Homogeneous, Micelle Quenching Fluoroimmunoassay for Detecting Amphetamines in Urine

Clarke J. Halfman and Dennis W. Jay

We developed a homogeneous fluoroimmunoassay for detecting amphetamines in urine. Only fluorescence intensity need be measured because the emission of non-protein-bound fluorescein-labeled amphetamine is preferentially quenched by detergent micelles. In a previously reported prototype assay system for measuring gentamicin in serum we used fluorescein and dodecyl sulfate (Anal Chem 1985; 57:1928–30). We have found that favorable hydrophobic and (or) ionic character of the analyte and unfavorable polar and (or) ionic character of the fluor are important determinants of the desired interactions. An anionic detergent and fluorescein, therefore, should be appropriate for apolar or cationic analytes, such as gentamicin and amphetamines. A greater [H+] at the anionic micelle surface is important for quenching emission from the fluoro moiety. Millimolar concentrations of dodecyl sulfate rapidly denature immunoglobulin unless happen is bound with sufficiently high affinity. Affinity was sufficiently high for the antibody used in the prototype gentamicin assay but not for the amphetamine antibody. Thus for the amphetamine assay, we used a non-denaturing detergent, dodecyl(oxyethylene)₁₂ sulfate. The assay requires 30 μL of specimen in 2 mL of total assay volume. Amphetamine (d-, dl-, and meth-), at a concentration of 1 mg per liter of urine, is readily detected.

Additional Keyphrases: methamphetamine · effects of detergents on immunoglobulins · dodecyl(oxyethylene)₁₂ sulfate

A homogeneous fluoroimmunoassay response is provided when a fluorescence property of bound labeled analyte is substantially different from that of free labeled analyte. Fluorescence intensity is the simplest and most sensitive property to measure, but usually is not considerably different for free and bound labeled analyte. Although polarization is generally greater for bound labeled analyte, and is therefore a useful response variable in immunoassays for analytes of small molecular size (1), a general means for inducing an intensity difference between bound and free labeled analyte would provide for a simpler, potentially more sensitive, less expensive assay. Three such means have been reported in the literature: conjugating antibody with a complementary acceptor dye quenches emission, by energy transfer, specifically from bound labeled analyte (2); using an additional antibody to fluorescein quenches emission specifically from free labeled analyte, when steric hindrance from binding by analyte antibody prevents antifluor binding (3); and detergent micelles can preferentially quench emission from free labeled analyte (4).

We demonstrated the last approach with an assay for measuring gentamicin in serum, using dodecyl sulfate (DDS) and fluorescein-labeled gentamicin.¹ The general utility of the method was not established, but we postulated that the preferential quenching of emission from free labeled analyte was due to micelles interacting directly with the analyte moiety but not with the fluor moiety. Thus, if the analyte moiety were enveloped within the immunoglobulin binding site, then the fluor moiety would not interact with micelles, even though exposed to the bulk solvent, and emission would not be quenched. We suggested that the requisite interactions could be achieved by using a detergent and a labeling fluor with the same ionic charge (and opposite to that of the analyte, when charged).

However, molecular features other than ionic charge are evidently responsible for the lack of interaction between fluorescein and DDS micelles, because other ionic fluoros clearly interact with detergent micelles regardless of respective ionic charges (5). To evaluate the potential general utility of detergent micelles for providing a homogeneous fluoroimmunoassay response, we needed to elucidate the molecular features that inhibit the interaction of some fluoros, such as fluorescein, with ionic detergents, and that would assure interaction of the analyte moiety with micelles. For this purpose we compared the interaction of various ionic fluoros with a cationic and an anionic detergent.

Furthermore, we suspected that intensity might not always be quenched when labeled analyte interacted with micelles. Indeed, it was surprising that fluorescein emission was altered, because the emission of other fluoros appeared to be altered by the incorporation of the fluoros into the hydrophobic micelle interior (6–8). We therefore conducted studies to examine the mechanism of the micelle-induced quenching.

DDS is known to denature proteins (9), including immunoglobulins (10). However, we recently reported that hapten binding prevented detergent denaturation of immunoglobulins so long as binding affinity for hapten was sufficiently high (11). The affinity of the immunoglobulin used in the prototype gentamicin assay (4) was apparently sufficiently high, but the lower-affinity amphetamine antiserum was rapidly denatured by DDS (11). For use with this amphetamine antiserum we therefore found a less-denaturing detergent, dodecyl(oxyethylene)₁₂ sulfate [DD(OE)₁₂S], which provided a homogeneous distinction between free and bound fluorescein-labeled methamphetamine.

Materials and Methods

Chemicals. We used glass-distilled, de-ionized water. All other reagents were at least reagent-grade quality and were used without further purification. The sodium salt of DDS and the bromide of dodecyl trimethyl ammonium (DDTA) were purchased from Aldrich Chemical Co., Milwaukee, WI; the sodium salt of DD(OE)₁₂S was obtained from Henkel.

¹ Nonstandard abbreviations: DD(OE)₁₂S, dodecyl(oxyethylene)₁₂ sulfate; DDS, dodecyl sulfate; DDTA, dodecyl trimethylammonium; FA, fluorescein-labeled methamphetamine.
Inc., Fort Lee, NJ. Fluorescein was the "Kromex" product from J. T. Baker Chemical Co., Phillipsburg, NJ. Phosphate-buffered saline, containing 10 mmol of NaH₂PO₄, 150 mmol NaCl, and 15 umol of DSS per liter, was adjusted to the desired pH (usually 7.4) with HCl or NaOH. The low concentration of DSS maintained stability of solutions containing low concentrations of fluorophores. Borate-buffered saline contained 10 mmol of sodium borate, 150 mmol of NaCl, and 15 umol of DSS per liter, and was adjusted to the desired pH as above. Sheep antiserum to amphetamine, a generous gift from Syva Co., Palo Alto, CA, was stored in aliquots at -80 °C until needed.

Preparation of fluorescein-labeled methamphetamine. Fluorescein-labeled methamphetamine (FA), with a butyramine bridging group, was prepared from fluorescein isothiocyanate and methamphetamine, both from Sigma Chemical Co., St. Louis, MO, under reaction conditions described by Smith (12). The N-4-(aminobutyl) methamphetamine was synthesized by reacting methamphetamine with 4-(bromoacetetyl)phthalimide (Aldrich) as described by Aoki and Kuriova (13). The purity of the isolated product was evaluated by thin-layer chromatography on precoated silica-gel F-254 plates (E. Merck, Westbury, NY) with a developing solvent of chloroform/methanol (70/30 by vol). A single, well-defined fluorescein spot was observed under long-wavelength ultraviolet illumination (Rf = 0.12; the Rf of fluorescein isothiocyanate under those conditions was 0.32). We based FA concentrations on absorbance measurements at 492 nm and the molar absorbity of 9.0 × 10³ mol⁻¹ cm⁻¹, given by the manufacturer, of fluorescein at this wavelength.

Fluorescence measurements. Fluorescence properties were measured with a dual-channel, Model 4000 SLM spectrofluorometer (SLM Instruments, Urbana, IL) as described previously (14). We used an excitation wavelength of 494 nm and matched Turner 2A12 filters (transmission above 500 nm), purchased from Sequoia-Turner Corp., Mountain View, CA, in the detection beams for measurements of fluorescein. The appropriate excitation wavelength and matched cutoff filters were used for measuring the other fluorescein dyes. Glann-Thompson polarizers, supplied with the instrument, were used in the excitation and emission beams when necessary. Vertical (V) and horizontal (H) emission components were measured simultaneously on the two individual detection channels with vertically polarized excitation. We normalized the signal strengths of the two detection channels by setting V = H, with horizontally polarized excitation for polarization determinations. When V - H was used to monitor fluor binding (15, 16), we set V = H, with vertically polarized excitation for the appropriate fluor reference solution.

Design of the assay. The anti-amphetamine serum was characterized to optimize the assay response. We determined binding-site concentration and affinity by titrating two dilutions of antiserum with FA (17) and measuring V - H as the response to binding (15, 16). The titration data were fit by nonlinear, least-squares regression analysis (18) with binding-site concentration and affinity for FA as the variables. Binding-site concentration of the antiserum was 61 (SD 2) μmol/L and the equilibrium dissociation constant was 7.5 (SD 0.5) nmol/L. The equilibrium dissociation constant for methamphetamine, 10.8 (SD 1.4) nmol/L, was determined by fitting data from competitive binding between the drug and FA (18). The concentration of antibody-binding sites (40 nmol/L) and of FA (52 nmol/L) for the assay were chosen to provide a response with a maximum initial relative slope and a midpoint at a drug concentration of 1 mg/L, with use of 30 μl of specimen and a total assay volume of 2.0 mL (19). Assays were conducted by adding 30 μL of standards or samples to 1.96 mL of FA in phosphate-buffered saline, pH 6.5, containing 50 mmol of DD(OE)HS per liter; initial intensity (I₀) was measured several minutes later. Final intensity (I₁) was determined several minutes after adding 20 μL of a 13-fold dilution of antiserum. Assay response (ΔI) was I₁ - I₀.

Results and Discussion

Detergent–Dye Interactions

We gained information about the molecular features of the co-reactants responsible for the requisite interactions by examining the influence of micelles of a cationic detergent, DDTA, and of an anionic detergent, DDS, on the fluorescence polarization of several ionic fluorescent dyes (see Figure 1). As shown in Table 1, all of the fluor interacted to some extent with each detergent above the critical micelle concentration. However, the anionic fluor interacted more strongly with the cationic micelles and the cationic fluor interacted more strongly with the anionic micelles. Rhodamine B interacted strongly with both detergents. With DDS, fluorescein interacted the most weakly; with DDTA, the pyronin Y interaction was the weakest. The stronger interaction between other like-charged fluor and detergent was apparently due to the presence of apolar groups, i.e., the benzoyl ester moiety of methyl fluorescein and of rhodamine 6G. Indeed, fluorescein interacted with DDS micelles at pH values less than 5.5 when the benzoate moiety was neutralized—behavior consistent with results from previously reported studies of other dye–detergent systems.

The solubilization of apolar fluor such as pyrene by detergent micelles clearly demonstrates that apolar compounds, or groups, are readily incorporated into the hydrophobic micelle interior (7, 20). The presence of a polar group at one end of a co-reacting compound would limit the extent of penetration of the apolar moiety because the energetically favorable location of the polar group would be at the micelle surface exposed to the polar, bulk aqueous solvent, as would be the polar head group of the detergent monomers composing the micelle. When the detergent and the co-reacting species are ionic and of the same charge, the depth of

![Fig. 1. Structures of fluorescent dyes](image-url)
penetration of an apolar moiety of the co-reacting molecule is further limited by electrostatic repulsion. When the ionic charge of the detergent and of the co-reacting species is opposite, then electrostatic attraction adds to the interaction energy and mixed micelles may even form at a detergent concentration less than the critical micelle concentration (21).

Interestingly, fluorescein did not interact with DDS micelles when the tricyclic dihydroxy xanthene group was neutralized ($pK_a = 6.2$). The high polarity of the oxygen heteroatom, rather than the rigid, planar structure of the xanthene group, probably prevented incorporation of this neutral group into the hydrophobic micelle interior. This conjecture was substantiated by the observation that pyronin Y did not interact with DDAnmicelles, although acridine orange did (Table 1).

In light of this behavior, we concluded that a molecule would be inhibited from interacting with micelles only if like ionic charges and (or) polar groups were appropriately located to prevent incorporation of apolar groups into the hydrophobic micelle interior. Apolar groups of such a molecule would not be incorporated into the micelle interior, even when ionic charges are opposite to the micelle charge, but the molecule would be “adsorbed” at the micelle surface by electrostatic attraction, just as counter-ions are concentrated at the micelle surface (22, 23). Fluorescein and pyronin Y, as well as rhodamine B, were therefore probably adsorbed at the surface of oppositely charged micelles. Apparently, this was also the mode of interaction of gentamicin with DDS micelles in the prototype assay (4).

Interaction of the other fluor (Table 1) involved incorporation of apolar portions into the micelle interior. The lack of interaction of fluorescein with DDS micelles was accounted for by several molecular features: the carboxylate of the benzoate moiety, the ionized quinoid group of the dihydroxy xanthene tricyclic ring system, and the oxygen heteroatom of the middle ring preventing interaction, even when the quinoid group was neutralized.

The combination of fluorescein as labeling fluor and an anionic detergent such as DDS would be well suited for use in assay systems for any apolar or cationic analyte. Although a cationic detergent such as dodecyl trimethyl ammonium would be suitable in assays for any apolar or anionic analyte, a candidate fluor is more problematic. Pyronin Y did not interact with micelles of a cationic alkyl chain detergent, but reactive derivatives for conjugation to analytic are not available. Reactive derivatives of rhodamine B are available, but this fluor interacted with DDATA micelles (Table 1), apparently by electrostatic attraction of the benzoate group. Possibly, when conjugated to analytic and bound to antibody, steric hindrance may prevent the benzoate group of rhodamine B from interacting with micelles. Otherwise, synthesis of the choline ester should provide a suitable labeling fluor.

**Drug Detection**

Detection of abused drugs in urine is a useful application of the method, most drugs of abuse, except the barbiturates, being cationic amino compounds; thus fluorescein and an anionic detergent (e.g., DDS) would be appropriate components of an assay system. We developed an assay system for amphetamines first. The fluorescein–iso-thiocyanate adduct of butyamine-bridged methamphetamine (FA) interacted with DDS micelles in phosphate-buffered saline (pH 7.4) as expected. Evidently, the amphetamine moiety was responsible for the interaction, because fluorescein itself did not interact. The interaction quenched fluorescein emission by 75%.

The mechanism for the micelle-induced quenching was suggested by the effect of DDS micelles on the pH dependence of emission from the fluor moiety (Figure 2). The titration of FA in the absence of DDS (curve a) was essentially identical to that of fluorescein ($pK_a$ of 6.2); DDS micelles shifted the $pK_a$ to 8.0 (curve b). The concentration of counter-ions, including $H^+$, may be several orders of magnitude greater at the micelle surface than in the bulk solvent (22, 23). The results from the titrations (Figure 2) therefore suggested that the micelle-induced quenching was

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**Table 1. Interaction of I onic Fluorescent Dyes with a Cationic and an Anionic Alkyl Chain Detergent in Phosphate-Buffered Saline (pH 7.4)**

<table>
<thead>
<tr>
<th>Fluorescent dye (6 mmol/L)</th>
<th>DDS concn, <em>$^\text{a}$</em> mmol/L</th>
<th>DDTA concn, <em>$^\text{a}$</em> mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.001</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>polarization</td>
<td>intensity$^b$</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>0.014</td>
<td>1.00</td>
</tr>
<tr>
<td>Methyl fluorescein</td>
<td>0.016</td>
<td>0.47</td>
</tr>
<tr>
<td>Acridine orange</td>
<td>0.030</td>
<td>0.53</td>
</tr>
<tr>
<td>Pyronin Y</td>
<td>0.027</td>
<td>0.35</td>
</tr>
<tr>
<td>Rhodamine 6G</td>
<td>0.025</td>
<td>3.0</td>
</tr>
<tr>
<td>Rhodamine B</td>
<td>0.070</td>
<td>3.0</td>
</tr>
</tbody>
</table>

$^a$The critical micelle concentrations for DDS and DDTA are approximately 1 and 5 mmol/L, respectively.

$^b$Relative to the intensity in 0.001 mmol/L detergent.
due to protonation of the fluorophoric group because of a 60-fold greater H$^+$ concentration at the micelle surface.

By this mechanism, the assay response magnitude is obviously pH dependent. Assuming that the intensity of antibody-bound FA is equivalent to the intensity in the absence of DDS, the absolute response magnitude would be greatest at pH 7.1. The magnitude of the more pertinent relative response would be greater the lower the pH. In practice, the minimum pH to be used would be limited by the signal strength needed to avoid appreciable values for specimen blanks.

DS was unsuitable for the amphetamine assay because the detergent rapidly denatured the antibody used, hapten-binding ability being rapidly lost when DDS was added (final concentration 10 mmol/L) to antibody-bound FA. We reported earlier that bound hapten could prevent DDS denaturation of immunoglobulin so long as affinity was sufficiently great, or dissociation rate was sufficiently slow (11). The binding of fluorescein-labeled thyroxin to thyroxin antibody, with a dissociation half-time of 1.25 h, protected from DDS denaturation. Evidently, the affinity of the antibody used in the prototype gentamicin assay (4) was also sufficiently great. However, the dissociation rate ($t_{1/2}$ = 75 s) of the anti-amphetamine immunoglobulin we used was too rapid to afford adequate protection from DDS denaturation.

Of several other anionic detergents evaluated for their non-denaturing ability, DD(OE)$_{12}$S seemed most suitable. Micelles of this detergent interacted with FA and consequently quenched emission (Figure 3). The effect of micelles of this detergent on the pH-dependence of FA intensity was similar to that of DDS (Figure 2) except that the pK$_a$ was shifted only 1.3 pH units, representing a somewhat lesser increase in H$^+$ concentration, about 20-fold, at the micelle surface.

This detergent, however, was less than ideal because its micelles also interacted with antibody-bound FA. We suspected micelle interaction with the fluorescein moiety and indeed demonstrated interaction of DD(OE)$_{12}$S micelles with fluorescein (Figure 4). The micelle interaction increased the pK$_a$ of the fluorogenic group by about 1.0 pH unit. Apparently, the neutralized dihydroxy xanthene group favorably partitioned within the (octylenylene)$_{12}$ micelle shell by about ninefold more than in the bulk aqueous solvent. We did not observe this behavior with the completely hydrophobic DDS micelles. Evidently, only the weakly fluorescent, neutralized form of the dihydroxy xanthene group was incorporated into the polar shell of the DD(OE)$_{12}$S micelles; otherwise, polarization would have been increased at higher pH. Polarization was increased only at lower pH, where the neutralized, micelle-incorporated form contributed substantially to the total emission.

Even though DD(OE)$_{12}$S micelles interacted with the fluorescein moiety of FA bound to amphetamine antibody, the interaction was stronger with free FA. The relatively greater quenching of free FA emission by DD(OE)$_{12}$S micelles increased with decreasing pH; at a pH of 6.5 or less, the intensity of free FA was less than 60% of bound FA. This extent of differential quenching by DD(OE)$_{12}$S micelles was sufficient to provide a homogeneous distinction between free and bound FA in an immunoassay. A typical response curve for methamphetamine is shown in Figure 5. Analysis of 50 different urine specimens, to which we added 1 mg of d-,, d,l-, or methamphetamine per liter, demonstrated an adequate

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![Fig. 3. Fluorescent titration of FA with DD(OE)$_{12}$S](image)

![Fig. 4. Effect of DD(OE)$_{12}$S on the pH dependence of fluorescent properties of fluorescein](image)

![Fig. 5. Response curve for methamphetamine fluoroimmunoassay](image)
intensity difference from that for unsupplemented controls (Table 2). Sensitivity (detection limit) was ±0.05 mg/L, and precision at 1 mg/L was ±0.2 mg/L.

The antisera exhibited highest affinity for methamphetamine, in contrast to the EMTR assay (Syva Co.) with the same antisera, which exhibits the greatest response for l-amphetamine. The different relative affinities probably resulted from the different labels used in each assay system.

At the pH of 6.5 used to obtain a reasonable magnitude of relative response, fluorescein emission was only a fraction of its value at higher pH, so that intensities of sample blanks were about 20% of the test signal. Conducting the assay at a higher pH would substantially minimize the relative signal strength of sample blanks, thus obviating their measurement and subtraction. Other quenching ions, such as Cu++, are also concentrated at the surface of oppositely charged micelles (22, 23) and we are currently investigating whether their use will permit conducting the assay at higher pH.

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References