Fluorescein and Fluorescein Glucuronide Pharmacokinetics After Intravenous Injection

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The permeability of the blood-retinal and blood-aqueous barriers to fluorescein (F) and the rate of aqueous flow can be estimated by measurements of F in the vitreous, aqueous, and plasma after systemic administration. F is commonly measured by fluorescence, but fluorescein glucuronide (FG), a metabolite of F, also fluoresces. To assess the influence of FG on the quantitation of F by fluorescence, we studied the pharmacokinetics of F and FG for 38 hr in the plasma of five normal subjects given 14 mg/kg of sodium fluorescein intravenously. The plasma and the plasma ultrafiltrate were measured by fluorescence and by high performance liquid chromatography. In our fluorophotometer, FG was 0.124 times as fluorescent as F. F was rapidly converted to FG, and within 10 min the concentration of unbound FG exceeded that of unbound F. The terminal half-lives of F and FG in the plasma ultrafiltrate were 23.5 and 264 min, respectively, so that FG contributed almost all of the plasma fluorescence after 4–5 hr. Because FG was less bound in the plasma than F, the ratio of the fluorescence of the plasma ultrafiltrate to that of the plasma increased with time. The greatest proportion of the total F available to penetrate into the ocular compartments occurred shortly after injection. We concluded that FG is an important contributor to the fluorescence of the plasma ultrafiltrate after intravenous injection and that accurate quantitation of physiologic parameters calculated from the plasma F requires taking this factor into account. Invest Ophthalmol Vis Sci 27:1107–1114, 1986

Measurement of the permeability of the blood-retinal barrier (BRB) or blood-aqueous barrier (BAB) can improve our understanding of ocular function in both normal and disease states. The permeabilities of the BRB and BAB to fluorescein (F) have been studied frequently because F has two enormous advantages: 1) it can be administered to humans as well as to animals, and 2) it can be measured in the eye noninvasively by optical techniques, such as fluorophotometry. To quantitate the inward permeability of the BRB to F, or to another indicator, one must know for a given period of time how much F actually crossed the barrier and how much F was available to cross the barrier. Specifically, one must know not only the amount of F in the vitreous, but also the time course of free F in the plasma. In fact, the inward permeability coefficient is the former divided by the latter. Similar principles apply to measurements of the BAB, but the analysis must also include the flow of aqueous. The concentrations of F in the eye and plasma are usually measured by fluorescence. This assumes that F is the only important source of the fluorescence.

Fluorescein monoglucuronide (FG), a fluorescent metabolite of F formed in the liver, recently has been identified. FG contributes substantially to the plasma fluorescence after intravenous or oral fluorescein. It enters the anterior chamber in substantial quantities. Previously, we have described an improved method to measure F and FG using high performance liquid chromatography (HPLC).

Previous studies of F and FG after intravenous fluorescein in humans reported on a single subject or measured only during the first hour after injection. Accordingly, we undertook a study of the pharmacokinetics of F and FG in the plasma in five normal human subjects for the first 38 hr after an intravenous injection of F. Our results confirmed that, by 1 hr after injection, a substantial percentage of the plasma fluorescence arises from FG. The kinetic data will be published in a more complete form elsewhere.

Materials and Methods

Five normal subjects, four men and one woman, were studied. The mean and standard deviation of their ages were 28.6 ± 5.6 yr. A 14 mg/kg dose of sodium fluorescein was rapidly injected intravenously. Blood
was taken from a vein 11–24 times within 38 hr after injection. The samples were obtained more frequently immediately after the injection. For these early samples, a butterfly needle was placed in the antecubital vein contralateral to that into which the F had been injected. Samples of 5 ml were acquired as rapidly as possible in the first 4 min after injection. The plasma was obtained by centrifugation. Determinations on the fluorescence of the whole plasma and the plasma ultrafiltrate were performed on a commercially available fluorophotometer (Fluorotron Master; Coherent, Palo Alto, CA). The whole plasma samples were measured after diluting them 100 times in phosphate buffer (pH = 7.4), which previously had been found to minimize binding and quenching by the plasma proteins. Plasma ultrafiltrates were prepared using a membrane micropartition system (MPS-1 YMT membranes; Amicon, Danvers, MA). Plasma samples (approximately 0.5 ml) were centrifuged at 2000 g for 20 min at room temperature through the system as previously described. Determination of the F and FG concentrations in the plasma ultrafiltrate were performed by HPLC.

Briefly, the chromatographic method used a carrier of methanol and water with 5 mM PIC-A on a C-18 reverse phase column. Detection was by fluorescence. The ultrafiltrate was injected directly onto the column. Pure F and FG were used as standards. The latter had been specially synthesized by a chemist. The fluorescence of both F and FG are dependent on the excitation and detection wavelengths, and their ratio should be determined for each fluorophotometer. The relative fluorescence of FG to F per unit concentration at pH = 7.4 on our fluorophotometer (R) was evaluated by measuring the fluorescence of known concentrations of pure FG and F.

The decline of the plasma values for each subject was fit to standard pharmacokinetic equations on a computer using nonlinear regression analysis. Concentration-time data were fit to the appropriate 1-, 2-, or 3-compartment models. The ratio of the ultrafiltrate fluorescence to the total plasma fluorescence (U/P) was plotted versus time. This value was related to the binding of the fluorescent compounds to plasma macromolecules. The unbound fraction of F (Bp) or FG (BFG) was determined by the ratio of the fluorescence in the ultrafiltrate to that in the whole plasma when the concentration of the other fluorophore was unmeasurable by HPLC. The unbound fractions can be converted to unbound percentages by multiplying by 100%. If the binding of each dye was uninfluenced by the presence of the other, U/P measured when both dyes were present in the plasma should conform to the ratio calculated according to the following equation:

$$\frac{u}{p} = \frac{F_u + RFG_u}{(F_u/B_F) + (RFG_u/B_{FG})},$$

where $u/p$ is the calculated ratio of the ultrafiltrate fluorescence to that of the whole plasma (in contrast to the ratio actually measured, $U/P$), and $F_u$ and $FG_u$ are the concentrations of F and FG in the plasma ultrafiltrate in ng/ml. A linear correlation between $U/P$ and $u/p$ was performed.

In order to judge the rapidity of conversion of F to FG, we calculated the molarity of F and FG in the whole plasma, $F_m$ and $FG_m$, respectively, for each time:

$$F_m = \frac{F_u}{B_F}$$

$$FG_m = \frac{FG_u}{B_{FG}}$$

The quantity of F available to cross the blood–ocular barriers, which is necessary in the calculation of the inward BRB or BAB permeability, is the ultrafiltrate F concentration versus time integral, that is, the area under the curve (AUC) between the time of injection and the time of measurement in the eye. Pharmacokinetic models assume that the distribution of dye in the plasma occurs instantaneously. That is, the peak concentration is at time zero, after which it declines. Experimentally, the venous plasma dye concentration rose to a maximum at about 3 min after injection and then declined. To estimate the area under the curve within the first 3 min, we used the trapezoidal method. The data points were connected by straight lines and the area of the trapezoid, $TA$, under each line was calculated as:

$$TA = \frac{(A + B) \cdot T}{2},$$

where A and B are successive plasma measurements acquired the time interval $T$ apart. The areas of the trapezoids were then summated for each curve.

We evaluated AUC for the total plasma fluorescence, the ultrafiltrate fluorescence, the ultrafiltrate F concentration, and the ultrafiltrate FG concentration. The last two were calculated in both mass and molar units. To determine which time intervals contributed most to the total AUC within the first 60 min (the most common time used in vitreous fluorophotometry), we determined AUC from 0–3, 3–10, 10–30, 30–60, and 60–120 min after injection. These values were expressed as the percentage of AUC from 0–60 min that occurred during each interval.

We calculated the error that results from estimating the ultrafiltrate F AUC by using the plasma fluorescence without measuring F. Two ratios were quantitated to indicate the error if FG is ignored: 1) the total plasma fluorescence AUC over the ultrafiltrate F AUC,
Fig. 1. Time course of fluorescence of the plasma, plasma ultrafiltrate, ultrafiltrate fluorescein, and ultrafiltrate fluorescein glucuronide after intravenous injection of fluorescein in one representative subject.

The total plasma fluorescence and the fluorescence of the ultrafiltrate rose rapidly, peaked about 3 min after injection, and then declined slowly. The fluorescence due to FG rose more slowly than the other components, since it was generated by metabolism. After peaking at about 30 min, it declined slowly, similar to the plasma ultrafiltrate fluorescence. The F fluorescence rose very quickly and then declined much more rapidly than the ultrafiltrate fluorescence or the total plasma fluorescence, so that there was very little F fluorescence in the late phases (more than 4–5 hr after injection). It is clear that the ultrafiltrate fluorescence and total plasma fluorescence in the late phases were due predominately to FG rather than F.

The same four factors over the first 2 hr after injection have been displayed in Figure 2 to illustrate more clearly their rapid changes during that interval. The ultrafiltrate FG fluorescence exceeded that due to the ultrafiltrate F even in the first hour.

In Figure 3, F and the FG are expressed in terms of concentration rather than fluorescence. However, the curve for F is the same as in Figures 1 and 2 because the ratio of concentration to fluorescence was one. The plasma fluorescence and the ultrafiltrate fluorescence are presented as in Figure 2. About 10 min after injection, the concentration of FG (ng/ml) in the ultrafiltrate exceeded the ultrafiltrate F concentration. Fur-
thermore, within the first 30 min, $F_{Gm}$ became greater than $F_m$. This indicates very rapid transformation of $F$ to $F_G$.

The plasma fluorescence and ultrafiltrate fluorescence were best fit by a three-compartment model (triexponential decay). However, the ultrafiltrate $F$ was best fit by a two-compartment model (biexponential decay). The $F_G$ decay phase appeared to be monoe xponential). The half lives for each decay phase are given in Table 1.

![Fig. 2. Time course of fluorescence of the plasma, plasma ultrafiltrate, ultrafiltrate fluorescein, and fluorescein glucuronide in the first 2 hr after injection in a subject.](image)

![Fig. 3. Time course of plasma ultrafiltrate concentration of fluorescein and fluorescein glucuronide in the first 2 hr after injection of fluorescein in a subject. The fluorescence of the plasma and the plasma ultrafiltrate are also shown.](image)
The unbound fraction of F often has been estimated by taking the ratio of the plasma ultrafiltrate fluorescence to the plasma fluorescence, which assumes only one fluorescent substance is present. However, Figure 4 shows that this ratio changed dramatically with time after injection. In the first several minutes after injection, when no FG was detectable, the mean and standard deviation for this fraction was 0.10 ± 0.023. In contrast, it was 0.41 ± 0.10 more than 8 hr after injection, when no F was measurable. The correlation between U/P and u/p was 0.823 (P < 0.001), indicating that the change in the ratio of the ultrafiltrate fluorescence to the total plasma fluorescence within the first 8 hr was due to the changing ratio of F to FG in the plasma over time with the unbound fractions remaining constant. Furthermore, we found no evidence that the venous plasma binding sites became substantially saturated after injecting the dose we used.

The mean and standard deviation of AUC for total plasma fluorescence, ultrafiltrate plasma fluorescence, F, and FG between 0–60, 0–120, and 0–∞ minutes after injection are given in Table 2. Within the first hour after injection, only 25% of the moles to which the barrier was exposed was to F. However, F contributed 63% of the potential fluorescence to which the barrier was exposed. These values were lower at later times. Table 3 indicates the proportion of the AUC from time 0–1 hr contributed during specific intervals. For the ultrafiltrate F, the greatest exposure of the barrier was between 3–10 min. Between 0–60 minutes, only 19% of the ultrafiltrate F AUC occurred in the last 30 min in contrast to over half of the total of ultrafiltrate FG AUC. Furthermore, in the second hour, only an additional 15% of the first hour's ultrafiltrate F AUC occurred, yet almost as much ultrafiltrate FG AUC occurred in the second hour as in the first.

We calculated how well the ultrafiltrate F AUC could be estimated using fluorescence without performing a chemical determination for F (Table 4). If only the AUC of the plasma fluorescence had been measured, the result would have been 14 times too high. Furthermore, if ultrafiltration of the plasma had been performed to eliminate the effect of binding, the value

### Table 1. Half-lives of decay phases

<table>
<thead>
<tr>
<th>Decay phase</th>
<th>Total plasma fluorescence</th>
<th>Ultrafiltrate fluorescence</th>
<th>Ultrafiltrate fluorescein</th>
<th>Ultrafiltrate fluorescein glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Half-life (min)</td>
<td>6.46</td>
<td>4.14</td>
<td>2.93</td>
<td>—</td>
</tr>
<tr>
<td>First</td>
<td>47.4</td>
<td>67.6</td>
<td>23.5</td>
<td>264</td>
</tr>
<tr>
<td>Second</td>
<td>301</td>
<td>317</td>
<td>296</td>
<td>264</td>
</tr>
</tbody>
</table>

### Diagram

**Fig. 4.** Time course of the ratio of ultrafiltrate fluorescence to total plasma fluorescence in one subject. The first values reflect the binding of fluorescein, the last values indicate the binding of fluorescein glucuronide, and the intermediate values represent mixtures of the two.
Table 2. Plasma area under the time curve (mean and SD)

<table>
<thead>
<tr>
<th>Minutes</th>
<th>0-60</th>
<th>0-120</th>
<th>0-∞</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total plasma fluorescence (µg-min/ml equivalents)</td>
<td>6,300 ± 1,300</td>
<td>7,800 ± 1,600</td>
<td>11,000 ± 1,900</td>
</tr>
<tr>
<td>Ultrafiltrate fluorescence (µg-min/ml equivalents)</td>
<td>740 ± 77</td>
<td>990 ± 100</td>
<td>2,200 ± 290</td>
</tr>
<tr>
<td>Ultrafiltrate fluorescein mass (µg-min/ml)</td>
<td>460 ± 100</td>
<td>530 ± 130</td>
<td>570 ± 150</td>
</tr>
<tr>
<td>Ultrafiltrate fluorescein glucuronide mass (µg-min/ml)</td>
<td>2,220 ± 570</td>
<td>4,200 ± 880</td>
<td>11,000 ± 3,900</td>
</tr>
<tr>
<td>Percent of ultrafiltrate due to fluorescein mass</td>
<td>17</td>
<td>11</td>
<td>4.9</td>
</tr>
<tr>
<td>Percent of ultrafiltrate due to fluorescein moles</td>
<td>25</td>
<td>16</td>
<td>7.2</td>
</tr>
<tr>
<td>Percent of ultrafiltrate due to fluorescein fluorescence</td>
<td>63</td>
<td>50</td>
<td>29</td>
</tr>
</tbody>
</table>

The concentrations of F and FG in the rabbit vitreous 5 hr after injection were 950 and 816 ng/ml, respectively. These corresponded to the respective fluorescence values of 950 and 101 ng/ml equivalents for F and FG.

Discussion

Our study of the pharmacokinetics of F and FG in the plasma resulted in several major conclusions. First, intravenously administered F is rapidly converted to FG in humans by conjugation with glucuronic acid, presumably in the liver. These findings confirm and extend those of others. This process would be of little interest in evaluating the BRB or BAB to F except that FG fluoresces. Glucuronidation is one of the major mechanisms by which the body metabolizes drugs and certain physiologic molecules, such as bilirubin. This process generally has the physiologic advantage of rendering the molecule less toxic and more water soluble, which usually reduces its volume of distribution and enhances renal excretion. The glucuronidation of F occurred so rapidly that, within 30 min after an intravenous injection of F, the molar concentration of FG exceeded that of F in the whole plasma. As time went on, the differing rates by which F and FG were cleared from the plasma caused the ratio of F to FG to drop continuously; F represented less than 1% of the total FG plus F 3-4 hr after intravenous injection. Since R is dependent on the spectrum used for both excitation and detection, the fluorescence measurements and quantities derived from them in this study using the Fluorotron Master may differ from those obtained with other fluorophotometers. Nevertheless, our findings are of wide interest, because this is the most frequently used fluorophotometer, and other fluorophotometers often use similar wavelengths.

Second, the apparent unbound fraction of F determined by U/P rose with time for several hours after the administration of F. This also has been observed by others. This increase in U/P is expected, because the saturation of binding sites is low below a concentration of about 200 µg/ml; indeed, it is low enough that the unbound fraction of F is practically independent of con-
centration. Furthermore, FG has a lower tendency to bind to plasma proteins than F, even if they bind to the same sites. The plasma F levels in the antecubital vein sometimes exceeded 200 μg/ml when they peaked about 3 min after injection. Thus, in the early phases after injection, the unbound fraction of F may have been slightly greater than at lower concentrations as the plasma binding sites became saturated. However, we did not detect this experimentally. Grotte et al. found little difference in the binding of F and FG in the plasma using the method of fluorescence polarization in a single subject. The reasons their result differ from ours and others are not immediately apparent.

Third, most F entered the eye shortly after injection, because the amount crossing the barrier over time from the plasma is proportional to the ultrafiltrate F AUC. Our calculations showed that over two thirds of the ultrafiltrate F AUC for this interval in a single subject. The reasons their result differ from ours and others are not immediately apparent.

Fourth, substantial errors are introduced in estimating the ultrafiltrate F AUC by measuring the plasma with fluorescence alone, even after ultrafiltration, because FG is ignored. These errors increase with time as FG is synthesized and F is eliminated. If F is used to estimate the permeability of the blood–ocular barriers, these errors should be taken into account, for example, by plasma ultrafiltration and performing HPLC.

Fifth, FG penetrated into the rabbit vitreous and reached a concentration approaching that of F 5 hr after injection. Recently, Kitano and Nagataki injected rabbits with pure FG intravenously and found that FG contributed substantially to the vitreous fluorescence within 1 hr after injection. They also calculated the ratio of the inward permeability of F to that of FG to be 3.37. If this applied to the humans in our study, FG would have contributed about 15% of the vitreous fluorescence 60 min after injection. Further information is needed to define the extent to which FG contributes to the vitreous fluorescence in humans, and how this should influence the interpretation of previously reported fluorophotometry results. However, vitreous fluorophotometry data have fit reasonably well to mathematical models of the pharmacokinetics of F in the vitreous, even though they did not consider FG. Thus, FG may not greatly influence vitreous fluorophotometry results in normal humans 60 min after injection.

Measurements taken several hours after a dose of F have been used to make inferences about the outward permeability of the BRB to F, the rate of loss of F from the aqueous by diffusion, and the rate of flow of aqueous. Since the ultrafiltrate FG AUC becomes progressively larger than the ultrafiltrate F AUC with time, FG is likely to contribute importantly to the intraocular fluorescence at these times.

Sixth, simple ways of estimating F and FG in both the blood and the eye are needed. We have shown that FG in the plasma introduced significant errors in the usual techniques of measuring F by fluorescence. Beta glucuronidase has been used to determine F and FG, but it adds a multistep procedure to the evaluation of F in the plasma. HPLC is a relatively expensive technique not familiar to many people interested in evaluating the eye by ocular fluorophotometry. Neither of these methods can be used to make measurements in the eye noninvasively. McLaren and Brubaker have described a method of measuring mixtures of F and FG in the plasma and aqueous humor by fluorescence based on measurements made using two wavelengths of an argon laser. We look forward to the establishment, simplification, and ready availability of this or other methods for measuring F and FG in the intraocular compartments and plasma.

**Key words:** fluorescein, fluorescein glucuronide, blood–ocular barrier permeability, ocular fluorophotometry, pharmacokinetics

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**References**


