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# Postmortem detection of benzodiazepines

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## Abstract

More than 2,000 different Benzodiazepines (BZD) have been synthesized. There are about three-dozen BZD derivatives that are also available on the market. These drugs have been implicated in sudden and unexplained deaths especially when co-ingested with alcohol. Interpretation of forensic postmortem toxicological data can be very difficult and should be done with a thorough knowledge of the case history, including autopsy results, reports from the scene, and available medical history. Experienced forensic toxicologists rely on their own case experience as well as the unique circumstances of each case under examination. Even armed with detailed toxicological data, it is still difficult to pinpoint the cause of death when multiple agents are ingested at the same time.

## Keywords

Pharmacology, Forensic Toxicology, Benzodiazepines, Clinical Pharmacology, Emergency Medicine, Psychiatric Drugs, Postmortem Forensic Examination, Forensic Medicine

## 1. Introduction

An Austrian chemist working for the pharmaceutical giant Hoffmann-La Roche was the first scientist to discovered BZD. His name was Dr. Leo Sternbach. He discovered the drug in 1954. The efficacy and superiority of this class of drugs were not immediately known. But the superiority of BZD over other related drugs became known shortly after its discovery. This led to the first BZD being patented in 1959. In 1960, a new drug called Librium (chlrodiazepoxide) was introduced to the world. Interest in the BZD drug and its medicinal properties continued to grow. Researchers continued with additional testing of related chemical compounds, which eventually led to the discovery and introduction of the drug, diazepam (Valium). Valium made its debut in 1963 and is still a widely prescribed drug to treat the symptoms of anxiety. Valium has a high abuse potential and is a drug abused extensively, particularly among the health care professionals. [1]

Diazepam is an anti-anxiety agent and it is shown to be approximately three times more potent than chlrodiazepoxide with greater muscle relaxing properties. The research on BZDs has continued and has lead to the discovery of several BZD derivatives such as nitrazepam (Mogadon), flurazepam (Dalmane) and alprazolam (Xanax). Xanax is also a popular drug of abuse. By around the mid-1970s, over 8000 tons of BZDs were being sold every year. Today. More than 2,000 different BZDs have been synthesized. There are about three-dozen BZD derivatives that are also available on the market. [3]

## 2. Epidemiology

BZDs are encountered with some frequency in overdose surveys. BZDs are usually consumed in combination with other drugs. These drugs rarely cause fatal outcomes. Overdoses involving BZD are likely most prevalent in suicide attempts rather than from unintentional consequences of recreational use. In this regard, such overdoses are similar to those typically seen with psychotherapeutic drugs and do not resemble typical cases that are the benchmark for the drugs of abuse. Because of their widespread use, the BZD class of drugs has a high potential for abuse. Moreover, BZD are frequently used in cases of overdose, either as single substance exposure or in combination with other substances. Alcohol is frequently implicated as a synergist in the incidents of BZD overdose.

[27] In 2008, a total of 78,443 BZD single-substance exposures were reported to US poison control centers. Of the reported cases, 332 (0.004%) resulted in major toxicity and 8 (0.0001%) resulted in death. In the UK during the 1980s, the overall rate of mortality was 5.9 per one million prescriptions for BZDs. The exposure to temazepam and flurazepam was associated with the most toxic effects in the reported cases. Survey data from the United States have documented continuing declines in nonmedical use of BZD in the general population.[28] Therapeutic use has largely shifted from the older, longer-acting BZDs to the shorter-acting agents that have become available more recently. In this article I discuss the challenges faced by forensic toxicologists in detecting BZD in postmortem biological fluids and establishing the analyte in question is indeed the cause of death.

## 3. Chemistry

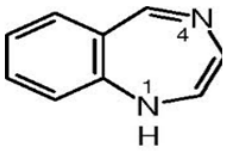


***Figure 1.***

The term BZD is the chemical name for the heterocyclic ring system, which is formed by the joining one benzene and one diazepine ring. Benzene is an organic chemical compound that has a molecular formula C6H6. [1]Benzene is a natural component of crude oil, and it represents one of the most basic petrochemicals. Benzene is an aromatic hydrocarbon, the second [n]-annulene, and a cyclic hydrocarbon with a continuous pi bond. It is also related to the functional group of aromatic hydrocarbons known as arene that is a generalized structure for benzene. [1] Diazepine is a seven-member heterocyclic compound with two nitrogen atoms. When combined with a benzene ring, it forms the structural basis for the BZD class of drugs. In these drugs, the nitrogen atoms are at the 1 and 5 positions as, for example, in clobazam. Depending on the position of the fused benzene ring, the nitrogen atoms may also be given numbers 1 and 4. BZD drugs are substituted 1,4-benzodiazepines, but this chemical designation is not specific enough because this designation may also refer to other compounds that do not have any active pharmacological properties. [2]

BZD drugs may be differentiated based on their varying side chains that are attached to the central structural skeleton. The different side chains affect the affinity of the molecule for binding to thegamma-aminobutyric acid(GABA) receptors. It is this binding that modulates the pharmacological properties of BZDs. Many of the pharmacologically active BZD drugs contain the 5-phenyl-1H-benzo[e][1,4]diazepin-2(3H)-one structure. Mechanism of Action ofGamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the CNS. BZDs mediate their effect by potentiating the activity of GABA. When BZD binds to a specific receptor on the GABA receptor complex, it facilitates the binding of GABA to the specific receptor site. BZD binding results in frequent opening of the chloride channel that is part of the GABA receptor. When Chloride channel opens, it results in membrane hyperpolarization, which in turn inhibits cellular depolarization. The increase in GABA neurotransmission results in sedation, muscle relaxation, anxiolysis, and anticonvulsant effects. When peripheral nervous system (PNS) is stimulated by GABA neurotransmission those PNS GABA receptors may cause reduced cardiac contractility and vasodilatation. These changes have the potential to alter tissue perfusion. [6,7]

The rate at which BZD mediates its onset of action is influenced by the drug's ability to cross the blood-brain barrier. There are some BZDs that are relatively lipophilic. For this reason, these BZDs usually produce a faster onset of action compared to the relatively water-soluble BZDs. BZD effects can be enhanced with the co-ingestion of ethanol. Peak blood concentrations of most BZD drugs occur within 1-3 hours after oral administration. After a single dose administration, the lipophilic BZDs will have a shorter duration of action (shorter CNS effect) than water-soluble BZD agents. This is due to the rapid redistribution from the CNS to peripheral sites such as the adipose tissue. Hence, lorazepam, a water-soluble BZD, has a longer CNS duration of action than diazepam, which is more lipophilic. However, diazepam metabolizes to active intermediates that prolonged its half-life and thus extending this drug's therapeutic effects.[8] BZDs are metabolized primarily in the liver. This is accomplished through a process of oxidation and/or conjugation. When the body metabolizes most BZDs, they produce pharmacologically active metabolites. These metabolites often have longer half-lives than their original parent compounds. [8]

## 4. Postmortem Forensic Examination of Benzodiazepines

Drug detection in postmortem specimen is routinely performed as part of a forensic autopsy. The results of the toxicological examination often assist the law enforcement in arriving at the cause of death. Initially, toxicological analysis will assist the coroner, medical examiner, or equivalent in establishing evidence of drug use. Alternatively, toxicological data may help refute drug use as the cause of death. The latter is an important factor in forensic autopsies because a pathological examination alone will not reveal evidence of drug use. Drug use may only be confirmed by appropriate toxicological procedures performed on appropriately collected sample(s). Clearly, in cases of sudden or unexplained death, evidence of drug use may serve to establish the cause of death, or at the very least, point to evidence indicating drug misuse, drug abuse,

or even suicide. [12]

Drug misuse here is defined as those cases where inappropriate doses or inappropriate drug combinations have been ingested. Drug abuse on the other hand denotes cases of deliberate and usually recreational use of illicit drugs. Neither term here is meant to imply a suicidal intent. Toxicological analysis is particularly important in cases of homicide, where drugs may have been given by the assailant to reduce the victim's consciousness. Toxicological data is also valuable in cases in which the victim used drugs in an apparent suicide. In this latter situation, influences of the drug on the victim's behavior may be important in criminal trials, not necessarily to mitigate the intent by the defendant, but primarily to reconstruct, as nearly as possible, the chain of events that led to the act. [15]

Such reconstruction may involve corroboration by witnesses who may have observed the victim's drug-using behavior. Typical drugs used in such cases are alcohol, amphetamines, and cocaine. But in cases of suicide, toxicologists often encounter one or more of the BZDs such as alprazolam, diazepam, flunitrazepam, etc. Blood is the most useful specimen and it is the biological fluid commonly collected during the course of an autopsy to be used for toxicological analysis. Blood is useful because drugs present in this fluid can best be related to physiological effects observed in the deceased and may be used to assess the likelihood of a recent drug use or exposure to toxic chemicals.

There are problems associated with the collection of blood during postmortem examination. The major problem is the phenomena of postmortem drug redistribution. This refers to the processes by which the movement of drugs and other chemical poisons occur between tissues, organs, and body fluids after death.Another problem is biotransformation after death. This is particularly true of certain classes of BZD to be discussed later.

Urine is another specimen source and is often collected for toxicological analysis. Since concentrations of drugs and their metabolites are usually much higher in the blood than the urine, urine concentration levels provide a more realistic benchmark for assessing drug use over the preceding day or two. Urine may be collected during the autopsy by the opening of the abdomen, or by direct puncture to the bladder. However for the detection of BZD in postmortem examinations, liver and blood are the most preferred methods. The urine and serum are least preferred in postmortem examinations as urine and serum are used most often in antemortem analysis. There are several methods for detecting drugs in the urine. The most frequent method is an enzyme immunoassay (EIA), or radioimmunoassay (RIA), and florescence polarization immunoassay (FPIA). There are additional more sophisticated methodologies that may be performed on extract of urine. These are analysis performed using TLC, TLC liquid chromatography (HPLC) or gas chromatography/mass spectrometry (GS/MS). The only accepted procedures for urine analysis which also incorporate the definition of the National Institute of Drug Abuse (NIDA), and the Department of Defense (DOD), are immunoassays followed by gas chromatography/mass spectrometry confirmation.

The liver is a favored tissue for toxicological analysis because drugs are often found in higher concentrations there than in the blood. Liver is readily homogenized and it is the main organ for xenobiotic transformation. A liver sample must be collected in all cases of suspected drug use. A 100 g aliquot is sufficient for most toxicological analysis. The right lobe of the liver is the preferred site for sample collection. The right lobe is least susceptible to postmortem diffusion of drug from the bowel contents or from the mesenteric circulation. Drug content following analysis is normally reported as milligrams per kilogram of wet tissue weight. The specimens analyzed in postmortem cases are most often blood and liver instead of urine and serum. The use of blood, liver, and all other postmortem specimens require separate validation that may be markedly different than those used in antemortem examination. The toxicological methods used require modification in postmortem analysis to ensure a reliable extraction recovery, a low level of interference, and reproducible quantitative results. Special attention must be paid to these factors so that partly or fully putrefied specimens are free from interference that usually originates from endogenous substances.

Cutoff values often used in workplace, sports, and drugs testing are no longer valid in postmortem examination that involves specimens other than urine. Moreover care must be exercised to avoid postmortem urine examination that relies on cutoff limits used for antemortem drug testing. The reason for this modification is necessary because the presence of even a small concentration of drug may have forensic significance in a postmortem examination. The same may not be the case in routine antemortem drug testing. It is essential for the drug-screening procedure to encompass the widest category of drugs. Examination of urine or blood using one of the commercial immunoassays, or even thin layer chromatography (TLC), is usually the recommended first step for the main classes of drugs. These drug classes include amphetamines, barbiturates, BZD, cannabinoids, cocaine metabolites, and morphine-like opiates.

The use of a solvent extraction technique at acidic pH, or simple precipitation of blood proteins with acetonitrile allows the more potent BZDs to be detected with the aid of gradient HPLC with multiwavelength or photo-diode array detection. A basic extraction procedure using butyl chloride or a solid-phase extraction procedure with octadecyl-bonded cartridges or mixed-phase cartridges provides a fairly clean extract from postmortem blood or other tissues that may be suitable for analysis by capillary gas chromatography (GC) with flame ionization detector (FID).

The use of amass spectrometry (MS) detector is preferred in postmortem examination of blood samples.. This will

allow simultaneous detection and confirmation, although a nitrogen-phosphorus detector will provide a higher sensitivity for many substances compared to full scan MS. Electron capture detectors (ECD) are extremely useful for detection of BZDs. The confirmation utilizing gas chromatography/mass spectrometry is required because the screening methodology using immunoassay can give false positive results secondary to cross reactivity. This problem is due to the fact that screening assays cannot specifically identify the drug. Instead, the antibodies recognize substances that may have similar chemical structure and are immunologically or enzymologically reactive but are other than the drug of interest. For example, immunoassays for amphetamines show cross reactivity with drugs structurally related to amphetamines such as over-the-counter sympatomedicoamines, phenylpropanolamine, and ephedrine. They also show cross reactivity with over-the-counter legal medications available for nasal congestion, cold, and appetite suppressant. For these reasons, confirmation is required by gas chromatography/mass spectrometry.Thecombination of gas chromatography/mass spectrometry provides an extremely high index of reliability when properly preformed. Although the above procedures are usually used in connection with antemortem urine specimens, if they are used on a postmortem urine sample, these methods must be adjusted and revalidated as discussed earlier. Also as mentioned elsewhere, in postmortem drug analysis, the most commonly used sample matrix is the whole blood. Postmortem changes can denature the matrix, resulting in a loss or degradation of drugs. This, in turn, may result in erroneous analytical findings. [10]

A number of drugs are capable of undergoing post death chemical changes in the body. These chemical changes may be either metabolically mediated or may be caused by spontaneous degradative processes. Nitro-containing drugs such as the BZDs, nitrazepam, clonazepam, nitrazepam, flunitrazepam, and others, undergo rapid biotransformation after death. This biotransformation yields the respective drug's amino metabolites. These rapid changes are due to the action of certain bacteria known as "obligate anaerobes". Toxicologists must focus their examination on these products of BZD biotransformation instead of attempting to isolate the parent drug. Chemical instability occurs in a large class of drugs and their metabolites. [9]

This is true even in cases where the specimens were stored frozen under appropriate storage conditions. Some BZD and BZD metabolites show time dependent losses. In one study, GC and immunoassay techniques used for blood and urine specimens were compared for their effectiveness as screening tools for detection of BZDs in post-mortem forensic toxicology. The researchers found the GC method for blood analysis in postmortem cases to be a good alternative to the common combination of urine immunoassays followed by GC separation of blood specimen. [9]The authors noted "in post mortem forensic toxicology, the present GC method for blood seems to be a good alternative to the common combination of urine immunoassay followed by quantitative analysis of blood by chromatography". A disadvantage of the present GC method is the fact that the amino groups of the 7-amino metabolites of clonazepam and flunitrazepam do not silylate in the present silylation procedure, and consequently the detection limits of these metabolites are high". [16]

One of the most important factors that affect the interpretation of postmortem drug concentrations is the phenomenon known as "postmortem redistribution". The term 'postmortem redistribution' is used to describe the movement of drugs within the body after death. This redistribution effect results in the blood concentration of a drug being significantly higher at autopsy than that, which is immediately present after death. Postmortem redistribution is a complex phenomenon, and probably involves several mechanisms to a varying degree. The first, and probably the major contributor in most cases is the release and diffusion of the drug after death from tissues or organs that contain high concentrations of the drug (usually the lungs and liver) into nearby cardiac and pulmonary blood vessels. This mechanism has been clearly identified for several drugs. The exact mechanism at a molecular level has not been identified, but it is known there are changes in pH and protein structure that occur after death, and thereby disrupt the protein binding characteristics of drugs. The drugs such as the tricyclic antidepressants that concentrate in the major organs through binding to protein and other molecules are more likely to undergo redistribution by diffusion and enter the nearby blood vessels. [36]

It should be noted that although some toxicologists may refer to postmortem redistribution from the heart, the bulk of the redistribution occurs from the lungs and the liver. In contrast to the tricyclic antidepressants, the BZDs undergo very little postmortem redistribution because they are not highly concentrated in the major organs relative to blood. [17] In general, it is well established that vitreous humor is less affected by changes observed in the whole blood. [24] To assess the usefulness of vitreous humor for the analysis of BZD drugs, Scott and his colleagues obtained postmortem vitreous humor and whole blood from 27 postmortem cases. They investigated three BZD drugs. These drugs were temazepam, diazepam, and demethyldiazepam. For temazepam and diazepam, the researchers found some correlation between the matrices ($R2 = 0.789$ and 0.724, respectively). But for demethyldiazepam, no correlation was detected ($R2 = 0.068$). Regression analysis on plots of vitreous humor versus blood concentrations revealed gradients of less than 1.0 indicating the levels in whole blood were higher than the corresponding levels in vitreous humor. [24]

Femoral blood is commonly accepted as the most reliable specimen for drug analysis in postmortem forensic toxicology.[43] There is considerable data suggesting that the drug concentrations in the peripheral blood samples are

closer to the antemortem level than the concentration in cardiac blood. [13] In those cases where the finding of overdose is to be introduced as evidence in court proceedings, a single sample of blood for toxicological examination may be considered insufficient. In these cases, analysis of several samples of blood and tissue will help to increase the possibility of reaching a correct conclusion. [13]

There are other factors to consider in determining the quantification of BZD in postmortem blood. For example when the stability of BZDs lorazepam, estazolam, chlordiazepoxide, and ketazolam were considered in post- mortem blood stored at different temperatures for at least six months, it was found that stability of these agents remained unchanged in temperatures varying between 20°C and -80°C. But in the case of Estazolam, it proved to be the most stable of all BZDs studies. The most unstable of the BZDs studied was ketazolam because it left no traces after about two weeks in any of the sample bloods. Ketazolam was lost at room temperature and over 8 or 12 weeks at 4°C, with the simultaneous detection of diazepam. Chlordiazepoxide also suffered complete degradation in all samples. Before storage of these blood samples, A solid-phase extraction technique was used on all the studied samples, and benzodiazepine quantification was performed by high-performance liquid chromatography-diode-array detection.[50]. These results suggests that in postmortem blood samples left for long periods in varying storage conditions, the presence of some classes of BZD should be viewed with caution.

It should be noted reference values on drug concentrations in tissues are seldom present. The above data suggests that there is a post-mortem diffusion of drugs along a concentration gradient from compartments of high concentration such as solid organs into the blood with the resulting artefactual increase of drug concentration levels in the blood. The highest drug concentration levels are usually found in central vessels such as pulmonary artery and vein, and lowest levels are found in peripheral vessels such as subclavian and femoral veins. This is due to the postmortem redistribution effect as discussed previously.

Most common analytical methods for detection of BZD may be summarized as shown in the table below:

Summary of Analytical Tests for Quantification of Benzodiazepines in Post-mortem Blood or Liver

***Table 1.***

| Procedure Name | Antemortem | Postmortem | Efficacy |
|---|---|---|---|
| Radioimmunoassay | urine/serum | | XXX |
| Enzyme immunoassay (EIA) | urine/serum | Blood/liver | XX |
| Fluorescence polarization immunoassay (FPIA) | Urine/serum | | XXX |
| Gas chromatography/ mass spectrometry[1] | Urine/serum | Blood/liver | XXXX |
| TLC | Urine/serum | Blood/serum | XXX |
| TLC liquid chromatography (HPLC) or gas | Urine/serum | blood/liver | XXX |
| Electron capture detectors (ECD) | | Blood/liver | XXX |
| HPLC | | Blood/liver | XXXX |
| GC | | Blood/liver | XX |
| LC/MS/MS | Urine/serum | Blood/liver | XXXX |

It should be noted that BZD drugs are relatively resistant to postmortem redistribution effect. [37] A HPLC method has been developed for the analysis of several BZDs including some of their metabolites in the blood, plasma and urine. The method requires a liquid-liquid extraction with n-hexaneethylacetate, a gradient elution on a C8 reversed phase column with non-electrolyte eluent, and photo diode array detection.[40] This method provides for a rapid detection, purity check, identification as well as quantitation of the eluting peaks. The detection limit for this method is 10 to 30 ng and the limit of quantitation is 0.05 and 956g/mL, using 1 mL of blood, plasma, or urine. This analytical procedure is applied routinely in forensic toxicological examinations of blood, stomach content, urine, and organ samples. The lack of electrolyte buffer in the eluent allows for a more robust procedure with shorter rinsing time and fewer technical problems. [40]

Although GC may be recommended as a suitable method for the analysis of most benzodiazepines, several of them, particularly the 3-hydroxyderivatives, undergo thermal degradation and rearrangements.[47] Chlordiazepoxide, cloxa- zolam, lormetazepam, haloxazolam, oxazolam, ethyl loflazepate and temazepam yield multiple peaks, which leads to difficulty interpreting the data[47]

GC-MS is one of the most commonly used techniques for the identification and quantitation of forensic drug samples. As a “hyphenated” technique, it combines the separation power of a GC with the analyte specificity of a spectroscopic technique, providing highly specific spectral data on individual compounds in a complex mixture of compounds often without prior separation. [46] Identification is accomplished by comparing the retention time and mass spectrum of the analyte with that of a reference standard. All compounds identified by GC-MS and reported must be compared to a current mass spectrum of the appropriate reference standard, preferably obtained on the same instrument, operated under identical conditions. [47]

Because of the diversity of chemical structure among benzodiazepines, the use of a single HPLC method to separate all possible compounds is difficult. [46]

By comparison, there is now a faster more accurate forensic toxicology assay, which provides an easy and rapid technique to enable simultaneous multi-drug quantitation and identification from various sample matrices. These could be saliva, urine, or serum and even whole blood samples.The sensitivity of LC/MS/MS (tandem MS) quantitative methods is capable of detecting and quantifying drugs of abuse for forensic toxicology at levels significantly lower than the current

cut-off levels. Application of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for quantitative testing of drugs in urine, blood, serum/plasma, and meconium has generated rapid multi-analyte tests with unheralded sensitivity and specificity. [48]

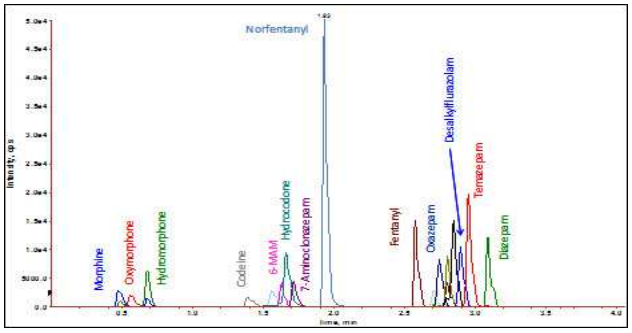


*Figure 2.*

Combined benzodiazepine and opiate analysis. Samples of 9 opiates and 10 benzodiazepines were analyzed in a single method. Data shown are from a 10-ng/mL calibrator (1 ng/mL for fentanyl), and the total run time was six minutes.

## 5. Conclusion

The BZD's are widely prescribed by clinicians for variety of ailments. The widespread availability of BZD raises some concerns about not only the addictive properties of this family of drugs but also their potential for accidental overdose as well as their use as an instruments of suicide. Although BZD's are not widely abused as compared to other drugs, stimulant addicts increasingly use them as parachute drugs. This fact alone and the easy availability of these drugs by prescription should raise the abuse and toxicity profile of BZDs amongst researchers.

Interpretation of forensic postmortem toxicology data can be very difficult and should be done with a thorough knowledge of case history, including autopsy results, reports from the scene, and available medical history. It is not too difficult for a toxicologist to interpret a high blood strychnine concentration in a person found dead in close proximity to an open container of strychnine-containing rodent poison together with a note indicating suicide. But the situation becomes increasing more difficult when multiple substances such as BZD and alcohol are found in the postmortem biological fluids or tissue specimen. The determination of the cause of death and the relative responsibility of each individual substance to the fatal outcome is sometimes impossible to determine within the limits of scientific certainty. There is some temptation for forensic toxicologists and others to refer to tables and charts in order to determine the therapeutic and toxic concentrations of the drug under investigation. Although these reference tables are of some value in clinical toxicology, such references are of dubious value when analyzing postmortem toxicology results.

In fact reliance on charts and tables of therapeutic and fatal drug concentrations can result in misleading and erroneous conclusions. Often times these table references rely extensively on clinical data. They seldom take into account tolerance levels that develop differently from person-to-person. The reference charts for toxicity threshold never account for phenomena such as postmortem redistribution. Experienced forensic toxicologists rely on their own case experience as well as the unique circumstances of each case under examination. This information may be supplemented by compilations of drug monographs where references to the original published work are available. Even armed with toxicological data, it is still difficult to pinpoint the cause of death when multiple agents are ingested at the same time.

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