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Comparative pharmacokinetics and bioavailability of intranasal and rectal midazolam formulations relative to buccal administration in rabbits

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Abstract:

Midazolam (MDZ) is effective in treating seizures in a medical emergency service. However, intravenous (i.v.) administration requires skillful trained personnel. Alternative routes such as rectal, buccal, sublingual, or intranasal administration are established choices for drug application in an out-patient service. The aim of this work was to use rabbit as a model to compare the pharmacokinetics of midazolam and its 1’-hydroxy metabolite via i.v., rectal, intranasal and buccal administration with novel formulations. A single-dose, randomized, open-label, four-period crossover pharmacokinetic study was conducted with a three-day washout period between each segment. CYP3A activities were compared by studying the enzyme kinetics of midazolam in rabbit (CYP3A6) and human (CYP3A4/5) liver microsomes to qualify rabbit as a model species to predict human metabolic activity. From this study, a comparable apparent $K_m$ (7.86 vs. 8.66 $\mu$M) but a slightly higher $V_{max}$ (2117 vs. 1361 pmol/min/mg) was observed in rabbits, and this resulted in a 1.72-fold higher intrinsic clearance. Midazolam had comparable bioavailability among 4 routes tested (60-70%) which is higher than oral (35-44%). The absorption was faster in intranasal and rectal (~10 min) than the buccal administration (~20 min). The in vivo study also indicated that female rabbits had around 2-fold higher activity (1’-OH-MDZ/MDZ) in CYP3A6 than the male rabbits suggested that male rabbits may be closer to human in CYP3A activity. Overall, the rectal and intranasal formulations under current development might have the potential for administering midazolam in an out-patient emergency service when i.v. administration is not an option.
Key words: Midazolam; Pharmacokinetics; Rabbit; Intranasal; Buccal; Rectum
Graphical abstract:

Comparative PK study of MDZ formulations in rabbit

Comparative enzyme kinetics in rabbit and human liver microsomes
1. Introduction

It is estimated that more than 50 million individuals worldwide are suffering from epilepsy. The World Health Organization (WHO) has documented that the highest number of epilepsy cases occur in the developing countries. Current epidemiological investigation in China showed the morbidity from epilepsy is around 0.5%, with the first attack usually happening in childhood or teenagers. Because of lack in access to a convenient medical service, especially for patients in rural areas, the treatment is often not applied timely and effectively. Status epilepticus (SE) is the most common seizure that occurs in pediatric epilepsy patients. Mortality and severe neurologic outcome assessment showed a direct correlation to how fast the patients received a treatment. Immediate treatment is proved to be very critical for seizures and can save lives. On the other hand, a good anticonvulsant should be easy of administration, fast-acting, effective, and safe. Many clinical reports have described the successful use of midazolam (MDZ) to control refractory status epilepticus without any adverse effects in infants and children. However, for young children, i.v. administration requires skillfully trained personnel. Therefore, alternative routes such as rectal, buccal, sublingual, and intranasal (i.n.) administration could provide a practical alternative for drug administration in out-patient setting. However, up to now, only buccal midazolam (Epistatus®) has been developed and acquired marketing license for treating pediatric status epilepticus. Although rectal and intranasal midazolam are commonly used in clinic practice as an off-label medication, suitable formulations are not commercially available and the injectable solution of MDZ (5mg/ml) is
commonly used for all these routes. An issue is that the volume of spray administered into the nares ranged from 1-4 ml, well beyond the volume of a nasal cavity (100 µl). To meet the requirement of clinical therapy, especially in a rural area or in a poorly equipped hospital, the intranasal and rectal midazolam formulations were developed in our institute.

When developing a new formulation for a marketed drug, the most important requirement is to demonstrate good absorption and bioavailability compared to the current formulation, for which a comparative pharmacokinetics study is generally conducted. Although midazolam has shown a great potential in the treatment of SE, the pharmacokinetic experiments on the clinical or nonclinical side are confined to limited reports in the literature. Walberg et al. demonstrated that midazolam had bioavailability of 78% following intranasal administration in children. Towne et al., reviewed the pharmacokinetics of midazolam through intramuscular and showed the mean absolute bioavailability of 87% in adult epileptic patients, 87% in children, and about 90% in healthy adult volunteers. Wermeling and his coworkers reported a comparison study on midazolam nasal spray formulation to an i.v. injection in healthy volunteers. They found that midazolam was rapidly absorbed following intranasal administration, with a median $T_{\text{max}}$ of 10 min and bioavailability of 60 ± 23%. Veldhorst-Janssen et al., found that midazolam serum concentration of 30 ng/ml (the effective concentration to treat epilepsy in humans) reached within 5 minutes of intranasal administration in a group of 9 healthy volunteers. Kaartama et al., investigated the pharmacokinetics of sublingual administration of midazolam in
rabbits. They reported that the maximum plasma concentration of 1’-hydroxymidazolam (1’-OH-MDZ) was about 12% of that of midazolam systemic concentration. Considering the above isolated studies, a thorough cross-route administration comparative pharmacokinetics study of midazolam via intranasal, rectal, buccal, and i.v. routes has not yet been investigated or reported. Hence, the aim of this study is to investigate the pharmacokinetics of MDZ and its major metabolite 1’-OH-MDZ after rectal, intranasal, and buccal administration in rabbits with their respective formulations. The results could help to develop these formulations for clinical therapy, especially in pediatric population.

2. Material and Methods

2.1 Chemicals, standards and midazolam formulations

The reference standard of midazolam (99.8% purity) was obtained from National Institution for Food and Drug Control (Beijing, China). The 1’-OH-MDZ was purchased from Sigma-Aldrich (St. Louis, MO). The internal standard (IS), L-phenylalanine hydrochloride (L-8021) was provided by Beijing Institute of Pharmacology and Toxicology (Beijing, China). Pooled human liver microsomes and rabbit liver microsomes were purchased from BD Biosciences (Woburn, MA, USA). NADPH was obtained from Roche Molecular Biochemicals (Indianapolis, Indiana, USA). Acetonitrile was of chromatographic grade (Fisher Company, USA). Formic acid, sodium chloride and other reagents were of analytical grade (Sinopharm Chemical Regent Co. Int., Shanghai, China). Ultrapure water (generated by Milli-Q®
Academic A10 Milipore system) was used throughout the study. Midazolam nasal spray (midazolam-NS) formulation with the concentration of 25 mg/ml and midazolam rectal gel (midazolam-RG) formulation with the concentration of 5mg/g were provided by the department of pharmaceutics (Beijing Institute of Pharmacology and Toxicology, Beijing, China). The inactive ingredients of the intranasal formulation included propylene glycol, ethanol, hydroxypropyl β-cyclodextrin (HP-β-CD) and sterile water. The inactive ingredients of the rectal gel formulation included propylene glycol, ethanol, hydroxyl ethyl benzene, bio-adhesive polymers hydroxypropyl methyl cellulose (HPMC) and sterile water. Midazolam i.v. injection and buccal solution with the concentration of 5mg/ml were purchased from Jiangsu Enhua Pharmaceutical co., LTD (Xuzhou, Jiangsu).

2.2 Animals

Six adult New Zealand white rabbits (3 males and 3 females) with the average weight of 2.4 ± 0.27 kg (mean ±SD) were obtained from Beijing Keyu Laboratory Animal Centre (Beijing, China) and housed individually in stainless steel cages in the Laboratory Animal Centre of the institution under standard conditions. All the animal experiments were conducted in the Beijing Center for Drug Safety Evaluation Center and were in agreement with the protocol of the Institutional Animal Care and Use Committee of the Centre, which was in compliance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). One week before the pharmacokinetics experiment, all the rabbits underwent jugular vein intubation operation successfully. Aseptic conditions
with extreme caution during the surgery and postoperative care play significant roles to keep the maintenance of catheter patency. In the morning of interval between the pharmacokinetic experiments, the catheter should be gently flushed with sterile saline.

2.3 Study Design

A single-dose, randomized, open-label, four-period crossover study was carried out with a three days washout period. All rabbits received doses in random order, so that every rabbits received four routes of administration at the same dose of 0.5mg/kg: bolus injection, buccal administration, drops into both nostrils, and rectal administration. Rabbits were fasted for 12 h prior to dosing and remained fasted for 2 h after administration, with free access to water. Midazolam solution was administered as a bolus injection to the marginal auricular vein of the right ear, or to the upper right buccal site of the cheek using an Eppendorf® Micropette with around 200 µl of volume. The midazolam-NS was administrated with about 20 µl into each nostril. The head of rabbits were held facing straight upward for 1 min after administration. For the rectal administration, each rabbit was held in a supine position for administration of midazolam-RG. Blood samples (0.2 ml) were collected from the jugular vein intubation before and at 2, 5, 10, 15, 20, 30, 45 min, and 1, 1.5, 2, 3, 4, 6, and 8 h after administration. The collected blood was replaced with same volume of saline buffer. The blood samples were collected into heparinized tubes and immediately centrifuged for 10 min at 4°C, 3,000×g. Plasma was harvested and stored at -20°C until analysis.

2.4 In vitro Enzyme Kinetics Assay
Enzyme kinetics studies of 1’-OH-MDZ formation were performed using pooled liver microsomes from humans and rabbits. In brief, MDZ concentrations of 0.5, 1, 2, 5, 10, 2.5, 5, 10, and 20 µM were prepared in triplicate with microsomes in 100 mM phosphate buffer. The microsomal protein concentrations in the incubations were 0.5 mg/ml for both species. After preincubation at 37°C for 5 min, the reactions were initiated by the addition of NADPH (1 mM) solution. The reactions were terminated after 5 min by the addition of acetonitrile containing internal standard. The formation rate of 1’-OH-MDZ was measured by LC-MS/MS and calculated with the standard curve. Estimation of the maximum rate of 1’-OH-MDZ formation (V_{\text{max}}) and the Michaelis constant (K_{\text{m}}) was performed by nonlinear regression analysis (GraphPad Prism version 5.0; GraphPad Software Inc., La Jolla, CA, USA).

2.5 Bioanalytical method

HPLC experiments were conducted utilizing a Finnigan Surveyor™ HPLC system (Thermo Electron, San Jose, CA, USA). The mass detection was carried out using a Finnigan TSQ Quantum Discovery max system (Thermo Electron) composed of an electro spray ionization source. Data acquisition was performed with Xcalibur 1.2 software (Thermo Finnigan, USA). Peak integration and calibration were performed using LCQuan software (Thermo Finnigan, USA). The samples were separated on a BetaBasic-C_{18} analytical column (50 mm × 2.1 mm i.d., 5 µm particle size, Thermo, USA), and eluted with aqueous solution (A) and acetonitrile contains 0.1% formic acid (v/v) (B) under a linear gradient mode. The mobile phase program was as follows: 0-1 min, from 85% to 5% A; 1-3 min, from 5% to 85% A; 3-4.5 min, kept at 85% A.
An aqueous solution of 15 µL was injected for the analysis. The mobile phase was delivered at a flow rate of 0.45 ml/min during the first 3 mins then reduced to 0.3 ml/min in the following time window. The column and autosampler tray temperature were set at 25°C and 4°C, respectively.

The mass spectrometric detector was operated in the ESI positive ion selected reaction monitoring (SRM) mode. The parameters were set as follows: auxiliary gas pressure 12 psi; sheath gas pressure 45 psi; ion transfer capillary temperature 320°C; ion spray voltage 4800V. Quantification was performed using selected reaction monitoring of 

$m/z$ 326.1→222.0 for MDZ, $m/z$ 342.1→168.047 for 1’-OH-MDZ, $m/z$ 358.0→156.0 for L-8021 (IS), respectively.

The standard stock solutions of MDZ, 1’-OH-MDZ and IS were prepared in acetonitrile at a concentration of 1 mg/ml. All stock solutions were kept at -30°C prior to use. The mixed solutions were prepared by diluting MDZ and 1’-OH-MDZ standard stock solutions with acetonitrile to obtain the ideal concentrations. The IS standard working solution was at the concentration of 50 ng/ml in acetonitrile. In order to achieve a series of concentration calibration standards, the desired volumes of mixed solutions were spiked into the blank rabbit plasma. The final concentrations of calibration samples for MDZ/1’-OH-MDZ in rabbit plasma were of 0.8/0.2, 2/0.5, 8/2, 20/5, 80/20, 400/100, 800/200 ng/ml. Quality control samples were prepared independently containing 1.6/0.4, 40/10, 600/150 ng/ml of MDZ /1’-OH-MDZ, respectively.

All plasma samples were thawed at room temperature prior to analysis. A 50µL
aliquot of each sample was added with 100 µl IS working solution to precipitate protein. After vortexing vigorously for 1 min, the sample was centrifuged at 15,000×g for 10 min. Following the current guidance of FDA and subsequent Bio-analytical Methods Validation Workshop white paper \(^{15}\). The method was fully validated for its linearity, selectivity, precision and accuracy, sensitivity, matrix effect, recovery and stability.

2.6 Pharmacokinetic calculations

Pharmacokinetic parameters of MDZ and 1'-OH-MDZ were determined by fitting concentration-time data with a non-compartmental analysis using the WinNon-Lin 6.2 software (Pharsight, USA). The area under curve up to the last sampling point (AUC\(_{(0-t)}\)) and up to infinity (AUC\(_{(0-\infty)}\)) were calculated by the linear trapezoidal rule. The terminal elimination rate constant (\(\lambda_Z\)) was calculated by log-linear regression of the terminal part of the plasma concentration-time curves using at least three time points, and the half-life \(t_{1/2}\) was calculated as \(\ln 2/\lambda_Z\). In the case of intravenous administration, the total body clearance (CL) and the volume of distribution (V) were calculated from \(CL = \text{Dose}/AUC\) and \(V = CL/\lambda_Z\). In the cases of extravenuous administration, time to reach peak concentration (\(T_{\text{max}}\)) and peak concentration (\(C_{\text{max}}\)) were the observed values. The mean residence time (MRT) was calculated by the equation of MRT=AUMC/AUC. The mean absorption time (MAT) of individual formulation was obtained by calculating the difference between the MRT from the special routes to the MRT from i.v. administration. The bioavailability (F) of midazolam was calculated from the ratio of AUC following extravenuous over i.v.
administration.

2.7 Data analysis

The SPSS 13.0 statistical software package for Windows was used for statistical analysis. The pharmacokinetic parameters of i.n. and rectal routes of administration were compared to each other and also to the buccal route by the paired Student's t test, respectively. A p value < 0.05 was considered statistically significant.

3. Results

3.1 LC-MS/MS assay

Representative chromatograms of blank plasma, spiked plasma and real samples are shown in Fig. 1. The assay was free of interference from endogenous substances or contaminating peaks in rabbit plasma. A typical regression equation for the calibration curves was \( y = 0.0150 \times X_{(C)} + 0.0102, r^2 = 0.999 \) for MDZ over the range of 0.8-800 ng/ml; \( y = 0.0039 \times X_{(C)} + 0.0003, r^2 = 0.998 \) for 1'-OH-MDZ over the range of 0.2-200 ng/ml, using a weighted (\(1/x^2\)) linear regression. The low limit of quantification (LOQ) of the method used was 0.8 ng/ml and 0.2 ng/ml for MDZ and 1'-OH-MDZ, respectively. The intra-day precision and inter-day precision (error from the true value) of MDZ were found to be less than 5.6% and 6.2%, respectively, at the QC concentrations. Similarly, the intra-day precision and inter-day precision of 1'-OH-MDZ were found to be less than 9.6% and 9.0%, respectively. The accuracy ranged from 93.4% to 107% for the intra-day and inter-day runs for MDZ, and from 100% to 107% for intra-day and inter-day runs for 1'-OH-MDZ for QC samples. The average recoveries of MDZ and 1'-OH-MDZ were over the range of 83.6% to 94.8%
at low, middle and high QC levels. The enhanced matrix effects were observed for both MDZ and 1’-OH-MDZ in this method. The signals were higher compared to the reference with the range of 96-112%. MDZ and 1’-OH-MDZ were stable in rabbit plasma kept at ambient temperature for 12 h, or at -80 °C for 15 days after three cycles of freeze/thaw (for full validation data, please see the supplemental data).

Considering MDZ is not only commonly used in clinic, it is also a well known CYP3A4 substrate that often used in drug-drug interaction studies, the quantitative determination of MDZ and its major metabolite in bio-matrix is well developed in laboratories worldwide. Compared with the published analytical methods, the method of our current assessment of midazolam and its metabolite using LC-MS/MS is proved to be both accurate and robust.

3.2 Pharmacokinetic study

The time courses of mean MDZ and 1’-OH-MDZ plasma concentrations following i.v., i.n., rectal and buccal administrations are shown in Figs. 2 and 3. Several characteristics of midazolam pharmacokinetic behavior can be inferred from Figs. 2 and 3. The uptakes of midazolam into systemic circulation via i.n., rectal and buccal administrations are all rapid. Considerable concentrations of midazolam can be detected in all the samples at 2 min after dosing. The elimination was fast after all routes of administration, with the t_{1/2} of approximately 1-1.5 h. The concentrations of midazolam after 8h administration were all lower than the low detection limit of 0.8 ng/ml. The terminal elimination velocity of midazolam was unaffected by route of administration with similar slopes. The pharmacokinetic profiles of 1’-OH-MDZ
Fig.1. Representative MRM chromatograms of (A) blank plasma, (B) blank plasma spiked with MDZ (8 ng/ml), 1’-OH-MDZ (2 ng/ml) and IS, (C) real plasma sample obtained from a rabbit at 2 h after buccal administration of 0.5 mg/kg MDZ.

showed that the concentrations of this major metabolite were much lower than the parent drug, which is consistent with the literature reports. In addition, the inter-individual variability (reflected as large standard deviation) of 1’-OH-MDZ concentrations was much higher than that of the parent drug which suggested some variability in metabolism among the animals.
Fig. 2 Plasma concentration-time profiles of parent drug in male and female combined rabbits, after single i.n., buccal, rectal or intravenous administration MDZ at 0.5 mg/kg. Data were shown as mean ± SD (n = 6)

Fig. 3 Plasma concentration-time profiles of metabolite (1’-OH-MDZ) in male and female combined rabbits, after single i.n., buccal, rectal or intravenous administration MDZ at 0.5 mg/kg. Data were shown as mean ± SD (n = 6)

The pharmacokinetic parameters of MDZ and 1’-OH-MDZ were calculated and are presented in Table 1. The mean absorption times (MATs) of MDZ through intranasal, rectal, and buccal formulations were 0.23 h, 0.20 h, and 0.34 h in rabbits, respectively, which demonstrated that the absorption was rapid. In addition, i.n. and rectal routes may result in a faster absorption compared to the buccal administration. The bioavailabilities of MDZ via intranasal, rectal, and buccal route were around 60-70%
with no significant difference among these three different administration routes. The paired $t$-test analysis showed that the major pharmacokinetic parameters of MDZ between i.n. vs. buccal or i.n. vs. rectal administration were statistically similar. However, the $C_{\text{max}}$, $T_{\text{max}}$, MRT and MAT of MDZ following rectal administration showed statistically significant difference compared to that of buccal administration. Among the three administration route studied, the pharmacokinetic parameters showed that rectal administration would result in better absorption.

Table 1-1 Pharmacokinetic parameters of MDZ in rabbit after single intranasal, buccal, rectal or intravenous administration MDZ at 0.5 mg/kg. Data were shown as mean ± SD (n = 6)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Unit</th>
<th>Values (mean ±SD)</th>
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<tbody>
<tr>
<td></td>
<td>i.v.</td>
<td>buccal</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>h</td>
<td>1.80±0.18</td>
</tr>
<tr>
<td>$CL$</td>
<td>ml/min/kg</td>
<td>27.5±7.94</td>
</tr>
<tr>
<td>$V$</td>
<td>ml/kg</td>
<td>4329±1398</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>ng/ml</td>
<td>176±47.2</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>h</td>
<td>0.25±0.13</td>
</tr>
<tr>
<td>$MRT$</td>
<td>h</td>
<td>0.96±0.33</td>
</tr>
<tr>
<td>$MAT$</td>
<td>h</td>
<td>0.34±0.14</td>
</tr>
<tr>
<td>$AUC$</td>
<td>ng·h/ml</td>
<td>324±107</td>
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<tr>
<td>$F$</td>
<td>%</td>
<td>67.0±20.5</td>
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Table 1-2 Pharmacokinetic parameters of 1'-OH-MDZ in rabbit after single intranasal, buccal, rectal or intravenous administration MDZ at 0.5 mg/kg. Data were shown as mean ± SD (n = 6)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Unit</th>
<th>Values (mean ±SD)</th>
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<tbody>
<tr>
<td></td>
<td>i.v.</td>
<td>buccal</td>
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<tr>
<td>$t_{1/2}$</td>
<td>h</td>
<td>1.76±0.71</td>
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<tr>
<td>$C_{\text{max}}$</td>
<td>ng/ml</td>
<td>74.9±47.0</td>
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<tr>
<td>$T_{\text{max}}$</td>
<td>h</td>
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<tr>
<td>$MRT$</td>
<td>h</td>
<td>1.34±0.35</td>
</tr>
<tr>
<td>$AUC$</td>
<td>ng·h/ml</td>
<td>54.8±34.8</td>
</tr>
<tr>
<td>$F$</td>
<td>%</td>
<td>55.7±44.0</td>
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</table>

$^*P<0.05$, $^*^*P<0.01$, i.n. vs buccal administration;
$^*P<0.05$, $^*^*P<0.01$, rectal vs buccal administration;
$^*^*P<0.05$, $^*^*^*P<0.01$, i.n. vs rectal administration.
3.3 In vivo gender-dependent pharmacokinetics and metabolism differences.

To investigate the gender-dependent differences in pharmacokinetics and metabolism of MDZ, the substrate of CYP3A, the plasma concentration-time profiles of MDZ and 1'-OH-MDZ from the four routes in male and female rabbits are separated and presented in Fig. 4. The individual pharmacokinetic parameters showed that plasma exposure of MDZ in male rabbits is around 1.4-fold higher than that in the female rabbits via i.v. administration; whereas the exposure of metabolite in male rabbits is around 1.3-fold lower than that in the female rabbits. In addition, the plasma exposures to MDZ in male rabbits through the three other routes all exhibit slightly higher than that in female rabbits, whereas the exposures to metabolite are, on another hand, lower. The observed greater $\text{AUC}_{1'-\text{OH-MDZ}}/\text{AUC}_{\text{MDZ}}$ ratios in female rabbits via i.v. and alternative routes (Fig. 5) are consistent with those previously reported \(^{20}\). The in vivo pharmacokinetic study indicated rabbit CYP3A6 activity possesses a slight gender-dependent difference, with higher activity observed in female rabbits.
Fig. 4 Plasma concentration-time profiles of MDZ and 1’-OH-MDZ in male (diamond) and female (square) rabbits, after single intravenous (A), i.n.(B), buccal (C), or rectal (D) administration MDZ at 0.5 mg/kg. Data were shown as mean ± SD (n = 3).

Fig. 5 AUC$_{1’-OH-MDZ}$/AUC$_{MDZ}$ ratio of male and female rabbits, after single i.n., buccal, rectal or intravenous administration MDZ at 0.5 mg/kg. Data were shown as mean ± SD (n = 3).

3.4 Comparative enzyme kinetics of 1’-OH-MDZ formation in rabbit and human

Results from of the enzyme kinetics study of the formation of 1’-OH-MDZ in rabbit and human liver microsomes are presented in Fig. 6 and Table 2. The apparent Michaelis-Menten constant (K$_{m}$) was similar between species in 0.5 mg/ml microsomal incubations (7.86µM for rabbit and 8.66µM for human). V$_{max}$, the
maximum rate of 1’-OH-MDZ formation was a bit higher (1.56-fold) in rabbit (2117 pmol/min/mg protein) compared with that in human (1361 pmol/min/mg protein). Overall, the estimated intrinsic clearance ($V_{\text{max}}/K_{m}$) of 1’-OH-MDZ formation was 1.72-fold higher in rabbit than that in human liver microsomes.

Fig. 6 Enzyme kinetic study, monitored by 1’-OH-MDZ formation, was performed in human (A), rabbit (B) liver microsomes with NADPH.

Table 2 Enzyme kinetics parameters for the hydroxylation of midazolam by human and rabbit liver microsomes (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Rabbit</th>
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<td>$K_m$ (µM)</td>
<td>8.66±0.55</td>
<td>7.86±0.77</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (pmol/min/mg)</td>
<td>1361±21</td>
<td>2117±60</td>
</tr>
<tr>
<td>$CL_{\text{int}}$ (µl/min/mg)</td>
<td>158±10</td>
<td>271±26</td>
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4. Discussion:

Midazolam's unique chemical structure confers a number of properties that distinguish it from other benzodiazepines. It exhibits lipophilicity and penetrates the central nervous system quickly. It has a short onset and short duration of actions and is water soluble in acidic aqueous solution makes it an ideal agent for intravenous administration with less venous complications. Many clinical reports have described the successful use of midazolam in controlling refractory status epileptics because of
its remarkable anticonvulsant action\textsuperscript{21-22}. Due to the extensive oral first-pass effect, parenteral route administration which facilitates its clinical outcomes had been investigated. In China, buccal, intranasal and rectal preparations of midazolam have not been commercial available. The aim of our current study was to determine the pharmacokinetics of intranasal and rectal administration of midazolam in comparison with the buccal administration in rabbits with the ultimate goal of applying knowledge to help develop in future Imidazolam for administration in pediatric population.

The midazolam pharmacokinetics study via alternative routes had been performed in several laboratory animals including dogs\textsuperscript{23}, piglets\textsuperscript{24} and rabbits\textsuperscript{25}, but buccal administration was only conducted in children or volunteers\textsuperscript{26}. In the present study, the proper selection of test animal species and design fit for purpose study is important. Rats or hamsters are commonly used for \textit{in vivo} buccal drug permeability studies\textsuperscript{27}, however, such study design has limitations. Unlike humans, buccal epithelial tissues in most preclinical species animals are keratinized. In this study, rabbit was selected as the most suitable species for human (children) buccal drug delivery as they are small with non-keratinized buccal mucosa similar to humans\textsuperscript{28}. It is known that non-keratinised lining areas like the floor of the mouth and buccal mucosa are generally most permeable compared with the keratinised regions such as the hard palate and gingival\textsuperscript{29}. Due to the fact that rabbits are not the well studied preclinical species for drug metabolism enzymes, especially for common CYP3A substrate like midazolam, enzyme kinetics of midazolam was firstly compared in human (CYP3A4/5) and rabbit (CYP3A6) liver microsomes to assess if rabbit would
have similar human CYP3A mediated intrinsic clearances. Our data showed that rabbit and human have a similar *in vitro* clearance determined by comparable $K_m$ and a bit higher $V_{\text{max}}$ in rabbit. The rabbit CYP3A6 had a bit greater midazolam 1’-hydroxylation activity than human CYP3A4/5 based on the $CL_{\text{int}}$ value of 271.1µl/min/mg and 157.7µl/min/mg, respectively (about 1.7-fold).

It is well known that rats exhibit great gender-related difference in CYP3A2 activity with male rats exhibiting higher activity than female rats \(^{30}\). On the other hand, the activities of CYP3A4 could vary greatly in range of 14-fold among individual human liver microsomes without obvious gender difference \(^{31, 32}\). Through the current *in vivo* pharmacokinetics comparison, it was found that rabbit showed a slight gender-related difference in CYP3A6 activity, with higher activity in female compared to male rabbits. The mean 1’-OH-MDZ/MDZ ratio in female rabbits via i.v., i.n., buccal, and rectal routes are 1.95, 1.38, 3.34, and 3.58-fold higher than that in male rabbits, which partly explained that female steroid treated rabbits have more incidences to the development of osteonecrosis than male rabbits \(^{17}\). Although rabbit is not a commonly used laboratory animal for drug metabolism investigations, it has been used in a large number of pharmacology and toxicology studies, including atherosclerosis research \(^{33}\), the orthopedic related study \(^{34}\), reproductive toxicity \(^{35}\) or dermotoxicity evaluations.

Furthermore, recent data concerning the molecular clock, which regulates the circadian rhythmicity in mammals, provide better insight into the nature of the circadian rhythms in rabbits, which led to the circadian fluctuation of midazolam’s clearance \(^{36}\). Hence, the comparisons of CYP3A activity between rabbits and human
and the gender differences between male and female rabbits are of great value. According to our current findings, it can be assumed that the hepatic CYP3A activity of male rabbit is closer to that of humans than female rabbits.

Considering repeat blood sampling is a challenge task especially via marginal auricular vein duration a four-period crossover study, six rabbits with indwelling external jugular vein catheterization were employed. Blood sampling through the jugular catheter offers the advantage that accurate volume of blood can be withdrawn and the lost volume can easily be replaced as well. Our practice provided such a system ideally for multi-cross-over pharmacokinetic studies with minimal requirement of animal handling and minimized stress on animal.

It was observed in our study that the absolute bioavailability of MDZ via i.n., rectal, or buccal routes in rabbits were similar at around 60-70%. That is higher than the oral administration (35-44%) previously reported. It is well-known that MDZ is the substrate of CYP3A4 and subject to extensive intestinal and hepatic metabolism. Oral bioavailability (F) is a function of the fraction absorbed (F_{abs}), gastrointestinal or gut wall availability (F_{G}), and hepatic availability (F_{H}). The bioavailability via parenteral routes avoids the first-pass metabolism in gut and liver. The ratio of AUC_{(1'-OH-MDZ)/AUC_{MDZ}} in i.v. administration was 16.9%, while that after buccal, rectal and i.n. administration was 14.4%, 14.3%, and 12.5%, respectively. Extravenous bioavailability is closely related to the absorption barrier and the way enter the bloodstream. The absorption of drugs in the nasal cavity, mucosal membrane lining the cheeks, and rectal mucosal surface are facilitated by a relatively large
surface area and rich vascularisation, such as the rectal route $^9,^{40, 41}$. The absorbed drug then enters the reticulated veins, which directly accesses systemic circulation, bypassing the portal vein. In the present study, it was also demonstrated that the $T_{\text{max}}$ and $\text{MAT}$ of midazolam via intranasal and rectal administration (~10min) exhibited faster absorption than buccal (~20min). Rectally and intranasally administered formulation of midazolam, currently in development, might have the potential for use in out-patient emergency service, and therefore might contribute to decrease in the morbidity and mortality from status epilepticus, when intravenous access is difficult to administer. Future research should be directed to assess the clinical pharmacokinetics following these alternative routes of administration.

5. Conclusions

Our present study demonstrated that the pharmacokinetic profiles via i.n., rectal, and buccal routes in rabbits were similar with higher absolute bioavailabilities than that after single oral administration of midazolam. Intranasal and rectal administration exhibited faster absorption than buccal administration. In vivo pharmacokinetic study showed that rabbit CYP3A6 exhibited a slight gender difference with higher biotransformation activity of midazolam in females. In addition, rabbit CYP3A6 had comparable but slightly higher intrinsic clearance of midazolam compared to human CYP3A4 in pooled liver microsomal incubations, and that makes the male rabbit a better model for human regarding the CYP3A activity. Rectally and intranasally administered formulation of midazolam currently under development might have the potential to be an effective treatment of seizures in an out-patient emergency service.
Declaration of interest

The authors report no declarations of interest.

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