Detection of Phenazepam in Impaired Driving

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Phenazepam is a potent 1,4-benzodiazepine that has gained notoriety among recreational drug users. First synthesized in Ukraine in the 1970s, it is one of the most commonly prescribed benzodiazepines in Russia and other commonwealth of independent state nations, where it is used therapeutically as a prescription drug. Reports of abuse are widespread and several European countries have taken steps to control its use. However, in the USA, phenazepam is not approved for use by the Food and Drug Administration, nor scheduled under the Federal Controlled Substances Act. Phenazepam is widely available on the Internet, and recreational drug users report a potency 10-fold greater than that of nordiazepam. We report a case of a 24-year-old male driver who was apprehended for impaired driving following a two-vehicle crash. The subject exhibited slurred speech and profound psychomotor impairment. Toxicology testing revealed phenazepam at a concentration of 76 ng/mL in blood, with no other drugs detected. This case report not only demonstrates the potential for adverse traffic safety consequences following the misuse of phenazepam, but also highlights the importance of analytical factors such as immunoassay cutoff concentration, cross-reactivity and comprehensive screening using chromatographic-based techniques for impaired driving investigations.

Introduction

Phenazepam [7-bromo-5-(2-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepine-2-one] is a 1,4-benzodiazepine that is structurally related to bromazepam (Figure 1). It was first synthesized in Ukraine in 1975 and more recently, it has emerged as a drug of abuse (1, 2). It is reported to be one of the most commonly prescribed benzodiazepines in Russia and other commonwealth of independent state countries (3). Although it has no legitimate clinical uses in the USA, it has been used therapeutically as a sedative hypnotic, anticonvulsant, muscle relaxant, anxiolytic and for the treatment of neurological disorders and alcohol withdrawal (1, 3–5). When used therapeutically, it is available as 0.5 and 1 mg tablets, injectable solutions (0.1 and 0.3%) and 1–4 mg transdermal patches (Phenapercuten). Oral doses of 0.5 mg (2–3 times daily) may be prescribed, but doses up to 10 mg/day are reported (3).

Phenazepam is controlled in several countries, particularly Eastern Europe and Scandinavia, where abuse of the drug has been most widespread. Presently, it is controlled in Estonia, Latvia, Lithuania, Moldova, Finland, Norway, Sweden and the Republic of Ireland. In 2011, the UK passed legislation to ban importation of the drug and in 2012, it was controlled in an amendment to the Misuse of Drugs Act. In the USA, phenazepam is not controlled at federal level, although two states have taken steps to control its use: In 2012, phenazepam was classified as a Schedule I drug in both Louisiana (www.pharmacy.la.gov) and Arkansas (http://www.arkleg.state.ar.us). Illicitly, it is sold in powder, tablet and liquid form via the Internet. A recent report suggests that the major distribution centers are located in Cameroon, mainland China and the USA (1). Retail prices for 100 mg phenazepam powder average $40–55 and typical users are males in their 20–30s (4). Phenazepam has also been encountered in the form of lysergic acid diethylamide (LSD) mimic blotters in some parts of the USA (6). Recreational users report doses of 2–10 mg of phenazepam powder (7). Although phenazepam is sometimes encountered as counterfeit diazepam, it is also promoted as a ‘legal high’ under names such as ‘Bonsai’ and ‘Bonsai Super-Sleep’ (8).

The pharmacology of phenazepam has not been widely studied in humans, largely due to the limited geographical use of the drug for legitimate purposes. Initial clinical studies of the drug in the former Soviet Union in the 1980s are sparse, but suggest extensive metabolism by aromatic oxidation, hydroxylation in the 3 position and subsequent glucuronidation (9, 10) (Figure 2). Phenazepam and its metabolite (3-hydroxyphenazepam) are reported to be full gamma aminobutyric acid (GABA)A agonists, having greater potency than diazepam (10). Recreational drug users report phenazepam to have 10 times the potency of nordiazepam (11) and in one double-blind clinical study, the tranquilizing effect of the drug was reported to be more pronounced and of longer duration compared with diazepam (12). Phenazepam is characterized as a powerful anxiolytic, having strong sedative, anticonvulsive and hypnotic properties (13). In phenazepam cases where both blood and urine were available, parent drug and 3-hydroxyphenazepam were reported following enzymatic hydrolysis (4). However, following controlled administration of 3 and 5 mg in humans, 3-hydroxyphenazepam was not detected in the blood (5). Other reports among drug users confirm it to be a minor metabolite in blood (4). However, an early Russian study suggested that 3-hydroxyphenazepam may thermally degrade during gas chromatography (GC) analysis (14), so some caution is necessary.

There are still relatively few pharmacological or toxicological studies involving phenazepam in humans. In a very early report, doses of 3–5 mg produced peak blood concentrations of 24–38 ng/mL at ~4 h with a half-life estimated to be 60 h (5). Oral doses of the drug are reported to result in significant first pass effect and the overall elimination kinetics appeared comparable with other long-acting benzodiazepines. When 2 mg doses were administered intramuscularly in epileptic patients, the half-life was estimated to be as short as 15 h (15).

A recent review article highlights the adverse health consequences associated with the increased popularity of phenazepam in Europe and the USA (1). Effects may include somnolence, dizziness, incoordination, slurred speech, confusion, memory loss,
ataxia, disorientation, asthenia and hallucinations. Although clinical pharmacokinetic studies are limited, dated and in some cases contradictory, there are numerous reports of adverse consequences following use (1, 2, 4, 16–19). Effects are not always short-lived. In one recent report in the UK, prolonged neuropsychiatric toxicity was described ~60 h after recreational use (8). In another study of 61 individuals in Sweden, as many as 14 subjects were still experiencing symptoms for >5 days after ingestion of the drug (20).

Not surprisingly, the GABAergic and soporific effects of phenazepam have attracted attention, particularly in countries where misuse of the drug is widespread. In reports from Finland, as many as 3.5% of all driving under the influence of drug cases were found to contain phenazepam (4, 18). Although not yet

Figure 1. Structural similarity of 1,4-benzodiazepines: phenazepam and bromazepam. Prazeplam was used as the IS.

Figure 2. Metabolism of phenazepam [7-bromo-5-(2-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepine-2-one] (A) to 5-bromo-(2-chlorophenyl)-2-aminobenzophene (B), 6-bromo-(2-chlorophenyl)-quinazoline-2-one (C) and hydroxylation to 3-hydroxyphenazepam (D) (10).
as widespread in the USA, we report a case of a phenazepam-impaired driver exhibiting profound impairment. The case is also used to highlight the importance of analytical factors associated with impaired driving investigations.

Case report
At ~1:10 pm, a police officer who was responding to a traffic accident on a rural highway observed a second, unrelated two vehicle crash on the same road. A 24-year-old white male failed to stop at an intersection and ran into the rear of another vehicle that was stopped at a red light. The subject (152 lb) had slurred speech and was very unsteady on his feet. The subject refused to perform field sobriety tests, but agreed to provide a blood sample. As the subject walked, he staggered from side to side and was in need of assistance to prevent himself from falling down. The subject almost fell to the ground after being asked to stand up from a seated position. The subject was cooperative and when asked what was wrong, he stated he had taken ‘vicodin and antibiotics for a bad tooth’ the night before. While being transported to the hospital for blood collection, the subject required assistance, was disoriented and attempted to walk in the wrong direction.

Materials and methods

Chemicals and reagents
Phenazepam, oxazepam, prazepam and pentobarbital-D₅ were purchased from Cerilliant (Round Rock, TX, USA). PolyCrom Clin II (6 cc) solid-phase extraction (SPE) columns (Catalog #691-0506) containing 35 mg polymeric sorbent were obtained from SPEware (Baldwin Park, CA, USA). Deionized water was purified through a Millipore Milli Q water system (Billerica, MA, USA). Acetic acid, hexane, ethyl acetate, methanol, methylene chloride and isopropl alcohol were obtained from Mallinckrodt-Baker (Hazelwood, MO, USA). Ammonium hydroxide and sodium chloride were obtained from Fisher Scientific (Pittsburgh, PA, USA) and mepivacaine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Monobasic and dibasic sodium phosphate purchased from VWR (West Chester, PA, USA) were used to prepare a 100 mM phosphate buffer (pH 6). All inorganic reagents and solvents were ACS and HPLC grade or better, respectively. The SPE elution solvent (prepared daily) consisted of 2% concentrated ammonium hydroxide in a mixture of methylene chloride:isopropl alcohol (80:20, v/v).

The Benzodiazepine Direct ELISA Kit (catalog # 214-0192) was purchased from Immunoassay (Poloma, CA, USA). All immunoassay reagents used were provided in the kit, with the exception of phosphate-buffered saline (PBS), which consisted of 100 mM phosphate buffer (pH 7.0) in 150 mM saline. The internal standard (IS) solution consisted of prazepam (0.01 mg/mL), mepivacaine (0.01 mg/mL) and pentobarbital-D₅ (0.01 mg/mL) in methanol. Independently prepared methanic phenazepam standards were used for the preparation of all calibrators and controls. Drug-free bovine blood containing 1% sodium fluoride (w/v) and 0.2% potassium oxalate (w/v) was obtained from Quad Five Materials (Ryegate, MT, USA).

Enzyme-linked immunosorbent assay
Blood samples were routinely screened for the presence of benzodiazepines in accordance with the manufacturer’s instructions and the standard operating procedure of the laboratory at a cutoff concentration equivalent to 50 ng/mL oxazepam. Blood (100 µL) was prediluted (1:10) with PBS (900 µL) prior to screening. Automated screening was performed using a Tecan Freedom EVO 75 (San Jose, CA, USA), Tecan HybridFlex plate washer and Tecan Sunrise plate reader. Briefly, 50 µL of prediluted blood (1:10 in PBS) was added to microtiter wells with 100 µL enzyme conjugate. After incubation for 60 min and rinsing thoroughly with deionized water, 100 µL of tetroethyl benzidine substrate solution was added. After an incubation for 30 min, 100 µL of stop reagent (1 M hydrochloric acid) was added and the absorbance was measured at 450–620 nm.

To determine the cross-reactivity of the immunoassay toward phenazepam, an eight-point dose–response curve was generated using whole blood calibrators between 1 and 1,000 ng/mL. The percent binding (%B) was defined as 100 × (A/A₀), where A and A₀ were the absorbance of the sample and that of the drug-free matrix (blank), respectively. A second dose–response curve was generated for oxazepam (the target analyte for the immunoassay) in an identical fashion. The concentration of phenazepam that produced an absorbance reading equivalent to 50 ng/mL oxazepam (Cphenazepam) was used to estimate the percent cross-reactivity as follows: % Cross-reactivity = 100 × (50/Cphenazepam).

GC–MS instrumentation
GC–mass spectrometry (MS) analysis was performed using an Agilent HP 5975 MSD/7890 GC (Santa Clara, CA, USA) equipped with an HP-5MS Agilent J&W (30 m × 0.25 mm × 0.25 µm) capillary column purchased from VWR (West Chester, PA, USA). The injector and interface were set at 250 and 280 °C, respectively. Injections (2 µL) were made in split mode with a 10:1 split ratio. Ethyl acetate and methanol were used as the wash solvents. The oven temperature was held at 150 °C for 0.50 min, ramped to 180 °C at a rate of 20 °C/min with a hold time of 2 min, ramped to 230 °C at a rate of 5 °C/min with a hold time of 1 min, ramped to 250 °C at a rate of 10 °C/min with a hold time of 0.5 min and then ramped to 290 °C at a rate of 30 °C/min with a final hold time of 6.5 min. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The MS was operated in the electron impact ionization mode. The ion source and quadrupole were set at 230 and 150 °C, respectively. Data were initially acquired using full-scan acquisition for initial qualitative identification of acidic, basic and neutral drugs. Selected ion monitoring (SIM) was subsequently used for quantitative analysis of phenazepam (350, 319 and 315), using prazepam (324 and 295) as the IS (quantitation ions are shown bold). Although isotopically labeled IS are generally preferable, deuterated phenazepam was not commercially available at the time of analysis.

Solid-phase extraction
Phenazepam was identified using a SPE procedure that is routinely used in our laboratory to identify basic, acidic and neutral drugs. A methanic working standard was used to prepare all calibrators and controls. After addition of IS solution (100 µL) to
a 2-mL blood sample, 4 mL of 100 mM, pH 6.0, phosphate buffer was added. Buffered blood samples were added to PolyCrom Clin II SPE columns and successively rinsed using 1 mL of deionized water and 1 mL of 1 M acetic acid. Columns were dried under full vacuum for 5 min and rinsed with 1 mL of hexane. Acidic and neutral drugs were eluted into conical glass tubes using 1 mL of ethyl acetate. After removal of the collection tubes, columns were then washed with 1 mL of methanol and dried under full vacuum for an additional 5 min. Collection tubes were replaced and basic drugs were eluted using 1 mL of 2% ammonium hydroxide in 80 : 20 (v/v) methylene chloride : isopropyl alcohol. Extracts were evaporated to dryness under nitrogen at 50°C, reconstituted in 30 μL of ethyl acetate and transferred to autosampler vials for analysis. The total extraction time was < 1 h.

Results and discussion

The blood sample obtained from the subject arrested for impaired driving was previously tested at another facility and no alcohol or drugs were detected. Enzyme multiplied immunoassay technique for common drugs of abuse was used to screen the blood, and the cutoff concentration for the benzodiazepine assay was reported to be 100 ng/mL. The sample received no further analysis.

The sample was submitted to our laboratory for additional testing, because the observations were inconsistent with the toxicology results. The sample was treated in accordance with our standard protocol for impaired driving cases. Blood samples were routinely screened by enzyme-linked immunosorbent assay for opiates (20 ng/mL of morphine), methamphetamine (20 ng/mL of d-methamphetamine), benzodiazepines (50 ng/mL of oxazepam), cocaine metabolite (50 ng/mL of benzoylecgonine) and cannabinoids (10 ng/mL of l-11-nor-9-carboxy-Δ9-tetrahydrocannabinol). Presumptive positive results were confirmed by GC–MS, and comprehensive testing for basic, acidic and neutral drugs using SPE and GC–MS was routinely conducted on samples that screen negative by immunoassay.

Phenazepam was readily identified using full-scan GC–MS analysis. No other drugs were detected. A representative whole blood extract containing 10 ng/mL phenazepam is shown in Figure 3. Quantitative analysis using SIM from whole blood fortified with phenazepam (0–100 ng/mL), yielded an $R^2$ value of 0.999 and a concentration of 76 ng/mL phenazepam. The limit of quantitation (LOQ) of the assay was 1 ng/mL using three different sources of blood over 3 days. At the LOQ, accuracy was 91–109% ($n = 9$), signal-to-noise ratios ranged from 31 : 1 to 102 : 1 and interassay coefficient of variation (CV) was 9.0% ($n = 9$). At 100 ng/mL in blood, accuracy was 102% and the interassay CV was 10.2% ($n = 15$).

Phenazepam is a basic drug that is easily detected using routine toxicological procedures. SPE or liquid–liquid extraction protocols that are commonly used for the isolation of other alkaline drugs should be effective, when coupled with GC–MS, liquid chromatography (LC)–MS or LC–MS–MS. Reported limits of quantitation range between 3 and 28 ng/mL (16–18) by LC–MS–MS and 10–20 ng/mL (21, 22) by GC–MS. The presence of both chlorine and bromine in phenazepam are responsible for the highly characteristic mass spectrum, which enhances detectability, without the need for chemical derivatization.

The blood sample from the driver screened positive for benzodiazepines using a 50-ng/mL cutoff concentration. Dose–response curves were used to determine the cross-reactivity of the Immunalysis benzodiazepine assay toward phenazepam. The concentration of phenazepam in blood that produced an absorbance equivalent to the cutoff concentration of 50 ng/mL oxazepam was ~20 ng/mL. This corresponds with a cross-reactivity of 250%. The effective concentrations for 50% binding (EC50) for oxazepam and phenazepam were 90 and 60 ng/mL, respectively. The cross-reactivity of commercial benzodiazepine immunoassays toward phenazepam was previously reported to be 43–176% using cutoff concentrations in urine between 200 and 300 ng/mL (23). Affinity constants determine the strength of the binding between the antibody and the drug, and ultimately the characteristics of the dose–response curve. The measured cross-reactivity at a specific cutoff is highly dependent on the dose–response characteristics and for this reason, it is important to determine cross-reactivity using the specific conditions and cutoff concentrations that are used in routine casework.

Phenazepam has been detected in impaired drivers overseas over a wide range of concentrations. Although benzodiazepines

![Figure 3. Ion chromatogram of phenazepam (10 ng/mL) in blood.](https://academic.oup.com/jat/article-abstract/37/8/605/778827/figure3)
can negatively influence driving skills in a dose-dependent manner, the possibility of tolerance makes it difficult to determine impairment based on drug concentration alone. The scientific literature specific to phenazepam and driving impairment is limited. Nevertheless, its effects on driving can be inferred from studies of the benzodiazepine class as a whole, and these have been widely studied and reviewed (24–27). In particular, the use of benzodiazepines with long half-lives has been associated with an increased risk of road traffic accidents (28). The long half-life of phenazepam may increase the impairment window relative to other short-acting benzodiazepines and more importantly, increase the potential for cumulative effects with repeated dosing.

Assessment of the consequences for driving is often complicated by multi-drug use. Concentrations reported in recreational drug users overlap with those reported in fatalities (4). Phenazepam abuse is widespread in Finland due to its geographical proximity to Russia, where ~3.5% of all apprehended drivers tested positive for phenazepam. The median blood concentration in impaired drivers was 61 ng/mL and the range was 4–3,600 ng/mL (4). Of the 141 phenazepam positive cases, only 7 involved phenazepam alone. Concentrations ranged from 44 to 3,000 ng/mL and effects were not correlated with concentration. In another study from Finland, concentrations of 5–3,000 ng/mL were reported in blood from apprehended drivers, and the overall prevalence of the drug was 3.4% (18). In cases where phenazepam was the only drug, observations included unsteady gait, difficulty walking, confusion, impaired balance, slurred speech, memory loss, ataxia and pupils that are slow to react to light (3, 18). In a US report of 11 impaired driving investigations reported in the State of Georgia (17) between March 2010 and August 2011, the median phenazepam concentration was 170 ng/mL. Five cases involved phenazepam alone, and these ranged from 40 to 3,200 ng/mL. Reported driving behaviors included striking fixed objects such as trees or other vehicles at stop lights, failure to maintain lane and running off the road. Impairment and severity of driving behavior or crash conditions were not correlated.

In Norway, the Ministry of Transport and Communications proposed a phenazepam impairment threshold of 1.8 ng/mL to be equivalent to a legal per se blood alcohol concentration (BAC) of 0.02%. They further recommended low and high impairment limits of 5 and 10 ng/mL phenazepam, respectively, as equivalent to BACs of 0.05 and 0.12% (29). These recommendations stemmed from the Norwegian Institute of Public Health's statistical analysis of fatally injured drivers between 2006 and 2008. Although statutory approaches of this type are not favored in the USA, it serves to highlight the serious concerns regarding phenazepam and traffic safety that exist in countries that have had wider experience with the drug.

Conclusions

This case report highlights the importance of comprehensive toxicological testing in impaired driving investigations. It also demonstrates issues related to cross-reactivity and cutoff concentrations of immunoassay screening tests that are commonly used in laboratories. Failure to use cutoff concentrations that are sufficiently low, understand the limitations of cross-reactivity or perform chromatographic-based screening, can have serious toxicological consequences.

Although benzodiazepines are largely considered to have low toxicity, their potential for driving impairment is well documented. In particular, increased crash risks associated with benzodiazepines are more pronounced for drugs with longer half-lives. The concentration of phenazepam reported in this case (76 ng/mL) is consistent with the median concentration reported by Kriikku et al. (61 ng/mL) (4) and is considerably higher than therapeutic blood concentrations of 24–38 ng/mL (5). The relatively sparse pharmacokinetic data and the possibility of tolerance complicate the pharmacological interpretation of quantitative blood drug toxicology. Reports to date suggest that phenazepam may have potent and long-lasting sedative effects, and further study of its pharmacology and toxicology is warranted.

References


