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Original Article

Application of a UPLC–MS/MS method to the protein binding study of TM-2 in rat, human and beagle dog plasma ☆

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ABSTRACT

TM-2 known as a potential antitumor drug is a novel semi-synthetic taxane derivative. As drug–protein interactions contribute to insights into pharmacokinetic and pharmacodynamic properties, we elucidated the binding of TM-2 to plasma protein. In this study, a simple, rapid and reliable method was developed and validated employing equilibrium dialysis for the separation of bound and unbound drugs and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) for the quantitation. Protein binding reached equilibrium within 24 h of incubation at 37 °C. After liquid–liquid extraction with methyl tert-butyl ether, the samples were separated on Thermo Syncronis UPLC® C_{18} (2.1 mm × 50 mm, 1.7 μm), and acquisition of mass spectrometric data was performed in multiple reaction monitoring (MRM) mode via positive electrospray ionization. The assay was linear over the concentration rang of 5–2000 ng/mL. The intra- and inter-day precisions were 0.1%–14.8%, and the accuracy was from −6.4% to 7.0%. This assay has been successfully applied to a protein binding study of TM-2 in rat, human and beagle dog plasma. TM-2 showed high protein binding of 81.4% ± 6.5% (rat), 87.9% ± 3.6% (human) and 79.4% ± 4.0% (beagle dog). The results revealed that there was an insignificant difference among the three species.

1. Introduction

Chemotherapy for cancer with the taxanes such as docetaxel and paclitaxel has played an important role in oncology as cancer tends to be the number one killer of human beings [1]. The lack of sufficient antitumor activity of these agents may be a primary and constitutive deterrent to clinical use. Great attempts have been made to develop novel derivatives with improved activity and reduced toxicity. 13-(N-Boc-3-i-butylisoserinoyl-4,10-β-diacetoxy-2-α-benzoyloxy-5-β-20-epoxy-1,13-α-dihydroxy-9-oxo-19-norcyclopropa[g]tax-11-ene (TM-2) (Fig. 1) is a novel semi-synthetic taxane derivative on the basis of the modification of larotaxel. Compared with docetaxel or larotaxel, TM-2 has been assessed in a variety of human tumor lines *in vitro* and shown to display high anticancer efficacy. Improved cytotoxic activity was noted especially on cell lines resistant to multi-drug resistance (MDR, KB/VCR, human cervical adenocarcinoma resistant to vincristine and MCF-7/ADR, human breast cancer resistant to adriamycin) [2]. The *in-vivo* study on antitumor activity of TM-2 against A549 (human lung cancer xenografts) in nude mice demonstrated that TM-2 exhibited the tumor control rate value of > 82.24% and the T/C (the relative tumor proliferation rate) value of 17.87%. Our previous study showed that TM-2 is a good candidate for further development. The physicochemical properties, degradation kinetics and the preparation of TM-2 have already been investigated in detail [3]. The pharmacokinetic studies of TM-2 in both rats and dogs have also been studied. Also, the major metabolic pathway of TM-2 in rats was hydroxylation of the taxane ring or the lateral chain [4–7].

The efficacy and toxicity of the drugs are influenced by several factors. Plasma protein binding (PPB), one of the factors, plays an important role in the pharmacokinetics (PK) and pharmacodynamics (PD) of a drug and the PPB data are useful for designing optimal therapeutic dose and estimating safety margins during drug development. The accurate determination of unbound fraction of drug in plasma is essential in the therapeutic monitoring because the remaining free fraction closely relates to the pharmacological effect, and this is especially important for highly protein-bound drugs. It is evident that taxanes such as paclitaxel, docetaxel and felotaxel are highly bound to plasma protein [8–10].

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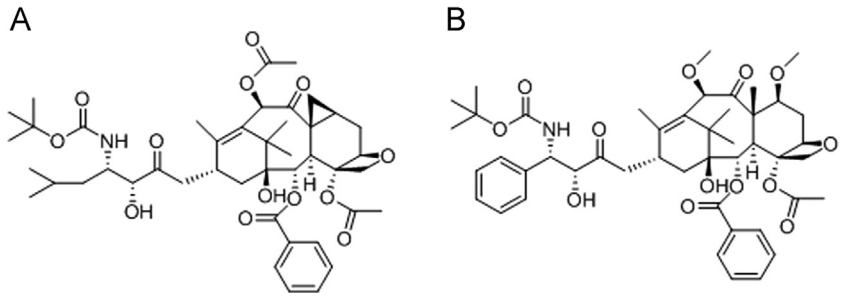

Fig. 1. Structures of (A) TM-2 and (B) the IS.

Though some basic preclinical information of TM-2 is thus accessible, no data of PPB is available yet. Therefore, the determination of the PPB of TM-2 becomes one of the important issues for the complete research and evaluation of the preclinical study.

Various methodologies have been established for determination of PPB, among which equilibrium dialysis (ED) and ultrafiltration (UF) are the most frequently employed methods [11–16]. Most methods use equilibrium dialysis instead of ultrafiltration for the taxanes such as docetaxel, paclitaxel and cabazitaxel (internal standard, IS, Fig. 1). The objective of the project was to develop a relatively simple and reliable method for quantifying TM-2 in rat, human and beagle dog plasma. Ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) is currently considered the best choice for supporting preclinical studies due to its selectivity, sensitivity and high output. In light of the foregoing, equilibrium dialysis and UPLC–MS/MS were employed in our study and the fully validated method was successfully applied to a plasma protein binding study of TM-2.

2. Experimental

2.1. Chemicals and reagents

TM-2 (purity > 98.0%) and cabazitaxel (purity > 98.0%) were synthesized in the School of Pharmacy, Fudan University (Shanghai, China). Acetonitrile and methanol of HPLC-grade were obtained from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade ammonium acetate was provided by Dikma Co., Ltd. (Beijing, China). Methyl tert-butyl ether of analytical-grade was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). MD27 equilibrium dialysis membranes with a molecular weight cut-off of 8000–14,000 Da were purchased from Viskase (Darien, IL, USA). Blank adult human plasma was collected from healthy female or male volunteers (Blood Centre of Liaoning, Shenyang, China). Drug-free rat and beagle dog plasma used in the experiment were collected from different rats and beagle dogs and stored at −80 °C until use (The Experimental Animal Center of Shenyang Pharmaceutical University, Shenyang, China). In the experiment of the method validation, blank plasma from single source (6 lots of blank matrix) was used for the sample preparation of selectivity and matric effect. Blank plasma used in the other parts of the method validation was pooled (rats and beagle dogs) or mixed-gender (human).

2.2. Instruments and UPLC–MS/MS conditions

The experiment was performed on an ACQUITY™ UPLC system (Waters Corp., Milford, MA, USA) and a Waters TQD triple quadrupole mass spectrometer (Waters Corp., Manchester, UK) equipped with an electrospray ionization source. All data were acquired in centroid mode by the Masslynx™ NT4.1 software and analyzed by QuanLynx™ program (Waters Corp., Milford, MA, USA). The analyte was separated on Thermo Syncronis UPLC® C_{18} (2.1 mm × 50 mm, 1.7 μm) with a thermostated column oven setting at 35 °C. The mobile phase was composed of solvent A (acetonitrile) and solvent B (2 mM ammonium acetate) at the flow rate of 0.2 mL/min according to the following linear gradient: 0–1.5 min, 60%–90% A; 1.5–2.7 min, 90% A; 2.7–2.8 min, 90%–60% A; 2.8–3.5 min, 60% A. The autosampler was conditioned at 8 °C, and the injection volume was 5 μL.

Mass spectrometric analysis was performed in positive ion mode under multiple reaction monitoring (MRM) with ion transitions at *m/z* 812.39→551.35 and 836.36→555.26 for TM-2 and the IS, respectively. Spectrometric parameters were as follows: desolvation temperature of 400 °C, source temperature of 100 °C, desolvation gas of 550 L/h, cone gas of 40 L/h, dwell time of 0.2 s and capillary voltage of 3.50 V. The cone voltage were 30 V for TM-2 and 25 V for the IS, and collision energy was 10 eV for both TM-2 and the IS.

2.3. Preparation of working solutions and standard curves

Individual stock solutions of TM-2 and IS were prepared in methanol both at 1.0 mg/mL. The stock solution of TM-2 was then serially diluted with methanol to obtain the working solutions ranging from 0.05 to 20 μg/mL. The IS working solution of 1 μg/mL was also prepared with methanol from the primary stock solution. All the solutions were stored at 4 °C and brought to room temperature before use.

Calibration standards and quality control (QC) samples used to estimate precision and accuracy of the method were prepared from two separate sets of solutions in parallel with three different matrices. Drug-free rat, human and beagle dog plasma was used to prepare calibration standards and QCs for total TM-2 measurement, and drug-free buffer (phosphate-buffered saline (PBS) buffer, pH 7.4) was used to prepare calibration standards and QCs for free TM-2 measurement. Calibration standard samples of TM-2 (5, 10, 50, 100, 500, 1000, 2000 ng/mL) were obtained by spiking 10 μL of the appropriate working solutions to 100 μL drug-free buffer (calibration curve in PBS buffer) or blank rat, human and beagle dog plasma (calibration curve in plasma). QC samples at low, middle and high concentrations (10, 100 and 1600 ng/mL) were prepared separately in the same fashion.

2.4. Sample preparation

A 10 μL aliquot of the IS solution (1000 ng/mL) and 10 μL of methanol were added to 100 μL of plasma sample (fluid within the dialysis bags) or the PBS buffer (fluid outside the dialysis bags) in a 5.0 mL plastic centrifuge tube. After vortex-mixing for 1 min, the mixture was extracted with 2 mL of methyl tert-butyl ether by shaking for 10 min. The organic and aqueous phases were then separated by centrifugation at 10,000 rpm (20 °C) for 10 min.

All supernatants were transferred to a clean plastic tube, and evaporated to dryness under nitrogen stream at 35 °C. The residue was reconstituted with 100 μL of acetonitrile : water (3:2, v/v) and centrifuged again at 13,000 rpm at 4 °C for 5 min, and then 5 μL aliquot of the resulting solution was injected into the UPLC–MS/MS system for analysis.

2.5. Quantitative bioanalysis method validation

Validation of the quantitative UPLC–MS/MS method was assessed including specificity, linearity, precision and accuracy, recovery, matrix effect and stability according to U.S. Food and Drug Administration (US-FDA) Guidance for Industry: Bioanalytical Method Validation, European Medicines Agency Guideline on Bioanalytical Method Validation, and China Food and Drug Administration (CFDA) Technical Guideline for Non-clinical Pharmacokinetic Study of Chemical Drugs [17–19].

2.5.1. Selectivity

The specificity of the method was evaluated by analyzing six blank plasma or PBS buffer samples and spiked plasma or PBS buffer samples at the lower limit of quantitation (LLOQ). The peak areas of the endogenous interference co-eluted with the analytes should be less than 20% of the peak area of the LLOQ standard and less than 5% of the peak area of the IS.

2.5.2. Linearity

Calibration standards were prepared and analyzed by plotting the peak area ratios of the analyte to the IS versus the nominal concentration using a linearly weighed ($1/x^2$) least squares regression method [20] in duplicate on three consecutive days. Calibration curves were considered acceptable when the correlation coefficient (r) was greater than 0.99 and the bias of the calculated concentrations was within ± 15% of the nominal concentrations, except the LLOQ with an allowed deviation of ± 20%.

2.5.3. LLOQ, precision and accuracy

LLOQ was established by analyzing six blank plasma samples spiked with 5.0 ng/mL of TM-2 with acceptable precision and accuracy (less than 20% for each criterion). Precision and accuracy were determined by assessing six replicates of the QC samples at three levels (10, 100, 1600 ng/mL) on three consecutive validation days. Intra- and inter-day precisions (RSD, relative standard deviation) were required not to exceed 15%, and accuracy (RE, relative error) should be within ± 15%.

2.5.4. Recovery and matric effect (ME)

Recovery values were calculated at three QC levels with six replicates by comparing the peak areas of the regularly processed QC samples (A) with those of spiked post-extraction samples (B), expressed as: $A/B \times 100\%$. Blank plasma from six lots was extracted and then spiked with the analyte and the IS to evaluate the ME of the analyte and IS. The peak area in spiked plasma post-extraction samples (B) was then compared with those of standard solutions containing the analyte at equivalent concentrations (C), expressed as: $B/C \times 100\%$.

2.5.5. Stability

The stability of the analyte in rat, human and beagle dog plasma or PBS buffer was evaluated by analyzing three replicates of rat, human and beagle dog plasma or PBS buffer samples at three QC levels under the following conditions: stored at −80 °C for 1 month, exposed at room temperature for 4 h, undergoing three freeze–thaw cycles and kept in autosampler tray at 8 °C for 12 h. The samples were considered stable when the assay RE was within ± 15% of the nominal concentration.

2.5.6. Carry-over

Carry-over effect was investigated by injecting blank samples after calibration standard at the upper limit of quantification (ULOQ). The measured peak area should be less than 20% of the peak area of the analyte at the LLOQ.

2.6. Equilibrium dialysis

In order to determine the plasma protein binding rates, the equilibrium dialysis method was used. TM-2 at different designed concentrations (50, 500, 2000 ng/mL for rat, 50, 200, 1000 ng/mL for human and 50, 200, 500 ng/mL for beagle dog, the concentrations were designed depending on the peak concentration, distribution phase concentration and elimination phase concentration in rats and beagle dog [4,6]) was *in-vitro* spiked to the PBS buffer and the blank rat, human or beagle dog plasma was added into the semi-permeable membrane bag. Prior to analysis, each of the resulting samples was incubated at 37 °C for 24 h to achieve equilibrium between plasma and PBS buffer. The fluid outside and inside the dialysis bags was collected after the incubation. The concentration in the dialysis bag was determined by UPLC–MS/MS using standard curves as the total concentration, i.e., the unbound concentration plus the concentration of drug bound to protein, and the concentration of the fluid outside the dialysis bags was measured as the unbound fraction. The percentage of protein binding was calculated using the following formula: Protein binding ratio F_u (%)$=(D_t-D_f)/D_t \times 100$, where D_t represents the total drug concentration in the plasma compartment, and D_f is the concentration of the free fractions in the PBS buffer compartment.

2.7. Data analysis

The results are expressed as mean ± standard deviation (SD). The paired Student's t-test was used for statistical analysis. Differences associated with P-values lower than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Non-specific binding (NSB) experiment

TM-2 at different designed concentrations (50, 500, 2000 ng/mL) was spiked to the PBS buffer and 2 mL of drug-free PBS buffer was added into the semi-permeable membrane bag. After incubation at 37 °C for 24 h, both the fluids inside and outside the dialysis bag were pretreated according to the sample preparation procedure. The non-specific binding of TM-2 to the membrane is expressed as NSB (%)$=[C_nV_{out}-C_d\ (V_{out}+V_{in})]/C_nV_{out} \times 100$, where C_n is the nominal concentration of TM-2 spiked to the PBS buffer, C_d is the concentration of determination, V_{out} represents the volume of the PBS buffer spiked with TM-2 outside of the dialysis bag, and V_{in} represents the PBS buffer volume added into the dialysis bag. The mean NSB ratio was less than 10%, suggesting that the non-specific binding of TM-2 to the membrane was considered to be negligible.

3.2. Attainment of plasma protein binding steady state

This experiment was conducted to learn when binding of TM-2 reached equilibrium. 20 mL aliquot of PBS buffer spiked with TM-2 at three levels (50, 500, 2000 ng/mL, 50, 200, 1000 ng/mL and 50, 200, 500 ng/mL for rat, human and beagle dog plasma, respectively), and 2 mL of rat, human or beagle dog plasma added into the dialysis bag were incubated in a brown container for periods of 8, 12, 24 and 48 h. Each timed sample was then prepared and

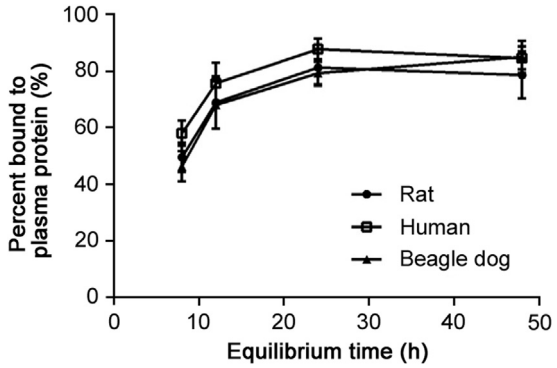

Fig. 2. Time to attain a steady state of protein binding of TM-2 to rat, human and beagle dog plasma.

analyzed according to the above method. The plasma protein binding ratios of TM-2 at three concentration levels and different equilibrium time are illustrated in Fig. 2. As shown in Fig. 2, the percentage of TM-2 bound progressively increased during the first 24 h of incubation, and remained relatively constant throughout the 24–48 h study period ($P > 0.05$). The results demonstrated that the plasma protein binding reached a steady state or equilibrium after 24 h incubation. Therefore, a dialysis time of 24 h was used for all subsequent equilibrium dialysis experiments.

3.3. Method validation

3.3.1. Specificity

Figs. 3–6 depict the typical chromatograms of blank plasma (rat, human and beagle dog) sample, PBS buffer and blank plasma (rat, human and beagle dog) sample spiked with TM-2 at LLOQ and the IS, and PBS buffer and plasma samples collected after equilibrium dialysis. No significant endogenous interference co-eluting with the analyte and the IS was observed in the blank rat, human and beagle dog plasma and PBS buffer, suggesting that the validated method was highly selective for the analyte.

3.3.2. Linearity and LLOQ

The calibration curve was linear over the range of 5–2000 ng/mL in plasma and PBS buffer. The following equations of the calibration curve were used: in rat plasma: $y=1.36x+2.02$ ($r=0.9940$); in human plasma: $y=1.73x+2.38$ ($r=0.9958$); in beagle dog plasma: $y=1.43x+5.43$ ($r=0.9958$); in PBS buffer: $y=1.82x+3.05$ ($r=0.9941$), where y represents the peak area ratio of each analyte to IS, and x is the nominal concentration of the analyte. The LLOQs were 5 ng/mL with acceptable precision (RSD) of 14.8% (rat plasma), 12.5% (human plasma), 18.3% (beagle dog plasma), 15.0% (PBS buffer) and accuracy (RE) of −4.0% (rat plasma), −4.0% (human plasma), −6.0% (beagle dog plasma), and −6.0% (PBS buffer).

3.3.3. Precision and accuracy

The accuracy and precision values of TM-2 in rat, human and beagle dog plasma and PBS buffer at three QC levels (10, 100 and 1600 ng/mL) are shown in Table 1. The intra- and inter-day precision values for TM-2 in rat, human and beagle dog and PBS buffer were not more than 14.8%, while the accuracy values were −6.4% to 7.0% at three QC levels. The results demonstrated that the method was reliable and reproducible for the determination of TM-2 in rat, human and beagle dog plasma and PBS buffer.

3.3.4. Recovery and matrix effect

The mean recoveries of the analyte ranged from 69.9% to 101.3% across the concentration range in rat, human and beagle dog plasma and PBS buffer (Table 1). In addition, the recoveries of the

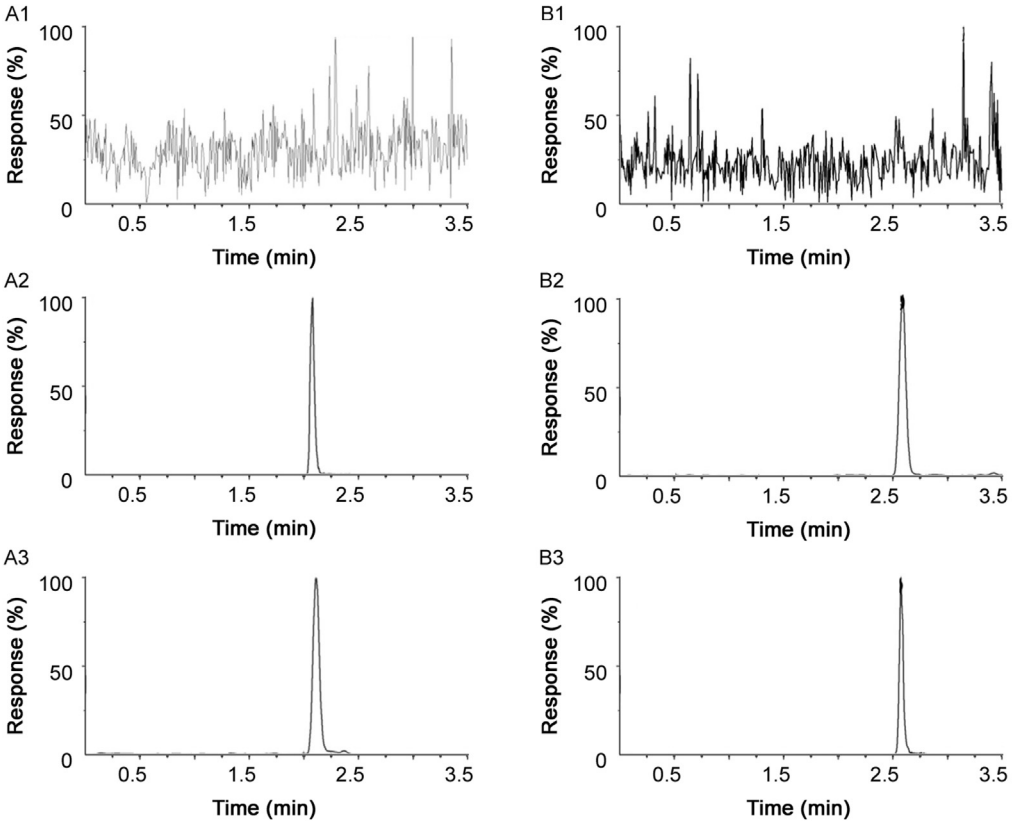

Fig. 3. Typical MRM chromatograms of (A) IS and (B) TM-2 (1: blank rat plasma; 2: blank rat plasma spiked with TM-2 (5 ng/mL) and IS (100 ng/mL); 3: rat plasma sample (220.7 ng/mL) collected in the dialysis membrane after 24 h equilibrium dialysis of rat plasma at 50 ng/mL).

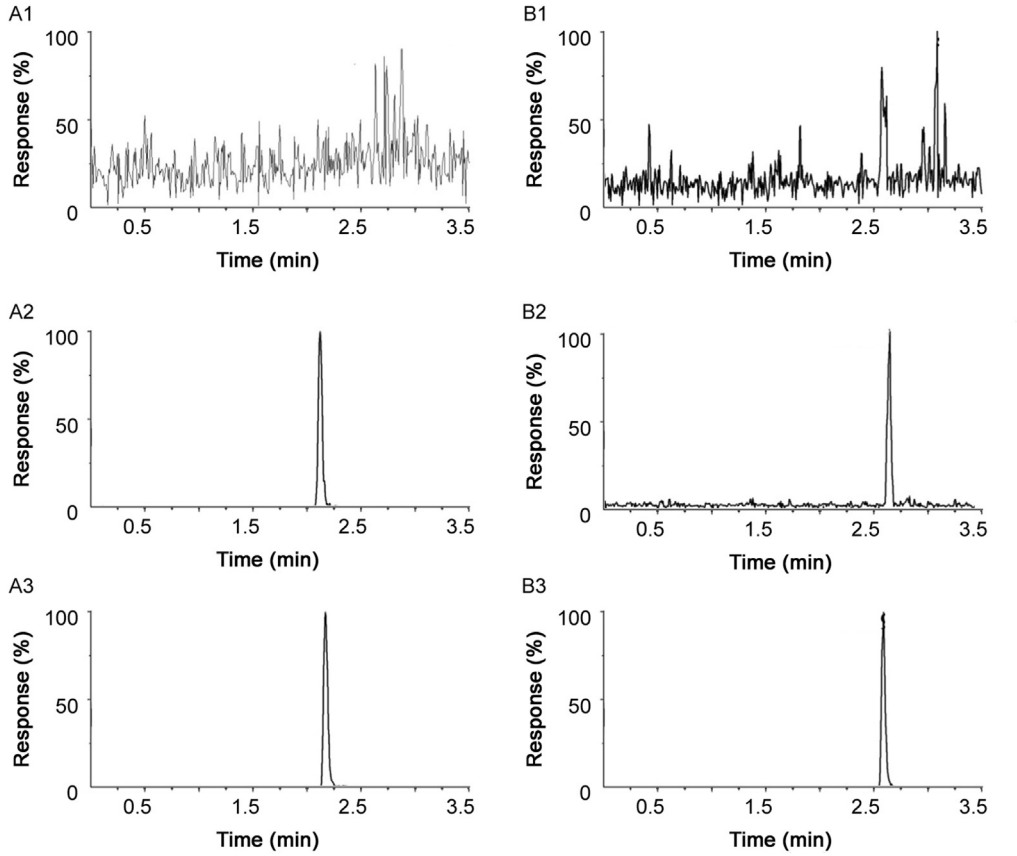

Fig. 4. Typical MRM chromatograms of (A) the IS and (B) TM-2 (1: blank human plasma; 2: blank human plasma spiked with TM-2 (5 ng/mL) and the IS (100 ng/mL); 3: human plasma sample (242.7 ng/mL) collected in the dialysis membrane after 24 h equilibrium dialysis of human plasma at 50 ng/mL).

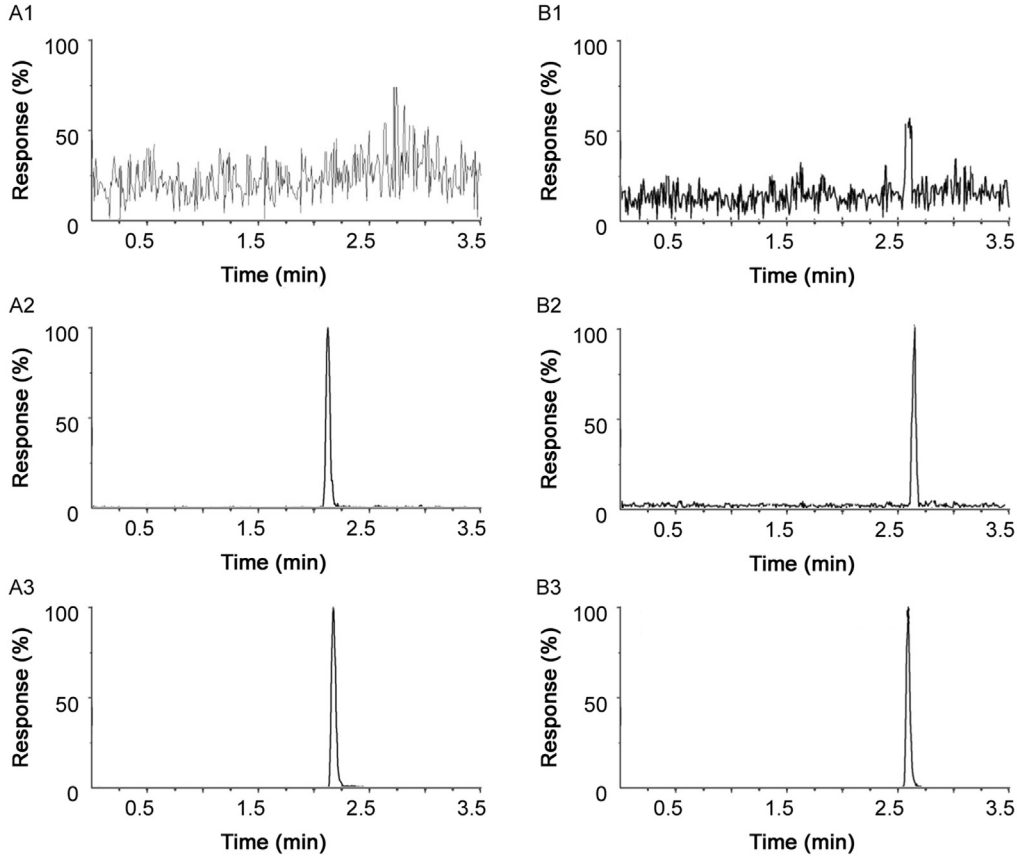

Fig. 5. Typical MRM chromatograms of (A) the IS and (B) TM-2 (1: blank beagle dog plasma; 2: blank beagle dog plasma spiked with TM-2 (5 ng/mL) and the IS (100 ng/mL); 3: beagle dog plasma sample (125.2 ng/mL) collected out of the dialysis membrane after 24 h equilibrium dialysis of beagle dog plasma at 50 ng/mL).

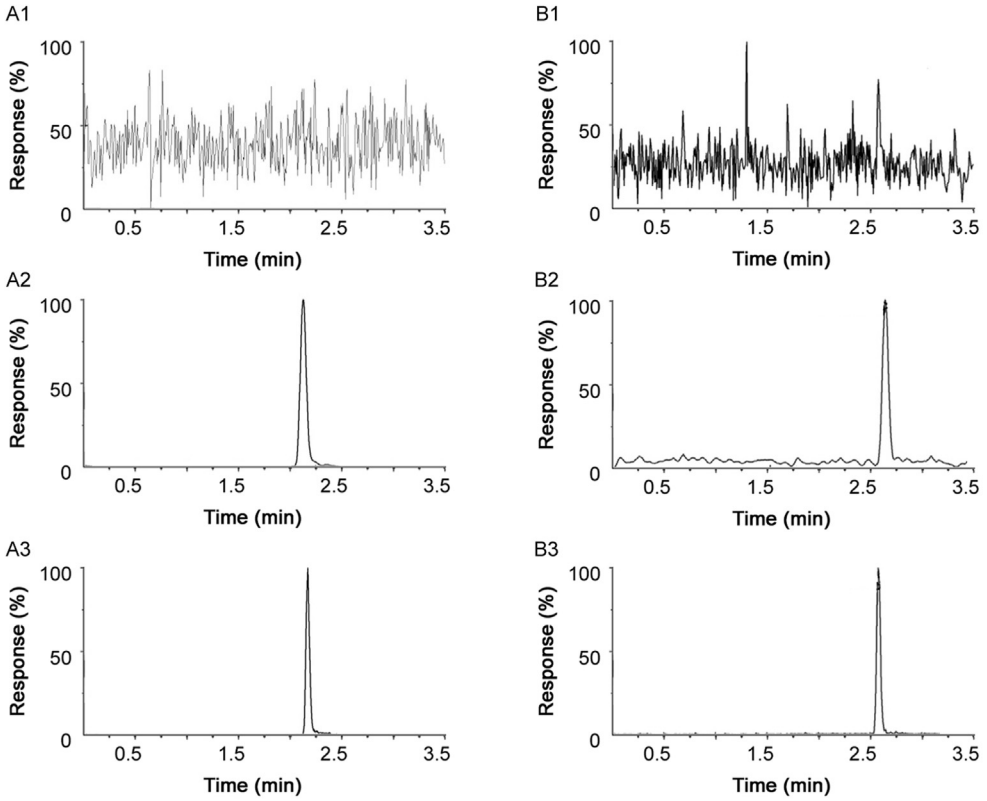

Fig. 6. Typical MRM chromatograms of (A) IS and (B) TM-2 (1: blank PBS buffer; 2: blank PBS buffer spiked with TM-2 (5 ng/mL) and IS (100 ng/mL); 3: PBS buffer sample (25.7 ng/mL) collected out of the dialysis membrane after 24 h equilibrium dialysis of PBS buffer at 50 ng/mL).

Table 1
Precision, accuracy, recovery and matrix effect of TM-2 in rat, human and beagle dog plasma and PBS buffer ($n=6$).

Matrices	Concentration (ng/mL)	Precision (RSD, %)		Accuracy (RE, %)	Recovery (mean ± SD)	Matrix effect (mean ± SD)
		Intra-day	Inter-day			
Rat plasma	10	11.4	11.9	2.0	69.9 ± 6.6	90.7 ± 4.3
	100	2.3	8.4	−0.5	74.5 ± 5.7	92.0 ± 2.6
	1600	6.5	7.1	−2.6	73.6 ± 7.6	100.2 ± 4.7
Human plasma	10	3.3	8.7	6.0	91.2 ± 10.8	94.4 ± 13.4
	100	14.8	7.9	5.3	82.6 ± 2.9	112.2 ± 4.4
	1600	3.5	10.4	−6.4	85.6 ± 8.6	94.0 ± 1.6
Beagle dog plasma	10	11.8	8.7	4.0	84.3 ± 8.1	108.8 ± 8.9
	100	8.3	7.2	3.9	90.4 ± 6.5	93.9 ± 5.5
	1600	7.8	11.7	−2.8	101.3 ± 8.0	110.5 ± 5.3
PBS buffer	10	14.1	0.1	2.0	75.7 ± 11.2	113.5 ± 19.2
	100	5.7	6.6	7.0	86.7 ± 7.6	87.4 ± 8.6
	1600	14.5	9.2	−0.6	84.2 ± 5.0	110.0 ± 7.0

IS were between 79.1% and 92.1%. The average ME of the analyte ranged from 87.4% to 113.5% over the corresponding concentration ranges in rat, human and beagle dog plasma and PBS buffer (Table 1). Moreover, the ME of the IS was between 98.9% and 107.4%. These data indicated that the ME (ion suppression or enhancement) from the rat, human and beagle dog plasma and PBS buffer was negligible under the current conditions.

3.3.5. *Stability*

The results of stability experiments under the mentioned conditions are presented in Table 2. TM-2 was stable in plasma (rat, human and beagle dog) and PBS buffer after standing at room temperature for 4 h, in the ready-to-inject samples in autosampler tray at 8 °C for 12 h, undergoing three freeze–thaw cycles and stored at −80 °C for 1 month. The stock solutions of both analyte and the IS stored at −20 °C were stable for 1 month.

3.3.6. *Carry-over*

Carry-over was not found for the analyte and the IS in extracted double blank plasma (without analyte and the IS) after subsequent injection of the ULOQ (1600 ng/mL).

Table 2
Stability of TM-2 in rat, human beagle dog plasma and PBS buffer under various storage conditions ($n=3$).

Conditions	Concentration (ng/mL)	Rat plasma		Human plasma		Beagle dog plasma		PBS buffer	
		RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)
Three freeze–thaw cycles	10	2.2	15.7	3.7	−7.0	10.0	0	19.3	−5.3
	100	7.1	−5.1	6.9	−5.5	12.3	5.9	6.0	7.1
	1600	3.7	−3.3	7.8	1.0	8.7	5.4	4.8	11.3
Room temperature for 4 h	10	13.2	3.3	18.4	−6.3	2.6	16.0	12.8	−6.3
	100	3.7	−0.7	2.3	−10.8	15.0	−1.3	7.5	−10.8
	1600	9.6	−4.8	10.9	−1.2	5.0	0.6	14.9	−1.2
Frozen at −80 °C for 30 days	10	2.6	12.7	5.8	−11.3	3.4	6.0	14.0	−7.0
	100	5.1	4.6	3.3	12.0	8.8	−7.9	10.7	−3.0
	1600	10.1	1.9	12.3	0.5	2.8	5.2	8.2	−2.7
Autosampler at 8 °C for 12 h	10	11.1	7.3	9.6	−1.3	11.5	4.0	9.6	6.0
	100	4.5	−6.3	7.2	3.0	11.9	−8.2	4.6	−9.1
	1600	5.3	−5.4	2.7	−13.1	4.4	−5.8	10.1	2.8

3.4. Plasma protein binding

The present *in-vitro* study was carried out to determine the extent of plasma protein binding of TM-2. The bound and unbound drug from spiked plasma was separated by the equilibrium dialysis technique. Rat plasma protein binding of TM-2 was 82.2% ± 10.8%, 82.8% ± 3.9% and 79.3% ± 3.9% at 50, 500 and 2000 ng/mL, respectively. Human plasma protein binding of TM-2 was 89.1% ± 0.3%, 85.7% ± 6.3% and 88.9% ± 1.5% at 50, 200 and 1000 ng/mL, respectively. Beagle dog plasma protein binding of TM-2 was 84.2% ± 0.8%, 79.6% ± 3.8% and 78.2% ± 2.3% at 50, 200 and 500 ng/mL, respectively. Concentration dependent rat, human and beagle dog plasma protein binding was not observed over the selected concentration range (50–2000 ng/mL for rat plasma, 50–1000 ng/mL for human plasma and 50–500 ng/mL for beagle dog plasma). All the results revealed that TM-2 was bound extensively to plasma protein ($\geq$ 78.2% bound) and there was no significant difference in bound fractions among these three species (rat, human and beagle bog).

4. Conclusion

A rapid, sensitive and simple method based on equilibrium dialysis and UPLC–MS/MS was developed and validated for the determination of rat, human and beagle dog plasma protein binding of TM-2, which could meet the current requirements of bioanalytical method validation. The results showed that TM-2 had high plasma protein binding in the physiological conditions of rat, human and beagle dog and there were no obvious differences among the three species. The method and the results will prove to be potentially valuable for further study of TM-2.

Acknowledgments

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