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An atmospheric pressure chemical ionisation liquid chromatographic–tandem mass spectrometry method for the analysis of benzodiazepines in urine

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ABSTRACT

The objective of this work was to establish an analytical method for the analysis of 7 Benzodiazepines (diazepam, oxazepam, temazepam, nordiazepam, desalkylflurazepam, alprazolam and α-hydroxyalprazolam) in urine specimens taken from drivers suspected of driving under the influence of drugs. The specimen, calibrator and control preparation involved hydrolysis of conjugated benzodiazepines using β-glucuronidase in sodium acetate buffer, with incubation at 60 °C for 2 h. Specimens were then centrifuged, before being diluted 1 in 5 (total dilution 1 in 10), with 10% acetonitrile in water. Specimens were analysed using a Shimadzu Prominence UPLC coupled to an AB Sciex 4000 QTrap LC–MS-MS. The chromatographic column was a Shim-pack XR ODS 2.2 µm. 3.0 × 50 mm column and the mobile phase was a binary gradient system comprising of mobile phase A which was an ammonium formate/formic acid buffer dissolved in water and mobile phase B which was an ammonium formate/formic acid buffer dissolved in Acetonitrile. APCI was selected as the ionisation technique and the MS was operated in MRM mode, monitoring 2 transitions per analyte. The validation of the method is described. The method was found to be linear, accurate and precise (within day and between day) for diazepam, oxazepam, temazepam, nordiazepam, desalkylflurazepam, alprazolam and α-hydroxyalprazolam. The results of 480 cases are reviewed and show that alprazolam use was found in 35% of cases. Use of benzodiazepines resulting in oxazepam, nordiazepam or temazepam were found ca. 70% of cases analysed.

1. Introduction

It is illegal to drive while under the influence of an intoxicant in the Republic of Ireland [1]. An intoxicant is defined as alcohol and/or a drug or drugs. A study conducted in the Republic of Ireland in 2000/2001 showed that driving under the influence of drugs was a significant problem and that the most prevalent drugs found were cannabis and benzodiazepines [2]. Whilst benzodiazepines are legally prescribed drugs for the treatment of anxiety, insomnia and other conditions, they are also widely abused [3]. They are known to impair the skills required for driving, and studies have shown that benzodiazepine users are at a significantly increased risk of motor vehicle collisions [4,5]. Indeed, a French study looked at the association between the use of benzodiazepines and risk of road traffic accidents, found an increased risk of being responsible for a road traffic accident in drivers that were dispensed benzodiazepine hypnotics (Odds Ratio = 1.39) [6] and it follows that there is a higher risk when benzodiazepines are misused.

Drivers who are arrested under the Road Traffic Act, under suspicion of driving under the influence of an intoxicant are required to provide either a breath specimen for the purpose of alcohol analysis and/or a blood or a urine specimen for the purpose of alcohol and/or drug analysis [1]. Where a urine specimen is collected and drug analysis is required specimens of urine are tested for the presence of benzodiazepines using an Alere benzodiazepines ELISA (urine cut-off 200 ng/ml) and any presumptive positives require confirmation by LC–MS-MS.

Benzodiazepines undergo conjugation to their glucuronides during phase II metabolism and in order to analyse total benzodiazepine content, hydrolysis is required [7]. Hydrolysis can be achieved using treatment with an alkaline solution or using enzymatic hydrolysis [8,9]. Literature review shows that specimens have been prepared using liquid-liquid extraction [10–12] and solid phase extraction [13,14]. Analysis using GC–MS^n [15–17] and LC–MS^n [8,18–24] are common. Modern LC–MS-MS instrumentation is highly selective and allows the analysis of specimens which have been diluted and have not been

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subjected to a concentration step [9]. The method described here includes a simple 'one tube' hydrolysis step followed by dilute and shoot analysis.

Ionisation modes used for LC–MS include ESI (electrospray ionisation) and APCI (atmospheric pressure chemical ionisation), [25] and many instruments now facilitate easy switching between the two techniques. With ESI, ionisation occurs in the liquid phase and is appropriate for the determination of analytes with high molecular mass or high polarity. [26] ESI is probably the most frequently used ionisation mode in the field of analytical toxicology [27]. With APCI, ionisation occurs in the gas phase, and is reported to be suitable for the determination of analytes with moderate polarity and molecular mass. [25] Benzodiazepines have been analysed using both ESI [8,18,20,22,28] and APCI [23,24,29–31]. Some authors have noted improved sensitivity whilst using APCI [29,32], and others preferred it due to its lower susceptibility to matrix effects [21,23,24,33]. APCI was found to provide a significant improvement in signal-to-noise in comparison to ESI for this method.

Previous unpublished analyses by the MBRS show that the most common benzodiazepine compounds found in the urine of drivers arrested under the influence of drugs on Irish roads are diazepam, oxazepam, temazepam, nordiazepam, desalkylflurazepam, alprazolam and α-hydroxyalprazolam and so these were the target analytes for the method development presented here. The method described involves a simple dilution preparation which incorporates a hydrolysis step followed by analysis using HPLC-APCI-MS-MS operating in MRM mode.

With the application of the method described here, a breakdown of the benzodiazepines detected in Irish drivers can now be provided. Data from 480 cases indicate that the most prevalent benzodiazepines detected are Alprazolam and Diazepam.

2. Experimental

2.1. Chemicals and reagents

All standards including; diazepam, oxazepam, temazepam, nordiazepam, desalkylflurazepam, alprazolam, α −hydroxyalprazolam, α −hydroxyalprazolam D_5, oxazepam D_5, diazepam D_5 and oxazepam glucuronide were manufactured by Cerilliant (Austin, TX) and purchased from LGC Promochem (Teddington, UK). Medidrug external control 'Drug U-Confirmation Cut-off −25%' product code 40571, containing oxazepam and nordiazepam (75 ng/ml), purchased from LGC Promochem (Teddington, UK), UTAK external control 'Benzodiazepine Plus 100 ng/ml Urine Toxicology Control' product number 12100, containing 22 benzodiazepines, purchased from Grifols (Cambridge, U.K.). β-glucuronidase type L-II (from Limpets), ammonium formate and formic acid were purchased from Sigma (Arklow, Ireland). Sodium acetate trihydrate, concentrated acetic acid, acetonitrile (gradient grade), methanol (HPLC grade) and isopropyl alcohol were purchased from VWR (Dublin, Ireland). De-ionised water (15 MΩ) was generated on-site using an ELGA Purelab 75 system.

2.2. Instrumentation

The UPLC system was a Shimadzu Prominence UPLC system consisting of a Shimadzu DGU-20A3 degasser, 2 Shimadzu LC-20AD XR pumps, a Shimadzu SIL-20AC XR Auto sampler, a Shimadzu CBM-20A Controller and a Shimadzu CTO-20A Oven. This system was coupled to an ABSciex 4000 QTrap triple quadrupole linear ion trap, hybrid Mass Spectrometer. The CAD gas and curtain gas was N_2 which was generated by a Peak Scientific NitroGen N300DR (supplied by ABSciex, Ireland). The UPLC and MS were controlled using the Analyst 1.5 software. A Harvard Apparatus, 11 Plus syringe pump and Hamilton 1 ml Glass Syringe (p/n 81320/02) were used for compound optimisation and matrix effect studies. All pipetting was carried out using Gilson positive displacement pipettes. The incubator was a Memmert INB 200 (Supplied by Lab Unlimited, Ireland) and the centrifuge was Hettich Rotina 420 (Supplied by Lennox, Ireland).

2.3. Preparation of reagents

The 0.1 M sodium acetate buffer pH 4.0 containing 4500 units of β-glucuronidase type L-II was prepared by dissolving 0.68 g of sodium acetate trihydrate in 50mls of de-ionised water. The pH was adjusted to 4.0 pH units with acetic acid using a calibrated pH electrode. The appropriate amount of β-glucuronidase type L-II equivalent to 4500 units/0.5 ml [8] was weighed out and added to the buffer.

The 1 M ammonium formate solution was prepared by dissolving 15.75 g of ammonium formate in 250mls of deionised water.

Mobile Phase A was prepared by adding 4mls of 1 M ammonium formate solution and 4mls of Formic Acid to a 2L volumetric flask made up to the mark with de-ionised water. Mobile Phase B was prepared by adding 2mls of 1 M ammonium formate solution, 2mls of formic acid and 10mls of isopropyl alcohol to a 1 L volumetric flask made up to the mark with acetonitrile (Gradient Grade).

The UPLC rinse pump solution was an 80:20 mixture of de-ionised water and acetonitrile (HPLC Grade). The rinse port solution was a 1:1:1 ratio of acetonitrile (HPLC Grade), isopropyl alcohol and de-ionised water.

2.4. Preparation of calibrators, internal standards and controls

Working standards for the calibrators were prepared for diazepam, oxazepam, temazepam, nordiazepam, desalkylflurazepam, alprazolam and α −hydroxyalprazolam from the 1 mg/ml reference standards purchased. Composite calibrators containing these target analytes were prepared at 20, 50, 150, 275, 400 and 500 ng/ml. Working standard for the internal standards for hydroxyalprazolam D_5, oxazepam D_5 and diazepam D_5 were prepared from 1 mg/ml reference standards. Composite working standards for the controls containing the seven target analytes were prepared from the 1 mg/ml reference standards. Working standard for the hydrolysis control was prepared from the 100 μg/ml oxazepam glucuronide reference standard. UTAK and Medidrug external controls were prepared according to manufacturer's instructions.

2.5. Specimen preparation

Specimens were stored under refrigeration and were allowed to equilibrate to room temperature before sampling. 500 μl of specimen and 55 μl of Methanol (to equalise methanolic content with fortified samples) were added to a test tube. All specimens were prepared in duplicate, and following fortification with 55 μl relevant spiking solution, controls and calibrators were prepared in the same way as the specimens from this point. A volume of 20 μl of the 2500 ng/ml composite working internal standard was added to each tube. To this was added 500 μl of the buffered β-glucuronidase solution. The tubes were vortex mixed and then transferred to an incubator at 60 °C, for 2 h. After incubation the tubes were removed and allowed to come to room temperature. They were then centrifuged at 2500 rpm for 5 min. A 200 μl aliquot of supernatant was transferred from each tube to a HPLC vial and an 800 μl volume of 10% Acetonitrile in de-ionised water was added. The vials were capped, vortexed and placed in the autosampler for analysis.

2.6. LC–MS-MS procedure

The injection volume was 20 μl. The flow-rate was set at 0.8 ml/min. The column oven temperature was 40 °C. The binary gradient starts with 5% Mobile phase B and holds this up to 1 min, increasing to 10% Mobile Phase B at 1.5mins, 25% Mobile Phase B at 2.5 min, 30% Mobile Phase B at 6.5mins, 35% Mobile Phase B at 8.5mins, 90%