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Editor's Choice

The Evolution Toward Designer Benzodiazepines in Drug-Facilitated Sexual Assault Cases

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Abstract

Drug-facilitated sexual assault (DFSA) is a crime where the victim is unable to provide sexual consent due to incapacitation resulting from alcohol or drug consumption. Due to the large number of substances possibly used in DFSA, including illicit, prescription and over-the-counter drugs, DFSA faces many toxicological challenges. Benzodiazepines (BZDs) are ideal candidates for DFSA, as they are active at low doses, have a fast onset of action and can be easily administered orally. The last decade has seen the emergence of designer benzodiazepines (DBZDs), which show slight modifications compared with BZDs and similar pharmacological effects but are not controlled under the international drug control system. DBZDs represent an additional challenge due to the number of new entities regularly appearing in the market, their possibly higher potency and the limited knowledge available on their pharmacokinetic and pharmacodynamics properties. Many BZDs and DBZDs have a short half-life, leading to rapid metabolism and excretion. The low concentrations and short time windows for the detection of BZD in body fluids require the use of highly sensitive analysis methods to enable the detection of drugs and their respective metabolites. This review discusses the current state of the toxicological analysis of BZDs and DBZDs in forensic casework and their pharmacokinetic properties (i.e., absorption, distribution, metabolism, and elimination), as well as their analysis in biosamples typically encountered in DFSA (i.e., blood, urine and hair).

Introduction

Drug-facilitated sexual assault (DFSA) can be defined as a type of drug-facilitated crime (DFC) in which a victim is unable to object or resist a sexual act due to incapacitation resulting from alcohol or drug consumption. DFSA can lead to modified behavior and/or impaired perception, memory or decision-making capacity and a loss of motor function or drowsiness, as well as inability to ward off the attack. The voluntary or involuntary intake of the substances is classified into two categories, namely (i) proactive or predatory DFSA, in which victims are surreptitiously or forcefully administered an impairing or disinhibiting chemical, and (ii) opportunistic DFSA, where victims have voluntarily intoxicated themselves to the point of near or actual unconsciousness and the perpetrator takes advantage of this situation to engage in non-consensual sexual activity (1). This distinction usually relies on information provided by the victims and potential witnesses and is relevant as it can help in proving intent from the assailant in proactive DFSA cases, which is crucial for the charges in criminal prosecution (2).

There is no single substance that can unequivocally be associated with DFSA. Numerous substances have been reported in DFSA cases, including illicit, prescription and over-the-counter drugs. International listings of DFSA drugs are published by institutions such as the Society of Forensic Toxicologists (SOFT) (3) or the United Nations Office on Drugs and Crime (UNODC) (4). Systemic reviews (1, 2) and country-based surveys (5–12) highlight the typical and less common drugs used for chemical submission purposes. Alcohol remains the most frequently detected substance in DFSA cases, either alone or in co-consumption with other drugs such as cannabis (5–7), cocaine (7–9) or benzodiazepines (BZDs) (5, 7, 9, 13). BZDs (alone or in combination with ethanol) have been mentioned in all cited reports, while media-labeled "date rape drugs," such as ketamine or γ-hydroxybutyric acid, are encountered to a lesser extent. Except ethanol and cannabis, the most frequently detected substances in DFSA cases are typically correlated with the most prevalent drugs of abuse in a specific geographical area. Other prescription medications such as barbiturates, antidepressants (e.g., citalopram) or antipsychotics have also been reported (1, 6).

BZDs are ideal substances for DFSA, as they are active at low doses, have a fast action and can be easily administered orally. Moreover, some BZDs have a short half-life, leading to rapid metabolism and excretion. The trace concentrations and small time frame for detection of BZD in body fluids require the use of highly sensitive analysis methods to be able to detect

the drugs and their metabolites (13). Moreover, DFSA crimes are typically characterized by delayed reporting of the event by the victim—or even no reporting at all, either due to the amnesic properties of the drugs or because of the associated shame, blame, fear or self-denial (2). This obviously leads to additional challenges for the detection of the drug.

The last decade has seen a significant rise in new psychoactive substances (NPS), which represent another important challenge in DFC. NPS are synthetic drugs designed to circumvent national and international laws by minor modifications of the chemical structure of controlled substances. An increasing number of designer benzodiazepines (DBZDs), such as clonazolam and flubromazepam, have been reported by the European Union Early Warning System on NPS, with more than 80% being detected for the first time between 2014 and 2020 (14). DBZDs are not approved for medical use but can be easily obtained via various easily accessible (online) marketplaces. Similar to other NPS classes, little data are available on the clinical effects and pharmacological properties of DBZDs. Even though the effects of these DBZDs can partly be predicted by their structure and comparison with registered variants, their pharmacological effects and toxicity remain largely unknown. Some of the newer variants are highly potent, which can result in more serious intoxication events (15). Moreover, DBZDs are often mixed with other NPS such as synthetic cannabinoids or synthetic opioids, leading to additional health risks for DFSA victims (16). Similar to other NPS classes, many DBZDs are typically not detected by conventional toxicological screening techniques, leading to false-negative results in the case of DFSA cases. Finally, the lack of reference standards can hamper the confirmation of identity of DZBDs and their accurate quantitation.

This literature review presents an overview of the BZDs and DBZDs encountered in the context of DFSA. It first discusses the prevalence of these drugs worldwide, highlighting the evolution and trends in consumption. Pharmacokinetic properties of both conventional BZDs and DBZDs are presented, focusing on Phase I and Phase II metabolism. Finally, the different analytical approaches in assessing BZDs and DBZDs in body fluids are discussed, illustrating the associated challenges in the detection, identification and quantitation of these drugs.

Conventional BZDs

Effects and misuse in the context of DFSA

BDZs are therapeutic drugs prescribed for their anxiolytic, myorelaxant, sedative and anticonvulsant effects. They are also used as premedication in anesthesia and in the treatment of alcohol withdrawal syndrome. BZDs bind to the γ-aminobutyric acid type A ($GABA_A$) receptor, which is composed of five subunits forming an integral chloride channel. GABA, the major inhibitory neurotransmitter in the central nervous system (CNS), binds to a site located between the α and β subunits, while BZDs bind to a specific site between subunits α and γ, inducing a conformational change of the receptor, resulting in a higher affinity for GABA (17). This leads to a more frequent chloride channel opening, resulting in an enhanced conductance of the chlorine ions (Cl^-). Higher concentration of Cl^- in the postsynaptic cleft lowers the membrane potential, in turn decreasing neuronal firing and resulting in CNS depression. In physiological terms, this translates into sedation and relaxation (13).

BZDs act on $GABA_A$ receptors located in the spinal cord, brain stem, cerebellum and limbic and cortical areas. The resulting hyperpolarization triggers a cognitive altered state characterized by drowsiness, slurred speech, confusion, disinhibition and impairment of motor coordination and higher brain functions such as memory (17). These effects are typically advantageous for DFSA since they incapacitate the victim, who is unable to object or resist sexual acts. Even being a relatively rare symptom, disinhibition causes the victim to be more susceptible to engage in activities that would otherwise not be undertaken in a normal state of consciousness. At even stronger sedation and motor impairment effects, a victim can lose all wakefulness, alertness and, thereby, the ability to respond to the invasive actions of the perpetrators. Moreover, the memory loss of the event after drug intake, caused by the anterograde amnesia effect, can either limit the value of the testimony of the victim or prevent or delay the accusation (18).

BZDs are often associated with ethanol co-consumption, which intensifies their psychomotor effects due to both pharmacokinetic and pharmacodynamic interactions. Ethanol also binds the $GABA_A$ receptor, which creates a synergistic effect with BZDs (19). Moreover, ethanol and BZDs are both metabolized by cytochrome P450 enzymes, which can lead to drug interaction and delayed clearance of BZDs (17). In the context of DFSA, the victim then becomes even more vulnerable and faces an increased risk of severe toxicity, loss of consciousness, respiratory depression or even death (1).

BZDs reported in DFSA forensic casework

As listed in Table I, numerous BZDs have been reported in toxicological screenings in the context of drug-related sexual incidents (1). It is worth mentioning that the listed studies sometimes report the class without specifying the exact substance, which explains why the information provided in Table I is not exhaustive.

The prevalence of each BZD in DFSA strongly depends on the country. For instance, clonazepam is encountered more frequently in China (10), France (21) and the USA (7), lorazepam in Canada (27), flunitrazepam in Taiwan (24), oxazepam in Norway (11) and diazepam in Italy (5), Netherlands (8), New Zealand (6) or the UK (12). These regional differences maybe be explained by different country-specific prescription practices.

The large majority of BZD are typically administered orally as tablets except for one case where an injection was used in a medical setting (30). In some cases, the victim declared having taken the drug on the basis of a prescription (e.g., (11, 26)), while in other cases it was forcefully administered by the perpetrator (e.g., (20, 22, 25)) or spiked into drinks (e.g., (20, 21)). In most cases, the type of DFSA is not specified since contextual information is missing, such as the testimony of the victim, or because the police report is incomplete. The same issue arises regarding self-reported ethanol consumption that could be associated with BZD addition to alcohol beverages in a predatory DFSA context or self-intoxication by the victim in opportunistic DFSA.

The reported BZDs have usually been detected in blood and urine and occasionally in hair when a negative result was obtained with conventional matrices. In many cases, the matrix (i.e., blood or urine) was not specified, nor was the time between the reported administration and the sampling.

Table I. Prevalence and Context Information of BZDs Detected in Biological Matrices in DFSA-Related Criminal Cases (H = hair; B = blood; N/S = not specified; U = urine)

BZD	Galenic form	Countries (time span)	Matrices and detection windows	Type of DFSA	Self-reported alcohol intake in individual cases	References
Alprazolam	Tablet	Denmark (N/S)	Blood (20 h)	Proactive	Yes	(20)
		France (2003–2007); (N/S)	Blood, urine and hair (range: 10–108 h); hair (N/S)	Proactive	N/S; no	(21, 22)
		Italy (2010–2018)	Blood and/or urine (N/S)	Proactive	N/S	(5)
		New Zealand (2015–2018)	Blood and/or urine (mean: 16 h, median: 10 h, range: 2–93 h)	N/S	N/S	(6)
		Norway (2003–2010)	Blood and/or urine (mean: 29.6 h, median: 12.5 h, range: 1 h-16 days)	Opportunistic	N/S	(11)
		Sweden (2003–2007)	Blood and/or urine (N/S)	N/S	Both	(23)
		Taiwan (2011–2015)	Urine (N/S)	N/S	N/S	(24)
		USA (2015–2016)	Blood and/or urine (N/S)	N/S	Yes	(7)
Bromazepam	Tablet	France (2003–2007); (N/S)	Blood, urine and hair (range: 12–96 h); blood and hair (Case 1: 20 h (B), 1 month (H); Case 2: 18 h (B), 3 weeks (H), Case 3: 6 h (B and U), Case 4: 6 weeks (H))	N/S; proactive	N/S; no	(21, 25)
		Netherlands (2004–2006)	Blood and/or urine (N/S)	N/S	N/S	(8)
Clonazepam	Tablet	Australia (2003)	Blood (Case 14: 12 h, Case 15: 28 h)	Opportunistic	N/S	(26)
		Canada (2005–2007)	Urine (<24 h)	Proactive	N/S	(27)
		China (2017); (N/S)	N/S (N/S); blood and hair (18 h (B) and 5 weeks (H))	N/S	N/S; no	(10, 28)
		France (2003–2007)	Blood, urine and hair (range: 2–168 h)	Proactive	N/S	(21)
		Italy (2010–2018)	Blood and/or urine (N/S)	Proactive	N/S	(5)
		New Zealand (2015–2018)	Blood and/or urine (mean: 16 h, median: 10 h, range: 2–93 h)	N/S	N/S	(6)
		Norway (2003–2010)	Blood and/or urine (mean: 29.6 h, median: 12.5 h, range: 1 h-16 days)	Both	N/S	(11)
		Denmark (N/S); (2009–2016)	Blood (Case 1: 40 h, Case 18: 24 h); hair (N/S)	Proactive; N/S	Both; N/S	(20, 29)
		Taiwan (2011–2015)	Urine (N/S)	N/S	N/S	(24)
		USA (2015–2016)	Blood/and or urine (N/S)	N/S	Yes	(7)
Diazepam	Tablet	Australia (2003)	Blood and/or urine (Case 4: 3 h, Case 6: 24 h, Case 8: 46 h, (B), Case 12: 4 h (B), Case 13: 24 h (B)	Both	Both	(26)
		Canada (2005–2007)	Urine (<24 h)	Proactive	N/S	(27)
		China (2017)	Nonspecified (N/S)	N/S	N/S	(10)
		Denmark (N/S)	Blood (Case 1: 40 h; Case 17: 20 h)	Proactive	Yes	(20)
		France (2003–2007)	Blood, urine and hair (range: 2–47 h)	Proactive	N/S	(21)
		Italy (2010–2018)	Blood and/or urine (N/S)	Proactive	N/S	(5)
		Netherlands (2004–2006)	Blood and/or urine (N/S)	N/S	N/S	(8)
		New Zealand (2015–2018)	Blood and/or urine (mean: 16 h, median: 10 h, range: 2–93 h)	N/S	N/S	(6)
		Norway (2003–2010)	Blood and/or urine (mean: 29.6 h, median: 12.5 h, range: 1 h—16 days)	Both	N/S	(11)
		Sweden (2003–2007)	Blood and/or urine (N/S)	N/S	Both	(23)
		Taiwan (2011–2015)	Urine (N/S)	N/S	N/S	(24)
		UK	Blood and/or urine (N/S)	Both	N/S	(12)
		USA	Blood and/or urine (N/S)	N/S	Yes	(7)
	Injection solution	Belgium (N/A)	Blood and/or urine (30 h)	Proactive	No	(30)

(continued)

Table I. (Continued)

BZD	Galenic form	Countries (time span)	Matrices and detection windows	Type of DFSA	Self-reported alcohol intake in individual cases	References
Flunitrazepam	Tablet	Canada (2005–2007)	Urine (<24 h)	Proactive	N/S	(27)
		France (2003–2007)	Blood, urine and hair (range: 1–168 h)	Proactive	N/S	(21)
		Italy (2010–2018)	Blood and/or urine (N/S)	Proactive	N/S	(5)
		Japan (N/S)	Urine (24 h)	Proactive	No	(31)
		Netherlands (2004–2006)	Blood and/or urine (N/S)	N/S	N/S	(8)
		Norway (2003–2010)	Blood and/or urine (mean: 29.6 h, median: 12.5 h, range: 1 h—16 days)	Both	N/S	(11)
		Taiwan (2011–2019)	Urine (N/S)	N/S	N/S	(24)
Flurazepam	Tablet	UK (2000–2002)	Blood and/or urine (N/S)	Opportunistic	N/S	(12)
		USA (2015–2016)	Blood and/or urine (N/S)	N/S	No	(7)
Lorazepam	Tablet	Canada (2005–2007)	Urine (<24 h)	Proactive	N/S	(27)
		China (2017)	Nonspecified (N/S)	N/S	N/S	(10)
		France (2003–2007)	Blood, urine and hair (range: 48–67 h)	Proactive	Yes	(21)
		Italy (2010–2018)	Blood and/or urine (N/S)	Proactive	N/S	(5)
		New Zealand (2015–2018)	Blood and/or urine (mean: 16 h, median: 10 h, range: 2–93 h)	N/S	N/S	(6)
		Taiwan (2011–2015)	Urine (N/S)	N/S	N/S	(24)
		UK (2000–2002)	Blood and/or urine (N/S)	Both	N/S	(12)
		USA (2015–2016)	Blood and/or urine (N/S)	N/S	Yes	(7)
Midazolam	Tablet	France (2003–2007)	Blood and/or urine (range: 47–72 h)	Proactive	N/S	(21)
		Italy (2010–2018)	Blood and/or urine (N/S)	Proactive	N/S	(5)
		Netherlands (2004–2006)	Blood and/or urine (N/S)	N/S	N/S	(8)
		Taiwan (2011–2015)	Urine (N/S)	N/S	N/S	(24)
		USA (2015–2016)	Blood and/or urine (N/S)	N/S	N/S	(7)

(continued)

Table I. (Continued)

BZD	Galenic form	Countries (time span)	Matrices and detection windows	Type of DFSA	Self-reported alcohol intake in individual cases	References
Nordiazepam (desmethyl-diazepam)	Tablet	Australia (2003)	Urine (24 h)	Proactive	Yes	(26)
		Denmark (N/S)	Blood (Case 1: 40 h, Case 17: 20 h)	Proactive	Yes	(20)
		France (2003–2007)	Blood and hair (range: 10–72 h)	Proactive	N/S	(21)
		Italy (2010–2018)	Blood and/or urine (N/S)	Proactive	N/S	(5)
		Netherlands (2004–2006)	Blood and/or urine (N/S)	N/S	N/S	(8)
		Norway (2003–2010)	Blood and/or urine (mean: 29.6 h, median: 12.5 h range: 1 h–16 days)	Both	N/S	(11)
		Taiwan (2011–2015)	Urine (N/S)	N/S	N/S	(24)
		USA (2015–2016)	Blood and/or urine (N/S)	N/S	Yes	(7)
Nitrazepam	Tablet	Canada (2005–2007)	Urine (<24 h)	Proactive	N/S	(27)
		Norway (2003–2010)	Blood and/or urine (mean: 29.6 h, median: 12.5 h range: 1 h–16 days)	Both	N/S	(11)
		Taiwan (2011–2015)	Urine (N/S)	N/S	N/S	(24)
		UK (2000–2002)	Blood and/or urine (N/S)	Both	N/S	(12)
Oxazepam	Tablet	Denmark (N/S); (2009–2016)	Blood (mean: 23.5 h); hair (median: 38 days, range: 29–180 days)	Both; N/S	No; Yes	(20, 29)
		France (2003–2007)	Blood, urine and hair (range: 1.5–72 h)	Proactive	Yes	(21)
		Italy (2010–2018); (2016)	Blood and/or urine (N/S); hair (35 h and 7 months)	Proactive	N/S; Yes	(5, 32)
		Netherlands (2004–2006)	Blood and/or urine (N/S)	N/S	N/S	(8)
		Norway (2003–2010)	Blood and/or urine (mean: 29.6 h, median: 12.5 h range: 1 h–16 days)	Both	N/S	(11)
		Taiwan (2011–2015)	Urine (N/S)	N/S	N/S	(24)
		USA (2015–2016)	Blood and/or urine (N/S)	N/S	No	(7)
Temazepam	Tablet	France (2003–2007)	Blood and urine (45 h)	Proactive	N/S	(21)
		Italy (2010–2018)	Blood and/or urine (N/S)	Proactive	N/S	(5)
		Netherlands (2004–2006)	Blood and/or urine (N/S)	N/S	N/S	(8)
		Taiwan (2011–2015)	Urine (N/S)	N/S	N/S	(24)
		USA (2015–2016)	Blood and/or urine (N/S)	N/S	No	(7)
Triazolam	Tablet	Denmark (2009–2016); (N/S)	Urine and hair (N/S); urine and hair (20 h (U) and 37 days (H))	N/S; proactive	N/S; yes	(29, 33)
		France (2003–2007)	Urine and hair (48 h (U) and 4 months (H))	Proactive	N/S	(21)
		USA (2015–2016)	Blood and/or urine (N/S)	N/S	No	(7)

When detected in blood, most BZDs were detected within a 24-h time window, with some exceptions (i.e., up to 40 h (20)) Therefore, for a correct interpretation and use of case findings, authors of case studies are strongly recommended to include this relevant information in future work, and reviewers and editors are urged to request such information in case reports.

Pharmacokinetic properties of BZDs

Absorption

BZDs can be administered using different routes, namely intravenous, intramuscular, oral, sublingual, nasal or rectal (17), which determines the bioavailability of the drug. Table II reports the conventional BZDs available in the European market, including their oral bioavailability, onset time and half-life.

BZDs are well absorbed upon oral administration with bioavailability in the range of 70–90%, although midazolam and triazolam yield lower values. The therapeutic dose depends on the potency of the drug, ranging from 0.125 mg (triazolam; Table II) for high-potency BZDs up to 120 mg (oxazepam; Table II) for low-potency substances. Higher doses of BZDs can lead to toxicity, even though there is little known on the specific doses that result in toxic adverse effects (2).

Distribution

Lipophilicity is one of the key parameters in the distribution and accumulation of BZDs in lipid-rich areas such as CNS. Indeed, the substances with higher lipophilicity show higher rates of absorption and rapid onset of effects, which make them ideal candidates for chemical submission. For example, diazepam and flunitrazepam (Table II) reach the CNS faster than more polar BZDs such as oxazepam or temazepam that have a hydroxyl group in their chemical structure (34). Furthermore, an increased lipid solubility is also related to a higher risk for anterograde amnesia. For instance, midazolam produces significantly greater anterograde amnesia than alprazolam. Moreover, clonazepam and lorazepam are less lipid soluble than alprazolam and, thus, present a lower risk of amnesic effects (17). The volume of distribution depends on the molecular size and degree of protein binding of the BZDs. Highly lipophilic compounds typically exhibit greater plasma protein binding, which affects their pharmacokinetic properties as only the free fraction has the ability to partition through the membranes. Plasma protein binding varies greatly among BZDs; for instance, diazepam and bromazepam are 99% and 60% protein bound, respectively (42).

Metabolism

The core chemical structure of BZDs consists of the combination of a benzene ring with a diazepine ring, with positions 1, 2, 5 or 7 available for substitution (Figure 1). Typically, the 5 position of the diazepine ring contains an aryl substituent. Additional substituents in the 1 and 4 positions of the diazepine ring lead to different 1,4-benzodiazepins. Table III lists the route of metabolism and metabolites produced for the common BZDs. BDZs are usually first oxidized to form Phase I metabolites, with the exception of clonazepam and nitrazepam that undergo nitro reduction. Phase I reactions mostly encompass hydroxylation and *N*-demethylation or nitro reduction of the 7-amino compound

Table II. Administration, Absorption, Therapeutic Dose and Half-life of Common BZDs (IV = intravenous; IM = intramuscular)*

Compound	Typical routes of administration	Oral bioavailability [%]	Therapeutic daily oral doses [mg]	Plasma concentrations [mg/L]	Rate of absorption[a]	Onset time [min]	Lipid solubility	Half- life [h]	Duration of effects [h]
Alprazolam	Oral	84–91	1–3 (max. 10)	0.005–0.05 (–0.08)	Intermediate	30	Moderate	6–20	Variable
Bromazepam	Oral	84	3–18 (max. 60)	(0.05–) 0.08–0.2	Intermediate	10–20	Low	8–22	4–8
Clonazepam	Oral, IV and IM	>80	4–8	(0.004–) 0.02–0.08	Slow	20–40	Low	20–60	8–12
Diazepam	Oral, IV, IM, rectal	90	5–30	0.1–2 (–2.5)	Rapid	15–60	High	24–48	>12
Flunitrazepam	Oral	64–77	0.5–2	0.005–0.015	Rapid	15–30	High	10–20 (–30)	4–8
Flurazepam	Oral	83	15–30	0.02–0.1	Intermediate	45–90	High	2–3	12–16
Lorazepam	Oral, IV and IM	90	1–10	(0.02–) 0.08–0.25	Slow	20–30	Low	10–40	Intermediate
Midazolam	Oral, nasal, IV, IM, rectal	40–50	7.5–15 for sleep disorders	0.04–0.1 (–0.25)	Rapid	10–20	High	1.5–3	Short
Nitrazepam	Oral	53–94	5–10	0.03–0.1	Rapid	30–60	Low	20–30	6–8
Nordiazepam	Oral	N/A	Up to 15	0.2–0.8– (1.8)	Rapid	45–120	High	40–100	10–20
Oxazepam	Oral	92	30–120	0.2–1.5	Slow	60–120	Low	6–20	6–8
Temazepam	Oral	90–100	10–20 for insomnia 20–40 for premedication	0.02–0.15 (–0.9)	Slow	20–90	Moderate	6–25	6–10
Triazolam	N/A	44	0.125–0.250	0.002–0.02	Rapid	10–20	Moderate	2–5	Short

*Information retrieved from references (34–41)
[a]Rate of absorption: slow: >2 h; intermediate: 1–2 h; rapid: <1 h.

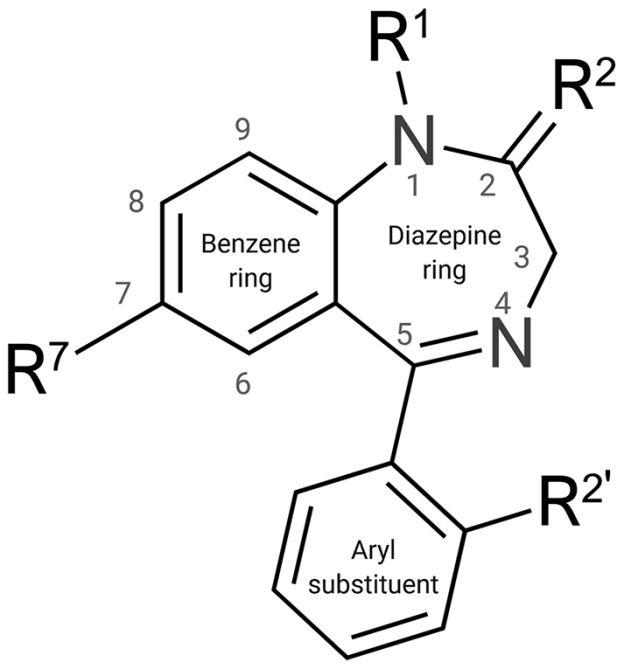

Figure 1. General chemical structure of a 1,4-benzodiazepine (core formed by the combination of a benzene ring and a diazepine ring) with a 5-aryl substituent.

in the case of nitrobenzodiazepines as illustrated in Figure 2 for diazepam and clonazepam, respectively. For many BZDs, Phase I metabolism produces active metabolites that extend the effects of the drug. Phase II metabolites are usually formed via glucuroconjugation of the Phase I metabolite or the parent drug or—for the nitrobenzodiazepines—via acetylation of the amine function of the reduced nitro metabolites. BZDs with hydroxyl groups such as lorazepam, oxazepam or temazepam experience direct glucuronidation without first-pass metabolism, leading to inactive metabolites that are rapidly excreted and, therefore, showing a shorter duration of action (43).

Excretion

The half-life of BZDs is an important factor to estimate the "incident concentration", that is, the level at the time of the alleged sexual assault as estimated from the analysis of a sample taken during a given time period after the incident. However, it does not reflect the time of recovery from the effects of the drug and is therefore insufficient to estimate the duration of action of the ingested drug. A compound is considered nearly completely cleared from the body after approximately five elimination half-lives (17). The half-life of some active metabolites can be significantly longer than that of the parent BZD, such as nordiazepam ($t_{1/2} = 40$–100 h) compared to diazepam ($t_{1/2} = 24$–48 h) and desalkylflurazepam ($t_{1/2} = 40$–144 h) versus flurazepam ($t_{1/2} = 2$–3 h), respectively. Nordiazepam and desalkylflurazepam are both active metabolites, which extends the overall duration of effects.

The most prevalent excreted form is usually the glucuronidated metabolite with a variable fraction of the parent drug being excreted in its unchanged form—usually in minute amounts (except for alprazolam, up to 20%). Triazolo or imidazole BDZs such as alprazolam, triazolam and midazolam are mainly excreted as glucuronides of the hydroxylated metabolites, and the α-hydroxy metabolite predominates over the 4-hydroxy metabolite (Table III). Finally, for nitrobenzodiazepines, the 7-amino metabolite is generally the most abundant form detected in urine.

Designer Benzodiazepines

DBZDs and potential misuse in DFSA

The market of NPS has drastically increased in recent years, with an average of 50 new drugs being annually reported for the first time in Europe since 1997 according to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (47). The synthesis of NPS aims at circumventing list-based national or international laws via a slight modification of the structure of established licit pharmaceutical compounds or illicit drugs. The class of NPS showing a structure based on BZDs is typically referred to as DBZDs. The first DBZD notified to the EMCDDA was phenazepam in 2007; until now, 30 DBZDs have been documented (47). Most of the DBZDs available in the market have never been licensed as therapeutic drugs, with some exceptions such as premazepam and etizolam, which are being prescribed in Russia, Estonia, Latvia, Lithuania and Belarus, as well as India, Japan and Italy (48).

DBZDs are synthesized following different strategies, namely (i) selection of 1,4-BZDs from literature or patent applications or synthesis of a logical combination of substituents (e.g., diclazepam); (ii) synthesis of active metabolites (e.g., fonazepam and nifoxipam); (iii) addition of triazole groups (e.g., flubromazolam) and (iv) modification of etizolam (e.g., deschloroetizolam and metizolam) (48, 49).

According to the EMCDDA, no new BZDs have been reported for the year 2020 (47), but 64% of NPS identified in toxicology cases from January 2019 to April 2020 were BZD-type substances (UNODC report) (50). Because of their novelty, country-based reports listing DBZDs remain scarce in scientific literature. Table IV lists the published cases. Most reports concerned acute or fatal intoxication incidents (i.e., clinical admissions or postmortem autopsies) and driving under the influence cases, with high prevalence in males (51). Interestingly, the use of DBZDs in DFSA has only been reported in China and Canada so far (Table IV). Similar to conventional BZDs, the prevalence of DBZDs varies per country. However, the most commonly reported DBZDs worldwide are currently clonazolam, diclazepam, etizolam, flubromazolam and flualprazolam.

Effects of DBZDs

Even though the mechanism of action of DBZDs remains understudied, quantitative structure–activity relationship predictive modeling suggests that DBZDs effects are also mediated through $GABA_A$ receptors but that the designer drugs appear to have a greater binding affinity compared to prescription BDZs (57). Similar to conventional BZDs, co-consumption of DBZDs with other CNS depressants such as alcohol can increase the risk of respiratory depression and death (51). The potency of DBZDs can also be estimated via the functional groups in the benzene and diazepine rings that can either increase or decrease the activity. For instance, substituents at the R_7 position increase the affinity

Table III. Metabolism of Common BZDs via Phase I and Phase II Metabolism*

BZD	Class	Phase I metabolism and key metabolites (activity and half-life)	Phase II metabolism and key metabolites (activity and half-life)	Target analytes detected in urine (Bold text: key analytes)
Alprazolam	1,2-triazobenzodiazepine	*4-hydroxylation (primary)* 4-hydroxyalprazolam (active, 6–20 h) *1-hydroxylation* α- hydroxyalprazolam (active, 1–2 h)	*Conjugation with glucuronic acid*	*Parent drug* Alprazolam (12–20%) *Metabolites* 5-chlorobenzophenone (17%) **α-hydroxyalprazolam** (15–17%) 4-hydroxyalprazolam (0.3%) Conjugates of the previous
Bromazepam	1,4-benzodiazepine (2-pyridinyl ring at 5-position)	*Ring cleavage and 3-hydroxylation* 3-hydroxybromazepam (active, ca. 17 h)	*Conjugation with glucuronic acid* 3-hydroxybromazepam glucuronide	*Parent drug* Bromazepam (2%) *Metabolites* Benzophenone (0.4%) **3-hydroxybromazepam glucuronide** (27%) Hydroxylated conjugated benzophenone (40%)
Clonazepam	7-nitro-1,4-benzodiazepine	*Reduction of nitroso group and N-acetylation* 7-aminoclonazepam (active, N/S) 7-acetamidoclonazepam *3-hydroxylation* 3-hydroxyclonazepam	*Conjugation with glucuronic acid*	*Parent drug* Clonazepam (0.5%) *Metabolites* **7-aminoclonazepam** (major) 7-acetamidoclonazepam 3-hydroxyclonazepam
Diazepam	1,4-benzodiazepine	*N-demethylation (N-dealkylation)* Nordiazepam (*N*-desmethyldiazepam) (active, 40–100 h) *3-hydroxylation* Oxazepam (active, 5–15 h) Temazepam (active, 8–15 h)	*Conjugation with glucuronic acid*	*Metabolites* Nordiazepam **Oxazepam, Temazepam** Conjugates of oxazepam and temazepam
Flunitrazepam	7-nitro-1,4-benzodiazepine	*Reduction of nitroso group and N-acetylation* 7-aminoflunitrazepam (active, up to 120 h) 7-acetamidodesmethylflunitrazepam (inactive) *N-demethylation (N-dealkylation)* Desmethylflunitrazepam (moderately active, 23–33 h) *Hydroxylation* 3-hydroxyflunitrazepam	*Conjugation with glucuronic acid*	*Parent drug* Flunitrazepam (<0.2%) *Metabolites* **7-aminoflunitrazepam** (major) 7-aminodesmethylflunitrazepam 7-acetamidodesmethylflunitrazepam 3-hydroxyflunitrazepam
Flurazepam	1,4-benzodiazepine	*N/A* Hydroxyethylflurazepam (active, 2–4 h) *N*-desalkylflurazepam (active, 40–144 h)	*Conjugation with glucuronic acid*	*Metabolites* **N-desalkylflurazepam** (29–55%) Conjugated *N*-1-desalkyl-3-hydroxy-flurazepam (1–2%)

(continued)

Table III. (Continued)

BZD	Class	Phase I metabolism and key metabolites (activity and half-life)	Phase II metabolism and key metabolites (activity and half-life)	Target analytes detected in urine (Bold text: key analytes)
Lorazepam	1,4-benzodiazepine		*Conjugation with glucuronic acid* Glucuronide conjugate of lorazepam (ca. 16 h)	*Metabolites* **Lorazepam glucuronide** (75%)
Midazolam	1,2- imidazobenzodiazepine	*1-hydroxylation* α -hydroxymidazolam or 1-hydroxy-midazolam (active, ca. 1 h) *4-hydroxylation (primary)* 4-hydroxymidazolam	*Conjugation with glucuronic acid*	*Parent drug* Midazolam (<1%) *Metabolites* **1-hydroxymidazolam glucuronide** (60–80%) 4-hydroxymidazolam glucuronide (3%) 1,4-dihydroxy midazolam (1%)
Nitrazepam	7-nitro-1,4-benzodiazepine	*Reduction of nitroso group and N-acetylation* 7-aminonitrazepam (inactive) 7-acetoamidonitrazepam (inactive)	*Conjugation with glucuronic acid*	*Parent drug* Nitrazepam (1 %) *Metabolites* **7-aminonitrazepam** and conjugate (31%) 7-acetamidonitrazepam (21%) and conjugate 3-hydroxy-2-amino-5-nitrobenzophenone (ca. 5%)
Nordiazepam	1,4-benzodiazepine	*3-hydroxylation* Oxazepam (active, 5–15 h)	*Conjugation with glucuronic acid*	*Parent drug* Nordiazepam (in trace quantitites) *Metabolites* **Oxazepam conjugated** (major)
Oxazepam	1,4-benzodiazepine		*Conjugation with glucuronic acid* Glucuronide conjugate of oxazepam (inactive)	*Parent drug* Oxazepam (in trace amounts) *Metabolites* **Oxazepam conjugate** (70–80%)
Temazepam	1,4-benzodiazepine		*Conjugation with glucuronic acid* Glucuronide conjugate of temazepam (inactive)	*Parent drug* Temazepam (<%) *Metabolites* **Temazepam as conjugate** (73%) **Oxazepam as conjugate** (6%)
Triazolam	1,2-triazobenzodiazepine	*1-hydroxylation* α-hydroxytriazolam (active, up to 35 h) *4-hydroxylation* 4-hydroxytriazolam	*Conjugation with glucuronic acid*	*Parent drug* Triazolam (2%) *Metabolites* **α-hydroxytriazolam, principally conjugated** (60–80%) 4-hydroxytriazolam, principally conjugated (10%) α-hydroxy-4-hydroxy triazolam (2%) Others (2%)

* Information retrieved from references (17, 41, 44–46)

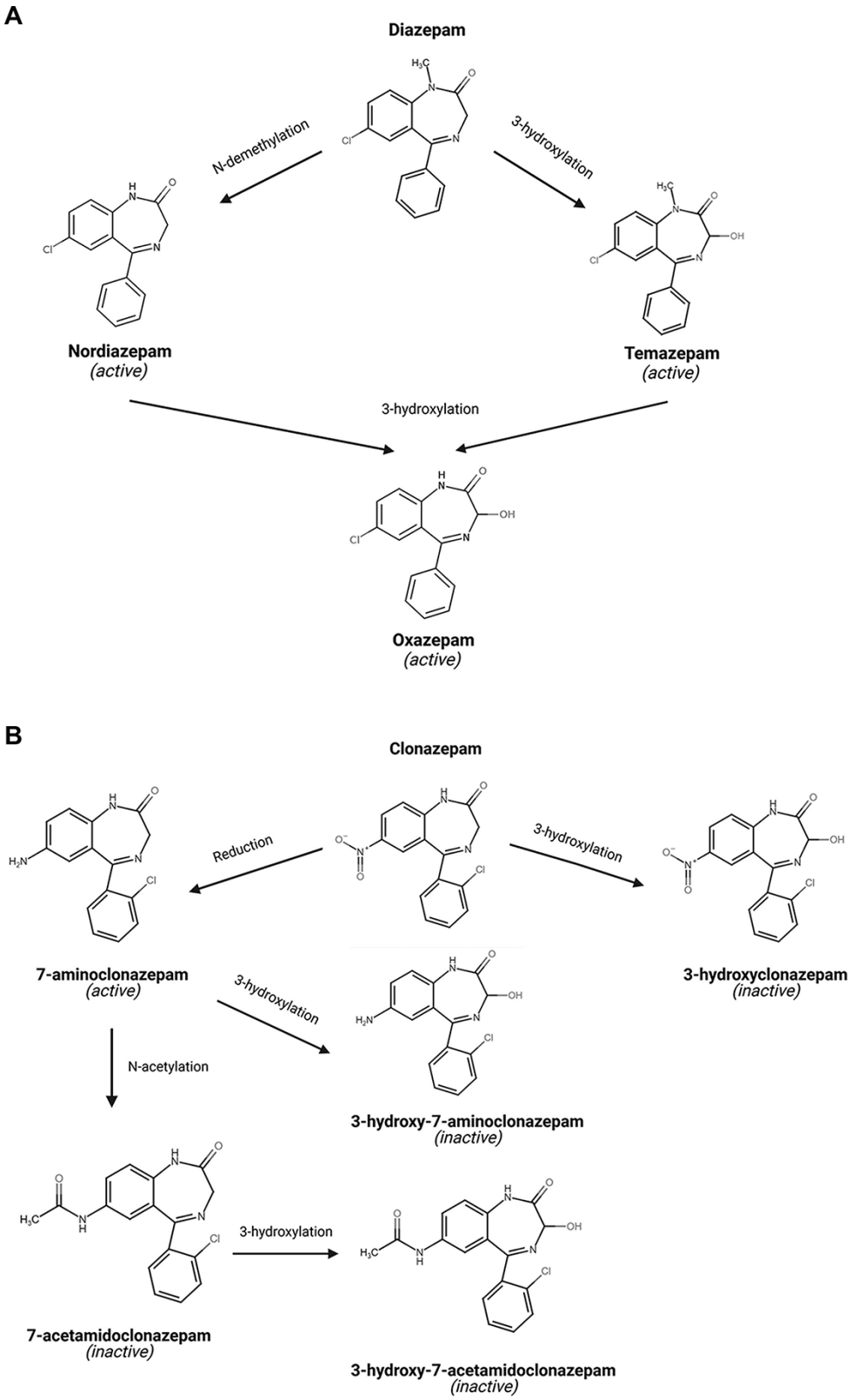

Figure 2. Illustration of the Phase I metabolism of BZDs. A. Primary metabolic transformation pathways of the 1,4-benzodiazepine diazepam and chemical structures of its major metabolites. B. Primary metabolic transformation pathways of the 7-nitro-1,4-benzodiazepine clonazepam and chemical structures of its major metabolites.

to the $GABA_A$ receptor, following the order $CH_2F_3 > I > Br$ (15). Since DBZDs also lead to the production of active metabolites, structure–activity relationship studies should be complemented with additional (clinical) studies. *In vivo* data on pharmacological effects and potency of DBZDs remain scarce, as DBZDs have not been evaluated in (pre)clinical

Table IV. DBZDs Reported in Forensic Casework and Intoxication Reports (DUID = Driving under the Influence of Drug)

Country	Years	Type of cases/samples	Biological matrices	DBZDs detected (number of detection)	Reference
Canada	2019–2020	DUID, postmortem cases, DFSA	Blood and urine	Flubromazomal (79) Flualprazolam (70) Etizolam (43)	(52)
China	2017	DFSA	Blood/urine/hair	Diclazepam (6) Flualprazolam (1)	(10)
Netherlands	2013–2017	Forensic samples (seized drugs), consumer drug samples and samples from Poisons center	N/S	Clonazolam Diclazepam Etizolam Flubromazepam Flunitrazolam Phenazepam Pyrazolam	(53)
Norway	2016–2019	DUID, postmortem cases, other (not specified)	Blood	Diclazepam (334) Phenazepam (138) Etizolam (40) Clonazolam (22) Flubromazolam (20) Flualprazolam (10) Flubromazepam (5)	(54)
Sweden	2012–2016	Admissions at hospital	Urine	Flubromazolam (92) Flubromazepam (33) Pyrazolam (33) Meclonazepam (26) Etizolam (20) Clonazolam (16) 3-Hydroxyphenazepam (8) Nifoxipam (5) Diclazepam (4) Metizolam (4) Deschloroetizolam (1) Phenazepam (1)	(55)
USA	2014–2017	Admissions at hospital	N/S	Etizolam (162) Clonazolam (50) Flubromazolam (13) Diclazepam (4) Flubromazepam (3) Meclonazepam (1) Norflurazepam (1)	(56)

trials. El Bahlkhi et al. investigated the possible correlation between the effects described by recreational users with the structure–activity relationships for 10 different DBZDs (15). Among the physical effects relevant in the context of DFSA (i.e., sedation, amnesia and disinhibition), the potency of the selected DBZDs was ranked as follows:

- Sedation: flubromazepam > meclonazepam > diclazepam > metizolam > flubromazolam > clonazolam, nifoxipam > deschloroetizolam > etizolam > pyrazolam.
- Amnesia: flubromazolam > clonazolam > meclonazepam > flubromazepam, etizolam > diclazepam, nifoxipam > metizolam > pyrazolam.
- Disinhibition: clonazolam > diclazepam > etizolam.

Clonazolam, flubromazolam, diclazepam and flubromazepam were considered the most potent DBZDs. Interestingly, these DBZDs are also frequently encountered in intoxication and forensic reports (Table V).

Pharmacokinetics of DBZDs

Absorption

DBZDs can be purchased as pills, tablets or capsules, pellets, blotting paper, powder or in a liquid form either from an illegal supplier or on the dark web as "research chemicals" (even though implicitly intended for human consumption). Oral administration is the most prevalent route of administration. Typical administered doses, indicated in Table V, were derived from Internet posts of experienced recreational users on websites such as TripSitFactSheets (39). Therapeutic doses are also known for etizolam and phenazepam. Flunitrazolam has a reported common dose of 0.08–0.14 mg, which is significantly lower than that of flunitrazepam (0.5–2 mg), one of the most potent pharmaceutical BZDs that has been associated with DFSA. The potency of flunitrazolam is expected to be greater due to the presence of the triazolo ring on the flunitrazepam backbone (58).

Distribution

DBZDs include short-acting drugs, such as clonazolam or etizolam with an elimination half-life of approximately 3–7 h but also long-acting drugs such as diclazepam (46 h), meclonazepam (80 h) or flubromazepam (106 h). Norflurazepam is the active metabolite of flurazepam and shows a significantly increased half-life of 40–250 h vs. 2–3 h for the parent compound. Other parameters related to the elimination half-life such as protein binding, volume of distribution and lipophilicity are described by Manchester et al. (42).

Table V. Oral Dosage, Onset Time, Duration of Effects and Half-life of Selected DBZDs (59–62)

Compound	"Recreational" oral doses [mg]	Onset [min]	Duration of effects [h]	Half- life [h]
3-Hydroxyphenazepam	0.5–4	30–90	24	N/A
Clonazolam	0.2–1	20–60	6–10	3.6
Deschloroetizolam	4–6	1–5	8–10	N/A
Diclazepam	2–4	15–90	8–12	42
Etizolam	0.25–3 Therapeutic: 0.5–2.0	30–120	5–8	3.4–7.1
Flualprazolam	0.125–2.5	10–30	6–14	9.5–12
Flubromazepam	4–8	15–90	12–18	106
Flubromazolam	0.15–0.25	20–45	Sedation for 10 h, Partial amnesia >24 h	Possibly 30 h
Flunitrazolam	0.08–0.15	10–30	4–5	N/A
Meclonazepam	2–3	20–60	9–15	80
Metizolam	2	30–90	5–8	N/A
Nifoxipam	0.5–3.0	45–120	10–75	N/A
Norflurazepam (*N*-desalkylflurazepam)	5–10	45–120	10–16	40–250
Phenazepam	3–5 Therapeutic: max. 10	15–60	>18	15–60
Pyrazolam	0.5–4	10–25	5–8	17

DBZDs are relatively lipophilic, ranging from phenazepam (highest lipophilicity) to pyrazolam (lowest lipophilicity). Furthermore, similar to conventional BZDs, they exhibit a high degree of plasma protein binding (90% on average), with the exception of pyrazolam (78%).

An overview of DZBD dosage, onset time, direction of effects and half-life is given in Table V.

Metabolism and excretion

Similar to BZDs, DBZDs undergo Phase I and Phase II reactions before excretion. The biotransformation reactions and products can therefore be predicted based on the reactions established for their respective conventional BZD analogs.

Numerous *in vitro* and *in vivo* strategies have been conducted to investigate the metabolism of DBZDs as reported in Table VI. Only etizolam and phenazepam have been tested in clinical trials. *In vitro* experiments, the most frequently used approach, consists of incubating the drug of interest with human liver microsomes (HLMs), leading to the production of both Phase I and Phase II metabolites. Metabolites can then be identified using high-resolution mass spectrometry (MS) based techniques and nuclear magnetic resonance. HLMs have been used to study the metabolism of clonazolam (63), diclazepam (63), deschloroetizolam (63), etizolam (63), flualprazolam (64), flubromazepam (63), flubromazolam (63, 65), flunitrazolam (66), meclonazepam (63, 67), metizolam (68), nifoxipam (63) and norflurazepam (66). Another strategy relies on the analysis of urine samples obtained from a large cohort of users. One of the most relevant examples of this approach is the STRIDA project in Sweden (55) that has led to the detection of clonazolam (69), flubromazolam (70), meclonazepam (69), nifoxipam (69) and pyrazolam in urine samples. Finally, self-administration studies, where one of the principal investigators of the study ingested a certain dose of the drug have also been reported. Results of such studies are reported for diclazepam (71), fubromazepam (72), fubramazolam (73), flunitrazolam (74), metizolam (68) and pyrazolam (70). Since these studies include one single subject, they do not provide information on the interindividual variability in the metabolism of these drugs. It is worth mentioning that *in vitro* findings obtained with HLM do not always correlate with *in vivo* findings. A striking example is flunitrazolam (Table VI), where the *in vitro* experiments conducted by Moosman et. al (66) identified hydroxylation reactions that could not be confirmed *in vivo* in the study of Ameline and colleagues (74). Conversely, the metabolite 7-acetamidoflunitrazolam could be detected *in vivo* but not *in vitro*. Such findings illustrate the need to confirm the metabolite routes observed in HLM-based studies.

Information on excretion is detailed in Table VI. This information has been compiled from large cohort studies based on urine collection or from self-administration studies. The parent compound is typically detected in small traces in urine, except for pyrazolam, which is mostly excreted in its parent form.

Analysis of (Designer) Benzodiazepines

Analytical challenges in the analysis of BZDs and DBZDs

Detection of (D)BZDs is crucial in the investigation of DFSA. The presence of (D)BZDs in a sample from a victim who does not take prescribed medication to treat anxiety, insomnia, panic disorder or seizures should be considered a relevant finding. Additionally, the blood concentration of (D)BZDs is also important as higher doses are generally correlated with a higher degree of impairment (17).

One of the major analytical challenges associated with the analysis of (D)BZDs in body fluids is the low concentrations of the parent drug. The highly potent flunitrazepam has a therapeutic dose of 0.5–2 mg (Table II), which translates to a blood concentration of ca. 0.005–0.015 ng/mL for the therapeutic range and a lower limit of 0.05 ng/mL for the toxic range (37). Furthermore, some (D)BZDs exhibit a short elimination half-life as illustrated with the half-life of midazolam, i.e., ca. 1.5–3 h (Table V). Finally, the concentrations are sometimes below the limits of detection by the time the biological samples are collected, especially when a long time has elapsed between the alleged assault and the moment that the victim contacts the authorities.

The number of BZDs and DBZDs that can be used in a sexual assault represents another challenge. Indeed, a large number of (D)BZDs with a different structure, metabolism and excretion are available in the (black) market, requiring very selective analytical methods capable of discriminating

Table VI. Structural Relation of DBZDs to Other BZDs/DBZDs, Metabolism and Target Analytes for Detection

Compound	Class	Relation with BZDs or DBZDs	Phase I metabolism and key metabolites (activity, half-life)	Phase II metabolism and key metabolites (activity, half-life)	Target analytes for detection in biological matrices (detection window). Bold text, key analytes	Reference
3-Hydroxyphenazepam	1,4-Benzodiazepine	Metabolite of phenazepam	N/A		N/A	(75)
Clonazolam	Triazolobenzodiazepine	Triazole analog to clonazepam	*Reduction of nitroso group and demethylation* 8-aminoclonazolam (also stated as 7-aminoclonazolam) 8-acetamidoclonazolam (also stated as 7-acetamidoclonazolam) *Hydroxylation* Hydroxyclonazolam	*Conjugation with glucuronic acid* 8-aminoclonazolam glucuronide 8-acetamidoclonazolam glucuronide Hydroxyclonazolam glucuronide	*Blood* Parent compound *Urine* More abundant: **7-aminoclonazolam** Less abundant: Parent compound	(59, 63, 69)
Deschloroetizolam	Thienotriazolodiazepine	Dechlorinated analog of etizolam	*Hydroxylation* Hydroxydeschloroetizolam Dihydroxydeschloroetizolam	*Conjugation with glucuronic acid* Deschloroetizolam glucuronide	*Urine* More abundant: **Hydroxy-deschloroetizolam** Less abundant: Hydroxydeschloroetizolam Dihydroxydeschloroetizolam	(63, 76)
Diclazepam	1,4-Benzodiazepine	2′-chloroanalogue of diazepam	*Demethylation (dealkylation)* Delorazepam (active, 78 h) *Hydroxylation* Lormatazepam (active, 13 h) *Demethylation/hydroxylation* Lorazepam (active, 12 h)		*Serum* Parent compound (99 h) Delorazepam (10 days) *Urine* More abundant: **Delorazepam** (6 days) Less abundant: Lorazepam (19 days) Lormatazepam (11 days) Traces of parent compound	(71)
Etizolam	Thienotriazolodiazepine	BZD analog Benzene ring replaced by a thiophene ring	*Hydroxylation* α-hydroxyetizolam 8-hydroxyetizolam	*Conjugation with glucuronic acid* Etizolam *N*-glucuronide	*Blood* Parent compound *Postmortem blood* α-hydroxyetizolam 8-hydroxyetizolam	(63, 76, 77)
Flualprazolam	Triazolobenzodiazepine	2′-fluoro derivative of alprazolam	*1-hydroxylation* α-hydroxy flualprazolam *4-hydroxylation* 4-hydroxy alprazolam	*Conjugation with glucuronic acid* α-hydroxy flualprazolam glucuronide 4-hydroxy flualprazolam glucuronide *N*-glucuronide Dihydroxy flualprazolam glucuronide	*Plasma* More abundant: Parent compound Less abundant: α-hydroxyflualprazolam 4-hydroxyflualprazolam *Urine* More abundant: **α-hydroxyflualprazolam glucuronide** 4-hydroxyflualprazolam glucuronide *N*-glucuronide Less abundant: Hydroxy metabolites Parent compound	(65)

(continued)

Table VI. (Continued)

Compound	Class	Relation with BZDs or DBZDs	Phase I metabolism and key metabolites (activity, half-life)	Phase II metabolism and key metabolites (activity, half-life)	Target analytes for detection in biological matrices (detection window). Bold text, key analytes	Reference
Flubromazepam	1,4-Benzodiazepine	Related to flunitrazepam by change of nitro group by bromine atom	*Hydroxylation* 3-hydroxyflubromazepam Debromoflubromazepam Hydroxyflubromazepam		*Serum* Parent compound (23 days) Hydroxyflubromazepam (19 days) Debromoflubromazepam (7 days) *Urine* More abundant: **Hydroxyflubromazepam** (5 h—28 days) Debromoflubromazepam (5 h—6 days) Less abundant: Parent compound (2 h—6 days)	(72)
Flubromazolam	Triazolobenzodiazepine	Triazole analog of flubromazepam	*Hydroxylation* 4-hydroxyflubromazolam α-hydroxyflubromazolam Dihydroxyflubromazolam	Conjugation with glucuronic acid Flubromazolam glucuronide α-hydroxyflubromazolam glucuronide 4-hydroxyflubromazolam glucuronide	Urine **α-hydroxyflubromazolam** (8 days) 4-hydroxyflubromazolam Flubromazolam glucuronide α-hydroxyflubromazolam glucuronide 4-hydroxyflubromazolam glucuronide Dihydroxyflubromazolam Flubromazolam glucuronide (6 days)	(65, 70, 73, 78, 79)
Flunitrazolam	Triazolobenzodiazepine	Triazole analog of flunitrazepam	*Reduction of nitroso group* 7-aminoflunitrazolam 7-acetamidoflunitrazolam (only *in vivo*) *N/A* Desnitroflunitrazolam (only *in vivo*) *Hydroxylatio*n (only *in vitro*) Hydroxyflunitrazolam Dihydroxyflunitrazolam	*Conjugation with glucuronic acid* Flunitrazolam *N*-glucuronide	*Urine* More abundant: **7-aminoflunitrazolam** (37 h) **7-acetamidoflunitrazolam** Desnitroflunitrazolam Hydroxyflunitrazolam Less abundant: Parent compound (21 h)	(66, 74)
Meclonazepam	1,4-Benzodiazepine	Similar in structure to clonazepam	*Reduction of nitroso group and deacetylation* 7- Amino-clonazepam 7-Acetamido-clonazepam		*Urine* **7-aminomeclonazepam** **7-acetaminomeclonazepam** Parent compound	(67)
Metizolam	Thienotriazolodiazepine	Demethylated analog of etizolam	*Hydroxylation* 2-hydroxymetizolam *N*-hydroxymetizolam	*Conjugation with glucuronic acid* 2-hydroxymetizolam glucuronide *N*-hydroxymetizolam glucuronide	*Urine* More abundant: **2-hydroxymetizolam** (8 h) **2-hydroxymetizolam glucuronide** (8 h) ***N*-hydroxymetizolam** (30 h) ***N*-hydroxymetizolam glucuronide** (30 h) Less abundant: Parent drug (46 h)	(68)

(continued)

Table VI. (Continued)

Compound	Class	Relation with BZDs or DBZDs	Phase I metabolism and key metabolites (activity, half-life)	Phase II metabolism and key metabolites (activity, half-life)	Target analytes for detection in biological matrices (detection window). Bold text, key analytes	Reference
Nifoxipam	1,4-Benzodiazepine	Active metabolite of flunitrazepam	*Reduction of nitroso group and dealkylation* 7-aminononifoxipam *N/A* Denitro-nifoxipam	*Conjugation with glucuronic acid* Nifoxipam *N*-glucuronide	*Urine* More abundant: **Nifoxipam glucuronide** Less abundant: 7-aminonifoxipam 7-acetaminonifoxipam	(63, 69, 76)
Norflurazepam (*N*-desalkylflurazepam)	1,4-Benzodiazepine	Active metabolite of flurazepam Precursor in synthesis of midazolam	*Hydroxylation* 3-hydroxynorflurazepam 4′-hydroxynorflurazepam 3,4′- dihydroxynorflurazepam		N/S	(66)
Pyrazolam	Triazolobenzodiazepine	Combines structural elements of alprazolam and bromazepam	*Hydroxylation* Hydroxy metabolite Isomer 1 Hydroxy metabolite Isomer 2	*Conjugation with glucuronic acid* Parent glucuronide Isomer 1 Parent glucuronide Isomer 2 Hydroxy metabolite glucuronide Isomer 1 Hydroxy metabolite glucuronide Isomer 2	*Serum* Parent compound (50 h) *Urine* More abundant: **Parent compound** (6 days) Less abundant: If hydrolysis of urine: **Pyrazolam glucuronide** If no hydrolysis of urine: **Mono-hydroxy metabolite**	(70, 80)
Phenazepam	1,4-Benzodiazepine	Prodrug of nordazepam	*Hydroxylation* 3-hydroxyphenazepam		Urine **3-hydroxyphenazepam** bromo-(2-chlorophenyl)-2- aminobenzophenone (ABPH) bromo-(2-chlorophenyl) quinazoline-2-one (QNZ)	(81)

and identifying these compounds. Moreover, some of the (D)BZDs are positional isomers (e.g., diclazepam and 4-chlorodiazepam), and their separation requires highly selective methods.

DBZDs pose additional challenges in the analysis and interpretation of the data. First, reference standards are often not commercially available. Moreover, the limited amount of clinical data available on the effects of DBZDs makes it difficult to establish cut-off values for the detection of a one-time drug intake for the purpose of chemical submission. Finally, little is known on the metabolism of DBZDs and the associated metabolites, which renders the development of comprehensive analytical approaches very challenging, especially for the analysis of urine samples.

Analytical methods

Immunoassays

Several immunoassays can be considered for the detection of BZDs, including the enzyme multiplied immunoassay technique (EMIT), enzyme-linked immunosorbent assay, homogenous enzyme immunoassay (HEIA), kinetic microparticle immunoassay, and cloned-enzyme donor immunoassay (CEDIA). Due to their speed and ease of use, these immunoassays are typically used as a screening method prior to confirmation using gas chromatography (GC) or liquid chromatography (LC) combined with MS.

Immunoassays are broadly used in forensic toxicology due to their ease of use, rapidity, flexibility, and the possibility to obtain semi-quantitative results. Many of these assays are flexible enough to react to different BZDs structures. However, in some cases, the large diversity of chemical structures of BZDs leads to false-positive or false-negative results due to the variable immunoreactivity of the antibodies used for the immunoassays. As an example, the EMIT® II Plus benzodiazepine assay (5) uses polyclonal sheep antibodies targeting diazepam as binding site. The drug present in the biological sample compete with a labeled diazepam that is added as substrate in the assay. BZDs such as bromazepam, lorazepam and, especially, Phase II glucuronide metabolites exhibit low cross-reactivity, that is, they have less affinity than the labeled diazepam for the polyclonal antibody, which increases the chance of obtaining false-negative results (82). Moreover, even if assays are able to distinguish between positive and negative specimens, the semi-quantitative information is approximate, as it reflects the cumulative concentrations of the drugs and their metabolites, which cross-react with the assay (82).

Since the structures of DBZDs are similar to those of BZDs, immunoassays may also enable their detection in biological samples. Pettersson Bergstrand et al. evaluated four different assays, CEDIA, EMIT II plus, HEIA and KIMS II, for the detection of 13 DBZDs in urine samples (83). Generally, a high cross-reactivity was shown for DBZDs in all assays, demonstrating that conventional kits can be used for the standard urine immunoassay screening of DBZDs. However, flutazolam, meclonazepam and nifoxipam showed a low cross-reactivity in the EMIT II plus assay, similar to deschloroetizolam, etizolam and flutazolam when using the HEIA assay. Nifoxipam led to unfavorable results with the KIMS II. O'Connor et al. investigated the detection of six DBZDs in blood samples and reported sufficient cross-reactivity for all target analytes (84).

It is worth mentioning that DBZDs with a more divergent structure compared to conventional BZDs might remain undetected during the screening phase. Moreover, these assays have been evaluated using parent drugs; some metabolites may not cross-react using these antibody-based tests. Finally, even in the presence of a sufficient cross-reactivity, the low concentrations expected from potent DBZDs in blood after an alleged DFSA incident might not be detected by these methods—similar to BZDs.

MS-based techniques

GC–MS and LC–MS are typically used for confirmation after the initial immunoassay-based screening, as those techniques provide the high sensitivity, selectivity and accuracy needed for the quantitation of drugs and associated metabolites in biological samples. Nowadays, many routine laboratories also use MS-based approaches as initial screening techniques. Table VII lists the MS-based analytical methods developed in the last decade for the analysis of BZDs in DFSA samples, which are blood, urine and hair. The large majority of applications report the use of LC–MS, which does not require the tedious and time-consuming sample preparation required prior to GC–MS analysis.

Table VII highlights that most of the developed methods commonly target 18–24 BZDs of the 50 currently known. In addition to BZDs, the presented methods also analyze other drugs (drugs of abuse and prescribed medication) commonly associated with DFSA. Such multianalyte screening methods can enable the simultaneous analysis of up to 200 analytes as demonstrated by Rossi et al. (95).

Compared to the relatively extensive literature that covers BZD analysis, very little has been published regarding the analysis of DBZDs in the context of alleged DFSA cases. The first articles reporting the analysis of DBZDs using MS-based strategies were self-administration studies focusing on the detection of a specific compound. This has evolved toward the development of multitarget methods, which are listed in Table VIII. The studies reported in Table VIII were linked to forensic casework that included suspected DFSA samples or where DFSA was mentioned as a possible modus operandi. All methods focused on blood or urine samples; studies reporting the analysis of DBZDs in hair samples focused exclusively on a single compound, flubromazolam (73) and metizolam (96), and were not included.

With the exception of the study of Mei et al. (97), the reported methods targeted both BZDs and DBZDs, the latter representing a small fraction of the compounds analyzed. The limits of detection obtained for the analysis of DBZDs were comparable to those reported for the BZDs.

Biosamples and sample preparation

Conventional matrices used for the detection of BZDs and DBZDs include blood and urine (Tables VII and VIII). Since many (D)BZDs and their respective Phase I metabolites are conjugated with glucuronic acid during their Phase II metabolism, a deconjugation step is often performed prior to the sample preparation to enhance the detection of the non-conjugated analyte and improve the overall sensitivity. This deconjugation step typically consists of enzymatic hydrolysis

Table VII. Analytical Methods Applied to the Analysis of Benzodiazepines in Biological Matrices from Alleged DFSA Samples

Target analytes	Biological matrix	Sample pretreatment	Analyte extraction	Recovery of BZDs [%]	Analytical method	Limits of detection	Reference
18 BZDs (1 metabolite) and other drugs Total: 46 compounds	Whole blood	Adjust to pH 4 (ammonium acetate buffer)	SPE Sorbent: Oasis® MCX Elution: ethyl acetate (5% ammonium hydroxide)	17–135	UHPLC–TOF-MS	LOQ: 0.1–3 ng/g	(20)
24 BZDs (7 metabolites) and other drugs Total: 65 compounds	Whole blood	Deproteinization with methanol Adjust to pH 9 (saturated carbonate buffer)	DLLME Extractant: Chloroform Disperser: Methanol with addition of sodium chloride	Mean: 8– 69	UHPLC–MS-MS Acquisition: SRM	LOD: 2 ng/mL LOQ: 5 ng/mL except for alprazolam and chlordiazepoxide	(85)
23 BZDs (2 metabolites) and other drugs Total: 96 compounds	Whole blood		PP and PLR Protein precipitation with acetonitrile/methanol (95:5, v/v), supernatant: formic acid 1% in acetonitrile (v/v) on Phree® Phospholipid Removal Plates	Mean: 69 for all drugs	HPLC–MS-MS Acquisition: SRM	LOD: 0.1–10 ng/mL	(86)
18 BZDs (1 metabolite) and other drugs Total: 128 compounds	Urine	Hydrolysis with β-glucuronidases/arylsulfatase at pH 5.5 (acetate buffer) at 55°C for 1 h	SPE Sorbent: Oasis® HLB Elution: methanol and methanol/isopropanol (3:1, v/v)	16–108	GC–EI-MS Derivatization: BSTFA + 1% TCMS in ethyl acetate/acetonitrile (1:1, v/v)	LOD: 120–12,000 ng/mL LOQ: 200- 20 000 ng/mL	(87)
21 BZDs (4 metabolites) and Z-drugs Total: 23 compounds	Urine	Hydrolysis with β-glucuronidase at pH 6.0 at 55°C for 1 h, then alkalization at pH 7.5 with phosphate buffer	LLE Dichloromethane/propan-2-ol mixture (85:15, v/v)	Mean: 55.5–98.3	HPLC–MS-MS Acquisition: SRM	LOD: 0.5–30 ng/mL LOQ: 2–100 ng/mL	(88)
5 BZDs (2 metabolites)	Urine	Adjust to pH 9.5 (carbonate buffer)	LLE Ethyl acetate	70.5– 96.7	HPLC–MS-MS Acquisition: MRM	LOD: 0.125–1 ng/mL LOQ: 0.25–5 ng/mL	(89)
22 BZDS (4 metabolites) and other drugs Total: 91 compounds	Urine	Both hydrolyzed and nonhydrolyzed samples Hydrolysis with β-glucuronidase at 55°C for 30 min	Directly (without extraction)	Mean: 95 for all drugs	HPLC–MS-MS Acquisition: MRM	N/S	(90)
24 BZDs (5 metabolites) and other drugs Total: 54 compounds	Urine	Adjust to pH 9.5 (sodium bicarbonate buffer)	LLE Ethyl acetate	89.6–110.3	HPLC–MS-MS Acquisition: MRM	LOD: 0.5–5 ng/mL LOQ: 1–10 ng/mL	(24) (Methods in (91))

(continued)

Table VII. (Continued)

Target analytes	Biological matrix	Sample pretreatment	Analyte extraction	Recovery of BZDs [%]	Analytical method	Limits of detection	Reference
18 BZDs (6 metabolites)	Hair	Segmentation and rinsing twice with dichloromethane. Drying and segments cut into pieces <3 mm and pulverized in phosphate buffer (pH 8.4 at RT for 1 h)	LLE Dichloromethane	39.4–102.6	HPLC–MS-MS Acquisition: MRM	LOD: 0.0005–0.002 ng/mg	(28)
18 BZDs (2 metabolites) and other drugs Total: 35 compounds	Hair	Decontamination with two washes with water followed by two washes with dichloromethane. Drying and segmentation into <1 mm. Incubation overnight at RT with phosphate buffer pH 5.	LLE Dichloromethane/ether (70:30, v/v).	N/S	HPLC–MS-MS Acquisition: MRM	LOD: 0.0005–0.01 ng/mg LOQ: 0.0005–0.01 ng/mg	(92)
13 BZDs (3 metabolites) and other drugs Total: 52 compounds	Hair	10 mg hair, one wash with isopropanol and two with H_2O. Drying and extraction. Segmentation in 1–2 mm segments or pulverization. Incubation overnight at 37°C	Extraction Methanol:acetonitrile:ammonium formate (pH 5.3) Filtration PTFE filter	87–102	UHPLC–TOF-MS	LOD: 0.01–0.04 ng/mg LOQ: 0.05 ng/mg	(29) (Methods in (93)
20 BZDs (6 metabolites) and 3 Z-drugs Total: 23 compounds	Hair	Decontamination with H_2O, acetone and hexane. Pulverization in a bench-top mill.	Extraction under shaking 1st step: methanol 2nd step: methanol/ammonium formate buffer (1:1, v/v) at pH 3.5	N/S	UHPLC–MS-MS Acquisition: MRM	LOQ: 0.0005–0.01 ng/mg	(94)
28 BZDs (9 metabolites) and other drugs Total: 200 compounds	Blood	Deproteinization with methanol, adjust to pH 9 (saturated carbonate buffer)	DLLME Extractant: chloroform Disperser: methanol with addition of sodium chloride	Mean: 8.0–69.3	UHPLC–MS-MS Acquisition: MRM	LOD: 2 ng/mL LOQ: 5 ng/mL except for alprazolam and chlordiazepoxide	(95) (Methods for blood in (85)
	Urine	Hydrolysis with β-glucuronidase in acidic medium	Dilution and shoot Aqueous formic acid	N/S	UHPLC–MS-MS Acquisition: MRM	LOD: 1–5 ng/mL (not determined for hydroxymidazolam)	
	Hair	Decontamination with two washes with aqueous tween 80 and one wash with acetone. Segmentation into 1 mm.	Extraction Aqueous formic acid at 40°C overnight	N/S	UHPLC–MS-MS Acquisition: MRM	N/S	

DLLME: dispersive liquid–liquid microextraction; LLE: liquid–liquid extraction; LOD: limit of detection; LOQ: limit of quantitation; PP: protein precipitation; RT: room temperature; SRM: selected reaction monitoring.

Table VIII. Analytical Methods Applied to the Analysis of DBZDs in Biological Matrices Including DFSA Samples

Target analytes	Biological matrix	Internal standards for DBZDs	Sample pretreatment	Analyte extraction	Recovery of DBZDs [%]	Analytical method	Limits of detection of DBZDs	Reference
8 DBZDs, 10 BZDs and Z-drugs Total: 20 compounds	Whole Blood	$^{13}C_6$-diazepam	Adjust to pH 7.5 (phosphate buffer)	PALME SLM: 2-undecanone: dihexyl ether: (1:1, W/W) and 1% trioctylamine (w/w) Acceptor solution: dimethyl sulfoxide and formic acid (75:25, v/v).	Mean: 52–101	UHPLC–MS-MS Acquisition: MRM	LOD: 0.1–5 ng/mL for all drugs LLOQ: 2 ng/mL	(98)
13 DBZDs	Whole blood	Mixture of: Chlordiazepoxide-d_5 Diazepam-d_5 Nordiazepam-d_5 Temazepam-d_5	Adjust to pH 4.5 (sodium acetate buffer)	SPE Sorbent: Clean Screen® XCEL I Elution: ethyl acetate:ammonium hydroxide (98:2)	Mean: >50–83.9 (except for nifoxipam and 3-hydroxy-phenazepam)	HPLC–MS-MS Acquisition: MRM	LOD: 0.5 ng/mL for all drugs LOQ: 1 ng/mL	(97)
14 DBZDs, 26 BZDs (11 metabolites) and 3 Z-drugs Total: 43 compounds	Blood and urine	α-Hydroxymidazolam-d_4 (bromazolam) *N*-desalkylflurazepam-d_4 (*N*-desalkylflurazepam) Alprazolam-d_5 (flubromazolam) Diazepam-d_5 (diclazepam) Estazolam-d_5 (clonazolam, estazolam, flunitrazolam, nitrazolam) Etizolam-d_3 (deschloroetizolam, etizolam) Phenazepam-d_4 (phenazepam)	*Urine* Hydrolysis with genetically modified β-glucuronidase at 55°C for 30 min	PP with methanol: acetonitrile (10:90, v/v), adjust to pH 6 (sodium phosphate buffer) SPE Sorbent: Screen-C® Ethyl acetate containing 2% (v/v) ammonium hydroxide	N/S	HPLC–MS-MS Acquisition: MRM	LOD: 1.0–10 ng/mL (not accurate for 3-hydroxy flunitrazepam and methylclonazepam)	(99)

using β-glucuronidase or a mixture of β-glucuronidase and arylsulfatase. Alternatively, the conjugated metabolites can also be analyzed, rendering the deconjugation step unnecessary (89, 91).

Hair is an interesting alternative matrix for the analysis of (D)BZDs as it allows for a longer detection window. Compared with blood and urine matrices, hair requires an extensive pretreatment procedure before the extraction of the target analytes. A decontamination step of hair fibers is required, prior or after segmentation or pulverization of the hair. Hair pulverization usually leads to higher extraction recoveries (100, 101). The pulverized or segmented hair is then incubated with different solvent mixtures (up to 24 h) prior to the extraction step (102).

The sample preparation step relies on common procedures, including protein precipitation, liquid–liquid extraction (LLE), solid-phase extraction (SPE) and—for urine samples—dilute and shoot approaches. With the basic pKa of (D)BZDs being in the range of approximately 1.5–3.5 (42), alkalization of the samples prior to LLE is typically performed, prior to extraction using an adequate solvent, often ethyl acetate or dichloromethane. Rossi et al. used a miniaturized approach, that is, dispersive liquid–liquid microextraction (DLLME) for the extraction of BZDs and other relevant drugs (95). DLLME uses a combination of an extracting solvent (e.g., dichloromethane) and a dispersion solvent miscible with water (e.g., methanol), which is added to the sample. This ternary system leads to the formation of microdroplets, which strongly enhance the surface area and allows for instantaneous equilibrium partitioning of analytes between the aqueous sample and the extraction solvent, resulting in a drastically reduced extraction time (i.e., a couple of seconds) (103).

Another interesting miniaturized approach is offered with parallel artificial liquid membrane extraction (PALME), used by Vårdal et al. for the extraction of BZDs, DBZDs and Z-drugs in whole blood (98). In PALME, targeted analytes present in an aqueous donor solution are transferred through an organic supported liquid membrane to an acceptor solution. By modifying the pH of the donor and acceptor solutions, target analytes can be extracted and preconcentrated in a miniaturized and semi-automated format. By combining this setup with ultra-high pressure liquid chromatography–MS-MS analysis, limits of detection down to 0.1 ng/mL were achieved.

Separation and detection

As illustrated in Tables VII and VIII, a large majority of (D)BZDs analysis methods is based on LC–MS. Only one study reports the use of GC–MS for the analysis of BZDs (and other date-rape drugs) in urine samples (87). Most of the studies included the quantitation of (D)BZDs and, therefore, frequently include tandem MS (MS-MS) approaches. Very few articles report the use of high-resolution MS, for instance, time-of-flight (TOF) or Orbitrap mass analyzers.

The developed methods typically aim for the detection of concentrations lower than the therapeutic level, which seems to be easily achievable with state-of-the-art triple quadrupole instruments and adequate sample preparation.

It is worth mentioning that the isotopically labeled internal standards used for the accurate quantitation of DBZDs were most frequently not analyte-specific, which may be explained by the low number of commercially available labeled DBZDs standards. The exceptions include *N*-desalkylflurazepam, estazolam, etizolam and phenazepam.

Discussion

Together or in combination with ethanol, BZDs belong to the most frequently used drugs in DFSA. Among all BZD prescription drugs, one-third have been reported in DFSA cases. Currently, more than 30 DBZDs have been noted, with the first substance already detected in 2007. Surprisingly, the use of DBZDs for DFSA remains relatively rare as only diclazepam and flualprazolam have been reported in DFSA casework in China so far. This may be explained by the screening and/or confirmation methods currently used in DFSA investigations, which typically only target prescription BZDs (95). Equally important, drug-screening tests should always be performed in sexual assault cases where ethanol may be involved as ethanol and (D)BZDs lead to similar toxicological effects.

BZDs exhibit a large variability in their pharmacokinetic properties. Notably, the metabolism of these drugs shows significant differences, with some BZDs leading to the formation of active metabolites, while others only forming inactive species. Moreover, the excretion half-life of some of these active metabolites is significantly higher than for the parent drug as illustrated with the half-life of desalkylflurazepam ($t_{1/2} = 40$–144 h) compared to flurazepam ($t_{1/2} = 2$–3 h). Furthermore, the concentrations of BZDs in biosamples also depend on the sex, weight and age of the victim, which additionally complicates the interpretation of the measured concentrations. Finally, the (voluntary or involuntary) possible combination of BZDs with other drugs or alcohol increases the complexity of the interpretation of BZDs concentrations in DFSA casework.

For most of the DBZDs reported, little is known about the regular dose, their effects and their pharmacokinetic properties. As for BZDs, they are expected to span a broad range of psychoactive effects and metabolic properties. Most of the information reported in this review has been obtained from drug users in a noncontrolled setting or from self-administration studies, which does not provide insights into the interindividual pharmacokinetic differences. Moreover, the data currently available still requires scientific confirmation as highlighted by, for instance, pyrazolam for which metabolites have been only tentatively identified or flunitrazolam for which discrepancies between *in vitro* and *in vivo* results have been found. Clinical studies with human volunteers remain difficult to implement as the potency and toxicity of these designer drugs remain poorly understood. Animal models may therefore be explored to study the pharmacokinetic properties of these compounds. Even though the conclusions obtained may not always be directly extrapolable to humans, it allows for a more rigorous experimental design.

Blood and urine are the most commonly used matrices for the detection of (D)BZDs in DFSA cases. Blood-based samples show the highest correlation between the analyte concentration and the psychoactive effects, which—together with the information on the administered dose and/or the victim statement with respect to the incapacitating effect of the drug at the time of the alleged incident—can assist in a correct

forensic interpretation. It is worth mentioning that the administered dose is frequently unknown but this information is often requested by the prosecutor. The interpretation of blood concentrations of DBZDs is even more challenging due to the scarce information available on the metabolism of DBZDs in humans. In the case of BZDs, most of the studies focusing on blood-based matrices report the analysis of both parent drugs and metabolites. However, only parent drugs have been quantified in studies dealing with DBZDs. For short-acting (D)BZDS, such as midazolam or triazolam, blood may not be an adequate matrix if the time elapsed between the incident and the sampling is too long, typically exceeding 12 h (20, 86). In this case, urine or hair should be considered, or the blood sample should be screened for the presence of potential metabolites.

Urine is considered the matrix of choice in DFC or DFSA cases according to the SOFT (3) and the UNODC (4) guidelines. The detection window can indeed span up to 120 h after the assault. The analysis should be performed within the first 72 h; otherwise, the sample should be stored at −80°C (95). Both parent drug(s) and metabolites should be included in the analysis. Table VII indicates that the expected metabolites are not always analyzed within the reported studies probably because most of these methods included the enzymatic hydrolysis of Phase II metabolites, which favors the parent drug concentration. With such enzymatic approaches, incomplete hydrolysis or variable hydrolysis efficiencies may be observed, which can lead to results that are not representative for casework samples (95). In this respect, an indicator to evaluate and monitor the hydrolysis efficiency should be added to the workflow.

Frequently, sexual assault cases are reported after several days, weeks or even months. Under such circumstances, hair can be considered as an attractive alternative matrix for blood and urine, as it offers a much longer detection window— up to several months after the event—for both the parent drug and the metabolites. As an example, flunitrazepam and oxazepam have been detected in hair samples 7 months after a sexual assault (32). Hair segmental analysis provides relevant information on whether the drug was taken regularly before the alleged incident or if it was administered once at the moment of the incident. Moreover, hair samples can be stored and transported at room temperature (4). Hair is also considered an interesting matrix for drugs with a short half-life, such as midazolam and triazolam, which are rapidly excreted from the body. Xiang et al. (100) and Wang et. al (29) reviewed the drug concentrations in hair in DFC cases, showing the relevance of using hair to obtain supporting information.

The use of immunoassays in DFSA investigation is currently becoming obsolete for diverse reasons. First, many routine forensic laboratories are now equipped with adequate state-of-the-art GC–MS or LC–MS instruments, which are used for both the screening and confirmatory steps. Moreover, immunoassays lead to high risks of obtaining false-negative results, as the commercially available tests do not enable the detection of all compounds potentially present in the sample. In the case of DBZDs, the high cross-reactivity obtained with immunoassays for BZDs allows the detection of many but not all DBZDs.

Screening approaches for the analysis of BZDs typically consist of multitarget LC–MS-MS methods analyzing not only BZDs but also other classes of drugs. The vast majority of studies reported are based on a triple quadrupole mass analyzer, acquiring MS-MS data using selected reaction monitoring. Surprisingly, very few studies report the use of high-resolution MS (using TOF or an Orbitrap mass analyzers, for instance), which allows for retrospective data analysis, a feature considered highly useful for the discovery of nontargeted and/or novel DBZDs and related metabolites.

Most of the LC–MS-MS methods developed for the analysis of BZDs as reported in Table VII demonstrate limits of detections below the expected therapeutic range, with a few exceptions. Due to the lack of information regarding the dose–effect relationship of most of the DBZDs, it is difficult to estimate whether the limits of detection and quantitation reported in Table VIII are fit-for-purpose. The higher potency of some DBZDs compared with conventional BZDs suggests lower expected concentrations and, therefore, the need for even lower limits of detection and quantitation. Finally, most methods developed for the analysis of DBZDs use isotopically labeled internal standards of conventional BZDs, due to the lack of labeled DBZDs standards, which may lead to inaccurate quantitation of the targeted analytes.

Due to the serious legal consequences associated with DFC and DFSA, a correct interpretation of the drug concentrations measured in biosamples may be beneficial to support a victim's story, even though the mere presence of (D)BZDs is in most cases very helpful for a case elucidation. Interpretation of drug concentrations remains challenging for both conventional BZDs and designer BZDs. As an example, diazepam is metabolized into nordiazepam (desmethyldiazepam), oxazepam and temazepam. These metabolites can also be ingested as drugs, mostly oxazepam and temazepam, as nordiazepam is only prescribed in a limited number of countries. Moreover, oxazepam is an active metabolite not only of diazepam but also of nordiazepam and temazepam. The introduction of DBZDs further complicates the situation as some DBZDs are metabolites of conventional BZDs. For instance, the presence of nifoxipam (3-hydroxydesmethylflunitrazepam) or fonazepam (desmethylflunitrazepam) can mistakenly be associated with flunitrazepam intake. Furthermore, 3-hydroxyphenazepam is erroneously linked to phenazepam; no data on its metabolism are available to elucidate which drug has been ingested. Finally, diclazepam is metabolized into lorazepam, lormetazepam and delorazepam, which are all prescribed BZDs. Overall, this shows the importance of merging the information obtained from the analysis of biosamples with the drug history and testimony of the DFSA victim and possibly other witnesses, as well as other supporting evidence (e.g., the Internet orders of the incapacitating agents by the suspects or packaging found at the scene of the incident).

Conclusions

A large number of BZDs have been reported in DFSA-related forensic casework over the last decade. Due to their potency, fast onset of action, oral administration and short half-life, BZDs are ideal candidates for DFSA. DBZDs present additional advantages for perpetrators as these substances are freely available in the market and typically remain undetected in conventional multitarget screening approaches. Over the past years, there has been a significant increase in DBZDs available in the drug market, with more than 80% of these substances being reported for the first time between 2014 and

2020. DBZDs give rise to concerns regarding both individual and public health due to the limited knowledge on their pharmacology and toxicity, the lack of information regarding the dose, the potential presence of unknown adulterants and the lack of knowledge regarding the effects of the co-consumption of DBZDs with other drugs or alcohol.

The low concentrations and small detection windows of (D)BZDs and their metabolites in body fluids require the use of highly sensitive state-of-the-art analytical approaches. Moreover, DFSA crimes are typically characterized by delayed reporting or even reluctance to report the event by the victim, which poses additional challenges in the detection of these substances. In this context, both blood and urine samples should be collected as early as possible after the alleged incident to provide qualitative and quantitative data. If more than 72 h have elapsed since the incident, taking a hair sample from the victim should also be considered.

The reviewed literature also showed the lack of standardization in the information collected during alleged DFSA cases, such as the estimated time elapsed between the incident and the sampling, the drug history of the victim or ethanol consumption. This information is of utmost importance for an accurate interpretation of the analytical results and shows the relevance of adequate training of early responders on how to approach and inform DFSA victims. Indeed, police officers or medical staff should be encouraged to collect as much contextual information as possible and convince victims to grant permission for the early collection and analysis of biological specimens.

Finally, the growing complexity of the DBZD market highlights the need to continuously strengthen state-of-the-art analytical strategies for the detection, identification and quantitation of such substances, including the use of untargeted high-resolution MS approaches, the use of chemical libraries to monitor substance use and the commercial availability of regular and isotopically labeled standards.

Declarations

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