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Determination of benzodiazepines in ante-mortem and post-mortem whole blood by solid-supported liquid–liquid extraction and UPLC–MS/MS☆

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ABSTRACT

A solid-supported liquid–liquid extraction ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method was developed and validated for the determination of benzodiazepines commonly found in Norway, for use in cases with suspected driving impairment and autopsy cases by analysis of human whole blood samples. The following compounds were included: alprazolam, bromazepam, clonazepam, diazepam, flunitrazepam, lorazepam, midazolam, nitrazepam, nordiazepam (metabolite of diazepam), oxazepam and phenazepam. Aliquots of 500 μL whole blood were added 500 μL of borate buffer pH 11 and extracted by solid-supported liquid–liquid extraction on ChemElut® columns using three times 2.5 mL of methyl *tert*-butyl ether. Deuterated analogues were used as internal standards (IS) for all analytes, except for midazolam, phenazepam and bromazepam which had no commercially available deuterated analogues at the time the method was developed, and therefore used diazepam-d_5, flunitrazepam-d_7 and nitrazepam-d_5, respectively. The analytes were separated using UPLC with a 2.1 × 100 mm BEH C_{18}-column, 1.7 μm particle size, and quantified by MS/MS using multiple reaction monitoring (MRM) in positive mode. Two transitions were used for the analytes and one transition for the IS. The run time of the method was 8 min including equilibration time. The concentrations of the benzodiazepines in the method span a broad range varying from the lowest concentration of 0.005 μM for flunitrazepam to the highest of 20 μM for oxazepam. The calibration curves of extracted whole blood standards were fitted by second-order calibration curves weighted $1/x$, with R^2 values ranging from 0.9981 to 0.9998. The intermediate precision had a CV (%) ranging between 2 and 19%. Recoveries of the analytes were from 71 to 96%. The LLOQs for the analytes varied from 0.0006 to 0.075 μM and the LODs from 0.005 to 3.0 nM. Matrix effects were studied by post extraction addition and found to be between 95 and 104% when calculated against an internal standard. A comparison with two other LC–MS methods was performed during method validation. Good correlation was seen for all analytes. The method has been running on a routine basis for several years, and has proven to be very robust and reliable with good results for external quality samples. The method also meets the requirements of the legislative limits for driving under the influence of non-alcohol drugs to be introduced in the Norwegian legislative system from 2012.

1. Introduction

Benzodiazepines are amongst the most frequently prescribed psychoactive drugs world wide [1]. Due to their hypnotic, anxiolytic, anticonvulsant and muscle-relaxant properties they are used for the therapy of anxiety, convulsive attacks and sleeping disorders. The sedative and amnestic properties of some benzodiazepines are also considered useful in anaesthesia. In addition, the benzodiazepines have a rapid onset of action combined with low acute toxicity. Benzodiazepines are, however, also associated with abuse and some can be toxic at higher blood drug concentrations. Their use might lead to development of dependence, and the benzodiazepines are commonly used in combination with other substances of abuse [1]. Studies have indicated that benzodiazepines

Abbreviations: DUI, driving under the influence of drugs; UPLC–MS/MS, ultra-performance liquid chromatography–tandem mass spectrometry; LLE, liquid–liquid extraction; LC–MS/MS, liquid chromatography with tandem mass spectrometry; HPLC, high performance liquid chromatography; MS/MS, tandem mass spectrometry; R^2, the correlation coefficients; S/N, signal-to-noise ratio; LOD, limit of detection; LOQ, limit of quantification; ME, matrix effects; RSD, relative standard deviation; CV, coefficient of variation; NIPH, Norwegian Institute of Public Health.

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impair psychomotor, cognitive and driving performance, and especially in combination with alcohol and/or illicit drugs thus represent a risk factor in traffic safety [2–5]. Some benzodiazepines are also known to be used to facilitate sexual assault (date rape) [6,7].

In Norway benzodiazepines are frequently detected in blood samples from drivers apprehended under the suspicion of impaired driving [8,9]. In the 5-year period from 2000 to 2005 one or more benzodiazepines were found at frequencies varying between 38 and 57% of the total number of blood samples received for analysis at the Norwegian Institute of Public Health (NIPH) [9]. In a roadside study from 2005 to 2006 a sample material of 10,816 oral fluid samples provided by Norwegian motor vehicle drivers were analysed, of which a total of 1.4% were positive for benzodiazepines [10].

Due to their importance in forensic toxicological and clinical settings, there are numerous analytical procedures for the determination of benzodiazepines to be found in the literature. Gas chromatography coupled to mass spectrometry has for very many years been a method of choice in clinical and forensic toxicology. During the last 15 years, however, liquid chromatography mass spectrometry or tandem mass spectrometry has become a mature technique finding many applications in the same fields [11–13]. The use of liquid chromatography reduces the need for derivatization and is very useful for hydrophilic, thermolabile, and non-volatile substances. With the advance of columns with sub-2 μm particles and the instrumentation necessary to handle the large back-pressures that follows, i.e. ultra performance liquid chromatography (UPLC) or ultra high performance liquid chromatography (UHPLC), better separation and shorter run times are achieved as well.

Many analytical methods have been reported for the detection of benzodiazepines in various biological matrices by LC–MS or LC–MS/MS and recently by UPLC–MS(/MS) and the subject was recently extensively reviewed by Nakamura [14]. The aim of the present work is to describe a fully validated, rapid confirmation method for determination of benzodiazepines common on the Norwegian market for use in impairment cases as well as in forensic autopsy cases using solid-supported liquid–liquid extraction and UPLC–MS/MS on whole blood samples.

2. Experimental

2.1. Chemicals and reagents

The reference substances were purchased from the following manufacturers: clonazepam, flunitrazepam and nitrazepam from Alltech (Lexington, KY, USA), phenazepam from Chiron AS (Trondheim, Norway), bromazepam from Sigma–Aldrich (St. Louis, MO, USA), alprazolam, diazepam, lorazepam, midazolam, nordiazepam and oxazepam from Lipomed (Arlsheim, Switzerland). The IS alprazolam-d_5, clonazepam-d_4, diazepam-d_5, flunitrazepam-d_7, nitrazepam-d_5, nordiazepam-d_5, oxazepam-d_5 and lorazepam-d_4 were all purchased from Cerilliant Corp. (Round Rock, TX, USA).

The chemicals *di*-sodium tetra borate decahydrate (GR), sodium hydroxide (pellets, GR), ammonium acetate, acetic acid and methyl *tert*-butyl ether were provided by Merck KGaA (Darmstadt, Germany). Acetonitrile was obtained from Lab-Scan (Dublin, Ireland). Purified water was obtained with a Milli-Q system (Millipore, Billerica, MA, USA). The ChemElut™ 1 mL cartridges were obtained from Varian Inc. (Palo Alto, CA, USA).

2.2. Biological samples

For the preparation of controls and calibrators, whole blood (containing 2 g sodium fluoride, 6 mL heparin and 10 mL water per 450 mL blood) was obtained from the blood bank at Ullevål University Hospital (Oslo, Norway), and screened for drugs and alcohol before use by immunoassay and chromatographic methods. Confirmation analysis of benzodiazepines in whole blood samples at NIPH are predominantly done in impairment cases and forensic autopsy cases. The samples are then received in 4 mL BD Vacutainer® Plus Plastic Blood Collection Tubes (BD Vacutainer Systems, Frankling Lake, NJ, USA) containing 10 mg sodium fluoride and 8 mg potassium oxalate, and 25 mL Sterilin tubes (Sterilin, Caerphilly, UK) containing 200 mg potassium fluoride, respectively.

Collected samples are stored at 4 °C prior to processing. Aliquots of 500 μL are then transferred to separate 5 mL polypropylene tubes (Sarstedt AG, Rommelsdorf, Germany) which are stored at 4 °C until the time of analysis.

2.3. Standard solutions

For each compound two separate stock solutions were prepared in methanol, identified as calibration and quality control (QC), respectively. From the stock solutions aqueous work solutions were prepared containing all the benzodiazepines. Calibration and QC samples were prepared in batches adding aqueous calibration or control solution to drug free whole blood and dispensing in aliquots of 500 μL after thoroughly mixing. The aliquots were stored in 5 mL polypropylene tubes (Sarstedt AG) in a freezer at −20 °C for up to 12 months. The calibrators (n = 6–7) ranged from sub-therapeutic to high dose/toxic levels and the control samples (n = 4–5) were distributed to cover the calibration range. A mix of internal standards with concentrations ranging from 1.25 to 50 μM was prepared in water and stored at 4 °C until empty, typically 2–3 months.

2.4. Sample preparation

A 500 μL aliquot of whole blood was added 50 μL IS and 500 μL saturated borate buffer pH 11, and mixed on a multitube vortexer for 60 s. The mixture was transferred to a ChemElut™ cartridge and the analytes eluted with three aliquots of 2.5 mL methyl *tert*-butyl ether. The eluate was evaporated at 40 °C until dryness under nitrogen at a pressure of 5 psi using a Caliper TurboVap (Caliper Life Sciences, Hopkinton, MA, USA) and reconstituted in 100 μL acetonitrile:5 mM ammonium acetate buffer pH 5.0 (25:75, v/v) prior to injection into the UPLC–MS/MS-system.

2.5. UPLC conditions

A Waters Acquity UPLC module (Waters Corp., Milford, MA, USA) was used for separation. Gradient elution was performed on an Acquity UPLC BEH C18 (2.1 × 100 mm, 1.7 μm) column with an Acquity UPLC BEH C18 VanGuard Pre-Column (2.1 × 5 mm, 1.7 μm) in front, both from Waters (Wexford, Ireland). A two level five factor full factorial design experiment studying the factors pH, temperature, flow, ion strength of the buffer and the percentage of acetonitrile at gradient starting point was used to aid in method development. The retention time of each of the benzodiazepines and number of separated peaks as found by manual inspection were used as responses. The parameters were optimized to get the maximum number of resolved peaks achievable with a retention time of less than 10 min. A flow rate of 0.6 mL/min with acetonitrile (mobile phase A) and 5 mM ammonium acetate buffer pH 5.0 (mobile phase B) as solvents was used in a convex ramp, giving a slower gradient at the beginning and a steeper at the end compared to a linear profile. The gradient is shown in Table 1. The total cycle time of the method was 8 min. The column temperature was held at 65 °C and the injection volume was 5 μL using partial loop injection with a needle overfill flush. Weak wash and strong wash