Ines B. Collison,[1] Ph.D.; Vina R. Spiehler,[2] Ph.D.; Steve Guluzian;[1] and Paul R. Sedgwick[1]

Setting Cutoff Concentrations for Immunoassay Screening of Postmortem Blood

REFERENCE: Collison IB, Spiehler VR, Guluzian S, Sedgwick PR. Setting cutoff concentrations for immunoassay screening of postmortem blood. J Forensic Sci 1998;43(2):390–394.

ABSTRACT: The objective of this study was to establish the optimum immunoassay cutoff concentrations for screening postmortem blood from coroner's cases for drugs of abuse with a coated tube radioimmunoassay (RIA) to ensure that the results with the coated tube RIA would be equal to or better than those with the previously used double antibody RIA. Immunoassay results (positive or negative) blood were compared to confirmed results on those cases by GC/MS alone or in combination with GLC using either a NPD or FID detector. Four to seven potential cutoff concentrations were evaluated for the drug classes opiates, amphetamines, cocaine and metabolites, and barbiturates. Specimens were 350 postmortem blood specimens and liver homogenates.

The cutoffs chosen for the coated tube RIA using this approach were 5 ng/mL morphine, 25 ng/mL methamphetamine, 500 ng/mL benzoylecgonine, and 500 ng/mL secobarbital. These cutoffs corresponded to a sensitivity and specificity of 94% and 96% for opiates, 93% and 86% for amphetamines, 91% and 96% for cocaine and metabolites and 91% and 87% for barbiturates. The double antibody RIAs were run on the same specimens with cutoffs of 20 ng/mL morphine, 50 ng/mL methamphetamine, 50 ng/mL benzoylecgonine and 1000 ng/mL phenobarbital. The sensitivity and specificity's for the double antibody immunoassay were: >99% and 96% for opiates, 83% and 89% for amphetamines, 98% and 97% for cocaine, 79% and 95% for barbiturates.

KEYWORDS: forensic science, radioimmunoassay, postmortem blood, cutoffs, sensitivity, specificity, forensic toxicology

The choice of a threshold or cutoff concentration for screening of postmortem blood and tissue homogenate specimens for drugs affects both the sensitivity and specificity of the screening tests (1). Cutoffs which minimize the possibility of false positives are often chosen for screening urine from employees or employee applicants, or specimens from living persons accused of a crime. In investigation of death the possibility of false negatives is also of concern. In this study Receiver Operating Characteristic (ROC) analysis (2–4) was used to choose a cutoff concentration for the coated tube radioimmunoassays (RIA) which optimized diagnostic sensitivity, specificity and predictive value. Gas chromatography/mass spectrometry (GC/MS) analysis was used as the measure of presence of the drug (5). In this study GC/MS, gas chromatography with a nitrogen phosphorus detector (GC-NPD) or Toxilab B thin layer chromatography were used as the measure of the absence of drugs or metabolites in the drug class. Specimens were screened for opiates, amphetamines, cocaine and metabolites and barbiturates by a coated-tube RIA and a double antibody RIA. A positive radioimmunoassay result which was confirmed by a second chromatographic test was considered a true positive. A positive immunoassay result which was not confirmed was considered a false positive. A negative immunoassay result on a case for which an acid or a base screen by GC-NPD was also negative was considered a true negative. A negative immunoassay result for which the chromatography screening revealed a drug present was defined as a false negative. The sensitivity and specificity obtained with the optimal cutoff concentrations for the coated tube radioimmunoassay were compared to those previously used in this laboratory with the double antibody radioimmunoassay on post mortem specimens to ensure that the results with the coated tube RIA would be equal to or better than those with the previously used double antibody RIA.

[1]Orange County Sheriff-Coroner, Forensic Science Services, Santa Ana, CA.

[2]Spiehler and Associates, Newport Beach, CA.

Received 22 May 1997; and in revised form 9 July 1997; accepted 10 July 1997.

Methods

Radioimmunoassays—The coated tube radioimmunoassays were obtained from Diagnostic Products Corporation, Los Angeles, CA. They were performed according to manufacturers instructions but 100 μL of whole blood or liver homogenate was analyzed in place of urine and the incubation time was increased to two hours (6).

The double antibody radioimmunoassays were obtained from Roche Diagnostics, Nutley, NJ. They were performed according to manufacturer's instructions except that 100 μL of whole blood or liver homogenate was analyzed in place of urine, 1 mL of labeled antigen was used and the incubation time was increased to one hour (7).

GC-NPD—Basic drugs screen: Promazine (1000 ng/mL) or N-propyl amphetamine (1000 ng/mL) were added to the samples as internal standards. The samples were adjusted to basic pH with NaOH and extracted with butyl chloride. The extracts were dried and then reconstituted in methanol and injected into a gas chromatograph equipped with a nitrogen phosphorus detector (NPD) or into the GC/MS.

GC/MS—An HP 5890 gas chromatograph coupled with and HP 5970 or HP 5972 mass spectrometer was used.

Opiates—A modification of a previously published method (8) was used. The extracts were derivatized with TFA. Deuterated morphine and codeine were used as internal standards. Calibration standards were 1000, 100, and 20 ng/mL (cutoff) morphine and

codeine. The ions monitored were 367 amμ and 477 amμ for morphine and 395 amμ and 282 amμ for codeine.

Cocaine—A modification of a previously published method (9) was used. The extracts were derivatized with DMFdiPA. Deuterated cocaine, benzoylecgonine and cocaethylene were used as the internal standards. Calibration standards were 500, 200, and 20 ng/mL (cutoff) cocaine, benzoylecgonine and cocaethylene. The ions monitored were 182 amμ, 303 amμ and 272 amμ for cocaine; 210 amμ, 331 amμ and 272 amμ, for benzoylecgonine and 196 amμ, 317 amμ and 272 amμ for cocaethylene.

Amphetamines—A modification of a previously published method (10) was used. The extracts were derivatized with TFA. Deuterated amphetamine and methamphetamine were used as the internal standards. Calibration standards were 1000, 500, and 25 ng/mL (cutoff) amphetamine and methamphetamine. The ions monitored were 140 amμ and 118 amμ, for amphetamine and 154 amμ and 118 amμ for methamphetamine.

Barbiturates—A previously published method (11) was used. After solid phase extraction the extracts were injected into a gas chromatograph with a flame ionization detector or a GC/MS for full ion scan. Various internal standards were used depending on the identity of the barbiturates in the sample. Calibration concentrations were 10 μg/mL barbiturate and a full calibration curve was run for quantitation.

ROC Analysis—The number of true positives TP, false negatives FN, false positives FP and true negatives TN, was determined for four to seven possible cutoff concentrations (for example at 50, 100, 500, 1000, 2000, 3000, and 4000 ng/mL benzoylecgonine equivalents) by comparison of the radioimmunoassay result to the result by GC/MS or GC-NPD for opiates, amphetamines and cocaine. For the barbiturates the confirmation methods were GC/FID, GC/MS full scan or Toxilab B. A sample was considered a true positive (TP) if both the radioimmunoassay and the chromatographic results were in concordance, i.e., both positive for cocaine (cocaine present above the putative cutoff concentration by GC/

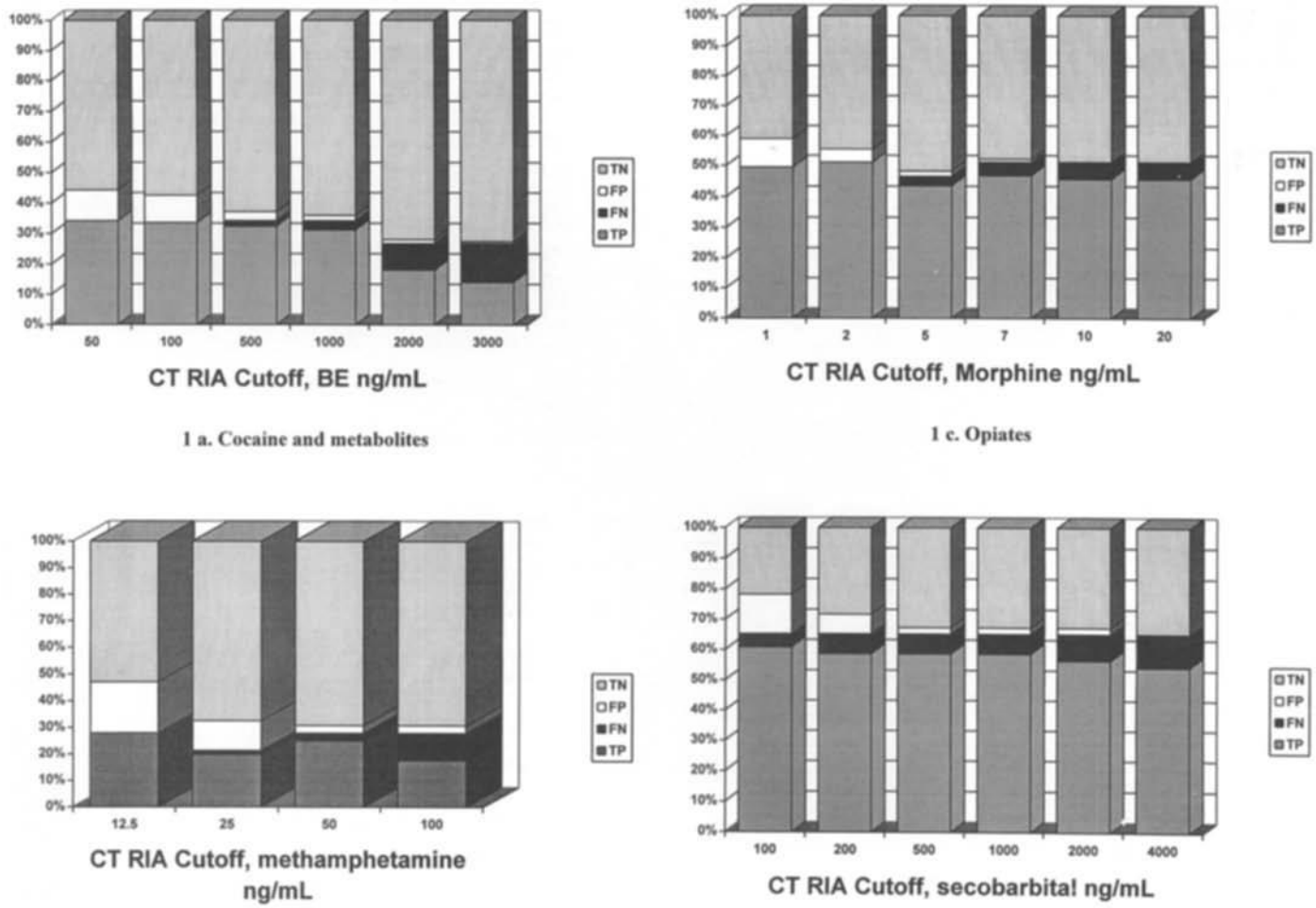

FIG. 1—*TP, FN. FP. TN Analysis for Different Cutoffs for CT RIA. The percent true positives (positive by both the coated tube RIA and by GC/MS), true negatives, false positives and false negatives are shown for a.) cocaine, benzoylecgonine and cocaethylene are shown for 57 positive blood and liver specimens and 110 negative specimens at cutoffs of 50, 100, 0.500, 1000, 2000, or 3000 ng/mL; b.) methamphetamine for cutoffs at 12.5, 25, 50, and 100 ng/mL methamphetamine* (n = *141); c.) opiates for cutoffs at 1, 2, 5, 7, 10* (n = *170) and d.) barbiturates, for cutoffs at 100, 200, 0.500, 1000, 2000, and 4000 ng/mL secobarbital* (n = *73).*

TABLE 1—*Sensitivity and specificity for the two immunoassays.*

	CT RIA			DA RIA		
Screen	Cutoff	Sensitivity	Specificity	Cutoff	Sensitivity	Specificity
Opiates	5 ng/mL	94%	96%	20 ng/mL	100%	96%
Amphetamines	25	93%	86%	50	83%	89%
Cocaine	500	93%	96%	50	98%	97%
Barbs	500	91%	87%	1000	79%	95%

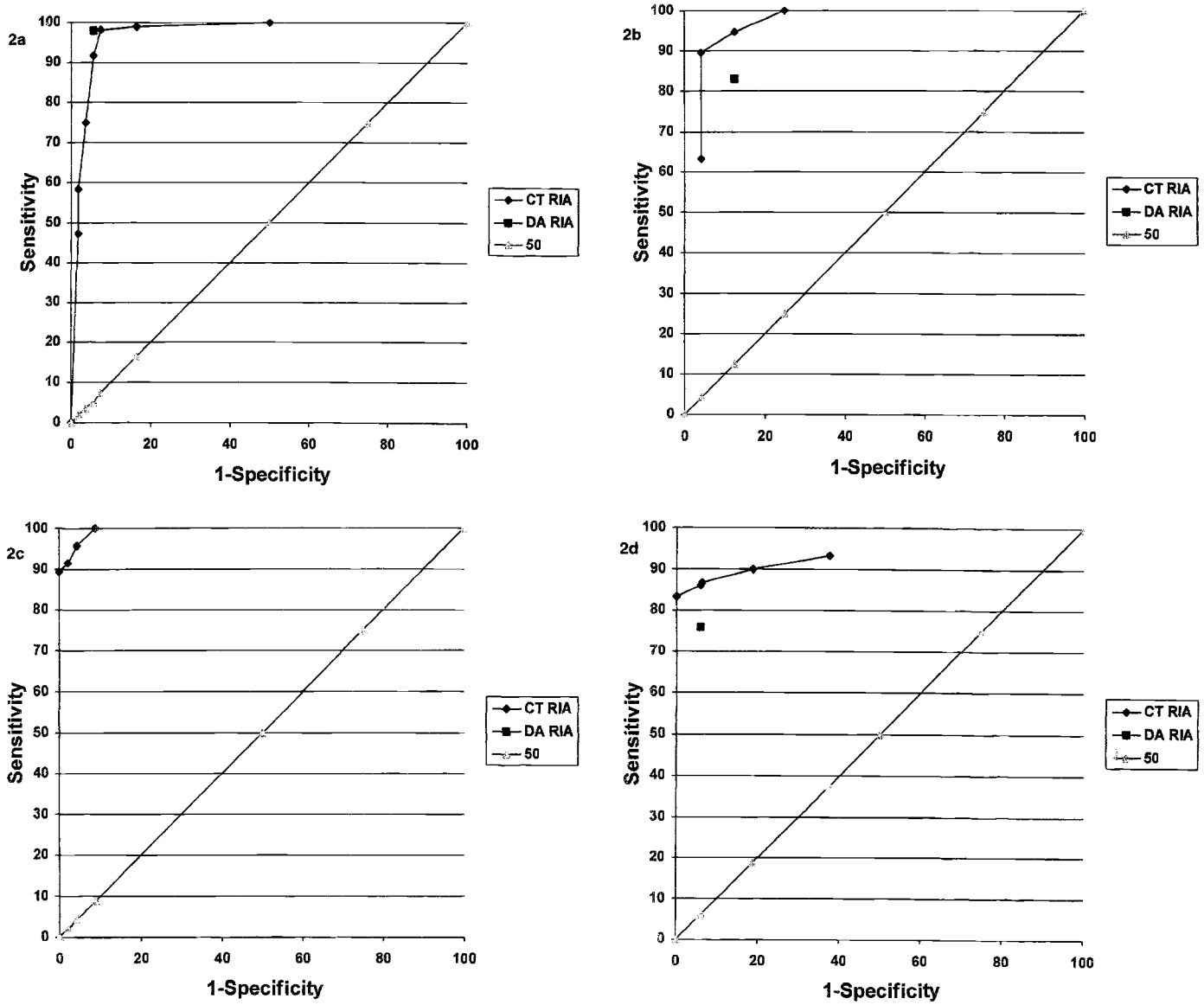

FIG. 2—*ROC Graph for Two RIAs for Different Cutoffs. The percent sensitivity (percent true positives) was plotted vs 1-specificity (the false positives rate) for the two radioimmunoassays. a.) cocaine the ROC curve for cutoffs of 50, 100, 500, 1000, 2000, and 3000 ng/mL benzoylecgonine for the coated tube assay and 50 ng/mL benzoylecgonine for the double antibody assay calculated from comparison to GC/MS* (n = *167); b.) methamphetamine, the ROC curve for cutoffs at 12.5, 25, 50, and 100 ng/mL methamphetamine for the coated tube assay and for 50 ng/mL methamphetamine for the double antibody assay calculated from comparison to GC/MS* (n = *141); c.) opiates, the ROC curve for cutoffs at 1, 2, 5, 7, 10, and 20 ng/mL morphine for the coated tube assay and for 20 ng/mL morphine for the double antibody assay calculated from comparison to GC/MS* (n = *170) and d.) barbiturates, the ROC curve for cutoffs at 100, 200, 500, 1000, 2000, and 4000 ng/mL secobarbital for the coated tube assay and 1000 ng/mL phenobarbital for the double antibody assay* (n = *73).*

MS), or a true negative (TN) if both were negative for cocaine (no drugs found by GC/MS or GC-NPD). Samples for which the radioimmunoassay was positive (cpm below the mean of the cutoff calibrator cpm) but the GC/MS result revealed cocaine concentrations negative or below the LOQ were defined as false positives (FP). Samples for which the radioimmunoassay result was negative (cpm above the mean of the cutoff calibrator) but the GC/MS result showed cocaine concentrations above the LOQ of the assay were defined as false negatives (FN). Sensitivity and Specificity were calculated according to the following formulas (3):

$$\text{Sensitivity} = TP/(TP + FN)$$

$$\text{Specificity} = TN/(TN + FP)$$

Sensitivity was plotted vs 1-specificity for the six possible cutoff concentrations to obtain ROC curves (4).

Results

The optimum cutoff for the immunoassay was judged to be the analyte concentration for the cutoff calibrator which minimized the false positive and false negative results. Figure 1 shows the relative true positive, false positive, false negative and true negative results for cocaine, opiates, barbiturates and methamphetamine for different cutoff concentrations. The optimum cutoff values for the coated tube radioimmunoassay were: cocaine 500 ng/mL BE; opiates 5 ng/mL morphine; barbiturates 500 ng/mL secobarbital; and methamphetamine 25 ng/mL methamphetamine.

Sensitivity was calculated as the true positive rate or the confirmation rate by chromatographic methods. Specificity was calculated as the true negative rate. The cutoffs, sensitivity and specificity for the two immunoassays for all four screens at their optimum cutoff concentrations are shown in Table 1.

The Relative Operating Characteristic curves (sensitivity vs 1-specificity) were plotted for the immunoassays to determine the relative accuracy. The ROC plot for the immunoassays for cocaine and metabolites, opiates, barbiturates and methamphetamine are shown in Fig. 2.

The agreement between the coated tube and the double antibody radioimmunoassays is shown in Table 2. Overall, in four screens on 350 specimens the two radioimmunoassays agreed in 1305 and disagreed in 89 results. In general at these cutoffs the coated tube RIA had greater specificity and the double antibody RIA had greater sensitivity. This is shown for each screen in Fig. 3.

Discussion

In the Orange County Sheriff-Coroner's Forensic Science Service toxicology laboratory the double antibody Abuscreen radioimmunoassay was used for screening postmortem specimens and the coated tube radioimmunoassay was used for screening blood from living persons accused of crimes (driving under the influence of alcohol or drugs, being under the influence of a controlled substance, felonies, etc.). Because the Abuscreen double antibody radioimmunoassay has been discontinued, the laboratory evaluated the possibility of using the coated tube radioimmunoassay for screening postmortem specimens. The cutoff concentrations established for screening blood from living persons were set to reduce the possibility of false positive results. The cutoff concentrations in use for screening blood of living persons with the coated tube RIA were: 5 ng/mL morphine for opiates, 50 ng/mL methamphetamine for amphetamines, and 2000 ng/mL benzoylecgonine for cocaine and metabolites. The objective of screening in postmortem cases was to minimize both false positive and false negative results. From the analysis carried out in this study, the cutoff concentration for cocaine was found to be too high and were reduced to 500 ng/mL benzoylecgonine in analysis of postmortem specimens to reduce the possibility of false negative results. The values for opiates and for methamphetamine used in living persons were found to perform acceptably for postmortem cases and were not changed. The cutoff concentration for barbiturates was set to 500 ng/mL secobarbital.

In addition to the different objectives in postmortem and antemortem screening which require setting different cutoff concentrations, different cutoffs are required for the radioimmunoassays from different manufacturers because the two different radioimmunoassays have a different range of cross-reactivities with drugs, drug metabolites and related chemical compounds. This is a result of different sources for the antibodies, different procedures for

TABLE 2—*Agreement between the CT RIA and the DA RIA.*

Screen	R+/D+	R+/D(−)	R(−)/D+	R(−)/D(−)	n
Amphetamines	55	6	10	278	349
Opiates	90	23	0	237	350
Cocaine*	61	0	12	126	199
Cocaine†	17	2	2	130	151
Barbs‡	31	31	3	134	199
Barbs§	7	0	0	139	146

*vs CT RIA using 2000 ng/mL cutoff. †vs CT RIA using 500 ng/mL cutoff.
‡vs. CT RIA using 1000 ng/mL phenobarbital cutoff.
§vs CT RIA using 500 ng/mL secobarbital cutoff.

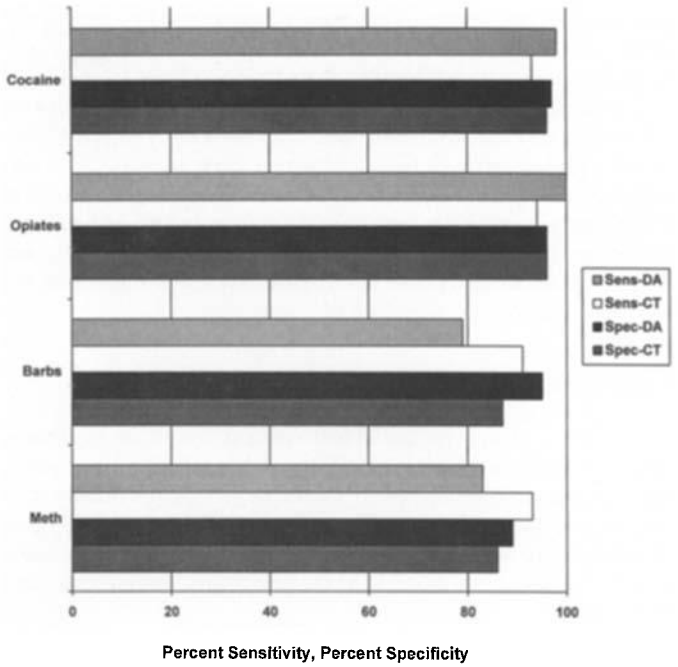

FIG. 3—*Sensitivity and Specificity for the Two Radioimmunoassays. Sensitivity or true positive rate (light gray bars for double antibody RIA, white bar for coated tube RIA) and Specificity or true negative rate (black bars for double antibody and dark gray bars for coated tube RIA) are shown for the four drug classes. These values are for the following cutoffs: cocaine 50 and 500 ng/mL BE; opiates 20 and 5 ng/mL morphine; barbiturates 1000 ng/mL phenobarbital and 500 ng/mL secobarbital; and methamphetamine 50 and 25 ng/mL methamphetamine for the double antibody and coated tube radioimmunoassays respectively.* n = *167 cocaine, 170 opiates, 73 barbiturates and 141 methamphetamine.*

preparing the drug-protein carriers which were used for production of the antibodies and changes to the antibodies which occur in bonding to the tube wall in the coated tube radioimmunoassays.

The sensitivity and specificity values obtained in this study are conservative in that we included a large number of selected cases which had discrepancies between the two immunoassays and cases which were expected to prove difficult for screening tests such as those involving multiple opiates other than morphine and codeine. Therefore, the positive predictive value or confirmation rate was not calculated since the specimens analyzed in the study were not random or sequential sampling of cases.

Conclusion

By choice of appropriate cutoff concentrations for the coated tube radioimmunoassay, a sensitivity and specificity on postmortem specimens comparable to the those of the previously used double antibody radioimmunoassay was obtained. This ensures that the results with the coated tube RIA on postmortem specimens will be equivalent to those with the previously used double antibody RIA.

References

1. Ferrara SD, Tedeschi L, Frison G, Brusini G, Castagna F, Bernardelli B, et al. Drugs of abuse testing in urine: statistical approach and experimental comparison of immunochemical and chromatographic techniques. J Anal Toxicol 1994;18:278–91.
2. Zweig MH, Campbell G. Receiver-Operating Characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 1993;39:561–77.
3. NCCLS GP-10P. Assessment of clinical sensitivity and specificity of laboratory tests. Villanova, PA; National Committee for Clinical Laboratory Standards, 7(6), April 1987.
4. Galen RS, Gambino SR. Beyond normality. New York, NY: John Wiley & Sons, 1975;13.
5. Spiehler VR, O'Donnell CM, Gokale DV. Confirmation and certainty in toxicology screening. Clin Chem 1988;33:1535–9.
6. Appel T, Wade N. Screening of blood and urine for drugs of abuse utilizing DPC's Coat-A-Count Radioimmunoassay kits. J Anal Toxicol 1989;13:274–6.
7. Spiehler VR, Sedgwick P. Radioimmunoassay screening of whole blood samples for drugs of abuse. J Anal Toxicol 1985;9:63–70.
8. Reed D. Comparison of spectrofluorometric and GC/MS procedures for the quantitation of morphine in blood and brain. Clin Toxicol 1979;14(2):169–80.
9. Spiehler VR, Reed D. Brain concentrations of cocaine and benzoylecgonine in fatal cases. J Forensic Sci 1985;30:1003–11.
10. Jain N, Sneath T, Budd R. Amphetamines in methodology for analytical toxicology ed. Irving Sunshine, CRC Press, Cleveland, OH, 1971;24–6.
11. Chen XH, Franke J-P, Wijsbeek J, DeZeeuw RA. Isolation of acidic, neutral and basic drugs in whole blood using a single mixed-mode solid-phase extraction column. J Anal Toxicol 1992;16:351–5.

Additional information and reprint requests:
Vina Spiehler, Ph.D.
422 Tustin
Newport Beach, CA 92663