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Evaluation of Seven Immunoassays Not Commonly Used in Toxicology

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Abstract

Several abused drugs are routinely screened by forensic laboratories. However, there are other commonly abused drugs that are not routinely screened. Diphenhydramine, dextromethorphan, methadone, sertraline, fluoxetine, tricyclics, and carisoprodol are all compounds that are seen in toxicological samples but are rarely screened. The implementation of a screening method is desirable in order to decrease laborious tasks and amount of sample needed. In order to determine if screening these compounds by enzyme-linked immunosorbent assay (ELISA) should be adopted, a prevalence study of these compounds was conducted on 1304 previously tested forensic samples. It was observed that dextromethorphan, methadone, sertraline, fluoxetine, tricyclics, and carisoprodol were seen in less than 2% of forensic samples; while diphenhydramine was observed in 4% of forensic samples.

Keywords: Forensic science; Prevalence study; Enzyme-linked immunosorbent assay, Diphenhydramine; Dextromethorphan; Methadone; Sertraline; Fluoxetine; Tricyclics; Carisoprodol

Introduction

At the Orange County Crime Lab (OCCL), forensic samples are tested for several drugs that are commonly abused in driving under the influence of drugs (DUID), drug facilitated sexual assault, and postmortem (PM) casework. Cases are routinely screened for seven ELISA assays: methamphetamine, opiates, tetrahydrocannabinol/carboxy- tetrahydrocannabinol, oxycodone/oxymorphone, benzodiazepines, cocaine, and zolpidem. Urine and blood samples are screened for these compounds by using the competitive enzyme-linked immunosorbent assay (ELISA) technique. If positive, they are confirmed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) or gas chromatography coupled to a mass spectrometer detector (GC-MS). Even though these seven classes encompass the majority of drugs encountered in cases, there are still various drugs that are considered to be commonly used or abused by the public, including diphenhydramine, dextromethorphan, methadone, sertraline, fluoxetine, tricyclics, and carisoprodol. Currently, these drugs must be requested by the submitting agency for more extensive screening. Most of these compounds have been known to cause impairment and all have been seen in overdose cases [1-12].

Diphenhydramine is an antihistamine agent used commonly for the relief of allergy symptoms, and it is also used as a sedative or an antiemetic [1,2,13]. Even though diphenhydramine is considered to be non-toxic and no dependence or withdrawal effects have been observed with recommended doses, a number of intoxication cases due to overdosage have been documented [1-13]. Dextromethorphan is a non-opioid antitussive added to cold medication for the treatment of coughs [2,13]. This drug is commonly abused due to its hallucinatory, numbness, and disassociation effects; and people also take it as an alternative to methamphetamine or ecstasy [9,11,13]. Methadone is a narcotic analgesic used for the treatment of moderate to severe pain and is commonly used for the treatment of withdrawal symptoms associated with opioid addiction [2,10,13]. In recent years, fatalities in adults due to methadone overdose have increased due to the widespread availability of this drug [5,10,13]. Some of these fatalities are due to accidental overdoses [5], while others are due to the illegal misuse of methadone for its sedative and analgesic effects [2]. Sertraline is a selective serotonin reuptake inhibitor (SSRI) that is used as a relatively safe antidepressant [13]. Fluoxetine is another SSRI that is used for the treatment of depression [13], as is the tricyclic drug group [13]. Tricyclics are widely used and have been known to account for up to 18% of all national poisoning deaths [6]. A few of the most common tricyclics in forensic toxicology are amitriptyline and nortriptyline. Carisoprodol is a muscle relaxant and sedative prescribed for acute, musculoskeletal pain [2,13]. This particular drug is known to be frequently abused because of its sedative-hypnotic effects [2,4,8].

In addition to knowing their abuse potential, these drugs have been known to impair drivers. For example, due to the sedative effect of diphenhydramine, it has been shown to diminish cognitive and psychomotor performance in healthy individuals [1], increase weaving behavior, decrease break reaction time, and increase sleepiness in drivers in a control study [14]. The administration of a single maximum dose of 120 mg of dextromethorphan to healthy individuals in a controlled study did not seem to disturb their driving ability [15]; however, with high doses one can expect gross cognitive and psychomotor impairment which would deem the person unfit to drive [2]. The administration of methadone to healthy non-methadone users has been observed to impair driving ability [2]. In addition, despite the consumption of sertraline being relatively safe, the side effects that usually accompany its ingestion have contributed to several aviation and motor vehicle accidents [7,16]. Several studies agree that fluoxetine has little effect on driving [17,18]. Ingestion of tricyclic antidepressants has been known to cause dilated pupils, blurred vision, myoclonic jerks, agitation, and drowsiness which are effects that would make an individual unfit for driving [6]. Additionally, driving under the influence of carisoprodol is a well known problem where signs of psychomotor and cognitive impairment have been observed in individuals who were driving under the influence of this drug [2]. In another study, a concentration effect relationship between blood carisoprodol concentration and impairment was found where no impairment was observed in individuals consuming less than 700 mg of carisoprodol. Impairment was observed at larger doses [19].

Even though these drugs are considered to be commonly used or abused compounds, they are not currently screened by the OCCL unless specifically requested by an agency. When testing for these compounds is requested, an alkaline drug screen extraction is performed. This is a time intensive liquid-liquid extraction (LLE) process which requires 4 mL of sample to confirm a drug by gas chromatography with a Nitrogen-Phosphorous detector (GC-NPD) and GC-MS. The adoption of ELISA immunoassay kits for the screening of these compounds would decrease the amount of sample needed to do the analysis which is highly desirable. However, before this technique can be implemented, it was important to determine if these compounds are commonly observed in samples. For this reason, a prevalence study of these compounds was conducted on 1,304 previously tested forensic samples.

Methods

Samples

Crime laboratory samples previously tested by the lab were obtained from police agencies or the Sheriff Coroner Division. These samples included 1,009 ante-mortem (AM) blood samples, 286 post-mortem (PM) blood samples, and 9 AM urine samples. AM blood samples are contained in vials with 2% sodium fluoride and 0.25% potassium oxalate; PM blood samples are contained in 200 mL amber bottles with 4 grams sodium fluoride and 1.5 grams potassium oxalate; and AM urine samples are contained in clear urine containers with 2% sodium fluoride.

Reagents and Chemicals

Orphenadrine (1 mg/L) was obtained from Riker Laboratories Inc. (Northridge, CA). All other chemicals

and reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO).

Immunoassay

Immunoassays were conducted for all seven targeted compounds following the OCCL's validated ELISA screening method [20]. A total of 1,304 samples were screened using the TECAN® EVO instrument. Samples were diluted 1:10 with phosphate buffer saline (Immunalysis Corp. Pomona, CA). The solution was vortexed, centrifuged, and placed on the TECAN® EVO instrument. Control standards can be seen in Table 1 for each assay and were prepared the same way as samples. Direct ELISA kits for carisoprodol, diphenhydramine, dextromethorphan, fluoxetine, methadone, sertraline, and tricyclics were obtained from Immunalysis Corp. (Pomona, CA). Immunoassay analysis was conducted on a TECAN EVO-200 containing 8 tips, coupled to a Hydroflex washer, and to a Sunrise reader; as well as on a TECAN EVO-150 containing 4 tips, coupled to a Columbus Pro washer, and to a Sunrise reader. Standards (1 mg/mL) for all seven targeted compounds were obtained from Cerilliant® (Round Rock, TX). The volumes used for the samples; the 3,3',5,5' tetramethylbenzidine (TMB) substrate; the 1 M HCl stop solution; and the conjugate; as well as the cutoff and high positive concentrations for each compound are summarized in (Table 1).

Compound	Sample Volume (µL)	TMB Volume (µL)	Stop Solution Volume (µL)	Conjugate Volume (µL)	Cut-off Concentration (ng/mL)	High Positive Concentration (ng/mL)
Diphenhydramine	10	100	100	100	100	100,000
Carisoprodol	10	100	100	100	250	250,000
Methadone	10	100	100	100	50	100,000
Sertraline	100	100	100	100	100	100,000
Fluoxetine	100	100	100	100	100	100,000
Dextromethorphan	20	100	100	100	50	50,000
Tricyclics	25	100	100	100	25	50,000

Table 1: Volumes and Concentrations Used for ELISA Screening.

Once on the instrument, the first set of standards is dispensed in each well, followed by the forensic samples and ending with the second set of standards. The corresponding conjugate is then added into each well. After a 70 minute incubation period at 37 °C, the wells are washed six times with DI water, and TMB substrate is dispensed to all samples/standards and incubated at ambient temperature for about 25 minutes. A 1 M HCl stop solution is then added to each well to stop the color change reaction. Finally, the plate is loaded into the reader where each well is read and the absorbance is measured at a dual wavelength of 450 nm and 620 nm. Qualitative data analysis of the results from the immunoassay analysis was conducted using the instrument's software application.

Confirmation of ELISA Results by GC-NPD

An LLE was performed for all confirmations and quantitations of the ELISA positive samples. All sample types were run in duplicate for quantitation so an average could be reported. A calibration curve was created using validated standards obtained from Cerilliant® (Round Rock, TX). The standards used to make the calibration curve ranged from 0.1 mg/L to 20.0 mg/L. Two quality control standards were also extracted at 0.3 mg/L and 3.0 mg/L. The quality control samples were diluted separately from the standards to check the accuracy of the calibration curve. The method has been validated following the Scientific Working Group for Forensic Toxicology (SWGTOX) Guidelines for Method Validation [21].

Prior to extraction, 1 mL of the internal standard and 1 mg/L orphenadrine were added to 2 mL of each sample and standard. The samples were salted out using 2.5 mL of a saturated borate buffer solution. Diluted sodium hydroxide was added to bring the samples to a pH of approximately 8.5. Butyl chloride was then added to each tube and the tubes were shaken and centrifuged. The organic layer was then placed into a separate tube to which 4mL of 0.25 M sulfuric acid was added. The tubes were again shaken and centrifuged, and the organic layer was aspirated and discarded. Saturated sodium hydroxide was added drop-wise to each tube until the pH was greater than 10. Once basic, 6 mL of butyl chloride was added to each tube. After shaking and centrifuging the tubes, the organic layer was transferred to a new test tube and a drop of 1 M HCl in methanol was added. The samples were dried down and reconstituted with ethanol. All samples were run on GC-NPD and GC-MS.

Results

Immunoassay

Out of the 1,304 samples that were screened using the TECAN® EVO, most samples were tested for all seven of the targeted compounds, and some samples were tested for fewer than seven as seen in (Table 2). Once samples were screened through ELISA, the number of positive samples for each assay was determined and their corresponding percentage was calculated as can be seen in (Table 2). All positive samples were then confirmed.

Assay	Samples Tested	Positive samples	Percent Positive (%)
Diphenhydramine	1200	64	5.33
Dextromethorphan	1280	21	1.64
Sertraline	1176	15	1.28
Tricyclics	1216	34	2.8
Methadone	1272	24	1.89
Carisoprodol	1288	42	3.26
Fluoxetine	1128	39	3.46

Table 2: ELISA Results.

When considering the sample type (PM blood, AM blood, and AM urine), the following observations were seen. Out of the 1304 samples ran; 286 were PM bloods, 1009 were AM bloods, and 9 were AM urine samples. From these; 53 (18.53%), 74 (7.33%), and 9 (100%), respectively were found to be positive. Once the positive samples were run on the GC-NPD, it was observed that 7 (13.21%) of the 53 positive PM blood samples, 20 (27.03%) of the 74 positive AM blood samples, and 7 (77.78%) of the 9 AM positive urine samples were false positives. These results are expected as false positives are commonly observed for urine samples due to the fact that they are run using blood cut-off concentrations. In order to circumvent this problem, labs usually run urine samples with a higher cut-off concentration.

Confirmation of ELISA Results by GC-NPD

For confirmatory purposes, all 136 ELISA positive samples were run on the GC-NPD, the number of confirmed positive samples was determined, and their corresponding percentage was calculated along with the number of false positives and false negatives. A summary of all these results is in (Table 3).

Discussion

Our results suggest that most of the tested compounds are observed in less than 2% of forensic samples. It was observed that some of the tested compounds presented a high number of false positives as can be seen by (Table 3). We have identified three main reasons why such a high number of false positives were observed: cross-reactivity with a new compound, high detection limits, and stability problems. Despite the number of false positives, diphenhydramine was one of the compounds that were commonly observed in forensic samples. Before forensic samples were tested for all the targeted compounds, the assays were tested for compounds with the highest percentage of cross-reactivity as described in the inserts provided with the ELISA kits. Compounds tested for cross-reactivity for each assay can be seen in (Table 4). No work was conducted on compounds that were not mentioned in the inserts.

Assay	GC-NPD Confirmed Positive Samples	Positive Sample Percentage (%)	False Positives	False Positive Percentage (%) from ELISA Positive Results	False Negatives
Diphenhydramine	48	4	16	25	1
Dextromethorphan	17	1.33	4	19.05	0
Sertraline	9	0.77	6	40	0
Tricyclics	24	1.97	10	29.41	0
Methadone	24	1.89	0	0	0
Carisoprodol	21	1.63	21	50	0
Fluoxetine	16	1.42	23	58.97	0

Table 3: Confirmation Results.

Assay	Compound	% Cross reactivity
Diphenhydramine	Diphenhydramine	100
	Cyclobenzaprine	200
Carisoprodol	Carisoprodol	100
	Meprobamate	118
Methadone	Methadone	100
Dextromethorphan	Dextromethorphan	100
	Dextrorphan	83
Tricyclics	Nortripyline	100
	Amitriptyline	200
	Desipramine	200
	Imipramine	200
	Trimipramine	50
	Clomipramine	40
	Doxepin	15
	Nordoxepin	15

	Cyclobenzaprine	83
	Chlorpromazine	40
	Diphenhydramine	0.25
	Quetiapine	0.25
Sertraline	Sertraline	100
	Nor-Sertraline	5
Fluoxetine	Fluoxetine	100
	Nor-Fluoxetine	25

Table 4: Cross-Reactivity with Related Drugs.

Quetiapine, amitriptyline, and cyclobenzaprine all cross-reacted with both the diphenhydramine and tricyclics plates. In addition, doxylamine and clomipramine were observed to cross-react with the diphenhydramine plate, while diphenhydramine was observed to cross-react with the tricyclics plate. These observations were expected as all of these compounds were previously tested for cross-reactivity; however, citalopram, which was not tested for cross-reactivity, was a compound that was thought to be cross-reacting with the diphenhydramine plate. For diphenhydramine, 16 samples (25%) of the 64 ELISA positive samples were found to be false positives. When samples were analyzed using GC-NPD, it was observed that in 7 of these false positive samples, citalopram in a range of 0.73-4.5 mg/L was present. Due to the high number of false positive samples containing citalopram, it is believed that citalopram might be cross-reacting with the diphenhydramine plate causing false positives to be observed. Similarly, tricyclics were also observed to have a high number (29.41%) of false positives. As mentioned before, there are many known compounds that cross-react with the tricyclics plate including diphenhydramine. Being that diphenhydramine cross-reacts with the tricyclics plate, it is believed that citalopram might also be cross-reacting with this plate and attributing to some of the false positives. Sertraline and carisoprodol were two of the compounds with some of the highest number of false positives with 40% and 50% false positives, respectively. For both of these compounds, the cutoff concentration used (100 ng/mL for sertraline and 250 ng/mL for carisoprodol) was similar to the limit of detection (LOD) used by the GC-NPD (100 ng/mL for sertraline and 250 ng/mL for carisoprodol). Therefore, the high number of false positives for both compounds is attributed to the fact that the GC-NPD could not detect the positive samples that had a lower screen concentration than the established LOD for that drug. To solve this problem, the cut-off used in ELISA could be raised, or LC/MS/MS could be used for the confirmation of these drugs.

Fluoxetine showed the highest number (58.97%) of false positives. While working with this compound, it was observed that at low concentrations fluoxetine would break down in pig's blood in a short period of time. For this reason, it is likely that cases with low concentrations of fluoxetine would also break down in human blood. In addition, being that confirmations of this drug using GC-NPD took place about two months after the initial screening of the samples via ELISA, it is believed that fluoxetine was not stable enough in the samples containing low concentrations of this drug. Fluoxetine might have degraded in these samples; thus contributing to a higher number of samples being positive for fluoxetine when screened by ELISA as opposed to the lower number of samples confirmed by the GC-NPD instrument. This observation is validated by the fact that fluoxetine is known to be unstable in plasma at room temperature and has been known to show significant loss in as little as two weeks [22]. It is worth mentioning that, since each sample was extracted to test for all seven drugs, not all 136 ELISA positive samples were positive for all assays tested; therefore, the negativity of these samples was also confirmed when they were run on the GC-NPD. Out of the 673 negative results observed, only 1 (0.15%) was found to be a false negative. All other negative results were confirmed to be negative.

Conclusion

In summary, this work reveals that six of the targeted drugs (dextromethorphan, fluoxetine, carisoprodol, methadone, sertraline, and tricyclics) were observed in less than 2% of the forensic samples tested, while diphenhydramine was observed in more than 2% of the samples tested. False positives were observed for most of the compounds tested and these observations were rationalized based on the compounds' cross-reactivity, detection limits, and stability. The ELISA negative results were confirmed to be negative with only 1 (0.15%) result being a false negative. Based on these results, it was concluded that because diphenhydramine is widely used, has a high impairment potential, and had a high percentage of positive samples (4%) -even higher than some drugs that are currently being screened routinely- it is recommended for laboratories to implement diphenhydramine along with the cross-reacting compounds cyclobenzaprine, amitriptyline, doxepin, clomipramine, imipramine, bromopheniramine, chlorpheniramine, doxylamine, nortriptyline, norclomipramine, protriptyline, trimipramine, despiramine, pheniramine, nordoxepin, and possibly citalopram into routine screening tests.

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