Multiple Drugs of Abuse in Urine Detected with a Single Reagent and Fluorescence Polarization

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We describe a simple polarization fluoroimmunoassay for the detection of several commonly abused drugs in urine. The single reagent used, prepared by mixing four different antisera and three fluorescein-labeled derivatives, produces a polarization signal that is the average of the individual signals of the derivatives. One adds urine to the pre-mixed reagent, incubates at room temperature for a few minutes, then measures fluorescence polarization. The presence of any of several abused drugs, at concentrations of 1 mg/L or more, noticeably decreases the average signal. Although other combinations are possible, the present assay detects the presence of a cocaine metabolite, amphetamine, and (or) several barbiturates.

Urine is screened for abused drugs by qualitative techniques followed, if necessary, by a confirmatory assay. Among the screening techniques developed are radioimmunoassays involving separation, such as the Roche Abuscreen®, and non-separation enzymoimmunoassays, such as the EMIT-d.a.u.® from Syva. Rapid, easily automated assays are desirable and, for simplicity, several investigators have attempted to develop combination assays capable of detecting any of several abused drugs (1, 2). We recently described the development of fluorescence polarization immunoassays (FPIA) for individual drugs of abuse (3–5). We describe here the use of a combination reagent capable of detecting benzoylecgonine (a cocaine metabolite), amphetamine, and several commonly abused barbiturates in urine. The use of a single assay tube to detect the presence of one or more of several drugs simplifies the initial screening procedure and reduces costs.

Materials and Methods

Reagents. We prepared fluorescein-labeled derivatives as described previously (3–6).

Immunogens. These were also described as prepared previously (3–6) and used to immunize three mature ewes with each immunogen, by a previously described protocol (7). Serum from the first or second bleed from the sheep giving the highest titer was used in the assay development described below.

FPIA single reagent. We prepared the working assay reagent in sodium borate buffer (100 mmol/L, pH 9.1) containing, per liter, 1 g of gelatin and 1 g of sodium azide. We combined the antiserum to benzoylecgonine (diluted 630-fold), to amphetamine (diluted 3220-fold), to secobarbital (diluted 1450-fold), and to phenobarbital (diluted 1450-fold), in assay buffer with fluorescein-labeled benzoylecgonine (5 nmol/L), fluorescein-labeled amphetamine (2.2 nmol/L), and fluorescein-labeled secobarbital (8.5 nmol/L). We stored the working reagents at 4°C, in the dark, until required.

Assay standards. For assay standards, we prepared stock 1 g/L solutions of amobarbital, butobarbital, cyclobarbital, pentobarbital, secobarbital, amphetamine, and benzoylecgonine, then diluted these in assay buffer to give concentrations of 0.5, 1.0, 2.0, and 5.0 mg/L for each analyte.

Polarization fluorometer. We used either a Model 4000 polarization fluorometer (SLM Instruments, Urbana, IL 61801) described elsewhere (6), or a Model PF1-20 polarization fluorometer (Perkin-Elmer Corp., Beaconsfield, Bucks, U.K.). The latter instrument was fitted with a 480-nm interference excitation filter and a 520-nm-cutoff emission filter. It had a fixed, vertically oriented polarizer in the excitation light beam, the polarization being determined by driving a vertical, then a horizontal, polarizer into the emission beam and measuring the corresponding fluorescence signals. The Perkin-Elmer instrument was equipped with a flow cell (7 μL illuminated volume), a peristaltically-pumped sample-sipping system, and an autosampler that delivered 10 μL of urine into 1.5 mL of single reagent.

Specimens. We obtained urine specimens from the Department of Chemical Pathology, Hackney Hospital, London, U.K., where they had been screened for drug abuse by thin-layer chromatography for barbiturates (3), gas–liquid chromatography for amphetamine (4), and EMIT-d.a.u. for cocaine (5).

FPIA procedure. We performed the assays at room temperature as follows: To 1.5 mL of working reagent in a 50 × 10 mm glass cuvette (no. 9518; Abbott Diagnostics Division, Basingstoke, Hampshire, U.K.) add 10 μL of urine or standard, vortex-mix, and allow to stand for 30 min before reading the fluorescence in the fluorometer. A sample was considered to be positive for one of the drugs if it gave a polarization value lower than the cutoff value (set at the fluorescence for secobarbital, 1.0 mg/L).

Results

Standard curve. The assay is designed as a qualitative screen using only one standard as a positive/negative cutoff. However, to give an indication of the degree of displacement, we include Figure 1, which depicts the standard curves for amphetamine, benzoylecgonine, and secobarbital obtained with the FF1-20.

Assay optimization. We calculated the standard deviation of the polarization readings from 20 replicates of a zero standard (assay buffer). With the SLM instrument the mean polarization value was 173.6 mP with a standard deviation (SD) of 0.966 and a coefficient of variation (CV) of 0.55%. The minimum detectable concentration of secobarbital at the 95% confidence level (8) was estimated as 0.25 mg/L, for amphetamine 0.2 mg/L, and for benzoylecgonine 0.2 mg/L. This procedure does not take into account interference from endogenous fluorophores. Therefore, we assayed 20 urines from members of staff simultaneously with standard curves for benzoylecgonine, amphetamine, amobarbital, butobarbital, cyclobarbital, pentobarbital, and secobarbital. The mean polarization reading of the 20 normal urines was 173 mP (SD 3.14, CV 1.81%). The polarization value 3 SDs below this mean for each individual standard curve was taken as the detection limit for that drug. The detection limit for

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benzoylcegonine was 0.9 mg/L; for amphetamine, 0.8 mg/L; and for secobarbital, 1.0 mg/L. Because all had cutoff points close to 1 mg/L this was considered a convenient value to use as a cutoff for a positive result. For the PF1-20 the detection limit for benzoylcegonine was 1.0 mg/L; for amphetamine, 1.0 mg/L; and for secobarbital, 2.5 mg/L.

Because the assay is primarily a positive/negative screen, the important factor is the variation in polarization readings (3). We assayed 10 replicates of a normal urine. The mean polarization reading was 173.1 mP (SD 2.51, CV 1.45%). We then assayed a positive urine with a concentration close to the cutoff value. The mean polarization value was 153.3 mP (SD 0.82, CV 0.53%). When we repeated the calculation using concentration units, the mean value was 1.16 mg/L (SD 0.11, CV 9.3%).

Specificity. The cross-reactivity (9) of each individual antisera has been described previously: anti-secobarbital and anti-secobarbital (3); anti-amphetamine (4); anti-benzoylcegonine (5). We carried out repeat studies with the combination reagent and obtained similar results.

Correlation with other screening methods. We assayed 175 urine samples that had been screened by either thin-layer chromatography (barbiturates), gas chromatography (amphetamine), or 

Discussion

Initial screening for drug abuse requires a simple, inexpensive, qualitative assay. Attempts have been made to facilitate such screening by detecting several drugs in a single assay (1, 2). Earlier, we described (3–5) the use of fluorescence polarization to detect individual drugs, and we describe here the combination of these reagents. In the FPJA a mixture of four specific antisera and three fluoresce-in-labeled derivatives is used, and the assay is made possible by the rapid dissociation kinetics of tracer from antibody binding (3). All that is required is the addition of a small amount of urine to the single reagent, followed by a brief incubation and the measurement of fluorescence polarization.

The assay is easily automated (5) and capable of screening large numbers of samples. The fluorescence polarization signal obtained from the single reagent is an average of the individual signals, and the presence of any one of the abused drugs in the sample causes a decrease in this average signal.

The dilution of antiserum giving 80% binding for the poorest assay (for benzoylcegonine) was chosen and dilutions of the other antisera were selected to give a similar polarization value. Various concentrations of each label were tested and the concentration giving the best sensitivity in the combination reagent was chosen for assay development.

We used the full 30-min incubation to allow for equilibration. However, initial dissociation is rapid and a few minutes of incubation is satisfactory for all samples other than those containing drugs in concentrations near the cutoff point. The precision data quoted are for the full incubation period. When shorter incubation times are used, poorer precision may be expected. The assay was carried out at room temperature; should shorter incubation times be needed the incubation temperature can be increased somewhat, to partly compensate.

Human urine contains some natural fluorophores, and the interference they cause is the major factor limiting assay sensitivity. The assay was originally developed with the SLM instrument and a sensitivity of 1 mg/L was attainable with a fluorescent-label concentration of 10 nmol/L—a sensitivity we find satisfactory for our use, but which could be improved by decreasing the label concentration or by use of blank correction.

The SLM instrument is too expensive for routine laboratory use. The PF1-20 is in a more suitable price range, but does have the disadvantage of less sophisticated optics and wavelength selection. Using the PF1-20, we found that some normal urines gave false-positive results; these urines were found to contain high concentrations of riboflavin. Riboflavin is highly fluorescent with the maximum in a similar spectral region to fluorescein. Its fluorescence is almost completely quenched on binding to an equimolar concentration of riboflavin-binding protein (10). When riboflavin-binding protein (Sigma Chemical Co., Poole, Dorset, U.K.) was added to the single reagent to give a concentration of 9.7 mg/L, and the PF1-20 was used for the assay, assay performance was nearly as good as with the SLM.

The reason for the false-positive results with two urines containing high concentrations of phenothiazines and cyclizine is not clear. There is presumably some interference (possibly fluorescent) from their metabolites.

The reagent we used can detect the presence of cocaine, amphetamine, and any of the five most commonly abused barbiturates (11), but the combination reagent could be expanded to include a wider range of drugs—or specially tailored for choice. The single reagents have proved to be exceptionally stable. A single reagent for amphetamine has remained stable for more than two years at room temperature when stored in a brown glass bottle.

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References

Bence Jones Proteinuria in Multiple Sclerosis
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We report our findings of Bence Jones proteins (monoclonal free light chains of immunoglobulins) in concentrated urines of patients with multiple sclerosis, by using agarose electrophoresis and immunofixation. The lack of such findings in urines from healthy subjects and patients with other neurological disorders should stimulate further investigation.

Additional Keyphrases: immunofixation, electrophoresis

agarose gel

A characteristic, although not exclusive, laboratory finding in at least 90% of patients with clinically definite multiple sclerosis (MS) is the oligoclonal immunoglobulin pattern consisting of multiple homogeneous bands in the γ-globulin region on electrophoresis of cerebrospinal fluid (CSF) (1). The number and mobility of oligoclonal IgG bands appear to be specific for the individual MS patient and remain relatively constant throughout the duration of the disease (1-3). With the adoption of more-sensitive techniques, such as isoelectric focusing, most MS patients with CSF oligoclonal immunoglobulins exhibit similar oligoclonal bands in serum (3-6). However, the significance of the serum oligoclonal banding in MS remains to be established.

Little attention has been paid to the possible occurrence of immunoglobulin abnormalities in the urinary electrophoretic pattern of MS patients. We performed such an investigation and found homogeneous (monoclonal) free light chains of immunoglobulins, i.e., Bence Jones proteins, in some MS patients who had no evidence of any concurrent B-lymphoproliferative disease.

Materials and Methods

Serum, urine, and CSF samples, simultaneously collected from 10 consecutive unselected patients—two men and eight women, ages 16 to 42 years (mean 32 years)—with clinically definite MS were studied by using high-resolution agarose gel electrophoresis combined with immunofixation as previously described in detail (7). Monospecific antisera to heavy and light chains of immunoglobulins were from Dakopatts, Copenhagen, Denmark, and Behringwerke, Marburg, F.R.G. Before electrophoresis, all urine samples were concentrated up to 600-fold in collodion bags (Sartorius Membranfilter, Göttingen, F.R.G.) by extraction under reduced pressure (8). The same procedure was adopted to concentrate the CSF samples 80- to 100-fold.

Results and Discussion

No homogeneous immunoglobulin band was found in the serum samples from any of the 10 patients. The CSF oligoclonal pattern, consisting of three to nine discrete bands, was recognized in eight patients. The two patients without detectable CSF oligoclonal banding had had MS for more than five years, had received prior steroid therapy, and had no clinical evidence of disease activity in the preceding 12 months.

Homogeneous (single or multiple) bands reacting exclusively with one of the two light-chain-type antisera were observed in urines from three patients with newly diagnosed MS, who were in a phase of active disease and had not received prior therapy. The monoclonal free light chains were of κ type in two patients, and one patient had simultaneously bands of κ and λ type exhibiting different electrophoretic mobilities (Figure 1). The oligoclonal IgG bands in the CSF sample from this latter patient displayed light chains of κ as well as λ type, in contrast to all the other CSF samples, which contained IgG bands of κ type. The urinary excretion of monoclonal free light chains [calculated from the combined data on total protein content and the densitometric evaluation of the monoclonal bands (8)] ranged from 0.02 to 0.07 g/L.

Up to now we have used the described procedure (7) to investigate more than 1000 urine samples for the presence