Moving beyond immunoassays for poisoned patients

Amy Carpenter Aquino

April 2020—Bypassing immunoassays as the first step in toxicology testing can minimize clinician calls to the laboratory about negative toxicology reports for apparently overdosed patients and save diagnostic time.

“Immunoassay is not the right approach to monitor the compliance of the patient with benzodiazepines and opioids,” because it may not detect all drugs in a class due to poor cross-reactivity, Amitava Dasgupta, PhD, DABCC, professor of pathology and laboratory medicine, University of Texas McGovern Medical School, said in a session at last year’s AACC annual meeting. He presented several cases illustrating the limitations of immunoassays in toxicology screening.

Amphetamine immunoassays suffer from interferences with over-the-counter cold medications and poor cross-reactivity with amphetamine-like designer drugs, which cannot be detected until the concentration reaches a level of 20,000 ng/mL—“a severe overdose,” he said. And patients poisoned with novel psychoactive substances also show negative toxicology reports.

Dr. Dasgupta’s co-presenter was Kara Lynch, PhD, DABCC, co-chief of the core laboratory at Zuckerberg San Francisco General Hospital and associate professor of laboratory medicine at the University of California, San Francisco. Dr. Lynch talked about testing strategies beyond immunoassays and how her laboratory uses high-resolution mass spectrometry with a quadrupole time-of-flight instrument to crack its toughest toxicology cases.

In one case Dr. Dasgupta presented, a physician called the lab because an initial drug screen for a possible overdose patient was negative for opiates but a second screen performed hours later was positive. The clinician had prescribed naloxone after the initial screen, but it did not reverse the patient’s overdose symptoms, indicating they were not opioid-related. The patient was a known marijuana abuser, so the clinician was also surprised to see a negative THC result.

“A sample was sent to a reference laboratory, which confirmed the presence of naloxone and JWH-073, a common designer synthetic cannabinoid,” he said. The positive result in the second urine screen was due to the naloxone, which is an opioid and cross-reacts with opiate immunoassays at high concentrations. “Any patient who gets naloxone will test positive.”

Dr. Dasgupta

Another patient known to take tramadol was admitted to the emergency department in November with a drug overdose. The patient’s initial drug screen result was negative for opiates but positive for phencyclidine (PCP). “That confused the clinician because the patient said he took too much tramadol,” for which the patient was given and responded to naloxone therapy. Confirmation tests were negative for PCP and positive for tramadol. Why was the PCP positive?

“I usually see PCP-positive results in January, February, and March because of dextromethorphan,” a cough suppressant, Dr. Dasgupta said. But this case was in November. “We learned that tramadol can also cause a false-positive PCP result.”

He referred to a case in the literature in which a 42-year-old man died at home (Hull MJ, et al. *Am J Forensic Med*

Pathol. 2006;27[4]:359–362). The man's postmortem urine drug screen was positive for PCP with the Syva Emit II Plus phencyclidine assay, and toxicology analysis showed a tramadol level of 14 mg/L, "which is very high because therapeutic level is 100 to 300 ng/L or 0.1 to 0.3 mg/L," Dr. Dasgupta said. "But there is no PCP confirmed, so the authors speculated that tramadol was causing this false-positive PCP level."

Dr. Dasgupta shared the finding of an Australian study on cross-reactivity in the CEDIA buprenorphine immunoassay by opiates (Saleem M, et al. *Ann Clin Biochem.* 2017;54[6]:707–711). "Is the opioid buprenorphine cross-reacting with the opioid immunoassay?" he said. "When you use this assay at a 5 µg/L cutoff, do you need to do any opiate testing?"

The authors found that cross-reactivity in the CEDIA buprenorphine immunoassay by opiates at concentrations less than 2,000 µg/L will not cause a false-positive buprenorphine result. "The HHS increased the cutoff level from 300 to 2,000 in 1998," he said. "However, some private employers still use 300."

In another case, a patient who presented to the ED in an overdosed state was given naloxone and later died. "When he was still alive, we did the opiate screen using ELISA mass spec and we didn't find anything," Dr. Dasgupta said. "But it was an opiate-related death, so the case went to the medical examiner's office where they have a much broader screen." The forensic test results showed that the patient had taken the over-the-counter antidiarrheal drug loperamide. The suggested maximum dose is four tablets. "When the family members showed up, we found that he probably took 30 to 50 tablets to get high."

Loperamide is an opioid with poor bioavailability but when taken in excess, it crosses the blood-brain barrier and produces euphoria (Eggleston W, et al. *Ann Emerg Med.* 2017;69[1]:83–86). "If it goes to the bloodstream, it's very potent, as potent as fentanyl," Dr. Dasgupta said, calling the drug "the poor man's methadone." The therapeutic level is 0.24 to 3.1 ng/mL. Death from cardiac dysrhythmia has been reported in patients with loperamide levels as low as 77 ng/mL and as high as 140 ng/mL.

In one of two cases that illustrate the problems in detecting benzodiazepine by immunoassay, a clinician complained to the laboratory when a patient on lorazepam had a negative toxicology screen. GC-MS confirmed lorazepam at a level of 1,566 ng/mL; the EMIT (enzyme multiplied immunoassay technique) cutoff for lorazepam is 600 ng/mL. What went wrong?

"We thought that most of the lorazepam was conjugated with glucuronic acid, and when a benzodiazepine is conjugated, it has a much lower cross-reactivity" with the immunoassay, Dr. Dasgupta said.

He and his colleagues tested a theory that the EMIT benzodiazepine immunoassay sensitivity could be increased. "We incubated urine with beta-glucuronidase to break up the glucuronic acid conjugate," he said (Dixon RB, et al. *Ther Drug Monit.* 2015;37[1]:137–139).

Thirty-one urine specimens collected from patients taking benzodiazepines were treated with beta-glucuronidase for two hours at 47°C, and then tested with the EMIT benzodiazepine immunoassay on the Vista 1500 analyzer (Siemens Healthineers) and with an LC-MS/MS assay. All 31 specimens showed benzodiazepine levels above the 200 ng/mL cutoff concentration by LC-MS/MS, but 11 of the 31 specimens showed a negative response by the EMIT, for a false-negative test result rate of 35.5 percent.

"Even if you break the conjugated compound and you are using the benzodiazepine immunoassay, it's not adequate for monitoring compliance," Dr. Dasgupta said. He advises labs to tell clinicians who want to test for compliance to skip the benzodiazepine screen and go right to the confirmation assay.

In another case, a 58-year-old woman was brought to the ED after she attempted suicide by taking 12 2-mg lorazepam tablets. Her urine benzodiazepine test result was negative using an immunoassay with a cutoff of 200 ng/mL. The clinician doubted the result and ordered a second urine benzodiazepine screen 14 hours later, which was also negative. Both urine samples were then tested by GC-MS, and those results showed a benzodiazepine level of more than 20,000 ng/mL (Wenk RE. *Arch Pathol Lab Med.* 2006;130[11]:1600–1601).

“This is a prozone or hook effect,” Dr. Dasgupta said. “There was so much benzodiazepine that it oversaturated the binding site, causing a false-negative result. We know that the hook effect causes falsely lower values with sandwich assays for big molecules such as hCG. This is the only case I know where a small molecule like benzodiazepine gave a falsely low value due to hook effect.”

Another case highlighted the challenges presented by synthetic cannabinoids, which standard drug screens don’t detect (Bassir Nia A, et al. *J Psychopharmacol.* 2016;30[12]:1321–1330). The patient was admitted to the ED with a suspected overdose, but the toxicology report was negative for all drugs except the inactive cannabis metabolite THC-COOH. “The clinical picture indicated a life-threatening overdose not consistent with THC-COOH because the THC value [55 ng/mL] was very low,” Dr. Dasgupta said. “We thought it was maybe a synthetic cannabinoid.”

The clinician ordered a synthetic cannabinoid panel (sent to a reference laboratory), and the patient had a positive result for JWH-018, one of several types of synthetic cannabinoid compounds (Yeruva RR, et al. *Innov Clin Neurosci.* 2019;16[1–2]:31–32).

“Synthetic cannabinoids account for 39 percent of all psychoactive drugs, and there are more than 177 compounds reported,” Dr. Dasgupta said, noting they’re far more dangerous than marijuana. “It’s a nightmare.”

Several companies offer synthetic cannabinoid immunoassays, Dr. Dasgupta said, but the problem is that they detect the most common synthetic cannabinoids. “They cannot detect everything, so you need an LC-MS/MS assay. And the limitation of developing an LC-MS/MS assay is the lack of a pure standard.”

Bath salts, too, present a problem. Many fatalities have been associated with overdoses of synthetic cathinone, of which bath salts are a synthetic derivative. They cannot be detected with amphetamine/methamphetamine immunoassays, Dr. Dasgupta said.

Randox Toxicology was the first company to offer an immunoassay to detect bath salts in urine. Its Drugs of Abuse V Biochip Array assay has two synthetic cathinone antibodies: Bath Salt I targets mephedrone/methcathinone, and Bath Salt II targets 3’,4’-methylenedioxypyrovalerone (MDPV)/3’,4’-methylenedioxy-α-pyrrolidinobutiophenone (MDPBP).

“You cannot detect everything,” Dr. Dasgupta said, “but at least you can detect the most commonly abused bath salts and synthetic cannabinoids.”

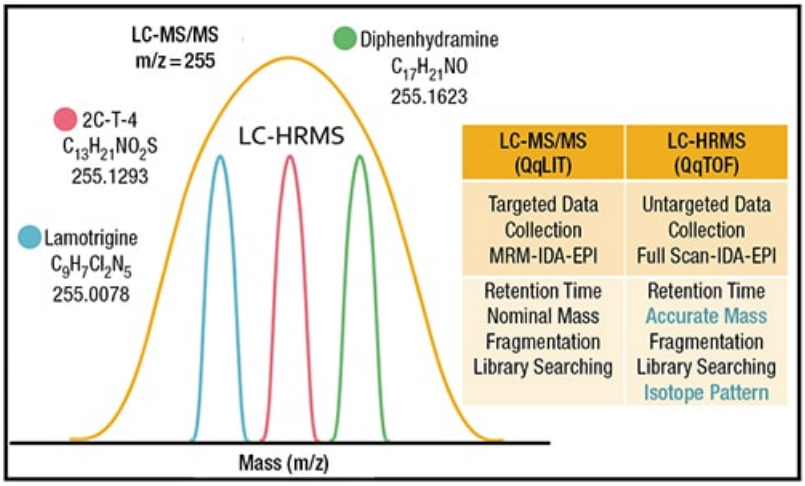
At Zuckerberg San Francisco General Hospital, to solve the most challenging toxicology cases, the laboratory uses liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) on a quadrupole time-of-flight mass spectrometry (QTOF-MS) system. The laboratory serves as a regional toxicology laboratory, taking on difficult cases from other Bay area hospitals referred through the Northern California Poison Control Center.

“Urine drug testing is just not adequate to cover what’s going on when we talk about toxic exposures,” Dr. Lynch agreed. A urine drug screen will detect only a small subset—psychostimulants, narcotics, heroin, cocaine, alcohol—of the more than 2.7 million agents responsible for the more than 2 million toxic exposures reported annually to the American Association of Poison Control Centers, she said. “Pharmaceutical events are typically the cause, but we can’t detect most pharmaceutical agents using a urine drug test.”

In a case of an unidentified overdose, an untargeted data acquisition approach to detecting compounds by an LC-HRMS method on a QTOF-MS leads to the most accurate results, Dr. Lynch said.

GC-MS—"the old gold standard"—has its advantages, among them the reproducibility of the mass spectra created for each compound and the large, transferable mass spectral libraries, but "the disadvantages are huge when it comes to the clinical laboratory," Dr. Lynch said. GC-MS cannot detect nonvolatile, polar, and thermally labile compounds, and "almost all drugs fall into these categories." Lengthy sample preparation delays results.

High-Resolution Mass Spectrometry



LC-MS/MS (QqLIT)	LC-HRMS (QqTOF)
Targeted Data Collection MRM-IDA-EPI	Untargeted Data Collection Full Scan-IDA-EPI
Retention Time Nominal Mass Fragmentation Library Searching	Retention Time Accurate Mass Fragmentation Library Searching Isotope Pattern

Multiple reaction monitoring (MRM) is the most common mass spectrometry method used for compound detection. The drawback is the number of MRM transitions—in which selected precursor ions undergo fragmentation to become product ions—required to detect large numbers of compounds. "You have to do a lot work to set up the method, and it gets bulky with all of these different transitions."

Another MRM approach uses information dependent acquisition (IDA). With IDA, parameters can be set up to tell the instrument which compounds to fragment, and then all the fragments are collected for a full mass spectrum. "It scans all the product ions, and you get an actual spectrum for that particular compound that can be searched against the library," Dr. Lynch said. "It's still a targeted data acquisition approach because you have to tell the instrument what you want to detect."

With LC-HRMS, there are different types of platforms and options for scanning for compounds. Dr. Lynch and colleagues use an untargeted data acquisition approach on a QTOF, which provides the most information about unknown compounds. The process begins with a full scan, and then the first quadrupole in the instrument acts as a mass filter, selecting a narrow mass range. Users can set the method in different ways, "for example, picking the top 20 ions at any moment in time," she said. The selected mass is fragmented by collision-induced dissociation in the second quadrupole and sent through a flight tube. "The time it takes for the ion to travel through the flight tube is proportional to the mass-to-change ratios. It gives an accurate measure of the mass of both the precursor ion and the product ions," resulting in a high mass resolution for more precise detection.

A nominal mass instrument does not help differentiate among compounds with similar mass peaks, Dr. Lynch explained. For example, lamotrigine, diphenhydramine, and 2C-T-4 (2,5-dimethoxy-4-isopropylthiophenethylamine), a designer amine, all have a nominal mass of 255 by LC-MS/MS. "With a high-resolution mass instrument, you get very tight mass peaks and the accurate mass of a compound," she said. "We would get distinct mass peaks for these three different compounds because they have different formulas and different exact masses." (See box above.)

An accurate mass helps determine the compound's formula, narrowing the possibilities. Isotope pattern analysis is also possible. "You have several different ways in which to identify a compound when using this technology."

LC-HRMS analysis begins with an untargeted data collection. The analysis itself can be performed by one of three methods: targeted, suspect, and untargeted.

For the targeted method, the ZSFG laboratory has a list of about 350 compounds with analytical standards. "We have tested them on our method and know what our limit of detection is and the matrix effects. We've validated the assay for those compounds. We have those spectra in our library, so we know everything about it and how well we can detect it." The process is set up for quick results.

Suspect data analysis is useful when a clinician has an overdose patient and a sample of the drug the patient took

but doesn't recognize the name, which is often the case with new designer drugs. "We can say, 'We don't have a standard for that compound. We've never seen that compound before,' and still query the data and see if we see a peak and have acquired spectra for that compound," Dr. Lynch said. If the drug's formula is available, it can be used to search the data to see if there is a peak for that formula. If no spectra for that compound are in the library, "we can search the literature and there are other ways to identify or confirm if the compound is there." Eventually, the laboratory orders an analytical standard to confirm the compound's presence.

Dr. Lynch said her laboratory tries to steer clear of untargeted data analysis. "It's like trying to find a needle in a haystack. If you tell the software to pull out the thousand most abundant masses in a particular sample, it will pull out 1,000 masses and give you 1,000 formulas. Then you have to figure out if any of those formulas corresponds to a drug that may have caused the presentation."

Dr. Lynch is often asked if high-resolution mass spec is as good as tandem mass spec, LC-MS/MS, in terms of its sensitivity. She and colleagues compared the two (Thoren KL, et al. *Clin Chem.* 2016;62[1]:170–178).

"We took our subset of drugs that we were most interested in detecting, spiked them into drug-free urine in increasing concentrations, and looked for the limit of detection for the two different methods," she said. Both technologies had the same limit of detection for 76 out of 169 compounds tested. The tandem mass spec method had a lower LOD for 60 compounds, and the LC-HRMS method had a lower LOD for 33 compounds. The differences in LOD between the two technologies were minimal, she said, usually 5 ng/mL or 10 ng/mL.

Dr. Lynch

Regarding specificity, both technologies found 469 of 562 confirmed compounds in 100 urine samples. The LC-HRMS method identified fewer drugs (515) compared with tandem mass spec (596), but LC-HRMS had a 97 percent confirmation rate while tandem mass spec had an 89 percent confirmation rate. "No method is perfect," she said.

Of the 62 drugs that the LC-HRMS method missed, 33 were unique drugs, and few misses were due to LOD. "For the majority, it was because no LC-MS/MS spectra were acquired. We could see the mass peak, but the instrument failed to acquire the spectra." She and her colleagues tried different technologies and succeeded in acquiring identification of five additional compounds using a variable SWATH (sequential window acquisition of all theoretical fragment-ion spectra) method (Whitman JD and Lynch KL. *Clin Chem.* 2019;65[7]:862–870).

Dr. Lynch presented a case from a few years ago in which a 27-year-old male and a 37-year-old female arrived at the Zuckerberg San Francisco General Hospital ED with complaints of extremity weakness and paresthesias. They had fallen asleep after ingesting alcohol, cocaine, and Xanax (alprazolam) with a friend. When they woke up six hours later, their friend was dead. Standard laboratory test results showed similar symptoms in both patients: demand cardiac ischemia, slightly elevated troponin levels (1.43 ng/mL for the female patient, 1.9 ng/mL for the male patient), rhabdomyolysis with elevated CK >1,000 U/L, and compression neuropathy. The male patient also had acute kidney injury (creatinine: 1.68 mg/dL).

Over the next two months, nine additional patient cases and four deaths (the medical examiner investigated the latter) were associated with what turned out to be counterfeit Xanax. Using LC-HRMS untargeted data collection on samples of urine, serum, and pills from eight patients, Dr. Lynch and colleagues determined there was no alprazolam in the samples but detected (along with cocaine and its metabolite and cocaethylene) fentanyl and etizolam. The latter is a designer thienodiazepine and a benzodiazepine analog that produces serious central nervous system depressant effects and is "six times more potent than diazepam and stronger than most of the

prescribed benzodiazepines," Dr. Lynch said. A public health advisory alerted the community.

Overdoses from synthetic opioids other than methadone—nonpharmaceutical fentanyl, illicitly manufactured fentanyl, fentanyl analogs, and novel synthetic opioids—have increased sharply nationwide since 2013. "We have addressed this by ordering a lot of the fentanyl analogs and validating our assay to be able to detect these. So these compounds have been added to our targeted list," Dr. Lynch said. With a high-resolution platform and an assay, "it's pretty easy to add to it," she said. "All you do is order a standard and determine the limit of detection and matrix effects if you're doing a qualitative assay." High matrix effects can be expected because of the simple sample preparation required to get quicker results. "It's a little bit of a tradeoff."

In an opioid-related case, a 33-year-old male with a history of heroin addiction was found in a bathroom at his worksite. The patient was pulseless but received CPR and naloxone on site and showed slight improvements. The patient later said he had been smoking a drug a friend bought online and admitted to frequently smoking fentanyl. He used the same amount this time because he thought the drug was fentanyl, but his urine drug screen was positive only for amphetamines and THC. LC-HRMS analysis revealed carfentanil in the urine and the drug. Often used to sedate elephants, carfentanil is showing up in adulterated heroin, cocaine, methamphetamine, and illicit Xanax, though it's a bigger problem on the East Coast and in the Midwest than on the West Coast, Dr. Lynch said, then added, "Our people are just buying and using fentanyl, unfortunately."

The patient's blood test results were not positive for carfentanil. "We often request a blood and a urine," she said. "Sometimes we'll see something in the urine that we don't see in the blood, or see something in the blood we don't see in the urine. When you think about metabolites and windows of detection, the two together are beneficial in helping to determine what's going on for a patient." The patient, who was discharged after 24 hours in the ICU, likely had a better outcome because he was not opioid naive.

Another opioid case involved a 29-year-old male found unresponsive after injecting an unknown drug. The patient regained consciousness on the way to the ED and showed symptoms of opiate toxidrome and complained of polydipsia. Standard laboratory results revealed elevated neutrophils and creatinine and a normal eGFR. The urine drug screen was positive for benzodiazepines but not for opiates, despite his presentation.

Audience members thought the mystery drug could be a designer amphetamine, but biological samples tested by LC-HRMS showed positive results for U-47700 (240 ng/mL) and phenazepam (1.4 mg/L). U-47700, also known as Pink, is an opioid seven times more potent than morphine and has been responsible for overdose deaths but often in combination with other drugs, Dr. Lynch said.

"We were curious about the phenazepam because the level was pretty high," she said. The patient admitted post-discharge to having taken phenazepam, a non-FDA-approved benzodiazepine with a half-life of 49 to 301 hours, a few days before the overdose. He had purchased the phenazepam and U-47700 on the darknet.

While it may be tempting for laboratories to save diagnostic time by ordering several new compound standards for their toxicology libraries, Dr. Lynch cautions against it. "It's not financially feasible for a clinical laboratory," she said. Her best advice: "We let the cases lead us." If she and colleagues get a case and find a compound, they will order the standard and the confirmation.

"There are always new designer drugs, but you may never see a case at your institution, so buying a $500 standard isn't financially reasonable." With high resolution, she said, a compound can be identified without a standard. "Follow up with confirmation by ordering that particular standard."

Amy Carpenter Aquino is CAP TODAY senior editor.