K16 A Rapid Method for the Determination of Benzodiazepines in Postmortem Blood by HPLC-MS-MS

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Attendance at this presentation will enable the participant to learn about a new method for the qualitative determination of benzodiazepines in postmortem blood specimens by HPLC-MS-MS. This method is a comprehensive screen for these drugs and it is rapid, robust and reproducible over a wide range of concentrations.

The Forensic Toxicology Service offers a National screening and quantification toxicology service to Coroners and Forensic Pathologists, as well as to Police Forces. As a result, screening for benzodiazepines is required in a large number of postmortem specimens followed by quantification of those detected. Benzodiazepines remain the U.K.'s most commonly abused prescription drugs. Examples include diazepam (Valium), lorazepam (Ativan), nitrazepam (Mogadon - "moggies") and temazepam ("jellies," "egg"). In order to be able to offer a more rapid, reliable and robust method for their identification and quantification, a method for heir analysis by HPLC-MS-MS using a minimal amount of postmortem blood has been developed.

The analytes of interest are extracted from postmortem blood as follows: in a 2mL polypropylene tube 100IL of the blood specimen is added, 250IL of phosphate buffer (pH 7.0), 100IL of prazepam (1mg/L) as internal standard and 1mL methyl tert-butyl ether (MTBE). The contents are then mechanically mixed for 5 minutes and centrifuged for 1 minute at 12000 rpm. The top, organic, layer is then transferred to a clean 4.5mL polypropylene tube and evaporated to dryness using a Savant SpeedVac SC200 coupled to a Savant RT 4104 refrigerated condensation trap. The residue is reconstituted in 250IL of 80% methanol, vortex mixed for 20 seconds and transferred to a polypropylene auto-sampler vial. 10IL of the sample is then analyzed by HPLC-MS-MS. The total run time on the HPLC-MS-MS for each specimen is less than 4 minutes.

The analytical column used is a 15cm x 4.6mm (id) Supercosil LC18-DB (5Im particle size) ODS column maintained at 50ºC using a Perkin Elmer series 200 column oven. Isocratic solvent delivery is achieved using a Perkin Elmer series 200 pump set at 1 mL/min. Sample injection, 10IL, is performed by a Perkin Elmer series 200 auto-injector. The mobile phase consists of methanol/water (85:15, by volume) supplemented with ammonium acetate solution to achieve a final concentration of 2 mmol/L.

Detection is by tandem mass spectrometry (HPLC-MS-MS), using a Sciex API2000 triple quadrupole mass spectrometer (Applied Biosystems). A turbo ion spray (heated electrospray) source heated to 300°C is used to introduce the sample into the mass spectrometer. A postcolumn splitter (10:1) is installed just before the ion spray interface. The mass spectrometer is operated in positive ionization, multiple reaction mode (MRM, MS-MS), with the resolution set to unit resolution (±0.5m/z). High purity air is used as the nebulizer gas and high purity nitrogen as the collision gas.

The Applied Biosystems Sciex Analyst software is used to control the HPLC-MS-MS, record the output from the detector, integration of peak areas and calculation of peak area. In assays requiring quantification, the Analyst software is used to calculate the peak area ratios, produce the calibration line using $1/x^2$ weighed through zero regression and to calculate the concentration of each analyte.

A reference mixture of control substances made in drug-free human blood is extracted and run with all unknown samples as part of every assay. These reference concentrations were selected as they approximate the lower levels of the respective substance in blood following their therapeutic intake, as noted from the literature. The table below presents

compounds contained in the current reference mixture, their respective final concentrations, together with the target masses used for their identification.

 ** Presenting Author*

Drug Name	Concentration	Q1 m/z	Q3 m/z
Alprazolam	0.05	308.9	280.9
Chlordiazepoxide	0.50	299.9	227.1
Chlordiazepoxide Lactam	0.30	286.8	180.1
Citalopram	0.05	325.2	109.1
Clobazam	0.10	300.9	259.0
Desmethylclobazam	0.20	286.9	245.0
Desmethyldiazepam	0.20	271.0	165.1
Diazepam	0.10	284.9	153.9
Lormetazepam	0.05	345.0	289.2
Lorazepam	0.05	320.8	275.0
Loprazolam	0.05	465.2	111.3
Nitrazepam	0.05	281.9	236.0
Oxazepam	0.10	287.0	156.1
Prazepam (Internal Standard)	N/A	325.1	271.1
Temazepam	0.40	300.9	255.0

The method has performed satisfactorily for samples from cases involving either therapeutic use or overdose with these agent. Due to the high sensitivity of the instrument used, acute overdoses require reconstitution in a larger volume of solvent prior to injection. The limit of detection for the chosen analytes was better than 1/10th of the respective lower therapeutic level as analyzed in the reference mixture. The limits of detection and quantification can be improved by increasing the injection volume, from 10IL to 50 or even 100IL, if required. Following the general screen by the method described herein, the remaining extracts are analyzed by a different HPLC-MS-MS method for benzodiazepines with lower concentration ranges such as flunitrazepam.

The method described here is rapid (total run time on the HPLC-MSMS is less than 4 minutes for each specimen), reproducible and robust and can be applied in forensic toxicology laboratories for the screening of benzodiazepines, a family of sedative hypnotic drugs commonly used and abused worldwide.

Forensic Toxicology, Benzodiazepines, HPLC-MS-MS

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