Amphetamines in Hair by Enzyme-Linked Immunosorbent Assay*

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Abstract

Human hair was collected from the occipital crown region of the head from several subjects; these hair samples were presumptively positive for amphetamines by a previously evaluated immunosassay. Hair was washed briefly with methanol to remove external contamination, then extracted with hot methanol for 2 h to recover the drugs. The extracts were evaporated to dryness, reconstituted in buffer, and analyzed using a new enzyme-linked immunosorbent assay (ELISA) technique adapted for the detection of amphetamines in hair. Gas chromatography–mass spectrometry was used as the reference technique. Cross-reactivity of several related compounds was evaluated by equating the inverse of the ligand concentration at 50% antibody binding to the affinity constant for each compound. The ratio of a compound's affinity constant to that for d-methamphetamine was used to derive percent crossreactivity. These experiments yielded values of 30.8% for d-amphetamine, 7.4% for l-methamphetamine, 4.3% for phentermine, 2.9% for l-amphetamine, and <1% for ephedrine, methylenedioxyamphetamine, and methylenedioxymethamphetamine. Cross-reactivity of unrelated compounds was found to be non-existent. The optimum cutoff concentration was determined by receiver operating characteristic curve analysis to be 300 pg/mg and the observed limit of detection was 60 pg/mg. Intra-assay precision at 300 pg/mg was 3.3% (coefficient of variation, CV), and the interassay CV was 10.5%. The sensitivity and specificity of the method were 83% and 92%, respectively.

Introduction

The use of hair to test for exposure to drugs of abuse has quickly gained the attention of employers and government agencies around the world. Much of this popularity is due to the several advantages hair has over more conventional sample types such as urine. Briefly, these advantages are the following: hair collection can be done in a simple and non-invasive manner, thus eliminating many invasion-of-privacy objections to urine collections, particularly those that are observed; hair samples can document a longer time period of drug exposure and identify current users; hair may be more difficult to adulterate and to "beat" than urine; and, lastly, drugs in hair seem to be remarkably stable, allowing for retesting and even recollection to produce equivalent results to a prior test. Drugs and metabolites become incorporated into human hair via the bloodstream, sweat, sebum, or external exposure and can remain intact in the hair for several months (1-3). A positive drug analysis from a portion of hair proximal to the scalp is thought to reflect a window of drug detection of several weeks (4-6). A remarkable example of the long-term stability of drugs in hair is the finding of the cocaine metabolite benzoylecgonine in prehistoric Chilean mummies approximately 2000 years old (7).

Among the difficulties with detection of drugs in a hair sample, however, is the need for exceptional sensitivity to detect extremely low levels, typically in the picogram-per-milligram range. Additionally, there is the need for an efficient screening method that can cope with large numbers of samples. It is even more desirable that the technique lend itself to automation. Automated enzyme-linked immunosorbent assays (ELISAs) fill these requirements very well.

The Diagnostix Single Step™ ELISA is a solid-phase microtiter plate immunoassay originally designed to detect drugs of abuse in blood, serum, and urine. The test is performed in microwells coated with a high affinity capture antibody to the desired drug (antigen or analyte). A sample is added to the well, followed by the enzyme conjugate. During this initial phase, the enzyme conjugate competes with the analyte in the sample for binding sites on the antibody-coated microwells. A wash solution is then applied to remove any unbound materials such as excess conjugate and residual sample. Enzyme substrate is then added for the final color-development process. Color intensity is inversely proportional to the amount of analyte present in the sample. Therefore, samples that contain
drug or analyte will inhibit binding of the enzyme conjugate to the antibody, resulting in little substrate binding and less color development than in the negative calibrator.

In this study, we describe the application of an ELISA technique developed for amphetamines to the analysis of hair samples for employment screening purposes.

Methods

Materials

A Single Step ELISA kit for amphetamines from Diagnostix Inc. (Mississauga, ON, Canada) was used throughout the study and contained the following elements: a 96-well antibody coated plate, enzyme conjugated to horseradish peroxidase (HRP), enzyme diluent, wash solution (Tween-20 in phosphate buffered saline solution) and substrate solution containing 3,3',5,5'-tetramethylbenzidine (TMB) (8). Methanol (high-performance liquid chromatographic [HPLC] grade, Baxter Scientific, McGaw Park, IL) and ethyl acetate (HPLC grade, Burdick & Jackson, Muskegon, MI) were purchased commercially, stored at ambient temperature, and used as received. Phosphate buffer (K2HPO4, 46mM, pH 7.0) and stopping reagent (3N sulfuric acid) were prepared in the laboratory.

The stock drug standards for methamphetamine were purchased as 1-mg/mL methanolic solutions from Radian (Austin, TX) and Alltech (State College, PA) for preparing calibrators and controls, respectively. The internal standards used for mass spectral confirmation of ELISA-positive results were amphetamine-d5 and methamphetamine-d5 (Radian, Austin, TX). Drugs used in the interference studies were purchased from Sigma (St. Louis, MO) or eluted from TOXI.DISCS | provided in the TOXI.DISCS Library II (Ansys, Irvine, CA). The derivatizing agent used for gas chromatographic–mass spectrometric (GC–MS) confirmation of methamphetamine and amphetamine was N-methyl-bis-trifluoroacetamide (MBTFA, Pierce, Rockford, IL).

Equipment

A Mark V automatic pipettor from Diagnostic Products Corp. (Los Angeles, CA) was used to pipet all samples, calibrators, and controls into the microtiter plate. Following the completion of the assay, the plates were read using a Syva Microtrak® ELA autoreader from Syva Corp. (Palo Alto, CA) with available detection wavelengths of 450 and 630 nm. Microplates were washed with a “Wellwash 4” automatic ELISA plate washer from Denley Instruments, Ltd. (London, England). A TekTator V serological rotator from TekPro/American Hospital Supply Corp. (Evanston, IL) was used to facilitate the reaction process.

Instrumentation

All hair sample extracts screened by ELISA were analyzed on a Hewlett-Packard (Palo Alto, CA) 5890 GC interfaced with a Hewlett-Packard 5972 mass selective detector equipped with a 7673A automatic liquid sampler and a RTX-5 capillary column (30 m x 0.25-mm i.d., 0.25-μm film thickness, Restek Co., Bellefonte, PA).

Hair samples

Hair was collected from the occipital crown of donors, snipping as closely to the scalp as possible. Eighty-four hair specimens ranging in weight from approximately 80 to 120 mg were collected and placed in aluminum foil carriers, oriented to identify the root end. These were then placed in collection envelopes with tamper-evident seals and transported to the laboratory.

Samples were chosen for inclusion in this study based on positive results obtained from a previously evaluated screening assay that proved to lack specificity. Of these 84 samples, only 43% were positive by GC–MS confirmation, resulting in a 57% false-positive rate. Because of this inappropriately high number of false positives, it was determined that this would be an excellent sample group with which to validate the new ELISA technique.

Preparation for analysis

Samples were removed from the shipping envelopes one at a time, and the identity and integrity of the sample was verified. A 3.9-cm (~1.5 in.) segment of hair, which was assumed to approximate a growth period of about 90 days (9), was clipped from the root end. This portion was separated and used for subsequent analysis. The portion to be tested was subdivided by repeated cutting into segments between 2 and 5 mm in length. These segments were then mixed to ensure homogeneity and used for immunochromatographic analysis.

A 20-mg portion of each subdivided and mixed hair sample was weighed out and placed in a labeled 16 × 100-mm disposable borosilicate glass culture tube that was placed in a test tube rack for screening. For decontamination, 2.0-mL portions of methanol were added to each tube, and the tubes were allowed to incubate for 10 min at ambient temperature, swirling occasionally. At the end of the period, the tubes were swirled once more and the solvent decanted. In addition to removal of frank drug contamination on the external surfaces of the hair samples, the treatment was adequate to remove residues of gels and shampoos. To conduct the extraction, a new 2.0-mL portion of methanol was added to each washed hair sample, and the tubes were heated for 2 h at 70–75°C. At the conclusion of the incubation period, the tubes were cooled to ambient temperature, and the methanol layers were transferred to clean, labeled 12 × 75-mm glass culture tubes. The extracts were evaporated just to dryness under a stream of dry nitrogen in a heating block at 37°C. Once dried, the residues were reconstituted with 600 μL of pH 7.0 phosphate buffer, vortex mixed, and submitted for immunological testing.

Hair specimens that had previously tested negative by immunassay were pooled, and the described washing and incubation procedure was repeated. Before use in the study, the pooled negative hair was screened for amphetamines and four other drug groups by immunoassay. If the results were again negative, the negative hair matrix was certified for use.

Immunassay

Each ELISA run contained negative controls, low controls (200 pg/mg hair), high controls (400 pg/mg hair), and cutoff calibrators (300 pg/mg hair). A set of each was placed at the
washed hair samples in the same manner as previously were inversely proportional to the quantity of amphetamine was read on the plate reader at 450 nm, using a wavelength of injection port temperature, 240~ interface o.e.

was then injected into the GC-MS instrument. 1.o were then cooled to ambient temperature and diluted with 50 pL of this period, the reaction was stopped by the addition of 100 MBTFA by heating at 90~ for 15 rain. The derivatized extracts were prepared by spiking into certified-negative hair matrix m/z with the following mass ions being monitored:

cubated at ambient temperature for I2 min. At the conclusion of this period, the reaction was stopped by the addition of 100 pL of 3.0N sulfuric acid. The resulting yellow chromophore substrate reagent was added to each well, and the plate was incubated at ambient temperature for 12 min.

At the conclusion of this period, the reaction was stopped by the addition of 100 pL of 3.0N sulfuric acid. The resulting yellow chromophore was read on the plate reader at 450 nm, using a wavelength of 630 nm for background correction. Final absorbance readings were inversely proportional to the quantity of amphetamine-like compound present in the original samples.

**GC-MS**

Methamphetamine and amphetamine were extracted from washed hair samples in the same manner as previously described for screening, with the exception of the inclusion of internal standards methamphetamine-d_4 and amphetamine-d_3 at 1000 pg/mg. Calibration samples contained 1000 pg/mg, and controls in negative hair matrix were prepared at 0, 300, and 1200 pg/mg. Samples were extracted manually on solid-phase extraction columns (Clean Screen® extraction columns, 130 mg/3 mL, World Wide Monitoring/United Chemical Technologies, Inc., Bristol, PA) using an extraction procedure previously described for urine samples (10) and derivatized with 50 ~L of MBTFA by heating at 90°C for 15 min. The derivatized extracts were then cooled to ambient temperature and diluted with 50 pL of ethyl acetate. One microliter of the extract was then injected into the GC-MS instrument.

The GC–MS parameters were as follows: splitless injection with a purge time of 0.4 min; injection port temperature, 240°C; interface (auxiliary) temperature, 300°C; initial temperature 90°C ramped to 120°C at 30°C/min, then ramped to 150°C at 5°C/min, and then ramped to 250°C at 35°C/min for a final hold time of 1 min. Selective ion monitoring (SIM) was used with the following mass ions being monitored: m/z 154, 118, and 110 for methamphetamine-trifluoroacetyl (TFA); m/z 158 and 113 for methamphetamine-d_7-TFA; m/z 140, 118, and 91 for amphetamine-TFA; and m/z 144 and 92 for amphetamine-d_2-TFA. Quantitation was accomplished by calculating the area of mass ion ratios (bold ions) of analyte to the respective internal standard using a single-point calibration at 1000 pg/mg. The limit of quantitation (LOQ) for both methamphetamine and amphetamine was 50 pg/mg.

**LOD and assay precision for ELISA**

The limit of detection (LOD) was defined using the signal-to-noise ratio of the negative drug calibrator. Seventy-two negative controls in hair matrix were run, and the mean and standard deviation (SD) of the absorbance were calculated. The LOD was determined by calculating the mean negative calibration absorbance (A_0) minus three times the SD (LOD = A_0 − 3SD).

Intra-assay precision of the absorbance was determined by running a series of spiked hair samples at concentrations of 0, 200, 300, and 400 pg/mg (n = 24 each) throughout the plate for 3 consecutive days and determining the final mean coefficient of variation (CV) (%) for each concentration. Interassay precision of the absorbance was determined by calculating the mean CV (%) for each of these concentrations for 24 consecutive days.

**Receiver operating characteristic (ROC) analysis**

Assay sensitivity and specificity were used to create an ROC graph (Figure 1) (11–13). In this graph, sensitivity was plotted versus 1 – specificity for several possible cutoff values for methamphetamine. The optimum cutoff for the assay was determined by selecting the concentration in the plot closest to the upper left corner of the graph. Qualitatively, the closer the plot to the upper left corner, the higher the overall accuracy of the test (11). To compute the sensitivity and specificity, the number of true positives (TP), false negatives (FN), false positives (FP), and true negatives (TN) was determined for eight prospective cutoff concentrations (100, 150, 200, 250, 300, 350, 400, and 450 pg/mg methamphetamine equivalents) by comparison of the immunoassay result for each of the 84 samples to the corresponding GC–MS result (Figure 2). A true positive was defined as a sample yielding positive results for both immunoassay and GC–MS. A true-negative sample was defined as a sample testing negative for both assays. A sample for which the immunoassay was positive (absorbance below the cutoff and assay precision for ELISA) and the GC–MS result was negative was
defined as a false positive. Samples for which the immunoassay result was negative (absorbance above the mean of the cutoff calibrator) and the GC–MS result positive was defined as a false negative. Sensitivity and specificity were calculated conventionally as follows (12,14):

\[
\text{Sensitivity} = \frac{TP}{TP + FN}, \quad \text{Specificity} = \frac{TN}{TN + FP}
\]

Once these values were determined for each of the eight presumed cutoff values, they were plotted on the ROC graph to obtain an ROC curve (Figure 1). After the optimum cutoff was determined from inspection of the ROC curve, both the positive and negative predictive values (in relation to the chosen cutoff) were calculated from the following formulae (12,14):

\[
\text{PPV} = \frac{TP}{TP + FN} \cdot \frac{\text{Prevalence}}{1 - \text{Prevalence}}
\]

\[
\text{NPV} = \frac{TN}{TN + FP} \cdot \frac{1 - \text{Prevalence}}{\text{Prevalence}}
\]

An arbitrary value of 50% was chosen for "prevalence" in this equation in order to compare with the PPV and NPV of other immunoassays reported in the literature.

The efficiency of an assay was defined by Spiehler et al. (13) as "the proportion (percent) of specimens correctly classified as containing or not containing the analyte of interest". Efficiency was calculated using the following formula:

\[
\text{Efficiency} = \frac{TP + TN}{TP + TN + FP + FN}
\]

Cross-reactivity

In order to compare cross-reactivity data between drugs, antibody-binding data were collected for the various compounds of interest. These were initially classified into two groups: (A) those which were considered amphetamine analogues and (B) other common drugs without obvious similarity to amphetamines. Studies for each amphetamine analogue employed a series of binder concentrations intended to bracket the concentration which would produce 50% saturation of the antibody. Methanolic standards of the amphetamine analogues were evaporated to dryness, reconstituted in buffer, and serially diluted to provide the seven concentrations (5000, 2500, 1250, 625, 312, 156, and 78 ng/mL) tested initially in the cross-reactivity study. It was necessary to normalize these data and express them in a consistent format. This was done in two stages: first, each data point was normalized to the blank reading (zero drug) and second, data for the strong binder d-methamphetamine was extrapolated to infinite binder concentration using a double reciprocal plot of inverse absorbance versus inverse binder concentration to determine the absorbance endpoint at maximal saturation of antibody sites. Once this was done, a value for fractional saturation (f) of antibody-binding sites was calculated for each binder concentration (whether of d-methamphetamine or another drug). Fractional saturation was expressed as a number between 0 and 1 (or, when multiplied by 100, as a percentage, as in Figure 3).

If a compound binds tightly enough in a particular concentration range to substantially saturate the antibody binding site, the linear portion of the curve will cross the 50% saturation line (i.e., f = 0.5). The binder concentration corresponding to this point can then be read off the concentration axis and, when converted to molarity, is proportional to the inverse of the affinity constant or equilibrium constant for binding (15).
The percent cross-reactivity of the structural analogues of the amphetamines was expressed as the ratio of binding constant for the analogue to that for \(d\)-methamphetamine, multiplied by 100\% (Table I). If a compound's binding isotherm did not cross the 50% saturation line, a 100-fold greater concentration of the binder was used. If the binding isotherm from this experiment still did not cross the 50% saturation line, the cross-reactivity of the compound was assigned a value of <1%.

To evaluate the assay's cross-reactivity for a series of structurally unrelated drugs, the following protocol was employed: compounds were extracted from TOXI-DISCs provided in the TOXI-DISCs Library II (Ansys, Irvine, CA) to a final concentration of 10 pg/mL and analyzed with the ELISA assay. If a compound yielded a negative ELISA result, it was determined to have no cross-reactivity with the kit.

### Results and Discussion

#### Analytical precision and LOD of ELISA

The LOD of the ELISA assay was 60 pg/mg. The intra-assay precision of calibrator absorbances averaged over 3 days (\(n = 24\) each day) was 3.8\% at 0 pg/mg, 2.9\% at 200 pg/mg, 3.3\% at 300 pg/mg, and 3.5\% at 400 pg/mg. The interassay precision of the absorbance over 24 days was 9.6\% at 0 pg/mg, 10.0\% at 200 pg/mg, 10.5\% at 300 pg/mg, and 10.8\% at 400 pg/mg.

#### Diagnostic accuracy

The aim of this study was to modify and validate an immunoassay for the detection of amphetamines in human hair. Unlike more conventional sample matrices, such as blood or urine, no traditional or regulatory cutoff levels exist. Therefore, cutoff levels were based on the assay's diagnostic sensitivity and specificity as recommended by Zweig and Campbell (11).

The optimum cutoff for the assay was calculated by ROC analysis (11–13). This treatment required determination of the assay's sensitivity and specificity as defined in Methods. Using data for each of the eight cutoff concentrations, the sensitivity and specificity were plotted on the ROC curve (Figure 1). A theoretical cutoff concentration that yields no FPs or FNs would result in values for sensitivity and specificity of unity. Visual inspection of the ROC curve shows that the closest point on the curve to 1.0 for sensitivity and 0 for 1 – specificity lies between the 250 and 300 pg/mg cutoff concentrations. The more conservative cutoff of 300 pg/mg was selected to minimize the incidence of FPs.

The histograms in Figure 2 depict the number of TP, FN, FP, and TN at various cutoff levels from 100 to 450 pg/mg. Figure 2 may provide a more intuitive visualization of assay performance than the ROC curve (Figure 1). One goal of a properly constructed screening assay is to minimize the number of FPs and FNs. As shown in Figure 2, this is a function of the chosen cutoff value, and one cannot in practice eliminate both simultaneously. Clearly, the 200-, 250-, and 300-pg/mg cutoff concentrations yield the least total number of FPs and FNs (Figure 2).

At the 300-pg/mg cutoff, a total of 74 specimens were either TP (30 samples) or TN (44 samples) (Figure 2) resulting in an efficiency of 88\% (74/84). The sensitivity and specificity of the assay at 300 pg/mg was determined to be 83\% (30/36) and 92\% (44/48), respectively. Using these values, the positive predictive value (PPV) for an immunoassay result in hair at 50% prevalence was 91\%, and the negative predictive value (NPV) was 84\%. These values compare favorably to the work of Fay et al. (16) who reported a PPV and NPV of 86\% and 75\%, respectively, (using a 50% prevalence) for the detection of methamphetamine in sweat with an enzyme immunoassay.

#### Cross-reactivity

Typically, cross-reactivity information for several analytes is provided by manufacturers of commercial immunoassay kits. Often, this is reported as percent cross-reactivity of each analyte at one or more different concentrations resulting in multiple cross-reactivity values per analyte. This approach provides useful information for compounds that have little or no cross-reactivity but is incomplete for highly cross-reactive compounds. In addition, major inaccuracies in estimated cross-reactivities occur when the binding affinity of the interferent differs greatly from the target analyte. For example, if a compound has a much tighter binding affinity for the antibody than the target compound, then the cross-reactivity can be underestimated because of the difficulty of measuring slight differences in saturation in the flat part of the binding curve. Calculating the actual affinity constant for each compound provides a more accurate estimation of cross-reactivity. In the present study, this was accomplished by producing binding isotherms for compounds structurally similar to \(d\)-methamphetamine, the target analyte (Figure 3). This resulted in more accurate and singular cross-reactivity values for each analyte tested.

The cross-reactivity of the assay to isomeric forms of amphetamine and methamphetamine (based upon 100\% for \(d\)-methamphetamine) was 30.8\% for \(d\)-amphetamine, 7.4\% for...
l-methamphetamine, and 2.9% for l-amphetamine (Table I). These findings were important because the l-isomer of methamphetamine, often labeled desoxyephedrine, is available in over-the-counter nasal spray preparations (17,18). This can potentially produce false-positive results because non-chiral mass spectrometric confirmation methods for amphetamines do not distinguish the d- or l-form of methamphetamine. It is therefore desirable to have a screening test that is as specific as possible for the d-isomer which may forestall the necessity for chiral derivatization.

Some amphetamine-like drugs commonly encountered in this laboratory include fenfluramine, ephedrine, phentermine, and phenylpropanolamine. These compounds showed relatively low cross-reactivities of 15.4%, 11.4%, 4.3%, and 3.4%, respectively.

Labetalol showed a significantly high cross-reactivity to d-methamphetamine at 74.3% (Table I). This finding is not particularly surprising because the molecule possesses a structural moiety remarkably similar to the methamphetamine molecule. This compound could potentially increase the incidence of ELISA false positives and has been identified as the source of amphetamine immunoassay false positives by Branum et al. (19). However, labetalol, an alpha and beta adrenergic antagonist used in the treatment of hypertension, is not frequently encountered in employment populations. Further consideration of the structure reveals two hydroxyl groups, which suggests that, because of its polar nature, it may not incorporate very efficiently into hair. This expectation is supported by the fact that there is a much lower metabolite to parent drug ratio in hair compared to blood or urine samples when the metabolites are more polar than the parent analyte (20,21). It is therefore highly unlikely that positive amphetamine ELISA results produced by labetalol in hair specimens are of real concern. However, if a false positive due to labetalol were to arise, then the absence of a methamphetamine positive in the GC–MS confirmation would lead to a negative reported result. None of the 33 other common drugs and chemicals tested at 10 μg/mL showed any significant cross-reactivity (Table II).

Table II. Non-Cross-Reactive Compounds in Amphetamine ELISA Tested at 10 μg/mL

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cross-Reactivity</th>
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<tbody>
<tr>
<td>Carisoprodol</td>
<td>Quinine</td>
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<tr>
<td>Acetaminophen</td>
<td>Methacqualone</td>
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<tr>
<td>Meperidine</td>
<td>Diphenhydramine</td>
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<tr>
<td>Meprobamate</td>
<td>Triamterene</td>
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<tr>
<td>Phencyclidine</td>
<td>Cocaine</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Amoxapine</td>
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<tr>
<td>Nortripryline</td>
<td>Desipramine</td>
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<td>Amitriptyline</td>
<td>Benzoylcegonine</td>
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<tr>
<td>Nicotine</td>
<td>Propanoylpropionine</td>
</tr>
<tr>
<td>Methadone</td>
<td>Trazodone</td>
</tr>
<tr>
<td>Codeine</td>
<td>Mephine</td>
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<tr>
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<td>Lidocaine</td>
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<td>Pentazocine</td>
<td>Chlorpheniramine</td>
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<tr>
<td>Imipramine</td>
<td>Naproxen</td>
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<tr>
<td>Phenolphthalein</td>
<td>Trimeprazine</td>
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<tr>
<td>Carbamazepine</td>
<td>Erythromycin</td>
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<td>Doxepin</td>
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Conclusion

In summary, the described ELISA assay has proven to be both a reliable and accurate initial test for the presence of methamphetamine and amphetamine in hair. The diagnostic sensitivity and specificity values of 83% and 92%, respectively, are consistent with other immunoassays employed for detection of drugs of abuse in unconventional sample matrices (16,22,23) and provide the laboratory with a workable tool for screening employment hair specimens for amphetamines.

References

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Manuscript received March 23, 1998; revision received May 26, 1998.