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Determination of oxymatrine and its metabolite matrine in rat blood and dermal microdialysates by high throughput liquid chromatography/tandem mass spectrometry

Hong Zheng[a,b,*], Guangchun Chen[c], Lifu Shi[b], Ziyang Lou[a], Fangjian Chen[b], Jinhong Hu[b,**]

[a] School of Pharmacy, Second Military Medical University, Guo He Road 325, Shanghai 200433, PR China
[b] Department of Pharmacy, Changhai Hospital, Second Military Medical University, Changhai Road 174, Shanghai 200433, PR China
[c] University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642, USA

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ABSTRACT

Oxymatrine (OMT) and matrine (MT) are the major quinolizidine alkaloids found in certain *Sophora* plants, which have been extensively used in China for the treatment of viral hepatitis, cancer, cardiac diseases and skin diseases (such as atopic dermatitis and eczema). A precise, sensitive and high throughput LC–MS/MS was developed to determine OMT and its metabolite MT in rat blood and dermis collected using microdialysis technique. Microdialysis probes were inserted into the jugular vein/right atrium and dermis of Wistar rats, and 3% OMT gel (1 g) was administered via topical application. The samples were collected and then injected into the LC–MS/MS system after adding the internal standard (codeine, CDN). Chromatographic separation was achieved in a run time of 2 min on a reversed phase short-column (50 mm × 2.1 mm, 3.5 μm). The mobile phase for column separation was methanol–ammonium formate (pH 5.0; 25 mM) (70:30, v/v) with a flow rate of 0.3 mL/min. A diverter valve was installed post-LC column for desalting. Detection of analytes and IS was done by tandem mass spectrometry, operating in positive ion and multiple reaction monitoring (MRM) acquisition mode. The protonated precursor to product ion transitions monitored for OMT, MT and IS was m/z 265.0 → 247.3, 249.1 → 148.3 and 300.0 → 215.2, respectively. The lower limit of quantification (LLOQ) for OMT and MT was 0.5 ng/mL. The calibration curves were linear over the range of 0.5–1000 ng/mL for OMT and MT with a coefficient of determination >0.999. This selective and sensitive method is useful for the determination of OMT and MT and in the pharmacokinetic studies of these compounds. The blood and dermal concentration–time profile of OMT and its metabolite MT suggest that the limiting factor for dermal metabolism is the low capacity of enzymes in the skin rather than the quantity of penetrated OMT.

1. Introduction

Microdialysis is a technique used to monitor the *in vivo* concentration time course of drugs and endogenous substances in the tissue's extracellular fluid [1]. Among several applications, microdialysis has gained popularity to study drug pharmacokinetics in dermis in the past few years [2,3]. Dermis is the target of several diseases and the site from which drugs are absorbed into blood from transdermal delivery systems. Some of the advantages of microdialysis over existing methods to study pharmacokinetics in skin are the capability to perform sampling at the same site for several hours, the physiological conditions of the sampling, and the low invasiveness of this method [4]. A better understanding of the pharmacokinetic of drug in dermis will promote the development of improved therapy schedules, drug formulations or molecular structure for drugs meant to act on skin diseases. In addition, the simultaneous estimation of the pharmacokinetic parameters in dermis and blood will help in the understanding of the kinetic relationship between the two sites, sorting out the role of skin in transdermal delivery and dermal metabolism.

Oxymatrine (OMT) and matrine (MT) are the major quinolizidine alkaloids from the root of *Sophora flavescens* Ait. (kushen), but also from *Sophora subprostrata* (shandougen), and from the above ground portion of *Sophora alopecuroides* [5,6]. Chemical structures of two constituents are shown in Fig. 1. They have been extensively used in China for treatment of viral hepatitis, cancer, cardiac diseases (such as viral myocarditis), and skin diseases (such as psoriasis and eczema) [7–10].

* Corresponding author at: School of Pharmacy, Second Military Medical University, Guo He Road 325, Shanghai 200433, PR China. Tel.: +86 21 25070400; fax: +86 21 25070400.
** Corresponding author. Tel.: +86 21 25070665; fax: +86 21 25070665.
E-mail addresses: zhenhg226@yahoo.com.cn (H. Zheng), hujh@smmu.edu.cn (J. Hu).

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Due to the high pharmacological activities of OMT and MT, several analytical methods of the two alkaloids have been developed for medical research. These methods include fluorescence quenching [11], thin layer chromatography (TLC) [12], high-performance capillary electrophoresis (HPCE) [13] and high-performance liquid chromatography (HPLC) [14–16]. But most of them with UV detection at 218 nm for OMT and/or MT in plasma with a limit of quantitation of about 0.5 μg/mL are not practical and sensitive enough for detecting the metabolite MT in plasma. Although there are a few reports about OMT can be reduced to MT, which might have pharmacological and toxicological implications in biological samples with LC–MS [17,18] methods at low ng/mL concentration levels for animal or human studies after oral or i.v. administration of OMT, there is little information about the pharmacokinetics of OMT and its active metabolite MT after topical application of OMT up until now.

In this paper, a more precise and sensitive LC–MS/MS method is described to determine OMT and its active metabolite MT in a small volume (5 μL) of microdialysates collected alternatively from two probes implanted in the jugular vein/right atrium and dermis in rats. The samples were submitted to a short-column to achieve high throughput LC–MS/MS assay. Five microliters of microdialysates was separated and monitored by an LC–MS/MS system for 2.0 min per sample. Finally the method reported was successfully applied to a pharmacokinetic study of OMT and its active metabolite MT in rat dermis and blood after topical application of OMT to five rats.

2. Experimental

2.1. Materials and reagents

OMT, MT and the internal standard (IS, codeine, CDN, Fig. 1(C)), were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and ammonium formate (HPLC gradient grade) were obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Saline (0.9%) was obtained from Baite Medical Co. Ltd., Shanghai, China. MQ water, which is in-house deionized water, further purified in a Milli-Q Academic system (Millipore, Bedford, MA, USA) was used. All other reagents were of analytical grade.

2.2. LC–MS/MS instrumentation

A 1200 series high throughput HPLC–MS/MS system (Agilent Technologies, Santa Clara, CA, USA) consisted of high-pressure binary gradient pump, high-performance autosampler, thermostatted column compartment, on-line degaser, diode-array detector, MassHunter workstation and 6410 triple quadrupole mass spectrometer equipped with an electrospray ionization source.

2.3. Liquid chromatographic conditions

The separation was achieved with an Agilent SB C_{18} column, (3.5 μm, 50 mm × 2.1 mm, Agilent Technologies, Santa Clara, CA, USA) and a mobile phase of methanol–ammonium formate (pH 5.0; 25 mM) (70:30, v/v) at a flow rate of 0.3 mL/min. The column temperature was maintained at 40 °C and the injection volume was 5 μL. The analysis time was 2 min per sample. A diverter valve was installed post-LC column to divert the substances eluted early to waste. The diverter valve was inline with the MS instrument at 0.5 min and offline at 2 min. The switch was used after the analytical column to avoid salt overload in the mass spectrometer.

2.4. Mass spectrometer conditions

Ionization was achieved using electrospray in the positive mode with the spray voltage set at 4000 V. Nitrogen was used as nebulizer gas and nebulizer pressure was set at 40 psi with a source temperature of 105 °C. Desolvation gas (nitrogen) was heated to 350 °C and delivered at a flow rate of 10 L/min. For collision-induced dissociation (CID), high purity nitrogen was used as collision gas at a pressure of 0.1 MPa. Quantification was performed using multiple reaction monitoring (MRM) mode at *m/z* 265.0 → 247.3 for OMT, *m/z* 249.1 → 148.3 for MT and *m/z* 300.0 → 215.2 for CDN. The collision energies for collision-induced dissociation for the aforementioned transitions were 32, 35 and 28 eV, for OMT, MT and CDN, respectively with a dwell time of 200 ms for each channel. Product ion mass spectra for OMT, MT and CDN were shown in Fig. 2.

2.5. Preparation of stock solutions, calibration standards and quality control samples

The standard stock solutions of OMT, MT and CDN were prepared in water to final concentrations of 3, 3 and 1 μg/mL, respectively. Calibration standards were prepared by serial dilution of stock solutions OMT and MT with saline (0.9%) to obtain OMT and MT concentrations both in the range of 0.5–1000 ng/mL. Quality control samples were prepared separately in an analogous manner as for the calibration standards at concentrations of 1, 10, 100 and 1000 ng/mL both for OMT and MT. The solvent for stock solutions was water, for calibration standards and quality control samples was saline (0.9%), respectively. All samples were stored at −80 °C.

2.6. Dialysate sample preparation

To 5 μL of each microdialysis sample, 5 μL of CDN solution (1 μg/mL) and 40 μL saline (0.9%) were added and vortexed for 3 min. Five microliters aliquots of the mixture were injected onto LC–MS/MS system.

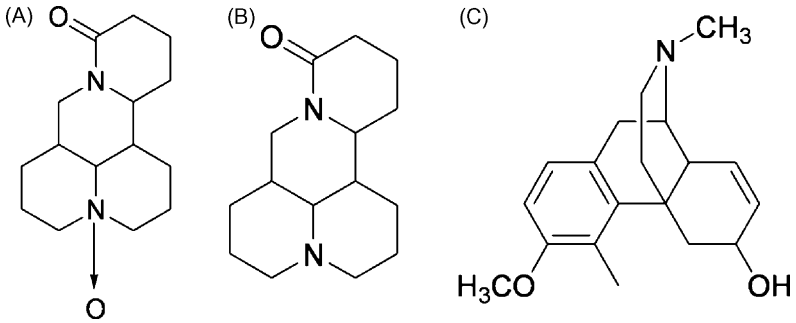

Fig. 1. Chemical structures of OMT (A), its metabolite MT (B), and the internal standard CDN (C).

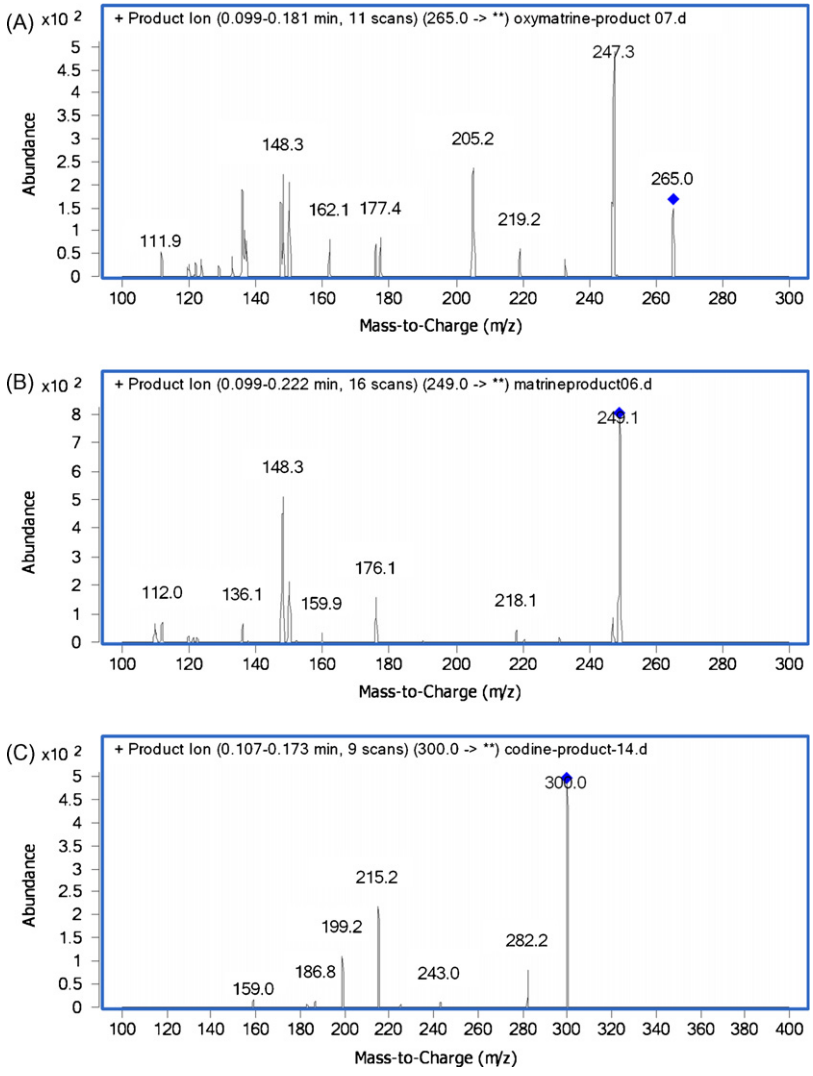

Fig. 2. The product ion scan spectra of $[M+H]^+$ ions of: (A) Oxymatrine with CID at 32 eV, (B) Matrine with CID at 35 eV, (C) Codeine with CID at 28 eV, respectively.

2.7. Assay validation

Standard solutions for the calibration curves, containing OMT and MT, were made daily in eight different concentrations 0.5, 1, 5, 10, 50, 100, 500, 1000 ng/mL by diluting the stock solutions in saline (0.9%). Calibration curves in saline (0.9%) were calculated by linear least-square regression of the peak area ratios of each analyte to IS versus microdialysates concentrations. OMT and MT concentrations in unknown samples were determined by interpolation from the calibration curves.

The intra-day precision and accuracy of the method were determined by analyzing six replicates at 1, 10, 100 and 1000 ng/mL levels of OMT and MT carried out in a single day, while using six replicates at these four concentrations over 6 days of the validation period to demonstrate the inter-day precision and accuracy, respectively. The accuracy was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentration (C_{obs}) as follows: accuracy (Bias %) = $[(C_{nom} - C_{obs})/C_{nom}] \times 100$. The precision coefficient of variation (C.V.) was calculated from the standard deviation and observed concentration as follows: precision (%C.V.) = [standard deviation (SD)/C_{obs}] × 100.

Two sets of the samples were prepared to evaluate the matrix effect in the quantitative bioanalytical method.

Set 1. The samples were prepared by mixing 5 μL saline (0.9%) with 5 μL of CDN solution (1 μg/mL) and 40 μL of the appropriated concentrations of standard solutions of OMT and MT to the target concentrations of 10, 100, 1000 ng/mL for OMT and MT. After mixing, the solutions were transferred into autosampler vials, and 5 μL was injected onto the LC–MS/MS system.

Set 2. The samples were prepared by mixing 5 μL dialysate collected from microdialysis with 5 μL of CDN solution (1 μg/mL) and 40 μL of the appropriated concentrations of standard solutions of OMT and MT to the target concentrations of 10, 100, 1000 ng/mL for OMT and MT. After mixing, the solutions were transferred into autosampler vials, and 5 μL was injected onto the LC–MS/MS system.

By comparing the peak areas of set 1 and set 2, the ion suppression or enhancement associated was assessed.

2.8. In vivo microdialysis experiments

Adult male Wistar rats (Shanghai Slac Laboratory Animal Co. Ltd., Shanghai, China), weighing 280–350 g, were anesthetized with urethane (1.25 g/kg, i.p.) during the surgical procedure. The rat was placed on a temperature-controlled heating-pad to maintain body temperature at 37–38 °C with the back of the body facing

the pad. The fur in abdominal region of rat was removed with an electric animal hair clipper one day before the study. Before the pharmacokinetic study, first a laboratory-made blood microdialysis probe [10 mm membrane length, and 5000 molecular mass (M_r) cut-off] was implanted in the jugular vein with the use of a guide cannula and positioned toward the right atrium. Then, a laboratory-made dermal microdialysis probe (30 mm membrane length, 5000 molecular mass cut-off) was implanted in the dermis of the abdominal region of the rat [19,20]. Both probes were connected to a microinjection pump (BAS Microdialysis, Indianapolis, IN, USA) and perfused with saline (0.9%) at a flow rate of 2 μL/min. This work was done in accordance with the Principles of Laboratory Animals Care (NIH Publication #85-23, revised 1985).

2.9. Drug administration

After 1 h of stabilization, 3% OMT gel (1 g) was applied topically to the skin at a dose of 7.09 μmol/cm^2 at the point where the microdialysis probe was cutaneously implanted. The skin surface was covered with polyethylene film during the experiment (occlusive dressing technique). After 5 h of application, the remaining gel was gently removed with paper cloths without rubbing of the skin. Both of the blood and dermal dialysis samples were collected in a 200 μL Eppendorff tube at 20 min intervals for about 12 h and then the samples were stored at −80 °C until analysis.

2.10. Recovery of microdialysate

For *in vivo* recovery, the blood and dermal probes were inserted into the rat jugular vein/right atrium and dermis under anesthesia with urethane (1.25 g/kg, i.p.). Perfusion solutions containing OMT and MT (50, 100 and 200 ng/mL) were passed through the microdialysis probe into rat blood and dermis, respectively, at a constant flow rate of 2 μL/min using an infusion pump (BAS Microdialysis). Following a stabilization period of 1 h post probe implantation, the perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of OMT and MT were determined by LC–MS/MS. The *in vivo* relative recovery (R_{dial}) of OMT and MT across the microdialysis probe was calculated by the following equation: $R_{dial} = (C_{perf} - C_{dial})/C_{perf}$. OMT and MT microdialysate concentrations (C_m) were converted to unbound concentration (C_u) as follows: $C_u = C_m/R_{dial}$.

3. Results and discussion

3.1. LC–MS/MS optimization

OMT, MT and CDN (IS) were at first characterized by MS^2 scan and MS–MS product ions to ascertain their precursor ions and to select product ions for use in MRM mode, respectively. To get the richest relative abundance of precursor ions and product ions, the parameters for fragmentor energies and collision energies were optimized, and the MRM transition were chosen to be m/z 265.0 → 247.3 for OMT, m/z 249.1 → 148.3 for MT and m/z 300.0 → 215.2 for CDN.

Under the chromatographic conditions used, the mobile phase yielded retention times of less than 0.8 min for all species allowing high sample throughput. Typical chromatograms of blank dermal and blood dialysates are shown in Fig. 3. No additional peaks due to endogenous substances that could have interfered with the detection of the compounds of interest were observed. Fig. 4 shows the chromatograms of OMT and MT in blood and dermal dialysates collected 200 min after application administration of OMT. The retention times were 0.745 min for OMT, 0.809 min for MT, and 0.750 min for CDN, respectively.

3.2. Linearity, precision and accuracy

Linear responses were obtained for both OMT and MT ranging from 0.5 to 1000 ng/mL with correlation coefficient values >0.999.

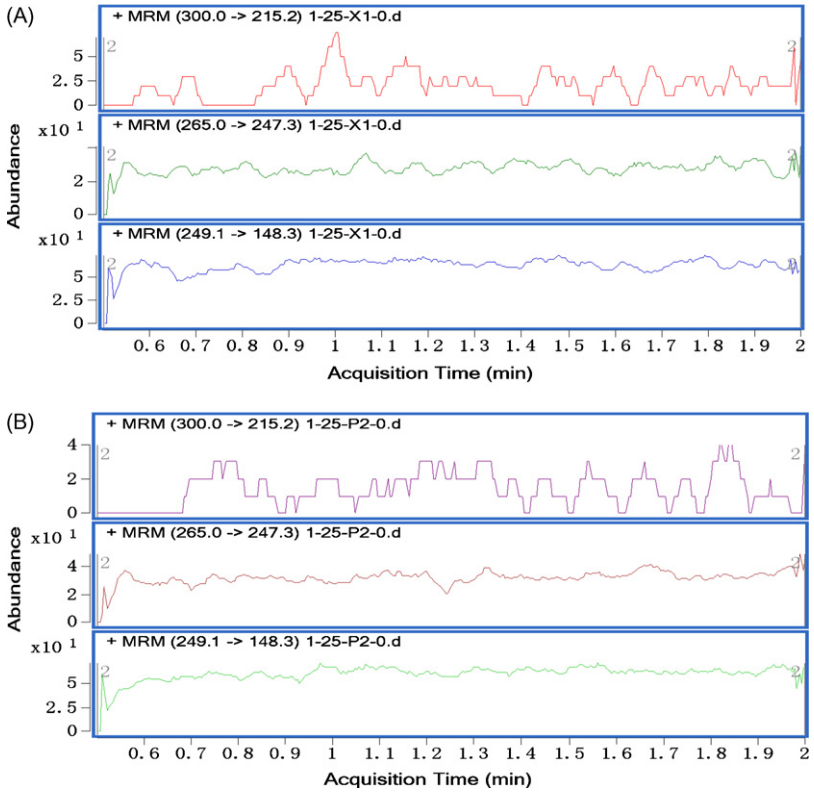

Fig. 3. Chromatograms of (A) blank blood dialysate, (B) blank dermal dialysate.

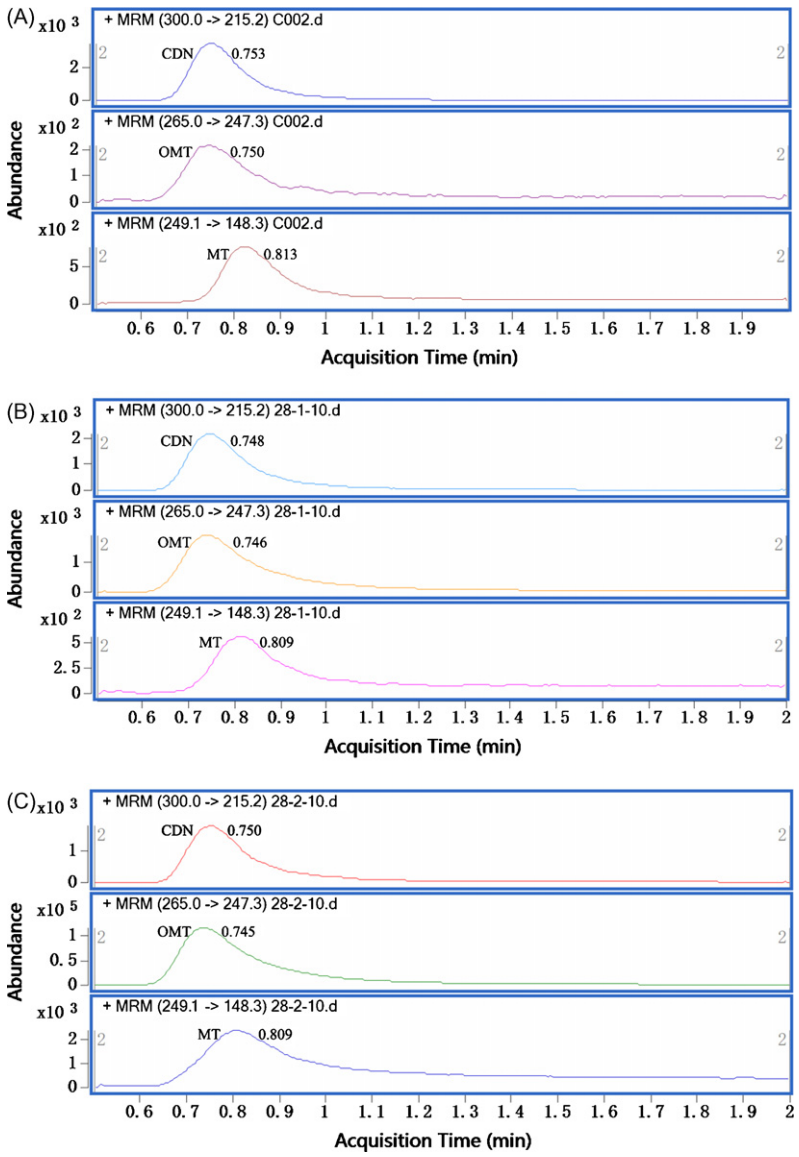

Fig. 4. Chromatograms of (A) saline containing 1 ng/mL OMT and MT, (B) blood dialysate 200 min after the application administration of OMT, (C) dermal dialysate 200 min after the application administration of OMT.

The limit of detection (LOD) was determined at a signal-to-noise ratio of 3. The lower limit of quantitation (LLOQ) based on at least ten times of signal-to-noise ratio. For OMT and MT the LOD is, respectively, 0.2 and 0.1 ng/mL. While LLOQ used in the construction of the calibration curves was set at 0.5 ng/mL for both. The intra- and inter-day precision (%RSD) and accuracy (%Bias) of OMT and MT are shown in Table 1. The overall precision ranged from 2.0% to 13% and accuracy ranged from −4.2% to 12% for OMT and the precision ranged from 1.2% to 15% and accuracy ranged from −2.8% to 6% for MT, respectively.

3.3. *In vivo recovery of OMT and MT from microdialysis probe*

Average *in vivo* recovery levels of OMT and MT were 31.1 ± 7.6% and 39.6 ± 4.7% in blood, 26.7 ± 0.85% and 41.1 ± 1.7% in dermis, respectively (Table 2). No difference in recovery values for same region was observed after the addition of OMT and MT to the perfusate. The recoveries from the microdialysis probes in rat blood and dermis were independent of the concentration for these experiments.

3.4. *Matrix effect of dialysate*

The peak area ratio of the standard solutions mixed with saline (0.9%) compared to that spiking into dialysates expresses the matrix effect in the sample matrix. When the ratio value equals 1 represented the response in saline and in the dialysate was the same and no matrix effect was observed. The results listed in Table 3 showed that the mean matrix effect of OMT and MT was 0.96 ± 0.02 to 0.97 ± 0.05 and 1.02 ± 0.07 to 0.98 ± 0.08, respectively. The value is close to 1, the matrix effect is low and in the quantitative analysis the matrix effect could be ignored.

3.5. *LC–MS/MS analysis of OMT and its metabolite MT in rat biological samples.*

Representative concentration–time profiles of OMT and its metabolite MT in rat blood and dermis after the application administration of OMT gel (3% OMT) are shown in Fig. 5. As shown in Fig. 5, both OMT and MT were detected in dermis and blood

Table 1
Intra- and inter-assay precision (RSD) and accuracy (Bias) of the LC–MS/MS method for the determination of OMT and MT.

Nominal concentration (ng/mL)	Observed concentration (ng/mL)	RSD (%)	Bias (%)
Intra-day of OMT			
1	1.05 ± 0.10	9.5	5
10	10.2 ± 0.73	7.2	2
100	98.2 ± 3.8	3.9	−1.8
1000	1045 ± 29	2.8	4.5
Inter-day of OMT			
1	1.12 ± 0.15	13	12
10	9.58 ± 0.87	9.1	−4.2
100	103 ± 5.93	5.8	3
1000	1022 ± 20.5	2.0	2.2
Intra-day of MT			
1	1.02 ± 0.08	7.8	2
10	10.1 ± 0.66	6.5	1
100	100 ± 4.33	4.3	0
1000	1007 ± 16.0	1.6	0.7
Inter-day of MT			
1	1.06 ± 0.16	15	6
10	9.72 ± 0.84	8.6	−2.8
100	105 ± 2.57	2.4	5
1000	1004 ± 11.9	1.2	0.4

Data are expressed as mean ± SD ($n = 6$).

Table 2
In vivo microdialysate recovery (%) of OMT and MT in rat blood and dermis.

Concentration (ng/mL)	Recovery (%)			
	In rat blood		In rat dermis	
	OMT	MT	OMT	MT
50	28.5 ± 2.8	35.8 ± 4.7	27.5 ± 7.2	42.9 ± 3.7
100	25.1 ± 3.4	38.2 ± 2.5	25.8 ± 7.4	40.9 ± 1.8
500	39.6 ± 1.8	44.9 ± 1.4	26.8 ± 5.4	39.6 ± 1.8
Average	31.1 ± 7.6	39.6 ± 4.7	26.7 ± 0.85	41.1 ± 1.7

Data are expressed as mean ± SD ($n = 6$).

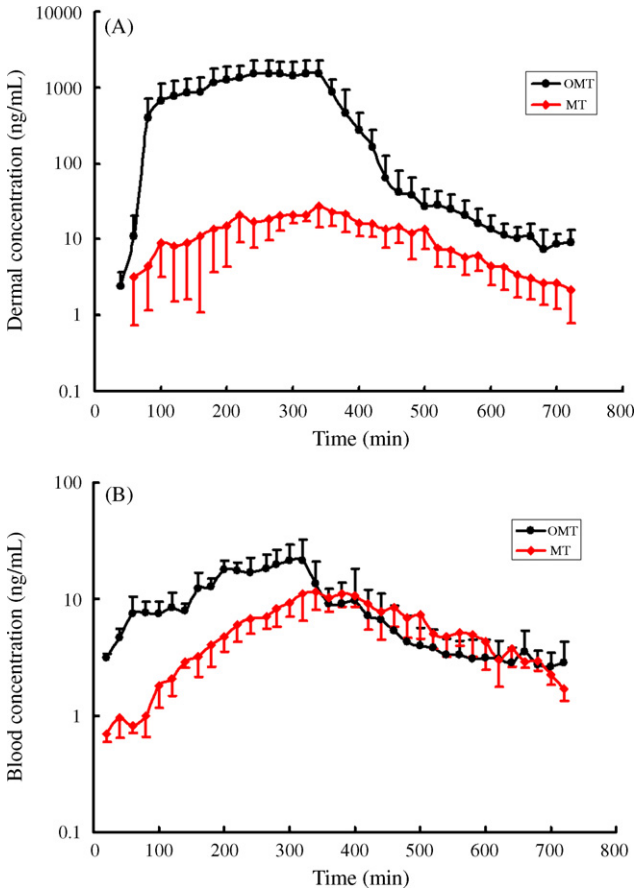

Fig. 5. Unbound concentration–time profiles of OMT and MT in rat dermal (A) and blood (B) dialysates after the application administration of OMT gel ($n = 5$).

after topical application of OMT, indicating that the reduction of OMT rapidly occurred. The blood/dermal ratio of OMT unbound concentrations was small, being about 1%, indicating severe hindrance of skin barrier to the uptake of OMT in the circulation system. The effluent concentrations varied between catheters and between application areas of dermal within test animals. Some study indicated that the variability might not be a problem of the microdialysis technique itself, but rather might be due to variations regarding the dermal concentration after the penetration of an exogenous compound [19]. The reasons for variability in dermal concentrations might lay in the biological variations of the human skin barrier function, the differences of skin metabolism and the individual ability of dermal clearance [20]. It is well known that skin has the capacity for local metabolism of chemicals [21,22]. Microdialysis is a reliable and useful technique for investigation of percutaneous absorption and dermal metabolism. For instance, two unknown substances, suspected to be the metabolites of sinomenine, mostly demethyl and hydroxylated derivatives of sinomenine were found in skin microdialysates from the rats which were administered sinomenine [23,24]. Our results, although determined only in five rats, showed that the extent of metabolised OMT in the skin was rather low in relation to the amount which penetrated through the skin. We found that the ratio of MT and OMT in rat dermis was about 1%. This finding can be explained that the capacity of enzymes in the skin is limited. Therefore, the limiting factor for dermal metabolism is the low capacity of enzymes in the skin rather than the quantity of penetrated OMT.

Table 3
Matrix effect (%) data for OMT and MT in blood and dermal dialysate.

Nominal concentration (ng/mL)	Matrix effect (%)			
	In rat blood		In rat dermis	
	OMT	MT	OMT	MT
10	95.37 ± 10.18	98.23 ± 7.41	97.14 ± 3.37	90.05 ± 7.11
100	96.33 ± 5.34	110.38 ± 2.74	102.44 ± 8.32	96.85 ± 5.60
1000	98.74 ± 1.84	97.27 ± 6.21	92.86 ± 4.18	107.18 ± 5.07
Average	96.81 ± 1.74	101.96 ± 7.31	97.48 ± 4.80	98.03 ± 8.63

Data are expressed as the ratio (%) of the mean peak area of a dialysate spiked with standard solution to the mean peak area of standard solution prepared in saline, and are expressed as mean ± SD ($n = 3$).

4. Conclusions

The study of OMT and MT pharmacokinetics in skin and blood (via microdialysis) appears to be a valuable tool to understand transdermal delivery and dermal metabolism of drug. To our knowledge, this is the first report, which elucidated the *in vivo* time-course profiles of OMT and its metabolite MT in rat dermis and blood after topical application of OMT using microdialysis coupled with high throughput LC–MS/MS technique, of which 2 min of a total run time per sample and 5 μL of the sample were enough for the determination. Current data obtained from rats suggest that the dermal metabolism of OMT seems to be low in quantity compared to the systemic metabolism. The limiting factor for dermal metabolism is the low capacity of enzymes in the skin rather than the quantity of penetrated OMT.

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