A Rapid and Sensitive UPLC–MS/MS Method for Determination of Remimazolam and Its Main Metabolite in Human Urine and Its Application in Clinical Urine Recovery Study

Ying Zhou^{1*}, Ming Lu² and Ji Jiang^{2*}

¹Department of Pharmacy, The First Affiliated Hospital of Nanchang University, Nanchang 330006, China

²Clinical Pharmacology Research Center, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100730, China

Received: 16 February 2017; accepted: 14 March 2017

Remimazolam is a new chemical entity belonging to the benzodiazepine class of sedative drugs. A sensitive and rapid method based on ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) has been developed and validated for the determination of remimazolam and its major carboxylic acid metabolite (M1) in human urine. Urine samples were prepared by dilution and analyzed using an isocratic chromatographic separation. Inter- and intra-batch results for remimazolam were within 10.7% for accuracy and 5.5% for precision, and for M1, within 5.8% for accuracy and 4.2% for precision, respectively. This study represents the first reported example for the quantification of remimazolam and its main metabolite in human urine. Furthermore, this method has been successfully applied for the urine recovery study of remimazolam in Chinese healthy subjects. Only about 0.01% of the administered remimazolam dose was eliminated in the urine over the 24 h period in the form of unchanged remimazolam, and more than 75.1% of the administered dose was eliminated in the form of M1. Remimazolam is excreted mainly in the form of M1 in urine after intravenous administration, and there is no excessive accumulation in vivo after administration of remimazolam.

Keywords: Remimazolam, metabolite, UPLC–MS/MS, urine recovery study, analytical method

1. Introduction

Remimazolam, which is designed to undergo rapid hydrolysis in the body by nonspecific tissue esterases to its pharmacologically inactive carboxylic acid metabolite (M1), is a new chemical entity belonging to the benzodiazepine class of sedative drugs [1]. Preclinical and clinical studies showed that remimazolam permitted a faster onset, a shorter duration of sedative action, and a more rapid recovery than currently available short-acting sedatives [2, 3]. Therefore, because of its organ-independent metabolism and fast-acting onset and recovery, remimazolam appears to have potential advantages when used as an intravenous sedative agent for the induction and maintenance of anesthesia.

Remimazolam tosylate was approved by China Food and Drug Administration as an investigational new drug for the potential use in anesthesia in 2013 and is currently being evaluated in phase 3 trials. As we know, it is necessary to know about the route and extent of urinary excretion of a new drug, and the above information expresses the drug accumulation in vivo which is closely related to safety of the drug in human. Thus, it is necessary to develop a rapid and sensitive method to determine remimazolam and its main metabolite in human urine. To the best of our knowledge, there have been no other reports in the literature pertaining to the systematic determination of remimazolam and its main metabolite M1 in urine.

In this study, a sensitive and rapid method based on ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) has been developed and validated for the determination of remimazolam and its major carboxylic acid metabolite (M1) in human urine [4]. The method was successfully applied for the pharmacokinetic studies of remimazolam to in-

vestigate the route and extent of its urinary excretion in Chinese healthy subjects.

2. Materials and methods

2.1 Chemicals and reagents. Remimazolam tosylate and its internal standard [¹³C₂] remimazolam, carboxylic acid metabolite of remimazolam, and its internal standard [¹³C₂] M1 (chemical structures are shown in Figure 1) were provided by Jiangsu Hengrui Medicine Co., Ltd. (Jiangsu, China). Methanol and acetonitrile (HPLC grade) was purchased from Burdick & Jackson. Ammonium formate and formic acid (analytical grade) were supplied by Sigma-Aldrich, respectively. HPLC-grade water was obtained using a Milli-Q system (Millipore, Bedford, USA). Drug-free human urine was obtained from six different healthy subjects who were drug-free for at least 2 weeks.

2.2 Chromatographic and MS conditions. The chromatographic separation was carried out on an Acquity UPLC Core system (Waters Co., MA, USA). Isocratic elution was applied at a flow rate of 0.4 mL/min using an Acquity UPLC CSH C₁₈ (50 mm × 2.1 mm, 1.7 μm) column at 25 °C. Mobile phase A was acetonitrile, and mobile phase B was water containing 10 mM ammonium formate and 0.1% formic acid (A–B = 40:60, v/v). The run time was 1.5 min, and the injection volume was 7.5 μL.

Mass spectrometric analysis was performed using Xevo-TQS triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source in the positive mode (Waters Co., MA, USA). The detection was operated in the multiple reaction monitoring (MRM) mode, and the MRM transitions of remimazolam and its IS were m/z 439 → 362 and m/z 444 → 412, and those of M1 and its IS were m/z 425 → 407 and m/z 430 → 412, respectively. The ionization source conditions were cone voltage,

* Author for correspondence: zyyiva@163.com, pk.frosh@gmail.com

This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (<https://creativecommons.org/licenses/by-nc/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium for non-commercial purposes, provided the original author and source are credited, a link to the CC License is provided, and changes - if any - are indicated.

Figure 1. Chemical structures of remimazolam tosylate (a), M1 (b), [¹³C₂] remimazolam (c), and [¹³C₂] M1 (d)

45 V; capillary voltage, 3 kV; source temperature, 150 °C; and desolvation temperature, 600 °C. The optimized collision energy of remimazolam and its IS, and M1 and its IS were 30 V and 30 V, and 20 V and 18 V, respectively. The cone and desolvation gas flow rates were 150 and 1000 L/h, respectively. Data acquisition and processing were performed using MassLynx software (version 4.1).

2.3 Preparation of calibration standard (CS) and quality control (QC) samples. Stock solutions of remimazolam and M1 for CS and QC were prepared separately in methanol to a final concentration of 1 mg/mL for each analyte, and stock solutions of their IS (1 mg/mL) were also prepared separately in methanol. These stock solutions were further diluted to yield working solutions at several concentration levels.

Calibration standards and QC samples in urine were prepared by diluting corresponding working solutions with drug-free human urine, respectively. The final concentrations of calibration standards of remimazolam were from 0.5 to 1000 ng/mL and those of M1 were from 20 to 40,000 ng/mL. The final concentrations of QC samples for the evaluation of intra- and inter-batch precision and accuracy, matrix effect, and stability were 1.5, 30, and 750 ng/mL for remimazolam and 60, 1200, and 30,000 ng/mL for M1, respectively. IS working solutions (100 ng/mL for remimazolam and 80 ng/mL for M1) were prepared with mobile phase (A–B = 40:60, v/v). All the solutions were stored at –30 °C and brought to room temperature (25 °C) before use. Urine samples were stored at –70 °C.

2.4 Sample preparation. Urine samples were pretreated by dilution procedure with different dilution factors. Urine samples for the quantitative determination of remimazolam were prepared by diluting 50 µL of urine sample with 250 µL of IS working solution (100 ng/mL [¹³C₂] remimazolam in mobile phase). Urine samples for the quantitative determination of M1 were prepared by diluting 50 µL of urine sample with 950 µL of mobile phase. Then, 30 µL of the diluted urine sample was combined with 270 µL of IS working solution (80 ng/mL [¹³C₂] M1 in mobile phase). Following vortex mixing (for 1 min) and centrifugation (for 5 min at 16,242 × g) to settle any particulate matter, 7.5 µL of the supernatant was injected to the UPLC–MS/MS system.

2.5 Method validation. The method was validated for selectivity, sensitivity, linearity, precision and accuracy, matrix effects, and stability according to the US Food and Drug Administration (FDA) (2013) [5] and China Food and Drug Administration (CFDA) guidelines (2005) [6] for the validation of bioanalytical methods.

The selectivity of this method was evaluated by comparison of the chromatograms of remimazolam and M1 at the LLOQ to those of six individual human blank urine samples.

Calibration standards in human urine were prepared and analyzed in three independent runs. The relative standard deviation (RSD) was calculated for all slopes of calibration curves. The LLOQ is defined as the lowest concentration on the calibration curve. Intra- and inter-batch precision and accuracy were determined by measuring the concentrations of analyte in urine in five replicates of QC samples at low, medium, and high concentrations for three separate batches.

Matrix effects were evaluated to identify any suppression or enhancement of signal from an interfering substance around the retention time of remimazolam and M1. The matrix effects were investigated at three levels (1.5, 30, and 750 ng/mL for remimazolam; 60, 1200, and 30,000 ng/mL for M1) by comparing the peak areas of post-extraction blank urine samples from six different subjects spiked with analyte with that of the standards in mobile phase at the same concentration level. The inter-subject variability of matrix effects at each concentration level should be less than 15% [7–9].

The stabilities of remimazolam and M1 in biological matrices and in working solution at different storage conditions were evaluated as follows: The short-term stability of analytes in human urine was assessed after 24 h of storage at room temperature. The long-term stabilities of analytes in human urine and in working solution were assessed after 291 days of storage in a freezer at –70 °C and 44 days of storage in a freezer at –30 °C, respectively. The stability of analytes was assessed after three freeze–thaw cycles (–70 °C to room temperature). The stability of analytes in extracts was also tested after 24 h at 10 °C.

2.6 Application of the method to urine recovery study. The validated method was applied to quantify urine

concentrations of remimazolam and M1 in a urine recovery study. Thirty-six healthy subjects were enrolled in this single ascending-dose study (doses ranging from 0.10 mg/kg to 0.35 mg/kg) of remimazolam administered as a 1 minute intravenous (IV) injection. Urine samples were continuously collected up to 24 h prior to and in the following intervals: 0 to 4, 4 to 8, 8 to 12, and 12 to 24 h. Aliquots of urine samples were stored at -70°C until analysis. The study was conducted in accordance with International Conference on Harmonization Good Clinical Practice guidelines, all applicable subject privacy requirements, and the ethical principles outlined in the Declaration of Helsinki. The study was approved by the Ethics Committee of Peking Union Medical College Hospital, and all subjects signed the informed consent form before the study.

3. Results

3.1 Validation procedure

3.1.1 Selectivity and sensitivity. No peaks interfering were observed at the retention times of remimazolam, M1, and the internal standards in the blank urine samples. Typical MRM chromatograms obtained from blank urine, blank urine spiked with remimazolam or M1 (LLOQ) and their relative internal standards, and a subject's urine sample obtained at interval of 0–4 h after 1 minute IV administration of 0.35 mg/kg remimazolam tosylate injection are shown in Figure 2.

3.1.2 Linearity, precision, and accuracy. Calibration curves ranging from 0.5 to 1000 ng/mL for remimazolam and from 20 to 40,000 ng/mL for M1, respectively, were established. Calibration curves were regressed using linear equation with a weighting factor of $1/x^2$. Correlation coefficients of all calibration curves were more than 0.99. The deviations of back-calculated

concentrations of calibration standards from their nominal values were between -0.5% and 1.6% for LLOQ, and between -6.5% and 4.4% for all other calibration levels.

Precision and accuracy values were determined on three different batches by measuring five replicates of QC samples at three concentration levels. The results are listed in Table 1. It indicated that the method provided adequate precision and accuracy for the determination of two analytes since RSD% was $<15\%$ for all the investigated concentrations and RE% was within $\pm 15\%$.

3.1.3 Matrix effects. Matrix effects and inter-subject variability data from three concentrations of QC urine samples are summarized in Table 2. The inter-subject variabilities were no more than 10.2% for both analytes. This indicated little or no difference in ionization efficiency of remimazolam and M1 from different urine lots. The urine assay was not tested for recovery as the sample preparation involved a simple dilution step only.

3.1.4 Stability. The stability tests of the analytes were designed to cover expected conditions of handling of clinical samples. The results demonstrated that remimazolam and M1 were stable in

Table 2. Results of matrix effects of remimazolam and M1 in human urine ($n = 6$)

Compound	Nominal Concentration (ng/mL)	Matrix effects% (inter-subject variability% ^a)
Remimazolam	1.50	84.8 (3.6)
	30.0	89.7 (2.9)
	750	88.5 (1.7)
M1	60.0	110.7 (4.7)
	1200	108.3 (3.0)
	30,000	107.3 (10.2)

^aExpressed as RSD %.

Figure 2. Representative MRM chromatograms of remimazolam, M1, $[^{13}\text{C}_2]$ remimazolam, and $[^{13}\text{C}_2]$ M1: (1) blank urine sample, (2) blank urine sample spiked with two analytes at LLOQ and two internal standards (100 ng/mL for remimazolam and 80 ng/mL for M1), and (3) urine sample collected from a healthy subject at interval of 0–4 h after 1 minute IV administration of 0.35 mg/kg remimazolam tosylate injection

Table 1. Accuracy and precision of intra- and inter-batch for the detection of remimazolam and M1 in human urine ($n = 5$)

Compound	Nominal concentration (ng/mL)	Intra-batch accuracy (%)	Inter-batch accuracy (%)	Intra-batch precision (RSD%)	Inter-batch precision (RSD%)
Remimazolam	1.50	94.0	89.3	3.6	3.9
	30.0	103.3	97.3	4.3	5.5
	750	97.9	95.9	3.6	3.0
M1	60.0	97.3	96.3	3.8	4.2
	1200	95.0	94.2	3.8	3.1
	30,000	99.0	98.3	2.5	2.1

Table 3. Results of stability of remimazolam and M1 under different storage conditions ($n = 5$)

Storage condition	Nominal concentration (ng/mL)		Measured mean concentration (RE%) (ng/mL)	
	Remimazolam	M1	Remimazolam	M1
Short-term stability ^a	1.50	60.0	1.52 (1.7)	64.7 (7.8)
	30.0	1200	28.8 (−4.2)	1259 (4.9)
	750	30,000	736 (−1.8)	30,757 (2.5)
Long-term stability ^b	1.50	60.0	1.65 (10.1)	55.1 (−8.1)
	30.0	1200	30.7 (2.5)	1140 (−5.0)
	750	30,000	763 (1.7)	27,937 (−6.9)
Freeze–thaw stability ^c	1.50	60.0	1.45 (−3.4)	65.1 (8.5)
	30.0	1200	32.7 (9.0)	1275 (6.3)
	750	30,000	823 (9.8)	30,795 (2.7)
Auto-sampler stability ^d	1.50	60.0	1.68(12.1)	65.0 (8.4)
	30.0	1200	29.6 (−1.5)	1312 (9.3)
	750	30,000	738 (−1.5)	32,211 (7.4)

^aStored at room temperature (25 °C) for 24 h.^bStored at −70 °C for 291 days.^cAfter three freeze–thaw cycles.^dKept at 10 °C for 24 h.**Table 4.** Urinary excretions of remimazolam and M1 in Chinese healthy subjects after 1 minute IV administration of remimazolam tosylate injection of different doses ($n = 6$)

Collection interval (h)	0.10 mg/kg	0.15 mg/kg	0.20 mg/kg	0.25 mg/kg	0.30 mg/kg	0.35 mg/kg
Remimazolam						
0–4	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
4–8	–	–	–	–	–	–
8–12	–	–	–	–	–	–
12–24	–	–	–	–	–	–
Total	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
M1						
0–4	54.7 ± 8.91	55.0 ± 5.58	58.5 ± 8.90	58.3 ± 7.05	63.4 ± 3.17	50.6 ± 10.5
4–8	16.4 ± 4.23	17.1 ± 5.86	19.6 ± 4.61	19.2 ± 5.21	19.4 ± 3.05	17.0 ± 2.75
8–12	5.70 ± 2.10	5.74 ± 3.38	6.47 ± 1.76	5.07 ± 2.45	4.79 ± 2.34	5.66 ± 2.41
12–24	2.17 ± 1.19	1.97 ± 0.83	1.98 ± 0.49	1.95 ± 1.12	1.87 ± 0.30	1.83 ± 0.98
Total	79.0 ± 7.98	79.8 ± 13.1	86.5 ± 6.04	84.5 ± 7.05	89.5 ± 4.07	75.1 ± 9.16

Results are expressed as percentage of the dose (mean ± SD).

–, The concentrations of remimazolam in urine are below the level of quantitation.

human urine after three freeze–thaw cycles, at room temperature and 10 °C in autosampler post-extraction for 24 h and in a freezer set to −70 °C for 291 days. Working solutions of remimazolam and M1 stored at −30 °C for 44 days also showed good stability. The stability results are summarized in Table 3.

3.2 Application of the method to urine recovery study.

The present UPLC–MS/MS method was successfully applied to investigate the urine pharmacokinetic profile of remimazolam after 1 minute IV administration of remimazolam tosylate injection to Chinese healthy subjects. Table 4 shows the urinary excretion of remimazolam and M1 in subjects treated with different doses of remimazolam tosylate injection. Only about 0.01% of the administered remimazolam dose was eliminated in the urine over the 24 h period in the form of unchanged remimazolam, and more than 75.1% of the administered dose was eliminated in the form of M1. In the meanwhile, the majority of remimazolam and M1 were excreted during the 0 to 4 h collection interval (about 67.4% of total amount of remimazolam and M1 excreted in the urine). The maximum rate of remimazolam excretion occurred during the 0 to 4 h collection interval.

4. Discussion

4.1 Sample preparation. Direct dilution was used to prepare urine samples because urine samples are cleaner compared with plasma samples and no interference was found in the blank urine sample. Urine samples were diluted with different dilution factors to bring concentrations to a linear range.

4.2 UPLC–MS/MS optimization. An UPLC–MS/MS method for the detection of remimazolam and M1 in human urine was developed based on the previous study. The $[M + H]^+$ were chosen as the precursor ions for all analytes, and the fragment ions having only about 20% of the abundance of that

of the molecular ions were chosen as the precursor ions for $[^{13}C_2]$ remimazolam and $[^{13}C_2]$ M1 in order to avoid the “cross-talk” effect caused by isotopic peak of bromine. Also, we chose the product ions based on the most abundant peak, cleaner chromatogram, and higher S/N ratio.

During the development of chromatographic conditions, mobile phase systems with different pH values and different organic solvents, such as methanol and acetonitrile, were investigated. When acetonitrile–water containing 10 mM ammonium formate and 0.1% formic acid (40:60, v/v) pumped at a flow rate of 0.4 mL/min at 25 °C was used as eluent, a good chromatographic profile and sensitivity were achieved. After careful comparison of several columns, an Acquity UPLC CSH C₁₈ (50 mm × 2.1 mm, 1.7 μm) column was proved to be more suitable as it can provide sufficient retention and better peak shape for analytes.

4.3 Urine recovery study. Urine recovery study showed that only about 0.01% of the administered remimazolam dose was eliminated in the urine over the 24 h period in the form of unchanged remimazolam, and more than 75.1% of the administered dose was eliminated in the form of M1. The rest 25% of the initial dose were eliminated in the forms of oxidated and glucuronide metabolites [10]. The maximum rate of remimazolam excretion occurred during the 0 to 4 h collection interval. Based on these data, it can be concluded that remimazolam is excreted mainly in the form of M1 in urine after intravenous administration and there is no excessive accumulation in vivo after administration of remimazolam.

5. Conclusion

A simple, sensitive, and specific UPLC–MS/MS method has been developed and validated for the determination of

remimazolam and its main metabolite M1 in human urine, which requires a very low sample volume and takes a very short time. Based on the results of the validation of the current study, it is envisaged that this simple, highly sensitive, and specific method for determination of remimazolam and M1 in human urine could apply a better understanding of the urinary excretion of remimazolam, which gives important information about accumulation and safety in Chinese healthy subjects after intravenous administration of remimazolam. This study represents the first reported example for the quantification of remimazolam and its main metabolite in human urine and has been successfully applied for the PK study of remimazolam to investigate the route and extent of its urinary excretion in Chinese healthy subjects.

6. Ethical approval

The study was conducted in accordance with International Conference on Harmonization Good Clinical Practice guidelines, all applicable subject privacy requirements, and the ethical principles outlined in the Declaration of Helsinki.

7. Informed consent

Informed consent was obtained from all individual participants included in the study.

Acknowledgments. The author thanks all of the subjects who participated in the clinical study.

This study was financially supported by Jiangsu Hengrui Medicine Co., Ltd. This work was also supported by a grant from the National Program on Key Research Project of New Drug Innovation (No. 2012ZX09303006-002).

References

1. Kilpatrick, G. J.; McIntyre, M. S.; Cox, R. F.; Stafford, J. A.; Pacofsky, G. J.; Lovell, G. G.; et al. *Anesthesiology* **2007**, *1*, 60.
2. Rogers, W. K.; McDowell, T. S. *IDrugs* **2010**, *12*, 929.
3. Upton, R. N.; Somogyi, A. A.; Martinez, A. M.; Colvill, J.; Grant, C. *Br J Anaesth* **2010**, *6*, 798.
4. Zhou, Y.; Wang, H.; Jiang, J.; Hu, P. *J Chromatogr B Analyt Technol Biomed Life Sci* **2015**, 976–977, 78.
5. US FDA, *Guidance for Industry Bioanalytical Method Validation*, **2013**, www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf, Accessed 22 Aug 2016.
6. China Food and Drug Administration, *Guideline on clinical pharmacokinetic study of drugs*, **2005**, www.sfda.gov.cn/directory/web/WS01/images/u6Rp9KpzuB2bSy0qm0+ravwabRp9HQvr+8vMr11ri1vNStlPlucGRm.pdf, Accessed 22 Aug 2016.
7. Taylor, P. J. *Clin Biochem* **2005**, *38*, 328.
8. Matuszewski, B. K.; Constanzer, M. L.; Chavez-Eng, C. M. *Anal Chem* **2003**, *75*, 3019.
9. Matuszewski, B. K. *J Chromatogr B Analyt Technol Biomed Life Sci* **2006**, *830*, 293.
10. Zhou, Y.; Hu, P.; Jiang, J. *J Pharm Biomed Anal* **2017**, *137*, 78.