Fluorescence Polarization Immunoassay Evaluated for Screening for Amphetamine and Methamphetamine in Urine

Yale H. Caplan, Barry Levine, and Bruce Goldberger

We studied the recently developed Abbott fluorescence polarization immunoassay (FPIA) for amphetamine and methamphetamine in urine and compared the results with those of the Syva enzyme-multiplied immunoassay technique (EMIT) and a gas-chromatographic assay. The FPIA method showed a limit of quantification of 0.3 mg/L, comparable with the lower cutoff of the EMIT assay. FPIA demonstrated greater specificity than the EMIT assay: phenylpropanolamine and ephedrine showed extremely limited cross reactivity with the FPIA antibody. Analysis of 249 urine specimens by all three methods clearly demonstrated the FPIA method to be acceptable for screening for amphetamine and methamphetamine in urine.

Additional Keyphrases: abused drugs • drug assay • enzyme immunoassay and gas-chromatographic assay compared

Amphetamine (β-phenylisopropylamine) was first synthesized as a pharmacological agent in 1887, methamphetamine in 1919. Many of the pharmacological effects of the drug, including bronchodilator, respiratory stimulant, and analeptic properties, were noted by Alles (1) in 1933. Large-scale abuse of amphetamines was first observed during the Second World War, when soldiers used the drug to combat fatigue. By the early 1960s, heroin addicts in the San Francisco Bay area were using amphetamines, and the abuse of these drugs continued in the United States throughout the 1960s. The development of tighter government regulation and the reduction in legally produced amphetamines led to declines in their abuse in the 1970s. Nevertheless, amphetamines are still produced in clandestine laboratories in the United States and made available to those intent on drug abuse (2).

The continued concern regarding amphetamine and methamphetamine use and abuse necessitates that the laboratory maintain the capability of testing for these drugs in biological fluids. Among analytical methods available, immunoassay represents the fastest and least complicated means for detecting amphetamines in urine. The Roche Abuscreen® RIA and the Syva EMIT® assays are widely used for such screening. Over the past few years, the TDx® analyzer, manufactured by Abbott Diagnostics, Inc., has become an integral part of many clinical toxicology laboratories. Based on fluorescence polarization immunoassay (FPIA), it can rapidly and accurately measure concentrations of therapeutic drugs being monitored by these laboratories. The FPIA technique has currently been applied to drug-abuse testing in urine. Here we report our evaluation of the TDx Abused Drug Assay for amphetamine by comparison with the Syva EMIT and a gas-chromatographic (GC) reference method.

Materials and Methods

Materials

Urine specimens to be analyzed for amphetamine and methamphetamine were obtained from two sources: (a) 100 random (unselected) urine specimens, provided by the Maryland Medical Laboratories, Inc. (MML specimens), and (b) 149 urine specimens containing amphetamine, methamphetamine, or amphetamine-like compounds, provided by Abbott Diagnostics, Inc. (Abbott specimens).

We prepared the following aqueous standards: d-amphetamine hydrochloride and methamphetamine hydrochloride, each 100 mg/L as free base; and mephentermine, 100 mg/L as free base, these drugs being obtained from SmithKline Beckman Co. (Philadelphia, PA 19101), Abbott Laboratories (Abbott Park, IL), and Wyeth Laboratories (Philadelphia, PA 19101), respectively. The mephentermine solution served as the internal standard.

Hydrochloric acid, sodium hydroxide, and potassium hydroxide were all reagent grade. Dichloromethane and methanol were pesticide-grade solvents.

Extrrelut QE columns for liquid and gas chromatography were obtained from EM Science, Cherry Hill, NJ 08034.

Procedures

Abbott TDx FPIA: The Abbott TDx was operated in accordance with the operator's manual. The specific variables used for the amphetamine assay were established by Abbott. The instrument was calibrated at the beginning of this evaluation with six calibrators (provided by Abbott) ranging from 0 to 3.0 mg/L. Thereafter, no other calibration was performed or necessary throughout the evaluation.

Syva EMIT assay: The EMIT-dau Amphetamine Assay and Low Calibrator A were purchased from Syva Co. (Palo Alto, CA 94304), and prepared as recommended. The reconstituted EMIT antibody/co-enzyme reagent A and enzyme reagent B were diluted 16-fold with EMIT drug-assay buffer. Once diluted, the reagents were stable for 72 h when refrigerated. Before analysis, the calibrator and subject samples were diluted and mixed in a 0.4-mL disposable beaker with use of a pipettor-dilutor (Fisher Scientific, Springfield, NJ 07081) set to deliver 100 μL of specimen with 250 μL of EMIT drug-assay buffer.

Negative calibrator was pipetted into rotor positions 2 and 3 of a Multistat III Microcentrifugal Loader (Instrumentation Laboratory, Lexington, MA 02173), and the positive calibrator was placed in positions 4, 5, and 20. Subject samples were pipetted into positions 6 through 19. Diluted antibody/coenzyme reagent A was placed into the "2nd Reagent" position and enzyme reagent B was placed into the "Reagent" position on the loader. The settings on the loader were as follows: sample volume 20, sample total volume 95, reagent volume 30, reagent total volume 30, and second reagent volume 95.

We placed the loaded rotor into the centrifugal analyzer for assay at 30 °C, in the absorption mode at 340 nm. The
EIA-DAU cassette program tape was edited to include a 10-s delay time and 105-s incubation time.

Any specimen giving a response greater than that obtained with the low calibrator was considered positive. All specimens that were positive by EMIT and negative by GC were re-analyzed with the EMIT confirmatory kit, which involves pretreating urine specimens to destroy many of the phenethylamines present in the specimen (3).

Gas-chromatographic analysis: For amphetamine and methamphetamine quantification, we used a Model 3920 gas chromatograph (Perkin-Elmer, Norwalk, CT) equipped with a nitrogen–phosphorus detector, a Sigma 1 data station, and a 2 m × 2 mm (i.d.) column packed with 10% Apiezon L with 20 g/L KOH (Supelco, Bellefonte, PA 16823). Helium was the carrier gas, flowing at 30 mL/min. The oven was operated isothermally at 140 °C; the injector temperature was 200 °C; and the detector temperature was 300 °C.

To 1 mL of standard or unknown urine specimen we added 1 mL of 1 mol/L NaOH and 50 μL of internal standard solution. After vortex-mixing, we applied the contents to an Extrelut QC column. After 5 min, each column was eluted with two 8-mL portions of dichloromethane into a 12-mL centrifuge tube containing two drops of 0.1 mol/L HCl in methanol. After evaporating dichloromethane extract under nitrogen at 45 °C, we reconstituted the residue with 100 μL of methanol and 3 μL plus 1 μL of 0.1 mol/L KOH in methanol (added directly to the syringe) and injected this into the GC. To quantify the unknown urine specimens for amphetamine and methamphetamine, we compared the GC results with those for drug-free urine specimens that had been fortified with known amounts of amphetamine and methamphetamine.

Precision Study

The precision of the FPIA assay was evaluated by analyzing each of three TDx controls five times per day for 10 days over a two-week period. The three controls (threshold (T), low (L), and high (H)) contained amphetamine at concentrations of 0.3, 0.6, and 2.0 mg/L, respectively.

Specificity Study

The antibody in the FPIA assay reacts equally to amphetamine and methamphetamine. To assess the ability of the FPIA immunoassay to react with compounds similar to amphetamine and methamphetamine we added aqueous standards of these compounds to drug-free urine, then analyzed the urine specimens by the FPIA and EMIT assays.

Analysis of Specimens

We analyzed the 100 MML specimens in duplicate by the FPIA and EMIT assays. Any urine specimen that was positive by at least one method was analyzed by GC. We analyzed the Abbott specimens in duplicate by the FPIA and EMIT assays and in singlicate by GC. A urine specimen was considered positive by GC if the total concentration of amphetamine and methamphetamine exceeded 0.3 mg/L.

Results

Precision Study

The within-coefficients of variation (CVs) for the three controls were T 5.2%, L 2.8%, and H 1.7%; the between-day CVs were: T 7.4%, L 4.9%, and H 4.9%. Because all within-day CVs were less than 6% and all between-day CVs were less than 8%, we consider the precision acceptable throughout the range of quantification.

Specificity Study

Table 1 lists a group of amphetamine-like drugs and their respective concentrations in urine, which we studied by FPIA to determine their cross reactivity with the FPIA amphetamine assay. Also included is the response of the EMIT assay, which we calibrated with the negative and low calibrators supplied by Syva. Only fenfluramine, phenmetrazine, phentermine, and phenethylamine appeared to show significant cross reactivity with the FPIA method. Phenylpropanolamine and ephedrine, two drugs found in a wide variety of over-the-counter preparations, did not cross react with the FPIA assay.

Random Urine Specimens

Of the 100 random urine specimens (MML specimens) screened for amphetamine by the FPIA and EMIT methods, none was positive by FPIA but four were positive by EMIT. Of the four that were positive by EMIT, GC analysis confirmed that none of the specimens contained amphetamine or methamphetamine. Therefore, there were four "false positives" by EMIT. Three of these specimens were subsequently found to be negative for amphetamine and methamphetamine when tested with the EMIT confirmatory procedure.

Abbott Urine Specimens

A total of 149 urine specimens were analyzed by the FPIA, EMIT, and GC methods. Ninety-six were positive and 53 were negative by GC for amphetamine and methamphetamine. However, most of the GC-negative specimens contained amphetamine-like compounds. Of these 53 specimens, 39 were negative by both immunoassay techniques, five were positive by FPIA and negative by EMIT, eight were positive by EMIT and negative by FPIA, and one was positive by both methods. However, if 0.4 mg/L (instead of 0.3 mg/L) were used as the cutoff for a positive result, only two of the six false positives generated by the FPIA technique would have been considered positive; that is, four of the six false positives would have been considered negative.

Table 1. Cross Reactivity of Amphetamine-like Compounds to the FPIA and EMIT Amphetamine Assays

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc.</th>
<th>TDx value</th>
<th>% Cross Reactivity EMIT</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ephedrine</td>
<td>10</td>
<td>low</td>
<td></td>
<td>pos.</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>low</td>
<td></td>
<td>pos.</td>
</tr>
<tr>
<td>Fenfluramine</td>
<td>10</td>
<td>1.4</td>
<td>14</td>
<td>pos.</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.8</td>
<td>6</td>
<td>pos.</td>
</tr>
<tr>
<td>Methylenedate</td>
<td>10</td>
<td>low</td>
<td></td>
<td>neg.</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>low</td>
<td></td>
<td>neg.</td>
</tr>
<tr>
<td>Phenmetrazine</td>
<td>10</td>
<td>low</td>
<td></td>
<td>neg.</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>low</td>
<td></td>
<td>neg.</td>
</tr>
<tr>
<td>Phenylpropanolamine</td>
<td>10</td>
<td>0.4</td>
<td>4</td>
<td>pos.</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.5</td>
<td>2</td>
<td>pos.</td>
</tr>
<tr>
<td>Phentermine</td>
<td>10</td>
<td>0.5</td>
<td>5</td>
<td>pos.</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.8</td>
<td>5</td>
<td>pos.</td>
</tr>
<tr>
<td>Phenethylamine</td>
<td>10</td>
<td>0.2</td>
<td>2</td>
<td>pos.</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.4</td>
<td>1</td>
<td>pos.</td>
</tr>
</tbody>
</table>

*Results are the average of two determinations. "Low" is defined as any response less than that obtained with the TDx A calibrator. *A positive response was any response greater than that observed with the EMIT low calibrator.
positives were near the cutoff concentration. Subsequent re-
analyses of the nine specimens positive by EMIT and negative
by GC were found to be negative by the EMIT confirmatory
technique.

The other 96 urine specimens contained amphetamine and/or methamphetamine in a total concentration >0.3 mg/L. The FPIA method produced a positive result each time, whereas the EMIT method gave a false negative one time—for an amphetamine concentration of 0.3 mg/L, which is the same concentration as the EMIT low calibrator.

For 35 urine specimens, amphetamine/methamphetamine values by GC were between 0.3 and 2.9 mg/L. Of these the FPIA values agreed with the GC values within 25% in 29 cases, but differed by more than 25% in six cases. GC values were >3.0 mg/L 61 times, while the FPIA method reported values >3.0 mg/L 59 times. Because the FPIA assay does not report values greater than 3.0 mg/L, no other quantitative comparisons could be made. The fact that FPIA values were observed equal to or greater than GC values was not unexpected because untreated urine specimens may contain various quantities of minor metabolites with unknown cross reactivity to the less-specific immunomassay method. In contrast, the GC method, which involves sample extraction and chromatographic separation, provides more specific quantification.

Discussion

Ordinarily, drugs are rarely quantified in urine because of the difficulties in interpreting the values obtained. However, in assessing the validity of an assay, quantification is required. Generally, an evaluation of the sensitivity, specificity, and precision of a method necessitates comparing these parameters with those of more established methods. Abbott arbitrarily sets the FPIA limit of quantification at 0.3 mg/L, the same limit of quantification as in the EMIT assay, even though the FPIA limit of detection is 0.08 mg/L. By altering the threshold parameter, this limit of quantification may be increased, but not decreased. Ideally, the cutoff should be the lowest drug concentration that can be clearly distinguishable from a zero drug concentration, but this must be independently established by each laboratory.

The breakdown of concentrations measured by the FPIA method (Table 2) suggests that the 0.3 mg/L cutoff recommended by Abbott is quite reasonable. By the 0.3 mg/L cutoff, 100% of the MML urine specimens and 87% of the Abbott urine specimens that were negative by GC were also negative by FPIA. The MML urine specimens were chosen at random, whereas the Abbott urine specimens had been specifically identified to contain amphetamines and methamphetamine-like compounds. This accounts for the fact that the proportion of drug-negative urines among the Abbott specimens was much less than that for the MML specimens.

We found the FPIA method less cross reactive to amphetamine-like compounds than was the routine EMIT method, especially with regard to phenylpropanolamine and ephedrine. Unlike serum assays, where low cross reactivity is relatively insignificant, urine-screening assays having comparable cross reactivities may lead to erroneous conclusions, given the possibility of much higher drug concentrations in urine than in serum. For example, in this study, we detected amphetamine concentrations of approximately 30 mg/L in some urine specimens. An amphetamine-like compound present at that concentration and 1% cross reactive with the amphetamine assay would give an apparent amphetamine concentration of 0.3 mg/L in the assay, producing a false-positive test result. Also, a urine specimen containing phentermine (an appetite suppressant subject to abuse by anorectics) at 10 mg/L would show an apparent amphetamine concentration of 0.5 mg/L by FPIA. Phentermine is metabolized to a lesser extent than amphetamine (4), so that higher concentrations of the parent drug in urine would be expected.

The advantages realized in using the TDx assays for therapeutic drug monitoring also apply to drug-abuse screening with urine samples. Only 50 μL of urine is required in the sample well, 6 μL is used for the analysis; no sample pretreatment or temperature equilibration is required; urine samples can be taken directly from the refrigerator to the instrument; a batch of 20 samples and controls may be prepared and analyzed within 25 min; and the instrument need not be recalibrated each time the assay is used. We performed these experiments over a six-week period; after the initial calibration, no re-calibrations were performed or required. One difference between the therapeutic drug monitoring methods and the urine screening methods is that, in the former, samples with concentrations exceeding that of the highest calibrator can be re-analyzed by using a decreased sample volume. However, this "dilution protocol" cannot be implemented automatically by the instrument for a urine screening method; if necessary, a manual dilution may be performed.

Because the TDx is not calibrated with each batch, the precision of the method is quite important, especially at the threshold concentration, which is used to identify positive specimens. Precision (CV) at the threshold concentration of 0.3 mg/L was 7.4%, and was similar at higher amphetamine concentrations also: 4.9% at 0.6 and 2.0 mg/L.

We conclude that the Abbott Abused Drug amphetamine assay is an acceptable method for screening urine for the presence of amphetamine and methamphetamine. The precision and sensitivity of the FPIA assay are highly acceptable, and its specificity exceeds that of the EMIT amphetamine assay. Despite this increased specificity, we emphasize the absolute requirement to confirm all positive results by an alternative technique based on a different analytical principle.

References