TECHNICAL NOTE

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Evaluation of the Abuscreen Online Assay for Amphetamines on the Hitachi 737: Comparison with EMIT and GC/MS Methods

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ABSTRACT: The performance of the ONLINE Assay for Amphetamines on the Hitachi 737 was compared to the Syva Emit d.a.u. Assay and GC/MS. Randomly screened (n = 2964) patient urine samples were assayed using ONLINE and Emit d.a.u. assays concurrently, using d-amphetamine, 1000 ng/mL and d-methamphetamine, 1000 ng/mL as the screening cutoff for ONLINE and Emit d.a.u. assays, respectively. All presumptive positives were confirmed by GC/MS. The specificity was 99% (2937/2964) for ONLINE and 97% (2873/2964) for Emit. Agreement with GC/MS was 80% (110/137) for ONLINE and 55% (110/201) for Emit.

KEYWORDS: toxicology, amphetamines, methamphetamines, immunoassay, cost, ONLINE, Emit

Amphetamines, including amphetamine (AMP) and methamphetamine (MAMP), continue to be prescribed and abused drugs in many countries around the world [*1,2*]. In military and civilian forensic drug testing programs, two independent methods are required to report a sample as positive [*3*]. Immunoassay methods are frequently used as an initial screen to detect the presence of AMP and MAMP in urine [*2,4–10*]. The recommended method for confirmation of amphetamines is GC/MS [*11,12*].

There are several factors that can complicate immunoassay screening for amphetamines in urine. One of the most important considerations is assay specificity. The presence of stimulants, hallucinogens, and over-the-counter (OTC) medications in use for diet aid (phenylpropanolamine) and cold remedies (ephedrine) or their metabolites in urine that are structurally similar to AMP and MAMP [*13*] can result in presumptive positive samples requiring confirmation that do not contain AMP and/or MAMP. These compounds are frequently present in urine samples submitted for drugs of abuse testing and are often in very high concentrations, increasing the probability of cross-reacting in an immunoassay and yielding false positive results [*6*].

Cost is an important consideration in selecting a method for the initial assay in screening for drugs of abuse. Confirmation of presumptive positive samples requires the use of additional labor, supplies, and instrumentation—often at a considerable cost. Thus, screening methods need to have a high specificity. Otherwise, an unacceptably large number of urine samples will screen positive in the initial screen and negative at confirmation, resulting in a potentially large and unnecessary expenditure of time and money.

The following study compared the specificity and cost-effectiveness of two homogeneous assays on the Hitachi 737 analyzer for their ability to detect urine samples containing only AMP and/or MAMP. Samples identified as positive by one or both assays were confirmed by GC/MS.

Experimental

Reagents and Materials

ONLINE kits and standards were provided for evaluation by Roche Diagnostic Systems (Branchburg, NJ). Emit kits and standards were purchased from Syva (Palo Alto, CA). Raichem glucose-6-phosphate (G-6-P)/NAD buffered substrate was purchased from Reagents Applications Inc. (San Diego, CA). All reagents and solvents used for the analysis of amphetamines by GC/MS were of analytical grade. AMP-D5 and MAMP D-5 were purchased from Radian Corporation. d-AMP and d-MAMP were purchased from Altech-Applied Science. Mixed standards of d-MAMP and d-AMP were prepared using pooled urine that was shown not to contain drug by immunoassay and GC/MS. The concentrations of these mixtures were confirmed by GC/MS.

Instrumentation

ONLINE and Emit were run on the Hitachi 737 analyzer (Boehringer Mannheim, Indianapolis, IN) according to manufacturer's

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protocols. The analyzer was equipped with a data management system which was used for the qualitative analysis of amphetamines.

Mass spectra were obtained on a Hewlett Packard Model 5890A with a 5970A Mass Selective Detector (MSD). The data system used was an HP 98561A computer equipped with a HP 9133L disc drive. Data acquisition and calculation were performed using Target (Thru-Put, Inc.) software. The MSD was operated with electron impact ionization at 70 ev in the selected ion monitoring (SIM) mode. The instrument was autotuned daily with perfluorotributylamine (PFBTA). A fused silica capillary column, 10 m × 0.18 mm × 0.4 μm DB-5 (J&W Scientific, Inc.) was used.

Extraction and Derivatization

Deuterated internal standards (AMP-D5 and MAMP-D5) in methanol (50 μL, 10 mcg/mL) were added to urine samples (4 mL) in a Toxi-Tube A Extraction tube. The amphetamines were extracted into the organic solvent, acidified with methanolic hydrochloric acid, and concentrated by evaporation under a stream of nitrogen to dryness at 50°C. The extract was derivatized with trifluoracetic anhydride (TFA)(50 uL ethyl acetate + 50 uL TFA), dried, reconstituted with ethyl acetate (50 uL), and injected (1 uL) into the GC/MS. The ions monitored were 140, 118, 117 (AMP); 154, 118, 110 (MAMP); 144, 123 (AMP-D5); and 158, 113 (MAMP-D5). The quantitation of AMP and MAMP was determined from a calibration curve derived from negative urine samples spiked with known concentrations of AMP and MAMP.

Analytical Procedures

The Abuscreen ONLINE® Amphetamine Assay was performed according to the manufacturer's instructions. The Syva Emit® d.a.u. Amphetamine Assay Reagent A and Reagent B were diluted with Raichem G-6-P/NAD Buffered Substrate and Emit® d.a.u. Buffer, respectively. Briefly, 5 mL Reagent A was diluted with 210 mL reconstituted G-6-P/NAD (Tris Buffer, 55 mmol/L, pH 8; G-6-P, 7.2 mmol/L; NAD, 7.2 mmol/L), and 5 mL Reagent B was diluted with 30 mL Emit® d.a.u. Buffer (Tris Buffer, 0.825 m/L, pH 8.0) and 135 deionized (DI) water.

The Hitachi 737 was operated in precision mode. The ONLINE® reaction was determined in the endpoint (ENDP 10–18) rate mode, and absorbances were measured at 505 nm. The sample, Reagent 1, and Reagent 2 volumes were 10 uL, 175 uL, and 85 uL, respectively. The Emit® d.a.u. reaction was determined in the endpoint (ENDP 11–20) mode, and wavelengths 1 and 2 were 340 nm and 415 nm, respectively. Sample, Reagent A, and Reagent B volumes were 10 uL, 120 uL, and 120 uL, respectively. The Hitachi 737 was calibrated with d-amphetamine, 1000 ng/mL and d-methamphetamine, 1000 ng/mL for the ONLINE® and Emit® d.a.u. assays, respectively. To ensure that the instrument was calibrated and functioning correctly, negative and positive calibrators, controls and cutoff calibrators were run and monitored with each batch (n = 99) of urine samples.

All GC/MS assays included a low (250 ng/mL) and high (1000 ng/mL) calibrator, a limit of quantitation (LOQ, 125 ng/mL), a positive and negative control with each batch (n = 16) of urine samples. Calibrators and controls had to agree within 20% of their target concentrations both qualitatively and quantitatively for the ions monitored.

Standard Curves

A plot was generated for ONLINE using 0, 500, 1000, and 2000 ng/mL d-AMP standards versus milliabsorbance units. Similar plots were generated with Emit using 0, 1000, and 3000 ng/mL d-MAMP standards. For comparison purposes, two Emit plots were generated, one using Emit reagents and the other using Emit reagents supplemented with G-6-P.

Precision Study Methods

Standards were run five times each day for the five days of the study every 100 samples. The standards used to evaluate the ONLINE assay were 0, 500, 800, 1000, 1200, 1500, and 2000 ng/mL of d-AMP. The standards used to evaluate the Emit assay were 0, 1000, and 3000 ng/mL of d-MAMP (Negative, Calibrator A Level 1, Calibrator A Level 2). The instrument gave results in absorbance units and these results were converted into transformed numbers for analysis.

Analytical Sensitivity

The zero calibrator was run five times each day for five days. An average and standard deviation were calculated from the 25 readings. The analytical sensitivity was then determined by adding two standard deviation units (95% confidence level) to the average.

Comparative Study

Randomly screened (n = 2964) patient samples were assayed concurrently with the ONLINE assay for amphetamines and the in-house (supplemented) Emit d.a.u. monoclonal amphetamine/methamphetamine assay on the Hitachi 737. The screening cutoff was 1000 ng/mL of d-AMP for the ONLINE assay and 1000 ng/mL of d-MAMP for the Emit assay.

All urine samples yielding positive screening results, by one or both of the screening assays, were confirmed by GC/MS. A sample containing 500 ng/mL or greater of AMP or 200 ng/mL or greater of AMP in the presence of 500 ng/mL or greater of MAMP by GC/MS was identified as positive for AMP.

Results and Discussion

Standard Curve Comparison

Results of the standard curves for ONLINE, Emit, and extended Emit are shown in Fig. 1. The 1000 ng/mL cutoff was assigned a zero milliabsorbance value, and readings above and below the cutoff were expressed in milliabsorbance (mA) units.

The dynamic range of the ONLINE assay between 0 and 2000 ng/mL of d-AMP was greater than 700 mA. In contrast, the dynamic range of the Emit assay between 0 and 3000 ng/mL of d-MAMP was 18 mA for unextended reagents and 65 mA for extended reagents. Due to the greater discrimination between levels with the extended Emit reagents over the unextended reagents, precision and clinical data were generated with the extended Emit reagents.

The response of ONLINE with pure d-MAMP is 50% at 1000 ng/mL and 0.5% at 200,000 ng/mL. However, 500 ng/mL of d-MAMP in the presence of 200 ng/mL of d-AMP gives a response equivalent to 1000 ng/mL of d-AMP. Furthermore, 500 ng/mL of d-MAMP in the presence of 500 ng/mL of d-AMP gives a response greater than the 2000 ng/mL d-AMP standard. This enhancement effect is plotted in Fig. 2 along with the response for d-AMP alone.

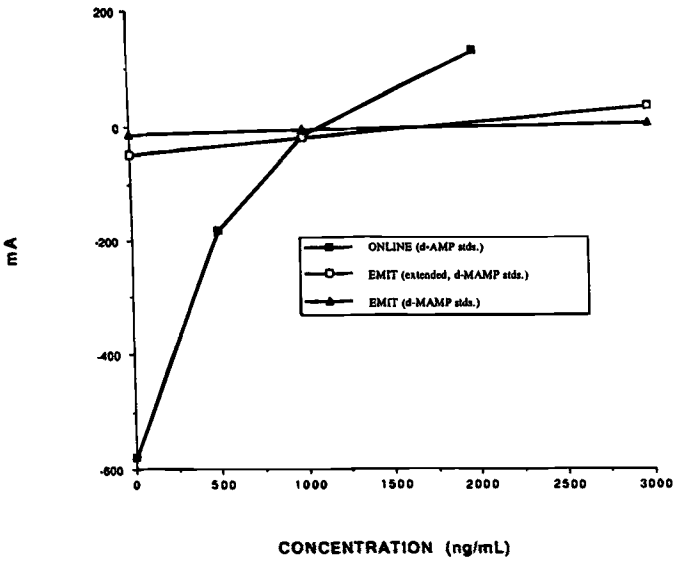

FIG. 1

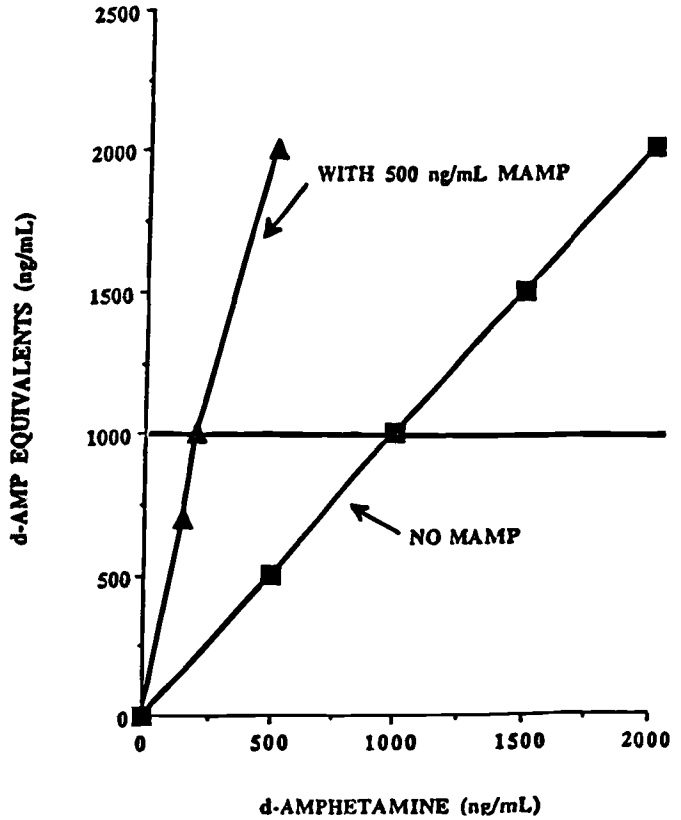

FIG. 2

As Figure 2 indicates, mixtures of d-AMP and d-MAMP give a response in the assay that is greater than the sum of the concentrations. This enhancement effect has also been reported in the Abbott assay for amphetamines [9].

Precision Study

Precision data were generated for both ONLINE and Emit on transformed numbers. The results for ONLINE are in Table 1 and the within-run CV precision at all levels tested was less than 6%. The between-run CV precision over the five-day period ranged from 10% at the low range of the curve (500 ng/mL) to 1.4% at the high range (2000 ng/mL) of the curve. The CV precision at the 1000 ng/mL d-AMP level was 4.5% within-run and 3.6% between-run. There was no overlap with the cutoff observed at 800 and 1200 ng/mL of d-AMP over the five-day study. The data indicate that a curve could be generated and stored on day one and the values could be read off the initially stored curve over the five-day period. Under these conditions, no overlap of the 800 and 1200 ng/mL controls with the cutoff was observed.

TABLE 1—*ONLINE precision study results over a five-day period.*[a]

Calibrator Level d-amphetamine (ng/mL)	Between-Run CV %	Mean Within-Run CV %	Range Within-Run CV %
500	10.0	4.6	3.8–5.6
800	4.8	4.7	3.4–5.5
1000	3.6	4.5	3.7–5.4
1200	2.3	4.4	3.3–5.2
1500	1.7	3.6	1.5–5.5
2000	1.4	3.0	2.5–5.2

[a]Precision was run $n = 5$ for five days and was calculated on transformed numbers (ng/mL).

The Emit precision data were generated using Calibrator A Level 1 (1000 ng/mL d-MAMP) and Calibrator A Level 2 (3000 ng/mL d-MAMP). These results show (Table 2) within-run CV precision on transformed numbers to be between 23.6% at the low range (1000 ng/mL) of the curve and 5.5% at the higher range (3000 ng/mL) of the curve. The between-run CV precision was somewhat higher ranging from 32.9% at the low range (1000 ng/mL) of the curve and 10.2% at the higher range (3000 ng/mL) of the curve. Controls at 800 and 1200 ng/mL of d-MAMP were not run. Due to the shifting values at the cutoff, it would not be possible to store a curve on day one and use it throughout the study. In this laboratory, the Emit calibration is checked every 100 samples and adjusted as necessary.

Analytical Sensitivity

When compared, there was a threefold difference in analytical sensitivity between ONLINE and Emit. The analytical sensitivity for ONLINE was calculated to be 148 ng/mL and for Emit was calculated to be 446 ng/mL. It is interesting to note that the standard deviation on absorbance was 26.5 mA for ONLINE and 7.8 mA for Emit. Since the absorbance difference between 0 ng/mL and the cutoff is 566 mA for ONLINE and 23 mA for Emit, the conversion to ng/mL resulted in a smaller range of transformed numbers for ONLINE and a larger range of transformed numbers for Emit.

TABLE 2—*Emit precision study results over a five-day period.*[a]

Calibrator Level d-methamphetamine (ng/mL)	Between-Run CV %	Mean Within-Run CV %	Range Within-Run CV %
1000 (Level 1)	32.9	23.6	16.0–30.8
3000 (Level 2)	10.2	5.5	0.6–7.3

[a]Precision was run $n = 5$ for five days and was calculated on transformed numbers (ng/mL).

Comparative Study Results

The results of a random screen of 2964 samples are given in Table 3. Both ONLINE and Emit identified 110 samples positive that were confirmed by GC/MS by the criteria described under the experimental section. The 110 samples were the same for each technology. In addition, ONLINE falsely identified 27 samples as positive and Emit falsely identified 91 samples as positive. All 27 false positive samples by ONLINE were a subset of the 91 false positive samples of Emit. Both technologies identified 2763 samples as being negative. The agreement of ONLINE versus GC/MS was 80%. In contrast, the agreement of Emit versus GC/MS was 55%.

The distribution of the false positive samples is described in detail in Table 4. Only 7 of the 27 false positive samples screened with ONLINE and 33 of 91 false positive samples screened with Emit did not contain any AMP or MAMP. As shown in Table 4, the remaining false positive samples by each technology did contain measurable levels of AMP, MAMP, or both. It is of interest that although 6 samples—two positive with ONLINE and Emit and four positive with Emit only—had combined concentrations of AMP and MAMP greater than 1000 ng/mL, they failed to meet the GC/MS cutoff minimum of 200 ng/mL of AMP.

Conclusions

The ONLINE assay demonstrated several significant analytical advantages when compared to the Emit assay. The dynamic range for ONLINE is 700 mA compared to 65 mA for Emit. The ONLINE assay yielded a 596 mA span to cutoff compared to 23 mA for Emit. In addition, the CV precision at the cutoff ranged from 3.7% to 5.4% for ONLINE as compared to 16.0% to 30.8% for Emit. These analytical advantages resulted in a greater discrimination between a negative sample and the cutoff, providing a more specific assay for AMP and MAMP.

TABLE 3—*Results of clinical evaluation* (n = *2964) of ONLINE and emit assays for amphetamines.*

Assay	# Positive Samples	# Confirmed Positives[a]	# False Positives
ONLINE	137	110	27
Emit	201	110	91

[a]Positive by GC/MS criteria: 500 ng/mL of amphetamine or 500 ng/mL of methamphetamine with 200 ng/mL amphetamine.

TABLE 4—*Distribution of ONLINE and Emit false positives*[a] *on a Random Clinical Study* (n = *2964).*

Combined AMP and MAMP Concentration (ng/mL) by GC/MS	ONLINE False Positives	Emit False Positives
None detected	7	33
Less than 500	4	30
Less than 1000	14	22
Greater than 1000	2	6
Total Number	27	91

[a]Containing less than 500 ng/mL of amphetamine or containing less than 500 ng/mL methamphetamine in the presence of at least 200 ng/mL amphetamine.

The Syva Emit d.a.u. Monoclonal Amphetamine Assay used in this study was modified by the addition of G-6-P/NAD. Similar to the comparison between the analytical advantages of ONLINE compared to the in-house Emit assay, the in-house assay displayed significant analytical improvements when compared to the undiluted Syva Emit d.a.u. Monoclonal Amphetamine Assay. These improvements included a greater dynamic range (65 mA v. 18 mA) and an increased span to cutoff (23 mA v. 8 mA). These findings are in agreement with previous studies [*14,15*] that demonstrated that the addition of G-6-P/NAD not only is cost-effective by "extending" the reagents, but it also increases the analytical sensitivity and performance of the Emit d.a.u. assays.

In screening urine samples for amphetamines, the immunoassay should be able to detect both AMP and MAMP. The ONLINE assay contains two monoclonal antibodies—for AMP and MAMP, respectively. The MAMP antibody in the absence of AMP has a very low response to MAMP, leading to a very low cross-reactivity to OTC medications. However, in the presence of low concentrations of AMP, the MAMP response is enhanced. The low cross-reactivity of the ONLINE assay to over-the-counter medications was demonstrated in the finding that only seven of the presumptive positives contained no detectable amount of AMP and/or MAMP. In contrast, 33 of the Emit presumptive positive samples contained no detectable amount of amphetamines. Additionally, these findings demonstrate that an immunoassay standardized on d-AMP (ONLINE) can detect MAMP as well as an assay standardized on d-MAMP (Syva, Emit d.a.u.).

The results of the study demonstrated that the ONLINE Amphetamine Assay has a high degree of specificity. When compared to the Syva Emit d.a.u. assay, ONLINE produced 70% (27/91) less presumptive positive samples. This represents a significant difference in the number of confirmations to be performed. The ONLINE assay screening resulted in a total of 137 confirmations compared to 201 confirmations for urine samples screened with the Syva Emit d.a.u. assay. Assuming a cost of $15.00 per GC/MS confirmation for amphetamines, including materials and labor, there would be a realized savings of up to $960.00 ($3015 to $2055) in this study (assuming that the immunoassay costs were the same). This increase in amphetamine specificity can provide an even higher cost reduction when medium to high volume chemistry analyzers are used.

It is noteworthy that both the ONLINE and Emit assays were able to detect samples containing amphetamine alone or in combination with methamphetamine that did not meet the current Substance Abuse and Mental Health Services Administration (SAMSA, formerly NIDA) reporting guidelines for amphetamines [*16*]. The guidelines specify that a sample is reported positive when it contains at least 500 ng/mL MAMP in the presence of 200 ng/mL or greater amphetamine, or when the sample contains at least 500 ng/mL AMP. Seventy-four percent (20/27) and 64% (58/91) of the patient urine samples that were negative by the GC/MS administrative cutoff for AMP and/or MAMP contained measurable amounts of amphetamines.

On the basis of this study, it appears that ONLINE provides a reliable and cost-effective screening for amphetamines in urine. Compared to Emit, it has a lower probability of producing false positive AMP screening results. When used as an initial screening method in a drug testing laboratory, ONLINE would result in a lower number of presumptive positive samples requiring costly secondary confirmation testing. The information in this study could be useful in selecting a cost-effective drug screening program and selecting a methodology to ensure that the combination of screen-

ing and confirmation assays does not yield a reportable false positive result.

References

[1] Ellenhorn, M. J. and Barceloux, D. G., *Medical Toxicology*, Elsevier, New York, 1988, pp. 625–641.

[2] Baselt, R. C. and Cravey, R. H., *Disposition of Toxic Drugs and Chemicals in Man*, Third Edition, Year Book Medical Publishers, Inc., Chicago, 1989, pp. 49–52; 516–519.

[3] Federal Register, Part IV, Department of Health and Human Services; Alcohol, Drug Abuse, and Mental Health Administration; Mandatory Guidelines for Federal Workplace Drug Testing Programs; Final Guidelines: Notice, v. 53, #69, pp. 11978–11989, Monday, April 11th (1988).

[4] Godolphin, W., In *Methodology for Analytical Toxicology*, Vol. 2, I. Sunshine and P. I. Jatlow, Eds., CRC, Boca Raton, Florida, 1982, pp. 205–214.

[5] Sunshine, I., In *Methodology for Analytical Toxicology*, Vol. 2, I. Sunshine and P. I. Jatlow, Eds., CRC, Boca Raton, Florida, 1982, pp. 205–214.

[6] D'Nincoula, J., Jones, R., Levine, B., and Smith, M. L., "Evaluation of Six Commercial Amphetamine and Methamphetamine Immunoassays for Cross-Reactivity to Phenylpropanolamine and Ephedrine in Urine," *Journal of Analytical Toxicology* Vol. 16, 1992, pp. 211–213.

[7] Dasgupta, A., Saldana, S., Kinnaman, G., Smith, M., and Johansen, K., "Analytical Performance Evaluation of Emit® II Monoclonal Amphetamine/Methamphetamine Assay: More Specificity than Emit® d.a.u.™ Monoclonal Amphetamine/Methamphetamine Assay," *Clinical Chemistry*, Vol. 39, No. 1, 1993, pp. 104–108.

[8] Cody. J. T., "Cross-Reactivity of Amphetamine Analogues with Roche Abuscreen Radioimmunoassay Reagents," *Journal of Analytical Toxicology*, Vol. 14, 1990, pp. 50–53.

[9] Prekop, M. A., Manno, J. E., Kunsman, G. W., Cockerham, K. R., and Manno, B. R., "Evaluation of the Abbott ADx Amphetamine/Methamphetamine II Abused Drug Assay: Comparison to TDx, Emit and GC/MS Methods," *Journal of Analytical Toxicology*, Vol. 15, 1991, pp. 323–326.

[10] Poklis, A., Saady, J. J., Fitzgerald, R. L., and Bogema, S. C., In *Proceedings of the International Congress on Clinical Toxicology, Poison Control and Analytical Toxicology LUX TOX '90*, R. Wenning, Ed., Bull. Soc. Med. Grand Duche Luxemb., 127 Suppl.: 1990, pp. 74–87.

[11] Hornbeck, C. L. and Dzarny, R. J., "Quantitation of Methamphetamine and Amphetamine in Urine by Capillary GC/MS. Part I. Advantages of Trichloroacetyl Derivatization," *Journal of Analytical Toxicology* Vol. 13, 1989, pp. 144–149.

[12] Taylor, R. W., Le, S. D., Philip, S., and Jain, N. C., "Simultaneous Identification of Amphetamine and Methamphetamine Using Solid-Phase Extraction and Gas Chromatography/Nitrogen Phosphorus Detection or Gas Chromatography/Mass Spectrometry," *Journal of Analytical Toxicology*, Vol. 13, 1989, pp. 293–295.

[13] Thurman, E. M., Pedersen, M. J., Stout, R. L., and Martin, T., "Distinguishing Sympathomimetic Amines from Amphetamine and Methamphetamine in Urine by Gas Chromatography/Mass Spectrometry," *Journal of Analytical Toxicology*, Vol. 16, 1993, pp. 19–27.

[14] Sung, E. and Neeley, W. E., "A Cost-Effective System for Performing Therapeutic Drug Assays. 1. Optimization of the Theophylline Assay," *Clinical Chemistry*, Vol. 31, 1985, pp. 1210–1215.

[15] Yu, S. S., and Osterloh, J., "A Cost Effective Emit d.a.u. Assay with Improved Sensitivity," *Clinical Chemistry*, Vol. 33, 1987, pp. 976–979.

[16] NIDA Notice to all DHHS/NIDA certified laboratories, December 19, 1990.

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