8-4-2021

UPLC-MS/MS Analysis of the Michaelis-Menten Kinetics of CYP3A-mediated Midazolam 1'- and 4-hydroxylation in Rat Brain Microsomes

Devaraj Venkatapura Chandrashekar
Chapman University

Barent Dubois
Chapman University

Reza Mehvar
Chapman University, mehvar@chapman.edu

Recommended Citation

Chandrashekar DV, DuBois B, Mehvar R. UPLC-MS/MS analysis of the Michaelis-Menten kinetics of CYP3A-mediated midazolam 1'- and 4-hydroxylation in rat brain microsomes. *J. Chromatogr. B.* 2021;1180:122892. https://doi.org/10.1016/j.jchromb.2021.122892

UPLC-MS/MS Analysis of the Michaelis-Menten Kinetics of CYP3A-mediated Midazolam 1'- and 4-hydroxylation in Rat Brain Microsomes

UPLC-MS/MS analysis of the Michaelis-Menten kinetics of CYP3A-mediated midazolam 1'- and 4-hydroxylation in rat brain microsomes

Devaraj Venkatapura Chandrashekar, Barent DuBois[1], and Reza Mehvar*

Department of Biomedical and Pharmaceutical Sciences, School of Pharmacy, Chapman University, Irvine, USA

* Corresponding author at: Department of Biomedical and Pharmaceutical Sciences, Rinker Health Science Campus, Chapman University, 9401 Jeronimo Road, Irvine, CA 92168, USA. Tel.: +1 714 516 5490; fax: +1 714 516 5481.; E-mail address: mehvar@chapman.edu.

[1] Current Address: Novo Nordisk Research Center Indianapolis, Indianapolis, Indiana 46241, United States.

ABSTRACT

Midazolam (MDZ) is a short-acting benzodiazepine with rapid onset of action, which is metabolized by CYP3A isoenzymes to two hydroxylated metabolites, 1'-hydroxymidazolam and 4-hydroxymidazolam. The drug is also commonly used as a marker of CYP3A activity in the liver microsomes. However, the kinetics of CYP3A-mediated hydroxylation of MDZ in the brain, which contains much lower CYP content than the liver, have not been reported. In this study, UPLC-MS/MS and metabolic incubation methods were developed and validated for simultaneous measurement of low concentrations of both hydroxylated metabolites of MDZ in brain microsomes. Different concentrations of MDZ (1-500 μM) were incubated with rat brain microsomes (6.25 μg) and NADPH over a period of 10 min. After precipitation of the microsomal proteins with acetonitrile, which contained individual isotope-labeled internal standards for each metabolite, the analytes were separated on a C_{18} UPLC column and detected by a tandem mass spectrometer. Accurate quantitation of MDZ metabolism in the brain microsomes presented several challenges unique to this tissue, which were resolved. The optimized method showed validation results in accordance with the FDA acceptance criteria, with a linearity ranging from 1 to 100 nM and a lower limit of quantitation of 0.4 pg on the column for each of the two metabolites. The method was successfully used to determine the Michaelis-Menten (MM) kinetics of MDZ 1'- and 4-hydroxylase activities in rat brain microsomes ($n = 5$) for the first time. The 4-hydroxylated metabolite had 2.4 fold higher maximum velocity ($p < 0.01$) and 1.9 fold higher ($p < 0.05$) MM constant values than the 1'-hydroxylated metabolite. However, intrinsic clearance values of the two metabolites were similar. The optimized analytical and metabolic incubation methods reported here may be used to study the effects of various pathophysiological and pharmacological factors on the CYP3A-mediated metabolism of MDZ in the brain.

Keywords

UPLC-MS/MS

CYP3A

Midazolam hydroxylation

Brain

Microsomes

Michaelis-Menten kinetics

1. Introduction

Cytochrome P450 (CYP) enzymes are a superfamily of enzymes that are responsible for the metabolism of most drugs and endogenous compounds. Although the main site of expression of CYP enzymes is in the liver, they are also found in extrahepatic tissues, such as the intestines, kidneys, and brain [1, 2]. Among extrahepatic tissues, the brain has been shown to express a number of CYP enzymes, which contribute to the local metabolism of drugs and endogenous compounds [2-5]. However, the content of total brain CYP is 0.5%–2% of that in the liver [5], resulting in much lower activities in the brain. Additionally, brain CYP enzymes are more prone to degradation and loss of activity during the preparation of microsomes for in vitro assays [6]. Therefore in vitro characterization of brain CYP enzymes are more difficult than that of the liver enzymes, requiring the use of more sophisticated and sensitive methods.

Cytochrome P450 3A (CYP3A) is one of the most important subfamilies of CYP enzymes involved in the metabolism of a majority of clinically-used drugs and various endogenous substances in humans [7, 8]. Among CYP enzymes, CYP3A isoenzymes are the most abundant CYP in the liver hepatocytes [7] and intestinal epithelial cells [9]. Therefore, they act as a major determinant of the oral bioavailability and systemic clearance of drugs that are metabolized by CYP3A. In addition to the liver and intestine, CYP3A enzymes are also expressed in different areas of the brain at the mRNA, protein, and functional level [10, 11]. Additionally, metabolism of CYP3A substrates, like testosterone, erythromycin, and alprazolam, by rat and human brain microsomes has been reported [11-14]. Despite their lower expression in the brain, CYP3A isoenzymes may significantly contribute to the local metabolism and physiologic, pharmacologic, and toxicologic actions of many centrally-acting drugs and endogenous compounds [2-4, 14].

Therefore, it is necessary to characterize CYP3A enzymatic activity in the brain to understand the role of these enzymes in the pharmacotherapy of central nervous system diseases.

Midazolam (MDZ) is a short-acting benzodiazepine with rapid onset of action, which is used in the treatment of epilepsy and as a sedative before surgery or medical procedures. The drug is a specific substrate for CYP3A and is commonly used as a marker of CYP3A activity in rat and human liver microsomes [15-17]. In both humans and rats, MDZ is converted to two hydroxylated metabolites, 1'-hydroxymidazolam (1'-OH-MDZ) and 4-hydroxymidazolam (4-OH-MDZ), by CYP3A isoenzymes (Fig. 1). Although both human CYP3A4 and CYP3A5 isoenzymes produce both metabolites, the two enzymes exhibit different regioselectivity [15]. Despite its wide use as a centrally-acting drug and a marker of CYP3A activity in the liver, metabolism of MDZ to 1'-hydroxy and 4-hydroxy metabolites in the brain tissue has not yet been characterized. This may be due to the relatively low enzymatic activity of CYP3A in the brain and lack of appropriate metabolic incubation and analytical methods, specifically developed for the characterization of the CYP3A-mediated metabolism of MDZ in the brain tissue.

Several analytical methods like HPLC [18], GC-MS [19, 20], and LC-MS/MS [21-24] have been reported for quantifying MDZ and/or one or two of its hydroxylated metabolites in plasma samples. Furthermore, some LC-MS/MS methods have been used to quantify MDZ metabolites in the liver microsomes [24, 25]. In the present communication, we report the development of a sensitive and reproducible UPLC-MS/MS assay that is specifically validated for the simultaneous measurement of the low concentrations of both hydroxylated metabolites of MDZ in the brain microsomes. Additionally, we report on the optimization of enzymatic incubation conditions to evaluate the Michaelis-Menten (MM) kinetics of the CYP3A-mediated metabolism of MDZ in the rat brain microsomes. The advantages of our methods are their high

analytical sensitivity (1 nM), low incubation volume (25 µL), use of stable isotope-labeled internal standards for the individual hydroxylated metabolites, relatively low microsomal protein amounts (6.25 µg), and a short incubation time (10 min).

2. Materials and methods

2.1. Chemicals

Midazolam (MDZ) base (MDZ-Base) and its HCl salt (MDZ-HCl) were purchased from the United States Pharmacopeial Convention (Rockville, MD, USA). The 1'-hydroxy (1'-OH-MDZ) and 4-hydroxy (4-OH-MDZ) metabolites of midazolam were procured from Cerilliant Corporation (Round Rock, TX, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. The stable isotopes 1'-hydroxymidazolam-d_5 (1'-OH-MDZ-d_5) and 4-hydroxymidazolam-d_5 methanoate (4-OH-MDZ-d_5) were purchased from Toronto Research Chemicals (North York, ON, Canada). Acetonitrile, methanol, and water of LC-MS grade were procured from Fisher Scientific (Hampton, NH, USA). All other reagents and chemicals were of high purity and purchased from commercially available sources.

2.2. Instrumentation and chromatographic conditions

The UPLC system (Bruker Scientific LLC; Billerica, MA, USA) was equipped with a binary pump, integrated column oven, degasser, and auto-sampler (CTC-PAL). Separation of the analytes was achieved using a Kinetex 1.7 µm C_{18} (100 x 2.1 mm, 100 A) column (Phenomenex Inc; Torrance, CA, USA), connected to a Phenomenex C_{18} Security Guard ULTRA (2.1 mm) pre-column, which were maintained at 35°C. Two different mobile phases and gradient systems were used during the method development process. The initial mobile phase/gradient condition (initial method) consisted of a mobile phase with 5 mM ammonium formate with 0.05% formic acid in water (A) and acetonitrile: methanol (95:5) with 0.05 % formic acid (B). This mobile phase was

delivered at a flow rate of 0.3 mL/min using a gradient system consisting of 0–1.2 min, 20% B; 1.2–5.0 min, linear gradient 20–75% B; 5.0–6.5 min, 75% B; and 6.5–9.5 min, 20% B. The final mobile phase/gradient condition (final method) consisted of a mobile phase with 5 mM ammonium formate in water (A) and acetonitrile (B). This mobile phase was delivered at a flow rate of 0.25 mL/min using a gradient system consisting of 0–1.2 min, 30% B; 1.2–5.0 min, linear gradient 30–70% B; 5.0–6.0 min, 70% B; 6.0 min, 30% B; 6.0–7.5 min, 30% B.

The detection was performed using an EVOQ Elite triple quadrupole mass spectrometer (Bruker Scientific LLC; Billerica, MA, USA) equipped with an ion source interface. Ionization was achieved by electrospray in positive mode, and the mass spectrometer was operated in multiple-reaction monitoring (MRM) mode with m/z transitions of 342 to 202.9 for 1'-OH-MDZ, 342 to 233.9 for 4-OH-MDZ, 346.9 to 207.9 for 1'-OH-MDZ-d_5, and 346.9 to 234.9 for 4-OH-MDZ-d_5. The heated probe gas and nebulizer gas flow rates were set at 40 and 50 psi, respectively. The probe temperature was set at 400°C, and the ion spray voltage was 3000 V. The collision energy for the individual analytes was optimized accordingly. Data acquisition and processing were controlled by the Bruker MSWS-8 software.

2.3. Preparation of stock and working solutions

The primary stock solution of 4-OH-MDZ (1 mg/mL) was prepared in methanol, while for 1'-OH-MDZ, the commercially-available stock (100 µg/mL) in methanol was used. For the internal standards, the stock solution of 4-OH-MDZ-d_5 (2 mg/mL) was prepared in DMSO, while the commercially-available stock solution in methanol (100 µg/mL) was used for 1-OH-MDZ-d_5. The primary stock solutions of 1'-OH-MDZ and 4-OH-MDZ were further diluted with 100 mM Tris-HCl buffer (pH 7.4) to make working solutions of 10, 20, 50, 100, 250, 500, 750, and 1000 nM for the preparation of calibration curves and 10, 30, 300, and 800 nM for quality control (QC) samples.

The primary stock solutions of the internal standards were further diluted in acetonitrile to prepare a 10 nM solution.

2.4. Preparation of calibration curves

For calibration standards, the working solutions of the two metabolites were added to Tris-HCl buffer (pH 7.4), containing rat brain microsomes (0.25 mg/mL) and $MgCl_2$ (20 mM) in a final volume of 25 µL to make final concentrations of 0 (blank), 1, 2, 5, 10, 25, 50, 75, and 100 nM for each of the two metabolites. The microsomal proteins were then precipitated by the addition of 75 µL of acetonitrile, containing 10 nM of both 1-OH-MDZ-d_5 and 4-OH-MDZ-d_5 as internal standards. After vortex-mixing, the samples were centrifuged at 13,400 rpm for 5 minutes (4°C), and a 5 µL aliquot was subjected to the UPLC-MS/MS analysis. Calibration curves for each of the two MDZ metabolites were constructed by plotting the peak area ratios of each analyte: internal standard against the nominal concentrations of the metabolite in the samples using a weighting factor of 1/x, where x is the added concentration.

2.5. Assessment of metabolite impurities in MDZ substrate from different sources

Solutions (500 µM) of MDZ-Base and MDZ-HCl were prepared in incubation buffers (*n* = 3) and analyzed for their content of 1'-OH-MDZ and 4-OH-MDZ impurities. Additionally, to assess the potential effects of impurities on the application of the assay, triplicate samples of MDZ-Base or MDZ-HCl (500 µM) were incubated with brain microsomes (see section 2.9 for details), and the concentrations of the generated metabolites were quantitated immediately (zero) and at 10 min after the incubation.

2.6. Substrate effect on the analyte signal

The substrate interference study was performed to evaluate if the presence of high concentrations of the substrate (MDZ) would influence the detector response to 1'-OH-MDZ, 4-

OH-MDZ, or their respective isotope-labeled internal standards. The samples containing rat brain microsomes (0.25 mg/mL), 1'-OH-MDZ (10 nM), and 4-OH-MDZ (30 nM) in the presence and absence of MDZ (500 μM) were prepared by protein precipitation with the acetonitrile containing both internal standards (10 nM) (n = 3). The processed samples were analyzed using two different mobile phases and gradient systems (initial and final methods), described above in section 2.2, and the effect of substrate on the peak areas of the analytes and their internal standards were investigated.

2.7. Method validation

The analytical method was validated with respect to selectivity, carryover, precision, accuracy, sensitivity, recovery, and matrix effects, consistent with the 2018 FDA guidelines for bioanalytical method validation [26].

2.7.1. Selectivity and carryover

Selectivity of the method was evaluated by analyzing blank brain microsomal samples prepared from six different brain samples without any spiked analyte (blank) or with analytes spiked at the LLOQ to ensure the matrix is free from the endogenous interference at the retention times of the analytes and internal standards. The acceptance criteria for the selectivity were: (1) no interfering peaks at the retention times of the analytes or internal standards in the blank samples, (2) measured concentrations of the spiked samples within ± 20% of LLOQ, and (3) internal standards responses in the blank samples of ≤ 5% of that in the calibration and QC standards.

To evaluate the carryover effect, six replicates of blank brain microsomal samples were intermittently injected into the system, each immediately after the injection of a sample spiked with the upper limit of quantification (ULOQ) concentration (100 nM). Additionally, six replicates

of samples spiked with LLOQ concentration (1 nM) of analytes were analyzed. For all analytes, the carryover in the blank samples should not exceed 20% of LLOQ.

2.7.2. Precision and Accuracy

Precision and accuracy of the assay were evaluated by analyzing three independent runs, each consisting of a set of calibration standards and four QC levels with six replicates per each QC level. The QC samples, which were prepared similar to the calibrators, were run at the lower limit of quantitation (LLOQ, 1 nM) and low (LQC, 3 nM), medium (MQC, 30 nM), and high (HQC, 80 nM) concentrations of both metabolites in the calibration curve. Within-run precision and accuracy were estimated by analyzing six replicates of each QC sample against the calibration standards on the same day. Between-run precision and accuracy were determined by preparing and analyzing calibration standards and QC samples in six replicates on three different occasions. The precision was calculated as percent relative standard deviation (%R.S.D.) of the QC replicates. The accuracy was calculated as percent relative error (%R.E.) of the measured concentrations versus the theoretical concentrations. The acceptance criteria for the precision were %R.S.D. values of ≤ 15% for all the QC levels except for the LLOQ samples, which should be within 20%. The acceptance criteria for the accuracy were %R.E. values of ≤ ± 15% for all the QC levels, except for the LLOQ samples, which should be within ± 20%.

The acceptance criteria for calibration curves require that at least 75% and a minimum of six non-zero calibrators should be within ± 15% (for all calibrators except LLOQ) or ± 20% (for LLOQ) of the theoretical concentration.

2.7.3. Recovery and matrix effect

Recovery and matrix effects of 1'-OH-MDZ and 4-OH-MDZ were determined at three QC levels of LQC (3 nM), MQC (30 nM), and HQC (80 nM) (n = 6 for each level), whereas for the

internal standards (1'-OH-MDZ-d_5, 4-OH-MDZ-d_5), a concentration of 10 nM was used. Three sets of samples were prepared by adding the analytes to the microsomal sample (pre-precipitation), supernatants after protein precipitation of blank microsomal samples (post-precipitation), or a mixture (75:25) of acetonitrile and buffer (neat). After analysis of the samples, peak areas of the analytes and the internal standards were determined. Percent recovery was calculated by dividing the peak area response of the analytes in the pre-precipitation samples by the respective mean area in the post-precipitation samples and multiplying the results by 100. Matrix effect was determined by calculating a matrix factor, which was obtained by dividing the peak area response of the analytes in the post-precipitation samples by the respective mean area in the neat samples. A matrix factor of 1 indicates no matrix effect, whereas the matrix factors of less than or higher than 1 indicate ion suppression or ion enhancement, respectively.

2.7.4. Autosampler stability

Autosampler stability was assessed by analyzing replicates of QC samples (n = 6) processed at concentrations of 3, 30, and 80 nM, which were kept in the auto-sampler at 10^{o}C over a period of 24 h. The samples were injected immediately or at 4, 8, or 24 h after storage in the autosampler, and the accuracy (percent error relative to the immediate injection) and precision (R.S.D.) of the measurements at different times (4-24 h) were determined. Additionally, the absolute peak area counts of the analytes and internal standards obtained after the immediate injection was used as the reference to evaluate the stability of the analytes and their internal standards individually at the tested time points (4, 8, and 24 h). Benchtop stability was not conducted because the samples had to be prepared immediately after microsomal incubations (Section 2.9), which require immediate protein precipitation using acetonitrile at the end of the 10-min incubation period.

2.8. Preparation of rat brain microsomes

Animal studies were approved by the Institutional Animal Care and Use Committee of Chapman University. Adult (8-12 weeks), male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA). The rats were housed in a temperature- and humidity-controlled room under a 12 h light-dark cycle with free access to food and water ad libitum. Under isoflurane anesthesia, a 22-gauge needle was inserted into the left ventricle of the heart (inlet), followed by an incision of the right atrium (outlet). The heart was then perfused with a cold saline solution at a flow rate of 25 mL/min for 6 min to remove the blood from the brain and other organs. Perfusion of tissue removes contamination of microsomes by hemoglobin in the residual blood, which causes a uniform decrease in the specific activities of CYP enzymes [27]. The whole brain was then removed, snap-frozen in liquid nitrogen, and stored at -80^0C.

Rat brain microsomes were prepared by differential centrifugation method, according to the procedure described before [28]. The final microsomal pellet was re-suspended in a storage buffer (250 mM Mannitol, 0.1 mM EDTA, 5 mM HEPES, 20% glycerol, 0.1 mM dithiothreitol, 22 μM butylated hydroxytoluene, and 0.1 mM phenylmethylsulfonylfluoride; pH 7.4) and stored at –80°C for later experiments. Total protein concentrations were estimated by the Bradford method using bovine serum albumin as standard.

2.9. Determination of the midazolam hydroxylase activity

MDZ stock solutions (50 mM) were prepared by dissolving the MDZ-Base powder in 105 mM HCl, followed by subsequent dilutions in 5 mM HCl. To ensure that formation of metabolites was in the linear range for both reaction time and microsomal protein, time linearity (0–15 min) and protein linearity (0.25, 0.5, and 1.0 mg/mL) experiments were performed at a substrate concentration of 250 μM in triplicates. Subsequently, a protein concentration of 0.25 mg/mL and

an incubation time of 10 min were used to determine MDZ hydroxylation activity in the rat brain microsomes. The Michaelis-Menten (MM) kinetics experiments were performed at MDZ concentrations of 0, 1, 2.5, 10, 25, 50, 100, 250, and 500 µM. To ascertain inter-animal variability, the MM curves were constructed using brain tissues obtained from five different animals. In a final volume of 25 µL of 100 mM Tris-HCl buffer (pH 7.4), microsomal protein (0.25 mg/mL or 6.25 µg) was pre-incubated at 37°C for 5 min along with $MgCl_2$ (20 mM) and NADPH (1 mM). The reaction was initiated by the addition of the substrate. The substrate vehicle (2.5 µL of 5 mM HCl) had minimal, if any, effect on the final pH of the 25 µL incubation mixture (7.3-7.4). After 10 min of incubation at 37°C, reactions were terminated by the addition of 75 µL of acetonitrile containing 10 nM of both internal standards. Samples were centrifuged and subjected to the UPLC-MS/MS analysis. The maximum velocity (V_{max}) and MM constant (K_M) parameters were obtained by nonlinear regression analysis (GraphPad Prism; La Jolla, CA, USA) of the metabolism rate-substrate concentration data using a one-enzyme MM model.

3. Results and discussion

Given the relatively low CYP contents of the brain [5] and higher susceptibility of the brain microsomes to degradation [6], it is necessary to devise sensitive and reproducible analytical and metabolic incubation methods to characterize the CYP-mediated metabolism of drugs and endogenous compounds in this tissue. As shown below, we have developed, optimized, and validated such methods to characterize the CYP3A-mediated hydroxylation of MDZ in the rat brain.

3.1. UPLC-MS/MS optimization

The mass spectrometric conditions were optimized in the positive ionization mode for the simultaneous analysis of the two hydroxylated metabolites of MDZ and their respective

isotopically-labeled internal standards. Figure 2 depicts fragmentation patterns of 1'-OH-MDZ (A), 1'-OH-MDZ-d_5 (B), 4-OH-MDZ (C), and 4-OH-MDZ-d_5 (D), along with the selected Q1/Q3 m/z signals. For both analytes and their respective internal standards, the signal representing an m/z of 18 units less than that of the parent ion was the most prominent fragment (Fig. 2). However, this fragment had the same m/z ratio for the two hydroxylated metabolites (1'-OH-MDZ and 4-OH-MDZ) or their internal standards (1'-OH-MDZ-d_5 and 4-OH-MDZ-d_5). Therefore, we selected the next prominent fragments, which were not shared between the two structural isomers, for their quantitation. The substrate MDZ was not quantified but was monitored with the m/z transition of 326 to 291.

For chromatographic conditions, different solvents and columns were used in our preliminary experiments to optimize the peak shapes, linearity, and sensitivity of the assay. The chromatographic conditions were initially optimized using gradient elution of a mobile phase consisting of 5 mM ammonium formate with 0.05% formic acid in water (A) and acetonitrile: methanol (95:5) with 0.05 % formic acid (B) at a flow rate of 0.3 mL/min (initial method). This method resulted in linear calibration standards, including consistent and similar internal standard signals across the calibration standards. However, when the metabolic incubation samples were analyzed using this method, there was a progressive decrease in the detector's signal for the 1'-OH-MDZ-d_5 internal standard when the substrate (MDZ) concentration in the samples was increased. Therefore, to determine the possible role of MDZ on these results, the detector's signals for the two metabolites and their respective internal standards were quantitated in the presence and absence of a 500 μM concentration of the substrate (MDZ). When the initial method was used, presence of substrate caused ~25% reduction in the peak areas of both 1'-OH-MDZ and its internal standard, whereas the areas of 4-OH-MDZ and its internal standard were not affected. This

reduction in detector's signal intensity was associated with a partial co-elution of 1'-OH-MDZ with MDZ (Fig. 3A), whereas the 4-hydroxylated metabolite eluted before the substrate peak (Fig. 3B), suggesting ion suppression of the 1'-hydroxylated metabolite and its internal standard by the high concentrations (500 μM) of the parent drug MDZ (MDZ:1'-OH-MDZ concentration ratio of 50,000). Consequently, the chromatographic method was further optimized (final method) to completely separate both metabolites from the substrate, with the substrate eluting last in the chromatogram (Figs. 2C and 2D). Using the optimized method (final method), presence of a high concentration (500 μM) of the substrate (MDZ) did not affect the detector's response to either of the hydroxylated metabolites or their internal standards. Therefore, the final method was validated and employed for CYP3A assay standardization in the rat brain microsomes.

The ion suppression of metabolites by the substrate is especially important for the brain microsomal studies where, due to low enzymatic activity of the brain, the ratio of substrate: metabolite concentrations could reach as high as 250,000 [28, 29]. Therefore, the substrate and generated metabolite should be well separated in the LC-MS/MS analysis of the brain CYP studies.

3.2. Chromatograms

Figure 4 shows the chromatograms of blank, the lowest (1 nM) and the highest (100 nM) standards in the calibration curves, the metabolic incubation samples at the lowest (1 μM) and the highest (500 μM) concentrations of the substrate, and the internal standard for 1'-OH-MDZ. Similar data are presented for 4-OH-MDZ in Fig. 5. As demonstrated in these figures, 1'-OH-MDZ (and its internal standard) and 4-OH-MDZ (and its internal standard) eluted at ~4.1 and 3.8 min, respectively, as sharp peaks with relatively low baseline noises (Figs. 4 and 5).

Previous LC-MS/MS studies quantitating one or both hydroxylated metabolites of MDZ have used internal standards with similar structures, such as prazepam [22], flurazepam [21],

phencynonate [25], or deuterated MDZ [24]. Also, deuterated 1'-OH-MDZ was used as an internal standard for quantitating both metabolites of MDZ [23]. Our LC-MS/MS method uses deuterated internal standards for each of the hydroxylated metabolites of MDZ.

3.3. Selectivity and carryover

No interfering peaks were observed at the retention times of the analytes or their respective internal standards in the six different blank brain microsomal samples injected, indicating selectivity of the analytical method. Additionally, after injection of the highest concentration of the analytes in the calibration curve, no detectable peaks could be found in a subsequent blank chromatogram for any of the six replicates, indicating no carryover.

3.4. Calibration curves

The calibration curves were linear and reproducible over the standard concentration range of 1–100 nM for both 1'-OH-MDZ and 4-OH-MDZ, with the coefficient of determination (r^2) values of ≥ 0.99. Representative equations, calculated from the inter-day calibration curves, were y = 0.05597x-0.0184 for 1'-OH-MDZ, and y = 0.0350x-0.0125 for 4-OH-MDZ, where y and x refer to the analyte: internal standard peak area ratio and the concentration of analytes, respectively. The errors associated with the back-calculated concentrations for all the eight non-zero calibrators in all the precision and accuracy experiments were between -9.15% to 6.61% for 1'-OH-MDZ and -7.60% to 8.68% for 4-OH-MDZ, well within the acceptance criteria for the calibration curves.

3.5. Precision and accuracy

Intra- and inter-run precision and accuracy data for 1'-OH-MDZ and 4-OH-MDZ were determined by analyzing six replicates of QC samples at four different concentrations, as shown in Tables 1 and 2, respectively. Precision and accuracy values for both intra- and inter-run

experiments were within the acceptable limits. For 1'-OH-MDZ, the intra- and inter-run R.E. values ranged from –6.05% to 7.81% with R.S.D. values of ≤ 13% (Table 1). For 4-OH-MDZ, the range of R.E. values was between –6.49%% and 10.4%, and R.S.D values were ≤ 13.8% (Table 2). Based on the precision and accuracy values reported in Tables 1 and 2, the lower limit of quantitation (LLOQ) of 1'-OH-MDZ and 4-OH-MDZ in our assay was 1 nM (341 pg/mL), which translates to 0.4 pg of analytes on the column. Based on the per column sensitivity data, our UPLC assay is around 1000 fold more sensitive than a previously reported HPLC-MS/MS assay that quantitated 1'-OH-MDZ in plasma [22]. However, our sensitivity is only ~4-fold higher than a more recently reported method that measured both 1'-OH-MDZ and 4-OH-MDZ in human plasma and liver microsomes [24].

3.6. Recovery and matrix effect

Previously validated LC-MS/MS methods for the quantitation of hydroxylated metabolites of MDZ in biological samples have used liquid-liquid [21, 22] or solid-phase [23] extraction or protein precipitation followed by evaporation of the supernatant and reconstitution of the residue [24]. Our studies used a simple acetonitrile precipitation method, followed by the direct injection of the supernatant.

The recoveries of 1'-OH-MDZ, 4-OH-MDZ, and their respective internal standards from the brain microsomal proteins after the protein precipitation method are presented in Table 3. Average recoveries ranged between 97.4% and 106% for all the analytes and their internal standards at the tested concentrations (Table 3). These data suggest an almost complete recovery of the analytes and their standards using the acetonitrile protein precipitation method.

The matrix factors for all the analytes and their internal standards (Table 4) ranged from 0.948 to 1.05, indicating no notable effect of the microsomal matrix on the detector's response to the analytes or their internal standards in our method.

Our sample preparation method (direct injection after protein precipitation) is faster than the other sample preparation methods reported in validated LC-MS/MS methods for hydroxylated metabolites of MDZ [21-24]. The complete recovery of the analytes and lack of matrix effect in our direct injection method is most likely due to the low protein concentrations (0.25 mg/mL) in our microsomal samples compared to plasma or serum samples, which contain relatively high protein concentrations.

3.7. Autosampler stability

Both hydroxylated metabolites of MDZ and their internal standards in the samples containing 3, 30, or 80 nM of each analyte and 10 nM of the internal standards were stable in the autosampler (10°C) for up to 24 h. The relative error values were in the range of –4.7% to 2.6% (1'-OH-MDZ) or –5.1% to 3.0% (4-OH-MDZ), and the precision (R.S.D.) values were in the range of 2.48% to 7.50% (1'-OH-MDZ) or 2.37% to 14.0% (4-OH-MDZ) for all the tested time points and concentrations. Additionally, the absolute peak areas at 4, 8, and 24 h of storage in the autosampler fluctuated between 94.6% and 109% (1'-OH-MDZ), 95.1% to 111% (1'-OH-MDZ-d_5), 95.6% to 109% (4-OH-MDZ), and 96.1% to 113% (4-OH-MDZ-d_5) of the areas at time zero. These data indicate that both hydroxylated metabolites and their internal standards are stable in the autosampler (10°C) for at least 24 h.

3.8. Metabolites impurity in the MDZ substrates from different sources

Our previous experience with brain microsomes [28] showed that, if left uncorrected, even very low impurities of the metabolite in the substrate might add significant errors to quantitation

of metabolic rates for this tissue. The reason for this observation is that the brain metabolic rates are generally much lower than those in the liver. Therefore, although a very low metabolite impurity in the substrate may not add a significant error to the overall metabolite generation rate in the liver microsomes, the error might be significant for the brain microsomes, especially at high substrate concentrations. Initially, we used the HCl salt of MDZ (MDZ-HCl), which is considerably more water soluble than the MDZ base, as the substrate for the microsomal incubation studies. However, we observed measurable concentrations of both 1'-OH-MDZ (2.82 ± 0.06 nM) and 4-OH-MDZ (3.76 ± 0.32 nM) in stock solutions containing 500 μM MDZ-HCl. Subsequently, we tested the level of impurities in the analytical reference standard of MDZ-Base, which was solubilized in diluted HCl solutions. In contrast to the observations with MDZ-HCl, the impurities in the 500 μM MDZ-Base solutions were below the limit of quantitation of the assay. To confirm these results, we prepared metabolic incubation mixtures with both 500 μM MDZ-HCl or MDZ-Base and analyzed the samples both immediately (zero) or 10 min after the incubation (Fig. 6). As shown in Fig. 6, the zero samples of MDZ-HCl contained significant peaks of both 1'-OH-MDZ (Fig. 6A) and 4-OH-MDZ (Fig. 6C), which on average constituted 16-21% of the peak areas after 10 min of incubation. This impurity would result in significant overestimation of the formation rates of 1'-OH-MDZ and 4-OH-MDZ when MDZ-HCl is used. Conversely, when MDZ-Base was used as the substrate, metabolite impurities were below the limit of quantitation in the zero incubation samples (Figs. 6B and 6D). Therefore, we used MDZ-Base as the substrate for our incubation studies with brain microsomes. However, if the employed substrate contains measurable amounts of the metabolites, like MDZ-HCl tested here, incubations have to be run both at zero and 10 min, and the formation rate corrected for the presence of the metabolites in the zero samples [28].

3.9. *Estimation of midazolam hydroxylation kinetics in the rat brain microsomes*

The optimization of the metabolic incubation method, in terms of linearity of the metabolite formation with respect to the microsomal protein concentration and incubation time, along with the Michaelis-Menten (MM) plots for both hydroxylated metabolites of MDZ, are depicted in Figure 7. At a substrate concentration of 250 μM, the formation rates of both 1'-OH-MDZ and 4-OH-MDZ were linear with respect to both incubation time (up to 15 min) and microsomal proteins when the protein concentrations were 0.25 or 0.5, mg/mL (Figs. 7 A and B). However, at a protein concentration of 1.0 mg/mL, some evidence of non-linearity was observed at incubation times beyond 10 or 12.5 min (Figs. 7 A and B). Therefore, a brain microsomal protein concentration of 0.25 mg/mL and an incubation time of 10 min were set for the MM kinetic studies.

The MM kinetics of hydroxylation of MDZ to 1'-OH-MDZ (Fig. 7C) and 4-OH-MDZ (Fig. 7D) were assessed in the rat brain microsomes and substrate concentrations ranging from 1 to 500 μM. Nonlinear regression analysis of the data revealed that a single-enzyme MM model would adequately fit the data presented in Figs. 7C and 7D for both metabolites with V_{max}, K_M, and intrinsic clearance ($Cl_{int} = V_{max}/K_M$) values, which are presented in Table 5. In the rat brain microsomes, the 4-hydroxylated metabolite had 2.4 fold higher V_{max} ($p < 0.01$) and 1.9 fold higher ($p < 0.05$) K_M values than the 1'-hydroxylated metabolite (Table 5). However, the Cl_{int} values of the two metabolites were similar (Table 5).

The MM kinetics of MDZ hydroxylation have been previously reported in the rat liver microsomes [17]. In contrast to the relatively close Cl_{int} values for the hydroxylated metabolites in the brain (Table 5), the Cl_{int} of 1'-OH-MDZ in the rat liver microsomes was 4-fold lower than that for 4-OH-MDZ [17], suggesting different regioselectivity for the CYP3A-mediated hydroxylation of MDZ in the liver and brain. The lower Cl_{int} of 1'-OH-MDZ, relative to 4-OH-MDZ, in the rat

liver microsomes was mostly due to its substantially lower V_{max}, while the K_M values for the two metabolites were relatively similar [17]. In terms of brain metabolism of MDZ, we are only aware of one preliminary study [30], which reported minimal formation of 1'-OH-MDZ in the presence of large concentrations of rat brain microsomal protein (1500 μg in 300 μL) and substrate (300 and 500 μM). Therefore, the MM kinetic data reported here appear to be the first report on the CYP3A-mediated kinetics of MDZ hydroxylation in the brain tissue.

4. Conclusions

Despite the wide use of MDZ as a centrally-acting drug and as a probe for CYP3A activity in the liver, reports on its metabolism in the brain are scarce or non-existent. Here, we report a sensitive UPLC-MS/MS method, which is specifically developed and validated for the simultaneous analysis of both hydroxylated metabolites of MDZ in the brain microsomes. Additionally, we have optimized a metabolic incubation method to analyze the formation rate of the hydroxylated metabolites of MDZ in the brain microsomes. The optimized analytical and metabolic incubation methods reported here allowed us to characterize the complete MM kinetics of both metabolites in the rat brain using relatively low protein concentrations and short incubation times.

Acknowledgments

This study was supported by funding from Chapman University School of Pharmacy.

References

[1] U.M. Zanger, M. Schwab, Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation, Pharmacol. Ther., 138 (2013) 103-141.

[2] D. Fanni, F. Pinna, C. Gerosa, P. Paribello, B. Carpiniello, G. Faa, M. Manchia, Anatomical distribution and expression of CYP in humans: Neuropharmacological implications, Drug Dev. Res., DOI 10.1002/ddr.21778 (2021).

[3] C.S. Ferguson, R.F. Tyndale, Cytochrome P450 enzymes in the brain: emerging evidence of biological significance, Trends Pharmacol. Sci., 32 (2011) 708-714.

[4] D.M. McMillan, R.F. Tyndale, CYP-mediated drug metabolism in the brain impacts drug response, Pharmacol. Ther., 184 (2018) 189-200.

[5] W. Kuban, W.A. Daniel, Cytochrome P450 expression and regulation in the brain, Drug Metab. Rev., 53 (2021) 1-29.

[6] S.V. Bhagwat, M.R. Boyd, V. Ravindranath, Rat brain cytochrome P450. Reassessment of monooxygenase activities and cytochrome P450 levels, Drug Metab. Dispos., 23 (1995) 651-654.

[7] O. Burk, L. Wojnowski, Cytochrome P450 3A and their regulation, Naunyn Schmiedebergs Arch. Pharmacol., 369 (2004) 105-124.

[8] F.P. Guengerich, Cytochrome P-450 3A4: regulation and role in drug metabolism, Annu. Rev. Pharmacol.Toxicol., 39 (1999) 1-17.

[9] L.S. Kaminsky, Q.Y. Zhang, The small intestine as a xenobiotic-metabolizing organ, Drug Metab. Dispos., 31 (2003) 1520-1525.

[10] C. Woodland, T.T. Huang, E. Gryz, R. Bendayan, J.P. Fawcett, Expression, activity and regulation of CYP3A in human and rodent brain, Drug Metab. Rev., 40 (2008) 149-168.

[11] Z. Jayyosi, K.O. Cooper, P.E. Thomas, Brain cytochrome P450 and testosterone metabolism by rat brain subcellular fractions: presence of cytochrome P450 3A immunoreactive protein in rat brain mitochondria, Arch. Biochem. Biophys., 298 (1992) 265-270.

[12] H. Rosenbrock, C.E. Hagemeyer, I. Singec, R. Knoth, B. Volk, Testosterone metabolism in rat brain is differentially enhanced by phenytoin-inducible cytochrome P450 isoforms, J. Neuroendocrinol., 11 (1999) 597-604.

[13] S. Yadav, A. Dhawan, P.K. Seth, R.L. Singh, D. Parmar, Cytochrome P4503A: evidence for mRNA expression and catalytic activity in rat brain, Mol. Cell. Biochem., 287 (2006) 91-99.

[14] H.V. Pai, S.C. Upadhya, S.J. Chinta, S.N. Hegde, V. Ravindranath, Differential metabolism of alprazolam by liver and brain cytochrome (P4503A) to pharmacologically active metabolite, Pharmacogenomics J., 2 (2002) 243-258.

[15] J.C. Gorski, S.D. Hall, D.R. Jones, M. VandenBranden, S.A. Wrighton, Regioselective biotransformation of midazolam by members of the human cytochrome P450 3A (CYP3A) subfamily, Biochem. Pharmacol., 47 (1994) 1643-1653.

[16] L.L. von Moltke, D.J. Greenblatt, J. Schmider, S.X. Duan, C.E. Wright, J.S. Harmatz, R.I. Shader, Midazolam hydroxylation by human liver microsomes in vitro: inhibition by fluoxetine, norfluoxetine, and by azole antifungal agents, J. Clin. Pharmacol., 36 (1996) 783-791.

[17] A. Ghosal, H. Satoh, P.E. Thomas, E. Bush, D. Moore, Inhibition and kinetics of cytochrome P4503A activity in microsomes from rat, human, and cdna-expressed human cytochrome P450, Drug Metab. Dispos., 24 (1996) 940-947.

[18] J. Jurica, M. Dostálek, J. Konecný, Z. Glatz, E. Hadasová, J. Tomandl, HPLC determination of midazolam and its three hydroxy metabolites in perfusion medium and plasma from rats, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 852 (2007) 571-577.

[19] J. Martens, P. Banditt, Simultaneous determination of midazolam and its metabolites 1-hydroxymidazolam and 4-hydroxymidazolam in human serum using gas chromatography-mass spectrometry, J. Chromatogr. B: Biomed. Sci. Appl., 692 (1997) 95-100.

[20] C.B. Eap, G. Bouchoux, K. Powell Golay, P. Baumann, Determination of picogram levels of midazolam, and 1- and 4-hydroxymidazolam in human plasma by gas chromatography-negative chemical ionization-mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 802 (2004) 339-345.

[21] V.A. Frerichs, C. Zaranek, C.E. Haas, Analysis of omeprazole, midazolam and hydroxy-metabolites in plasma using liquid chromatography coupled to tandem mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 824 (2005) 71-80.

[22] M.R. Shiran, A. Gregory, A. Rostami-Hodjegan, G.T. Tucker, M.S. Lennard, Determination of midazolam and 1'-hydroxymidazolam by liquid chromatography-mass spectrometry in plasma of patients undergoing methadone maintenance treatment, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 783 (2003) 303-307.

[23] S. Ghassabian, S.M. Moosavi, Y.G. Valero, K. Shekar, J.F. Fraser, M.T. Smith, High-throughput assay for simultaneous quantification of the plasma concentrations of morphine, fentanyl, midazolam and their major metabolites using automated SPE coupled to LC-MS/MS, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 903 (2012) 126-133.

[24] M. Dostalek, J.S. Macwan, S.D. Chitnis, I.A. Ionita, F. Akhlaghi, Development and validation of a rapid and sensitive assay for simultaneous quantification of midazolam, 1'-

hydroxymidazolam, and 4-hydroxymidazolam by liquid chromatography coupled to tandem mass-spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 878 (2010) 1629-1633.

[25] S. Xiong, J. Li, W. Zhang, X. Wang, Z. Zhang, Induction of CYP3A by morroniside in rats, J. Pharmacol. Sci., 127 (2015) 414-418.

[26] U.S. Food and Drug Administration, Guidance for Industry: Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, 2018.

[27] R.E. Pearce, C.J. McIntyre, A. Madan, U. Sanzgiri, A.J. Draper, P.L. Bullock, D.C. Cook, L.A. Burton, J. Latham, C. Nevins, A. Parkinson, Effects of freezing, thawing, and storing human liver microsomes on cytochrome P450 activity, Arch. Biochem. Biophys., 331 (1996) 145-169.

[28] B.N. DuBois, R. Mehvar, UPLC-MS/MS analysis of dextromethorphan-O-demethylation kinetics in rat brain microsomes, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 1096 (2018) 66-72.

[29] B.N. DuBois, F. Amirrad, R. Mehvar, Kinetics of dextromethorphan-O-demethylase activity and distribution of CYP2D in four commonly-used subcellular fractions of rat brain, Xenobiotica, 49 (2019) 1133-1142.

[30] P. Voirol, M. Jonzier-Perey, F. Porchet, M.J. Reymond, R.C. Janzer, C. Bouras, H.W. Strobel, M. Kosel, C.B. Eap, P. Baumann, Cytochrome P-450 activities in human and rat brain microsomes, Brain Res., 855 (2000) 235-243.

Table 1

Intra- and inter-run precision and accuracy of 1'-hydroxymidazolam quality control samples in rat brain microsomes.

Nominal Concentration (nM)	Measured Concentration			
	Run	Mean (nM)	Precision (%R.S.D.)	Accuracy (%R.E.)
Intra-run (n = 6)				
1.00 (LLOQ)	1	0.96	13.0	–3.78
	2	0.95	7.89	–4.63
	3	0.94	6.79	–6.05
3.00 (LQC)	1	3.22	2.55	7.17
	2	3.10	2.65	3.33
	3	3.39	0.91	12.9
30 (MQC)	1	31.7	1.13	5.80
	2	30.9	3.57	2.98
	3	31.7	4.24	5.59
80 (HQC)	1	76.3	3.01	–4.64
	2	75.5	2.58	–5.59
	3	80.1	3.80	0.15
Inter-run (n = 18)				
1.00 (LLOQ)	1, 2, 3	0.95	9.13	–4.80
3.00 (LQC)	1, 2, 3	3.23	4.27	7.81
30.0 (MQC)	1, 2, 3	31.4	3.31	4.79
80.0 (HQC)	1, 2, 3	77.3	4.02	–3.36

Table 2

Intra- and inter-run precision and accuracy of 4-hydroxymidazolam quality control samples in rat brain microsomes.

Nominal Concentration (nM)	Measured Concentration			
	Run	Mean (nM)	Precision (%R.S.D.)	Accuracy (%R.E.)
Intra-run (n = 6)				
1.00 (LLOQ)	1	0.95	5.23	–5.12
	2	0.94	13.8	-5.95
	3	0.96	6.23	-4.00
3.00 (LQC)	1	2.99	4.01	–0.22
	2	3.10	4.73	3.36
	3	3.31	6.69	10.2
30 (MQC)	1	30.8	4.10	2.68
	2	31.0	3.25	3.43
	3	33.1	3.16	10.4
80 (HQC)	1	75.5	1.53	-5.58
	2	74.8	3.45	–6.49
	3	80.0	4.63	0.00
Inter-run (n =18)				
1.00 (LLOQ)	1, 2, 3	0.95	8.70	–5.02
3.00 (LQC)	1, 2, 3	3.13	6.59	4.44
30.0 (MQC)	1, 2, 3	31.6	4.72	5.49
80.0 (HQC)	1, 2, 3	76.8	4.50	–4.02

Table 3

Recovery data (mean ± SD, n = 6) for 1'-hydroxy (1'-OH-MDZ) and 4-hydroxy (4-OH-MDZ) midazolam and their respective internal standards, 1'-OH-MDZ-d_5 and 4-OH-MDZ-d_5. Samples were prepared in the rat brain microsomes at low (3.0 nM), medium (30 nM), and high (80 nM) concentrations of the metabolites. The concentrations of the internal standards (1'-OH-MDZ-d_5 and 4-OH-MDZ-d_5) were the same (10 nM) in all the samples.

Concentration (nM)	1'-OH-MDZ	1'-OH-MDZ-d_5	4-OH-MDZ	4-OH-MDZ-d_5
3.0 (LQC)	97.4 ± 14.8	100 ± 12.7	97.8 ± 14.9	99.6 ± 11.3
30 (MQC)	104 ± 7.64	102 ± 9.03	102 ± 8.94	102 ± 11.4
80 (HQC)	102 ± 4.81	99.2 ± 4.08	106 ± 7.61	105 ± 7.96

Table 4

Matrix factor (mean ± SD, n = 6) for 1'-hydroxy (1'-OH-MDZ) and 4-hydroxy (4-OH-MDZ) midazolam and their respective internal standards, 1'-OH-MDZ-d_5 and 4-OH-MDZ-d_5. Samples were prepared in the rat brain microsomes at low (3.0 nM), medium (30 nM), and high (80 nM) concentrations of the metabolites. The concentrations of the internal standards (1'-OH-MDZ-d_5 and 4-OH-MDZ-d_5) were the same (10 nM) in all the samples.

Concentration (nM)	1'-OH-MDZ	1'-OH-MDZ-d_5	4-OH-MDZ	4-OH-MDZ-d_5
3.0 (LQC)	1.00 ± 0.12	1.01 ± 0.09	1.05 ± 0.138	1.04 ± 0.124
30 (MQC)	0.949 ± 0.051	0.963 ± 0.046	1.00 ± 0.084	0.994 ± 0.101
80 (HQC	0.996 ± 0.111	1.01 ± 0.119	0.948 ± 0.137	0.961 ± 0.133

Table 5

Maximum velocity (V_{max}), Michaelis-Menten constant (K_M), and intrinsic clearance (Cl_{int}) values (mean ± SD, $n = 5$ brains) of midazolam metabolites in rat brain microsomes.

Parameter	1'-OH-MDZ	4-OH-MDZ
V_{max} (pmol.min^{-1}.mg^{-1})	3.48 ± 0.91	8.37 ± 2.17**
K_M (μM)	10.2 ± 4.1	19.4 ± 4.1*
Cl_{int} (μL. min^{-1}.mg^{-1})	0.383 ± 0.171	0.441 ± 0.131

*, $p < 0.05$; **, $p < 0.01$; two-tailed, paired t-test.

LEGENDS TO FIGURES

Fig. 1. Chemical structures of midazolam and its CYP3A-mediated hydroxylated metabolites, 1'- and 4-hydroxymidazolam.

Fig. 2. LC-MS/MS fragmentation pattern of 1'-hydroxymidazolam (A), 1'-hydroxymidazolam-d_5 (B), 4-hydroxymidazolam (C), and 4-hydroxymidazolam-d_5 (D) with their Q1 (solid circles) and Q3 (dashed circles) m/z highlighted.

Fig. 3. Representative chromatograms of 1'-hydroxymidazolam (A and C) and 4-hydroxymidazolam (B and D) in the rat brain microsomes containing 500 μM of the substrate analyzed by two different methods, consisting of different mobile phases and gradient conditions. Composition of mobile phases and their gradient conditions for the initial and final methods are described in Section 2.2.

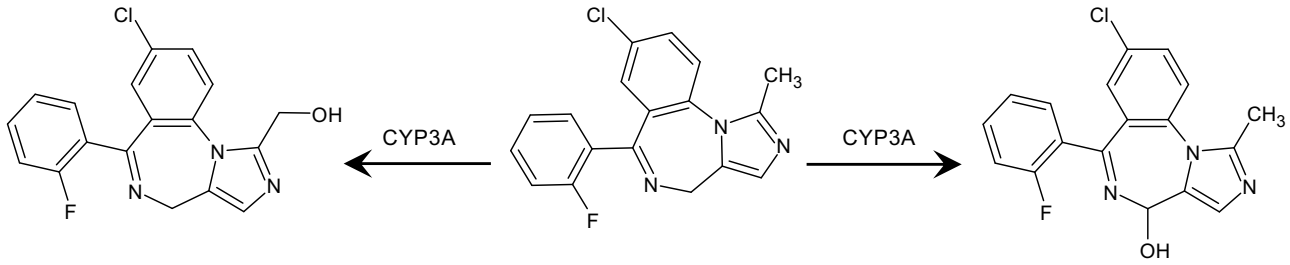
Fig. 4. Typical MRM chromatograms of 1'-hydroxymidazolam in blank brain microsomal matrix spiked with zero (A), 1 (B), or 100 (C) nM of 1'-hydroxymidazolam or in metabolic incubation mixtures at the substrate (midazolam) concentrations of 1 (D) and 500 (E) μM. The concentrations of 1'-hydroxymidazolam were 2.20 and 12.3 nM in D and E, respectively. For metabolic incubations, rat brain microsomes (0.25 mg/mL) were incubated with midazolam for 10 min. For comparison, the chromatogram of the internal standard is also shown (F).

Fig. 5. Typical MRM chromatograms of 4-hydroxymidazolam in blank brain microsomal matrix spiked with zero (A), 1 (B), or 100 (C) nM of 4-hydroxymidazolam or in metabolic incubation mixtures at the substrate (midazolam) concentrations of 1 (D) and 500 (E) μM. The concentrations of 4-hydroxymidazolam were 2.24 and 25.2 nM in D and E, respectively. For metabolic incubations, rat brain microsomes (0.25 mg/mL) were incubated with midazolam for 10 min. For comparison, the chromatogram of the internal standard is also shown (F).

Fig. 6. Representative chromatograms of 1'-hydroxymidazolam (A and B) and 4-hydroxymidazolam (C and D) in the rat brain microsomes immediately (T_0) and 10 min (T_{10}) following microsomal incubation with 500 μM MDZ-HCl (A and C) or MDZ-Base (B and D).

Fig. 7. The time and protein linearity of the metabolite formation rates (A and B) and MM plots (C and D, *n* = 5) of 1'-hydroxymidazolam (A and C) and 4-hydroxymidazolam (B and D) in the rat brain microsomes. The time (0-15 min) and protein (0.25, 0.5, or 1.0 mg/mL) linearity studies (A and B) were conducted in triplicates using brain microsomes containing 250 μM of MDZ-Base. The MM studies (C and D) were conducted using 0.25 mg/mL microsomal protein with MDZ-Base concentrations of 0–500 μM and incubation time of 10 min (*n* = 5 different brains). The symbols and error bars represent the mean and SD, respectively, and the lines represent the linear (A and B) or nonlinear (C and D) regression fit of the experimental data.

Fig. 1



1'–OH–Midazolam **Midazolam** **4–OH–Midazolam**

Fig. 2

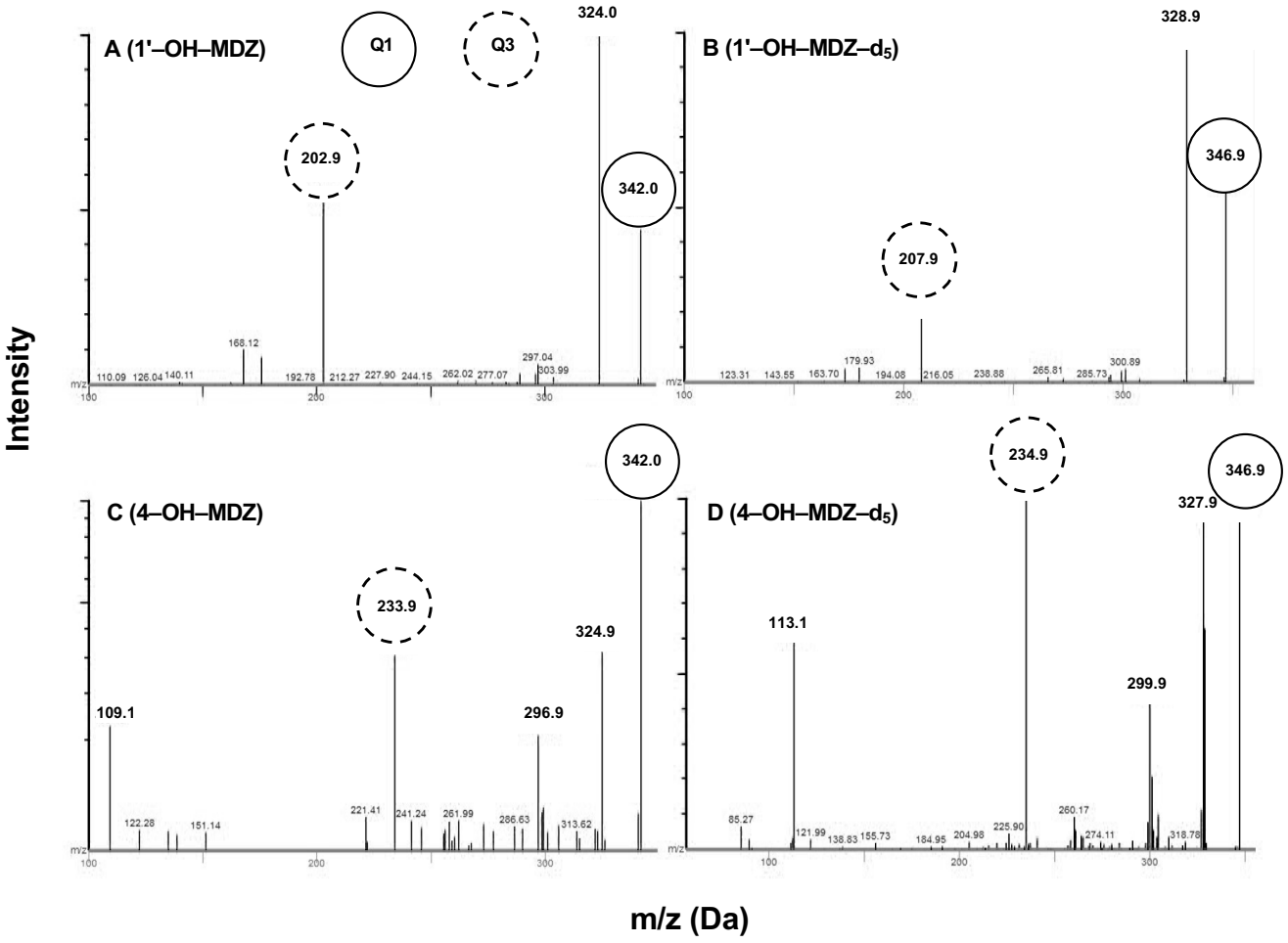

A (1'–OH–MDZ)
Q1
Q3
324.0
202.9
342.0
168.12
110.09 126.04 140.11 192.78 212.27 227.90 244.15 262.02 277.07 297.04 303.99
B (1'–OH–MDZ–d5)
328.9
346.9
207.9
123.31 143.55 163.70 179.93 194.08 216.05 238.88 265.81 285.73 300.89
C (4–OH–MDZ)
342.0
233.9
324.9
109.1
296.9
122.28 151.14 221.41 241.24 261.99 286.63 313.62
D (4–OH–MDZ–d5)
234.9
346.9
327.9
113.1
299.9
85.27 121.99 138.83 155.73 184.95 204.98 225.90 260.17 274.11 318.78
Intensity
m/z (Da)

Fig. 3

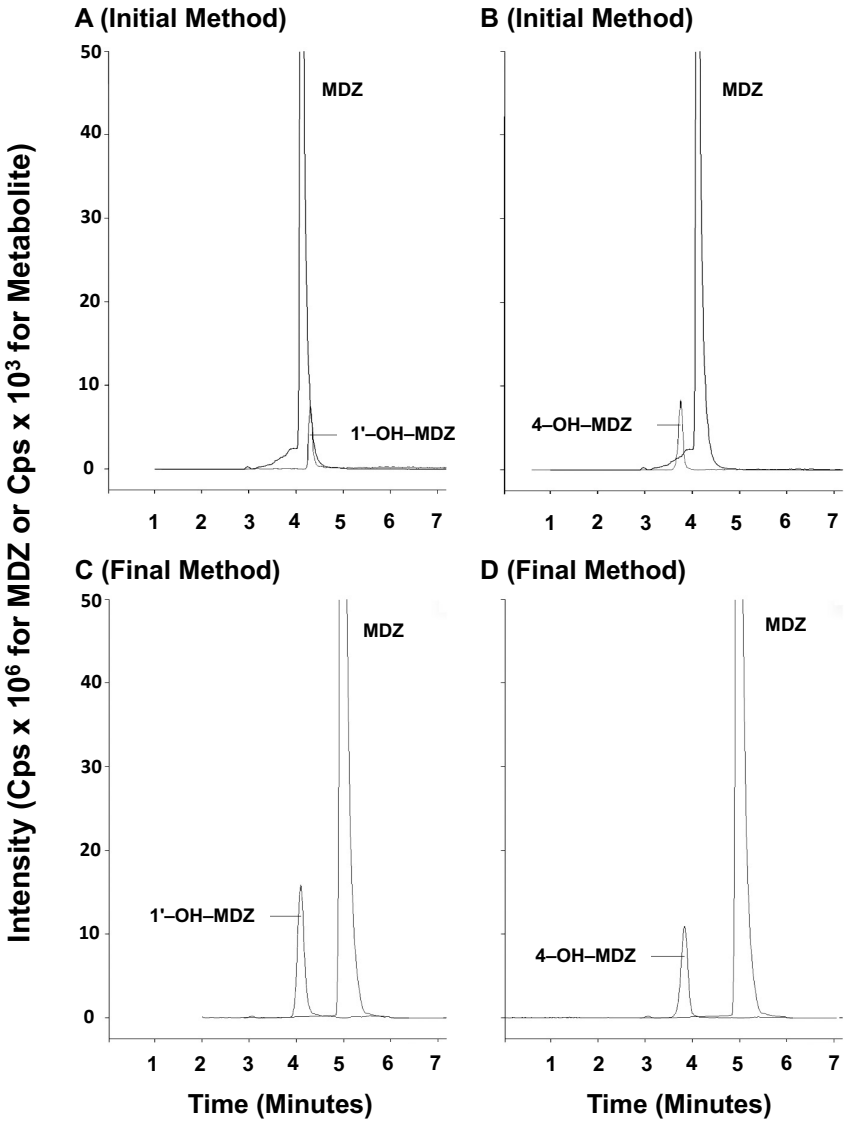

A (Initial Method)
B (Initial Method)
C (Final Method)
D (Final Method)
MDZ
1'–OH–MDZ
4–OH–MDZ
Intensity (Cps x 10^6 for MDZ or Cps x 10^3 for Metabolite)
Time (Minutes)
0
10
20
30
40
50
1
2
3
4
5
6
7

Fig. 4

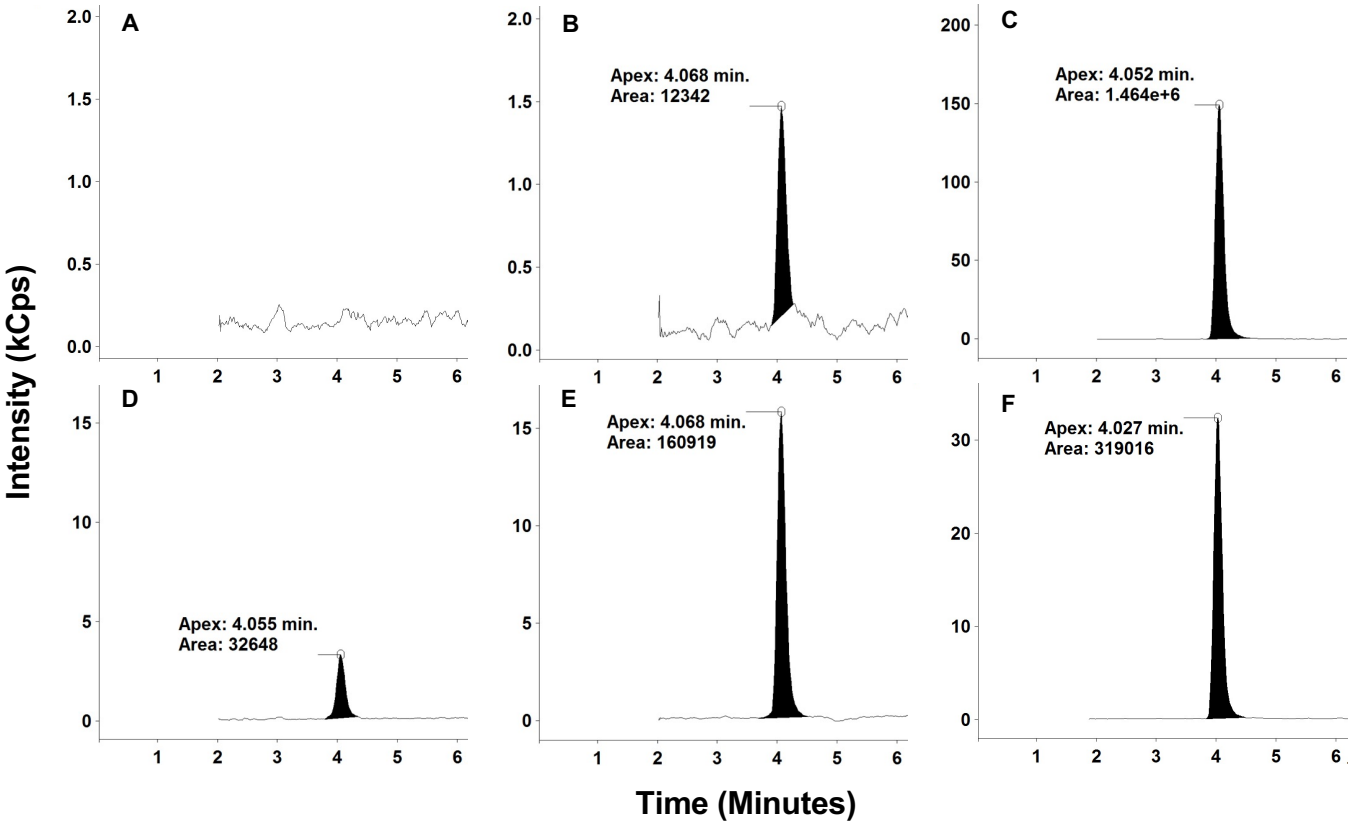

A
B
Apex: 4.068 min.
Area: 12342
C
Apex: 4.052 min.
Area: 1.464e+6
D
Apex: 4.055 min.
Area: 32648
E
Apex: 4.068 min.
Area: 160919
F
Apex: 4.027 min.
Area: 319016
Intensity (kCps)
Time (Minutes)

Fig. 5

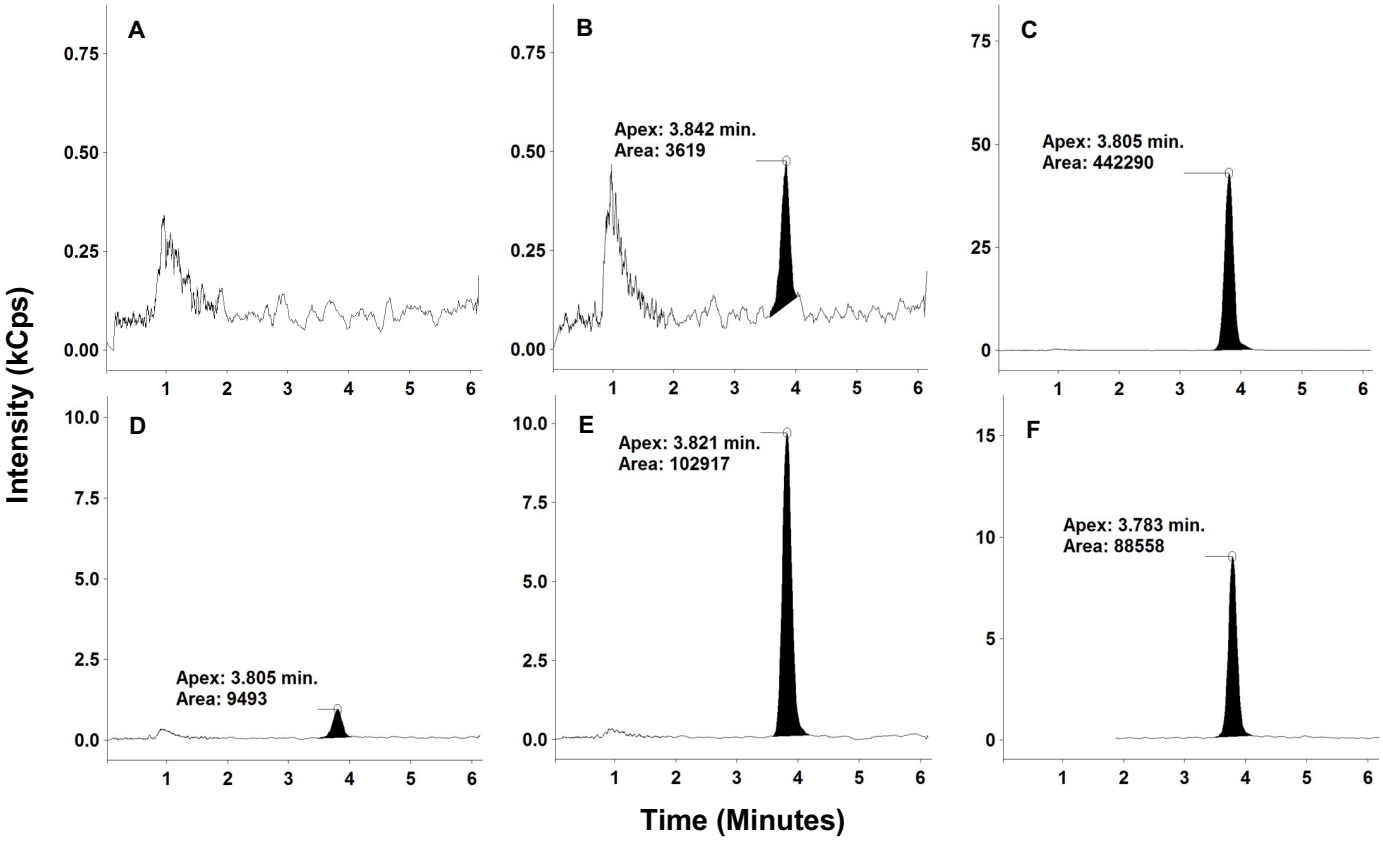

A
B
C
D
E
F
Apex: 3.842 min.
Area: 3619
Apex: 3.805 min.
Area: 442290
Apex: 3.805 min.
Area: 9493
Apex: 3.821 min.
Area: 102917
Apex: 3.783 min.
Area: 88558
Intensity (kCps)
Time (Minutes)

Fig. 6

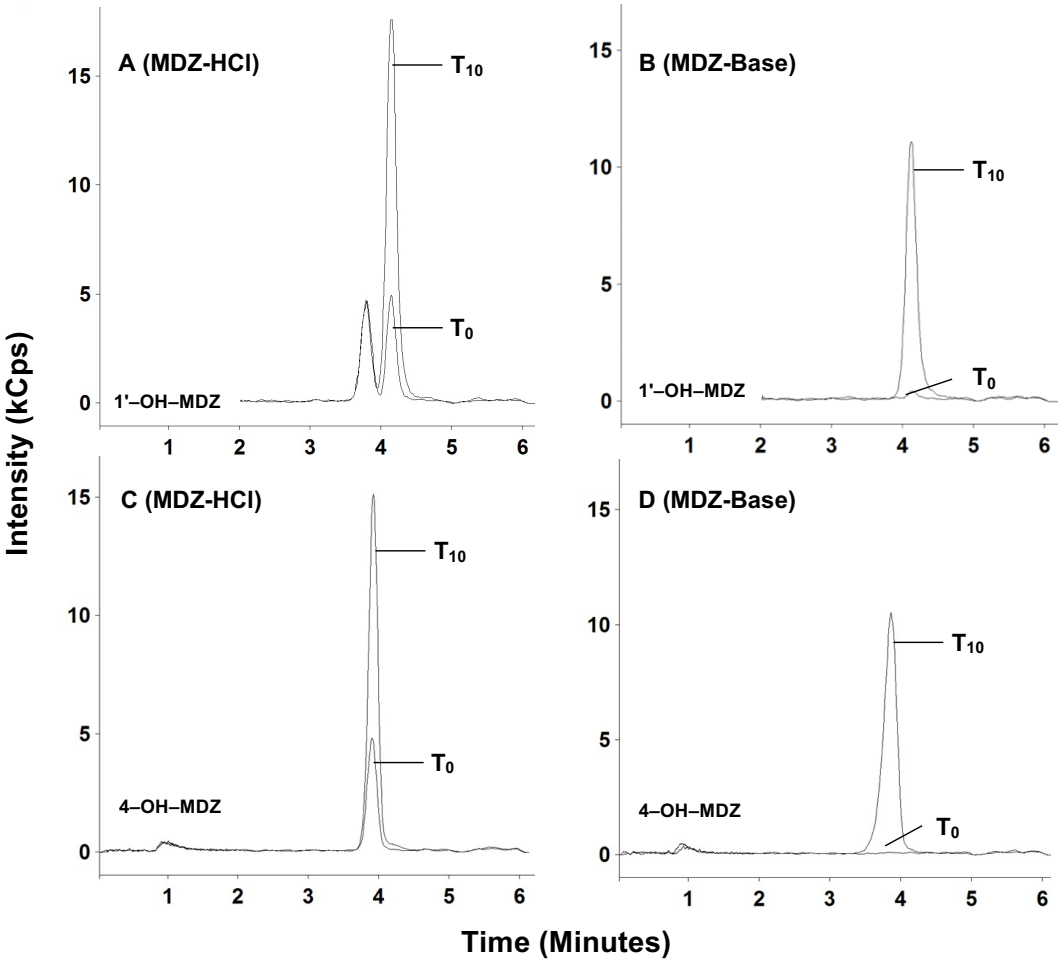
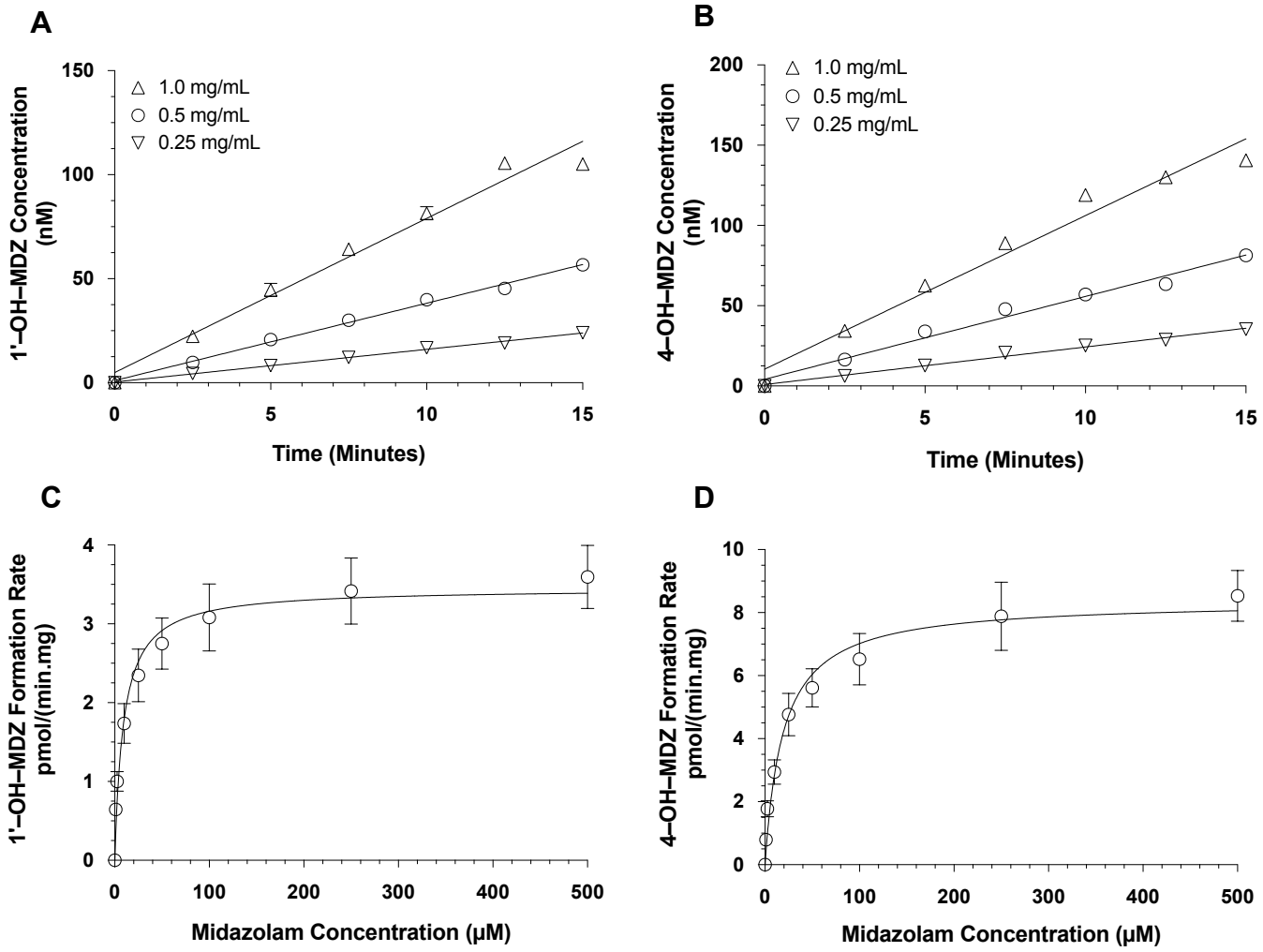

A (MDZ-HCl)
T10
T0
1'–OH–MDZ
B (MDZ-Base)
T10
T0
1'–OH–MDZ
C (MDZ-HCl)
T10
T0
4–OH–MDZ
D (MDZ-Base)
T10
T0
4–OH–MDZ
Intensity (kCps)
Time (Minutes)

Fig. 7


A
1'–OH–MDZ Concentration (nM)
1.0 mg/mL
0.5 mg/mL
0.25 mg/mL
Time (Minutes)
B
4–OH–MDZ Concentration (nM)
1.0 mg/mL
0.5 mg/mL
0.25 mg/mL
Time (Minutes)
C
1'–OH–MDZ Formation Rate pmol/(min.mg)
Midazolam Concentration (μM)
D
4–OH–MDZ Formation Rate pmol/(min.mg)
Midazolam Concentration (μM)