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NONINVASIVE FLUORESCENCE DETECTION OF HEPATIC AND RENAL FUNCTION

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ABSTRACT

A noninvasive *in vivo* fluorescence detection scheme was employed to continuously monitor exogenous dye clearance from the vasculature. Differentiation between normal and impaired physiological function in a rat model was demonstrated for both liver and kidney. A fiber optic transmitted light from source to ear; a second fiber optic positioned near the ear transmitted the fluorescent light to a detector system. Two model dye systems were employed in this initial study. Indocyanine green, known to be exclusively cleared from the blood stream by the liver, was excited *in vivo* with laser light at 780 nm. The fluorescence signal was detected at 830 nm. A characteristic clearance curve of normal hepatic function was obtained. After a partial hepatectomy of the liver, the clearance curve was extended in time, as would be expected from reduced hepatic function. In addition, fluorescein labeled poly-D-lysine, a small polymer predominantly cleared from the blood stream by the kidney, was excited *in vivo* with laser light at 488 nm. The fluorescence signal was detected at 518 nm. A characteristic clearance curve of normal renal function was obtained. After a bilateral ligation of the kidneys, the clearance curve remained elevated and constant, indicating little if any clearance. Thus, the feasibility of a new noninvasive method for physiological function assessment was established. [S1083-3668(98)01703-1]

Keywords fluorescence; near infrared radiation; indocyanine green; hepatic clearance; renal clearance; dyes.

1 INTRODUCTION

Medical care often requires rapid assessment of excretory organ function. However, current tests of hepatic and renal function have several shortcomings.[1–6] First, many tests currently employed in clinical practice (such as serum creatinine and liver enzymes) are unable to demonstrate rapid changes occurring in organ function. Second, some tests require collecting specimens over an extended period of time. Renal function, for example, is often measured by determining the creatinine clearance rate, which requires collecting urine over a 24 h time period. Third, tests which lack the first two constraints often have additional limitations. Radiotracer studies, for example, can be used to measure hepatic and renal function.[7,8] However, these tests are expensive, and require specialized handling of multiple samples.

A liver function test sporadically employed in the clinic involves use of the dye indocyanine green (ICG). ICG is known to be exclusively cleared from the bloodstream by the liver and is excreted into the bile. Thus a measurement of the ICG blood clearance time profile is directly related to liver function.[9]

The ICG test has undergone an evolution in technology. In its first incarnation, blood was withdrawn from the subject at several times following an IV bolus injection. The blood samples were then processed spectrophotometrically to determine ICG concentration.[10–12] The need to obtain a series of blood samples limited its utility. Subsequently, a noninvasive technique employing ear densitometry was developed.[13] Problems associated with the clinical development of this device recently led Japanese researchers to improve upon the ear densitometry technique. This newer method, termed the finger-piece method, employs transmitted light of two wavelengths measured through a finger to deduce ICG concentration.[14–18]

Both ear densitometry and the finger-piece method involve measuring absorption of light by ICG. In this article, we demonstrate the evolution of this technology to the next logical step, fluorescent determination of ICG *in vivo*. This may be an advantage since the *in vitro* fluorometric determination of ICG in plasma has been demonstrated to be more sensitive than absorption determination by at least an order of magnitude.[19] The methodology to assess liver function based on ICG fluorescence[20–22] is described herein.

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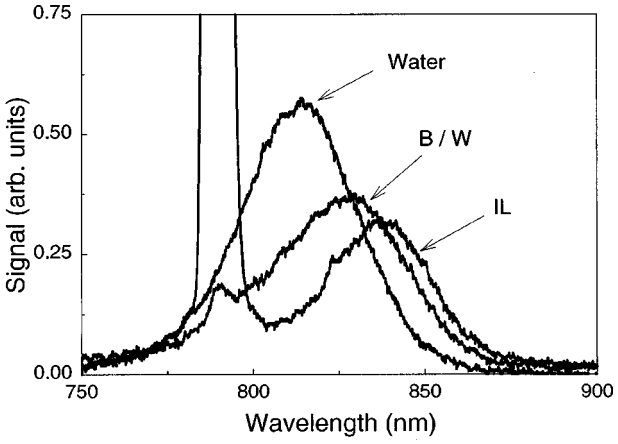

Fig. 1 Fluorescence emission spectra of ICG in three aqueous based environments; water, 0.5% intralipid (IL), and a rat blood/water (B/W) mixture. Note that scattering due to the excitation light at 790 nm is easily detected in the intralipid solution (composed of fat emulsion particles on the order of 1–10 μm), and in the water/blood mixture due to its particulate nature also.

This methodology is generalized to include the fluorescence detection of an agent which is cleared from the bloodstream by the kidneys. Radiotracer studies have shown recently that poly-D-lysine clears through the kidneys by glomerular filtration, followed by tubular reabsorption.[23] The noninvasive fluorescence methodology is applied to fluorescein labeled, succinylated poly-D-lysine. Thus assessment of renal function by *in vivo* fluorescence detection is also shown to be feasible.

2 MATERIALS AND METHODS

2.1 INDOCYANINE GREEN PREPARATION AND EMISSION WAVELENGTH SELECTION

ICG in powdered form was obtained from Sigma (St. Louis, MO). Solutions were made by diluting the appropriate amount of ICG in 10 mL of distilled water. Concentrations less than 1 mM were made by serial dilution of a 1 mM stock solution.

To establish the appropriate wavelength for detection of *in vivo* ICG fluorescence, fluorescent emission spectra were collected (using a Perkin Elmer MPF-44B fluorescence spectrophotometer scanning at 60 nm/min and the detector slit width set to 4 nm) for ICG in three biologically relevant media. These spectra are shown in Figure 1. The ICG concentration in each respective solvent was 11 μM. An excitation wavelength of 790 nm was employed for each spectra. The emission peak for ICG in water is near 815 nm, which yields a Stokes shift of 25 nm. The emission peak wavelength for the water/blood mixture is near 825 nm, and that for the intralipid solution (which is widely used as a tissue phantom to mimic optical properties[24]) is near 840 nm. Thus, for the experiments described below, an emission wavelength of 830 nm was chosen to collect the fluorescent light.

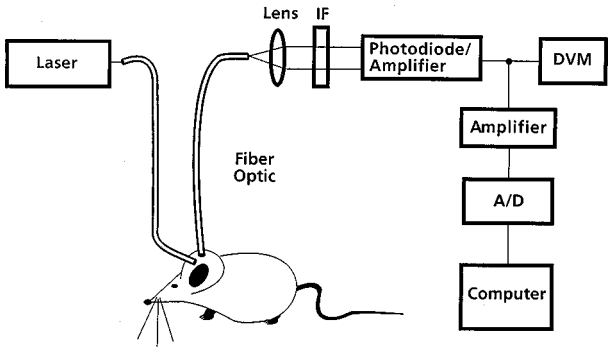

Fig. 2 Schematic of *in vivo* fluorescence detection apparatus.

2.2 FLUORESCEIN LABELED, SUCCINYLATED POLY-D-LYSINE PREPARATION AND EMISSION WAVELENGTH SELECTION

Poly-D-lysine of molecular weight 1000–4000 and fluorescein isothiocyanate were obtained from Sigma (St. Louis, MO). A reaction was implemented for a 2:1 molar ratio of polymer to dye at 4°C overnight. The fluorescein conjugate was next separated from unreacted starting material by gel filtration. Succinic anhydride was then used to succinylate the fluorescent polymer.[25] The reaction mixture was subsequently dialyzed against saline using a 3.5 kD cutoff membrane to retain the desired polymer conjugate. The resultant 220 μM fluorescein concentration equivalent was used directly for this investigation.

The nominal maximum emission wavelength for *in vitro* fluorescein fluorescence, 518 nm, was employed.[26] No further optimization for possible *in vivo* effects was attempted.

2.3 APPARATUS

A schematic of the apparatus for noninvasive *in vivo* detection of fluorescence is shown in Figure 2. For ICG fluorescence detection, a nominal 780 nm collimated solid state laser source was employed (LaserMax Inc. model No. LAS-300-780-5). For fluorescein fluorescence detection, an argon ion laser (Coherent Innova model 90) tuned to the 488 nm line was used. Either laser source is directed into the end of a 3.2 mm diameter glass fiber optic bundle (Oriel No. 77526). The other end of this laser delivery bundle is placed approximately 1 cm from the rat ear at an approximate 45° angle. A second similar fiber optic bundle for use as the fluorescence detection conduit is placed approximately 1 cm from the ear at an approximate 30° angle.

The exit end of the detection fiber bundle is positioned at the focal length of a 20 mm focal length lens. The output light is thus directed toward the detector after exiting the bundle and passing through the lens. A narrow band interference filter

is the next element in the optics train (CVI Laser Corporation), allowing light of the appropriate wavelength to pass on to the detector. For the ICG fluorescence experiment, an 830 nm filter [10 nm full width at half maximum (FWHM) bandwidth] was used. For the fluorescein fluorescence experiment, a 518 nm filter (3 nm FWHM bandwidth) was used.

The detector is a small silicon photodiode (UDT model PIN-10DP) connected to a transimpedance amplifier (Graseby Optronics model TRAMP®). A digital voltmeter monitors the output signal. A subsequent voltage amplifier (Tektronix AM-502) boosts the signal if needed. The amplifier output is connected to a National Instruments BNC-2080 breakout board, which is interfaced to a National Instruments DAQCard-700 data acquisition board (A/D). LabVIEW® data acquisition software collects the experimental raw data.

2.4 ANIMAL PREPARATION

For the ICG studies, female Fischer 344 rats weighing 173–195 g were used. These animals were first anesthetized with urethane (1.35 g/kg) administered via intraperitoneal injection. The anesthesia dose was administered so as to minimize the variability of the anesthetic plane achieved by the individual rats. For the renal agent clearance studies, normal Sprague-Dawley rats weighing 250–328 g were used. These animals were anesthetized in the same manner as in the ICG clearance studies.

After the animals had achieved the desired plane of anesthesia, a 21 gauge butterfly with 12 in. tubing was placed in the lateral tail vein of each animal and flushed with heparinized saline. The animals were placed onto a heating pad and kept warm throughout the entire study. The lobe of the left ear was fixed to a glass microscope slide.

The dye was subsequently administered via the indwelling catheter, and the clearance of the agent monitored. Clearance curves were obtained from an $n=3$ or 6 population.

3 RESULTS AND DISCUSSION

3.1 THE HEPATIC FUNCTION INVESTIGATION

3.1.1 ICG Clearance Study

An injection of 500 μL of 1.045 mM ICG was made yielding a 0.21–0.24 mg/100 g body weight dose for the rats employed in this study. A measurement of the time dependence of fluorescence at the ear pre- and postbolus injection of the ICG solution for six rats was performed. Three of the rat data sets are shown in Figure 3. The experiment can be described in terms of three stages. Stage 1 consists of approximately the first 30 seconds of data, which is gathered prebolus injection. These data are constant and represent the baseline value for the forthcoming experiment.[27] Stage 2 occurs several seconds

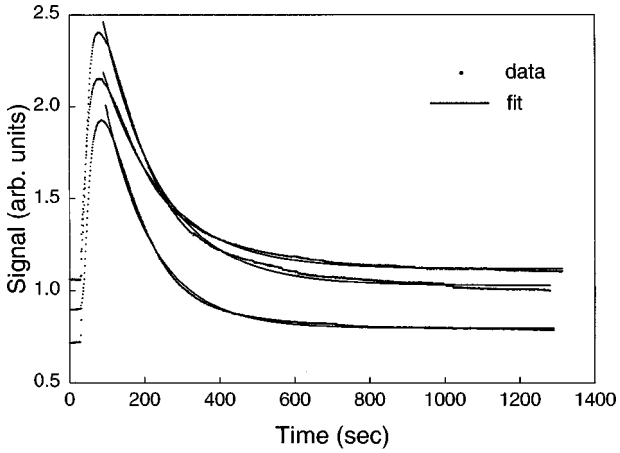

Fig. 3 *In vivo* fluorescence time dependence for three rats with normal liver function after a bolus injection of ICG. The solid lines are single exponential fits to the measured data (see Table 1).

postinjection, the signal rapidly rises to a maximum as the dye is reaching the ear and equilibrating in the blood pool. In the third stage, the fluorescence signal decays with time as the liver filters the ICG out of the blood stream. Visually, the decay rates are similar for all three data sets, and well within biological variability. After 15 min, approximately 90% of the initial signal is lost.

The apparent rapid distribution (equilibration) of ICG into the bloodstream (steep rise of Stage 2 in any of the data sets) and the apparent exponential decay of ICG from the bloodstream (Stage 3 data) suggest that the data may follow an open one compartment pharmacokinetic model. The characteristics of such a model are no absorption, entire drug dose in systemic circulation, rapid distribution of drug between bloodstream and tissue, instantly attained equilibrium (steady state), and that the drug concentration decrease is dependent on excretion.[28]

The fluorescent signal (arising from the dye concentration in the blood) as a function of time was fit to a single exponential decay appropriate to an open one compartment pharmacokinetic model. The equation employed to fit the Stage 3 data was

$$S = Ae^{-t/\tau} + B, \tag{1}$$

where S is the fluorescent light intensity signal measured, and t is the time point of the measurement. The quantity of interest τ, which is the decay time, and constants A and B are deduced from the fitting procedure.

The nonlinear regression analysis package within SigmaPlot® (Jandel Scientific Software, Rafael, CA) was employed to fit data to Eq. (1). The Stage 3 data from all six rats were fit and the numerical results are listed in Table 1. Fits to the data sets depicted in Figure 3 are also shown in Figure 3.

The goodness of fit (correlation coefficient $r^2 = 0.996$–0.998), and hence appropriateness of this model is seen by the fit to data in Figure 3. Thus the

Table 1 Results of fits to Eq. (1) for Stage 3 data from rats with a normal functioning liver. Data shown in Figure 3 from Rat Nos. 1, 3, and 5. Data shown in Figure 5 from Rat No. 2.

Rat No.	τ (s)
1	163±1
2	149±1
3	155±1
4	153±1
5	127±1
6	122±1

assumption that the measurement is observing ICG clearance via fluorescence determination is self-consistent.

The average time constant τ for all six rat data sets is 145±16 s. Inverting this number to get the decay rate (k) yields $k=41\%\pm5\%/\text{minute}$. This agrees well with a literature ICG clearance study in rats (using the blood sampling procedure), for which a decay rate of $k=45\%\pm6\%/\text{minute}$ was found for a dose of 0.25 mg/100 g body weight.[10]

3.1.2 Control ICG Clearance Study

To verify that the measurement was indeed that of ICG fluorescence, a control study was performed. A rat was injected, as above, with 500 μL of 1.41 mM ICG. A normal fluorescence time course was obtained, and is labeled as ICG-1 in Figure 4. Then the same rat was injected with 500 μL of 1.41 mM fluorescein (Sigma, St. Louis, MO). As shown in Figure 4, no fluorescence signal is detected. As a second negative control injection, 500 μL of normal saline solution (Baxter, Deerfield, IL) was injected into the same rat. Again, no detectable signal was obtained.

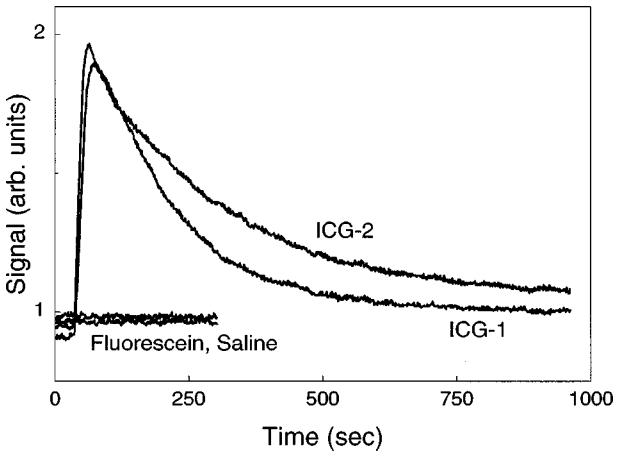

Fig. 4 The *in vivo* fluorescence time dependence of a succession of bolus injections in one rat. In chronological order; ICG (ICG-1), fluorescein only, saline only, ICG again (ICG-2).

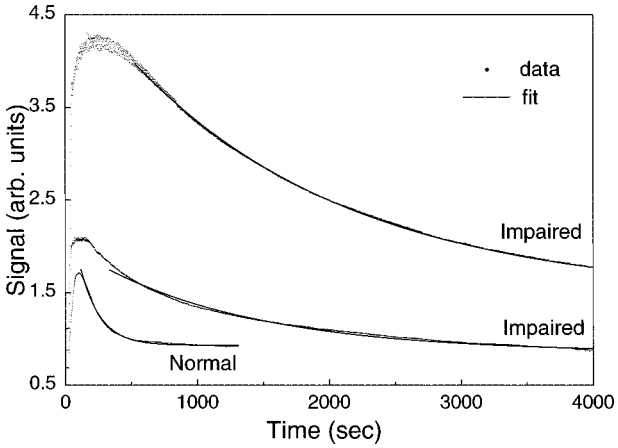

Fig. 5 The *in vivo* fluorescence time dependence after a bolus injection of ICG for rats with partial hepatectomies (impaired), and a comparison to a rat with normal liver function (normal). The solid lines are single exponential fits to the measured data (see Table 2).

Finally, the rat was once again injected with 500 μL of 1.41 mM ICG, and a second ''normal'' curve was obtained, labeled as ICG-2 in Figure 4. Thus, only fluorescent light at or near 830 nm is being detected.

The injections described above were done sequentially within a short time frame. Some saturation of the liver's ability to remove ICG is evident from the longer apparent time constant in ICG-2 with respect to ICG-1.

3.1.3 ICG Clearance in Partially Hepatectomized Rats

To verify that these fluorescence decay curves were related to liver function, and to assess this methodology's feasibility to determine impaired liver function, the following experiment was done. A partial hepatectomy was surgically performed on three rats.[29] Once the surgery was complete, the rat was allowed to equilibrate for 10 min. The rat was then injected with 500 μL of 1.007 mM ICG solution. A measurement of the time dependence of fluorescence at the ear pre- and postbolus injection of the ICG solution on these three rats followed.

Two of these data sets are shown in Figure 5, along with a measurement from a normal liver functioning rat for comparison. The capability of the liver to remove ICG from the blood pool is drastically altered. The fluorescence decay rate is much slower in the rats with partially hepatectomized livers than that of normal liver functioning rats.

The data from our rats with partial hepatectomies were also fit to Eq. (1). The fits were good (correlation coefficient $r^2=0.994$–1.000), and the time constants deduced are listed in Table 2. The fits to the data sets in Figure 5 are also shown in Figure 5. The decay time for the impaired liver function is almost an order of magnitude longer than the normal liver function decay time. Upon sacrifice, the livers were extracted and weighed. The amount of ligated liver

Table 2 Results of fits to Eq. (1) for Stage 3 data from rats with a partial hepatectomy. Data shown in Figure 5 from Rat Nos. 1 and 4.

Rat No.	τ (s)
1	1225±4
2	1334±1
4	1659±2

(nonfunctional) ranged from 33%–38% of the total liver weight. Thus, an order of magnitude change in the decay time resulted from a reduction of the functioning liver mass by approximately one-third. The capability to discriminate an even smaller reduction in functioning liver mass by this technique may be reasonably expected. The lower limits of sensitivity and resolution have not been explored.

3.2 THE RENAL FUNCTION INVESTIGATION

A rat with normal kidney function was injected with 500 μL of the 220 μM fluorescein labeled poly-D-lysine solution. The time dependence of fluorescence measured at the ear pre- and postbolus injection is shown in Figure 6. This clearance curve is qualitatively similar to the ICG clearance curve in that it exhibits the same three stages. The resultant fit of this data to Eq. (1) is also plotted in Figure 6, and yielded $\tau = 1643 \pm 9$ s. The quality of the fit suggests that the open one compartment model is appropriate for this process also. Inverting the decay time yields a decay rate of $k \sim 3.7\%/\text{min}$. This may be favorably compared to a literature renal clearance study in Sprague-Dawley rats employing a somewhat analogous compound, fluorescein isothiocyanate-inulin, for which a decay rate $k \sim 8\%/\text{minute}$ may be deduced.[30]

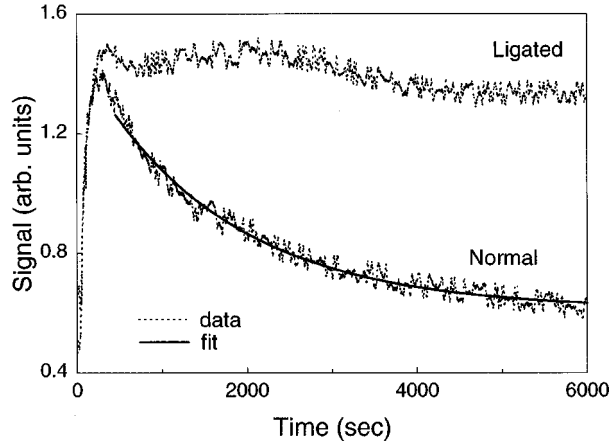

Fig. 6 The *in vivo* fluorescence time dependence after a bolus injection of fluorescein labeled poly-D-lysine in a single rat prekidney ligation (normal) and postkidney ligation (ligated). The solid line is a single exponential fit to the measured data.

To verify that the measurement was indeed that of fluorescein fluorescence, a control study was performed. A rat was injected with the fluorescein labeled polymer, followed by an injection of ICG, followed by an injection of saline, followed lastly by another injection of the fluorescein labeled polymer. Signals were obtained for the first and last injections only, no signal was obtained for the ICG or saline injection. Thus, only fluorescent light at or near 518 nm is being detected.

Finally, to verify that these fluorescence decay curves were related to renal function, a 500 μL dose of a 220 μM fluorescein labeled poly-D-lysine solution was injected into a rat following bilateral ligation.[31] The time dependence of fluorescence measured at the ear pre- and postbolus injection is also shown in Figure 6. Stages 1 and 2 are similar to the normal functioning kidney data; however, the signal in Stage 3 remains elevated and nearly constant. Thus little, if any, clearance of the injected dose is occurring.

4 SUMMARY

The feasibility of employing noninvasive *in vivo* fluorescence detection to monitor hepatic and renal function is demonstrated in an animal model using two model dye systems. Further development of this methodology should include improvements with respect to instrumentation (better detectors, better delivery and collection of light via fiber optics, optimizing laser power, etc.) for the purpose of increasing the sensitivity and signal-to-noise ratio over that reported herein. The dependence of the absolute fluorescent signal intensity on laser power and beam size, dye quantum efficiency, and *in vivo* capillary bed density needs to be further investigated also.

Based on the ease of use and the rapid time scale of measurement, application of this monitoring technique may be especially useful within the critical care unit, at the bedside, and/or in the physician's office. Development of dye systems for measurement of specific aspects of hepatic or renal function (such as glomerular filtration rate or effective renal plasma flow) would have the greatest initial clinical utility.

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27. The value of the baseline should be zero, since no fluorescence is occurring during this stage. However, the lens employed in the optical train depicted in Fig. 2 is too small in diameter to collimate all of the light emitted from the fiber optic. Light impinging the bandpass interference filter nonnormally is not properly filtered, and therefore is partially passed through the filter. A larger diameter lens or an altered optical setup would eliminate this artifact.
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