Measurement of Fluorescein and Fluorescein Monoglucuronide in the Living Human Eye

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Fluorescein monoglucuronide is a fluorescent metabolite of fluorescein, and is 1/3 to 1/34 as fluorescent as fluorescein, depending on the wavelength of excitation. After systemic administration, fluorescein glucuronide reaches concentrations many times greater than fluorescein. In order to study the effect of fluorescein glucuronide on the measurement of ocular dynamics, we devised a technique to measure fluorescein and fluorescein glucuronide in the anterior segment of the living human eye. Concentrations of each fluorophore were determined by differential spectrofluorophotometry from measurements at excitation wavelengths of 457.9 nm and 488.0 nm. Measurements were made on normal volunteers after oral and intravenous administration of fluorescein. Fluorescein was the dominant fluorophore during the first hour, while fluorescein glucuronide became dominant after 3 hours. By 6 hours there was 10 to 30 times more fluorescein glucuronide than fluorescein in the anterior chamber after oral administration, and three to ten times more after intravenous administration. The blood aqueous diffusion coefficient k_d estimated from the apparent concentration of fluorescein measured at 457.9 nm was consistently greater than k_d estimated from measurements at 488.0 nm. Estimates of k_d, which were made on the basis of concentrations of fluorescein determined from measurements at both wavelengths, were lower than estimates based on measurements at either wavelength. These results indicate that wavelength of excitation may influence the determination of ocular parameters when systemic fluorescein is used. Care must be taken in the interpretation of measurements when metabolites of a fluorophore can interfere with measurement of the fluorophore itself. Invest Ophthalmol Vis Sci 27:966–974, 1986

When fluorescein is given systemically, it is rapidly metabolized to other compounds.[1–4] Three of these metabolites have been examined and characterized by Chen et al.[1,2] The metabolite of most importance in ocular fluorophotometry is fluorescein monoglucuronide (FG), since it is highly fluorescent, although not as fluorescent as fluorescein, and it appears in relatively high concentration. In the blood, concentrations of FG are several times greater than concentrations of fluorescein several hours after oral or intravenous administration of fluorescein.[5] A significant portion of the fluorescence measured from the plasma after a systemic dose of fluorescein may originate from FG because of its fluorescence and relative abundance.

It is not clear how much FG crosses the blood-ocular barrier in the vitreous body or anterior segment. One might expect that, due to its lower lipid solubility,[5] it might cross the blood–ocular barrier less freely than fluorescein. On the other hand, since there is a high molar ratio of FG to fluorescein in the blood after a systemic dose, there may be a greater difference in concentration of FG across the blood–ocular barrier. The extent to which FG contributes to ocular fluorescence after a systemic dose is unknown.

The concentration of FG in a solution can be determined by measuring fluorescence before and after breakdown with β-glucuronidase.[1,2] This technique is well suited for measuring FG in blood and urine, but for obvious reasons is not suited for measuring ocular concentrations of FG in human subjects. A noninvasive technique would be preferable, such as that proposed by Grotte and his associates,[5] which utilizes differential spectrofluorophotometry.

This technique makes use of differences in excitation spectra of fluorescein and FG. Excitation of both fluorophores peaks near 495 nm. At this wavelength at pH 7.3, the molar fluorescent intensity of fluorescein is about 34 times that of FG.[5] Excitation of fluorescein decreases rapidly at shorter wavelengths, while excitation of FG decreases more gradually. At 445 nm this ratio is only about 5. Because of these spectral differences, the extent to which FG contributes to fluorescence measured from a mixed solution of the two fluorophores is greatly dependent upon wavelength of excitation.

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If the ratio of fluorescence is known at two wavelengths, the concentration of both fluorophores in a mixture can be determined from the fluorescence at the two wavelengths. In the present study, we used this principle to measure concentrations of fluorescein and FG in the living human eye after a systemic dose of fluorescein. Concentrations of the two fluorophores were measured with time, and dynamic properties of each were examined.

Materials and Methods

Concentrations of Fluorescein and Fluorescein Glucuronide

A scanning ocular fluorophotometer described previously[6] was used to measure ocular fluorescence at two excitation wavelengths available from the argon laser, 488.0 nm and 457.9 nm. The power of the excitation beam at each wavelength was adjusted to 70 μW at the eye, and the system standardized by scanning a fluorescent glass plate each time the wavelength was changed.

In a mixture of fluorescein and FG, both fluorophores contribute to the total fluorescence. The following equations describe the fluorescence at two wavelengths:

$$I_m^S = [F]E_F^S + [FG]E_{FG}^S \quad (1)$$

$$I_m^L = [F]E_F^L + [FG]E_{FG}^L, \quad (2)$$

where I_m^S and I_m^L are the intensities of fluorescence of the mixture of fluorescein and FG at the short and long wavelengths respectively, E_F^S and E_F^L are the micromolar fluorescence intensities of fluorescein (intensity of fluorescence of a 1 micromolar solution) at the short and long wavelengths respectively, E_{FG}^S and E_{FG}^L are the micromolar fluorescence intensities of FG at the short and long wavelengths respectively, and [F] and [FG] are the micromolar concentrations of fluorescein and FG.

Equations 1 and 2 may be solved for [F] and [FG]:

$$[FG] = \frac{I_m^S/E_F^S - I_m^L/E_F^L}{E_{FG}^S/E_F^S - E_{FG}^L/E_F^L} \quad (3)$$

$$[F] = I_m^L/E_F^L - [FG]E_{FG}^L/E_F^L \quad (4)$$

Notice that equations 3 and 4 contain the ratios of micromolar fluorescence intensity (E_{FG}/E_F) and the ratios of the intensity of fluorescence of the mixture to the micromolar fluorescence intensity of fluorescein (I_m/E_F) at each wavelength. The ratio E_{FG}/E_F can easily be determined from solutions of fluorescein and FG of known concentration. The ratio I_m/E_F is the apparent concentration of fluorescein that is measured when the fluorophotometer is standardized to fluorescein.

Molar fluorescence intensities of fluorescein and FG were measured from 1 μmol/L solutions maintained at pH 7.3 in a phosphate buffer. Sodium fluorescein was diluted from a 10% solution (Funduscein®, CooperVision Pharmaceuticals Inc., San German, Puerto Rico). Fluorescein monoglucuronide, given to us by Dr. Shigetoshi Nagataki of Tokyo University, was dissolved in phosphate buffer solution and diluted to 1 μmol/L. The FG was synthesized by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan. Purity was specified by the manufacturer to be 99.421% FG and 0.105% fluorescein (Lot. V306H11). The scanning ocular fluorophotometer was calibrated at both wavelengths by measuring serial dilutions of the 10% fluorescein solution. As a result, calibrated measurements of a solution of fluorescein gave the same concentration at both wavelengths even though fluorescence at 488.0 nm is about four times greater than at 457.9 nm.

Fluorophotometry

Ten normal volunteer subjects were studied. Informed consent was obtained from each subject after the nature of the study had been explained and an eye exam had been performed to insure a normal anterior chamber and fundus. On the morning of the study, an intravenous catheter (Angiocath®, Deseret Medical, Inc., Sandy, UT) was inserted into the antecubital vein and kept open with an obturator. Four of the subjects were then given an oral dose of fluorescein (Funduscein®, CooperVision Pharmaceuticals Inc., San German, Puerto Rico) (7 mg/kg) mixed in a cola beverage and six were given fluorescein (Funduscein®, CooperVision Pharmaceuticals Inc., San German, Puerto Rico) by intravenous injection (4 mg/kg). Measurements of fluorescence in the anterior segment were made at 15 min, 30 min, 60 min, 90 min, 120 min and hourly thereafter for a total of 8 hr. Scans were made at both wavelengths with approximately 5 min between. Prior to each scan a blood sample of approximately 3 cc was withdrawn through the catheter, and the plasma separated by centrifugation. A small amount of the plasma was diluted 1:100 in phosphate buffer solution (pH 7.3) and its fluorescence measured at both wavelengths.

The use of the two-dimensional scanning fluorophotometer provided an opportunity to visualize and measure concentrations of fluorophore in the "pupillary bubble" of aqueous humor which enters the anterior chamber through the pupil from the posterior chamber. The pupillary bubble was enhanced in one eye by reducing thermal mixing of the aqueous humor in the anterior chamber. This was accomplished by asking the subject to assume a face-down position by staring at a fixation target on the floor, and to hold one hand lightly over the eye being studied. Maintaining

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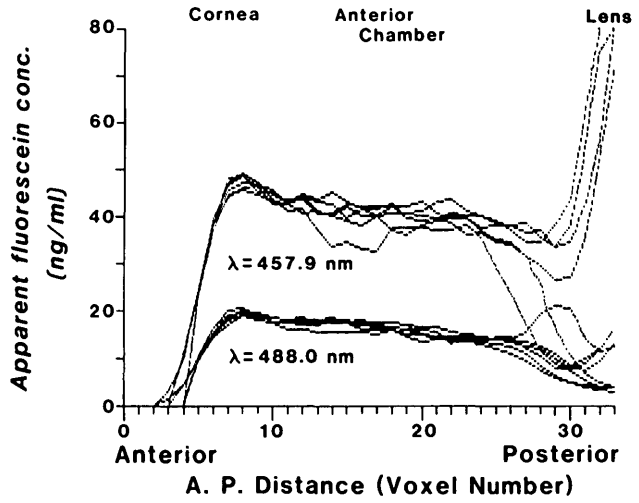

Fig. 1. Apparent concentration of fluorescein at 457.9 nm and 488.0 nm graphed as a function of anterior-posterior distance (same subject and time displayed in Figure 2). The mean concentration measured in the anterior chamber and cornea was more than twice as high when measured at 457.9 nm than when measured at 488.0 nm. When fluorescein was applied topically, this difference was not seen.

the optical axis in a vertical position placed the thermal gradient across the anterior chamber in an anterior to posterior direction, which reduced lateral movement of the pupillary bubble, while warming the cornea with the hand reduced the thermal gradient. Subjects followed this procedure for 3 min prior to the scan. In addition, containment of the pupillary bubble was improved by constricting the pupil to a diameter of about 2 mm, by applying ½% pilocarpine topically, one drop every 2 hr.

The apparent concentrations of fluorescein in the anterior chamber, pupillary aqueous, and cornea were determined by calculating the mean concentration in the corresponding voxels (volume element of measurement as determined by the excitation and detection pathways of the scanning ocular fluorophotometer) of the cross-sectional scan. Concentration was graphed as a function of anterior–posterior distance with the help of an interactive computer program. Scans were aligned on the rising signal from the cornea and consecutive graphs superimposed, as displayed in Figure 1. When enough scans were plotted to cover the area of interest (usually 10–15 scans for measurements of concentration in the aqueous humor outside the pupillary bubble), a graph of the mean concentration at each voxel was plotted. The region of interest (eg cornea, anterior chamber, pupillary bubble) in this final scan was then selected by using a pair of cursors, and the mean concentration between cursors stored on a computer disk. The operator had the opportunity to reject any anterior-posterior scan which appeared to be artifactious or to be outside the region of interest.

When measuring concentrations in the anterior chamber (C_a) the operator avoided the pupillary bubble, which could be seen as a hyper- or hypofluorescent region in front of the pupil, either by rejecting those scans or by using the cursors to select portions of the final graph outside the bubble. Concentrations within the pupillary bubble were measured in a similar way. Scans through the pupillary bubble were selected on the basis of their appearance, and the concentrations in one to nine voxels at the center of the bubble were averaged. If the concentration in the bubble was nearly the same as the surrounding aqueous, as it is during the first 1–2 hr, the bubble could sometimes not be seen on individual anterior-posterior scans, and measurements were averaged from a region in front of the pupil where the bubble was usually found.

Low concentrations in a small volume are partially masked by the spread of fluorescence from a surrounding solution of higher concentration. The effect of this spread function on measurements of the pupillary bubble was evaluated by scanning a test chamber made from a contact lens and filled with a fluorescein solution. A bubble of silicone oil without fluorescein was injected into the rear of the chamber through a 0.5 mm diameter tube, and the chamber was scanned. As the volume of the oil drop increased from 0.5 μl to 16 μl, the apparent fluorescence measured from the center of the droplet decreased from 7.5% to 2% of the surrounding fluorescence. Pupillary bubbles that were measured appeared to be 0.5 μl or larger. The spread function places a lower limit on concentration that can be measured in the pupillary bubble relative to the surrounding aqueous. Measurements in the pupillary bubble were not corrected for this spread function.

During the first 3–4 hr after a systemic dose, the concentration in the anterior chamber was greater than the concentration in the cornea, and fluorescence in the central cornea was hidden by the spread of higher fluorescence in the anterior chamber. For this reason, attempts were not made to measure concentrations in the cornea until they were greater than concentrations in the anterior chamber and the appropriate voxels could easily be selected. After this time, one or two voxels in the cornea were averaged across several scans through the central region of the cornea. The center of the cornea was selected to avoid the influx of dye from the limbus.

Blood-Aqueous Diffusion Coefficient

One way to determine the ease with which a substance crosses the blood-aqueous barrier is to calculate a diffusion coefficient. The diffusion coefficient is dependent on the chemical nature of the substance and, if more than one substance are present, its measured value may be dependent on wavelength. The method

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used to determine the blood aqueous barrier diffusion coefficient k_d in this study is described here.

Mathematically, k_d may be defined by its use in a three compartment model which is a modification of the Kinsey-Palm model[7] and was suggested by Friedenwald and Becker.[8] This model may be described by two differential equations:

$$\frac{dC_a}{dt} = k_d(C_p - C_a) + k_f(C_h - C_a) + k_{a\cdot ca}(C_c/r_{ca} - C_a) \quad (5)$$

$$\frac{dC_c}{dt} = k_{c\cdot ca}(C_a r_{ca} - C_c), \quad (6)$$

where C_a is the concentration of fluorescein in the aqueous humor, C_p is the concentration of unbound fluorescein in the plasma, C_h is the concentration of fluorescein in the pupillary aqueous, C_c is the measured concentration of fluorescein in the cornea (unbound plus bound), k_d is the blood-aqueous diffusion coefficient, k_f is the coefficient of loss of fluorescein due to outflow, $k_{a\cdot ca}$ is the aqueous to cornea diffusion coefficient referred to the volume of the anterior chamber, r_{ca} is the ratio of concentration of fluorescein in the cornea to concentration in the aqueous humor at steady-state, and $k_{c\cdot ca} = k_{a\cdot ca}\frac{v_a}{v_c r_{ca}}$, where v_a is the geometric volume of the anterior chamber and v_c is the geometric volume of the cornea.

If normal values of the constants k_f, $k_{a\cdot ca}$ and r_{ca} are assumed, measured values of $C_h(t)$ and $C_p(t)$ are used, and a value of k_d is approximated, then equations 5 and 6 can be solved numerically for C_a as a function of time. The task is to find the value of k_d such that the time dependent concentration of tracer in the anterior chamber predicted by numerical integration of equations 5 and 6 most closely coincides with observed values of C_a at the times measured.

An initial value of k_d was selected, and equations 5 and 6 were integrated by using a simple Euler method[9] to determine $C_a(t)$ between 0 and 120 min. A time interval of 1 min was used in the integration. The sum of the squares of differences between the predicted C_a and measured C_a was then calculated. This calculation was repeated as k_d was systematically varied, until the k_d was found that gave the minimum sum of squares. This value was accepted as the k_d of best fit.

It was assumed that $k_f = 0.014$ min^{-1}, $k_{c\cdot ca} = .003$ min^{-1}, and $r_{ca} = 1.6$. The value of k_f was the mean value found in a series of subjects studied by topical method and is in agreement with published results.[10,11] No attempt was made to determine k_f by using this technique. The model is not appropriate to determine k_f, since it does not have a component to account for the influx of fluorescein to the anterior chamber by diffusion across the limbus and through the cornea. The values used for $k_{c\cdot ca}$ and r_{ca} have been published,[11,12] v_a was determined photogrammetrically and v_c was assumed to be 70 μl. The unbound fraction of fluorescein in the plasma was determined by measuring polarization of fluorescence in the undiluted plasma as described previously.[13] C_p and C_h were assumed to change linearly between times when they were measured. Values of C_h were not corrected for the optical effects of fluorescence in the anterior chamber, since they were relatively close to C_a during this time period.

The model described by equations 5 and 6 does not require measurement of C_c. Instead, the cornea is assumed to be a dead-end compartment, and concentrations of tracer in the cornea are generated by the numerical integration. Influx of fluorescein from the limbal tissues is assumed to be negligible. This model was chosen because of the difficulty in measuring concentration of fluorophore in the cornea during the first several hours of the experiment while concentrations in the anterior chamber are much higher than in the cornea. In practice, exact knowledge of C_c provides little information regarding k_d, since the calculated k_d changes only slightly as $C_c(t)$ is varied. For example, if the value of $k_{c\cdot ca}$ is increased by 50%, giving the cornea more weight in the calculation, the least squares value of k_d increases by less than 5%.

In a similar way, the assumption of values of r_{ca} and k_f are not critical for the calculation of k_d. During the first 120 min, C_a is dominated by diffusion of fluorescein into the anterior chamber, and large changes in k_f or r_{ca} lead to small changes in the least squares value of k_d.

All fluorophotometric measurements were made at either 457.9 nm or 488.0 nm, and then immediately repeated at the other wavelength. As much as 5 min were required between scans at different wavelengths to examine and store the data, scan the other eye, change wavelength of the laser, and complete the maneuver to enhance the pupillary bubble. The blood–aqueous barrier diffusion coefficient, k_d, was determined from the apparent concentrations of fluorescein measured at 457.9 nm and at 488.0 nm, and from the concentrations of fluorescein determined by using equations 3 and 4. Since the unbound fraction of FG in the plasma was not known, a transfer coefficient of FG was determined with the assumption that all FG measured in the plasma was unbound. When the free fraction is established, the true k_d of FG can be approximated by dividing the values obtained here by the free fraction.

Results

The ratios of molar fluorescent intensity of fluorescein to FG were 22.9 at 488.0 nm and 5.3 at 457.9 nm.

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Table 1. Measurement of mixtures of fluorescein and fluorescein monoglucuronide

Nominal concentration of fluorophore in sample		*Ratio*	*Apparent concentration of fluorescein* (ng/ml)*		*Concentration of fluorophore calculated by means of equations 3 and 4*	
[F] (μM)	*[FG] (μM)*	*[FG]/[F]*	*488.0 nm*	*457.9 nm*	*[F] (μM)*	*[FG] (μM)*
1.0	0	—	368.4	369.3	1.00	0.017
0	1.0	—	16.1	69.8	0.0	1.00
1.0	0.1	0.1	365.5	369.1	0.99	0.067
0.1	0.1	1	33.8	44.8	0.083	0.21
0.1	1.0	10	49.0	104.2	0.088	1.03
0.1	2.0	20	66.2	171.1	0.094	1.96
0.1	3.0	30	84.9	246.4	0.098	3.02
0.1	10.0	100	195.7	711.0	0.11	9.64
0.01	0.1	10	5.2	10.9	0.0095	0.11
0.01	0.2	20	6.7	18.0	0.0089	0.21
0.01	0.3	30	8.4	24.8	0.0094	0.31
0.01	1.0	100	19.4	71.7	0.0099	0.98

* Calibrated to fluorescein.

In order to test the accuracy of this measurement system, fluorescein and FG were mixed at different ratios, the fluorescence of the mixtures measured at both wavelengths, and the measurements used to calculate the concentration of fluorescein and FG in the mixture. Results of this experiment are shown in Table 1. It was clear that the dominant fluorophore was most accurately measured. Thus, the concentration of FG can be most accurately determined when it is several times greater than the concentration of fluorescein.

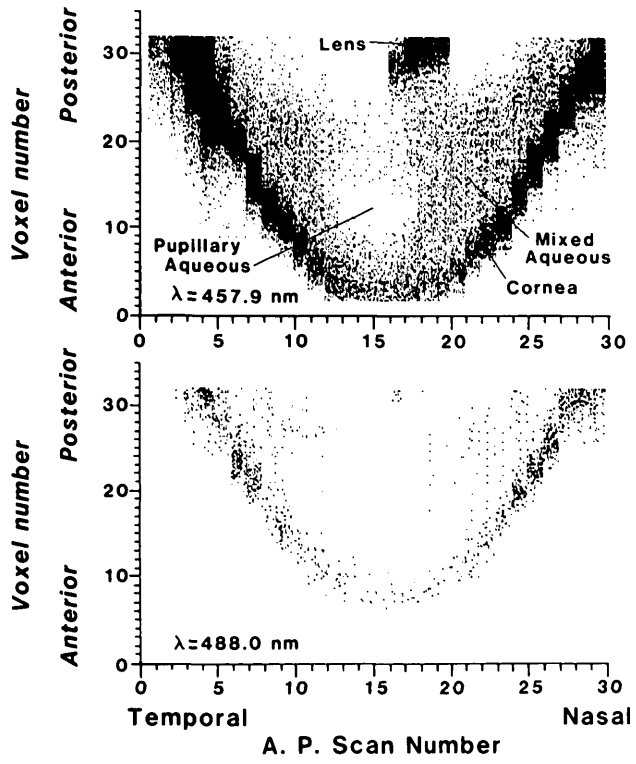

Fig. 2. Scans across the anterior chamber and cornea with excitation wavelengths 457.9 nm and 488.0 nm. Scans were made 6 hr after oral administration of 7 mg/kg sodium fluorescein. The intensity scale of each scan ranges from 5 ng/ml (white) to 100 ng/ml (black) and is linearly related to apparent concentration of fluorescein.

After fluorescein was given systemically, the apparent concentration of fluorescein depended greatly on the wavelength of excitation. The difference at two wavelengths can be seen in the scans that were made 6 hr after administration of oral fluorescein (Fig. 2). This difference is illustrated quantitatively in the graph of superimposed anterior-posterior scans (Fig. 1). In this example, the apparent concentration of fluorescein in the anterior chamber measured at 457.9 nm was approximately twice that measured at 488.0 nm.

In two subjects, fluorescein .25% (Fluress®, Barnes-Hind Pharmaceuticals, Inc., Sunnyvale, CA) was administered topically and measurements were made in the anterior chamber and cornea after 8–14 hr. No difference in concentration was measured at the two wavelengths. One would not expect a difference after topical administration, since it is doubtful that ocular tissues can conjugate fluorescein.

Concentrations of fluorescein and FG in the aqueous humor, pupillary bubble (posterior chamber aqueous), cornea, and plasma were calculated from consecutive measurements at 488.0 nm and 457.9 nm. An example of intraocular fluorescein and FG concentrations is shown in Figure 3 for one subject. Mean concentrations at several times are presented in Tables 2 (oral) and 3 (intravenous). In some measurements, when the concentration of FG was much less than the concentration of fluorescein, equation 3 yielded a value of [FG] less than zero. This was the result of small errors in measuring apparent concentrations that were almost equal. When this occurred, [FG] was assumed to be equal to zero.

During the first 30 min after administration, fluorescence in the pupillary aqueous was near or slightly

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greater than fluorescence in the anterior chamber. After the first hour, concentrations in the pupillary aqueous were less than those in the anterior chamber, and appeared to decrease in parallel with concentrations in the plasma (Fig. 3) as noted by others.[14,15] Measurements of fluorescein and FG in the pupillary aqueous were the least consistent of all ocular measurements, as can be seen from the variability in Tables 2 and 3. This is primarily the result of difficulties in creating a uniform pupillary bubble prior to each scan and the time lag between the measurements at the two wavelengths.

Two differences were consistently seen in the dynamics of fluorescein and FG in the eye. First, fluorescein and FG were at a maximum in the anterior chamber at different times. After the oral dose, fluorescein was at a maximum in 2 to 3 hr, while FG was at a maximum in 4 to 6 hr. After the intravenous dose, fluorescein was at a maximum in 1 to 2 hr while FG required 2 to 4 hr. Second, after the first 2 hr, concentrations of FG were considerably higher than concentrations of fluorescein in the anterior chamber and plasma. Six hr after ingestion, molar concentrations of FG were 12 to 30 times greater than fluorescein in the anterior chamber, and 17 to 50 times greater in the plasma. After intravenous injection, these ratios were not as high. Six hr after injection, concentrations of FG were 2–10 times higher than fluorescein in the anterior chamber, and 12–30 times higher in the plasma.

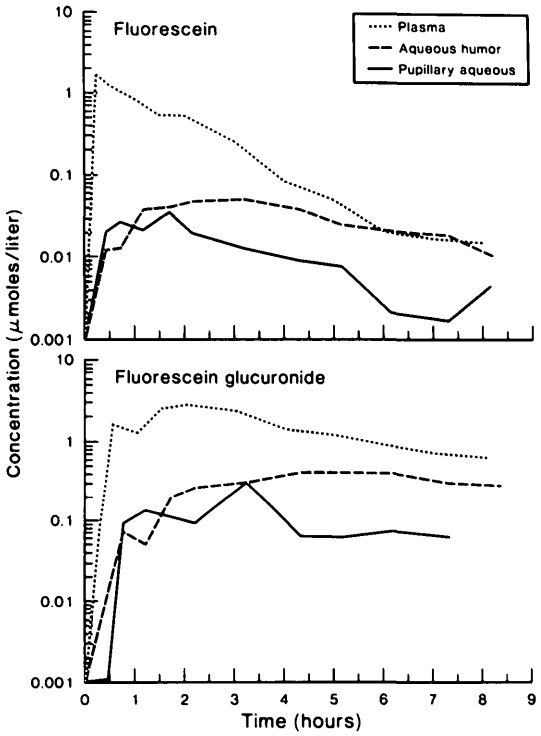

Fig. 3. Concentrations of fluorescein and fluorescein glucuronide after oral fluorescein were calculated by using equations 3 and 4. Notice that concentrations of fluorescein were at a maximum earlier than FG. After the first hour, concentrations of FG were considerably greater than fluorescein in all three compartments.

The blood-aqueous transfer coefficient is usually determined from measurements of fluorescence in the aqueous humor and plasma. In order to study the effect of excitation wavelength on the calculated value of the transfer coefficient, k_d was determined from fluorescence at 457.9 nm and 488.0 nm, as well as from the

Table 2. Mean concentrations of fluorescein and fluorescein glucuronide after an oral dose of 7 mg/kg in four subjects

		Anterior chamber		*Pupillary aqueous*		*Cornea*		*Plasma*	
Time (Hr)		*F*	*FG*	*F*	*FG*	*F*	*FG*	*F*	*FG*
		(μM)		*(μM)*		*(μM)*		*(μM)*	
.5	Mean	0.015	0	0.021	0	*	*	1.67	0.47
	SD	0.012		0.018				1.43	0.59
1	Mean	0.047	0.16	0.021	0.081	*	*	0.73	2.3
	SD	0.035	0.15	0.0083	0.061			0.36	1.4
2	Mean	0.052	0.39	0.017	0.089	*	*	0.47	3.1
	SD	0.031	0.25	0.0061	0.041			0.17	1.3
3	Mean	0.058	0.38	0.012	0.16	*	*	0.28	2.8
	SD	0.031	0.26	0.0051	0.12			0.15	1.2
4	Mean	0.031	0.53	0.0076	0.073	*	*	0.13	2.0
	SD	0.010	0.33	0.0043	0.053			0.077	0.8
5	Mean	0.034	0.38	0.0066	0.059	*	*	0.057	1.5
	SD	0.017	0.25	0.0033	0.011			0.031	0.7
6	Mean	0.015	0.44	0.0036	0.080	0.041	0.46	0.047	1.2
	SD	0.010	0.21	0.0033	0.036	0.029	0.20	0.054	0.7
7	Mean	0.019	0.31	0.0052	0.038	0.040	0.49	0.025	0.96
	SD	0.0072	0.16	0.0057	0.019	0.024	0.22	0.023	0.58
8	Mean	0.0093	0.33	0.0031	0.029	0.027	0.62	0.020	0.77
	SD	0.0058	0.16	0.0025	0.025	0.0054	0.27	0.018	0.51

* Concentrations in the cornea were not measured until they were greater than concentrations in the anterior chamber.

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Table 3. Mean concentrations of fluorescein and fluorescein glucuronide after an intravenous dose of 4 mg/kg in six subjects

		Anterior chamber		*Pupillary aqueous*		*Cornea*		*Plasma*	
Time (Hr)		*F*	*FG*	*F*	*FG*	*F*	*FG*	*F*	*FG*
		(μM)		*(μM)*		*(μM)*		*(μM)*	
.5	Mean	0.052	0.093	0.057	0.18	*	*	9.8	0.35
	SD	0.029	0.22	0.033	0.42			4.2	0.84
1	Mean	0.098	0.12	0.053	0.070	*	*	1.0	2.2
	SD	0.028	0.06	0.029	0.087			0.30	0.7
2	Mean	0.089	0.23	0.023	0.069	*	*	0.30	1.4
	SD	0.022	0.06	0.013	0.10			0.07	0.3
3	Mean	0.067	0.20	0.035	0.034	*	*	0.13	0.97
	SD	0.017	0.16	0.026	0.036			0.02	0.25
4	Mean	0.044	0.23	0.013	0.11	*	*	0.063	0.69
	SD	0.010	0.12	0.012	0.084			0.010	0.16
5	Mean	0.038	0.17	0.016	0.038	*	*	0.035	0.51
	SD	0.008	0.09	0.011	0.041			0.006	0.14
6	Mean	0.026	0.16	0.0055	0.041	0.069	0.36	0.020	0.41
	SD	0.004	0.11	0.0047	0.033	0.020	0.11	0.006	0.13
7	Mean	0.023	0.13	0.010	0.030	0.078	0.27	0.016	0.31
	SD	0.007	0.05	0.006	0.027	0.019	0.21	0.006	0.11
8	Mean	0.017	0.12	0.0044	0.082	0.069	0.27	0.0096	0.27
	SD	0.007	0.05	0.0037	0.10	0.016	0.12	0.0040	0.06

* Concentrations in the cornea were not measured until they were greater than concentrations in the anterior chamber.

concentration of fluorescein calculated from fluorescence at both wavelengths. The results of these calculations are presented in Table 4. The transfer coefficient k_d was $0.83 \pm .39 \times 10^{-3}$ min^{-1} (mean ± S.D.) and $0.67 \pm 0.30 \times 10^{-3}$ min^{-1} at 457.9 nm and 488.0 nm respectively (IV and oral). The difference is significant ($P < .005$, paired t-test). The k_d of fluorescein by itself was $0.61 \pm 0.27 \times 10^{-3}$ min^{-1}, slightly lower than k_d determined from fluorescence at 488.0 nm.

Table 4. k_d measured at different wavelengths and k_d of fluorescein (equation 4)

	$k_d \times 10^{-3}$ min^{-1}		
Subject	*457.9 nm*	*488.0 nm*	*Fluorescein only (equation 4)*
Oral fluorescein			
1	0.49	0.45	0.44
2	1.25	0.88	0.75
3	1.42	1.14	0.98
4	0.65	0.50	0.45
Mean, oral fluorescein	0.95	0.74	0.66
SD	0.45	0.33	0.26
IV fluorescein			
5	1.25	1.12	1.08
6	0.49	0.38	0.34
7	0.55	0.53	0.51
8	0.91	0.64	0.56
9	0.96	0.77	0.72
10	0.28	0.25	0.24
Mean, IV fluorescein:	0.74	0.62	0.58
SD	0.36	0.31	0.30
Mean, oral and IV	0.83	0.67	0.61
SD	0.39	0.30	0.27

Calculation of k_d of FG is complicated by the uncertainty of the unbound fraction of FG in the plasma and the uncertainty of the cornea-aqueous transfer coefficient, $k_{c \cdot ca}$, of FG. Nagataki and Matsunaga[16] have recently shown that the unbound fraction of FG in the plasma is greater than the unbound fraction of fluorescein. One can approximate the value of k_d of FG by first calculating a transfer coefficient with the assumption that all FG in the plasma is unbound, and then dividing by the true unbound fraction of FG when it is determined. The mean transfer coefficient determined with the assumption that all FG was unbound was $0.16 \pm 0.10 \times 10^{-3}$ min^{-1} with oral and $0.19 \pm 0.06 \times 10^{-3}$ min^{-1} with intravenous administration. It was assumed that $k_{c \cdot ca}$ of FG was equal to .003 min^{-1} (same as with fluorescein). Although this assumption may not be correct, a large error in $k_{c \cdot ca}$ has little impact on the calculation of the transfer coefficient. For example, a 50% change in $k_{c \cdot ca}$ gives approximately a 5% change in the calculated transfer coefficient of FG.

Discussion

Differential spectrofluorophotometry is a relatively simple, noninvasive means of measuring concentrations of two fluorophores in the eye and plasma. The scanning ocular fluorophotometer used here is well-suited for measuring fluorescein and FG due to the simplicity of changing between well-spaced, narrow-band excitation wavelengths in the appropriate exci-

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tation spectra. When using other fluorophotometers, the same measurement can be made if an appropriate combination of excitation filters is chosen to give a significant difference between the ratio of molar fluorescence of fluorescein and FG.

One source of experimental error in this system is the time that elapses between measurements at the two wavelengths. Ideally, simultaneous measurements would be made, since a change in the concentration of either fluorophore during the delay reduces the accuracy of the calculation of [F] and [FG] with equations 3 and 4. This error is greatest early in the experiment when concentrations change rapidly.

The presence of protein in a solution of fluorescein and FG might also affect the ability to separate the two fluorophores. When fluorescein is bound to albumin, its excitation spectrum shifts towards longer wavelengths and its fluorescence efficiency decreases. When FG is bound, its spectrum is slightly red shifted, but fluorescence is not quenched (*unpublished observations*).[16] As a result, the ratios of molar fluorescence (E_{FG}/E_F) used in equations 3 and 4 could change in the presence of protein. In this experiment, no attempt was made to correct for the presence of protein in the solutions measured. Protein would have little effect in the diluted plasma, where the free fraction of fluorescein was estimated to be about 95%, or in the aqueous humor, where the protein concentration is normally very low. Measurements of fluorescein and FG in the anterior chamber of subjects with flare or in the cornea could be affected by the presence of relatively high concentrations of protein.

Mean values of k_d determined in this experiment are within the range reported by others. Nagataki[14] reported $0.97 \pm .32 \times 10^{-3}$ min^{-1} (±S.D.) measured with intravenous fluorescein while Araie[17] reported $0.68 \pm .37 \times 10^{-3}$ min^{-1} measured with oral fluorescein. Araie also presented a value of 0.45×10^{-3} min^{-1}, corrected for the presence of FG in the plasma and the entry of fluorescein into the anterior chamber through the limbus and cornea. Our mean values of $0.67 \pm .30 \times 10^{-3}$ min^{-1} at 488.0 nm and $0.83 \pm .39 \times 10^{-3}$ min^{-1} at 457.9 fall between these values. Our value of k_d of fluorescein alone, $0.61 \pm .27 \times 10^{-3}$ min^{-1}, is in agreement with Araie's corrected value.

The blood–aqueous transfer coefficient has typically been determined by relating fluorescence of tracer in the aqueous humor to fluorescence in the plasma after systemic administration. The wavelength dependence of fluorescence in the aqueous humor and plasma due to the prescence of the metabolite FG is clearly demonstrated in this experiment. The question of whether or not the appearance of FG would influence measurement of the blood–aqueous barrier transfer coefficient led us to measure k_d at 457.9 nm and 488.0 nm. Our results indicate that k_d appears greater when measured at shorter wavelengths where a significant portion of the fluorescence is from FG.

If permeability is due only to simple diffusion through cells that form the blood–aqueous barrier, one might expect that the transfer coefficient of FG should be lower than that of fluorescein, since FG has a considerably lower lipid solubility. In the present experiment, if measurements at the short wavelength more closely reflect movement of FG, then the k_d of FG appears to be greater than the k_d of fluorescein. This is consistent with the hypothesis that movement of fluorophore between the eye and plasma is partially a transport mediated process.[15] If fluorescein is a better substrate for outward transport than FG, it may have a lower k_d. Alternatively, an underestimate of the unbound fraction of FG in the plasma[16] would lead to an overestimate of k_d.

In this experiment and others, the presence of high concentrations of FG in the plasma might compromise the polarization measurement used to determine the unbound fraction of fluorescein. This technique is based on the assumption that all of the fluorescence in the plasma is from a single fluorophore. Brubaker et al[13] noted, however, that the unbound fraction appeared to gradually increase several hours after a systemic dose of fluorescein. This time course corresponds to the appearance of relatively high concentrations of FG. Nagataki and Matsunaga[16] noted that FG was more loosely bound to plasma proteins and that there was a greater unbound fraction of FG than fluorescein. This condition would lead to an overestimate of unbound fluorescein when measured by polarization and an underestimate of the k_d of fluorescein. The influence of FG on the measurement of the free fraction of fluorescein in the plasma by the polarization method was not pursued further here, since k_d was determined from measurements made during the first 2 hr when the ratio of FG to fluorescein in the plasma was relatively low.

The presence of FG in the eye and plasma after a systemic dose of fluorescein may influence ocular fluorophotometry in two ways. First, since the fluorescence signal originates from two fluorophores with different spectral properties, concentrations determined from fluorescence measured at a single wavelength may be in error when the fluorophotometer is calibrated against a standard of one of the fluorophores. The extent of the error is heavily dependent on wavelength as may be seen in the two scans shown in Figures 1 and 2. The different values of k_d obtained by using the apparent concentration of fluorescein measured at each wavelength illustrate the importance of this wavelength dependence. Errors can be minimized by making measurements in the eye and plasma with the same instrument at the same excitation wavelength.

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Second, it is not known whether fluorescein and FG move across the blood–ocular barrier with the same freedom. Thus, differences in parameters measured by ocular fluorophotometry under two treatments or in disease states may be the result of alteration of the dynamics of fluorescein, FG, or both. In addition, changes in measured ocular dynamics may be related to altered ratios of the unbound form of the two fluorophores in the plasma. One must therefore use care in interpreting changes that are observed under experimental conditions and in disease states.

Key words: fluorescein, fluorescein glucuronide, fluorophotometer, anterior chamber, aqueous humor

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