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TECHNICAL NOTE

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Screening Postmortem Whole Blood for Oxycodone by ELISA Response Ratios*

ABSTRACT: The objective of this study was to investigate the accuracy of screening postmortem whole blood for oxycodone using the ratio of the oxycodone immunoassay response to the response for the specimen obtained with a general opiate-class immunoassay. Fifty eight specimens which were negative for opiates and 158 postmortem whole blood specimens positive for opiates including 66 specimens known to contain oxycodone were assayed. Specimens were diluted 1:5 with assay buffer and analyzed by both the Neogen Oxymorphone/Oxycodone ELISA and the Neogen Opiate Group ELISA (Neogen Corporation, Lexington KY). The oxycodone equivalents in ng/mL from the Oxymorphone/Oxycodone ELISA were divided by the morphine equivalents in ng/mL from the Opiates ELISA to obtain an Oxycodone/Opiates Response Ratio. This ratio was compared with the GC/MS data for all specimens and for opiate positive specimens. Receiver Operating Characteristic (ROC) analysis suggested that optimum relative response ratio was 2.0. The sensitivity of the ELISA response ratio for the presence of oxycodone at a response ratio cutoff of 2.0 was 89.4% ± 3.8% and the specificity was 88.1% ± 3.2%. Specimens with a ratio of 2.0 or higher had a greater than 50% probability (positive predictive value) of containing oxycodone in a population with a greater than 15% prevalence of oxycodone.

KEYWORDS: forensic science, oxycodone, ELISA, differential immunoassay, opiates

A number of cases of diversion of OxyContin®, and related prescription opiate narcotics, for illegal use and abuse have been in the national press this past year (1). As a result of the popularity of these drugs and the diversion to street use, oxycodone may be increasingly encountered in driving under the influence, abuse and overdose cases (2,3). Oxycodone and related semisynthetic thebain derivatives may be missed by general opiate screens, most of which are only weakly cross-reactive with the C6-keto-opioids (3,4), and by confirmation procedures which use GC/MS Selected Ion Monitoring (SIM) parameters set for morphine and codeine (5).

Differential immunoassay has been used to compare the response of a compound-specific directed immunoassay to the response of a class-specific immunoassay (6,7). Differential immunoassay using relative response ratios can be used to identify which opiate positive specimens may contain oxycodone or related C6-keto-opioids. By dividing the response of a second, oxycodone-directed, immunoassay by the specimen response in a general opiate screen immunoassay, a relative immunoassay response ratio is obtained. Oxycodone-involved cases can be identified by response ratios above an empirical cutoff threshold. This elevated response ratio indicates which specimens should be confirmed for oxycodone, oxymorphone, hydrocodone and/or hydromorphone in addition to the confirmation for morphine and codeine. This paper describes the validation of a relative response ratio for identification of oxycodone-containing whole blood specimens using the Neogen Oxymorphone/Oxycodone ELISA and the Neogen Opiates ELISA (Table 1).

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Methods

Fifty eight specimens which were negative for opiates and one hundred fifty eight postmortem whole blood specimens positive for opiates including sixty-six specimens known to contain oxycodone were assayed. Thirty eight oxycodone positive specimens were obtained from the Office of the Medical Examiner of Travis County, Austin, TX. The remainder of the oxycodone positive, opiate positive and opiate negative specimens were obtained from the Office of the Chief Medical Examiner, Oklahoma City, OK.

Neogen Microtiter Plate Assays

Specimens were diluted 1:5 with assay buffer and analyzed by both the Neogen™ Oxymorphone/Oxycodone ELISA and the Neogen™ Opiate Group ELISA (Neogen™ Corporation, Lexington KY) (Table 1). Both immunoassays are microtiter plate-based ELISAs using horseradish peroxidase-labeled drug and anti-drug antibody immobilized to the microplate wells. Spiked whole blood calibration standards, specimens, the manufacturer's EIA standard, and negative and positive synthetic urine based controls were run on each plate. For opiates, standard concentrations were 0, 1, 5, 10, 20, 50 and 100 ng/mL morphine. For oxymorphone/oxycodone, the spiked standard concentrations were 0, 1, 5, 10, 20, 50 and 100 ng/mL oxymorphone. Diluted drug-enzyme conjugate was

TABLE 1A—*Cross-reactivity of the Neogen Oxymorphone/Oxycodone ELISA.*

Compound	Percent Cross-reactivity
Oxycodone	400%
Oxymorphone	100%
Hydrocodone	30.80%
Hydromorphone	12.30%
Codeine	5.30%
Ethylmorphine	5.30%
Thebaine	3.40%
Morphine	1.70%
Levorphanol	1.40%
Noroxymorphone	0.53%
Naloxone	0.23%
Norcodeine	0.02%
Etorphine	<0.02%
Amitriptyline	0.01%

TABLE 1B—*Cross-reactivity of the Neogen Opiates Group ELISA.*

Compound	Percent Cross-reactivity
Ethylmorphine	1043%
Codeine	730%
Hydrocodone	228%
Morphine	100%
Thebaine	72%
Hydromorphone	35.60%
Levorphanol	23.10%
Oxycodone	5.20%
Norcodeine	1.94%
Morphine-3-B-D-Glucuronide	1.93%
Nalorphine	0.56%
Normorphine	0.28%
Oxymorphone	0.22%
Levallorphan	0.19%
Meperidine	0.07%
Amitriptyline	0.04%
Triimipramine	0.03%
Dextromethorphan	0.03%
Imipramine	0.03%
Doxepin	0.02%
Chloropromazine	0.02%
Nortriptyline	0.01%

added to the microtiter plate wells and the mixture incubated at room temperature for 45 minutes. After incubation the plate was washed five times with wash buffer (phosphate buffer with Tween 20) using a Bio-Tek Elx50 Microplate Strip Washer (Bio-Tek Instruments, Highland Park, Winoski, VT) to remove any unbound sample or drug-enzyme conjugate. K-Blue® substrate (tetramethylbenzidine (TMB) plus hydrogen peroxide) was added and after a 30-min substrate incubation, the reaction was halted with the addition of Red Stop Solution (a non-acid peroxidase stop solution). The test was read using an Elx800 Universal Microplate Reader equipped with a 650 nm filter (Bio-Tek Instruments, Highland Park, Winoski, VT).

Calibration curves were plotted as log concentration vs the logit of the ratio of the mean absorbance at each concentration divided by the mean absorbance of the zero standard (B/B_o). The oxymorphone or morphine equivalents were estimated from the calibration curve using the ratio of the mean absorbance of the specimen to the mean absorbance of the zero standard.

The oxymorphone equivalents in ng/mL from the Oxymorphone/Oxycodone ELISA were divided by the morphine equivalents in ng/mL from the Opiates ELISA to obtain an Oxycodone/Opiates Response Ratio. This ratio was compared to the GC/MS data for all specimens and for opiate positive specimens.

Assay Precision

Intraassay precision was determined from 40 replicate analysis of the 1:5 dilution of the 20 ng/mL oxymorphone standard. Interassay precision was calculated from the absorbance ratios for the standards and controls run at the beginning and end of each microtiter plate experiment. The targets for the urine controls were zero and 100 ng/mL oxymorphone.

Oxycodone, Hydrocodone and Other Drugs by GC/MS

The analysis for oxycodone, as well as other drugs, was performed by a previously published method for screening basic drugs (8). Modifications to the published method were made to the extraction (9). At the Office of the Chief Medical Examiner, Oklahoma City, OK, following the addition of internal standard, ammonium hydroxide (0.5 mL) and 7.5 mL chlorobutane were added to 2.5 mL of blood and urine or 1.0 g of a tissue preparation (1:4 homogenate). The organic layer was removed following mixing and centrifugation and 2.5 mL of 1N sulfuric acid was added. Following mixing and centrifugation, the organic layer was aspirated and 0.5 mL ammonium hydroxide was added to the acid layer; 2.5 mL chlorobutane was added and the samples were mixed and centrifuged. The organic layer was transferred to a glass conical test tube and the tubes were placed in a 40°C waterbath. The solvent was placed under a gentle stream of nitrogen. Methanolic HCl (20 μL) was added when about 0.5 mL of the chlorobutane layer remained. The remaining solvent layer was then dried to residue. Fifty μL methanol was then added and 1 μL was analyzed by GC/MS with electron impact ionization in full scan mode. This method is a general screen and detects codeine, hydrocodone, oxycodone, oxymorphone and benzodiazepines as well as other drugs. The LOQ and LOD for oxycodone are approximately 25 ng/mL and 50 ng/mL.

Free morphine and 6-monoacetylmorphine (6-MAM) were analyzed by GC/MS following isolation by solid phase extraction and derivatization with trifluoroacetic anhydride (TFAA). The samples (1.0 μL) were injected on a Hewlett-Packard 5890/5970 GC/MS equipped with a capillary column (15 meter HP – 1; Hewlett-Packard). The injector temperature was 250°C and the detector temperature was 280°C. The initial oven temperature was 150°C. The oven temperature was ramped at 12°C/min to 300°C. The GC/MS was run in the selected ion monitoring (SIM) mode monitoring three ions for morphine (m/z 364, 477, 311) and 6-MAM (m/z 364, 411, 311). A single ion was monitored for the d3-morphine internal standard (367). Quantitation was based on the area ratio between the 364 ion of the analytes and the 367 ion of the d3-morphine. A calibration curve for the morphine is extracted simultaneously with the samples. The method is linear between 50 and 1,600 ng/mL, (LOQ 50 ng/mL).

At the Office of the Medical Examiner of Travis County, Austin, TX, a similar modification of the Forester method (8) was used for an acid-neutral-alkaline drug screen. Two ml of blood containing internal standards (2 μg SKF-525 and 6 ug mephentoin) were extracted with 5 mL n-butyl chloride before and after addition of ammonium hydroxide. Alkaline drugs were back extracted from the n-butyl chloride into 5 mL of 1.0 N HCl, then the n-butyl chloride was evaporated to dryness at 50°C under a nitrogen stream. The acid and neutral drug residue was reconstituted in hexane and acetonitrile for manual injection into the GC/MS. Alkaline drugs were extracted into 100 μL chloroform after addition of 0.8 ml

conc. ammonium hydroxide to the HCl layer. Two μL of the chloroform button was injected into the HP5973 GC/MS using pulsed splitless injection, injector at 250°C, initial oven temperature 70°C for 2 min then ramped at 25°C/min to 290°C. The HP5970 was run in full scan mode (m/z 37–550 amu). For oxycodone quantification, calibrators at 50, 100, 250, 500 and 1000 ng/mL spiked into blood were analyzed in parallel with the unknown blood specimens. The relative retention time for oxycodone was 1.083 average with a 5% range of 1.078–1.084. Using the peak area of the 315 amu ion for oxycodone relative to 86 amu ion for SKF-525, a limit of detection of 25 ng/mL and a limit of quantitation of 50 ng/mL was obtained.

Sensitivity and Specificity

Sensitivity, the true positive rate, was calculated from the tally of true positives and false negatives determined by comparison of the GC/MS findings as: Sensitivity = TP/(TP + FN). Specificity was calculated as: Specificity = TN/(TN + FP) (10,11). Because sensitivity and specificity are probabilities, the standard error (SEp) is equal to SEp = square root [p(1 − p)/n]. Receiver Operating Curves (ROC) were obtained by plotting the sensitivity at each putative response ratio cutoff vs. (100 – % specificity) at that cutoff value (12). The positive predictive value was calculated as fp/[fp + (1 − f)(1 − q)] where f is the prevalence in the population to be tested, p is the sensitivity and q is the specificity (10,11).

Results

Oxymorphone/Oxycodone ELISA Precision

The intra-assay precision of the Neogen Oxymorphone/Oxycodone ELISA for forty replicates of spiked whole blood at 20 ng/mL oxymorphone was calculated from the absorbance with a mean optical density (OD) 0.334 ± .029 SD for a % CV of 8.7%. The intra-assay precision of the B/B_o ratio had a mean of 37.8 ± 2.3 (6.2% CV) and for estimated concentration, the intra-assay mean was 18.8 ng/mL ± 3.5 ng/mL (18.9% CV).

The inter-assay precision of the ratio of the absorbance of the 20 ng/mL oxymorphone whole blood calibrator to that of the zero whole blood calibrator (B/B_o) had a mean of 36.54 ± 2.04 SD or a % CV of 5.59% (n = 8). The inter-assay mean for estimated concentration was mean 18.96 ± 2.00 (10.6% CV, n = 8). The inter-assay precision calculated from the B/B_o ratios of the urine controls run in each assay had a mean of 89.8 ± 8.8, n = 8, (9.9% CV) for the negative control and a mean of 9.13 ± 0.75, n = 8 (8.29% CV) for the positive control. The positive control was always positive and the negative urine control was always negative. The data for inter-assay precision was taken from eight different runs performed over a three-month period. The plates were manually pipetted.

Oxymorphone/Oxycodone ELISA Cutoffs, Sensitivity and Specificity

The oxymorphone equivalents from the Neogen Oxymorphone/Oxycodone ELISA were compared to GC/MS results on oxycodone positive specimens (66 oxycodone positive specimens) and specimens known to contain no opiates (58 specimens). The true positives, false negatives, false positives and true positives for the ELISA in this comparison for possible cutoff levels are shown in Fig. 1*a*. From the true positives and false negatives, the sensitivity or true positive rate for each cutoff was calculated. From the true negatives and false positives, the sensitivity or true negative rate at each cutoff was calculated. The sensitivity was plotted vs 100% minus the specificity to obtain a Receiver Operating Characteristic (ROC) curve. The optimum cutoff for the assay was determined to be 20 ng/mL from the inflection point of the ROC curve. The sensitivity and specificity of the 20 ng/mL oxymorphone equivalents cutoff was 98.4% ± 1.5% and the specificity was 98.3% ± 1.7% (Table 2).

TABLE 2—*Sensitivity and Specificity Neogen Oxymorphone/Oxycodone ELISA.*

Oxycodone Positive and Opiate Negative specimens				
1:5 Dilution, 20 ng/mL cutoff			ELISA Results	
			+	−
	Result by GC/MS	+	65	1
		−	1	57
	Sensitivity = 65/66 = 98.4% ± 1.5%			
	Specificity = 57/58 = 98.3% ± 1.7%			
All specimens				
	Result by GC/MS	+	65	1
		−	29	121
	Sensitivity = 65/66 = 98.4% ± 1.5%			
	Specificity = 121/150 = 80.7% ± 3.2%			

However, the Oxymorphone/Oxycodone ELISA also produced responses in opiate positive specimens containing no oxycodone which ranged from 0.0 to 650 ng/mL oxymorphone equivalents depending on the concentrations and the cross-reactivity of the opiates present in each specimen. Other opiate values in these specimens ranged from trace present to 13,600 ng/mL morphine, 1,900 ng/mL codeine and 650 ng/mL hydrocodone. Repeating the above analysis on all data (58 opiate negative specimens and 158 opiate positive specimens) revealed that a cutoff of 20 ng/mL oxymorphone equivalents would have erroneously identified 28 specimens as positive which according to GC/MS analysis did not contain oxycodone (false positives) (Table 2 and Fig. 1*b*). To find a parameter to identify which opiate positive specimens might contain oxycodone, the relative response ratio was investigated.

Relative Response Ratio

Specimens containing oxycodone produced large responses in the oxycodone-directed immunoassay and positive but weaker responses in the general Opiate Group immunoassay. A relative response ratio was calculated by dividing the oxymorphone equivalents in ng/mL from the Oxymorphone/Oxycodone ELISA by the morphine equivalents in ng/mL from the Opiates ELISA to obtain an Oxycodone/Opiates Relative Response Ratio. Response ratios were calculated for 189 specimens; ratios involving specimens with a zero result could not be calculated. The median relative response ratio for oxycodone-containing specimens was 12.9; the mean was 33.7 (n = 66). The median response ratio for all opiate positive specimens not containing oxycodone was 0.055 and the mean was 7.3 (n = 123).

ROC analysis was used to find the optimum response ratio cutoff value for identifying opiate positive specimens containing oxycodone and to determine the probability that a specimen with this ratio would contain oxycodone or a related C6-keto-opiate. The true positives, false negatives, false positives and true negatives for different relative response ratios from 0.1 to 10 are shown in Fig. 2. From the true positives and false negatives, the sensitivity or true

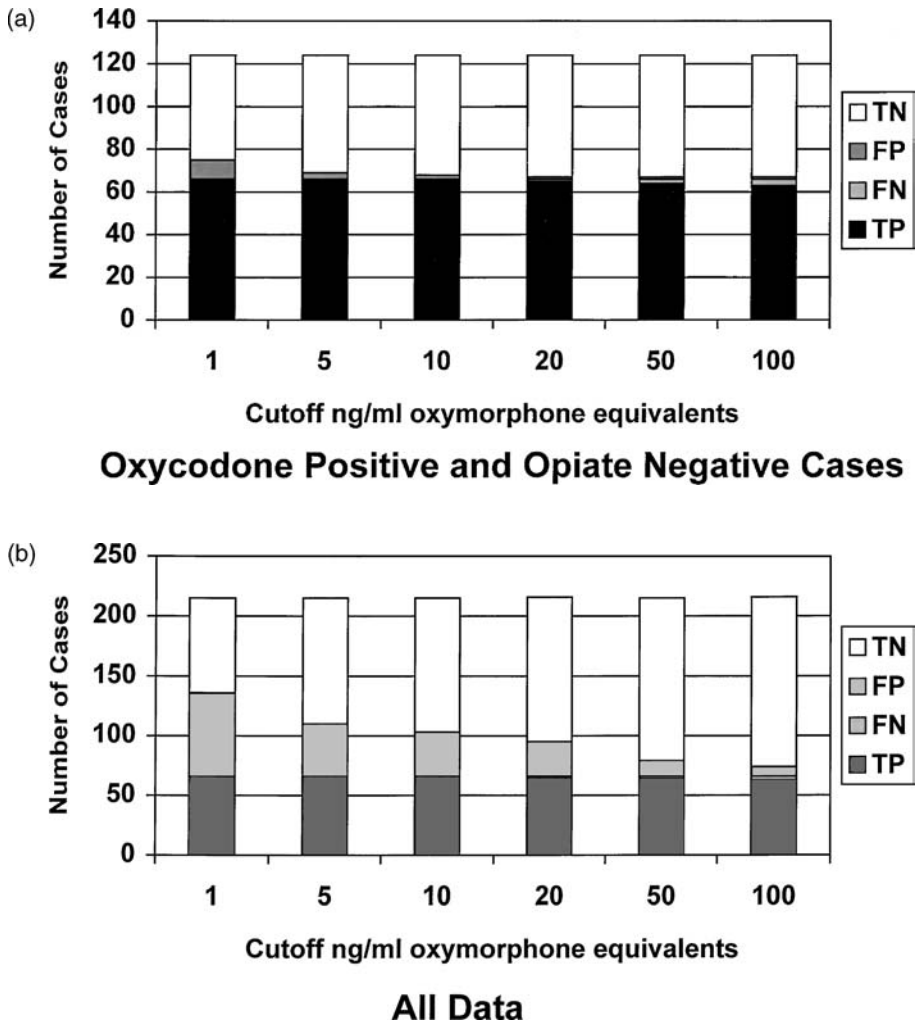

FIG. 1—*TP, FN, FP and TN's for oxycodone for cutoffs 1–100 ng/mL oxymorphone equivalents, a) for oxycodone positive and opiate negatives cases and b) for all data.*

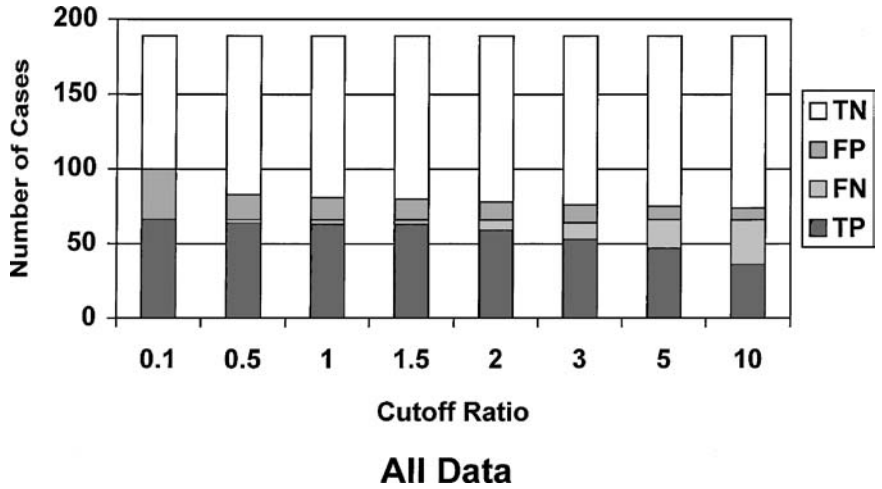

FIG. 2—*TP, FN, FP, TN's for Relative Response Ratio for cutoffs 0.1–10.*

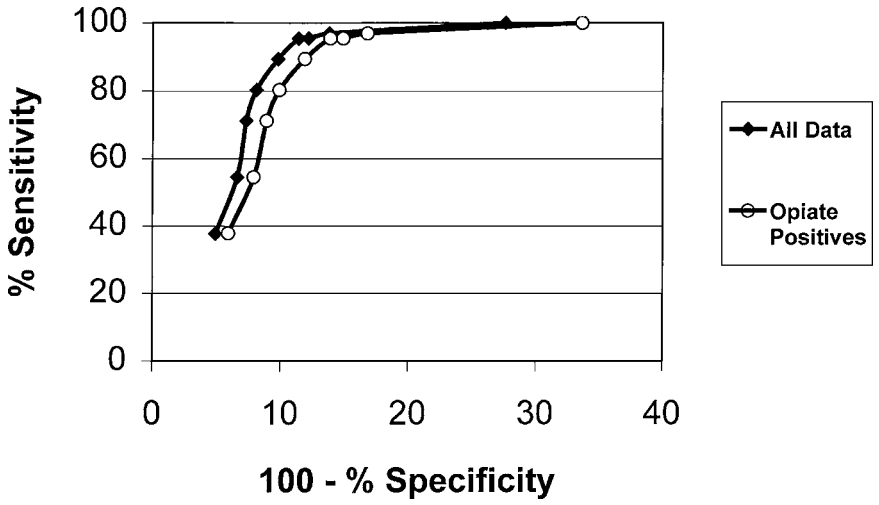

FIG. 3—*ROC Curves for ELISA Relative Response Ratios.*

TABLE 3—*Sensitivity and Specificity: Relative Response Ratio Oxycodone/Opiates*

Cutoff Ratio 2.0			ELISA Ratio	
Opiate Positive Specimens			+	−
	Result by GC/MS	+	59	7
		−	12	89
	Sensitivity = 59/66 = 89.4% ± 3.8%			
	Specificity = 89/101 = 88.1% ± 3.2%			
All Specimens				
	Result by GC/MS	+	59	7
		−	12	111
	Sensitivity = 59/66 = 89.4% ± 3.8%			
	Specificity = 111/123 = 90.2% ± 2.7%			

positive rate for each ratio cutoff was calculated. From the true negatives and false positives, the sensitivity or true negative rate was calculated. The sensitivity was plotted vs one hundred percent minus the specificity to obtain a Receiver Operating Characteristic (ROC) curve (Fig. 3). The optimum relative response ratio was 2.0. Specimens with a relative response ratio of 2.0 or higher had a greater than 50% probability (positive predictive value) of containing oxycodone. The sensitivity of the ELISA response ratio for the presence of oxycodone at a response ratio cutoff of 2.0 was 89.4% ± 3.8% and the specificity was 88.1% ± 3.2% (Table 3). For the one hundred and fifty eight consecutive opiate positive cases from the Oklahoma Medical Examiner, 28 contained oxycodone or oxymorphone (prevalence of 17.7%). The positive predictive value (PPV) for a prevalence of 15% oxycodone/oxymorphone-involved cases was:

$$PPV = fp/[fp + (1 - f)(1 - q)]$$

$$PPV = 0.15 \times 0.894/0.15 \times 0.894 + (1 - 0.15)(1 - 0.881)$$

$$= 0.568 \text{ or } 56.8\%$$

Discussion

In large part the relative response ratios were as expected. For example, in a heroin-involved death case in which the post mortem blood contained 130 ng/mL morphine with 6-monoacetyl morphine present by gas chromatography/mass spectrometry, the ELISA results were 1.6 ng/mL oxymorphone equivalents and 140 ng/mL morphine equivalents for a relative response ratio of 0.011. In an oxycodone-involved death which had only 150 ng/mL oxycodone present, the ELISA results were 351 ng/mL oxymorphone equivalents and 20 ng/mL morphine equivalents for a relative response ratio of 17.570. Specimens containing other opiates gave intermediate results. For example, in a hydrocodone-involved death, the blood contained 160 ng/mL hydrocodone by gas chromatography/mass spectrometry and the ELISA results were 465 ng/mL oxymorphone equivalents and 191 ng/mL morphine equivalents for a relative response ratio of 2.44. In a case with only a trace codeine present, the ELISA results were 64.8 ng/mL oxymorphone equivalents and 293 ng/mL morphine equivalents for a relative response ratio of 0.221. Multiple opiates in post mortem specimens are not uncommon. The discrepant results were investigated for the presence of other opiates but there was no consistent correlation.

Relative response ratios or differential immunoassay using the response from a more specific immunoassay compared to the response from a general group-specific immunoassay have been reported to differentially screen out a subset of opiate group drugs by Hand et al. (6) and by Cassani and Spiehler (7). Hand et al. (6) used the relative response of radioimmunoassays employing iodinated morphine and three different antisera to measure the concentrations and pharmacokinetics of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human plasma.

Cassani and Spiehler (7) used the ratio of the response of the Diagnostic Products Corporation (DPC) Opiate Screen Radioimmunoassay (RIA) on hair digests to the response of the DPC Morphine RIA and the DPC 6-Acetyl Morphine RIA on the same hair digests to differentiate hair from patients receiving medical morphine or codeine from hair of users of heroin. The Opiate RIA/Morphine RIA ratios from hair results were approximately 1 for medical morphine, ranged from 1 to 1.48 for heroin users and were greater than 100 for codeine patients. The ratio of the 6-AM RIA/Morphine RIA ranged from 1.5 to 7.5 in hair from heroin addicts. The present study shows that this approach can be used to screen post mortem blood for oxycodone with the Neogen™ ELISA immunoassays.

The Neogen™ Oxymorphone/Oxycodone ELISA has a cross-reactivity of 400% for oxycodone and 100% for oxymorphone,

30.8% for hydrocodone and 12.3% for hydromorphone (Table 1). Oxycodone is metabolized to oxymorphone by cytochrome P450 2D6. The cross-reactivity with codeine is only 5.3% and for morphine 1.7%. The Neogen™ Opiates Group ELISA is a broadly cross-reactive opiate group immunoassay (Table 1). Neither assay had a response within the calibration curve range with the negative whole blood specimens. However, some decomposed specimens caused false positive results with the ELISA assays. While the Neogen™ Oxymorphone/Oxycodone ELISA immunoassay does not have sufficient selectivity to identify OxyContin®- and other oxycodone-involved cases from the positive or negative results at a 20 ng/mL cutoff, it is sufficiently specific to identify possible C6-keto-opioid-contining specimens by using the ratio of the relative response to the Neogen™ Opiate Group ELISA result. Using a response ratio of 2.0 resulted in fewer false positives than using the 20 ng/mL ELISA cutoff to identify specimens containing oxycodone or oxymorphone (twelve vs twenty nine). However using a response ratio of 2.0 would have resulted in seven false negatives while use of the 20 ng/mL ELISA cutoff resulted in only one false negative.

In conclusion, by using relative response ratios, the Neogen™ Oxymorphone/Oxycodone ELISA can be used as a second immunoassay to identify which opiate-positive specimens should be confirmed for oxycodone and related C6-keto-opioids.

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