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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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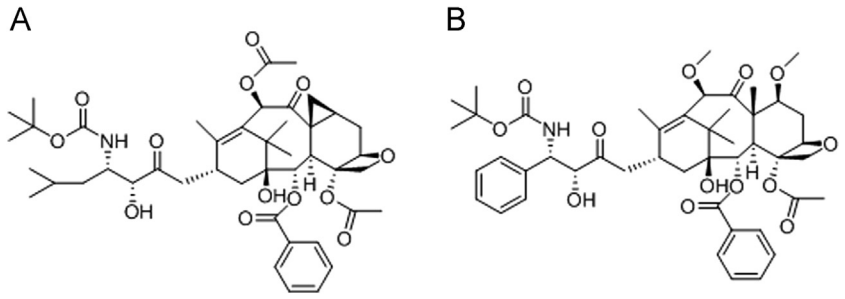

Fig. 1. Structures of (A) TM-2 and (B) the IS.

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.