Indirect enzyme-linked immunosorbent assay for the quantitative estimation of lysergic acid diethylamide in urine

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A new antibody to lysergic acid diethylamide (LSD) was used to develop a novel indirect ELISA for the quantification of drug in urine. Evaluation of the new assay with the commercially available LSD ELISA (STC Diagnostics) shows improved performance. The test requires 50 μL of urine, which is used to measure concentrations of drug in the μg/L to ng/L range. The limit of detection was 8 ng/L compared with 85 ng/L in the commercial assay, and analytical recoveries were 98–106%. Our test detected 0.1 μg/L of LSD in urine with an intraassay CV of 2.4% (n = 8) compared with 6.0% for a 0.5 μg/L sample in the commercial assay (n = 20). The upper and lower limits of quantification were estimated to be 7 μg/L and 50 ng/L, respectively. Specificity was evaluated by measuring the extent of cross-reactivity with 24 related substances. Drug determination using the new assay offers both improved sensitivity and precision compared with existing methods, thus facilitating the preliminary quantitative estimation of LSD in urine at lower concentrations with a greater degree of certainty.

The continued use of lysergic acid diethylamide (LSD)³ for recreational drug use has persisted for over 30 years. The detection of the drug in body fluids is made difficult by the low dose ingested and its rapid biotransformation (1,2). Analytical sensitivity is essential to detect concentrations of the drug in urine, which are typically in the low- to sub-μg/L range.

In the past, the most frequently used techniques for the detection of LSD in urine and biological matrices have been thin layer chromatography, RIA, high performance liquid chromatography–fluorescence, and gas chromatography–mass spectrometry. As a general rule, the latter methods require large sample volumes, are technically demanding, and are poorly suited for high sample throughput. Immunoassays are advantageous in terms of their potential sensitivity, small sample volume requirement, and large sample capacity. In general, an immunoassay is the first test in the routine investigation of biological specimens for drugs of abuse according to recognized guidelines (3). However, the positive identification of LSD on the basis of an immunoassay is not considered conclusive for legal purposes because of low assay specificity. It is quite common for antibodies raised against the parent drug to undergo cross-reactions with structurally similar molecules, including metabolites, some of which are as yet unidentified. Those samples that screen positive must be confirmed using a more rigorous technique that has both adequate sensitivity and specificity.

The most frequently used screening technique for LSD has been RIA, of which there are a number of commercially available kits in both Europe and North America. The detection limits of different RIAs for LSD have been reported in the literature in the range 0.2, 0.4, 1.0, and as high as 5 μg/L (4–7). The cutoff concentration recommended by the manufacturer, above which a sample is considered positive, is usually 0.5 μg/L (8). This is because, after a typical dose of ~100 μg, the concentration of LSD can fall below the cutoff concentration within 24 h (9). Ideally, the cutoff value should be set at a reasonable concentration that reflects the urinary elimination of the drug (3). However, forensic samples may contain LSD below this value, which frequently causes the test to be used at concentrations lower than recommended, e.g., 0.1 μg/L (8). This is because, after a typical dose of ~100 μg, the concentration of LSD can fall below the cutoff concentration within 24 h (9). Ideally, the cutoff value should be set at a reasonable concentration that reflects the urinary elimination of the drug (3). However, it must also reflect the sensitivity that is attainable using current analytical techniques.

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³Nonstandard abbreviations: LSD, lysergic acid diethylamide; TMB, 3,3',5,5'-tetramethylbenzidine; SM, skim milk; and PBS, phosphate-buffered saline.

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The need for a highly sensitive immunoassay for LSD is probably the result of increasingly sensitive confirmatory procedures, the relatively short detection time for the drug, and the renewed interest in LSD caused by a resurgence of abuse among young people (10). The main advantage of a RIA is its inherent sensitivity when it is used with a radiolabeled drug that has a high specific activity. However, recent trends have been towards nonisotopic procedures, which are safer, have longer shelf-lives, and do not need special disposal and laboratory facilities. Several recent advances have been made regarding immunoassay screening technologies for LSD drug use, some of which can be used in a quantitative mode. These include the OnLine latex-based aggregation assay (Roche Diagnostic Systems), the coupled enzyme-donor immunoassay, CEDIA® (Boehringer Mannheim), and the enzyme-multiplied immunotechnique, Emit® II (Behring Diagnostics). One disadvantage is that these methods require the use of large and expensive automated analyzers. An ELISA is available for small-scale testing (STC Diagnostics) that has a detection limit of 0.085 μg/L LSD in urine (11).

In the past, confirmatory analysis using techniques such as high performance liquid chromatography–fluorescence and gas chromatography–mass spectrometry suffered from relatively poor detection limits, typically around 0.5 μg/L in urine (8, 9, 12, 13). This is the result of common background interferences observed with biological specimens, the need for prior extraction, and derivatization, as well as thermal instability, low volatility, and the tendency of the drug to undergo absorptive losses during chromatographic procedures. However, recent analytical advances, such as tandem mass spectrometry-coupled procedures, have substantially improved the sensitivity of emerging confirmatory analyses. An increasing number of reports now describe mass spectrometric confirmation of LSD at concentrations as low as 10–50 ng/L (12, 14–18).

The aim of this work was to develop an ELISA for LSD in urine that was as sensitive as the emerging confirmatory techniques. A competitive-binding assay that utilized a novel polyclonal drug antibody was used to quantify LSD in urine. Free LSD in the urine specimen and immobilized LSD on the surface of a polystyrene microtiter well compete for a limited number of antibody-binding sites. Anti-LSD bound to the immobilized drug is detected with peroxidase-labeled antibody and subsequent tetramethylbenzidine color reaction, in which the absorbance is inversely related to the concentration of LSD in the urine. The primary objective was to develop and optimize conditions for the detection of LSD in urine and to characterize immunoassay performance in terms of precision, accuracy, sensitivity, and specificity. It should be noted that quantitative estimates of LSD in biological fluids are typically higher with immunoassays compared with confirmatory techniques, which is attributed to the cross-reactivity of the antibody with drug metabolites present in urine (6, 19, 20). Therefore, quantitative estimates obtained by immunoassay more closely represent the concentration of LSD and related compounds in the urine, depending on the specificity of the antibody reagent. For the purpose of this study, the detection limit of the immunoassay is described exclusively with respect to the parent drug, LSD. For evaluation purposes, drug-free urine samples were supplemented with a pure standard of d-LSD tartarate and were, therefore, free of metabolites or related LSD-like substances that might be present in the urine of someone who had ingested the drug.

Materials and Methods

REAGENTS
Disposable 96-well polystyrene plates were obtained from Corning. LSD tartrate was kindly supplied by Dr. Haro Avdovich of the Bureau of Drug Research, Health Canada (Ottawa, ON). Goat anti-rabbit IgG horseradish peroxidase and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma Chemical Co. Inorganic salts, hydrogen peroxide, acids, and dimethyl sulfoxide supplied by Fisher Scientific were certified ACS grade. Iso-LSD was provided by Dr. Kevin Gormley through the National Institute on Drug Abuse (NIDA) Drug Supply Program (Rockville, MD), and 2-oxo-3-hydroxy-LSD was purchased from Radian International. Dr. Haro Avdovich of the Bureau of Drug Research (BDR), Health Canada (Ottawa, ON) supplied additional controlled substances. These included N,N-dimethylyryptamine, α-lysergamide, lysergic acid, lysergic acid amide, mescaline, and psilocin. Coramine, 5-hydroxytryptamine, α-ergocryptine, ergonovine maleate, ergotamine tartrate, hordenine hemisulfate, L-tryptophan, lysergol, and N-demethyl-LSD (nor-LSD) were purchased from Sigma. Research Biochemicals supplied ergocornine, ergocristine, methylergonovine maleate, and methysergide maleate. The TMB substrate solution was prepared immediately before use; it consisted of 16 mL of deionized water, 4 mL of 200 mmol/L acetate/citrate buffer, pH 6.0, 200 μL of TMB in dimethyl sulfoxide (10 g/L), and 20 μL of hydrogen peroxide (30 mL/L). The acetate/citrate buffer was made by adding 200 mmol/L citric acid to 200 mmol/L sodium acetate to give a final pH of 6.0. A 50 g/L solution of Carnation nonfat skin milk (SM) powder in 150 mmol/L phosphate-buffered saline, pH 7.4 (PBS), was routinely used as the blocking agent (SM-PBS). Antibodies to the drug were obtained from rabbits that were immunized with LSD that was covalently attached to the carrier protein keyhole limpet hemocyanin (21).

Immunoassay Procedure
ELISA plates were coated overnight at 4 °C with 100 μL of LSD-derivatized bovine serum albumin coating antigen (25 ng/L in PBS), which was prepared by the Mannich reaction (4). Uncoated sites on each microtiter well were preblocked with 175 μL of 50 g/L SM-PBS for 30 min at 37 °C. Plates were washed five times with PBS between
each of the following incubation steps. LSD was diluted in undiluted urine to give concentrations between 20 and 0.002 μg/L, and immune rabbit serum was diluted in 100 g/L SM-PBS. Equal volumes of urine and the antibody solution were added to microtiter wells in that order, such that the final volume was 100 μL. Pre-immune rabbit serum was used to estimate the degree of nonspecific binding in the assay. After gentle agitation, the plate was incubated for 2.5 h at 37 °C. Goat anti-rabbit IgG horseradish peroxidase (100 μL) diluted 1:1000 in 50 g/L SM-PBS was added, and the plates were incubated for 30 min at 37 °C. After the final plate wash, the color reaction was initiated with 100 μL of TMB substrate solution followed by 50 μL of 1 mol/L sulfuric acid to stop the reaction 5 min later. The absorbance was measured at 450–620 nm using an SLT EAR 400AT plate reader (SLT Lab-Instruments). Results were displayed as the percentage of antibody that remained bound (% bound) relative to the zero calibrator, which was drug-free urine. Calibration curves were plotted using the mean of quadruplicate measurements for all LSD calibrators and the blank.

**ASSAY SENSITIVITY**
The limit of detection was calculated from the mean response of the zero calibrator minus 3 SD. This value was based on quadruplicate measurements for a blank urine specimen that was known to contain no LSD. The upper and lower limits of quantification were calculated from the mean ± 3 SD of negative urine samples obtained from healthy drug-free volunteers (n = 24), which were stored at −20 °C for up to 3 months before use.

**ACCURACY AND PRECISION**
Urine specimens were supplemented with LSD to give high (10 μg/L), medium (1.0 μg/L), and low (0.1 μg/L) concentrations. The concentration of LSD in each supplemented sample (n = 8) was interpolated from the calibration graph, using a four-parameter logistical equation that was calculated using Microcal Origin. The analytical recovery of drug, 95% confidence limits, and CVs were calculated for each sample.

The intraassay CVs of supplemented urine samples were measured over the entire calibration range. Interassay CVs (between-run precision) were calculated for 12 assays that were performed over 4 months. These results were used to assess the reproducibility of quantitative measurements and to record any differences caused by reagent changes.

**ASSAY SPECIFICITY**
The specificity of the assay was evaluated by measuring the degree of cross-reactivity of various compounds that were used in place of LSD in the immunoassay described earlier. Duplicate measurements were made for each compound over a range of concentrations (1 × 10⁻⁵ to 1 × 10⁻¹² mol/L), using LSD as the reference. The amount of antibody that was bound at each inhibitor concentration was calculated as a percentage, relative to the measured absorbance when no drug was present. Inhibition curves were compared for each compound of interest, relative to LSD. The approximate percentage of cross-reactivity was calculated from the amount of compound that produced a signal equivalent to 0.5 μg/L LSD, which is the widely accepted immunoassay cutoff concentration for a positive urine specimen (22).

**Results and Discussion**

**LIMITS OF DETECTION AND QUANTIFICATION**
Typical calibration data obtained using the competitive binding assay are depicted in Fig. 1. The limit of detection was 8 ng/L by interpolation, more than an order of magnitude lower than the commercial ELISA, which reports a detection limit of 85 ng/L by extrapolation (11). The nonspecific binding, which was estimated using pre-immune rabbit serum, was 3%. The estimated upper and lower limits of quantification were 7 μg/L and 50 ng/L LSD in urine, respectively (n = 24). Commercially available immunoassays generally recommend that 0.5 μg/L be used to discriminate positive from negative specimens to reduce the possibility of false positives. However, typical concentrations of LSD in urine are below this amount. The overall working range of the immunoassay extends from as low as 50 ng/L to as high as 7 μg/L, which is well within the region of forensic interest.

Overall assay sensitivity is largely dependent on the affinity of the antibody (23, 24), although the conditions
used in the ELISA affect the optimum assay range. It is known that the method used for coupling hapten to the carrier protein influences the dose–response behavior. Heterologous assays in which the immunogen and immobilized antigen are different, with exception of the hapten, are known to produce the most sensitive results. This is of particular importance for the detection of small haptens by ELISA, in which the antigen attached to the solid phase should use a different carrier protein, chemical linkage, and site of attachment with respect to the immunizing antigen. This immunoassay, which shows improved sensitivity, uses a unique heterologous system whereby the hapten-protein conjugate used for immunization was prepared with a unique chemical attachment that is distinct from that of the surface immobilized hapten-carrier conjugate.

**ACCURACY**

A four-parameter logistical curve fit was calculated according to the following equation:

$$y = \frac{A_1 - A_2}{1 + (x/x_0)^p} + A_2$$

where optimal fit was obtained using $A_1 = 97.6$, $A_2 = 1.8$, $x_0 = 0.79$, and $p = 0.618$, and $x$ and $y$ are the concentration of LSD in urine in $\mu g/L$ and percentage bound, respectively. The analytical recovery of LSD from urine ranged from 98% to 106% over three orders of magnitude (Table 1). Interpolated concentrations of LSD were 10.3, 0.98, and 0.11 $\mu g/L$ for 10.0, 1.00, and 0.10 $\mu g/L$ LSD in urine, respectively. The indirect nature of the assay, which produces increased absorbance with decreased concentration of drug in the urine, is reflected in the precision of replicate measurements. The CV values for 10.0, 1.0 and 0.1 $\mu g/L$ LSD in urine ($n = 8$) were 5.1%, 2.5%, and 2.4%, respectively. This compared favorably with the commercial ELISA, which reports a CV of 6% for 0.5 $\mu g/L$ LSD added to urine ($n = 20$). When the ELISA described herein is used, the analytical recovery of 0.1 $\mu g/L$ LSD in urine was 106%, and the CV was only 2.4% ($n = 4$). This illustrates that even in the sub-$\mu g/L$ region of forensic interest, precision and accuracy are not compromised.

**Table 1. Accuracy determination for the detection of LSD in urine by ELISA.**

<table>
<thead>
<tr>
<th>Expected concentration, $\mu g/L$</th>
<th>Interpolated concentration $\pm$ 95% CL, $\mu g/L$</th>
<th>Recovery, %</th>
<th>%CV ($n = 8$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>10.30 ± 2.00</td>
<td>103</td>
<td>5.1</td>
</tr>
<tr>
<td>1.0</td>
<td>0.98 ± 0.15</td>
<td>98</td>
<td>2.5</td>
</tr>
<tr>
<td>0.10</td>
<td>0.11 ± 0.02</td>
<td>106</td>
<td>2.4</td>
</tr>
</tbody>
</table>

LSD was added to normal human urine at 10.0, 1.0, and 0.10 $\mu g/L$ ($n = 8$). Calibration standards ($n = 4$) were used to interpolate concentrations directly from the graph using a four-parameter logistical equation.

**Table 2. Inter- and intraassay precision of LSD in urine by ELISA.**

<table>
<thead>
<tr>
<th>Concentration of LSD in urine, $\mu g/L$</th>
<th>Intraassay CV, % ($n = 4$)</th>
<th>Interassay CV, % ($n = 12$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.0</td>
<td>7.57</td>
<td>22.59</td>
</tr>
<tr>
<td>2.0</td>
<td>4.05</td>
<td>11.30</td>
</tr>
<tr>
<td>0.2</td>
<td>3.71</td>
<td>6.25</td>
</tr>
<tr>
<td>0.02</td>
<td>2.69</td>
<td>4.10</td>
</tr>
<tr>
<td>0.002</td>
<td>2.78</td>
<td>4.64</td>
</tr>
</tbody>
</table>

The CV is shown for LSD calibration standards run in the same assay ($n = 4$) and in different assays ($n = 12$).

**PRECISION**

Intraassay precision was estimated from the CV values of calibration standards that were run in the same assay. As expected, the overall precision decreases as the concentration of analyte increases because of the indirect nature of the assay. Table 2 shows precision measurements over a wide range of LSD concentrations. The precision profile of the immunoassay, in which intra- and interassay CV values are plotted against the concentration of drug, are shown in Fig. 2. This clearly shows that optimum precision is achieved at sub-$\mu g/L$ concentrations of LSD in urine. Table 2 also shows the interassay precision of calibration standards run in different assays over 4 months ($n = 12$). The precision profile indicates the same overall trend for between- and within-run CV values, the former of which are somewhat more exaggerated, as expected.

Concentrations >4 $\mu g/L$ lead to significantly decreased precision (interassay CV >15%), of which accept-

![Fig. 2. Precision profile for the determination of LSD in urine.](image-url)
able limits are usually 10–20% (27). The concentration range that produced inter- and intraassay CVs <15% was 0–4 μg/L LSD in urine, which is the suggested working range of the assay. Reagent changes such as new bovine serum albumin-LSD coating antigen, TMB stock solution, or enzyme-labeled antibody did not adversely affect precision over the range of concentrations that were tested.

SPECIFICITY
Assay specificity was estimated by measuring the degree of cross-reactivity of various compounds of interest. The concentration of derivative that caused a decrease in signal that was equivalent to 0.5 μg/L LSD was used to estimate the approximate cross-reactivity as a percentage (Table 3). Of the 24 compounds cited here, only 3 were found to cross-react substantially. These were nor-LSD (52%), lysergic acid methyl-n-propylamide (34%), and 2-oxo-3-hydroxy-LSD (3.4%). The first of these is the only confirmed human metabolite to date (16), although the last compound has also been tentatively identified. As such, cross-reactivity with nor-LSD, and to a lesser extent with 2-oxo-3-hydroxy-LSD, might be considered advantageous from a drug-screening perspective. Minor cross-reactions were also observed with iso-LSD (0.05%), ergonovine (0.016%), and methylerygonovine (0.008%). The remainder of compounds tested, which included structurally related analogs such as lysergic acid, lysergic acid amide, ergotamine, and methysergide among others, did not exhibit any measurable cross-reactivity (<0.008%).

A new immunoassay for LSD in urine that compares favorably with existing methods is described. This sensitivity is probably the result of the high affinity antibody to LSD that is used in the procedure. Results obtained thus far indicate that the method may be useful for drug determination in the sub-μg/L region of forensic interest.

This method offers a substantial improvement in the detection limit compared with a number of commercially available immunoassays currently available. This approach facilitates the detection of LSD in urine at concentrations that were previously only attainable by RIA but with less expense and without the need for radioisotopes.

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