showed a lower overall reading, especially in the wavelength range below 400 nm. At the higher concentrations, a bright fluorescence was observed at the front of the cuvette, but not at the back. Similar results were obtained for samples containing propylene glycol as the solvent.

The fluorescence of 1 μg/ml to 10 μg/ml 8-MOP in propylene glycol are shown in figure 3.2. The spectrograph micrometer was set at 0.125 and the laser was pulsing at its maximum repetition rate of 20 Hz (the detector gathered data for approximately ten seconds). The height of the spectral peaks increased with 8-MOP concentration. Unfortunately, concentrations below 2 μg/ml were indistinguishable and inconsistent. In

these early experiments, plastic cuvettes were used as the sample containers.

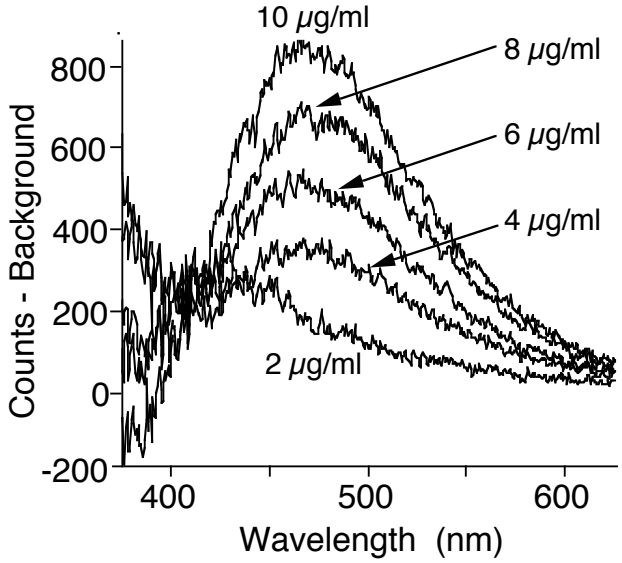

Figure 3.2: Spectra of 8-MOP in propylene glycol of various concentrations using a perpendicular excitation/detection arrangement.

The apparatus was altered to illuminate and detect from the same side of the cuvette. One attempt coupled the laser into three 1 mm fibers arranged in a triangular pattern around the detector probe (see figure 3.3(a)), but too much sensitivity was lost. Another attempt used a beam splitter centered between the spectrograph and the cuvette; part of the excitation light was reflected into the sample and the emitted fluorescence passed through a filter into the spectrograph (see figure 3.3(b)). This failed because too much excitation light was lost by the beam splitter. Yet another attempt used a front surface mirror to reflect the excitation light into the sample that was placed directly in front of the spectrograph's entrance slit (see figure 3.4). This also produced inconsistent as well as illogical results.

The problem was the cuvette. Plastic cuvettes had been used as sample containers; however, it was discovered that these cuvettes were fluorescing. Figure 3.5 shows spectra of two empty cuvettes: plastic and glass. The fluorescence peak from the plastic cuvettes is located at roughly the same wavelength as the peak for 8-MOP; it therefore interfered

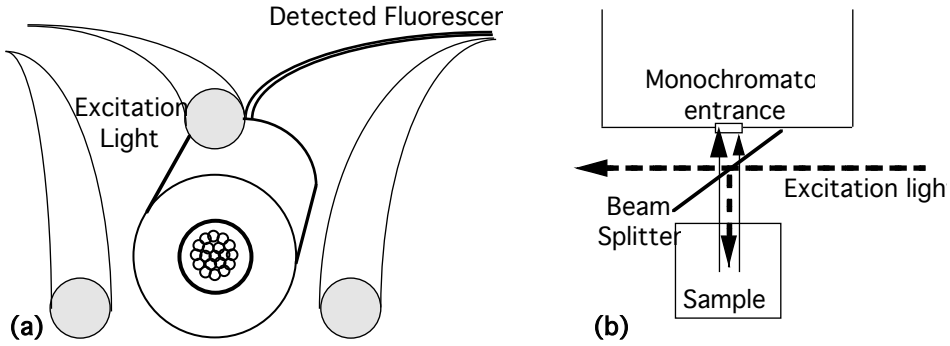

Figure 3.3: Two inadequate detection schemes using plastic cuvettes: (a) shows the cuvette's view of the triangular arrangement of the fibers for frontal illumination and detection (b) diagrams the beam splitter attempt.

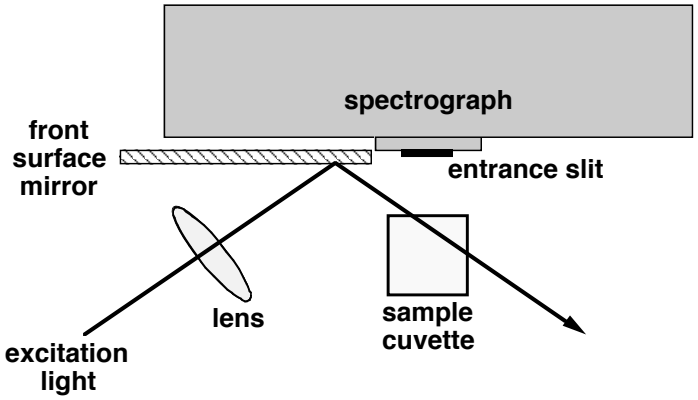

Figure 3.4: An arrangement using a front surface mirror to reflect the excitation light into the cuvette.

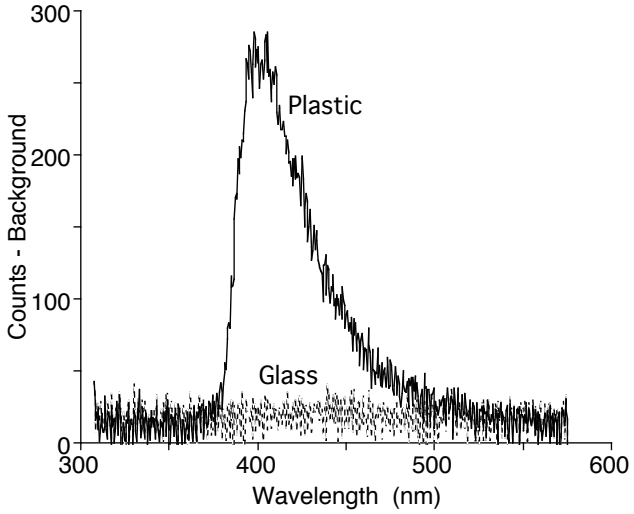

Figure 3.5: Spectra comparing a plastic cuvette and a glass cuvette (using 337 nm excitation and 389 nm cutoff filter).

with detecting the psoralen fluorescence. The glass cuvette did not fluoresce.

The experiment was improved by replacing the plastic cuvettes with glass cuvettes. A silica bifurcated fiber was used for both excitation and detection. The KV389 filter was again placed just inside the entrance port of the spectrograph. Figure 3.6 shows the fluorescence spectrum obtained using ethanol as the solvent. Peak fluorescence can be seen at 470 nm (using 337 nm excitation and no slit). Fluorescence decreases with decreasing concentration. For the first time, the fluorescence spectra were in the correct order down to the low concentration of 200 ng/ml. The reason for the double peaks seen near 400 nm and 425 nm has not been confirmed. They are probably due to Raman Scattering; energy from the excitation light can be converted into vibrational energy in the molecules causing the emitted light to decrease in frequency.

To quantify the relationship between fluorescence intensity and 8-MOP concentration, the averages of the counts gathered from 465 to 475 nm were plotted as a function of concentration. Figure 3.7 shows two different experiments examining the same concentrations of 0.1, 0.2, 0.5, 1, 2, and 5 μg/ml. A linear relationship between fluorescence and 8-MOP concentration in ethanol was obtained. Furthermore, the two different ex-

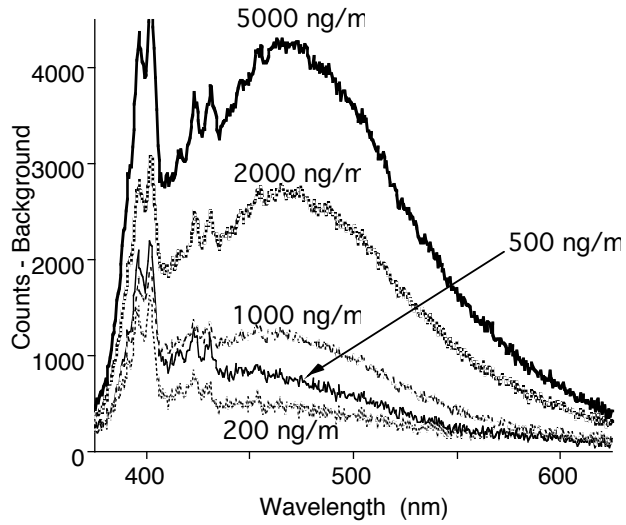

Figure 3.6: Different concentrations of 8-MOP in ethanol using glass cuvettes (baseline subtracted).

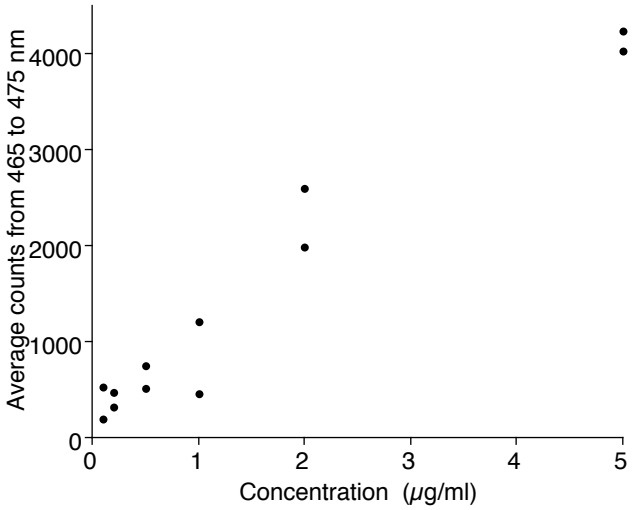

Figure 3.7: The relationship between fluorescence and concentration of 8-MOP in ethanol using glass cuvettes (baseline subtracted).

periments show consistency in the data. The two plots are relatively close to one another for each concentration.

Another experiment was performed using quartz cuvettes. The 337 nm nitrogen laser was used with the triggering system discussed in subsection 2.1.6. No slits were attached to the spectrograph. Fluorescence spectra were taken from samples ranging from 50 to 4000 ng/ml of 8-MOP in ethanol (2 ml of solution per cuvette). To differentiate between different spectra, plots were made comparing concentration to the average of the fluorescence in the range from 450 – 550 nm (see figure 3.8(a)) or 465 – 475 nm (see figure 3.8(b)). Subtracting the ethanol only spectrum, which eliminates the background, was another attempt to give clarity to the data (figures 3.9(a) and 3.9(b) show these results).

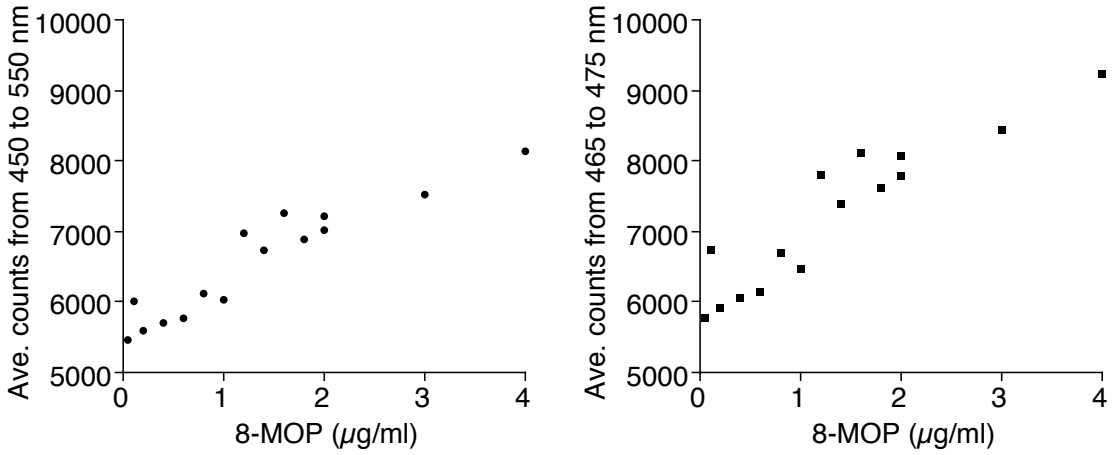

Figure 3.8: The average counts in the ranges of (a) 450 to 550 nm and (b) 465 to 475 nm with no subtraction (using 337 nm excitation).

The fluorescence of 8-MOP shifts as the solvent changes. In ethanol, the peak fluorescence is at about 470 nm; in water the peak of 8-MOP red-shifts to approximately 500 nm (see figure 3.10). The fluorescence peak in serum diluted with water is close to 500 nm as well.

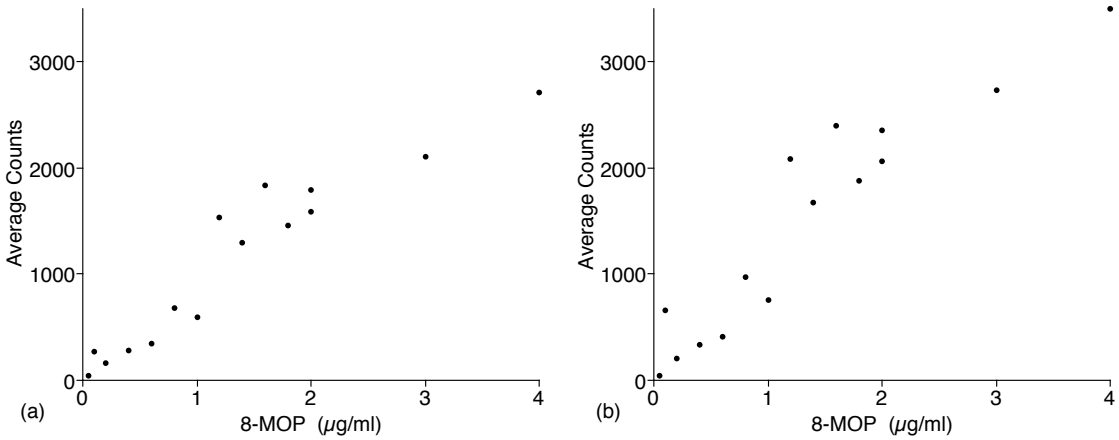

Figure 3.9: The average counts in the range of (a) 450 to 550 nm and (b) 465 to 475 nm with the ethanol only spectra subtracted.

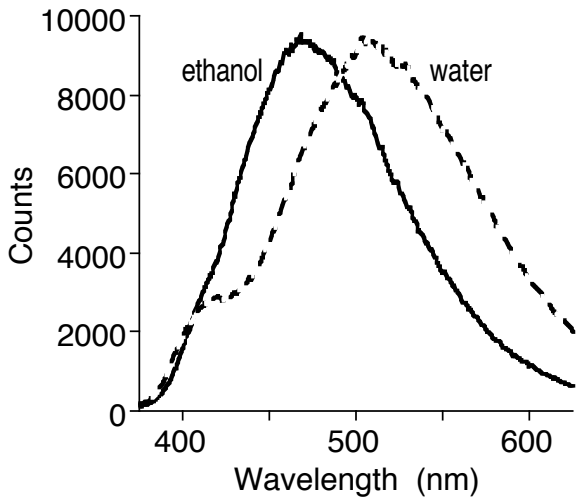

Figure 3.10: Different solvents of 8-MOP (both are 100 μg/ml) produce fluorescence peaks at different wavelengths. Excitation light is the 364 nm line of the argon laser using 100 μm slit and 389 nm filter.

3.2 Measurement of 8-MOP in serum

Spectra of 8-MOP in porcine serum were obtained using the nitrogen laser at 337 nm, the triggering system, the bifurcated fiber, and the quartz cuvettes (no slits). A strong serum autofluorescence signal was observed. It was close to impossible to distinguish the

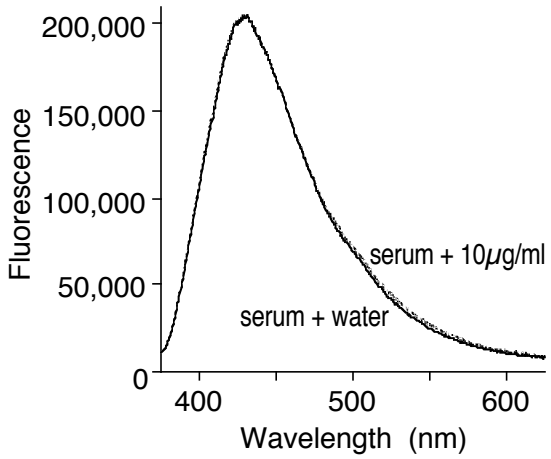

Figure 3.11: Yes, there are two spectra shown. One is of 600 μl of serum diluted with 600μl of water and the other is 600 μl of serum diluted with 600 μl of 10 μg/ml of 8-MOP in water. The nitrogen laser was used as the excitation wavelength.

8-MOP fluorescence from the serum fluorescence (see figure 3.11). Diluting the samples with water, a potassium buffer, or ethanol did not help.

A different excitation wavelength was tried. An ideal excitation wavelength would be one that is well absorbed by 8-MOP and poorly absorbed by serum. Unfortunately, this ideal wavelength does not exist. Below 337 nm, 8-MOP fluorescence increases, but serum fluorescence also increases. Above 337 nm, serum fluorescence drops off, but 8-MOP fluorescence decreases as well. Figure 3.12 shows the absorbance of serum diluted with water versus the absorbance of 8-MOP in water. This figure shows that the peak absorbance of serum is around 275 to 280 nm while the peak absorbance of 8-MOP is between 300 and 305 nm. The absorbance of light suggests that an excitation wavelength just above 337 nm would be better than below because the serum absorbance in water is

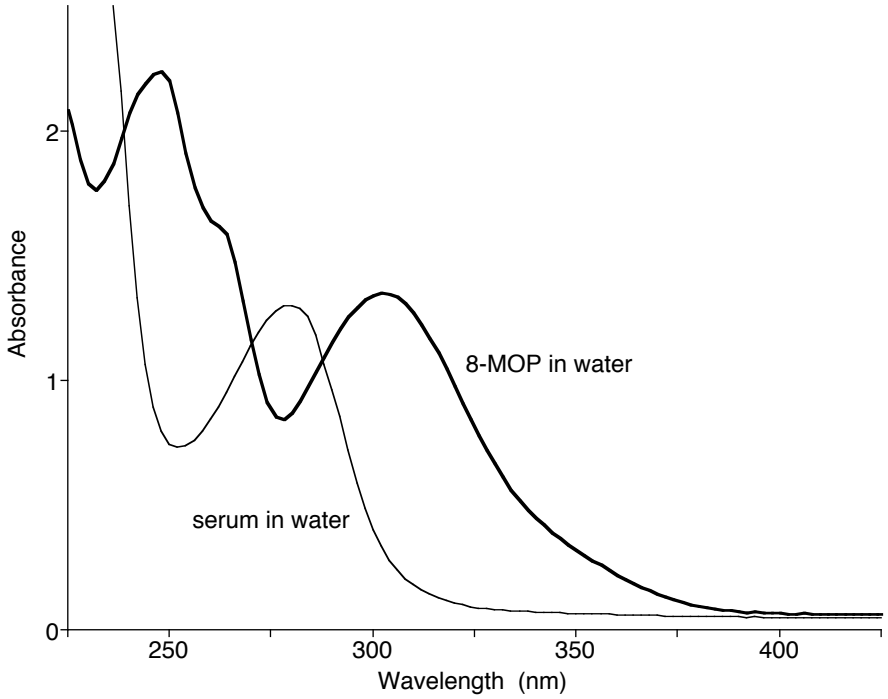

Figure 3.12: This graph shows the UV-A absorbance spectrum of serum diluted with water (1:64) versus 8-MOP in water ($1\,\mu$g/ml).

lower than the 8-MOP absorbance at wavelengths above 337 nm. Distilled water in the quartz cuvette does not have a significant absorption spectrum to change results in any way. The closest wavelength attainable with an available laser was 365 nm.

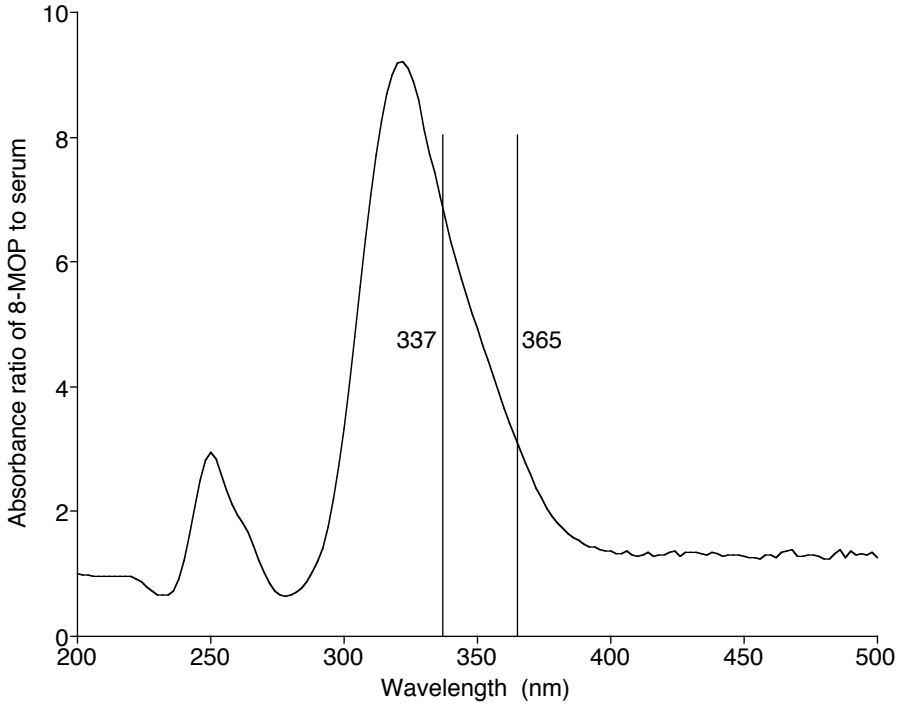

Figure 3.13: The ratio of the absorbance of 8-MOP in water to the absorbance of serum in water shows that both 337 nm and 365 nm are good wavelengths for exciting 8-MOP and not serum (absorbance data taken from figure 3.12).

The nitrogen laser is equipped with a dye module (Laser Science, Inc. model DLM-120) which allows a dye to be pumped using the 337 nm line. The laser dye PBD (Exciton # 03660) has a peak output at 365 nm and a range from 357 to 390 nm. After maximizing the 365 nm output of the nitrogen pumped dye laser, samples of 8-MOP in water were analyzed (figure 3.14). The highest signal detected was for an empty cuvette that reflected the unattenuated excitation light from the rear cuvette face back into the bifurcated fiber. Cuvettes with samples in them would absorb some of the excitation light and the light from the rear face was diminished. Despite the presence of the 389 nm cut-off filter inside the spectrograph, the excitation source could be observed out to

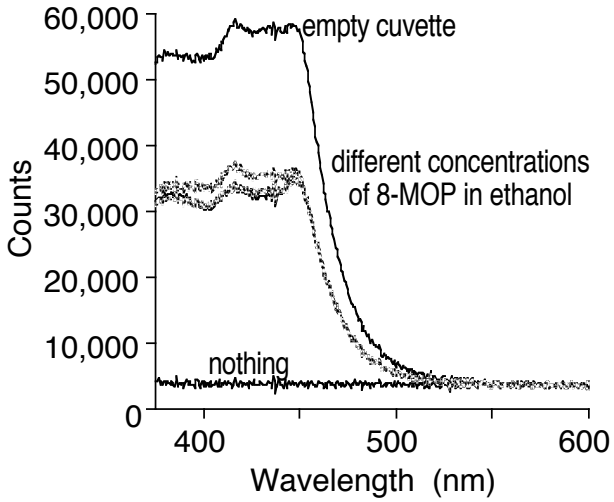

Figure 3.14: This spectra shows the reflection of the excitation light from the cuvette. An empty cuvette reflects the most light. The other samples all reflect about the same amount. With nothing in the cuvette holder, only background noise was observed.

500 nm. This contamination was due to the wide band of the excitation light. Different slits did not help control this contamination. The nitrogen laser pumped dye-module has a 10 nm linewidth at half its peak, but it has a much wider band at one-hundredth of its peak, which is broad enough to be detected 100 nm from the peak. This broad linewidth, plus the fact that the excitation pulse power was rather low (probably less than 10 μJ per pulse), prohibited detecting any 8-MOP fluorescence measurements.

The 364 nm line of an argon laser was tried next. This is the actual laser used for the PUVA experiments, so it makes good sense to use it. A quartz prism and a circular aperture were used to separate the 364 nm line from the 351 nm (see section 2.3). The aperture allows a narrow spectral band of light (less than 10 nm) to be coupled into the bifurcated fiber. Approximately 28 mW of continuous wave output was delivered from the 364 nm line through the bifurcated fiber. This power leads to beautiful 8-MOP fluorescence in both water (figure 3.15) and serum.

Although 25, 50, and 100 μm slits were tested (as well as no slit), only the results from the 100 μm slit are shown. This slit provided the greatest fluorescence without saturating

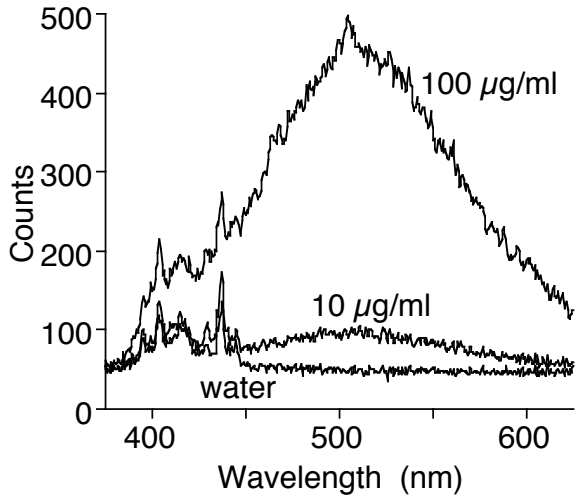

Figure 3.15: Fluorescence of different concentrations of 8-MOP in water (100 μm slit with 365 nm excitation).

the detector. The absence of the slit produced counts in the detector near saturation. Figure 3.16 shows five samples. Each sample contains 500 μl of serum and 1000 μl of the diluting solution (water or 8-MOP/water) shown in the graph. For clarity, the higher concentrations of 10 μg/ml, 50 μg/ml, and 100 μg/ml have been omitted, although they produced consistent spectra also. The water-diluted spectra is subtracted from the others in figure 3.17. The average counts detected from 450 to 550 nm (with background subtraction) are plotted versus the 8-MOP concentration in the serum samples in figure 3.18. The linear relationship expected is not present. The counts seem to flatten out as the 8-MOP level increases. This phenomena may be due to the re-absorption of the fluorescence emitted from the center of the cuvette. Low concentrations do not absorb light as well as higher concentrations.

To find the lowest detectable concentration, the serum was diluted with 8-MOP in water in the range of 100 to 700 ng/ml. The results were exciting. In this low range of 8-MOP in serum, a relatively linear relationship of fluorescence to concentration could be observed. Figures 3.19, 3.20, and 3.21 show promising results of a consistent relationship of 8-MOP fluorescence to concentration.

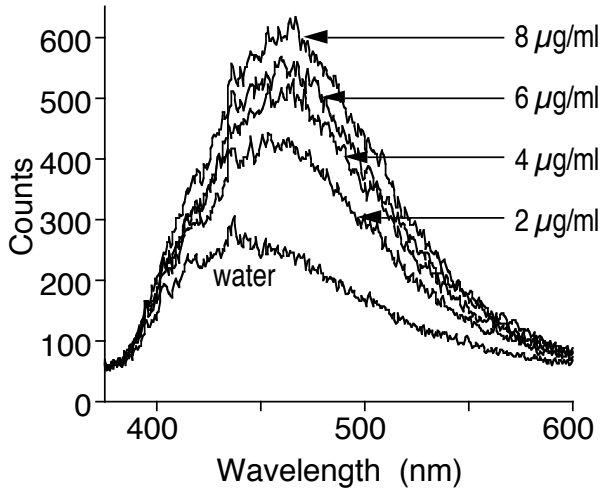

Figure 3.16: Fluorescence of serum diluted with different concentrations of added 8-MOP (the concentrations shown were then mixed with serum).

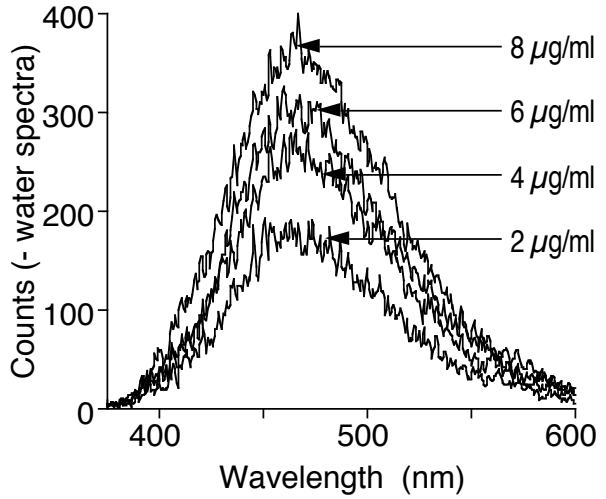

Figure 3.17: The water-diluted sample was subtracted from these 8-MOP concentrated dilutions of serum (365 nm excitation).

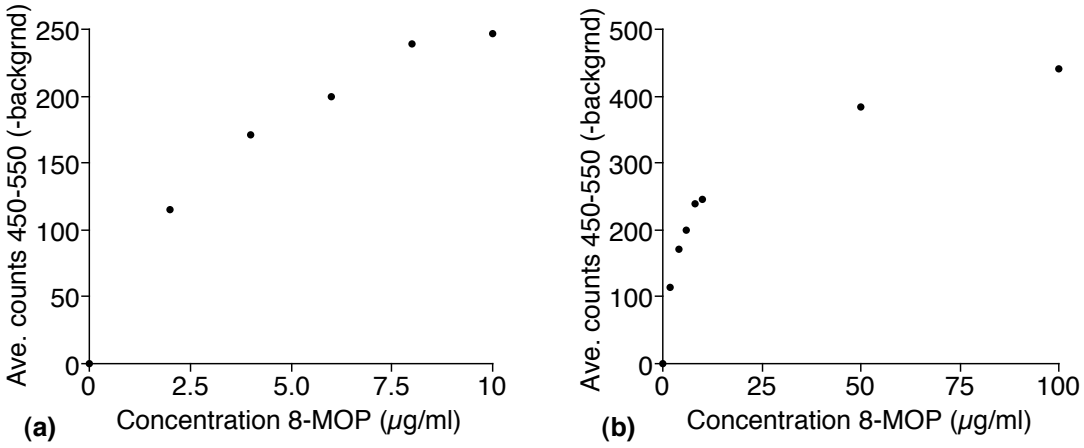

Figure 3.18: The relationship between background-subtracted fluorescence signal and 8-MOP concentration used to dilute serum samples appears to be linear for concentrations below 10 μg/ml in (a) but does not appear to be linear above 10 μg/ml in (b).

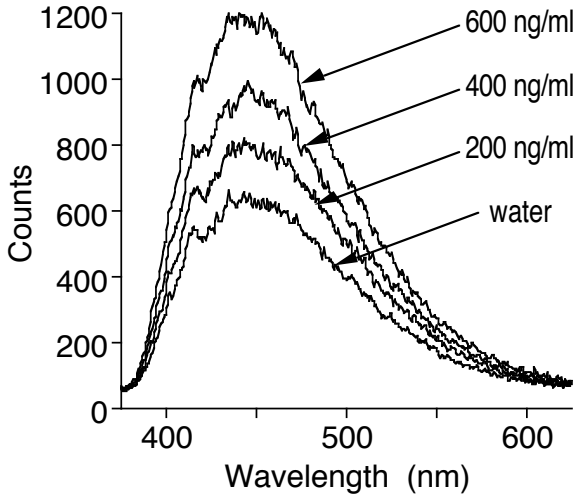

Figure 3.19: Low 8-MOP concentrations in serum can be easily differentiated (100, 300, 500, and 700 ng/ml are not shown for clarity)

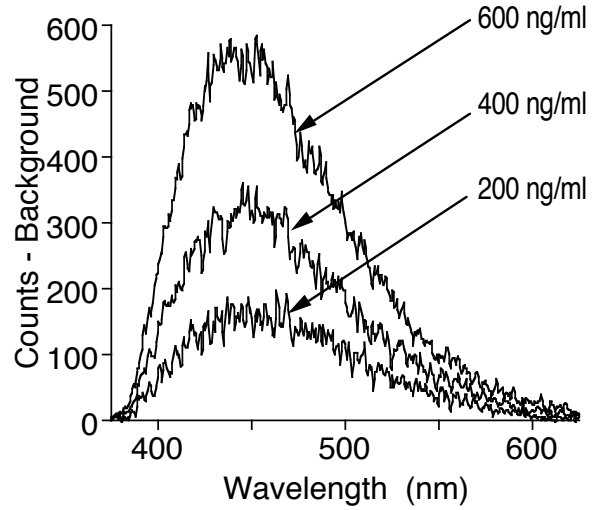

Figure 3.20: With the water diluted spectra subtracted, the difference among these low concentrations is obvious.

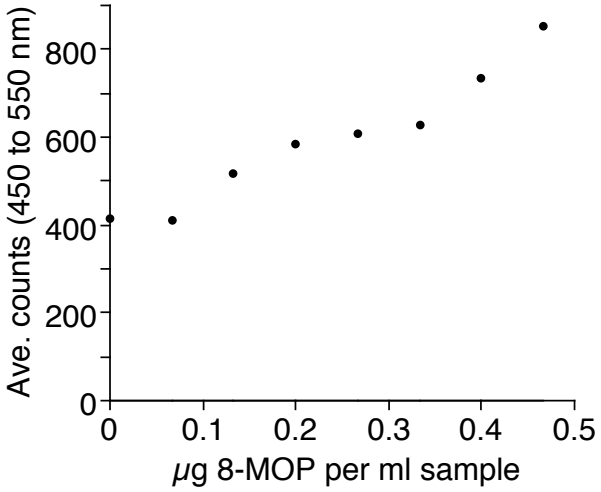

Figure 3.21: The relationship of fluorescence signal to 8-MOP concentration seems to be linear at these low concentrations.

Another way to look at data of this sort is by comparing the slope of the spectra. The slope of two points on a line is usually obtained by dividing the change in the height of a curve by its change in width, i.e. $\Delta y/\Delta x$. An alternate method of comparing lines that lead to a common point is using equation 3.1.

$$[F(450) - F(550)]/F(550) = ratio \tag{3.1}$$

In this equation, F(450) would be the counts at 450 nm, or an average around this wavelength. Figure 3.22 shows a comparison of ratios obtained from the 8-MOP in serum data at low concentrations using the 100 μm slit. An average of the counts was taken within 5 nm of the two wavelengths. The results are similar to figure 3.21 which examined an average rather than a ratio of averages.

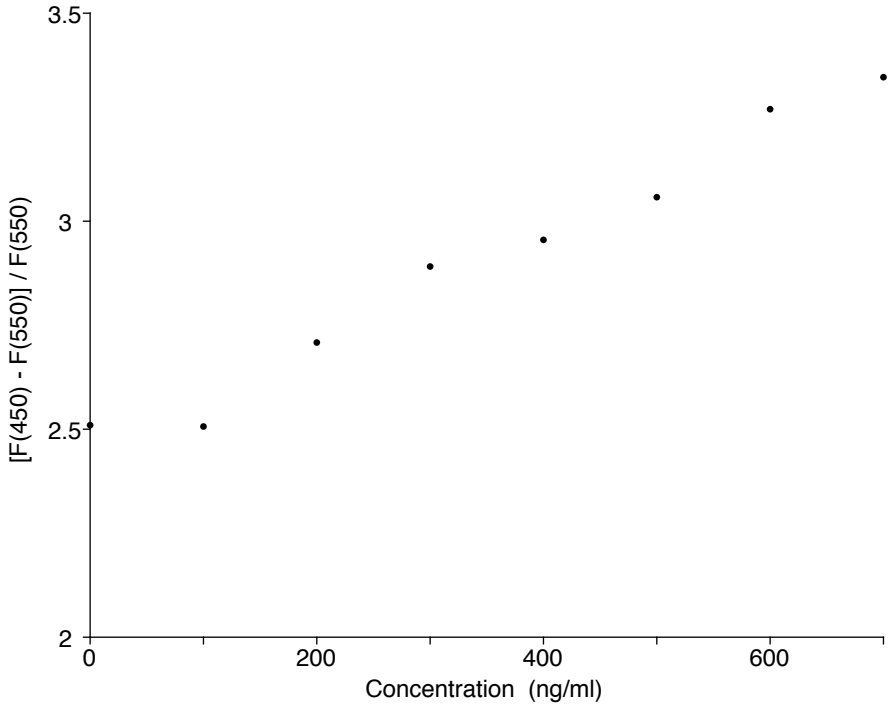

Figure 3.22: The relationship of fluorescence signal to 8-MOP concentration can also be examined by looking at the ratios of two points on each spectrum.

When the experiment was redone for concentrations varying from 0 to 1000 ng per 1.5 ml of serum and water in increments of 100 ng, the results were fairly consistent.

Although the data for no slit came out better, the fluorescence was too close to saturating the detector and was therefore not used for the set-up.

Chapter 4

Discussion

4.1 Problems conquered in 8-MOP experiments

A number of significant obstacles were encountered during this project. These hurdles included: the inner filter effect, timing, selectivity of wavelength, and unwanted fluorescence. These will each be discussed in the following pages.

4.1.1 Overcoming the inner filter effect

The inner filter effect is evident when the sample strongly absorbs the excitation light so that fluorophores at the back are not illuminated. Recall (from section 3.2) that in highly concentrated samples, strong fluorescence could be observed in the solution at the front of the cuvette, but little or no fluorescence was seen at the back of the cuvette. The solution at the front of the cuvette absorbs the majority of the excitation light resulting in the bright emission; however, the excitation light does not reach the solution further back in the sample (see figure 4.1). It was for this reason that frontal illumination and detection was desired. The method that worked best involved the use of a bifurcated fiber. The bifurcated fiber delivered the excitation light from the laser to the sample and delivered the emitted fluorescence from the sample to the detector. Results were a tremendous improvement over the perpendicular arrangement first used.

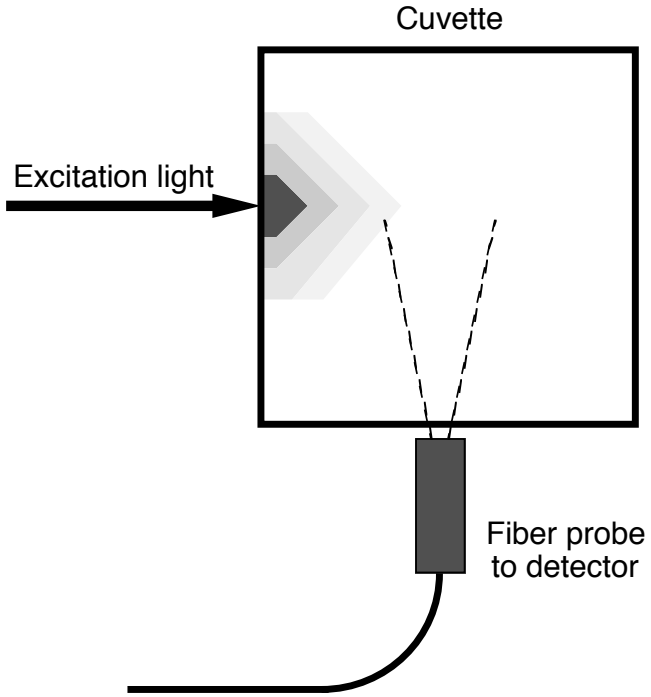

Figure 4.1: Less light can reach the back of the cuvette in a high concentrated solution because of the inner filter effect. The probe may not see the fluorescence in this set up because most of the fluorescence occurs near the front face.

4.1.2 Triggering the set up

Because the nitrogen laser has a 3 ns pulse and the 8-MOP fluorescence lasts less than 2 ns, timing was of the essence in the first phase of this experiment. It was crucial that the detector was reading data while fluorescence was occurring. An ideal set-up would turn the detector on at the moment the laser fired and would turn the detector off when the fluorescence terminated, approximately 5 ns later. Although these short times were not possible with the equipment used in this experiment, it was still important to have the detector on for a minimal amount of time. If the detector were on when no 8-MOP fluorescence was being emitted, then the detector would only be measuring emission from other light sources. The triggering system permitted the detector to be on for a minimum of 70 ns (refer to figure 2.8). Internal delays in the equipment had to be factored into the triggering times. The nitrogen laser had a 700 ns delay and the detector had a 350 ns delay. It was only important to trigger the fluorescence measurements with the 8-MOP in the non-serum solvents because the laser used for the experiments with serum was continuous wave.

4.1.3 Examining only the desired fluorescent light

Since the fluorescence signal being measured was only a minute fraction of the excitation light's intensity, it was vitally important to detect only the desired fluorescent light. In ethanol, 8-MOP fluorescence was emitted at and around 470 nm using a 337 nm excitation source. In serum, 8-MOP fluorescence was emitted at and around 500 nm using a 364 nm excitation source. The KV389 filter prohibited the excitation light from entering the detector where saturation would have otherwise been highly likely. The spectrograph was crucial in selecting the desired wavelength range to be examined. The setting of 0.125, which permitted a spectra from approximately 363 to 635 nm to be analyzed, was used for both phases of the experimental research.

Another important parameter was the elimination of any light entering the detector other than the fluorescence obtained from the sample. This was accomplished through

the use of the spectrograph's slits, black tape, and black boxes. Turning the room lights off was important in the serum experiments where the OMA was taking continuous readings. The room lights were not as important when triggering the system because the 70 ns time frame of each run is so quick. Although this is not much time to make a difference in signal, the lights were nevertheless left off.

4.1.4 Eliminating unwanted fluorescence

An unfortunate problem with this project was that the 8-MOP was not the only substance which fluoresced. The obstacle which took the longest to overcome was the fluorescence of the plastic cuvettes. Once the difference in fluorescence signals using glass cuvettes compared to plastic cuvettes (see figure 3.5) was discovered, glass containers, and eventually quartz, were used. After this switch, good results of the 8-MOP measurements in ethanol were obtained. The largest problem associated with the 8-MOP in serum experiments was the strong fluorescence of the serum. This unwanted serum fluorescence continues to be a problem which needs improvement. Diluting the serum with water seemed to help the experiments, but there may be a different solvent (or solvents) that could improve the 8-MOP to serum fluorescence ratio.

It was important to use UV-A transparent optical devices in the set-up. The cuvettes, prism, lenses, and bifurcated fiber should not absorb any of the excitation light because they could produce their own fluorescent signal. The peak around 400 nm seen in figure 3.6 could possibly be a fluorescent signal from the bifurcated fiber used that was constructed of bundles of high grade fused silica.

4.2 Discussion of 8-MOP measurements in ethanol

Figure 3.1 shows the first 8-MOP in ethanol experimental results. The y-axis of this graph is labeled "Counts - Background". The counts refer to the number of photons detected by each of the photodiodes in the array. The background is subtracted so the difference can be seen. Usually, including this first case, the background spectrum was

from a cuvette filled exclusively with the solvent. A spectra taken from a cuvette full of ethanol, for example, would be set as the background. When negative numbers are obtained from the background subtraction, it would mean that less light at that wavelength was reaching the detector than the light obtained in the ethanol only sample. This was probably caused by an absorption of the excitation light at the front of the sample in the cuvette; recall that this first set up had the detector arranged perpendicularly to the excitation source. Lower counts were obtained for higher concentrations when the frontal illumination and detection orientation was used because the majority of the fluorescent signal came from the plastic cuvette being used. The higher concentrations absorbed more of the excitation light so less light would be reflected from the back of the cuvette.

The results of the first experiment (described at the beginning of section 2.3; also see figure 3.1) demonstrate the inner filter effect. The low concentration of 100 ng/ml had data close to zero because there was little difference from the ethanol background. A concentration of 1 μg/ml produced a fluorescence peak at 470 nm. The 10 μg/ml concentration had a strong fluorescence peak. 100 μg/ml also demonstrated good fluorescence, but its peak was lower than the 10 μg/ml peak. This phenomena seen in the perpendicular arrangement (see figure 2.11) was due to screening (another word for the inner filter effect). The higher concentration absorbed more of the light at the front of the cuvette so that the detector, which was examining the center of the cuvette, did not see all the fluorescence. The 1 mg/ml concentration allowed just a small amount of light past its front surface so that little fluorescence was recorded by the OMA.

The spectra in figure 3.2 demonstrate a good ordering of the different concentrations. Only the even concentrations are shown for clarity; however, the odd concentrations did in fact fill in the gaps nicely. A problem with this technique is that it does not allow good measurements of lower concentrations, i.e., below 2 μg/ml. It was the intent of this project to be able to measure differences in concentration as low as 50, or even 20 ng of 8-MOP per ml of solvent.

Although the perpendicular arrangement works decently in solvents that do not absorb much (or any) of the excitation light, this experiment would eventually be using serum as the solvent. Serum absorbs UV-A light strongly and would cause serious problems with the inner filter effect in measuring 8-MOP fluorescence if the perpendicular set-up was used. The frontal illumination and detection arrangement was adopted in hopes of making 8-MOP diagnostics in serum.

An interesting observation can be made from both figures 3.1 and 3.2. There is an increase in signal below 400 nm occurring in opposite order of concentration. This is most probably due to the signal from the plastic cuvettes fluorescing. For low absorbing samples, the plastic will fluoresce on both the front and the back sides of the cuvette. For higher absorbing samples, the back of the cuvette will not receive as much excitation light; consequently, it will not fluoresce as much. Both of these spectra were taken using plastic as the sample containers and without the 389 nm cut-off filter in place.

When the improvements of the cut-off filter, the bifurcated fiber, the triggering system, and the glass cuvettes were finally employed, exciting results were obtained (see figure 3.6). In an attempt to define the relationship between fluorescence and concentration, the average counts obtained between 465 and 475 nm were plotted in figure 3.7. There appeared to be a linear correlation between this average and the concentration of 8-MOP in ethanol. Good results are also seen in figures 3.8 and 3.9, which are from an experiment that used quartz cuvettes.

There are a number of ways to analyze the data obtained in an experiment. All of the analyzation techniques tried yielded similar results; however, the optimal method of comparing fluorescence with concentration is not known for sure. The data can be compared by looking at the average counts around the fluorescence peak. The average could be taken of the background subtracted spectra. A ratio of counts at two wavelengths can be taken to compare the slope of the spectra, but determining which two wavelengths produce the best results is not confirmed.

The figures showing average counts versus concentration seemed to be an adequate indication that the relationship between fluorescence signal and 8-MOP concentration

in ethanol exists and can be determined. The goal of this research project, however, was to determine the concentration of 8-MOP in serum, not ethanol.

4.3 Discussion of 8-MOP measurements in serum

The results of 8-MOP fluorescence in serum using the 337 nm excitation of the nitrogen laser were poor. The only fluorescence signal observed was from the serum, not the 8-MOP. It was obvious that the fluorescence was not a result of the 8-MOP because serum diluted with 10 μg/ml of 8-MOP in water yielded an identical spectrum to serum diluted with 1 μg/ml solution. The fluorescence peak was close to the cut-off filter's wavelength which indicates that the fluorescence peak was below the cut-off filter. The results of 8-MOP fluorescence in serum using the 365 nm excitation from the nitrogen-pumped dye laser may have been worse than the 337 nm excitation. Spectra for serum in different concentrations of 8-MOP were inconsistent.

The 364 nm line of the argon laser produced beautiful fluorescence of the 8-MOP in serum. The two main reasons that the argon laser does a better job is that it is much more powerful and 364 nm is absorbed much better in psoralen than in the serum (see figures 3.12 and 3.13). Another factor that can affect fluorescence, but that did not come into play, was quantum efficiency. The quantum efficiency is approximately the same for both 8-MOP and serum at the two wavelengths used [30].

Tests of serum diluted with different 8-MOP concentrations were truly positive when the argon laser was used as the source. Figure 3.16 shows four spectra taken from serum diluted with water, 200, 400, and 600 ng/ml. Not only are these spectra in their correct order of concentration, but there is a good relationship of their average counts to their concentration (see figure 3.18). Lower concentrations of 8-MOP in water also showed consistency. A linear relationship exists between low concentrations of 8-MOP in diluted serum to average counts between 450 and 550 nm (see figure 3.21).

The goal of this research was reached in determining that a relationship exists between 8-MOP fluorescence and 8-MOP concentration in both clear solvents and serum. The

testing is not perfected, but large strides have been made to a new method of diagnosing levels of 8-MOP in blood quickly, inexpensively, and accurately.

Chapter 5

Transferring files

Spectra from the OMA are saved as an MS DOS file on a 5.25 inch floppy disk. These files are saved on the disk in the lower of the two OMA drives by typing "`2:FILENAME.DAT`" when prompted in the disk operating system. It is necessary to specify drive two so the information can be transferred to another disk using an MSDOS computer. The name of the file can be up to eight characters long, but must begin with a letter, not a number. The file must end with .DAT because it is a data file. The 5.25 inch disk can hold about sixteen files before the user risks losing information. These disk files can be copied onto 3.5 inch disks, which can be used on our Macintosh computers for analysis. Opening the file using a text editor program shows a list of numbers retrieved from the OMA. The spreadsheet program Excel (version 4.0) was used to reproduce and analyze the data.

Absorbance and transmission spectra could be obtained using a diode spectrophotometer (Hewlett Packard model: 8452A). These files are saved as `FILENAME.WAV`. Files are copied to disk by the command `C: COPY C/HPUV/DATA/FILENAME.WAV B:` where disk B is the destination of the copied files. Multiples files are representing by substituting an asterisk for the name, `COPY A:*.DAT` for example. Files are deleted on disk A by the command `DEL A:FILENAME.DAT`. The command `DIR` gives a directory listing of files on the selected disk.

Chapter 6

Nitrogen gas

It was imperative (according to the solid state detector manual) that the detectors should not be operated without first being flushed with dry gas for at least one minute. The dry gas has to be applied during operation of the detector to prevent atmospheric moisture from condensing inside the unit. Condensation could result in serious damage to the detector. Nitrogen gas was used as the dry gas because it was readily available. The gas was administered at at rate of approximately 1000 cc per minute. Some precautions had to be observed when handling the tank of nitrogen gas. Although nitrogen gas in not explosive, when it is compressed in a tank it has a potential of becoming a rocket if the tank is punctured or the valve is broken. If a tank of nitrogen (or other) gas develops a leak, the contents will try to equilibrate with their surroundings by releasing extra pressure. A full tank would release the extra pressure so fast that the tank would be forced in the opposite direction. It is important to prohibit the tank from falling over because that could damage the container. Any accidents were prevented by always securing the nitrogen tank with straps or chains, even if the tank was thought to be empty.

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Biographical Note

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