Determination of midazolam in rabbit plasma by high performance liquid chromatography with diode array detection

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Abstract: Developing HPLC assays that avoid the use of dangerous solvents for drug extraction and require only small volumes of blood are important improvements to assay methodology. This paper describes a simple midazolam assay which uses solid phase extraction with strong cation exchange solid phase extraction cartridges instead of liquid-liquid extraction with organic solvents and commonly available equipment to measure midazolam concentrations in only 300µL of plasma. The small volume of sample required for this makes it useful for application in small animal and neonate clinical studies.

Keywords: HPLC; midazolam, solid phase extraction, small volume assay

Introduction
Midazolam is a short acting benzodiazepine used as premedication and sedation during procedural and intensive care settings. It is generally administered as an intravenous bolus or a short-term infusion. It is weakly basic (pKa=6), water soluble at pH4 but highly lipophilic at physiological pH [1].

Different methods for analysing midazolam in serum or plasma have been reported in the literature. These include gas chromatography methods requiring derivatisation prior to analysis, HPLC with UV/diode array detection (HPLC-UV/DAD) [2-13] and HPLC with mass spectrometry (HPLC-MS)[14].

Most methods employing HPLC-UV/DAD detection use liquid-liquid extraction procedures. Organic solvents such as diethyl ether [3, 4, 6, 10, 11], toluene [2], chloroform [12] or cyclohexane [8, 9] are used to extract midazolam from the sample matrix. The organic solvent is then evaporated under nitrogen to dryness or a back extraction step is used.

Few assay techniques for midazolam have reported the use of SPE cartridges for sample clean up. These have the advantage of avoiding the use of potentially dangerous organic solvents. Cartridges can be selected application to on the basis of individual drug characteristics to improve analyte retention. Methods previously reported using SPE cartridges for extraction of midazolam have used either C1 [1] or C18 [5] cartridges. Strong cation exchange cartridges present benefits of being able to target and concentrate analytes without evaporation. These have not previously been used for midazolam.

Methods employing HPLC-UV/DAD reported in the literature usually require at least 1mL of serum or plasma [1, 4-6, 8, 10-13]. To obtain 1mL of serum or plasma, a volume of about 2mL of blood is often required. In some clinical situations, such as neonatal use and where midazolam is being studied in small animals it is not possible to withdraw multiple samples of this size due to their small total blood volume. With the growing need for pharmacokinetic studies in children, particularly neonates, assays requiring a small amount of blood will become increasingly important. While HPLC/MS methodologies can use smaller blood volumes [14] access to this expensive equipment can be limited.

Here a new, simple, rapid and sensitive HPLC-UV method for analysis of midazolam using strong cation exchange cartridges is described. The method requires only 300µL of plasma and uses a solid phase extraction clean up procedure reducing the need for potentially dangerous organic solvents.

Experimental Section

Materials
Midazolam ampoules (1mg/mL) were obtained from Roche Products (Dee Why, NSW Australia). The internal standard, clonazepam, was obtained from PCCA (Houston, Texas). Drug free plasma was obtained from rabbits that had not received midazolam. Experimental procedures were approved by the Animal Care and Ethics Committee of the University of Newcastle (A-2009-113).

Methanol and Acetonitrile (HPLC grade) were obtained from VWR International (EC). Deionised water was generated with a Synergy Millipore water purification system with a 0.22µm Simpak filter (France). Formic acid
was obtained from Fluka (Sigma Aldrich, Australia). Glacial Acetic Acid, Sodium Acetate, ammonium hydroxide and orthophosphoric acid were obtained from (Ajax Finechem Pty Ltd, Australia).

**Preparation of Stock Solutions**

Working stock solutions of midazolam were prepared by diluting 1mg/mL midazolam in deionised water on each day of analysis. For each analytical run, calibration standards were prepared in the drug free rabbit plasma at final plasma concentrations of 1000, 500, 100 and 50ng/mL. Quality control samples were prepared separately with final plasma concentration of 1000ng/mL and 100ng/mL. The internal standard solution was prepared by weighing 10mg of clonazepam and dissolving in isopropanol (Ajax Finechem Pty Ltd, Australia). This solution was stored in the refrigerator at 4°C. An aliquot of this solution was taken and diluted 1 in 10 with deionized water for each analytical run.

**Sample Preparation**

Oasis MCX® SPE cartridges (1mL, 30mg) (Waters Australia) were conditioned with 1mL of methanol and 1mL of water. To 300μL of plasma sample was added 25μL of internal standard solution and 300μL of 4% orthophosphoric acid solution. The sample was vortexed for 10 seconds and transferred to the SPE cartridge. The sample was then aspirated slowly through the sorbent. Following washing of the cartridge with 1mL deionized water, 1mL of 2% formic acid in water and 1mL of methanol, the analytes were then eluted with 1mL of 5% ammonium hydroxide in methanol and collected in glass tubes. The eluents were evaporated under air at room temperature using a Techne Dri-Block® sample evaporator (Cambridge, United Kingdom). Prior to HPLC analysis, the residues were dissolved in 140μL of mobile phase and 40μL of sample injected into the HPLC system.

**Chromatography**

The chromatography system consisted of a Shimadzu SIL-20A HT auto-injector, Shimadzu LC-10AT VP pump and a Shimadzu SPD-M10A VP Diode Array UV detector. The UV wavelength was set at 254nm. The chromatographic separation of midazolam and clonazepam was accomplished using a Synergi® Polar-RP 4μm 150x4.6mm column (Phenomenex, Australia). Chromatographic data were collected and compiled by Shimadzu Class VP7.4 software. Mobile phase used was a 200:245:555 (v/v) mixture of acetonitrile:methanol:sodium acetate buffer. The buffer was adjusted to pH 3.4 after mixing with the organic phase. The mobile phase was pumped isocratically at 1mL/min.

**Linearity**

The ratio of the peak area of midazolam to the peak area of clonazepam was plotted against the midazolam concentration to generate a calibration curve for each run. Calibration curves were generated by least-squares regression.

**Accuracy and precision**

Accuracy and precision were evaluated by determining midazolam at two different concentrations of QC samples in 4 replicates on 4 different days. Analysis of Variance was performed on the data and used to determine the between and within day coefficient of variation. Accuracy was defined as the percentage difference between the mean observed concentration and the nominal concentration.

**Recovery**

The recovery of midazolam from plasma was determined using 4 samples at two concentrations (1000ng/mL and 100ng/mL). The plasma samples were extracted and the peak areas for midazolam were compared to the peak area of a direct injection of midazolam.

**Stability**

The stability of midazolam in plasma was determined by preparing plasma samples containing 1000ng/mL and 100ng/mL of midazolam. Samples were assayed on day of preparation and then stored at -4°C for 14 days. The samples were then re-assayed.

**Clinical Application**

The applicability of this assay for measuring a wide range of midazolam concentrations in plasma was demonstrated by analyzing plasma samples drawn from adult female New Zealand White Rabbits before, during and after 0.78 mg/kg (1mg/mL) bolus injection followed immediately by IV infusion of midazolam at 0.011 mg/kg/min for a known period of time (approximately 110 minutes). Blood samples (2mL) were collected from an ear artery catheter at known periods and placed into heparin sodium tubes (Vacuette® 2mL LH Lithium Heparin tubes). The samples were stored in a container of ice in the refrigerator till the completion of the experiment then centrifuged at 3000 rpm for 15mims in a single batch. Plasma samples were collected from the tubes then stored at -80°C until assayed.
Results and Discussion

The retention times for midazolam and clonazepam (internal standard) were 16.25min and 19.5min respectively (Figure 1). No interfering peaks were observed at the retention time of midazolam or the internal standard. The lower limit of quantitation observed for this assay was 10ng/mL when an aliquot of 100μL of reconstituted sample was injected.

Calibration curve for midazolam was linear over the range of 50 to 2000ng/mL with a correlation coefficient (R²) of 0.9998. The between-day assay coefficient of variation was 2.7% at 100ng/mL and 4.5% at 1000ng/mL. The within-day assay coefficient of variation was 3.3% at 100ng/mL and 4.4% at 1000ng/mL. The assay was demonstrated to be accurate with observed values being within +/- 5 % of the nominal concentration. The mean recovery of midazolam was observed to be 88.5% +/- 3.8% at 1000ng/mL and 90% +/- 1.2% at 100ng/mL. Stability study demonstrated that plasma midazolam samples are stable for at least 14 days when stored at 4°C. Values obtained were within 5% of the original concentration.

When developing the assay it was noted that the retention time of midazolam and clonazepam was highly dependent on the pH of the mobile phase. Greater separation and faster run times were achieved by adjusting the pH (Figure 2). Midazolam direct injection were trialled at final pH 3.2, 3.4 and 3.8 and showed that faster run times for the cation exchange SPE column for sample clean up. Previous methods have used cartridges with different packing material. The samples can be quickly prepared and the method has the ability to determine levels as low as 10ng/mL. While HPLC-MS methods are able to achieve lower limit of quantitation, many researchers do not have access to this expensive equipment. This method offers another approach that may be useful when studying the pharmacokinetics of midazolam in this animal model (Figure 4).

Conclusions

This method offers a number of advantages over previously published assays. The assay requires only a small volume of plasma (300μL) compared to the 1mL often required with other techniques. It also avoids the use of organic solvents such as diethyl ether, chloroform and toluene. The extraction method is unique in that it uses a solid-phase extraction column for sample clean up. Previous methods have used cartridges with different packing material. The samples can be quickly prepared and the method has the ability to determine levels as low as 10ng/mL. While HPLC-MS methods are able to achieve lower limit of quantitation, many researchers do not have access to this expensive equipment. This method offers another approach that may be useful when studying the pharmacokinetics of midazolam- especially in small animal models or neonates.

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References

Figure 1: Chromatogram of plasma sample containing 50ng/mL midazolam

Figure 2: Effect of pH on midazolam retention time
Figure 3: Chromatograms (a) Typical chromatogram of drug free plasma (NZ White Rabbit 3.27kg); (b) Actual sample (453.1ng/mL) obtained from NZ White Rabbit receiving a 0.78mg/kg bolus midazolam injection immediately followed by an IV midazolam infusion at 0.011mg/kg/min.

Figure 4: Concentration over time in rabbit plasma- Rabbit (3.27kg) was given a 0.78mg/kg i.v. bolus of midazolam at 0 mins followed immediately by an i.v. infusion of 0.011mg/kg/min. Infusion was stopped at 110 mins. Ordinate = measured plasma levels of midazolam.