Differentiation of Amphetamine/Methamphetamine and Other Cross-Immunoreactive Sympathomimetic Amines in Urine Samples by Serial Dilution Testing, Allison Woodworth, Al N. Saunders, John W. Koenig, Thomas P. Moyer, John Turk, and Dennis J. Dietzen (Departments of Pediatrics and Pathology and Immunology, Washington University School of Medicine, St. Louis, MO; Drug Analysis Laboratory, Barnes-Jewish Hospital, St. Louis, MO; Department of Laboratory Medicine and Pathology, Mayo Clinic and Mayo Foundation, Rochester, MN; address correspondence to this author at: Department of Pediatrics, Box 8208, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110; fax 314-286-2892, e-mail Dietzen_D@kids.wustl.edu)

Background: Immunoassay-based screening for amphetamines has a variable positive predictive value (PPV) for detecting amphetamine abuse. The lack of immunoassay specificity necessitates confirmatory testing by gas chromatography–mass spectrometry (GC/MS), but the technical complexity and expense of GC/MS limit its availability. Physicians may make decisions regarding patient disposition based on unverified results. In this study we assessed the utility of using dose–response properties to distinguish urine samples containing amphetamines from samples containing cross-immunoreactive species.

Methods: Urine was supplemented with known concentrations of amphetamine, methamphetamine, methylendioxymethamphetamine (MDMA), or pseudoephedrine. Using a series of dilutions, we determined the maximum change in rate over the fractional change in concentration for each compound in the Emit II amphetamine/methamphetamine immunoassay. Patient urine samples that screened positive for amphetamines were diluted 1:1, 1:10, and 1:20, and maximum slope estimates within the dynamic assay range were determined. An optimal slope cutoff that differentiated samples containing (meth)amphetamine from those containing cross-reacting species was determined by ROC analysis.

Results: The slope of the dose response was largest for amphetamine and methamphetamine, followed by MDMA and pseudoephedrine. The optimum slope cutoff for identifying patient specimens containing (meth)amphetamine was 320 (sensitivity, 96%; specificity, 90%; PPV, 92%). High concentrations of less reactive compounds may mask low concentrations of amphetamines.

Conclusions: Use of the slope of the dose–response relationship in patient urine specimens can enhance the PPV of presumptive positive immunoassay results but does not exclude the presence of low amphetamine concentrations in samples containing high concentrations of cross-reactive species.

Amphetamines and their analogs are a family of noncatecholamine stimulants that are among the most commonly abused drugs in the United States (1). Urine drug screening for amphetamines is performed in both clinical and workplace settings. The positive predictive value (PPV) of amphetamine immunoassays to detect amphetamine abuse varies from 0% (2) to 90% (3) because these assays recognize a wide variety of sympathomimetic amines (4–7) (see Table 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol52/issue4). Positive immunoassay results should, therefore, be verified by alternative methods such as gas chromatography–mass spectrometry (GC/MS). The technical complexity and expense of GC/MS analysis limit its availability in many clinical laboratories, however. Physicians thus must often make decisions regarding patient disposition based on unverified screening immunoassay results.

Amphetamine immunoassays display precise dose–response properties from concentrations below the Substance Abuse and Mental Health Services Administration (SAMHSA)-stipulated cutoff to concentrations well above cutoff (8). Within this dynamic range, amphetamine and methamphetamine may exhibit dose–response properties that are distinguishable from other cross-immunoreactive compounds. The AACC has encouraged the US Food and Drug Administration to require reagent manufacturers to specify drug specific dose responses without knowledge of their utility (www.aacc.org/govt/2004/doa_comments.doc). This AACC document states that: “the dilution characteristics of each drug in terms of signal linearity when diluted with negative urine . . . will allow the user to differentiate between a member of the class and other potential cross-reactants without the need for a more specific procedure such as GC/MS.” The aim of this study was to assess the utility of using dose–response properties to distinguish urine samples containing amphetamine and/or methamphetamine from samples containing cross-immunoreactive species.

All analyses were performed with the Syva Emit II PLUS monoclonal amphetamine/methamphetamine assay (Dade-Behring). The cutoff concentration for a positive result at the Barnes-Jewish Drug Analysis Laboratory was 300 μg/L d-methamphetamine. The imprecision of the instrument signal was 3.3% for a control containing 0 μg/L methamphetamine (mean response = 831 mA/min) and 1.4% for a control containing 360 μg/L methamphetamine (mean response = 1078 mA/min). Both controls were run daily over the course of the study and spanned 2 lots of Emit amphetamine reagent. We first generated urine pools containing saturating amounts of highly reactive species (amphetamine and methamphetamine), a moderately reactive species [methylendioxymethamphetamine (MDMA)], and a weakly reactive species (pseudoephedrine). Urine pools containing 10 mg/L d-amphetamine sulfate (Sigma), 10 mg/L D-methamphetamine-HCl (Alltech), 100 mg/L MDMA (Alltech), or 5000 mg/L L-pseudoephedrine (Sigma) were analyzed undiluted and after serial dilution to 80%, 60%, 40%, 20%, 10%, 6%, and 4%.
5%, and 2.5% of the original concentration. Within the dynamic range of the immunoassay (850–1400 mA/min), differences in the dose–response relationship between amphetamine, methamphetamine, MDMA, and pseudoephedrine were readily apparent (Fig. 1A). These differences were quantitatively expressed by determining the maximum change in signal between 2 consecutive dilutions divided by the fractional change in concentration (maximum incremental slope). Across reagent lots, the mean (SD) of maximum incremental slopes were 451 (24) (n = 6), 399 (28) (n = 6), 263 (16) (n = 4), and 202 (7) (n = 4) for methamphetamine, amphetamine, MDMA, and pseudoephedrine, respectively (Fig. 1A, right). The results for methamphetamine and amphetamine were clearly distinct from those for MDMA and pseudoephedrine.

We next analyzed 42 patient urine specimens containing amphetamine, methamphetamine, and a variety of other cross-immunoreactive substances. Samples from physician-ordered drug tests that screened near or above cutoff concentrations for amphetamines were obtained from the Barnes-Jewish Hospital Drug Analysis Laboratory (St. Louis, MO), Christian Hospital Northeast (St. Louis, MO), or Mayo Medical Laboratories (Rochester, MN). The substances responsible for the presumptive positive immunoassay results were identified by various methods. Amphetamine, methamphetamine, and pseudoephedrine were detected by thin-layer chromatography (Toxilab) or GC/MS as described by Luzzi et al. (8) and Poletinni et al. (9). MDMA was identified by GC with nitrogen-phosphorus detection (Barnes-Jewish Hospital Drug Analysis Laboratory), and phentermine, chlorpheniramine, and phenylpropanolamine were identified by GC/MS (Mayo Medical Laboratories).

For immunoassay analysis, urine samples were analyzed undiluted and after 1:1, 1:10, and 1:20 serial dilution. In general, this combination of dilutions generated at least 2 points within the dynamic range of the assay regardless of the starting signal. However, in some samples containing very high concentrations of amphetamines, only a single dilution was contained in the dynamic assay range. To prevent underestimation of the slope in these circumstances, we used the change in response between the lowest dilution and drug-free urine to calculate the maximum incremental slope. Maximal incremental slopes for the patient samples with amphetamine and methamphetamine ranged from 300 to 1000. Samples containing only the least reactive sympathomimetic amines exhibited maximum incremental slopes <200. Samples containing moderately reactive species such as phentermine and MDMA exhibited maximum incremental slopes of 300–400 (Fig. 1B). Optimal cutoffs for identifying specimens containing (meth)amphetamine were derived from ROC analysis (see Fig. 1 in the online Data Supplement). A cutoff of 300 yielded 100% sensitivity and 85% specificity. At a cutoff of 450, sensitivity and specificity were 67% and 100%, respectively. Optimum sensitivity (96%) and specificity (90%) were achieved at a cutoff of 320. The area under the curve was 0.975. These data were consistent across 2 consecutive reagent lots, but these cutoffs may vary with subsequent reagent lots.

As observed in our patient data set, amphetamines are often present in combination with other cross-reactive substances. The presence of weakly reactive substances has the potential to lower the determined slope and produce false negatives. We therefore prepared a series of 500 μg/L methamphetamine calibrators containing increasing concentrations of pseudoephedrine and analyzed these undiluted and after dilution. This presents a worst-case situation in which methamphetamine concentrations are slightly above cutoff and a very weakly reactive substance is present in large concentrations. The maximum incremental slopes of the methamphetamine samples were reduced in a dose-dependent manner by increasing pseudoephedrine concentrations (see Fig. 2 in the online Data Supplement), but even in the presence of 2500 mg/L pseudoephedrine (an 8000-fold excess over methamphetamine), the maximum slope remained >200, distinct from those specimens containing only pseudoephedrine.

We observed considerable variability in slope estimates in different patient samples containing the same compound. This variability results not only from the presence of weakly reactive substances but also from the limited dilution scheme we used. More precise identification of the constituents of the urine sample may be possible with schemes that use a greater number of dilutions or dilutions that are tailored based on the initial assay response. The present scheme permits the best possible slope estimates with the fewest dilutions. Excessive numbers of dilutions are impractical for routine clinical use and would not prevent underestimation of slope caused by the presence of weakly cross-reactive substances (identified or unidentified) in the sample.

Initial clinical toxicology results obtained by immunoassay are reported as "presumptive". This study was carried out to determine whether the use of sample dose–response characteristics could strengthen this presumption. As far as we know, this study is the first of its kind to examine the utility of such an approach. We chose to first investigate the properties of amphetamine screening assays because the target compounds are few and many of the potential cross-reactants are well defined. The technique displayed considerable power to identify specimens containing (meth)amphetamine. In our data set, the PPV of the immunoassay to detect amphetamine abuse without the dilution protocol was 57%. Use of the dilution protocol increased the PPV to 92%. Our dilution approach cannot, however, guarantee a 100% negative predictive value. High concentrations of less reactive substances may reduce the dose–response slope in rare specimens and mask the presence of low amphetamine concentrations.

Widespread use of this sample dose–response approach in other commercial amphetamine immunoassays poses some obstacles. Antibody cross-reactivity is variable; therefore, slope cutoffs will be assay specific. Manufacturers would likely have to define acceptable cutoffs for
Fig. 1. Dose–response characteristics of amphetamine analogs, sympathomimetic amines, and patient urine samples on the Emit II Plus Monoclonal AMP/MAM Assay.

(A, left), drug-free urine was supplemented with amphetamine (●), methamphetamine (○), MDMA (■), and pseudoephedrine (♦) and diluted to the indicated concentrations. Data points represent the means of 2–4 determinations with a single lot of reagent. The cutoff for a positive test was set to 1000 mA/min. The top x axis is for methamphetamine and amphetamine (0 to 10 mg/L); the bottom x axes are for MDMA (0 to 100 mg/L) and pseudoephedrine (0 to 5000 mg/L). (Right), maximum incremental slopes for individual dose–response curves were determined over a span of 10 months with 2 different reagent lots. Individual slope values for amphetamine (n = 6), methamphetamine (n = 6), MDMA (n = 4), and pseudoephedrine (n = 4) calibrators are displayed. The mean values of the slopes for amphetamine vs methamphetamine, methamphetamine vs MDMA, and MDMA vs pseudoephedrine were all statistically different as assessed by two-tailed, unpaired t-test (P < 0.01). (B), patient samples containing (meth)amphetamine and/or other sympathomimetic amines were analyzed by immunoassay undiluted and after 1:1, 1:10, and 1:20 dilution with drug-free urine. Maximum incremental slope was determined for each sample and plotted on the left. Constituents of the urine samples determined at Barnes-Jewish Hospital or Mayo Medical Laboratories are indicated on the right.
laboratories without access to drug standards on a lot-specific basis. The utility of this approach in immunoassays for other drugs of abuse (e.g., opiate, benzylecgonine, cannabinoids, and phencyclidine) will depend on the selectivity of the antibodies, the degree to which similar cross-reacting drugs are present in urine specimens, and the degree to which unchanged parent drug is excreted in the urine. Extensive conversion of parent compound to less reactive metabolites will decrease the ability of sample dose–response characteristics to distinguish highly reactive from less reactive substances in urine.

We have shown here that sample dose–response characteristics can distinguish highly reactive amphetamines from moderately reactive compounds (e.g., MDMA) and weakly reactive compounds (e.g., pseudoephedrine) in urine samples that contain a single species of drug. Clinical urine specimens, however, contain mixtures of these compounds; consequently, slope values reflect the relative concentrations of cross-reacting compounds and their relative affinity for antibody. The value of the approach described here is to increase the PPV of the initial presumptive result in a time frame more rapid than is typically available when GC/MS is used. In a pediatric emergency setting, this technique may quickly indicate that a urine specimen contains illicit amphetamines rather than an over-the-counter cold remedy, preventing discharge back into an abusive environment before the results of GC/MS analysis are completed. It must be emphasized, however, that this dilution protocol is not intended to replace the need for confirmation of immunoassay results by a more definitive method such as GC/MS, particularly in workplace or forensic settings.

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References


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