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LC/MS Provides New Capabilities in Toxicology

By Brad Hall and J. Rod McCutcheon

Mass spectrometry (MS) is considered the "gold standard" detector for the identification of drugs and poisons in forensic and clinical toxicology. The requirements of using a mass spectrometer as a detector for the two most common separation techniques in a forensic toxicology laboratory, gas chromatography (GC) and liquid chromatography (LC), are quite different, with GC considered less complex.

Mass spectrometers operate under low-pressure conditions to minimize ion/molecule and molecule/molecule collisions, thus requiring high-capacity vacuum pumping systems. The coupling of gas chromatography to mass spectrometry has traditionally been less complex because of lower gas flows exiting the chromatographic column, especially with the development of capillary gas chromatographic columns. In addition, substances eluting from the gas chromatographic column are already in the gas phase, thus amenable to traditional ionization methods such as electron ionization.

In contrast, the introduction of a liquid into a mass spectrometer was historically considered more complex because, under the vacuum requirements of mass spectrometers, the flow of vapor from the LC would be too high for the pumping system to handle. Robust, commercialized gas chromatography/mass spectrometry (GC/MS) systems appeared in laboratories in the latter quarter of the 20th century.

GC/MS has proven to be an excellent tool widely employed in forensic and clinical laboratories for the identification of substances in physiological fluids and tissues. However, the high thermal requirements of the gas chromatograph cause some molecules to break down in the heated injector, thus making them undetectable or unsuitable for quantitative analysis. In addition, polar molecules typically respond poorly in a gas chromatograph, requiring derivatization of the polar groups, if possible, in order to obtain suitable chromatography. Liquid chromatography, on the other hand, is routinely conducted at room temperature or slightly above and thus is amenable to a wider range of drugs.

Ultraviolet/visible, electrochemical, or fluorescence detectors have been the traditional detection methods for liquid chromatography. These detectors have the disadvantage of being non-specific, that is, they give no specific mass spectral information.

Electrospray ionization

It was not until the development of electrospray ionization (ESI), a technique that allows the introduction of ions from a liquid into a mass spectrometer, that the practical use of liquid chromatography/mass spectrometry (LC/MS) became possible. ESI has proven to be one of the most significant advances in analytical mass spectrometry. The pioneering work of Fenn and co-workers in the development of electrospray and the application to large biomolecules was recognized by award of the 2002 Nobel Prize in chemistry (1, 2).

ESI was found to be applicable to a wide range of molecules, from small drugs to proteins. The commercialization of LC/MS with modern computer data systems occurred in the 1990s, when reliable, robust instruments were developed. Today, pharmaceutical companies use LC/MS to determine therapeutic drug concentrations and metabolites (3). LC/MS paves the way for the discovery of more sophisticated drugs because the analytes can be measured in physiological specimens by mass spectrometry. It is likely the number of therapeutic drugs to which LC/MS has been applied in the past five years, mainly by the pharmaceutical industry, ex-

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ceeds the number of drugs for which GC/MS methods were published in the past few decades (4).

LC/MS applicability

LC/MS enhances a laboratory's ability to analyze for drugs and poisons that are not detectable by GC/MS. In addition, because of the inherent sensitivity of LC/MS, methods can be moved from the GC/MS to achieve better detection limits and/or decrease the extraction and analysis time, thus improving efficiency.

Until recently, there were few LC/MS methodology papers investigating drugs and poisons of interest in a forensic or clinical laboratory. LC/MS methods appearing in the clinical literature include analysis of cardiac glycosides, such as digoxin, digitoxin, and lanatoside C (4). The analysis of the mushroom toxins alpha-amanitin and beta-amanitin in urine by LC/MS is another example where no mass spectrometric method was previously possible (5). The analysis of intact neuromuscular blocking agents in biological fluids is greatly enhanced by LC/MS. Simple LC/MS screening and quantitative procedures for atracurium, pancuronium, vecuronium, mivacurium, rocuronium, and mebezonium have been published (6, 7). The measurement of peptide drugs and anticancer drugs are two examples among many other applications of LC/MS (8, 9). Many review articles have been written concerning LC/MS, with a few specific to forensic and clinical toxicology (4, 10, 11).

Applications in our laboratory

In our laboratory, the drugs for which we had no previous method, but which we now analyze by LC/MS, include risperidone, ziprasidone, gabapentin, baclofen, clonidine, sildenafil, and morphine glucuronides (direct). Analyses that we moved from GC/MS to LC/MS include propoxyphene, norpropoxyphene, fluoxetine, norfluoxetine, benzodiazepines, quetiapine, paroxetine, albuterol, blood delta-9-tetrahydrocannabinol (THC), and 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (carboxy-THC). Moving methods from GC/MS to LC/MS eliminates the need for derivatization and usually results in simpler extraction, lower detection limits, and shorter run times.

Benzodiazepines: The measurement of highly polar benzodiazepines and metabolites at low concentrations by GC/MS is difficult. Benzodiazepine testing by LC/MS is much easier. An important application of benzodiazepine analysis in our laboratory involves cases of suspected drug-facilitated sexual assault. Most benzodiazepines exert a sedative, hypnotic effect rendering a victim more susceptible to sexual assault or more willing to consume alcohol or other drugs. In many cases, the victim may present to the sexual assault nurse examiner several hours or even a day after the incident. By then, the drug may have metabolized to low concentrations, making detection more challenging. Besides diazepam and nordiazepam, which are still analyzed on the GC/MS because the performance is suitable for the concentrations typically encountered, our laboratory has a urine screening protocol for fourteen benzodiazepines: temazepam, triazolam, lorazepam, clonazepam, α-hydroxytriazolam, midazolam, 7-aminoclonazepam, flunitrazepam, flurazepam, alprazolam, 7-aminoflunitrazepam, desalkylflurazepam, α-hydroxyalprazolam, and oxazepam.

We use a simple liquid–liquid extraction at alkaline pH and evaporate the resulting extract to dryness. The residue is reconstituted in mobile phase and injected. It is important to conduct enzymatic hydrolysis on urine specimens prior to extraction of the benzodiazepines. Although all these substances can be detected in one method on our triple-quadrupole LC/MS, we typically perform two injections of the same extract to lower the detection limits. We routinely see at least 1–2 ng/mL of the benzodiazepines. The chromatographic run time is 6 minutes on a C18 LC column (50 mm x 2.1 mm, 5 micron).

The analysis of alprazolam in blood is a good example of how LC/MS can significantly improve detection and reduce analysis time. We previously analyzed alprazolam by full-scan electron-impact GC/MS with a routine limit of quantitation of 25 ng/mL. In comparison, Figure 1 shows a product-ion chromatogram (m/z 309.1⇒ 281.0) for alprazolam by LC/MS/MS at a concentration of 0.5 ng/mL. The retention time for alprazolam in the GC/MS procedure was approximately 12.5 minutes, compared with a 3.3-minute analysis time with the LC/MS method. Improved sensitivity can also reduce the amount of sample needed for analysis.

Case history

The decedent was last seen the day before by neighbors outside the residence. The next day a realtor arrived and used a lockbox key to show the residence to potential buyers. The decedent was found unresponsive in a small bed in an office.

Emergency services personnel declared the person dead on the scene. Several open medication vials were nearby. No suicide note was found. The decedent's pants were lying near the bed with a large kitchen knife placed on top. No blood was noted on

the knife or the decedent. Suspected drugs at the scene were clonazepam and baclofen.

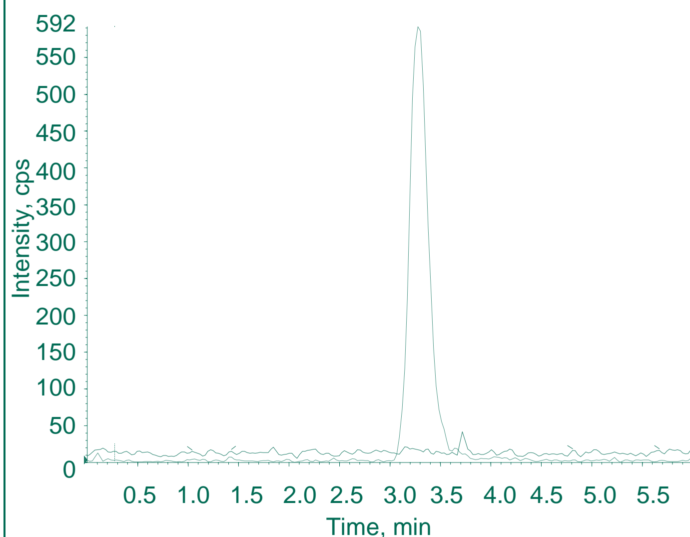
Autopsy results were unremarkable other than congestion in the lungs. The stomach contained 30 mL of turbid brown liquid. No tablets, capsules, or residues were identified within the gastric contents. The small intestine, the colon, and their vascular supply were unremarkable.

Initial enzymeimmunoassay screening produced an indicated response for benzodiazepines (below the cutoff concentration). A blood alkaline drug GC/MS screen produced a small peak that matched the clonazepam metabolite 7-aminoclonazepam in the mass spectrum library. This finding of a high level of 7-aminoclonazepam was unusual. No baclofen was detected by the screening procedures.

The clonazepam and 7-aminoclonazepam were quantified by LC/MS/MS to be 46 ng/mL and 1100 ng/mL in peripheral blood, respectively. This very high level of metabolite is most likely due to postmortem conversion. Nitrobenzodiazepines are known for their instability in postmortem blood, and this case illustrates the need for metabolite quantification. The result indicates that the amount of clonazepam ingested actually was higher than the detected 46 ng/mL concentration.

Baclofen possesses zwitterionic characteristics, which lead to very poor extraction efficiency into organic solvents. Sample preparation for LC/MS requires no extraction, involving only a protein precipitation step followed by evaporation and reconstitution in the LC mobile phase. Baclofen ions are efficiently transferred from the solution after LC separation to the gas phase and into the mass spectrometer via electrospray. Using this method, a baclofen concentration of 25 mg/L was measured in the peripheral blood. The cause of death was determined to be a baclofen overdose.

Figure 1. Blood extract containing alprazolam at 0.50 ng/mL overlayed with a blank blood chromatogram.



Method development strategies and concerns

It is common to say that electrospray transports ions from the solution phase to the gas phase. However, the process of droplet formation, the competition for charges in the droplet, and the efficiency with which the analyte is converted to gas-phase ions all illustrate that the electrospray process is actually very complex. During method development it is important to have a basic understanding of the electrospray process and to investigate the many issues that can improve detectability of an analyte. For example, competition for charges can dramatically affect the detection of a target drug. Improving sample preparation and extraction methods to yield clean extracts free from high concentrations of endogenous substances or salts reduces this problem. Liquid–liquid extraction is an excellent method for isolating organics from salts.

In addition, our laboratory often employs a hexane wash of the reconstituted extract residue to remove unwanted lipophilic components from entering the LC column and eventually the electrospray source. Because LC/MS calibration curves can often be established at low levels, suitable dilution of tissue homogenates or other difficult specimens can reduce matrix effects of these specimens, thus improving detectability. Finally, lower volumes of specimen also tend to reduce matrix effects as well as conserve specimen.

Certain aspects of the separation technique and subsequent ionization are also important to consider. Electrospray is most efficient when high concentrations of methanol or acetonitrile are present in the mobile phase. Keeping the water content below 20 percent is ideal; however, chromatographic separation may suffer. A common method for overcoming these trade-offs is to use gradient LC programs.

Importance of separations

The importance of LC chromatographic separation should not be overlooked. Because the mass spectrometer offers an extra degree of specificity, users may be tempted to think that separation is not always necessary. However, separating the drugs of interest from the interfering endogenous substances will most likely enhance the drug signal.

At higher LC flow rates, electrospray is characterized as a concentration-sensitive technique; therefore, a linear-response relationship is possible for at least one order of magnitude and higher flow rates do not affect the signal intensity. Most of the elec-

trospray methods in our laboratory use flow rates from 200–300 µL/min. The linear regression calibration graphs are typically established from the 1–10 ng/mL range up to the 200–500 ng/mL range for most drugs with alkaline characteristics. Establishing the calibration graphs on the lower end or below the therapeutic range allows dilution of the case specimens and therefore better matching of the matrix with the calibration standards.

Although ESI has gained status as a "universal" ionization source for LC/MS, a technique known as atmospheric pressure chemical ionization (APCI) is another source commonly supplied with commercial LC/MS instruments. APCI is generally regarded to be better for lower polarity molecules and to have a wider dynamic range than electrospray. However, analytes must be thermally stable up to around 150 ºC and must be volatile. Our laboratory uses positive-ion APCI, after solid-phase extraction, for the determination of THC and its metabolite, carboxy-THC, in blood. The separation is conducted on a 50-mm C18 column with a 5-micron particle size. A mobile phase consisting of a formic acid buffer at pH 4 is used at a flow rate of 400 mL/min. THC and carboxy-THC are eluted separately from the solid-phase extraction column, and in turn are injected separately on the LC/MS. We use a lower limit of quantitation of 2 ng/mL and 5 ng/mL for THC and carboxy-THC, respectively.

Commercially available LC/MS systems

The number of LC/MS instruments in labs performing routine toxicology assays has increased significantly in the past few years. The number of articles in peer-reviewed journals reporting LC/MS as the analytical technique has increased correspondingly. In 1995, the *Journal of Analytical Toxicology* and *Clinical Chemistry* each had only one article referencing LC/MS as the analytical technique used. The projected number of LC/MS-related articles in 2004 is more than 20 for each of these journals.

Another reason for the increased use of LC/MS instrumentation is that it has become more affordable. A few years ago the cost of a triple-quadrupole LC/MS instrument was more than $300,000. Now these systems start at around $200,000. Ion-trap systems start at $150,000, and single-quadrupole systems are $125,000. Their size has also decreased, with benchtop units having a footprint of less than nine feet including the data station.

When choosing a mass analyzer for LC/MS in a forensic or clinical toxicology laboratory, it is important that the instrument be able to perform MS/MS for selectivity and also provide accurate quantitative results. A triple-quadrupole mass spectrometer is one type of mass analyzer that balances performance and cost to achieve these two goals. In a triple-quadrupole mass spectrometer, a precursor ion is isolated by quadrupole one. In quadrupole two, this precursor ion is accelerated and subjected to collisions with a background nitrogen or argon gas. This process results in the fragmentation of the precursor ion. Quadrupole three is then used to scan the resulting product ions either individually or in a full-scan mode. Therefore, by starting with an individual ion from a drug, the product ions provide a mass spectral identification of that drug. The more product ions that are observed, the more selective the identification.

The technique of monitoring individual product ions is called multiple-reaction monitoring or selected-reaction monitoring. The correct identification of a drug or poison is of paramount concern in forensic and clinical toxicology. A mass spectrometer capable of MS/MS and the monitoring of more than one product ion is necessary to achieve this goal, especially when LC/MS provides the only means of detection of a particular substance.

Other mass analyzers, including single quadrupole and quadrupole ion trap, have been successfully coupled to liquid chromatography and are commonly found in forensic and clinical toxicology laboratories. Single-quadrupole LC/MS instruments do allow fragmentation of a precursor ion in the source; however, the fragmentation energy is applied to all ions exiting the LC column at a particular time. Single quadrupoles therefore suffer from a higher background signal reaching the detector, thus often resulting in higher detection limits with lower specificity. Quadrupole ion trap LC/MS systems do allow MS/MS or even MS^n and are commonly used in laboratories performing structural elucidation studies of drugs and metabolites.

When evaluating a particular instrument, concern should be given to the designs of the atmospheric pressure ionization source(s) and the vacuum interface, because these features are specific to a particular instrument company and may be protected by patents. The features of each instrument should be evaluated to see if they meet the desired capabilities for one's laboratory. Finally, reputation, cost, and completeness of the package, including the LC system components, service contracts, and training, may give one manufacturer an advantage over another.

Conclusion

Today's LC/MS instruments are robust and reliable with control achieved through well-written software. A basic understanding of the theory regarding ion formation and transmission from the solution to

the gas phase for ESI and APCI, as well as a keen understanding of the operation of the mass spectrometer and interpretation of the results, will give one a good basis for developing or implementing successful LC/MS methods. For those with only a GC/MS background, training classes offered by the LC/MS manufacturer are highly recommended. Some have speculated that LC/MS instruments will eventually replace GC/MS instruments in the analytical toxicology laboratory. We recognize that LC/MS has advantages for several types of analytes; however, we view the techniques as complementary.

References

1. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray ionization-principles and practice. Mass Spec Rev 1990;9:37–70.
2. Cho A, Normile D. Nobel Prize in chemistry: mastering macromolecules. Science Oct 18 2002;298(5593):527–8.
3. Olah TV, McLoughlin DA, Gilbert JD. The simultaneous determination of mixtures of drug candidates by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry as an in vivo drug screening procedure. Rapid Commun Mass Spec 1997;11:17–23.
4. Maurer HH. Liquid chromatography-mass spectrometry in forensic and clinical toxicology. J Chromatog B Biomed Sci Appl 1998;713:3–25.
5. Maurer HH, Schmitt CJ, Weber AA, Kraemer T. Validated electrospray liquid chromatographic-mass spectrometric assay for the determination of the mushroom toxins alpha- and beta-amanitin in urine after immunoaffinity extraction. J Chromatog B Biomed Sci Appl 2000;748:125–35.
6. Cirimele V, Villain M, Pepin G, Ludes B, Kintz P. Screening procedure for eight quaternary nitrogen muscle relaxants in blood by high-performance liquid chromatography-electrospray ionization mass spectrometry. J Chromatog B Analyt Technol Biomed Life Sci 2003;789:107–13.
7. Sayer H, Quintela O, Marquet P, Dupuy J-L, Gaulier J-M, Lachâtre G. Identification and quantitation of six non-depolarizing neuromuscular blocking agents by LC–MS in biological fluids. J Anal Toxicol 2004;28(2):105–10.
8. Wan H, Umstot ES, Szeto HH, Schiller PW, Desiderio DM. Quantitative analysis of [Dmt(1)]DALDA in ovine plasma by capillary liquid chromatography-nanospray ion-trap mass spectrometry. J Chromatog B Analyt Tech Biomed Life Sci 2004;803(1):83–90.
9. Stokvis E, Rosing H, Crul M, et al. Quantitative analysis of the novel anticancer drug ABT-518, a matrix metalloproteinase inhibitor, plus the screening of six metabolites in human plasma using high-performance liquid chromatography coupled with electrospray tandem mass spectrometry. J Mass Spec 2004;39:277–88.
10. Bogusz MJ. Liquid chromatography-mass spectrometry as a routine method in forensic sciences: a proof of maturity. J Chromatog B Biomed Sci Appl 2000;748:3–19.
11. Marquet P. Progress of liquid chromatography-mass spectrometry in clinical and forensic toxicology. Ther Drug Mon 2002;24:255–76.

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Propoxyphene: Still Popular After Five Decades of Use

By Alphonse Poklis

Propoxyphene is the name given to the alpha-rademate isomers of the diastereomeric chemical 4-dimethylamino-3-methyl-1,2- diphenylpropionoxy-butane. Generally, propoxyphene refers to the dextrorotatory isomer, d-propoxyphene (dextropropoxyphene) that has analgesic activity and was initially available under the trade name Darvon. The levorotatory isomer of propoxyphene is without analgesic activity, but was formerly available as a cough suppressant under the trade name Novrad (Darvon, spelled backwards).

An effective analgesic acting on the central nervous system, propoxyphene is used for the treatment of mild-to-moderate pain. When taken orally, a 100-mg dose is about as effective as a 65-mg oral dose of codeine. However, propoxyphene has a much lower incidence of gastrointestinal side effects such as nausea, anorexia, constipation, and abdominal pain. The drugs are equally likely to cause drowsiness.

When given parenterally, a 130-mg dose of propoxyphene produces analgesia equivalent to that produced by 50 mg of meperidine (1). However, like methadone, it causes burning and local tissue irritation and is not administered therapeutically by injection.

Propoxyphene has no antipyretic, anti-inflammatory, or antitussive action. It is available in oral formulations as either the hydrochloride (65 mg) or the napsylate salt (50 or 100 mg), and is often found in combination with aspirin and caffeine or acetaminophen. Daily oral doses range from 128–390 mg for the hydrochloride and 200–600 mg for the napsylate.

Pharmacokinetics

Propoxyphene is absorbed after oral or parenteral administration; but, like codeine, it is considerably less effective by the oral route. Following absorption, propoxyphene is distributed throughout the body tissues and fluids. It has a very large volume of distribution, ranging from 12 to 26 L/Kg. Propoxyphene is metabolized primarily via n-demethylation to norpropoxyphene, which is one-fourth to one-half as active an analgesic as propoxyphene. The contribution of norpropoxyphene to the efficacy of the parent drug has not been thoroughly established. Norpropoxyphene is further converted to several inactive dealkylated, cyclic amide, and ring hydroxy-

lated metabolites (2). Only about 10% of an administered dose can be recovered unchanged in the 24-hour urine (1).

Steady-state plasma concentrations after daily doses of 195 mg of hydrochloride average 0.42 mg/L propoxyphene and 1.45 mg/L norpropoxyphene two hours post administration. Plasma half-lives average 15 hours (range, 8–24 hours) for propoxyphene and 27 hours (range, 24–34 hours) for norpropoxyphene. The minimum lethal dose of propoxyphene in adults has been estimated at 500–800 mg. Generally, blood propoxyphene concentrations exceeding 1 mg/L are considered life-threatening, and concentrations >2 mg/L are lethal (3).

However, significant tolerance can develop with chronic use of high doses, particularly in those addicted to opiates. Asymptomatic opiate addicts on daily doses of 800–1600 mg propoxyphene napsylate for the maintenance of withdrawal had serum concentrations ranging from 0.13–1.07 mg/L and 0.82–2.64 mg/L for propoxyphene and norpropoxyphene, respectively (4)!

Toxicity

At the usual therapeutic doses propoxyphene produces no significant effects on the cardiovascular system nor does it produce a significant decrease in the response to respiratory stimulation by CO_2 (5). Moderately toxic doses usually produce central nervous system and respiratory depression, with large overdoses complicated by convulsions, hypotension, and cardiac arrhythmias with neurological signs progressing from stupor, coma, and pulmonary edema to death by respiratory depression or circulatory collapse (6). In rats, a narcotic antagonist will not only reduce the lethality of toxic doses of propoxyphene, but also prevent or reduce the incidence of convulsions (7). However, the use of an antagonist is ineffective in human overdoses.

Norpropoxyphene is partially responsible for the toxic effects of propoxyphene, especially the delayed cardiotoxicity. Norpropoxyphene toxicity is resistant to naloxone. In cases of overdose of combination forms of propoxyphene and acetaminophen, the delayed toxicity of acetaminophen must be considered and treated. The effects of acetaminophen may be missed with the slowing of gastrointestinal motility due to propoxyphene or formation of a bezoar (8).

Early success

Now available under several different trade names or as a generic, propoxyphene was originally introduced by Eli Lilly in 1957 as Darvon, an analgesic to bridge the gap between aspirin and potent opiates. For the next 20 years, propoxyphene was one of the most commonly prescribed drugs in America. Its immediate and sustained success was related to several claims and practical factors. Initially, it was claimed to be "equal to codeine, milligram per milligram," as an analgesic, without being addictive.

When very large doses were used in morphine-tolerant addicts, some respiratory depression was seen, suggesting that there was not a high degree of cross-tolerance between propoxyphene and morphine. However, abrupt discontinuation of chronically administered propoxyphene (up to 800 mg per day given for almost two months) resulted in mild abstinence phenomena.

Large oral doses (300–600 mg) produced subjective effects that were considered pleasurable by post-addicts, but were not necessarily identified as morphine-like (9). The wide popularity of propoxyphene in clinical situations in which codeine had been previously used seemed to be a result of an unrealistic concern about the addictive potential of codeine. Additionally, physicians were generally not aware of propoxyphene toxicity or the possibility of lethal overdose (10).

Changing impressions

By the late 1970s, popular impressions of propoxyphene pharmacology and usefulness were in question. With the advent of improved analytical methodologies in the early 1970s, forensic toxicologists were detecting propoxyphene in drug overdose deaths at a rate that was increasing at a faster pace than the increase in total drug deaths (11). The publication of numerous surveys of propoxyphene-related deaths, particularly in major medical journals (12, 13), alerted the medical community to the toxicity of this drug that was previously considered relatively benign.

In addition, it was established that propoxyphene could cause addiction, although most addicts were in the population of prescription-drug and alcohol abusers (similar to today's oxycodone abusers). Indeed, in 1978 a leading textbook in clinical pharmacology stated, "The most prominent effect [of propoxyphene] may be its addictive quality" (14).

The popular form of propoxyphene was a mixture with aspirin in a bullet-shaped capsule, called Darvon Pulvule. Propoxyphene hydrochloride was in the capsule as a small round pink pill that abusers could easily remove, dissolve in water, and inject. Although propoxyphene was not widely used by intravenous drug users, the manufacturer responded to this concern by introducing insoluble tablets as the napsylate salt, Darvon N.

During this time, the efficacy of propoxyphene came into question, as many studies indicated it was

no more effective as an analgesic than aspirin and much less effective than codeine in pain control. Indeed, in 1971 the first edition of the American Medical Association's book, *Drug Evaluation*, stated, "[Propoxyphene's] popularity is probably due to the fact that it does not require a narcotic prescription, rather than its effectiveness as an analgesic." These issues resulted in regulatory action in 1977, when prescribing of propoxyphene was limited for the first time by placement in Schedule IV of the Controlled Substances Act.

Despite these concerns, propoxyphene remains a popular drug today. Twenty years later in 1999, it was among the 15 most commonly encountered drugs in emergency room incidents (17).

Analysis

Because propoxyphene has been available for almost 50 years, all the analytical advances in toxicology have been applied at one time or another to its detection in biological specimens. Indeed, recently gas chromatography/mass spectrometry (GC/MS) has been used to detect propoxyphene and norpropoxyphene in hair (16). (Can publication of a high performance liquid chromatography/MS/MS method be far behind?) However, the College of American Pathologists (CAP) toxicology proficiency surveys from 1990 to the present reveal that by far the most popular screening tests for propoxyphene are immunoassay and Toxi-Lab thin-layer chromatography (TLC). GC and GC/MS are popular methods for confirmation and/or quantification.

All the popular commercial propoxyphene immunoassays display good analytical sensitivity, including Triage Plus PPY immunoassay, DRI enzymeimmunoassay, Emit II plus enzymatic immunoassay, Abuscreen Online microparticle immunoassay, Abbott TDx, Axsym fluorescence polarization immunoassay, and cloned enzyme donor immunoassay (CEDIA) calibrated with propoxyphene at 300 ng/mL. During the past 10 years of the CAP-UDS survey, more than 99% of respondents testing with these assays correctly reported the presence of propoxyphene when challenged. However, the tests vary in sensitivity to norpropoxyphene: Triage Plus PPY has a cutoff of 400 ng/mL, while DRI, TDx, OnLine, and CEDIA have a cutoff of ~500 ng/mL. Emit has the poorest sensitivity with a cutoff of 800 ng/mL.

This lack of sensitivity to norpropoxyphene was noted in a recent study that included 98 urine specimens positive for propoxyphene and/or norpropoxyphene by GC/MS (17). In the study, there was a 98.8% agreement of positive or negative results between Triage Plus PPY and both the DRI and OnLine assays. The two discordant specimens contained norpropoxyphene concentrations within ±20% of the cross-reactivity cutoff value for norpropoxyphene for the three assays. However, there was only an 88% agreement of positive or negative results between these three assays and the Emit assay. Twenty urine specimens yielding positive results were negative by Emit. The discordant results were due to poor cross-reactivity of Emit to norpropoxyphene concentrations of 1000 ng/mL or less.

In general, these immunoassays display good selectivity; however, elevated urine concentrations of diphenhydramine or tricyclic antidepressants may yield false-positive results. Although propoxyphene is chemically related to methadone, neither methadone nor its metabolites cross-react with propoxyphene immunoassays. Also, the d- and l-isomers of propoxyphene can be differentiated by the popular commercial immunoassays.

Propoxyphene is readily isolated from urine by liquid-liquid Toxi-tube A extraction and identified by Toxi-Lab-A TLC. Propoxyphene (Rf 0.82) yields a blue spot in Stage 1 visualization with formaldehyde vapor and sulfuric acid. Propoxyphene use is readily indicated by the additional presence of the norpropoxyphene metabolite, which produces a characteristic tear-drop streak between Rf 0.2–0.5 and appears olive or gray in Stage 1 visualization. At very low concentrations, the norpropoxyphene spot may appear as a weak, blanched, yellowish discoloration similar to sympathomimetic amine drugs.

Chromatography

All popular GC and electron impact GC/MS methods for propoxyphene analysis treat the sample with a strong base before the final extraction to convert norpropoxyphene into a cyclic amide to improve its chromatographic properties. This reaction was first described in 1968 by Wolen and Gruber (18). Propoxyphene and the cyclic amide are readily extracted from alkaline media by liquid/liquid extraction or by numerous commercial solid-phase extraction methods. Propoxyphene and the cyclic amide both chromatograph well in capillary columns on fused-silica-bonded phases, in general displaying better peak shape on the intermediately polar phenylmethylsilicone phases than on nonpolar methylsilicone phases. Both analytes give good response with a nitrogen phosphorus detector.

For MS analysis deuterated internal standards are available. Typically a detector is operated in the selective ion monitoring mode using the following ions: propoxyphene, 250, 265, and 178; norpropoxyphene cyclic amide, 105, 220, and 77; 2H5-propoxyphene 255 and 270; and 2H5-norpropoxyphene cyclic amide, 239 and 240. The lower limit of quantification of

each analyte in a typical urine drug-testing confirmation procedure is 150 ng/mL.

Today, the use of semi-synthetic opiates such as oxycodone and hydrocodone has decreased the prescribing of propoxyphene for pain treatment. However, given its resilience in the face of past criticism, no doubt propoxyphene will continue to be a concern to toxicologists on its coming fiftieth birthday.

REFERENCES

1. Lasagna L. The clinical evaluation of morphine and its substitutes as analgesics. Pharmaco Rev 1964;16:47–83.
2. McMahon RE, Sullivan HR, Due SL, Marshall FJ. The metabolite pattern of d-propoxyphene in man. The use of heavy isotopes in drug metabolism. Life Sci 1973;12:463–73.
3. Baselt RC. Disposition of toxic drugs and chemicals in man, 6th ed. Foster City, California: Biomedical Publications, 2003.
4. Hartman B, Miyada DS, Pirkle H, Sedgwick P, Cravey RH, Tennant Jr FS, Wolen RL. Serum propoxyphene concentrations in a cohort of opiate addicts on long term propoxyphene maintenance therapy. Evidence for drug tolerance in humans. J Anal Toxicol 1998;12:25.
5. Burget Jr DJ, Greene MN. Dextropropoxyphene and ventilatory response to carbon dioxide in man. Yale J Biol Med 1962;35:185.
6. Nicotra MB, Rivera B, Pool JL. Dextropropoxyphene overdose: clinical and pharmacological observations. Clin Toxicol 1981;18:599–613.
7. Chapman JE, Walaszek EJ. Antagonism of some toxic effects of dextropropoxyphene by nalorphine. Toxicol Appl Pharmacol 1962;4:752.
8. Spiller HA. Persistently elevated acetaminophen concentration for two days after an initial four-hour non-toxic concentration. Vet Human Toxicol 2001;43:218.
9. Fraser HF, Isbell H. Human pharmacology and addiction liability of *dl*- and *d*-propoxyphene. Bull Narcotics 1960;12:9.
10. Public Citizen, press release, Nov 21, 1978, citing Intern J Addiction 1977;12:43.
11. Finkle BS, McClosky KL, Kiplinger GF, Bennett IF. A national assessment of propoxyphene in postmortem medicolegal investigation, 1972–1975. J Foren Sci 1976;21:706.
12. Sturner WQ, Garriott JC. Deaths involving propoxyphene: a study of 41 cases over a two-year period. JAMA 1973;223:1125.
13. McBay AJ, Hudson P. Propoxyphene overdose deaths. JAMA 1975;233:1257.
14. Melmon KL, Morrelli HF. Clinical pharmacolgy: basic principles in therapeutics, 2nd ed. New York: McMillan, 1978.
15. Year-end 1999 emergency department data. Drug Abuse Warning Network, National Institute on Drug Abuse, Rockville, Maryland, 1999.
16. Gaillard Y, Pepin G. Gas chromatographic-mass spectrometric quantitation of dextropropoxyphene and norpropoxyphene in hair and whole blood after automated on-line solid-phase extraction. Application in twelve fatalities. J Chromatog Biomed Sci Appl 1998;709:69.
17. Poklis A, Poklis JL, Trani LD, Backer RC. Evaluation of the Triage PPY on-site testing device for detection of dextropropoxyphene in urine. 31st Ann Mtg Society Forensic Toxicology, Dearborn, Michigan, Oct 15, 2002.
18. Wolen RL, Gruber Jr CM. Determination of propoxyphene in human plasma by gas chromatography. Anal Chem 1968;40:1243.

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