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Development and validation of a sensitive assay for analysis of midazolam, free and conjugated 1-hydroxymidazolam and 4-hydroxymidazolam in pediatric plasma: Application to Pediatric Pharmacokinetic Study☆

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ABSTRACT

Pharmacokinetic, pharmacodynamic and pharmacogenomic studies of midazolam are currently being performed in critically ill children to find suitable dose regimens. Sensitive assays using small volumes of plasma are necessary to determine the concentrations of midazolam and its respective metabolites in pediatric studies. Midazolam is metabolized to hydroxylated midazolam isomers, which are present as free as well as the corresponding glucuronide conjugates. A high-performance liquid chromatographic method with tandem mass spectrometry has been developed and validated for the quantification of midazolam, and free and total 1-hydroxymidazolam and 4-hydroxymidazolam metabolites in small volumes of plasma. Cleanup consisted of 96-well μ-elution solid phase extraction (SPE). The analytes were separated by gradient elution using a C_{18} analytical column with a total run time of 5 min. Multiple reaction monitoring was employed using precursor to product ion transitions of *m/z* 326.2 → 291.3 for midazolam, *m/z* 342.1 → 203.0 for 1-hydroxymidazolam, *m/z* 342.1 → 325.1 for 4-hydroxymidazolam and *m/z* 330.2 → 295.3 for $^{2}H_{4}$-midazolam (internal standard). Since authentic hydroxymidazolamglucuronide standards are not available, samples were hydrolyzed with β-glucuronidase under optimized conditions. Assay conditions were modified and optimized to provide appropriate recovery and stability because 4-hydroxymidazolam was very acid sensitive. Standard curves were linear from 0.5 to 1000 ng/mL for all three analytes. Intra- and inter day accuracy and precision for quality control samples (2, 20, 200 and 800 ng/mL) were within 85–115% and 15% (coefficient of variation), respectively. Stability in plasma and extracts were sufficient under assay conditions. Plasma samples were processed and analyzed for midazolam, and free 1-hydroxymidazolam and 4-hydroxymidazolam metabolites. Plasma samples that were hydrolyzed with β-glucuronidase were processed and analyzed for midazolam, and total 1-hydroxymidazolam and 4-hydroxymidazolam metabolites under the same assay conditions. The difference in concentration between the total and free hydroxymidazolam metabolites provided an estimate of conjugated hydroxymidazolam metabolites. The combination of 96-well μ-elution SPE and LC–MS/MS allows reliable quantification of midazolam and its metabolites in small volumes of plasma for pediatric patients. This assay is currently being successfully utilized for analysis of samples from ongoing clinical trials.

1. Introduction

Midazolam is a short acting benzodiazepine that is routinely used in the treatment of critically ill children. There is a growing need to understand the pharmacokinetics, pharmacodynamics and pharmacogenomics of midazolam disposition in critically ill children [1–3]. For children who are mechanically ventilated, there is a need to quantitatively define the heritable and non-heritable factors that underlie the variability in midazolam and morphine exposure and response. The Pharmacologic Impact on Sedation Assessments (PISA, NCT01105663) study, which was recently conducted, examined heritable polymorphisms on drug exposure, metabolite formation and pharmacodynamic response in critically ill children. Hypothermia's Impact on Pharmacology (HIP, NCT01560338) study is being conducted to estimate the impact of hypothermia on the variability in midazolam pharmacokinetics in children after cardiac arrest and to estimate the impact of

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genetic factors on the variability in midazolam pharmacokinetics. The results will be valuable to optimize the prospective treatment of critically ill children. Hence, there is a need to develop a sensitive bioanalytical method for the quantification of midazolam, morphine and their metabolites in pediatric plasma samples.

Midazolam is metabolized by cytochrome P450 enzymes 3A4 and 3A5 [4,5] to 1-hydroxymidazolam and 4-hydroxymidazolam. Of these, 1-hydroxymidazolm is the major pharmacologically active metabolite and 4-hydroxymidazolam is the minor metabolite. Each hydroxylated metabolite is further metabolized by UGT to form the corresponding glucuronide conjugate. There are several LC–MS/MS methods reported for the analysis of midazolam and 1-hydroxymidazolam in human plasma and other biological samples [6–26]. However, there are few LC–MS/MS methods reported for the analysis of 4-hydroxymidazolam, which is an acid sensitive metabolite [8,9,16,23]. Three of the reported methods [8,16,23] require a larger volume of plasma samples (0.5–1.0 mL), which is not feasible in pediatric studies where blood draws are limited to smaller volume to minimize risk. A recent study utilized 1-hydroxymidazolam glucuronide that was isolated from human urine as a standard for quantitation [6]. The goal of our study was to develop a sensitive assay for the measurements of midazolam, and free and glucuronide conjugated 1-hydroxymidazolam and 4-hydorxymidazolam in a small volume of pediatric plasma samples. However, authentic analytical standards of 1-hydroxymidazolam and 4-hydroxymidazolam glucuronides were not readily available. Hydrolysis with β-glucuronidase has previously been shown to provide quantitative hydrolysis of glucuronide metabolites [27]. Therefore, we employed β-glucuronidase hydrolysis to measure total 1-hydroxymidazolam and 4-hydroxymidazolam in plasma samples, obviating the need for the glucuronide standards. By analyzing plasma samples with and without hydrolysis utilizing our validated LC–MS/MS method, we were able to quantitate the levels of free and total 1-hydroxymidazolam and 4-hydroxymidazolam metabolites in small volumes for clinical samples. The respective difference between the total hydroxylated midazolam metabolites after hydrolysis and free hydroxylated midazolam metabolites concentration before hydrolysis provided a measure of 1-hydroxymidazolam and 4-hydroxymidazolam glucuronides in the plasma samples.

2. Materials and methods

2.1. Chemicals and reagents

Midazolam, 1-hydroxymidazolam, and $^{2}H_{4}$-midazolam (internal standard) were purchased from Cerilliant (Round Rock, TX, USA), and 4-hydroxymidazolam (Fig. 1), reagent-grade formic acid (~98%), and β-glucuronidase from Helix promatia were purchased from Sigma-Aldrich (St. Louis, MO, USA). The different individual (either male or female) lots of drug-free (blank) human plasma prepared with citrate phosphate dextrose (CPD) as an anti-coagulant were obtained from the blood bank at The Children's Hospital of Philadelphia. Six different lots of drug-free (blank) human plasma prepared with lithium heparin as an anti-coagulant were obtained from BioreclamationIVT (Baltimore MD). HPLC grade methanol and acetonitrile were purchased from Fisher-Scientific (Pittsburgh, PA, USA). Deionized water from Barnstead Nanopure™ water purifying system (Thermo Fisher Scientific, Marietta, OH, USA) was used for all experiments.

2.2. LC conditions

The Shimadzu HPLC system consisted of two LC-20AD delivery pumps, a DGU-20A5 Shimadzu vacuum degasser, a SIL-20AC Shimadzu autosampler and a CBM-20A system controller (Shimadzu Scientific Instruments; Columbia, MD, USA). HPLC separations were performed on a Kinetex C_{18} analytical column (2.6 µm 100 A, 2 × 50 mm, Phenomenex Torrance, CA). For chromatographic separation,

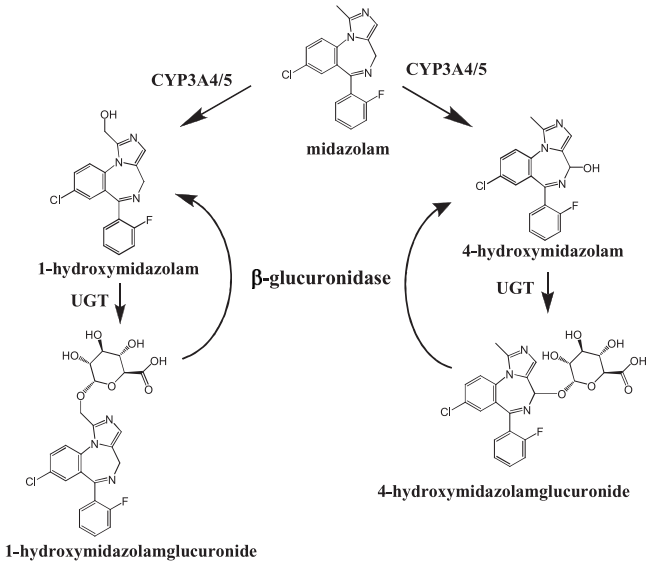

Fig. 1. Major metabolic pathways of midazolam.

acetonitrile/water (10:90, v/v) with 0.1% acetic acid was used as mobile phase A and acetonitrile with 0.1% acetic acid was used as mobile phase B. The initial mobile phase condition was 95% mobile phase A and 5% mobile phase B. The linear gradient was as follows: 0.00–0.51 min mobile phase A 83%, mobile phase B 17% with divert valve off; 0.51–2.00 min mobile phase A 83%, mobile phase B 17%; 2.00–3.00 min mobile phase A 20%, mobile phase B 80%; 3.00–3.50 min mobile phase A 95%, mobile phase B 6%; and initial gradient maintained until 5 min. The flow rate was 0.30 mL/min and 2 µL of the sample was injected for each analysis. The column and autosampler were maintained at 40 °C and 10 °C, respectively. The LC flow was diverted to the waste for the first 1.5 min using an electronic valve actuator with a Rheodyne selector valve when the data acquisition was not taking place.

2.3. MS conditions

An AB Sciex 4000 triple quadrupole mass spectrometer equipped with Turbo Ionspray was used for sample analysis. Software for controlling this equipment, acquiring and processing data was Analyst (Version 1.6.2; AB Sciex; Framingham, MA). The positive ionization mode for MS/MS analysis was utilized. Nitrogen was used as the nebulizer, auxillary, collision and curtain gases. Analytes were detected by tandem mass spectrometry using multiple reaction monitoring (MRM) with a dwell time of 100 ms. To determine the mass of the precursor and product ions, a solution of 5 µg/mL of midazolam, 1-hydroxymidazolam, 4-hydroxymidazolam, or internal standard $^{2}H_{4}$-midazolam in mobile phase (acetonitrile: water (55/45, v/v) with 0.1% acetic acid) was infused directly into the ion sources with a Harvard Apparatus syringe pump at a flow rate of 10 µL/min. The following precursor-to-fragment transitions were monitored for quantitation: *m/z* 326.2 → 291.3 for midazolam, *m/z* 342.1 → 203.0 for 1-hydroxymidazolam, *m/z* 342.1 → 325.1 for 4-hydroxymidazolam and *m/z* 330.2 → 295.3 for $^{2}H_{4}$-midazolam (internal standard). To further confirm the identity of 1-hydroxymidazolam and 4-hydroxymidazolam, a precursor-to-fragment transition of 342.1 → 297.0 was utilized.

The conditions for ionization of midazolam, 1-hydroxymidazolam, 4-hydroxymidazolam, and $^{2}H_{4}$-midazolam were optimized using individual standard solutions, each at 500 ng/mL in mobile phase (acetonitrile: water (55/45, v/v) with 0.1% acetic acid) at 10 µL/min. Midazolam, 1-hydroxymidazolam, 4-hydroxymidazolam, and $^{2}H_{4}$-midazolam were infused by a syringe pump alone or through a Tee device at a flow rate of 10 µL/min into the stream of mobile phase