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Original Article

Development and optimization of a RP-HPLC method to quantify midazolam in rat plasma after transdermal administration: validation and application in pharmacokinetic study

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Abstract: A reversed phase high performance liquid chromatographic assay coupled with UV detection (239 nm) has been developed and validated for the determination of midazolam in rat plasma samples after transdermal administration. A liquid-liquid extraction was used to extract the compound from plasma sample. The separation was performed on a Hypersil ODS C18 column using a mobile phase composed of acetonitrile and 0.1% triethylamine aqueous solution (52:48, V/V), pumped at a flow rate 1.0 mL min^{-1}. The calibration curves showed good linearity with correlation coefficient higher than 0.998 for all analytes in the range 0.10-10.0 μg mL^{-1}. Accuracy in the measurement of quality control (QC) samples was in the range 95-107% of the nominal values. The intra-day and inter-day precisions in the measurement of QC samples were less than 10% coefficient of variation. The developed method is suitable for quality control of midazolam in their mixtures and in transdermal delivery system pharmaceutical preparations. The validated assay was found to be suitable for the pharmacokinetic study of midazolam in rats with transdermal administration.

Keywords: Midazolam, transdermal, pharmacokinetic, liquid chromatography

Introduction

Midazolam (MDZ, CAS [59467-70-8]) is a short-acting benzodiazepine compound and its chemical structure is shown in **Figure 1**. MDZ is commonly used in intravenous anesthesia induction, short-term sedation and anticonvulsant [1]. The compound shows the inhibitory action on the central nervous system results from its interactions with the GABAA receptors, which are present in several brain regions [2], therefore it is widely used as a premedicant medicine before surgery as for its relative safety when compared with barbiturates as it do not lead to coma when used in high doses [3-5]. While the most important drawback of the drug applying in clinic is the necessity of repeated intravenous or intramuscular administration since the duration of elimination half-life in human is relatively short, which is disadvantage for patient compliance [6].

A transdermal patch contained Midazolam is developed in our laboratory. The transdermal drug delivery systems (TDDS) have been successfully developed to treat a variety of conditions from smoking cessation to pain [7], and the TDDS has a number of advantages over other administration, as it provides continuous administration of drug through the skin, which maintains constant plasma drug levels [8]. Continuous delivery of drug may reduce systemic side effects associated with high plasma drug levels [9]. The multiday dosing that is made possible by the sustained delivery of drugs with short half-life, which would require frequent dosing if given intravenous or intramuscular administration, improves patient compliance [10].

Several analytical methods have been published for the determination of MDZ with or without its metabolites in different biological

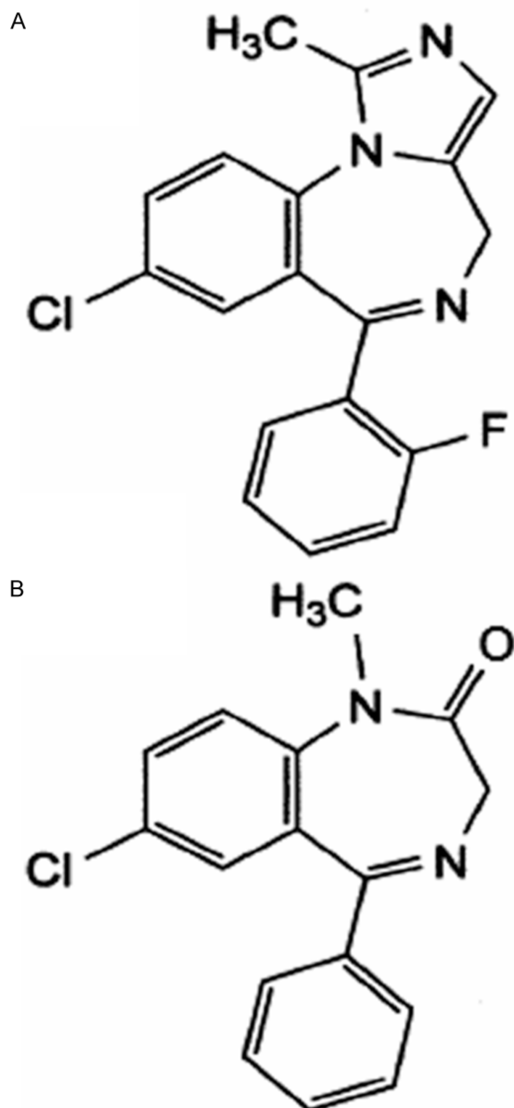

Figure 1. The chemical structures of (A) midazolam and (B) diazepam.

fluids, such as in serum, plasma, urine or whole blood. These techniques utilized high performance liquid chromatography (HPLC) with UV detection [11-14], HPLC-mass spectrometry (MS) [15-17], and gas chromatography-mass spectrometry (GC-MS) [18]. Although these methods utilizing MS detectors are more specific and sensitive than HPLC-UV assays, and provide very low limits of detection, the essential equipment may not be available in many laboratories. Most of the HPLC-UV methods suffer from various limitations, including inadequate sensitivity; use of expensive solid phase extraction cartridges, long run times, or rigorous operating requirement of mobile phase [11-14, 19].

Herein, we present a HPLC-UV method for the assay of MDZ in rat plasma and its validation in transdermal drug delivery system. The method offers the advantage of simplicity, specificity, sensitivity, and low sample volume to perform pharmacokinetic studies of midazolam. The simplicity of our proposed method is facilitated by using a single-step liquid extraction procedure rather using the expensive solid phase extraction cartridges. The mobile phase was modified with organic alkali rather than phosphate, which made the two similar compounds, midazolam and diazepam, separate well. The sensitivity was good enough to monitor MDZ level in plasma for 24 h after transdermal administration. Therefore, we report an application of the method to determine MDZ pharmacokinetics in rat after a single transdermal administration. To our knowledge this is the first method described for the quantification of MDZ in transdermal drug delivery system.

Experimental

Chemicals and reagents

Midazolam was purchased from the Enhua Pharmaceutical Co. Ltd. (Xuzhou, Jiangsu Province, China). The internal standard (I.S.) diazepam was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methylene dichloride, acetonitrile (all HPLC grade), triethylamine, and sodium hydroxide were purchased from Dahua Chemic Regent Company (Guangzhou, China). Methanol was of HPLC grade from SIYOU Co. Ltd. (Tianjin, China). All other reagents and solvents used were of analytical grade and water was milli-Q grade.

Apparatus and chromatographic conditions

Liquid chromatography was carried out on an Agilent 1200 series HPLC apparatus which consisted of a quart pump, a degasser, a Rheodyne model 7125 injector with 20 μL loop (Rheodyne Inc., Cotata, CA, USA), a UV detector, a column oven and a LC1200 workstation (Agilent, PA, USA). The separations were performed on a Hypersil ODS C_{18} column of 250×4.6 mm i.d., with 5 μm particle size (Thermo, USA). The mobile phase consisted of a mixture of acetoni-

trile and 0.1% triethylamine aqueous solution (52:48, V/V), at a flow rate of 1.0 mL min^{-1}. The mobile phase was prepared daily and filtered under vacuum through a 0.45 μm membrane filter before use. The eluate was continuously monitored using a UV detector at wavelength 239 nm. Separations were maintained at 25°C.

Preparation of calibration standards and quality control samples

A stock solution of MDZ (2 mg mL^{-1}) was diluted to a series of working standard solutions of MDZ containing 1, 2, 4, 10, 20, 40, 100 μg mL^{-1} in methanol, and the internal standard working solution of 2.0 μg mL^{-1} was prepared in methanol.

Twenty microliters of these working MDZ solutions were added to 0.18 mL drug-free plasma to prepared the calibration standard samples containing 0.1, 0.2, 0.4, 1.0, 2.0, 4.0, 10.0 μg mL^{-1} of MDZ. Quality control (QC) samples with low, medium, and high concentrations of 0.4, 4.0, and 10.0 μg mL^{-1} of MDZ were prepared in the same.

Extraction of MDZ from plasma

0.2 mL plasma (blank plasma, sample plasma, and plasma spiked with MDZ for preparation of calibration and QC samples) were added 10 μL I.S. working solution, 10 μL of 2 mol L^{-1} sodium hydroxide, and 1 mL mixed organic solvents (n-hexane: methylene dichloride: isopropanol = 64:33:3, V/V/V) in turn. The mixture was vortex mixed for 1 min and centrifuged at 3,000 g for 10 min, and then the organic phase was transferred into another tube and evaporated to dryness at room temperature with the aid of a gentle stream of nitrogen. The residue was dissolved in 400 μL of methanol, and aliquot of 20 μL was injected into the LC system for analysis.

Method validation

Specificity: Chromatograms obtained from blank plasma, spiked plasma, and plasma from treated rat, were compared to determine the level of interference of endogenous components with MDZ and I.S.

Linearity and sensitivity: For the evaluation of linearity, calibration curves were constructed by plotting the peak area ratio of MDZ to I.S. vs concentration of MDZ, and fitted by the least square method with a $1/C^2$ weighting factor. Calibration curves containing seven points were assayed in duplicate on three consecutive days over the range 0.10-10.0 μg mL^{-1}.

The LOQ was defined as the limit of quantification with a signal-to-noise ratio of >10, a precision of RSD <15% and RE <10%, and verified by five replicates [20].

Precision, accuracy and extraction recovery: QC samples at three different concentrations (0.4, 4.0, and 10.0 μg mL^{-1} for MDZ) were prepared and analyzed. The intra- and inter-day assays were repetitively carried out on the plasma samples five times a day and once a day for three sequential days, respectively. The accuracy was calculated as relative error (RE, %) between nominal and measured concentrations. The precision was determined as the relative standard deviation (RSD, %) of the measured concentrations. The intra- and inter-day precisions were required to be below 15%, and the accuracy to be the range from -20% to 10%. (the VICH Steering Committee, European Medicines Agency, 2009).

To assess the plasma extraction recovery, two series of five replicates of QC samples at low, middle and high concentration were processed as described above, one with plasma and another with mobile phase. The extraction recovery was obtained by calculating the area ratio of spiked plasma samples to the corresponding samples with mobile phase instead.

Pharmacokinetic study

A transdermal patch containing MDZ were prepared in our laboratory with the method as follow. Briefly an acrylic resin composition (Hecheng Pharmaceuticals trade Ltd., Nanjing, China) was dissolved in ethyl acetate at ambient temperature. MDZ was solvent in a mixture solvent of ethanol and propanediol, and Azone as the penetration enhancer were then added to the above solution that was agitated at room temperature for 20 min, and then homogenized for 10 min. The mixture was applied to the surface of a flexible backing membrane (Kangbeide Pharmaceuticals, Ltd., Beijing, China), placed at static state for 30 min, and dried at 40°C for 0.5 h and at 60°C for 1.5 h. After cooling, the

patch was cut to pieces (4×4 cm^2, containing 9 mg of MDZ), covered by a protective membrane layer, and packed by aluminum-plastic membrane.

Forty-five Sprague-Dawley rats (body weights 250±20 g) purchased from Guangdong Medical College Laboratory Animal Center (Zhanjiang, China), were used to study the pharmacokinetics of MDZ patch. All experimental procedures were performed in accordance with guidelines on experimental (pre-clinical) study of new pharmacological substances (Sino Food and Drug Administration, 2007). Rats were fixed and patch was applied to the shaved skin. Blood samples were collected at specific time points till 48 h, and centrifuged at 13,000 rpm for 10 min. Plasma (200 μL) was separated and stored at -20°C till analyzed [21].

The plasma concentrations of MDZ at different times were expressed as means ± SD, and the mean concentration-time curve was plotted. All calculations of pharmacokinetic data were performed with the program of Drug and Statistics 3.0 (DAS, T.C.M. Shanghai, China).

Result and discussion

Optimization of chromatographic conditions

MDZ and diazepam, the I.S, are both typical benzodiazepine compounds with basic group in the molecules as for the presence of a imidazole ring in the 1, 2-position (**Figure 1**). The nitrogen in the 2-position provides sufficient basicity (pKa 6.15). In strong acidic solutions the diazepine ring reversibly opens between the positions 4 and 5, producing a polar water-soluble primary amine derivative. At physiological pH values, approximately 96% of MDZ is bound to plasma proteins [22]. Therefore, during the extraction of MDZ and I.S. from plasma with organic solvents, sodium hydroxide solution (2 mol L^{-1}) was added to increase the efficiency of the extraction procedure. The extraction recovery of MDZ was increased up to 92% when alkali was added into the plasma samples, in contrast it was only 68% when no basification of the samples, and the extraction recovery of I.S. was increased from 64% to 90%.

The extraction solvents used by most of the HPLC reports for MDZ include using either diethyl ether, ethyl acetate, n-hexane or diethyl ether/methylene chloride [23-25], while the combination of methylene dichloride and n-hexane in different proportions were previously tested and the mixed organic solvents (n-hexane: methylene dichloride: isopropanol = 64:33:3, V/V/V) was found be an ideal extraction solvent. In reported liquid-liquid extraction method, the extraction solvent was diethyl ether [14]; moreover, the dosage of extraction solvent was 4 ml per sample, which was profuse in organic solvent though the recovery is high up to about 100%. In this method, the dosage of extraction solvent was 1 ml per sample, and the extraction recovery of MDZ and the I.S. was up to 92% and 90%, with a CV less than 13% and 11%, respectively. The recovery was suitable in the application of pharmacokinetic assay and the precision and accuracy were accordance with the claim (the VICH Steering Committee, European Medicines Agency, 2009). Compared with other methods utilizing liquid-liquid extraction [14, 26], this method showed better or at least similar results in the determinations of MDZ.

Diazepam was used as the internal standard because its molecular structure was similar with MDZ, but unfortunately, the two similar compounds were hardly separated. So a suitable mobile phase was necessary. For a sharp and symmetrical peak, the mobile phase was should be alkali, 15 mM KH2PO4 was used to adjust the pH value in reported method [14]. In our preliminary experiments, several mixtures of organic solvents and phosphate solution were tested as mobile phases, the results were not satisfactory. When methanol or acittrile and phosphate solution were mixed on line, the base line of pump pressure was not smooth, it went down sharply and reverted quickly, always unbalanced. The phenomenon could be explained for the dissolution of phosphate. When phosphate aqueous solution were mixed with organic solvents, the solubility changed and some tiny crystal of phosphate separate out the solution, so the pump pressure is not balanced. It also was validated by the following investigation, when the organic solvents and phosphate solution were mixed beforehand in breaker, it was transparent or cloudy related with the order of interfusing the solvents or aqueous solution. Since the phosphate solution is unsatisfactory in the method, some other alkaline solutions were considered and

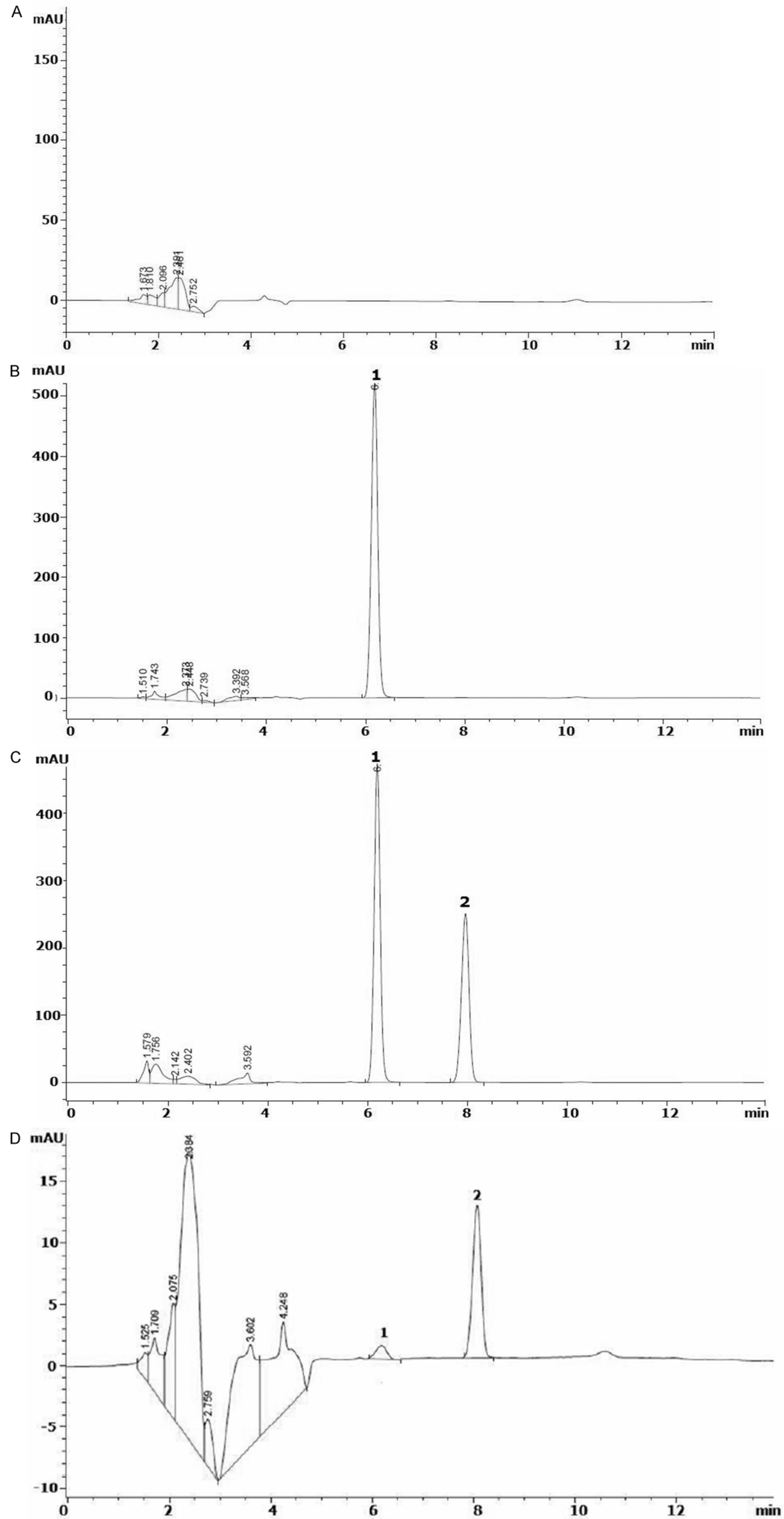
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Figure 2. LC chromatograms obtained from: A. A blank rat plasma; B. A rat plasma spiked with Midazolam reference at 50 μg mL^{-1}; C. A rat plasma spiked with Midazolam and Diazepam at 40 μg mL^{-1} and 1.0 μg mL^{-1}, respectively; D. A rat plasma collected at 2 h after a transdermal administration of 9 mg Midazolam per patch, spiked with I.S. at 0.1 μg mL^{-1}, Peaks: 1 = Midazolam, 2 = Diazepam (I.S.).

Table 1. Intra- and inter-day accuracy and precision (*n* = 6)

Nominal concentration (μg mL^{-1})	Measured concentration (Mean ± SD, μg mL^{-1})	Accuracy (RE, %)	Precision (RSD, %)	Recovery (%)
Intra-day				
0.4	0.43±0.02	7.5	3.6	80.2
4.0	4.24±0.06	6.0	1.3	84.6
10.0	9.47±0.14	-5.3	1.4	86.4
Inter-day				
0.4	0.36±0.02	-10	6.8	--
4.0	4.18±0.33	4.2	8.0	--
10.0	10.62±0.78	6.2	7.3	--

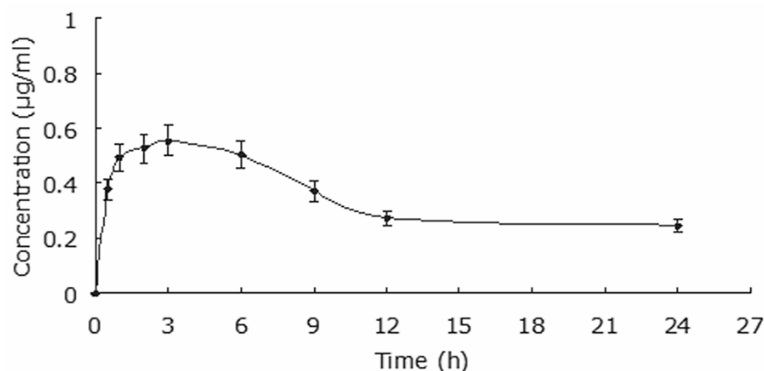

Figure 3. Mean plasma concentration-time profile for Midazolam in rat plasma after a transdermal administration of 9 mg Midazolam per patch.

triethylamine was selected finally. The mixture of acetonitrile and 0.1% triethylamine aqueous solution (52:48, V/V) was confirmed for the optimal mobile phase; MDZ and I.S. were separated well with this mobile phase.

Method validation

Specificity: The specificity of the method was demonstrated by comparing chromatograms of samples from blank plasma, drug-spiked plasma and sample plasma. The typical chromatograms were obtained with a drug-free plasma, a naive plasma spiked with MDZ and a plasma sample containing 0.56 μg mL^{-1} of MDZ (**Figure 2**). No peaks from endogenous plasma components or MDZ metabolites interfering with MDZ or internal standard were observed. The retention times of MDZ and diazepam (internal standard) were 6.1 and 7.9 min, respectively. The total run time for an assay was approximately 15 min.

Calibration curve and sensitivity: The calibration curve was constructed with a peak area ratio of MDZ to I.S. vs 5F plasma concentration. The mean regression equation is $y = 0.0475x+0.1071$ ($r^2 = 0.9983$), in which x is the MDZ plasma concentration, with a linear range of 0.10-10.0 μg mL^{-1}. The LOQ was 0.02 μg mL^{-1} (S/N = 32, RSD = 2.6%, RE = 1.6%).

Precision, accuracy and extraction recovery: The intra- and inter-day precision, accuracy and extraction recovery were listed in **Table 1**, showing the method to be good of precision, accuracy and extraction recovery. Plasma samples were efficiently extracted with the mixed organic solvents under the confirmed conditions.

Pharmacokinetic study

In vivo evaluation of plasma concentrations of MDZ was carried out in Sprague-Dawley rats on ventral surface, and the mean plasma concentration-time curve profile was illustrated in **Figure 3**. The corresponding pharmacokinetic parameters were calculated and listed in **Table 2**. Plasma concentrations of MDZ after intravenous administration in rats were reported [27], and the values of AUC_{0-t}, C_{max}, t_{max} and $t_{1/2}$ were listed in **Table 2** to compare with that of transdermal administration.

The results in **Table 2** showed that significant difference existed between some parameters although the values of C_{max} in the two administrations were similar. The distribution half-live ($t_{1/2\alpha}$) of TDDS (0.37 hr) was longer a lot than

Table 2. Comparison of MDZ pharmacokinetics between Transdermal administration and Intravenous administration in rats

Parameter	Transdermal administration	Intravenous administration*
C_{max} (μg/ml)	0.56	0.52
t_{max} (h)	3.00	0.083
$T_{1/2\alpha}$ (h)	0.37	0.067±0.052
$T_{1/2\beta}$ (h)	11.85	0.6617±0.268
CL_t (L h^{-1})	1.08	0.69±0.21
AUC_{0-t} (μg ml^{-1} h)	8.34	--

*Data from [27].

that (0.06 hr) of intravenous administration, which could be attributed to the skin barrier for drug permeation. The elimination half-live ($t_{1/2\beta}$) was about 11 hr in the TDDS applied in rats, by comparison, the $t_{1/2\beta}$ in intravenous administration was about 0.6 hr, it indicated that the TDDS can significantly prolong the effective therapeutic time. And more notably, there is two steady-state plasma levels was observed in the TDDS and the steady state levels were maintained hereabout the C_{max} during 3-6 hr and the sustaining concentration of approximately 0.3 μg mL^{-1} during 12-24 hr. After 6hr, the concentrations of MDZ in plasma were determined decreasing and the drug was metabolized and eliminate until 24 hr, while it was clear after 2 hr in intravenous administration. These results indicated the TDDS could be controlled released and maintained a long effective drug concentration, which are advantages for improving patient compliance and avoiding to frequently intravenous administration.

Conclusion

The described LC method with UV detection is the simple analytical method for the determination of MDZ in rat plasma. It has been linear, precise and accurate in the concentration range 0.10-10.0 μg mL^{-1}; therefore it is suitable for pharmacokinetic study of MDZ in trasdermal delivery drug system. The transdermal patch was found to have a prolonged release of MDZ and proved to be useful as a sustained release drug delivery system when compared to intravenous administration.

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Disclosure of conflict of interest

None.

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