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A Sensitive Solid-Phase Fluoroimmunoassay for Detection of Opiates in Urine

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Abstract

An automated flow fluorometer designed for kinetic binding analysis was adapted to develop a solid-phase competitive fluoroimmunoassay for urinalysis of opiates. The solid phase consisted of polymer beads coated with commercial monoclonal antibodies (MAbs) raised against morphine. Fluorescein-conjugated morphine (FL-MOR) was used as the fluorescein-labeled hapten. The dissociation equilibrium constant (K_D) for the binding of FL-MOR to the anti-MOR MAb was 0.23 n*M*. The binding of FL-MOR to the anti-MOR MAb reached steady state within minutes and was displaced effectively by morphine and other opiates. Morphine-3-glucuronide (M3G), the major urinary metabolite of heroin and morphine, competed effectively with FL-MOR in a concentration-dependent manner for binding to the antimorphine MAb and was therefore used to construct the calibration curve. The sensitivity of the assay was 0.2 ng/mL for M3G. The assay was effective at concentrations of M3G from 0.2 to 50 ng/mL, with an IC_{50} of 2 ng/mL. Other opiates and heroin metabolites that showed >50% crossreactivity when present at 1 µg/mL included codeine, morphine-6-glucuronide, and oxycodone. Methadone showed very low crossreactivity (<5%), which is a

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benefit for testing in patients being treated for opiate addictions. The high sensitivity of the assay and the relatively high cutoff value for positive opiate tests allows very small sample volumes (e.g., in saliva or sweat) to be analyzed. A double-blind comparison using 205 clinical urine samples showed good agreement between this single-step competitive assay and a commercially performed enzyme multiplied immunoassay technique for the detection of opiates and benzoylecgonine (a metabolite of cocaine).

Index Entries: Morphine; urinalysis; monoclonal antibody; polymethyl methacrylate; beads; KinExA™.

Introduction

Competitive fluoroimmunoassays have a broad spectrum of application for the detection of small and large molecules. Early problems with low sensitivity caused by high background fluorescence *(1)* have been overcome by the development of methods that separate bound fluorescent molecules from those remaining free in solution. One such method, particle concentration fluorescence immunoassay (PCFIA) *(2,3)*, employs inexpensive and easily handled microbeads as a solid phase and reduces background fluorescence by separating bead-bound fluorescence from unbound fluorescent molecules in the liquid phase. The fiber-optic biosensor (FOB) accomplishes a de facto separation of bound and free fluorescent molecules by taking advantage of the very limited range of the evanescent light emitted by optical fibers *(4,5)*. Only those molecules that are bound to antibodies, or other macromolecules immobilized on the optical fiber, are close enough to be excited by the evanescent light. This feature eliminates the need to manipulate the sample to separate bound from free fluorescent molecules, reduces the background fluorescence, and greatly increases the speed and sensitivity of individual measurements. Developed almost concurrently with PCFIA, FOB devices allow an additional benefit of direct observation of the binding of the analyte and subsequent calculation of the kinetics of the binding reaction. FOBs have been used extensively in our laboratory to detect and quantitate pesticides *(6,7)*, polychlorinated biphenyls *(8)*, and cocaine and its metabolites *(9–11)*. Current FOB devices, however, require the continuous attention of the user, either for the installation of fresh fibers or the regeneration of a previously used fiber, thereby increasing the cost and user input of each analysis beyond the point of usefulness for high-throughput applications.

The recently developed KinExA fluoroimmunoassay instrument (Sapidyne, Boise ID) features many of the best traits of both formats. It combines the low cost and ease of handling of polymer microbeads with the real-time monitoring of binding of FOBs *(12,13)*. In the present study, the instrument was adapted for fluoroimmunoassay of opiates, and 205 clinical urine specimens were tested in a double-blind experiment for evidence of heroin and cocaine abuse. The results were compared to data obtained by a commercial laboratory using the enzyme multiplied immunoassay technique (EMIT) test *(14–17)*.

Materials and Methods

Reagents

Antimorphine monoclonal antibodies (MAbs) and antibenzoylecgonine (anti-BE) MAbs were obtained from Fitzgerald Industries (Concord, MA) and Biostride (Palo Alto, CA), respectively. Fluorescein-labeled BE (FL-BE) and morphine (FL-MOR) were obtained from The Binding Site (San Diego, CA). Opiates, cocaine, related compounds, and other drugs of abuse were obtained from the National Institute on Drug Abuse (NIDA). Polymethylmethacrylate (PMMA) microbeads (98 µm) were obtained from Sapidyne.

Microbead and Specimen Preparation

Antibodies were immobilized on PMMA beads for use as the sensing element by incubating 200 mg of beads in 1.0 mL of MAb solution (25 µg/mL in phosphate-buffered saline [PBS], pH 7.4) for 1 h at room temperature. Antibodies were immobilized on PMMA beads solely by adsorption. High-purity (>98%) drug and metabolites were diluted in PBS. Urine specimens were prepared by diluting 100 µL of specimen in 3 mL of PBS (approx 3%) to perform triplicate analyses.

KinExA Competitive Fluoroimmunoassay

Beads, coated with MAb, were placed in the bead reservoir of the instrument, and the single-step fluoroimmunoassay was performed as described *(13)*. In brief, 6.67 mg of MAb-coated beads was drawn into a flow cell embedded in a light emitter/detector. The instrument then mixed a 1.0-mL aliquot of a diluted urine specimen with an equal volume of PBS containing 3.75 n*M* FL-MOR. This mixture was drawn through the packed beads in the flow cell. Unlabeled opiates, if present in the sample, competed with FL-MOR for binding to the MAbs coating the beads. The fluorescence of the bead pack was monitored continuously and recorded each second by the computer operating the instrument. The amount of analyte was quantified by observing the decrease in fluorescent signal caused by competition between analyte in the urine and FL-MOR. The calibration curve for urinalysis was performed using diluted negative urine to which morphine-3-glucuronide (M3G) was added. Experiments for the determination of rate and affinity constants for the binding of FL-MOR to the antimorphine MAb were performed in PBS. The assay for BE was essentially the same as that for opiates, except that the fluorescent competitor was FL-BE, the volume of diluted urine analyzed was 0.75 mL, and the FL-BE concentration was 2.5 n*M*. Opiates and cocaine metabolites were quantified as M3G and BE equivalents, respectively. The assay time was 3.5 min/specimen.

Urinalysis

Urine specimens were collected by the University of Maryland Drug Treatment Center from patients in the methadone maintenance program

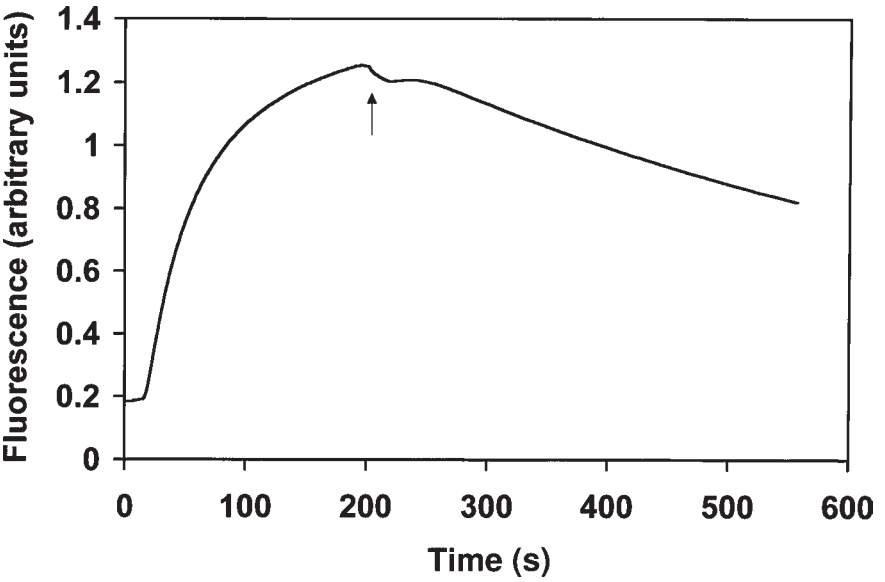

Fig. 1. Time course of the association and dissociation of fluorescein-labeled morphine (FL-MOR) from antimorphine MAb immobilized on the surface of microbeads in the KinExA fluoroimmunoassay instrument. The FL-MOR solution was replaced with buffer (PBS) after 200 s to observe the rate of dissociation of FL-MOR from the MAb. A sudden drop in fluorescence that lasted 10–20 s was frequently observed on initiation of the PBS wash (arrow).

who voluntarily submit to random urinalysis. Aliquots of urine specimens were provided for biosensor analysis at the time the specimens were sent for EMIT testing at Friends Medical Laboratory (Lutherville, MD). The EMIT results were not revealed by the center until our analysis was complete; therefore, the fluoroimmunoassays were double blind. Each specimen was prepared as described under Materials and Methods and tested in triplicate. A mean percentage of inhibition of fluorescence was calculated and compared to the calibration curves to determine the concentration of M3G equivalents in each specimen.

Results

Rate and Affinity Constants for Binding of FL-MOR to Immobilized MAb

Rate constants for the binding of FL-MOR to and dissociation from the MAb immobilized on the microbeads were calculated from the time course of association and dissociation (Fig. 1). The dissociation rate constant (k_{-1}) was calculated from the half-life ($T_{1/2}$) of dissociation 5 min after the beginning of the experiment, following withdrawal of FL-MOR. Fluorescence values over 120 s during dissociation of FL-MOR from the beads were used to construct the dissociation curve. A semilogarithmic plot of fluorescence

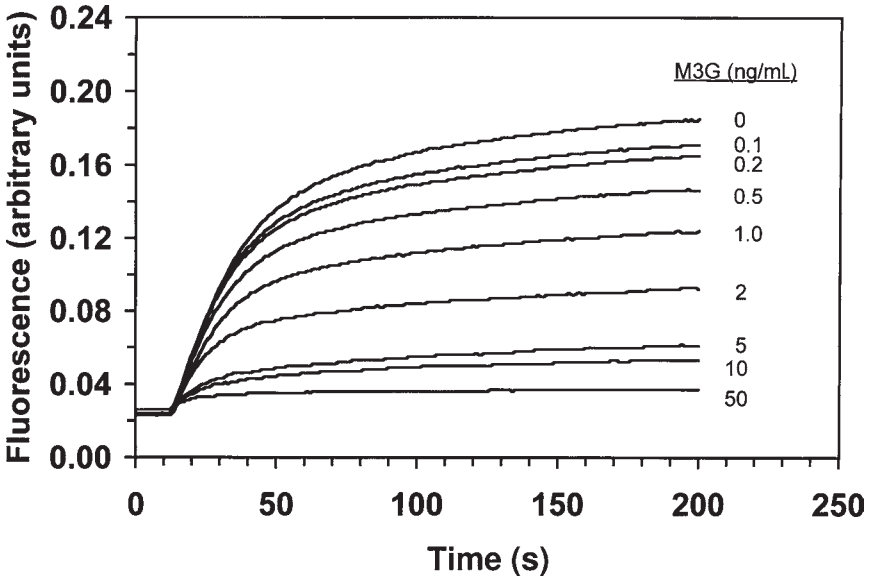

Fig. 2. Calibration of the KinExA fluoroimmunoassay for M3G in negative urine. Increasing concentrations of M3G decreased the fluorescent signal by competing with fluorescein isothiocyanate–morphine for binding to bead-bound antimorphine MAb. Concentrations given are the final M3G concentrations. The corresponding M3G concentrations in negative urine specimens are 33-fold higher.

vs time was used to obtain $T_{1/2}$, which gave a dissociation rate constant of 0.0815/min. An apparent association rate constant (k_{app}) of 1.38/min was obtained as the slope of the linear plot of ln ($Fl_{ss}/Fl_{ss} - Fl_t$), in which Fl_{ss} is the fluorescent signal at steady state and Fl_t is the signal at time t (a quantity that directly reflects the fractional occupancy of MAb binding sites by FL-MOR vs time) during the initial 200 s of the assay. An association rate constant (k_1) of 1.7×10^8 M/min was obtained from the formula $k_1 = (k_{app} - k_{-1})/$[FL-MOR] *(18)*, and according to the law of mass action, the K_D ($=k_{-1}/k_1$) was 0.23×10^{-9} M.

Detection of M3G

The presence of M3G was detected by its ability to compete with FL-MOR for binding to anti-MOR MAbs bound to the surface of the polymer beads (Fig. 2). Increasing concentrations of M3G, ranging from 0.1 to 50 ng/mL, produced decreasing near-steady-state signals from 0.18 (no M3G) to 0.03 mV (50 ng/mL). A calibration curve in diluted negative urine was thereby generated for the quantitation of M3G equivalents. Plotting the percentage of inhibition of fluorescent signal vs M3G concentration yielded the calibration curve (Fig. 3). The IC_{50} for M3G was 2 ng/mL and the K_i was 0.11 ng/mL. The assay for M3G had approximately a 10-fold better detection limit than a similar assay performed to detect the cocaine metabolite BE (Fig. 3).

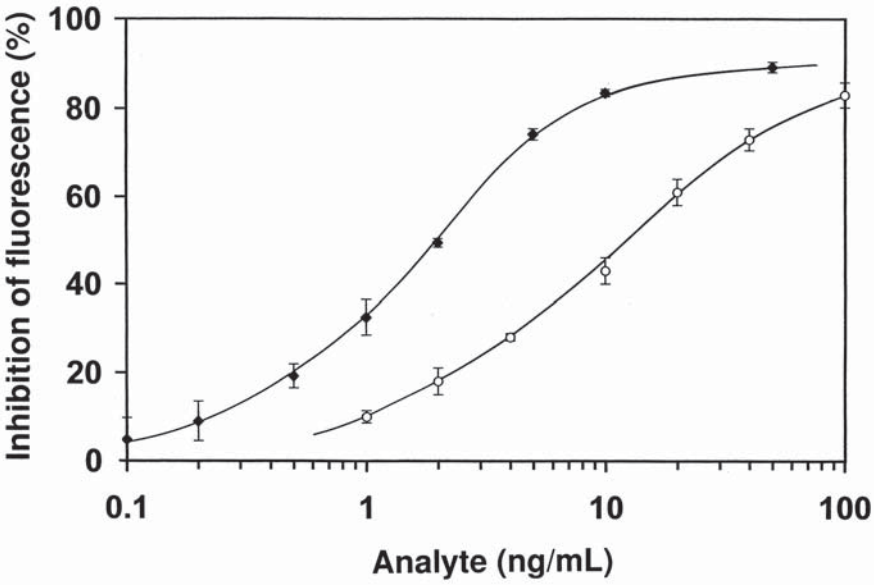

Fig. 3. Plot of percentage of inhibition of fluorescence as a function of M3G (◆). A similar curve was previously generated for BE (○) *(12)*. The K_D for FL-BE was 2.2 n*M*, the IC_{50} for BE was 12 ng/mL, and the limit of detection for BE was 2 ng/mL.

Specificity of Opiate Fluoroimmunoassay

Of the compounds tested, morphine was bound by the antimorphine MAb with the highest affinity. Morphine and M3G displaced FL-MOR from the MAb with IC_{50} values of 50 and 66 ng/mL of urine, respectively. The inhibition of FL-MOR binding by 1 µg/mL of morphine was assigned a value of 100%. Morphine-6-glucuronide (M6G), codeine-6-glucuronide (C6G), and oxycodone displayed crossreactivity (Table 1), whereas methadone, etorphine, and buprenorphine-6-glucuronide were poor inhibitors of FL-MOR binding to the immobilized MAb. None of the nonopiate compounds tested displayed significant crossreactivity (Table 1). Morphine, M3G, and M6G are all metabolites of heroin that are found in urine *(19)*; therefore, the presence of each in urine would properly contribute to a positive reaction with the anti-MOR MAb. Similarly, the anti-BE MAb was found to be specific for cocaine, BE, and cocaethylene, whereas ecgonine and ecgonine methyl ester were poorly recognized *(13)*. Morphine, M3G, M6G, C6G, and oxycodone were poor inhibitors of FL-BE binding to the anti-BE MAb, as were the other compounds tested (data not shown). We therefore concluded that the likelihood of error owing to nonspecific binding of analytes by each antibody was low. However, these results also highlight the importance of the choice of MAb(s) when designing an immunoassay.

Analysis of Clinical Urine Samples for Opiates

The NIDA concentration value for positive drug test results was 300 ng/mL in urine for opiates at the time the experiments were performed.

Table 1
Crossreactivity of an Immobilized MAb Against Morphine with Other Drug Metabolites and Drugs of Abuse

Compound	Crossreactivity (%)[a]
Morphine	100.0 ± 2.1
M6G	89.5 ± 1.4
Oxycodone HCl	88.9 ± 1.3
M3G	86.5 ± 1.3
C6G	80.4 ± 0.1
BE	5.5 ± 1.3
Cocaine HCl	0[b]
Cocaethylene	0
Ecgonine	0
Ecgonine methyl ester	0
Methadone	4.0 ± 1.3
Etorphine	1.2 ± 1.2
Methaqualone	0.4 ± 0.4
Buprenorphine-6-glucuronide	0
Amphetamine HCl	0
Methamphetamine HCl	0

[a]Values are the mean of three replicate measurements and represent the inhibition of binding of FL-MOR to the antibody by 2.5 µ*M* of each compound, ± SEM. The inhibition of FL-MOR binding in competition with morphine was taken as 100%.

[b]Zero denotes no detectable inhibition of fluorescence, or fluorescence greater than that observed with buffer alone.

Table 2
Comparison of KinExA Fluoroimmunoassay Data (M3G Acid Equivalents) to Commercial Laboratory EMIT Testing for Presence of Opiates in Urine[a]

KinExA	EMIT	Number of samples
Positive	Positive	29
Negative	Negative	166
Positive	Negative	1
Negative	Positive	9
Total		205

[a]Agreement for 195 of 205 specimens in detecting M3G equivalents = 95.1%.

The assay was very sensitive, with 50 ng/mL of M3G eliminating nearly all the fluorescent signal. Therefore, urine specimens were diluted approx 30-fold to bring the cutoff value into the dynamic range of the assay. Opiates at a concentration of 300 ng/mL in undiluted urine yielded a diluted concentration of approx 9 ng/mL and an 83% inhibition of fluorescence (Fig. 3).

Two hundred and five clinical urine specimens received from patients in the University of Maryland Drug Treatment Center were tested for the

Table 3
Comparison of KinExA Fluoroimmunoassay Data (BE Equivalents) to Commercial Laboratory EMIT Testing for Presence of Cocaine Metabolites in Urine[a]

KinExA	EMIT	Number of samples
Positive	Positive	49
Negative	Negative	153
Positive	Negative	2
Negative	Positive	1
Total		205

[a]Agreement for 202 of 205 specimens in detecting BE equivalents = 98.5%.

presence of opiates as M3G equivalents. The results showed good agreement with the EMIT data (Table 2). The fluoroimmunoassay agreed with the commercial test in 195 of 205 cases, or 95.1%. The specimens were also tested for the presence of BE using FL-BE and beads coated with anti-BE MAb to compare further the results of the fluoroimmunoassay with clinical data (Table 3). The fluoroimmunoassay for BE agreed with the commercial EMIT test in 202 of 205 samples, or 98.5%.

Discussion

The fluoroimmunoassay we have described is extremely sensitive, with the presence of 50 ng/mL of M3G resulting in a very strong inhibition of the fluorescent signal. Therefore, only a small volume of urine was necessary for an accurate quantitation of opiates. Because NIDA recently raised the cutoff value for positive opiate tests to 1 µg/mL (to accommodate non-abuse-related opiate intake with foods), specimens as small as 1–5 µL may be sufficient for testing. Such high sensitivity might not be required for urinalysis, in which large specimen volumes are available, but may be quite useful for detecting opiates in body fluids usually available only in small volumes (e.g., saliva, sweat, or tears).

M3G was chosen as the standard for the assay because it is the major metabolite of morphine and heroin. More than 50% of the opiates in urine is typically M3G, and therefore most of the signal generated by the clinical samples tested was likely owing to the presence of this compound. The ability of the antimorphine MAb to crossreact with metabolites of heroin and morphine as well as oxycodone and the codeine metabolite C6G provides the ability to detect the presence of several of the most widely abused opiates. Conversely, the very low crossreactivity of methadone in this assay reduces interference by this drug, a benefit in testing in the clinical setting in which the level of methadone in urine will be high in patients being treated for opiate addiction.

The overall agreement between the EMIT analysis and the fluoroimmunoassay was 397 in 410 assays, or 96.8% (205 specimens assayed for

two drugs/metabolites). Of the 13 mismatches between the two methods, 7 measurements using the fluoroimmunoassay method were very close to the cutoff limits. If these results had agreed with the EMIT test data, the success rate would have been 98.8% (5 mismatches in 410 assays). Mismatches close to the cutoff limits were likely owing to variability in the two assays. In the fluoroimmunoassay, the variability among replicate measurements of prepared standards using the same batch of MAb-coated beads was approx 2%, and the variability among measurements using independent preparations of both standards and MAb-coated beads was also approx 2%, in measurements of standards ranging from 0.3 to 100 ng/mL (data not shown). The EMIT test is qualitative, giving only positive or negative results calibrated to the NIDA cutoff values, and has a false-positive rate of 3–5% *(20,21)*. For this reason, and to identify which drug or metabolite is present in the specimen, positive EMIT results were confirmed by retesting by EMIT and also fluorescence polarization immunoassay in the case of cocaine metabolites, or retesting by EMIT and subsequent separation of urine opiates by thin-layer chromatography (R. Kokoski, personal communication). Many laboratories also confirm positive EMIT data gas chromatography mass spectroscopy with (GC-MS) analysis *(19)*, depending on the application.

The fluoroimmunoassay described herein shares with fiber-optic instruments the inherent advantage of separation of free from bound fluorescent molecules during the binding event. This allows the user to observe the kinetics of binding in real time, unlike enzyme-linked immunosorbent assay, radioimmunoassay, or PCFIA, in which free-labeled molecules must be removed after the binding reaches equilibrium. The assay is applicable to urinalysis of any chemical for which antibodies and a fluorescent conjugate can be obtained, and its sensitivity can approach that of GC-MS. However, it cannot distinguish and quantitate multiple opiate metabolites simultaneously, as might be useful when determining the time elapsed from the last dose of a drug. A separate batch of coated beads is required for each metabolite of interest, and the specificity of the assay is determined (and limited) by the availability of antibodies that can distinguish among them.

The KinExA instrument has also been used to detect the herbicide 2,4-D *(22)* and to measure antibody/antigen affinities *(23–25)*, its designed purpose. In its current configuration, the KinExA instrument is capable of performing automated replicate binding analyses of up to 12 samples plus a reference. Instruments in development will handle larger numbers of samples and automate sample dilutions and loading, and a handheld instrument is planned (S. Lackey, Sapidyne, personal communication). The technology displays a flexibility that has so far escaped FOBs, which currently require manual operation, more expensive materials, and more complex protocols for immobilizing antibodies. The instrument can likely be expanded to include multiple bead reservoirs and flow cells to increase the number of analytes detected simultaneously. Alternatively, multiple

analytes could be detected in a single flow cell/bead pack if multiple fluorescent tags that absorb/emit in different wavelengths were used, and emitters/detectors of the corresponding wavelengths installed. Future studies on the commercial usefulness of this method must include GC-MS analysis of specimens to determine the accuracy and precision of the assay, especially for samples containing drug/metabolite concentrations close to the positive/negative limit. However, the extensive agreement between our method and the widely accepted commercial EMIT testing indicates that this technology, given a good selection of high-affinity antibodies and a design for increased throughput, is likely to prove both sensitive and accurate enough for large-scale testing and have the additional advantage over EMIT of quantitation of the analytes.

Acknowledgments

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