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Minimal Standards for the Performance and Interpretation of Toxicology Tests in Legal Proceedings

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ABSTRACT: There have been several high profile criminal and civil cases that have been litigated in recent years involving toxicologic analyses and interpretations of blood, urine, and other specimens for drugs of abuse. Disputes have erupted between prominent toxicologists and laboratory scientists as to the validity and interpretation of the data presented. The disputes centered around the fact that the procedures used in these cases had not been properly validated with analytical noise being misinterpreted as a positive result. As with any analyses, forensic tests must be conducted in a manner such that they meet the minimum standards accepted within the toxicology community. No conclusions as to presence or absence of drug, its concentration, or its physiologic effects can be made if there is a failure to meet these basic standards. Several cases are presented where these standard tenets may not have been followed.

KEYWORDS: forensic science, drugs-of-abuse testing, enzyme immunoassay, gas chromatography/mass spectrometry (GC/MS), signal-to-noise ratio

Need for Confirmation of Immunoassay Screening Results

Commercial immunoassays, used for screening for drugs of abuse, are designed to provide presumptive information about the presence or absence of different drug classes in urine. There is great variability in the sensitivity and specificity of these assays. The cocaine metabolite assay, for example, is very specific for benzoylecgonine (BE) in urine, with essentially no other drugs or metabolites producing positive results. On the other hand, the amphetamines class assay produces positive results for the presence of illicit amphetamines, as well as many over-the-counter and prescription sympathomimetic amines. Due to the potential for interferences, results of immunoassays must be confirmed with more definitive techniques such as GC/MS. Unlike clinical toxicology, where results of presumptive immunoassay tests are used in conjunction with the patient's presenting signs and symptoms, in forensic toxicology, the analytical results may be the only evidence available. Therefore, it is imperative that these results be irrefutable. Confirmatory analyses are, therefore, necessary to achieve this level of confidence. For workplace drug testing, GC/MS analysis is required by the Substance Abuse and Mental Health Services Administration (SAMHSA) (1). Likewise, results of immunoassay screening tests should not be admissible in court cases unless defendable confirmation procedures are performed.

Need for Validation Studies

Most commercial immunoassay tests for drugs of abuse are not designed for use in serum, blood, bile or other tissues. Validation studies have shown that these tests may be used to analyze serum and blood under certain conditions. Radioimmunoassay (RIA) and Enzyme Immunoassay Technique (EMIT) have been used to test for cocaine, benzoylecgonine, Δ^9-9-carboxy-tetrahydrocannabinol-9-carboxylic acid (THCA, marijuana metabolite), barbiturates, benzodiazepines, opiates, and other drug classes in blood (2,3). These studies validated the extraction procedures used, the stability of drugs in blood under various storage conditions, the effect of different anticoagulants used following phlebotomy, and analytical performance of these assays, in terms of its sensitivity and specificity compared to GC and GC/MS.

To date, there have been only a few validation studies conducted on dried blood stains for drug testing. Rattenbury and Taylor measured theophylline in dried blood by EMIT, and noted potential interference from hemoglobin in the eluates (4). Bergqvist et al. measured mefloquine and metabolites using high pressure liquid chromatography (HPLC), and found substantial degradation when samples were stored at 37°C (5). In these studies, blood was deposited onto filter paper, and carefully stored prior to analysis. Without separate validation studies, it is inappropriate to extrapolate these results to other specimens or storage conditions. An important part of all validation studies is the need to document the limits of detection or assay sensitivity. It is also necessary to validate the stability of drug samples in alternate specimens. In dried blood, for example, DuBey et al. found that THCA was not stable at room temperature after 12 weeks of storage (6). In a similar manner, no studies have been conducted on the use of radioimmunoassay for nasal swabs, postmortem bile, or on samples that are diluted into culture media designed for the detection of infections in blood. In fact, blood culture media contains digestive tissue broths designed to inhibit coagulation and leukocyte phagocytosis which

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may interfere with the precipitation and detection steps necessary in the RIA technique. Bile may also not be a suitable specimen for RIA because of the presence of bile acids and salts which may potentially prevent the proper reaction between antigens and antibodies.

Investigational studies have been conducted to characterize the extent to which salicylates interfere with commercial EMIT II assays for urine drugs of abuse testing (7,8). The interfering substance has been identified as the metabolite salicyluric acid. This compound reduces the molar absorptivity of NADH in a concentration-dependent fashion. In vitro fortification studies have demonstrated the quantitative effect of salicylates ingestion on analysis of BE.

Minimum GC/MS Standards for Qualitative Identification

When GC/MS confirmation procedures are used, detection and quantitative identification criteria must be pre-established. Full scan analysis is the best approach, especially when mass spectra are compared against standards that are contemporaneously extracted and injected under identical analytical conditions. When standards are not injected at the same time, the analyst may perform spectral searches against large library databases to identify an unknown in a sample. This approach has limited utility because many libraries do not contain drug metabolites or spectra of derivatized drugs. Selected ion monitoring is used to improve the sensitivity of the analysis. The acceptable minimum of ions is three for the analyte and two for the internal standard (9). Appropriate negative and positive controls and calibrators prepared in the matrix of the sample being tested are required. Chemical ionization (CI)-GC/MS assays using single ion monitoring can be employed if the laboratory is experienced in this methodology, and there is tight control over the extraction and analysis conditions. Although the SAMHSA guidelines permit use of negative or positive ion CI, the National Laboratory Certification Program (NCLP) states that "positive specimens must show analyte peaks free from potentially interfering peaks, and the number of monitored ions must be sufficient to instill confidence in the identification" (9). The main criteria for the identity of a compound in single ion monitoring are the chromatographic retention time and the appropriate ratio of the ion being monitored. Single ion monitoring may be acceptable if there are other corroborating analytic data to substantiate the analysis, such as positive results by immunoassay screening, the presence of the parent compound, or a metabolite of the compound, or the analysis of other biological fluids collected at or near the same time (e.g., urine).

Limits of Detection and Quantitation

The terms "analytical sensitivity," and "limits of detection (LOD)" are usually considered to be synonymous; i.e., the lowest concentration of an analyte that can be statistically distinguished from a sample that is devoid of that analyte (10). The limit of quantitation (LOQ) is the lowest concentration of analyte that the amount of the analyte present can be determined with a predetermined degree of confidence (e.g., 95%). There are two basic approaches for the experimental determination of an assay's LOD and LOQ; the statistical and the empirical approaches.

In the statistical approach, a specimen devoid of the analyte in question is prepared and analyzed multiple times by the assay in question (e.g., $n = 10$) (11). The mean result and the standard deviation (SD) of the mean are determined. Using this approach, it is generally accepted that the statistical LOD is the mean plus 3 times the SD (99% confidence), and the statistical LOQ is the mean plus 10 times the SD. In addition, the LOD should have a signal-to-noise ratio (S/N) of 3:1, and LOQ S/N of 10:1 (15).

The empirical approach is more widely accepted, and involves the use of serial dilutions of a positive sample with an appropriate diluent (e.g., the matrix being tested) (11,12). Dilutions are prepared and tested down to the concentration where the results first fail to meet basic acceptance criteria for the method. In the case of GC/MS, minimum criteria include qualitative values of S/N, retention time, peak shape, ion ratios (for selected ion monitoring or SIM analysis) and library spectral match criteria (for full scan analysis) (10). For LOQ, these criteria must be met 100% of the time, while for LOD, they must be met for a majority of the determinations (e.g., 85 to 95%). Certification agencies require the validation of LOD and LOQ prior to use of these assays on forensic samples (9).

Appropriateness of Standards and Controls

Proper calibration of qualitative and quantitative assays is necessary for the accurate interpretation of results. For immunoassays, a direct calibration or verification should be performed with each analysis. Calibrators should bracket the concentration range of the forensic results to be interpreted. The laboratory needs to demonstrate with the analysis batch that it can detect and quantify the drug concentration found. For GC/MS analysis, calibration should be performed contemporaneously with the analysis of unknowns, and not several days before or after.

Another basic tenet of toxicology is the appropriate use of negative and positive controls. Control materials must be prepared in the matrix of the specimens to be tested, and be analyzed in the same run as the unknown sample. Use of a urine control material to test a dried blood stain, blood culture vial, or bile fluid is unacceptable. In addition, at least one positive control should be assayed wherein the drug concentration is at or near the cutoff concentration of the assay for determining a positive result. For SAMHSA regulated testing, controls at 25% above and below the cutoff concentration are routinely employed.

Analysis of a drug free negative control in any analytical method is also essential. The negative control is used for two major purposes. The first is to identify the presence of endogenous compounds that may give a positive response. Even if it appears that the analytical method is highly specific, there is always the possibility that interferences may occur. This is especially true when the signals being measured are at or near the noise level of the instrument being used. Some endogenous compounds may generate a relatively small signal that may be interpreted as essentially zero response when the analyte is present at high concentrations, but represents a significant response in the absence of the analyte. Secondly, if high concentrations of calibrators or positive controls of the analyte have been analyzed prior to the analysis of the sample, some of the analyte may carry over into the next sample analysis. Solvent blanks analyzed between the calibrators and/or controls may demonstrate that carryover occurred. However this procedure is not infallible and in GC analysis, analytes from a previous analysis may be adsorbed on the column and not be desorbed by a solvent blank. An endogenous substance in the negative control can displace the analyte from the column and produce a positive response. If the analyte is not cleared from the analyzer, then the displacement of column-bound analyte would be affected by the endogenous substance in the sample causing an erroneously positive result. Analyzing a negative con-

trol just prior to analyzing a sample will verify that carryover has not occurred.

Correlation of Quantitative Results to Impairment

Urine testing for many drugs of abuse classes is predicated on the detection of metabolites and not the parent drug. Because of the variability in rates of metabolism, renal clearance, and variations in water excretion rates, an accepted fact in urine drug testing is that quantitative results for urine metabolites cannot be correlated to specific blood concentrations of the metabolites or parent drugs (13). Without a reliable blood drug concentration, conclusions regarding impairment of the subject is difficult. In the specific cases of cocaine and THC, measurement of *inactive* metabolites in urine in the absence of parent drug precludes any conclusions of impairment. Experimental studies have shown that urine drug concentrations for BE can be detected for several days and THCA can persist for several weeks, long after the immediate psychoactive effects of the drug have terminated (14,15).

Impairment by a drug can be assessed by contemporaneous examination of the subject in question by qualified individuals. When blood or serum concentrations are available, impairment can also be inferred if the concentration of the active drug is reliably determined, and the effects of the drug have been extensively studied. For example, the correlation of blood alcohol concentrations with degrees of impairment is used in courts (16). For other drugs of abuse, this correlation is dependent on several factors. Dubowski (17) listed three interrelated variables and three additional confounding factors that influence a drug's effect or potential for impairment: the drug concentration in the biological fluid, its dose, the elapsed time between intake and specimen collection, tolerance, habituation to the drug by the individual, and interactions with other drugs.

Case Summaries

Four cases are presented where certain of the tenets were not adhered to. Transcripts of the proceedings were obtained and reviewed to ensure the accuracy of these summaries.

Case #1

A blood stain that was deposited onto the rug of an automobile over ten years prior to the trial was analyzed for drugs of abuse. The carpet with the dried stain was stored at room temperature during this time. A portion of the carpet containing a blood spot was excised from the vehicle, along with a second portion of the carpet containing no visually apparent blood stain. Each sample was extracted with ammonium hydroxide and dimethyl formamide (DMF). The aliquots were reduced to near-dryness, reconstituted with water, and tested by EMIT for BE, THCA (Table 1), and eight other commonly encountered drugs of abuse. Although results in this case were well below the respective calibrator concentrations for BE and THCA, and there were statements in that laboratory's procedure manual that such results must be reported as negative, the consulting toxicologist reported positive results for these metabolites. Results for the other drugs were never made available.

TABLE 1—*Results of EMIT testing of dried blood stain for cocaine and THC metabolites** (Case #1).

Sample	Cocaine ΔA	THC ΔA
Stained rug (0.7 g)	0.2469	0.4501
Unstained rug (1.0 g)	0.2439	0.4493
Negative standard	0.2439	0.4416
Stain minus negative standard	0.0030	0.0121
Normalized results†	0.0043	0.0044
Standard (300 for cocaine, 20 for THCA)	0.3687	0.4940
Amount found (ratioed from the standard)	5.17 ng	0.85 ng
Concentration (amount ÷ blood volume)	59 ng/mL	9.8 ng/mL

*Based on measurement of iron by inductively coupled plasma spectrometry, the amount of blood present in the stain was determined to be 0.087 mL.

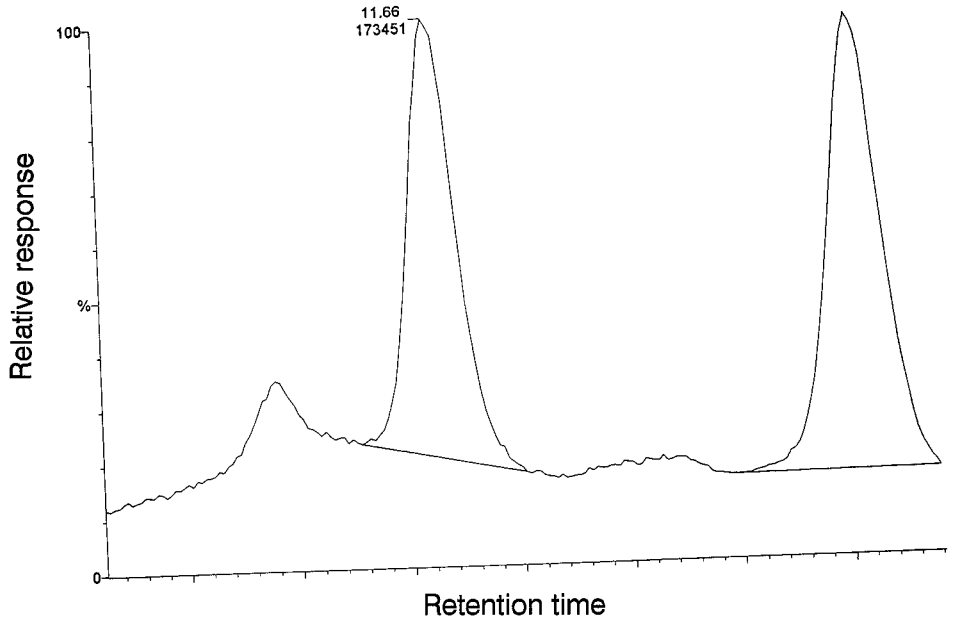
† ΔA of stain divided by amount taken minus unstained carpet divided by amount taken.

No GC/MS confirmations were performed, because the entire sample was consumed in the initial testing.

Case #2

A toxicological analysis was performed on a portion of a pair of woman's panties that appeared to contain a dried blood stain. After addition of a trideuterated THCA internal standard, the stain was simultaneously extracted and methylated in acidic methanol with heating. The extract was filtered and further derivatized in chloroform and trifluoroacetic anhydride (TFA). The extract was dried, dissolved in water/methanol and extracted with hexane. The hexane phase was separated, dried, rederivatized in chloroform with TFA, and dried. The residue was reconstituted in acetone. The sample was injected into a GC/MS/MS (tandem) with negative ion CI, detection using only single stage analysis. Although a similar assay was previously validated and reported from another lab for THC and THCA in whole blood, plasma, and urine (18), no validation studies were conducted by the lab testing the undergarment. Single ion monitoring was performed on the parent ions at *m/z* 457 and 454 for the internal standard and THCA, respectively. The resulting chromatograms are shown in Figs. 1*a* and *b*. The chromatograms were smoothed with a digital Savitski-Golay algorithm (19). The unsmoothed chromatogram of THCA is shown in Fig. 1*c*. Calibrators were prepared by extraction and derivatization of aqueous standards and analyzed several days later. A positive control was prepared by fortifying a blood sample at 10 ng/mL, placing it onto a similar material as the stained garment, and dried. The control was analyzed using the procedure described above. Other than a solvent blank, no negative blood stain or garment control was used. The amount of blood extracted from the panties was estimated by comparing the weight of blood stain and unstained cuttings from the garment. Because the two cuttings were not identical in size, the "area density" of the unstained material was determined by producing and weighing magnified photographs of the cuttings and calculating an unstained area density. From this, an appropri-

FIG. 1—*GC/MS analysis of the extracted dried blood stain for* Case #2. *(*a*) Digitally smoothed ion response at m/z 457.4 corresponding to the deuterated THCA internal standard. (*b*) Digitally smoothed ion response at m/z 454.4 corresponding to the THCA. (*c*) Unsmoothed ion response at m/z 454.4. The retention time in minutes (top number) and area (bottom) are given next to the peak of interest.*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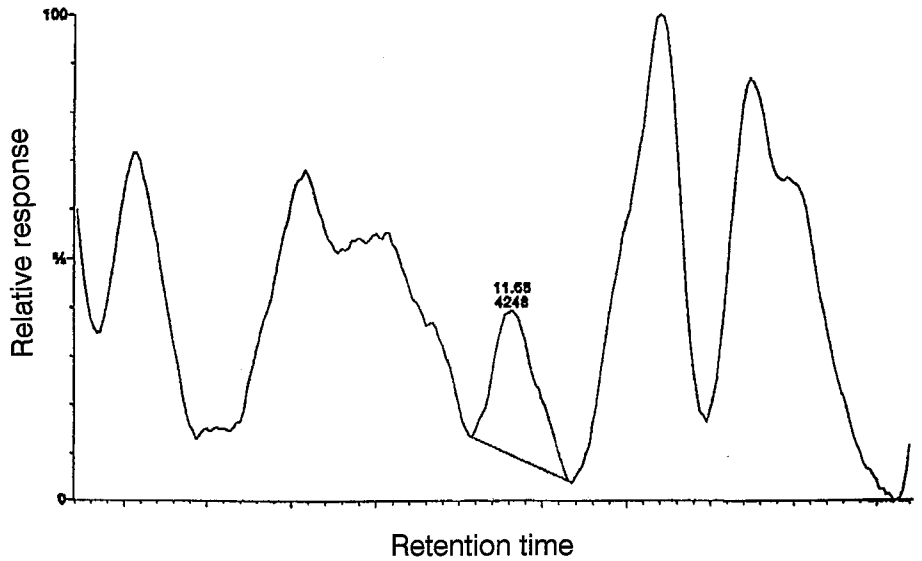
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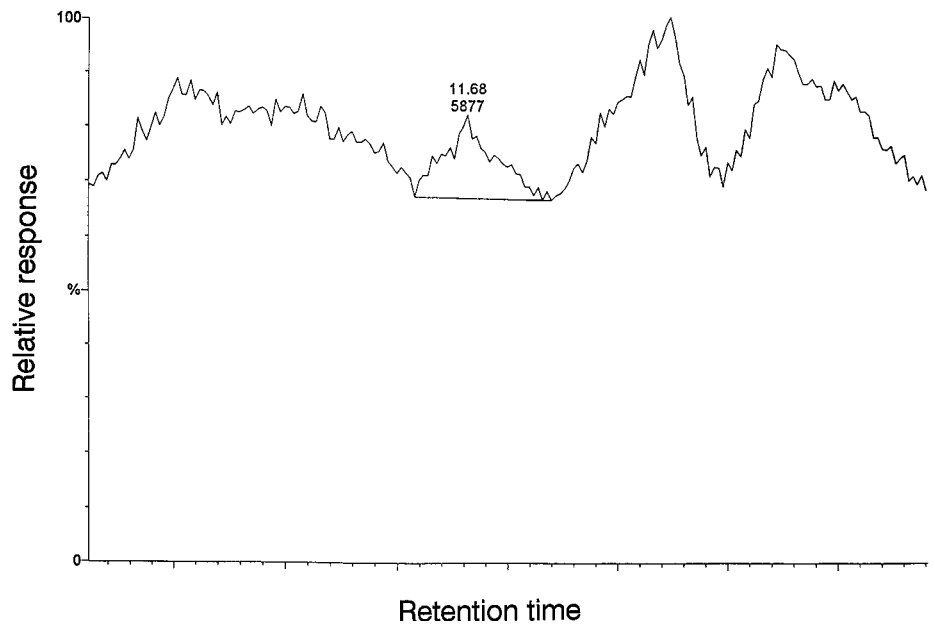
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ate correction for the blood stained cutting was made. It was assumed that the dried blood weighed 18% of the original undried blood. Under these assumptions, the amount of blood in the stain was estimated to be between 25 to 50 μL. Using the calibration curve, the analyst reported a recovery of 650 pg THCA from the panties. This resulted in an estimated THCA concentration in the blood stain of between 12.2 to 27.2 ng/mL.

Case #3

A laboratory reported a quantitative value for THCA of 17.3 ng/mL in blood by GC/MS. The active component, THC, was not detected. The serum concentration of THCA was estimated to be 23.9 ng/mL, by multiplication of the whole blood value by 1.38. This factor was based on a published report that correlates serum to whole blood concentrations for THC, not THCA (20). (There are no comparable conversion factors published for the THCA metabolite.) From the serum THCA concentration, an estimate was computed by a toxicologist as to the time that marijuana was ingested, using a linear regression model for the absorptive phase, and a nonlinear model for the elimination phase of THCA. Parameters for the model were obtained from data generated from a controlled human THC consumption study (21). The consultant used data from 9 of the 10 subjects reported in the study, having rejected data from the tenth subject as being an outlier. He further assumed that the subject in Case #3 fully smoked one marijuana cigarette containing 1% THC, although there was no evidence that any smoking was conducted by the subject. The regression models used by the consultant produced an administration time of either 26 or 78 min prior to blood collection, for the absorption and elimination phases, respectively. From these calculations, he concluded that the subject was impaired by the effects of marijuana at the time that blood was lost from that individual.

TABLE 3—*Raw data for immunoassay results from* Case #4.

Sample	Radioactivity or EMIT Result*		Interpretation†
Radioimmunoassay			
Drug-free urine	28,228	28,738	
Positive urine control (300 ng/mL)	3,338	3,728	
Blood culture vial	10,069	8,498	positive
Swab	17,668		trace positive
Drug free urine	20,672	19,424	
Positive urine control (300 ng/mL)	4,388	3,980	
Bile	17,304	17,164	? negative
Drug free urine	20,192	18,944	
Positive urine control (300 ng/mL)	4,140	4,104	
Postmortem blood	21,992	20,541	negative
EMIT			
Drug free urine	53	54	
Positive urine control (300 ng/mL)	88	88	
Postmortem urine	48		negative

* Radioactivity in counts per minute; EMIT in ΔA.
† Official interpretation from medical examiner report.

TABLE 2—*Summary of toxicology results from* Case #4.

Sample	Drugs Tested	Methodology†	Result
Antemortem			
Blood*	cocaine	RIA	positive
	meperidine, basic drugs, cocaine	GC	negative
	opiates	RIA	negative
	barbiturates	LC	negative
	salicylates	fluor	2.8 mg/dL
Urine	amphetamines, barbs, BE, THC, opiates	EIA	negative
Postmortem			
Blood	ethanol	Conway	negative
	cocaine, opiates	RIA	negative
	meperidine & other basic drugs	GC	negative
	barbiturates	LC	negative
	salicylates	fluoro	20.6 mg/dL
Urine	basic, acidic drugs	TLC	negative
	amphetamines, BE, THC	EIA	negative
Liver (100 g)	pheniramine derivative	TLC	positive
	phenelzine, haloperidol, basic drugs	TLC	negative
Bile	cocaine, opiates	RIA	negative
Stomach contents	salicylates	chemical test	negative
	acetaminophen	TLC	trace
	basic drugs	TLC	negative
Swab	cocaine	RIA	trace

* Culture media, dilution 1:10 with blood.
† GC, gas chromatography, RIA, radioimmunoassay, fluoro, fluorometry, LC, liquid chromatography, EIA, EMIT I (Behring Diagnostics) enzyme immunoassay, Conway, Conway microdiffusion test, TLC, thin-layer chromatography.

Case #4

A toxicologic analysis was performed on biological fluids obtained on a hospital patient, and on fluids and tissues obtained after death. A summary of the findings are shown in Table 2. All samples were deemed by the medical examiner to be negative except for a trace of acetaminophen in stomach contents, salicylates in antemortem and postmortem blood, pheniramine derivative detected by thin-layer chromatography, and BE in the antemortem blood that had been diluted 1:10 with a culture media (originally used to test for a possible blood infection), and a trace of cocaine metabolite on a swab (presumed to be nasal although there was no documentation as to its body site of collection). A GC confirmation for cocaine was performed on the blood culture and produced a negative result. No controls were used for the measurement of BE on the swab. A toxicologist independent of the testing lab was asked to review the results. Using the raw data made available (Table 3), the consulting toxicologist made an interpretation that the antemortem blood in culture media, postmortem bile, and the swabs were positive by RIA for BE. He argued that the BE result for ante and postmortem urine samples were falsely negative by EMIT due to the presence of salicylates (7), and falsely negative by GC because the appropriate BE derivative was not used prior to GC analysis. The toxicologist concluded that the data were consistent with a typical nasal cocaine dose of 100 mg, and that the dose was 12 to 16 h prior

to death. It was inferred that cocaine use by the decedent contributed to her death.

Summary

The need to analyze new and challenging samples and drugs will continue. It is not possible to predict all of the needs that will be placed on the laboratory. Therefore, each laboratory should develop a policy for addressing these situations. If the laboratory accepts these samples for testing, the policy should include criteria for validating or verifying the testing methods to be used. One cannot assume that methods designed for urine testing will necessarily work for other biological materials. This is particularly true for rarely encountered samples such as dried blood stains, bile, or blood diluted with media designed for culturing. Validation studies for nontraditional specimens are time consuming and expensive, and it may be difficult for laboratories to justify conducting these studies for particular cases, especially when the need for testing these types of specimens may never again arise. Nevertheless, important decisions are made on these tests, and at least a minimal validation should be performed. For both drug screening and confirmation testing, calibrators and control samples should be prepared in a matrix that is as similar as possible to the matrix to be tested. These should include drug-free calibrators and control samples as well as samples fortified with the drug(s) being tested. These data should be used to establish the linear range of the method, identify potential matrix effects and most importantly to establish a reporting cutoff.

Workplace drug testing is a highly regulated arena designed with many safeguards to minimize the chance of false positive results. As such, cutoff concentrations are administrative, i.e., there is a built in "buffer zone" that enables a clear distinction between noise, and a true positive analytical signal. No such safeguards exist in forensic toxicology where a quantitative result between the LOD and LOQ, can be reported as positive. However, as in all forensic analyses, the LOD and LOQ for the matrix and environment of the sample should be validated wherever possible, through literature reports or by the testing laboratory. No sample should be reported positive unless the laboratory can demonstrate that a calibrator or control with a concentration less than or equal to that of the sample being tested also tests positive. Both the tested sample and the quality control (QC) samples should meet all qualitative acceptance criteria such as S/N, ion ratios, and retention times. If a quantitative result is reported, the tested sample and the QC materials should meet all quantitative criteria for reporting.

The testing itself should reflect the needs of the case and must include confirmation. Because there is usually a limited amount of sample, testing should be targeted towards suspected drugs, while maintaining enough sample for confirmation of presumptive findings. Most toxicologists consider GC/MS as the "gold standard" for forensic testing, and most attorneys consider results as irrefutable. However, GC/MS can have limitations depending upon the mode of operation, the experience of the operator performing the test, and the toxicologist interpreting the results (22) (as shown). Data can be manipulated to arrive at an erroneous conclusion. The use of single ion monitoring should be performed only under tightly controlled prevalidated conditions, as it is not as definitive as full scan or selected ion analysis. Testing unknown samples concurrently with calibrators and controls enables the documentation of the extraction, derivatization and instrument performance with the unknown sample. Data from controls, calibrators and test samples tested on a different day should not be used.

The consequences of erroneously produced or interpreted test results in criminal or civil cases are no less serious than for workplace testing. Therefore, toxicologists should be conservative when concluding that a test result is positive. In our opinion, an individual should be presumed to be devoid of drugs unless there is overwhelming scientific evidence to the contrary. Corroborative data from the case history, other types of validated analyses, and additional specimens are important, and should be consistent with the facts and history of the case. Circumstantial, hearsay, or undocumented evidence should not be admissible. The cases presented here suggested that the opposite approach might have been taken, i.e., the slightest suggestion of a positive result interpreted as proof of overt drug use. Once in evidence, further steps were taken to suggest impairment. Expert witnesses have an ethical responsibility that should not be taken lightly. Scientific evidence is becoming increasingly complex and technical while making it difficult for the courts and juries to comprehend. One guest editorial of the *Journal* stated that "juries are increasingly making determinations on the credibility of a forensic scientist's evidence, not on scientific fact but on how it is presented" (23). Witnesses should ensure that their opinions are congruent with current scientific standards, and not be manipulated into extending their testimony to support a particular side of a case.

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