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Technical Note

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ELISA Detection of Phenazepam, Etizolam, Pyrazolam, Flubromazepam, Diclazepam and Delorazepam in Blood Using Immunalysis® Benzodiazepine Kit

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Abstract

Phenazepam and etizolam were the first uncontrolled benzodiazepines available for sale in the UK. Pyrazolam, flubromazepam and diclazepam are not used medicinally anywhere in the world; they are produced exclusively for the uncontrolled, recreational market. It is important to know whether potentially abused drugs like these can be detected in routine toxicological screening tests. The purpose of this study was to evaluate whether the Immunalysis® Benzodiazepines ELISA kit could detect phenazepam, etizolam, pyrazolam, flubromazepam, diclazepam and its metabolite delorazepam. Their cross-reactivity was assessed by comparing the absorbance of the drug with that of oxazepam, the reference standard. This study found that these uncontrolled benzodiazepines cross-react sufficiently to produce a positive result with the Immunalysis® Benzodiazepine ELISA kit. Cross-reactivity ranged from 79 to 107% for phenazepam, etizolam, pyrazolam, flubromazepam, diclazepam and delorazepam fortified into blood. The results show that it is possible to detect these newer benzodiazepines with traditional forensic toxicology laboratory tools and it is important to include these benzodiazepines in the confirmation tests.

Introduction

Benzodiazepines are one of the most commonly used drugs in Scotland. The most commonly prescribed benzodiazepine in Scotland is diazepam. In the financial year 2013–2014, diazepam was dispensed 891,005 times. The second most dispensed benzodiazepine was temazepam, followed by nitrazepam. These figures include repeat prescriptions as well as prescriptions written in hospital that were dispensed within the community but not prescriptions dispensed within hospitals (1). Phenazepam was the first uncontrolled benzodiazepine to emerge on the UK recreational market; police seized phenazepam in white powder form in Scotland in October 2008. From 2008 to 2011, phenazepam was detected in blue pills seized in the UK; these pills often had markings that suggested they were being sold as diazepam (2). Phenazepam was subsequently controlled in the UK in 2012 (3). Phenazepam has been a prescribed drug in Russia since the 1970s for neurological disorders, epilepsy and alcohol withdrawal (4).

Etizolam has been prescribed in Japan since 1983 for anxiety and used as a strong muscle relaxant. The drug was first reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) in December 2011 after four blue tablets were seized from an individual in the UK who had purchased them online (5). Etizolam has become increasingly popular recreationally in the UK and remains unscheduled. Etizolam abuse has been reported as a problem in Japan (6).

Pyrazolam emerged on the online recreational market in 2012. Unlike phenazepam and etizolam, it is not marketed by any pharmaceutical company anywhere in the world; therefore, it is considered as the first designer benzodiazepine produced for the legal market. Pyrazolam was first reported to the EMCDDA in August 2012 by Finnish Customs in Helsinki who seized 10 white tablets from an incoming item of mail in August 2011. A white tablet analyzed by TICTAC Communications in July 2012 is the first reported instance of pyrazolam in the UK (5). Pyrazolam's structure can be thought of as a combination of alprazolam and bromazepam (7).

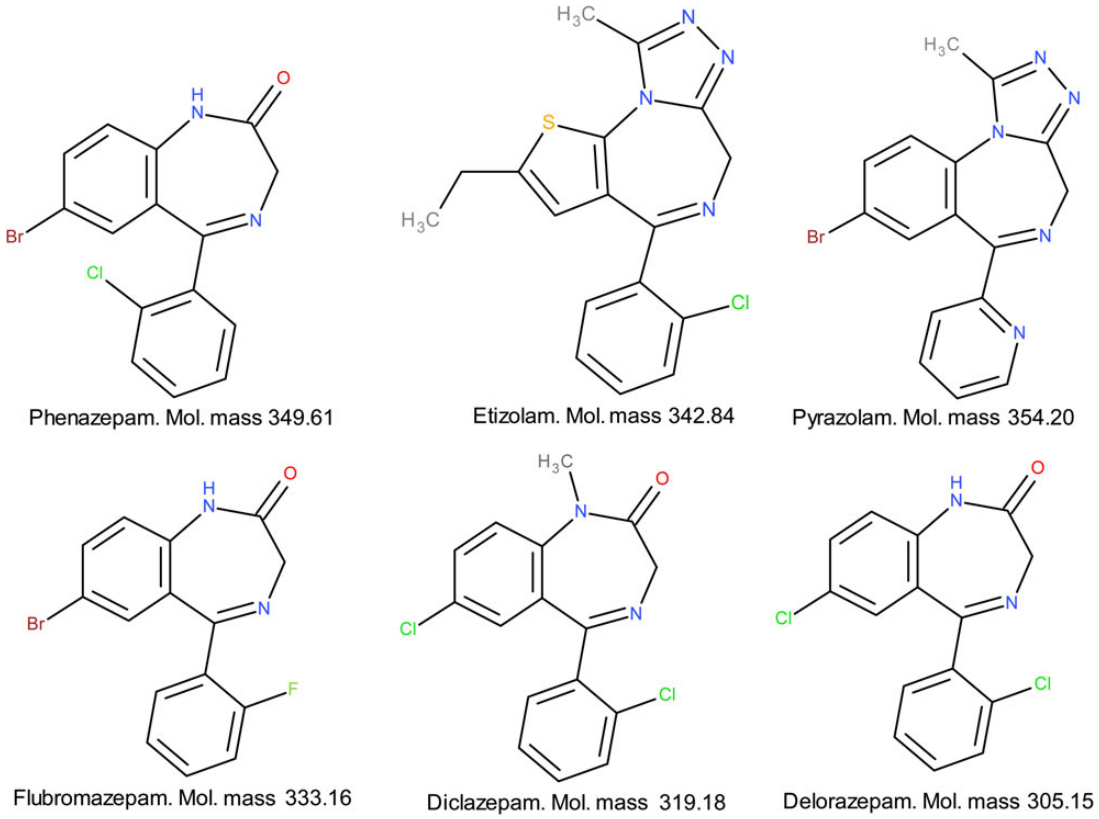

Figure 1. The chemical structure of phenazepam, etizolam, pyrazolam, flubromazepam, diclazepam and delorazepam.

The second designer benzodiazepine to appear on the legal market was flubromazepam in late 2012. Flubromazepam is structurally related to phenazepam; the chlorine atom in phenazepam is substituted for fluorine in flubromazepam (8). Germany was the first to report flubromazepam to the EMCDDA; Germany reported 10 capsules of flubromazepam collected in March 2013. In June 2013, the UK then reported a sample of 500 mg flubromazepam powder collected in April 2013 in Guernsey, which had been sent from the UK (5).

Diclazepam emerged as a research chemical in 2013. It was first reported to the ECMDDA by the Department of Forensic Toxicology, the University Medical Center Freiburg, Germany, which analyzed 20 diclazepam tablets. The UK reported a collection of diclazepam samples bought online to the ECMDDA in September 2013 (5). Diclazepam has the structure of diazepam with an additional chlorine atom (see Figure 1) (9). Delorazepam, lorazepam and lormetazepam are active metabolites of diclazepam. These metabolites are prescription drugs in the UK with the exception of delorazepam.

The purpose of this study was to evaluate whether the Immunalysis® Benzodiazepines ELISA kit (part number: 214-0480) could detect phenazepam, etizolam, pyrazolam, flubromazepam, diclazepam and its metabolite delorazepam. Their cross-reactivity was assessed by comparing absorbance of the drug with that of oxazepam, the reference standard.

Experimental

Reagents

Phenazepam, etizolam and oxazepam were purchased from Sigma-Aldrich, UK. Pyrazolam and diclazepam were purchased from Chiron AS, Trondheim, Norway. Flubromazepam and delorazepam were purchased from LGC Standards, Teddington, UK. The blank blood used was purchased from the Scottish National Blood Transfusion Service, Gartnaval Hospital, Glasgow, UK. The Immunalysis® Benzodiazepine ELISA kit and the PBS buffer (pH 7.0) were purchased from Alere Toxicology, Abingdon, UK. HiPerSolv Methanol was purchased from VWR International Ltd., Leicestershire, UK.

Sample preparation

Individual stock solutions containing 1 µg/mL of drugs were prepared in methanol. Oxazepam used for the cross-reactivity comparison was also prepared at 1 µg/mL in methanol. Blank blood and PBS buffer were fortified with oxazepam at concentrations of 0, 5, 10, 100 and 300 ng/mL, and these were used as calibrators. These concentrations were chosen to cover a suitable range. Individual aliquots of blank blood were fortified with phenazepam, etizolam, pyrazolam, flubromazepam, diclazepam and delorazepam at the same concentrations. All fortified blood, calibrators and controls were diluted 1 : 4 with PBS buffer.

Instrumentation

The Tecan Miniprep 75, Columbus plate washer and Sunrise plate reader (all manufactured by Tecan Group Ltd., Switzerland) were used for this study.

Immunoassay procedure

Every fortified blood sample was prepared in duplicate, and every sample was pipetted into two separate wells providing four results for each concentration. Ten microliters of calibrator, control or fortified blood were added into each appropriate well. The samples were added in the order of increasing concentration. The immunoassay was carried

Table I. Cross-Reactivity with PBS Buffer and Blank Blood Calibrators

Drug	Concentration (ng/mL)	Calibrators in PBS buffer, % cross-reactivity	SD ($n = 4$)	Calibrators in blank blood, % cross-reactivity	SD ($n = 4$)
Phenazepam	5	107	0.01	96	0.01
	10	110	0.02	109	0.03
	100	143	0.01	84	0.01
	300	93	0.02	69	0.02
Etizolam	5	111	0.03	100	0.03
	10	96	0.05	96	0.06
	100	84	0.01	126	0.01
	300	143	0.01	107	0.01
Pyrazolam	5	90	0.06	94	0.10
	10	75	0.04	88	0.13
	100	139	0.03	82	0.03
	300	111	0.02	81	0.01
Flubromazepam	5	98	0.08	91	0.01
	10	103	0.11	98	0.03
	100	82	0.02	75	0.02
	300	105	0.01	73	0.01
Diclazepam	5	106	0.08	85	0.04
	10	91	0.07	98	0.03
	100	122	0.04	72	0.04
	300	95	0.00	62	0.01
Delorazepam	5	89	0.09	87	0.08
	10	90	0.07	93	0.05
	100	72	0.03	70	0.03
	300	93	0.01	69	0.01

out according to the Immunalysis protocol (10). The absorbance was measured at 450 nm using the plate reader. The absorbance is inversely proportional to the concentration of the drug. Sensitivity, precision and cross-reactivity data were provided by Immunalysis.

Results and discussion

In forensic toxicology, it is important to keep up with emerging drugs of abuse and be aware of the drugs that give us a positive response at the screening step. It is crucial that laboratory confirmation methods are capable of matching the range of drugs that can be detected at the screening step in order for the screen to be a valuable test. This study found that these benzodiazepines do cross react with the Immunalysis® Benzodiazepine ELISA kit (Table I). When the calibrators were made up in PBS buffer, the cross-reactivity was 113, 109, 104, 97, 104 and 86% for phenazapam, etizolam, pyrazolam, flubromazepam, diclazepam and delorazepam, respectively. When the calibrators were prepared in blank blood, the cross-reactivity was 90, 107, 86, 84, 79 and 80% for phenazepam, etizolam, pyrazolam, flubromazepam, diclazepam and delorazepam, respectively. All results were considered acceptable with a percentage of covariation below 15. The cross-reactivity of diclazepam's other metabolites, lorazepam and lormetazepam, was not determined as these details are included in the manufacture's instruction manual. The cross-reactivity of lorazepam is stated as 90% at a concentration of 50 pg/well and 85% at a concentration of 100 pg/well. The cross-reactivity of lormetazepam is stated as 120% at a concentration of 500 pg/well.

Case study

This case study describes the death of a 20-year-old male who had been diagnosed with a psychotic disorder. He had a history of drug abuse, in particular legal drugs, which he appeared to be purchasing from websites. At the time of his death, he was prescribed lymecycline, fluoxetine, pregabalin and zuclopenthixol. The deceased was found face down in bed. There were various powders, tablets and 'legal high' packets found inside his house. The deceased's blood triggered a positive ELISA response using the Immunalysis® Benzodiazepine ELISA kit. The confirmation tests in this case were carried out by using the 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with the Agilent 6410 Triple Quad LC/MS System (Agilent Technologies). The etizolam and pyrazolam methods had a limit of detection of 0.0025 mg/L and a limit of quantitation of 0.005 mg/L. The sample was analyzed for 10 benzodiazepines including diazepam, desmethyldiazepam, oxazepam, temazepam, lorazepam, 7-aminoflunitrazepam, nitrazepam, chlorodiazepoxide, phenazepam and an etizolam screen. Etizolam was confirmed at a concentration of 0.01 mg/L. A test for pyrazolam was also carried out due to pyrazolam tablets being present during the study. Pyrazolam was found in the blood at a concentration of 0.07 mg/L. All of the other benzodiazepines mentioned were negative. This suggests that the Immunalysis® Benzodiazepine ELISA kit will identify etizolam and/or pyrazolam in postmortem blood in real cases.

Conclusion

This study has illustrated that all six drugs tested have good cross-reactivity with the Immunalysis® Benzodiazepine ELISA kit. The benzodiazepine drug group is vast and has the potential for many uncontrolled drugs to be added to the legal market. As the prevalence of these drugs is unknown, it is critical that confirmatory toxicological methods expand to include the newer emerging drugs in order to stay relevant. Including these new drugs in routine analysis will allow the prevalence of this drugs to be monitored.

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